Received: 8 July 2025 | Revised: 4 August 2025 | Accepted: 5 August 2025

DOI: 10.1111/php.70022

RESEARCH ARTICLE

Experimental assessment of photodynamic therapy effects on 4NQO-induced rat tongue carcinogenesis: Clinical, morphological, and immunohistochemical analysis

Lauren Frenzel Schuch[1,2,3] | **Tuany Rafaeli Schmidt**[4] | **Daniela Campagnol**[5] | **Vivian Petersen Wagner**[3,6] | **Tuane Nerissa Alves Garcez**[5] | **Alexia Antunes Deluca**[4] | **Felipe Martins Silvera**[2,3] | **Ronell Bologna-Molina**[2,3] | **Chris Krebs Danilevicz**[7] | **Cristiane Helena Squarize**[3,8] | **Rogerio Moraes Castilho**[3,8] | **Marco Antonio Trevizani Martins**[3,4,9] | **Vanderlei Salvador Bagnato**[10] | **Cristina Kurachi**[10] | **Pablo Agustin Vargas**[1,3] | **Manoela Domingues Martins**[1,3,4]

[1]Oral Diagnosis Department, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil

[2]Department of Diagnosis in Pathology and Oral Medicine, School of Dentistry, Universidad de la Republica, Montevideo, Uruguay

[3]National Institute of Science and Technology in Translational Biophotonics in Dentistry (BioPHOTO Dent), Brazil

[4]Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[5]Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

[6]Department of Dentistry, School of Dentistry, Universidade de São Paulo, São Paulo, Brazil

[7]Department of Pharmacology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[8]Michigan University, School of Dentistry, Department of Periodontics and Oral Medicine, Ann Arbor, Michigan, USA

[9]Department of Oral Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[10]Department of Physics and Materials Science, São Carlos Institute of Physics, Universidade de São Paulo, São Carlos, São Paulo, Brazil

Correspondence
Manoela Domingues Martins, Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil.
Email: manomartins@gmail.com and manoela.martins@ufrgs.br

Abstract

The present study aimed to evaluate the safety of photodynamic therapy (PDT) during oral carcinogenesis using a 4-nitroquinoline 1-oxide (4NQO) rat model, which serves as a surrogate for tobacco-induced oral cancer. Forty male Wistar rats received 4NQO (25 ppm) in drinking water for either 12 or 20 weeks to induce oral lesions. Animals were divided into two groups: Control and PDT. PDT was applied weekly from the beginning of the protocol. A 5-aminolevulinic acid (5-ALA) solution was topically applied to the posterior tongue, followed by laser irradiation guided by fluorescence detection 2 h post-application. Clinical, histopathological, and immunohistochemical (Ki-67) evaluations were performed. Lesions on the posterior dorsum of the tongue manifested in all animals, initially presented as white patches, and over time, progressed to erosive lesions, nodules, and ulcers. Histopathological

Abbreviations: PDT, photodynamic therapy; 4NQO, 4-nitroquinoline-1-oxide; OPMD, oral potentially malignant disorder; OSCC, oral squamous cell carcinoma; DMSO, dimethylsulfoxide; 5-ALA, 5-aminolevulinic acid; LSD, least significance difference; LS, least squares; CI, confidence intervals; nm, wavelength; mW, milliwatt; W/cm^2, power density; S, seconds; J/cm^2, energy density; J, joule.

analysis revealed that epithelial dysplasia predominated at 12 weeks, while squamous cell carcinoma was more frequent at 20 weeks in both groups. No significant differences were observed between the Control and PDT groups concerning clinical appearance or microscopic analysis at either time point. However, Ki-67 expression was significantly lower in the PDT group ($p < 0.05$), indicating reduced cell proliferation. Cellular proliferation was also downregulated at distant sites, specifically in the anterior tongue, similar to that observed in 4NQO-treated posterior tongue in the PDT group relative to controls at 12 and 20 weeks, suggesting that the antiproliferative effect of PDT extends beyond the irradiated site. Although PDT did not significantly influence lesion progression, it was found to markedly reduce Ki-67 expression levels – even at non-irradiated sites. This observation suggests a potential suppressive effect of PDT on cellular proliferation, which may play a role in modulating tumor growth during the carcinogenesis process.

KEYWORDS

Oral carcinogenesis, oral dysplasia, oral potentially malignant disorders, photodynamic therapy

INTRODUCTION

Photodynamic therapy (PDT) represents a noninvasive therapeutic modality employed for the treatment of both malignant and noncancerous conditions.[1] Its origins trace back to the early 20th century when Oscar Raab, a medical student, made the initial discovery.[2] Shortly thereafter, in 1913, Dr. Friedrich Meyer-Betz conducted the inaugural clinical study on humans, wherein he applied porphyrins to his own skin, terming it photoradiation therapy.[3] The first investigation into the effects of PDT on tumors occurred in 1942. This study involved the injection of hematoporphyrin into tumor-bearing mice, followed by exposure to a quartz-halogen lamp to examine tumor necrosis and fluorescence.[4]

This therapeutic approach encompasses three pivotal elements: (1) oxygen, (2) a photosensitizer, and (3) a specific wavelength of visible light.[5] During this process, the light activates the photosensitizer, initiating a cascade of photobiological and photochemical reactions. These reactions lead to irreversible damage, and ultimately, the demise of the target cells.[6–10] Given that PDT operates as a cold photochemical method, it avoids tissue heating. Notably, connective tissues, including elastin and collagen, at the treated site exhibit no discernible alterations. Consequently, in comparison to thermal laser techniques and other invasive approaches, there is a notably diminished risk of compromising the integrity of fundamental operative structures.[5]

PDT was initially introduced in the palliative treatment of metastatic breast cancer in 1975.[11] In dermatology, PDT finds extensive use for the treatment of superficial skin cancers, including but not limited to actinic keratoses, superficial or nodular basal cell carcinomas, Bowen's disease, and in situ squamous cell carcinomas.[11,12] More recently, it has been applied in the treatment of inflammatory and infectious dermatoses, including granuloma annulare, warts, and acne.[11] Within the domain of oral medicine, several studies have explored the effects of PDT on oral potentially malignant disorders (OPMD) and oral cancer.[13–17]

Our team conducted a comprehensive overview to systematically assess and critically evaluate the existing evidence concerning the impact of PDT on the management of OPMD and oral squamous cell carcinoma (OSCC).[18] In brief, regardless of the observed effects that are both satisfactory and secure of PDT against OPMD and OSCC, further well-designed cohort studies are required to establish its efficacy as a first-line treatment. Additionally, its safety in mucosa altered by carcinogenic processes remains undetermined, and its utilization in clinical centers is not yet widespread.[19] Given these considerations, the current study aims to assess the impact of PDT on oral carcinogenesis induced by 4NQO in a rat model. Specifically, we investigate whether this therapy interferes with cell proliferation during the carcinogenic process, using clinical, morphological, and immunohistochemical analyses.

MATERIALS AND METHODS

Ethical approval

The ethical approval for this study was granted by the Hospital de Clínicas de Porto Alegre's Ethics Committee,

Brazil, under protocol number 2021–0614. All steps were conducted in strict adherence to the guidelines outlined in Brazilian Law 11.794 and the Brazilian Guideline for the Care and Use of Animals, as established by the National Council for Animal Experimentation Control. The experiment was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Animal model

Forty male albino Wistar rats (8 weeks; 250-300 g) were chosen for the research. The animals were maintained under controlled conditions (i.e., 22 ± 2°C, humidity at 55% ± 5%, and a 12-h light–dark cycle). Throughout the experiment, the rats had unrestricted access to selected solid feed and autoclaved water, with or without the addition of 4NQO. The rats were individually housed in ventilated polycarbonate microisolators, featuring a floor lined with flocked pine shavings and a maximum density of three animals per box. All handling of the boxes and animals adhered to controlled conventional practices, utilizing materials subjected to a prior sterilization process.

Sample size

For the sample size, a software developed by the University of British Columbia (https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html) was used. In this calculation, prevalence data for OSCC in groups treated with 4NQO alone and 4NQO + chemoprotective agent were sourced from prior studies employing similar dosage and application duration of the carcinogen. Specifically, data from Soares et al., 2018 (60% vs. 0%, respectively), and Thandavamoorthy et al., 2014 (83% vs. 16%, respectively), were used.[20,21] Based on a desired power of 0.8 and an alpha value of 0.05, a sample size of eight animals per group was determined. To account for potential loss due to adverse effects of the carcinogen, estimated at up to 30% of the sample,[22] the final established sample size was 11 animals per experimental group for each day of euthanasia (at both 12 and 20 weeks). The selection of two euthanasia time points is crucial for evaluating the carcinogenesis process at distinct intervals.[23]

Experimental design

Figure 1 illustrates the methodological procedures. The experimental animals were divided into four groups,

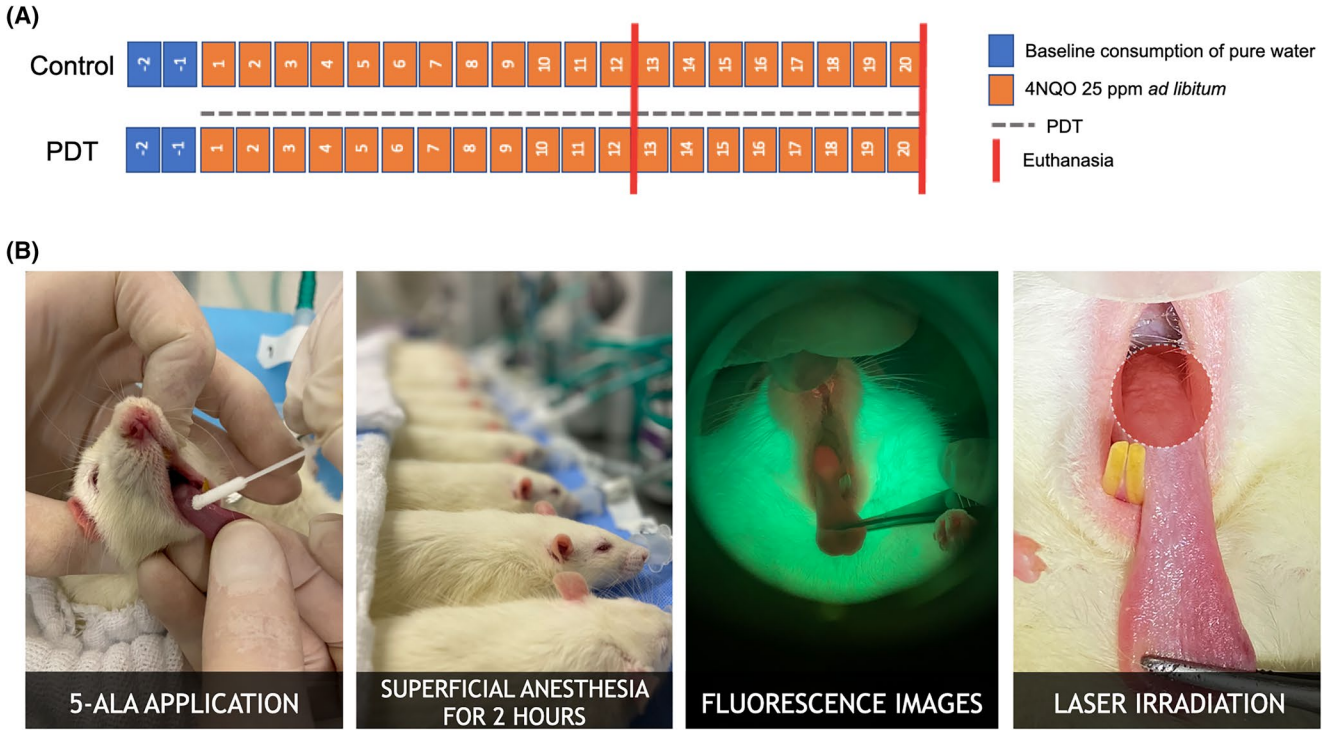

FIGURE 1 Schematic representation of the experimental design. (A) Both control and PDT groups received 4NQO (25 ppm) for 20 weeks, with euthanasia performed at two time points (Week 12 and Week 20). (B) Images illustrate the procedure for the PDT group: Topical application of 5-ALA to initial tongue lesions, followed by superficial anesthesia of the rats for 2 h, fluorescence imaging of the targeted region, and subsequent laser irradiation.

which were organized according to the two times of euthanasia (i.e., 12 and 20 weeks):

Control Group ($n = 22$): Control animals were treated with a normal diet and provided 4NQO drinking water for 12 weeks ($n = 11$) and 20 weeks ($n = 11$).

PDT Group ($n = 18$): PDT protocol in animals treated with a normal diet and provided 4NQO drinking water for 12 weeks ($n = 11$) and 20 weeks ($n = 7$).

Water and 4NQO consumption control

In accordance with the animals' initial consumption of pure water, water intake was monitored before the administration of 4NQO. To calculate the average water consumption per box, the drinkers were weighed twice a week over a 2-week period (Figure 1A).

Oral carcinogenesis model

All animals received treatment with a 25 ppm solution of 4NQO (LOT 711645086-1-1, Toronto Research Chemicals Inc., Toronto, Canada) diluted in drinking water. The formulation involved a calculation based on 25 mg of 4NQO, 1 mL of the dimethylsulfoxide solvent (DMSO – Sigma, St. Louis, MO, USA), and 1 liter of autoclaved water. Before reaching the designated concentration, a graduated scheme of 10 ppm was administered in the first week.[24]

The rats had free access to water with carcinogen during all the experimental periods. To minimize the adverse effects caused by the 4NQO, the animals received pure water twice a week and a 5% glucose solution once a week (100 mL per animal). Moreover, during the five initial weeks, a hypercaloric diet once a day [2 pellets (2 g) per animal] was offered.

After the experimental period (12 and 20 weeks), all rats were euthanized by excess isoflurane inhalation.

Administration of 5-ALA and PDT protocol

The PDT protocol was based on previous studies.[25,26] PDT was performed with the animal sedated once a week. Inhalational anesthesia was administered with isoflurane, diluted in oxygen, supplied by a vaporizer at a concentration of 5% for induction and 1%–2% for maintenance of anesthesia. The photosensitizer 5-aminolevulinic acid (5-ALA) was topically applied at 5% by dissolving powdered 5-ALA (Sigma Aldrich, USA) in EDTA (ethylenediaminetetraacetic acid), homogenized with lanolin and Vaseline. A swab impregnated with 5-ALA solution was rubbed on the posterior region of the dorsum of the tongue, covering an area of approximately 3 × 3 mm. This region was selected based on the higher prevalence of lesions in this site.[27] After sedation, 5-ALA was applied to the back portion of the tongue's dorsum. A time of 2 h is necessary until the fixation of the 5-ALA in the mucosa—the rat remained anesthetized at a maintenance dose. During this period, the animals were maintained under superficial anesthesia (0.5%–1% isoflurane diluted in oxygen), receiving basic care such as the use of a heating bed and compresses to avoid hypothermia. Body temperature was measured using a digital thermometer introduced in the rectal region.

After 2 h, a point on the posterior region of the tongue demarcated by the dye was irradiated with the following laser protocol: a 635 nm laser (85 mW) with a 0.03 cm^2 spot size (2.83 W/cm^2) was applied for 23 s, delivering 65 J/cm^2 and 1.95 J per point. The irradiation parameters were adjusted based on the parameters utilized in a previous study.[28] Figure 1B illustrates these steps.

Widefield fluorescence imaging

Before the laser irradiation step, fluorescence images from the tongue were acquired in a dark room (Figure 1B). The objective of the fluorescence image analysis was to assess both the production and uniform distribution of PpIX on the dorsum of the tongue. To capture the images, a diagnostic system designed for Widefield fluorescence was employed (EVINCE, MM Optics, Sao Carlos – Brazil). The images obtained via Widefield fluorescence were qualitatively examined using red color visualization.

Animal monitoring

The physical and behavioral health of the animals was checked daily. Every 2 days, rats were weighed. Once a week, the tongues of the animals were photographed for clinical follow-up. At the end of the experiment, a blind evaluator scored the clinical appearance based on a previous study.[29]

Histological analysis of tongues

After euthanization, the whole tongue was removed and fixed in a neutral-buffered formalin solution for 24 h. The formalin-fixed tongues were then processed and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin for histopathological analysis. A blinded, experienced oral pathologist conducted the microscopic evaluation. All samples were blinded regarding both the experimental group and the

time point of analysis; they were classified based on the presence or absence of epithelial dysplasia, carcinoma in situ, or invasive carcinoma.

Immunohistochemical staining and assessment

For the immunohistochemical analysis, 3-μm thick sections were prepared from paraffin-embedded tissue blocks and mounted on polarized slides. The slides underwent antigen retrieval using a heat-induced epitope retrieval solution (Reveal Decloaker, RTU; Biocare Medical). Endogenous peroxidase activity was quenched with 0.9% hydrogen peroxide for 5 min. The tissue sections were then incubated with the monoclonal anti-ki67 antibody (clone RM360, Rabbit; BioSB, Santa Barbara, CA, USA; 1:50). Following primary antibody incubation, the sections were treated with a biotinylated anti-mouse/anti-rabbit secondary antibody and a streptavidin–horseradish peroxidase (HRP) complex for 40 min each (mouse/rabbit ImmunoDetector Biotin Link and HRP Label; Bio SB). Negative control samples were processed without the primary antibody, whereas positive control samples utilized breast cancer tissues. Detection was accomplished using 3,3′-diaminobenzidine (DAB) as the chromogen (Biocare Medical), and the sections were counterstained with Harris hematoxylin.

All slides were scanned using a MoticEasyScan One digital scanner (Motic Asia, Hong Kong) and examined digitally with Pathomation software (Pathomation BV, Belgium). Photographs were taken at 40× magnification, focusing on the posterior region of the tongue where PDT was applied, as well as the anterior portion for comparison of the carcinogenesis process. The Ki-67 labeling index was calculated as the percentage of Ki-67-positive cells relative to the total number of nuclei in three selected hot-spot areas from both the posterior (PDT-treated) and anterior tongue regions. Cell counting was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), which involved manual delimitation of the regions of interest, followed by automatic quantification of positive (brown nuclear staining) and negative nuclei.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software version 10.0 (GraphPad Software, San Diego, CA, USA). Differences between groups at each experimental time point were evaluated using multiple t-tests. Significant differences are indicated by different lowercase letters in both graphs and tables ($p < 0.05$). Moreover, a two-way repeated measures ANOVA was used to evaluate the effects of treatment group (control vs. PDT) and anatomical site (lesion site vs. anterior area) on the percentage of Ki-67–positive cells. In this design, the treatment group was treated as a between-subjects factor (column factor), while the anatomical site was treated as a within-subjects factor (repeated measures, time factor). A similar analysis was conducted using histopathological diagnosis as the between-subjects (column) factor to assess whether Ki-67 expression varied by diagnosis across different anatomical sites. For the first model, post hoc pairwise comparisons were performed using Fisher's least significant difference (LSD) test without correction for multiple comparisons, as interaction effects were not statistically significant. For the second model, Tukey's multiple comparisons test was used for post hoc analysis. Data are presented as mean differences of predicted least squares (LS) means, with corresponding 95% confidence intervals (CI). Statistical significance was defined as $p < 0.05$.

RESULTS

Clinical evaluation of oral lesions

The animals from the control and PDT groups developed lesions on the tongue mucosa localized mainly along the posterior dorsum of the tongue, corresponding to the region where the PDT protocol was applied. No lesions were observed in other locations. The lesions began in Week 5, observed as white patches. Over time, erosive lesions, white plaques, nodules, and ulcers could be observed. Nonsignificant differences were noted at 12 weeks ($p = 0.26$) and 20 weeks ($p = 0.32$) between the Control and PDT Groups.

Widefield fluorescence imaging

During the image acquisition, clinical differences in PpIX production could be observed since Week 8. File S1 shows an example of the qualitative analysis of the PpIX accumulation formed in 2 h according to the Widefield fluorescence imaging.

PDT does not interfere with the clinical and morphological cancer progression; it reduced cell proliferation

The clinical features are illustrated in Figure 2 (weeks 7, 12, 19, and 20). The morphological findings are

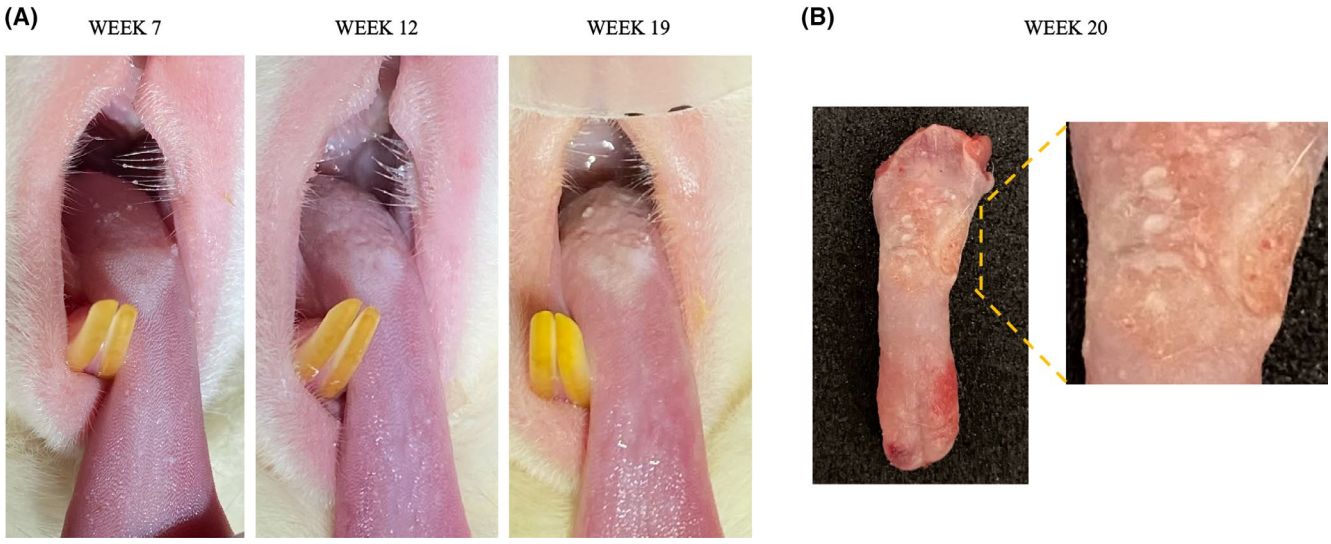

FIGURE 2 Follow-up images of an animal from the PDT group. (A) Macroscopic evaluation of tumor initiation shows evidence of cancer progression, with white spots on the dorsum of the tongue at Week 7, followed by the formation of a verrucous plaque on the posterior dorsal tongue at Weeks 12 and 19. (B) Macroscopy of the same animal's tongue after euthanasia at the Week 20 time point reveals an ulcerated lesion on the dorsal tongue.

TABLE 1 Histological diagnosis after 12 and 20 weeks of experiment.

	Hyperplasia, acanthosis and hyperkeratosis	Epithelial dysplasia	In situ carcinoma	Carcinoma
12 weeks				
Control Group, $n=11$	0	8	3	0
PDT Group, $n=11$	2	8	1	0
20 weeks				
Control Group, $n=11$	0	1	1	9
PDT Group, $n=7$	0	0	2	5

summarized in Table 1 and illustrated in Figure 3. The figure shows representative histological images of epithelial hyperplasia (Figure 3A,B) showing preserved epithelial architecture without atypia, epithelial dysplasia (Figure 3C,D) with nuclear pleomorphism, hyperchromatism, and basal cell disorganization, and invasive squamous cell carcinoma (Figure 3E, F) which presents as full-thickness atypia, cellular pleomorphism, stromal infiltration, and keratin pearls. No statistical differences were observed between control and PDT groups in 12 weeks ($p<0.54$) or 20 weeks ($p<0.43$). At the end of 12 weeks, 8 (72.7%) animals of each group (PDT and control) exhibit epithelial dysplasia. After 20 weeks of experiment, the majority lesions in the control and PDT group were squamous cell carcinoma.

Figure 4 illustrates the distribution of immunostaining in the control and PDT groups at both 12 and 20 weeks. The Mann–Whitney Test demonstrated that Ki-67 expression was significantly lower in the PDT group ($p<0.05$); suggesting that PDT may have a suppressive effect on cellular proliferation within the treated region (Figure 5A,B).

PDT broadly downregulates cellular proliferation in the tongue

At the 12-week time point, two-way repeated measures ANOVA revealed significant main effects of anatomical site ($p<0.0001$) and treatment group ($p=0.0040$) on Ki-67 expression. No significant interaction was observed between the two factors ($p=0.4376$), indicating that the effect of treatment was consistent across anatomical sites. Post hoc comparisons using uncorrected Fisher's LSD test showed that Ki-67 positivity was significantly reduced in the PDT group compared with the control group, both at the lesion site (mean difference = 11.30%, 95% CI: 1.31–21.29, $p=0.0278$) and at the anterior area (mean difference = 15.71%, 95% CI:

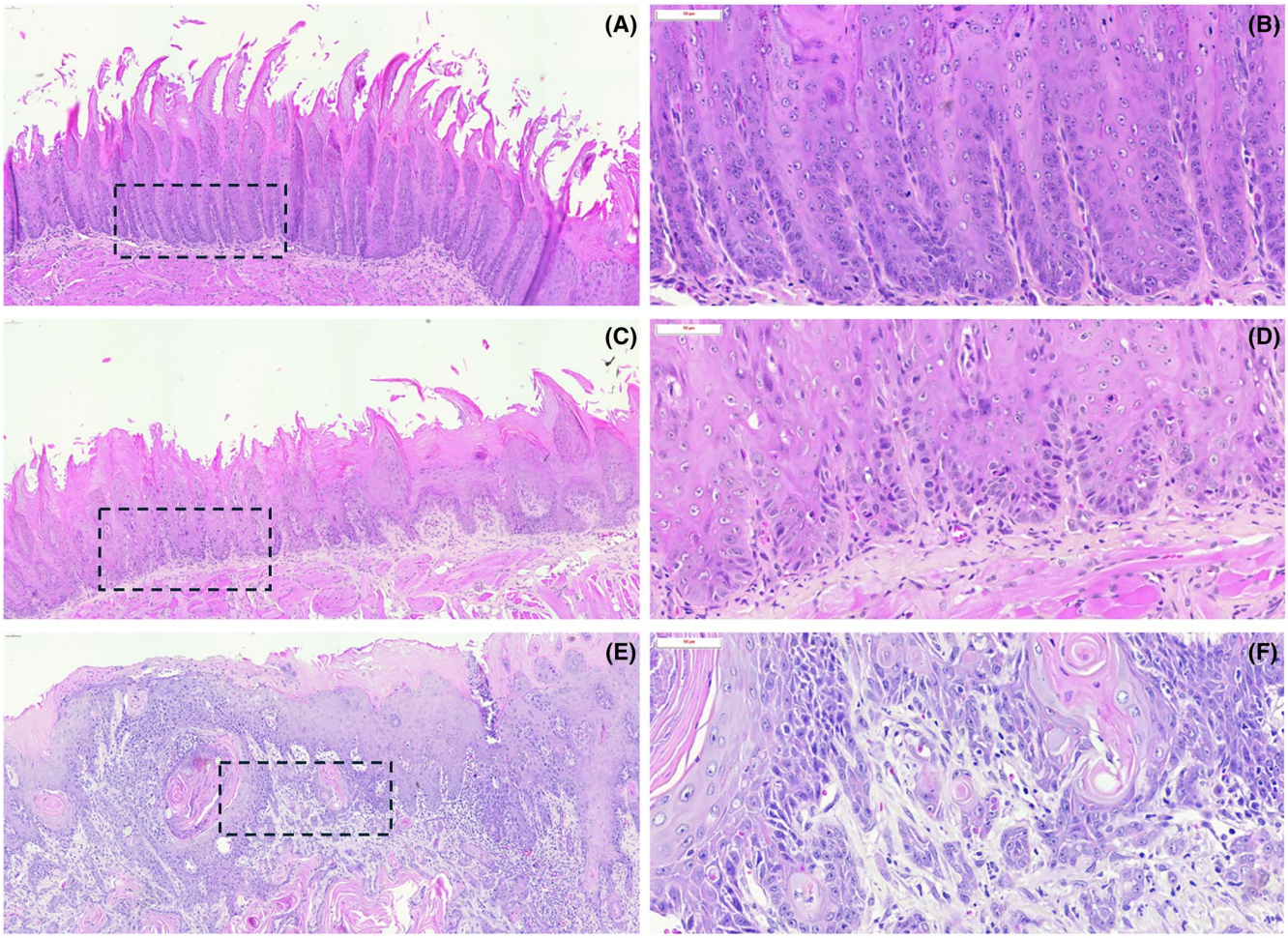

FIGURE 3 Representative histological images of epithelial hyperplasia (A and B), epithelial dysplasia (C and D), and squamous cell carcinoma (C and D) at 9× magnification (left panel) and 40× magnification (right panel).

5.72–25.70, $p=0.0030$). Additionally, within each treatment group, Ki-67 expression was significantly higher at the lesion site than at the anterior area, both in the control group (mean difference = 12.89%, 95% CI: 4.40–21.38, $p=0.0052$) and in the PDT group (mean difference = 17.30%, 95% CI: 9.24–25.36, $p=0.0003$).

Similarly to the findings at 12 weeks, the 20-week analysis revealed significant main effects of both anatomical site ($p<0.0001$) and treatment group ($p=0.0003$) on Ki-67 expression, with no significant interaction between factors ($p=0.3886$). Post hoc analyses using uncorrected Fisher's LSD confirmed that the PDT group showed significantly reduced Ki-67 positivity compared with controls at both the lesion site (mean difference = 25.76%, 95% CI: 14.11–37.41, $p<0.0001$) and the anterior area (mean difference = 20.86%, 95% CI: 9.21–32.51, $p=0.0010$). Within-group comparisons indicated that Ki-67 expression remained significantly higher at the lesion site than in the anterior area in both the control group (mean difference = 17.90%, 95% CI: 10.35–25.45, $p=0.0001$) and the PDT group (mean difference = 13.00%, 95% CI: 3.98–22.02, $p=0.0078$).

Notably, the difference in Ki-67 expression between anatomical sites was more pronounced in the PDT group at 12 weeks (mean difference = 17.30%, 95% CI: 9.24–25.36, $p=0.0003$) compared with the control group (12.89%, 95% CI: 4.40–21.38, $p=0.0052$), suggesting a stronger early regional modulation of proliferation in response to treatment. However, by 20 weeks, the control group exhibited a larger regional difference (17.90%, $p=0.0001$) than the PDT group (13.00%, $p=0.0078$), potentially reflecting a more homogeneous normalization of epithelial proliferation across sites in treated animals over time.

In a final analysis considering the histopathological diagnosis (no epithelial dysplasia, epithelial dysplasia, in situ carcinoma, or invasive carcinoma) as the column factor, a significant main effect of anatomical site ($p<0.0001$), but no significant effect of diagnosis ($p=0.7146$), nor a significant interaction between factors ($p=0.2250$) were observed. Post hoc analysis using Tukey's multiple comparisons test indicated no statistically significant differences in Ki-67 expression among the three diagnostic categories at either the lesion site or anterior area in the group of

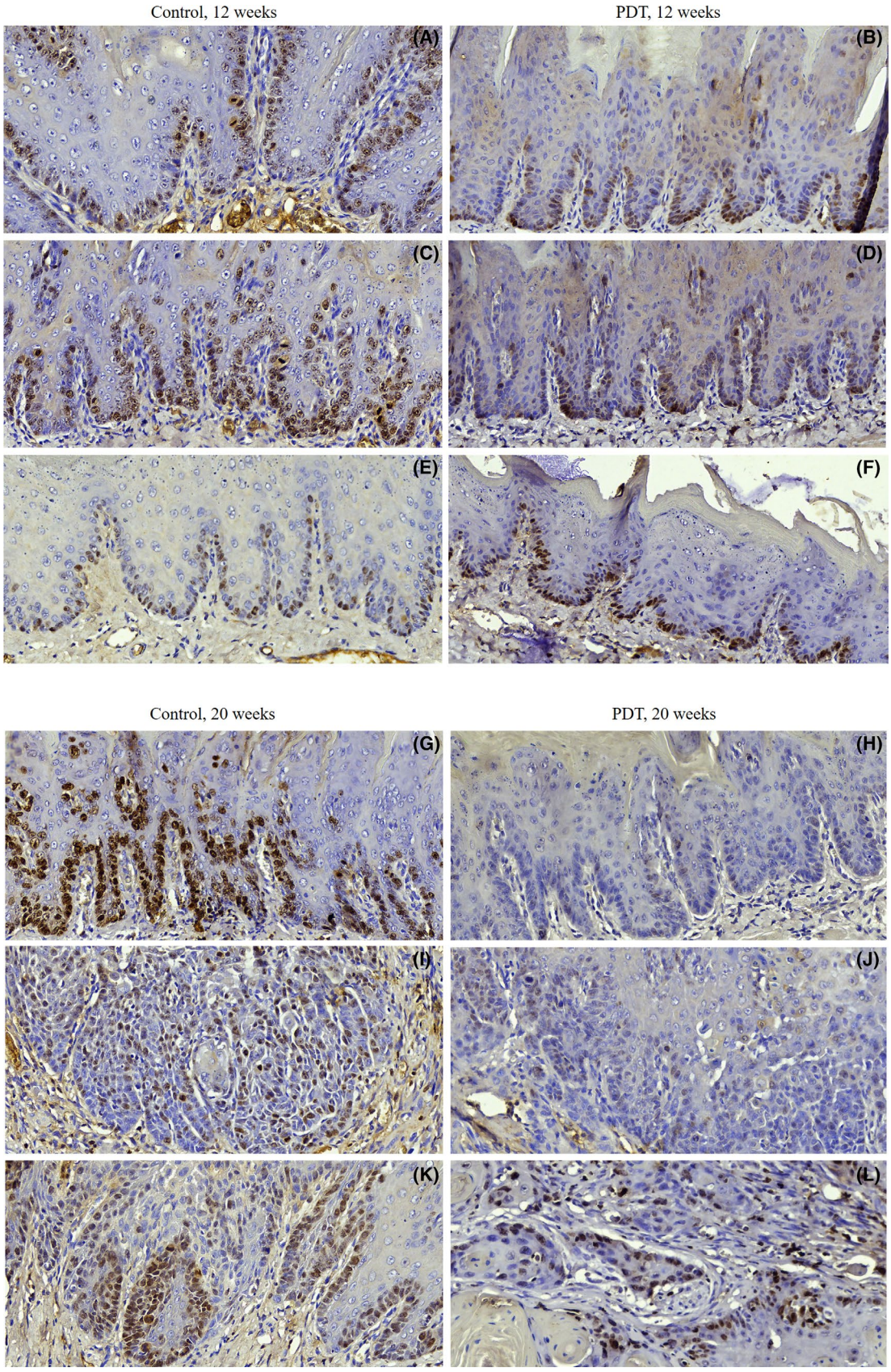

FIGURE 4 Immunohistochemical analysis of proliferative cells from control and PDT groups using the Ki-67 antibody. Note the reduced number of proliferative cells in the PDT group at 12- and 20-week time points, as observed at 20× magnification, in epithelial dysplasia (A–H), in situ carcinoma (I–J), and squamous cell carcinoma (K–L), compared with the control illustrative images.

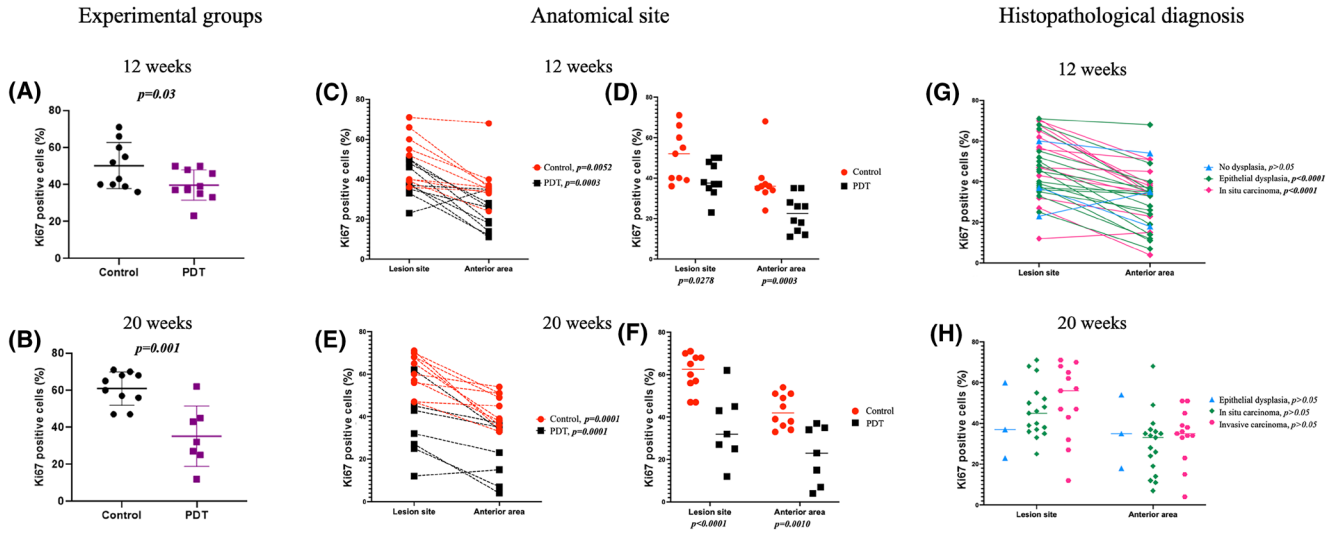

FIGURE 5 Statistical analysis of Ki-67 expression quantification. (A–B) Quantification of Ki-67-positive cells (%) in the control and PDT-treated groups at 12 (A) and 20 (B) weeks posttreatment, indicating a significant reduction in cellular proliferation by PDT. Statistical analysis of Ki-67 expression is presented as follows: (A–B) Panels A and B show the quantification of Ki-67-positive cells (expressed as a percentage) in both control and PDT-treated groups at 12- and 20-week posttreatment, respectively. PDT treatment is associated with a significant reduction in cellular proliferation at both time points. (C–F) Panels C–F illustrate Ki-67 expression in two anatomically distinct regions, the lesion site versus the anterior area, at 12 weeks (Panels C and D) and 20 weeks (Panels E and F). In each group, proliferative indices are significantly lower in the anterior area compared with the lesion site. Panels C and E depict paired comparisons from individual samples, whereas Panels D and F summarize the grouped data. (G–H) Panels G and H stratify the data by histopathological diagnosis at 12 and 20 weeks, respectively. At 12 weeks (Panel G), cases of epithelial dysplasia and in situ carcinoma exhibit significantly reduced Ki-67 expression in the anterior area. By 20 weeks (Panel H), no significant differences are observed between the regions.

20 weeks (all $p > 0.5$). However, when comparing anatomical sites within each diagnosis in 12 weeks, Ki-67 expression was significantly higher at the lesion site compared with the anterior area in both epithelial dysplasia (mean difference = 16.24%, 95% CI: 10.60–21.87, $p < 0.0001$) and in situ carcinoma (mean difference = 16.85%, 95% CI: 10.40–23.29, $p < 0.0001$), while no significant difference was found for epithelial dysplasia (mean difference = 4.33%, 95% CI: −9.09–17.76, $p = 0.5147$). These findings suggest that the regional pattern of proliferation becomes more distinct with increasing histopathological severity, particularly in cases of carcinoma.

Animal monitoring

The animals maintained their well-being, as evidenced by daily assessments and weight gain over the weeks. The weight and 4NQO consumption are illustrated in File S2. Regarding adverse effects, vocalization ($n = 3$), blood in urine ($n = 1$), nose secretion during the sedation period probably due to the carcinogen ($n = 2$) and acute oral ulcer in the first week of the experiment ($n = 5$) were observed. We had no losses with the 4NQO model.

DISCUSSION

Since the 1970s, PDT has been recognized as an effective treatment for neoplastic conditions affecting various anatomical sites, including the bladder, stomach, breast, skin, and mouth. Animal model studies published to date have assessed PDT as a potential treatment for chemically induced oral lesions.[26,28,30] However, limited knowledge exists regarding its impact on tissues during carcinogenesis. From our understanding, this study stands as the pioneer in examining the effects of PDT during ongoing exposure to a carcinogen, as well as evaluating its safety within the context of continuous carcinogenic stimulation. Our findings not only highlight PDT's ability to reduce epithelial proliferation but also suggest its potential to negatively modulate tumor growth at a regional level. These results suggest that within the specific conditions of this preclinical research, PDT may offer a potential therapeutic role in the management of early-stage oral carcinogenesis, possibly preventing the progression to more aggressive forms of the disease.

The 4NQO rat model is considered an important model that demonstrates all stages of oral carcinogenesis, which is histologically and molecularly like human oral

carcinogenesis.[27] Consistent with previous reports, our study confirmed that 12 weeks of 4NQO exposure predominantly resulted in epithelial dysplasia, whereas OSCC became the most prevalent lesion by 20 weeks.[23] While PDT did not significantly alter the clinical or histopathological characteristics of these lesions, it was associated with a significant reduction in Ki-67 expression. Given that Ki-67 is a well-established marker of cellular proliferation, this finding suggests that PDT may exert an inhibitory effect on tumor cell growth within the carcinogenic field, even though it did not prevent lesion progression under continuous carcinogen exposure.[31]

Several studies have explored PDT as a therapeutic intervention for oral carcinogenesis, primarily focusing on treating established lesions following 4NQO exposure. Barcessat et al.[25] exposed animals to 4NQO for 16 weeks before treatment with PDT. Laser irradiation was performed 2 h after 5-ALA application (660 nm, 40 mW, 90 J/cm^2, 0.04 spot area, for 3 min). A second PDT cycle was conducted 3 days after the initial application, following the same protocol. Analysis of cell dynamics in potentially malignant lesions after two sessions of 5-ALA-mediated PDT revealed fluctuations in apoptosis and DNA repair processes among epithelial cells, as reported by the authors. In the same way, Khozeimeh et al.[30] administered 4NQO for 12 weeks before treatment with PDT (laser parameters: 660 nm, 300 mW, 100 J/cm^2, for 5 min). The authors performed laser topical application of ALA on two occasions, with a 2-h interval, followed by light exposure 4 h after the initial ALA administration. Their findings revealed that ALA-mediated PDT was an effective therapeutic approach for oral dysplasia. Recently, Fu and coauthors[32] conducted a photodynamic experiment using an animal model with application of a solution of dimethylbenz[a] anthracene (DMBA) in the buccal pouches of hamsters treated with one PDT session. Their results showed that for superficial precancerous lesions like oral leukoplakia, dual-wavelength PDT [653 nm light (18.342 J/sq. cm) + 410 nm light (18.342 J/sq. cm)] offered a paradigm shift by balancing efficacy and safety.

A key difference between these studies and our methodology lies in the PDT protocol, particularly in the timing and frequency of treatment. While previous studies applied PDT in one or two sessions using higher energy densities (90–100 J/cm^2), we administered PDT at a lower energy dose (65 J/cm^2) once per week over 12 and 20 weeks. This protocol was intentionally selected to prioritize safety during continuous carcinogen exposure. Nonetheless, it is plausible that higher cumulative light doses or more frequent applications could enhance therapeutic effects. Therefore, future studies should explore alternative protocols, including dose escalation and intensified application schedules, to determine the optimal parameters for efficacy without compromising safety. Additionally, as PDT may be more effective once the carcinogenic stimulus is withdrawn,[25,30] further investigations comparing regimens with and without 4NQO exposure are warranted to clarify the conditions under which PDT most effectively modulates carcinogenesis. We hypothesize that continuous 4NQO exposure creates a pro-tumorigenic environment that may diminish the observable effects of PDT, particularly its antiproliferative and immunomodulatory actions. This limitation is important when translating findings to clinical settings, where PDT is typically applied after risk factor cessation. Future studies should include a carcinogen withdrawal phase to better simulate preventive or adjuvant scenarios and more accurately assess PDT's therapeutic potential during early neoplastic progression.

Although PDT did not alter lesion progression, the significant reduction in Ki-67 expression suggests a potential suppressive effect on cell proliferation. This finding aligns with in vitro studies, such as Jin et al.,[33] who demonstrated that ALA-PDT inhibits oral precancerous cell growth by modulating the TGF-β signaling pathway, with an enhanced suppressive effect observed when combined with LY2109761. A review conducted by Naharro-Rodriguez et al.[34] also reported that PDT significantly reduces Ki-67 expression in precancerous skin lesions, indicating a decrease in cellular proliferation typically associated with lower lesion aggressiveness. Given that Ki-67 is a validated biomarker of mitotic activity and has been associated with malignant potential and unfavorable outcomes in oral cancer, its reduction may reflect early biological modulation of carcinogenesis. Although histopathological changes were not observed, the downregulation of Ki-67 may represent a subclinical preventive effect of PDT, suggesting a role for the therapy in impeding lesion progression. Also, an interesting and somewhat unexpected observation in this study was the reduction of Ki-67 expression in the untreated anterior region of the tongue, suggesting that PDT may exert regional biological effects beyond the irradiated tissue. One possible explanation is the diffusion of reactive oxygen species (ROS) from the treated area, influencing adjacent or distant tissues.[35] Additionally, PDT may stimulate immune responses, such as the release of cytokines or antigen presentation, or involve vascular or lymphatic mediators that spread therapeutic effects across untreated regions.[36] These bystander effects indicate that PDT may modulate cellular proliferation on a regional level, even in areas not directly exposed to the treatment. To elucidate these mechanisms, future studies should incorporate cytokine profiling (e.g., IL-1β, IL-6, TNF-α, IL-10), evaluation of systemic and local oxidative stress markers (such as 8-OHdG), and immunophenotyping of tissue-infiltrating immune cells using flow

cytometry or immunohistochemistry. Despite these findings, the precise mechanisms underlying this phenomenon remain unclear; therefore, such integrative molecular and immunological analyses will be critical to determine whether the antiproliferative effects of PDT are mediated by ROS-dependent signaling, immune modulation, or a combination thereof in the context of carcinogenesis.

Some limitations of our experimental study should be considered. First, despite the literature describing the posterior dorsum of the tongue as the region most prone to lesion development in the 4NQO model, the entire mucosa of the animal would be affected by the carcinogen's effects. Thus, we could have more areas with potential for cancer development. However, we believe this would not alter the results obtained. Secondly, regarding the technique, our analysis relies solely on weekly photographs of the animals' tongues, and acute adverse effects of the PDT technique could have developed in the initial days of application and disappeared before the next photographic record. However, based on what is reported in the literature, adverse effects of PDT have not yet been reported.[30] This is further endorsed by an overview conducted by our group demonstrating that such effects are scarcely reported in humans.[18] Also, although the use of intermediate histological evaluations could help capture transient morphological changes, performing incisional biopsies during the carcinogenesis protocol in rodents presents significant technical limitations. The small size of the tongue, combined with the difficulty in clinically identifying altered areas at early stages, makes this approach challenging and may risk compromising the animal's health or the progression of lesions. Finally, we emphasize that this is a pioneering but preliminary study on the safety of PDT during the carcinogenic process. Further studies, expanding the analysis techniques as well as euthanasia times, may help in understanding this issue.

Our findings reinforce the safety of PDT, as no adverse effects were observed and the treatment did not exacerbate the carcinogenic process. However, further studies are needed to confirm its long-term safety and therapeutic potential in clinical settings. To enhance translational relevance, future research should explore models with greater anatomical and physiological similarity to humans, such as patient-derived xenografts, to optimize light delivery and treatment parameters. Moreover, well-designed pilot clinical trials in patients with inoperable lesions could help assess the clinical applicability of PDT in complex scenarios. Despite technological advances, PDT has not yet been established as a standard therapy, even in fields where conventional treatments offer limited outcomes.[37,38] Nonetheless, evidence suggests that PDT may eventually be integrated into routine cancer care– either as part of a multimodal approach or as a standalone option for early-stage or palliative treatment.[39] In this context, preclinical studies like ours provide a necessary foundation for the development and refinement of clinical protocols.

CONCLUSION

Our data indicate that PDT does not alter the histopathological trajectory of oral lesions during malignant transformation. Nevertheless, the significant reduction in Ki-67 expression observed in PDT-treated animals—including in anatomically distant, non-irradiated sites—suggests a robust antiproliferative effect that extends beyond the immediate treatment area.

ACKNOWLEDGMENTS

This study received funding from the Postgraduate Research Group of Hospital de Clínicas de Porto Alegre (GPPG/FIPE: 2021-0614). The authors express their gratitude to the Coordination of Improvement of Higher Education Personnel (CAPES, Finance Code 001) in Brazil. L.F.S. and T.R.S. were recipients of fellowships. Additionally, the Brazilian National Council for Scientific and Technological Development provided financial support (CNPq). V.S.B., C.K., M.D.M., and P.A.V. are recognized as research fellows of CNPq. L.F.S., F.M.S., and R.B.M., are research fellows supported by the Agencia Nacional de Investigación e Innovación/Sistema Nacional de Investigadores (ANII/SNI), Uruguay. This study was developed within the scope of the National Institute of Science and Technology in Translational Biophotonics in Dentistry (BioPHOTO Dent) (Grant n° 408830/2024-7).

CONFLICT OF INTEREST STATEMENT

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ronell Bologna-Molina https://orcid.org/0000-0001-9755-4779
Cristiane Helena Squarize https://orcid.org/0000-0002-6782-3429
Rogerio Moraes Castilho https://orcid.org/0000-0001-5358-612X

Vanderlei Salvador Bagnato https://orcid.org/0000-0003-4833-239X
Manoela Domingues Martins https://orcid.org/0000-0001-8662-5965

REFERENCES

1. Kwiatkowski S, Knap B, Przystupski D, et al. Photodynamic therapy - mechanisms, photosensitizers and combinations. *Biomed Pharmacother*. 2018;106:1098-1107.
2. Raab O. On the effects of fluorescent substances on infusoria. *Z Biol*. 1900;39:524-526.
3. Meyer-Betz F. Untersuchungen uber die Biologische Wirkung des haematoporphyrin und anderer derivative des blut-und Gallenfarbstoffs. *Dtsch Arch Klin Med*. 1913;112:476-503.
4. Auler H, Banzer G. Untersuchungen über die Rolle der Porphyrine bei Geschwulstkranken Menschen und Tieren. *Z Krebsforsch*. 1942;53:65-68.
5. Olek M, Machorowska-Pieniążek A, Olek K, Cieślar G, Kawczyk-Krupka A. Photodynamic therapy in the treatment of oral squamous cell carcinoma - the state of the art in preclinical research on the animal model. *Photodiagnosis Photodyn Ther*. 2021;34:102236.
6. Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. *Lancet Oncol*. 2000;1:212-219.
7. Prates RA, Yamada AM, Suzuki LC, et al. Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. *Photomed Laser Surg*. 2011;29(12):835-844.
8. Sharma SK, Mroz P, Dai T, Huang YY, St Denis TG, Hamblin MR. Photodynamic therapy for cancer and for infections: what is the difference? *Isr J Chem*. 2012;52(8–9):691-705.
9. Prażmo EJ, Kwaśny M, Łapiński M, Mielczarek A. Photodynamic therapy As a promising method used in the treatment of Oral diseases. *Adv Clin Exp Med*. 2016;25(4):799-807.
10. van Straten D, Mashayekhi V, de Bruijn HS, Oliveira S, Robinson DJ. Oncologic photodynamic therapy: basic principles, current clinical status and future directions. *Cancers (Basel)*. 2017;9(2):19.
11. Caccavale S, Iocco A, Pieretti G, Alfano R, Argenziano G. Curettage + microneedling + topical ALA-PDT for the treatment of acral resistant warts: our experience. *Photodiagnosis Photodyn Ther*. 2019;27:276-279.
12. Jiang W, Liang M, Lei Q, Li G, Wu S. The current status of photodynamic therapy in cancer treatment. *Cancers (Basel)*. 2023;15(3):585.
13. Saini R, Poh CF. Photodynamic therapy: a review and its prospective role in the management of oral potentially malignant disorders. *Oral Dis*. 2013;19(5):440-451.
14. Cerrati EW, Nguyen SA, Farrar JD, Lentsch EJ. The efficacy of photodynamic therapy in the treatment of oral squamous cell carcinoma: a meta-analysis. *Ear Nose Throat J*. 2015;94(2):72-79.
15. Alkindi M. Therapeutic efficacy of photodynamic therapy in oral squamous cell carcinoma: a systematic review. *Biosci Biotechnol Res Commun*. 2020;13(1):187-194.
16. Romano A, Di Stasio D, Gentile E, Petruzzi M, Serpico R, Lucchese A. The potential role of photodynamic therapy in oral premalignant and malignant lesions: a systematic review. *J Oral Pathol Med*. 2021;50(4):333-344.
17. Binnal A, Tadakamadla J, Rajesh G, Tadakamadla SK. Photodynamic therapy for oral potentially malignant disorders: a systematic review and meta-analysis. *Photodiagnosis Photodyn Ther*. 2022;37:102713.
18. Schuch LF, Schmidt TR, Kirschnick LB, et al. Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: an overview of systematic reviews. *Photodiagnosis Photodyn Ther*. 2023;42:103531.
19. Zhang W, Chen S, Bai Z, et al. Photodynamic therapy for Oral squamous cell carcinoma: current status, challenges, and prospects. *Int J Nanomedicine*. 2024;19:10699-10710. doi:10.2147/IJN.S481901
20. Soares GR, de Moura CFG, Silva MJD, et al. Protective effects of purple carrot extract (Daucus carota) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *Med Oncol*. 2018;35(4):54.
21. Thandavamoorthy P, Balan R, Subramaniyan J, Dhas G. Alleviative role of Rutin against 4-Nitroquinoline-1-oxide (4-NQO) provoked Oral squamous cell carcinoma in experimental animal model. *J Pharm Res*. 2014;8:899-906.
22. Wagner VP, Spuldaro TR, Nör F, et al. Can propranolol act as a chemopreventive agent during oral carcinogenesis? An experimental animal study. *Eur J Cancer Prev*. 2021;30(4):315-321.
23. Ribeiro DA, Salvadori DM. Gingival changes in wistar rats after oral treatment with 4-nitroquinoline 1-oxide. *Eur J Dent*. 2007;1(3):152-157.
24. Schuch LF, Campagnol D, Schmidt TR, et al. Proposal of a secure and efficient protocol for a murine oral carcinogenesis model induced by 4-nitroquinoline-1-oxide (4NQO). *Pathol Res Pract*. 2023;247:154547.
25. Barcessat AR, Huang I, Rosin FP, Dos Santos Pinto D Jr, Maria Zezell D, Corrêa L. Effect of topical 5-ALA mediated photodynamic therapy on proliferation index of keratinocytes in 4-NQO-induced potentially malignant oral lesions. *J Photochem Photobiol B*. 2013;126:33-41.
26. Rosin FC, Barcessat AR, Borges GG, Ferreira LG, Corrêa L. Vascular alterations after photodynamic therapy mediated by 5-aminolevulinic acid in oral leukoplakia. *Lasers Med Sci*. 2017;32(2):379-387.
27. Zigmundo GCO, Schuch LF, Schmidt TR, et al. 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: a systematic literature review. *Pathol Res Pract*. 2022;236:153970.
28. Hsu YC, Yang DF, Chiang CP, Lee JW, Tseng MK. Successful treatment of 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch precancerous lesions by topical 5-aminolevulinic acid-mediated photodynamic therapy. *Photodiagnosis Photodyn Ther*. 2012;9(4):310-318.
29. Schuch LF, Campagnol D, Schmidt TR, et al. Investigating the safety of photobiomodulation in oral carcinogenesis: insights into cell proliferation, invasion, and apoptosis via the 4NQO model. *Sci Rep*. 2024;14(1):28303. doi:10.1038/s41598-024-78763-y
30. Khozeimeh F, Ziaei S, Khalesi S, Allameh M, Jahanshahi G. The efficacy of photodynamic therapy in rat tongue dysplasia. *J Clin Exp Dent*. 2019;11(7):e587-e592.
31. Li LT, Jiang G, Chen Q, Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol Med Rep*. 2015;11(3):1566-1572. doi:10.3892/mmr.2014.2914

32. Fu S, Zhu T, Chen L, Zhou G, Sun J. Assessment of bimodal laser photodynamic therapy at Wavelenths of 410 nm and 653 nm for Oral precancerous lesions: an in vitro and in vivo study. *Photodiagnosis Photodyn Ther*. 2025;53:104564. doi:10.1016/j.pdpdt.2025.104564
33. Jin JQ, Wang Q, Zhang YX, Wang X, Lu ZY, Li BW. Effect of ALA-PDT on inhibition of oral precancerous cell growth and its related mechanisms. *Lasers Med Sci*. 2022;37(9):3461-3472.
34. Naharro-Rodriguez J, Bacci S, Fernandez-Guarino M. Molecular biomarkers in cutaneous photodynamic therapy: a comprehensive review. *Diagnostics (Basel)*. 2024;14(23):2724. doi:10.3390/diagnostics14232724
35. Hu D, Li Y, Li R, et al. Recent advances in reactive oxygen species (ROS)-responsive drug delivery systems for photodynamic therapy of cancer. *Acta Pharm Sin B*. 2024;14(12):5106-5131. doi:10.1016/j.apsb.2024.10.015
36. Kazemi KS, Kazemi P, Mivehchi H, et al. Photodynamic therapy: a novel approach for head and neck cancer treatment with focusing on Oral cavity. *Biol Proced Online*. 2024;26(1):25. doi:10.1186/s12575-024-00252-3
37. Moghissi K. PDT: the plight. *Photodiagnosis Photodyn Ther*. 2007;4(4):223.
38. Benov L. Photodynamic therapy: current status and future directions. *Med Princ Pract*. 2015;24(Suppl 1):14-28.
39. Algorri JF, Ochoa M, Roldán-Varona P, Rodríguez-Cobo L, López-Higuera JM. Photodynamic therapy: a compendium of latest reviews. *Cancers (Basel)*. 2021;13(17):4447.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Schuch LF, Schmidt TR, Campagnol D, et al. Experimental assessment of photodynamic therapy effects on 4NQO-induced rat tongue carcinogenesis: Clinical, morphological, and immunohistochemical analysis. *Photochem Photobiol*. 2025;00:1-13. doi:10.1111/php.70022