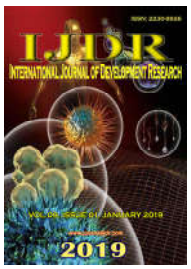
ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 09, Issue, 01, pp.25408-25412, January, 2019



ORIGINAL RESEARCH ARTICLE OPEN ACCESS

CAFFEINE EFFECT ON NON-MOTOR SYMPTOMS IN AN ANIMAL MODEL OF PARKINSON DISEASE

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ARTICLE INFO

Article History:
Received 17th October, 2018
Received in revised form
20th November, 2018
Accepted 24th December, 2018
Published online 30th January, 2019

Key Words:
Parkinson's Disease,
Non-motor Symptoms,
MPTP,
Caffeine, Basal Ganglia.

ABSTRACT

It has been shown that A2A receptor antagonist drugs have beneficial effects on neuroprotection and control of the motor symptoms of Parkinson's disease; however, their effects on non-motor symptoms are virtually unknown. This study aimed to evaluate the effect of caffeine on non-motor symptoms in an animal model of Parkinson's Disease. The experiment was composed of two groups, the saline and the MPTP group, each composed of three subgroups. The animals received saline or MPTP for five consecutive days and after one day were treated with saline, caffeine 10 mg / kg or 20 mg / kg; after the tests of Elevated plus maze, Open field and Tail suspension test as well as the Discrimination Olfactory test were performed and recorded. The data and statistical analysis were performed by the Kruskal-Wallis test with Dunn post-test, with a significance level of $p < 0.05$. It was observed that there was a small significant difference only in the CA and LCE tests, by which one cannot affirm any of the hypotheses raised, since the differences occurred among the random groups. Therefore it was not possible to verify the efficacy of caffeine on these symptoms.

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Citation: Hudman Cunha Ortiz and Albert Schiaveto de Souza, 2019. "Caffeine effect on non-motor symptoms in an animal model of parkinson disease", *International Journal of Development Research*, 09, (01), 25408-25412.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting 1% of the population over 55 years old and is characterized by the progressive degeneration of the dopaminergic neurons of the substantia nigra and nigro-striatal dopaminergic pathways (Lees *et al.,* 2009). The motor symptoms of PD appear after the loss of approximately 50-70% of these neurons and are their distinguishing symptoms which serve as basis from which diagnosis and treatment are initiated, and are now recognized as being preceded by a "pre-motor" phase composed largely of a range of different non-motor symptoms (NMS) (Blesa *et al.,* 2012; Sauerbier *et al.,* 2016). NMS may appear for years, perhaps decades, before motor symptoms (Taylor *et al.,* 2010) including: olfactory loss (Ubeda-Banõn *et al.,* 2014), cognitive dysfunction (Lindgren and Dunnett, 2012), dementia (Zurkovsky *et al.,* 2013), sleep disorder, autonomic dysfunction, urinary complaints constipation, depression, anxiety (Bonito-Oliva *et al.,* 2014; Tanveer *et al.,* 2018) and altered circadian cycle, as well as manifestations in other systems or external organs such as skin (Planken *et al.,* 2017), which progressively affect the quality of life and independence of individuals (Lageman et al.,2014). In short, its increasing prevalence of complications is very complex, marking a new concept scenario for PD, so specific NMS treatments are needed (Del Rey *et al.,* 2018). The fact that most drugs currently available for PD treatment (such as levodopa - L - DOPA) is more efficient in relieving motor than cognitive alterations, has led many researchers to postulate non-dopaminergic mechanisms for the cognitive symptoms of PD and It seems that caffeine and selective A2A adenosine receptor antagonists may be particularly useful in restoring learning and memory processes and even the olfactory process (Prediger, 2010). Thus the aim of this study was to analyze the caffeine effect on the non-motor symptoms of PD, specially anxiety, depression and olfactory disturbance in an animal

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model of Parkinson disease (1-methyl-4phenyl-1,2,3,6-tetrahydropyridine – MPTP + probenecid) in mice.

MATERIALS AND METHODS

Animals: Male Swiss mice were used, from the Federal University of Mato Grosso do Sul (UFMS), weighing between 30-40g. The animals were kept in the vivarium with water and food *ad libitum* until the beginning of the experiments. The light cycle (12/12 h, lights switched at 6:00 am) and ambient temperature (23 ± 1 ° C) were controlled.

Drugs: Non-selective adenosine receptor antagonist: caffeine (1,3,7-trimethylxanthine), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), both dissolved in 0.9%, in addition to probenecid, which was dissolved in dimethylsulfoxide (DMSO). All drugs were given intraperitoneally (i.p.).

Experiment: The study was approved by the Ethics Committee on the use of animals / ECUA of the Federal University of Mato Grosso do Sul, with protocol number 670/2015. The animals were divided into two groups: saline group and MPTP group, both were divided into three subgroups consisting of 7 or 8 animals and each animal was administered 0.9% saline or probenecid (250 mg / kg) 30 minutes before MPTP (25 mg/kg) for 5 consecutive days via ip, once a day, on the seventh day the treatment with saline or caffeine (10 or 20 mg / kg) was initiated once daily, via ip, for 15 consecutive days, at the twenty-second day the tests of Elevated plus maze (EPM), Open field test (OFT) and the Tail suspension test (TST) were performed, and only on the 23rd day, the Olfactory discrimination test (ODT) was performed, so that there was no interference in the previous tests by the fasting to which the animals were subjected for its accomplishment. The illustrative scheme of the experiment is shown in Figure 1.

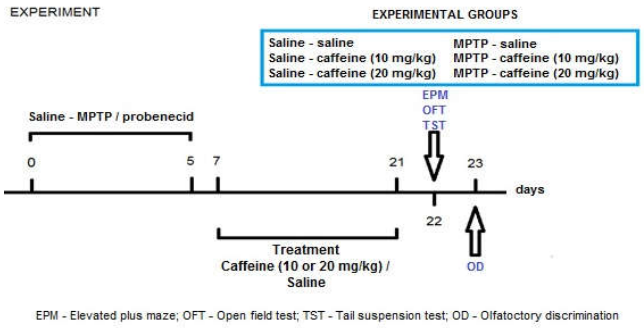

Figure 1. Illustrative scheme of the experiment

Animals evaluation

Elevated plus maze: This test is considered as the gold standard method for assessing anxiety in basic research, and was based on a previous study (Komada *et al.,* 2008). Each animal remained in the labyrinth for 5 min, being initially placed in the labyrinth with the face turned to the closed arms. The tests were recorded and the number of entries, time spent on the closed arms and on the open arms as well as the percentage of time spent and entries in the open arms were measured later.

Open field test: This test based on the work of Whimbey and Denenberg (1967) and later modified by Walsh and Cummins (1976), can measure the anxiety as well as the animal's motor behavior. In our study, the following parameters were evaluated: the frequency of horizontal exploratory behaviors, which is the number of quadrants traveled in the central, peripheral and total area, vertical exploration by the number of erections performed by the animal (Rearing), number of fecal boli and number of times the animal was self-cleaned (Grooming), length of stay in the central and peripheral areas and percentage of time spent in the peripheral quadrant.

Tail suspension test: TSC was proposed by Stéru et al. (1985) and is a validated test for evaluating the efficacy of antidepressant drugs and places the mice in a moderate but unavoidable stress situation. It consists of the animal suspension by the tail with the use of adhesive tape, in an apparatus constructed according to the measures mentioned in the study by Can et al. (2012). The tests were recorded for later measurement of the latency time among the escape movements until the first moment in which the animal surrenders remaining immobile and its total time of immobility and mobility when hanging.

Olfactory discrimination test: This test measures the animal's ability to find buried food using olfactory cues and follows the protocol used in the study by Cunha et al. (2012). The time spent to find the food and start eating it was measured. Each test was recorded for later counting of the time with the use of a stopwatch. A maximum time of 15 min was used for cutting, and mice reaching the cut-off point were included in the analysis as if they had found the food after 15 min.

Statistical analysis: The analyzed variables were subjected to the Shapiro-Wilk statistical test, considering p> 00.5. After the test, it was observed that the samples were classified as non-normal (non-parametric) using the Kruskal-Wallis test, followed by the Dunn post-test, for data comparison among the experimental groups. Statistical analysis was performed using the SigmaPlot program, version 12.5, considering a level of significance of 5% (Norman and Streiner, 1994; Shott, 1990).

RESULTS

None of the variables analyzed in the OD test and TST showed a statistically significant difference. In the EPM test there was a significant difference only in the variables: time of stay in the center and in the closed arms (CA), as shown in table 1. In the OFT there was a significant difference in the variables described in Tables 2 and 3.

Table 1 - Results regarding the two variables analyzed in the EPM test: length of stay in the central part and closed arms (CA) in each of the experimental groups

ELEVATED PLUS MAZE		
Groups	Center	CA
SAL – SAL	71.46±9.85ab	181.06±12.59ab
SAL – Caf 10 mg	83.09±8.11ab	126.18±13.21b
SAL – Caf 20 mg	72.6±7.09ab	202.99±12.00a
MPTP – SAL	79.57±6.93ab	156.36±24.51ab
MPTP - Caf 10 mg	101.51±2.64[a]	168.6±9.76ab
MPTP - Caf 20 mg	69.24±16.32b	191.14±25.24ab
P	0.027	0.034

Results are presented as mean ± standard error. Variable time expressed in seconds. Different lowercase letters in the columns indicate significant difference among the experimental groups (Dunn post-test, p <0.05).

Table 2 - Results concerning the three variables analyzed in the OFT, number of fecal boli (FB), number of grooming and rearing, in each of the experimental groups

OPEN FIELD			
GROUPS	**FB**	**GROOMING**	**REARING**
SAL – SAL	4.14±0.83ab	3.14±0.55a	32.71±6.23ab
SAL – Caf 10mg	4.00±0.60a	1.63±0.18ab	53.38±6.26a
SAL – Caf 20mg	3.88±0.77ab	3.75±0.86a	27.75±6.34ab
MPTP – SAL	2.86±0.4ab	1.00±0.22b	38.14±8.59ab
MPTP - Caf 10mg	2.14±0.51ab	2.00±0.44ab	26.29±7.34ab
MPTP - Caf 20mg	1.00±0.49b	1.71±0.89ab	15.00±4.05b
P	0.010	0.002	0.011

Results are presented as mean ± standard error. Variable time expressed in seconds. Different lowercase letters in the columns indicate significant difference among the experimental groups (Dunn post-test, $p < 0.05$).

Table 3 - Results regarding four variables analyzed in the OFT: number of central quadrants traveled (CQ), number of peripheral quadrants traveled (PQ), total number of quadrants traveled and percentage of peripheral quadrants traveled (%PQ) compared to the total, in each of the experimental groups

OPEN FIELD TEST				
Grupos	**CQ**	**PQ**	**Total**	**% PQ**
SAL - SAL	22.71±2.85ab	83.57±9.26ab	106.29±11.61ab	77.74±1.91a
SAL – Caf 10mg	36.63±3.05a	92.00±4.56a	131.13±7.02a	70.37±1.77a
SAL – Caf 20mg	23.00±5.87ab	64.00±6.16b	87.00±11.00ab	76.70±3.93a
MPTP - SAL	35.57±2.65a	81.29±7.26ab	116.86±7.89ab	68.96±2.77a
MPTP - Caf 10mg	27.14±4.49ab	79.14±9.72ab	06.29±11.36ab	73.91±3.86a
MPTP - Caf 20mg	15.71±3.98b	66.29±8.01ab	82.00±9.83b	80.76±3.83a
p	0.005	0.037	0.010	0.036

Results are presented as mean ± standard error. Variable time expressed in seconds. Different lowercase letters in the columns indicate significant difference among the experimental groups (Dunn post-test, $p < 0.05$).

DISCUSSION

According to our results on the EPM test, which refers to anxiety, there was no significant difference between the control group and the MPTP group, only intra group difference, as in the variable time of permanence in the center of the apparatus, with significant difference between MPTP + Caf 10 mg/ kg and the MPTP + Caf 20 mg / kg groups, and time of stay in the closed arms in which the Sal + Caf 20 mg / kg group remained longer time than the Sal + Caf group 10 mg / Kg. Based on these results, it cannot be affirmed that the animals treated with MPTP showed anxiety and if there was improvement or not with the administration of caffeine. Vučković et al. (2008) used C57BL/6 mice in their study, making 4 injections of MPTP intraperitoneally on the same day, every two hours, to evaluate changes in behavior and memory due to damage in the basal ganglia, and it was observed that there was 68% of loss of TH + neurons in the substantia nigra pars compacta (SNc) with the development of associative memory and fear deficits, however, there were no signs of anxiety or depression. However, the studies performed by Wang et al. (2009), Sy et al. (2010) and Ho et al. (2011), using Wistar rats, with MPTP infusion (1 μmol in 2 μmol saline solution), in the SNc, by estereotaxy, demonstrated increased anxiety in rats, evaluated as a shorter stay in the open arms in compared to the controls in the EPM test. Regarding the variables analyzed in the OFT, there was a difference between the Sal + Caf 10 mg and MPTP + CAf 20 mg groups, in the variable Grooming, between Sal + Sal, Sal + Caf 20 mg and MPTP + Sal groups, but both results do not allow us to affirm that the treated group or the control group presented higher level of anxiety than the other, because of the difference that occurs among the random groups. Just as in the variables that relate to the motor part, only the item Rearing showed a significant difference among the control group and the group treated with MPTP, the number of quadrants traveled, both central and peripheral, as well as the total number, despite showing difference among the groups, it is not possible state that MPTP-treated animals presented higher motor impairment than the control group, also because of the difference that occurs among unrelated groups. Similar results were found in the study by Ferro et al. (2005), in which Wistar rats were used, with bilateral intracerebral MPTP infusion, in which the animals showed few motor alterations due to the reduced number of dopaminergic cell loss detected in their assay, compared to 6-OHDA treated rats. These results differ from two studies carried out by the same group of researchers (Patil *et al.,* 2014a,b): the first, in which Swiss albino mice were injected with MPTP injections at a dose of 25 mg / kg + probenecid for 5 consecutive days, once a day, it is observed a significant decrease in locomotor activity, rearing and grooming, increased immobility time in the OFT, and only 16% of tyrosine hydroxylase (TH +) containing cells were found in the substantia nigra compared to the control group; in the second study following the same animal model there were similar results regarding the motor part with the smallest number of TH + cells, only 7%. In the TSC, in the three analyzed variables (latency, mobility time and immobility), no significant differences were found among the groups, demonstrating that the animals did not exhibit depressive behavior with the animal model used here.

Our findings are in line with research conducted by Santiago *et al.* (2010) who compared the depressive behavior in different animal models of PD and also did not verify the presence of depressive behavior in Wistar rats, in which bilateral intracerebral MPTP was infused. Gorton et al. (2010) that even using different methodology of our study, using C57BL / 6 mice with 4 injections of intraperitoneal MPTP on the same day, every two hours of interval, also did not verify any depressive behavior with the use of TSC. In contrast, the results of Mori et al. (2005) who used the same protocol to perform their study on the neural mechanisms underlying motor dysfunction detected by the TST, demonstrated a longer time of immobility in the test than the control group which may be due to depressive behavior. Olfactory disorders are one of the first non-motor symptoms observed in PD, hyposmia can be multifaceted and not restricted to one modality (Taylor *et al.,* 2010), and however in the study herein there was no significant difference among the evaluated groups. The patients with PD have demonstrated deficiencies in odor detection, differentiation and identification; on the other hand, the olfactory disorder is one of the most difficult NMS to replicate with MPTP use in rodents (Mcdowell and Cheselet, 2012) and in humans. On the other hand Schintu et al. (2009) who induced PD with chronic MPTP use in conjunction with probenecid in mice observed that the animals presented characteristics typical of PD, including impairment of motor and olfactory functions associated with partial loss of TH + neurons SNc. In addition to dopaminergic loss, Dluzen (1992) proposes that olfactory deficit in PD may be associated with the reduction of noraepinefrin in the olfactory bulb, according to the results of her study. In trials that used intranasal MPTP administration (Prediger, 2006; Prediger, 2010) there was impairment of the olfactory function with decrease of TH+

levels in the olfactory bulb, striatum and substantia nigra by apoptotic mechanisms, reducing the concentration of dopamine in different brain structures such as olfactory bulb, striatum and prefrontal cortex but not in the hippocampus.

MPTP is widely used in mice due to the low cost and greater ease in handling the animal, as well as in primates to test drug treatment protocols prior to the study in humans, since they resemble more closely human DP and researches have already been performed with the use of MPTP for both motor symptoms and NMS (Blesa *et al.,* 2012; Le *et al.,* 2014). For unknown reasons mice are resistant to MPTP applied systemically and mouse strains vary widely in their sensitivity to the toxin (Bové et al. 2005). Many factors are known to influence the reproducibility of the lesion, including lineage of the mice (and even supplier), age, sex and weight (Le *et al.,* 2014). In the research herein, a subacute administration protocol was used according to a study published by Duty and Jenner (2011) and still the death rate due to MPTP exposure was high (53%) for no apparent reason. High doses of the acutely applied toxin can be lethal because they result in neuro or cardio peripheral toxicity. The mortality risk is dose-dependent and usually occurs within 24 hours after the first MPTP dose, and the high mortality rates (up to 50%) seen after acute bolus administration can be reduced to acceptable levels (<20%), with reductions in the administered dose and can be almost completely avoided with the use of alternative protocols in which the same dose or even higher dose is given in fractional doses for a longer period of time (Duty and Jenner, 2011).

Conclusion

The results of the study herein were inconclusive on the animal model's ability used here to reproduce non-motor symptoms such as anxiety, depression and olfactory PD disorder. It was not possible to ascertain the therapeutic potential of caffeine on the non-motor symptoms of PD and it is suggested that future studies be carried out to further elucidate the MPN in the MPTP animal model of PD in mice and the caffeine action on them.

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