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COMPARISON OF THE ANTIOXIDANT AND BEHAVIORAL EFFECTS OF CANNABIDIOL WITH SYNTHETIC DRUGS IN AN ANIMAL MODEL FOR VALPROIC ACID-INDUCED AUTISM

COMPARAÇÃO DOS EFEITOS ANTIOXIDANTES E COMPORTAMENTAIS DO CANABIDIOL E DE FÁRMACOS SINTÉTICOS EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPRÓICO

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ABSTRACT

Autism spectrum disorder (ASD) is a prevalent and complex neurodevelopmental condition characterized by challenges in social communication and interaction, sensory sensitivities, repetitive behaviors, and intellectual disability. This study aimed to compare the antioxidant effects of cannabidiol extract with synthetic drugs (Risperidone, Aripiprazole and Fluoxetine) in an animal model for autism induced by valproic acid and confirm its possible improvement in the behavioral symptoms arising from the disorder and its lesser side effects. Therefore, the animals received treatment with cannabidiol for 15 consecutive days orally (gavage) at a dose of 60 mg/kg, with an interval of 24 hours between one treatment and another. Other groups received, respectively, Risperidone (0.1mg/kg), Aripiprazole (1.5mg/kg) and Fluoxetine (5mg/kg) for 15 consecutive days orally (gavage), following an interval of 24 hours between treatments and others. Twelve hours after the last treatment, the animals were subjected to behavioral tests of social interaction and repetitive behavior and subsequently sacrificed by decapitation with removal of the nervous tissue for analysis of oxidative stress. The behavioral variables (crossed squares, locomotion time, and standing time) analyzed in the open field test did not present statistically significant differences, nor did the three-chamber test. Comparing the results to the control group, both the autistic and the Risperidone and fluoxetine groups showed increased formation of substances reactive to thiobarbituric acid levels.

Regarding superoxide dismutase (SOD) activity, the autistic group showed a decrease in enzyme activity compared to the control group, and none of the synthetic drugs and cannabidiol alone altered SOD activity. Fluoxetine and CBD demonstrated a reversal of the reduction caused by autistic behavior on enzyme activity. There were no behavioral changes when comparing

animals treated with CBD with those treated with synthetic drugs. Data suggests that CBD has better antioxidant action when compared to synthetic drugs.

Keywords: Autism Spectrum Disorder; Cannabidiol; Valproic Acid; Oxidative Stress; Animal Behaviors; Synthetic drugs.

RESUMO

O transtorno do espectro autista (TEA) é uma condição neurodesenvolvimental complexa e prevalente, caracterizada por dificuldades na comunicação e interação social, sensibilidades sensoriais, comportamentos repetitivos e deficiência intelectual. Este estudo teve como objetivo comparar os efeitos antioxidantes do extrato de canabidiol com drogas sintéticas (Risperidona, Aripiprazol e Fluoxetina) em um modelo animal de autismo induzido por ácido valpróico, bem como verificar sua possível melhora nos sintomas comportamentais decorrentes do transtorno e seus menores efeitos colaterais. Os animais receberam tratamento com canabidiol por via oral (gavagem) durante 15 dias consecutivos, na dose de 60 mg/kg, com intervalo de 24 horas entre as administrações. Outros grupos receberam, respectivamente, Risperidona (0,1 mg/kg), Aripiprazol (1,5 mg/kg) e Fluoxetina (5 mg/kg) também por via oral (gavagem) durante 15 dias consecutivos, com intervalo de 24 horas entre os tratamentos. Doze horas após o último tratamento, os animais foram submetidos a testes comportamentais de interação social e comportamento repetitivo e, em seguida, sacrificados por decapitação para remoção do tecido nervoso e análise do estresse oxidativo. As variáveis comportamentais (número de quadrados cruzados; tempo de locomoção; tempo em pé) analisadas no teste de campo aberto não apresentaram diferenças estatisticamente significativas, assim como o teste das três câmaras. Comparando-se os resultados ao grupo controle, tanto o grupo autista quanto os grupos tratados com Risperidona e Fluoxetina apresentaram aumento na formação de substâncias reativas ao ácido tiobarbitúrico. Em relação à atividade da superóxido dismutase (SOD), o grupo autista apresentou diminuição da atividade enzimática em comparação ao grupo controle, e nenhum dos fármacos sintéticos nem o canabidiol isoladamente alterou a atividade da SOD. Fluoxetina e CBD demonstraram reversão da redução causada pelo comportamento autístico na atividade enzimática. Não foram observadas alterações comportamentais ao comparar os animais tratados com CBD com aqueles tratados com drogas sintéticas. Os dados sugerem que o CBD apresenta melhor ação antioxidante quando comparado aos fármacos sintéticos.

Palavras-chave: Transtorno do Espectro Autista; Canabidiol; Ácido Valpróico; Estresse Oxidativo; Modelo Animal.

INTRODUCTION

Autism spectrum disorder (ASD) is a prevalent and complex neurodevelopmental condition characterized by challenges in social communication and interaction, sensory sensitivities, repetitive behaviors, and intellectual disability[1]. It is known to have a vital genetic component; after two identical twins had the same condition, they can present with diverse medical conditions and can be caused by environmental factors[2]. Given the rising prevalence of autism diagnoses, insights into the pathophysiology, and therapeutic approaches to ASD, animal models for autism need to be developed[3].

Compounds such as valproic acid carry teratogenic risks. They may develop ASD-like phenotypes in rats, including impairments in social communication and repetitive behavior when used in

maternal treatment of the rodents[4,5]. Therefore, animal models of ASD are essential not only for the characterization of controlling pathogens but also provide a preclinical platform for developing therapeutic approaches for ASD[6].

Among the treatments for ASD, Cannabidiol (CBD), a prominent component of Cannabis sativa, has been extensively studied and pointed out as a practical option to treat the symptomatic conditions found in autism[7]. In addition to stimulating 5HT1A receptors involved in mood and anxiety regulation and potentiating anti-inflammatory pathways, CBD regulates inhibitory neurotransmission in the brain through its function as a positive modulator of GABAA receptors and by increasing endocannabinoid levels, improving levels of endocannabinoids that assist in mood, sleep, and brain function regulation[8]. CBD has been investigated in recent decades, not for its psychoactive properties, since if used in isolation it does not have the psychoactive effects of marijuana, but rather for its clinical effects that are mainly attributed to its potential neuroprotective action such as antioxidant, in addition to its anxiolytic and antidepressant effects[7–9].

Aripiprazole is a quinolinone atypical antipsychotic with promising results in reducing irritability, hyperactivity, communication and repetitive behaviors in patients with ASD and with fewer side effects than other atypical antipsychotics. The drug exhibits a high affinity for dopamine D2 receptors and serotonin 5-HT2A receptors, acting as a partial agonist at the D2 receptors, leading to a lower level of receptor activation compared to dopamine itself, and as an antagonist at the serotonin 5-HT2A receptors, moderately blocking the signaling pathway[9,10,11].

Risperidone is also an atypical antipsychotic approved for treating irritability in ASD. However, its mechanism involves blocking dopamine D2 and serotonin 5-HT2A receptors, which helps reduce irritability and aggressiveness. Additionally, risperidone is associated with more significant adverse effects than aripiprazole, including sedation, weight gain, metabolic issues, and a higher risk of causing neuroleptic malignant syndrome[10,12].

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) - the most widely prescribed drug class in ASD in the USA overall - that has shown promise in reducing primarily obsessive-compulsive behavior in ASD. However, its use remains a topic of debate due to limited evidence supporting its efficacy in treating core symptoms of ASD, such as repetitive and restricted behaviors (RRBs). Additionally, SSRI in the wrong doses can cause adverse effects, including disinhibition, hypomania, and agitation, which may worsen specific symptoms in individuals with ASD[13,14].

Considering the paucity of studies on alternative therapies, such as CBD, for the treatment of autism, the study aims to compare the antioxidant effects of CBD extract with synthetic drugs (Risperidone, Aripiprazole and Fluoxetine) in an animal model for autism induced by valproic acid and confirm its possible improvement in the behavioral symptoms arising from the disorder and its lesser side effects.

EXPERIMENTAL PROCEDURES

ANIMALS

Young male and pregnant Wistar rats obtained from the Central Animal House of the Regional University of Blumenau, Blumenau, Brazil, were used in the experiments. The animals from our breeding stock were maintained on a 12 h light/12 h dark cycle at a constant temperature (22±1°C), with

free access to water and commercial protein chow. All experiments were carried out in accordance with the provisions of Law No. 11,794, of October 8, 2008, and other rules applicable to the use of animals in teaching and/or research, especially the Normative Resolutions of the National Council for the Control of Animal Experimentation - CONCEA. The experimental protocol (003/2021) was approved by the Ethics Committee for Animal Research at the Regional University of Blumenau.

Female Wistar rats aged 60 days had their cycle controlled, and when they were in proestrus, they were placed to mate with a male of the same breed and age during the night. The confirmation of copulation occurred through observation in the morning of the vagina plug, consisting of solidified semen that generates the tamponade of the female's vaginal canal. Females with observed vaginal plugs were considered as E0 (day 0 embryo)[3,10]. Pregnant females received a single intraperitoneal injection with 600mg/kg of valproic acid or saline on the 12th day of gestation [3,5,11]. After birth, only male puppies were selected for the study, given the higher prevalence of autism in males[12,14].

After weaning the animals and reaching a developmental age of 27 days, they were divided into 10 groups: control 1, control 2, G1a, G1b, G2a, G2b, G3a, G3b, G4a and G4b. The animals in control groups 1, G1a, G2a and G3a were born to mothers induced by saline, therefore considered without autism. The animals in control groups 2, G1b, G2b and G3b were animals born to mothers induced by valproic acid and thought to have autism. These previously mentioned groups were treated for fifteen days orally with sesame oil (Control 1 and Control 2), CBD extract at dose of 60mg/kg (G1a, G1b), Risperidone 0,1 mg/kg (G2a, G2b), Aripiprazole 1,5 mg/kg (G3a, G3b) or Fluoxetine 5 mg/kg (G4a, G4b) [9].

The animals were subjected to behavioral tests of social interaction and open field post-treatment to verify rodents' motor and psychotherapeutic response to treatments.

BEHAVIORAL TESTS

Three Chamber Test

The social interaction test (TCT) is used in the animal model of autism to analyze social behavior, the search for social novelties, and the social interaction of animals[16,17]. The animals were tested for their ability to socialize and social novelty, and their preference and social interactions were evaluated when they spent more time with the new animal. The impairment in social interaction can be demonstrated when there is less interaction with the unknown animal.

A custom box with three chambers (120x80x40cm^3) was used, divided by two walls, allowing the passage from one chamber to another. One of the chambers had a new animal for the animal to be tested, and the other chamber had a new non-social object. The test animal was placed in the central chamber and remained free to explore the different compartments for 10 minutes.

Fig. 8. Animal model of autism during Three Chamber Test.

The test was performed in a specific room for behavioral experiments. The variables of time in each chamber, time with the new animal, and time with the new non-social object were analyzed.

Open Field Test

The open field test (OFT) for animal models of autism is one of the most recognized for assessing exploratory behavior and locomotion of the rats studied. The test was performed in a custom box (100x100x40cm^3) divided into 25 squares, during a 10-minute test session for each animal. The test animal is placed in the center of the box and for the duration of the session, allowed to explore the entire area[16,17,18].

Fig. 9. Animal model of autism during Open Field Test.

The variables defecation, movement in the open field, number of crossed squares (crossings), exploratory behavior, self-cleaning and support on two paws were analyzed through the frequency and time during the test execution.

TISSUE PREPARATION

For the analysis of oxidative stress parameters, the rats were sacrificed by decapitation in the absence of anesthesia; the brain was quickly removed and kept on ice with a sodium phosphate buffer. The homogenate was prepared in a suitable buffer using a Potter-Elvehjem homogenizer (5 pulses), subsequently centrifuged at 3,000 rpm, at 4°C for 15 minutes to remove cellular residues. Then, the supernatant was stored in aliquots at -80°C for later determination of antioxidant enzyme activity, total sulfhydryl content, and formation of substances reactive to thiobarbituric acid (TBA-RS).

OXIDATIVE STRESS ANALYSES

Thiobarbituric Acid Reactive Substances (TBA-RS) Measurement

TBA-RS was determined according to the method described by Ohkawa et al. (1979)[18]. TBA-RS measures malondialdehyde (MDA), a lipoperoxidation product mainly caused by free hydroxyl radicals. Briefly, the homogenate in 1.15% KCl was mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. The absorbance determined TBA-RS at 535 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each point of the curve was subjected to the same treatment as the supernatants. TBA-RS were calculated as nanomoles of malondialdehyde formed per milligram of protein.

Total sulfhydryl content measurement

Total thiol group concentration was determined by Aksenov and Markesbery's method (2001)[19]. Briefly, 50 µL of homogenate were added to 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 1 mM EDTA. The reaction was started by adding of 30 µL of 10 mM 5,5´-dithiobis(2-nitrobenzoic acid) (DTNB) and incubated for 30 min at room temperature in a dark room. Total sulfhydryl content was determined by measuring the absorbance at 412 nm. The analysis of a blank (DTNB absorbance) was also performed. Results are reported as nmol 3-thio-2-nitrobenzoic acid (TNB)/mg protein.

Catalase Assay (CAT)

CAT activity was assayed by Aebi method (1984)[20] using a UV–visible Shimadzu spectrophotometer. The method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 25µL of sample and 600 µL of 10 mM potassium phosphate buffer, pH 7.0, 20 mM H_2O_2. One CAT unit is defined as 1µmol of H_2O_2 consumed per minute, and the specific activity is calculated as CAT units/mg protein.

Glutathione Peroxidase (GSH-Px) Activity Assay

GSH-Px activity was measured using Wendel's method (1981)[21], which had tert-butyl hydroperoxide as a substrate. The decomposition of NADPH was controlled in a UV-vis Shimadzu spectrophotometer at 340 nm for 3 minutes and 30 seconds. 90 µL of each sample was added to the medium containing 800 µL of buffer, 20 µL of 2.0 mM GSH, 30 µL of 0.15 U/mL GSH reductase, 10 µL of 0.4 mM azide, and 10 µL of 0.1 mM NADPH. The absorbance was counted every 10 seconds for 1 minute and 30 seconds. Then, 50 µL of 0.5 mM *tert*-butylhydroperoxide was added, and the absorbance was read

for another 2 minutes. One GSH-Px unit is characterized as 1 µmol of NADPH consumed per minute, and the specific activity is defined as GSH-Px units/mg of protein.

Superoxide Dismutase (SOD) Assay

The method used to assay SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide ($O^{\bullet -}$), which is a substrate for SOD Marklund (1985)[22]. Briefly, to 15µL of each sample, 215µL of a mixture containing 50.0 µM Tris buffer, pH 8.2, 1.0 µM EDTA and 30.0 µM CAT were added. Subsequently, 20.0 µL of pyrogallol was added, and the absorbance was immediately recorded every 30 seconds for 3 minutes at 420 nm using a UV–visible Shimadzu spectrophotometer. The inhibition of the autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be indirectly assayed spectrophotometrically. A calibration curve was performed with purified SOD as a reference to calculate the activity of SOD present in the samples. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation, and the specific activity is reported as SOD units/mg protein.

Protein Determination

Protein was measured by the method of Lowry et al. (1951)[23], using serum bovine albumin as standard.

STATISTICAL ANALYSIS

For behavioral tests, Kruskal-Wallis analyzed data. For biochemical analyses, data were analyzed by ANOVA followed by the Duncan multiple range test when the F-test was significant. All analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) for Windows version 20.0, using a PC compatible computer (IBM Corp., Armonk, NY, USA). Values of $p<0.05$ were considered to be significant.

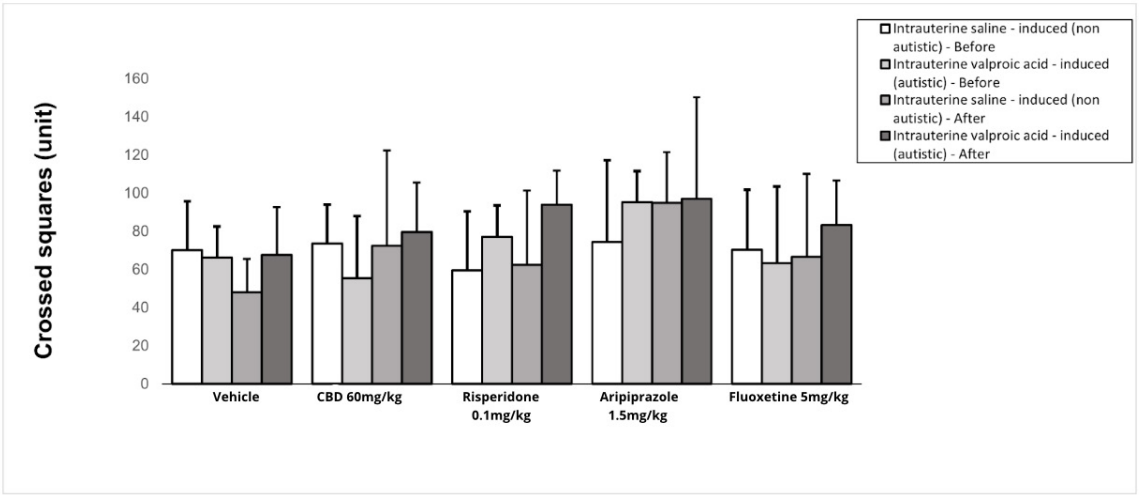
RESULTS

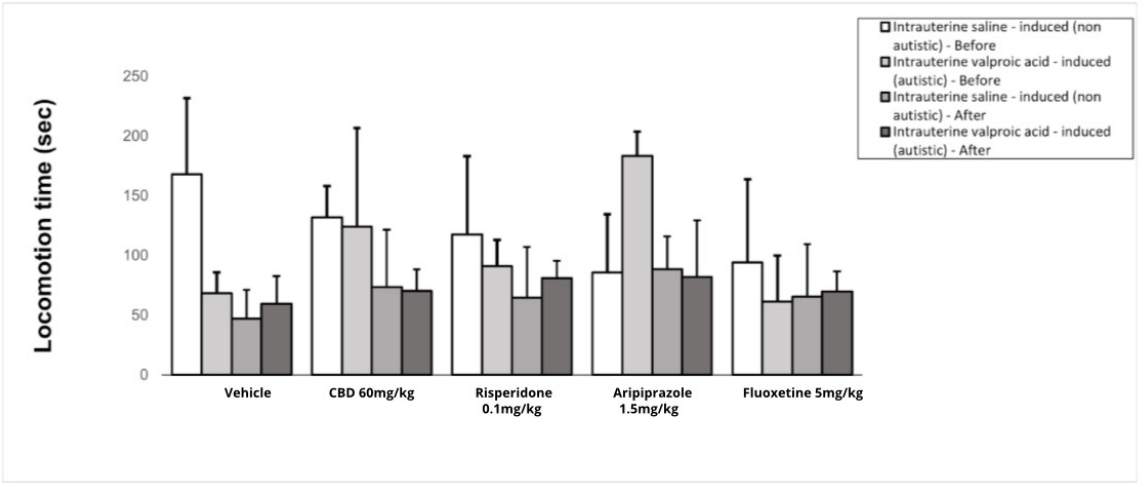
BEHAVIOR ANALYSIS

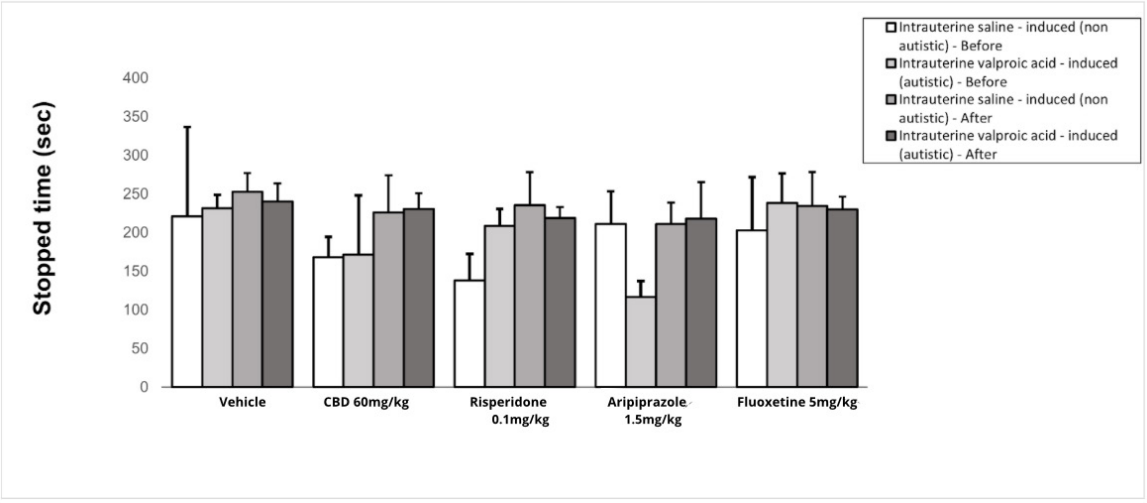
Open Field Test (OFT)

The variables of number of squares completely crossed, locomotion time and time without locomotion analyzed in the OFT (Figure 1) showed no significant differences in crossed squares, locomotion time or stopped time, before and after treatment. Compared to control rats, autism model rats displayed lower activity levels, as evidenced by fewer square crossings and increased immobile time. The administered treatments did not significantly alter the observed behaviors in either control or autism model rats. The variables of urination and defecation were not presented as a graph due to their data distribution and statistical insignificance. The variables jumping, digging, and biting the tail did not occur during the OFT.

Fig. 1. Analysis of the variables number of squares crossed, locomotion time, time without locomotion, time in repetitive behaviors and behavior of remaining on two paws observed during the open field test. Time of locomotion, time without locomotion, and time in repetitive behaviors were analyzed in seconds. Results are expressed as mean ± SE. Different from control, ***p<0,001, n=11.





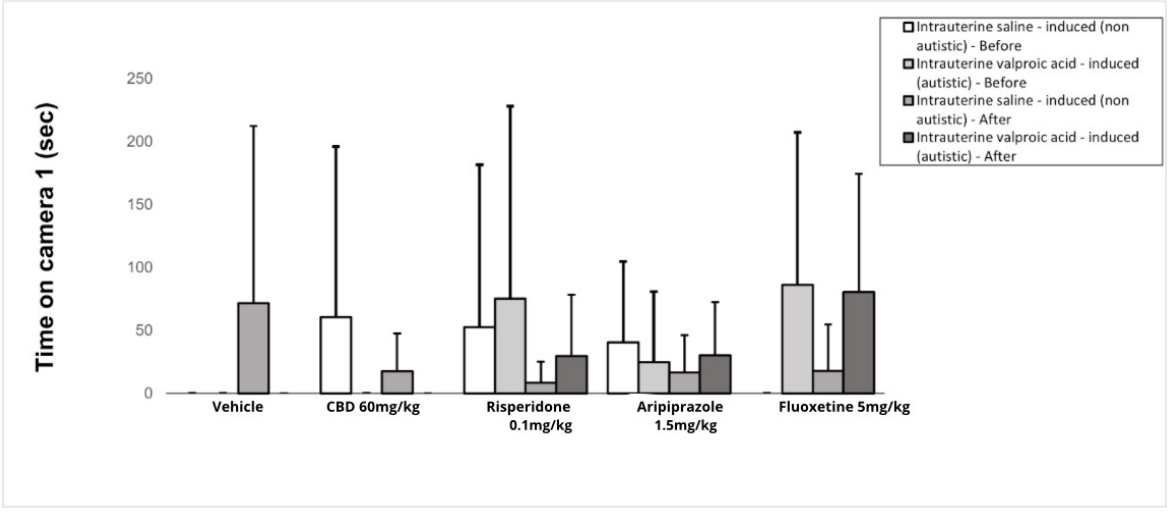


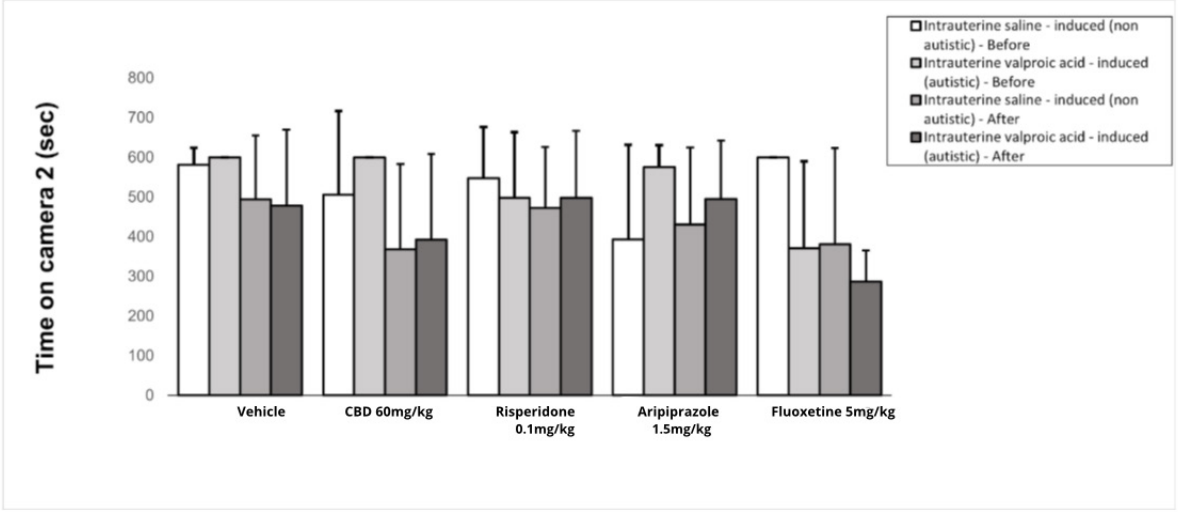
The behavioral variables analyzed in the OFT did not present statistically significant differences compared to the non-autistic control (intrauterine saline), nor were intragroup differences observed comparing the results before and after drug treatment.

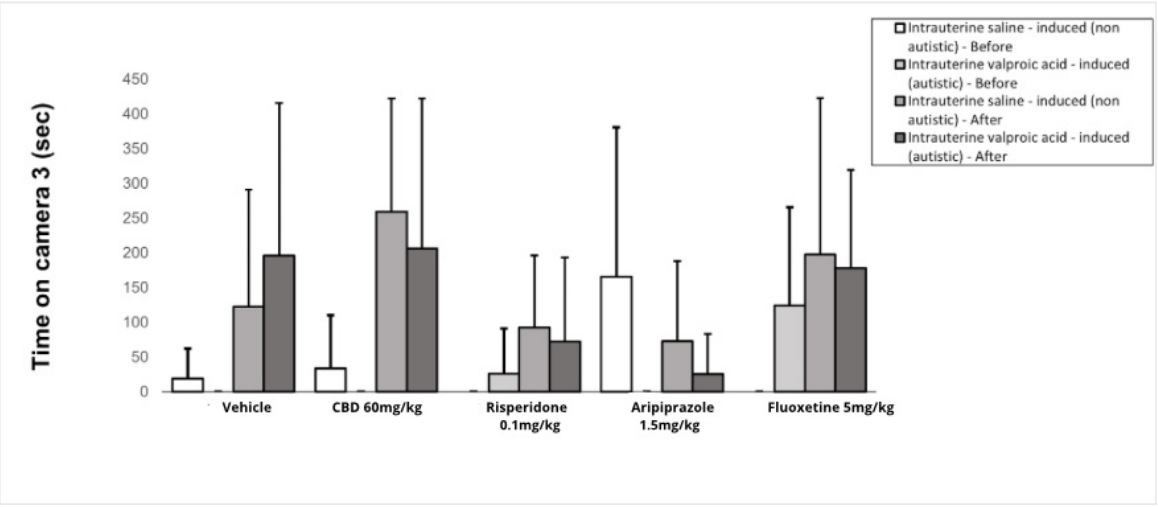
Three Chamber Test (TCT)

In the TCT, the variables analyzed are time in chambers 1, 2, and 3, and time of socialization with a new animal and object. Chamber 1 contained the new object. Chamber 2 was the central chamber, where the animal was placed at the beginning of the test. Chamber 3 contained a new animal unknown to the test animal.

Fig. 2. Analysis of the variables time in chamber number 1, time in chamber number 2, time in chamber 3, time of socialization with a new object and time of socialization with a new animal observed during the three-chamber test. Results are expressed as mean ± SE. Different from control, $^{***}p<0,001$, n=11.







The behavioral variables (time in chamber number 1, time in chamber number 2, and time in chamber 3) analyzed in the Three Chambers Test (TCT) did not present statistically significant differences compared to the non-autistic control (intrauterine saline), nor were intragroup differences observed comparing the results before and after drug treatment.

OXIDATIVE STRESS PARAMETERS

As seen in Figure 3, comparing the results to the control group, both the autistic and non-autistic groups treated with Risperidone or fluoxetine showed increased TBARS levels. Regarding treatment with CBD and Aripiprazole, they were able to reverse the increased levels of TBA-RS observed in the autistic group.

Regarding SOD activity (Figure 4), the autistic group showed a decrease in enzyme activity compared to the non-autistic group. Also, none of the synthetic drugs or CBD alone altered SOD activity on non-autistic, but treatment with Fluoxetine and CBD demonstrated a reversal of the reduction caused by autistic behavior on enzyme activity. As for CAT and GSH-Px activity (Figures 5 and 6), neither the autistic group nor the tested antipsychotics and fluoxetine caused changes in enzyme activity compared to the control group.

Furthermore, concerning total sulfhydryl content (Figure 7), CBD and Risperidone showed higher levels than the control group, indicating a protective effect. Fluoxetine, on the other hand, exhibited reduced sulfhydryl levels compared to the control group.

Fig.3. Effects of Cannabidiol (CBD), Risperidone, Aripiprazole and Fluoxetine as a function of the presence or absence of autism on superoxide dismutase activity (SOD) in the cerebral cortex of male Wistar rats. One unit of superoxide dismutase (SOD) is defined as the amount of SOD required to inhibit 50% of the auto-oxidation of pyrogallol. Results are expressed as mean ± SD. Different from control, ***p<0,001 n=7.

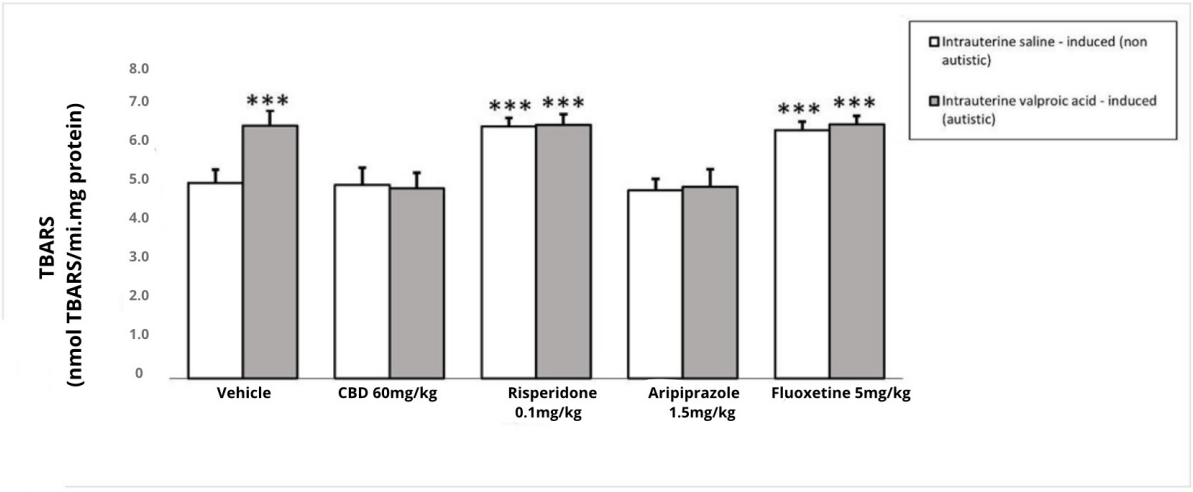


Fig.4. Effects of Cannabidiol (CBD), Risperidone, Aripiprazole and Fluoxetine as a function of the presence or absence of autism on superoxide dismutase activity (SOD) in the cerebral cortex of male Wistar rats. One unit of superoxide dismutase (SOD) is defined as the amount of SOD required to inhibit 50% of the auto-oxidation of pyrogallol. Results are expressed as mean ± SD. Different from control, ***p<0,001 n=7.

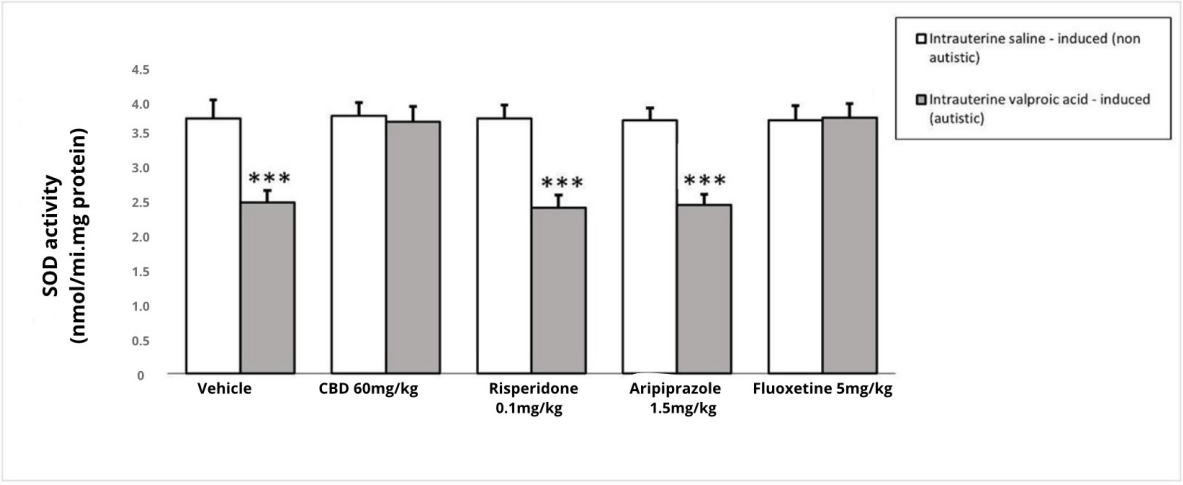
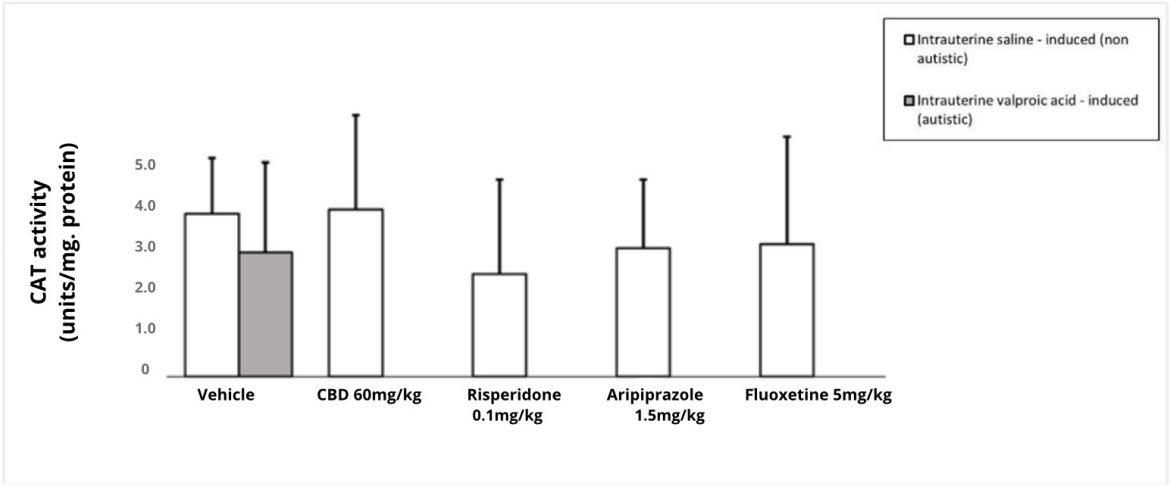


Fig. 5 and 6. Effects of Cannabidiol (CBD), Risperidone, Aripiprazole and Fluoxetine as a function of the presence or absence of autism on catalase (CAT) and on glutathione peroxidase (GSH-Px) activities in the cerebral cortex of male Wistar rats. One unit of catalase (CAT) is defined as 1 µmol of H2O2 consumed per minute. One unit of glutathione peroxidase (GSH-Px) is defined as 1 µmol of NADPH consumed per minute. Results are expressed as mean ± SD. Different from control, ***p<0,001 n=6.



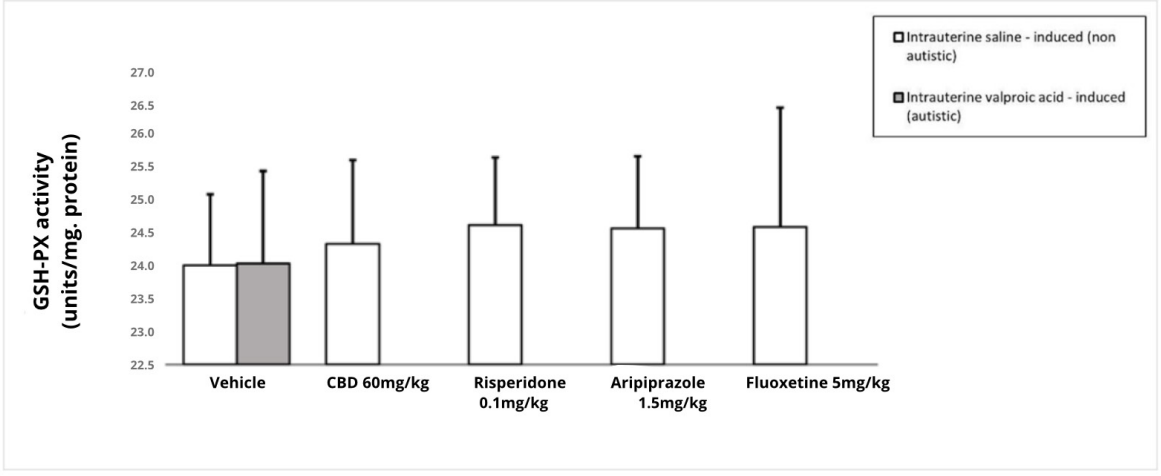
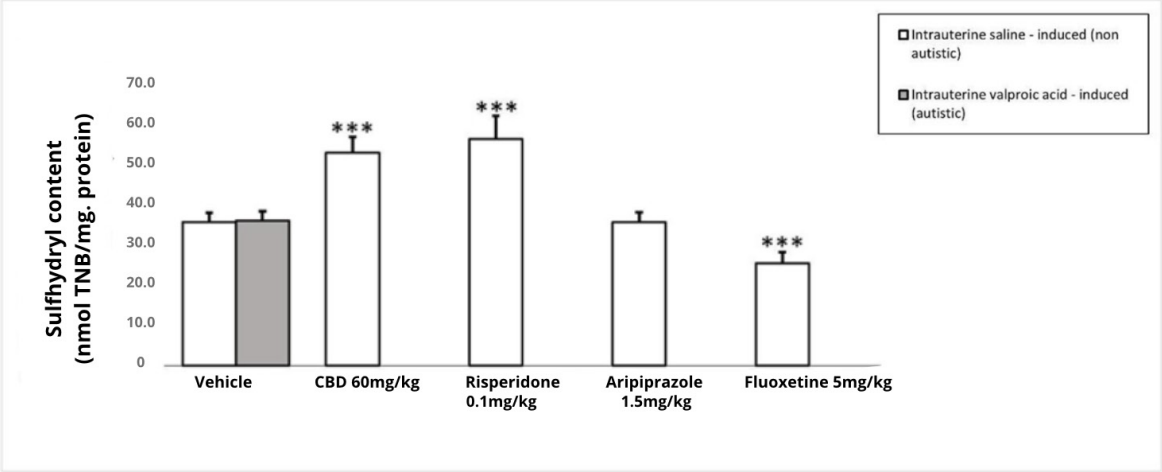


Fig. 7. Effects of Cannabidiol (CBD), Risperidone, Aripiprazole and Fluoxetine as a function of the presence or absence of autism on the total content of sulfhydryls in the cerebral cortex of male Wistar rats. The total sulfhydryl content is expressed as nmol of TNB per mg of protein. Results are expressed as mean ± SD. Different from control, ***p<0,001 n=7.



DISCUSSION

The brain is highly vulnerable to oxidative stress due to its elevated oxygen demand, abundance of polyunsaturated fatty acids, and limited antioxidant defenses. These factors facilitate lipid peroxidation and cumulative oxidative damage, which are strongly implicated in neurodegenerative diseases and neurodevelopmental disorders. The enzymatic antioxidant defense system is critical for maintaining redox homeostasis and preventing oxidative damage. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, which CAT or GSH-Px subsequently detoxifies. The coordinated activity of these enzymes is significant in neural tissue, where their impairment enhances vulnerability to oxidative stress–related damage.

Regarding oxidative stress, it was noticed that TBA-RS, a test used to measure a product of lipid peroxidation mainly caused by the presence of free hydroxyl radicals, called malondialdehyde (MDA), CBD, and Aripiprazole showed satisfactory results due to a reversal of its increase, indicating a protection against lipid peroxidation. This process is particularly critical in the central nervous system due to its high lipid content and oxidative metabolism. Furthermore, concerning total sulfhydryl content, the groups of CBD and Risperidone, appearing at elevated levels, also demonstrated a positive result by showing a protective effect against protein damage, unlike Fluoxetine, which showed a reduced effect. Total sulfhydryl content is a key indicator of cellular antioxidant capacity and redox balance. Reduced levels reflect depletion of antioxidant defenses and increased susceptibility to oxidative stress.

Ultimately, no statistical changes were observed regarding CAT and GSH-Px activities compared to the control group. As for the SOD activity results, Fluoxetine and CBD seemed to reverse the reduction in autistic behavior in enzymatic activity, suggesting improved antioxidant defense in the cerebral cortex of autistic behavior of animals.

As for the autistic-like behavior of the animals, the OFT tests and the three-chamber test were used. The OFT, introduced by Hall in 1934, is a direct observation instrument most used in the study of animal behavior since its precision in measurements in different types of behavior can be easily developed[1,2]. Autism is a critical assessment tool for animals' exploratory behavior and locomotion. The behavioral observations of the animals with and without autism in our study were made through the frequencies of the number of squares crossed, locomotion time, time without locomotion, time spent in repetitive behaviors, behavior of remaining on two legs, defection and grooming. According to the literature, defecation shows the emotional state of the test animal, especially the feeling of anxiety, which is common in autism. The self-cleaning movements of animals can demonstrate stereotypical, commonly performed in autism by repetitive movements. However, these behaviors were not significant in our work.

Rearing, the behavior of standing on two legs is considered an exploratory behavior generated by the novelty of an environment, with the hippocampus being one of the brain regions involved in this type of behavior. This type of behavior may be related to anxiety and depression in animals in an environment considered new to them[1,2]. In our study, the administered treatments, including CBD, did not produce significant changes in the behaviors of the animals in either the control or autism model groups. The variables of urination, defecation, and behaviors of jumping, digging, and tail biting did not show statistical relevance or occurrence during the test.

The TCT is used in autism studies for the analysis of social behavior, the search for social novelties, the recognition of social cues, and the social interaction of animals[3]. Social preference and interaction are defined by spending more time with the new animal than in the opposite camera, which has only one new object for the test animal. Social interactions with the young animal include nose-to-snout sniffing, anogenital inspection, flank exploration, and following behavior[2]. The amount and duration of scoring with the young animal and with the object are scored, the total number of entries into the test chamber, and the time of reciprocal social interaction.

In autism, impairment in social interaction is a hallmark feature that is evidenced through difficulty participating in group activities, affective detachment, and often a lack of social empathy. In the animal model for autism, animals tend to have a shorter interaction time with the new animal, like human behavior[1]. However, the behavioral variables (time in chamber number 1, time in chamber number 2, and time in chamber 3) analyzed in the TCT did not present statistically significant differences compared to the non-autistic control (intrauterine saline), nor were intragroup differences observed comparing the results before and after drug treatment.

In contrast to our study, which was conducted with Wistar rats induced with valproic acid at a dose of 600 mg/kg on the 12th day of gestation, the study by Ali et al. (2005)[24] investigated the therapeutic potential of CBD oil supplementation in a valproic acid-induced autism model using albino BALB/c mice exposed to valproic acid (600 mg/kg) on the 13th day of gestation. The results showed that valproic acid-treated mice exhibited a significant increase in repetitive behavior. In contrast, animals treated with CBD or risperidone displayed a considerable reduction in this behavior compared with the valproic acid group. Moreover, valproic acid-exposed mice showed longer latency to withdraw the hind paw in response to a painful stimulus, indicating impaired nociceptive response. CBD treatment reduced latency and improved the behavior of the animals. When evaluating anxiety and locomotor activity, valproic acid treatment induced hyperlocomotion and anxious behavior, associated with sensorimotor deficits, cognitive fragmentation, and impaired habituation. However,

CBD oil treatment significantly reduced anxious behavior by lowering stress levels. In the novel object recognition test, valproic acid exposure increased the time spent with familiar objects, suggesting recognition deficits. CBD treatment reversed this effect by increasing exploration of novel objects and social preference, and significantly elevating the discrimination index, indicating improved exploratory memory. In the social interaction test, CBD oil treatment reversed the effects of valproic acid, restoring normal behavior, and significantly increasing the frequency of social interactions. In contrast, mice treated only with valproic acid spent less time with unfamiliar animals than the control group. Additionally, the study showed that prenatal exposure to valproic acid increased oxidative stress, with decreased activity of glutathione-S-transferase, SOD, and CAT, reduced levels of reduced glutathione, and increased lipid peroxidation and nitric oxide levels, resulting in neuronal damage and impaired early brain development. In contrast, treatment with CBD oil, as well as with risperidone, reduces oxidative stress by restoring antioxidant enzyme activity and decreasing lipid peroxidation and nitric oxide levels in valproic acid-treated mice. With respect to oxidative stress, these findings are consistent with our data, as we also observed reduced SOD activity and increased lipid peroxidation.

The absence of significant results in our behavioral tests can be partially attributed to the limitations of the valproic acid-induced animal model used. As discussed by Kuo and Liu (2022)[9], the valproic acid model represents only a subset of the environmental risk factors associated with Autism Spectrum Disorder (ASD), and maternally valproic acid-exposed ASD patients constitute a small portion of the total ASD population. Therefore, the valproic acid-induced pathophysiology may not encompass the full complexity of ASD. Furthermore, the heterogeneity of ASD, with its diverse genetic and environmental etiologies, makes it challenging to faithfully replicate all aspects of the disorder in a single animal model[9], demonstrating the animal model's complexity and the difficulty in standardizing results. These limitations may have contributed to the lack of significant effects of the treatments tested in our experiments. Since no significant behavioral effects were observed at the dose used in our study, we consider the use of a higher dose of valproic acid in future investigations. In our case, the elevated standard error indicates substantial inter-individual variability, which may have obscured potential differences between groups and limited the statistical detection of significant effects. Thus, the evaluation of the impact of the different treatments remains valid. This factor may have masked potential differences between groups, not necessarily indicating a genuine lack of effect. Therefore, the results still provide relevant information and should be interpreted cautiously.

In conclusion, although Aripiprazole and Risperidone showed some positive effects, CBD was the drug that had the most significant result on the antioxidant effect, as it showed protection against lipid peroxidation, increased sulfhydryl levels and helped reverse the reduction in SOD enzyme activity. Furthermore, the limitation of the animal model used, representing only a fraction of autism risk factors, likely influenced the absence of significant behavioral results. The complexity of ASD, with its diverse causes, makes faithful reproduction in a single animal model challenging. Therefore, it is crucial to interpret the results cautiously and seek more comprehensive models in future research.

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