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Comet Assay to Determine DNA Damage Induced by REM Sleep Deprivation in Rats

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Abstract: The aim of this work was to evaluate by comet assay the possible induction of DNA strand breaks in Peripheral Blood Mononuclear Cells (PBMC) of REMd sleep deprived (REMd) rats. Wistar male rats were submitted to 72h of REMd using a narrow platform inside a water tank and then allowed to recover at different times (0, 24,48 and 72 h). Wide platform technique was used as "wet control" (WC). Both groups were treated similarly before and after being put on either the narrow or wide platforms. Blood aliquots for comet assay were obtained before, during and after REMd and in WC groups at the times cited above. REMd and WC presented a significant increase ($p<0.05$) in the number of comet units compared with it's base line level. The increase was proportional to REMd period and time on wide platforms, but after being taken off it progressively returned to base line level after 72 h. REMd and WC rats presented similar induction of DNA strand breaks. Possible mechanisms of DNA damage are discussed.

Key words: Comet assay, DNA lesions, rats, REM sleep deprivation, oxidative stress

INTRODUCTION

In 1964, Jouvet *et al.*[1] developed a technique for REM (Rapid Eye Movements) sleep deprivation in cats, which was adapted for rats. The inverted flower pot technique or water-tank technique, consists of placing the animal on top of a narrow platform surrounded by water. This procedure allows muscle atony associated with REM sleep, when the animal falls in the water this is the basis of selective deprivation of REM sleep (REMd).

To compare environmental conditions associated with the sleep deprivation of rats in narrow platform (6.5 cm of diameter) technique[2], some studies used as control, a wide (14 cm diameter) platform procedure[3-5]. In these experiments animals were placed on individual platforms surrounded by water at the same environmental conditions. Control groups were also kept in cages under non-stressful conditions. In another method the animals were submitted to REMd on multiple platforms without movement restriction, isolation and social instability[6,7].

Studies of REMd in animals and humans, demonstrate important behavioral and physiological alterations such as: cardiovascular and respiratory changes[8,9]; cognitive deficits and fatigue[10]; increased sexual activity[11]; increased motor activity[12]; irritability and aggressiveness[13]. In addition, REMd animals present altered modulation of neurotransmitters in the peripheral and central nervous system[14-19].

Sustaining sleep deprivation for 2 or 3 weeks in rats is fatal and it was suggested that waking neural activity, if not interrupted by periods of restorative sleep, may damage brain cells through excytotoxic or oxidative mechanisms. Some studies suggest that sleep deprivation over long periods may lead to cell death in the brain[20,21]. Gene expression studies in rat brain show that c-fos expression was higher in manually sleep deprived rats for 3 to 24 h[22-24] and in cat brain[25]. Sleep deprivation also increased induction of arylsulfotransferase, a enzyme related to catabolism of monoamine[23]. Other data did not show DNA fragmentation[26], necrosis and/or apoptosis in brain cells in REMd rats[27,28]. Data about induction of oxidative stress after REMd are controversial, showing no induction of oxidative stress[27] and the presence of oxidative stress in hypothalamus[29].

Until now the possible effects of REM sleep deprivation or stress in causing DNA lesions evaluated in peripheral blood mononuclear cells (PBMC) by the comet assay has not been studied.

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In comet assay, a test developed by Ostling and Johnson[30], the nucleus of PBMC can be investigated by electrophoresis in agarose gel which evaluates lesions and the repair of the DNA in individual cells. With this method DNA damage either by chemical or physical agents can be evaluated[31-33].

The aim of this work was to verify the possible induction of DNA strand breaks in peripheral blood mononuclear cells during and after REM sleep deprivation submitted to the water tank technique, using the comet assay.

MATERIALS AND METHODS

Animals: Adult male Wistar naive rats (n=10, 3-4 months of age, body weight 250-350 g) from our own colony were used for this study. They were housed in a room under controlled environment: normal light/dark cycle conditions (12 h light/12 h dark; lights on at 6:00 a.m.). Animals had free access to water and food and ambient temperature was kept at 23±2°C. Experiments were conducted in accordance with the Department Committee of Animal Care.

Sleep deprivation: Rats (n=5) were placed in individual deprivation chambers (24x24x34 cm) including the narrow circular platform (6.5 cm diameter) surrounded by water (changed daily) up to 1 cm below the platform surface (REMd rats), where they remained for 72 h. "Wet controls" (WC) rats were placed in individual deprivation chambers consisting of a wide platform (14 cm diameter) surrounded by water up 1 cm below platform surface, where they also remained for 72 h. During these experiments all animals had free access to food and water. The same animals used on the narrow and wide platforms were the normal control rats prior to this procedure (base line).

Sleep deprivation recovery: The recovery of the animals (n=5) was performed after discontinuation of REM sleep deprivation or wide platform procedures in a standard box (33x40x17 cm), which allowed the sleep rebound and rest for 72 h in the room under controlled environment: normal light/dark cycle conditions (12 h light/12 h dark; lights on at 6:00 a.m.) and free access to water, food and under controlled ambient temperature (23 ± 2°C).

Aliquot procedure: The aliquots of peripherical blood were obtained at different time intervals of deprivation and recovery (0, 24, 48, 72 h) by simple puncture in posterior limb fingers and immediately mixed with heparin by comet assay procedure.

Comet assay procedure: Glass microscope slides, frosted at one end, were precoated with 1.5% normal agarose in milli-Q water type. These slides were allowed to air dry overnight prior to use. A 0,5% low-melting-point agarose (LMP) mixture in PBS was melted and maintained at 37°C; 120 µl of LMP was then added to 10 µl of blood sample and the resultant mixture was pipetted onto a precoated glass microscope slide and allowed to gel for 5 min before being transferred to an ice tray. The slides were then immersed in ice-cold lysing solution (100 mM EDTA, 10 mM Tris, 1% Triton X100, 1% DMSO, 2.5 M NaCl) for 1 h. After lysis the slides were placed onto a flat bed electrophoresis (1.0 V cm^{-1}, 25 min) tank and covered (5-6 mm) with alkali unwinding solution (50 mM NaOH, 1 mM EDTA, pH 12.5, 25 min). The slides were then stained with ethidium bromide (25 µg ml^{-1}), covered with slipped and examined at 250 x magnification under a fluorescent microscope (Zeiss-Jenamed) using green light from a 50 W mercury source with a 580 nm reflector and a 590 nm barrier filter set. Fifty randomly cells selected per sample were analyzed in each of three independent experiments. Comets were visually scored according to tail length into four classes (from undamaged class 0 to maximally damaged class 3). Thus, the total score for each group of 50 comets ranged from 0 (0 X 50 all undamaged) to 150 (3 X 50, all maximally damaged).

Statistical analysis: The comet scores, in arbitrary units, in rat blood cells subjected to different techniques and time of REMd or recovery were analyzed by ANOVA, followed by Bonferroni post test with a significance level of $p<0.05$. InStat Graphpad software was used to perform statistical analysis (GraphPad InStat version 3 for Windows 95, GraphPad Software, San Diego California, USA).

RESULTS AND DISCUSSION

The comet values PBMC in REMd rats submitted to narrow platform and recovered at different times are shown in Fig. 1 with a significant and progressive increase ($p<0.05$) of comet scores with the time of REMd, returning progressively to base line (comet scores before platform technique), after 72 h of sleep recovery.

Figure 2 shows the comet scores in WC rats submitted to wide platform technique and recovery at different times. This figure also shows that the comet scores increased progressively and significantly ($p<0.05$) with the time over wide platform, returning at the control level (base line) after 72 h of recovery.

In addition, these data show that the comet scores in rat PBMC reach similar levels in REM sleep deprived

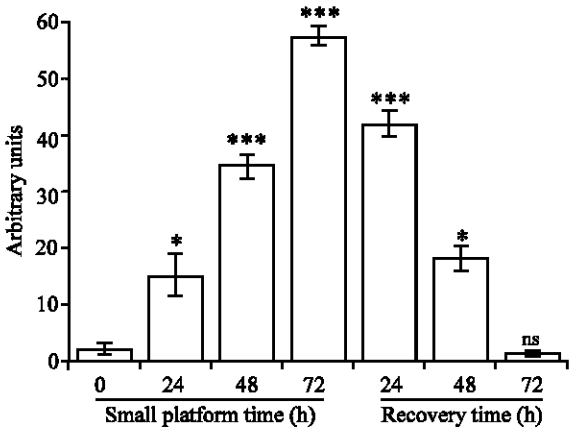

Fig. 1: Follow up of comets in blood cells of the rats subjected to REM sleep deprivation in water tank by small platform technique. Wistar rats were subjected to deprivation for 72 h and recovered until 72 h. Blood samples were obtained for each time (0, 24, 48 and 72 h) and comet assay performed (*p<0.05; ***p<0.001). Columns represent comet moments in blood cells of the five rats. Results are means of triplicate determinations (each rat generated three slides); error bars represent standard deviations

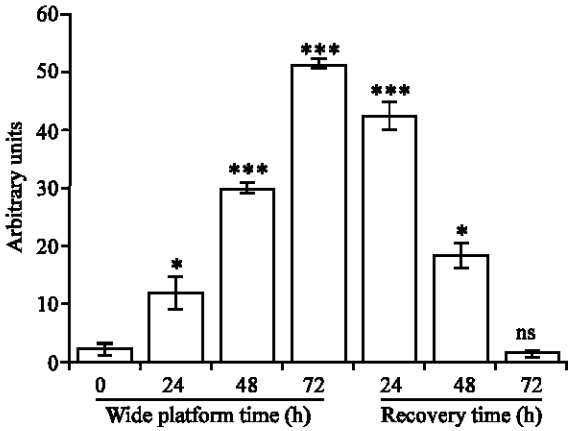

Fig. 2: Follow up of comets in blood cells of the rats subjected to water tank by wide platform technique " wet controls" (WC). Wistar rats were subjected to wide platform for 72 h and recovered until 72 h. Blood samples were obtained for each time (0, 24, 48 and 72 h) of recovery and comet assay performed. (*p<0.05; ***p<0.001). Columns represent comet moments in blood cells of the five rats. Results are means of triplicate determinations (each rat generated three slides); error bars represent standard deviations

animals (REMd group) as in rats submitted to the wide platform technique (WC group). Using the comet assay we evaluated DNA strand breaks in peripheral blood mononuclear cells (PBMC), which constitute a part of pool leucocytes, before, during and after REM sleep deprivation (REMd) on narrow platform and a "wet" control (WC) on wide platform. After REM sleep deprivation and the wide platform procedures a sleep rebound and a stress recovery occurred.

The comet assay is a method that can be used to estimate the extent of DNA damage[31]. The major advantage of comet assay over other methods of measuring DNA damage, such as pulse-field gel electrophoresis, is that the information acquired considers the distribution of DNA damage among individual cells within a sample population. In addition, only a small number of cells are required, allowing examination of a large number of experimental conditions from a single-treated population of cells.

The results obtained in this study show that in REMd rats, the comet scores in PBMC are proportional to deprivation time (Fig. 1). Besides, it was verified that after 72 h of REMd rebound, the animals showed a progressive count of comets similar to base line (before deprivation).

The analysis of Fig. 2 shows that WC rats submitted to wide platform technique present a comet induction profile similar to that observed in rats submitted to narrow platform technique (p>0.05).

Others studies have demonstrated that the narrow platform technique causes reduction of slow wave activity[7,34] and stress in animals[6,7]. Indicative parameters of stress such as: body weight loss; reduced thymus, spleen and liver weight, increased adrenal weight, increased basal and ACTH-stimulated corticosterone levels have been described in REMd animals[35-37]. Stress was also associated with the use of wide platform[2,35]. On the other hand, wide platform has been demonstrated to produce sleep deprivation when parameters such as latency, total time spent in REM sleep and duration of REM episodes was also shown to be altered for a period of 90 h on wide platforms[7]. In this study, it was suggested that modified multiple platform is an effective method to REM deprivation (narrow platform). They also proposed that the introduction of a grid instead of the classical wide platform should be considered as an adequate environmental control.

Moreover, REMd rats present a progressive increase in whole body energy expenditure, manifested by dramatic increase in food intake with loss of body weight[38,39]. Body temperature increases slightly by less than 0.5°C above normal during the REMd[39].

Sleep deprived rats develop necrotic skin lesions that do not become inflamed, which are similar to dermatoses present in immuno-compromised states[40]. In fact, clinical and experimental studies demonstrated that sleep deprivation reduced lymphocyte stimulation and depressed granulocyte phagocytosis[41] as well alter activities of natural killer cells and plasma interleukin-I[42]. These findings support the proposition that sleep has an important role in immune function[37].

In addition it was as also verified that infections develop more frequently during or after periods of sleep deprivation and suggests that REMd can impair immune function[37,43]. The decreased resistance to infection may allow lethal opportunistic organisms to enter the bloodstream and trigger a toxic reaction cascade[44].

In addition, oxidative stress has been demonstrated to occur in leucocytes under stress conditions[45-48]. Total glutathione, an antioxidant enzyme, was shown to be reduced in hypothalamus of REMd rats[29]. Thus, the possibility that DNA damage observed in PBMC of REMd and WC rats could be explained by oxidative stress. However, other data do not show altered levels of catalase and superoxide dismutase[27], indices of necrosis and apoptosis[28] or homocysteine plasma concentrations[49].

It is not possible to separate sleep deprivation and stress in narrow and wide platform techniques. Other experiments are necessary to clarify this question about DNA damage in PBMC. Nevertheless, the results of this study using a comet assay, show that sleep deprivation and/or stress induces DNA strand breaks in peripheral blood mononuclear cells.

Present data demonstrated that comet assay can be used to evaluate the induction of DNA strand breaks in peripheral blood mononuclear cells of rats submitted to stress conditions and suggest that stressful situations, including REM sleep deprivation, may have important immunological implications.

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