EFFECTS OF CHRONIC REM SLEEP RESTRICTION ON ANXIETY-LIKE BEHAVIOR AND THE IMMUNE SYSTEM OF RATS

EFEITOS DA RESTRIÇÃO CRÔNICA DE SONO REM SOBRE O COMPORTAMENTO ANSIOSO E O SISTEMA IMUNE DE RATOS

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ABSTRACT

The objective of this study was to investigate the effects of partial chronic rapid-eye-movement (REM) sleep restriction (SR) (18 hours daily, 21 days) on anxiety-like behavior and the immune system in rats. Male Wistar rats were randomly allocated to sleep-restricted (n=14) and control (n=14) groups. During the experiment, the rats were immunized twice with chicken immunoglobulin Y (days 01 and 15). Blood samples were collected on days 01, 08, and 22, and the levels of antibodies (IgM, IgG1, and IgG2a) were measured. On day 22, the animals were tested in the elevated plus-maze (EPM). The results showed that sleep restriction increased adrenal weight and decreased body and spleen mass in rats. No changes in thymus weight were observed. Behavior in the EPM and antibody production were not altered by sleep restriction. Thus, one can infer that SR influenced the energy metabolism and spleen mass but does not interfere in antibody production and in the behavioral parameters of the animals investigated in the present study.

Key Words: sleep deprivation; animal behavior; immunology.

RESUMO

O objetivo deste estudo foi investigar os efeitos da restrição parcial crônica do sono (RS) de movimento rápido dos olhos (REM) (18 horas/dia, por 21 dias) sobre o comportamento ansioso e o sistema imune em ratos. Ratos machos Wistar foram alocados aleatoriamente em grupos RS (n = 14) e controle (n = 14). Durante o experimento, os ratos foram imunizados duas vezes com imunoglobulina Y de galinha (dias 01 e 15). Amostras de sangue foram coletadas nos dias 01, 08 e 22, e os níveis de anticorpos (IgM, IgG1, e IgG2a) foram mensurados. No dia 22, os animais foram testados no labirinto em cruz elevado (LCE). Os resultados mostraram que a restrição do sono aumentou o peso da glândula adrenal e diminuiu a massa corporal e a massa do baço dos ratos. Nenhuma alteração no peso do timo foi observada. O comportamento ansioso avaliado pelo LCE e produção de anticorpos não foram alterados pela restrição do sono. Assim, podemos inferir que a RS influenciou o metabolismo energético e a massa do baço, mas não interferiu na produção de anticorpos e nos parâmetros comportamentais dos animais investigados no presente estudo.

Palavras-Chave: privação de sono; comportamento animal; imunologia.

INTRODUCTION

When an individual sleeps below usual, with possible damage to the performance of some tasks, it is called sleep restriction (SR) (1). SR represents probably the most common sleep disturbance. Many factors can be related to it, such as socio-environmental issues (e.g. labor demand or family responsibility), pharmacological treatments, and a sedentary lifestyle (2).

Several studies have pointed out to the vital role of sleep, especially rapid-eye-movement (REM) sleep, which is related to development, energy conservation, and psychological and cognitive functioning (3,4). In this sense, sleep disturbances can be closely related to the onset of some...
dysfunctions which can involve behavioral (e.g., mood and anxiety disorders) and physiological disorders, including cardiovascular (e.g., hypotension and bradycardia) and immune system disorders (e.g., immunosuppression) (5,6).

Sleep may serve as a prognostic indicator in infectious disease, thus indicating better chances of survival (7). The immunomodulatory role of sleep can be exemplified by a study, which showed that 21 days of SR led to a reduction in total circulating leukocytes, especially lymphocytes, in rats (8). This immunosuppression caused by SR in rats is justified, in parts by the fact that the animals produce less melatonin, which is a potent stimulator of the immune system (9). However, in relation to production of total antibodies, it has been shown that chronic SR may potentiate the production of immunoglobulins M (IgM), G (IgG), and A (IgA) antibodies in rats (8,10). Regarding antibody production, it is a consensus in that individuals produce primary and secondary antibodies. The primary antibodies are IgM, which are produced by B lymphocytes after first contact with the antigen. Secondary antibodies, for example, immunoglobulins G1 (IgG1) and G2a (IgG2a), are produced by B lymphocytes after the second contact with the same antigen, being more specific than the IgM in the antigenic recognition (11).

Since SR may be stressful and affect aspects of both emotional functioning and the immune system, the objective of the present study was to evaluate the effects of chronic REM sleep restriction on anxiety-like behavior and the immune system in Wistar rats.

**MATERIALS AND METHODS**

**Animals**

Twenty-eight adult (90-day old) male Wistar rats were used (body weight = 345 ± 56 g). The animals were supplied by the Central Vivarium of the State University of Londrina (UEL). The rats were housed in groups of five per polypropylene cage (40 cm x 34 cm x 17 cm). Water and commercial rat chow were available *ad libitum* throughout the experiment. The temperature in the vivarium was maintained at approximately 23 °C and a 12/12 h light/dark cycle was established (lights on at 7:00). The animals were randomly distributed between two groups, control (C, n=14) and SR (n=14). The experiment was carried out in accordance with the recommendations of the Brazilian Society of Neurosciences and Behavior and was approved by the ethics committee of UEL (CEEA/UEL 66/09).

**Sleep restriction**

For REM SR, the procedures and animal care were adapted from Zager et al. (8). The rats submitted to SR were maintained for 18 h (beginning at 04:00 PM) daily in black polyvinylchloride tanks (100 cm x 50 cm x 50 cm), with seven animals per tank, for 21 days. After each 18-h sleep deprivation period, the rats could sleep for 6 h (sleep window beginning at 10:00 AM). The tanks contained platforms (5 cm high and 7 cm in diameter), which the animals climbed to avoid contact with water that was 4 cm in depth. The other rats were kept in their cages during these periods.

**Antigen preparation and immunization**

The immunoglobulin Y (IgY) antibody used was not sourced from a commercial company, instead, the authors obtained the IgY antibody from egg yolk of laying hens, following the protocol described by Akita and Nakai (12). The animals (C and SR) were immunized intramuscularly twice during the experiment (1st and 15th day). At each immunization, the rats received 100 µl of solution containing 100 µg of chicken IgY diluted (v/v) in phosphate-buffered saline (PBS) solution and Freund’s complete (first immunization) or incomplete adjuvant (second immunization).

**Blood collection**

Blood samples were collected at three moments; on the first day of the study (before the beginning of the sleep restriction), on the eighth day (seven days after the first immunization), and on the twenty-second day (seven days after the second immunization), always being performed after the behavioral tests. The collections were performed through cardiac puncture, after the animals had been anesthetized intramuscularly with an association of ketamine hydrochloride 1 ml/kg (Dopalen®, Sespo, Brazil) and xylazine hydrochloride 1 ml/kg (Anasedan®, Sespo, Brazil). Approximately 500 µl of blood was
drawn per rat at each collection. The blood was stored in 1.5 ml tubes containing 50 µl of 5% EDTA. After this procedure, the blood was centrifuged for separation and the plasma stored at -20 ºC.

Behavioral analysis
On the 22nd day of the study, at the end of the SR, the animals were assessed on the EPM. This is an apparatus used to study anxiety in rats. The EPM used had a wooden floor, two open arms (50 cm x 12 cm) surrounded by a 1 cm high acrylic edge, and two enclosed arms (50 cm x 12 cm) with 40 cm high acrylic walls. A central quadrant (12 cm x 12 cm) connects the four arms at right angles. The whole apparatus was raised 50 cm off the room floor. Approximately one hour before the testing, the rats were transported in their home-cages to a room adjacent to the testing room. No previous habituation to the apparatus was performed. Before testing, the apparatus was cleaned with paper towels and a 5% (v/v) ethanol solution. Each animal was gently placed on the central quadrant facing one of the enclosed arms and allowed to freely explore for five min. Entries into the arms were recorded. An entry was recorded each time the four paws stepped into an arm. Time spent in the open arms was also recorded. Entries into the open arms were converted to the percentage of the total number of entries into any type of arm.

Collection and weighing of organs
On the 22nd day of the study, after the behavioral test, the animals were euthanized with an anesthetic overdose (ketamine hydrochloride 3 ml/kg and xylazine hydrochloride 3 ml/kg), and the spleen, thymus, and adrenal glands were collected and weighed (wet weight was used for analysis).

Enzyme-linked immunosorbent assay (ELISA)
The evaluation of antibody production (IgM, IgG2a, and IgG1) was performed using a solution containing 1 µg/ml of chicken IgY to sensitize the plate in a volume of 100 µl per well. The plasma dilution was 1:100. Dilutions of the anti-IgM (03-9820, Zymed, Carlsbad, USA), anti-IgG2a (03-9620, Zymed, Carlsbad, USA), and anti-IgG1 (A110-106P, Bethyl, Montgomery, USA) peroxidase conjugates were 1:10000, 1:20000, and 1:100000, respectively. The ELISA was conducted as described by Lobato et al. (13).

Statistical analysis
Statistical analysis was performed using the statistical package SPSS 17®. After normality was established by the Kolmogorov-Smirnov test, the results were grouped as mean and standard error values of the mean. The Student's t-test was used to compare the groups regarding behavior, variation in body mass, weight of the spleen, thymus, and adrenal gland, and the production of antibodies. The significance level adopted was p < 0.05.

RESULTS
Figure 1A shows the variation in the mean body mass of the rats during the 21 days of chronic SR. The Student's t-test showed that SR decreased the body mass of the rats when compared to C animals, which presented increases in body mass throughout the study (p < 0.01). This fact points to a metabolic alteration in the rats caused by the restriction of sleep.

It was observed that chronic SR enlarged adrenal glands (p < 0.01) (Figure 1B). This increase in adrenal weight is related to the behavioral changes in the rats affected by the SR.

Figure 1C presents the mean spleen weights of animals submitted to sleep restriction or not. The Student's t-test showed that shorter sleep led to a reduction in the mean weight of the spleen (p < 0.01). No effect of sleep restriction was observed on the weight of the thymus (Figure 1D, p > 0.05).

In the elevated plus-maze test (Table 1) and production of IgM, IgG1, and IgG2a-specific antibodies (Figure 2), no significant differences were observed between animals submitted to chronic sleep restriction and controls (p > 0.05). In addition, no significant differences were observed in terms of antibody production before and after immunizations (data not shown).
Figure 1. Variation in body mass (A), adrenal mass (B), mass of the spleen (C), and mass of the thymus (D). The animals were submitted to chronic sleep restriction for 21 days (18 hours/day). C, control animals (n = 14). SR, animals submitted to sleep restriction (n = 14). Data are presented as means and standard error of the means, *difference between groups, p < 0.01 (Student's t-test for independent samples).

Table 1. Behavioral measures obtained in the elevated plus-maze.

<table>
<thead>
<tr>
<th>Behavioral measure</th>
<th>Control</th>
<th>Sleep restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed arm entries</td>
<td>7.6 ± 0.9</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>Open arm entries (%)</td>
<td>25.0 ± 4.9</td>
<td>29.0 ± 4.1</td>
</tr>
<tr>
<td>Time in open arms (s)</td>
<td>32.4 ± 8.5</td>
<td>39.7 ± 6.9</td>
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DISCUSSION

The reduction in body mass observed in animals submitted to chronic sleep restriction was in accordance with the findings of Machado et al. (14) The fact that rats lose body mass during the chronic SR period may be related to the level of stress involved, which is suggested by the increase in adrenal mass in the animals of this study and also reported elsewhere (15). In relation to spleen weight, it was observed that SR was involved in its reduction; these data are in agreement with the findings of Zager et al. (8) who observed a decrease in spleen weight of Wistar rats.

The SR performed in the present study, despite the stress (evidenced by the adrenal increase and reduction in body mass), did not influence the emotional parameters (anxiety-like behavior) or the locomotion of the rats, evaluated by the EPM test (percentage of entries into and time spent in the open and entries in the closed, respectively). The effects of chronic SR on anxiety-like behavior in rats are controversial. Similarly, another study (16), although using a more stringent restriction regimen (20 h/day), reported no effect on rat anxiety (open-field and EPM tests) even after four weeks of SR. However, anxiogenic effects have been reported in some other studies (17,18). In one study, a two-day interval between the end of the SR and behavioral evaluation was not enough to neutralize anxiogenic effects (17,18). These discrepant results indicate that chronic SR effects on anxiety-like behavior deserve further evaluation.

Regarding antibody production, as the animals in the present study produced anti IgY, IgM, IgG, IgG2a, and IgG1 antibodies normally, regardless of sleep restriction, we can conclude that the short sleep time during...
the study was not able to modulate the humoral immune response. Similarly, it has been shown that chronic SR, in addition to not preventing the production of specific antibodies, also results in an increase in the production of total antibodies of the classes IgM, IgG, and IgA (8,10).

Although there was no change in the antibody production of the rats in the present study, the literature shows that melatonin acts directly on the humoral immune response. Immune cells (e.g., spleen) exhibit the melatonin MT2 receptor which, upon activation, triggers an increase in splenocyte proliferation, a condition favoring the humoral immune response (19). In this sense, since the production of antibodies in immunized rats is not affected by SR, it may indicate that there is a species-dependent factor, once in humans, antibody production is directly influenced by quality and time of sleep and clinical studies should control the variable sleep (20).

CONCLUSION

Chronic REM SR resulted in loss of body mass, an increase in adrenal mass, and a reduction in spleen mass in rats. On the other hand, no effect of SR was observed on the thymus mass, anxiety-like behavior, or production of specific antibodies. Thus, we can infer that animals submitted to SR presented alterations in the energy metabolism and spleen mass but does not interfere in antibody production and in the behavioral parameters of the animals investigated in the present study.

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