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Immunomodulation by classical conditioning in NZB/W (F1) mice: Lifespan and diurnal variation

Mario André Leocadio Miguel[a,*], *Luiz Menna-Barreto*[b,1]

[a]*Departamento de Fisiologia, Universidade Federal do Rio Grande do Norte, Avenida Salgado Filho, S/N, 59078-970, Brazil*

[b]*Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, Rua Arlindo Betio, 1000, 03828-000, Brazil*

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ABSTRACT

Systemic Lupus Eritematosus (SLE) is a systemic inflammatory disease often treated with the agent cyclophosphamide (CY), known by provoking important adverse reactions to the organism. Ader and Cohen have demonstrated an alternative way of administrating this agent based on pavlovian conditioning, in order to reduce the aggression caused by CY. Considering the influence of the temporal organization on learning and memory processes, the purpose of this study was to understand the temporal aspects involved in the conditioned immunomodulation. In a search for circadian modulation, we selected NZB/W (F1) female mice, a strain that spontaneously develop SLE. Divided into two major groups, the animals were submitted, in different phases of day, to a classical conditioning immunomodulation protocol, consisting in weekly parings of saccharin solution and CY injections. The success of the paradigm was evaluated by comparing lifespan among the groups. Simultaneously, it was monitored the water intake behavior, in order to correlate the stability of two rhythmic parameters, amplitude and spectral power density of the 24-h rhythm, with the progression of SLE. Our results indicate that mice could benefit from the conditioning task performed either in the light phase or in the dark phase of the LD cycle, as expressed by an increased lifespan. Concerning the rhythmic parameters, there was evidence of association between the rhythmic stability and the evolution of SLE, demonstrated by the maintenance of healthy levels of amplitude and spectral potency of the 24-h rhythm in animals exposed to the conditioning paradigm.

1. Introduction

Since Metalnikov and Chorine [1], pioneers who described the relationship between the central nervous system (CNS) and the immune processes in the 1920's, neuroimmunomodulation has been extensively explored by different research groups aiming to unveil how the immune system interacts with the CNS, from cellular [2] to behavioral [3] levels. Particularly, It has been found that cellular and humoral immunity may be influenced by Pavlovian

*Corresponding author. Tel.: +5584 999905890.
E-mail addresses: mmiguel@cb.ufrn.br (M.A.L. Miguel), menna@usp.br (L. Menna-Barreto).
[1]Tel.: +5511 30918831.

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conditioning. Pairing a novel drinking solution (conditioned stimulus-CS) with an immunomodulatory drug (unconditioned stimulus-US) results in a modulation of the immune function. Findings in conditioned immunosuppression [4] raised the question of whether the development of an immune disorder could be delayed by the classical conditioning paradigm. To answer this question, Ader and Cohen [5] investigated the progression of proteinuria and the lifespan of spontaneous lupus prone mice under a classical conditioning paradigm in which a saccharin-flavored drink is paired with the injection of an effective immune-suppressing drug cyclophosphamide. The results were consistent with their hypothesis, confirming the biological impact of conditioned immunomodulation revealed by the modulation of the lifespan.

Although immune system conditioning has been extensively studied, the temporal aspect of this form of associative learning has been widely overlooked. A growing body of evidence indicates daily modulation in learning and states that both morphological and physiological integrity of circadian timekeeping system are critical for learning and memory processes [6–9]. Davies et al. [10] demonstrated a 24-h rhythm in passive avoidance behavior. Rats trained and tested with a 4-h interval throughout a 24-h cycle exhibited more pronounced memory retention during the light phase of the LD cycle than during the dark phase. Moreover, the "time-stamp" phenomenon, which comprises the strong relationship between the performance in a learning task and the circadian phase of prior training, has been reported both for reward-conditioned place preference and for avoidance-based place leaning in hamsters [11,12] even for animals carrying the Tau mutation and displaying a short 20 h-period of rest-activity rhythm [13].

In addition to the interaction between learning and the circadian timekeeping system, immune function has also been found to be sensitive to daily temporal modulation. Daily physiological variations in circulating B and T cells [14], cell migration [15] and response of T cells to antigen [16] are examples of essential immune functions under the control of the circadian system. Macrophages and monocytes have been extensively studied due to their robust intrinsic clock machinery and high amplitude circadian output, leading to excellent time givers to peripheral tissues [17]. Moreover, recruitment of macrophages and monocytes during infection is based on the expression of adhesion molecules whose activity of under the control of the circadian timekeeping system [18]. However, possible effects of immune functions on circadian system have not received the same attention. In an effort to study such interaction, Marpegan et al. [19] found that *Escherichia coli* lipopolysaccharide (LPS) significantly altered the pattern of activity of mice by promoting a delay in the rest-activity cycle and, specifically, inducing a different cell expression in the core site of the circadian timekeeping system, the suprachiasmatic nuclei.

Taking into account the already described influence of the circadian timekeeping system on learning processes and the clear interface between immune function and the circadian timekeeping system, it would be important to address the significance of the circadian organization in the progression of immune diseases. For instance, Systemic lupus erythematosus (SLE) is an autoimmune disease that describes a whole range of systemic affections, including skin, joints, central nervous system and kidney. A recent British cohort study stated that although incidence presented an annual 1.8% decrease, prevalence changed from 64.99/100.000 to 97.04/100.00, from 1999 to 2012 [20]. In addition, despite substantial advances in the therapeutic approaches of this autoimmune disease, the mean age of death of patients with systemic lupus erythematosus is 44 y [21], thus representing a life threatening condition that impacts people during their high productive working-age.

Besides the well established kidney and vascular diseases associated with SLE, it is considered poorly understood its relationship with the circadian timekeeping system. Melatonin daily pattern, a well stablished and gold marker of the circadian phase, seems to be affected by SLE, as patients show significantly lower daily melatonin levels in comparison to healthy women during short photoperiod [22]. It is a significant finding since melatonin is considered one of the most prominent endogenous synchronizer needed to maintain the stability of phase relationship and reinforce the different circadian rhythms in the whole organism [23]. Moreover, the prevalence of sleep disorders in SLE as well as the contributing factors to their occurrence remain poorly understood, despite its prevalence rates ranging between noteworthy 55% and 85% according to distinct study approaches [24].

NZB/W (F1) mice offers a well-established mouse model of SLE since mice develop, spontaneously, the disease that resembles human SLE, including antinuclear antibodies, hemolytic anemia, proteinuria and a fatal progressive glomerulonephritis [25]. This model has also been submitted to classical conditioning immunosuppression protocols [5] and to different regimes of administration of immunosuppression drugs, including Cyclophosphamide (CY), a chemotherapeutic agent that exhibits a notably diurnal rhythm in toxicity [26]. All these features of the animal model and of the therapeutic agent open a wide range of opportunity to the study of a possible diurnal modulation in conditioned immunosuppression in a murine model of the Human Lupus Disease.

Therefore, our objective is to evaluate the interaction between the circadian organization and the survival of the NZB/W(F1) mice, as well as to search for a distinct effect of the conditioned immunosuppression according to the time of the day the protocol is administered, regarding both the progression of renal commitment and lifespan.

2. Materials and methods

2.1. Animals

Female New Zealand mice (NZBx NZW F1) were 6 months old at the beginning of the experiment. They were housed individually in standard polypropylene cages and were kept in an air-conditioned, soundproof holding room, at an ambient temperature of $22 \pm 2\,^{\circ}C$ and under a 12:12 h light-dark cycle (LD 12:12, lights on at 07:00 h). Food and water were available ad libitum. All experiments were performed in