

Sleep Loss Induces Differential Response Related To Genotoxicity in Multiple Organs of Three Different Mice Strains

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Abstract: The purpose of the present study was to determine the genetic damage induced by paradoxical sleep deprivation (PSD) in three different male mice strains in peripheral blood, heart, kidney and liver tissues by the single cell gel (comet) assay. Swiss, C57BL/6j and hairless (HRS/j) mice were submitted to PSD by the multiple platform technique for 72 hr, and DNA damage was evaluated. Statistically significant differences in DNA damage were found in blood cells of the Swiss mice strain when compared to negative controls. By contrast, no statistically significant differences were found in the C57BL/6j or hairless mice strains. With regard to the liver, extensive genotoxic effects were found in the Swiss strain. The hairless and C57BL/6j mice strains did not show any signs of genotoxicity in this organ. The same lack of effect was noted in kidney and heart cells of all strains evaluated. In conclusion, our results reveal that sleep deprivation exerted genetic damage in the form of DNA breakage in blood and liver cells of the Swiss mice strain only. This type of approach should be considered when studying noxious activities on genetic apparatus induced by sleep deprivation in mice since the Swiss strain is more suitable for this purpose.

The single cell gel (comet) assay is a straightforward and efficient method for detecting DNA damage in mammalian cells [1–5]. The assay allows any viable eukaryotic cell to be analysed for DNA damage. For these reasons, the comet assay is now extensively used in biomonitoring studies whose goals are to detect putative genotoxins in the environment [6]; for review see [5,7,8]. In fact, previous studies conducted by our group demonstrated that the single cell gel (comet) assay is a functional tool for detecting DNA breakage induced by pathological processes such as experimental carcinogenesis, parasitic infections and exposure to xenobiotics, in multiple organs [2,3,9–12]. Recently, we demonstrated that selective paradoxical sleep deprivation (PSD) induces genetic damage in blood and brain cells in male rats [13], indicating that lack of sleep leads to severe molecular damage [14].

In humans, the shortening of sleep can occur due to various factors such as social life, artificial light, shift work or sleep disturbances. Indeed, sleep loss is heightened in societies that have a frenzied lifestyle. Additionally, most sleep disorders, such as sleep apnoea and insomnia, lead to sleep deprivation, which disrupts vital biological processes that are necessary for physical health [15,16]. The sleep deprivation method performed in rodents mimics the sleep fragmentation due to repeated awakenings, and is thus a useful tool for

investigating the effects of sleep loss. Abbreviation of sleep due to spontaneous sleep curtailment or sleep disorders can be assumed to produce several deleterious and long-lasting effects on certain physiological processes. For instance, it is well documented that PSD exerts a wide variety of negative effects on the immune system [17–20] and leads to changes in cognitive, mood, behavioural, hormonal and brain functions [21–27]. However, to the best of our knowledge, no study has investigated the influence of PSD on the genome at the level of the single cell in any mice strain. Thus, the aim of this investigation was to examine whether the peripheral blood, liver, kidney and heart of male mice are particularly sensitive to DNA damage following PSD in three different strains: Swiss, C57BL/6j and hairless (HRS/j). The investigation was conducted in three different strains in order to investigate whether the magnitudes of the PSD responses in each strain are related to DNA damage.

Materials and Methods

Animals. Adult Swiss and C57BL/6j mice were provided by our institution (Federal University of São Paulo). Hairless (HRS/j) mice were provided by the animal facility of the Institute of Chemistry (University of São Paulo). The mice were acclimated to the laboratory for at least 7 days prior to experimentation. The animals were housed in a ventilated cage system (Techniplast, Italy) in a temperature-controlled room (22°C) with a 12:12 hr light–dark cycle (lights on at 0700 hr) and allowed free access to food and water. Ventilated cages provide low rates of infection, eliminate odours from excreta, and reduce noise that may stress the animals, providing better welfare and living conditions. Mice used in this study were maintained

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and treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals [28] and the experimental protocol was approved by the UNIFESP Ethical Committee (CEP 1711/08).

Paradoxical sleep deprivation and experimental design. Ninety-day-old male mice of each strain were randomly distributed into two groups: the home-cage control and paradoxical sleep-deprived (PSD) groups. The experimental groups were submitted to PSD for 72 hr using the modified multiple platform method, which consists of placing five mice in a ventilated cage (38 × 31 × 17 cm, Tecniplast, Buguggiate, Italy) containing 14 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. At the onset of each paradoxical sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus awakening. Food and water were available *ad libitum*. This procedure for PSD has been previously published by our group [19,29]. The home-cage control group was maintained in separate cages in the same ventilated cage system as the experimental mice. By housing both groups in the same environment, we are able to control for differences in housing conditions between the two groups as well as provide the PSD group with satisfactory conditions of the ventilated cages.

After being housed in the water cages (PSD groups) or home-cages (control group), mice were transported to an adjacent room and decapitated at 9.00 a.m. and at noon. Blood was collected into sterile tubes containing liquid EDTA and the organs (heart, kidney and liver) were immediately removed and minced in 0.9% NaCl. The supernatant was removed and the cellular suspensions (~10 µl) were used for the single cell gel (comet) assay.

Single cell gel (comet) assay. The protocol used for peripheral blood, heart, kidney and liver cells followed the guidelines outlined by Sasaki *et al.* [30] with some modifications. Briefly, a volume of 5 µl of peripheral blood was added to 120 µl 0.5% low-melting-point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. Similarly, the supernatants (cellular suspension, 10 µl) of the heart, kidney, liver and peripheral blood samples were added to 120 µl 0.5% low-melting-point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslips were removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM

Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for approximately 1 hr. The slides were placed in alkaline buffer (pH>13) for 20 min. and then electrophoresed for 20 min. at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored until analysis. In order to prevent additional DNA damage, all steps were performed under reduced illumination. Independent positive controls peripheral blood, liver, kidney and heart cells were treated *in vitro* with 10 µM H₂O₂ (Hydrogen peroxide) for 15 min. at 37°C in order to ensure reproducibility and sensitivity of assay.

Genotoxicity data analysis. A total of 50 randomly captured comets per animal (25 cells from each slide) [31] were examined blindly by one expert observer (DAR) at 400 × magnification. The observer used a fluorescent microscope (Olympus, Tokyo, Japan) that was connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Suffolk, Haverhill, UK), which was calibrated according to the manufacturer's instructions. The computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To measure DNA damage, two image analysis system parameters were considered: tail intensity (% migrated DNA) and tail moment (the product of the tail length and the fraction of DNA in the comet tail) [31]. Since none of the groups showed significant differences between these parameters, one parameter, tail moment, was chosen for the presentation of the results.

Statistical methods. The results obtained in the single cell gel (comet) assay were statistically evaluated using the Kruskal-Wallis non-parametric test followed by *post-hoc* Dunn's test using Sigma Stat for Windows (Jadel Scientific, San Rafael, CA, USA). Values are expressed as mean ± S.E.M. The level of significance was set at 5%.

Results

In this study, we evaluated genetic damage induced by PSD *in vivo* in different target organs using three mice strains. Statistically significant differences ($p < 0.05$) in DNA damage were found between the blood cells of the Swiss mice strain submitted to PSD for 72 hr and those of negative controls (i.e. specimens not exposed to PSD) (table 1 and fig. 1). By contrast, no statistically significant differences ($p > 0.05$) in blood cells were found in the C57BL/6j or Hairless (HRS/j) mice strains. These findings are summarized in tables 2 and 3.

Regarding the liver, extensive genotoxic effects were found in the Swiss strain only. The hairless (HRS/j) and C57BL/6j mice strains did not show any signs of genotoxicity for this organ. The same lack of effect was noted in kidney cells, i.e. no statistically significant differences were detected between experimental group and the negative controls for all strains evaluated ($p > 0.05$). These findings are of the same magnitude as that of the heart cells, in which PSD did not

Table 1.

DNA damage (tail moment) in peripheral blood, liver, kidney and heart cells of Swiss mice exposed to paradoxical sleep deprivation (PSD) protocol.

DNA damage (tail moment)			
Organs evaluated	Negative control	PSD group	Positive control ¹
Peripheral blood	0.60 ± 0.35	3.20 ± 1.61*	4.38 ± 1.25*
Liver	0.30 ± 0.20	4.60 ± 1.80*	5.20 ± 0.65*
kidney	0.40 ± 0.30	0.20 ± 0.10	4.30 ± 1.65*
Heart	0.55 ± 0.50	0.55 ± 0.30	3.40 ± 1.20*

*Significantly different from all other groups ($p < 0.05$).

¹ H₂O₂ (10 µM), $n = 5$ animals.

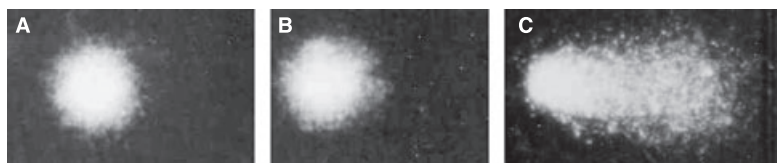


Fig. 1. Representative comet images of a blood cell from a negative control (A), a liver cell exposed to 72 hr of PSD (B), and an MMS-treated cell (positive control) (C). DNA was stained with ethidium bromide; 40 × magnification.

Table 2.

DNA damage (tail moment) in peripheral blood, liver, kidney and heart cells of C57BL/j mice exposed to paradoxical sleep deprivation (PSD) protocol.

DNA damage (tail moment)			
Organs evaluated	Negative control	PSD group	Positive control ¹
Peripheral blood	0.53 ± 0.35	0.80 ± 0.43	4.38 ± 1.25*
Liver	0.20 ± 0.15	0.15 ± 0.20	5.20 ± 0.65*
kidney	0.30 ± 0.25	0.45 ± 0.30	4.30 ± 1.65*
Heart	0.40 ± 0.24	0.30 ± 0.25	3.40 ± 1.20*

* Significantly different from all other groups ($p < 0.05$).

¹H₂O₂ (10 μM), $n = 5$ animals.

Table 3.

DNA damage (tail moment) in peripheral blood, liver, kidney and heart cells of hairless (HRS/j) mice exposed to paradoxical sleep deprivation (PSD) protocol.

DNA damage (tail moment)			
Organs evaluated	Negative control	PSD group	Positive control ¹
Peripheral blood	0.61 ± 0.15	0.25 ± 0.15	4.38 ± 1.25*
Liver	0.35 ± 0.25	0.30 ± 0.20	5.20 ± 0.65*
kidney	0.25 ± 0.25	0.45 ± 0.30	4.30 ± 1.65*
Heart	0.55 ± 0.30	0.55 ± 0.45	3.40 ± 1.20*

* Significantly different from all other groups ($p < 0.05$).

¹H₂O₂ (10 μM), $n = 7$ animals.

cause injury to the mouse genome. These findings are summarized in tables 1–3.

Mice blood, liver, kidney and heart cells were further assayed with H₂O₂ to ensure the sensitivity of the assay. A clear sensitivity was observed among experimental mice ($p < 0.05$) when compared to negative controls. No animal died unexpectedly during this experiment.

Discussion

The aim of the present study was to evaluate genetic damage induced by experimental sleep loss using three different mice strains. The investigation was conducted using the single cell gel (comet) assay. To the best of our knowledge, this approach has not been previously utilized in several organs using different mice strains.

The alkaline version of the single cell gel (comet) assay used is sensitive to a wide variety of DNA lesions. Among them are single- and double-strand breaks, oxidative DNA base damage, alkali-labile sites including abasic and incomplete repair sites, and DNA-DNA/DNA-protein/DNA-drug cross-linking in any eukaryotic cell [5]. Tail moment is a virtual measure, calculated by the computerized image analysis system that considers both the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method.

On the basis of tail moment data, the results of this study displayed that the alkaline single cell gel (comet) assay in our experimental conditions detected the presence of DNA damage in peripheral blood cells of mice from the Swiss strain submitted to PSD for 72 hr. By comparison, a recent study conducted by Kontogianni *et al.* [10] demonstrated higher basal levels of DNA damage and greater sensitivity to the effects of the DNA-damaging agents, such as hydrogen-peroxide, ethanol and gamma-irradiation, in lymphocytes isolated from obstructive sleep apnoea patients. Some authors have postulated that the effects of sleep loss on cellular and genomic activities could contribute to inflammatory cytokine activity. For instance, research has demonstrated that monocyte production of interleukin-6 and tumour necrosis factor alpha are significantly greater in the morning after a night of sleep loss, compared with morning levels following uninterrupted sleep [32]. This data was confirmed in a recent study conducted by the same research group [33]. Inflammation processes are associated with increased risk of several degenerative diseases, such as cardiovascular disorders, arthritis, hypertension and diabetes mellitus. This link is attributed to the fact that although such mechanisms help to eradicate several pathological conditions, they inevitably expose target organs to certain endogenous genotoxic agents [34]. Indeed, sleep is a key modulator of behavioural responses to immune activation and primarily exerts these effects through changes in neurotransmission and inflammatory protein expression. Recently, we demonstrated that PSD increased the susceptibility of mice to LPS-induced immobility [29]. Taken as a whole, our findings support the notion that PSD induces DNA damage in blood cells in the Swiss mice strain. Further studies examining DNA damage to the immune system should be conducted to explore the inflammation processes under a sleep deprivation paradigm.

The *in vivo* single cell gel (comet) assay guidelines [5] recommend that liver cells, in particular, should be analysed since the liver is a main organ for metabolism. To date, there are no data in the literature regarding the genotoxic potential of PSD on liver cells using *in vivo* murine models. Chang *et al.* [35] argued that PSD predisposes the liver to oxidative stress and phospholipid damage, leading to injury of the genetic apparatus. In fact, our results with the single cell gel (comet) assay detected DNA breakage in liver cells of the Swiss mice strain only. Overall, it seems as though this strain is more sensitive to genotoxic insult induced by PSD than other strains. However, it is not yet clear how and/or when the lack of sleep exerts these biological actions in this metabolic organ since the development of genetic damage in target cells depends not only on the initial levels of induced DNA damage and its repair, but also on other contributing factors (e.g. the production of reactive metabolites, their distribution, and their effects on cell proliferation). Furthermore, *in vitro* and *in vivo* genotoxicity tests detect compounds that induce genetic damage directly or indirectly by various mechanisms. Nevertheless, no single test is capable of detecting all genotoxic agents. Thus, for a more detailed

judgement on the genotoxic potential of PSD on liver cells, a battery of tests may be necessary.

The kidney is the main pathway for the elimination of absorbed ions as well as the main target organ of toxicity. Therefore, kidney cells were investigated for induced DNA breakage following PSD. Curiously, our results demonstrated the absence of an effect on DNA damage in mice submitted to PSD for all strains tested. Some authors have assumed that PSD modifies the 24 hr aldosterone profile by preventing the nocturnal increase in aldosterone release and leads to altered overnight hydromineral balance [36]. Moreover, others have affirmed an altered regulation of renal peripheral benzodiazepine receptors in sleep-deprived rats [37]. Therefore, our results are consistent with the notion that PSD does not induce genetic damage on mice kidney cells.

To further elucidate the possible outcomes of lack of sleep on the cardiovascular system, we evaluated genotoxicity in the heart cells. Loss of sleep induces elevations in circulating levels of cholesterol [28], stress-related hormones [38] and catecholamines [39–41], with attendant increases in blood pressure and heart rate [42]. Furthermore, habitual sleep loss and insomnia are markers of subclinical heart disease and are independent predictors of cardiovascular disease risk, particularly in males [43,44]. Our results show that PSD did not exert any detectable genotoxic activity on the heart for any of the mice strains evaluated. However, we speculate that despite the lack of genotoxic damage in heart cells, changes in the central nervous system may trigger cardiovascular dysfunction. Further studies are warranted to elucidate the issue.

In conclusion, our results reveal that PSD exerted genetic damage in the form of DNA breakage in blood and liver cells of the Swiss mice strain. Since DNA damage is an important step in events leading to genomic instability, which can promote the development of degenerative diseases, this study represents a relevant contribution to the evaluation of the potential health risks associated with sleep deprivation. Furthermore, this type of approach using multiple strains should be considered when testing noxious activities on genetic apparatus induced by sleep deprivation in mice since the Swiss strain is suitable for this purpose.

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