ORIGINAL ARTICLE

ANTIDEPRESSANT EXPLORATION OF MOXIDECTIN: EFFECTS ON BEHAVIOR, NEUROTROPHIC FACTORS AND OXIDATIVE STRESS BIOMARKERS IN MICE

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Highlights: (1) Moxidectin has an antidepressant effect in mice. (2) A single dose of Moxidectin increases *Bdnf* mRNA in the prefrontal córtex. (3) Moxidectin improves the oxidative stress profile of the CNS of mice. (4) Moxidectin may possess the same MoA as the novel antidepressant brexanolone.

PRE-PROOF
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ANTIDEPRESSANT EXPLORATION OF MOXIDECTIN: EFFECTS ON BEHAVIOR, NEUROTROPHIC FACTORS AND OXIDATIVE STRESS BIOMARKERS IN MICE

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ABSTRACT

Major depressive disorder is a reality to an increasing number of individuals worldwide, yet, it is still poorly understood and its treatment remains lackluster. Due to the subpar success rates of traditional monoaminergic pharmacotherapy, new targets have been explored. Here, the behavioral effects of a single dose of moxidectin (MOX) (1,5 mg/kg, 0,1 mL/10g, s.c.), a drug capable of allosterically modulating $GABA_A$ channels, was evaluated in male Swiss mice by the tail suspension (TST), splash (SPT), open field and elevated plus maze tests between 24 and 48 h post injection. MOX was capable of significantly decreasing the immobility time in TST and increasing grooming time in SPT, suggesting an antidepressant effect. To further explain the molecular pathways underlying MOX's observed behavioral changes, neurotrophic factors and oxidative stress biomarkers were identified. An increase in brain-derived neurotrophic factor (*Bdnf*) mRNA was observed, possibly explaining the behavioral changes. An increase in total antioxidant capacity and glutathione S-transferase (GST) in the prefrontal cortex and a decrease of GST in the hippocampus of MOX-treated animals may have also contributed to these results. To our knowledge, this is the first study demonstrating the antidepressant-like effect of MOX in mice. While these results are preliminary, they're promising. The utilization of $GABA_A$ modulators is an emerging and interesting new avenue to be explored in the treatment of neuropsychiatric ailments.

Keywords: depression, antiparasitic, BDNF, $GABA_A$, behavioral testing

INTRODUCTION

Depression affects around 5% of the world's adult population[1]. According to the DSM-5-TR, Major Depressive Disorder (MDD) is characterized by a depressed mood and/or anhedonia plus five other criteria that involve changes in weight, sleep, energy levels, low self-

esteem, suicidal ideation, and others in a manner that disturbs or interferes with the person's day to day life. Data on the overall efficacy of antidepressants is highly variable. One of the most complete and extensive studies to date relates a rate of remission of 36.8% on the first treatment attempt, with subsequent attempts reaching lower success rates, with an overall cumulative remission rate of 67%, leaving 33% of patients with unsatisfactory outcomes. Even when remission is achieved, MDD has very high relapse and recurrence rates[2–4].

Given the still unelucidated pathophysiology of MDD and the current lackluster performance of the monoaminergic therapy for its low success rates, various side effects, delayed onset, and limited efficacy, new targets have been explored, such as the NMDA receptor and its antagonist esketamine, which as already been approved as an antidepressant by the FDA[5]. Another promising target is the GABA receptor, namely via allopregnanolone (Allo). Allo is an endogenous neuroactive steroid, a progesterone metabolite, capable of positive allosteric modulation of the $GABA_A$ receptor. It has been observed that Allo levels are lower in patients with MDD, both in serum and cerebrospinal fluid in a symptom-related ratio, with levels rescued after successful treatment[6]. Two compounds derived from Allo, brexanolone and zuranolone, have already been approved by the FDA as treatments for postpartum depression (PPD)[7]. Another positive allosteric modulator of $GABA_A$ is moxidectin (MOX), an originally antiparasitic compound widely used in veterinary medicine[8,9], which recently has been shown to possess antidepressant effects in rats[10].

This paper aims to investigate the antidepressant potential of a single dose of MOX in male mice as well as identify some molecular pathways underlying it, including neurotrophic factors and oxidative stress biomarkers.

METHODOLOGY

Animals:

36 male Swiss mice, aged between 6 and 8 weeks old, weighing 30 to 40 g were utilized. Animals were purchased from Universidade Federal de Santa Maria's (UFSM) central vivarium. The animals were allowed to acclimatize to our vivarium for 6 days before the beginning of the experimental protocol. Animals were housed in standard shoebox cages with wood chip bedding, food and water *ad libitum* without any environmental enrichment. Standard vivarium humidity and temperature conditions with 12-hour light/dark cycle. All

administrations, manipulations and tests were performed under the light cycle, between 13:00 and 17:00 h. The animals were manipulated as little as possible.

Experimental design:

Animals were divided, at random, in 3 groups of 12 animals. This number was chosen based on similar literature[11,12]. Groups consisted of MOX, fluoxetine (FLX) (positive control), or control (naïve). 24 hours after administering the compounds, the open field test (OFT) and splash test (SPT) were carried out. On the following day, elevated plus maze test (EPM) and tail suspension test (TST), the last behavioral tests, were performed. On the next day, 72 h post injection, the animals were euthanized.

Drugs:

The MOX utilized was Cydectin 1% (Part.: 007/21, Zoetis, Brazil). It was diluted in 10% hydroalcoholic solution in a concentration that allowed the administration of 0,1 mL of solution for every 10 g of weight. Animals received 1,5 mg/kg of body weight, s.c. This dose is adapted from a previous study on rats[10]. The FLX utilized was Daforin 20 mg/mL (L2R9960, EMS, Brazil), diluted in saline in a concentration that allowed the administration of 0,1 mL of solution for every 10 g of weight. Animals received 10 mg/kg of body weight via gavage [11,12]. Mice from the control group did not receive any administration.

Euthanasia was performed by isoflurane sedation followed by exsanguination via intraventricular blood draw. The animals were then decapitated, the prefrontal cortex and hippocampus were collected and frozen at -80 ºC until analysis. The entire protocol was approved and overseen by our institution's Committee of Ethics on Animal Experimentation (CEUA/UFSM: 3435310322). All possible measures were taken to minimize animal suffering.

Behavioral tests:

The OFT was performed for 5 min, in which animals were recorded while alone in a room inside a circular 30 cm diameter apparatus[13]. The time spent in the middle of the apparatus or in the external zone was determined by ANY-Maze software. The inner 15 cm diameter was considered the central zone, the following 5 cm, a neutral zone, and the outer 5 cm, the periphery zone. The software was configured to account for the entire body with a head bias.

Immediately after, each animal underwent the SPT[14]. Animals were picked up, their back fur brushed with a finger, against the grain, once and a solution of 10% sucrose at room temperature was sprayed, with a standard cleaning spray nozzle, on their backs four times. This was done to ensure the animals were properly soaked, given the hydrophobic nature of mice fur. They were reallocated to the same apparatus in which they underwent the OFT and were again left alone in the room while being recorded for 5 min. The time spent grooming was determined manually. After the test, the animals were gently washed with warm water and towel-dried before being returned to their cages. We adopted this protocol for the SPT so that the animals were already familiar with the environment and would spend less time exploring. After the SPT, the apparatus was cleaned with ethanol 30% before receiving the next animal.

The EPM test[15] was performed in a standard, custom made, mice elevated plus maze, each arm measuring 30 cm, in which the animals were left alone in a room while being recorded. Checks in the middle of the 5 min were quickly performed to ensure no animal had jumped from the apparatus. ANY-Maze software evaluated the total distance traveled, time spent in the neutral zone, open arms or closed arms, and number of entries in each zone. After the test ended the apparatus was cleaned with ethanol 30% to receive the next animal.

After all animals underwent the EPM, the TST was carried out[16]. Animals were hung by their tails in a horizontal wooden rod using adhesive tape leaving 1 to 2 cm of tail above the tape. They were recorded while alone in a room, with a quick check in the middle to ensure no animal had fallen or climbed their tail. Animals who had, were repositioned and the clock restarted. The test lasted 6 min, with the first two minutes being disregarded. Total immobility time was evaluated manually.

Assays:

Thiobarbituric acid reactive substances (TBARS) determination was performed by homogenizing tissue samples with ice-cold 1.1% phosphoric acid and centrifuging at 1000 g for 5 min at 4 °C. The supernatants were used in the assay. It was performed by the addition of sample aliquots to tubes containing 7% phosphoric acid and a solution composed of 0.4% thiobarbituric acid, 20 mM NaOH, and 40 μM butylated hydroxytoluene. The tubes were heated in a boiling-water bath for 45 min. They were rapidly cooled on ice and then butanol was added. Finally, the tubes were vortexed and centrifuged at 1000 g for 5 min at room temperature. The organic layer was removed and placed in a cuvette. The absorbance was

measured at 532 nm. The TBARS levels are expressed as nmol/g tissue using ε = 156 mM−1 cm−1[17].

For the total antioxidant capacity (TAC) evaluation, a solution containing 5 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and 0.15 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 20 mM phosphate buffer pH 7.0 was incubated for 50 min at 45 °C. The resulting solution was afterwards rapidly cooled on ice and kept at 4 °C until use. The assay was carried out by the addition of sample aliquots to plates containing the ABTS/APPH solution at 22 °C. The absorbance was recorded at 734 nm for 1 min. TAC was calibrated against ascorbic acid standards and expressed as μmol/g tissue[18].

Glutathione S-transferase (GST) was measured according to Habig et al., (1974). Results are expressed as nmol/min/g tissue.

Nucleic acid extraction and qRT-PCR:

Prefrontal cortex and hippocampus samples were collected, immersed in TRIzol (ThermoFisher), and frozen at -80 ºC until analysis. All cited products were utilized as instructed by the manufacturer. The samples were extracted in TRIzol, followed by purification utilizing PureLink RNA MiniKit. Sample quality and quantity was analyzed by the NanoDrop-1000 Spectrophotometer (Thermo Scientific). Invitrogen's Superscript III was utilized for cDNA synthesis. Real-time qPCR was performed using SYBR fluorophore GoTaq1 Green Master Mix (Promega Corporation). All samples were run in duplicates with negative controls. Housekeeping genes utilized were β-actin[19] and HPRT[20]. The gene tested was brain-derived neurotrophic factor (*Bdnf*)[21]. β-actin was chosen as the control gene.

Statistical Analysis:

All data is expressed as mean ± SEM and were analyzed and graphed using GraphPad Prism (version 9.0.0, San Diego, USA). The Shapiro-Wilk normality test was utilized, and points considered non-normal were removed. All data was analyzed using one-way ANOVA followed by Dunnett's post hoc test, with $p < 0.05$ considered significant.

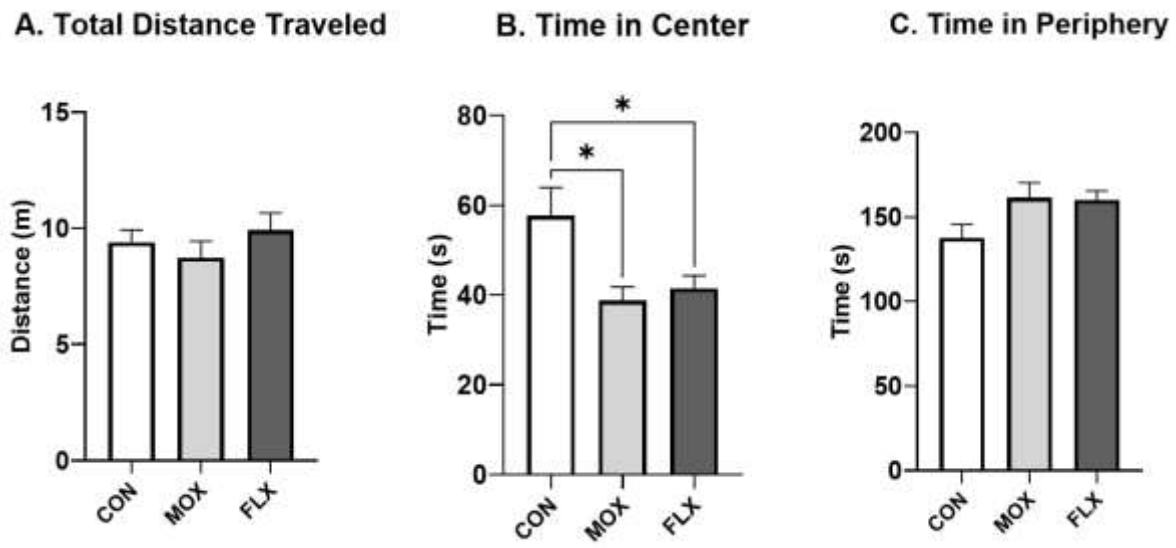
RESULTS

Behavioral Tests:

Single Moxidectin dose's Effect on Mice Behavior:

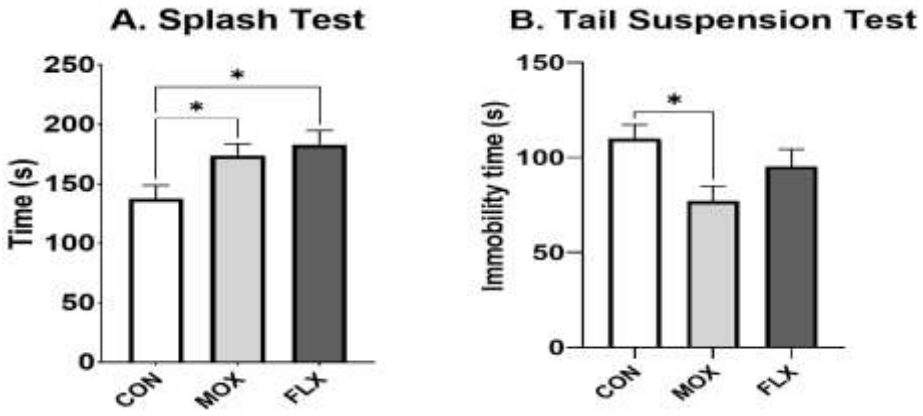
The behavioral response of mice exposed to MOX in the OFT demonstrated that in both groups, MOX and FLX, mice spent less time in the center (Fig. 1B), with p values of 0,0100 and 0,0430, respectively. No difference was observed in the total distance traveled (Fig. 1A) nor time spent in the peripheral zone (Fig. 1C) when compared to the CON group. Regarding the SPT, MOX and FLX-treated animals spent significantly more time grooming than the CON group, with p values of 0,0441 and 0,0158 respectively (Fig. 2A) Moreover, MOX decreased the immobility time in the TST, with a p-value of 0,0130, whereas FLX had no effect on it (p value of 0,3377) (Fig. 2B). Finally, in EPM, no significant changes were observed in relation to the CON group (Fig. 3).

Figure 1



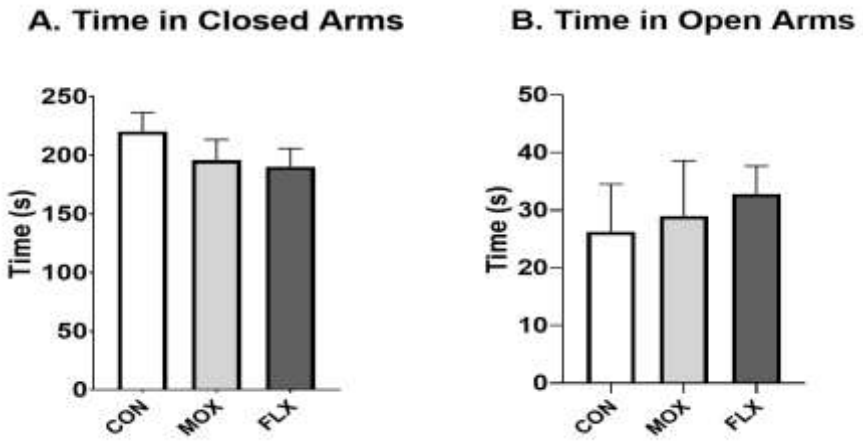
Open Field test. Results are presented as mean ± SEM, n = 8~11/group, * $p < 0,05$. (A) Total distance traveled; (B) Time spent in the central zone (C) Time spent in the periphery. FLX was used as positive control. Abbreviations: CON, control group; FLX, fluoxetine; MOX, moxidectin.

Figure 2



Results are presented as mean ± SEM, n = 8~11/group, * $p < 0,05$. (A) Time spent grooming on the SPT; (B) Time spent immobile in the TST. FLX was used as positive control. Abbreviations: CON, control group; FLX, fluoxetine; MOX, moxidectin; SPT, splash test; TST, tail suspension test.

Figure 3



Elevated Plus Maze test. Results are presented as mean ± SEM, n = 8~11/group, * $p < 0,05$. (A) Time spent in closed arms; (B) Time spent in open arms. FLX was used as positive control. Abbreviations: CON, control group; FLX, fluoxetine; MOX, moxidectin.

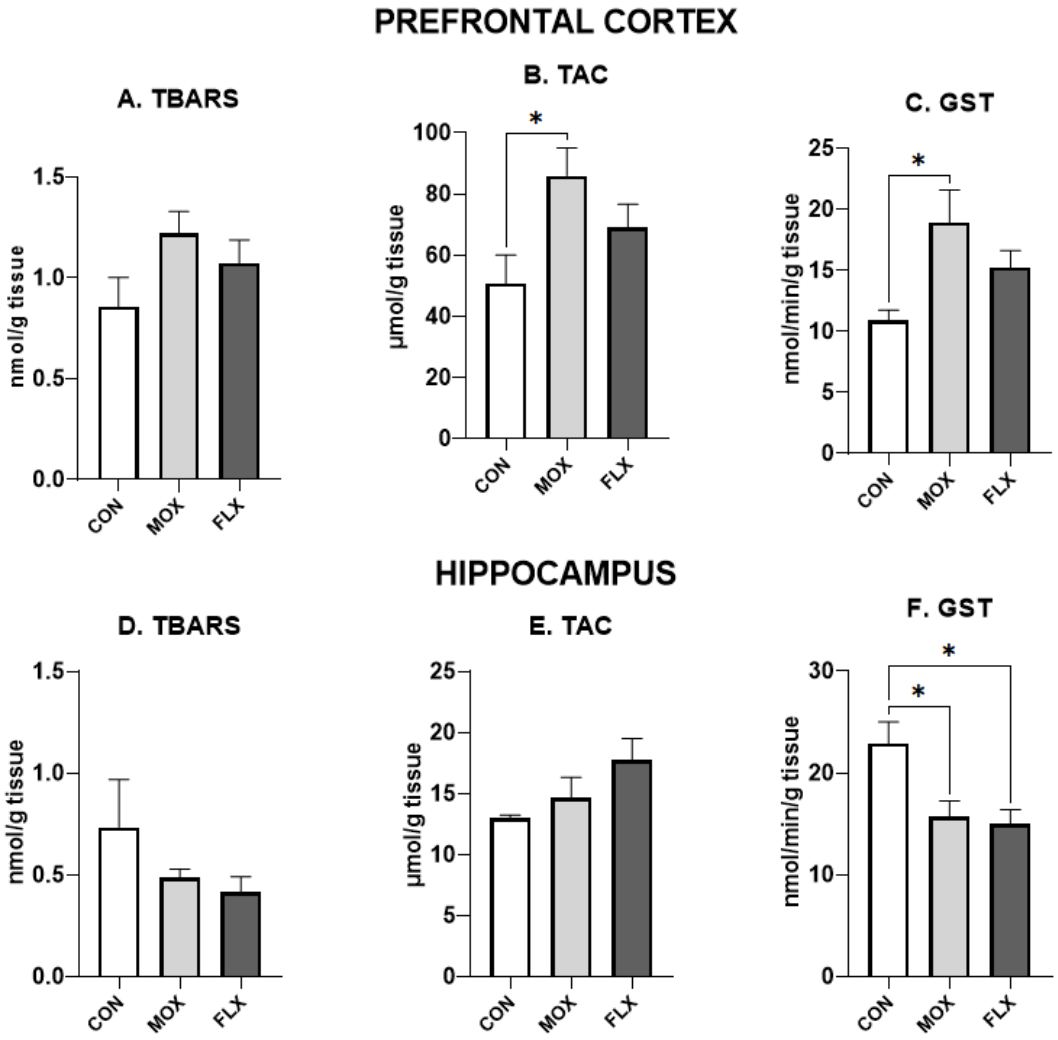
Single Moxidectin Dose's Effect on Oxidative Stress Biomarkers in the CNS:

Regarding the prefrontal cortex, no significant changes were observed in TBARS levels between groups (MOX p = 0,1430, FLX p = 0,4109) (Fig 4A). Furthermore, MOX increased TAC levels (p = 0,0300) (Fig 4B) and GST activity (p = 0,0192) (Fig 4C) FLX-

treated animals exhibited no significant changes in any of the analyzed biomarkers when compared to the CON group.

In the hippocampus, TBARS levels did not differ in any of the treated groups (MOX p = 0.4699, FLX p = 0,2685) (Fig 4D). Moreover, neither MOX (p = 0,6322) nor FLX (p = 0,0617) changed TAC levels (Fig 4E); treatment with either compound was linked to the decrease of GST activity in the hippocampus (MOX p = 0,0226, FLX p = 0,0130) (Fig. 4F).

Figure 4

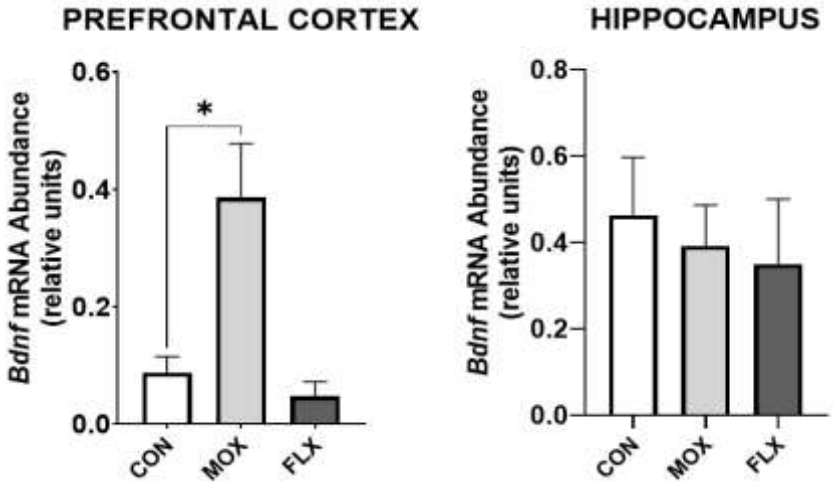


Oxidative Stress biomarkers. Results are presented as mean ± SEM, n = 4~5/group, * $p <$ 0,05. FLX was used as positive control. Abbreviations: CON, control group; FLX, fluoxetine; GST, glutathione S-transferase; MOX, moxidectin; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances.

Single Moxidectin dose's Effect on mRNA levels of Bdnf:

Bdnf mRNA levels were upregulated in the prefrontal cortex of the MOX-treated mice (p = 0,015) (Fig 5). Its levels did not change significantly in the FLX group when compared to the CON group (p = 0,8811). No changes were seen in *Bdnf* mRNA levels in the hippocampus (MOX p = 0,8745, FLX p = 0,7698) (Fig 5).

Figure 5



Results are presented as mean ± SEM, n = 3~5/group, * $p < 0,05$. FLX was used as positive control. Abbreviations: *Bdnf*, brain-derived neurotrophic factor; CON, control group; FLX, fluoxetine; MOX, moxidectin.

DISCUSSION

The current treatment of depressive disorders consists of psychotherapy and pharmacotherapy with compounds such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors, molecules that intervene with the metabolism of serotonin and other biogenic amines[22,23]. Even with the myriad of available compounds, treatment success rates are unsatisfactory, with up to 33% of patients not responding to four different treatment options[2,3].

This could perhaps be due to the still unelucidated pathophysiology of depressive disorders, which have been long theorized to be associated with a depletion of biogenic amines,

but this notion has been recently called into question, with some authors stating that there is no direct correlation between depressive disorders and serotonin[24]. Therefore, new targets have been explored. One of which is the recently approved compound for the treatment of PPD brexanolone, a $GABA_A$ positive allosteric modulator[25,26]

A compound that acts as a positive allosteric modulator of $GABA_A$ is MOX. Originally an antiparasitic, it was first marketed as an injectable for cattle in 1990, but nowadays it is widely utilized in most species in veterinary medicine[8] and is approved for human use by the FDA for onchocerciasis[27]. It acts by targeting the parasite's glutamate-activated chloride channels, leading to paralysis and eventual death. These channels are not found in mammals, however, which contributes to little to no side-effects in its users. In doses above the standard endectocidal, however, the compound can interact with mammalian ionotropic channels, such as the aforementioned $GABA_A$[8]. MOX has also shown promise in animal models of alcohol abuse disorder[28].

In this paper, we show that a single dose of MOX was able to significantly decrease the immobility time in the TST and increase grooming time in the SPT, suggesting an antidepressant effect.

The TST is useful for assessing the behavioral effects of antidepressant compounds and other pharmacological and genetic manipulations relevant to depression[29]. Higher immobility times are associated with a depressed mood, where this immobility may be analogous to the clinical observations that depressed patients often lack sustained expenditure of effort[30]. Therefore, decreased immobility time may insinuate a compound's antidepressant potential. MOX, in a single dose of 1,5 mg/kg, significantly decreased the immobility time in the TST. Our findings are congruent with data published by Getachew[10], which demonstrated that MOX in a single dose induced a 30% decrease in immobility in the forced swim test in rats at the dose of 2,5 mg/kg.

Anhedonia is a behavior defined as the inability to feel pleasure or the lack of pleasure-seeking behaviors, a hallmark of depressive disorders. In animals, this behavior can be evaluated by the SPT. The number of sprays, test location, time of testing, or even variable analyzed in the splash test is not always consistent throughout the literature, but the consensus is that decreased number of grooming bouts and/or decreased total grooming time in rodents is correlated with anhedonic behavior[31,32]. Our results show that a single dose of MOX could lower the anhedonic behavior of mice within 24 h. We observed increased grooming time for

both compounds, corroborating literature where FLX has been reported as capable of increasing grooming in mice[33].

Depressive disorders are often accompanied by anxiety. Therefore, the EPM test was performed to evaluate the anxiety levels of the treated animals. Albeit no significance was reached on either group, both showed tendencies to spend less time in open arms and more time in closed arms, an indication of anxiety. A review showed that FLX administration on naïve animals can have an anxiogenic effect, often shown by decreased time spent in open arms in the EPM[34].

The OFT is another popular test to measure animal anxiety-like behavior. Our results showed that both compounds were anxiogenic. MOX has been tested in rats in the OFT, but only the locomotor activity was reported as unchanged[10], this corroborates our findings. Changes in locomotion, observed as increased total travel distance and/or number of crossings in the open field test, can indicate altered neurological processes or abnormal brain function[35], therefore, the unaltered results found in the MOX group indicate no deleterious effects on the observed mice. FLX has been shown consistently as having little to no effect on locomotion in mice[34]. Even though there are no reports of MOX's effect on the time spent on each zone in the OFT, there is weak evidence that allopregnanolone, another positive allosteric modulator of $GABA_A$, can cause an increase in anxious behavior in the OFT[36].

To our knowledge, this is the first work of literature that evaluates animal response to MOX in the SPT, TST and EPM and the first to evaluate time spent in different zones in the OFT. As shown, MOX had similar effects to FLX in most behavioral parameters. The increased grooming time in the splash tests appoints to diminished anhedonia and therefore diminished depressive-like behavior, as does the decreased immobility time in the tail suspension test. These findings agree with the reports of Getachew (2019) in the forced swim test in Wistar-Kyoto rats. With the intent of exploring the molecular basis of this behavioral change, Getachew also reported increased levels of BDNF in the central nervous system of these animals via Western Blot.

BDNF is one of the main proteins of the neurotrophin family. Neurotrophins are proteins synthesized by neurons and glial cells, responsible for modulating cellular and synaptic replication, maturation, and survival, vital processes for memory and learning[37,38].

Animal and human studies show a positive correlation between BDNF levels and mood scores, mainly in the cortex and hippocampus[39]. Furthermore, BDNF mRNA abundance is

modulated by stress, therefore, this seems to be a molecule with an important role in MDD[40]. Stress causes a decrease in BDNF levels and interferes with the intracellular pathways activated by this neurotrophin, thus contributing to the atrophy and reduced activity of limbic structures[41].

The present study shows that MOX increases mRNA levels of *Bdnf* in the prefrontal cortex, but not hippocampus, of treated mice 72 h after administration. Getachew[10] showed, via Western Blot, that a single MOX dose (2,5 mg/kg) was capable of increasing the BDNF protein levels in the frontal cortex and hippocampus of rats at the 24 h mark. This rapid effect of MOX may constitute an important mechanism for fast-acting antidepressants, a current weakness of traditional treatments.

Another factor involved in neuropsychiatric disorders is oxidative stress. Oxidative stress is defined as the biologically damaging effects of free radicals such as reactive oxygen species (ROS). The presence of these molecules is normal and necessary for proper cellular functioning. However, when found in excess they may lead to cellular damage and even cellular death[42,43]. Mitochondrial dysfunction has been observed in many disorders, some of which are neuropsychiatric[44].

Some neuroactive compounds increase oxidative stress markers in the Central Nervous System[45] while others, such as FLX and other antidepressants, tend to decrease it[46–48]. Therefore, we sought to evaluate the oxidative stress biomarkers in prefrontal cortex and hippocampus of mice treated with MOX to further analyze its potential as a neuromodulator.

TBARS, is widely utilized as a generic metric of lipid peroxidation in biological fluids, often considered a good indicator of the levels of oxidative stress within a biological sample[49]. The non-significant results found in both tissues are a positive result, since they indicate that the compounds did not negatively affect the CNS of the animals. Another commonly utilized tool in the evaluation of the antioxidant capacity of tissues is TAC[50]. Our results showed MOX as capable of increasing the TAC of the mice's prefrontal cortex.

We observed a MOX-induced increase in cortical GST levels. The GSTs are a protein family of antioxidant enzymes that regulate stress-induced signaling pathways[51]. An isoform of GST, GST Mu, was found to be significantly decreased in the cortex of postmortem patients with MDD, schizophrenia and untreated bipolar disorder[52], the MOX-induced increase found in our animals may be an early indicator of a potential drug candidate for the treatment of these

ailments. Once more, to our knowledge, these are the first reported effects of MOX on the oxidative stress parameters in the CNS of mice.

The utilization of FLX, a well-established compound in the treatment of MDD, as a positive control, gives these results a more solid basis for understanding the behavioral effects of MOX. Albeit most behavioral results were similar, the mechanism of action of these compounds differs greatly. While FLX is a SSRI[53], this effect has not been demonstrated for MOX. MOX is a potent $GABA_A$ positive allosteric modulator[8], as well as a P2X4R modulator[28].

Looking to further understand MOX's role in behavior, it is important to note that the dysregulation of the hippocampus-pituitary-adrenal (HPA) axis has been implicated to play a crucial role in the pathophysiology of MDD. Changes in basal levels and in the pattern of release of corticosterone secondary to stress appear to be highly correlated with the susceptibility or resilience to depressive-like behaviors in mice[54]. Excess glucocorticoids have been shown to be neurotoxic, especially in the hippocampus, where apoptosis can be triggered by the activation of glucocorticoid receptors[55]. These events can contribute to the loss of hippocampal volume observed in MDD[56,57].

The corticotropin releasing hormone neurons receive input from a myriad of brain regions, with their activity ultimately regulated by GABAergic inhibition[58], it is estimated that a third of all inputs to that region are GABAergic[59]. In practice, microinjections of GABA antagonists into that area have shown to activate the HPA axis, while agonists decrease the circulating levels of stress hormones[60,61].

Furthermore, as commented previously, $GABA_A$ receptors are allosterically modulated by endogenous neurosteroids, such as allopregnanolone (Allo), a progesterone metabolite. Reduced levels of Allo and other neurosteroids have been observed and implicated to play a role in the pathophysiology of MDD, anxiety, Post Traumatic Stress Disorder and other neuropsychiatric ailments, while successful SSRI treatment rescues endogenous Allo levels[62,63]. Direct $GABA_A$ modulation is an emerging field in the treatment of neuropsychiatric disorders. It has gained traction mostly as a treatment for PPD with representatives such as brexanolone, the first FDA approved treatment specific for PPD[25,64].

Limitations of this study include the lack of monitoring of corticosterone levels, which could potentially confirm if MOX is capable of interfering with this pathway, and the use of

only male mice. Future studies should address these limitations and explore the effects of MOX in female mice and other neuropsychiatric disease models.

CONCLUSION

In summary, the administration of a single dose of MOX resulted in diminished depressive-type behaviors in the tested animals, demonstrated by decreased immobility time in TST and increased grooming time in the SPT. MOX also increased the antioxidant capacity in the prefrontal cortex and increased *Bdnf* mRNA levels, again indicating a potential antidepressant action with rapid onset. Albeit preliminary, these results are promising. More studies are necessary to further explore MOX's mechanism of action in a neuropsychopharmacological setting, as well as its efficacy in different paradigms, with the intention of confirming if this compound possesses clinical relevance.

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ANTIDEPRESSANT EXPLORATION OF MOXIDECTIN: EFFECTS ON BEHAVIOR, NEUROTROPHIC FACTORS AND OXIDATIVE STRESS BIOMARKERS IN MICE

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