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Changes in motor behavior during pregnancy in rats: the basis for a possible animal model of restless legs syndrome

Alterações no comportamento motor de ratas prenhes: bases para um possível modelo animal da síndrome das pernas inquietas

Keywords
Restless legs syndrome
Motor activity
Pregnancy
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Palavras-chave
Síndrome das pernas inquietas
Atividade motora
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Abstract

PURPOSE: Pregnant women have a 2-3 fold higher probability of developing restless legs syndrome (RLS – sleep-related movement disorders) than general population. This study aims to evaluate the behavior and locomotion of rats during pregnancy in order to verify if part of these animals exhibit some RLS-like features. **METHODS:** We used 14 female 80-day-old Wistar rats that weighed between 200 and 250 g. The rats were distributed into control (CTRL) and pregnant (PN) groups. After a baseline evaluation of their behavior and locomotor activity in an open-field environment, the PN group was inducted into pregnancy, and their behavior and locomotor activity were evaluated on days 3, 10 and 19 of pregnancy and in the post-lactation period in parallel with the CTRL group. The serum iron and transferrin levels in the CTRL and PN groups were analyzed in blood collected after euthanasia by decapitation. **RESULTS:** There were no significant differences in the total ambulation, grooming events, fecal boli or urine pools between the CTRL and PN groups. However, the PN group exhibited fewer rearing events, increased grooming time and reduced immobilization time than the CTRL group (ANOVA, $p<0.05$). **CONCLUSION:** These results suggest that pregnant rats show behavioral and locomotor alterations similar to those observed in animal models of RLS, demonstrating to be a possible animal model of this sleep disorder.

Resumo

OBJETIVO: Mulheres grávidas apresentam de duas a trêz vezes maior probabilidade de desenvolver a Síndrome das Pernas Inquietas (SPI – distúrbio do movimento relacionado ao sono) do que a população geral. O objetivo do estudo foi avaliar o comportamento e a locomoção de ratas durante a gravidez, a fim de verificar se esses animais apresentam algumas características SPI-like. **MÉTODOS:** Foram utilizadas 14 fêmeas Wistar de 80 dias de idade, pesando entre 200 e 250 g. Os ratos foram distribuídos em grupos controle (CTRL) e prenhes (PN). Após uma avaliação inicial do comportamento e da atividade locomotora em um ambiente de campo aberto, o grupo PN foi introduzido a prenhez, e sua atividade e comportamento locomotor foram avaliados nos dias 3, 10 e 19 de prenhez e no período pós-lactação em paralelo ao grupo CTRL. Os níveis de ferro e transferrina séricos nos grupos CTRL e PN foram analisados em sangue coletado após eutanásia por decapitação. **RESULTADOS:** Não houve diferenças significativas na deambulação total, nos eventos de *grooming*, bolos fecais e urina entre os grupos CTRL e PN. No entanto, o grupo PN apresentou menos eventos de *rearing*, aumento no tempo de duração de *grooming* e redução no tempo de imobilização em relação ao grupo CTRL (ANOVA, $p<0,05$). **CONCLUSÃO:** Esses resultados sugerem que ratas prenhes apresentam alterações comportamentais e locomotoras semelhantes às observadas em modelos animais de SPI, demonstrando ser um possível modelo animal desse distúrbio do sono.

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Introduction

Restless legs syndrome (RLS) is a sleep-related movement disorder with estimated prevalence between 5 and 10% of the general population[1] and is negatively associated with quality of life[2]. RLS is a sensory-motor disorder characterized by leg dysesthesia and restlessness while awake[3].

Among the so called secondary forms of RLS, the pregnancy-related one is one of the most common and well studied. Pregnant women have a 2–3 fold higher probability of developing RLS than general population[4,5].

Around 25% of women experience RLS during the third trimester of pregnancy. Recognized risk factors for RLS during pregnancy are multiparity, positive family history of RLS, previous history of RLS outside of pregnancy or in a previous pregnancy, other sleep disorders (insomnia, snoring), age and use of hypnotics. Possible etiologic factors implicated in this form of RLS are iron and folate deficiency, hormonal changes, mechanical stretching of the nervous lumbar roots, genetic predisposition, however the pathogenesis remains unknown[5-12]. The prevalence of RLS in pregnant women varies from between 11 and 30%, as assessed in groups of 100–642 women[5,6,13-16]. This variability in rates might be due to racial/geographical differences among the studies, the gestational stage of women recruited, and to the different criteria and method used for the diagnosis of RLS[5,17].

Low iron and folate levels may also be associated with the development of RLS during pregnancy, given that the demand for iron and folate increases considerably in pregnant women while plasma levels decrease[5].

The prevalence of RLS progressively increases from the third and fourth month of gestation, with a peak of intensity and frequency of the symptoms in the third trimester of gestation. The symptoms decrease during the ninth month and usually disappear or drop down around delivery[5]. An animal model of RLS might help in understanding the etiopathogenesis of the syndrome, and secondary forms of RLS can be a model to replicate in animals the RLS symptoms. However, to develop an animal model of RLS, several key components of the animal phenotype (behavior) of the syndrome must be defined and several studies have been performed to demonstrate an animal model that exhibits those characteristics[18-26].

Thus, due to the increased propensity of developing RLS during pregnancy, the objective of this study was to evaluate the behavior and locomotor activity of rats during pregnancy in order to verify if part of these animals exhibit some RLS-like features.

Methods

A total of 17 80-day-old female Wistar rats weighing between 200 and 250 g were obtained from the Center for the Development of Experimental Models for Medicine and Biology at the Universidade Federal de São Paulo (CEDEME/UNIFESP, São Paulo, Brazil). During the study period, the animals were maintained in the vivarium of the Departamento de Psicobiologia/UNIFESP. They were acclimatized to a room with a constant light-dark cycle (12/12h). The animals were given access to food and water ad libitum and were housed in collective cages. The rats used in this study were maintained and treated in accordance with the Council for International Organization of Medical Sciences (CIOMS) Ethical Code for Animal Experimentation[27] and the precepts of Colégio Brasileiro de Experimentação Animal (COBEA) (www.cobea.org.br). All procedures were approved by the Ethics Committee of UNIFESP (# 207/11).

The rats were divided into two groupsgroups: control (CTRL) and pregnant (PN), where the PN group was previously placed with male rats to induce pregnancy.

Experimental design

One week after the open-field test for environmental adaptation, which was used to evaluate the behavior and locomotor activity (each test lasted 10 minutes per animal), the animals began the baseline testing session. After the baseline session, the rats in the PN group were impregnated and then re-evaluated in the open-field tests on days 3, 10 and 19 of pregnancy and after their pups were weaned. The rats in the CTRL group were tested alongside the PN group. Analyses of the iron and transferrin levels were performed after the pups from the PN group were weaned.

Experimental procedures

Vaginal smears and breeding

To identify the phases of the estrous cycle, vaginal smears were performed daily on the rats during the same period of the day. The vaginal smears were performed by introducing saline into the vaginal opening of the rat with a 200-µl tip and aspirating the liquid containing the vaginal epithelial cells after a few moments. The liquid was spread onto a slide for microscopic examination at 40x magnification to determine the predominant cell type, which helps to identify the estrus phase. During this phase, the rats were placed with male rats to breed for a period of approximately 12 hours (19 h, day 1, 7 h, day 2). After being placed with the male rats, saline was again introduced into the female's vaginal cavity to aspirate the material contained following copulation. The aspirate was spread onto a slide for microscopic examination to detect spermatozoa[28-30].

Determination of serum iron and transferrin levels

The iron levels were determined using a two-point enzymatic method (VITROS®, Johnson & Johnson, NJ, USA) and were read at a wavelength of 600 nm. Transferrin, an iron transporter protein, was analyzed using an immunoturbidimetric reaction (Advia 1650®, Bayer, Holliston, MA, USA).

The open-field test

Locomotor activity was assessed by using the open-field paradigm, which consists of a circular wooden ring measuring 81 cm in diameter and enclosed by 41 cm high white walls. The ceiling was open, and the ground was divided into 19 quadrants. The open-field test is one of the most commonly used tests to assess locomotor activity in rodents. It has been already applied to quantify movement in the contest of previous attempts in obtaining animal model of RLS[18,23,31].

Each animal was individually placed in the center of the apparatus and observed for 10 minutes. However, only the final 5 minutes were evaluated. In line with the circadian pattern of the human RLS symptoms, which appear or worsen during night, all tests were performed between 9 a.m. and 11 a.m. because rats are nocturnal animals[23].

Observers quantified the following parameters: central ambulation (number of quadrants that the animal stepped on with four feet that were not close to the walls of the apparatus), peripheral ambulation (number of quadrants that the animal stepped on with four feet near the walls of the apparatus), total ambulation (the total number of quadrants that the animal stepped on with four feet), rearing frequency (the number of times that the animal supported itself with both hind legs), grooming frequency (the number of times that the animal put its mouth or paws on its body or head), total duration of grooming (the total amount of time that the animal put its mouth or paws on its body or head), total immobilization time (the total amount of time that the animal remained perfectly still, moving only the vibrissae) and total fecal boli and urine pools[32].

The same two observers participated in all open-field sessions. The first observer was responsible for recording the total time and observing the rearing events and total ambulation. The other observer was responsible for observing the total immobilization time and the duration and number of grooming events.

Statistical analysis

Repeated-measures analysis of variance (ANOVA) followed by the post hoc Tukey's test were used to analyze the behavior and locomotor activity over the course of the pregnancy. For the iron and transferrin analyses, Student's t-tests were used for independent measurements. The level of significance considered to reject the null hypothesis was $p<0.05$. The values are expressed as the means ± standard error.

Results

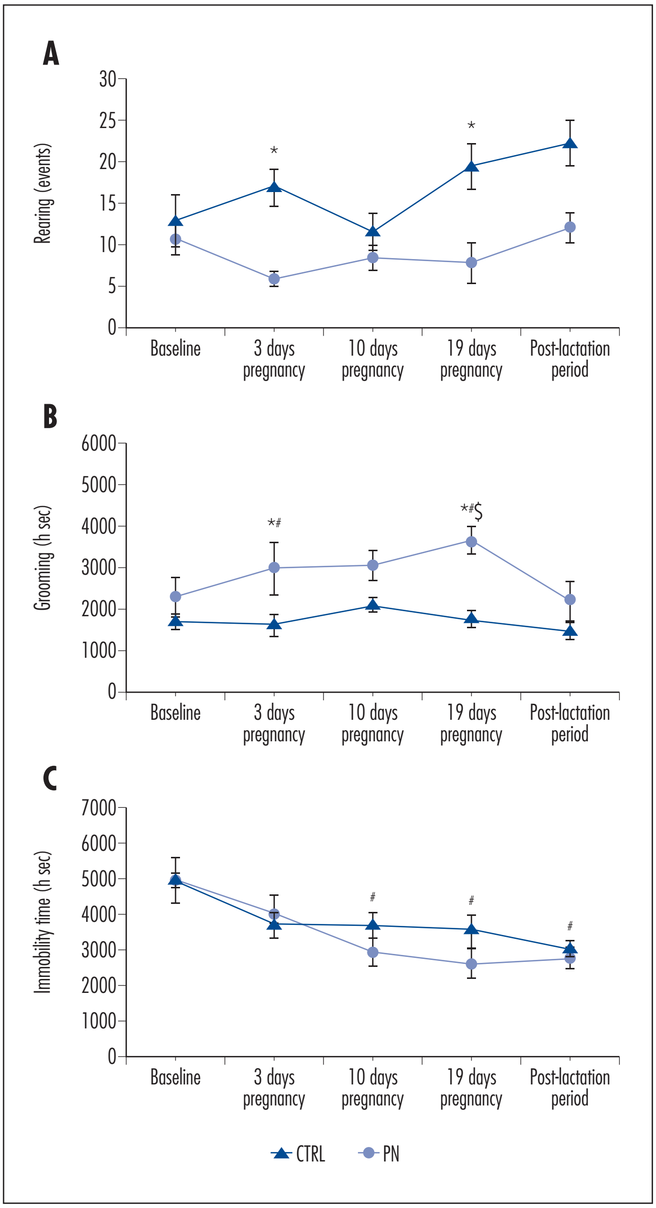
Table 1 presents the results for the CTRL and PN groups at baseline, on days 3, 10 and 19 of pregnancy and after the weaning period. There were no significant differences in the central, peripheral or total ambulation; grooming events; fecal boli or urine pools between the CTRL and PN rats.

Repeated-measures ANOVA indicated significant differences between the groups in the number of rearing events ($F_{(1,12)=}$ 8.137; p=0.01), duration of grooming ($F_{(1,12)=}$ 18.882; $p<0.001$) and duration of immobilization ($F_{(4,48)=}$ 8.889; $p<0.001$). The results presented in Figure 1 show that the

Table 1. Results of the open-field evaluations for the Control and Pregnant groups from the following sessions: basal, days 3, 10 and 19 of pregnancy and during the post-weaning period

Open-Field variables	Baseline		3 days pregnancy		10 days pregnancy		19 days pregnancy		Post-lactation period	
	CTRL	PN	CTRL	PN	CTRL	PN	CTRL	PN	CTRL	PN
CLA	8.00±3.14	5.85±1.69	6.42±3.16	2.28±0.80	5.00±2.10	2.00±0.95	8.14±4.59	3.42±3.10	15.00±2.91	5.71±1.89
PLA	29.71±9.80	34.57±9.59	40.57±8.28	23.42±6.24	28.57±7.68	22.42±5.56	33.14±4.31	23.14±5.91	47.71±8.49	24.42±5.00
LA	37.71±12.57	40.42±10.63	47.00±10.31	25.71±6.32	33.57±9.44	24.42±6.28	41.28±8.46	26.57±8.19	62.71±10.70	30.14±6.65
GRO	2.42±0.52	3.42±0.48	2.14±0.50	2.28±0.56	3.57±0.64	2.42±0.36	2.42±0.36	4.00±0.53	3.28±1.10	3.85±0.70
FB	0.57±0.57	0.42±0.20	2.28±1.01	2.42±1.34	1.57±1.11	0.85±0.45	1.28±0.89	0.42±0.29	0.00±0.00	1.00±0.69
UP	1.28±0.68	1.85±0.80	1.42±0.86	1.14±0.45	2.57±1.15	0.28±0.18	1.85±0.98	0.00±0.00	0.71±0.42	4.28±1.42

CTRL: control; PN: pregnant; CLA: central locomotor activity; PLA: peripheral locomotor activity; LA: locomotor activity; GRO: grooming (events); FB: fecal boli; UP: urine pools. ANOVA (p>0.05)



Repeated-measures ANOVA (p<0.05). *differs from the CTRL group in the same session; [#]differs from the baseline session of both groups; [$]differs from the post-weaning session for both groups. H sec: hundred seconds.

Figure 1. Rearing (A), grooming (B) and immobilization time (C) for the control (CTRL) and pregnant (PN) groups at the following time-points: baseline, days 3, 10 and 19 of pregnancy and post-weaning

PN group exhibited fewer rearing events, increased grooming time and reduced immobilization time compared to the CTRL group during the period of pregnancy, given that the duration of grooming returned to the baseline during the evaluation performed after the lactation period.

No significant differences in the serum iron (CTRL: 279.28±85.86 µg/dL; PN: 300.86±45.29 µg/dL) or transferrin (CTRL: 360.86±52.67 mg/dL; PN: 341.86±29.75 mg/dL) concentrations were observed between the CTRL group and the PN group, performed using blood collected six days after the pups from the PN group were weaned.

Table 2. Iron and transferrin serum levels in rats from the Control and Pregnant groups

Group	Iron (µg/dL)	Transferrin (mg/dL)
CTRL	279.28±85.86	360.86±52.67
PN	300.86±45.29	341.86±29.75

CTRL: control; PN: pregnant. Student's t-test (p>0.05)

Table 2 shows the results of the iron and transferrin evaluations, which were performed using blood collected six days after the pups from the PN group were weaned. No significant differences in the serum iron or transferrin concentrations were observed between the CTRL group and the PN group.

Discussion

This study assessed the behavior and locomotor activity of rats during pregnancy. Pregnant rats were shown to exhibit fewer rearing events, increased grooming time and less immobilization time compared to the CTRL group. However, no significant difference in ambulation and reduction in number of rearing events were observed, maybe due to anatomical reasons related to the pregnant condition in Wistar rats. Pregnant rats are relatively small considering the average number of pups per pregnancy (10 to 12 pups), and significant weight gain occurs over a short period (21 days). Similarly, pregnant women who experience RLS symptoms, especially during the third trimester of pregnancy[6], have some difficulty to relieve the symptoms by walking or stretching, preferring to move their limbs in bed.

The pregnant rats spent more time grooming compared to the CTRL rats, demonstrating that the condition of pregnancy affected this behavior and suggesting that this may have occurred because of increased limb discomfort. Like any other animal model in which the subjective elements of pathology cannot be evaluated, this model is also not able to assess the subjective need to move, which is necessary for a clinical diagnosis of RLS[22]. However, this model is consistent with the motor component of human RLS when particular attention is paid to the symptoms: the desire to move the limb, usually accompanied or caused by an uncomfortable sensation in the legs that begins or worsens during periods of rest or inactivity, when the patient is sitting or lying down, and where the symptoms are partially or totally relieved by movement such as walking or stretching[17]. Unpleasant sensory symptoms in the limbs of experimental animals can result in increased attention to the affected limb, which is manifested by licking, scratching, stretching or holding[24]. These behaviors were observed in the present study.

After analyzing the immobility time in the CTRL group, a clear decrease in the immobility time could be observed when the length on day 10 of the pregnancy (early symptoms of RLS in human pregnancy) and day 19 of pregnancy (peak RLS symptoms) were compared to the baseline session. This finding indicates that pregnancy can interfere with the rats ability to remain inactive.

In 2004, Manconi et al.[11] conducted a study on a group of 642 women throughout their pregnancies and reported a prevalence of 26.6% for RLS, of which 9.9% had been previously affected and 16.7% had not been previously affected. According to this study, the prevalence of the syndrome progressively increased during the third or fourth month of pregnancy, with the peak intensity and frequency of symptoms occurring during the third trimester, especially during the eighth month, followed by a reduction in symptoms in the ninth month and relief around delivery[11]. One month after delivery, the prevalence of RLS in the women evaluated decreased to 6.8% and remained between 5 and 6% in the 6 months following delivery. In 2001, Lee et al.[9] reported an increase in the prevalence of the syndrome in the last trimester of pregnancy. Initially, 32 pregnant women were evaluated, including 4 who experienced RLS symptoms at 12 weeks of pregnancy and 5 who reported symptoms at 24 weeks. In the 3rd trimester, 7 women reported symptoms of RLS, which corresponds to 23% (N was reduced to 30). One month after delivery, only one woman still presented with RLS symptoms[9]. This reduction in RLS symptoms in women after pregnancy is consistent with the results of the present study, which demonstrated that the duration of grooming in rats returned to baseline levels following the lactation period.

If we translate the human knowledge to the animal, we would expect that only about one quarter of rats will develop RLS during pregnancy, so this will decrease the statistical power of our final results.

The temporal relationship between RLS symptoms and the third trimester of pregnancy suggests that there are pregnancy-related factors that exacerbate RLS during this period, but the presence and duration of the syndrome depends primarily on the predisposition of each individual woman who experiences symptoms[5,11].

For the measurements of serum iron and transferrin levels 1 week after the pups in the PN group were weaned, the blood was analyzed approximately 1 month after delivery (21 days of feeding+6 days post-weaning). There was no significant difference between the CTRL and PN groups in the measured parameters because the degree of hemodilution that occurs during pregnancy in the PN group had already reverted to normal levels[9]. Similarly, the RLS symptoms most likely decreased because the secondary conditions related to the syndrome, such as pregnancy, were no longer present.

According to several studies that have analyzed serum and cerebral Fe, ferritin and transferrin levels in patients affected by RLS from either idiopathic causes or secondary to conditions such as pregnancy, most patients exhibited normal serum iron, ferritin and transferrin levels. However, the Fe and ferritin levels in the cerebrospinal fluid were reduced, and this dysfunction was probably due to the transport of elements from the blood to the brain via transferrin, resulting in an increase in transferrin in the brain[33,34].

In the 2001 study by Lee et al.[9] linking RLS and pregnancy, the group affected by RLS frequently manifested slightly higher serum Fe levels and lower ferritin levels, and these levels did not significantly change during pregnancy in either the affected group or in the group unaffected by RLS. In both groups, the lowest ferritin levels were measured in the third trimester when RLS is most prevalent. In the first month postpartum, both groups showed an improvement in the Fe, ferritin and hemoglobin levels due to the absence of hemodilution that occurs during pregnancy[9].

Technical difficulties prevented us in analyzing the animal blood to confirm that the hormone levels were altered by the pregnancy. The lack of this information is limitation of this study. Nonetheless, we have clearly demonstrated changes in the animals' behavior patterns.

In summary, our results suggest that pregnant rats exhibit behavioral changes that appear similar to an experimental model of RLS, demonstrating increased discomfort in the limbs and reduced immobilization time during pregnancy.

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