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FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
DOUTORADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO PATOLOGIA BUCAL

NATÁLIA BATISTA DAROIT

ALTERAÇÕES EPITELIAIS DA MUCOSA BUCAL: O USO DA CITOPATOLOGIA NA DETECÇÃO PRECOCE DO CARCINOMA ESPINOCELULAR

PORTO ALEGRE
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Linha de Pesquisa: Câncer Bucal

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RESUMO

O carcinoma espinocelular bucal (CEC) afeta de maneira agressiva e mutilante milhares de pessoas anualmente. Métodos de identificação precoce desta lesão vêm sendo desenvolvidos, e a citopatologia bucal enquadra-se nessa perspectiva. **OBJETIVO**: Avaliar o uso da citopatologia bucal na detecção precoce do carcinoma espinocelular nos diferentes estágios da carcinogênese. **MÉTODOS:** Inicialmemte foi realizada uma revisão da literatura para avaliar a capacidade do método citopatológico em detectar modificações precoces na mucosa bucal. Na etapa experimental, amostras citopatológicas de CEC, de leucoplasias bucais e da borda de língua de indivíduos expostos e não expostos ao tabaco e ao álcool foram avaliadas ultraestruturalmente por meio de microscopia eletrônica de transmissão (MET). Enfim, relatou-se um caso clínico de um CEC em estágio inicial no qual algumas técnicas citopatológicas foram utilizadas para avaliar alterações a níveis celulares nesta lesão. **RESULTADOS:** Diferentes metodologias podem ser aplicadas para a avaliação citopatológica da mucosa bucal com resultados positivos para detecção de alterações celulares na carcinogênese. Alterações ultraestruturais como perda das junções celulares, aumento do espaço intercelular e condensação de cromatina nuclear foram observados por MET nas células displásicas e neoplásicas. Mesmo em estágios iniciais da carcinogênese a citopatologia encontrou alterações citomorfométricas, no padrão de maturação celular, da velocidade de proliferação e de alterações metanucleares. **CONCLUSÃO:** A análise citopatológica bucal está em evolução tecnológica e possibilita a identificação de mudanças celulares que podem ser utilizadas para o acompanhamento de indivíduos de risco para o CEC e monitoramento de lesões potencialmente malignas.

PALAVRAS CHAVES: Citopatologia, Programas de Rastreamento, Leucoplasia Bucal, Carcinoma de Células Escamosas.

ABSTRACT

Oral squamous cell carcinoma (SCC) affects thousands of people annually, with an aggressive and mutilating behavior. Methods of early identification of this lesion have been developed, and oral cytopathology is a promising alternative. OBJECTIVE: To evaluate the use of oral cytopathology in the early detection of squamous cell carcinoma. METHODS: Initially a literature review was performed to evaluate the ability of the cytopathological method to detect early changes in the oral mucosa. In the experimental stage, cytopathological samples of SCC, oral leukoplakia, tongue border of individuals exposed and not exposed to tobacco and alcohol were evaluated ultra-structurally by transmission electron microscopy (TEM). Then, a clinical case of an early SCC was reported with the aid of different cytopathological techniques to evaluate changes at cellular levels. RESULTS: Different methodologies can be applied for the cytopathological evaluation of the oral mucosa with positive results to the detection of cellular alterations. Ultrastructural alterations such as a loss of cell junctions, an increase of the intercellular space and a condensation of nuclear chromatin were observed by TEM in dysplastic and malignant neoplastic cells. Even in the early stages of carcinogenesis cytopathology was able to detect cytomorphometric, cell maturation pattern, proliferation rate and metanuclear alterations. CONCLUSION: Oral cytopathological analysis is undergoing a technological evolution and allows the identification of cellular changes that can be used to monitor individuals at risk for SCC development and to monitor and follow-up potentially malignant lesions.

KEYWORDS: Cytopathology, Screening, Oral Leukoplakia, Squamous Cell Carcinoma.

SUMÁRIO

APRESENTAÇÃO

O escopo principal desta tese é realizar um estudo sobre a citopatologia como método de detecção de alterações precoces da mucosa bucal exposta a agentes carcinogênicos, em lesões leucoplásicas e no carcinoma espinocelular (CEC) bucal.

Inicialmente apresentaremos uma introdução sobre o assunto e a justificativa em estudar esse tema. Em seguida, apresentaremos os nossos objetivos e seguiremos apresentando três artigos científicos.

O artigo científico I trata-se de uma revisão sistematizada da literatura sobre o uso da citopatologia, suas diferentes análises metodológicas, vantagens e desvantagens de cada uma delas na detecção de alterações da mucosa bucal exposta a carcinógenos, nas lesões potencialmente malignas e no CEC.

O artigo científico II é a parte empírica desta tese, analisando ultra estruturalmente a morfologia das células esfoliadas da mucosa bucal normal, exposta a carcinógenos, em leucoplasias e no CEC bucal por meio da microscopia eletrônica de transmissão.

O artigo científico III é um relato de caso de um CEC em estágio inicial, demonstrando a aplicabilidade clínica da citopatologia na investigação de alterações precoces por meio de diferentes técnicas possíveis de avaliação.

INTRODUÇÃO

O câncer de boca apresenta uma incidência mundial de 300.373 novos casos a cada ano, a estimativa é que desses, ocorram 145.353 óbitos em decorrência da doença (WHO, 2012). No Brasil a última estimativa realizada pelo INCA apontou 15.490 novos casos dessa doença, sendo 11.140 em homens e 4.350 em mulheres. A região sul seria responsável pela incidência de cerca 16% destes casos (INCA, 2016). Dentre as neoplasias malignas bucais o carcinoma espinocelular (CEC) representa mais de 90% de todos os casos (FRONIE, 2013; INCA, 2016).

Além de altas taxas epidemiológicas dos CECs bucais, um aspecto importante é o impacto físico-psico-social, visto que esta enfermidade afeta um órgão multifuncional, que é responsável pela fala, mastigação, gustação, deglutição, como também estética. Além disso, o tratamento, frequentemente, é mutilador e representa grande morbidade para este indivíduo (WANG, 2013).

Neste sentido, ressalta-se a importância dos profissionais de saúde, pois o câncer de boca pode ser clinicamente detectável em suas fases iniciais. Entretanto, existem variáveis relacionadas à lesão como: múltiplos aspectos clínicos e velocidade de desenvolvimento. Como também relacionadas aos cirurgiões-dentistas como, por exemplo, falta de treinamento específico para conduta frente a suspeita de lesões malignas, pois sua ocorrência é incomum na rotina clínica de cada profissional, comparando-se com todas as outras doenças bucais – por exemplo, cáries e doenças periodontais (THOMSON, 2014). Esses fatores são, possivelmente, os mais significativos para o atraso no diagnóstico e encaminhamento para tratamento das neoplasias malignas bucais (FRONIE, 2013; TRAEBERT, 2015).

A carcinogênese é um processo celular de múltiplas etapas, com mutações acumuladas sobre o material genético (VOGELSTEIN, 1992). A maioria das patologias neoplásicas malignas bucais tem origem nas camadas basais dos ceratinócitos, os quais estão em constante renovação. Esta dinâmica tecidual representa uma potencial fonte de investigação para avaliar possíveis danos celulares (HAMILTON, 1974).

Tal como em outras doenças nas quais se realizam diagnósticos precoces e medidas preventivas (por exemplo: cárie dentária x aplicação tópica de flúor; doenças periodontais x orientação de higiene bucal) o método de citopatologia bucal também busca determinar alterações, quali e quantitativamente visando intervir preventivamente em fatores de risco para o câncer de boca (KAZANOWSKA, 2014). Desse modo, a identificação precoce do CEC reduziria as altas taxas de morbi-mortalidade causadas pelo tratamento desta doença em estágios mais avançados (VAN DER WAAL, 2013).

Estudos com amostras de esfregaços epiteliais da mucosa bucal exposta a carcinógenos, em lesões leucoplásicas e em CEC demonstraram alterações no padrão de maturação (ORELLANA BUSTOS, 2004; BURZLAF, 2007; BAUMGART, 2016), na atividade proliferativa (PAIVA, 2004; GEDOZ, 2007; FONTES, 2008), no padrão genético (BOHRER, 2004; KAMBOJ, 2007), na citomorfometria (tamanho celular e nuclear, relação núcleo/citoplasma) (EINSTEIN, 2005), na adesão e diferenciação celular (DA SILVA, 2017), no reparo de DNA (ALVES, 2017), na perda de heterozigozidade (ROSIN, 1997), na forma e superfície citoplasmática (KHAN, 2016) e na ploidia de DNA (SOUTO, 2010) comparando células epiteliais da mucosa bucal clinicamente normal.

O rastreamento de indivíduos de alto risco para o desenvolvimento do CEC e o acompanhamento longitudinal de lesões potencialmente malignas - pois não se sabe exatamente quais irão sofrer o processo de malignização - é um desafio para os cirurgiões dentistas e pode ser feito por meio de avaliações clínicas, histológicas e moleculares inclusive pela análise citopatológica (GÜNERI, 2017).

Desta forma, neste trabalho, explorou-se o uso da citopatologia bucal na avaliação de indivíduos de risco para o desenvolvimento do CEC, e em lesões potencialmente malignas, assim como, para avaliar as alterações celulares em CEC em estágios iniciais e mais avançados.

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OBJETIVOS

OBJETIVO GERAL

Pesquisar sobre o uso da citopatologia na detecção precoce de alterações epiteliais no processo de carcinogênese bucal.

OBJETIVOS ESPECÍFICOS

Revisar a literatura sobre as metodologias utilizadas sobre o material citopatológico na identificação de modificações na mucosa bucal, além análises aplicadas.

Avaliar se a microscopia eletrônica de transmissão permite a análise de células epiteliais esfoliadas da mucosa bucal de indivíduos expostos aos fatores de risco para o carcinoma espinocelular bucal, indivíduos com leucoplasia bucal e carcinoma espinocelular bucal. Comparar ultras estruturalmente características nucleares e citoplasmáticas dos grupos acima citados.

Relatar um caso clínico de CEC em estágio inicial no qual se utilizou a citopatologia bucal para avaliação epitelial por meio da Citomorfometria, do Padrão de Maturação Epitelial, do Teste de AgNOR e do Teste de Micronúcleos. Além disso, avaliar se este método é eficaz na detecção de alterações em estágios iniciais do processo de malignização.

ARTIGO CIENTÍFICO 1

Title: DOES CYTOPATHOLOGY IS ABLE TO DETECT EARLY ORAL MUCOSA DISTURBANCE? A REVIEW OF ANALYSIS APPLIED.

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ABSTRACT

Cytopathology is a microscopic evaluation of cell samples that are collected by scraping, washing, or sponging the surface of a tissue. The Papanicolaou test is an efficient method that has been used extensively for cervical cancer screening. A wide variety of techniques and analyses can be applied to study exfoliated cells in the oral mucosa. This study aimed to assess the capability of cytopathological techniques to identify oral mucosal changes for oral cancer screening, by recognizing and evaluating changes documented in relevant published studies. A search was performed in MEDLINE, for studies performed in humans that were published between 1997 and 2017. The search was structured as follows: “oral cancer” AND “screening” AND “cytopathology” OR “brush biopsy” OR “oral smears” OR “exfoliative cytology” AND the following techniques: Papanicolaou Test; Nucleolus Organizer Region; Micronucleus Tests; Cytomorphometric; Spectrum Analysis, Raman; Immunocytochemical; Loss of Heterozygosity; Immunofluorescence; Microscopy, Electron, Transmission; Microscopy, Electron, Scanning; Flow cytometry; and Ploidies for each methodological analysis. This search initially identified 861 studies; of these, 99 studies regarding oral cytopathology for oral cancer screening were included in this review. After considering the effectiveness of the techniques evaluated in this review, it is possible to infer that oral cytopathology detects cell disturbances before the clinical onset of oral lesions. Hence, the approach towards early oral cancer detection should couple some of these techniques with clinical examination in a longitudinal perspective.

Keywords: Mouth Neoplasms, Cytological Techniques, Early Detection of Cancer.

INTRODUCTION

The goal of oral cancer screening is to detect cellular disturbances even before clinical lesions occur[1]. Oral cytopathology, light-based detection, or oral spectroscopy were used as adjunctive tests for oral squamous cell carcinoma (OSSC) screening; none of these methodologies could be substituted for the standard method of final diagnosis that included a scalpel biopsy and histological assessment. However, the study of oral smears has the potential to achieve this objective[2]. Epithelial cell sampling by brushing the oral mucosa is a clinical approach used since many decades for monitoring cellular modifications[3]. These experimental studies usually evaluated risks in groups of individuals that used alcohol and tobacco[4–6], others agents such as crack cocaine,[7] peppers and hot meals[6], chemical compounds as formaldehyde, and n-hexane [8], and had oral potentially malignant lesions (OPML)[9,10]. This technique is non-invasive, relatively low-cost, and is well-accepted by patients, which allows the analysis of different cell samples with minimal adverse effects. However, important inconveniences are that it is observation time-consuming and does not provide information about the tissue architecture[11,12].

The sensitivity, specificity, and positive predictive value of this methodology are acceptable to detect changes in cell smears [13]. Cytopathology is a continuously evolving field;[5,14–16] the results obtained with Papanicolaou staining demonstrate the influence of carcinogens over maturation and morphometric parameters [17–19]. These findings, coupled with those of the AgNOR test, micronucleus test, immunocytochemical test, fluorescent staining, transmission electron microscopy, and loss of heterozygosity analysis improved the possibility of establishing a screening tool based on cell sampling without surgical intervention (Figure 1). The aim of this study was to evaluate and compare the results of these analyses and to delineate the future prospects of adjuvant methods for detecting modifications in the oral mucosa.

METHODOLOGY

The search identified all the studies matching the following key words in the title or abstract: “oral cancer” AND “screening” AND “cytopathology” OR “brush biopsy” OR “oral smears” OR “exfoliative cytology” AND the following techniques: Papanicolaou Test; Nucleolus Organizer Region; Micronucleus Tests;

Cytomorphometric; Spectrum Analysis, Raman; Immunocytochemical; Loss of Heterozygosity; Immunofluorescence; Microscopy, Electron, Transmission; Microscopy, Electron, Scanning; Flow cytometry; Ploidies; and Real-Time Polymerase Chain Reaction. The PubMed database was searched for studies conducted on humans, published between 1997 and 2017. Only studies in the English language were searched for, and duplicate studies were identified and excluded. After the initial filters were applied, the resulting articles were subjected to inclusion and exclusion criteria and reviewed by 6 reviewers (N.B.D., A.P.S., T.W.L., M.O.G., F.V., and P.V.R.). The articles that reviewers disagreed about were evaluated again until a consensus was reached.

Inclusion and Exclusion Criteria

Studies conducted in humans that investigated the use of cytopathology for oral cancer screening were included. Case reports documenting more than 10 cases, clinical studies, clinical trials, comparative studies, controlled clinical trials, multicenter and observational studies, and randomized controlled trials were included. Case studies conducted with less than 10 patients, literature reviews, and original studies without a control group were excluded.

RESULTS AND DISCUSSION

A search was performed for each oral cytopathological method; the search results and detailed reasons for exclusion are shown in the Figure 2. The results for each analytical method are presented below:

PAPANICOLAOU TEST

The Papanicolaou Test is usually used to analyze samples to determine if they are negative or positive for malignancy[20]; furthermore, it determines the presence of inflammatory cells [10] and the epithelial maturation pattern[19]. The different methods for obtaining cytopathological samples influence the results; the conventional brush collection method could be used to collect samples using a wooden spatula, metal spatula, smooth toothbrush, Cytobrush®, or Medibrush Plus®; samples could also be

collected by oral rinsing[6,10,19–21]. The results were better when the Cytobrush® or the oral rinse technique were used for sample collection[21].

The Papanicolaou test showed acceptable results for the detection of OSCC and leukoplakia; when DNA cytometry or cytomorphometric tests were additionally performed, the diagnostic accuracy increased significantly[9,10]. There was a moderately strong relationship observed between histopathological diagnosis and cytopathological analysis. Thus, this methodology is appropriate for the long-term monitoring of patients[22].

Oral mucosal cells of exposed individuals (tobacco/alcohol users and pepper/hot meal consumers) were evaluated by Papanicolaou Test; they showed the following cellular modifications: nuclear-cytoplasmic ratio alteration, hyperchromatism, chromatin clumping with prominent nucleoli, irregular nuclear membranes and bi- or multi-nucleation, scant cytoplasm, and variations in the size and/or shape of cells and nuclei; the frequency of atypical cells was exposure time dependent[6].

A quantitative approach for evaluating the effect of carcinogenic agents and oral lesions was to assess the maturation pattern of exfoliated oral cells. The exposed group showed a smaller number of superficial cells with nuclei; nevertheless, the keratinization index did not differ between the control and exposed groups. Additionally, an increase in the number of cells in the deeper epithelial layer was correlated to the severity of histopathological findings (i.e., leukoplakias without dysplasia, leukoplakias with dysplasia, and OSCC, gradually presenting a higher number of intermediate and parabasal cells)[19]. These findings are not in agreement with those of a previous study in which a greater percentage of anucleate superficial cells were found in smokers than in non-smokers[23]. In conclusion, the Papanicolaou technique could be used to detect oral mucosal disorders; nonetheless, when it was associated with a quantitative analysis, the results were more accurate.

AGNOR TECHNIQUE

The AgNOR test identifies nucleolar organizer regions (NORs) by silver (Ag) staining; NORs represent the transcriptional activity of DNA[24]. This marker evaluates the cellular proliferation velocity by examining the mean number of AgNORs per nucleus and the mean percentage of cells with more than three-five AgNORs per nucleus[5,25].

This technique was used to analyze cell proliferation in at-risk individuals such as quid chewers, tobacco and alcohol users, opium addicts, and crack cocaine users[26–30]. Quid chewers presented with a higher mean AgNOR per nucleus than non-chewers[28]. Sharma et al. found differences between the AgNOR counts of smokers and tobacco chewers and the AgNOR counts of the control group[31]; the epithelial proliferative activity was found to be higher in smokers than in nonsmokers[5,23,25–27,32].

Ahmed et al. observed that the AgNORs in alcohol consumers did not find statistical difference regarding control individuals, differently of smokers in which NORs mean was higher [6]. Unlikely, Paiva et al. and Matheus et al. analyzed the association between tobacco and alcohol, and found a greater mean number and area of AgNORs per nucleus in exposed groups[5,30]. A drastic difference was observed in the mean AgNOR counts of opium users and non-smokers after evaluating their oral smears[27]. A change in the oral epithelial proliferation pattern was observed in crack cocaine users [29]; in contrast, Matheus et al. have suggested that the use of crack cocaine does not affect oral epithelial cell proliferation[30]. There is a consensus among all of these studies regarding the usefulness of cytopathological evaluation using the AgNOR technique in at-risk individuals as a tool for monitoring and long-term evaluation, even before the appearance of clinical symptoms.

Concerning AgNOR evaluation in OPML and malignant neoplasias, Sowmya et al. showed that the proliferative index and AgNOR size increased gradually in the controls, oral leukoplakia (OL), oral submucous fibrosis, and OSCC[33]. The total number of AgNORs seems to be a biomarker for detecting neoplastic cells[10]; Remmerbach et al. were effectively able to detect OSCC, hyperkeratotic leukoplakias, and lichen planus cells using a semi-automated multimodal cell analysis and the AgNOR technique[34].

The mean AgNORs were compared in non-smokers, smokers without lesions, smokers with leukoplakia, and smokers with oral cancer, and the proliferative activity index was observed to increase gradually[35]. Importantly, to differentiate between benign and malignant cells, a cutoff value of 5.09 AgNORs per nucleus was established[34]; in contrast, Jajodia et al. suggested that the best cutoff value for differentiating between benign and malignant cells was 6.5[36]. The technique represents a proliferative marker in oral exfoliated cells and is a good assay for the early identification of changes occurring in these tissues.

MICRONUCLEUS TESTS

The micronucleus test has been used to analyze genotoxicity and cytotoxic events resulting because of occupational or environmental hazards; moreover, it could be useful for evaluating potentially malignant and neoplastic lesions[37,38]. In addition to micronucleated (MN), other cells exhibiting abnormalities such as condensed chromatin (CC), karyorrhexis (KR), broken eggs (BE), pyknotic nuclei (PN), karyolysis (KL), nuclear buds (NBUDs), and binucleated cells (BN) were also observed with this test[37]. Different staining procedures were performed to analyze metanuclear alterations by using acridine orange, Giemsa, Wright's stain, Feulgen associated or not with Fast Green, and DAPI plus propidium iodide[8,37,39–41]. The number of cells per sample that were studied varied between 500 cells/site and 3000 cells/site or more[7,8,39,42,43]. The Human MicroNucleus Project on Exfoliated Buccal Cells ($HUMN_{XL}$) project's specialized research group recommends the following: 1) avoid using non-specific DNA stains such as Giemsa or Aceto-orcein because they stain keratohyalin granules or bacteria, which can be counted as false positive micronuclei; 2) use the Feulgen nuclear method along with Fast Green as the standard method of staining ; 3) investigate a minimum of 2000 normally differentiated cells/sites[44].

Carcinogenic agents such as formaldehyde increased the occurrence of MN in nasal and oral mucosa of students after 8 weeks of exposure[40], though there was no difference in the MN frequency in the buccal mucosa after 2 weeks, in comparison with the controls[41]. Shoe-workers exposed to n-hexane, toluene, and methyl ethyl ketone also showed an increase in MN cells, which was correlated to the exposure time[8]. Wood dust exposure resulted in an increase in the occurrence of MN, KR, PN, KL and NBUDs[38]. The occupational hazards of welders caused an increase in the occurrence of CC and BN, in comparison to those in the controls[39]. The radiation emitted during panoramic radiographs revealed the increased occurrence of NBUDs, KR, and BN in at-risk patients; however, the MN frequency remained constant, demonstrating that there was no irreversible tissue damage[45]. *Beedi* smoke [46], tobacco smoke [15], smokeless tobacco [47], crack cocaine [7], alcohol exposure (alcohol-containing mouthwash with 26% ethanol)[48], and a combination of alcohol and tobacco use [43] also induce an increase in the number of MN cells in the oral mucosa.

The identification of MN and other nuclear alterations enabled the monitoring of OPML, screening of a singular group, and helping clinicians in the follow-up process[49]. The MN frequency and other nuclear abnormalities (BN, KL) were significantly higher in the oral white lesion cells than in normal mucosal cells [42]. Kamboj et al. evaluated oral exfoliated cells of oral leukoplakia (OL) and OSCC, and found that there was a greater increase in the number of MN cells occurring in lesion groups (2.30‰ in OL, 2.71‰ in OSCC) than in controls (0.64‰)[43]; besides MN, Pelliciolli et al also detected a progressive increase in the BE frequency within the same lesions[4].

Katarkar et al. additionally analyzed cases of oral lichen planus (OLP) and oral submucous fibrosis (OSF), comparing the cytogenetic damage in peripheral blood cells by using the comet assay for exfoliated oral cells and the MN test. The results affirm that the oral test can be used to extrapolate results similar to those of systemic analysis with the same effectiveness and greater efficiency. The greatest cytogenetic modifications frequencies were in cells in the following order: OSCC > OL > OSF > OLP[50]. This result was corroborated by that of Shah et al.; moreover, it indicated that increased mean MN frequencies were correlated to a higher histopathological grade of OSCC[51]. Another important observation is that patient age could influence the MN frequency[52]. Metanuclear alterations translate DNA content, identify early events, and can be used for monitoring the carcinogenic effects of chemical and physical agents and further OPML in oral mucosa.

CYTOMORPHOMETRIC ANALYSIS

Another application of the oral exfoliative technique is cytomorphometric analysis (CMM), which is a quantitative investigation used to evaluate certain cellular parameters such as the cytoplasmatic diameter (CD), nuclear diameter (ND), nuclear-to-cytoplasmatic ratio (N/C), nuclear area (NA), cytoplasmic area (CA), nucleus/cytoplasm area ratio (NA/CA), nuclear shape (NS), and perimeter (P)[53,54]. The patient age and sex influence CMM; the ND and CD were higher in females than in males (principally in older women), which could be because of the hormonal changes in this age group[55]. This observation is very important for efficient sample selection, which was extremely important; because the more paired the more reliable results.

Exfoliated oral cells of at-risk individuals were studied by using CMM. The use of alcoholic mouthwash caused a greater decrease in the NA and CA in smokers than in non-smokers, and increased the number of inflammatory cells[56]. The use of different forms of tobacco could induce alterations in cellular measurements; the CD progressively decreased and ND increased in non-users, tobacco chewers, and tobacco smokers; however betel quid chewers did not present with significant modifications in buccal cells[57]. Tobacco chewing influenced the oral mucosa without clinical lesions and also influenced the development of tobacco-lime lesions, OL and OSCC; a reduction in CD, an enlargement in ND, and a progressive increase of the N/C ratio were observed in these groups[17,58]. Such cellular modifications were also found in inhalant opium addicts and gutka chewers, who exhibited decreased CA and increased NA[18,59]. Woyceichoski et al. showed that crack cocaine users exhibited a decreased NA and NA/CA ratio in, and suggested that this can be associated with a higher degree of keratinization[60].

CMM in exfoliated epithelial squamous cells had been indicated to be an additional diagnostic test in early oral malignancy[61]. Compared to individuals with normal oral mucosa, individuals with epithelial dysplasia showed a reduced CA, signaling an early cytological dysplastic change[61]. Ogden et al. evaluated NA and CA in patients with oral cancer and cancer-free patients by the semiautomatic image analysis system, and observed a significant reduction in CA in the oral cancer group; however, no significant difference was seen in the NA values[62]. With regarding to the ND and CD, Ramaesh et al. determined an increase in ND and decrease in CD in malignant lesions[63]; a similar result was seen in a study by Shaila et al. conducted with atypical leukoplakia smears[64]. Metgud et al. showed that there was a significant reduction in both ND and CD mean as well as an increase in the N/C ratio mean in OL and submucous fibrosis patients, as compared to the normal oral mucosa[65]. Considering cellular perimeter and area in the OPML or lichen planus patients, leukoplakia, erythroplakia, and hyperkeratosis were higher compared with no lesion smear[54].

Comparing tobacco use with oral lesions by CMM analysis of oral keratinocytes, higher NA and ND, and lower CA and CD values were reported in OSCC patients were reported in comparison to those of individuals with tobacco chewing, smoking, and non-smoking habits, although gradual changes were found in individuals

exposed to tobacco[53]. These findings were corroborated by Sarkar et al., who added that the N/C ratio was significantly higher in smokers than in nonsmokers; and this trend was also observed in individuals with OPML as compared to those who were smokers[17]. Hence, we can conclude that there is a tendency for increased nuclear and decreased cytoplasmatic measurements to be obtained with the progression from normal to at-risk and malignant statuses; thus, it is considered a good practice to monitor the oral mucosa to determine the risk of cancer development.

RAMAN SPECTRUM ANALYSIS

Raman spectroscopy is a method of optical analysis that allows molecular-level tissue characterization. Using this method, the detection of biochemical changes which alter the optical and biological properties of dysplastic and cancerous tissues can be used as a biological parameter[66,67]. To our knowledge, only one experimental study has examined oral mucosal smears of exfoliated cells by Raman analysis[66].

The surface-enhanced Raman scattering by exfoliated oral cells showed an increased lipid content in normal cells, while exfoliated OSCC cells exhibited an increase in the protein and DNA content. This could be related to the increased number of dividing cells. In addition, the peak intensities can be used as diagnostic thresholds to differentiate OSCC from normal cells[67]. This analysis has very specific results for the exfoliated cell evaluation and is a promising tool for the longitudinal evaluation of patients exposed to carcinogens and OPML.

IMMUNOCYTOCHEMICAL ANALYSIS

The immunostaining of some proteins has been used to identify changes in oral brush biopsies[68,69]. The comparison of epithelial keratins K8, K19, K13, and K10 between OSCC and normal oral mucosa (contralateral side) was conducted. The main markers expressed in malignant lesions were cytokeratin 8 and 19. Immunocytochemical techniques, combined with assessment of DNA ploidies resulted in the correct identification of a large amount of malignant tumors[68]. Yamashina et al. evaluated CK13 and CK17 with cytomorphometry and observed a loss of CK13, which is associated with greater cellular atypia in the NA and N/C ratio, while the expression of CK17 was related to higher-grade cellular atypia[70].

Epithelial cell analysis using liquid-based cytology and AE1/AE3 immunostaining were used to evaluate OSCC samples; a positive reaction was observed in all cases. One advantage of these techniques in comparison with immunohistochemical methods is the small amount of antibodies required and reduced cost[71]. The staining of the tumor suppressor Fragile Histidine Triad (FHIT) was conducted in normal mucosa, at the parabasal and intermediate cells, and a strong positive nuclear and cytoplasmatic immunoreactivity was observed. This suggested that this marker would be effective for use in screening programs, previous OSCC patient follow-ups, and precancer lesion screening[72].

A comparative cytological study for evaluating Mcm-2/Mcm-5 levels between normal, dysplastic, and OSCC lesions showed that malignant cells were strongly stained and benign cells were unstained; thus, this immunostaining technique can indicate an ectopic cell cycle entry, which is present in epithelial dysplasia and malignant lesions[69]. The immunocytochemical evaluation of laminin-5 revealed that it had a sensitivity and specificity of over 90% in identifying oral malignant neoplasms; this protein is associated with invasion and metastasis processes[73].

The combination of some markers allows an increased OSCC risk assessment. Bloching et al. considered p53, Ki-67, PCNA, and cyclin D1 immunocytochemical analyses in a case control study. All the proteins expressions were different between tumor and control smears. High tobacco consumption was associated with increased Cyclin D1 and MIB1 nuclear expression. Furthermore, a highly positive cyclin D1 nuclear staining suggested a 62-fold increase in the upper aero-digestive tract tumor risk, and if the MIB1 threshold was exceeded, the risk increased 45 times. The use of a proliferation marker in combination with proto-oncogene immunocytological stains are promising for the identification of patients at high-risk of OSCC[74].

The genes and proteins related to the DNA repair mechanism were evaluated in tobacco-exposed individuals. Immunocytochemical stains for MLH1, MSH2 (involving in repair of errors in DNA base pairing), and ATM (a tumor suppressor gene) were negative in individuals who had never smoked, whereas they were rarely positive in smokers[75]. The immunocytochemical expression of E-cadherin (cell adhesion) was decreased in OL and OSCC in comparison to the controls and in at-risk individuals; also, it was associated with increased AgNORs in lesion groups, while Involucrin staining (cell differentiation) revealed no statistically significant differences[76].

Immunocytochemistry is a great way to evaluate the oral mucosa; it can specifically detect several epithelial characteristics. There are a few limitations regarding the analysis of deeper epithelial layers; long-term sample storage time may lead to a loss of immunoreactivity.

LOSS OF HETEROZYGOSITY

Loss of heterozygosity (LOH) is defined as an allelic loss at a heterozygous locus for a given marker[77,78]. The LOH assay evaluates polymorphic chromosomal regions that are near or within tumor suppressor genes and oncogenes. Inactivation of one of the two tumor suppressor gene alleles by a germ line or somatic mutation is a critical step in development of carcinogenesis, since only one more mutation is required before phenotypic expression occurs[79].

LOH has been shown to be a potent independent risk predictor for oral premalignant lesion progression[80–82]. This analysis is usually done by microdissection of formalin-fixed paraffin-embedded tissue (FFPE). Some researchers that glimpse LOH as a potential tool for oral cancer prevention proposed cytopathological sampling as an alternative sampling method for LOH analysis, since the technique is non-invasive and easier to perform [83,84].

To compare both sampling techniques, i.e., FFPE tissues and epithelial exfoliated cells in LOH analysis, Graveland et al. obtained exfoliated cells and performed biopsies from oral leukoplakia lesions. LOH was executed by PCR followed by fragment analysis. Exfoliated cells evaluated for LOH have a limited value for monitoring patients with leukoplakia, mainly in hyperkeratotic cases, because the thick keratin layer becomes a barrier for collecting cells from the deeper stratum of the epithelium[83]. Rosin et al. also explored the viability of exfoliated cells from OSCCs, carcinomas *in situ,* and dysplastic lesions to detect LOH by using PCR and autoradiography analysis. It was demonstrated that patterns of allelic loss observed in exfoliated cells and biopsies of the same region were similar [84]. The contradictory findings of the above mentioned studies can be due to the samples' histopathological features and methodological issues.

It is well-known that there is a LOH in *p53* (tumor suppressor gene) in fresh and FFPE oral squamous cell carcinoma specimens. Huang et al. demonstrated that exfoliated OSCC cells provide enough DNA for detecting *p53* LOH, leading to the conclusion that the non-invasive sampling method is feasible to determine tumor-specific allelic imbalances in patients with OSCC[85].

SCANNING ELECTRON MICROSCOPY

Ultrastructural examination was performed in oral cytopathology by scanning electron microscopy (SEM). Bizarre cell forms, irregular surface patterns, anisocytosis, no distinct borders, loss of cell contact, spherical blebs, cylindrical structures, surface evaginations, and slender strands were not observed in normal cells. These disturbances increased gradually in oral lichen planus (OLP), OL and OSCC. Filipodia was only observed in OSCC cells. A correlation between the SEM findings corresponds with the epithelial atypia detected by light microscopy[86].

Chomette et al. studied normal exfoliated epithelial cells by SEM and classified them as: flat (cells with microridges and microvilli with linear adhesions that correspond to the superficial layer), polygonal (cells with well-defined crests between junctions and some microvilli that correspond to intermediate cells) or round (cells totally covered by microvilli that corresponding to parabasal cells). OSCC cells showed enlarged polymorphous cells (round, globular, and elongated patterns) with irregular microvilli dispersed in their surfaces. Individuals with epithelial dysplasia showed polymorphous cells with edges separating their surfaces, which had irregular microvilli and ridges[87].

Khan et al. evaluated oral exfoliated epithelial cells in exposed individuals, OSCC, and normal tissues. Abnormal size-related variations, aberrant forms, and surface pattern irregularities were significantly higher in OSCC, tobacco smokers, and betel nut chewers when compared to control individuals. Cylindrical structures in the epithelial surface were more frequently observed in OSCC, tobacco, and betel nut chewers than in individuals with normal mucosa; surface evaginations were higher in individuals with OSCC than in at-risk groups. In opposition, the frequency of slender strands and filopodia was the highest in individuals with OSCC[88]. The SEM enables ultra-structural details of cellular changes to be viewed. Although it is an expensive and impractical technique for use in clinical practice, it allows a better understanding of the carcinogenesis process.

FLOW CYTOMETRY

Flow cytometry (FC) is a laser-based technology based on counting and classifying cells, determining biomarkers and proteins. Its use is appropriate in the clinical routine, and it is able to detect changes in clinically normal oral mucosa and non-dysplastic lesions[89]. When analyzed by FC, exfoliated keratinocytes from tobacco smokers presented an increased DNA content, aneuploidy, cells in synthesis percentage (S), G(2) + Mitosis (M), and apoptosis when compared to non-smokers[90].

An endocytotic quantitative study of squamous epithelial cells using fluorescence and FC evaluated oral cellular function when exposed to the effects of alcohol. The intracellular vesicles were identified as fluorescent microspheres; the capability for endocytosis was more reduced in the high alcohol user group than in children, adults who drink socially, or drink low levels of alcohol (smokers, non-smokers). This result can indicate a reduced ability for eliminating local carcinogens[91]. Some studies[92,93] also used flow cytometry to evaluate DNA ploidy, which would be explored in the next section.

DNA PLOIDIES

Ploidy analysis, also known as DNA-image cytometry, is an adjuvant technique that increases the sensitivity of oral cytopathology. Generally, cellular DNA content can be identified by the Feulgen reaction or DAPI stain. Results are interpreted by using flow cytometry or automated image analysis software. The number of cells analyzed varies between 100-300 cells per sample[9,92,94,95].

Ogden et al. evaluated DNA range profiles; commonly, normal cells were diploid and most OSCC smears had an abnormal DNA range profile (i.e., they were polyploid). However, when this analysis was combined with keratin immunostaining, the number of correctly diagnosed cases increased[68]. In another study clinically normal epithelial cells were diploid in individuals who were social drinkers and those who were chronic alcohol drinkers, although there was an increase in nuclear DNA content in some of the cells in the group that abused alcohol[96]. Souto et al. observed that the mean percentage of aneuploid cells was increased in smokers, and in the OL and OSCC groups as compared to non-smokers[95].

DNA-image cytometry in OSCC demonstrated an acceptable sensitivity and accuracy for cancer cell detection. This technique, in association with the qualitative Papanicolaou test, increases the diagnostic accuracy of malignant and pre-malignant lesions[14]. Another study using ploidy analysis found acceptable levels of predictive

values and low rates of false negative and positive cases, highlighting the technique as an useful adjuvant tool for monitoring neoplastic epithelial cells in OPML[97].

When the OLP cytopathological qualitative examination could not predict the presence of tumor cells in a sample with certainty, the ploidy test revealed DNA-polyploidy, and the cytologically “suspicious” samples were DNA-aneuploid. This results suggests that this combination is an auxiliary test to confirm if oral lesions exhibited malignancy and if scalpel biopsy was required[98].

In contrast to other reports in literature, OLP, OL, erythroplakia, and OSCC showed a high grade of diploid DNA patterns, whereas a minority of them was aneuploid. This variability in the ploidy pattern can be attributed to different methods for sample collection, the diploid reference considered, and the sample size studied. In addition, the histologic grade differentiation, tumor aggressiveness, and lymph node involvement can influence the ploidy pattern[54]. Another factor that can influence the accuracy of ploidy analysis is the lesion type. Early OSCC and hyperkeratotic lesion analysis can result in sampling errors due to a non-representative deeper epithelial layer sample; Kammerer et al. indicate this method as an adjunctive one, which was not a potential confirmatory method for excluding the possibility of malignancy[94].

One important observation made by Hirshberg et al. was that the proportion of non-diploid cells increased with a progression in the degree of epithelial dysplasia[99]. This finding was corroborated by other researches, demonstrating that non-dysplastic lesions were aneuploid in 22.8% and dysplastic in 56.7% of cases[93]. During malignancy, the frequency of aneuploidy increases; cases of OL without dysplasia were characterized by a single near-diploid aneuploid sublines, and dysplastic lesions and OSCC were characterized by high aneuploid sublines. This suggests a mechanism of “endoreduplication” and chromosomal loss in epithelial cells[92]. This methodology allows a genetic analysis that evaluates correct cell division, identifying mutations and failures along the cell cycle. Despite the easy execution, this test produces data requiring a laborious process of interpretation.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

The collection of cytological samples is critical to perform PCR; Reboiras-López et al compared instruments used for collection, and suggested that although

dermatological curettes achieve help to obtain a greater amount of RNA, Oral CDx is a less aggressive instrument that could obtain samples of acceptable quality and quantity[100]. The overexpression of cytokeratins was evaluated using reverse transcriptase polymerase chain reaction (RT-qPCR) in OSCC oral brush biopsy; among the studied proteins, CK 17 appears to be more promising than CK 19 and CK 20 for differentiating cancer cells from normal cells[101].

Advanced molecular analysis using real time PCR evaluated tobacco users, leukoplakias, and OSCC RNA expression; this technique could identify the B2M (immune system), CYP1B1(metabolic process), and KRT17(keratin sintesis) genes to differentiate between smears[102]. In another study, the expression of the V-type ATPase protein subunit C1 that modulated the acidification of intracellular compartments of cells was increased in individuals with OSCC[103]; nevertheless, the oral mucosa of smokers, former smokers, and nonsmokers demonstrated normal gene and protein expression[104].

Kugimoto et al also suggested the use of SCCA1 (proteinase inhibitor) as a biomarker for oral cancer screening; however, they suggested the use of multiple markers to improve the diagnostic accuracy[105]. MicroRNAs also could be analyzed by PCR, microRNA-21 (oncogenic) and microRNA-375 (anti-oncogenic) efficiently predicted OSCC in oral cytological smears[106].

FINAL CONSIDERATIONS

Considering the cytopathological applications available and the advantages and limitations of each technique (Table 1), it is possible to conclude that a combination of different analytical methods detects changes in the oral mucosa more precisely. If automatic computerized assisted testing, which evaluates different characteristics at the same time, is aimed at screening individuals at high risk for oral cancer in cross sectional or longitudinal studies, it may increase the accuracy of detection using non-invasive collection methods[16,107,108].

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FIGURE LEGENDS

Figure 1: Techniques applied in Oral Cytopathology. A) Papanicolaou Stain (40x), B) AgNOR technique (100x), C) Micronucleus Test (Feulgen Stain, 100x), D) E-cadherin cytoplasmatic immunocytochemical stain (20x), E) DAPI and Actin Green 488 fluorescence stains (Confocal microscopy, 63x), F) Transmission Electron microscopy (5000x), and G) Polyacrylamide gels visualized by autoradiography for loss of heterozygosity (LOH) analysis.

Figure 2: Flow chart of studies selection. PAPA: Papanicolaou Test; AgNOR: AgNOR Technique; MN: Micronucleus Tests; CMM: Cytomorphometric Analysis; Raman: Raman Spectrum Analysis; ICC: Immunocytochemical Analysis; LOH: Loss of Heterozygosity; IF: Immunofluorescence; TEM: Transmission Electron Microscopy; SEM: Scanning Electron Microscopy; FC: Flow Cytometry; Ploidies: DNA Ploidies; PCR: Real-Time Polymerase Chain Reaction.

Table 1: Comparative analysis between techniques applied in Cytopathological smears.

Figure 1

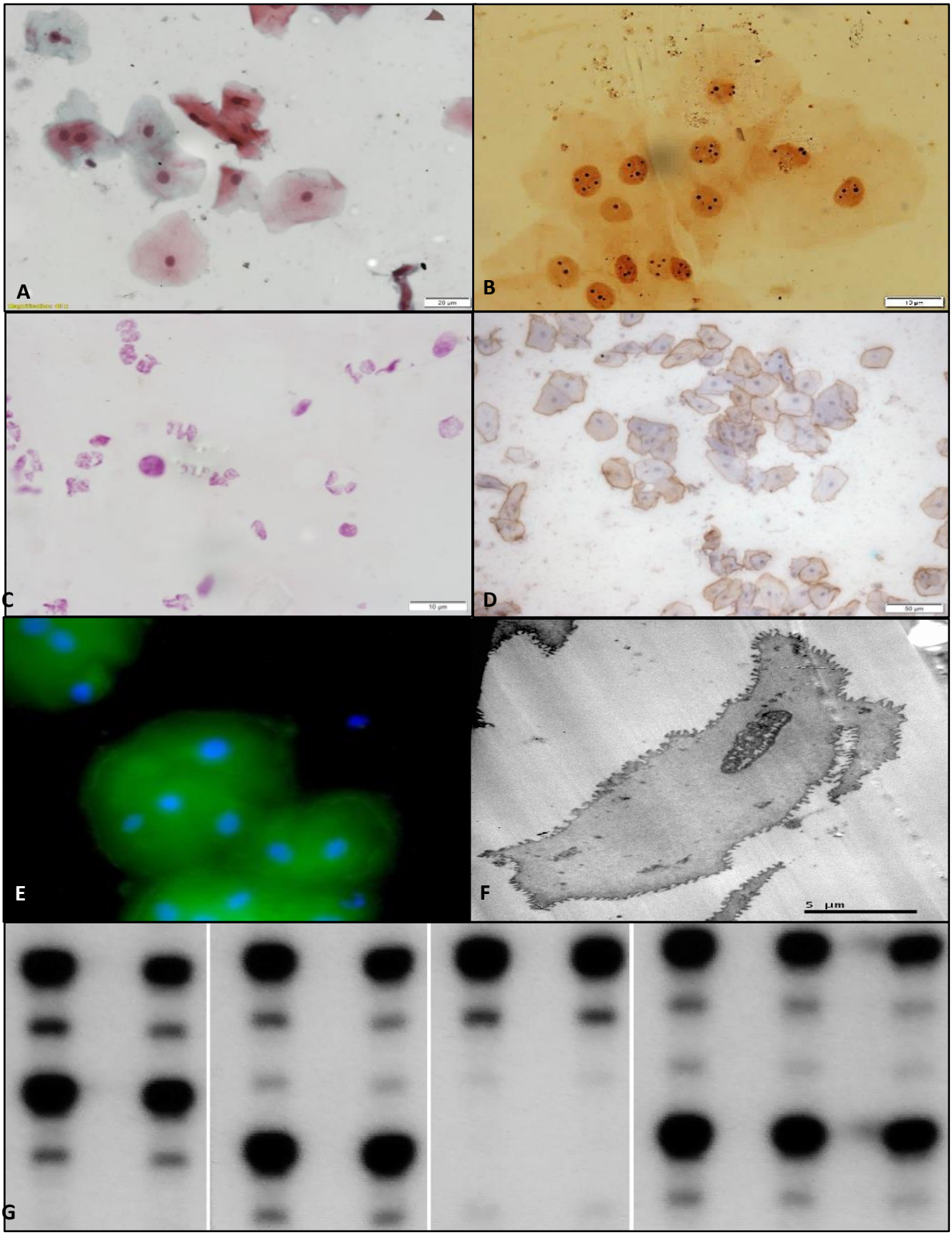

Figure 2

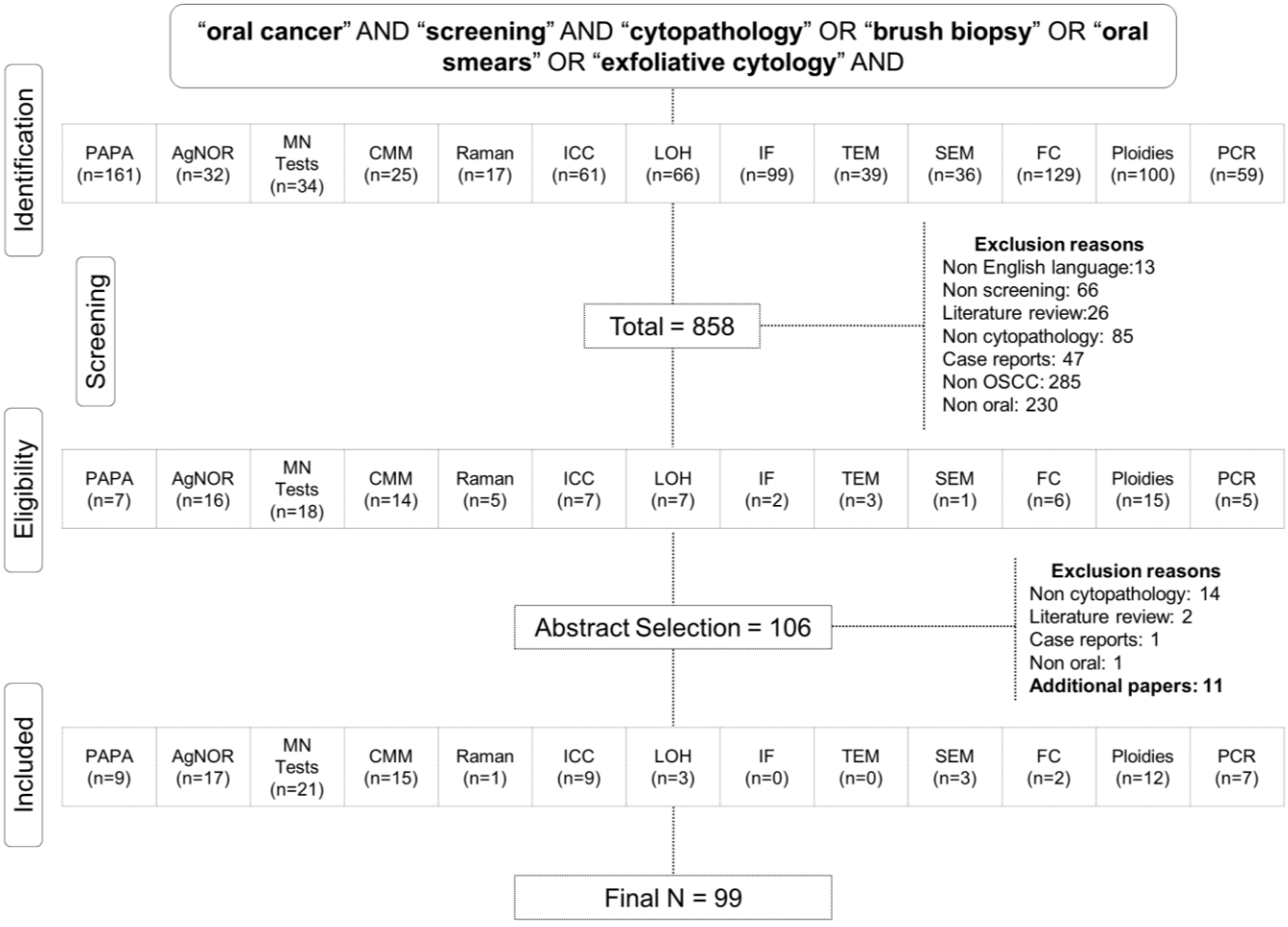
"oral cancer" AND "screening" AND "cytopathology" OR "brush biopsy" OR "oral smears" OR "exfoliative cytology" AND
Identification
PAPA (n=161)
AgNOR (n=32)
MN Tests (n=34)
CMM (n=25)
Raman (n=17)
ICC (n=61)
LOH (n=66)
IF (n=99)
TEM (n=39)
SEM (n=36)
FC (n=129)
Ploidies (n=100)
PCR (n=59)
Screening
Total = 858
Exclusion reasons
Non English language:13
Non screening: 66
Literature review:26
Non cytopathology: 85
Case reports: 47
Non OSCC: 285
Non oral: 230
Eligibility
PAPA (n=7)
AgNOR (n=16)
MN Tests (n=18)
CMM (n=14)
Raman (n=5)
ICC (n=7)
LOH (n=7)
IF (n=2)
TEM (n=3)
SEM (n=1)
FC (n=6)
Ploidies (n=15)
PCR (n=5)
Abstract Selection = 106
Exclusion reasons
Non cytopathology: 14
Literature review: 2
Case reports: 1
Non oral: 1
Additional papers: 11
Included
PAPA (n=9)
AgNOR (n=17)
MN Tests (n=21)
CMM (n=15)
Raman (n=1)
ICC (n=9)
LOH (n=3)
IF (n=0)
TEM (n=0)
SEM (n=3)
FC (n=2)
Ploidies (n=12)
PCR (n=7)
Final N = 99

Table 1

	Positive Aspects	Negative Aspects
Papanicolaou Test	Fast, low cost	Qualitative analysis is subjective–intra and inter examiner variability Sampling collection technique – depending on device and personal training Quantitative analysis requires internal validation for each anatomic site in the oral mucosa Keratinized sites are difficult obtain in the deeper cellular layers
AgNOR Test	Provides objective analysis	Technique is useful for individual and longitudinal analysis. Calibration is a pre-requisite Sensible for use at ambient temperatures, acceptable speed of the silver nitrate reaction in solution, and the acceptable sensitivity of silver nitrate to light
Micronucleus Test	Simple laboratory technique Worldwide-specific group for the MN test	Time consuming Calibration is a pre-requisite
Cytomorphometric Analysis	Simple, objective test	Software and microscopic-computer dependent. Gender and age variabilities can affect results
Raman Spectrum Analysis	Molecular information	Expensive equipment Fluorescent background
Immunocytochemical	Specific and sensible	Specific protocols must be established for each protein targeted Low stability of immunoreactive processes
Loss of Heterozygosity	Specific Predictive value established	Time consuming Calibration is a pre-requisite
Scanning Electron Microscopy	Ultrastructural information	Availability of equipment and resources Clinically uncertain relationship
Flow Cytometer	Practical	Availability of equipment and resources Sampling amount of cells dependent
Ploidy Analysis	Genetic information	Availability of equipment and resources
Real-Time PCR	Specific	Sample collection dependent Standardization is difficult

ARTIGO CIENTÍFICO 2

Title: ANALYSIS OF THE ULTRASTRUCTURE OF EXFOLIATED ORAL MUCOSA CELLS BY TRANSMISSION ELECTRON MICROSCOPY

*Artigo formatado de acordo com as regras da revista Cancer Cytopathology (A2, impact factor 3.818)

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ABSTRACT

The search for early cellular changes in oral epithelium is a major challenge for oral cancer diagnoses. Tobacco and alcohol are established risk factors for oral carcinogenesis; leukoplakia is another risk factor for oral squamous cell carcinoma (OSCC), few studies have evaluated the ultrastructural cellular characteristics of these individuals. This study evaluated exfoliated epithelial cells of the oral mucosa by transmission electron microscopy (TEM). The study groups were controls (individuals without lesions and not exposed to risk factors), individuals who use alcohol and tobacco, patient with homogeneous leukoplakia, and patient with OSCC. The ultrastructural features of the cells were analyzed in ultrathin cell sections. The cell-cell cytoplasmic connections in malignant cells lost the characteristic irregular pattern formed by the numerous interdigitations and the junctional process of normal cells and instead presented a straight cytoplasmic surface. The nucleus in leukoplakia and OSCC cells showed heterogeneous composition while non-lesional cells were homogeneous. The analysis of oral cytopathological smears by TEM contributes towards understanding during the process of malignancy of the cellular oral mucosa and this information can be applied in the early detection of OSCC.

Keywords: cytological techniques, mouth mucosa, carcinoma, squamous cell, microscopy, electron, transmission.

INTRODUCTION

Ultrastructural examination by TEM was performed in cytopathological samples extracted via fine needle aspiration and exfoliative cytology. Soft tissue, lymph node, bronchi alveolar lavage, pleural fluid, and urine smears are some examples of materials examined. TEM can be an adjunctive in the diagnosis of epithelial and mesenchymal tumors, non-neoplastic processes, and identification of microorganisms [1]. Cervical cells evaluated by TEM showed (i) loss of junctional complexes, such as desmosomes and (ii) degenerated nuclei with loss of the nuclear envelope in superficial layers cells; such evaluations demonstrate the enhanced resolution power of this technique [2].

TEM was used to evaluate oral leukoplakia showing discontinuous basal lamina, ruptured hemidesmosomes, pathologic cytoplasmic processes, indented nuclei, and sinusoidal dilatation of the nuclear membrane in non-homogeneous cell subtypes [3]. In oral squamous cell carcinoma (OSCC), the main changes observed by TEM were nuclei with irregular nuclear borders and prominent nucleolus and discontinued basal lamina with abundant cytoplasmic projections of basal cells with a decreased or total loss of hemidesmosomes [4].

It is possible to state there are changes in the ultrastructural characteristics between the normal epithelial cells and cells with lesions in the oral mucosa [5]. All these findings were observed in the tissue specimens. The purpose of this study was to evaluate ultra-structural characteristics of exfoliated oral squamous epithelial cells of individuals not -exposed to cancer risk factors, exposed to cancer risk factors (such as tobacco and alcohol), patients with leukoplakias, and patients with OSCC.

METHODS

The present study was conducted in accordance with Declaration of Helsinki and was approved by the Ethics Committee at the Universidade Federal do Rio Grande do Sul. The subjects of the study were healthy male patients. These individuals sought assistance at the Dental School and were invited to participate; all patients signed an informed consent form prior to their participation in the study. After being provided with the written informed consent, all participants answered a questionnaire to establish sociodemographic habits. Group I (CG) comprised controls (individuals not exposed to risk factors and with nonclinical lesions); group II (ATG) comprised alcohol and tobacco users without clinical lesions; group III (OLG) comprised oral homogeneous leukoplakia patients, and group IV (SCCG) comprised OSCC patients. The inclusion criteria followed the same criteria previously established in our study [6].

The exclusion criteria were as follows: patients younger than 18 years of age, patients with visible clinical oral lesions other than those observed in OSCC and leukoplakia, or patients with a previous diagnosis of OSCC or other potentially malignant lesions, such as erythroplakia.

Cytopathological smears were collected with rotating movements of the cytobrush (Absorve®, São Paulo, Brasil) with around the border of the tongue in non-lesional groups, over the lesions in the leukoplakia groups, and adjacent to the ulcer in the OSCC group. The cytobrush was stored in an Eppendorf® with 1.5 mL of glutaraldehyde fixative solution [glutaraldehyde 0.5% (Sigma Chemical Co., St. Louis, Missouri), paraformaldehyde 4% (Reagen, Brazil), and a 0.2 M sodium phosphate buffer, and a final pH fixing solution was 7.4] at 4 °C until the samples were processed. The collected sample was centrifuged to form a pellet, and the cytological brush was discarded. The samples were washed with sodium phosphate buffer (0.1 M, three times for 15 minutes each) and postfixed in 1% OsO_4 (Sigma Co.) in 0.1 M phosphate buffer (PB) for 1 hour. The samples were again washed with 0.1 M PB, dehydrated in a graded series of acetone, embedded in resin (Durcupan®; ACM-Fluka, Switzerland), and polymerized at 60 °C. In each of the steps above, the cells were pelleted by centrifuging the vials at 3000 rpm for 5 minutes and the excess reagent was removed. Ultrathin cross-sections (80–100 nm) were obtained using an ultramicrotome (MT 6000-XL; RMC, Tucson, Arizona) and a freshly made glass knife. The ultrathin sections were placed on copper grids and stained with 2% uranyl acetate for 20 minutes and lead citrate in distilled water for 10 minutes. The cytopathological analysis was performed by TEM (JEM 1200 EX II) at 80 kV voltages. For each sample, five cells were captured initially in a magnification that completely visualized the cell (± 5K). The magnification was then increased to verify the details in the cellular membrane (± 50K).

OBSERVATIONS AND RESULTS

The sociodemographic characteristics of the study groups are shown in table 1. A qualitative description was made considering the cellular membranes, cytoplasmic contents, and nuclear characteristics.

MORPHOLOGICAL ANALYSIS

The absence of cytoplasmic organelles such as the mitochondria, golgi complex, and endoplasmic reticulum was observed in all the samples evaluated.

Control Group (CG)

In the normal mucosal cells of the CG samples, a cytoplasmic membrane was present with well-defined microvilli and multiple cellular junctions with desmosomes. The nucleus was

dark colored, intact, and homogeneous. Bacterial colonies, lipid particles, tonofilaments, and keratohyalin granules were also observed among epithelial cells (Figure 1).

Alcohol-Tobacco Group (ATG)

The main morphological characteristics were similar to those observed in samples from the CG. Furthermore, the nuclear content was slightly heterogeneous (Figure 2).

Oral Leukoplakia Group (OLG)

Cells were elongated and thin. The membrane had irregular contours. The nuclear content was heterogeneous. Most of the cells observed were anucleated and keratohyalin granules were observed between the cells (Figure 3).

Oral Squamous Cell Carcinoma Group (SCCG)

The cytoplasmatic membrane was devoid of microvilli; with the absence of cell junctions there was an increase in the intercellular spaces. The nuclear content was distributed irregularly. Intracellular vacuolization, also known as the “Laking Effect,” was noted. The cells showed an increased nuclear/cytoplasmic ratio (Figure 4)**.**

DISCUSSION

This study used cytological sampling to demonstrate some important differences among normal, exposed, and carcinogenic cells, as observed by TEM analysis. A gradual loss of the cellular junctions and an increase in the intercellular spaces were observed in the present study, such that the maximum loss of cellular junctions and increase in intercellular spaces was observed in SCCG followed by OLG, ATG, and CG. These findings were reported by histological analysis of the tumor adjacent normal epithelia and OSCC using TEM [4]. In leukoplakias that present a dyskeratotic and dysplastic epithelium, a decrease in the number of junctional complexes and acantholytic widened intercellular spaces are observed [7]. In non-homogenous leukoplakias, Tamgadge et al. found ruptured desmosomes and widened intercellular spaces [3]. On comparing simplex, verrucosa, and erosive leukoplakia, it was found that intercellular spaces in erosive leukoplakia were significantly greater [8]. Moreover, Frithiof et al. observed a decrease in the number and size of desmosomes in OSCC cells [9]. These morphological findings are consistent with chemical studies evaluating cell adhesion proteins, whose levels decrease with the carcinogenesis process [6,10]. The loss of cell cohesiveness can lead to: 1) increased tissue permeability, which exposes cells from the deeper layers to the carcinogenic agents [11] and 2) invasion and metastasis processes in OSCC [12].

In present cytopathological samples of oral mucosa smears evaluated by TEM, no cytoplasmic organelles were found in states similar to those reported by Banoczy et al. The

histological analysis of leukoplakia by TEM showed that the mitochondria were observed in basal cells, but superficial layers with degenerated matured cells [8]. Due to the constant epithelial turnover, the superficial layer cells are in the final maturation stages; hence, these are not more feasible [13], as observed in Figures 1 and 2.

In the study sample, tonofilaments and keratohyalin granules were observed in all groups except SCCG. In non-homogenous leukoplakia analyzed by TEM, Tamgadge et al. demonstrated a decreased presence of tonofilaments and decreased or absent keratohyalin granules. This observation was compared with that in homogenous leukoplakia; abundant keratinization in the form of thick bundles of tonofibrils and increased number of keratohyalin granules of increasing size were observed in homogenous leukoplakia [3]. The presence of keratohyalin granules in epithelial superficial layers was correlated with anucleated cells [14]. Considering that samples from the non-lesional group were collected from the lateral border of the tongue, we expected to find keratohyalin granules since this is keratinized mucosa. Additionally, in the leukoplakia group, anucleated cells were found, probably, because it was a hyperortokeratinized lesions composed mostly of anucleated cells.

The pattern of chromatin condensed along the nuclear membrane increased from ATG to SCCG. In the CG, the nucleus presented a homogenous pattern with a dark color. We also observed that the more dysplastic was the lesion, the more irregular was the shape of the nucleus. Similar to the results reported in previous studies using TEM and histopathological analysis of human lung cells to detect changes that occur as chromatin condensed along the nuclear membrane [15], the dyskeratotic and dysplastic epithelium showed atypia of the nucleus, nucleolus, and mitotic apparatus [7]. In non-homogenous leukoplakia, an enlarged nucleolus with variation in size and shape was associated with nucleolar margination [3]. In erosive leukoplakia, an irregular (i) nuclear shape, (ii) chromatin distribution, and (iii) number of nucleoli were observed [8]. Irregular nuclear materials indicate early stages of apoptotic events [15]. Furthermore, an evident nucleolus is an important parameter signifying disturbance in cellular proliferation [16] and must be monitored in at-risk OSCC exposed individuals.

The “Laking effect” is seen as an intracellular vacuolization around the nuclear content. This can signal degenerative events in the cell [14]. Tamgadge et al. also observed this phenomenon in leukoplakia cases and suggested that this is a preneoplastic change [3]. In this study, was observed this alteration in a squamous carcinoma cell corroboring with previous above study.

FINAL CONSIDERATIONS

TEM was used to evaluate the exfoliated cells of the oral mucosa. The detailed morphological observations elucidate the changes induced on exposure to cancer risk

factors and the oral carcinogenesis process. TEM enables the understanding of the disturbances in epithelial differentiation during the process of malignancy.

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Figure Legends

Table 1: Sample sociodemographics and risk factors habits.

Figure 1: Control group oral epithelial cells sample. A) Cellular adhesion between a nucleated cell and another cell. B) Nucleated cell contended intracytoplasmatic lipid particle (in arrow). C) Dark homogeneous nuclear content. D) Dark rounded structures suggesting bacteria (in arrow). E) Desmosomal junctional process. F) Cytoplasmatic membrane showing keratohyalin tonofilaments (T). G) Cytoplasmatic membrane showing microvillus (M). H) Keratohyalin granules (K) in the intercellular space. Transmission electron microscopy, uranyl acetate, and lead citrate stains.

Figure 2: Tobacco-alcohol oral epithelial cells sample. A) Oral epithelial cells showing an irregular surface with microvillus. B) Homogeneous nuclear content (N). C) Heterogeneous nuclear content (N) and keratohyalin granules. Transmission electron microscopy, uranyl acetate, and lead citrate stains.

Figure 3: Leukoplakia oral epithelial cells sample. A) Clinical image showing homogeneous leukoplakia in right floor of the mouth. B) Photomicrograph showing squamous oral epithelial hyperortokeratinizated with bulbous projections into connective tissue (HE, 10x). C) Anucleated cells and nucleated superficial cells. D) Thin and elongated anucleated cells. E) Multiple cells attached with minimum intercellular space. F) Microvillus of the cytoplasmatic membrane and nucleus with condensed chromatin. G) Irregular contours of the cytoplasmatic membrane with interdigitations. H) Heterogeneous nuclear content. Transmission electron microscopy, uranyl acetate, and lead citrate stains.

Figure 4: Oral squamous cells carcinoma sample. A) Clinical image of an ulcerated lesion with a necrotic center in the ventral surface of the tongue. B) Malignant proliferation of epithelial squamous cell invading the connective tissue and exhibiting keratin pearls (HE, 10x). C) Epithelial cells attached but with increased intercellular space. D) Epithelial cells with altered nucleus/cytoplasm ratio. E) Pleomorphic epithelial cells. F)”Laking effect”. G) Absence of intercellular junctions. H) Flat surface without microvillus or interdigitations. Transmission electron microscopy, uranyl acetate, and lead citrate stains.

Table 1

	Group	Site	Age	Marital status	Education	Oral Hygiene	Tobacco	Alcohol
1	CG	Tongue	32	Single	University graduate	Good	NO	≤ once/week
2	CG	Tongue	43	Single	University graduate	Good	NO	≤ once/week
3	CG	Tongue	50	Single	Did not graduate university	Regular	NO	≤ once/week
4	CG	Tongue	63	Single	Did not graduate university	Poor	NO	≤ once/week
5	ATG	Tongue	65	Single	High school graduate	Regular	20 cigarettes/day for 40 years	300 mL “cachaça”/week for 40 years
6	OLG	Floor of the mouth	64	Divorced	Elementary school	Poor	60 cigarettes/day for 50 years	4L/week beer and wine
7	SCCG	Floor of the mouth	54	Single	Elementary school	Regular	20 “palheiros”/day for 43 years	500 mL “cachaça”/day for 20 years

Figure 1

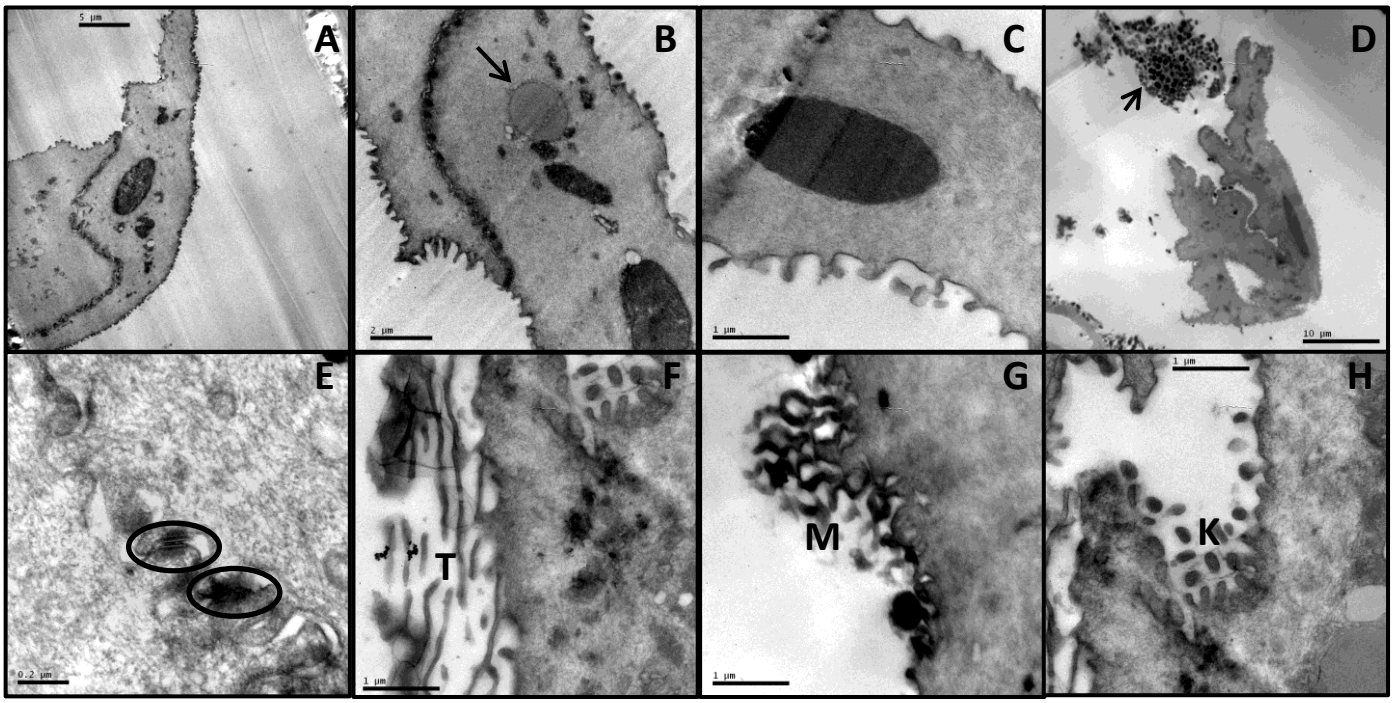


Figure 2

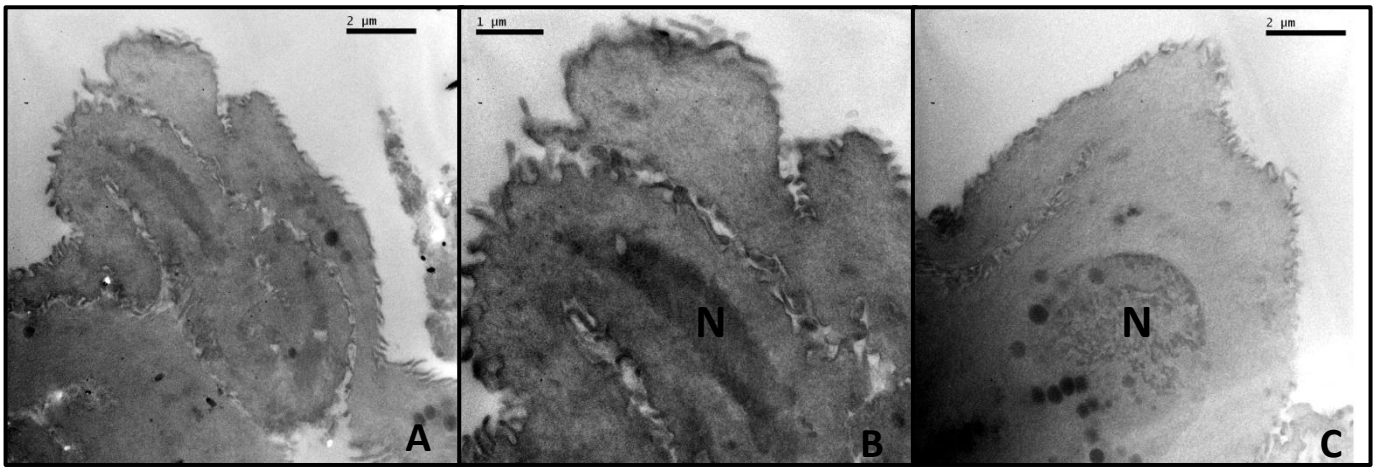


Figure 3

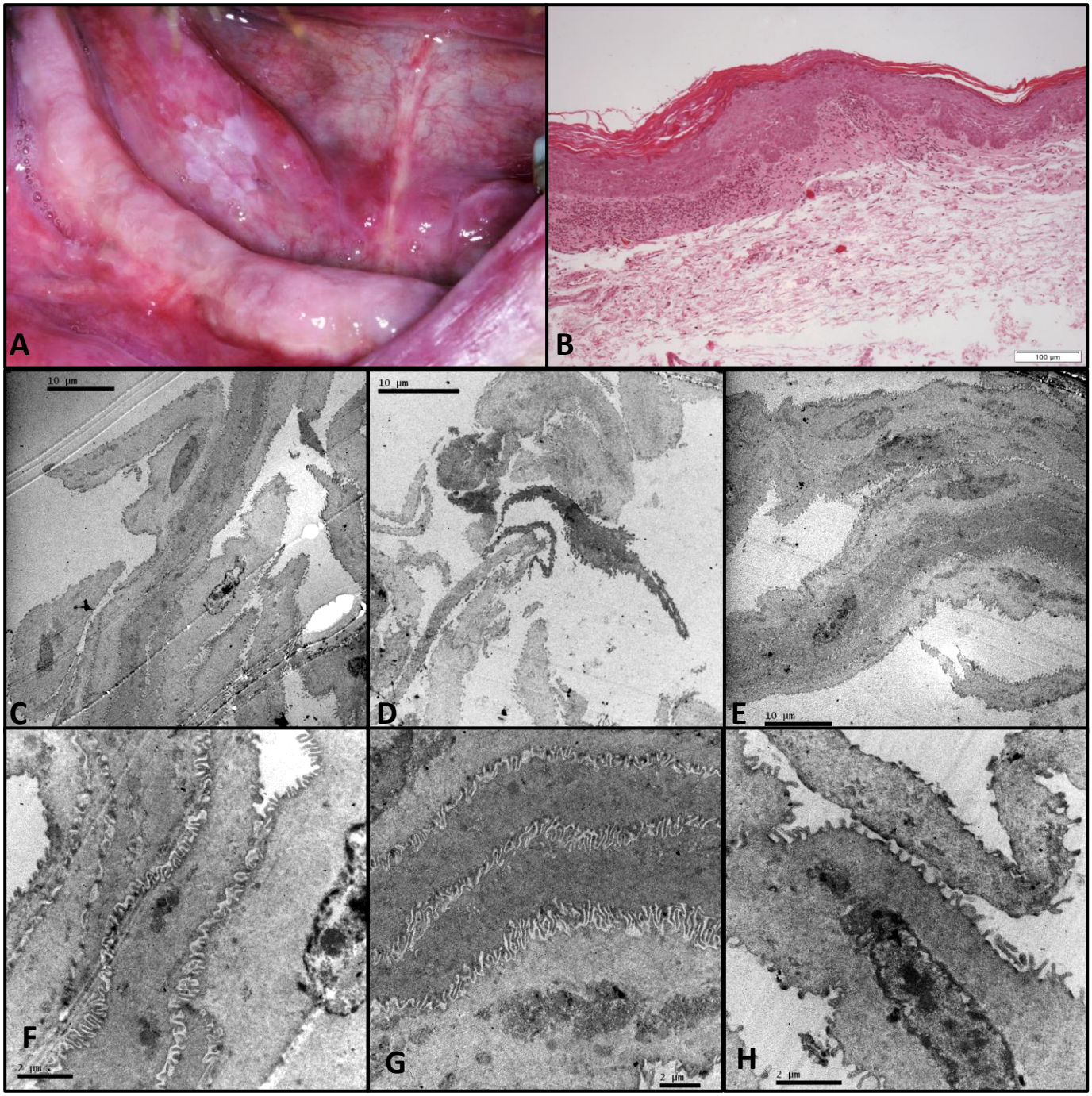
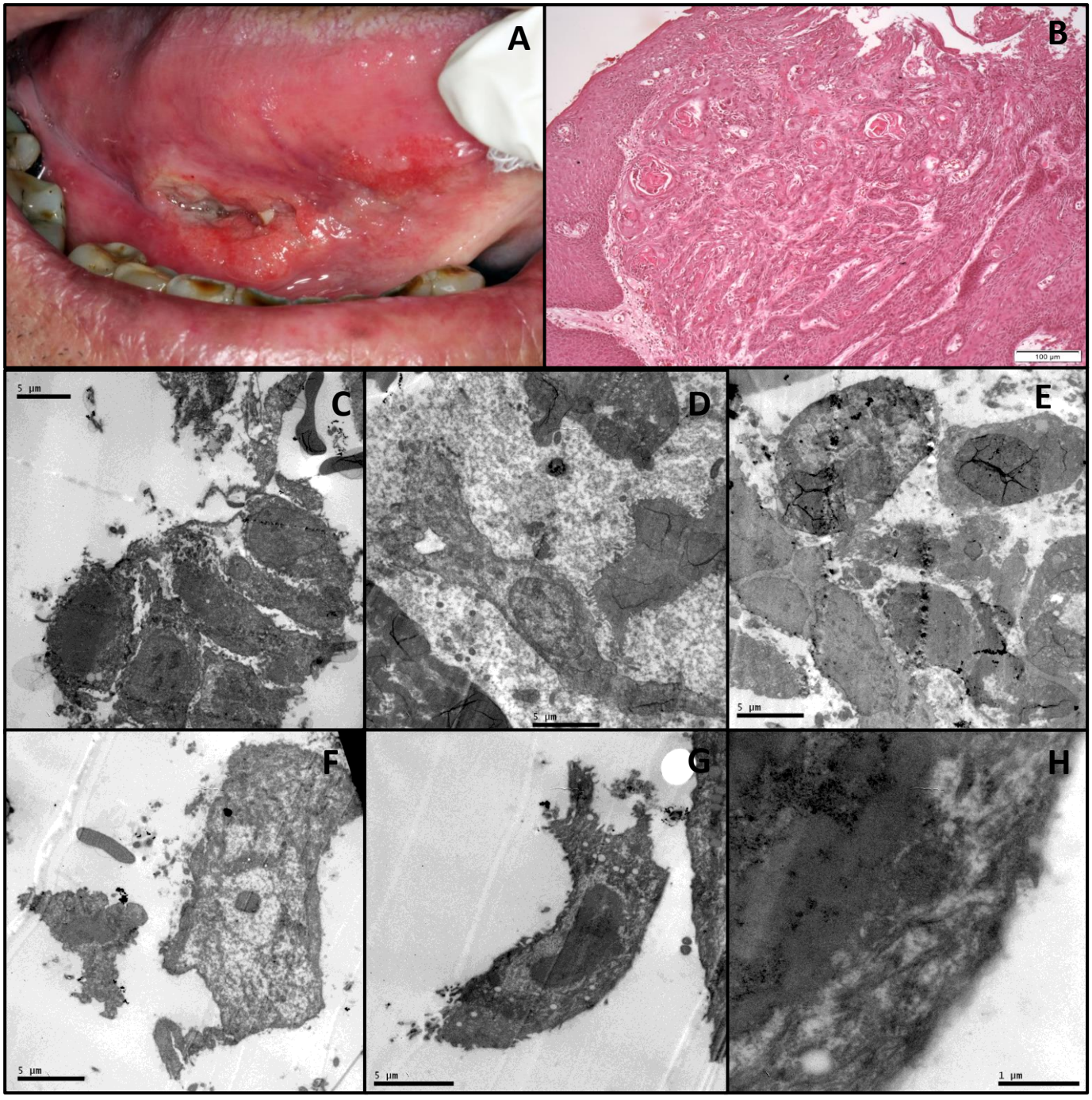


Figure 4



ARTIGO CIENTÍFICO 3

Title: THE USE OF CYTOPATHOLOGY AS AN AUXILIARY TOOL TO IDENTIFY ALTERATIONS IN EARLY CARCINOGENESIS STAGES OF ORAL SQUAMOUS CELL CARCINOMA.

*Artigo formatado de acordo com as regras da revista Diagnostic Cytopathology (Qualis B1, Impact Factor 1.161).

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Keywords: Early Diagnosis; Carcinoma, Squamous Cell; Cytological Techniques.

ABSTRACT

The diagnosis of oral squamous cell carcinoma (OSCC) in its early stages is a challenge for oral surgeons since its clinical features are not always classical. Cytopathological assays can contribute to identifying alterations at the cellular level. Objective: The present study reports a case of OSCC in a young male adult, without exposure to risk factors. Results: The histopathological examination showed a microinvasive carcinoma invading the connective tissue. The cytopathological results showed a higher percentage of deeper layer cells; the cytomorphometric examination revealed a nucleus/cytoplasm ratio of 0.14; the mean number of AgNOR per nucleus was 2.86, and the mean percentage with >2 nuclei was 58%. The micronucleus test found 3 micronucleated cells and several metanuclear aberrations. Conclusions: These findings support the hypothesis that cytological examination is an important adjunctive tool for monitoring clinically suspicious malignant oral lesions.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents more than 90% of oral malignant neoplasias. The traditional risk factors associated with the initiation and promotion of this disease are tobacco and alcohol exposure, or in cases specifically located on the lip, ultraviolet radiation[1]. Oral cancer is hardly detectable in the early stages of invasion, even though it is proven in the literature that there are early cellular changes before its clinical appearance[2]. Histologically, OSCC is classified according to invasion of adjacent tissues. If it occurs only in the lamina propria and does not yet involve submucosal tissues, then the tumor is denominated as microinvasive OSCC (miOSCC)[3].

The main purpose of the secondary prevention of OSCC is to diagnose oral potentially malignant lesions and/or to detect OSCC in its early stages like miOSCC[4]. In this sense, the screening of those lesions may be performed by cytopathological analysis[5]. Exfoliated oral epithelial cells may be analyzed by qualitative and/or quantitative methods: Papanicolaou staining[6,7], Argyrophilic nucleolar organizer region staining (AgNOR)[8], and the Feulgen reaction[9]. This study reports the cytopathological findings in miOSCC, diagnosed by histopathological examination, presented in a patient who neither used tobacco nor alcohol.

CASE REPORT

A 31-year-old male Caucasian patient, with no systemic impairment, without smoking and alcohol habits sought stomatology care referenced from his general dental clinician because of tongue erosion. This lesion persisted for 2 months, even after traumatic factors were completely removed. Upon intraoral clinical examination, an erosive leukoerythroplastic lesion was observed in the right border of the tongue (Fig. 1).

Cytopathological smears of the lesion site were collected with cytobrush (Absorve®, São Paulo, Brazil). The samples were distended on glass slides and stored in ethanol 99% at 4°C until processing. An incisional biopsy was performed; the specimen was stored in buffered formalin 10% and processed with hematoxylin/eosin technique. The oral smears were submitted to the following techniques: Modified Papanicolaou staining was performed according to the method of Burzlaff et al.[6], cytomorphometric analysis according with Shaila et al.[10], AgNOR staining according to Ploton et al.[11], and Feulgen staining according Pellicioli et al.[9].

RESULTS

According to maturation pattern, there were: 1 anucleated, 10 superficial with nuclei, 83 intermediate, 5 basal, and 1 binucleated cell in the first 100 cells analyzed. The cytomorphometric analysis showed an average of 50.03 µm (±27.4) for cellular diameter (CD), 7.07 µm (±4.11) for nuclear diameter (ND), and 0.14 (±0.05) for nuclear cytoplasm ratio (N/C). The mean number of AgNOR per nucleus was 2.86, the mean percentage of nuclei with >2 AgNOR was 58%, > 3 AgNOR was 24%, and >4 AgNOR was 8%. The micronucleus test found 3 micronucleated cells, 37 cells with broken-egg appearance, 24 cells with karyorrhexis, 16 binucleated cells, 22 multinucleated cells, and 15 cells with aberrant nuclei, out of a total of 1000 cells (Fig 2). The histopathological examination presented neoplastic epithelial squamous cell islands invading connective tissue but restricted to the lamina propria, and neoplastic cells exhibiting cellular pleomorphism, atypical mitotic figures, hyperchromatism, and keratin pearls (Fig 3). The tumor thickness was 450 µm in its maximum measurement. A final diagnosis of miOSCC was made.

The patient was referred to the oncologist for treatment, aiming at maximizing the surgical margins. The patient reported no major postoperative complications regarding speaking and swallowing. A clinical follow-up after 2 months did not reveal signals of recurrence (Fig 4).

DISCUSSION

Oral cytopathology aim to screen patients with the traditional OSCC risk factors and oral pre-malignant lesions[12]. The maturation pattern of oral epithelial cells showed an increased mean number of anucleated cells in oral leukoplakia and OSCC compared with clinically normal mucosa of the tongue border. The number of superficial cells with nuclei did not differ between groups, and intermediate cells was fewer in leukoplakia, comparing with OSCC and normal mucosa[13]. In another previous study the mean proportion of anucleated/superficial with nuclei/intermediate/basal cells in OSCC was 9.4/60.6/20.8/9.2; 41/55.5/3/0.5 in dysplastic leukoplakia; and 26.7/71.8/1.3/0 in non-dysplastic leukoplakia, respectively[6]. In the present case this proportion was 1/10/83/5, which corroborated with reports cited above in which dysplastic or malignant tissue presented higher frequency of intermediate and basal cells when compared with clinically normal mucosa.

Analysis of the cellular measurements is an important evaluation method to identify measurable changes in malignant and potentially malignant lesions[10]. CD in normal cells showed a mean of 51.78 (±0.11) µm, 45.73 (±0.16) µm in non-dysplastic leukoplakia, 41.32 (±0.13) µm in dysplastic leukoplakia, and 38.58 (±0.11) µm in OSCC. ND in normal cells presented a mean of 8.36 µm (±0.49), 8.31 µm (±0.68) in non-dysplastic leukoplakia, 9.04 µm (±0.46) in dysplastic leukoplakia, and 10.10 µm (±0.56) in OSCC[14]. In a more recent study Shaila et al. found a 52 µm mean for CD in normal mucosa, 53 µm in cases of leukoplakia without atypia, and approximately 48 µm in cases of atypical leukoplakia. The mean ND in the normal mucosa was 9.0 µm, 9.4 µm in leukoplakia without atypia, and 9.5 µm in atypical leukoplakia. The N/C ratio in normal mucosa was 0.022, 0.026 in leukoplakia without atypia, and 0.030 in atypical leukoplakia [10]. In a specific study evaluating normal oral exfoliated cells in male patients with ages ranging between 20-40 years, the mean ND was 7.99 µm (±0.87), the mean CD was 49.18 µm (±5.41), and N/C ratio was 0.1698 (±0.022)[15]. Although the results of the present case are in accordance with normal cytomorphometric parameters, high standard deviations were observed (50.03 µm [±27.4] for CD, 7.07 µm [±4.11] for ND, and 0.14 [±0.05] for N/C), which could indicate the subtle modification of the cells in the early stages of OSCC.

According to Paiva et al., the mean number of AgNORs per nucleus in clinically normal tongue was 2.2 for non-exposed individuals, 2.7 in smokers, and 2.9 in the smokers/alcohol group. The mean percentage of nuclei with >3 AgNOR was 12.2 among controls, 27.1 among smokers, 29.1 among smokers/alcoholics[16]. The mean number of AgNORs in oral leukoplakia was 3.58 and 4.16 in OSCC[13]. The present case showed a mean of 2.86 AgNORs per nucleus, and 24% of the cells presented >3 AgNORs/nucleus. These results showed a higher proliferative activity of the epithelial cells compared with normal non-exposed individuals and smokers, but lower compared with potentially malignant and OSCC lesions, probably because it is an initial stage of malignancy in a non-exposed individual.

The micronucleated cells (MN) frequency suggests chromosomal damage and it is able to quantify the breakage and chromosomal loss. The Human MicroNucleus Project on Exfoliated Buccal Cells (HUMNXL) estimated a 0.74‰ spontaneous MN frequency for subjects not exposed to genotoxic agents or radiation[17]. The mean MN in clinically normal tongue mucosa was 0.86‰; in tobacco-exposed individuals it was 0.82‰, and in tobacco/alcohol-exposed individuals it was 1.21‰ cells[18]. In another

study the metanuclear frequency was evaluated between non-exposed, exposed alcohol/tobacco, leukoplakia and OSCC, in men older than 30 years. In the non-lesion groups MN, cells with broken-egg appearance (BE) or karyorrhexis (KR) were not observed, compared to leukoplakia (MN=1; BE=0; KR=0.5) and OSCC (MN=2; BE=0.5; KR=0) groups[9]. In the present case the number of metanuclear alterations was increased, it is difficult to compare due a rarity of such early-diagnosed OSCC cases.

The recent proposal of a multivariate analysis model advocates that cytomorphometric evaluation provides relevant evidence to differentiate altered epithelial cells. Additionally, molecular biomarkers such as Ki67, MCM2, CD147, and EGFR expression increases the accuracy of this analysis, reinforcing the hypothesis that combined cytopathological evaluations are the greatest way to detect changes in the oral mucosa[19].

In this sense, a group denominated the Global Oral Cancer Forum (GOCF) was assembled in the effort to encourage programs focused on oral cancer screening. The goal of oral cancer screening is not to diagnose lesions, but to anticipate the appearance of clinical signs. Besides, to define the individuals who can have these alterations, with great potential to development of malignant neoplasias, their follow-up should be more rigorous[4]. Our study suggests actions focused on high-risk patients by using clinical examination associated with other tests as reported here in a case of miOSCC.

The detection of miOSCC is a good strategy for secondary prevention; less than 4 mm miOSCC invasion depth was related with a better prognostic and lower morbidity when compared to tongue OSCC that invaded more than 4 mm in depth[20]. This was observed in the present case in which the patient did not have impairment in function because the lesion was diagnosed at an early stage.

Clinically, miOSCC more frequently presents as potentially malignant lesions; the more prevalent affected sites are the tongue, following by buccal mucosa and floor of the mouth. The lesions may present as plaques and erosions, and tobacco and alcohol habits are less frequent in miOSCC comparing with classical OSCC [21], in accordance to the present case.

The incidence of OSCC among younger people is increasing, representing 4–6% of oral cancers[22]. OSCC in younger people can occur in the absence of, or even after a relatively short duration of exposure to tobacco and alcohol[23]. Other possible

carcinogenic causes have been studied to explain the possible initial mechanism, such as human papillomavirus and oral microbiota[24,25]. In a literature review regarding younger OSCC patients, the incidence was higher in men, on the tongue, gingiva and lower lip sites, in contrast to older OSCC patients in whom the floor of the mouth was the main affected site [26,27].

Recently, Liu et al. suggested one possible etiologic spectrum of head and neck SCC in young patients: compromised immunity that can correlate with human papillomavirus infection, genetic factors, and persistent irritation that generates inflammation and can lead to head and neck squamous cell carcinoma[28]. Within this possible explanation, further studies are needed to clarify these relationships.

FINAL CONSIDERATIONS

The findings presented here, mainly in the cytopathological pattern of epithelial maturation, AgNOR counts, and metanuclear abnormalities should be an early clinical warning of OSCC development. The laboratory results presented here coupled with other information as risk factor exposure, medical history and anatomic site may contribute to lower the morbidity related with OSCC. The approach presented in this specific case may be better understood with more reports considering larger groups with miOSCC and/or potentially malignant lesions.

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FIGURE LEGENDS

Figure 1: Clinical appearance. A) Initial view of the lesion at general dental clinician consultation, B) Clinical presentation 2 months after the initial examination.

Figure 2: Cytopathological findings. A) Intermediate cells, superficial cells with nuclei, and inflammatory cells (Papanicolaou, 40×), B) Nuclei with 3 and 4 AgNORs (AgNOR, oil immersion 100×), C) Broken egg and micronucleated cells (micronucleus test, oil immersion 100×), D) Cells with karyorrhexis (Micronucleus test, oil immersion 100×).

Figure 3: Photomicrographs of incisional biopsy. A) Mucosal fragment recovered by stratified squamous epithelium invading the lamina propria (H&E 4×), B) Malignant epithelial islands in the connective tissue (H&E, 10×), C) Keratin pearls and pleomorphic epithelial cells (H&E, 20×), D) Atypical mitosis inside the epithelial islands (black arrows) (H&E, 40×).

Figure 4: Surgical site showing scarring fibrosis following excision, without signs of recurrence in two months of follow-up.

Figure 1

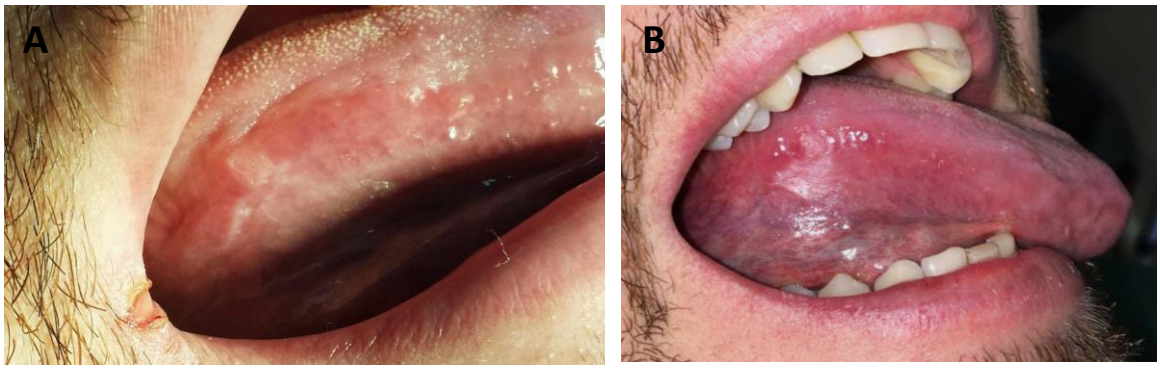


Figure 2

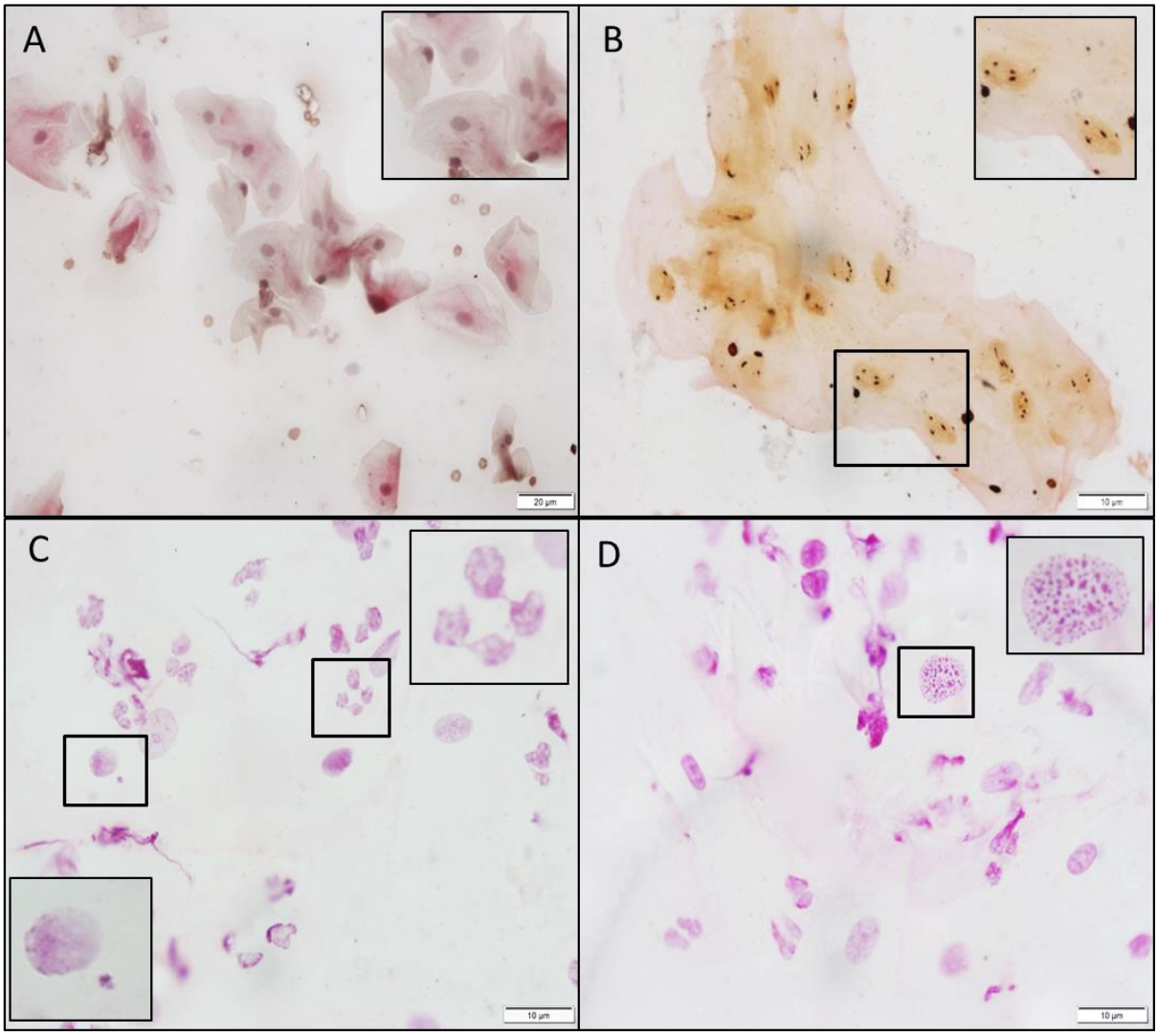


Figure 3

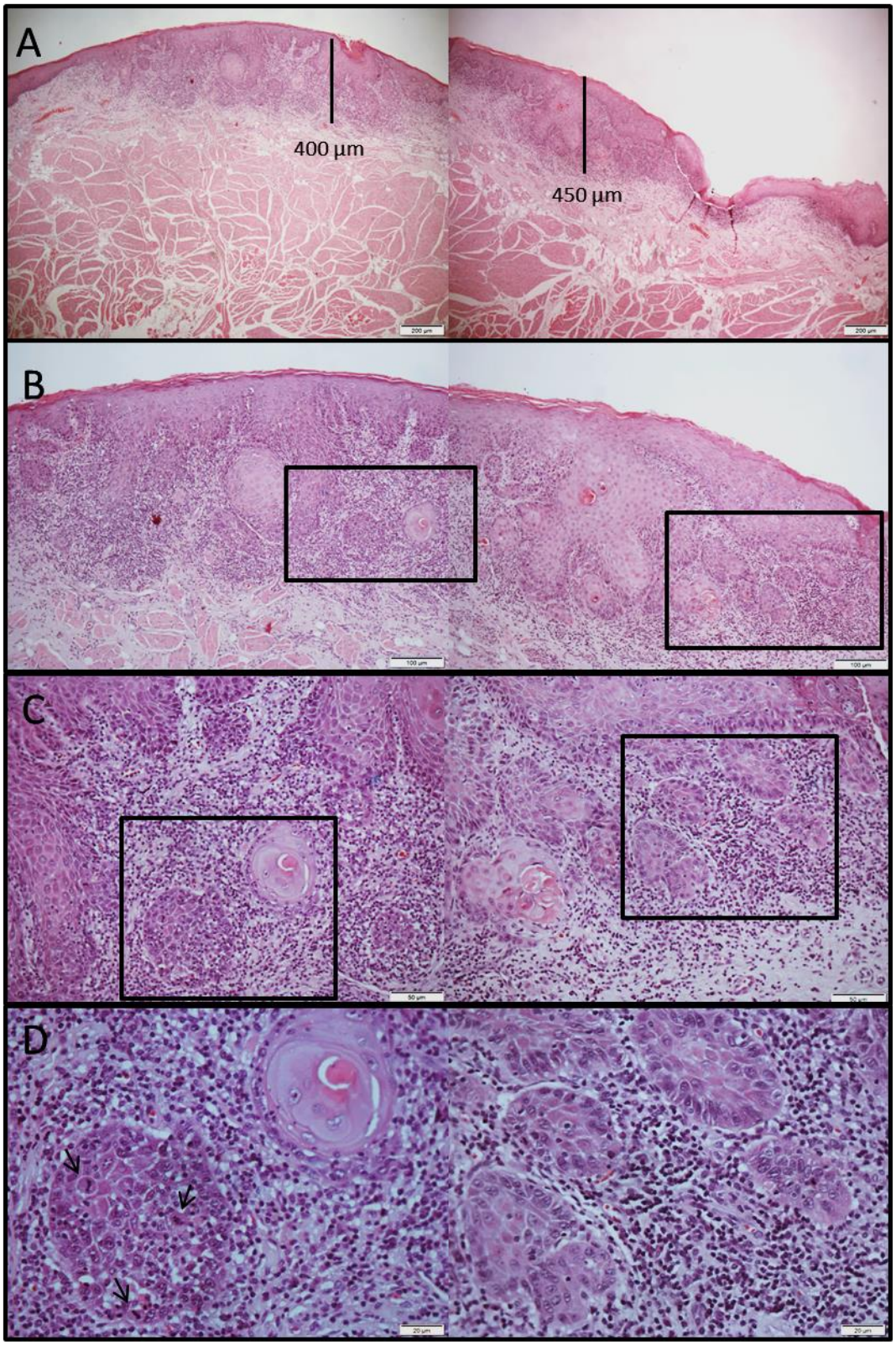
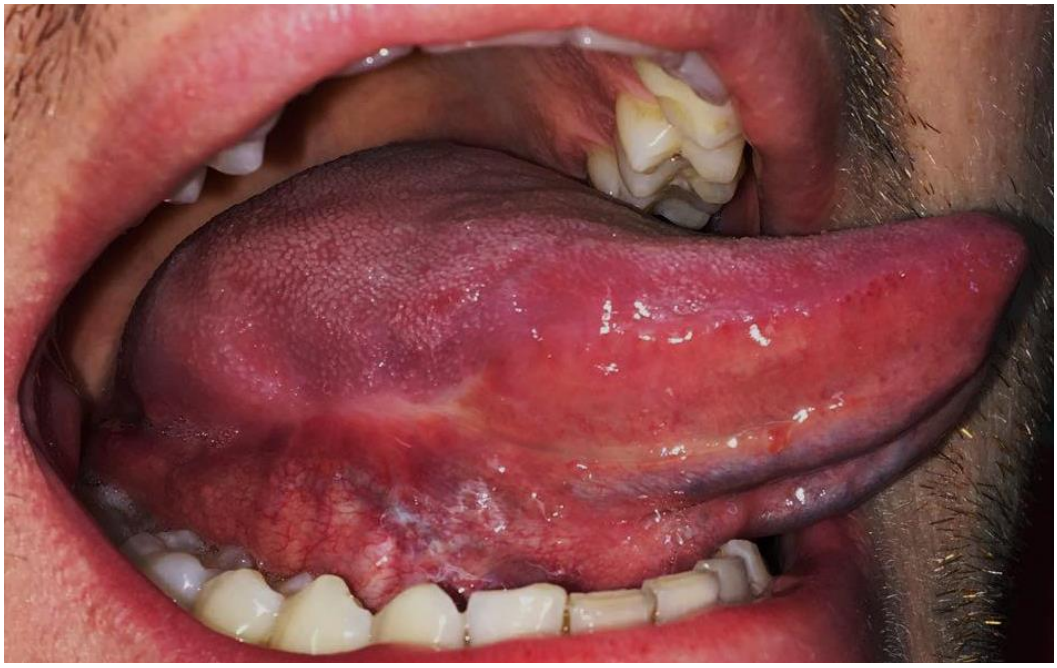


Figure 4



CONSIDERAÇÕES FINAIS

Os estudos que utilizam a análise citopatológica de células bucais foram capazes de detectar alterações epiteliais em indivíduos expostos aos fatores de risco, com lesões potencialmente malignas e com carcinoma espinocelular. Os resultados dos testes de acurácia entre eles especificidade, sensibilidade e valores preditivos positivos e negativos são variáveis. Esta variabilidade está relacionada com as diferentes técnicas de coleta da amostra e metodologias aplicadas sobre esse material obtido.

Entre as formas de coleta se destaca, positivamente, a citologia em meio líquido; entretanto, os métodos tradicionais de raspado ainda têm espaço, principalmente se considerarmos seu uso em grande escala. Os testes mais promissores para avaliação celular parecem ser: a citomorfometria, avaliações da velocidade da proliferação celular (AgNOR) e a quantificação de alterações metanucleares (Teste de Micronúcleos). A automatização das técnicas laboratoriais e a possibilidade de realizá-las concomitantemente em uma mesma amostra deverão, no futuro, melhorar ainda mais o desempenho na busca do diagnóstico cada vez mais precoce do CEC.

Mesmo com os progressos nos métodos avaliativos de raspados citopatológicos da mucosa bucal, há concenso na literatura de que havendo a suspeita clínica de lesão maligna, está indicada a realização de biópsia seguida do exame histopatológico – que permanece sendo o padrão ouro para diagnóstico do CEC.

A microscopia eletrônica de transmissão é útil para a análise ultraestrutural de células bucais esfoliadas demonstrando perda gradual das junções celulares como

também de microvilosidades, além da condensação da cromatina nuclear com a progressão do processo de carcinogênese. Embora sua aplicabilidade na rotina clínica do cirurgião-dentista seja difícil e onerosa, a realização desse tipo de estudo tem o potencial, ainda inexplorado, para o entendimento do comportamento biológico celular dos indivíduos de risco para o CEC (fumantes e alcolistas, assim como, indivíduos com lesões potencialmente malignas).

Mesmo em estágios iniciais do CEC em mucosa bucal, a análise citopatológica pôde determinar alterações morfométricas, na velocidade de proliferação e nos danos genéticos. Na literatura não são encontrados limites confiáveis para estratificar os pacientes de risco em alto e baixo grau para o desenvolvimento do CEC com relação a esses exames. Com a definição destes referenciais – sejam eles numéricos ou qualitativos - esses dados poderiam ser usados como indicadores de risco para mudanças de comportamento ou instituição de terapêuticas de reversão de dano nestes pacientes.

Estudos multicêntricos e de acompanhamentos longitudinais podem também contribuir para a obtenção destes parâmetros, com maior validade e poder amostral na medida em que se ampliam as probabilidades em surpreender os CEC bucais no princípio da evolução, devido à fugacidade deste estágio da doença.

ANEXO 1. APROVAÇÃO NO COMITÊ DE PESQUISA DA UFRGS

UFRGS

Universidade Federal do Rio Grande do Sul **Faculdade de Odontologia**

PARECER CONSUBSTÂNCIADO DA COMISSÃO DE PESQUISA

Parecer aprovado em reunião do dia 12 de junho de 2015

ATA nº 07/2015.

A Comissão de Pesquisa da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul após análise aprovou o projeto abaixo citado com o seguinte parecer:

Prezado Pesquisador PANTELIS VARVAKI RADOS,
Informamos que o projeto de pesquisa 29423 - ANÁLISE DA MORFOLOGIA, DA ADESÃO E DA PERMEABILIDADE DO EPITÉLIO BUCAL HUMANO EXPOSTO A CARCINÓGENOS encontra-se aprovado com o seguinte parecer:

Trata-se de um estudo que tem como objetivo avaliar a estrutura das células epiteliais da mucosa bucal, suas membranas plasmáticas, suas junções de ancoragem célula-célula e a expressão de moléculas de adesão celular frente sua exposição a agentes carcinogênicos. Para tal, utilizaremos raspados citológicos da mucosa bucal de indivíduos expostos e não expostos a agentes carcinogênicos, os quais serão avaliados por meio das técnicas de microscopia eletrônica de transmissão e imunocitoquímica com marcação para desmogleína-3. Em um segundo experimento, utilizaremos fragmentos de mucosa bucal removidos durante exodontia de terceiros molares inclusos de pacientes expostos e não expostos aos fatores de risco para o câncer de boca. Analisaremos junções celulares por microscopia eletrônica de transmissão, marcação de desmogleína-3, morfologia histológica por hematoxilina/eosina e permeabilidade celular por imunofluorescência. A partir de tais achados, espera-se elucidar os mecanismos que podem influenciar na permeabilidade do epitélio bucal exposto ao etanol e descrever os aspectos da adesão celular da mucosa bucal exposta a carcinógenos analisadas pela citopatologia. O projeto possui mérito científico, está bem descrito e delineado. Assim, sugerimos aprovação. Após isso, o mesmo deve ser encaminhado à Plataforma Brasil para apreciação ética.

Atenciosamente,
Comissão de Pesquisa.

Porto Alegre, 12 de junho de 2015.

Prof. Dr. Fabrício Mezzomo Collares
Coordenador da
Comissão de Pesquisa ODONTOLOGIA UFRGS