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AVALIAÇÃO DA VELOCIDADE DE PROLIFERAÇÃO CELULAR, CITOMORFOMETRIA E DANO GENÉTICO NO CAMPO DE CANCERIZAÇÃO BUCAL: UM ESTUDO CITOPATOLÓGICO.

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Tese apresentada à Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul como parte dos requisitos para obtenção do título de Doutor em Odontologia, área de concentração em Estomatologia Clínica.

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RESUMO

A citopatologia bucal pode ser aplicada como método de monitoramento em indivíduos expostos a fatores de risco ao câncer de boca. O campo de cancerização, representando as etapas iniciais da carcinogênese bucal, torna-se uma área atrativa ao estudo de biomarcadores que poderão ser utilizados para identificar indivíduos expostos a carcinógenos com maior risco ao desenvolvimento do câncer bucal. O primeiro artigo deste estudo visou comparar as características citopatológicas e histopatológicas da mucosa clinicamente normal adjacente ao carcinoma espinocelular bucal. Além disso, a área nuclear das células obtidas desta região, por meio de esfregaço citológico, foi mensurada e comparada à das células de indivíduos sem lesão expostos ao fumo e/ou álcool e a de pacientes não expostos a estes fatores de risco. Foram incluídos 90 pacientes de ambos os sexos com idade superior a 40 anos. Nos pacientes com carcinoma, além da citologia esfoliativa, foi obtido material para exame histopatológico da área adjacente ao tumor. Nos esfregaços, corados com Papanicolaou, foram mensurados os núcleos de 50 células intermediárias. Tanto os cortes histológicos, quanto os esfregaços citológicos foram classificados em baixo ou alto risco. Ao associar as características citopatológicas e histopatológicas, verificou-se sensibilidade, especificidade e acurácia de 100%, 75% e 75,86%, respectivamente. A média da área nuclear foi menor no grupo não exposto ao fumo e ao álcool, com diferença significativa ($p<0,05$) em relação aos demais. A associação cito-histopatológica da área adjacente ao câncer bucal apresentou boa sensibilidade, especificidade e acurácia. Além disso, constatou-se que a área nuclear é passível de ser utilizada

para detectar alterações celulares precoces na mucosa bucal exposta a carcinógenos, sendo a média percentual de núcleos com mais de 100 μm^2 o método de avaliação mais indicado. O segundo artigo objetivou avaliar o dano genético e a velocidade de proliferação celular no campo de cancerização, ou seja, na mucosa clinicamente normal adjacente ao câncer bucal. Os esfregaços citológicos foram corados com a técnica das AgNORs e reação de Feulgen, utilizando o mesmo raspado citológico obtido no primeiro artigo. A média das AgNORs/núcleo e do micronúcleo (MN) dos grupos fumo/álcool e carcinoma bucal foi superior, com diferença estatisticamente significativa ($p<0,05$) em relação ao grupo controle. Este, por sua vez, obteve média superior de botões nucleares (NBUDs) em relação aos grupos fumo/álcool e carcinoma bucal. Ambos os marcadores, número das AgNORs/núcleo e MN, evidenciaram a fase inicial da carcinogênese bucal, representada no campo de cancerização. Valores iguais ou superiores a 3.38 AgNORs/núcleo e/ou 3 MN/1000 células foram identificados como ponto de corte ideal para incluir um indivíduo exposto a carcinógenos no monitoramento longitudinal. Um modelo prospectivo dos marcadores foi sugerido.

Palavras-chaves: Mucosa Bucal; Citopatologia; Citomorfometria; AgNORs; Micronúcleo; Campo de Cancerização.

ABSTRACT

Oral cytopathology may be used to monitor individuals exposed to risk factor for oral cancer. In this context, the study of field cancerization, a phenomenon involved in the initial stages of oral carcinogenesis, has gained relevance for the establishment of biomarkers that may identify individuals exposed to carcinogens with the greatest risk for developing oral cancer. The first article of this study aimed to compare cytopathological and histopathological characteristics of the clinically normal mucosa adjacent to oral squamous cell carcinoma. The nuclear area of cells obtained by cytological smear of this region was also analyzed and compared to that of individuals without lesions but exposed to smoking and/or alcohol and of patients not exposed to these risk factors. Ninety patients of both sexes over 40 years old were included. In patients with carcinoma, in addition to exfoliative cytology, tissue was obtained from the area adjacent to the tumor for histopathological examination. In smears stained with Papanicolaou, the nuclei of 50 intermediate cells were measured. Both histological sections and cytological smears were classified as low or high-risk. The sensitivity, specificity and accuracy of the cytopathological diagnosis in relation to the histopathological diagnosis, considered the gold standard, were 100, 75 and 75.86%, respectively. The mean nuclear area was significantly lower ($p<0.05$) in the patients not exposed to the risk factors in relation to the others. The cyto-histopathological comparison of the area adjacent to oral cancer showed good sensitivity, specificity and accuracy. In conclusion, this article demonstrated that nuclear area can be used to detect early cellular changes in the oral mucosa exposed to carcinogens and that mean percentage of nuclei larger than 100 μm^2 is the most

indicated method for the assessment of these changes. The second article aimed to assess genetic damage and cell proliferation rate in the field of cancerization, i.e., the clinically normal mucosa adjacent to oral carcinoma. Cytologic smears from the same scrapes used in the first article were stained with silver and with the Feulgen reaction. The mean number of AgNORs/nucleus and micronuclei (MN) was significantly higher ($p<0.05$) in the Tobacco/Alcohol and Oral Cancer Groups than in the Control Group. Conversely, the mean number of NBUDs was higher in the Control Group compared with the other groups. The number of AgNORs/nucleus and MN/1,000 cells provide evidence of initial oral carcinogenesis at field cancerization areas. Cutoff values for inclusion of individuals exposed to carcinogens in longitudinal monitoring were ≥ 3.38 AgNORs/nucleus and/or ≥ 3 MN/1,000 cells. A prospective model including the biomarkers assessed in this study was proposed.

Keywords: Oral Mucosa; Cytopathology; Cytomorphometry; AgNORs; Micronucleus; Field Cancerization.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

4HNE	4- hidroxinonenal
AgNORs	Proteínas Associadas às Regiões Organizadoras Nucleolares
CDK4	Quinase Ciclina-Dependente 4
CDK6	Quinase Ciclina-Dependente 6
COX-2	Cicloxigenase-2
DB[a,l]PDE	dibenzo[a,l]pireno
DNA	Ácido Desoxirribonucleico
EGFR	Receptor do Fator de Crescimento Epidérmico
FASE G1	GAP 1
FASE G2	GAP 2
FASE S	Síntese
HPV	Vírus do Papiloma Humano
LOH	Perda de Heterozigosidade
MN	Micronúcleo
NNK	4-(metilnitrosamina)-1-(3-piridil)-1-butanona
NNN	N´-nitrosonornicotina
NORs	Regiões Organizadoras Nucleolares
NBUD	Botão nuclear
PCNA	Antígeno Nuclear de Proliferação Celular
PCR	Reação em cadeia da polimerase
pRb	Proteína codificada pelo gene Rb (Retinoblastoma)
RNAm	Ácido Riboxirribonucleico Mensageiro

RNS Espécies reativas de nitrogênio

ROS Espécies reativas de oxigênio

SUMÁRIO

1 INTRODUÇÃO

O desenvolvimento das neoplasias é um processo análogo à evolução Darwiniana no qual as células normais são substituídas por tumorais por meio de mutações que lhes conferem vantagem de crescimento. As neoplasias malignas expressam seis importantes características: autossuficiência dos sinais de crescimento, insensibilidade aos sinais anticrescimento, evasão da apoptose, potencial replicativo ilimitado, angiogênese contínua e capacidade de invasão e metástase (1). Determinou-se que o número de mutações necessárias ao surgimento de tumores varia de quatro a 12 (2).

A célula epitelial é regida por vias inibitórias e excitatórias que controlam a diferenciação, divisão e senescência (3). O epitélio bucal sofre ação de agentes externos capazes de ocasionar mutações em genes específicos envolvidos no processo da carcinogênese (4). Um dos agentes mais estudados, pelo seu efeito devastador à saúde, é o fumo, que ocasiona a morte de aproximadamente 6.000.000 de pessoas ao ano (5) e acarreta um elevado custo aos sistemas públicos de saúde (6).

A relação do tabaco com o câncer de boca é bem estabelecida na literatura (7,8). Xue et al. (9) descreveram os mecanismos pelos quais as substâncias presentes no tabaco promovem o surgimento de neoplasias malignas. A nicotina é uma substância não-carcinógena, porém ativa vias de sinalização responsáveis pela promoção, migração, invasão e angiogênese tumorais. A 4-(metilnitrosamina)-1-(3-piridil)-1-butanona (NNK) e a N´-nitrosonornicotina (NNN) são substâncias pró-carcinogênicas ativadas por citocromos em metabólitos reativos de DNA, que induzem à metilacão de nucleossomos e formam os adutos de DNA. Estes adutos

de DNA podem ser reparados por vários mecanismos. Caso isto não ocorra, existe a possibilidade de ocasionarem mutações em oncogenes e genes supressores tumorais. No total, foram identificadas aproximadamente 60 substâncias carcinógenas no tabaco. Por exemplo, o (DB[a,l]PDE), um potente carcinógeno presente nos hidrocarbonetos policíclicos aromáticos encontrados no fumo, foi capaz de induzir tumor em tecidos bucais de ratos, com aumento da expressão da proteína p53 e da enzima cicloxigenase-2 (COX-2). Outro dado interessante foi a capacidade do (DB[a,l]PDE) em alterar a expressão de diversos genes modulados pela p53 e relacionados com o ciclo celular, dano e reparo do DNA e apoptose (10). A proteína p53, frente ao dano do DNA, mantém a célula na fase GAP 1 (G1) do ciclo celular para que seja reparada, ou induz à apoptose (11). A associação da mutação deste gene com a exposição ao fumo e álcool foi avaliada em espécimes de carcinomas espinocelulares bucais por meio da reação em cadeia da polimerase (PCR). Maior porcentagem de mutação no gene p53 foi observada em indivíduos com carcinoma espinocelular expostos ao fumo, associado ou não ao álcool, em relação aos pacientes com câncer bucal não expostos a estes agentes (12).

O fumo pode também exercer seu papel carcinogênico por meio do estresse oxidativo, que se caracteriza pelo desequilíbrio entre os anti-oxidantes e oxidantes, ocasionando, assim, o dano celular. O estresse oxidativo pode agir em todas as etapas da carcinogênese bucal. As elevadas concentrações das espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) em indivíduos fumantes predispõem a danos em determinados genes, podendo ser um dos mecanismos que explicam a maior incidência de câncer bucal nestes pacientes (13).

O consumo de álcool também tem sido fortemente associado ao desenvolvimento do carcinoma espinocelular bucal. Há uma relação positiva e de

dose-resposta entre o uso de etanol ao longo da vida e o surgimento de tumores do trato aerodigestivo superior (14). Mesmo a pouca exposição ao álcool, consumo de até uma dose ao dia - aumentou significativamente o risco de desenvolvimento de tumores de boca e faringe (15). No Brasil, outro estudo epidemiológico reforça a relação do álcool ao constatar um aumento na incidência do carcinoma espinocelular de boca em homens, entre 1983 e 2003, porém com diminuição do câncer de pulmão, neoplasia estritamente relacionada ao tabaco (16).

O etanol exerce seu potencial carcinogênico em relação ao câncer bucal por meio da enzima álcool desidrogenase, presente no epitélio bucal, ou mesmo via determinados micro-organismos, que convertem o etanol em acetaldeído, substância carcinógena e mutagênica. O dano hepático ocasionado pelo álcool dificulta o metabolismo de nitrosaminas, estimulando a carcinogênese em diversos órgãos. O etanol ocasiona ainda imunossupressão, o que predispõe a infecções e determinadas neoplasias. Outros fatores sistêmicos predisponentes ao surgimento do câncer bucal em etilistas são: má nutrição, deficiência de vitaminas e cirrose hepática. O álcool ainda aumenta a permeabilidade do epitélio bucal a outros carcinógenos (17). Tal assertiva foi corroborada em um estudo que objetivou avaliar o efeito sinérgico do fumo e álcool na indução do câncer bucal, mensurando a formação de adutos carcinógenos no DNA em culturas de células epiteliais da mucosa jugal de ratos. O fumo foi o principal agente causador de dano ao DNA e o álcool aumentou a permeabilidade das células epiteliais a carcinógenos presentes no tabaco (18). Similarmente aos pacientes fumantes, nos etilistas também ocorre aumento do estresse oxidativo com maior concentração das ROS (13). Discute-se ainda o efeito citotóxico do álcool, que ocasiona a morte das células epiteliais mais superficiais da mucosa bucal, induzindo a divisão celular das células indiferenciadas

da camada basal. As mitoses sucessivas elevam a chance de dano ao DNA, aumentando o risco de aparecimento do tumor (19).

O efeito sinérgico do fumo e álcool no desenvolvimento do câncer bucal pode ser evidenciado em diversos estudos epidemiológicos realizados em diferentes regiões do Brasil como Paraíba (20), Minas Gerais (21), Paraná (22), Bahia (23) e Rio Grande do Sul (24). Independente da região na qual o estudo foi realizado, o carcinoma espinocelular foi o tumor mais prevalente na cavidade bucal, acometendo com mais frequência pacientes do sexo masculino, a partir da quinta década, tendo a língua e assoalho bucal como os sítios anatômicos mais envolvidos (21-24).

Recentemente, além do fumo e álcool, o papel do Vírus do Papiloma Humano (HPV) foi incluído na lista dos fatores etiológicos do carcinoma bucal. Em meta-análise, que selecionou 11 estudos compreendidos entre 1995 e 2015, totalizaram-se 1497 casos de carcinoma bucal ou de orofaringe e o HPV foi detectado em 39,27% das amostras (25). O HPV, mais especificamente o HPV-16, é um vírus de DNA, que por meio das suas oncoproteínas E7 e E6, ocasiona a perda do controle do ciclo celular nos pontos de checagem, o acúmulo de alterações genéticas e a inibição da apoptose (26).

A má condição do ambiente bucal, apesar de menos relevante, tem sido relacionada ao desenvolvimento do carcinoma espinocelular, principalmente quando associada ao fumo e álcool (27). Uma recente revisão de literatura citou a periodontite como um fator de risco ao surgimento de lesões potencialmente malignas e carcinoma espinocelular bucais (28). A relação da má higiene ao câncer bucal tem sido sugerida em decorrência da conversão, pelas bactérias, do etanol em acetaldeído, conhecido carcinógeno. Essas ainda produzem toxinas, as quais podem interferir na sinalização celular ou ocasionar dano direto ao DNA (29).

O consumo de mate em temperatura elevada também tem sido associado com o câncer de boca e de orofaringe (30). Por outro lado, existem diversos fatores protetores ao desenvolvimento do carcinoma espinocelular explicitados por meta-análises, como o consumo de chá (31), café (32), verduras e frutas (33). O desequilíbrio em prol dos carcinógenos sobre os fatores protetores resulta no surgimento do fenótipo maligno. No processo de carcinogênese bucal, ocorre o envolvimento de determinados genes que foram incluídos num modelo de progressão genética. A perda de heterozigosidade (LOH) em loci de cromossomos relacionados a genes supressores de tumor, 9p.21, 3p.21 e 17p.13 é um evento inicial da carcinogênese bucal. Já a LOH nos loci 11q.13, 13q.21 e 14q.31 é considerada um dano de fase intermediária. Por fim, nos estágios mais avançados da carcinogênese bucal, foi observada LOH nos loci 6p, 8p, 8q e 4q.26-28 (34). O surgimento do câncer bucal não se limita à LOH de genes supressores do tumor, considerando a sua complexidade e os vários estágios envolvidos nesse processo. Pode ocorrer o envolvimento de oncogenes, geralmente superexpressos nos carcinomas espinocelulares bucais, além de fatores relacionados ao mecanismo de imortalização celular, à estimulação da angiogênese, à evasão da apoptose e à facilitação da invasão e metástase (35,36).

Em 2004, Califano et al. (34) propuseram uma aplicação do modelo de progressão genética dos tumores de cabeça e pescoço, objetivando explicar o campo de cancerização, caracterizado como o fenômeno no qual todo o tecido sob a ação de carcinógenos apresenta alterações malignas ou pré-malignas. A sua fase inicial caracteriza-se por alterações genéticas em uma célula-tronco que as transmite para as células-filhas, envolvendo uma pequena área da mucosa (37). Num artigo de revisão mais recente é descrito um outro tipo, a célula-tronco cancerosa,

originada da célula-tronco adulta normal, capaz de auto-renovação e de originar células tumorais com diferentes fenótipos. Sugere-se que o crescimento tumoral seja mantido somente por este tipo celular. Um dos fatores que favorece esta hipótese é a longa vida da célula-tronco, permitindo que ocorram as mutações necessárias à transformação neoplásica (38). Após novas mutações nesta célula-tronco, ocorre lateralmente um aumento desta pequena área, originando um campo. Com o acontecimento de novas mutações, observa-se um processo de expansão e seleção clonais, ou seja, as células-filhas do clone dominante passam a se sobressair sobre as outras células. Com o aumento da instabilidade genética, durante a expansão do campo, ocorre o surgimento de um subclone com a capacidade de invasão e metástase. O campo de cancerização é um fator de risco ao desenvolvimento do carcinoma espinocelular bucal que pode estar presente mesmo na mucosa clinicamente normal adjacente ao tumor, representando uma área atrativa ao estudo de alterações incipientes da carcinogênese (37).

Apesar de todo conhecimento acerca do câncer bucal - fatores etiológicos, características clínicas, fatores genéticos, a maioria dos casos é diagnosticada tardiamente (20-23,39) devido ao caráter assintomático dos tumores em fase inicial (40). Tal constatação torna-se mais preocupante ao analisar os dados do Instituto Nacional do Câncer (INCA), que estimou 15.490 novos casos de câncer de boca entre homens e mulheres, no Brasil, para o ano de 2016. A segunda maior taxa bruta de incidência por 100.000 homens será localizada no Estado do Rio Grande do Sul, com aproximadamente 16,45 casos (41).

1.1 Citopatologia Bucal

A citopatologia bucal baseia-se na análise microscópica de células que descamam do epitélio. Apresenta vantagens que favorecem sua aplicação em âmbito populacional: rápida e fácil execução, indolor ao paciente durante o ato de raspagem e pouco invasiva, tendo um papel auxiliar no diagnóstico de lesões bucais, principalmente as potencialmente malignas e o câncer (42). Epstein et al. (43) avaliaram por meio de meta-análise a efetividade do exame clínico na detecção de displasias epiteliais e carcinomas espinocelulares bucais. Foram selecionados 24 estudos, com uma amostra de 1.956 casos. O exame clínico teve boa sensibilidade (0,93), porém baixa especificidade (0,31), indicando que não é capaz de predizer o diagnóstico histopatológico.

A citopatologia bucal foi avaliada, através de uma meta-análise, para a detecção do câncer e lesões potencialmente malignas bucais, tendo a histopatologia como padrão-ouro. Em 11 estudos, com uma amostra de 4.060 lesões, a sensibilidade variou de 78% a 100% e a especificidade variou de 28% a 100%, atestando seu uso no diagnóstico destas lesões. Em dois estudos que utilizaram a citologia em meio líquido, houve uma melhora destes parâmetros: a sensibilidade variou de 88,8% a 95,1%, enquanto a especificidade variou de 99% a 100% (44).

A técnica de obtenção do esfregaço citológico bucal vem sendo aprimorada com a utilização do citobrush em citologia de meio líquido, que demonstrou uma baixa percentagem de esfregaços inadequados para análise (45), além de maior especificidade e sensibilidade em comparação à citologia convencional (46). Com relação aos diferentes instrumentos de coleta para a citologia em meio líquido tais como citobrush, cureta dermatológica ou citobrush circular, foi constatado que não houve diferença quanto à celularidade, dispersão e tipos celulares (47). No entanto, o uso da citologia em meio líquido não garante a obtenção de células parabasais ou

basais que certificam a abrangência de todas as camadas do epitélio bucal (45,47). A dificuldade de obtenção de uma adequada amostra de células é um dos fatores discutidos como causa da baixa aplicabilidade da citologia bucal (48).

Outra importante alteração da citologia bucal, baseada no Sistema de Bethesda (utilizado na citologia cervical) (49), foi a adoção de uma nova forma de classificação das lesões bucais, que inclui um critério para considerar um esfregaço adequado para análise. Um círculo de 5 mm de diâmetro realizado na lâmina de vidro deve estar coberto por células em mais de 30% da sua área (50). Outro estudo considera os mesmos critérios adotados pelo Sistema de Bethesda para classificar um esfregaço citológico bucal como adequado: no mínimo 5.000 células na lâmina de vidro, baseando-se na contagem de dez campos descontínuos, em aumento de 40X, os quais devem ter no mínimo sete células por campo (45). Por outro lado, Navone et al. (48) classificam como satisfatório um esfregaço com apenas 30 células do tipo intermediária, sem a interferência de sangue ou infiltrado inflamatório.

Uma breve análise da citopatologia para detecção de neoplasias de colo uterino demonstrou que, independente do método de coleta utilizado, observaram-se casos insatisfatórios para análise (51). Outro estudo revisou os esfregaços negativos para malignidade em mulheres que, posteriormente, vieram a desenvolver câncer de colo de útero. As causas destes diagnósticos falso-negativos foram a falta de habilidade no momento da obtenção da amostra, o desconhecimento de alterações celulares importantes ou mesmo o desgaste físico do examinador (52). Mesmo assim, os métodos preventivos, dentre eles a citopatologia, contribuem significativamente na redução da incidência e mortalidade pelo câncer de colo uterino (53). O Sistema de Bethesda está em constante revisão frente a novas evidências científicas com relação às neoplasias do colo uterino, reforçando a

citopatologia como método de rastreamento e auxiliar no diagnóstico (54).

A citopatologia bucal, assim como a do colo uterino (51,52), apresenta desvantagens tais como dificuldade na obtenção de uma amostra adequada (48) e ocorrência de casos falso-negativos, principalmente em tumores com menos de dois centímetros (55). Outro ponto desfavorável é a falta de conhecimento entre os cirurgiões-dentistas sobre os aspectos básicos da técnica (56).

A citopatologia bucal deve ser aplicada como um método de rastreamento do câncer em âmbito populacional, direcionando seu uso em pacientes de risco - fumantes e etilistas, acima de 40 anos, e em sítios anatômicos mais suscetíveis ao carcinoma bucal (borda de língua, assoalho bucal, palato mole e região retromolar), com o intuito de aumentar sua efetividade (42,57,58). Baseado neste raciocínio, estudos citopatológicos com a técnica de Papanicolaou buscaram detectar alterações na mucosa bucal clinicamente normal de pacientes expostos ao fumo e álcool. Evidências de displasias epiteliais foram observadas em poucos casos (59-62) e, quando presentes, alterações celulares leves foram detectadas (63-65).

1.2.1 Coloração de Papanicolaou e Citomorfometria

As primeiras modificações na célula epitelial ocorrem em nível molecular, desencadeando reações que alteram, por fim, a sua morfologia (66). Na citomorfometria, realizada em esfregaços citológicos, uma evolução da técnica tem sido obtida tal como a utilização de um sistema semiautomático de análise de imagem (67). Outro ponto positivo é a possibilidade da utilização de diferentes meios fixadores sem influenciar a mensuração do núcleo e citoplasma (68).

Estudos utilizando a coloração de Papanicolaou têm investigado possíveis

alterações celulares prévias ao aparecimento de lesões potencialmente malignas ou carcinomas bucais. Um aumento da área nuclear das células sob ação do fumo tem sido detectado (69-71). Khandelwal e Solomon (66) investigaram as características citomorfométricas das células epiteliais obtidas da mucosa de indivíduos não fumantes/não etilistas, pacientes fumantes, bem como da mucosa de indivíduos com carcinoma bucal. Em esfregaços corados por meio de Feulgen e Papanicolaou, não houve diferença na área nuclear de fumantes e da mucosa clinicamente normal de indivíduos com carcinoma espinocelular. Outro estudo, além de corroborar o aumento da área nuclear sob o efeito do tabaco, menciona que este parâmetro foi proporcional ao tempo de uso e quantidade de cigarros consumidos (72). Evidenciando a importância do tempo de duração do tabagismo, não foi detectada diferença da área nuclear entre fumantes e não-fumantes com menos de 35 anos de idade (72,73).

O efeito do álcool sobre os parâmetros citomorfométricos tem sido pouco explorado. Ogden et al. (74) constataram, por meio de exame citopatológico, na mucosa jugal clinicamente normal, uma redução da área citoplasmática no grupo exposto ao álcool comparado ao controle. Discute-se que uma das causas da diminuição deste parâmetro tenha sido a deficiência nutricional dos pacientes etilistas. Em outros estudos, a redução da área citoplasmática foi o único parâmetro observado na mucosa jugal clinicamente normal de pacientes com câncer bucal em relação a indivíduos sem a neoplasia (75,76). A análise das alterações morfológicas, em citopatologia bucal, constatou o aumento da área nuclear e a redução da área citoplasmática como acontecimentos frequentes, sendo mais evidentes quanto maior o grau de displasia epitelial (67,77-79).

No entanto, as alterações citomorfométricas da mucosa bucal devem ser

interpretadas com cautela, considerando que fatores sistêmicos como a idade do paciente (80,81), diabetes tipo I ou II (82), anemia por deficiência de ferro (83), transplante renal (84) ou neoplasia em órgãos distantes da cavidade bucal (85) podem interferir nas suas mensurações. Inclusive fatores locais como candidíase bucal (86), localização anatômica na qual o esfregaço citológico é realizado (80) e síndrome de ardência bucal (87) influenciam a área nuclear e/ou citoplasmática.

1.2.2 Técnica das AgNORs e Proliferação Celular

A análise da proliferação celular também confere informações relevantes à carcinogênese bucal. Na fase de iniciação, a proliferação celular exerce função ao transmitir o dano inicial às células - filhas. No entanto, sua ação é mais evidente nas fases de promoção e progressão do tumor, com a expansão clonal das células que apresentam vantagens genéticas sobre as demais. O aumento da velocidade de proliferação celular, reduzindo o tempo para o processo de reparo do DNA, contribui para os múltiplos eventos genéticos da carcinogênese (88). As células em divisão celular são mais suscetíveis ao dano, pois a fita simples do DNA (estado presente nas células em divisão) é mais propensa às mutações do que a fita dupla. A célula em divisão permite recombinações mitóticas como a não disjunção ou conversão gênica, que resultam em alterações mais profundas do que a mutação única (89). A ativação de oncogenes por mutação, translocação ou amplificação, bem como a fixação da perda de heterozigosidade (LOH) de genes supressores do tumor ocorrem com a divisão celular (90).

As Regiões Organizadoras Nucleolares (NORs) são segmentos cromossomais que contém os genes ribossomais. Nas NORs estão presentes

proteínas não histônicas, que têm afinidade pelos íons de prata e são passíveis de serem visualizadas em amostras citológicas ou histológicas. Estas proteínas são denominadas de AgNORs e apresentam-se como pontos pretos dentro do núcleo (91). A sua quantificação é um marcador de velocidade de proliferação celular (92), considerando que as principais proteínas, nucleolina e B23, apresentam-se em maior quantidade na fase S-G2 e em menor na fase G1 do ciclo celular (93). Em espécimes de carcinomas espinocelulares bucais, o número das AgNORs/núcleo foi superior quando houve marcação positiva, tanto para o PCNA (antígeno nuclear de proliferação celular), quanto para a Ki-67 (proteína ki-67), reforçando seu papel como marcador de proliferação celular (94).

Avaliando a proliferação celular por meio da técnica das AgNORs foi possível discernir carcinomas da mucosa bucal normal (95), bem como as lesões benignas das potencialmente malignas e dos tumores bucais. Foram estipulados um valores ≥ a 4,8 AgNORs/núcleo ou mais de 70% das células do raspado com mais de 3 AgNORs/núcleo como indicativos de malignidade (96). Em outro estudo, a quantificação das AgNORs foi um método adjunto ao de Papanicolaou no diagnóstico de carcinomas bucais, estipulando uma média de 4 AgNORs/núcleo como sugestiva de alteração neoplásica (97).

Por meio da quantificação das AgNORs, foi possível observar o aumento da proliferação celular, mesmo antes da presença de alterações displásicas, em cortes histológicos de tecido da área adjacente ao carcinoma espinocelular, caracterizando-o como um marcador capaz de detectar modificações celulares incipientes (98). Diferente das lesões potencialmente malignas e carcinomas bucais, no epitélio normal as AgNORs apresentam-se como pontos bem definidos e em baixo número, reduzindo a subjetividade do método de contagem (99).

Ao comparar o número das AgNORs na mucosa bucal normal sob à ação do fumo, associado ou não ao álcool, foi observado aumento da atividade de proliferação celular em relação aos indivíduos não expostos a estes fatores (62,99-106). A capacidade de monitoração deste marcador em pacientes fumantes e etilistas foi confirmada por meio de estudos longitudinais (107,108).

1.2.3 Reação de Feulgen e Dano Genético

A quantificação do micronúcleo (MN) é um biomarcador para detectar dano genético (109). O MN é expresso em células em divisão que contêm cromossomos ou quebras de cromossomos acêntricos que não atingiram os polos do fuso durante a mitose. Forma-se um envelope nuclear ao redor deste fragmento cromossomal, que adquire a morfologia de um núcleo em interfase, porém menor (110). A avaliação deste biomarcador é um método simples, não invasivo e confiável para rastreamento populacional de indivíduos expostos a carcinógenos (111,112). Bonassi et al. (113), em um estudo de revisão, ao comparar diferentes métodos de coleta, constataram que a frequência de MN foi menor com o uso do citobrush. Estudos que utilizaram colorações não DNA específicas mostraram maior frequência de MN em relação à coloração DNA específica. Tanto o número de células avaliadas, como o critério para definir MN não influenciaram a sua frequência. Nersesyan et al. (114) corroboram a maior frequência de MN com colorações não DNA específicas. No entanto, Ceppi et al.(115) sugerem, para diminuir o intervalo de confiança, a contagem de 4000 células por lâmina.

Indivíduos que fumavam mais do que 40 cigarros/dia tiveram um aumento significativo da frequência de MN, que não foi influenciado pelo consumo diário de

álcool (113). Stich e Rosin (116) e Bohrer et al.(117), por sua vez, observaram maior frequência de MN somente com o sinergismo do fumo e álcool em relação ao grupo controle. A quantificação da frequência de MN também evidenciou o campo de cancerização (118-121).

Diferente do MN, o botão nuclear (NBUD), uma alteração metanuclear, tem significado e origem incertos (122). Thomas et al. (123) o caracterizaram como uma alteração com a mesma coloração do núcleo, que deve estar conectada ao núcleo principal, podendo variar da metade a um quarto do seu tamanho. O NBUD pode estar relacionado com genotoxicidade (124), com um processo degenerativo (125) ou ainda representar um processo adaptativo aos agentes clastogênicos (117). Bonassi et al.(113) não observaram associação dos NBUDs com o fumo.

A cariorrexe é uma alteração metanuclear relacionada à apoptose, sendo uma forma de eliminar células com dano genético (122), o que explicaria seu número aumentado em indivíduos expostos a carcinógenos (117,124). Outros estudos, no entanto, observaram maior frequência da cariorrexe em indivíduos não expostos ao fumo ou álcool (118,119).

O câncer bucal continua a ser diagnosticado em estágio avançado (20-23). Justifica-se, assim, a necessidade de métodos de detecção precoce passíveis de serem aplicados como rastreamento populacional, como a citopatologia (42) associada a técnicas sensíveis e de baixo custo (50,91,111). O campo de cancerização, representando as etapas iniciais da carcinogênese bucal (37), torna-se uma área atrativa ao estudo de biomarcadores que poderão ser utilizados para identificar indivíduos expostos a carcinógenos com maior risco ao desenvolvimento do câncer bucal (120). A citomorfometria e a frequência de MN já foram estudadas na área adjacente ao tumor, no entanto há uma grande variabilidade entre os

valores obtidos em diferentes estudos (66,75,121,119). A quantificação das AgNORs, por sua vez, foi realizada na região próxima ao carcinoma, mas somente em cortes histológicos (98).

2 PROPOSIÇÃO

2.1 Objetivo Geral

Avaliar, em células descamadas da mucosa bucal clinicamente normal, adjacente a carcinomas espinocelulares, alterações morfológicas, atividade de proliferação celular e dano genético, propondo a aplicabilidade desta análise na identificação e monitoramento de indivíduos expostos a carcinógenos.

2.2 Objetivos Específicos

- Determinar a área nuclear em esfregaços, corados pela técnica de Papanicolaou, da mucosa bucal do campo de cancerização. Estas características foram comparadas com as da mucosa de pacientes sem lesão, expostos ou não cronicamente ao fumo e álcool.

- Determinar a velocidade de proliferação celular por meio das AgNORs/núcleo da mucosa bucal do campo de cancerização. Esta variável foi comparada com a da mucosa de pacientes sem lesão, expostos ou não cronicamente ao fumo e álcool

- Determinar a frequência do número dos MN e outras alterações metanucleares da mucosa bucal do campo de cancerização, comparando-as com a da mucosa de pacientes sem lesão, expostos ou não cronicamente ao fumo e álcool, pela reação de Feulgen.

- Correlacionar os aspectos citopatológicos e histopatológicos da mucosa bucal do campo de cancerização.

- Determinar os pontos de cortes ideais dos métodos selecionados para identificar os indivíduos expostos a carcinógenos, sem lesão bucal, mais suscetíveis ao desenvolvimento do carcinoma espinocelular.

- Sugerir um modelo para o controle citopatológico longitudinal de indivíduos expostos a carcinógenos sem lesão bucal, segundo os pontos de cortes ideais estipulados.

3 ARTIGO DE PESQUISA 1

NUCLEAR AREA AND COMPARISON OF CYTO-HISTOPATHOLOGICAL CHARACTERISTICS OF ORAL FIELD CANCERIZATION

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Nuclear area and comparison of cyto-histopathological characteristics of oral field cancerization

Short title: Cytology of oral field cancerization

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ABSTRACT

This study aimed to compare the cytopathological and histopathological characteristics of the clinically normal mucosa adjacent to oral squamous cell carcinoma i.e., the field of cancerization. The nuclear area of cells obtained by cytological smear of this region was also analyzed and compared to that of individuals without lesions but exposed to smoking and/or alcohol and of patients not exposed to these risk factors. Ninety patients of both sexes over 40 years old were included. In patients with carcinoma, in addition to exfoliative cytology, tissue was obtained from the area adjacent to the tumor for histopathological examination. In smears stained with Papanicolaou, the nuclei of 50 intermediate cells were measured. Both histological sections and cytological smears were classified as low or high-risk. High-risk cellular changes were observed in eight cytological smears in the clinically normal mucosa adjacent to squamous cell carcinoma. The mean nuclear area was significantly lower ($p<0.05$) in the patients not exposed to the risk factors in relation to the others. The cytopathological-histopathological comparison of the area adjacent to oral cancer showed good sensitivity, specificity and accuracy. The nuclear area can be used to detect early cellular changes in the oral mucosa exposed to carcinogens. A model for its applicability for identification and monitoring of individuals at risk for the development of oral cancer was proposed. Prospective studies are suggested to validate the use of this model.

Key Words: papanicolaou smear, cell nucleus size, oral mucosa, field cancerization.

INTRODUCTION

Oral squamous cell carcinoma continues to be diagnosed at an advanced stage (1). In the majority of cases, this neoplasm occurs in individuals older than 50 years old, mainly on the side of the tongue and floor of the mouth (2). Delayed diagnosis of oral cancer worsens the patient's prognosis and survival (3), resulting in significant economic costs to the public health system, with long hospitalizations, radiotherapy and chemotherapy (4).

Clinical examination, when compared to histopathology, has good sensitivity but low specificity in the detection of potentially malignant lesions and oral cancer (5). Oral cytopathological examination, on the other hand, is an auxiliar method that can be applied at the population level in the diagnosis of these lesions, considering its advantages of being rapid and easy to perform and non-invasive and painless (6). By means of meta-analysis, a sensitivity of 80.2% and specificity of 96.7% were found for this technique using Papanicolaou staining, comparable to histopathological examination, in the detection of cancer and potentially malignant oral lesions (7). However, in the literature, there are no studies that compare cytopathological with histopathological examination in clinically normal oral mucosa, probably due to ethical reasons.

Cytopathology can be used in clinically normal oral mucosa as a cancer screening method in individuals at risk, such as those who smoke and drink alcohol and those over 40 years old, and in more susceptible anatomical sites (6). However, in cytopathological studies of clinically normal oral mucosa exposed to carcinogens, only slight cellular changes have been detected (8,9). Cytomorphometry using Papanicolaou staining demonstrated an increase in nuclear area of oral mucosa cells

in smokers compared to controls, suggesting a possible cellular change prior to the appearance of potentially malignant lesions (10,11). More sophisticated and sensitive techniques have been explored in oral cytopathology in individuals exposed to carcinogens (12), but their high cost limits their application at the population level, especially in underdeveloped or developing countries (13).

Using the field of cancerization, i.e., the clinically normal oral mucosa adjacent to the tumor, which is more susceptible to the occurrence of a second neoplasm, it is possible to evaluate early cellular changes in carcinogenesis even before the occurrence of potentially malignant lesions (14). Thus, this study aimed to compare the cytopathological and histopathological findings of the clinically normal mucosa adjacent to the oral squamous cell carcinoma. The nuclear area of cells obtained by cytological smear of the field of cancerization was analyzed and compared to that of individuals without lesions but exposed to smoking and/or alcohol and of patients not exposed to these risk factors. In addition, we suggested a model for its application in the screening and monitoring of individuals at risk for oral cancer.

MATERIALS AND METHODS

The present study was approved by the Ethics in Research Committee of our institution (Protocol 38019514.0.0000.5336). Each of the participants in the study signed an informed consent form. The sample comprised 90 male and female subjects, over 40 years old, who had been seen in the Oral Medicine Division of the Hospital, Specialized Dental Clinic and in a Health Care Unit. Patients with anemia

and/or diabetes were excluded. The patients were allocated into the three groups described below.

- Control Group: 30 patients without clinically visible oral lesions, who had no history of smoking and drank on average less than one alcoholic drink per day.
- Tobacco/Alcohol Group: 30 patients without clinically visible oral lesions, who smoked at least 20 filtered cigarettes per day for at least one year or more than 10 filtered cigarettes per day for more than 10 years. Alcohol consumption could be associated with smoking, and if so, it was quantified as drinks/day, regardless of the type of drink.
- Oral Cancer Group: 30 patients with oral squamous cell carcinoma. Vermillion lips lesions were excluded.

For each patient selected in the Oral Cancer Group, a person of the same sex and of approximate age was screened for the other groups. The anatomical sites subjected to cytological smears in Oral Cancer Group patients were the same areas scraped in individuals of the Control and Tobacco/Alcohol Groups, since one of the parameters evaluated, the nuclear area, can vary between keratinized and non-keratinized mucosa (15). Individual personal data, information about life style, diet, presence of systemic diseases and chronic use of drugs were registered on a clinical record. For patients in the Oral Cancer Group, the clinical features of squamous cell carcinoma were recorded.

Smears and Cytopathological Analysis

After anamnesis, patients with total or partial dentures were instructed to remove them and to rinse their mouth with saline. Sample collection was initiated with subjects from the Oral Cancer Group. Prior to biopsy, a cytological smear was

taken from the clinically normal mucosa adjacent to the lesion. A Velscope® Vx (LED Dental, Canada) was used to determine the site to be scraped. This device emits fluorescent light and indicates the presence of epithelial changes, which appear as a darkened area around the lesion. In cases where the device showed only the lesion, the smear was obtained approximately 10 mm from the tumor border. The cytological smear was performed with a cytobrush, using 10 to 15 spins to obtain cells from the deeper layers of the oral epithelium. The cytobrush was inserted in the container of the Liqui-PREP® system (Sirius, Brazil), and the samples were processed according to the manufacturer 's protocol.

In Control and Tobacco/Alcohol Groups a cytological smear was obtained following the same collection technique previously described. In these groups, the Velscope® Vx was not used, since the selection of the collection site depended on the sites evaluated in the Oral Cancer Group.

Cytological smears underwent Papanicolaou staining and classified according to Afrogheh et al. (13), by a single blinded examiner. To decrease the subjectivity of cytopathology, normal, atypical-probably reactive/low-grade and low-grade intraepithelial lesions were considered low-risk, while atypical-probably high-grade and high-grade lesions were grouped as high-risk. The slides were examined with a light microscope (model MB51OPT, Precision, China). The entire smear was examined at 100X magnification and areas suspected of showing epithelial atypia were analyzed at 400X. In the fields where there were evident cellular changes, a new analysis was performed together with an experienced cytopathologist to confirm the diagnosis.

In addition to the qualitative evaluation, the nuclear area of the first 50 non-overlapping cells obtained from the spinous and parabasal layers of the epithelium

was measured. (Figure 1F) The images were captured with a light microscope (Precision, model MB51OPT) coupled to a Moticam 580 5.0 MP camera (Motic-Hong Kong, China). The images were captured at 400X and saved in TIFF format. Afterwards, they were evaluated in the image processing and analysis software Image J. The cell nucleus was outlined using the freehand selection tool or oval/elliptical/brush selections and the area obtained in μm^2. With the use of a calibration ruler, 1 µm = 5.55 pixels was considered. The percentage of cells with a nuclear area greater than 90 and 100 μm^2 was calculated. A comparison of the mean nuclear area between the keratinized and non-keratinized mucosa was also performed

Biopsies and Histological Analysis

In the area adjacent to the tumor (Oral Cancer Group) where a cytological smear was previously performed, an elliptical fragment of 5 mm in diameter was removed after anesthetic infiltration (2% lidocaine with epinephrine 1:100,000). Following the same surgical steps, an incisional biopsy of the lesion was performed for diagnostic purpose. Both fragments, duly identified, were immersed in 10% formalin, subjected to routine histological processing and stained with hematoxylin and eosin (H&E).

Histopathological analysis of the normal mucosa adjacent to the tumor was performed by a previously calibrated examiner (kappa=0.72) according to the criteria of Warnakulasuriya et al. (16). The samples were classified as low-risk (no dysplasia and mild dysplasia) or high-risk (moderate or severe dysplasia) to reduce subjectivity. After histopathological classification of the fragments obtained from the area adjacent

to the lesion, a second experienced pathologist confirmed the analyses before the final diagnosis.

Data Analysis

For the cytopathological and histopathological comparison of the samples obtained in the clinically normal mucosa adjacent to the tumor, sensitivity, specificity and accuracy were determined considering histopathological examination as the gold standard. ANOVA followed by Tukey's post-hoc test was used to compare the mean nuclear area and the mean age of the groups. To compare the percentage of nuclei with more than 90 and 100 μm^2, a Kruskal Wallis followed by Nemenyi post hoc test was used. Student's t-test was used to compare the mean nuclear area of the keratinized and non-keratinized mucosa. The Mann-Whitney test was used for comparison of the Tobacco/Alcohol and Oral Cancer Groups regarding the mean number of cigarettes smoked and alcoholic drinks consumed per day, and of keratinized and non-keratinized mucosa regarding the percentage of nuclear areas larger than 100 μm^2. Binomial logistic regression was used to determine the most appropriate parameter to distinguish healthy mucosa from that likely to develop oral cancer, represented by Control and Oral Cancer Groups respectively, using the equation $log\{\pi(x)/1-\pi(x)\}=\beta 0+\beta 1x1+...+\beta pxp$. Models were selected in a stepwise fashion. After estimating the model, odds ratio (OR) was calculated using exponential coefficients. The results of the logistic model were shown in graphs describing the association between the parameter of interest and the likelihood of developing oral cancer. Diagnostic performance was assessed using receiver operating characteristic (ROC) curves. Optimal cutoff values were determined by the Youden index. The simple linear regression model was used to evaluate the association between life

style and dietary factors (smoking, consumption of alcohol, mate tea, coffee, vegetables, and fruits) and mean percentage of nuclei larger than 100 μm^2. Variables were selected in a stepwise fashion, with the removal of leverage points in Cook's distance graphs. Analyses were performed in the R 3.4.1 environment and $p<0.05$ was considered significant.

RESULTS

Characterization of Sample

Each group was composed of 21 males (70%) and 9 females (30%). The mean age (±SD) of the Control, Tobacco/Alcohol and Oral Cancer Groups was 61.3 ± 8.99, 58.86 ±9.84 and 64.33 ± 9.70 years, respectively, showing no significant difference ($p=0.09$). The mean daily cigarette consumption (±SD) in the Tobacco/Alcohol Group and Oral Cancer Group was respectively 24.16 ± 11.45 and 20.75 ± 14.49, with no significant difference ($p=0.24$) between the two groups. The mean alcohol intake in the Tobacco/Alcohol Group and Oral Cancer Group was 0.9/day ± 1.66 and 3.2/day ± 3.9, respectively, showing a statistically significant difference ($p<0.05$). Simple linear regression analysis showed that mean daily cigarette consumption was positively associated with increased mean percentage of nuclei greater than 100 μm^2. About 70% of the individuals in the three groups used chronic medication, with antihypertensive drugs being the most used. The characteristics of the sample and the anatomical locations of tumors and areas subjected to cytological smears are shown in Table 1.

Cytopathological and Histopathological Analysis

The distribution of cytopathological grades according to the classification of Afrogheh et al. (13) for the 3 groups is given in Table 2, and the morphological criteria are shown in Figure 1A-E. The sensitivity, specificity and accuracy of the cytopathological diagnosis in relation to the histopathological diagnosis, considered the gold standard, were 100, 75 and 75.86%, respectively (Table 3).

The mean nuclear area and the mean percentage of nuclei larger than 90 and 100 μm^2 were significantly higher in the Tobacco/Alcohol and Oral Cancer Groups than in the Control Group ($p<0.05$) (Table 4). Thirty-nine scrapes were performed on the keratinized mucosa and 51 scrapes on the non-keratinized mucosa. There was a statistically significant difference ($p<0.05$) between these two types of mucosa with regard to mean nuclear area. Means (±SD) of the non-keratinized and keratinized mucosa were 87.10±9.33 μm^2 and 83.88±12.26 μm^2, respectively. However, no statistically significant difference ($p=0.24$) between these two types of mucosa was observed regarding mean percentage of nuclei larger than 100 μm^2. Mean (±SD) percentage of nuclei larger than 100 μm^2 was 8.79%±9.58% in keratinized mucosa and 10.56%±9.16% in non-keratinized mucosa.

Logistic regression analysis revealed that mean percentage of nuclei larger than 100 μm^2 was the most appropriate parameter to distinguish a healthy mucosa from that likely to develop oral cancer, represented by Control and Oral Cancer Groups, respectively. Analysis of ROC curves revealed a good diagnostic performance, with an area under the curve (AUC) of 0.78 (minimum 0.665; maximum 0.895). Optimal cutoff point as determined by the Youden index was 8%, with a sensitivity of 0.866 and a specificity of 0.56 (Figure 2). OR was 1.079 and 95% confidence interval (95%CI) was 1.03 to 1.13. For longitudinal monitoring, results for the logistic models

were shown in a graph that described the association between the parameter considered the most appropriate for the analysis, i.e., mean percentage of nuclei larger than 100 μm^2, and likelihood of developing oral cancer (Figure 3).

Table 1. Characteristics of the sample regarding sex, age, anatomical site of oral cancer and area subjected to cytological smear.

Case	Sex	Age/years			Location	
		Control Group	Tobacco/Alcohol Group	Oral Cancer Group	Oral Cancer	Scrapes
1	M	69	59	58	Left lateral border of tongue	Left lateral border of tongue
2	M	53	52	58	Right ventral surface of tongue	Right ventral surface of tongue
3	F	68	65	72	Left floor of mouth	Left floor of mouth
4	F	79	72	78	Right soft palate	Right buccal mucosa
5	M	52	54	61	Right soft palate	Right buccal mucosa
6	M	71	50	62	Left retromolar trigone	Left buccal mucosa
7	M	75	83	86	Right lateral border of tongue	Right lateral border of tongue
8	M	60	66	64	Right buccal mucosa	Right hard palate
9	M	50	47	47	Right floor of mouth	Right lateral border of tongue
10	M	65	63	76	Right retromolar trigone	Right buccal mucosa
11	M	53	41	56	Left soft palate	Left hard palate
12	F	59	40	57	Right soft palate	Right hard palate
13	M	73	66	73	Left ventral surface of tongue	Left ventral surface of tongue
14	M	57	57	61	Left retromolar trigone	Left buccal mucosa
15	M	54	56	63	Left lateral border of tongue	Left lateral border of tongue
16	F	51	57	56	Lower alveolar ridge	Inner surface of lower lip
17	M	68	69	70	Right buccal mucosa	Right buccal mucosa
18	F	71	75	70	Right floor of mouth	Right lower buccal vestibule
19	M	51	56	65	Right lateral border of tongue	Right lateral border of tongue
20	F	61	64	75	Right lateral border of tongue	Right lateral border of tongue
21	F	70	61	58	Upper alveolar ridge	Right upper buccal vestibule
22	M	62	48	62	Right floor of mouth	Right ventral surface of tongue
23	M	54	55	50	Right floor of mouth	Right lateral border of tongue
24	M	62	64	59	Right soft palate	Right buccal mucosa
25	M	39	47	49	Left retromolar trigone	Left buccal mucosa
26	F	63	61	81	Right lateral border of tongue	Right lateral border of tongue
27	M	55	51	59	Right floor of mouth	Right ventral surface of tongue
28	M	63	58	59	Right retromolar trigone	Right buccal mucosa
29	F	66	71	77	Right lateral border of tongue	Right lateral border of tongue
30	M	66	58	68	Left lateral border of tongue	Left lateral border of tongue

Figure 1. Morphological criteria according to Afrogheh et al. (13). A) Normal Smear: Superficial with pycnotic nucleus and intermediate cells. B) Atypical-probably reactive/low-grade: Binucleated cell with perinuclear halo, increased nucleus/cytoplasm ratio and normochromatic nucleus. C) Low grade intraepithelial smear: *Cell with increased nucleus/cytoplasm ratio, slight nuclear hypercromasia and irregularity of nuclear membrane. D) Atypical - probably high - grade: *Cell with little cytoplasm, intensely hyperchromatic nucleus and irregularity of nuclear membrane. E) High - grade squamous intraepithelial lesion: Cell with nuclear hyperchromasia, increased nucleus/cytoplasm ratio and irregularity of nuclear membrane. F) Intermediate cells.

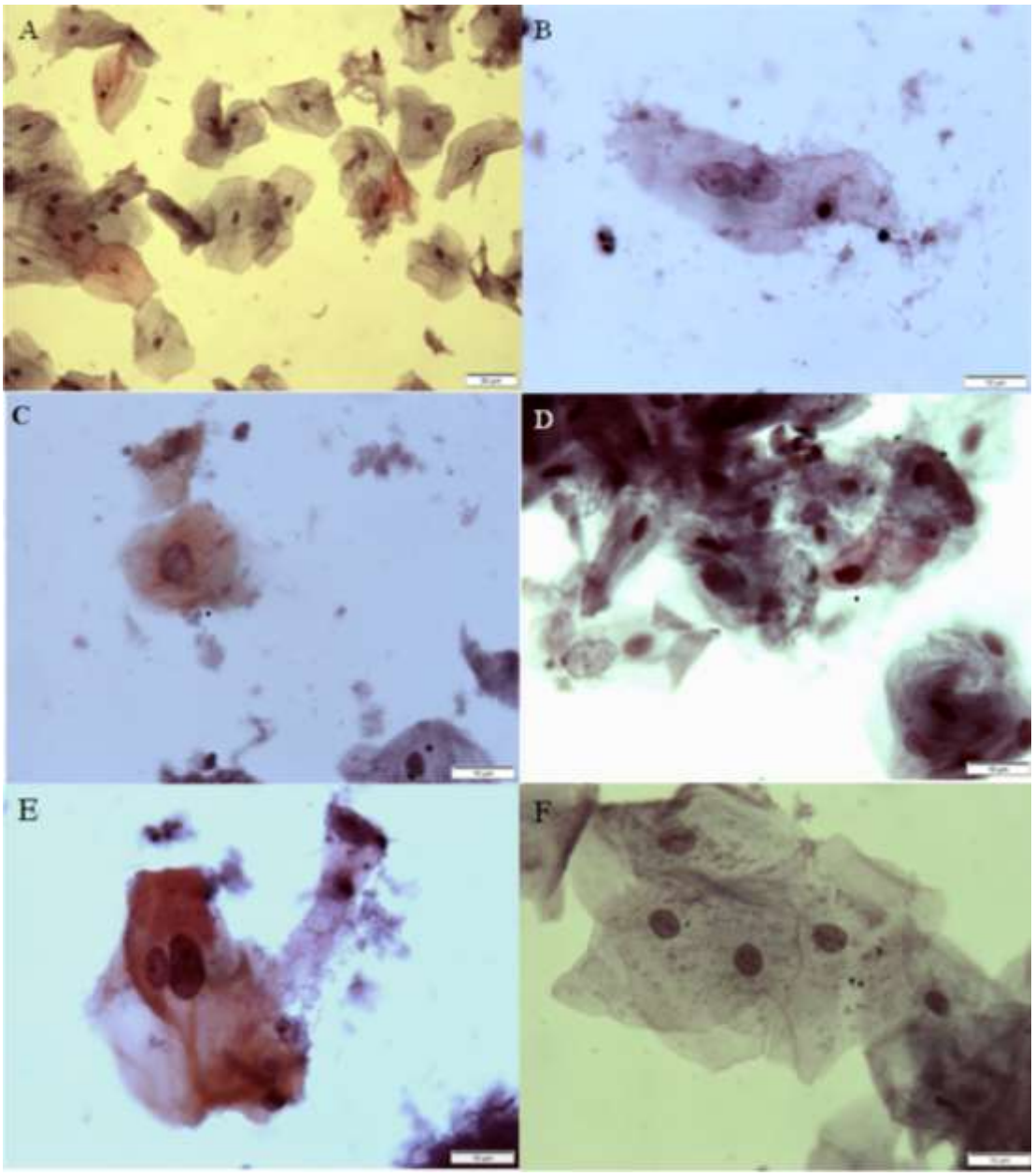


Table 2. Distribution of cytopathological grades in different groups.

Cytopathological grades	Control	Tobacco/alcohol	Oral Cancer	Total
Inadequate	-	-	1	1
Normal	29	24	9	62
Atypical-RL	1	6	9	16
LGSIL	-	-	3	3
Atypical-H	-	-	4	4
HGSIL	-	-	4	4
Total	30	30	30	90

Atypical-RL, Atypical probably Reactive/Low Grade; LGSIL, Low-Grade Squamous Intraepitelial Lesion; Atypical-H, Atypical Probably High Grade; HGSIL,High-Grade Squamous Intraepitelial Lesion.

Table 3. Comparison of cytopathological and histopatological grades of area adjacent to squamous cell carcinoma.

Cytopathology	Histopathology		
	Low Risk	High Risk	Total
Low Risk	21	0	21
High Risk	7	1	8
Total	28	1	29

Table 4. Mean nuclear area μm^2 (±SD) and mean percentage of nuclei greater than 90 and 100 μm^2 (±SD) in Control, Tobacco/Alcohol and Oral Cancer Groups.

	Control	Tobacco/alcohol	Oral cancer
#Nuclear Area μm^2	80.26±9.28 [a]	89.05±8.96 [b]	91.39±11.44 [b]
*> 90μm^2	23.46±18.68 [a]	44.66±25.24 [b]	47.53±27.01 [b]
*> 100μm^2	9.4±9.85 [a]	21.4±18.16 [b]	28±21.44 [b]

Different letters in rows indicate statistically significant difference. (#One-Way Anova, Tukey test / * Kruskal - Wallis, Nemenyi test - $p<0.05$).

Figure 2. Receiver operating characteristic curve for evaluation of the diagnostic performance with mean percentage of nuclei larger than 100 μm^2 and optimal cutoff point as determined by the Youden index.

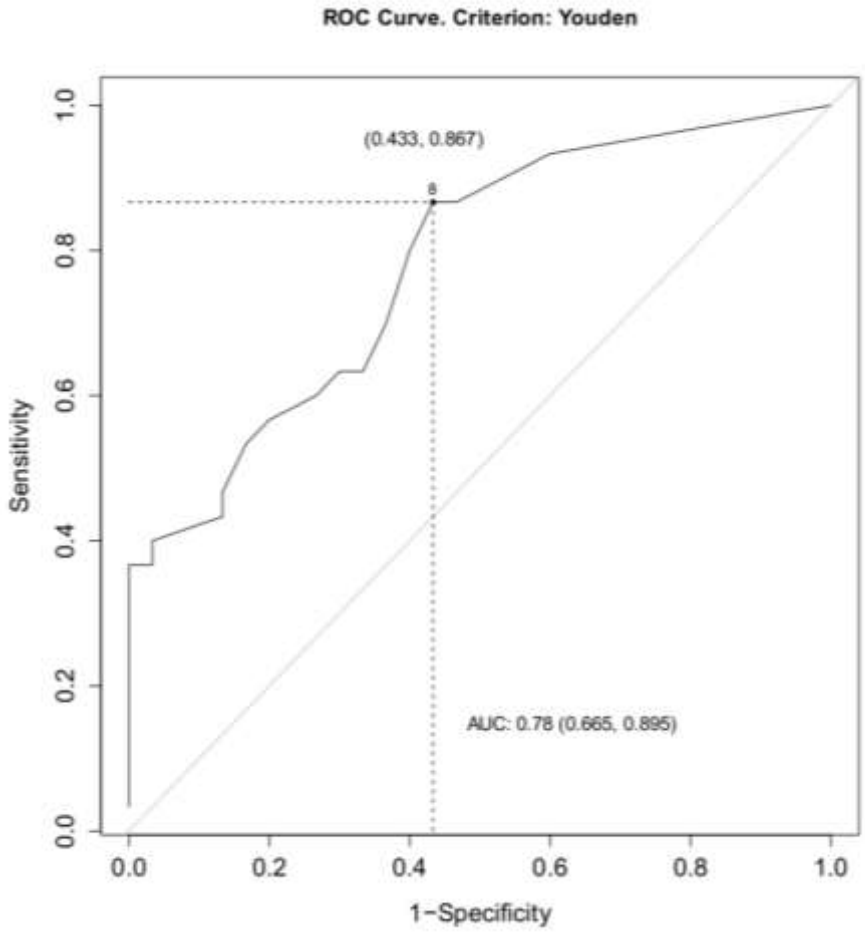
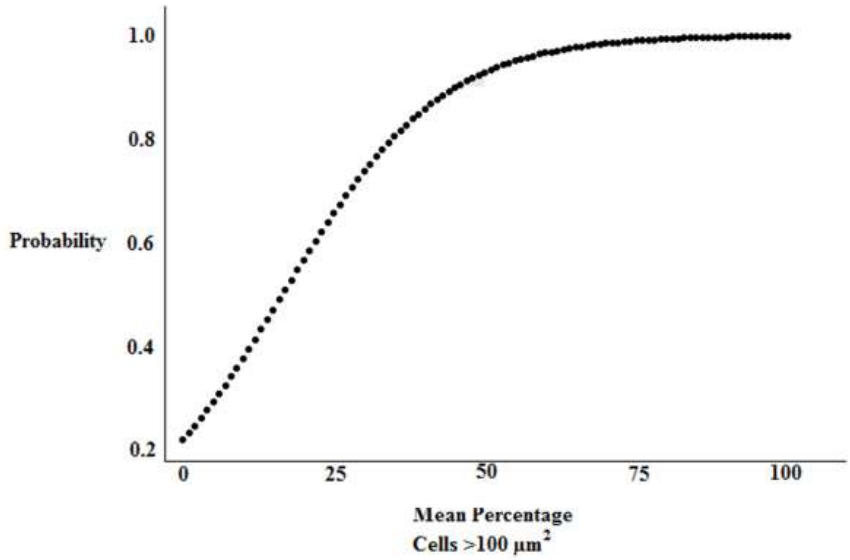


Figure 3. Likelihood of developing oral cancer according to mean percentage of nuclei larger than 100 μm^2.



DISCUSSION

Despite the inherent advantages in oral cytopathology, there is still resistance to its use as an auxilliary method in the diagnosis of oral cancer or as a means of monitoring individuals exposed to carcinogens, even though its applicability is proven for these purposes (7,8). In the present study, we used Papanicolaou staining in individuals exposed to smoking and alcohol over 40 years old, since more sophisticated techniques are costly and are not feasible at the population level (12).

As observed in other studies (8,9), no dysplastic changes, low - or high - risk, were seen in patients without lesions, exposed or not to carcinogens. Six cytological smears were classified as atypical-probably reactive/low-grade in the Tobacco/alcohol Group and only one in the Control Group. On the other hand, an interesting finding was the presence of high-risk cell changes (atypical high-grade and high-grade squamous intraepithelial lesion) in eight cytological smears of the

clinically normal oral mucosa adjacent to squamous cell carcinoma, reinforcing the concept of the field cancerization. Seven of these cases showed low-risk changes on histopathological examination, considering them as false positives. The wider scraped area in relation to biopsy or greater sensitivity of liquid - based oral cytology in detecting nuclear changes (enlarged size, hyperchromatic, irregular shape) are possible explanations for this result. Maraki et al. (17) suggested a greater sensitivity of cytopathology in the detection of incipient cellular changes in potentially malignant lesions when compared to histopathological examination.

To date, cytopathological and histopathological comparison studies have been performed on oral lesions (7). Using the field cancerization concept (14), in the present study we evaluated this comparison in the mucosa adjacent to oral cancer. To reduce the subjectivity of the histopathological classification, the epithelial changes were classified as low - or high - risk (16). Following the same reasoning, we opted to combine the cytopathological graduations. Like Afrogeh et al. (13), we found it difficult to distinguish the grades atypical-probably reactive/low-grade and low-grade intraepithelial lesion, and despite the lack of studies on the clinical implication in relation to different cytopathological grades, we believe that clinical management will only differ with a low-risk versus high-risk outcome.

Good specificity and accuracy and excellent sensitivity were found. However, the sensitivity result should be interpreted with caution, considering that it was based on only one case. Cankovic et al. (18), in a series of 30 cases, evaluated histopathologically the clinically normal mucosa adjacent to squamous cell carcinoma and found high-grade dysplastic changes in only three cases. We suggest further studies with a larger sample for better evaluation of sensitivity.

In this study, we used liquid-based oral cytology which, despite the higher cost compared to conventional cytology, yielded good-quality smears. The only smear considered inadequate was obtained from keratinized mucosa on the hard palate of an Oral Cancer Group patient, who reported discomfort when the sample was taken. It should be noted that the criterion used to determine the quality of the smear was based on Navone et al. (19). Considering the different characteristics of cellularity of the cervical-vaginal smear, we agree that oral cytopathology should have its own criteria. Another favorable point for liquid-based oral cytology is the possibility of the application of several techniques using a single scrape. We suggest the use of these techniques in specific cases after a low-cost and well-established initial screening with Papanicolaou staining. Smears classified as atypical-probably reactive/low-grade or low-grade intraepithelial lesion, in some cases after analysis with other techniques, may show changes that suggest a higher propensity for neoplastic transformation.

The nuclear area was analyzed taking advantage of the Papanicolaou staining. Like Khot et al. (10) and Ogden et al. (11), we found a statistically significant increase in this parameter in individuals exposed to carcinogens in relation to the Control Group. As expected, the mean nuclear area of the Oral Cancer Group also differed from individuals not exposed to tobacco or alcohol. However, no statistically significant difference was found between the Tobacco/Alcohol and Oral Cancer Groups, a finding reported by Khandelwal and Solomon (20), which associated the mean nuclear area of the cancerization field with exposure to smoking. However, unlike the study by Khandelwal and Solomon (20) who performed scrapes of the clinically normal mucosa contralateral to the tumor, we examined the area adjacent to the squamous cell carcinoma. With the greater proximity of the scraped area to the

tumor, it was expected that the changes present in the cancerization field could influence the nuclear area. However, the effects of smoking, present in both the Tobacco/Alcohol and Oral Cancer Groups appeared to be the main factors responsible for the increase in nuclear cytomorphometric parameters. The increase in nuclear area is an early cellular event, which is accentuated by the worsening of epithelial dysplasias (21). Such behavior favors its use as a quantitative parameter to monitor individuals exposed to carcinogens.

Of the methods assessed in this study, the most appropriate to distinguish a healthy mucosa from that likely to develop oral cancer was mean percentage of nuclei greater than 100 μm^2, which was used the remaining analyses. The ROC curve showed a good diagnostic performance. Weigum et al. (22) found that cytomorphometric parameters, such as cell nucleus size, were effective in distinguishing benign from malignant lesions and degrees of epithelial dysplasia, obtaining an AUC value of 0.93. Unlike our study, which assessed field cancerization, Weigum et al. (22) analyzed cytologic smears from dysplastic lesions and squamous cell carcinoma, probably increasing diagnostic performance. To optimize the use of oral cytopathology in individuals exposed to risk factors but with no oral lesions, this study determined a cutoff point of 8% of nuclei greater than 100 μm^2. For the longitudinal monitoring of patients with this cytologic profile, the logistic model was represented using a graph. For example, a reduction in mean percentage of nuclei greater than 100 μm^2 from 25% to 12% would reduce the risk for squamous cell carcinoma from nearly 70% to 40%. In the literature, there are no studies suggesting models to evaluate cytomorphometric parameters in normal oral mucosa.

For the prospective cytopathological assessment, we recommend assessing the same cell type. To detect early cellular changes in the clinically normal mucosa,

we selected intermediate and parabasal cells for the measurement of nuclear area (6). These cell types compose the deeper layers of the oral epithelium, where epithelial changes are found in mild dysplasias (16). Cytomorphometric studies do not specify which cell type - superficial, intermediate or parabasal - is being evaluated, making it difficult to determine any association between them (10,11,20,23). The mean nuclear area reported for non -keratinized mucosa in these studies (10,11,20,23) is lower than that observed in the present study. Probably, superficial cells with pyknotic nuclei were also included in the measurements of the former mentioned studies. In agreement with findings by Cowpe et al. (15), no statistically significant difference was observed in mean percentage of nuclei larger than 100 μm^2 between keratinized and non-keratinized mucosa. However, we found a statistically significant difference in mean nuclear area between the two types of mucosa. In order to promote methodological standardization, it is suggested that the same anatomical site should undergo cytological smear tests for longitudinal monitoring of patients.

Based on our results, we conclude that the cytopathology performed in clinically normal mucosa had a good specificity when it was compared to the histopathological examination and that dysplastic changes, even of high-grade, could be observed in the absence of lesions in the mucosa adjacent to the carcinoma. Considering the factors that influence the nuclear area, this quantitative parameter can be used to detect early cellular changes in the oral mucosa exposed to carcinogens, and mean percentage of nuclei larger than 100 μm^2 is the most appropriate for this assessment. Prospective longitudinal studies are suggested to validate the model proposed here for the use of cytomorphometric analysis to identify and monitor individuals at risk of oral cancer.

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4 ARTIGO DE PESQUISA 2

ASSESSMENT OF CELL PROLIFERATION RATE AND GENETIC DAMAGE IN ORAL FIELD CANCERIZATION: A CYTOPATHOLOGICAL STUDY

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ASSESSMENT OF CELL PROLIFERATION RATE AND GENETIC DAMAGE IN ORAL FIELD CANCERIZATION: A CYTOPATHOLOGICAL STUDY

Short title: Cytopathology of oral field cancerization

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ABSTRACT

Objective: To assess genetic damage and cell proliferation rate of clinically normal mucosa adjacent to oral cancer using cytopathological analyses and to establish a model for screening and monitoring of individuals with no oral lesion exposed to smoking/alcohol. **Materials and Methods:** Ninety male and female patients were allocated into three groups: Oral Cancer Group (patients with oral squamous cell carcinoma), Tobacco/Alcohol Group (patients with no oral lesions and exposed to these risk factors), and Control Group (individuals with no lesion and not exposed to tobacco and alcohol). A cytologic smear was obtained from the area adjacent to oral carcinoma in the Oral Cancer Group, and the same anatomical site was assessed in the other groups. Cytologic smears were stained by a silver-staining technique and by Feulgen reaction. The quantification of the number of AgNOR dots per nucleus and of micronuceli (MN) was carried out in the first 100 and 1000 smear cells, respectively. **Results:** Mean number of AgNOR dots per nuclei and of MN was significantly higher ($p<0.05$) in the Tobacco/Alcohol Group and Oral Cancer Group compared with the Control Group, whereas the number of nuclear buds (NBUDs) was significantly higher in the Control Group. **Conclusion:** Cutoff values for inclusion of individuals exposed to carcinogens in longitudinal monitoring were ≥ 3.38 AgNOR dots per nucleus and/or ≥ 3 MN/1 000 cells. A prospective model including the biomarkers assessed in this study was proposed but requires validation by longitudinal studies.

Key words: Biomarker, cytology, argyrophilic nucleolar organizer regions, field cancerization, oral cancer, prevention.

INTRODUCTION

The phenomenon of field cancerization was initially described by Slaughter et al. [1] to explain evidence of epithelial dysplasia in areas adjacent to oral squamous cell carcinoma. This phenomenon may be detected by markers of cell proliferation (AgNOR, ki-67 and BrdUrd/IdUrd) or genetic damage (loss of heterozygosity and micronuclei [MN]) and by cytomorphometric parameters [2–7]. In addition to the importance of field cancerization in the recidivation of oral carcinoma [8,9], the study of field cancerization has gained relevance for the establishment of biomarkers that may identify incipient carcinogenic changes [10]. Despite increased knowledge of the main causes of oral carcinoma [11,12] and of the profile of the most affected individuals [13], this neoplasm is still diagnosed at an advanced stage [14]. Oral cytopathology is a simple, painless, and noninvasive technique. These characteristics favor the use of oral cytopathology in field cancerization for the identification of population screening methods to monitor individuals at risk of developing oral cancer [15].

At the early stage of oral carcinogenesis, damage is transmitted to daughter cells, and increased cell proliferation rate reduces time for DNA repair [16]. Nucleolar organizer regions (NORs) are chromosomal segments containing ribosomal genes. These regions can be visualized by staining techniques that show argyrophilic nucleolar organizer regions (AgNORs). These are non-histonic proteins with affinity for silver ions that may be identified as black dots localized inside the nucleus [17]. The quantification of AgNOR is a marker of cell proliferation rate [18], since the amount of nucleolin and B23, the two major AgNOR proteins, is higher in the S-G2 phase and lower in the G1 phase of the cell cycle [19]. In oral mucosa without

lesions, smoking, whether associated or not with alcohol, has been found to increase cell proliferation activity in cytopathological analyses [20,21]. The ability of this marker to monitor smoking and alcoholic patients was assessed in a longitudinal study [22]. When assessed histological sections of areas adjacent to squamous cell carcinoma, Schwint et al. [3] observed increased cell proliferation even before the presence of dysplasic changes, thus representing a marker capable of detecting incipient cell changes. However, the use of AgNORs quantification in areas of field cancerization by oral cytopathology has not been well established yet.

Micronucleus (MN) is a reliable biomarker to assess chromosome breakage and loss and its morphology is similar to that of a interphase nucleus but smaller [23,24]. There has been an increasing interest in quantifying micronuclei to assess genetic damage in epithelial cells exfoliated from the oral mucosa in individuals exposed to genotoxic agents [25]. Bonassi et al. [26] assessed MN frequency in databases of different laboratories. Individuals who smoked more than 40 cigarettes/day showed a significant increase in MN frequency, whereas daily alcohol consumption did not influence MN frequency (26). Conversely, Stich et al. [27] found higher MN frequency with combined use of tobacco and alcohol. The quantification of MN frequency also evidenced field cancerization [6,28–30]. However, the great variability between laboratories [26,31] justifies the need for each facility to determine its reference values for MN and for other metanuclear anomalies, such as nuclear bud (NBUD) and karyorrhexis. NBUD has an unclear significance and origin [32,33], with controversial results in the literature. Bonassi et al. [26] did not observe any association between NBUD and smoking. Conversely, Nersesyan et al. [34] and Pellicioli et al. [6] found a higher number of NBUD in individuals exposed to smoking and in areas of field cancerization, respectively. Nevertheless, Bohrer et al. [35]

associated NBUD with DNA repair by observing that NBUD frequency was greater in the control group. Karyorrhexis, considered as a late stage of apoptosis [32], was more prevalent in oral mucosae exposed to carcinogens [26,34,35].

AgNORs has never been assessed by oral cytopathology in field cancerization, and there was great variation between studies with regard to MN quantification. Thus, the aim of this study was to assess genetic damage and cell proliferation rate in the incipient phase of carcinogenesis in areas of field cancerization and suggest a model for population screening and monitoring of individuals at risk for oral squamous cell carcinoma.

MATERIALS AND METHODS

The present study has been approved by the Ethics in Research Committee of our institution. Each of the participants in the study signed an informed consent form. The sample comprised 90 male and female subjects, over 40 years old, who came from Oral Medicine Division of the Hospital, Specialized Dental Clinic and in a Health Care Unit. Patients with anemia, diabetes, current or previous history of malignant neoplasm and chemotherapeutic or radiotherapeutic treatment were excluded. The patients were allocated into three groups:

- Control Group: 30 patients without clinically visible oral lesions who had no history of smoking and drank on average less than one alcoholic drink per day.
- Tobacco/Alcohol Group: 30 patients without clinically visible oral lesions who smoked at least 20 filtered cigarettes per day for at least one year or more than 10 filtered cigarettes per day for more than 10 years. Alcohol consumption could be

associated with smoking, and if so, it was quantified as drinks/day, regardless of the type of drink.

- Oral Cancer Group: 30 patients above 40 years of age with oral squamous cell carcinoma. Vermillion lips lesions were excluded. In this group, former smokers or alcoholics were those who reported not smoking or drinking alcohol at the time of anamnesis, regardless of the time they quit these habits.

For each patient selected in the Oral Cancer Group, a person of the same sex and of approximate age was screened for the other groups. The anatomical sites from which cytological smears were obtained in Oral Cancer Group patients were the same areas scrapped in individuals of the Control and Tobacco/Alcohol Groups. Individual personal data, information about life style, diet, presence of systemic diseases and chronic use of drugs were registered on a clinical record. For patients in the Oral Cancer Group, the clinical features of squamous cell carcinoma were recorded. After anamnesis, patients with total or partial dentures were instructed to remove them and to rinse their mouth with saline.

Smears and Cytopathological Analysis

Sample collection was initiated with subjects from the Oral Cancer Group. Prior to biopsy, a cytological smear was taken from the clinically normal mucosa adjacent to the lesion. A Velscope® Vx (LED Dental, Canada) was used to determine the site to be scrapped. This device emits fluorescent light and indicates the presence of epithelial changes, which appear as a darkened area around the lesion. In cases where the device showed only the lesion, the smear was obtained approximately 10 mm from the tumor border. The cytological smear was performed with a cytobrush, using 10 to 15 spins to obtain cells from the deeper layers of the

oral epithelium. The cytobrush was inserted in the container of the Liqui-PREP® system (Sirius, Brazil), and the samples were processed according to the manufacturer 's protocol.

In Control and Tobacco/Alcohol Groups a cytological smear was obtained following the same collection technique previously described. In these groups, the Velscope® Vx was not used.

Two slides were prepared, one by the silver-staining technique proposed by Ploton et al. [36] and the other by Feulgen reaction [37]. Slides were examined by a single blinded examiner with a light microscope (model MB51OPT, Precision, Nanjing, China).

AgNOR quantification

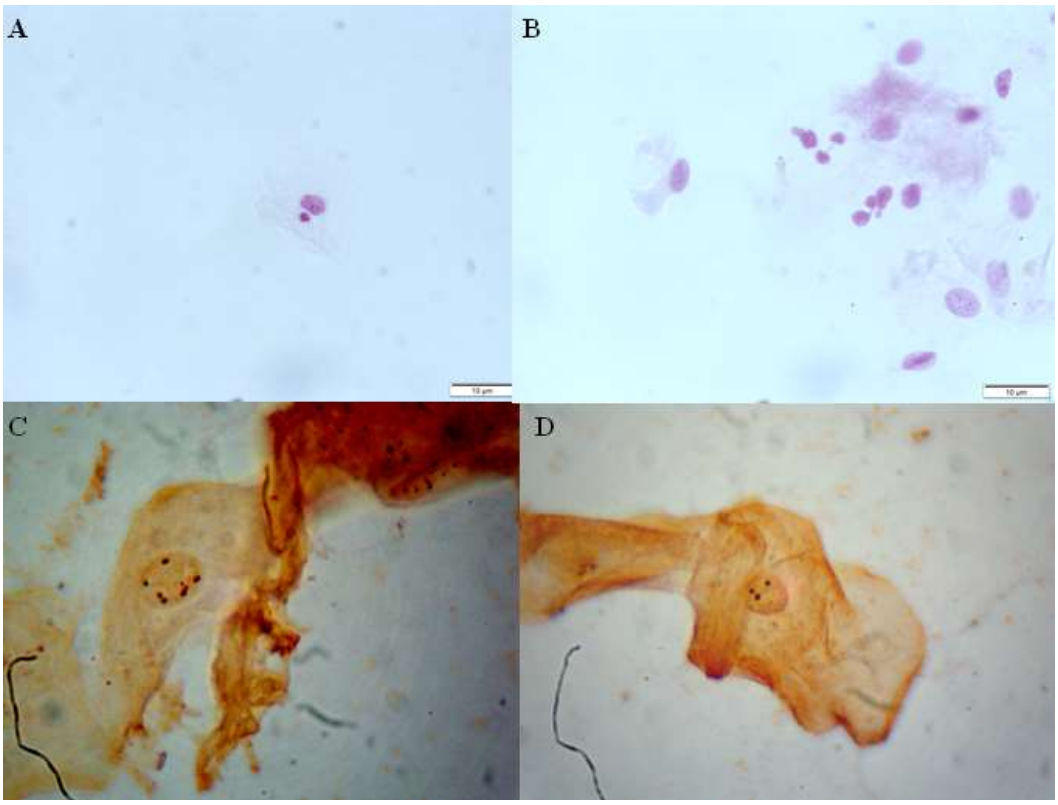
The number of AgNORs, represented as black dots inside the nucleus (Figura1C and D), per nucleus in the first 100 well-spread and non overlapping cells was quantified according to Crocker et al. [38], at 1 000X magnification with oil immersion lenses. Mean AgNORs dots/nucleus and mean percentage of nuclei with > 3 and > 5 AgNOR dots were assessed. Before AgNOR quantification, intra-examiner calibration was performed, with a kappa value of 0.782.

Quantification of MN, NBUDs, and karyorrhexis

After Feulgen reaction, the first 1 000 intermediate or parabasal epithelial cells with intact, well-spread, and non overlapping nuclei were initially counted at 400X magnification. Karyorrhectic and karyolytic cells or those with pyknotic nuclei were excluded from the counting of MN and NBUDs. MN and NBUD counts were confirmed at 1 000X magnification with oil immersion lenses. Additionally, the

number of karyorrhectic cells in the first 1 000 epithelial cells was also counted. For each slide, the number of MN, NBUDs, and karyorrhectic cells per 1 000 cells were calculated. MN counting was defined according to criteria by Tolbert et al. [32] (Figure 1A), and NBUD and karyorrhectic cell counting was defined according to criteria by Thomas et al. [39]. Structures similar to micronuclei but that were connected with the main nucleus were also considered NBUDs (Figure 1B).

Figure 1. Micronucleus (MN), nuclear buds (NBUDs), and AgNOR dots in epithelial cells obtained from cytologic smear. A: Epithelial cell from the Oral Cancer Group with one MN. B: Cytological smear from the Control Group with 3 NBUDs. C Epithelial cell from the Oral Cancer Group with 5 AgNOR dots in its nucleus. D Epithelial cell from the Tobacco/Alcohol Group with 3 AgNOR dots in its nucleus.



Data analysis

ANOVA followed by Tukey's post-hoc test was used to compare mean age and mean AgNOR dots per nucleus between groups. The Mann-Whitney test was used for comparison of the Tobacco/Alcohol and Oral Cancer Groups regarding the mean number of cigarettes and alcoholic drinks consumed per day and duration of smoking. The Kruskal-Wallis test followed by the Dunn test was used to compare the consumption of coffee (cups/day) and maté (gourds/day) between groups. A Kruskal-Wallis followed by Nemenyi post hoc test was used to compare mean percentage of nuclei with > 3 and > 5 AgNOR dots per nucleus between groups. The number of MN, NBUDs and karyorrhectic cells was compared using zero-inflated Poisson regression based on diagnostic analysis with Wald test, Cook's distance, and QQ-plot graph. Binomial logistic regression was used to determine the most appropriate parameter to distinguish healthy mucosa from that likely to develop oral cancer, represented by Control and Oral Cancer Groups respectively, using the equation $log\{\pi(x)/1-\pi(x)\}=\beta 0+\beta 1x1+...+\beta pxp$. Models were selected in a stepwise fashion. After estimating the model, odds ratio (OR) was calculated using exponential coefficients. The results of the logistic model were shown in graphs describing the association between the parameter of interest and the likelihood of developing oral cancer. Diagnostic performance was assessed using receiver operating characteristic (ROC) curves. Optimal cutoff values were determined by the Youden index. The simple linear regression model was used to evaluate the association between life style and dietary factors (smoking, consumption of alcohol, mate tea, coffee, vegetables, and fruits) and mean AgNOR dots per nucleus and mean MN count per 1 000 cells. Variables were selected in a stepwise fashion, with the removal of

leverage points in Cook's distance graphs. Analyses were performed in the R 3.4.1 environment and $p<0.05$ was considered significant.

RESULTS

Characteristics of the sample

Table 1 shows sample characteristics. Nearly 70% of subjects of the three groups were on chronic drug therapy, with antihypertensives being the most used class of medications. Table 2 describes the anatomic sites of squamous cell carcinomas and areas from which cytological smears were obtained.

Table 1. Sample characteristics.

Characteristic	**Control Group (n = 30)**	**Tobacco/Alcohol Group (n = 30)**	**Oral Cancer Group (n = 30)**	**p**
Gender	Male 21 Female 9	Male 21 Female 9	Male 21 Female 9	
Age, years (mean±SD)[a]	61.3±8.99	58.86±9.84	64.33±9.70	0.09
No. of cigarettes/day (mean±SD)[b]	-	24.16±11.45	20.75±14.49	0.5
No. of drinks/day (mean±SD)[b]	-	0.9±1.66	3.2±3.0	0.02[d]
Years of cigarette smoking (mean±SD)[b]	-	40.53±10.33	34.79±20.50	0.68
Cups of coffee/day (mean±SD)[c]	0.86±1.19	2.03±2.72	2.86±4.79	0.21
Gourds of mate/day (mean±SD)[c]	4.33±6.84	7.26±11.93	3.16±6.55	0.1

[a] One-way ANOVA, Tukey test

[b] Mann-Whitney

[c] Kruskal-Wallis, Dunn test

[d] Significant difference between groups ($p<0.05$)

Table 2. Characteristics of the sample regarding sex, age, anatomical site of oral cancer and area subjected to cytological smear.

Case	Location	
	Oral Cancer	**Scrapes**
1	Left lateral border of tongue	Left lateral border of tongue
2	Right ventral surface of tongue	Right ventral surface of tongue
3	Left floor of mouth	Left floor of mouth
4	Right soft palate	Right buccal mucosa
5	Right soft palate	Right buccal mucosa
6	Left retromolar trigone	Left buccal mucosa
7	Right lateral border of tongue	Right lateral border of tongue
8	Right buccal mucosa	Right hard palate
9	Right floor of mouth	Right lateral border of tongue
10	Right retromolar trigone	Right buccal mucosa
11	Left soft palate	Left hard palate
12	Right soft palate	Right hard palate
13	Left ventral surface of tongue	Left ventral surface of tongue
14	Left retromolar trigone	Left buccal mucosa
15	Left lateral border of tongue	Left lateral border of tongue
16	Lower alveolar ridge	Inner surface of lower lip
17	Right buccal mucosa	Right buccal mucosa
18	Right floor of mouth	Right lower buccal vestibule
19	Right lateral border of tongue	Right lateral border of tongue
20	Right lateral border of tongue	Right lateral border of tongue
21	Upper alveolar ridge	Right upper buccal vestibule
22	Right floor of mouth	Right ventral surface of tongue
23	Right floor of mouth	Right lateral border of tongue
24	Right soft palate	Right buccal mucosa
25	Left retromolar trigone	Left buccal mucosa
26	Right lateral border of tongue	Right lateral border of tongue
27	Right floor of mouth	Right ventral surface of tongue
28	Right retromolar trigone	Right buccal mucosa
29	Right lateral border of tongue	Right lateral border of tongue
30	Left lateral border of tongue	Left lateral border of tongue

AgNOR quantification

Table 3 presents mean and standard deviation (±SD) of AgNOR dots per nucleus and the percentage of nuclei with > 3 and > 5 AgNOR dots. Values were significantly higher in the Tobacco/Alcohol and Oral Cancer Groups than in the Control Group.

Table 3. Means (±SD) of AgNOR dots per nucleus and percentage of nuclei with > 3 and > 5 AgNOR dots in the three groups.

	Control	Tobacco/alcohol	Oral cancer
No. of AgNORs/nucleus#	2.86±0.56[a]	3.38±0.71[b]	3.45±0.58[b]
> 3 AgNORs/nucleus*	28.46±15.66[a]	44.16±19.86[b]	45.16±17.13[b]
> 5 AgNORs/nucleus*	2.86±3.02[a]	8.03±6.98[b]	7.73±7.71[b]

Different letters in rows indicate statistically significant difference ($p<0.05$)

#One-Way ANOVA, Tukey test

* Kruskal - Wallis, Nemenyi test

Quantification of MN and metanuclear anomalies

Due to lack of intermediate cells in cytologic smears, two samples of Control Group and one of the Oral Cancer Group were excluded. Comparison of mean number of MN, NBUDs, and karyorrhectic cells in 1 000 cells are shown in Table 4. MN and karyorrhectic cell counts were significantly higher in the Tobacco/Alcohol and Oral Cancer Groups than in the Control Group. Mean number of karyorrhectic cells was higher in the Oral Cancer Group than in the Tobacco/Alcohol Group, whereas mean number of NBUDs was significantly higher in the Control Group compared with the other groups.

Table 4. Means (±ME) of the number of MN, NBUDs, and karyorrhectic cells per 1 000 cells in the three groups.

Group	MN (±ME)	NBUDs (±ME)	Karyorrhexis (±ME)
Control (*n*=28)	0.75±0.35[a]	2.78±1.48[a]	4.1±2.02[a]
Tobacco/alcohol (*n*=30)	1.70±0.74[b]	0.83±0.65[b]	5.86±2.74[b]
Oral cancer (*n*=29)	1.72±0.55[b]	1.17±1.48[b]	8.34±1.48[c]

MN = micronuclei; ME = margin of error; NBUDs = nuclear buds.

Different letters in column indicate statistically significant difference ($p<0.05$).

Zero-inflated Poisson model.

AgNORs/MN: Screening and Monitoring Model

The binominal logistic regression model revealed that mean AgNOR dots per nucleus and mean number of MN per 1 000 cells were the most appropriate parameters to distinguish a healthy mucosa from that likely to develop oral cancer, represented by the Control and Oral Cancer Groups, respectively. Diagnostic performance of mean number of AgNOR dots per nucleus was good, based on ROC curves, with AUC of 0.80 (minimum 0,692, maximum 0,923). Optimal cutoff point as determined by the Youden index was 3.38, with sensitivity of 0.65 and specificity of 0.89. Diagnostic performance of mean number of MN per 1 000 cells was also good, based on ROC curves, with AUC of 0.68 (minimum 0.556, maximum 0.82). Optimal cutoff point as determined by the Youden index was 3, with sensitivity of 0.34 and specificity of 0.92 (Figure 2). OR for mean number of AgNOR dots per nucleus was 12.31 (95% CI = 3.17-75.39), and for mean number of MN per 1 000 cells was 2.06 (95% CI = 1.19-3.94). Graphs showing the probability of developing oral cancer according to the mean number of AgNOR dots per nucleus and to the mean number of MN per 1 000 cells are presented in Figure 3. Simple linear regression analysis

revealed a significantly positive effect ($p<0.05$) of daily cigarette consumption on mean number of AgNOR dots per nucleus and on mean number of MN.

Figure 2. ROC curve for evaluation of the diagnostic performance of mean number of AgNOR dots per nucleus (A) and of mean number of micronuclei per 1 000 cells (B), with their respective cutoff points as determined by the Youden index.

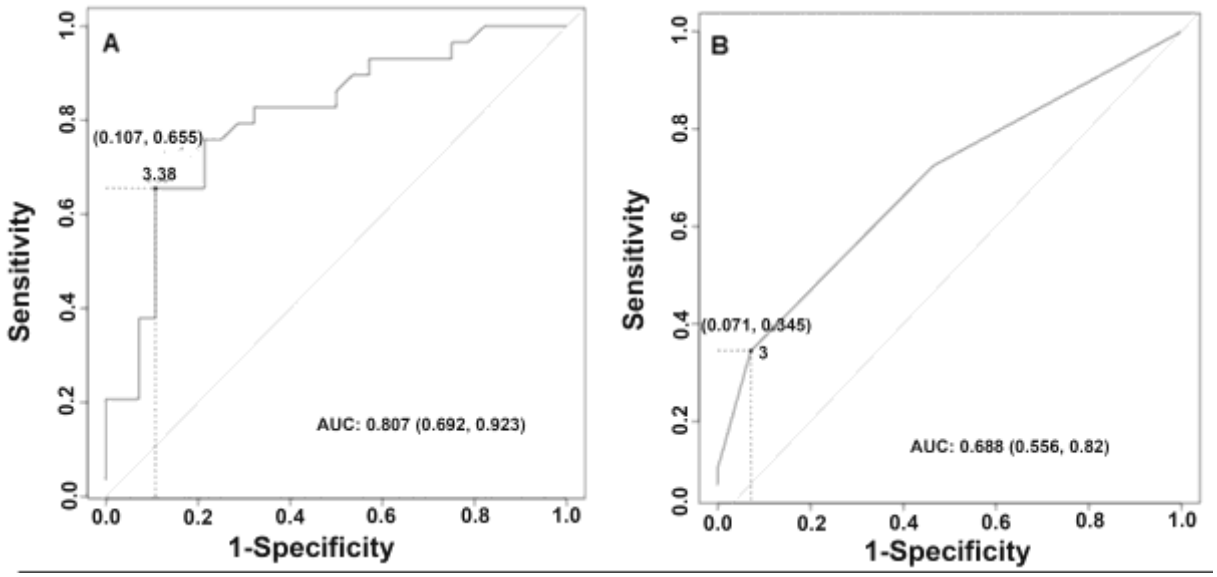
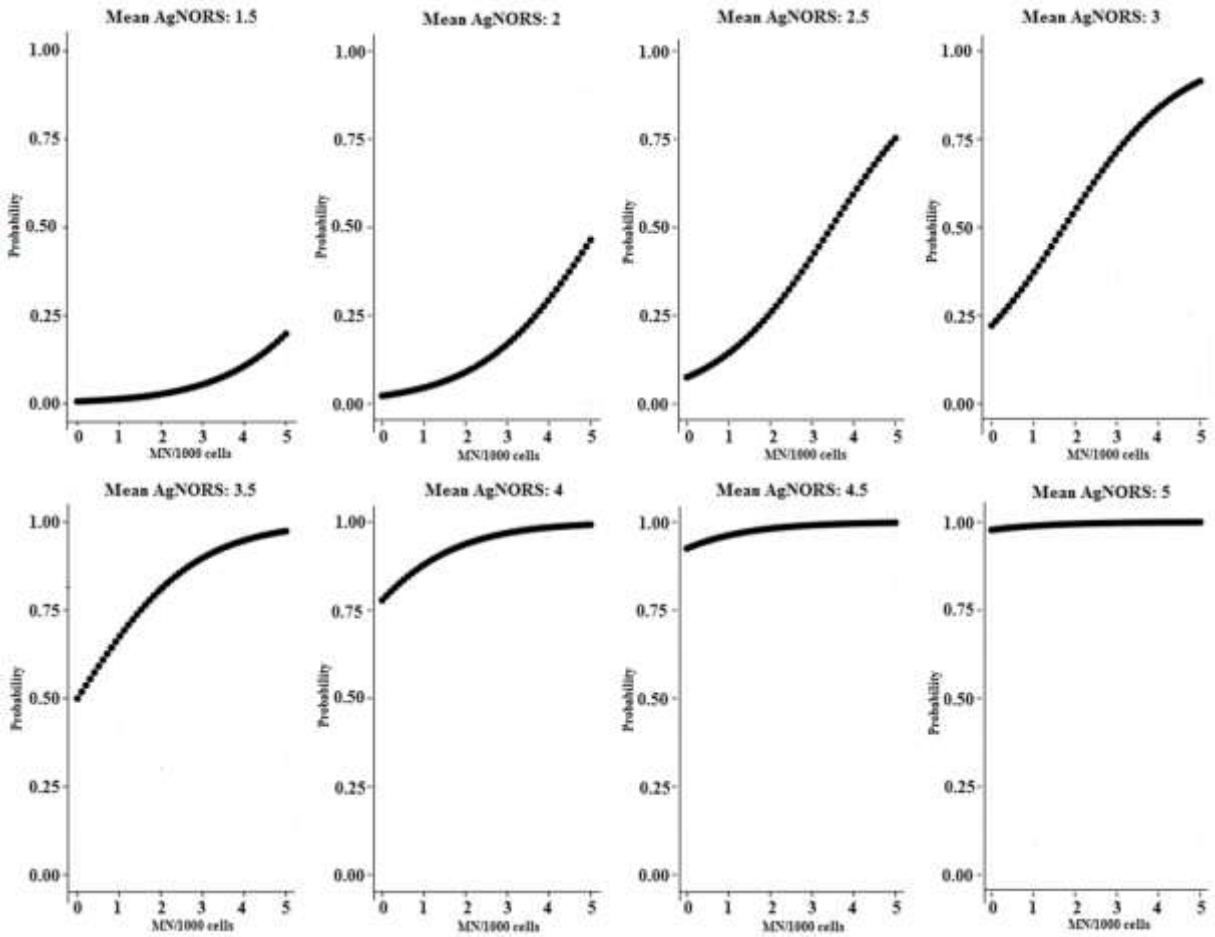


Figure 3. Probability of developing oral cancer according to mean number of AgNOR dots per nucleus and to mean number of MN per 1 000 cells.



DISCUSSION

In this study, we investigated cell proliferation rate and genetic damage in order to assess different aspects of early oral carcinogenesis. Cell proliferation activity (mean number of AgNOR dots per nucleus and mean percentage of nuclei with > 3 and > 5 AgNOR dots) was similar in the Tobacco/Alcohol and Oral Cancer Groups and lower in the Control Group. Smoking was a determining factor for this

finding, with simple linear regression analysis showing the significant positive effect of daily cigarette consumption on mean number of AgNOR dots per nucleus. Conversely, alcohol intake did not have an influence on this variable. This finding is corroborated by those of Paiva et al. [40], who found similar mean numbers of AgNOR dots per nucleus in smokers and in those who used tobacco and alcohol. However, Jindal et al. [41] observed higher cell proliferation with combined tobacco and alcohol use, which may be explained by the greater amount and duration of alcohol consumption found in their study [41] compared with the present study, resulting in increased mean AgNOR dots per nucleus.

With regard to MN quantification, the pattern of genetic damage was similar in the area of field cancerization from carcinoma patients and in the mucosa of individuals with no oral lesion exposed to carcinogens. MN counts were higher in the Tobacco/Alcohol and Oral Cancer Groups than in the Control Group. This finding may be explained by smoking, because a significant positive effect of daily cigarette consumption was observed only on the number of MN. Nersesyan et al. [34] and Wu et al. [42] also found greater genetic damage in smokers. The higher consumption of alcohol in the Oral Cancer Group compared with the Tobacco/Alcohol Group was not a determining factor for the number of MN. Similarly, Pellicioli et al. [6] and Lima et al. [43] did not find greater genetic damage with the combined use of tobacco and alcohol. Based on the findings obtained in this study showing greater genetic damage in areas adjacent to carcinoma and in individuals exposed to carcinogens, we agree with the hypothesis proposed by Stich et al. [44] that MN formation may be related to the failure of cells to repair themselves after intense aggression.

The present study also assessed the number of NBUDs. Shimizu et al. [45] relate NBUD with chromosome instability, with the elimination of amplified

DNA during S phase of the cell cycle or nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division [46]. Nersesyan [47] suggests that the presence of NBUDs in exfoliated epithelial cells is not related to genotoxicity and to findings based on studies with lymphocytes. Oral cytopathological studies presented controversial results with regard to NBUDs. Pellicioli et al. [6] found a higher NBDU frequency in areas adjacent to carcinoma and to leukoplakia. Nersesyant et al. [34] found a higher NBDU frequency in smokers of middle-tar and high-tar cigarettes compared with smokers of low-tar cigarettes. Conversely, Ramirez et al. [29] did not observe any difference in NBDU counts between areas of field cancerization in patient with oral carcinoma and the healthy mucosa of control patients. Bonassi et al. [26] assessed the number of NBUDs in databases from several laboratories and did not find any association with smoking. In agreement with findings by Bohrer et al. [35], we found a greater mean number of NBUDs in the Control Group than in the other groups. We suggest that this metanuclear anomaly is a defense mechanism related to low-intensity stimuli and not to the action of genotoxic agents in the oral cavity. As shown by Bohrer et al. [35], in some situations, band breakdown or Feulgen positive nucleoplasmic bridges would result in structures incompatible with MN. Lindberg et al. [46] confirm this hypothesis by observing that MN and NBUDs have different formation mechanisms in lymphocytes, using fluorescence in situ hybridization (FISH) assays. Conflicting literature results encourage the conduction of studies to establish the actual significance of NBUDs count for oral cytopatholgyical analysis.

In the present study, karyorrhexis was influenced by proximity to the tumor, with the Oral Cancer Group showing the highest mean and the Control Group the

lowest. However, Ramirez et al. [29] observed increased karyorrhexis in healthy mucosa compared with field cancerization. Pellicioli et al. [6] found that the frequency of this metanuclear abnormality did not differ between healthy mucosa and areas adjacent to tumors. The difference in age between control and oral cancer groups and the different anatomic sites assessed in the studies by Ramirez et al. [29] and Pellicioli et al. [6] may have contributed to these findings. In our study, we intended to control age and anatomic sites between the groups. Corroborating with our findings, Nersesyant al. [34] and Bohrer et al [35] found increased karyorrhexis in the mucosa exposed to carcinogens.

The most appropriate variables to distinguish healthy mucosa from cancerization field was mean number of AgNOR dots per nucleus and number of MN per 1 000 cells. We did not include metanuclear anomalies (NBUDs and karyorrhexis) in the analysis due to their unclear biological significance. The evaluation of cell proliferation rate had a good diagnostic performance, with an optimal cutoff point of 3.38. With regard to assessment of genetic damage, optimal cutoff point was established as 3 MN/1 000 cells. This value had low sensitivity (0.34) but excellent specificity (0.92). According to Ludwig & Weinstein [48], population screening markers require high specificity, in order to prevent an excessive number of false-positive cases resulting in unnecessary costs and patient anxiety. Our study found that individuals with no oral lesions exposed to carcinogens presenting with ≥ 3.38 AgNOR dots per nucleus and/or ≥ 3 MN/1 000 cells are required to undergo cytopathological longitudinal monitoring.

We observed that variation in the mean number of AgNOR dots per nucleus had a greater impact on the probability of developing squamous cell carcinoma than the number of MN per 1 000 cells. An increase of one unit in mean number of

AgNOR dots per nucleus leads to a nearly 12-fold increase in the likelihood of developing squamous cell carcinoma, whereas the increase of one unit in mean number of MN per 1 000 cells lead to a nearly 2-fold increase. Bloching et al. [28] quantified MN in areas of field canceration and found an 8-fold increase in the likelihood of developing oral cancer in smokers with no oral lesion who had > 0.0195 MN per cell. However, comparison between their findings and those of the present study is hindered by the fact that the study Bloching et al. [28] used Giemsa-staining, which tends to overestimate MN counts [26], rather than DNA-specific Feugen reaction. The graphs presented in Figure 3 should be used for the prospective monitoring of patients at risk of oral carcinoma. A decrease from 3 to 2 in the mean number of AgNOR dots per nucleus maintaining a count of 2 MN/1 000 cells would reduce the probability of developing oral cancer from nearly 50% to 10%.

Based on our results, both of the above markers were found appropriate to detect early oral carcinogenesis. Cutoff values for inclusion of individuals exposed to carcinogens in longitudinal monitoring were ≥ 3.38 AgNOR dots per nucleus and/or ≥ 3 MN/1 000 cells. A prospective model including the biomarkers assessed in this study was proposed but requires validation by longitudinal studies.

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DISCUSSÃO COMPLEMENTAR

Apesar das vantagens inerentes à técnica (42), do aprimoramento do método de coleta com o meio líquido (45,46) e de um sistema de classificação próprio (50), a citopatologia bucal tem uma baixa aplicabilidade clínica (56). O conhecimento da sua utilização como método auxiliar e não substitutivo ao exame histopatológico no diagnóstico do câncer e de lesões cancerizáveis (44), pode resultar na sua prática mais frequente. Sugerimos sua utilização como método adjunto ao exame clínico, orientando a decisão de biopsiar o paciente e encaminhá-lo a um especialista. Os aspectos positivos já mencionados em relação à técnica da citopatologia bucal (42) reforçam a importância da sua utilização na mucosa clinicamente normal para detectarem-se alterações celulares iniciais (57). A maioria dos carcinomas espinocelulares são diagnosticados tardiamente (22), dado constatado neste estudo, no qual 56,5% dos casos de câncer bucal mediam mais do que 4 cm.

O presente estudo foi o primeiro a associar os aspectos citopatológicos e histopatológicos na mucosa clinicamente normal adjacente ao carcinoma espinocelular bucal. Optamos pela área adjacente ao tumor uma vez que esta pode representar a fase inicial da carcinogênese bucal (37), sem a camada de ceratina das leucoplasias, que dificulta a obtenção de uma amostra adequada de células basais e parabasais do epitélio bucal (126). Na área adjacente ao tumor, sugerimos que a citopatologia foi mais sensível para identificar alterações celulares (relação núcleo/citoplasma, hipercromasia nuclear) do que a histopatologia. Nesta região, também foram observadas alterações morfológicas compatíveis com baixo grau - padrão citológico que deve ser investigado em indivíduos sem lesão bucal expostos

aos carcinógenos. Enfatiza-se, portanto, a importância do contínuo estudo das características qualitativas do campo de cancerização. O aumento amostral permitirá definir com maior propriedade a sensibilidade e especificidade do exame citopatológico em relação ao histopatológico.

A fim de selecionar a melhor área adjacente ao tumor a ser analisada, nos baseamos no estudo de Poh et al. (127). Estes autores concluíram que o uso do Velscope, equipamento que detecta a perda de autofluorescência do tecido alterado, foi útil na identificação do campo de cancerização subclínico, confirmado com análise histológica e molecular. Constatamos que nos carcinomas bucais de até 2 cm a área escurecida ao redor da lesão foi visualizada por meio do equipamento Velscope. Entretanto, nos tumores com mais de 4 cm, devido à dor e trismo apresentados pelo paciente, em muitas situações não foi possível a utilização desse equipamento. Estipulou-se, então, a realização da biópsia e esfregaço citológico a 10 mm da margem da lesão. Avaliar se o Velscope identificou uma área com mais alterações epiteliais não foi objetivo do nosso estudo.

Alguns autores indicam a associação de técnicas frente a um dúbio diagnóstico citopatológico (128). Considerando o baixo custo, acreditamos que métodos quantitativos devam ser aplicados em pacientes de alto risco ao desenvolvimento do carcinoma espinocelular bucal em conjunto com os aspectos qualitativos do esfregaço. Os métodos utilizados no presente estudo detectaram modificações citomorfométricas, de proliferação celular e de dano genético prévias ao aparecimento de lesão.

O uso da citomorfometria é atrativo pelo baixo custo e simplicidade da coloração de Papanicolaou, além da capacidade de detectar alterações celulares na mucosa clinicamente normal de indivíduos expostos ao fumo e álcool (69,71,74).

Acreditamos que a padronização dos aspectos metodológicos entre os estudos como área anatômica e tipos celulares avaliados, por exemplo, contribuiria para aplicação dos parâmetros citomorfométricos. A literatura carece de um estudo longitudinal para validar sua utilização como monitoramento de indivíduos expostos a carcinógenos. Por outro lado, um entrave à mensuração dos parâmetros celulares é o tempo despendido pra a sua análise, considerando seu uso em larga escala. Novas metodologias têm sido desenvolvidas com o intuito de reduzir este tempo, além de permitir a avaliação concomitante de diversos marcadores, tornando mais provável seu uso no cotidiano. Weigum et al. (129) e Abram et al.(130), utilizando a captura das células com o filtro da membrana associada a um nano - bio - chip, concluíram que os aspectos citomorfométricos (tamanho nuclear, celular e relação núcleo/citoplasma) foram tão efetivos na distinção de lesões benignas e malignas e graus de displasias epiteliais quanto os marcadores mais sofisticados. Este meio de análise poderia ser aplicado na mucosa clinicamente normal adjacente ao tumor, a fim de ter um papel mais preditivo ao aparecimento do carcinoma espinocelular.

A técnica das AgNORs não havia sido avaliada no campo de cancerização por meio da citopatologia. Observamos uma atividade de proliferação celular semelhante na área adjacente ao tumor e nos indivíduos expostos aos carcinógenos. A literatura ratifica o nosso achado, uma vez que maior número de AgNORs/núcleo tem sido associado ao fumo (103,60). Alguns pontos positivos desta técnica são o baixo custo, padronização da metodologia entre os estudos (103,100), pouco tempo dispendido (99), além da capacidade de monitoramente de pacientes expostos a carcinógenos (107). O aumento da proliferação celular predispõe a mutações com o encurtamento do ciclo celular (88), principalmente quando ocasionado por um agente iniciador da carcinogênese bucal (9). A existência de

diversos estudos que seguiram semelhantes critérios de contagem das AgNORs (131) e mesma técnica de impregnação pela prata (132) favoreceria a realização de uma metanálise com o intuito de determinar se há a influência do estilo de vida, além da exposição ao fumo, na quantificação das AgNORs, estipular valores de referência deste parâmetro em indivíduos sadios, dentre outros aspectos ainda não estabelecidos.

A contagem do MN já é estabelecida como um biomarcador para avaliar instabilidade cromossomal (133) e o crescente interesse da sua avaliação por meio da citopatologia bucal justifica-se pela facilidade da obtenção da amostra (111). Os resultados do presente estudo demonstraram que o dano genético do campo de cancerização foi semelhante ao dos indivíduos expostos a carcinógenos. Foi estipulado que para obter uma estimativa mais precisa da média de MN, seria necessário quantificar cerca de 4.000 células por esfregaço citológico (115). Concordamos que a contagem de um número maior de células pode tornar o dano genético mais evidente, entretanto, torna inviável sua aplicação, se realizada manualmente. Samanta e Dey (134) reforçam os aspectos positivos de avaliar-se a frequência de MN em células epitelias da mucosa bucal como baixo custo e simplicidade. Por outro lado, esses autores ressaltam a incompatibilidade da sua análise manual com o cotidiano do patologista e incentivam a automatização do ensaio do MN. Ao encontro desta perspectiva, Darzynkiewicz e Smolewski (135), com o uso da citometria de varredura a laser, constataram boa acurácia da quantificação de MN em células epiteliais bucais. Esta metodologia permite a análise de 1000 células entre 3 e 5 minutos. Os autores discutem a utilização deste meio automatizado para a avaliação de outras alterações metanucleares.

Considerando as alterações metanucleares avaliadas em nosso estudo,

observamos que o NBUD foi associado a um mecanismo de reparo na mucosa exposta a estímulos de baixa intensidade. Devido aos achados contraditórios da literatura com relação ao NBUD (117,124,125), decidimos não considerar o valor obtido no campo de cancerização como parâmetro sugestivo de predição ao desenvolvimento do carcinoma espinocelular.

Neste estudo, buscamos uma maior aplicabilidade clínica da citopatologia bucal, estipulando os melhores parâmetros a serem utilizados. Dentre todas as variáveis avaliadas, a média percentual de núcleos com mais de 100 μm^2, a média das AgNORs/núcleo e do número de MN/1000 células foram os mais adequados para discernir a mucosa saudável da passível de transformação maligna. Após, calculamos os pontos de corte ideais destes parâmetros para identificar os indivíduos mais suscetíveis ao desenvolvimento do carcinoma espinocelular e, por fim, foi sugerido um modelo de monitoramento destes pacientes. O próximo passo será a realização de estudos longitudinais que validem a metodologia aqui proposta.

Baseado nos nossos resultados, os marcadores de proliferação celular, dano genético e citomorfométrico detectaram alterações incipientes da carcinogênese bucal. O aperfeiçoamento de metodologias já em uso (129,130,135) e o estudo de modelos que aproximem a citopatologia bucal do cotidiano dos profissionais de saúde possibilitarão sua aplicação em larga escala para o rastreamento e monitoramento de indivíduos de risco ao surgimento do câncer bucal.

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ANEXO A

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 5896 Porto Alegre, 28 de agosto de 2014.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "PROLIFERAÇÃO E DANO GENÉTICO EM CÉLULAS DESCAMADAS DA MUCOSA BUCAL EXPOSTA A CARCINÓGENOS E ADJACENTE A CARCINOMAS ESPINOCELULARES" coordenado por FERNANDA GONCALVES SALUM. Caso este projeto necessite apreciação do Comitê de Ética em Pesquisa (CEP) e/ou da Comissão de Ética no Uso de Animais (CEUA), toda a documentação anexa deve ser idêntica à documentação enviada ao CEP/CEUA, juntamente com o Documento Unificado gerado pelo SIPESQ.

Atenciosamente,

Comissão Científica da FACULDADE DE ODONTOLOGIA

ANEXO B

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PROLIFERAÇÃO E DANO GENÉTICO EM CÉLULAS DESCAMADAS DA MUCOSA BUCAL EXPOSTA A CARCINÓGENOS E ADJACENTE A CARCINOMAS ESPINOCELULARES

Pesquisador: Fernanda Gonçalves Salum

Área Temática:

Versão: 2

CAAE: 38019514.0.0000.5336

Instituição Proponente: UNIAO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio
CNPQ

DADOS DO PARECER

Número do Parecer: 920.579

Data da Relatoria: 02/01/2015

Apresentação do Projeto:

O projeto PROLIFERAÇÃO E DANO GENÉTICO EM CÉLULAS DESCAMADAS DA MUCOSA BUCAL EXPOSTA A CARCINÓGENOS E ADJACENTE A CARCINOMAS ESPINOCELULARES é um trabalho de doutorado da Faculdade de Odontologia da PUCRS sob a orientação da Prof. Fernanda Gonçalves Salum.

Objetivo da Pesquisa:

Avaliar as células descamadas da mucosa bucal clinicamente normal e adjacente a carcinomas espinocelulares, as alterações citopatológicas, a atividade de proliferação celular, além dos danos genéticos,sugerindo um padrão citológico preditivo ao aparecimento do câncer bucal.

Avaliação dos Riscos e Benefícios:

Os possiveis riscos aos voluntários resultantes da pesquisa estão suficientemente descritos no projeto bem beneficios decorrentes.

Comentários e Considerações sobre a Pesquisa:

A pesquisa será desenvolvida selecinando 75 pacientes do sexo masculino, com idade superior a 40 anos,que serão distribuidos em tres grupos: Grupo I (controle): 25 pacientes sem lesões estomatológicas, semhistórico de tabajismo ou etilismo. Grupo II (fumo/alcool): 25 pacientes

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 920.579

tabajistas e etilistas mas que também não apresentem lesões na mucosa bucal. Grupo III (carcinoma espinocelular): 25 pacientes com carcinoma espinocelular bucal. Feita a coleta de dados dos tres grupos estes serão analisados com a finalidade de sugerir um padrão citológico preditivo ao aparecimento do câncer bucal.

Considerações sobre os Termos de apresentação obrigatória:
Toda a documentação foi apresentada satisfatoriamente.

Recomendações:
Não há recomendações.

Conclusões ou Pendências e Lista de Inadequações:
Não há pendências.

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:

PORTO ALEGRE, 18 de Dezembro de 2014

Assinado por:
Rodolfo Herberto Schneider
(Coordenador)

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ANEXO C

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO AOS VOLUNTÁRIOS DA PESQUISA

O senhor(a) está sendo convidado(a) a participar do estudo intitulado ***Proliferação e Dano Genético em Células Descamadas da Mucosa Bucal Exposta a Carcinógenos e Adjacente a Carcinomas Espinocelulares,*** que tem como objetivos avaliar alterações provocadas pelo álcool e pelo fumo nas células que revestem a boca.

Serão selecionados a participar do estudo pacientes fumantes e usuários de bebidas alcoólicas, além de não fumantes e não usuários de álcool, que não apresentam lesões na mucosa bucal. Será selecionado também um grupo de pacientes que apresentam lesão na mucosa bucal, com aspecto de carcinoma espinocelular, o tumor maligno mais comum da boca e que se origina das células de revestimento.

Se o Sr. concordar em participar, será solicitado um exame de laboratório para avaliar seu hemograma e glicemia. Além disso, será submetido à raspagem da mucosa bucal com uma pequena escova. Este procedimento é indolor e não invasivo. Não haverá custos ao participante quanto a esses procedimentos.

Nos pacientes que apresentam a lesão na mucosa bucal também será realizada biópsia parcial do tumor e coleta de um fragmento ao redor deste. Este procedimento cirúrgico consiste na remoção de um pequeno fragmento da lesão sob anestesia local para análise no microscópio, permitindo que seja estabelecido o diagnóstico. A inclusão de um pequeno fragmento ao redor da lesão não lhe trará prejuízos, pois o mesmo será englobado na cirurgia que o Sr. terá que realizar posteriormente para o tratamento desse tumor. Após o estabelecimento do diagnóstico, o paciente que apresentar neoplasia maligna será encaminhado a tratamento pelo Sistema Único de Saúde.

Nos pacientes que não apresentam lesões na mucosa bucal, caso sejam detectadas alterações das células analisadas, novas avaliações clínicas e laboratoriais serão realizadas dando seguimento ao acompanhamento desses individuos.

- Pelo presente consentimento informado, declaro que fui esclarecido de forma clara e detalhada, livre de qualquer forma de constrangimento e coerção, dos objetivos, da justificativa e dos procedimentos a que serei submetido pelo presente projeto de pesquisa.

- Fui igualmente informado:

* da garantia de receber resposta a qualquer pergunta ou esclarecimento a qualquer dúvida dos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa;

*da segurança de que não serei identificado e, que se manterá, o caráter confidencial das informações relacionadas com a minha privacidade;

* da liberdade de retirar meu consentimento, a qualquer momento, e deixar de participar do estudo, sem que isto traga prejuízo à continuação do meu cuidado e tratamento;

* do compromisso de proporcionar informação atualizada obtida durante o estudo.

O pesquisador responsável por esse projeto é a Profa. Dra. Fernanda Gonçalves Salum, tendo este documento sido revisado e aprovado pela Comissão Científica e de Ética da Faculdade Odontologia e pelo Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

Para qualquer esclarecimento ou dúvidas, antes e durante a pesquisa, entre em contato com a pesquisadora responsável Profa. Dra. Fernanda Gonçalves Salum (51) 8182-9945 ou com o Doutorando Ricardo Paiva (51) 9288-7263.

Caso necessário, o senhor (a) pode contatar o Comitê de Ética em Pesquisa da PUCRS, situado na Av Ipiranga 6690, prédio 60, sala 314, telefone: (51) 3320-3345. O horário de funcionamento do Comitê é de segunda a sexta das 8h às 12h e das 13h30min às 17h.

Pesquisador: _____.

Testemunha: _____.

Data: _____ Telefone: _____

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