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Impaired discriminative avoidance and increased plasma corticosterone levels induced by vaginal lavage procedure in rats

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ABSTRACT

Historically, females have been neglected in behavioral neuroscience research due to the alleged increased variability caused by hormonal fluctuations. More recently, there has been a tendency to include female subjects in the studies, in a majority of those cases with the condition that the hormonal variation is controlled. In rodent studies, the vaginal lavage procedure is a common method of collecting smears and determining the estrous cycle phase. However, little is known regarding the consequences of the procedure, although stress is often mentioned as a concern. Within the neuroscience field, spatial memory has been a relevant subject in terms of sex differences. The plus-maze discriminative avoidance task (PMDAT) allows for the concomitant evaluation of spatial memory, anxiety-like behavior, and locomotion, as well as possible interactions between these behaviors. The aim of the present study was to investigate the effects of the vaginal lavage procedure (VLP) on the performance of female rats in the PMDAT. We submitted adult female Wistar rats to VLP for 14 straight days and then to training and test sessions in the PMDAT. Additionally, another set of animals was submitted to the VLP procedure for determination of plasma corticosterone levels. Rats submitted to the vaginal lavage procedure did not discriminate the enclosed arms of the PMDAT apparatus, indicating impaired performance, but no anxiety-like alterations were found. VLP also resulted in a higher corticosterone level, suggesting it is a stressful manipulation. As such, the use of this method to control for hormonal variation should be restricted in behavioral studies.

1. Introduction

The natural fluctuation in females' hormone levels has been an argument for avoiding their inclusion in animal research for many years [1]. From a narrow point of view, including females in animal research could lead to increased data variability, lower statistical power, or the need for a large sample size [2]. However, even if females are more variable than males, this should not diminish the relevance of studying both sexes in biological and health research. Instead, this characteristic should propel further inquiries. Despite that, in 1977, the Food and Drug Administration (FDA) recommended excluding women and children from studies [3], reinforcing this historical negligence. It was only in the 1990's that funding agencies such as the National Institute of Health (NIH) demanded that women be included in clinical trials [4]. In parallel, the FDA reviewed the guidelines in 1993 to remove the rule obliging the exclusion of female subjects [3]. Almost 20 years later, the negligence of female subjects in animal research began to be questioned. In 2010, Nature and Science published articles reinforcing the relevance of studying females in basic research [1,3,5,6], leading to a recent tendency to include female animals in the studies.

The neuroscience field has been pointed out as one of the most neglectful fields on female sex representativeness. Researchers claim that variability of sexual characteristics is a substantial biological variable in their studies [7]. As previously mentioned, one oft-used justification for focusing on males is a regular variation in hormone production across the reproductive age. In this respect, recent studies

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suggest that basal hormonal variability is not exclusive to female subjects, as an intrinsic variability has also been observed in males [8–10]. In particular, when dealing with the subject of neuroscience, one should consider that the main sex hormones, estrogen and testosterone, are neuromodulators [11,12]. Additionally, sex hormones are not the only ones that vary across time or individuals. For example, corticosterone levels in dominant male rats are 5 times higher than in subordinates [13]. This variable should be as relevant as the female estrous cycle when studying behavior and other aspects of neural functioning in rodents. In summary, both female and male subjects display hormonal variations, which corroborate the fact that male and female rodents are similar regarding the variability of more than 140 traits [8,14]. Yet, it is essential to highlight that half of the traits analyzed in Dayton's study [14] revealed a sex difference. Therefore, besides the issue of unquestionable relevance, hormonal variation is not a valid reason for the exclusion of female subjects from biological sciences, particularly neuroscience. More importantly, considering all these facts we cannot expect male subjects to be the standard for both sexes.

Another issue regarding the inclusion of female subjects in neuroscience studies is the alleged inevitable need to monitor the natural hormonal cycle in order to control for the variations in behavior. However, although the determination of the hormonal cycle stage could be valuable to data interpretation [15–19], it is not always seen as a necessary procedure [9]. Indeed, the need for this procedure should take into account the relevance of hormonal variation to the focus of the study. For example, although in humans the menstrual cycle has been linked to emotional variations, there is little evidence of cognitive alterations [20].

In rodent studies, vaginal smear cytology is a method used to determine the estrous cycle stage according to the profile of cell types [21]. The procedure for sampling the vaginal smear is relatively non-invasive, although the repeated collection may induce pseudopregnancy [21,22]. Other common consequences of performing vaginal lavage are lesions in the vagina leading to chronic inflammation and anestrus cycles, but these are poorly documented. Stress is also frequently mentioned [21,23] as a problem involved in the collection of vaginal lavage, but studies do not specifically test this assumption by means of determining the corticosterone levels, for example. Furthermore, essentially no consideration has been given to possible adverse effects of vaginal smear evaluation on behavior.

Chronic exposure to stressful conditions is known to cause negative effects on brain structures [24]. Particularly, stress alters cognitive function, including learning, memory, and emotional processing [25]. Additionally, stress can also alter estrous cycling [26]. Recent data have shown that some types of repeated stress in female rats induce memory deficits in the plus-maze discriminative avoidance task (PMDAT) [19]. The PMDAT is a memory task used to investigate spatial memory within an aversive context, anxiety-like behavior, and locomotion, as well as possible interactions between these phenomena [27–31]. Although studies using female rodents have been performed with this task, none of these considered the effects of vaginal smear collection on behavioral performance [19,32–35]. The aim of the present study was to investigate the effects of vaginal lavage procedure on PMDAT performance. In addition, we sought to compare plasma corticosterone levels in females that were subjected or not to this procedure.

2. Methods

2.1. Animals

Three-month-old female Wistar rats (200–250 g) were kept in groups of 4 animals in regular cages (37 × 30 × 16 cm) in a room with acoustic isolation, controlled airflow and temperature (25 ± 1 °C) and a 12 h light/12 h dark cycle (lights on 06:30 a.m.). Food and water were provided ad libitum. The animals were handled according to the Brazilian law for the use of animals in scientific research (Law Number 11.794), and the local ethical committee approved all procedures (CEUA/UNIFESP Protocol # No. 9,117,090,917). All efforts were made to minimize pain, suffering, or discomfort caused to the animals, as well as reduce the number of animals used. Before the onset of any experimental protocol, all animals were gently handled for five minutes/day for five days.

2.2. Vaginal lavage procedure (VLP)

Collection of vaginal smears, the classical procedure to monitor estrous cycle phases, was conducted daily during the whole experimental protocol (16 days for experiments 1 and 2). In the first experiment, we conducted VLP for 14 days previously to the behavioral procedures and during the 2 days of the experiment. In these 2 days, VLP was conducted after the PMDAT sessions. In experiment 2, we collected vaginal smears for 16 days prior to euthanasia. The cage was removed from the cage rack and taken to a different room. After removal of the water bottle, the researcher opened the cage, and wrapped one hand around the back of the rat to pick it up. The rat was placed in the lid and the tail was lifted, exposing the vagina. The tip of a plastic pipette filled with 100 μL of distilled water was gently introduced into the vagina of the rat; the bulb of the pipette was slightly pressured 2 or 3 times, and the vaginal fluid collected. The tip of the pipette measured less than 2 mm in diameter. The procedure was performed carefully, the rats were handled gently to reduce stress to a minimum, and the whole collection procedure took no more than 30 s.

The material was placed on glass slides, dyed with methylene blue, and examined under a light microscope. The estrous cycle was identified according to cytological features: metestrus (or early diestrus) - a similar proportion of nucleated cells, leucocytes, and cornified cells; diestrus - the predominance of leucocytes; proestrus - the predominance of nucleated epithelial cells; and estrus - the predominance of cornified cells [34]. The estrous cycle phase was evaluated for the 14 days previously to the beginning of the behavioral experiment, as this is considered an adequate time window to ensure that females have regular cycles [16,34,36,37], with the observation of at least 2 complete cycles [21].

Control females (not submitted to VLP) were briefly handled for 5 consecutive days. Handling consisted of removing the animals individually from the home cage, gently holding them without restricting their movements. Naïve rats were not handled at all until the euthanasia.

2.3. Plus-maze discriminative avoidance task (PMDAT)

The apparatus employed is a modified version of the conventional elevated plus-maze, made of wood and painted black, with two enclosed arms (one aversive – *Av*- and one non-aversive – NAv; 50 cm in length × 15 cm wide × 40 cm high) opposite to two open arms (OA; 50 cm in length × 15 cm wide) (Fig. 1). The PMDAT was conducted over two sessions: training and test, each session lasting 10 min. In both sessions, the animals were individually placed in the center of the apparatus facing one of the open arms. In the training session, the aversive stimuli were turned on each time the animal entered with the whole body in the aversive enclosed arm and turned off when the animal left the arm. The aversive stimuli were an 80 dB noise and a 100 W light produced by speakers and lamps, respectively, placed above the *Av* arm. In the test session (24 h later), the animals were placed again in the central segment and allowed to explore the apparatus without presentation of the aversive stimuli for assessment of memory retrieval. Learning and memory were evaluated by the percentage of time spent in the aversive arm [%TAV = time in *Av* / (time in NAv + *Av*) × 100] throughout each session (in five blocks of 120 s each) and comparing the total time spent in the *Av* versus the NAv in the training and test sessions, respectively. The number of entries in the *Av* arm (in the training and test sessions) and the latency to enter the *Av* arm in the test session were also measured. Anxiety-like behavior was evaluated by the percentage of time spent in the open arms [%TOA = time in OA / (time in NAv + *Av* +

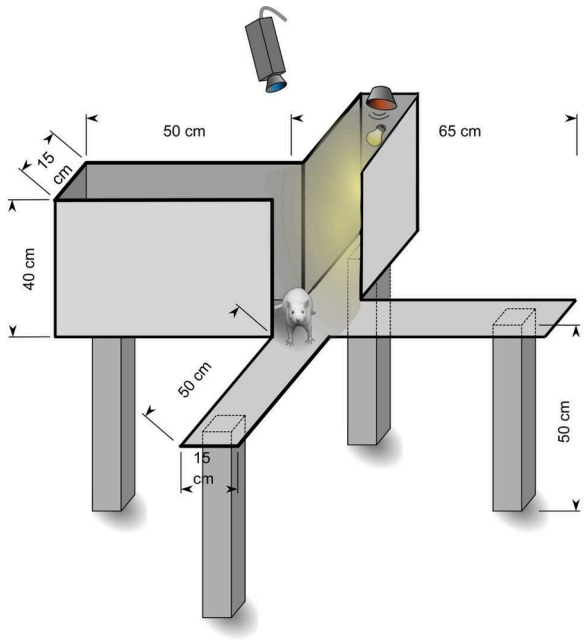

Fig. 1. Plus-maze discriminative avoidance task apparatus [39].

OA) × 100]. Similarly to the conventional elevated plus-maze, the lesser time spent in the open arms reflects higher levels of anxiety-like behavior. We also measured the frequency and time spent in risk assessment behaviors (stretched attend posture – SAP and head dipping). SAP is characterized by the animal stretching forward and then retracting to the original position, without doing any forward locomotion, to investigate a zone of the apparatus before entering it (or not). Head dipping is the orientation of the animal's head over the edge of the open arms of the maze, with the head pointing towards the floor. An increase in head dipping indicate less anxious behavior [38]. The distance traveled in the maze was used as a measurement of locomotion. The sessions were recorded by a digital camera placed above the apparatus, and the behavioral parameters were registered by a video-tracking software (Anymaze, Stoelting, USA). At the end of each behavioral session, the apparatus was cleaned with a 5% alcohol solution.

2.4. Determination of corticosterone levels

All animals were decapitated between 2 and 4pm, and trunk blood samples were collected in EDTA-containing tubes within 1 min after decapitation. Samples were centrifuged at 3000 rpm for 10 min. Plasma was collected and stored at −80 °C until the assays using an ELISA with commercial kit RE52151 (IBL International, Hamburg, Germany) and run in standard duplicates. The detailed procedure was conducted according to the instructions provided by the manufacturer. After the animals were euthanized, the subsequent collection of both blood samples and vaginal smears was performed.

2.5. Experimental designs

Experiment 1: Thirty-six rats were submitted ($n = 18$) or not ($n = 18$) to VLP for 14 days and then submitted to the training and test sessions in the PMDAT.On test days, VLP was carried out after the PMDAT session. We performed the analysis of risk assessment behavior on 16 of the rats (8 per group), and the remaining parameters were registered for all subjects.

Experiment 2: Thirty rats were submitted ($n = 10$) or not ($n = 20$) to VLP for 16 days and then decapitated to determine corticosterone plasma levels. The 20 females that were not submitted to VLP were allocated to the naïve group, which had their first contact with the researcher at the moment of the decapitation ($n = 10$), or to the control group, which was submitted to 5 days of handling prior to euthanasia ($n = 10$). The vaginal lavage of all rats was collected immediately *post mortem,* and we used the estrous cycle phase as a cofactor in the analysis, as this can interfere with the corticosterone levels [40, 41].

2.6. Statistical analysis

The analyses were conducted using SPSS software (version 22). We checked all data for normality with the Shapiro-Wilks test. Distance, time in the open and closed arms, entries on aversive arms,%TAV in total session, latency to first entry, head dipping and SAP were analyzed using non-parametric tests. TAV in blocks of 2 min and corticosterone levels were evaluated using parametric tests as indicated by the normality test. One control rat was excluded for not spending any time in the aversive arm during the training session. Pairwise comparisons between time spent in *Av* and NAv arms were performed using Wilcoxon matched-pairs signed-rank test. One-way ANOVA with repeated measures was run for the%TAV in 2-minute blocks across training and test

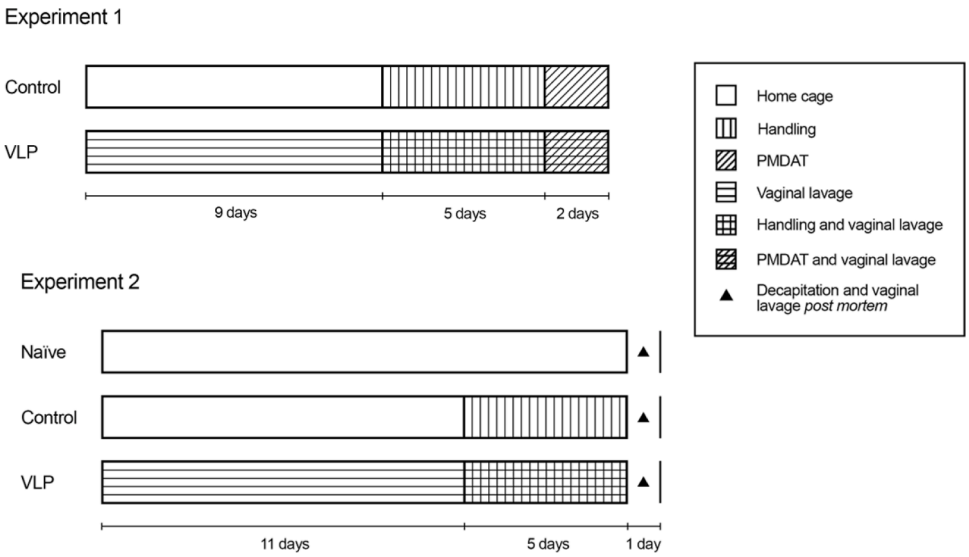

Fig. 2. Experimental design for experiments 1 and 2.

sessions with the presence or absence of vaginal lavage procedure as a between-subject factor. Mann-Whitney U tests were conducted to compare entries in *Av*, latency to enter *Av* in the test session, total distance, and risk assessment parameters (number and duration of head-dipping and SAP). One-way ANOVA was run for corticosterone levels between naïve, control, and VLP groups, and the estrous cycle phase was considered as a cofactor, Sidak post-hoc was also run. Effect size was measured by Eta-squared based (for Mann-Whitney U tests) or Eta-squared (for ANOVAs). All results were considered significant at $p \leq 0.05$.

3. Results

3.1. Learning and memory

Wilcoxon matched-pairs signed-rank tests were conducted to compare the time spent in *Av* and NAv arms for both the control and VLP groups. In the training session, both groups spent less time in *Av* than in NAv [control ($Z_{(16)}=-3.621$, $p = 0.000$); VLP group ($Z_{(17)}=-3.724$, $p = 0.000$)], suggesting that all the animals learned the task (Fig. 3A). Furthermore, in the training session, one-way ANOVA with repeated measures detected a time effect [$F_{(4136)}=12.022$, $p = 0.000$] for% TAV, suggesting increasing avoidance of *Av* across time. In summary, all animals learned the task, regardless of the VLP (Fig. 3C).

In the test session, Wilcoxon matched-pair signed-rank test showed a significant difference between the amount of time the control group spent in the aversive arm compared to the non-aversive arm [$Z_{(16)}=-3.101$, $p = 0.002$], but no significant difference was found in the VLP group (Fig. 3B). Moreover, females submitted to VLP spent less time in the NAv than control animals [$U_{(34)}=85.000$, $p = 0.025$; η^2 based=0.148]. ANOVA with repeated measures for% TAV revealed an effect of time [$F_{(1,35)}=6.88$, $p = 0.013$]. Although not statistically significant, VLP females tended to spend more time in the *Av* arm than control rats across time blocks (Fig. 3D). In addition, inf the test session, VLP females entered the aversive arm more than controls [$U_{(34)}=85.500$, $p = 0.025$; η^2 based=0.147]. No significant differences were seen in the latency for entering the *Av* for the first time in the test session ($p>0.05$) (Table 1).

3.2. Anxiety-like behavior and locomotor activity

In the training session, we did not observe significant differences ($p = 0.782$) in the percentage of time spent in the open arms (%TOA) between control and VLP groups (Fig. 4A). In the test session, VLP group spent more time in the open arms than the control group [$U_{(34)}=82.500$; $p = 0.020$; η^2 based=0.159] (Fig. 4B). In addition, no differences were seen in head dipping or SAP behaviors ($p>0.05$) (Table 2). Regarding locomotor activity, VLP group travelled a larger distance than control animals [$U_{(34)}=62.000$, $p = 0.002$; η^2 based=0.265] during the test session (Table 1).

3.3. Corticosterone levels

One-way ANOVA showed a significant difference in the corticosterone levels between conditions [$F_{(2,29)}=5.075$, $p = 0.014$; $\eta^2=0.519$]. Although we considered the estrous cycle as a cofactor, it was a non significant parameter ($p = 0.491$). Female rats submitted to the VLP showed higher corticosterone levels compared to control ($p = 0.021$)

Table. 1
Effects of vaginal lavage procedure on the number of entries into the aversive enclosed arm, latency to the first entry into the aversive arm in the test session and total distance traveled during training and test sessions. * $p<0.05$ compared to the control group (Mann-Whitney U test). Data are expressed as Mean±*S.D.*

Parameter	Group	Session	
		Training	*Test*
Aversive Arm entries	*Control*	08.24 ± 03.15	09.47 ± 03.68
	VLP	10.22 ± 03.26	11.83 ± 03.11 *
Latency (s)	*Control*	–	19.66 ± 28.53
	VLP	–	17.61 ± 15.71
Travelled distance (m)	*Control*	20.64 ± 06.60	24.77 ± 07.06
	VLP	21.77 ± 04.70	27.61 ± 06.56 *

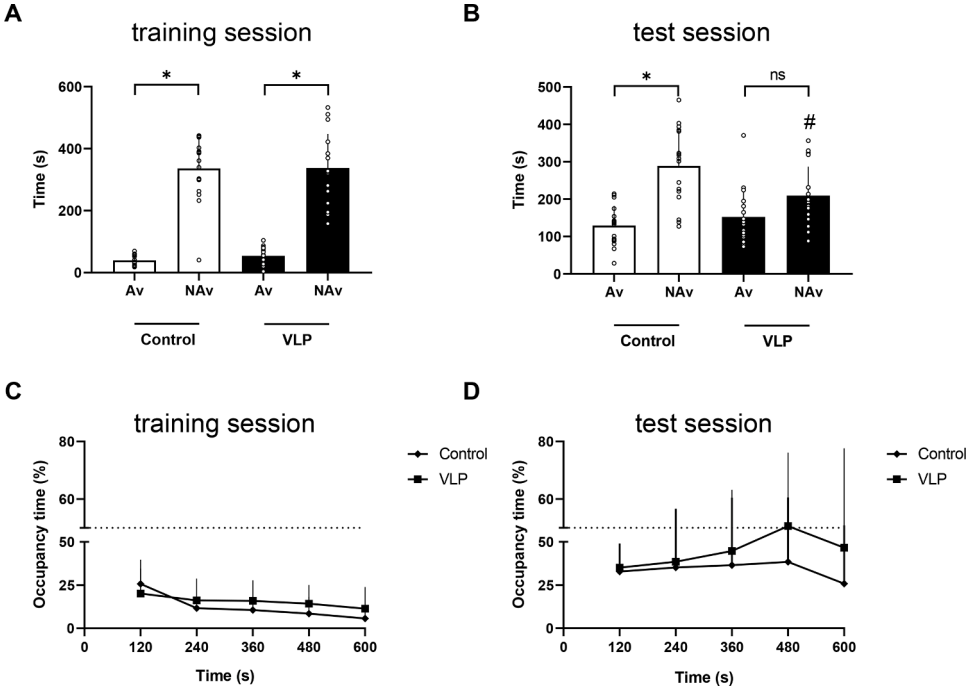

Fig. 3. Effects of the vaginal lavage procedure on time spent in the aversive (*Av*) and non-aversive (NAv) enclosed arms (A-B) and% time spent in the aversive arm across time (C-D) during training (A, C) and test (B, D) sessions. * $p<0.05$ compared to time spent in the non-aversive arm (Wilcoxon matched-pairs signed-rank test), # $p<0.05$ compared to control group (Mann-Whitney U test). Data are expressed as Mean+*S.D.*

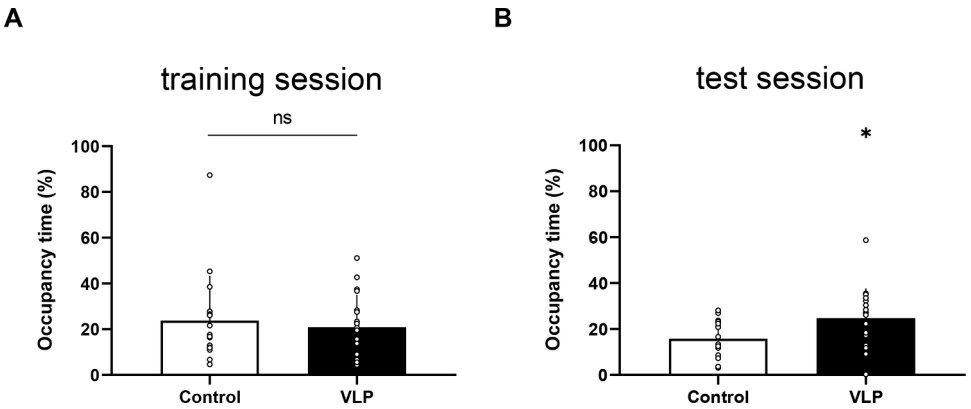

Fig. 4. Effects of vaginal lavage procedure on% time spent in the open arms during training (A) and test (B) sessions. * $p<0.05$ compared to the control group (Mann-Whitney U test). Data are expressed as Mean+*S*.D.

Table 2
Effects of vaginal lavage procedure on the number and duration (s) of head dipping and SAP in the open arms during training and test sessions. Data are expressed as Mean±*S*.D.

Parameter	Group	Session	
		Training	Test
Head dipping (number)	*Control*	20.00 ± 11.11	11.85 ± 04.05
	VLP	19.38 ± 09.86	14.88 ± 07.59
Head dipping (duration)	*Control*	16.81 ± 09.68	27.29 ± 34.08
	VLP	22.95 ± 12.15	19.68 ± 12.98
SAP (number)	*Control*	04.29 ± 03.73	06.57 ± 03.78
	VLP	04.75 ± 04.20	04.25 ± 03.33
SAP (duration)	*Control*	04.80 ± 03.73	07.86 ± 05.95
	VLP	04.41 ± 04.72	05.89 ± 02.99

and naïve ($p = 0.006$) rats (Fig. 5).

4. Discussion

The main results found in the present study were: (1) females submitted to VLP did not discriminate the aversive arm in the test session of PMDAT, indicating impaired performance, (2) no alteration on anxiety-like behavior was observed in the training session and (3) females submitted to VLP showed significantly higher corticosterone levels.

In the training session, all animals discriminated the aversive arm, indicating that VLP did not disrupt learning or acquisition of the task. Nevertheless, in the test session, females submitted to VLP explored both enclosed arms equally indicating an impairment of discriminative avoidance. However, VLP rats showed a tendency to increase aversive arm exploration over time in the test session. Therefore, we cannot exclude the possibility of a faster extinction of the task. Nevertheless, it was clear that VLP rats presented altered behavior during the test session compared to the control group.

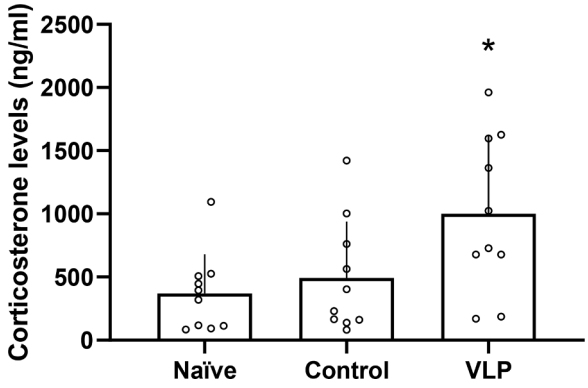

Fig. 5. Effects of vaginal lavage procedure on corticosterone level. * $p<0.05$ compared to naïve and control groups (ANOVA and Sidak post-hoc). Data are expressed as Mean+*S*.D.

Stress alters the performance in memory tasks [42,43]. Our data corroborate previous studies showing that 7 days of restraint, social isolation, and overcrowding-induced stress impaired the performance of female rats in the PMDAT [19]. Different manipulations can be chronic stress inducers (such as restraint, social isolation, overcrowding) as long as the animals go through some physiological and behavioral changes, and express, for example, higher corticosterone levels [44,45]. Therefore, although the vaginal lavage collection is not classically considered a stress-inducing procedure, it led to increased corticosterone levels and behavioral alteration. Besides the impairment in discrimination between the enclosed arms, rats that underwent VLP traveled a greater distance in the test (but not training) session. This outcome suggests a deficit in the habituation to the apparatus, although this group already presented a non-significant increase in locomotion in the training session.

In the training session, no differences in the exploration of the open arms were seen. Conversely, in the test session, rats that were submitted to the VLP explored the open arms more than controls. In this respect, studies have shown that the anxiety-like behavior can be evaluated only during a first exposure to the apparatus [27,46–50]. Therefore, the increase in open-arm exploration in the test session may have reflected another feature of the previously mentioned habituation deficit. Thus, the VLP did not alter anxiety-like behavior, even though it was a stressful manipulation. The lack of differences in risk assessment corroborates this result. The present data are in line with the study of do Nascimento [19], in which females were tested in the PMDAT after 3 different types of stressful conditions (restraint, social isolation, and overcrowding) and most groups did not display changes in distance traveled, time in open arms, and risk assessment behaviors in the training session.

Changes in anxiety-like behaviors in the elevated plus-maze by stressed females are controversial. In fact, decreased [51], increased [52] or unaltered [53] open-arm exploration in the plus-maze have been reported for female rats submitted to stressors. Consequently, the stress response of female rats in anxiety-like behaviors evaluated in plus-maze paradigms is not yet clear. Notwithstanding, in most of the studies (including all of the above), female rats were submitted to vaginal lavage or another procedure to evaluate the estrous cycle phase without a control group that was not submitted to the procedure.

Our results showed that female rats submitted to estrous cycle evaluation procedure had higher corticosterone levels and impaired performance in the PMDAT, suggesting that the vaginal lavage is not merely a tool, but a stressful manipulation that can alter behavior and induce physiological changes. This outcome has direct implications for

studies that verify sex differences on behavior when estrous cycle monitoring by vaginal lavage is performed. In other words, because only the female rats are submitted to this potentially stressful procedure, the interpretation of a sex-related altered performance might not be accurate.

The performance in the PMDAT requires the processing of spatial information [54] and most of the studies report that females perform worse than males in spatial tasks [55,56]. Nevertheless, other studies have shown that females react differently to stimuli involved in memory tasks, or even have different solution strategies when compared to males [32,56–58]. Additionally, females perform better than males when alternative parameters are taken into account in the analysis of performance [32,59]. Moreover, due to the research historical bias, every memory task was validated based on male performance, as well as the choice of parameters analyzed. Taken together, these observations suggest that a different performance does not always mean a memory deficit and that factors associated with female (and not male) behavior could interfere with memory evaluation. These remarks reinforce that males should not be considered the standard for comparison purposes.

As previously mentioned, the need for estrous cycle monitoring when studying behavior is not unequivocal. For example, Sprague-Dawley and Long Evans female rats show stability in memory performance and place cell activity across the estrous cycle phases [60,61]. Although the estrous cycle phases were not considered in the present experiment, previous studies with 3-month-old Wistar female rats have analyzed the PMDAT behavior across all the estrous cycle phases. In those studies, irrespective of the interventions applied, control groups consistently showed similar behavior across all phases [18,19,35].

Although no previous study had tested VLP-induced corticosterone secretion, our data corroborate that of Sharp and colleagues [62], who showed higher heart rates after the collection of vaginal smears. Another problem regarding vaginal lavage is the possibility of pseudopregnancy [23], and very little is known about pseudopregnancy-induced behavioral alterations. Moreover, previous studies showed that most vaginal lavage methods lead to alterations in the regularity of cycles [63–65], and may affect the quality of vaginal smear or injure the cervix. Noteworthy here is that minor differences in the vaginal lavage method may lead to different behavioral and physiological alterations [65].

One of the few previous studies assessing the behavioral effects of vaginal smear collection showed that this procedure attenuated cocaine-stimulated activity [16]. Importantly, the study showed that a few days of collecting vaginal lavage can mask an existing estrous cycle influence in cocaine effects [16]. Taking that study and the present results into consideration, we suggest that vaginal lavage induces behavioral and hormonal changes related to stress and that this should be taken into account when interpreting female results, especially when the focus of the study is the comparison with males. We also suggest that alternative methods should be applied when possible. For example, if the estrous cycle evaluation is essential for an experiment, one can use the visual method, in which the appearance of the mouse or rat vagina is analyzed (color, humidity, and opening size; [23,67,67[66]]). There is evidence arguing that this method is just as efficient and much less stressful than vaginal lavage [23 68]. Alternatively, single verifications by VLP after behavioral procedures or immediately after euthanasia could be applied. However, more studies comparing the methods are required to ensure their validity and relevance to data interpretation.

Finally, it is important to point out some limitations of our study. We assessed only one physiological stress parameter (corticosterone levels), and did not check for sustained corticosterone increase, although to that purpose another potentially stressful procedure would have to be included in the protocol (repeated blood collection). In addition, the control group did not receive the same amount of handling as the VLP group. However, it should be noted that the control handling procedure was similar to that usually applied to male subjects in behavioral studies.

5. Conclusion

Evaluation of estrous cycle by vaginal lavage is a stressful manipulation, leading to increased corticosterone plasma levels and alteration of behavior in the PMDAT. This fact may explain some of the sex differences in memory performance and some alleged memory deficits reported in female rats. Thus, this should not be a mandatory manipulation in studies with females, especially when the focus is the comparison between sexes, as it cannot be mimicked in male animals as a control procedure.

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