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EFFECT OF 7-(B-HYDROXYETHYL) THEOPHYLLINE AND 8-PHENYL THEOPHYLLINE ON THE ACQUISITION AND RETENTION ON THE SPACE MEMORY OF MICE

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ABSTRACT

Neurodegenerative diseases have an increasing prevalence in the population over 65 years of age. In this context, adenosine antagonists have a neuromodulatory role mainly in the hippocampus region, contributing to the maintenance and improvement of memory. Adenosine antagonists are promising in studies of preventive drugs for complications of neurodegenerative diseases. The aim of this study was to evaluate the effect of different concentrations of 7- (β-hydroxyethyl) theophylline and 8-phenyl theophylline, on the acquisition and retention of spatial memory of mice. Method: in this study were used male Swiss mice weighing 25-30 g and subdivided into two experiments, one for each drug. In each experiment two moments of application were performed, before and after the training / test section, with three dilutions for each drug evaluated. The mice were submitted to training sessions at the Morris Water Maze (MWM) for four days (three sessions per day) and were submitted to the test after 48 h of the last training, and the time for each animal to find the hidden platform was measured. Data were analyzed using Two-Way Reapeated-Measure ANOVA followed by Tukey post-hoc test where appropriate ($p < 0.05$). Results: The drugs did not reduce the latency for the animal to find the platform: group I ($p = 0.089$), II ($p = 0.641$), III ($p = 0.089$) and IV ($p = 0.290$) with significant effect in relation to the training day / test: group I ($p < 0.001$), II ($p < 0.02$), III ($p < 0.001$) and IV ($p < 0.001$). Conclusion: It is concluded that LAM is a useful test to evaluate the acquisition and retention of spatial memory in mice and that the experimental drugs tested had no effect on spatial memory, regardless of dosage and time of application.

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INTRODUCTION

Neurodegenerative diseases such as Parkinson's and Alzheimer's affect the Central Nervous System (CNS), the symptoms of Parkinson's are stiffness, agitation and slower movements.

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Alzheimer's has as main symptom memory impairment (Oliver and Rios, 2014). Currently there is no effective preventive therapy, only symptomatic medications to minimize the evolution of these diseases, being considered the most common cause of dementia, reaching 5% of the population over 65 years (Zhang *et al.,* 2013). The neuroprotective effects and the reduction of side effects produced with treatments that use adenosine receptor antagonists are proven by several studies. They have become promising in therapeutics for the treatment of neural degeneration, such as that induced by

spinal cord injury, stroke, Alzheimer's and Parkinson's diseases, as well as other diseases of CNS (Oliver e Rios, 2014). Studies with drugs that may act as adenosine antagonists represent an important target for research seeking to improve the symptoms of degenerative diseases, since the non-selective blockade of adenosine receptors by theophylline or other antagonists, as well as selective adenosine block A1 and receptors of A2A facilitates learning and memory retention in experimental studies (Acuña *et al.,* 2013). Scientific studies with animals have shown that xanthines have neuroprotective action, preventing the loss of dopaminergic neurons, delaying neuronal degeneration (Oliver and Rios, 2014). Caffeine facilitates synaptic connections between CNS cells including invertebrate animals (Chittka and Peng, 2014), as well as acting on the modulation of learning and memory functions, probably due to its non-selective action of adenosine receptor receptors (Alhaider *et al.,* 2010). Studies involving the relationship between theophylline and memory / learning, demonstrate a beneficial effect of theophylline on behavior and learning (Alzoubi *et al.,* 2013). In contrast, some studies have shown that there are no differences in their use (Xu *et al.,* 2007), some studies also suggest that the use of the drug may impair memory (Hirose *et al.,* 2004).

Few studies have demonstrated the action of 8-phenyl theophylline, but Ahlijanian and Takemori (1986) suggest that this drug has caffeine-like action, with caffeine being three times more potent than theophylline at presynaptic adenosine receptors without selectivity of receptor (Clanachan, 198). The choice of drug for use in this study was understood to be the importance of investigating an adenosine antagonist with its neuromodulatory action. In fact, it is perceptible in the literature review that there is a small number of research using theophyllines. Thus, when used, the application was to other systems of the body and with divergent results, from effective use to hippocampal impairment. From this perspective, this work aimed to study and compare the effects of 7β-hydroxyethyl theophylline and 8-phenylthiophilin, both adenosine receptor antagonists, on the acquisition and retention of spatial memory.

MATERIALS AND METHODS

128 Swiss male mice weighing 25 to 30 g were used, with approximately 60 days of life. The animals were kept in Plexiglas cages with water and ad libitum ration and 12h dark light cycle (7-AM and 7-PM) at room temperature of 25 ± 1 °C. All tests were performed in the afternoon period (13h. to 18h.). The experiments were performed with the 7β-hydroxyethyl theophylline and 8-phenylthiophilin drugs, both diluted in saline solution (0.9% NaCl) and administered intraperitoneally. For the spatial memory evaluation was used the Morris Water Maze (MWM) (Morris *et al.,* 1981). Four experiments were carried out in four experimental groups, with eight animals in each group, as described below.

Experiment I: Effect of 7β-hydroxyethyl theophylline on the acquisition of spatial memory of Swiss mice:

- Group 1 (n = 8): saline solution at 0.9% by volume of 10ml / kg animal weight, 30 minutes before training or MWM test.
- Group 2 (n = 8): 7β-hydroxyethyl theophylline diluted in 0.9% saline solution at a dose of 10mg / kg body weight, 30 minutes prior to training or testing in the MWM.
- Group 3 (n = 8): 7β-hydroxyethyl theophylline diluted in 0.9% saline solution at a dose of 20mg / kg body weight, 30 minutes prior to training or testing in the MWM.
- Group 4 (n = 8): 7β-hydroxyethyl theophylline diluted in 0.9% saline solution at a dose of 40mg / kg body weight, 30 minutes prior to training or testing in the MWM.

Experiment II: Effect of 7β-hydroxyethyl theophylline on the spatial memory retention of Swiss mice:

- Group 1 (n = 8): saline solution at 0.9% by volume of 10 ml / kg of animal weight, immediately after training or testing in the MWM.
- Group 2 (n = 8): 7β-hydroxyethyl theophylline diluted in 0.9% saline at a dose of 10 mg / kg body weight, immediately after training or testing in the MWM.
- Group 3 (n = 8): In this group the animals received intraperitoneal injection (i.p.), 7β-hydroxyethylthiophilin diluted in saline solution at 0.9% at a dose of 20mg / kg of animal weight, immediately after training or testing in the MWM.
- Group 4 (n = 8): 7β-hydroxyethyl theophylline diluted in 0.9% saline solution at a dose of 40 mg / kg body weight, immediately after training or testing in the MWM. MWM.

Experiment III: Effect of 8-phenylophylin in the spatial memory acquisition of Swiss mice:

- Group 1 (n = 8): In this group the animals received i.p., saline solution at 0.9% by volume of 10 ml / kg of animal weight, 30 minutes before training or in the MWM.
- Group 2 (n = 8): In this group the animals received i.p., 8-phenylthiophilin diluted in saline solution at 0.9% at the dose of 1mg / kg of animal, 30 minutes before training or in the MWM test.
- Group 3 (n = 8): In this group the animals received i.p., 8-phenylthiophilin diluted in saline solution at 0.9% at a dosage of 2mg / kg of animal weight, 30 minutes before training or in MWM .
- Group 4 (n = 8): in this group the animals received i.p., 8-phenylthiophilin diluted in saline solution at 0,9% at a dosage of 4mg / kg of animal weight, 30 minutes before training or in MWM .

Experiment IV: Effect of 8-phenylthyophylline on the retention of spatial memory of Swiss mice:

- Group 1 (n = 8): In this group the animals received i.p., saline solution at 0.9% by volume of 10 ml / kg of animal weight, immediately after the training or test in the MWM.
- Group 2 (n = 8): In this group the animals received i.p., 8-phenylthiophilin diluted in 0.9% saline at a dose of 1 mg / kg body weight, immediately after training or MWM testing.
- Group 3 (n = 8): In this group the animals received i.p., 8-phenylthiophilin diluted in 0.9% saline at a dose of 2 mg / kg body weight, immediately after training or LAM testing.

- Group 4 (n = 8): In this group the animals received i.p., 8-phenylthiophilin diluted in 0.9% saline solution at a dose of 4 mg / kg body weight, immediately after training or testing in the MWM.

Morris Water Maze (MWM)

The Morris Water Maze (MWM) test assesses the animal's ability to acquire, retain, and recall spatial memory by measuring latency for the animal to locate a platform submerged in a tank with water. In this manuscript, an adaptation of the protocol described by Morris *et al.* (1981) was used to evaluate the animals studied. The MWM was composed of a water box 119 cm in diameter and 90 cm deep, which was painted black and filled with water. The water temperature was maintained at 25 ° ± 1 ° C. Inside the labyrinth was placed a platform (19.5 x 11.0 cm) submerged 2 cm from the water surface, in the center of one of the four imaginary quadrants of the tank and kept in this place during all the training and tests. The animiais were oriented using visual flags at each of the four cardinal points of the tank wall.. In the test / training, the latency (in seconds) was measured so that the animal could find the platform hidden under the water, after being left in one of the quadrants. Three tests per day were performed during four consecutive days (Nunez, 2008). The animal has always been loosed facing the tank wall, leaving one of the three remaining (pseudorandom) cardinal points - other than the one on which the platform was. All groups were submitted to three daily training sessions for four consecutive days (D1, D2, D3 and D4), one day of rest (D5) and one test (D6), respecting the maximum latency time to find the 90 seconds. If the animal did not find the platform in this period, it was led to the platform. After each training the animal remains on the platform for 30 seconds for spatial storage, followed by 60 seconds of rest in an environment under light, performing the outputs of the three cardinal points, not repeating the exit of the same point in a given training / test. The spatial memory acquisition and retention were evaluated during the four training days and 48 hours after the training (test) in the MWM. In both cases, the latency (in seconds) was measured so that the animal could find the platform hidden under the water of the tank.

Statistical analysis

The results obtained through the behavioral tests were statistically analyzed using *SigmaPlot for Windows*® software, version 12.5, considering a level of significance of 5%. Time and dosage factors were compared by Two-Way Repeated-Measure ANOVA followed by Tukey post-hoc test where appropriate ($p < 0.05$).

RESULTS

Analysis of the results showed no effect of the experimental group on the dosage of the drug used (*two-way repeated measures ANOVA*, $p = 0.089$). However, there was a significant effect in relation to the day of the training / test ($p < 0.001$) without interaction between experimental group and day of training / test ($p = 0.605$). In the post-test, it was evidenced that the latency to find the platform in D4 (4th day of training) and D6 (test), independent of the group was lower than that of D1 and D2 (1st and 2nd day of training), -test Tukey ($p < 0.05$). In addition, the D4 latency (4th day of training) was lower than that observed in D3 (3rd day of training) ($p < 0.05$).

The analysis of the results showed that there was no effect of the experimental group in relation to the dosage of the drug used (*two-way repeated measures ANOVA*, $p = 0.641$). However, there was a significant effect in relation to the day of the training / test ($p < 0.02$) without interaction between experimental group and day of training / test ($p = 0.60$). In the post-test it was evidenced that the latency to find the platform in D6 (test), independent of the group, was lower than that in D1 (1st day of training), (Tukey post-test $p < 0.05$). The general analysis of the results showed that there was no effect of the experimental group in relation to the dosage of the drug used (*two-way repeated measures ANOVA*, $p = 0.089$), but there was a significant effect in relation to the day of the training / test ($p < 0.001$) without experimental group interaction and training / test day ($p = 0.605$). In the post-test, it was evidenced that the latency to find the platform in D4 (4th day of training) and D6 (test) independent of the group, was lower than that of D1 and D2 (1st and 2nd day of training), (Tukey post-test $p < 0.05$). In addition, the D4 latency (4th day of training) was lower than that observed in D3 (3rd day of training) ($p < 0.05$). The general analysis of the results showed that there was no effect of the experimental group in relation to the dosage of the drug (two-way repeated measures ANOVA, $p = 0.290$), but there was a significant effect in relation to the day of the training / test ($p < 0.001$) and interaction between the experimental group and training / test day ($p = 0.034$). The post-test evidenced that the control group did not present difference between the days of training / tests. The group with a dose of 1mg / kg had lower results in D6 (test) than D1 and D3 (1st and 3rd day of training). The group with a dose of 2mg / kg had a shorter time on D3, D4 and D6 (3rd and 4th day of training and test) than D1 (1st day of training). The group with dosage of 4mg / kg did not present difference between the days of training / tests (Tukey post-test $p < 0.05$). The post-test also shows that there was no interaction between the experimental groups and the training / test days (Tukey post-test $p < 0.05$).

DISCUSSION

The results obtained in this study demonstrated that the MWM memory evaluation protocol was able to evidence the acquisition and retention of the spatial memory in mice, since the animals of the experimental groups decreased the latency to find the platform during the training days and on the day of the test. The results presented in our study agree with other studies that used the labyrinth (MWM) and demonstrate that during the training, there is a reduction of the latency time for the animal to find the platform (Prediger *et al.,* 2005a). The results for the experimental groups of mice, 7β-hydroxyethyl theophylline and 8-phenylthiophilin at the different doses tested, applied before the training / test, showed no interaction with the control group, in relation to the decrease in latency time to find the platform. The same results were observed for the experimental group, 7β-hydroxyethyl theophylline, independent of the dosage tested for the post-training / test applications, when also compared to the control group. For the post-training / test applications, the results demonstrated an interaction between the experimental group per training / test day, but that was not evidenced by the post-test. An important point is that research related to xanthines commonly brings information about the action of the drug in the pulmonary, renal and cardiac systems. When studies bring their action into the CNS, the vast majority of the drug is caffeine.

Although several studies point to the CNS stimulant effect, adenosine antagonist drugs, especially xanthines, have not been shown in this study that the drug reduced the animal's latency time to find the platform. According to Xu *et al.* (2007), theophylline has no memory acquisition and retention effects, but the drug attenuated the degenerative effects of microwave radiation on memory acquisition. There are studies that demonstrate that theophylline at therapeutic doses may impair the learning and memory of developing animals and has no effects on developed animals (Hirose *et al.,* 2004). It is believed that theophylline action in memory impairment occurs by the induction of the substance to dopamine and the binding at the β-adrenergic receptor, where it contributes to memory attenuation (Hirose *et al.,* 2004). It can also be considered the activity of the substance as Cyclic nucleotide phosphodiesterases (PDEs), which are enzymes that catalyze the hydrolysis of cyclic AMP forming adenosine (Hirose *et al.,* 2004). According to other studies (Hirose *et al.,* 2004), the lack of theophylline effect in memory can be attributed not only to the A1 agonist but also to the non-competitive NMDA antagonists and H3 antagonists, these important receptors in synaptic plasticity.

In a retrospective study of the possible adverse effect of theophylline in asthmatic children, there is a significant correlation between theophylline use and inattention, hyperactivity, irritability and withdrawal behavior (Springer *et al.,* 1985). Hung *et al.* (2002) demonstrated that the use of aminophylline in rats with epileptic seizures contributes to spatial memory deficits; on the other hand, its association with another drug (lithium + pilocarpine), besides the memory deficit, the animals presented motor and morphological changes such as loss of neuronal cells. There is a clinical case report that presents theophylline as a cause of human intoxication (55 years old man) who developed amnestic syndrome after epileptic crisis, presenting bilateral lesion and hippocampal atrophy (Khol *et al.,* 2011). In addition, the theophylline has been used in the acquisition and retention of spatial memory, contrary to the results obtained in this study, although the memory test used was another, but they reinforce that the effect of theophylline can vary greatly with the time of the tests performed. Scientific evidence using the radial arm aquatic labyrinth in animals submitted to sleep deprivation and using MWM (Hu *et al.,* 2007) demonstrated that pentoxifylline, xanthine adenosine antagonist has beneficial spatial memory effects in rats (Alzoubi *et al.* al., 2013).

Florian (2011) reported that 8-cyclopentyl-1, 3-dipropylxanthine contributes favorably to spatial memory in rats submitted to sleep deprivation, and its association with cannabinoid has also brought about improvements in hippocampal synaptic plasticity (Assini *et al.,* 2012). The action and interaction of caffeine and sleep deprivation on cognitive function and synaptic plasticity in low and chronic caffeine doses do not interfere with memory construction but have protective effect on synaptic plasticity (Alhaider *et al.,* 2010). For authors such as Chu *et al.* (2012), the long-term use of caffeine contributes to memory maintenance in sleep-deprived animals. However, its use does not improve the motor performance (speed) of the animals submitted to MWM. In relation to speed, this study can not reach the conclusion, because in this research we do not monitor its speed. Caffeine had no beneficial effect on memory when associated with Tetrahydrocannabinol (THC). On the contrary, it potentiated the amnestic effects of THC (Panlilio *et al.,* 2011).

On the other hand, Sousa *et al.* (2011) states that caffeine at a concentration of 3 mg / kg significantly interfered in spatial memory performance in rats submitted to THC exposure. In relation to caffeine it is known that its action as an adenosine antagonist undergoes many variations in relation to dosage, time of administration and time of use. It is concluded that for the memory modulator effect there are moderate dose requirements, and in the case of overdose will present a reduction of the effect (Takahashi *et al.,* 2008). For a beneficial action of caffeine there is a need for a moderate dosage, but the classification on the dosage considered high is not consensual in the literature (Björklund *et al.,* 2008). A study carried out in 3047 participants evaluated the caffeine, alcohol and nutritional intake of a diverse population demonstrating that there are benefits of the cognitive domains, by the protective effect of caffeine and the adequate intake of nutrients, minimizing the compromise that occurs with the course of the age and with the use of alcohol (Beydoun *et al.,* 2014). Bottona *et al.* (2010) used mice with the objective of evaluating caffeine as a preventive drug in the dysfunction of memory consolidation. This study used 349 animals, which received caffeine (10mg / kg) for four consecutive days.

On the fifth day, the animal received scopolamine hydrobromide (2mg / kg) 15 minutes before or shortly after training. Bottona *et al.* (2010) evaluated short-term and long-term memory in object recognition, inhibitory avoidance time, and open field test. In their results, it was evidenced that the drug contributed to the acquisition of short and long term memory without causing hyperlocomotion in the open field test. As for memory retention, it was not as effective because the administration of the drug was suspended 48 hours before the retention test. Considering the use of the drug in humans, young adults, in whom the association of caffeine and glucose in memory and coordination tests was evaluated, the individual who used caffeine and glucose alone showed improvement in cognition in relation to simple, more connected activities to reasoning. The association of glucose and caffeine has proved effective for complex processes of attention and memory, without causing any behavioral change. (Adan e Grabulosa, 2010). The effects of xanthines on the hippocampus and on memory may be due to the drug used in the study, dosages, time of drug use and protocol chosen (Alhaider *et al.,* 2010). Other studies should be performed to understand the mechanisms of action of theophyllines, especially in the CNS, where research is scarce, considering that the majority of studies with this drug are focused on its action in the respiratory system. Another suggestion is that other studies are conducted with a larger sample of animals, where the effect of the drug may be more evident.

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