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Hypoestrogenism alters mood: Ketamine reverses depressive-like behavior induced by ovariectomy in rats

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ABSTRACT

Background: Estrogen deficiency is associated with the onset of depressive and anxiety symptoms, cognitive impairment, and adverse consequences. We investigated depressive-like behaviors in ovariectomized rats and ketamine's effect on this behavior.
Methods: Twenty-eight female Wistar adult rats were initially divided into two groups: ovariectomized (OVX) and sham surgery (SHAM). Hormonal status was verified by vaginal cytology, and the rats were subjected to a forced swimming (FS) test 18 days post-surgery, an open field (OF) test 28 days post-surgery, and an elevated plus maze (EPM) test 38 days post-surgery (Experiment 1). In addition, the effect of ketamine on depressive-like behavior of the female rats was evaluated (Experiment 2).
Results: OVX group exhibited anxiety-like behavior on EPM test (lower time spent and fewer entries in the open arms), without any difference in performance in the OF test. OVX rats showed depressive-like behavior (higher time of immobility) than SHAM rats in FS test. The SHAM group showed signs of hypoestrogenism (anestrus) at six months of age. Moreover, ketamine was able to reverse depressive-like behavior in the FS retest in both groups (OVX and SHAM).
Conclusion: Similar to the literature, we showed the antidepressant effect of ketamine in depressive female rats which was induced by ovariectomy; including in female rats submitted to sham surgery that interestingly presented a premature menopausal.

Introduction

Menopause is a physiological process caused by the loss of ovarian follicular activity leading to a decrease in the production of estrogens. Hypoestrogenism can cause a variety of physiological and psychological disorders such as changes in the menstrual cycle, vasomotor and genital symptoms, sleep problems, mood swings, and impaired cognitive function [1,2]. Age, menopausal status, chronic diseases and socio-demographic characteristics (income, ethnicity and educational level) have been identified as predictors of the frequency and severity of menopausal symptoms [2,3].

Data from two cohort studies in the United States showed increased risk of depression in women who enter menopausal transition [4,5]. However, the mechanisms responsible for the development of depression in perimenopausal women remain unclear [6,7]. On the other hand, Díaz-Véliz et al. suggest that ovarian hormones modulate anxiety levels and cognitive functions [8]. Anxiety may be a precursor for depression development [9] or may be accompanied by symptoms of depression [10], thus, it is important to consider equally anxiety and depression symptoms when investigating factors that may affect mood, such as hormonal status [11,12].

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Depressive and anxious symptoms can significantly reduce the quality of life of postmenopausal women [13]. The mechanisms that lead to the emergence of these symptoms in the menopausal transition are not well understood, and even the pathophysiology of depressive disorders has been widely debated. The monoamine theory posits that depression is caused by a decreased function of monoamines in the brain, and antidepressant drugs are designed to increase the supply of these substances by inhibiting reuptake (serotonin and norepinephrine reuptake inhibitors) or degradation (monoamine oxidase inhibitors) [14]. However, the long time to the onset of the therapeutic action and the low rates of remission has encouraged the search for more effective drugs. The observation that small doses of ketamine produce a rapid and transient antidepressant effect increased the interest in neurobiological systems that were not explored in depression [15]. Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist used in human and veterinary medicine, primarily for the induction and maintenance of general anesthesia. Berman et al. published the first report of ketamine's therapeutic effects on major depressive disorder [15]. After this randomized controlled trial (RCT), many studies provided evidence that a single, intravenous (*iv*), subanesthetic dose of ketamine may relieve depressive symptoms within hours [16]. Garcia et al. suggest that the increase of hippocampal brain-derived neurotrophic factor (BDNF) protein levels induced by ketamine might be necessary to produce a rapid onset of antidepressant action [17]. Another study from the same laboratory demonstrated that Ketamine alters behavior and decreases BDNF levels in the rat brain as a function of time after drug administration, suggesting that the effects of ketamine on behavior and BDNF levels are related to the time at which they were evaluated after administration of the drug [18].

To understand the pathophysiology of anxiety and depression disorders associated with the decline of endogenous estrogen levels and devise interventions aimed at attenuating these symptoms, it is very important to establish and study animal models of menopause. This study investigated depressive-like and anxiety-like behaviors and cognitive performance in ovariectomized rats and ketamine's effect on performance on a FS test.

Materials and methods

Animals

Twenty-eight female Wistar rats (90 days old, 200–300 g) were randomized by weight and housed in cages of polypropylene material (49 cm × 34 cm × 16 cm). They were housed four per cage and maintained with food and water available ad libitum on a 12 h light/dark cycle (lights on at 7:00 AM, and lights off at 7.00 PM) in a temperature-controlled environment (22 ± 2 °C). The animals were initially distributed into two groups: ovariectomized (OVX) and false surgery (SHAM) and subjected to the FS at 18th day post-surgery, open field test (OF) at 28th day post-surgery, and elevated plus maze test (EPM) at 38th day post-surgery. When the animals reached 180 days old, each group was subdivided into two more groups, which received ketamine or vehicle and were again subjected to the forced swimming test. The rats were handled for seven days prior to the experiments and remained in the laboratory for at least 30 min before being submitted to each test. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 110586) and were compliant with Brazilian guidelines involving the use of animals in research (Law Nº. 11.794). Additionally, all efforts were made to minimize suffering, pain and discomfort of the animals, as well as to reduce the number of animals.

Surgical procedures

One set of Wistar female rats underwent ovariectomy (surgical removal of the ovaries) and the other set underwent SHAM surgery (opening of the abdominal cavity and sewing it back). At 90 days of age, the rats were anesthetized with ketamine (80 mg/kg, *ip*; Syntec, Brazil) and Xylazine (20 mg/kg, *ip*; Sespo, Brazil) and underwent bilateral ovariectomy. The surgery consisted of a transversal dorsolateral incision of skin, between the last rib and pelvis and muscle dissection in order to expose the abdominal cavity. The ovary is located in a fat pad beneath the muscles. The periovarian fat was grasped to lift and exteriorize the ovary. The fallopian tube was crushed and ligated, and the ovary was removed by cutting above the clamped area. The muscle and the skin incision were closed with poligalactin and nylon suture. This procedure was repeated at the other side for bilateral ovariectomy. In SHAM surgery, rats underwent the same incisions, the ovaries and fallopian tubes were exposed and then put back in the abdominal cavity and the muscle and skin were closed. To reduce pain, all rats received dipyrone (25 mg/kg *ip*) after surgery, and recovered for 10 days.

Vaginal smear

Ten days after surgery, vaginal smear was daily obtained in both groups to verify hormonal status. Samples were obtained and analyzed as described by Goldman et al. [19].

Behavioral tests

Locomotor activity assessed by OF test

The behavioral assessment was performed in a varnished wood cage, measuring 60 cm × 40 cm × 50 cm with a glass front wall. The floor was covered with linoleum and divided up with dark lines: 12 squares of 13 cm × 13 cm each. The rats were gently placed in the left back corner and allowed to explore the surroundings for 5 min. The number of line crossings was taken as a measure of locomotor activity [20]. Rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touched the floor again [21], being evaluated as exploratory activity [22]. Grooming was defined as licking/washing of the head and body; it was assessed as a biological function of caring for the surface of the body [23]. The start of a trial occurred immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four paws crossed the boundary into a different, marked-out area. Five measures were taken during the five-min test sessions: latency to leave the first quadrant (time in seconds); number of line crossings (i.e. horizontal activity), outer and inner crossings; number of rearing behaviors (i.e. vertical activity); grooming (time in seconds); and number of fecal boluses. The box was thoroughly cleaned using 70% alcohol between each trial.

Anxiety-like behavior assessed by elevated plus-maze (EPM) test

The EPM test was used to evaluate anxiety-like behavioral state. The maze was made of black PVC and elevated to a height of 50 cm above floor level. The apparatus included two open arms and two closed arms (50 cm × 40 cm × 10 cm), which extended from a common central platform (10 cm × 10 cm). The animal was placed in the central area of the EPM, facing one of the closed arms. Next, the following behavioral measures were recorded during the five-min test sessions: number of protected head-dipping movements (PHD); number of non-protected head-dipping movements (NPHD); number of entries in the open arms (EOA); number of entries in the closed arms (ECA); time spent on the open arms

(TOA); time spent on the closed arms (TCA); time of grooming and number of rearing. Protected head dips involved dipping the head over the sides of the maze from within the central platform or a closed arm, whereas non-protected head dips were considered to occur when the animal dipped its head over the sides of the maze while on an open arm. In the EPM, entering a new area was recorded when all four paws crossed onto a new arm or into the central area [24]. After each test, the apparatus was cleaned thoroughly to remove any animal scent.

Depressive-like behavior assessed by FS test

The FS test involved three phases: training, test and retest (under drug influence). A glass square tank (dimensions 40 cm × 40 cm × 52 cm, divided into four squares of 20 cm × 20 cm to fit four rats) was filled with water (22–25 °C) to a depth of 35 cm, on such a way that the rats' tail could not touch the bottom of the tank. For the first exposure, the rats were placed into water for 15 min (training session). 24 h later, the animals were again placed in the water for a 5-min session (test session). The immobility time of rats was recorded in seconds, considering total immobility and/or movements to keep the head out of the water with no intention of escaping [25]. After the trial, rats were dried using soft towels and hair dryer, if necessary.

Experimental design

Experiment 1

Eighteen days after surgery (P108), groups OVX and SHAM were subjected to the sequence of FS, OF, and EPM tests. The tests were separate at least for one week between them. All female rats were submitted to all tests one by one.

Experiment 2

At P180, both groups (OVX and SHAM) were subdivided into two more groups that received ketamine (10 mg/kg, *ip* [17,26]; Cetamin®, Syntec, Brazil) (SHAM-K and OVX-K) or vehicle (SHAM-V and OVX-V, saline, *ip*), and were subjected to FS test (retest session) 30 min after the administration. All female rats were submitted to all tests one by one.

Statistical analysis

All data are presented as mean ± SEM. All analyses were performed using the Statistical Package for the Social Science (SPSS) software version 18.0. The normality test was performed by Kolmogorov-Smirnov, and the data presented normal distribution. Unpaired and paired Student's *t* tests were used to compare groups at the same age and to compare intragroup analysis (P108 and p180), respectively; and one-way ANOVA followed by Student–Newman–Keuls (SNK) after ketamine administration. Critical significance level used for all comparisons was 5%.

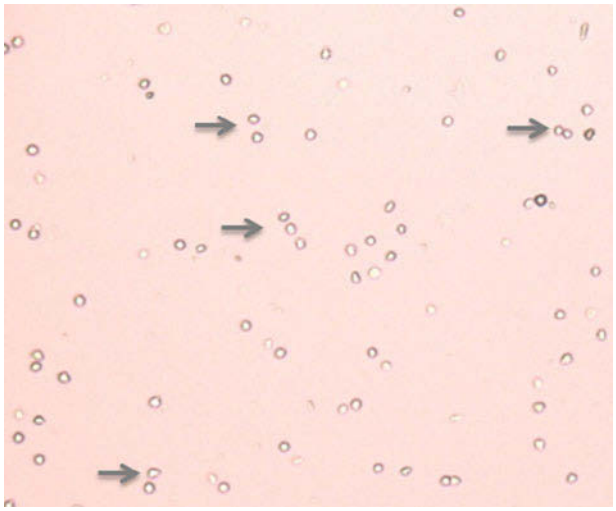

Fig. 1. Diestrus period of the oestrus cycle of female rats (the presence of diffuse few leukocytes).

Results

Experiment 1

The acyclic vaginal smears (void of nucleated (proestrus) cells) occurred in all ovariectomized rats (OVX), as verified since the beginning of vaginal smears procedures, 10 days after surgery, and continuing up to the end of the experiments (Fig. 1). SHAM group presented normal estrous cycling in the first 60 days after surgery. In addition, we did not observe any difference in the swimming time between SHAM and OVX; however, OVX group showed increased immobility time in the FS test as compared to SHAM group (Student's *t* test, $p > 0.05$ and $p < 0.05$, respectively; Fig. 2, Panels A and B; $n = 14$ per group). In the OF test, there was no significant difference between SHAM and OVX groups in all behaviors analyzed (Student's *t* test, $p > 0.05$, data not shown, $n = 10–11$ per group). In the EPM test, OVX group showed decreased time spent on open arms ($p < 0.02$), lower number of entries on open arms ($p < 0.02$) and lower number of NPHD ($p < 0.01$) as compared to SHAM group, suggesting an anxiety-like

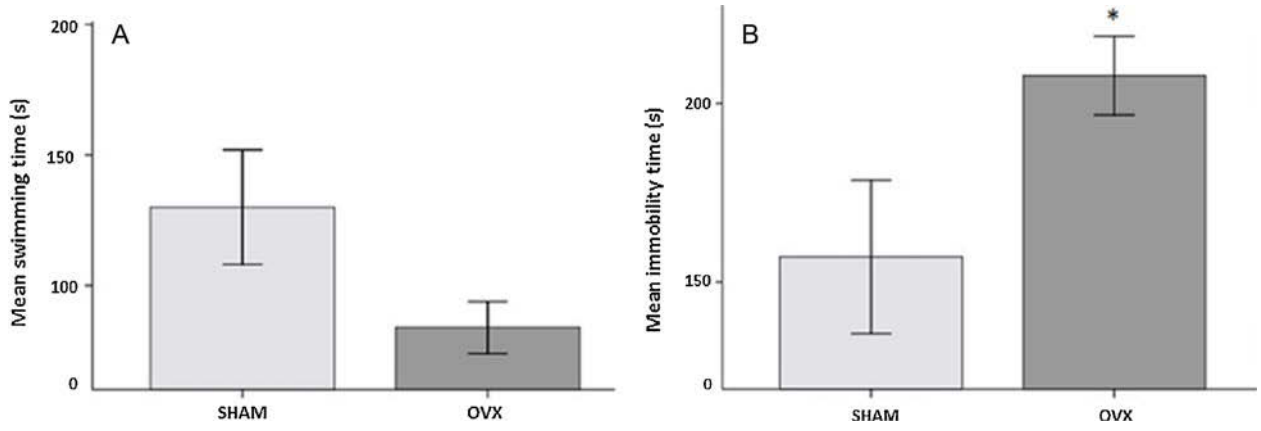

Fig. 2. Forced swimming test in Experiment 1. Data expressed as mean ± SEM, $n = 14$ animals/group. Time was expressed in seconds. Panel A: Time of swimming. Panel B: Time of immobility. *OVX was significantly different from SHAM (Student's *t* test, $p < 0.05$).

behavior in the OVX female rats (Student's *t* test, Fig. 3, Panels A–C, $n = 10–11$ per group).

Experiment 2

At P180, the SHAM group showing signs of hypoestrogenism as indexed by vaginal smears characterizing precocious menopause, increased immobility time on FS retest suggesting depressive-like behavior (paired Student's *t* test, $p < 0.05$, $n = 5–6$ per group, Fig. 4), associated to a strong tendency to be different in the swim time (paired Student's *t* test, $p = 0.05$, $n = 5–6$ per group, Fig. 4). In addition, both groups that received ketamine (SHAM-K and OVX-K groups) improved its performance and decreased the immobility time, i.e. ketamine reversed the depression-like behaviors in the FS retest (one-way ANOVA/SNK, $p < 0.05$, $n = 5–6$ per group, Fig. 5).

Discussion

In this study we showed, at the first moment, that ovariectomized female rats presented depressive-like and anxiety-like behaviors. It is interesting to note that the OVX female rats showed anxiety-like behavior in the EPM test without any impairment in the locomotion assessed in the OF test. Our findings corroborate previous studies showing the modulatory effects of ovarian hormones upon behavioral indices of anxiety [8,27]. Díaz-Véliz found different effects of diazepam on conditioning avoidance and motor activity in female rats, according to hormonal status [28]. Moreover, the anxiety-like behavior of OVX group on EPM test corroborates Kessler et al.'s studies (in 2005 and 2008) that showed association between depressive and anxiety disorders [10,29]. The estradiol injection, compared to vehicle, subcutaneously or into the hippocampus or amygdala of ovariectomized rats decreases anxiety-like and depression-like behaviors, as reported by Walf and Frye [27]. These authors also showed that chronic estradiol replacement to aged female rats reduces anxiety-like and depression-like behavior and enhances cognitive performance [30].

Interestingly, afterwards three months later, the SHAM surgery group presented the cessation of normal estrous cycling, exhibiting aberrant cycling patterns at 180 days old, including an increase in the number of days per cycle and a decrease in number of vaginal cells, on vaginal smears, followed by persistent diestrus. This result characterized a precocious menopause, and those also presented a depressive-like behavior. The OVX rats remained with similar behavior presented at P108. Moreover, the NMDA receptor antagonist (ketamine) was able to reverse this behavior in both groups evaluated (OVX and SHAM groups).

The precocity of the ovarian failure was an unexpected finding, for, in general, female rats only show no estrous cycle when they

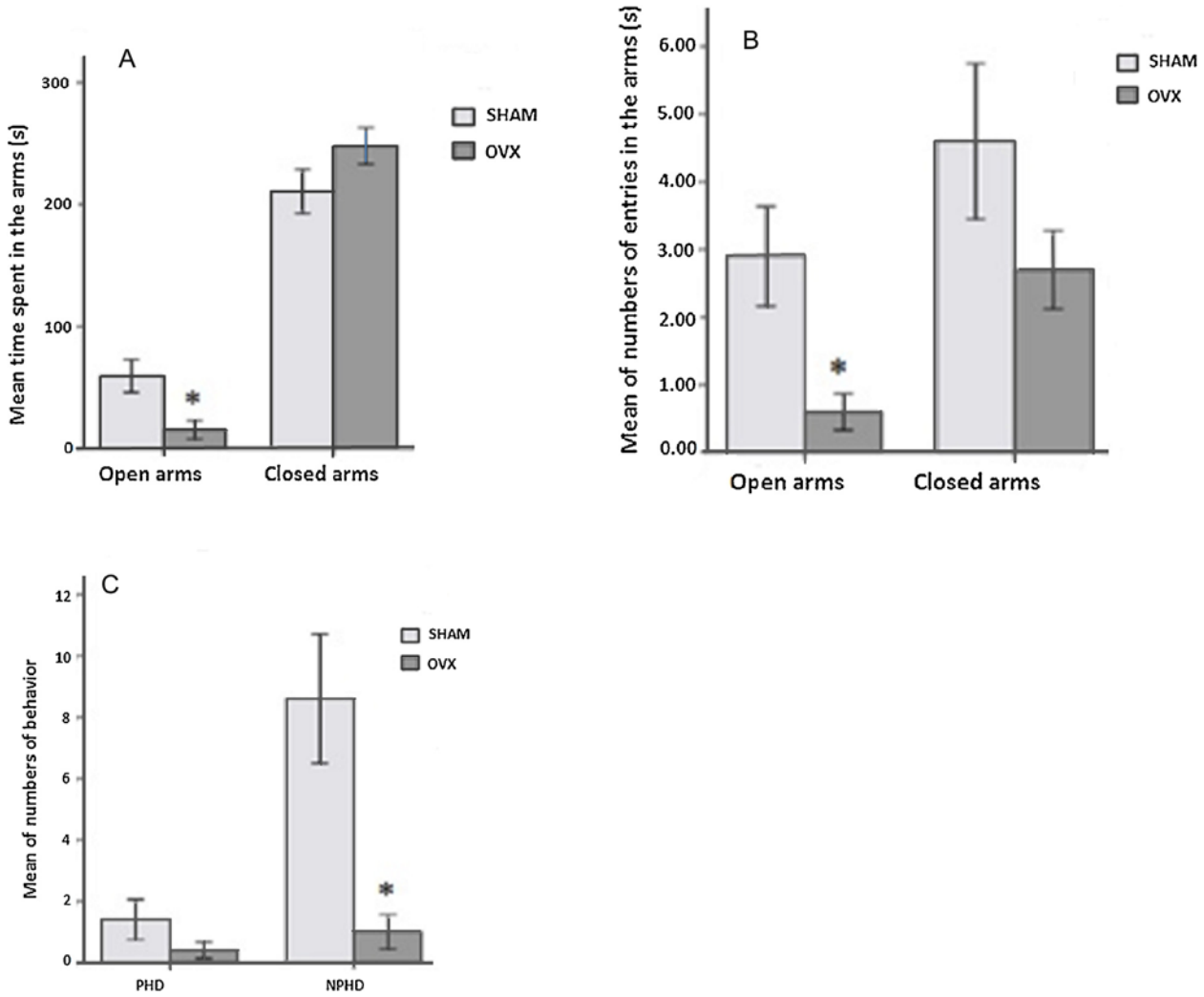

Fig. 3. Elevated Plus Maze test in Experiment 1. Data expressed as mean ± SEM. Panel A: Time spent in the arms was expressed in seconds. Panel B: Number of entries in the arms. Panel C: Number of PDH and NPHD behaviors. PHD: number of protected head dipping. NPHD: number of non-protected head dipping. *OVX different from SHAM group (Student's *t* test; Panel A, $p < 0.02$; Panel B, $p < 0.02$; Panel C, $p < 0.01$).

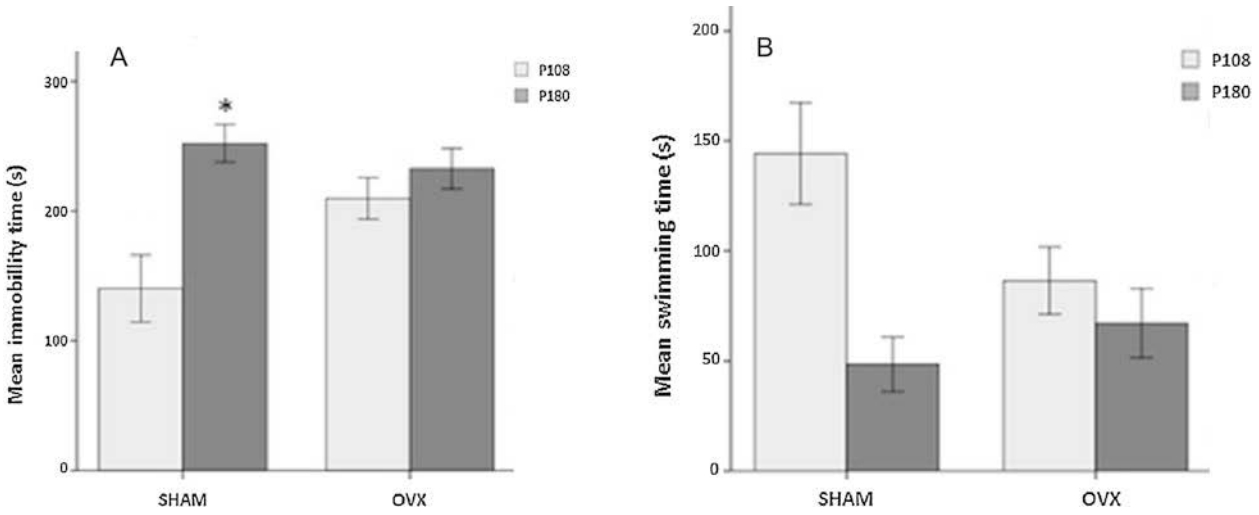

Fig. 4. Forced swimming test at P108 and P180 in OVX and SHAM groups. Time was expressed in seconds. Data expressed as mean ± SEM (n = 5–6 animals/group). Panel A: Time of immobility. Panel B: Time of swimming. *SHAM group different at P180 from P108 in the immobility time (paired Student's t test, $p < 0.05$).

reach about 12 months [31]. Reproductive senescence in rodents is similar to menopause in several critical aspects, including similar alterations in pulsatile LH release and the LH surge, variability of cycle length prior to acyclicity, and ultimate cessation of hormone cycling. We hypothesized that ovarian and fallopian tubes exposition through the small dorsolateral opening may have accelerated ovaries aging by some mechanism such as trauma or impairment of blood circulation, which possibly elicits irreversible degenerative responses or necrosis in the ovaries cells.

Depressive-like behavior on aged SHAM group was less evident than on OVX group. This finding indicates that depressive-like behavior is present on both, natural or artificial ovarian depletion in female rats, the latter being more evident, probably because of gradual lowering of hormone levels on aging, and this mimics natural menopause in humans. There are several transgenic mice models of ovarian senescence. However, in such cases, fetal development of the reproductive system has been affected and normal reproductive function has never been achieved [32]. These data suggested the possibility of a new animal model of menopause transition, in which there is a gradual ovarian failure, which can be used in researches on menopause symptoms, more closely mimicking the biology of natural menopause in humans.

Thus, female rats with surgical (OVX) and premature menopause (SHAM) showed depressive-like behavior probably related to hypoestrogenism. These results are consistent with the scientific evidence about neuromodulatory effect of estrogen on mood [6–8,13]. The amygdala and hippocampus are brain regions known to be involved in mood regulation and animal studies have reported that the amygdala has one of the highest densities of estrogen receptors in the brain [33]. Estrada-Camarena et al. studied ovariectomized female Wistar rats, using the FS test, and found that estrogens have antidepressant-like effect characterized by a reduced immobility and increased swimming time and facilitate the action of fluoxetine and desipramine [34,35]. However, these

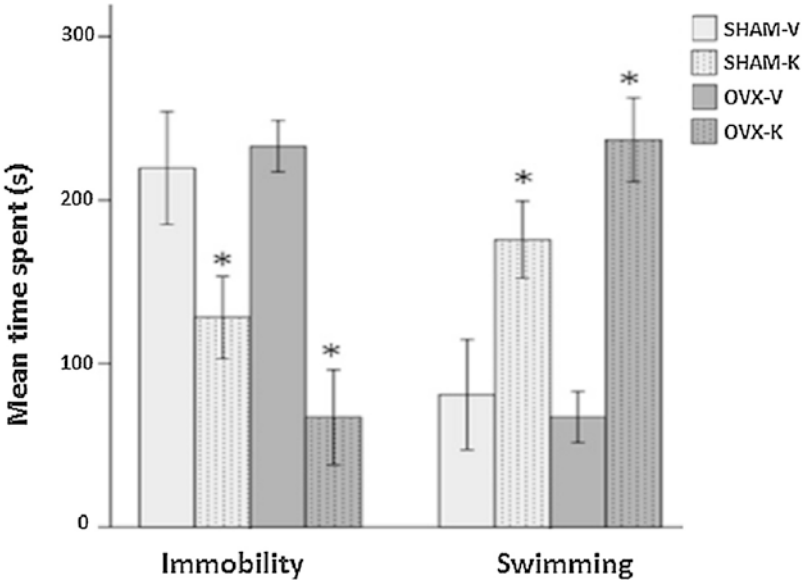

Fig. 5. Forced swimming test at six months in OVX and SHAM groups under use of ketamine or vehicle. Time was expressed in seconds. Data expressed as mean ± SEM (n = 5–6 animals/group). SHAM-V: rats under sham ovariectomy and vehicle (*ip*); SHAM-K: rats under sham ovariectomy and ketamine (10 mg/kg *ip*); OVX-V: rats under ovariectomy and vehicle (*ip*); OVX-K: rats under ovariectomy and ketamine (10 mg/kg *ip*). *SHAM-K and OVX-K different from SHAM-V and OVX-V groups (one-way ANOVA/SNK, $p < 0.05$).

effects were dependent on the type of estrogen used, and all combinations of estrogens and antidepressants decreased rats' locomotor activity when evaluated in the open field test [34,35].

In the present study, the depressive-like behavior was reversed in both groups (OVX and SHAM) by ketamine, a non-competitive antagonist of the NMDA receptor that has been studied as an antidepressant drug in the sub-anesthetic dose. Most of the experimental studies on antidepressant effects of ketamine use male rodents [36]. Carrier and Kabbaj demonstrated that intact female rats are more sensitive than male rats to the antidepressant effects of ketamine [37]. In addition, we used a dose of 10 mg/kg, as described by Garcia et al. [17] and Yang et al. [26], and the ovariectomized rats presented a significant reduction in the immobility time on the FS test. Our finding complements those of Carrier and Kabbaj study, showing a dose-dependent action of ketamine in ovariectomized female rats.

In general, rodent models use stress-induced impairment of hippocampal function to produce the depressive-like behavior and test new antidepressants [38]. Estrogens act through ERα and ERβ-receptor subtypes to modulate the transcription of genes, which, in turn, encode a variety of proteins. These proteins include many of the enzymes that play a key role in the synthesis and function of neurotransmitters including serotonin. Estrogens also have multiple effects on dopamine systems, including upregulation of dopamine D1A receptors [39] and increase of dopamine transporters' density [40]. Serotonin hetero-receptors are present in dopamine neurons creating multiple interaction points between estrogens, serotonin and dopamine neurotransmission that may be the link between depression and perimenopause in women with increased vulnerability. Yet, ketamine does not work through the "conventional" antidepressant mono-aminergic targets of serotonin and noradrenaline [41], since this effect is classically based on NMDA receptor antagonism. Then its action on a model of menopause depressive-like behavior launches new pathways into the study of the pathophysiology of depression in this period of the women's life. It is important to note that the action of estradiol on glutamatergic system, in relation to the lordosis behavior of female rats, has been well established in the works of McCarthy [42]. Thus, we can suggest that the reversion of depressive-like behavior in OVX rats makes a point of the role of glutamate in the depressive symptoms of menopause. The role of progesterone also has to be considered, since studies demonstrated proestrus increases in anxiolytic-like behavior in female rats that were coincident with elevated circulating and hippocampal progestin concentrations [43]. In addition, it is important to note that previous studies have been demonstrated that ketamine is able to increase some signaling proteins and synapses number and functions in the prefrontal cortex [44]. Other recent study showed that the antidepressant effect of ketamine can be linked to the synthesis of brain-derived neurotrophic factor (BDNF) [45].

It is important to point out an important limitation of our study that was the lack of control group. However, we need to consider that when the female rats were submitted to the SHAM ovariectomy surgery, we did not expect that those animals could present the hypoestrogenism. And, we believed that our results did not suffer any impairment with this decision, and add a new advance to animal model of menopause studies.

In conclusion, this study shows evidences about neuromodulatory effect of estrogen on mood, and ketamine's acute action on depressive-like behavior on a model of menopause. In addition, we observed an interesting finding, the premature induction of animal model of menopause induced by ovarian and fallopian tubes exposition. However, further researches are needed to clarify the mechanisms by which ovarian and fallopian tubes exposition through dorsolateral incisions may have caused precocious ovarian failure in female Wistar rats. Therefore, it is necessary to development new studies for better understanding about the intrinsic mechanisms related to depressive-like behavior induced by hypoestrogenism, and the involvement different receptor and signaling of the ketamine action.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the contents and writing of this paper.

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