Evaluation of the effects of limonene in a animal model of mania

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Abstract. Background: Patients with Bipolar Disorder (BD) present alternating episodes of depression, mania / hypomania and eutymia. In previous researchs, limonene showed anxiolytic effects and was able to reduce the hyperstimulation and dopaminergic neurotramission induced by psychostimulants. The objective of this study was to evaluate the effects of limonene administration on locomotion and exploration behavior in mice treated with methylphenidate (used for inducing mania in an animal model) using the Behavioral Pattern Monitor (MPC). Methods: Male Swiss mice (n = 75) were split in eight experimental groups. Firstly, saline, limonene or lithium were administered. Then, each mouse received saline or methylphenidate. After drug administration, locomotor and exploratory activity was observed. Data was analyzed by ANOVA followed by Duncan post-hoc test. Values $p \leq 0.05$ were considered significant. Results: Methylphenidate increased locomotory activity of the animals. The limonene-treated group didn´t show statistically significant results. Significant increase of the exploration wasn´t found in animals treated with methylphenidate compared to control group. A significant reduction of the holepokes and climbing was seen in lithium and limonene treated animals. Limitations: Video analysis were not done blindly. Conclusions: Limonene administration didn´t reduce methylphenidate-induced hyperlocomotion. Regarding exploratory activity, a significant reduction was observed in the limonene-treated group (200 mg / kg). However, administration of methylphenidate wasn´t able to the increase the exploratory activity, limiting the validity of the results. Thus, further studies are necessary to evaluate the effect of limonene on exploratory behavior in animal models of mania.

Keywords. Bipolar Disorder, Behavioral Pattern Monitor, Methylphenidate, Limonene

1. Introduction

Bipolar Affective Disorder (BAD) is defined by the Diagnostic and Statistical Manual of Mental Disorders and the International Code of Diseases (DSM-5 and ICD-10) as a cyclical disorder with abnormal mood swings. These changes can alternate between states of elevated mood, with increased activity (episodes of mania and hypomania), and lowered mood, with decreased activity (episodes of depression). Between these altered episodes there are often normal, euthymic mood states. For the characterization of the disease, ICD-10 considers the occurrence of two or more episodes of mood and activity disturbance, while DSM-5 considers one episode of mania sufficient for the diagnosis of bipolar disorder, without the need of a depression episode.

BAD usually appears in late adolescence or early adulthood and its treatment is focused on acute stabilization, reversing the manic or depressive episode to a euthymic, stable mood. This treatment has a degree of complexity, since drugs that relieve the symptoms of the depressive phase are capable of triggering episodes of mania or hypomania, and treatments aimed at the manic phase can be a trigger for depressive episodes (Geddes and Miklowitz, 2013; Takeshima, 2017).

Research into new therapeutic options for BAD becomes relevant, since suicide and suicidal behaviors, such as ideation, attempts, are more common in patients with BAD. It is estimated that 25 to 50% of these patients attempt suicide at least once in their lifetime, and 6% to 19% of these same patients come to death by consummated suicide (Beyer and Weisler, 2016). Furthermore, patients with this disorder have a higher relative risk of

developing substance abuse disorder compared to the rest of the population (Stokes et al., 2017). An American study analyzed the clinical outcome of children and adolescents with a diagnosis of BAD and substance abuse disorder, showing that this group had higher rates of hospitalization and suicide when compared to patients with BAD without the comorbidity (Cardoso et al., 2017). It has also been seen that early onset BAD, before the age of 18, is related to higher rates of aggressive behavior, risky behavior, and worse clinical outcome over time (Connor et al., 2017).

Animal models constitute an important approach for research on the pathophysiology and treatment of mental disorders (Andreatini, 2002; Andreatini et al., 2006; Young et al., 2011). Regarding the manic episode of BAD, there are few animal models, the main approach being the study of hyperlocomotion induced by psychostimulants (e.g. amphetamine, which increases dopaminergic, serotoninergic and noradrenergic neurotransmission). However, this approach has been questioned, as it would ignore other facets of the manic episode and be the same as that used in schizophrenia research (Wendler et al., 2016; Young et al., 2011). In this line, it is proposed that a better animal model of mania could be obtained using the an apparatus that would allow a richer/broader behavioral assessment (the Behavioral Pattern Monitor - BPM), specifically targeting the dopaminergic system (e.g. GBR12909, selective dopamine transporter inhibitor -DAT) for behavior induction (Perry et al., 2009; Young et al., 2011). In this regard, in a recent study it was observed that the employment of methylphenidate (a drug that blocks DAT) was able to induce increased locomotor activity, risk-taking behavior, and exploration in mice subjected to BPM (Moraes and Moraes, 2017).

Regarding dopaminergic neurotransmission, it was seen that the substance limonene was able to reduce increased dopamine levels in the nucleus accumbens and regulate methamphetamine-induced hypersensitivity of the postsynaptic dopamine receptor (Gu et al., 2019; Yun, 2014). Limonene is a substance found in citrus fruits and widely used by the food and pharmaceutical industry as a flavoring and flavoring agent. Due to its ease of obtaining, there are several studies encompassing its possible benefits as anti-inflammatory, antiallergic, anxiolytic, and antineoplastic (JIA et al., 2013; Kummer et al., 2013; Lima et al., 2013). Its anxiolytic effect was observed in mice subjected to limonene inhalation at concentrations of 0.5 and 1% using the elevated plus maze test. The treated animals spent less time in the closed arms of the apparatus (Lima et al., 2013). Limonene has also been shown to have the ability to reduce hyperlocomotion in animal models of depression (induced by moderate chronic stress), mania, and chemical dependence (induced by methamphetamine) (Gu et al., 2019; Yun, 2014; Zhang et al., 2019).

Therefore, the aim of this study was to evaluate the effects of acute limonene treatment in an animal model of mania using the Behavioral Pattern Monitor (BPM), which allows quantification of locomoroty and exploratory activity in mice.

2. METHODS

2.1 ANIMALS

Male Swiss mice (n=75), with a mean weight of 30 grams, born and raised in the vivarium of the Biological Sciences Sector of the Federal University of Paraná were used. The animals were kept under controlled conditions of temperature (21 ±1o C); 12-hour light-dark cycle (lights on at 7:00 am); food and water at will. All procedures were approved by the Ethics Committee for Animal Use (CEUA) of the Biological Sciences Sector of the Universidade Federal do Paraná.

2.2 DRUGS

Methylphenidate 5 mg/Kg (Ritalin, Novartis, São Paulo, SP, Brazil) was used for induction of hyperlocomotion, dissolved in saline and administered subcutaneously (Pereira et al., 2011).

Lithium carbonate (Eurofarma, Brazil) 100 mg/kg dissolved in saline, pH adjusted to 7.4 with HCl, administered intraperitoneally was used as a positive control.

Saline solution (NaCl 0.9%), administered intraperitoneally or subcutaneously, was used as a control in all experiments.

Limonene (dissolved in 0.9% saline and containing 5% polysorbate 80; 10 ml/kg, i.p.) was injected into the animals at concentrations of 200 and 400 mg/kg.

All drugs were administered in a constant volume of 10 ml/kg. The proposed doses were based on previous studies in our laboratory (Pereira et al., 2011; Sabioni et al., 2008) and on data from literature (Bhutada et al., 2010; Yun, 2014).

2.3 SPONTANEOUS MOVEMENT APPARATUS

The behavioral pattern monitoring system consists of a rectangular, transparent acrylic box measuring 306 mm wide by 612 mm long, which has a floor and side walls with, respectively, 3 and 8 holes with 1.2 cm in diameter. The floor was divided into 9 quadrants. The quadrants at each corner measure 96 mm x 172 mm, while the upper and lower central quadrants measure 96 mm x 268 mm. The central quadrant measures 114 mm x 268 mm, and the two remaining side quadrants measure 114 mm x 172 mm (Figure 1). All these measurements followed the model from the article by Tanaka et al (2012). The transparent equipment was placed inside a larger cardboard box, with the inside painted black, in order to avoid influence of the external environment

on the experiment.

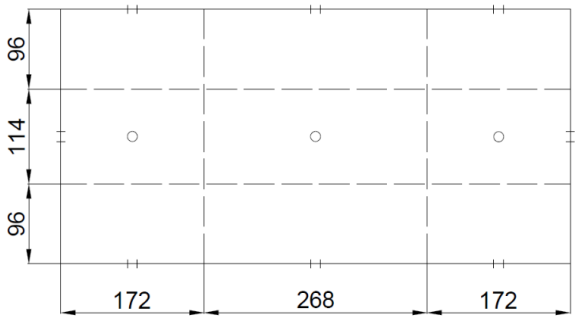

Figure 1 - Schematic of the behavioral pattern monitoring system. Note: values in the figure represent centimeters.

2.4 MEASUREMENT OF LOCOMOTION AND EXPLORATION

Mice were pretreated with limonene (200 or 400 mg/kg, i.p), lithium carbonate (100 mg/kg, i.p) or saline (0.9%, i.p). After 40 minutes, each animal received subcutaneous injection of saline (0.9%) or methylphenidate (5 mg/kg). After 30 minutes of the second injection, each mouse was individually placed in the apparatus of the behavioral pattern monitoring system (initially in the lower left quadrant, with the mice's head facing the wall), remaining for 60 minutes in the equipment. The permanence of each mouse in the apparatus was recorded by a digital camera. After the time had elapsed, the animals were removed from the apparatus and returned to their cages.

The videos were analyzed by the researchers. Locomotory activity was quantified by the number of quadrants crossed by each animal. Exploratory activity was measured by the number of times each mouse put its snout completely into one of the holes (holepokings), by the "lifts" without front-leg support (rearings), and by the number of times each animal lifted and supported itself using the side walls of the apparatus (climbs).

2.5 EXPERIMENTAL GROUPS

The 75 mice provided for the experiment were divided into eight experimental groups (Figure 2). One quarter of the animals were subjected to intraperitoneal injection of lithium, one quarter were subjected to intraperitoneal injection of saline, one quarter received intraperitoneal limonene at a concentration of 200 mg/kg and the last quarter received a concentration of 400 mg/kg. Subsequently each group was subdivided into two, one that received subcutaneous injection of saline and one that received subcutaneous injection of methylphenidate.

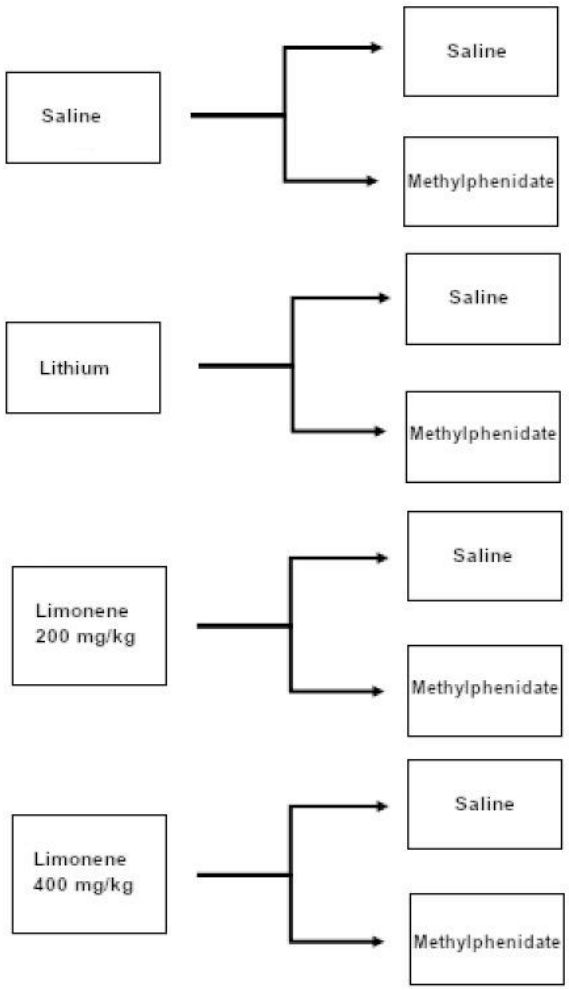

Figure 2 - Division of experimental groups.

2.6 GRAPHICAL REPRESENTATION

The program GraphPad Prism 8.2.0 was used for the construction of bar graphs.

2.7 STATISTICAL ANALYSIS

Statistical analysis of the methylphenidate experiments was performed by one-way ANOVA followed by Duncan's post-hoc test using the Statistica 8 program. Values of $p \leq 0.05$ were considered statistically significant.

3. RESULTS

3.1 LOCOMOTORY ACTIVITY

The group treated with saline + methylphenidate (5 mg/kg) showed a significant increase in locomotion compared to the one treated with saline + saline ($p=0.0358$). The group treated with lithium carbonate (100 mg/kg, i.p) + methylphenidate had a significant reduction in the number of quadrants traveled in comparison to the one treated with saline + methylphenidate ($p=0.0024$). No differences were seen between the groups treated with limonene (200 and 400 mg/kg, i.p) + methylphenidate and the group treated with saline + methylphenidate (Figure

3).

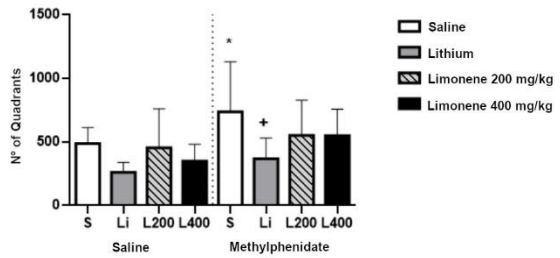

Figure 3 - effect of acute limonene administration on locomotor activity. Note: Effect of limonene (200 and 400 mg/kg, acute ip) on methylphenidate-induced hyperlocomotion (5 mg/kg, sc, single dose 30 min before test) of mice tested in the behavioral pattern monitoring system. Data represented by mean + standard deviation of the number of quadrants crossed. Left-side columns refer to saline treatment (control) and right-side columns refer to methylphenidate treatment. S: saline; Li: lithium; L200: limonene 200 mg/kg; L400: limonene 400 mg/kg. + p≤0.05 when compared to the the methylphenidate + saline group.

3.2 EXPLORATORY ACTIVITY

Duncan's post-hoc test showed no statistical difference in the number of rearings between the experimental groups (Figure 4).

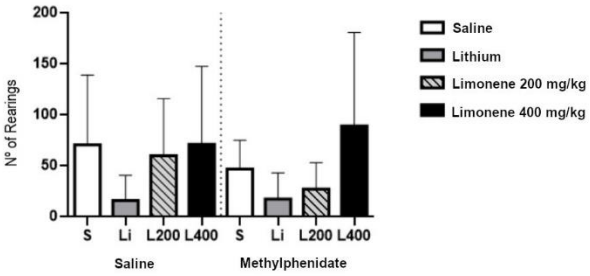

Figure 4 - Effect of acute limonene administration on exploratory activity (rearings). Note: Effect of limonene (200 and 400 mg/kg, acute ip) on exploratory activity (rearings) in methylphenidate-induced mania animal model (5 mg/kg, s.c, single dose 30 min before test) of mice tested in the behavioral pattern monitoring system. Data represented by mean + standard deviation of the number of rearings. Left-side columns refer to saline treatment (control) and right-side columns refer to methylphenidate treatment. S: saline; Li: lithium; L200: limonene 200 mg/kg; L400: limonene 400 mg/kg.

The groups treated with lithium carbonate (100 mg/kg, i.p) + methylphenidate and limonene (200 mg/kg, i.p) + methylphenidate obtained lower numbers of holepokings compared to the group treated with saline + methylphenidate (p=0.0002 and p=0.022, respectively). There was no difference between the groups treated with limonene (400 mg/kg, i.p) + methylphenidate and saline + saline compared to the one treated with saline + methylphenidate (Figure 5).

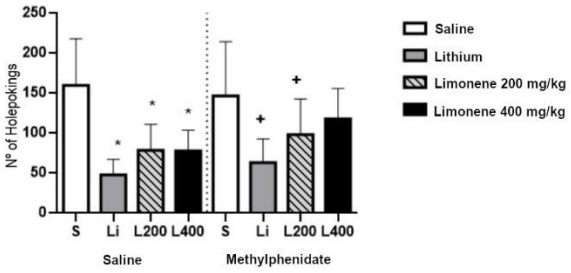

Figure 5 – Effect of acute administration of limonene on exploratory activity (holepokings). Note: Effect of limonene (200 and 400 mg/kg, acute ip) on exploratory activity (holepokings) in an animal model of mania induced by methylphenidate (5 mg/kg, s.c, single dose 30 min before test) mice tested in the behavioral pattern monitoring system. Data represented by mean + standard deviation of the number of holepokings. Left-side columns refer to saline treatment (control) and right-side columns refer to methylphenidate treatment. S: saline; Li: lithium; L200: limonene 200 mg/kg; L400: limonene 400 mg/kg. ** p≤0.05 when compared with saline + saline group. + p≤0.05 when compared to the methylphenidate + saline group.

The groups treated with lithium carbonate + methylphenidate and limonene (200 mg/kg, i.p) + methylphenidate showed lower number of climbings compared to the group treated with saline + methylphenidate (p=0.001 and p=0.016, respectively). No differences were observed between the groups treated with limonene (400 mg/kg, i.p) + methylphenidate and saline + saline compared to the one treated with saline + methylphenidate (Figure 6).

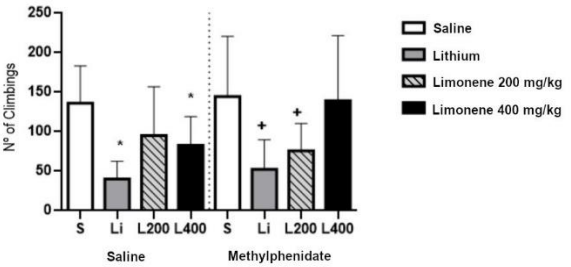

Figure 6 - Effect of acute limonene administration on exploratory activity (climbing). Note: effect of limonene (200 and 400 mg/kg, acute ip) on exploratory activity (climbing) in methylphenidate-induced mania animal model (5 mg/kg, sc, single dose 30 min before test) of mice tested in the behavioral pattern monitoring system. Data represented by mean + standard deviation of the number of climbings. Left-side columns refer to saline treatment (control) and right-side columns refer to methylphenidate treatment. S: saline li: lithium; l200: limonene 200mg/kg; l400: limonene 400 mg/kg. **p≤0.05 when compared to saline+saline group + p≤0.5 when compared to methylphenidate+saline group.

4. DISCUSSION

In this study, we evaluated the effects of a single administration of limonene (200 and 400 mg/kg, i.p) in mice treated with methylphenidate (5 mg/kg, s.c), with acute administration of lithium carbonate (100 mg/kg, i.p) as positive control and saline solution (NaCl 0.9% i.p) as negative control. The locomotory (by the number of crossed quadrants) and the exploratory activity (by the number of holepokings, rearings and climbings) of the animals were evaluated.

Methylphenidate, a substance that blocks dopamine reuptake, was shown to induce hyperlocomotion in mice. In the present study, this effect, as expected, was attenuated by acute administration of lithium carbonate. The administration of limonene (200 and 400 mg/kg i.p.) did not result in a statistically relevant decrease in locomotion when compared to the saline + methylphenidate group. A Korean study found different results, showing that the acute administration of limonene at a dose of 400 mg/kg was able to effectively block the effect of methylphenidate-induced hyperlocomotion in rats (Yun, 2014).

Contrary to the authors' expectations and countering previous studies (Perry et al., 2009; Souza et al., 2016; Moraes and Moraes, 2017), mice receiving methylphenidate did not show a significant increase in exploratory activity, holepokings, climbing and rearings, compared to the control group. Limonene treatment at 400 mg/kg concentration showed no significant changes in any of the variables analyzed. With 200 mg/kg, limonene significantly reduced the number of holepokings and climbing in mice treated with methylphenidate. Thus, there was no concentration-effect relationship regarding the administration of limonene. Acute use of lithium was able to reduce the number of climbing, rearings, and holepokings in the methylphenidate-treated mice, corroborating with recent research showing that lithium administration reduced methylphenidate-induced exploratory activity in the hole-board test (Souza et al., 2016) and the Behavioral Pattern Monitor for mice (Moraes and Moraes, 2017). However, satisfactory mania modeling for exploratory activity was not obtained using the saline + methylphenidate treated group. Therefore, no conclusions about the effect of lithium and limonene regarding this parameter can be drawn in the present study.

A limitation of this study was that the experiments and their analysis were not blinded to the researchers, resulting in possible observation bias. Although the effects of limonene did not show statistical significance, a reduction in both exploratory and locomotor activity was observed in the mice treated with methylphenidate. Thus, further studies would be necessary to verify whether the lack of significance was due to the great variability between results or if there really is no effect.

5. CONCLUSION

Limonene administration was not able to reduce methylphenidate-induced hyperlocomotion in mice. Regarding exploratory activity, a significant reduction was observed in the group treated with limonene 200 mg/kg. However, methylphenidate administration was not able to model the increase in exploratory activity, limiting the validity of the results. Thus, further studies are needed to evaluate the effect of limonene on exploratory behavior in this animal model of mania.

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