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Chronic caffeine use does not influence behavior and brain oxidative status in mice

O uso crônico de cafeína não influencia o comportamento e o status oxidativo cerebral em camundongos

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Abstract

Widely consumed in foods and as an energy supplement, caffeine has been studied given its pharmacological effects, especially on Central Nervous System (CNS). In this sense, the present study investigated whether and to what extent caffeine chronic use can influence the brain oxidative status and the behavioral activity of the C57BL/6 female mice. For this, fifteen animals were randomized into the following groups: Control (0.9% saline solution), Caf10 (10 mg/kg caffeine), and Caf50 (50 mg/kg caffeine). The animals received one daily caffeine dose by i.p route for 120 days. Twenty-four hours after the last administration, the animals were subjected to behavioral tests and euthanized. The blood was used by biochemical analysis and the brain to evaluate the oxidative status and micromineral levels. The caffeine did not influence the anthropometric parameters, lipid profile, and C-reactive protein levels. Further, superoxide dismutase (SOD) and glutathione-s-transferase (GST) activities maintained the same response profile. On the other hand, catalase (CAT) activity was decreased in both groups receiving caffeine compared to the control group. Despite this, malondialdehyde and carbonyl protein levels did not change among the groups, as well as the distribution micromineral levels. In the same way, no caffeine dose altered the findings of anxiety-like behaviors in the animals. Considering the time of caffeine administration, we believe that there was a cellular adaptation triggered by its use, tending to a protective effect on the brain.

Keywords: Caffeine. Reactive oxygen species. Animal Behavior.

Resumo

Amplamente consumida nos alimentos e como suplemento alimentar, a cafeína tem sido estudada devido aos seus efeitos farmacológicos, principalmente no sistema nervoso central (SNC). O presente estudo investigou se o uso crônico da cafeína pode influenciar o estado oxidativo do cérebro e a atividade comportamental de camundongos fêmeas C57BL/6. Para isso, quinze animais foram randomizados nos seguintes grupos: Controle (solução salina à 0,9%), Caf10

(10 mg / kg de cafeína) e Caf50 (50 mg / kg de cafeína). Os animais receberam uma dose diária de cafeína por via i.p. durante 120 dias. Vinte e quatro horas após a última administração, os animais foram submetidos a testes comportamentais e foram eutanasiados. O sangue foi utilizado para análises bioquímicas. No cérebro, foi avaliado o estado oxidativo e os níveis de microminerais. A cafeína não influenciou os parâmetros antropométricos, perfil lipídico e níveis de proteína C-reativa. Ademais, as atividades de superóxido dismutase (SOD) e glutationa-S-transferase (GST) mantiveram o mesmo perfil de resposta. Em contrapartida, a atividade da catalase (CAT) diminuiu em ambos os grupos que receberam cafeína. Já os níveis de malondialdeído e proteína carbonilada não se alteraram entre os grupos, assim como a distribuição dos microminerais. Nenhuma dose de cafeína desencadeou comportamento do tipo ansioso nos animais. Portanto, considerando o tempo de administração da cafeína, acreditamos que houve a adaptação celular desencadeada pelo seu uso, tendendo a um efeito protetor no cérebro. Além disso, o espaço amostral reduzido foi uma limitação para entendimentos mais acurados sobre os efeitos da cafeína no SNC.

Palavras-chave: *Cafeína. Espécies reativas de oxigênio. Comportamento Animal.*

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is present in the composition of a wide range of foods (e.g., coffee, tea, soft drinks, chocolate), energy drinks, and, commonly, it is used as an ergogenic feature for athletes (1). The effects of caffeine in the brain have been historically described as a psychostimulant, elucidated in behavioral investigations in both humans and rodents[1–4]. This molecule is a type of methylxanthine alkaloid and makes up the purine group, standing out for its antioxidant and stimulating potential of the central nervous system (CNS)[5,6]. Studies of the pharmacology of caffeine have pointed out that after its consumption and absorption by the organism, this natural molecule is quickly transported to the brain (half-life to 2-12 hours), being capable to influence the response of neurons (e.g., calcium release, ryanodine receptors agonist, trigger mitochondrial reactions), besides influencing the ionotropic receptor's activity (e.g., inhibition of 5'-nucleotidases and dephosphorylate the GABAA receptor)[7–11].

Structurally, caffeine is similar to adenosine, an endogenous neuromodulator purine nucleoside released on the CNS, and that acts as a hormone over different receptors[2,12]. Studies have shown that the effects of this molecule on the CNS can reflect its action as a nonselective antagonist of these adenosine receptors (A1 and A2), with consequent blockade of A2A receptor densely located in the striatum nucleus, showing a positive relationship with the neuroprotector[13,14]. These

receptors A2A, when active, have been potentially associated with the control of the N-methyl-D-aspartate (NMDA) pathway that results in the production of caspase-3 and reactive oxygen species (ROS) by mitochondrial pathway predisposing this way the tissues to suffer oxidative damage[9,10]. Additionally, the systemic effects of caffeine on A1 receptors could trigger lipolytic response profiles, which are usually related to modulation in serum lipid levels, such as cholesterol[15–17].

Due mainly to the influence of caffeine in the NMDA pathway, potential antioxidant effects of this compound have been reported in the nervous system[10,18]. This way, the imbalance between the reactive oxygen metabolites production and the downregulation of the endogenous antioxidant defense system has been closely associated with the oxidation and consequent functionality of lipids, proteins, and nucleic acids. Aligned with that fact, these mechanisms of oxidative stress besides predispose the loss of important cognitive and brain functions[8,19], maintaining a close relationship with reactivity to stress and anxiety disorders[20–23]. In this perspective, Rammal et al.[24] showed that anxious mice exhibited higher ROS accumulation in the neuronal and glial cells of three regions of the CNS (cortex, cerebellum, and hippocampus) when compared to the group of animals not linked to anxiety-related behaviors. Thus, the use of substances with antioxidant potential, as caffeine has been used as a pharmacotherapeutic co-adjuvant and neuroprotector, in the context of the neuronal and pathological changes in the CNS, including anxiety disorders and neurodegenerative conditions[18,25,26].

An efficient antioxidant mechanism is preponderant for minimizing the brain vulnerability of the brain against ROS attack. These facts have been commonly associated with the brain's high metabolic rates, its large concentration of polyunsaturated fatty acids (i.e., a target of free radical) with primordial regulatory functions, and lower levels of endogenous antioxidant enzymes of the brain tissue, compared to peripheral organs. Indeed, the brain cells undergo targets constant attacks by free radicals thus requiring the most effective defense mechanism[27–29]. In line with this fact, it is substantiated that the bioavailability of chemical elements as zinc (Zn), selenium (Se), and iron (Fe), besides participating as mediators in different cell signaling processes, when acting as enzymatic co-factors has the potential of the modulating the activity of antioxidant enzymes, thereby improving the system of defense brain against ROS attack[10,28,29]. Hereupon, it is notable that studies with rodents have shown that oxidative stress in the brain can reduce the bioavailability of these minerals[30,31].

In this context, there have been frequent approaches involving the use of exogenous antioxidant substances, dietary and pharmacological ones, as coadjutants in the improvement of the oxidative function of the different tissues and organs[32,33]. Due to the high frequency of caffeine consumption, from stimulant for athletes to coffee intake by the world population, in addition to its notable antioxidant profile associated with neuroprotective properties, the present study was carried out to evaluate if and to what extent its chronic use, in two different doses, has the potential to influence the brain cells oxidative status and the behavioral activity in C57BL/6 mice.

MATERIAL AND METHODS

Animals, treatments, and biometrics

Female C57BL/6 mice (8-week-old; n = 15) were kept in controlled environmental conditions (temperature 22 ± 2 °C, air humidity 60-70%, and 12/12 h daily light/darkness cycles). The animals were randomized into 3 groups with 5 animals per group as follows Control group (0,9% saline solution), Caf10 (10 mg/kg caffeine, and Caf50: 50 mg/kg caffeine. Food and water were provided *ad libitum*. The caffeine (Sigma, St Louis, MO, USA) was dissolved in 0.9% NaCl (*sodium chloride*) and provided daily by intraperitoneal (i.p.) dose for 120 days, a chronic exposition in rodents[10,34]. The experimental period and the caffeine doses were selected based on Hughes and Hancock[35]. Additionally, the doses administered were daily determined by animal weight measures. Twenty-four hours after the last caffeine dose administration, the behavioral activity of the animals was tested and they were anesthetized with tribromoethanol (250 mg/kg, i.p.) and euthanized by cardiac puncture for exsanguination. The blood was collected for biochemical analysis, while the whole brain was rapidly removed (< 1minute) and submerged on cold (4°C) phosphate buffer saline (PBS; 50mM, pH 7.0), followed by the cortex dissection and storage (-80°C). These frozen samples were subsequently used for enzymatic analysis to assess the oxidative brain profile and for the detection of microminerals levels in this tissue. The use of animals in this experiment was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Jequitinhonha and Mucuri Valleys (Protocol: 047/2016).

Food efficiency was evaluated during the experiment by obtaining parameters such as dietary intake (g/g mass/day), energy intake (kcal/g mass/day), and body mass (g). Dietary intake and body mass were recorded weekly. Energy intake was estimated based on the dietary intake using the reference values provided by each

chemical component of the diet (i.e., 4.0 kcal/g carbohydrate, 4 kcal/g protein, and 9 kcal/g fat). The Tail-Muzzle length of the mice was measured with an inelastic measuring tape (e = 0.1 cm). The Lee index (Lee) = $\left[\frac{3\sqrt{BW}}{NAL}\right]$ and Body Mass Index (BMI) = $\frac{BW}{NAL^2}$, were determined for obesity development assessment, where BW refers to the final body weight, and the naso-anal length[36].

Biochemical analysis

The biochemical analysis was accomplished by quantifying plasma levels of total HDL cholesterol, LDL cholesterol, and C-reactive protein. These biochemical parameters were analyzed on a spectrophotometer. Ultimately, the C-reactive protein was determined by using immunoturbidimetric assay, with the manufacturer's instructions (Bioclin, Belo Horizonte, Minas Gerais, Brazil).

Enzymatic antioxidant activity

The activity of antioxidant enzymes was investigated by using brain samples homogenized in ice-cold phosphate buffer (pH = 7.0) and centrifuged at 6200 rpm (5 °C, 15 min). The supernatant was used for the analysis of catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD)[37,38]. CAT activity was evaluated according to the method described by Aebi[39] by measuring the kinetics of hydrogen peroxide (H2O2) decomposition. SOD activity was estimated by the pyrogallol method based on the ability of this enzyme to catalyze the reaction of superoxide (O-2) and hydrogen peroxide (H2O2). GST activity was estimated by a spectrophotometer at 340 nm as described by Habig and Pabst[40] and calculated from the rate of nicotinamide adenine dinucleotide phosphate oxidation. Total protein levels in the supernatant were measured using the Bradford method[41].

Lipid and protein oxidation

Lipid peroxidation was evaluated from the quantification of thiobarbituric acid reactive substances (TBARS) according to a standardized biochemical colorimetric method. Briefly, the samples of the brain were homogenized in sodium phosphate buffer and centrifuged at 12.000 rpm for 10 min. The homogenate was collected and incubated with a thiobarbituric acid solution (0.375% thiobarbituric acid, 0.25N HCl, and 15% trichloroacetic acid) for 15min. TBARS levels were quantified spectrophotometrically at 535 nm[38,42]. Protein oxidation was estimated as the cerebral content of protein carbonyl (PCN)[43]. After homogenate removal, PCN content was measured in tissue pellets by the addition of 0.5 mL of 10 mM

dinitrophenylhydrazine (DNPH). The reaction is based on the derivatization of the carbonyl group with 2,4-DNPH, which leads to the complementary formation of a stable product (2,4-dinitrophenyl hydrazine). PCN content was measured spectrophotometrically at 370 nm[42].

Mineral microanalysis

Brain content of sodium (Na), calcium (Ca), potassium (K), copper (Cu), magnesium (Mg), iron (Fe), zinc (Zn), and selenium (Se) were quantified by mass spectrometry[44] - ICP-MS (Elan DRC II, Perkin Elmer, USA) to induce the high-temperature plasma (1000K), discriminating the ions by the intensity of released energy. For this process, 0.35 g brain samples were dehydrated in a drying oven at 80 °C for 5 days until reaching constant mass. Samples were manually macerated and digested for 24h in glass tubes containing 3 mL of sulfuric and nitric acid (1:1 v/v) at 30%. They were then heated in a microwave at 200 ºC for 20 minutes at 1000W. The samples were centrifuged at 2000 ×g, and 1 ml of the supernatant was dissolved in 20 ml of 1.68 mol L-1 HNO3 solution. Mineral standards for Na, Ca, K, Cu, Mg, Fe, Zn, and Se were purchased from Sigma-Aldrich (TraceCERT – ICP-MS, Sigma-Aldrich Inc., St. Luis, Missouri, USA)[38].

Animal behavior tests

Elevated plus-maze (EPM) test

The EPM consisted of an apparatus made of wood composed of two closed arms (30x5x30 cm) and two open arms (30x5 cm) arranged such that the two open arms were opposite to each other, besides a central area (5x5 cm). Each open arm has on its side acrylic supports (1 cm), to avoid possible animal falls. The maze was elevated to a height of 40 cm on the floor. The image monitoring and record for subsequent analysis were performed using a Sony® camera, Handycam model, positioned set up above the EPM apparatus. The procedure was made according to described by Pellow et al. (1985). The animals were conducted in the behavior room of the Laboratório de Nutrição Experimental (LabNutrex) 30 minutes before the test for acclimatization. For test realization, each animal was placed in the central area of EPM with the head facing one of the closed arms, remaining isolated in the behavior room and its movements in the apparatus were filmed for 300 seconds. The variables evaluated were: the number and the ratio of entries in each arm (i.e., closed or open), characterized when the animal entered with four legs, the permanence time in each

of the arms, the ratio of entries, and the time spent in each arm according to following equations.

Open field test

The open field apparatus consisted of a white wood box measuring 30 cm wide by 30 cm deep and 30 high. The box bottom was divided into 9 squares of 10 x 10 cm area each one, painted with black paint. This marking allowed the free movement of the animals by the total area of the box. The image monitoring and record for subsequent analysis were performed using a Sony® camera (Handycam model), positioned above the open field apparatus. The procedure was performed according to described by Teixeixa et al.[46]. The animals were placed in the box center, and their behavior was filmed for 600 seconds. The variables evaluated were: the number of the square that the animals crossed completely (i.e., with the four legs); the lifting of body and maintaining of weight over the hind legs or Rearing; the number and permanence of the entrance in the square center and self-cleaning or Grooming.

The hole-board test

The hole-board apparatus consisted of a white wood plate (40 cm long by 40 cm wide and 5 cm deep). The plate has 16 holes equidistant with 3 cm in diameter and is coupled in a glass box with 40 cm long by 40 cm wide and 30 cm high. Monitoring and recording of images for later analysis were performed by the same digital structure of the last behavior test. The camera was positioned one meter in front of the perforated plate apparatus. The procedure was performed as described by Kuru et al.[47]. The animals were placed in the center of the plate and their behavior was filmed for 300 seconds. The variable evaluated was the number of diving (i.e., times the animal dipped its head in the holes of the plate). It was considered valid when the head lowered at least enough for the eyes to be immersed by the hole.

Statistical analysis

The normality distribution of data was verified by the Shapiro-Wilk test. Data with parametric distribution were analyzed using a one-way analysis of variance (one-way ANOVA), followed by the Student-Newman-Keuls posthoc test. The non-parametric distributed data were transformed by logarithmic transformation before the analysis to normalize the distribution. Proportion data were transformed by angular transformation before the analysis given their nature as percentages. Data were expressed as mean ± standard deviation (mean ± S.D.). All results with $P \leq 0.05$

were considered statistically significant. Statistical analyzes were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Caffeine, in both concentrations, contributed to maintaining the anthropometric parameters associated with the final body mass and energy intake (kcal/day). Moreover, this response profile did not influence the Lee index, the body mass index (BMI), and the tail-muzzle length in the experimental animals ($p > 0.05$). Similarly, the relation between the biomolecules of lipidic profile HDL and LDL and the inflammatory biomarker CPR (C reactive protein) maintained their baseline values compared to controls animals ($p > 0.05$; Table 1). In the same way, the relation between the biomolecules of lipidic profile HDL and LDL and the inflammatory biomarker CPR (C reactive protein) further maintained their baseline values compared to controls animals ($p > 0.05$; Table 1). Finally, caffeine did not influence the animals' weight gain compared to the control group ($p > 0.05$; Figure 1).

Table 1 - Body mass, diet energy intake, biochemical analyzes, anthropometric measurements, and Lee index of experimental animals.

	Control	Caf10	Caf50
Final body mass (g)	21.32 ± 0.62a	21.65 ± 1.0a	22.63 ± 0.83[a]
Energy intake (kcal/day)	71.29 ± 16.60a	75.67 ± 25.32a	67.59 ± 11.40a
Lee Index (g/cm^3)	167.50 ± 2.55a	166.60 ± 7.52a	170.70 ± 2.94a
BMI (g/cm^2)	1.28 ± 0.03a	1.29 ± 0.05a	1.36 ± 0.03a
Tail-muzzle (cm)	16.56 ± 0.32a	16.76 ± 0.90a	16.58 ± 0.48a
C-reactive protein (mg/L)	0.53 ± 0.09a	0.50 ± 0.05a	0.510 ± 0.03a
HDL-cholesterol (mg/dL)	53.41 ± 4.44a	61.75 ± 14.0a	68.25 ± 4.14a
LDL- cholesterol (mg/dL)	47.98 ± 9.68a	46.00 ± 10.26a	53.16 ± 4.76a

HDL (High-Density Lipoprotein), LDL (Low-Density Lipoprotein), BMI (Body Mass Index). Control group, Caf10(10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the rows indicate the statistical difference between groups ($P < 0.05$).

Both doses of caffeine contributed to a significant decrease of catalase (CAT) levels in the brain tissue when compared to the control group ($p < 0.05$; Fig. 2B). On the other hand, the caffeine doses administered maintained the same baseline values of the cerebral superoxide dismutase (SOD) and glutathione-s-transferase (GST) of the control animals ($p > 0.05$; Figs 2A and C). Although we have observed a differential profile of response to the CAT enzyme, this was not sufficient to cause an imbalance between increased reactive oxygen metabolites and the enzymatic antioxidant defense system, once a time that no disorders associated with lipidic or protein oxidation (Fig. 3A and B) observed ($p > 0.05$).

Figure 1 - Body mass over the 17 weeks (mean ± SE) from controls and caffeine doses. Control: animals received saline solution; Caf10 (caffeine 10 mg/kg); Caf50 (caffeine 50 mg/kg). Statistical differences between groups (P < 0.05) were not observed on this parameter.

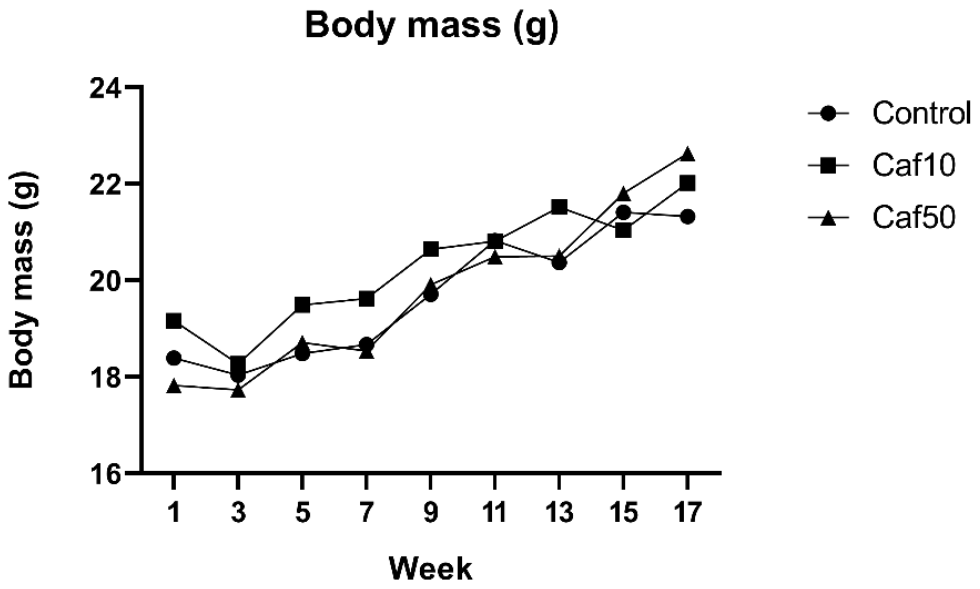


Figure 2 - Superoxide dismutase (SOD), glutathione-s-transferase (GST), and catalase (CAT) activities in the brain from controls and caffeine doses. Control: animals received saline solution; Caf10 (caffeine 10 mg/kg); Caf50 (caffeine 50 mg/kg). Different letters in the columns indicate the statistical difference between groups (P < 0.05).

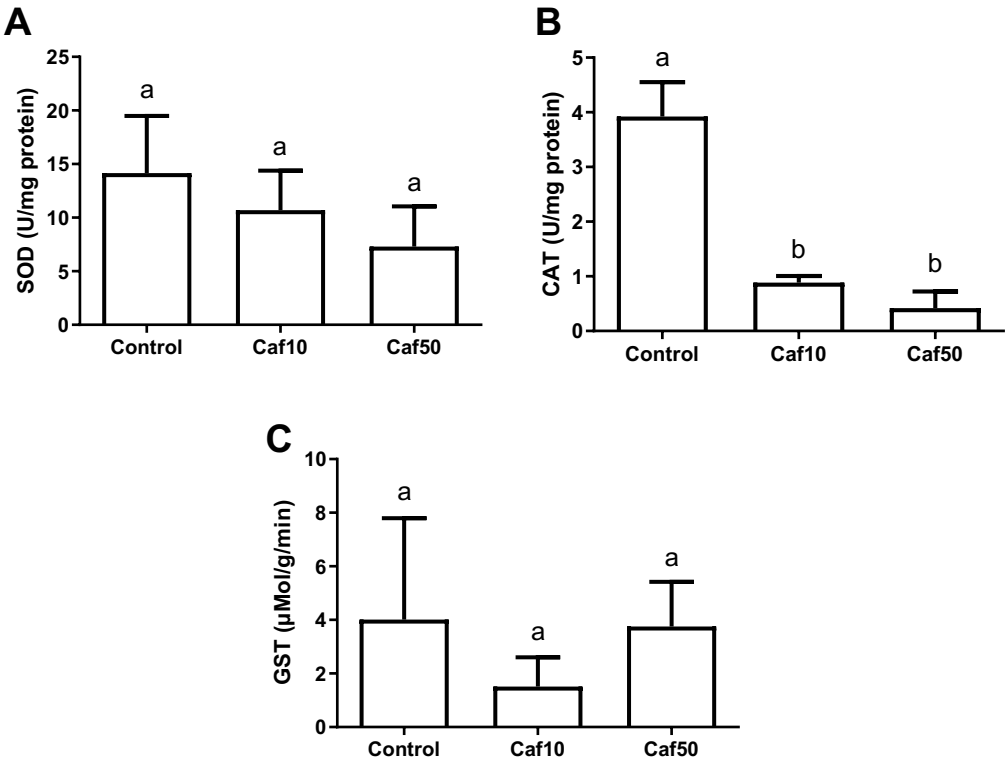
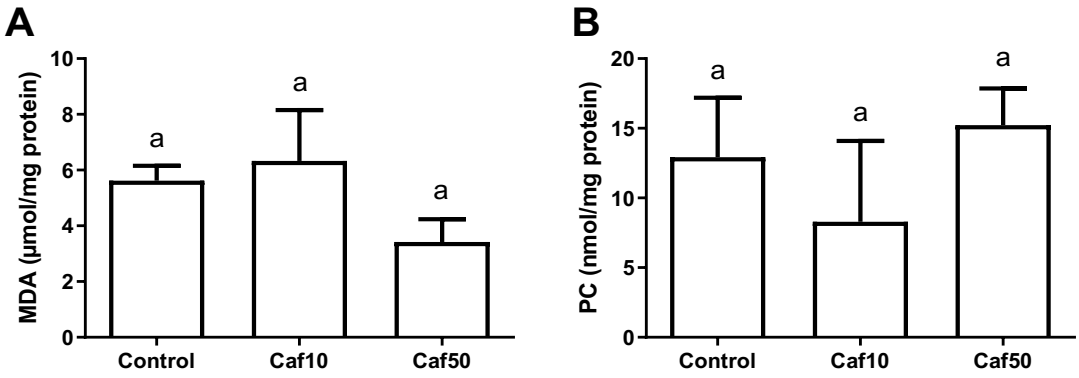


Figure 3 - Malondialdehyde (MDA) and carbonyl protein (PCN) levels in the brain from controls and caffeine doses. Control group: animals received saline solution, Caf10(10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the columns indicate the statistical difference between groups (P < 0.05).



In association with this response profile, the bioavailability of brain chemical elements, such as sodium (Na), potassium (K), phosphor (P), calcium (Ca), copper (Cu), magnesium (Mg), iron (Fe), zinc (Zn) and selenium (Se), did not present modifications after caffeine intake in both caffeine doses (10 and 50 mg/kg) by 120 days ($p > 0.05$; Table 2).

Table 2 - Mineral content in the brain of mice treated with different caffeine doses.

	Control	Caf10	Caf50
Na (mg/g)	3.3 ± 0.2a	3.5 ± 0.3a	3.6 ±0.2a
K (mg/g)	10.2 ± 1.5a	10.9 ± 1.7a	11.0 ± 1.2a
P (mg/g)	11.9 ± 1.4a	10.6 ± 1.1a	12.0 ± 1.3a
Ca (µg/g)	120 ± 7.1a	125 ± 8.8a	130 ± 10.6a
Cu (µg/g)	199 ± 10.4a	182 ± 9.0a	202 ± 11.7a
Mg (µg/g)	623 ± 11.7a	615 ± 13.9a	631 ± 15.2a
Fe (µg/g)	277 ± 14.0a	269 ± 11.3a	280 ± 11.5a
Zn (µg/g)	135 ± 7.5a	141 ± 6.3a	139 ± 7.0a
Se (µg/g)	0.43 ± 0.05a	0.40 ± 0.08a	0.46 ± 0.05a

Na(mg/kg), K (mg/g), P (mg/g), Ca (µg/g), Cu (µg/g), Mg (µg/g), Fe (µg/g), Zn (µg/g) and Se (µg/g) represent, respectively, sodium, potassium, calcium, copper, magnesium, iron, zinc and selenium concentrations. Control group, Caf10 (10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the rows indicate the statistical difference between groups (P < 0.05).

In the elevated plus-maze (EPM) test, the same entry frequency in the arm open or arm closed was observed between the animals independent of the caffeine doses administrated ($p > 0.05$; Fig 5A and 4B). The animals also remained by a similar

period in both open arms and closed arms compared to the control group ($p > 0.05$; Fig 5C and 4D).

Figure 4 - Quantitative analysis of the diving number of experimental animals in the Hole board test. Control group: animals received saline solution, Caf10(10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the columns indicate the statistical difference between groups (P < 0.05).

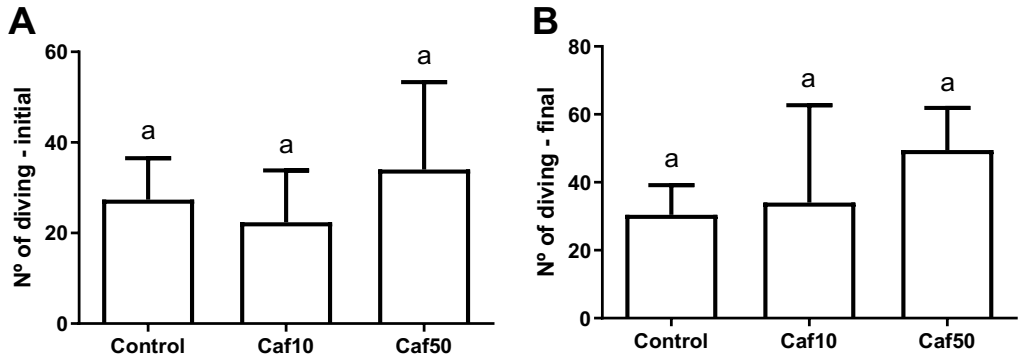
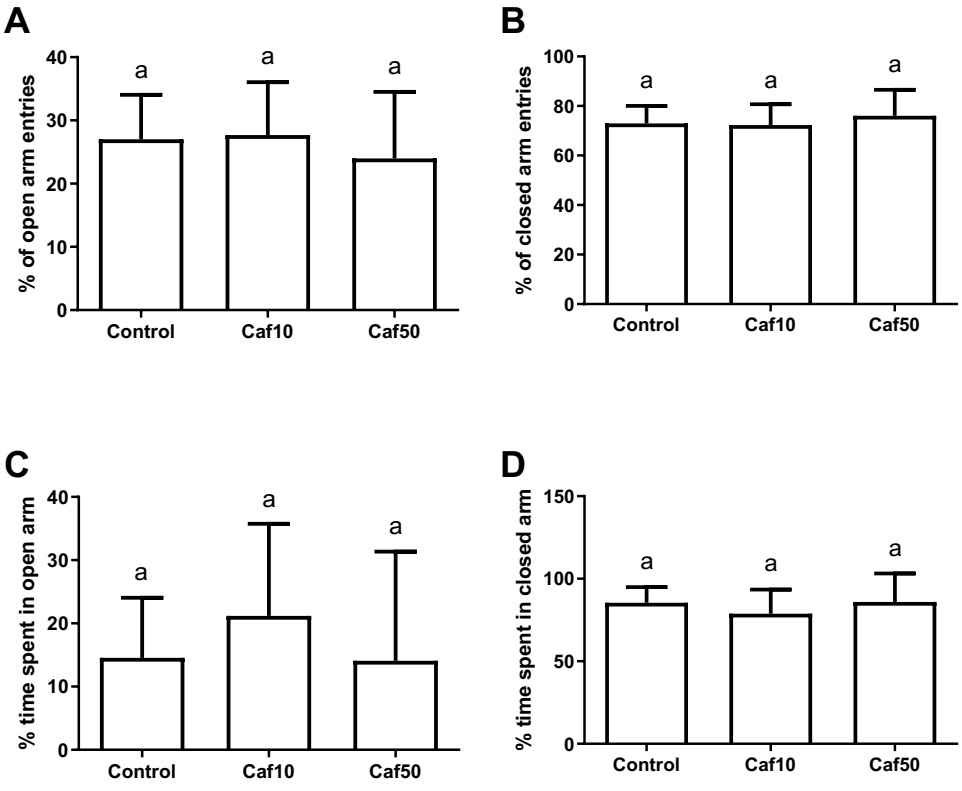


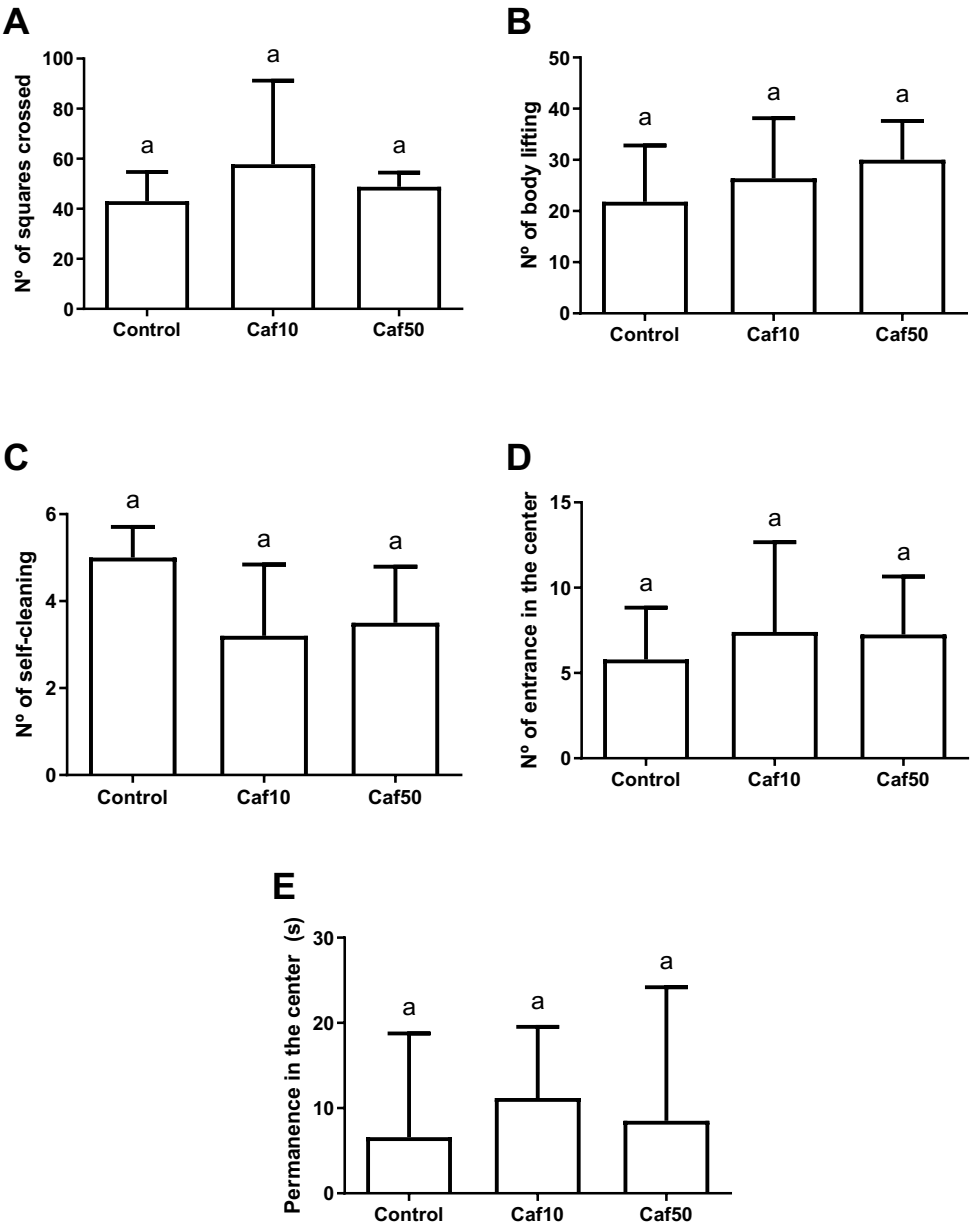
Figure 5 - Parameters in percentage obtained in elevated plus-maze (EPM) tests. Control group: animals received saline solution, Caf10(10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the columns indicate the statistical difference between groups (P < 0.05).



The quantitative parameters evaluated in the open-field test, such as body lifting, self-cleaning, squares crossed, entrances, and permanence in the square center were represented in Figure 6 (A-E). None caffeine dose was sufficient to cause the

significant modifications to these parameters in the experimental animals compared to the control group ($p > 0,05$). The same response profile of the other behavioral tests was observed to the Hole board test, which was presented in Figure 4 (A and B). The quantitative analysis of the diving number that experimental animals realized on the baseline was similar to the result after 120 days of consuming caffeine ($p > 0.05$).

Figure. 6 - The open field tests; Control group: animals received a saline solution, Caf10(10 mg/kg caffeine) and Caf50 (50 mg/kg caffeine). Different letters in the columns indicate the statistical difference between groups ($P < 0.05$).



DISCUSSION

The historical findings of antioxidant and neuroprotective activities of caffeine supported in literature its use in experimental conditions in the present investigation[4,35]. In this context, we evaluate the relation of caffeine chronic use (2 different doses daily by 120 days) over anthropometric parameters, serum biochemical analyzes, oxidative status, chemical elements bioavailability in the brain, besides behavioral tests in mice. The caffeine doses used in this murine study were designed considering the daily human consumption of this molecule. The lower dose (i.e., 10 mg/kg) was based on the global minimal caffeine consumption of about 2 to 7 cups (4 to 15 mg/kg/day respectively) and human supplementation (3-9 mg/kg), as an ergogenic feature. In contrast, the highest dose (50 mg/kg) determination was outlined from behavior findings in experimental models in the literature[10,18,34,35,48]. Ultimately, due to the experimental need to mitigate stressors for rodents during the course of the study, especially by previous studies on behavior or chronic use of caffeine[18,32,34,49,50], the decision to use the intraperitoneal route for the experimental protocol was determined.

The consumption of thermogenic aliments has been closely associated as an important ally in accelerating weight loss[51,52]. In humans, LeBlanc et al.[53] showed that the daily ingestion of a cup of coffee (4 mg/kg), is capable to increase the resting metabolic rate (RMR), suggesting the thermogenic effect of this substance. In experimental animals, Abreu et al.[10] when administering caffeine in 20 and 40 mg/kg doses by a period of 80 days for male rats didn't report any bodyweight changes nether in the energy intake by these animals. In this same perspective, Xu et al.[17] in their study with female rats ingesting 38,4 mg/kg of the caffeine by 13 weeks did not detect any interference associated with these same variables. Corroborating with these findings, in our investigation we also didn't find any changes associated with final body mass or energy intake (kcal/day) in the animals receiving caffeine at both doses. Equally the parameters of tail-muzzle and, especially, Lee Index and body mass index (BMI), both variables with sustained dependence of the weight variation[36], also maintained similar values compared to control animals, independently of the caffeine dose consumed.

Studies have shown that the direct consequence of the caffeine binding to the adenosine receptor (A1) has close relation with the increase of the Cyclic Adenosine Monophosphate (cAMP)[15], an important sign of the lipolysis activation pathway[16].

This caffeine lipolytic profile associated with increased levels of the lipid circulating in the blood (e.g., total cholesterol, low-density lipoprotein (LDL), and triglycerides) had already been reinforced in different experimental studies[54,55]. A recent study confirmed that the modulation of the expression of promoter regions of cholesterol synthesis genes was increased at different concentrations of caffeine (i.e., 30, 60, and 120 mg/kg). The epigenetic investigation was conducted from the intragastric administration of caffeine in pregnant female rats and observation of the offspring. The genetic influence of the molecule was associated with an increase in serum total cholesterol and LDL[17]. Although these studies indicate the interference of caffeine over lipid metabolism, in our experimental protocol, the 120 days of ingestion of this substance was not sufficient to support this affirmation. Contrary, its use contributed to keeping both the LDL and HDL cholesterol levels unchanged, independent of the dose consumed, maintaining them similar to the response profile presented by animals of the control group. We believe that caffeine chronic administration may have favored an adaptation mechanism associated with these variables

In this same perspective, caffeine use contributed to the maintenance of a homeostatic microenvironment associated with cerebral oxidative status in the experimental animals. According to Vieira et al.[5], the antioxidant activity of this substance is capable to reinforce the enzymatic response profile of SOD, GST, and CAT in overcoming the attack by free radicals (e.g., O2-, H2O2, OH-), maintaining the structural and functional integrity of tissues. Despite characteristically the caffeine keeps a positive relation influencing the tissue levels of antioxidant defense[32], in our investigation, this substance presented a differential response profile. Independent of consumed caffeine dose, the SOD and GST enzyme levels maintained similar values to those found in the control group. Nevertheless, metabolic modulation in the counterbalancing effect system to oxidative damage was also found among an enzyme evaluated in our study. The SOD activity is related to the conversion of superoxide radicals into hydrogen peroxide (H_2O_2)[56]. Hereupon, this enzyme is only effective when the peroxide is used as a substrate for the action of CAT, which converts it into water molecules, consolidating the antioxidant effect[57]. Besides, peroxide can interact with the superoxide anion and trigger the production of the highly reactive hydroxyl radical. In this sense, it would be possible that the reduction of catalase activity contributed to increasing the vulnerability of rodents' brains to oxidative stress. However, despite the enzyme

variation of CAT, it did not compromise the endogenous antioxidant defense system in the brain, respectively by tissue levels of the malondialdehyde and carbonyl protein. Finally, due to the counterbalance and attack system (i.e., MDA and PCN), these results suggest that caffeine did not predispose to oxidative stress.

This response profile of the antioxidant enzymes can be a relation to the maintenance of chemical element levels of microelements in brain tissue. According to Barbosa et al.[58] microelements like Zn, Cu, Fe, and Se, besides assisting in processes associated with the integrity of brain metabolism (i.e., effective mediators of innate immune activation[59], modulating the production of TNF-α, TIMP-1, and the blood capillary proliferation marker α-SMA)[60], many of these minerals also function as enzymatic co-factors. In this sense, Zn and Cu for example, are required by superoxide dismutase for dismutation of superoxide anion to oxygen and hydrogen peroxide, while Fe is essential for enzymatic CAT activity[61,62]. Also, Se is a cofactor for glutathione peroxidase involved in the reduction of hydroperoxides in the cell[63,64]. Despite the functionality of these antioxidant enzymatic groups and the relevance of the minerals' co-factors balance, the caffeine in the experimental conditions evaluated in our investigation was not capable of influence these variables, corroborating with the maintenance of the activities of these respective antioxidant defense enzymes evaluated.

It has been established that the balance between the endogenous antioxidant system and the attack by reactive oxygen and nitrogen metabolites are fundamentals for brain cells' integrity and, consequently, maintenance of the behavioral activities as the mobility and anxiety profile[10,35]. In this perspective, the review of Bishop et al.[65] showed a positive relationship between continuous oxidative damage in brain tissue (i.e., related to different ROS production pathways) and behavior activities functional decline. The modulation of the A2A receptor by caffeine has the potential to mitigate altered behavioral phenotypes in rats, as hyperlocomotion[66] and potentiation of anxiety-like behavior[67]. Despite this premise, Hughes et al.[35] when evaluating the behavior of animals who ingested 50 mg/kg/day caffeine by 20 days showed a hypomobility profile in the open field test compared to control, suggesting a differential response profile of this substance in cerebral activity. On the other hand, Abreu et al.[10] did not see any caffeine effect (20-40 mg/kg/day) added to the diet on locomotor and exploratory activities. Similar to this similar caffeine response profile, in our investigation we also none saw any change among variables analyzed to the open field test after chronic intake of this substance by animals.

Equally, though the caffeine use had been closely associated to decrease anxiety-like behaviors in rats[4,35], this substance was not capable of an induced change of these variables in the experimental animals in our study, evaluated by hole-board test. In this test, independent of caffeine dose, the frequency of diving in the apparatus was similar in all animals compared to the control group that received only water. In a study conducted by Pierard et al.[48], the application of an experimental protocol that administered 16 mg/kg of caffeine (i.p. route), evidenced an induction to explore the hole-board, indicating an improvement in anxiety-like behaviors parameters. On the other hand, Çakir et al.[4] evaluating the possibility of the protective effect of caffeine on anxiety in models of additional stress to rodents demonstrated that chronic (14 days) administration (3 mg/kg) of this molecule improved the level of anxiety before the exposure of additional stress.

The caffeine, in dose and time of administration in our investigation, was also not capable of influencing any parameters of the elevated plus-maze test, suggesting a disparate demeanor of its protective effect. Corroborating with this finding, Arendash et al.[68] did not see one cognitive change associated with anxiety levels in animals after a chronic (i.e., 6 weeks) daily intake of 1.5 mg of caffeine. On the other hand, Hughes et al.[35] evidenced a reduction of the time spent by animals in the open arm of the apparatus after intake 50 mg/kg/day of caffeine by 20 days suggesting an increase in anxiety signals.

Considering the exposure, although the literature is consistent in pointed the effectiveness of the intake of thermogenic drinks with considerable antioxidant effect as the caffeine, besides its psychostimulating potential, these studies, for the most part, are quite heterogeneous, especially related to the dose consumed and to time of caffeine administration. This fact has been reflected in differential response profiles consistent with positive, negative, and/or adaptive effects associated with using this substance. Lined up with these observations, in our investigation the caffeine at 10 and 50 mg/kg dose administered daily for 120 days showed no evidence of significant improvement on brain oxidative status. Despite this fact, we did not observe any significant oxidative damage. This response profile was also consistent with the behavioral analysis of the animals that maintained their baseline values, similar to the control animals. Considering the time of caffeine administration to the animals, we believe that there was a process of cellular adaptation triggered by its chronic use. However, to confirm or refute this inference believed to be a necessary realization of more detailed studies evaluating the consumption of caffeine variable

doses alternating of the time (e.g., acute, subacute, and chronic). Another important fact that can be associated with the limitation of this study refers to the sample number, despite the statistical adequacy of the analyzes performed. Despite this fact, in general, our findings reinforced the potential effects of caffeine over the oxidative status and brain functionality in mice.

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