

## Original Article

# The influence of sleep deprivation on expression of apoptosis regulatory proteins p53, bcl-2 and bax following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide

Juliana Noguti<sup>1</sup>, Tathiana Aparecida Alvarenga<sup>2</sup>, Mônica Levy Andersen<sup>2</sup>, Sergio Tufik<sup>2</sup>, Celina Tijuko Fujiyama Oshima<sup>2</sup>, Daniel Araki Ribeiro<sup>1,3</sup>

Departments of <sup>1</sup>Pathology, <sup>2</sup>Psychobiology, <sup>3</sup>Biosciences, Federal University of São Paulo, UNIFESP, SP, Brazil

## ABSTRACT

**Background:** The aim of this study was to evaluate whether paradoxical sleep deprivation could affects the mechanisms and pathways essentials for cancer cells in tongue cancer induced by 4-nitroquinoline 1-oxide in Wistar rats.

**Materials and Methods:** For this purpose, the animals were distributed into 4 groups of 5 animals each treated with 50 ppm 4 nitroquinoline 1 oxide (4 NQO) solution through their drinking water for 4 and 12 weeks. The animals were submitted to paradoxical sleep deprivation (PSD) for 72 h using the modified multiple platform method, which consisted of placing 5 mice in a cage (41 × 34 × 16 cm) containing 10 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. The investigations were conducted using immunohistochemistry of p53, Bax and Bcl-2 proteins related to apoptosis and its pathways. Statistical analysis was performed by Kruskal-Wallis non-parametric test followed by the Dunn's test using SPSS software pack (version 1.0). *P* value < 0.05 was considered for statistic significance.

**Results:** Although no histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure in all groups, in 12 weeks were observed pre-neoplastic lesions. Data analysis revealed statistically significant differences (*P* < 0.05) in 4 weeks group for p53 and for bcl-2 and for all immunomarkers after 12 weeks of 4NQO administration.

**Conclusion:** Our results reveal that sleep deprivation exerted alterations in proteins associated with proliferation and apoptosis in carcinogenesis.

**Key Words:** Apoptosis, carcinogenesis, rat, sleep deprivation

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Address for correspondence:  
Dr. Daniel Araki Ribeiro,  
Departamento de  
Biosciencias, Av. Ana Costa,  
95, Vila Mathias, Santos – SP,  
Brazil. Zip code: 11060-001  
E-mail: daribeiro@unifesp.br

## INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent pathologic type and accounts for over 80% of head and neck malignancies.<sup>[1]</sup> Tongue cancer is the most frequently encountered tumor of the oral cavity as compared with other cancers of the oral cavity and has a greater propensity for lymph

node metastasis.<sup>[2]</sup> The majority of the oral cancer patients are diagnosed at an advanced clinical stage and the rate of survival has not improved, despite advances in the treatment. About 40% of these patients die from uncontrolled despite advances in the treatment, about 40% of these patients die from uncontrolled loco-regional disease alone and 24% show metastases to distant sites.<sup>[3]</sup> Our research group, using experimental models, has employed the chronic administration of 4NQO in drinking water that stimulates rat tongue carcinogenesis in a manner similar to its human counterpart.<sup>[4,5]</sup> This model is based on the multi-step process of carcinogenesis, which is characterized by initiation, promotion, and tumor progression.<sup>[6,7]</sup>

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Over the past several decades, there has been a trend towards a voluntary reduction in sleep time. There was a decrease of about 1.5 h when compared to the average sleep duration during the early twentieth century.<sup>[8]</sup> It is currently recognized as the harmful effects of sleep deprivation (SD).<sup>[9]</sup> SD disrupts vital biological processes necessary for cognitive function and physical health.<sup>[10]</sup> Cancer, as we know it, evolves genetic alterations in specific genes (i.e., mutations and amplifications) in the DNA, alterations in the operation of cell survival mechanisms and/or modifications in the signal transduction pathways that regulate these processes appear to make cancer cells exempt from the normal constraints of cell proliferation and apoptosis.<sup>[11-13]</sup> Apoptosis is a genetically regulated cell death involved in the deletion of cells in normal, as well as malignant tissues. Bcl-2 and Bax are two important effectors genes during the apoptosis process in mammals. The human Tp53 gene has been reported to be a tumor suppressor gene. It is located on human chromosome 17 and rodent chromosome 10.<sup>[14]</sup> The genes regulated by p53 are involved in cell cycle inhibition, apoptosis and angiogenesis.<sup>[14]</sup> The development, growth progression and spread of cancer comprise a complex multistage process that involves intricate molecular and biochemical interactions that become somatic cells able to proliferate rapidly.<sup>[11]</sup> Therefore, we hypothesized that paradoxical sleep deprivation (PSD) could affect these mechanisms and pathways essentials for cancer cells and analyzed possible connections between carcinogenesis, especially in the initiation and promotion phases of the cancer cells and the chronic sleep loss.

## MATERIALS AND METHODS

### Animals and experimental design

All experimental protocols involving animals conformed to procedures described in the guiding principles for the use of laboratory animals and the study was approved by The Animal Committee of Paulista Medical School-UNIFESP. Twenty male Wistar rats (8 weeks old), weighing approximately 250 g were obtained from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME). The animals were maintained at 22°C with a 12:12 h light-dark cycle (lights on at 0700 h) and allowed free access to food and water inside standard polypropylene cages. The animals were

distributed into 4 groups of 5 each and were treated with 50 ppm 4NQO (Sigma Aldrich, St. Louis, USA) solution through water intake for 4 and 12 weeks.

### Paradoxical sleep deprivation

The animals were submitted to paradoxical sleep deprivation (PSD) for 72 h using the modified multiple platform method, which consisted of placing 5 mice in a cage (41 × 34 × 16 cm) containing 10 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. This method is based on the loss of muscle tone presented at each paradoxical sleep episode observed in mice.<sup>[15]</sup>

### Experimental procedure

Rats were randomly distributed into four groups: PSD and home-cage groups for 4 weeks and for 12 weeks following exposure to 4NQO at 50 ppm in drinking water. These time-points were chosen based on our previous studies.<sup>[16]</sup> Finally, the control rats were maintained in separate cages in the same room as the experimental rats during PSD and were euthanized on the same day as the other groups. By housing all groups in the same room, we controlled the environmental conditions. After residing in the water tanks (PSD groups) or home-cages (control groups), rats were brought to an adjacent room and decapitated between 09:00 h and noon with minimum discomfort. The tongues were longitudinally bisected for histopathological and immunohistochemistry examinations. The tissues were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks and stained with hematoxylin and eosin (H.E.; Merck).

### Histopathological analysis

Histopathological evaluation was performed by light microscopy. Analyses of the tongue sections were graded as normal, hyperplasia and/or hyperkeratosis (acanthosis of the spinous layer and/or increased thickness of the keratin layer), dysplasia (cellular changes including basal cell hyperplasia, nuclear pleomorphism, hyperchromatism, increased number of mitotic figures) per animal according to Ribeiro, *et al.*<sup>[17]</sup>

### Immunohistochemistry

Serial sections of 4 µm were deparaffinized in xylene and rehydrated in graded ethanol, then pretreated in a microwave with 10 mM citric acid buffer (pH = 6) for 3 cycles of 5 min each for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide

in PBS for 5 min for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum in PBS for 10 min. The specimens were then incubated with either anti-Bcl-2 monoclonal antibody (Santa Cruz, Biotechnology, USA) at a concentration of 1:200, anti-Bax monoclonal antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:100 and anti-p53 antibody (Santa Cruz Biotechnology, USA) which is able to detect both wild-type and mutant isoforms, at a concentration of 1:100. All incubations were carried out overnight at 4°C. This was followed by two washes in PBS and incubation with a biotinylated secondary antibody. The sections were washed twice with PBS followed by the application of preformed avidin biotin complex (vector Technologies, USA) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3-3-dianimobenzidine solution and counterstained with hematoxylin.

**Quantification of immunohistochemistry**

Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in control and “hot spot” areas. A total of 1000 epithelial cells were evaluated in 3 to 5 fields at ×400 magnification. All values were used as labeling indices. This protocol was established in previous studies by our group.<sup>[4]</sup>

**Statistical methods**

Statistical analysis was performed by Kruskal-Wallis non-parametric test followed by the Dunn’s test using SPSS software pack (version 1.0). *P* value < 0.05 was considered for statistic significance.

**RESULTS**

**Histopathological evaluation following 4NQO treatment**

No histopathological changes in epithelial cells were observed in the control group after 4 weeks-treatment with 4NQO. The primary histopathological change, i.e., hyperplasia and hyperkeratosis with the spinous cell layer gradually thickened, was evidenced after 12 weeks-treatment. In this period, epithelial dysplasia was also found in mild and moderate forms. The histopathological findings are summarized in Table 1.

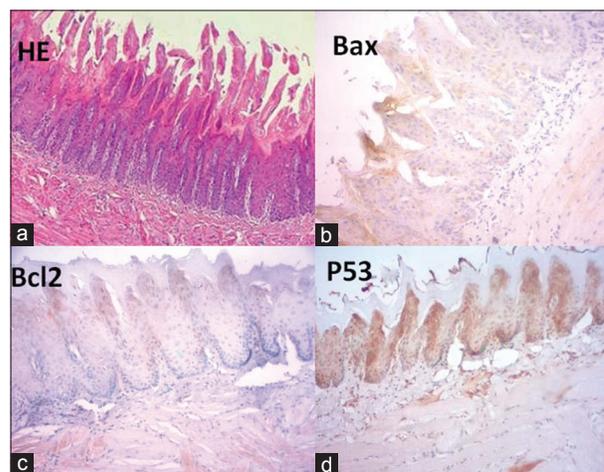
**Immunohistochemistry**

Immunohistochemical data for bcl-2, bax and p53 are summarized in Figures 1-5. Immunostaining

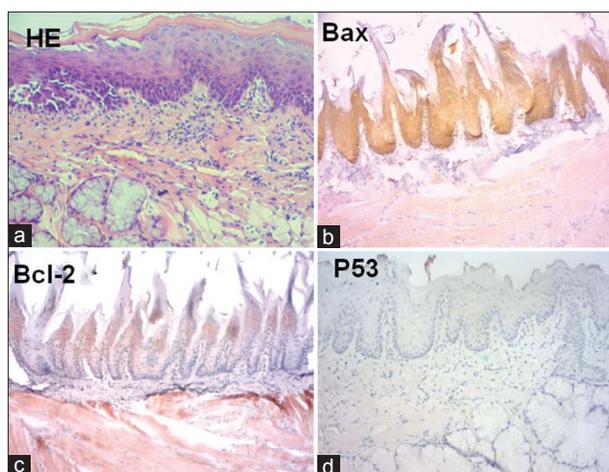
**Table 1: Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO) model for oral carcinogenesis submitted to acute sleep deprivation**

| Groups Weeks | No.of animals | Normal | Hyperplasia | Dysplasia | Hyperkeratosis |
|--------------|---------------|--------|-------------|-----------|----------------|
| 4 control    | 5             | 5      | 0           | 0         | 0              |
| 4 SD         | 5             | 5      | 0           | 0         | 0              |
| 12 control   | 5             | 2      | 2           | 2         | 1              |
| 12 SD        | 5             | 2      | 0           | 1         | 2              |

\*4NQO - 50 ppm by drinking water; SD: Sleep deprivation



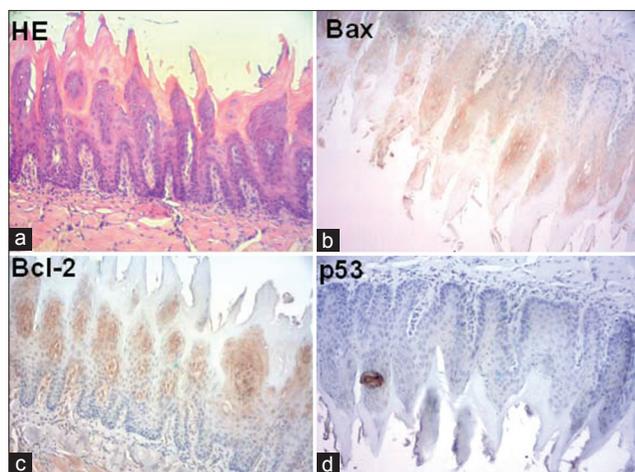
**Figure 1:** Photomicrographies showing the control group of rat tongue carcinogenesis after 4 weeks-treatment (a) hematoxylin-eosin stain; (b) immunohistochemistry for bax (c) immunohistochemistry for bcl-2; (d) immunohistochemistry for p53 (hematoxylin and eosin stain; 100× magnification)



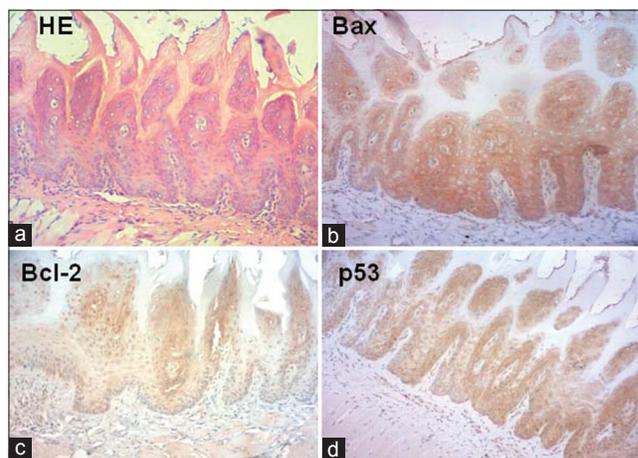
**Figure 2:** Photomicrographies showing the experimental group of rat tongue carcinogenesis after 4 weeks-treatment (a) hematoxylin-eosin stain; (b) immunohistochemistry for bax (c) immunohistochemistry for bcl-2; (d) immunohistochemistry for p53 (hematoxylin and eosin stain; 100× magnification)

for bcl-2 and p53 markers were detected in the cytoplasm with a granular pattern Figures 1-4. p53 expression was detected predominantly in the nuclei of the oral mucosa. Although no histological changes were induced in the epithelium after 4 weeks of carcinogen exposure, bcl-2 and bax were expressed in the superficial layers in control group, while p53 was expressed in all layers. Bcl-2 was over expressed in superficial layers of PSD group. p53 was found in all layers expressed weakly in PSD group. Following 12 weeks of carcinogen treatment, rat tongues showed early proliferative changes in the epithelium that were characterized by hyperplasia and dysplasia. These

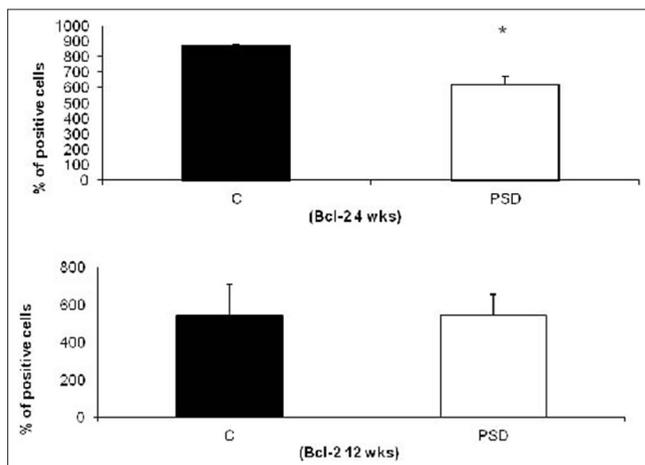
pre-neoplastic lesions contain high expressivity of bax. Control group showed expressivity of bax and bcl-2, especially in superficial layers, while p53 was weakly expressed. PSD group, we found p53 over expressed, especially in cells of tumor islands unlike the control group. Data analysis revealed statistically significant differences ( $P < 0.05$ ) in 4 weeks group for p53 ( $P = 0.02$ ), and bcl-2 ( $P = 0.02$ ). After 12 weeks of 4NQO administration, all immunomarkers investigated showed statistically differences. Such data are summarized in Figures 5-7 as depicted by positive epithelial cells following quantification of immunohistochemistry.



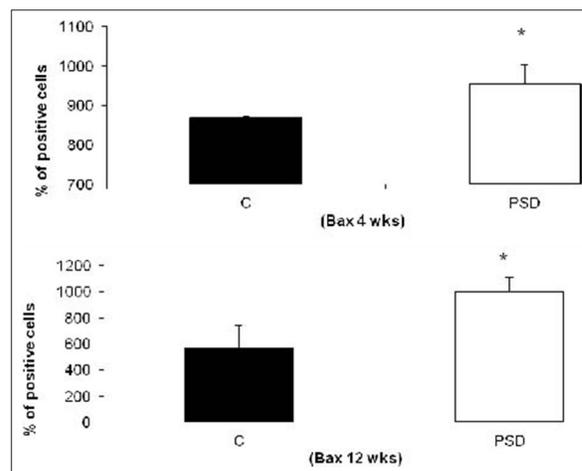
**Figure 3:** Photomicrographs showing the control group of rat tongue carcinogenesis after 12 weeks-treatment (a) hematoxylin-eosin stain; (b) immunohistochemistry for bax (c) immunohistochemistry for bcl-2; (d) immunohistochemistry for p53 (hematoxylin and eosin stain; 100x magnification)



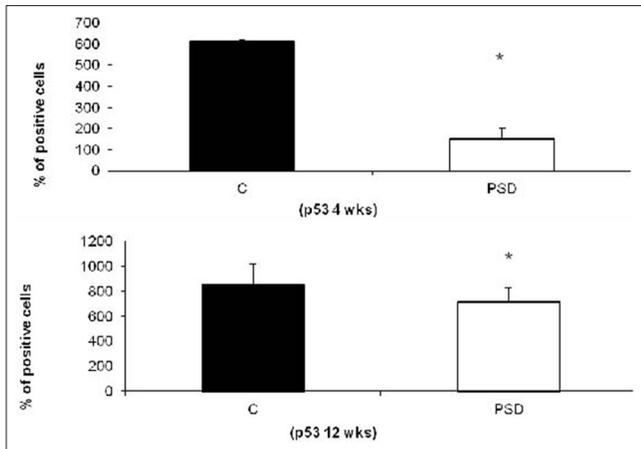
**Figure 4:** Photomicrographs showing the experimental group of rat tongue carcinogenesis after 12 weeks-treatment (a) hematoxylin-eosin stain; (b) immunohistochemistry for bax (c) immunohistochemistry for bcl-2; (d) immunohistochemistry for p53 (hematoxylin and eosin stain; 100x magnification)



**Figure 5:** p53 labeling index in the negative control and those exposed to 4-nitroquinoline 1-oxide for 4, and 12 weeks. Values were expressed as means±S.D. \* $P < 0.05$  when compared to negative control group



**Figure 6:** Bcl-2 labeling index in the negative control and those exposed to 4-nitroquinoline 1-oxide for 4, and 12 weeks. Values were expressed as means±S.D. \* $P < 0.05$  when compared to negative control group



**Figure 7:** Bax labeling index in the negative control and those exposed to 4-nitroquinoline 1-oxide for 4, and 12 weeks. Values were expressed as means $\pm$ S.D. \* $P < 0.05$  when compared to negative control group

## DISCUSSION

The present study was undertaken to evaluate the activity of sleep loss under acute conditions (PSD) in carcinogenesis of the rat tongue cancer induced by 4-nitroquinoline 1-oxide (4NQO). The investigations were conducted using immunohistochemistry of p53, Bax and Bcl-2, proteins related to apoptosis and its pathways. The major finding of the present study was that PSD could modulate the carcinogenesis process. To the best of our knowledge, this approach has not been demonstrated before.

An anti-apoptotic protein, bcl-2, prevents activation of caspases and apoptotic process, while bax, a pro-apoptotic proteins activates caspases leading to cell death.<sup>[17,18]</sup> In 4 weeks, the number of Bcl-2 positive cells was higher in control group when compared to the PSD group. Biswas, *et al.*,<sup>[19]</sup> found a number of bcl-2 positive neurons significantly lower when compared to bax. This indicates increased damage most likely increased apoptosis, however, this research was conducted only in brain cells without any interference of a chemical agent as 4 NQO. The expression of Bcl-2 oncoprotein may be regarded as a biological marker for neoplastic conversion.<sup>[20]</sup> Ribeiro, *et al.*<sup>[4]</sup> found similar results in 4 and 12 weeks control group, induced by 4 NQO, PSD was able to decrease bcl-2 expression in oral epithelial cells following 4NQO exposure. Therefore, it seems to be due genetic alterations, which may presumably play a critical role in loss of cell differentiation in

the process of malignant transformation, leading to conclude that in the control group Bcl-2 appears to be associated with progression of oral cancer, while PSD could improve the cancer progression. However, this suggestion should be verified by further investigation.

The expression of bax in PSD group did not alter after 4 weeks of 4NQO chronic administration, this results concur with studies performed by Ribeiro, *et al.*,<sup>[4]</sup> indicating a result of homeostatic pathways against the abnormal expression of bcl-2.<sup>[4]</sup> The expression of bax in control group was lower after 4 weeks of 4nqo chronic administration. Overexpression of Bax has been observed to promote apoptosis by increasing the susceptibility to anticancer drugs and radiation.<sup>[21,22]</sup> Naidoo, *et al.*<sup>[23]</sup> found that the endoplasmic reticulum (ER), major part of quality control system from the organism, stress response is compromised with aging and that the additional challenge of sleep deprivation further exacerbates ER stress and leads to induction of pro-apoptotic signaling.<sup>[23]</sup> Montez-Rodrigues, *et al.*<sup>[24]</sup> observed that the pre-frontal cortex was critically affected by PSD because bax increased and bcl-2/bax ratio was diminished. These data support the notion that sleep is important for brain functioning, as well as the interfere in the carcinogenesis process.<sup>[25,26]</sup>

p53 tumor suppressor gene not only participates in cell proliferation control but also plays a role in deletion of cells with DNA damage induction of apoptosis.<sup>[27]</sup> Loss of Tp53 tumor suppressor gene function due to mutation represents the most common genetic event known in human cancer.<sup>[28]</sup> Several works showed p53 expression higher in oral cancers.<sup>[29,30]</sup> It has been well documented that alterations of levels of p53 are early events in the development of tongue carcinoma. There is no doubt that p53 expression is an important early step in the development of carcinoma.<sup>[31]</sup> Expression of p53 in tongue squamous cell carcinoma induced with 4NQO showed in our control results agreement with studies performed using 4NQO in rats.<sup>[32]</sup> Interestingly, in 4 weeks of 4nqo administration, a number of p53 positive cells were lower in PSD group than in 12 weeks following 4 NQO PSD group. The Tp53 gene plays an important role in carcinogenesis, especially the early phases.<sup>[33]</sup> This abnormal expression of p53 between these groups could play a role in the development and biological behavior of oral neoplasms when associated with sleep disturbances. We suggest that

this observation will be useful in establishing a connection between cancer and sleep deprivation, to define the genetic alterations that could be involve in this process.

## CONCLUSION

In conclusion, our results reveal that sleep deprivation exerted alterations in proteins associated with proliferation and apoptosis in carcinogenesis, this observation have immense and far reaching implications and consequences on public health, especially on the night shift workers who often remain awake. This study represents a relevant contribution to the evaluation of the importance of carcinogenesis since, so far, there is no research demonstrating the relationship between the multistep of this process and sleep deprivation.

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