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Spontaneous alveolar bone loss after 4NQO exposure in Wistar rats

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ABSTRACT

Objective: This study evaluated the effect of an experimental carcinogenic, 4-Nitroquinoline 1-oxide (4NQO), in the spontaneous alveolar bone loss (ABL) in an animal model.

Design: Twenty-two male Wistar rats were included in this study. They were randomly divided into two groups: the control group (n = 10) received food and water *ad libitum*, and the test group (n = 12) receive the same food; however, 25 ppm of 4NQO was diluted in the drinking water. All animals were euthanized after 20 weeks, and the tongues were removed and analyzed macroscopically to determine the presence of oral mucosal lesions. All specimens were paraffin-embedded and histological sections were obtained. The microscopic analysis was based on routine procedure (haematoxylin and eosin stain). The analysis of spontaneous ABL was performed by a calibrated examiner using standardized photographs and imaging software. Differences in spontaneous ABL were assessed among the three resulting groups: control, 4NQO with oral squamous cell carcinoma (OSCC), and 4NQO without OSCC.

Results: In the 4NQO-treated group, nine animals developed OSCC. The animals in the 4NQO with OSCC group presented significantly more spontaneous ABL (0.65 ± 0.21 mm) than the control group (0.34 ± 0.05) ($p < 0.001$). The animals in the 4NQO without OSCC group showed a mean spontaneous ABL of 0.47 ± 0.13 mm, which was not statistically significant different when compared to the control group ($p = 0.096$).

Conclusions: It was concluded that the presence of OSCC enhanced spontaneous ABL in Wistar rats when compared to control animals. Additionally, it was shown that, solely, administration of 4NQO may not be considered responsible for alveolar bone destruction.

1. Introduction

Oral cancer has a multifactorial etiology and the most common known risk factors are exposure to tobacco and alcohol. Additionally, genetic, behavioral and environmental factors may be involved in its etiopathogenesis (Javed & Warnakulasuriya, 2016; Zeng et al., 2016). Among the most prevalent cancers, oral squamous cell carcinoma has a prevalence of 5% worldwide (Nielsen, Larsen, & Wolkoff, 2017). In Brazil, the incidence of oral squamous cell carcinoma is increasing. It was estimated that by the end of 2016, 11,140 new cases of oral cancer will be diagnosed (INCA, 2015). Approximately 50,000 new cases of oral cancer are expected around the world, of which 60% will occur in Asia, more specifically in India, Taiwan, China, Pakistan and Sri Lanka (Siegel, Miller, & Jemal, 2016).

Periodontal disease (PD) refers to the inflammatory processes that occur in the tissues surrounding the teeth in response to bacterial accumulation that may result in destruction of the periodontal tissues, such as bone loss, leading in the most severe cases to tooth loss (Mai et al., 2014). The literature has demonstrated associations between cancer and PD (Meyer, Joshipura, Giovannucci, & Michaud, 2008). The etiologic plausibility between the occurrences of both diseases is their shared etiological factors. Associations between PD and cancer, such as lung, pancreas, colon, rectum, brain, and skin cancer have been demonstrated in the literature (Michaud, Liu, Meyer, Giovannucci, & Joshipura, 2008). Additionally, a systematic review showed that the presence of periodontal disease increased the head and neck cancer risk

Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; ABL, alveolar bone loss; OSCC, oral squamous cell carcinoma; PD, periodontal disease; LABC, Laboratory Animal Breeding Center; HE, hematoxylin staining technique and eosin

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2.63-fold (Zeng et al., 2013).

For a proper understanding of the association between these diseases, studies using cancer induction in animal models, e.g., those using 4-nitroquinoline 1-oxide (4NQO), have been performed (Saitoh, Sato, Tonogi, Tanaka, & Yamane, 2016; Zhang, Hyer, Yu, D'Silva, & Kirkwood, 2014). However, the effect of 4NQO in periodontal disease/alveolar bone loss is poorly addressed. The objective of this study was to evaluate the experimental carcinogenic effect induced by 4NQO on spontaneous alveolar bone loss (ABL) in an animal model.

2. Materials and methods

This study was approved by the Animal Use Ethical Committee of the Federal University of Rio Grande do Sul (protocol #140049). Twenty-two male Wistar rats (2 months old), obtained from the Laboratory Animal Breeding Center (LABC) of the Federal University of Pelotas, were used in this study. Animals weighed approximately 300 g at the start of the study.

The animals were randomly allocated into two groups using a statistical website (randomization.com). Animals allocated to the control group (n = 10) received food and water *ad libitum*. Animals in the test group (n = 12) received similar food; however the liquid intake comprised 25 ppm of 4NQO diluted in drinking water (Sigma Aldrich, St. Louis, MO, USA). Water was provided in amber bottles because 4NQO is photosensitive.

The animals were maintained in the specific pathogen-free facility at the LABC of the Federal University of Rio Grande do Sul. They were placed in transparent polypropylene boxes (65 × 25 × 15 cm) with shavings, harboring four animals in each box. The temperature (20 °C ± 2 °C) and the humidity (50%–60%) were controlled. Additionally, adequate ventilation and light/dark cycles of 12 h were provided. The animals were weighed weekly and their health status assessed daily. All animals were euthanized after 20 weeks by exsanguination from a cardiac puncture after general anesthesia with isoflurane (5% in 0.5 l/min of O_2).

2.1. Sample size estimation

The sample size estimation for this study was based on previous published studies that used induced carcinogenesis by 4NQO in animal models. These studies allocated 10 animals to each experimental group (Carvalho et al., 2012; Fracalossi, Silva, Oshima, & Ribeiro, 2010; Minicucci et al., 2011; Ribeiro, Fracalossi, Uatari, Oshima, & Salvadori, 2009). Because another study reported a mortality rate of 28% in longer periods of 4NQO treatment (Dayan, Hirshberg, Kaplan, Rotem, & Bodner, 1997), two additional animals were allocated to the test group.

2.2. Clinical analysis

The frequency of tongue lesions (leukoplakic, papillary or ulcerated) was recorded for each animal.

2.3. Histopathological analysis

Subsequently, the tongues were removed and analyzed macroscopically to determine areas with and without lesions. The lingual lesions and normal mucosa fragments were separated for microscopic evaluation. Specimens were fixed in 10% buffered formalin solution. From the paraffin blocks, histological sections of 3 µm, were obtained that were submitted to hematoxylin and eosin (HE) staining for the production of the histological slides. Using these slides, morphological analysis was performed. Specimens were classified according to Miranda, Noguti, Carvalho, Oshima, and Ribeiro (2011) (Miranda et al., 2011) as: no microscopic changes, epithelial hyperplasia with/without hyperkeratosis, epithelial dysplasia, or oral squamous cell carcinoma. The frequency of each type of microscopic change was recorded for each group. The decision on the microscopic change was based on the consensus of three examiners (CCB, IPK, VCC), who were blinded to group allocation.

2.4. Alveolar bone loss analysis

The jaws were gently removed and placed in 10% buffered formalin. Subsequently, the pieces were submerged in 9% sodium hypochlorite for 4 h to completely remove all organic material. After that, the pieces were washed with water to neutralize the action of the hypochlorite. Jaws were then dried and dyed with 1% methylene blue to identify the cemento-enamel junction. Subsequently, the dyed pieces were prepared for standardized photographs, using an endodontic ruler and a wax to fix the pieces. The photographs were taken in a standardized manner using a professional camera (Nikon® D5300). For the photograph analyses, an imaging software (Image J®) was used, and a calibrated examiner (HRO) was used to perform the measurements from the cemento-enamel junction to the alveolar bone crest. Five measurements were performed, two in the mesial root, one in the furcation, and two in the distal root. All of these measurements were performed on the labial and palatal surfaces and on both the right and left hemiarch. Measurements were made on the basis of the number of pixels from the cemento-enamel junction to the alveolar crest and converted to mm (Oballe et al., 2014).

2.5. Intraexaminer calibration

All photos were analyzed by a trained and calibrated examiner (HJRO). A test for intraexaminer reproducibility was performed with five randomly chosen photographs measured twice by the same examiner after a one-week interval. The intra-class correlation coefficient was 0.99 for alveolar bone loss.

2.6. Statistical analysis

The mean value of all measurements was calculated for each animal both for the control and test groups. In the test group, 3 animals did not develop oral squamous cell carcinoma. This group was used for intragroup/intergroup comparison.

The normal distribution analysis was confirmed by the Shapiro-Wilk test ($p > 0.05$). Therefore, one-way ANOVA, followed by Tukey's post-hoc test, were used to assess the difference among the three groups, control, and 4NQO with and without OSCC. Afterwards, the post-hoc Tukey's test was used.

Regarding the analysis of weight, food and water consumption, only two groups (test and control) were considered, and a *t*-test for independent samples was performed. For all analyses, a p-value < 0.05 was considered statistically significant.

3. Results

Table 1 shows the baseline and final weight of all animals. No statistically significant differences between groups both at baseline and at the end of the study were demonstrated. However, in comparison to the

Table 1
Baseline, final weight, and weight gain in both experimental groups.

Group	Baseline weight (g)		Final weight (g)		Weight gain (%)	
	Mean (SD)	P-value	Mean (SD)	P-value	Mean (SD)	P-value
Control (n = 10)	362.42 (31.96)	0.76*	511.63 (43.43)	0.10*	41.32 (5.15)	< 0.01*
Test (n = 12)	373.65 (35.52)		416.67 (75.49)		11.15 (15.40)	

*t test for independent samples.

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