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EVALUATION OF OXIDATIVE STRESS IN EXFOLIATED CELLS FROM DIFFERENT REGIONS OF THE MUCOSA OF ORAL AND OROPHARYNGEAL CANCER PATIENTS

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Dissertação apresentada a Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista (Unesp), para obtenção do título de ‘’Mestre em Odontologia’’- Área de Concentração Estomatologia

Orientador: Prof. Assoc. Daniel Galera Bernabé

Coorientadora: Prof.ª Gisele Zoccal Mingoti

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“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana.”

- Carl Jung

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RESUMO

O câncer de cabeça e pescoço representa um desafio significativo para a saúde global, justificando investigações abrangentes dos seus mecanismos moleculares subjacentes. O objetivo do presente estudo foi avaliar os níveis de estresse oxidativo em células esfoliadas da região tumoral e duas regiões de mucosa não tumoral de pacientes com câncer de boca e orofaringe. **Pacientes e Métodos:** Para avaliar a expressão de espécies reativas de oxigênio (ROS) em células esfoliadas de diferentes áreas da mucosa de pacientes com carcinoma espinocelular (CEC) de boca e orofaringe, células epiteliais esfoliadas foram coletadas da região tumoral (T), peritumoral (PT) e contralateral (CL). As células provenientes das três regiões foram submetidas à mensuração da expressão intracelular de ROS por meio da sonda fluorescente H2DCFDA. Variáveis demográficas e clinico patológicas foram extraídas dos prontuários clínicos e suas associações com os níveis celulares de ROS foram avaliadas. **Resultados**: Houve uma tendência de aumento dos níveis de expressão intracelular de ROS nas células da região tumoral em relação as regiões PT e CL, mas este resultado não atingiu significância estatística ($p>0,05$). Não houve diferença na expressão intracelular de ROS entre as regiões PT e CL ($p>0,05$). Não foram encontradas diferenças significativas nos níveis intracelulares de ROS para nenhuma das 3 regiões analisadas entre os pacientes com tumores de boca em relação aos pacientes com tumores de orofaringe ($p>0,05$). As células esfoliadas da região do tumor dos pacientes fumantes e com a doença em estágio clínico avançado apresentaram um discreto aumento da expressão de ROS, sem atingir significância estatística. **Conclusão**: O método desenvolvido neste estudo preliminar foi eficaz na identificação e medição dos níveis de espécies reativas de oxigênio em células esfoliadas da região tumoral e áreas da mucosa não tumoral de pacientes com câncer de boca e orofaringe.

Palavras-chave: Câncer, Carcinoma espinocelular, Mucosa oral, Sonda fluorescente, estresse oxidativo, ROS, Radicais livres

Duarte, PVS. Evaluation of oxidative stress in exfoliated cells from different regions of the mucosa of oral and oropharyngeal cancer patients. 2024. 32 f. Dissertação (Mestrado) - Faculdade de Odontologia da Universidade Estadual Paulista, Araçatuba, 2024.

ABSTRACT

Head and neck cancer represents a significant challenge to global health, justifying comprehensive investigations into its underlying molecular mechanisms. The present study aimed to assess oxidative stress levels in exfoliated cells from the tumor region and two non-tumor mucosal regions of patients with oral and oropharyngeal cancer. **Patients and Methods:** To evaluate the expression of reactive oxygen species (ROS) in exfoliated cells from different areas of the mucosa of patients with oral squamous cell carcinoma (SCC) and oropharynx, exfoliated epithelial cells were collected from the tumor region (T), peritumoral (PT), and contralateral (CL) regions. Cells from the three regions underwent measurement of intracellular ROS expression using the fluorescent probe H2DCFDA. Demographic and clinicopathological variables were extracted from clinical records, and their associations with cellular ROS levels were evaluated. **Results**: There was a trend of increased intracellular ROS expression in cells from the tumor region compared to PT and CL regions, but this result did not reach statistical significance ($p>0.05$). There was no difference in intracellular ROS expression between PT and CL regions ($p>0.05$). No significant differences were found in intracellular ROS levels for any of the three analyzed regions between patients with oral tumors and those with oropharyngeal tumors ($p>0.05$). Exfoliated cells from the tumor region of smokers and those in advanced clinical stages showed a slight increase in ROS expression, without reaching statistical significance. **Conclusion**: The method developed in this preliminary study was effective in identifying and measuring levels of reactive oxygen species in exfoliated cells from the tumor region and non-tumor mucosal areas of patients with oral and oropharyngeal cancer.

Keywords: Cancer, Squamous cell carcinoma, Oral mucosa, Fluorescent probe, Oxidative stress, ROS, Free radicals

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CL	Contralateral
FR	Free radicals
H2DCFDA	6-carboxy 2′,7′-dichlorodihydrofluorescein diacetate
PT	Peritumoral
ROS	Reactive oxygen species
T	Tumor

SUMÁRIO

1 INTRODUCTION

Free radicals (FR) are atoms or molecules constantly produced in the body's normal metabolic process, with one or more unpaired electrons in the outermost layer characterizing their instability[1]. Some of these free radicals are reactive oxygen species (ROS).[1,2] When present in excess, ROS leads to oxidative stress resulting in the degradation of biological structures essential for cellular organic functioning, initiating or worsening diseases.[3] The body homeostasis relies on an antioxidant defense system to balance the harmful potential of ROS. Antioxidant activities favor the metabolic maintenance and organic balance of systems affected by ROS, contributing to healthy cell homeostasis.[3,4] Thus, oxidative stress is the cellular state in which ROS surpass the defense barrier of cellular antioxidants.[5]

Several studies have confirmed the association between the imbalance of reactive radicals and the formation and/or progression of various human diseases, including cancer[6]. Oxidative stress plays a significant role in the development of oral cancer, influencing all stages (Initiation, promotion, and progression), with the occurrence of DNA modifications caused by ROS in epithelial cells[6,7]. The initiation phase is triggered when a normal cell undergoes DNA mutation, resulting in the formation of an initiated cell[7,8]. In the promotion stage, initiated cells multiply clonally, promoting cell proliferation and inhibiting apoptosis, with oxidative stress playing a crucial role. ROS can temporarily modulate genes associated with cell proliferation or death, influencing transcription factors, leading to the activation of survival pathways and promoting tumor growth[8].

Oxidative stress also contributes to cancer progression, causing mutation, inhibiting antiproteases, positively regulating matrix metalloproteinases, and causing local tissue damage[6-8]. Thus, ROS is involved in all stages of oral cancer development,

acting through various events and pathways to damage cellular components and contribute to neoplastic transformation. In this context, oxidative stress in a microenvironment can create a pre-malignant field[8,10]. The term "field cancerization" was first described in 1953 by Slaughter and colleagues, who observed dysplastic epithelium adjacent to invasive oral cancers. The concept of a pre-cancerous field is supported by clinical and molecular evidence, comparing clinically normal epithelium with tumor tissues, revealing biochemical and structural changes in cells[10]. Although these alterations are generally attributed to growth factors and cytokines, chronic exposure to high levels intracellular ROS promotes the neoplastic transformation of normal cells, indicating the occurrence of a premalignant field defect by oxidative stress[10].

Considering that a tumor tissue exhibits a higher quantity of ROS, the regions proximal to the tumor also show oxidative alterations[8]. Oxidative stress is a known phenomenon in pre-malignant fields[10]. To date, no studies have assessed oxidative stress in shed cells from the tumor and different regions in the precancerous field of patients with oral cancer. Our study aimed to investigate the intracellular levels of ROS in exfoliated oral cells from the tumor and two different non-cancerous regions of the mucosa in patients diagnosed with oral and oropharyngeal cancer. Additionally, we evaluated the correlation between the ROS expression in the cells from the three distinct regions with clinicopathological characteristics of oncological patients.

2 MATERIALS AND METHODS

2.1 Ethic Statement

This study was approved by the Committee of Human Studies of the Sao Paulo State University (UNESP), School of Dentistry, Araçatuba, São Paulo, Brazil (nº. 5.719.154), and informed consent was obtained from all participants.

2.2 Patients

A total of 14 patients diagnosed with oral and oropharyngeal squamous cell carcinoma were recruited at the Oral Oncology Center of São Paulo State University (UNESP), School of Dentistry in Araçatuba, Brazil. The inclusion criteria for participants in the study were as follows: individuals aged over 18 years; having histopathological diagnosis squamous cell carcinoma; tumors located in oral cavity or oropharynx; and without any previous oncological treatment. Volunteers with any medical condition or cognitive deficit that could impede the completion of the clinical protocol were excluded from the study.

2.3 Demographic and Clinicopathological variables

Demographic, clinicopathological and biobehavioral data were extracted from patients' clinical records. Demographic variables (age and sex), clinicopathological variables (primary tumor size (T) and clinical staging), and biobehavioral data (history of tobacco consumption) were obtained from the oral or oropharyngeal cancer patients.

2.4 Collection of exfoliated epithelial cells from tumor area and two different regions

The collection of oral epithelial cells for the assessment of oxidative stress in the patients with oral and oropharyngeal cancer was conducted in the morning (8:00–11:00 am) in a previously prepared room. Patients began by rinsing their mouths with distilled water (three repetitions) to remove food residues, debris, and already shed mucosal cells before the material collection. Subsequently, cell exfoliation was performed in the region affected tumor (T), a peripheral region with intact mucosa adjacent to the tumor (Peritumoral, PT), and the opposite side of the lesion in the patient (Contralateral, CL). For example, in the case of a tongue border tumor on the right side, cells were collected from the left side. Exfoliation was carried out using a sterile and disposable mini-brush. Ten rotations of the brush against the target sites were performed, starting the sweep in the center of the region and gradually increasing the circumference to produce a spiral effect, thus avoiding continuous erosion in a single region. After the procedure, the brush head was placed in a test tube containing 4 ml of 1X PBS solution. The mini-brush containing the cells was rotated in the solution to release the cells. Along with the collection, the test tubes were labeled with the site of the collected sample.

2.5 Dichlorofluorescein Assay for Evaluation of Global Intracellular ROS Content

The intracellular content of reactive oxygen species (ROS) was quantified using the fluorescent probe H_2DCFDA (6-carboxy 2′,7′-dichlorodihydrofluorescein diacetate; Molecular Probes) following the manufacturer's instructions. In summary, cells suspended in 1X PBS were incubated with 5 µM H2DCFDA for 30 minutes in the dark at 37.0°C and 5% CO2 in air. Subsequently, they were washed twice with 1X PBS and mounted on slides.

2.6 Quantification of Fluorescence Intensity

The stained cells with the method described above were evaluated under an inverted microscope equipped with epifluorescence (Olympus, IX51), with excitation at 550nm and emission at 595nm for the H_2DCFDA probe. Images were captured and subsequently analyzed using the ImageJ software (National Institute of Health, Bethesda, MD, USA) to quantify the emitted fluorescence intensity, measured in pixels. Higher fluorescence intensity corresponds to increased expression of the assessed molecules. In each slide, a total of 100 cells were evaluated. The expression data for each molecule were presented as the average number of pixels per cell.

2.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.21 (GraphPad Software Inc., San Diego, CA, USA). All data were checked for normality. The analysis of variance (ANOVA) test with multiple comparisons was used to assess differences in intracellular ROS expression in the different analyzed regions. The association between clinicopathological data and ROS expression was also analyzed using the T-test or ANOVA. The level of statistical significance was set at $p < 0.05$. Data were reported as mean ± standard error.

3 RESULTS

3.1 Demographic and Clinicopathological variables

Fourteen patients met the inclusion criteria. The clinicopathological characteristics of cancer patients are described in Table 1. The majority of the patients (78.5%) were male, while 21.4% were female. Concerning smoking history, 71.4% of the cancer patients were smokers, 14.2% former smokers, and 14.2% non-smokers. Regarding tumor location, most of cases (64.2%) were located in oral cavity, while 35.7% of patients had the tumors located in oropharynx. Half of the patients had their primary tumors classified as T2, followed by T1 (28.5%), T3 (14.2%) and T4 (7.1%). Most patients (71.4%) had the disease in in the early stage at the time of cell collection, while 28.5% were diagnosed at an advanced stage.

Table 1 - Demographic and clinicopathological characteristics of cancer patients

Variable	Nº (%)
Age (years)	
Mean (SD)	65.78
Sex	
Male	11 (78.5)
Female	3 (21.4)
Smoking History	
Non-Smoker	2 (14.2)
Ex-smoker	2 (14.2)
Smoker	10 (71.4)
Tumor Location	
Oral	9 (64.2)
Oropharynx	5 (35.7)
Tumor Size:	
T1	4 (28.5)
T2	7 (50)
T3	2 (14.2)
T4	1 (7.1)
Clinical Staging	
Early (I/II)	10 (71.4)
Advanced (III/IV)	4 (28.5)

3.2 Intracellular ROS levels in buccal exfoliated cells from the tumor and two different regions

In this investigation we employed the specific fluorescent probe H2DCFDA to measure intracellular reactive oxygen species (ROS) in oral cells from patients with oral and oropharyngeal cancer. Exfoliated oral cells were collected from the tumor (T), peritumoral area (PT) and contralateral region (CL).

The results revealed elevated ROS levels, expressed as Arbitrary Fluorescence Units (AFU), in exfoliated cells from the T region (283.6 ± 25.19; $p< 0.27$), compared to PT (224.9 ± 22.88; $p< 0.4312$) and CL side of the tumor (242.3 ± 28.82; $p< 0.62$) but this result was not significant (Figure 1). Additionally, there was no significant difference in ROS expression between exfoliated cells from the PT region and those collected from the CL ($p< 0.62$) (Figure 1A). No differences were observed in ROS expression in the exfoliated cells from the patients with oral tumors compared to those with oropharyngeal tumors considering the three different analyzed regions (T, $p< 0.99$; PT, $p< 0.69$; CL, $p< 0.64$) (Figure 1B).

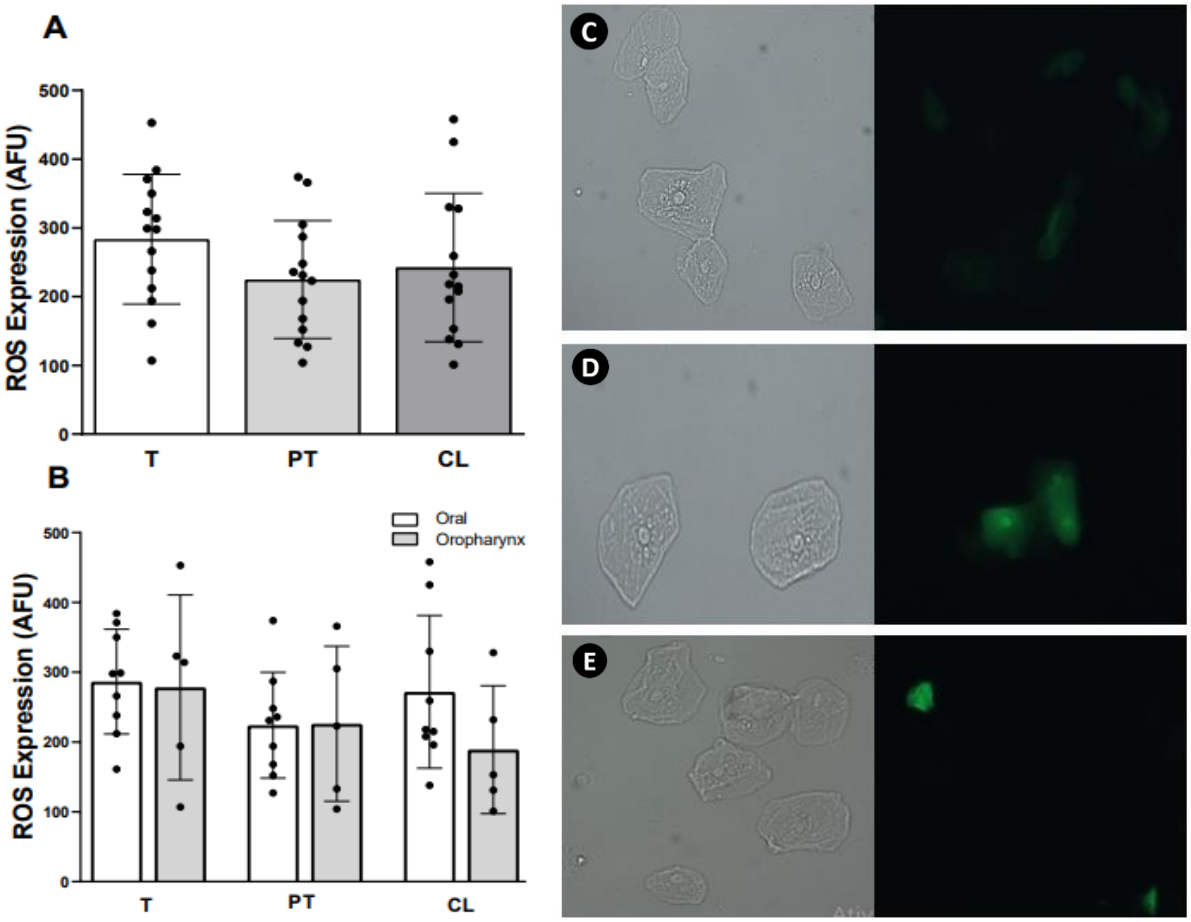

Figure 1 - Intracellular ROS levels in exfoliated cells from three different areas: tumor (T), peritumoral area (PT), and contralateral (CL). (A) The graph represents the mean intensity of fluorescent signals (pixels) from three different areas (T, PT and CL). (B) Representative photomicrographs show the H2DCFDA probe staining pattern in exfoliated oral cells (green) comparing the location of the primary tumor (oral cavity or Oropharynx) for the different three regions.

3.3 Associations between ROS expression and clinicopathological variables in cancer patients

When the association between ROS expression and clinicopathological was analyzed, the results showed that exfoliated cells from the T and PT area in smokers (T, 295 ± 25.24; PT, 249.7 ± 24.18) patients exhibited higher mean expression of ROS compared to non-smokers (T, 255 ± 66.84; PT, 162.8 ± 41.89). However, these results did not reach statistical significant ($p< 0.36$) (Figure 2A). When the patients were stratified according to clinical staging were not found significant differences in ROS expression in the three different regions analyzed (T, $p< 0.92$; PT, $p< 0.99$; CL, $p<0.62$). (Figure 2B). There were also no significant differences in ROS expression in exfoliated cells when the patients were classified according to tumor size in the T1/T2 versus T3/T4 (T, $p< 0.64$; PT, $p<0.98$; CL, $p<0.96$) (Figure 2C) or T1 versus T2/T3/T4 (T, $p<0.99$; PT, $p<0.92$; CL, $p<0.97$) (Figure 2D). Finally, although male patients showed higher ROS expression from exfoliated cells in the T region (252.7 ± 45.83), and women displayed increased expression for PT and CL (PT, 249.7 ± 71.31; CL, 237 ± 35.77), these results were not significant (T, $p<0.98$; PT, $p<0.99$; CL, $p<0.97$) (Figure E).

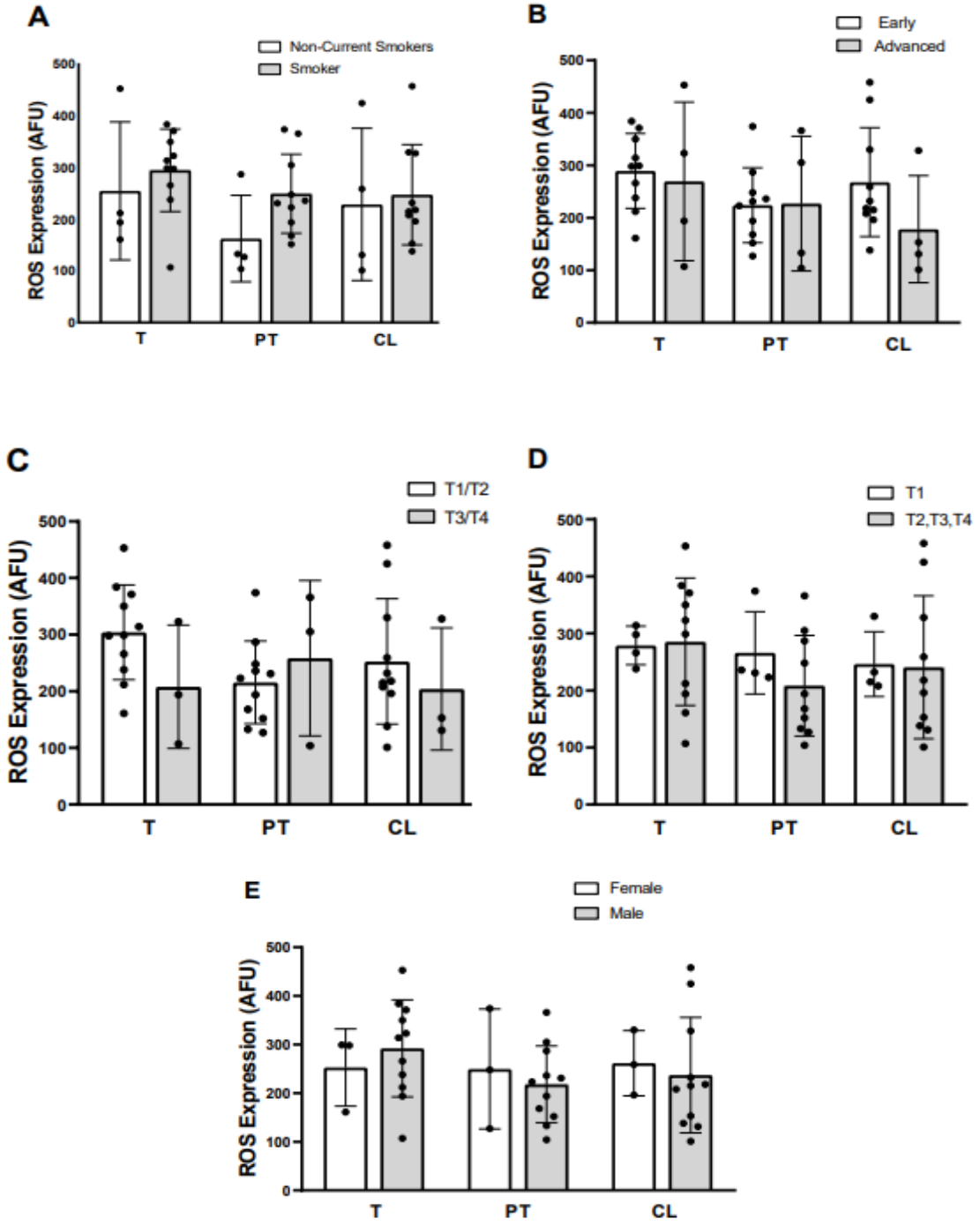

Figure 2 - Association between ROS expression in oral cells and clinicopathological characteristics. The graphs represent the mean ± standard error of the mean of the fluorescent signal intensities (pixels) measured in cells stained with H2DCFDA, in association with smoking history (A) clinical staging (B) tumor size (C and D) and sex (E)

4 DISCUSSION

In this study we evaluated the intracellular expression of ROS in exfoliated oral cells from patients with oral and oropharyngeal cancer. The ROS expression was compared among tumor cells (T) and two adjacent non-tumoral regions (PT and CL). Based on our current knowledge, no study has evaluated the expression of ROS in exfoliated cells from oropharyngeal and oral tumors. Additionally, there are no evidence regarding the expression of ROS in non-tumor regions, whether adjacent or distant from the tumor. However, although our results show a trend of increased ROS levels in tumor cells compared to cells from adjacent regions, this difference did not reach statistical significance. This observation suggests that oxidative stress may be a prominent feature at the tumor site, consistent with other studies that used the same probe as ours to trace the progression of oxidative stress and apoptosis induced by hydrogen peroxide and ethacrynic acid in vitro[23]. The mechanism by which oxidative stress participates in the carcinogenesis process and its relationship with external factors such as alcohol and tobacco are well-established in the literature[18]. The field cancerization in the oral mucosa can be initiated and propagated through various means, including an excess of ROS. Therefore, it would be expected that the peritumoral and contralateral regions would show a lower degree of intracellular ROS expression compared to the tumor region. However, the small sample size probably influenced this result not reaching significance in our study.

Our findings also revealed a similarity in the intracellular expression of ROS in the exfoliated cells of the mucosal regions of patients with oral cancer and those with oropharyngeal cancer. This homogeneity suggests that, regardless of the specific location of the tumor within the upper aerodigestive system, the oxidative profile in the examined cells can be relatively consistent. Ogden *et al* suggested that tobacco might

play a role in the oral alterations that lead to a premalignant field. However, they did not show a tendency for the influence of tobacco and alcohol on these morphological cell changes in other regions of the oral mucosa beyond the tumoral area of patients with head and neck squamous cell carcinoma (HNSCC)[29]. Despite the tumor region showing higher intracellular ROS expression compared to the other two locations for both smokers and non-smokers, our results showed no significant increase in ROS levels in the exfoliated oral cells of smokers compared to non-smoker patients. Burlakova *et al* investigated changes in antioxidant status induced by chronic smoking by collecting venous blood samples of 54 cancer patients and healthy subjects, they also did not find the influence of smoking on the oxidative stress parameter in cancer patients.[34]

Our study also found no significant association between intracellular ROS expression and clinicopathological variables of patients with oral and oropharyngeal cancer such as tumor size, clinical staging, and gender. There are no studies that have assessed the oxidative stress status in exfoliated oral cells from both healthy individuals and those with HNSCC, correlating with the clinical characteristics of each group. The lack of statistical significance in the association of ROS expression in cells from the three oral regions with the clinicopathological characteristics may be attributed to the limited sample size and variability of these clinical variables in our patients.

Additionally, the analysis of exfoliated cells may represent a momentary view of the oxidative stress status of oral mucosa. Longitudinal studies could offer a more dynamic understanding of these processes over time, evaluating the intracellular expression of ROS in cells from the different regions of the oral mucosa in different phases of the oncological treatment. Further studies with larger cohorts and more detailed analyses may provide additional insights into the dynamics of oxidative stress

in the field of cancerization of patients with HNSCC and its association with clinicopathological variables. This study provides for the first time an analysis of ROS levels in exfoliated cells from patients with oral and oropharyngeal cancer, exploring different regions of the oral mucosa.

5 CONCLUSION

The method developed in this preliminary study was effective in identifying and measuring levels of reactive oxygen species in exfoliated cells from the tumor region and non-tumor mucosal areas of patients with oral and oropharyngeal cancer.

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