FACULDADE DE ODONTOLOGIA

ANÁLISE CITOLÓGICA DA MUCOSA ORAL E CONCENTRAÇÕES SALIVARES E URINÁRIAS DE 1-HIDROXIPIRENO GLUCORONÍDEO EM TOMADORES DE CHIMARRÃO

LISIANE CÂNDIDO

2015

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

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CYTOLOGICAL ANALYSIS OF ORAL MUCOSA AND URINARY AND SALIVARY LEVELS OF 1-HYDROXYPYRENE GLUCURONIDE IN *CHIMARRÃO* DRINKERS

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Tese apresentada como requisito para obtenção do título de Doutor pelo Programa de Pós-Graduação em Odontologia, Área de Concentração Estomatologia Clínica, Faculdade de Odontologia, Pontifícia Universidade Católica do Rio Grande do Sul

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Porto Alegre

2015

C217a Cândido, Lisiane

Análise citológica da mucosa oral e concentrações salivares e urinárias de 1-hidroxipireno glucoronídeo em tomadores de chimarrão. / Lisiane Cândido. – Porto Alegre, 2014.

89 f. : il.

Tese (Doutorado) – Programa de Pós-Graduação em Odontologia, Faculdade de Odontologia, PUCRS.

Área de concentração: Estomatologia Clínica.

Orientadora: Profa. Dra. Karen Cherubini

1. Odontologia. 2. Estomatologia Clínica. 3. Citopatologia. 4. Cromatografia Líquida de Alta Performance. 5. Chimarrão. I. Cherubini, Karen. II. Título.

CDD 617.607

Ficha elaborada pela bibliotecária Anamaria Ferreira CRB 10/1494

Epígrafe

No meio do caminho tinha uma pedra
Tinha uma pedra no meio do caminho
Tinha uma pedra
No meio do caminho tinha uma pedra.
Nunca me esquecerei desse acontecimento
Na vida de minhas retinas tão fatigadas.
Nunca me esquecerei que no meio do caminho
Tinha uma pedra
Tinha uma pedra no meio do caminho
No meio do caminho tinha uma pedra.

Carlos Drummond de Andrade (1902 - 1987)

Dedicatória

À minha mãe, **Jucélia Nunes**, pelo exemplo de força e coragem, pela perseverança e incentivo aos estudos para a realização dos meus sonhos.

Agradecimentos

Agradeço ao meu marido, Eduardo Leandro Ignácio de Souza, pela compreensão, companheirismo, dedicação, amizade e confiança depositados em mim durante esta trajetória.

À minha filha, Laura Cândido de Souza, pelo carinho e amor incondicional que temos uma pela outra.

Aos meus irmãos, Jairo e Luciane, simplesmente por existirem e fazerem parte de mim, vocês dois são fortes exemplos para minha vida.

Aos meus cunhados, Rodrigo e Alexsandra, pelos churrascos, risotos, risadas e descontração durante o "curso".

Ao meu pai e a minha madrasta, Índio e Elenara. Por vocês fui muitas vezes fortalecida em momentos de fraqueza e não poderia deixar de lembrar a assessoria estatística e serviços de "baby sitting".

À minha sogra, Maria Ângela Zuchetto, pela valorização da minha busca pelo saber, pela presença durante a defesa da Dissertação de Mestrado e pelo amor que tens pela minha filha, obrigada!

Aos meus colegas de Pós-Graduação, Mariana Abreu, Juliana Cardoso, Victoria Trucci, Renata Boff, Felipe Martins, Jamil Saleh, Juliana Spanemberg, Ruchielli Borghetti, Clarissa Medeiros, Maria Noel, Vanessa Chidiac, Letícia Cuba, Monique Acauan, pelo companheirismo, coleguismo e amizade construídos durante o nosso Curso, mas especialmente a Victoria, Juliana Cardoso, Juliana Spanemberg, Mariana Abreu e Renata Boff agradeço pelos intermináveis "Momentos de Felicidade".

Ao meu amigo e professor Paulo Eduardo Kreisner, pela amizade, coleguismo e aprendizado em Implantodontia. Agradeço ainda por ter confiado a mim as tuas turmas de Pós-Graduação em Cirurgia Oral Menor e Implantodontia na docência das áreas conexas. Obrigada por todas as oportunidades, especialmente a de fazer parte do teu grupo.

Aos funcionários do Hospital São Lucas, amigos e pacientes, Cristiane Carloto, Antônio Luiz Ávila e Rosinéia Müller, pela amizade e confiança em mim depositadas.

Aos funcionários, Márcia Luisa Rollsing, Arthur Gazineu dos Santos, Lindomar Souza da Luz, pelo auxílio na execução dos procedimentos realizados no Ambulatório de Estomatologia.

Aos funcionários da Secretaria do Programa de Pós-Graduação, Kléber Melo, Davenir Brusch, Vanessa Xavier e Gabriel Jaques da Silva, pelo auxílio prestado durante o período do Doutorado.

Aos Professores Doutores Maria Martha Campos e Vinicius Duval da Silva e aos colaboradores Tiago Giuliani Lopes e Carlos Eduardo Leite, pela orientação, amizade e auxílio na execução dos procedimentos laboratoriais pertinentes à Tese.

Às Professoras Doutoras Maria Antonia Zancanaro de Figueiredo, Fernanda Salum e Liliane Yurgel por compartilharem seus conhecimentos e por viabilizarem aos alunos do Programa de Pós-Graduação uma excelente formação.

À minha orientadora, Professora Doutora Karen Cherubini, agradeço pela paciência, pela persistência, por ter acreditado no meu projeto e por ter-me guiado no caminho do término desta etapa. Obrigada pelos ensinamentos e pelo teu conhecimento.

Resumo

RESUMO

A carcinogênese é um processo complexo e multifatorial dependente de fatores que são inerentes ao indivíduo ou ambientais. A presença de hidrocarbonetos aromáticos policíclicos (HAPs) no meio ambiente tem sido apontada como um dos fatores de risco para o desenvolvimento de diferentes tipos de câncer, principalmente do trato aerodigestivo superior. O chimarrão é uma bebida quente à base de erva-mate que concentra HAPs incorporados à erva durante o processamento de secagem das folhas. Indivíduos com o hábito de tomar chimarrão estão expostos aos HAPs, exposição esta que pode ser mensurada por meio da quantificação de 1-hidroxipireno glucoronídeo (1-OHPG). O presente estudo teve por objetivo investigar alterações citomorfométricas do epitélio da mucosa oral em tomadores de chimarrão e correlacioná-las com os níveis salivares e urinários de 1-OHPG. Indivíduos adultos, de ambos os sexos e sem história de uso regular de álcool, foram distribuídos em quatro grupos: (1)=39 indivíduos tomadores de chimarrão, não-fumantes; (2)=25 fumantes tomadores de chimarrão, (3)=27 indivíduos fumantes não tomadores de chimarrão e (4)=27 indivíduos sem nenhum dos hábitos supracitados (grupo–controle). Amostras de citologia exfoliativa foram obtidas da mucosa do palato mole e mucosa jugal para avaliação qualitativa (Papanicolaou) e quantitativa (área nuclear, área citoplasmática e proporção núcleo/citoplasma). Amostras de saliva e urina foram coletadas para dosagem de 1-OHPG por meio de cromatografia líquida de alta performance (HPLC). Os resultados evidenciaram todas as amostras citológicas classificadas como classe I de Papanicolaou. As áreas nuclear e citoplasmática das células epiteliais do palato, bem como a proporção núcleo/citoplasma de ambos os sítios não diferiram significativamente entre os grupos. No grupo chimarrão, as células epiteliais da mucosa jugal exibiram área nuclear e área citoplasmática significativamente maiores que o grupo-controle. Foi observada correlação positiva entre área nuclear e citoplasmática, bem como entre concentrações salivares e urinárias de 1-OHPG.

Conclusão: Não foi observada associação do chimarrão com alterações citomorfométricas das células epiteliais do palato, ou com os níveis salivares e urinários de 1-OHPG. Embora área nuclear e citoplasmática tenham sido significativamente maiores no grupo chimarrão do que no controle, o presente estudo não permite inferir que essas alterações tenham tendência displásica.

Palavras-chave: carcinogênese; câncer; hidrocarbonetos aromáticos policíclicos; 1-hidroxipireno glucoronídeo; *Ilex paraguariensis;* cromatografia líquida de alta *performance;* citologia

Summary

SUMMARY

Carcinogenesis is a complex multifactorial process dependent on inherent to individual or environmental factors. Environmental polycyclic aromatic hydrocarbons (PAHs) have been pointed as a risk factor for different types of cancer, especially in upper aerodigestive tract. *Chimarrão* is a hot *maté* beverage containing PAHs that are incorporated to the herb during drying process of the leaves. Individuals that drink *chimarrão* are exposed do PAHs, which can be assessed by 1-hydroxypyrene-glucuronide levels (1-OHPG). The aim of the present study was to evaluate cytomorphometric alterations of oral mucosa epithelium in *chimarrão* drinkers correlating them to salivary and urinary levels of 1-OHPG. Adult males and females without history of regular alcohol use were allocated into 4 groups: (1)=39 *chimarrão* drinkers who did not smoke; (2)=25 *chimarrão* drinkers who smoked; (3)=27 smokers who did not drink *chimarrão*; and (4)=27 individuals who had neither of these habits. Mucosal scrapings were performed and subjected to qualitative (Papanicolaou) and quantitative (nuclear area, cytoplasmic area, nucleus/cytoplasm ratio) analysis. Urine and saliva samples were assayed for 1-OHPG by high-performance liquid chromatography (HPLC). All samples were classified into Papanicolaou class I. Nuclear and cytoplasmic areas of epithelial cells in soft palate smears did not significantly differ between the groups, whereas in buccal (cheek) mucosa they were significantly greater in the *chimarrão* group than in controls. The nucleus/cytoplasm ratio as well as salivary and urinary concentrations of 1-OHPG did not significantly differ. Urinary and salivary 1-OHPG concentrations were positively correlated to each other but they did not show any correlation with the cytometric variables. Nuclear and cytoplasmic areas were positively correlated to each other in either palate or buccal mucosa smears.

Conclusion: *Chimarrão* was associated with neither cytomorphometric alterations in epithelial cells of palate smears nor urinary and salivary 1-OHPG levels. Buccal smears showed higher nuclear and cytoplasmic area in the *chimarrão* group, but this result does not support an association with dysplasia.

Keywords: carcinogenesis; neoplasms; 1-hydroxypyrene-glucoronide; high performance liquid chromatography; cytology; *Ilex paraguariensis*.

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

A transformação de um tecido normal em neoplásico constitui processo complexo e multifatorial, que depende da exposição dos indivíduos a fatores de risco conhecidos. Tais fatores classificam-se em intrínsecos, como a susceptibilidade genética individual, e extrínsecos, em que o meio influencia a transformação tecidual. Dentre os fatores extrínsecos, destacam-se a exposição crônica à radiação solar, o tabagismo e o etilismo (WHO, 2011). A exposição crônica a alguns hábitos culturais de determinadas regiões geográficas, quais sejam, o tabaco mascado, nos Estados Unidos da América; a noz de Betel mascada, na Índia, e o Khat, na Península Arábica, podem atuar como indutores e/ou promotores da carcinogênese (Goldenberg *et al*., 2004).

O Sul da América Latina é uma região de clima propício à agricultura, onde a população predominantemente caucasiana explora economicamente o cultivo da planta *Ilex paraguariensis,* também conhecida como erva-mate, mate ou *yerba-maté*. Essa planta cresce, naturalmente ou por cultivo, na Argentina, no Paraguai, no Uruguai e nas regiões Sul e Centro-Oeste do Brasil e é usada para o preparo de bebidas como chimarrão, tererê, chás e refrigerantes. O consumo dessas bebidas também está crescendo nos EUA, no Canadá e na Europa (Filip *et al*., 2001).

O chimarrão é uma bebida quente à base de erva-mate que constitui hábito cultural amplamente difundido no Rio Grande do Sul, cuja associação com neoplasias malignas em diferentes sítios anatômicos tais como o trato respiratório superior, a boca, a orofaringe, a laringe e o esôfago tem sido questionada (Goldenberg *et al*.., 2004). Segundo Oreggia *et al*.. (1991), o chimarrão aumentaria em 2,5 vezes o risco de desenvolvimento de carcinoma de língua e, quando associado ao tabaco e/ou ao consumo de álcool, poderia aumentar entre três e cinco vezes o risco de desenvolvimento de carcinomas orais. Castellságue *et al.*

(2000) realizaram um estudo retrospectivo em cinco centros de referência para o tratamento de câncer de esôfago na América do Sul e sugeriram que quanto maior for a quantidade de chimarrão consumida, maior será a chance de desenvolvimento de câncer de esôfago.

A erva-mate possui componentes pró-carcinogênicos como hidrocarbonetos aromáticos policíclicos (HAPs) (Zuin *et al*., 2005; Kamangar *et al*., 2008; Vieira *et al*., 2010), que são incorporados a suas folhas durante o processamento, a partir da combustão de matéria orgânica (madeira) (Zuin *et al.*, 2005; Vieria *et al.*, 2010). Esses compostos, em sua maioria, são tóxicos e têm sido associados ao câncer do trato aerodigestivo superior (Roth *et al*., 2001). Um importante HAP carcinogênico encontrado na erva-mate é o benzopireno, cujo metabólito 1-hidroxipireno glucoronídeo (1-OHPG) é facilmente detectável na urina dos indivíduos expostos (Strickland *et al*., 1994). Estudos realizados com indivíduos saudáveis tomadores de chimarrão demonstram a associação entre o hábito e a presença do 1-hidroxipireno glucoronídeo (1-OHPG) na urina, e sugerem o envolvimento de bebidas derivadas da erva-mate (chimarrão e tererê) na alta incidência de câncer de esôfago na Região Sul do Brasil (Fagundes *et al.*, 2006; Abnet *et al.*, 2007; Kamangar *et al*., 2008). Por outro lado, há estudos que contestam o possível efeito carcinogênico da erva-mate, sendo que alguns deles apontam a alta temperatura da água usada no preparo do chimarrão como responsável pelo processo de carcinogênese, especialmente pela fase de promoção (Bastos *et al*., 2007; Sewran *et al*., 2003).

A citologia exfoliativa é uma manobra clínica que consiste na obtenção de células por meio de raspagem da superfície tecidual para posterior estudo citopatológico (Traut e Papanicolaou, 1943). O exame citopatológico, por sua vez, constitui técnica laboratorial de baixo custo que consiste em analisar células descamadas de superfícies teciduais, principalmente mucosas e pode revelar alterações celulares antes mesmo de sua

manifestação clínica (Rados *et al*., 1999). A partir da década de 40, com a coloração e classificação de Papanicolaou, a citologia exfoliativa começou a ser aplicada para diagnóstico precoce do câncer de colo de útero. O sucesso da técnica despertou pesquisadores para sua aplicação em outros sítios anatômicos, como a cavidade oral (Montgomery, 1951). A citomorfometria é um método que fornece informações sobre área citoplasmática e área nuclear, bem como sobre a relação entre ambas, o que possibilita a identificação de atipias celulares, como pleomorfismo nuclear e perda da proporção entre núcleo e citoplasma (Cowpe *et al*., 1988). O método de classificação de Papanicolaou tem sido complementado com a avaliação citométrica proposta por Cowpe *et al*. (1988). As avaliações dos parâmetros área citoplasmática, área nuclear e proporção núcleo/citoplasma aumentam a sensibilidade da técnica para o diagnóstico precoce do câncer de boca (Ogden *et al*., 1997). Além disso, a citologia exfoliativa constitui manobra clínica simples que obtém material biológico viável para outras análises tais como conteúdo de DNA celular e análises moleculares por meio de biomarcadores (Freitas *et al*., 2004).

Embora o hábito do chimarrão já tenha sido associado ao câncer de esôfago (Vassallo *et al.*, 1985; De Stefani *et al.*, 1990; Castelletto *et al.*, 1994; Pintos *et al.*, 1994; Rolon *et al.*, 1995; Sewran *et al.*, 2003; Szymańska *et al.*, 2010), sua participação na gênese do câncer de boca ainda constitui assunto controverso. O presente estudo tem por objetivo investigar se o hábito do chimarrão está associado a alterações citomorfométricas do epitélio da mucosa oral e correlacioná-las às concentrações salivares e urinárias do metabólito 1-hidroxipireno glucoronídeo. O trabalho está estruturado sob a forma de dois artigos científicos, sendo que o *artigo 1* apresenta uma revisão da literatura enfocando o papel dos HAPS na carcinogênese, e o *artigo 2* consiste na apresentação do experimento desenvolvido.

Artigo 1

2 ARTIGO 1

O artigo a seguir intitula-se ***Relevant topics on the relationship between polycyclic aromatic hydrocarbons and carcinogenesis*** e foi formatado de acordo com as normas do periódico ***Quality of Lyfe Research*** (Anexos A e B).

Relevant topics on the relationship between polycyclic aromatic hydrocarbons and carcinogenesis

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Running title: *Polycyclic aromatic hydrocarbons and carcinogenesis*

Keywords: carcinogenesis; polycyclic aromatic hydrocarbons; cancer; 1-hydroxypyrene-glucuronide

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds formed by hydrogen and carbon arranged in multiple aromatic rings. They are environmental contaminants derived from incomplete combustion of organic material and are commonly found in air, water, tobacco smoke, cooked food, and coal, among other sources. We present here a literature review focusing on the role of these compounds in the etiopathogenesis of cancer. Humans are exposed to PAHs during various professional and routine activities, which can be related to the development of tumors at different anatomic sites, especially in upper aerodigestive tract. Knowledge of the carcinogenic properties and mechanism of action of these compounds, as well as measures to minimize their effects on the human body, is essential to prevent cancer.

Keywords: Carcinogenesis; polycyclic aromatic hydrocarbons; cancer; 1-hydroxypyrene-glucuronide

Introduction

Carcinogenesis is a complex multifactorial process, which comprises three major steps: (a) initiation, (b) promotion and (c) progression [1]. Initiation corresponds to intracellular events where cell genotype alterations occur, determining malignant transformation. Promotion is characterized by phenotype formation and tumor cell survival. Progression involves the selection and growth of viable tumor cell clones capable of competing with normal cells [2]. Accordingly, the first sign of carcinogenesis is the disordered entry of cells into the cell cycle, and cancer cells develop from mutations that deregulate this cycle [3].

Inherent and environmental factors can be involved in carcinogenesis and are classified, respectively, into intrinsic and extrinsic factors. With regard to the environmental ones, the role of polycyclic aromatic hydrocarbons (PAHs) has been extensively discussed [4-17]. Toxicological studies report that animal exposure to PAHs results in toxicity to bone marrow [18], and cardiovascular [19,20] and reproductive [21] systems, as well as suppression of the immune system [22], with cancer being the major toxicological effect [5]. Such compounds have been associated with higher risk of developing malignancies of the lung [6], pancreas [7], bladder [8] and esophagus [9-17]. PAHs are activated to toxic and carcinogenic metabolites by the cytochrome P-450-monooxygenase system, and it is believed that extrahepatic expression of these enzymes plays a major role in the etiopathogenesis of respiratory and gastrointestinal tract cancer [23-25]. We present here a literature review focusing on important aspects of PAHs and their role in the carcinogenesis process.

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic compounds formed only by carbon and hydrogen atoms arranged in multiple aromatic rings [26], and originate from the incomplete combustion or pyrolysis of organic matter [27]. They are chemical pollutants found in air, water, food, fossil fuel, dyes, tobacco smoke, motor vehicle exhaust, waste, and also emissions from iron, steel and chemical factories [4]. For the general population, the major sources of exposure to PAHs are air and food [27]. These compounds can be absorbed by the respiratory and gastrointestinal tract and skin, and the majority of them are toxic. PAHs and their derivatives are classified into three groups according to their damage potential: group 1A, compounds carcinogenic to humans; group 2A, compounds probably carcinogenic to humans; and group 2B, compounds possibly carcinogenic to humans [4]. Fifteen of them show evidence of mutagenicity/toxicity in somatic cells *in vivo*, and they are benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, benzo[*a*]pyrene, chrysene, cyclopenta[*cd*]pyrene, dibenz[*a,h*]anthracene, dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,l*]pyrene, indeno[1,2,3-*cd*]pyrene and 5-methylchrysene [27]. These compounds do not act alone but rather as complex mixtures of diverse PAHs, promoting cell damage [28]. Because of their occurrence and also because they have shown clear carcinogenic effects in various types of bioassays in experimental animals, except for benzo[*ghi*]perylene, they are classified as priority contaminants [27,29].

PAHs include low-molecular weight or *small* compounds and high molecular-weight or *large* compounds, when they have respectively up to six or more than six aromatic rings [30]. The larger the number of aromatic rings in the chemical structure, the higher the PAH carcinogenic potential is to humans [4].

Mechanisms of action

To exert mutagenic and carcinogenic effects, PAHs need to be converted by cytochrome P450 enzymes (CYPs) into electrophilic metabolites, mostly PAH-diol-epoxide metabolite, whose reactivity with DNA varies widely [31]. Studies report their involvement in many steps of carcinogenesis, determining multiple simultaneous cellular events [31-34]. Hence, they are called complete carcinogens [4,35], which can exert their effects by indirect and direct pathways. PAHs produce reactive oxygen species (ROS) which cause cell membrane lipid peroxidation, altering both cellular permeability and architecture. Consequently, protein synthesis and exchanges with the extracellular environment are modified, allowing the entrance of substances that cause cell damage [36,37]. They can activate the intracellular arachidonic pathway, and this inhibits gap junctional intercellular communication (GJIC), favoring cell migration. Furthermore, the arachidonic acid pathway induces prostaglandin and leukotriene release, which in turn activates activator protein 1 (AP-1) transcription factor, proto-oncogene c-myc protein, and tumor necrosis factors (TNFs), stimulating cell proliferation. Also, after being activated by cytochrome P450 enzymes (CYP2A6/CYP2A13), PAH metabolites cause the formation of DNA adducts, which have been associated with emergence of *K-ras* mutations, where a proto-oncogene turns into an oncogene [7]. Studies show high rates of DNA mutation from G:C to A:T (Fig. 1). In general, such cell damage would be repaired, but in case of individual genetic propensity and long-term effects of carcinogenic products in the body, the defense system would be insufficient to limit the damage, allowing the formation of an altered cell clone that would proliferate and migrate. At this point, the tumor is established [36-39].

Contag [35] defends the hypothesis that PAHs are involved at two distinct moments of carcinogenesis initiation. These would be: (a) *initiation A*, in which cationic radicals and/or electrophilic metabolites of PAHs form covalent bonds with DNA bases resulting in

point mutations and promoting the activation of proto-oncogenes into oncogenes, such as *ras-like* oncogenes; (b) *initiation B*, where compounds from PAHs can change cell plasma membrane structure, causing changes in microviscosity and membrane fluidity. Such changes can modulate protein distribution and activity in the plasma membrane, which are major factors in the regulation of cellular proliferation. At first, those events would be reversible but the possible interaction between A and B initiation events has been considered, where the formation of initiation B complexes possibly has irreversible consequences if oncogenes are activated in the same cell and whose products (oncogenic proteins) act at the same site of the plasma membrane where B initiation complexes are found. It is possible that oncogenic proteins stabilize cell architecture alterations of B initiation by means of binding to or reacting with essential components, which leads to the irreversibility of the process.

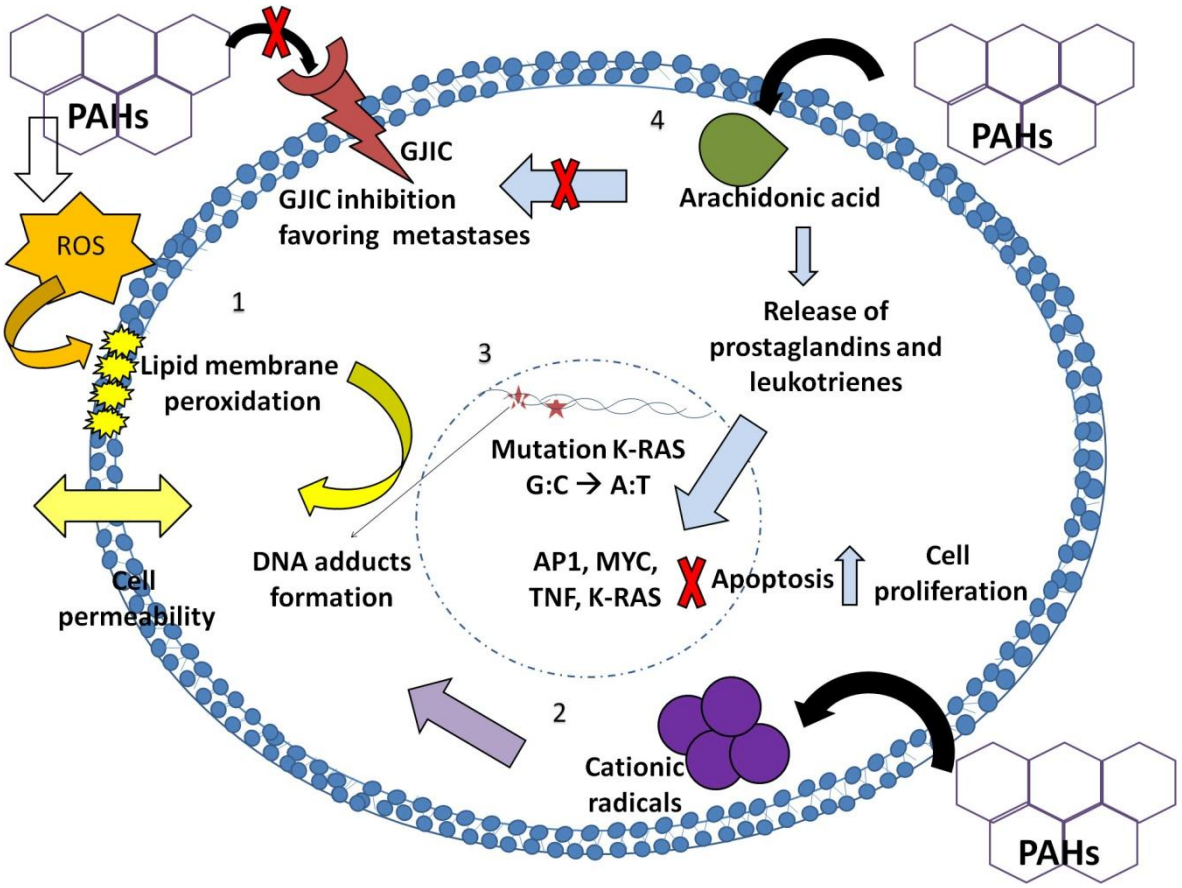

Fig. 1 Biomolecular alterations promoted by PAHs. PAHs lead to the production of reactive oxygen species (ROS), which cause lipid membrane peroxidation (1), changing cell architecture and protein exchanges between intra- and extracellular environments. Hydroxylated cationic radicals of PAHs enter the intracellular compartment and cause DNA adduct formation (2), which is associated with the emergence of *K-ras* mutations; G:C↦A:T mutations occur (3). PAHs activate the arachidonic acid pathway, releasing prostaglandins and leukotrienes (4), which inhibit tumor suppressor proteins, leading to apoptosis inhibition and cell proliferation. Also, PAHs inhibit gap junction intercellular communication (GJIC), either directly or through the arachidonic pathway, favoring neoplastic cell displacement and consequent metastasis.

Benzo[a]pyrene

Benzo[*a*]pyrene belongs to group 1A of PAHs, classified as carcinogenic to humans [4], and is composed of five aromatic rings with the chemical formula $C_{20}H_{12}$ [40]. Metabolic activation of this compound by cytochrome-P450 enzymes leads to the formation of benzo[*a*]pyrene diol epoxide and ROS, which cause DNA damage as well as DNA benzo[*a*]pyrene adduct formation [41], leading to carcinogenesis initiation [31]. Oxidative

stress induced by benzo[*a*]pyrene has effects on cell development, growth and survival by increasing lipid membrane peroxidation, oxidative damage to DNA and genetic mutation [32,41]. Hydroxylated cationic radicals of benzo[*a*]pyrene are the major agents involved in DNA adduct formation [38], which can represent the first step of initiation in carcinogenesis associated with PAHs [4].

Benzo[*a*]pyrene is the chemical most used to evaluate PAH toxicity, in either *in vivo* or *in vitro* studies, where it serves as a reference for investigating the toxicity of various PAH mixtures to which organisms have been exposed [28]. The major sources of benzo[*a*]pyrene, as with the other PAHs, are air, water, paints/dyes, tobacco smoke, vehicle emissions, iron and steel industry, chemical factories and waste [4]. It is also found in smoked/grilled/barbecued meat [42], and some reports have pointed to the presence of this contaminant in *yerba-maté* [43,44], black-tea [45], grain/cereals, vegetable oil, butter [46], embedded foods, toasted bread, potato chips and mashed potatoes [29], as well as kale and other vegetables [42].

1-Hydroxypyrene glucuronide (1-OHPG)

There are many sources of human exposure to PAHs, and 1-hydroxypyrene-glucuronide (1-OHPG) has served as a sensitive biomarker to evaluate the amount of such exposure [47]. That is because 1-OHPG is a stable metabolite derived from pyrene [13], which is excreted in the urine [48,49]. It is the non-hydrolyzed conjugated and detectable form of 1-hydroxypyrene, which determines recent exposure to PAHs [47], since its half-life ranges from 6 to 24 h [50]. 1-OHPG is a product formed from mammalian metabolism of pyrene and benzo[*a*]pyrene and their derivatives [51]. The presence and concentration of this metabolite can be evaluated by means of high performance liquid chromatography (HPLC). Many studies use urinary 1-OHPG as a biomarker of exposure to PAHs, as well as to correlate it to the risk of cancer development (Table 1). Accordingly, 1-OHPG seems

to be an effective and comprehensive biomarker for PAH exposure from both inhalation and ingestion [52]. Nevertheless, some concern has been noted as to the reliability of 1-OHPG to evaluate PAH exposure in epidemiologic studies of cancer, since many individual and environmental biases can interfere in the results of such investigation. Also, the absence of a gold standard method in this field makes it difficult to ascertain any misclassifications of results in studies using this biomarker, which still needs validation [53].

Reports on the relationship between PAHs and cancer

The association between PAH exposure and tumor development has been reported in the literature (Table 1). A cohort study conducted between 1918 and 1980 with Swedish chimney sweeps observed that these workers had higher risk of developing different types of cancer, possibly because of exposure to carcinogens from coal, coke and wood burning found in chimneys [9]. A follow-up of that study demonstrated once more that exposure to PAHs from toxic soot of chimneys fostered the development of cancer of the esophagus, lung, prostate and bladder in these workers [10]. According to a study that investigated the correlation between DNA adduct formation determined by PAHs and tumor mutation in SENCAR (sensitive to carcinogenesis) mice exposed to PAHs, depurinating adducts play a major role in PAH mutagenesis [54].

An association between exposure of asphalt-paving workers to PAHs and risk of bladder cancer was investigated in Denmark, Israel, Finland and Norway [8]. This historical cohort study followed paving workers from 1913 to 1999, over the technological evolution of asphalt (from components with 4 to components with 6 aromatic rings), matching them to bladder cancer records in those countries. Because of confounding

factors, it was not possible to conclude that PAHs from occupational exposure were responsible for bladder cancer in asphalt workers.

In a systematic review, Islami *et al.* [14] found that high temperature of beverages, such as tea, coffee and *chimarrão,* contributed to a high incidence of esophageal cancer. Nevertheless, they suggested that besides high temperature effects, there may be a concomitant effect of the chemical components in the genesis of such tumors. Afterwards, the authors investigated the association between the high incidence of esophageal cancer in Golestan Province (Iran) and occupational exposure to PAHs in different seasons of the year, analyzing urinary 1-OHPG levels and genetic polymorphisms [17]. They were not able to determine a cause/effect relationship between environmental PAH exposure and the high incidence of esophageal cancer because of confounding factors. On the other hand, Roth *et al.* [12] performed a study using similar methods and suggested that exposure to elevated levels of carcinogenic PAHs may be etiologically related to the high incidence of esophageal cancer in inhabitants of Linxian (China).

The ability of PAHs in *maté* beverages and tobacco to increase the risk of esophageal cancer was studied in southern Brazil's population [55]. Data obtained by means of questionnaires and quantification of urinary 1-OHPG suggested that exposure to PAHs from those sources increases the risk of esophageal squamous cell carcinoma. This idea was corroborated by Szymańska *et al*. [16].

Cioroiu *et al*. [6] investigated carcinogenic PAHs in lung tissue samples of patients with lung cancer and the association of these findings with ABO blood system phenotype, demographic status and smoking. There were high concentrations of carcinogenic PAHs in samples from individuals of A and O blood phenotypes. They also found higher levels of benzo[*a*]pyrene in samples from people who lived in urban areas compared to those from rural ones.

Abedi-Ardekani *et al.* [15] investigated the association between exposure to PAHs and esophageal cancer. Immune markers for BPDE (benzo[*a*]pyrene diol epoxide) were investigated in biopsy specimens of patients with esophageal squamous cell carcinoma and controls. In samples from esophageal cancer, only the non-tumor sites were considered for analysis. The authors found that PAHs and their metabolites were detectable in epithelial cells of the esophagus and that their levels were strongly associated with esophageal squamous cell carcinoma risk.

Wornat *et al.* [11] investigated PAHs in residual soot of wood ovens as a contributive factor to the high incidence of esophageal cancer in the population studied. They found mutagenic PAHs in soot from wood/coal burning, which suggests that these compounds contribute to esophageal cancer development. On the other hand, the association between PAH exposure and risk of colorectal cancer has not been confirmed [56].

Table 1 Reports on the relationship between polycyclic aromatic hydrocarbons (PAHs) and cancer

Subject	Study design/Method	Sample	Marker	Findings	Reference
Chimney soot and mortality cancer rates	Epidemiologic study Cohort	Swedish chimney sweeps	Mortality rate	Association between PAHs and high cancer mortality rates	Hogstedt *et al*. [9]
Chimney soot and mortality cancer rates	Epidemiologic study Cohort	Swedish chimney sweeps	Mortality rate	Association between PAHs and high prostate, bladder, esophageal and lung cancer mortality rates	Evanoff *et al*. [10]
PAHs and esophageal cancer	Cross-sectional Questionnaires HPLC	Non-smokers (Linxian-China) Urine	1-OHPG	Relationship between exposure to carcinogenic PAHs and esophageal cancer	Roth *et al*. [12]
PAHs and esophageal cancer	Descriptive HPLC	Soot deposits from the bottom surface of woks sitting on top coal-burning stoves	PAHs (20 benzenoid PAH, 6 fluoranthene benzologues, 1 cyclopentafused PAH, 1 indene benzologue, 3 oxygenated PAH, and 1 ring-sulfur-containing aromatic)	PAHs products may contribute to esophageal cancer development Two new compounds were identified: C24H14 napthol[1,2-*b*]fluoranthene and C30H16 tribenzo[*e,gh,i,k*]perylene	Wornat *et al*. [11]
PAHs and esophageal cancer	Case-control Questionnaires HPLC	*Maté* drinkers Urine	1-OHPG	Exposure to PAHs from tobacco and *maté* seems to contribute to increased risk of ESCC	Fagundes *et al*. [55]
PAH exposure in asphalt paving and bladder cancer risk	Cohort (1913–1999) Questionnaires Follow up	Asphalt workers	Relative risk (RR) Cancer incidence Estimated exposure to PAHs	Unable to find association between bladder cancer risk and exposure to PAHs	Burstyn *et al*. [8]
PAH-DNA adducts inducing mutations	*In vivo* study PCR	SENCAR mice treated with BPDE, BPDHD, anti-BPDE, DMBA, DB[*a,l*]P, anti-DB[*a,l*]PDE	H-ras mutations	Depurinating adducts play a major role in PAH mutagenesis	Chakravarti *et al*. [54]
PAHs and esophageal cancer	Case-control Cell culture Immunohistochemistry	Biopsied specimens from patients with ESCC and controls	BPDE-1	Evidence for a causal role for PAHs in esophageal carcinogenesis	Abedi-Ardekani *et al*. [15]

Subject	Study design / Method	Sample	Marker	Findings	Reference
Maté drinking and upper aerodigestive tract cancer	Case-control Questionnaires	Patients with upper aerodigestive tract cancer	Upper aerodigestive cancer and *maté* drinking rate	*Maté* drinking is associated with esophageal cancer, not because of hot temperature but probably because of carcinogens such as *N*-nitroso compounds and PAHs	Szymańska *et al*. [16]
Occupational exposure to HAPs and esophageal cancer	Cross-sectional Questionnaires HPLC PCR	Non-smokers from urban and rural areas (Iran) Urine, blood	1-OHPG Genetic polymorphisms	Unable to find association between exposure to PAHs and esophageal cancer risk	Islami *et al*. [17]
Smoking-induced genotoxicity and pancreatic carcinogenesis	Literature review	Not applicable	Not applicable	Correlation between cigarette smoking and pancreatic cancer risk, where the role of PAHs is pointed out	Momi *et al*. [7]
PAHs, lung cancer, demographic status and ABO phenotypes	Clinical study Questionnaires HPLC	Biopsied specimens from patients with lung cancer	PAHs [benzo(*a*)pyrene, benzo(*a*)anthracene, benzo(*b*)fluoranthrene, benzo(k)fluoranthrene]	High concentrations of carcinogenic PAHs in lung cancer biopsied specimens in patients with A and O blood phenotype from urban areas	Cioroiu *et al*. [6]
Risk of colorectal cancer and 1-OHPG	Case-control Questionnaires HPLC	Urine	1-OHPG	No association	Hofmann *et al*. [56]

HPLC= high performance liquid chromatography;1-OHPG= 1-hydroxypyrene glucuronide; ESCC=esophageal squamous cell carcinoma; PCR=polymerase chain reaction; SENCAR=sensitive to carcinogenesis; BPDE-1= benzo[*a*]pyrene diol epoxide antibody (clone 8E11); BPDHD= benzo[*a*]pyrene-7,8-dihydrodiol; anti-BPDE= antibody anti-benzo[*a*]pyrene diol epoxide; DMBA=7,12-dimethylbenz[*a*]anthracene; DB[*a,l*]P= dibenzo[*a,l*]pyrene; BPDE= benzo[*a*]pyrene diol epoxide

Final considerations

Cancer is the final result of numerous genotoxic injuries that cause DNA non-repaired mutations [57]. Genetic mutations can lead to the conversion of proto-oncogenes to onocogenes, causing disruption of cell cycle control and tumor suppressor gene inactivation, which allow neoplastic cell multiplication [3]. At first, DNA alterations are reversible, but depending on the cell damage degree, repair may not occur and then the altered cell clone population grows [2]. Chemical carcinogenesis needs an initiator product, capable of determining mutation, and a promoter agent, capable of spreading the preexistent mutation by stimulating cell replication [31]. Therefore, the more individuals are exposed to external chemical carcinogens, the higher the risk is of developing malignancies, even though genetic susceptibility is a determinant for cancer development.

PAHs are proven examples of carcinogens, which are present in the environment and to which population is exposed daily. Such compounds are constantly found in the air contaminated by vehicle emissions and the combustion of various organic products, or even in food. Exposure to them is frequent and constant, and people are usually not aware of that. Considering the carcinogenic potential of PAHs and their ubiquitous nature in the environment, it is crucial to develop further research to investigate the inherent risk of ingested food, habits, home and occupational environmental factors, aimed at minimizing population exposure to these carcinogens and promoting cancer prevention.

Acknowledgments

We thank Dr. A. Leyva (U.S.A.) for English editing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Artigo 2

3 ARTIGO 2

O artigo a seguir intitula-se **Cytomorphometry of oral mucosa epithelial cells and salivary and urinary 1-hydroxypyrene-glucuronide levels in *chimarrão* drinkers** e foi formatado de acordo com as normas do periódico *Oral Oncology* (Anexos C e D).

Cytomorphometry of epithelial cells of oral mucosa and salivary and urinary 1-hydroxypyrene-glucuronide levels in *chimarrão* drinkers

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Keywords: cytology; 1-hydroxypyrene-glucuronide; *chimarrão*; *maté*; oral cancer; oral mucosa

Running title: *Oral mucosa and chimarrão*

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ABSTRACT

Objective: To determine if *chimarrão* drinking is associated with cytomorphometric alterations of oral mucosa epithelial cells and to correlate these changes to 1-hydroxypyrene-glucuronide (1-OHPG) levels.

Material and methods: Adult males and females without history of regular alcohol use were allocated into 4 groups: (1)=39 *chimarrão* drinkers who did not smoke; (2)=25 *chimarrão* drinkers who smoked; (3)=27 smokers who did not drink *chimarrão*; and (4)=27 individuals who had neither of these habits. Mucosal scrapings were performed and subjected to Papanicolaou staining and cytomorphometric analysis. Urine and saliva samples were assayed for 1-OHPG by high-performance liquid chromatography (HPLC).

Results: Nuclear and cytoplasmic areas of epithelial cells in soft palate smears did not significantly differ between the groups, whereas in buccal (cheek) mucosa they were significantly greater in the *chimarrão* group than in controls. The nucleus/cytoplasm ratio as well as salivary and urinary concentrations of 1-OHPG did not significantly differ between the groups. Urinary and salivary 1-OHPG concentrations were positively correlated to each other but they did not show any correlation with the cytometric variables. Nuclear and cytoplasmic areas were positively correlated to each other in either palate or buccal mucosa smears.

Conclusion: *Chimarrão* was associated with neither cytomorphometric alterations in epithelial cells of palate smears nor urinary and salivary 1-OHPG levels. Buccal smears showed higher nuclear and cytoplasmic area in the *chimarrão* group, but this result does not support an association with dysplasia.

INTRODUCTION

Ilex paraguariensis, popularly known as *erva-maté*, *maté* or *yerba-maté*, is a plant commercially produced in South America. It grows naturally or by cultivation in Argentina, Paraguay, Uruguay and southern Brazil and is used in the preparation of beverages such as *chimarrão*, *tererê*, tea, and soda. Drinking *maté* beverages is a widely spread habit among populations of the southern region of South America, and the consumption of these beverages has been increasing in the USA, Canada and Europe [1].

Chimarrão is a hot beverage prepared with about 50 g of *yerba-maté* placed inside a dried calabash gourd called *cuia* and infused with water at 70 to 80°C. A *bomba*, which is a hollow metallic straw with a filter at one end, is positioned inside the herb and used to sip the beverage through it. It is a very common habit in southern Brazil, especially in the state of Rio Grande do Sul [2]. *Tererê*, in turn, is the cold version of the beverage, traditional in Argentina, Uruguay and Paraguay [3]. Paraguay, Argentina, Uruguay and Brazil consume, produce and export more than 300,000 tons of *yerba-maté* per year [2]. The plant contains bioactive compounds such as phenolic acids, flavonoids, saponins and caffeine, which can work as antioxidants in human body [4,5]. Nevertheless, it also has procarcinogen compounds such as polycyclic aromatic hydrocarbons (PAHs), and the high temperature of the water works as a solvent for chemical products that cause tissue damage such as tobacco carcinogens [6].

The association of *maté* with malignancies of different anatomic sites including upper respiratory tract, mouth, oropharynx, larynx, and esophagus has been discussed [2]. Oreggia *et al*.[7] investigated risk factors for tongue carcinoma development in Uruguay's population and found that *maté* drinking can increase the risk of squamous cell carcinoma of the tongue by 2.5-fold.

During manufacturing, *yerba-maté* leaves incorporate PAHs from incomplete combustion of wood used in the drying process [3,8,9]. Most of these compounds are toxic and have been associated with upper aerodigestive tract cancer [10]. Benzopyrene is an important carcinogenic PAH found in *yerba-maté*, whose major metabolite is 1-hydroxypyrene-glucuronide (1-OHPG). Studies performed with healthy *maté* drinkers showed an association between this habit and high levels of urinary 1-OHPG, suggesting the involvement of *maté* beverages (*chimarrão* and *tererê*) in the high incidence of esophageal cancer in southern Brazil [8,11,12]. 1-OHPG is found in the urine of people exposed to PAHs, representing the major marker of exposure to these contaminants [13-16].

Stefani *et al*. [17] conducted a study between 1990 and 2004 at the four major public hospitals of Montevideo (Uruguay), evaluating more than 13,000 patients (8,875 cases and 4,326 controls) with 13 different tumors. The authors found an association between *maté* drinking and cancer of the upper aerodigestive tract, esophagus, stomach, larynx, lung, uterine cervix, bladder and kidney. However, oral, pharyngeal, colon, rectum and breast cancer did not show any association with *maté* drinking. Bates *et al.* [18] evaluated 114 patients with bladder cancer paired with 114 healthy controls and also did not find any association with *maté* use. Nevertheless, other case-control studies have investigated the relationship between *maté* and oropharyngeal cancer [7,19-24], and all but one [20] found an association between *maté* drinking and increased risk of this malignancy. Moreover, several studies suggesting the involvement of *maté* drinking with higher risk for upper aerodigestive tract cancer comprise individuals who also smoke and drink alcohol. They estimated the adjusted relative risk considering these biases but did not isolate the variables into distinct groups according to strict inclusion and exclusion criteria [25-29].

Considering the controversies on this issue and also the proved participation of PAHs in carcinogenesis, as well as the need for disclosing possible cytological alterations of oral mucosa related to *maté* drinking, this study aimed to determine if *chimarrão*, the hot *maté* beverage, is associated with cytomorphometric alterations of epithelial cells of oral mucosa and to correlate them to salivary and urinary concentrations of 1-OHPG.

MATERIAL AND METHODS

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul. The sample comprised *chimarrão* drinkers and non-drinkers, 47 males and 71 females, ranging from 28 to 79 years of age (mean=54.48), who were allocated into 4 groups. Group 1 (n=39): *chimarrão* drinkers who did not smoke; group 2 (n=25): *chimarrão* drinkers who smoked; group 3 (n=27): smokers who did not drink *chimarrão*; and group 4 (control, n=27): persons who had neither of the two habits (*chimarrão,* tobacco). Inclusion criterion in group 1 (*chimarrão*) was drinking at least 1 L of *chimarrão* per day for 10 years or more [30]; in group 2, besides matching the *chimarrão* drinker criterion, they smoked at least 20 cigarettes per day for 10 years or more; in group 3 (smokers) they also smoked at least 20 cigarettes per day for 10 years or more. Exclusion criteria were: (1) regular alcohol intake (either daily or weekly) within the last 2 years; (2) use of alcohol-containing mouthwashes in the last 30 days prior to sample collection; (3) illicit drug use; (4) immunosuppressive conditions; (5) use of topical medicines on oral mucosa within 30 days before sample collecting. After explanation of the aims and procedures of the study, the participants signed a consent form. They then answered a questionnaire concerning personal data, habits, and medical history, and were subjected to clinical examination of the oral cavity and the subsequent procedures. The

final sample showed homogeneity in age and sex distribution of the individuals in the 4 groups.

Cytopathological scrapings

Mucosal scrapings were obtained by means of a conventional technique. First, a one minute mouthwash with water was done to remove food debris. The samples were then collected from soft palate and buccal (cheek) mucosa using a disposable cytobrush. The cytobrush was gently applied to the mucosa surface with 5 clockwise rotating movements at each site. Next, the brush was pressed on a microscope slide in rotating movements to spread the collected material on the slide surface, and the slides were immersed in 95% ethanol for fixation prior to Papanicolaou staining [31].

Capture and analysis of images

Images were captured using a Zeiss Axioskop 40 (Carl Zeiss, Oberkochen, Germany) light microscope with a 20x objective, connected to a videocamera (CoolSnap-Pro; Media Cybernetics, Bethesda, MD, USA) and to a microcomputer with Image Pro Capture Kit. Distinct fields (n=25 to 40) were captured in a standard manner over the whole slide, from left to right [32], until a minimum of 50 well-spread epithelial cells were obtained in each slide. Images were stored in uncompressed TIFF (Tagged Image File Format) and analyzed by a blind calibrated observer, using Image Pro Plus software 4.5.1 (Media Cybernetics). Calibration consisted of analyzing 10 slides, twice at two different moments, without knowing to which group each slide belonged. These two analyses were subjected to intraclass correlation coefficient, which showed $r = 0.965$.

Qualitative analysis of Papanicolaou smears

The smears were classified according to Papanicolaou cytopathological criteria [33]: (a) class 0 (insufficient or inadequate material for analysis); (b) class I (normal smear); (c)

class II (normal smear with inflammatory alterations); (d) class III (dysplastic alterations, suspicious smear); (e) class IV (strongly indicative, but not conclusive for malignancy); and (6) class V (malignancy).

Quantitative analysis of Papanicolaou smears

Fifty well-spread epithelial cells were quantitatively evaluated in each slide. Nuclear area (NA) and cytoplasmic area (CA) were measured using a specific tool in the Image Proplus software (Fig. 1). From these measures, the nucleus/cytoplasm ratio was also calculated.

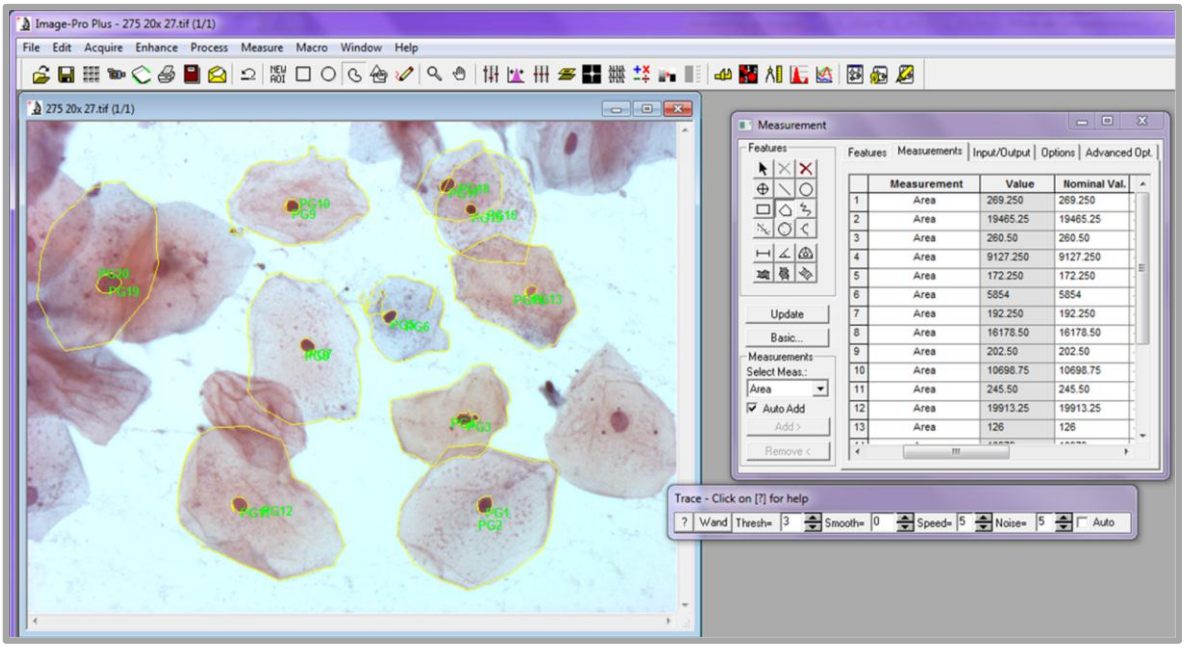

Figure 1: Nuclear and cytoplasmic areas in cytological smears of oral mucosa (Papanicolaou, 400x) in Image Pro Plus 4.5.1 (Media Cybernetics, Bethesda, MD, USA)

Salivary and urinary 1-OHPG

Urine and saliva samples were collected to determine 1-OHPG concentrations. Samples of total resting saliva were collected as described elsewhere [34], and urine samples according to a standard method [35]. Right afterwards, collected samples (saliva and urine) were frozen at -20ºC and stored until laboratory processing.

1-OHPG was assayed by high-performance liquid chromatography (HPLC) combined with fluorescence detector [36]. Samples were centrifuged at 10,000 rpm for 10 min (CELM Combate, Barueri, SP, Brazil), with saliva samples being centrifuged twice. Next, samples were diluted in acetate buffer, pH 5.0 (2 vol of buffer to 1 vol of sample), treated with 10 µL beta-glucuronidase (Sigma-Aldrich, St Louis, MO, USA), and incubated at 37°C for 2 h. Analyte extraction was done in (C18) solid phase extraction cartridges (Macherey-Nagel, Düren, Germany), previously conditioned with 2 mL of methanol and 5 mL of ultrapure water. After cartridge processing, samples were washed with 6 mL of 40 % methanol, and eluted with 2 mL of isopropanol. Samples were then evaporated, reconstituted with 200 µL of methanol and injected in a high-performance liquid chromatograph equipped with an isocratic pump, fluorescence detector, degasser, automatic injection system and computer loaded with ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The fluorescence detector was monitored with excitation at 242 nm and emission at 388 nm. Separation was carried out in a LiChrospher® 100 RP-18 (4.6x150 mm and 5 µm) column (Merck, Darmstadt, Germany) in a mobile phase consisting of methanol:acetonitrile:ultrapure water (35:35:30, v/v), at a flow rate of 1 mL/min.

Statistical analysis

Data were analyzed by means of descriptive and inferential statistics. Quantitative cytomorphometric variables and 1-OHPG were compared between the groups by using respectively ANOVA (complemented by Tukey test) and Kruskal-Wallis test. Spearman correlation coefficient was used to analyze the correlation between the variables. The analysis was performed in SPSS 17 (Statistical Product and Service Solution, Chicago, IL, USA), setting the level of significance at 5%.

RESULTS

Qualitative analysis in Papanicolaou

All samples analyzed were classified into class I (normal smear) of Papanicolaou (Fig. 2).

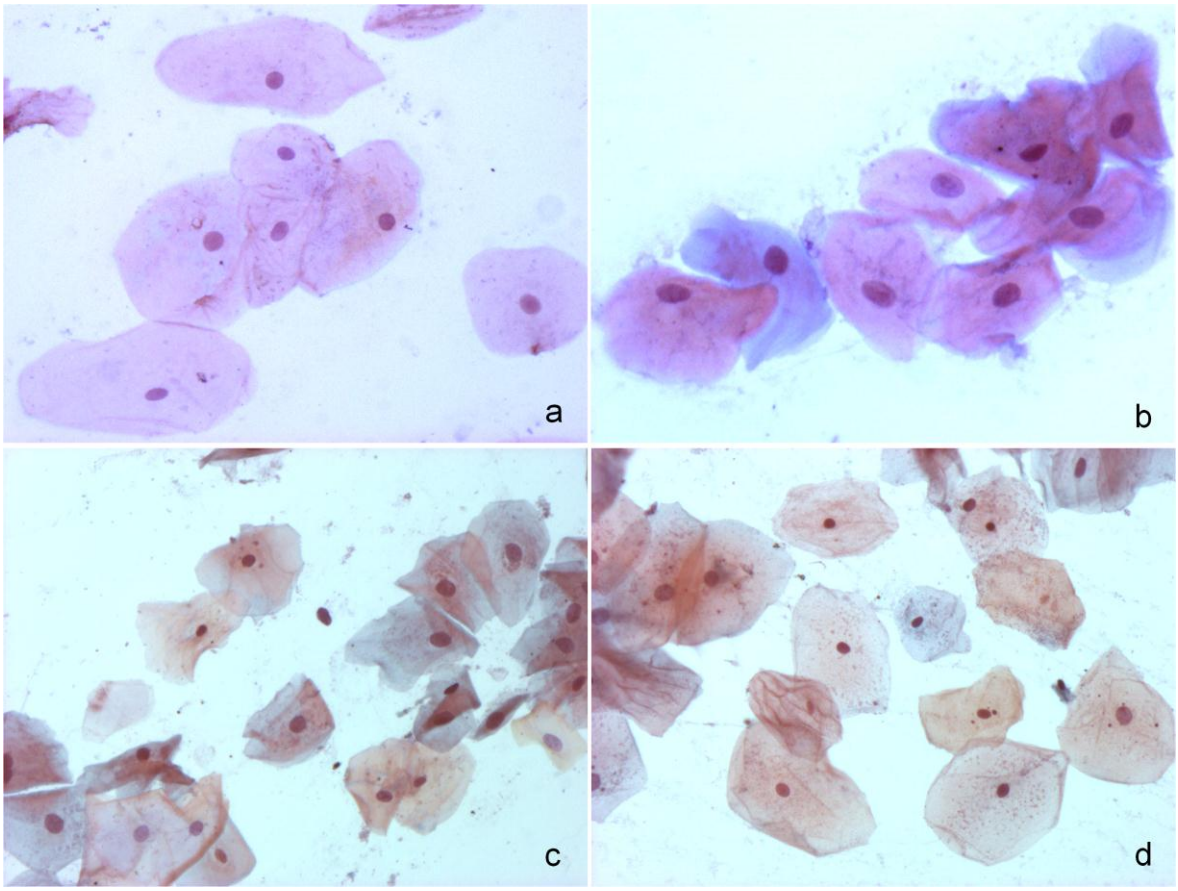

Figure 2 - Cytopathological examination. Papanicolaou staining (400 X) showing class I smears: (a) *chimarrão* group; (b) *chimarrão*/smoking group; (c) smoking group; (d) control group

Cytomorphometric analysis

Palate smears

Nuclear area (P=0.081), cytoplasmic area (P=0.407) and nucleus/cytoplasm ratio (P=0.676) of epithelial cells in palate smears did not significantly differ between the

groups analyzed, even though the *chimarrão* group showed the highest means for nuclear and cytoplasmic areas (ANOVA, Tukey test, Table 1).

Table 1 - Nuclear area, cytoplasmic area and nucleus/cytoplasm ratio of epithelial cells of palate smears in *chimarrão*, *chimarrão*/smoking, smoking and control groups

Group	Nuclear area (μm²)		Cytoplasmic area (μm²)		N/C ratio	
	Mean	SD	Mean	SD	Mean	SD
Chimarrão	72.88	18.96	2172.44	562.40	0.039	0.014
Chimarrão/smoking	62.41	21.10	1946.77	513.93	0.037	0.009
Smoking	69.20	14.90	2029.58	525.39	0.039	0.008
Control	63.86	14.97	2065.18	493.71	0.036	0.009
P*	0.081		0.407		0.676	

SD= standard deviation; N/C=nucleus/cytoplasm
**P* value for ANOVA, Tukey test, α=0.05

Buccal mucosa smears

Nuclear area of epithelial cells of buccal (cheek) mucosa was significantly higher in the *chimarrão* group than in controls (*P*=0.05), but no other significant differences were found between the groups for this variable (*P*>0.05). Cytoplasmic area of these cells was also significantly higher in the *chimarrão* group than in the control group (*P*=0.023), whereas it did not differ significantly between the other groups (*P*>0.05). The nucleus/cytoplasm ratio did not differ between the groups analyzed [*P*=0.097 (ANOVA, Tukey test, Table 2)].

Table 2 - Nuclear area, cytoplasmic area and nucleus/cytoplasm ratio of epithelial cells of buccal mucosa smears in *chimarrão*, *chimarrão*/smoking, smoking and control groups

Group	Nuclear area (μm²)		Cytoplasmic area (μm²)		N/C ratio	
	Mean	SD	Mean	SD	Mean	SD
Chimarrão	**72.40^{A}**	14.87	**2529.89^{A}**	553.18	0.033	0.009
Chimarrão/smoking	70.01^{AB}	16.94	2164.45^{AB}	629.17	0.038	0.009
Smoking	71.32^{AB}	14.24	2327.71^{AB}	570.09	0.036	0.008
Control	**62.46^{B}**	15.12	**2156.45^{B}**	459.40	0.033	0.009
P*	0.05		0.023		0.097	

SD= standard deviation; N/C=nucleus/cytoplasm
Means followed by different letters in a column showed a statistically significant difference.
**P* value for ANOVA, Tukey test, α=0.05

Salivary and urinary 1-OHPG concentrations

Salivary and urinary concentrations of 1-OHPG did not show any significant difference between the groups. Nevertheless, even though without statistical significance, either salivary or urinary 1-OHPG showed the highest values in the *chimarrão*/smoking group (Kruskal-Wallis, $P>0.05$, Table 3).

Table 3 - Salivary and urinary concentrations of 1-OHPG in *chimarrão*, *chimarrão*/smoking, smoking and control groups

Group	Saliva (ng/mL)			Urine (ng/mL)		
	Mean	SD	MD	Mean	SD	MD
Chimarrão	0.021	0.039	0.004	0.416	0.637	0.139
Chimarrão/smoking	0.028	0.032	0.021	0.984	1.376	0.436
Smoking	0.010	0.025	0.000	0.368	0.525	0.143
Control	0.012	0.017	0.000	0.270	0.307	0.219
P*		0.095			0.055	

SD=Standard deviation; MD=median
**P* value for Kruskal-Wallis test, $\alpha=0.05$

Correlations according to Spearman coefficient

In general analysis, urinary and salivary 1-OHPG levels were positively correlated to each other ($r=0.237$), but they did not show any correlation with the cytometric variables. Within the *chimarrão* group, salivary 1-OHPG was positively correlated with nuclear area in the palate ($r=0.444$) as well as with nuclear and cytoplasmic areas in buccal mucosa. In the smoking group, urinary 1-OHPG was inversely correlated with cytoplasmic area of epithelial cells of palate smears ($r=-0.475$). No other correlations were observed for 1-OHPG.

In general analysis, nuclear and cytoplasmic areas of epithelial cells were positively correlated to each other in either palate ($r=0.514$) or buccal mucosa ($r=0.520$) smears. Within groups, these correlations occurred in the *chimarrão* group [$r=0.431$(palate);

r=0.548 (buccal mucosa)] and *chimarrão*/smoking group [*r*=0.650 (palate); *r*=0.622 (buccal mucosa). In the smoking group, these two variables were correlated to each other in palate smears (*r*=0.636) but not in buccal mucosa ones, whereas in the control group, they did not show any correlation (Spearman correlation coefficient, α=0.05, Table 4).

Table 4 - "*r*" values in correlation analysis between the variables using Spearman coefficient

		Palate			**Buccal mucosa**			**1-OHPG**	
General		Nuclear area	Cytoplasmic area	N/C ratio	Nuclear area	Cytoplasmic area	N/C ratio	Saliva	Urine
Palate	Nuclear area	1	-	-	-	-	-	-	-
	Cytoplasmic area	0.514**	1	-	-	-	-	-	-
	N/C ratio	0.484**	-0.425**	1	-	-	-	-	-
Buccal mucosa	Nuclear area	0.683**	0.304**	0.404**	1	-	-	-	-
	Cytoplasmic area	0.429**	0.612**	-0.151	0.520**	1	-	-	-
	N/C ratio	0.112	-0.358**	0.470**	0.367**	-0.535**	1	-	-
1-OHPG	Saliva	0.054	-0.012	0.61	0.170	0.105	0.024	1	-
	Urine	-0.53	-0.73	0.116	-0.013	-0.121	0.094	0.237**	1
Chimarrão group									
Palate	Nuclear area	1	-	-	-	-	-	-	-
	Cytoplasmic area	0.431**	1	-	-	-	-	-	-
	N/C ratio	0.584**	-0.412**	1	-	-	-	-	-
Buccal mucosa	Nuclear area	0.724**	0.221	0.570**	1	-	-	-	-
	Cytoplasmic area	0.361*	0.504**	-0.069	0.548**	1	-	-	-
	N/C ratio	0.372*	-0.241	0.639**	0.453**	-0.397*	1	-	-
1-OHPG	Saliva	0.444**	0.153	0.308	0.506**	0.524**	0.012	1	-
	Urine	0.050	0.009	0.119	0.06	-0.017	0.103	0.124	1
Chimarrão/smoking group									
Palate	Nuclear area	1	-	-	-	-	-	-	-
	Cytoplasmic area	0.650**	1	-	-	-	-	-	-
	N/C ratio	0.439*	-0.242	1	-	-	-	-	-
Buccal mucosa	Nuclear area	0.649**	0.281	0.615**	1	-	-	-	-
	Cytoplasmic area	0.609**	0.592**	0.171	0.622**	1	-	-	-
	N/C ratio	-0.278	-0.550**	0.337	0.106	-0.660**	1	-	-
1-OHPG	Saliva	-0.067	-0.029	-0.125	0.153	0.120	-0.072	1	-
	Urine	0.140	0.092	0.147	0.217	-0.066	0.168	0.35	1

*Correlation is significant at the 0.05 level
**Correlation is significant at the 0.01 level
N/C= Nucleus/cytoplasm

		Palate			Buccal mucosa			1-OHPG	
Smoking group		Nuclear area	Cytoplasmic area	N/C ratio	Nuclear area	Cytoplasmic area	N/C ratio	Saliva	Urine
Palate	Nuclear area	1	-	-	-	-	-	-	-
	Cytoplasmic area	0.636**	1	-	-	-	-	-	-
	N/C ratio	0.165	-0.604**	1	-	-	-	-	-
Buccal mucosa	Nuclear area	0.557**	0.325	0.181	1	-	-	-	-
	Cytoplasmic area	0.359	0.708**	-0.510**	0.305	1	-	-	-
	N/C ratio	0.09	-0.372	0.571**	0.279	-0.720**	1	-	-
1-OHPG	Saliva	-0.136	0.184	-0.091	-0.224	-0.159	0.188	1	-
	Urine	-0.228	-0.475*	0.258	-0.236	-0.206	-0.044	0.300	1
Control group									
Palate	Nuclear area	1	-	-	-	-	-	-	-
	Cytoplasmic area	0.312	1	-	-	-	-	-	-
	N/C ratio	0.615**	-0.478*	1	-	-	-	-	-
Buccal mucosa	Nuclear area	0.643**	0.429*	0.132	1	-	-	-	-
	Cytoplasmic area	0.179	0.634**	-0.296	0.357	1	-	-	-
	N/C ratio	0.393*	-0.160	0.381*	0.618**	-0.394*	1	-	-
1-OHPG	Saliva	-0.097	-0.009	-0.058	-0.142	-0.025	-0.088	1	-
	Urine	0.095	0.283	0.011	0.030	0.103	-0.095	0.063	1

*Correlation is significant at the 0.05 level
**Correlation is significant at the 0.01 level
N/C= Nucleus/cytoplasm

DISCUSSION

In the present study, nuclear and cytoplasmic areas as well as nucleus/cytoplasm ratio of epithelial cells in palate and buccal smears were evaluated and compared between the groups, looking for signs of cell atypia. DNA may affect the nuclear volume because nuclear size would be directly proportional to the amount of DNA it contains and the extent to which that DNA is compacted [37-39]. Therefore, nuclear size alterations can be related to DNA content and activity, where dysplastic cells tend to have enlarged nuclei [40,41] and consequently lose the normal nucleus/cytoplasm ratio [39-41]. In this way, cell atypia could be presumed indirectly and early by means of cytometric changes [41], where enlargement of nucleus is the most important feature [42]. Anyway, in our results, palate smears did not show any significant cytometric alterations in either the nucleus or cytoplasm of epithelial cells, and therefore neither in the nucleus/cytoplasm ratio, which suggests *chimarrão* does not cause any atypia in these cells. Buccal mucosa smears, in turn, had some significant results, where epithelial cells in the *chimarrão* group showed greater nuclear and cytoplasmic areas compared to controls. It seems at first that buccal mucosa would be more prone than palate mucosa to suffer the effects of *chimarrão*, and also that these effects would represent cell damage. However, it is necessary to pay attention to the nucleus/cytoplasm ratio, which did not show any significant difference, suggesting that changes undergone by nucleus were accompanied by proportional changes in cytoplasm. The enlargement in these two cytological structures occurring in the same direction, as shown by the positive Spearman correlation coefficient, kept the nucleus/cytoplasm ratio from showing any significant alteration. Therefore, a physiological rule has been respected: nuclear and cytoplasmic volumes are somehow related to each other, a phenomenon referred to as the

karyoplasmic ratio [39,43], which is important for the maintenance of cell physiology [39].

According to Rashid and Haque [44] marked nuclear changes are suggestive of malignancy, whereas mild nuclear enlargement could be indicative of a regenerative process. In this way, one could assume that cytometric changes associated with *chimarrão* in our study would be regenerative instead of dysplastic, which would be corroborated by both the qualitative analysis of Papanicolaou smears we performed, which showed all samples classified into class I, and the positive nucleus/cytoplasm correlation. Moreover, such effects could be attributed to the reported beneficial properties of *yerba-maté* [45-49], which include DNA damage repair [49] and oxidative stress inhibition [45,46]. Actually, *yerba-maté* contains various bioactive compounds, where it is a rich source of phenolic acids, especially chlorogenic ones, which are potent antioxidant agents, and also purine alkaloids and triterpenoid saponins [47].

Still, in analyzing the cytometric results, it is curious that the *chimarrão* group had the highest nuclear area followed by the smoking group, albeit not significantly, whereas the *chimarrão*/smoking group had a nuclear area closer to control. It seems intriguing that the group combining the two injury factors was the one with the lowest nuclear and cytoplasmic areas. This finding could suggest some kind of interaction between *chimarrão* and smoking, other than synergistic injury effect that would be expected. Maybe this could be in line with the reported beneficial properties of *yerba-maté* [45-49].

Another point that deserved comment in cytometric analysis is that the smoking group did not show any significant difference when compared to either the *chimarrão* group or controls. Tobacco also caused no significant changes in buccal mucosa in our sample. This seems at first curious, since tobacco has been long recognized as a major

carcinogen in oral cancer [50-52]. However, it is important to emphasize that our sample did not have alcohol as an intervening variable, since the regular use of alcohol, including alcoholic-mouthwashes, was an exclusion criterion in our sample. Thus, this finding seems to reinforce the role of an increase in mucosa permeability and in solubility of tobacco carcinogens promoted by alcohol favoring tobacco carcinogenic effects [53,54]. Nevertheless, it is also important to consider if a larger amount of cigarettes smoked daily and especially over more years would have produced different results.

Salivary 1-OHPG levels were very low among the groups analyzed, whereas urinary concentrations showed values consistent with those reported in the literature. Although without statistical significance, both saliva and urine 1-OHPG were higher in the *chimarrão*/smoking group. It seems plausible that the association between *chimarrão* and smoking increased the levels of 1-OHPG more than two-fold if compared to the isolated habit (only smoking or only *chimarrão*). This finding corroborates the reports on the presence of benzopyrene in both tobacco smoke [11,55,56] and *yerba-maté* [8,11], and consequently the occurrence of higher levels of 1-OHPG in *chimarrão* drinkers as well as in smokers [11].

The absence of significant differences in either salivary or urinary levels of 1-OHPG between the groups and their lack of correlation with cytometric alterations in general analysis suggests that this metabolite is not related to the cytometric findings. That is, if *chimarrão* was capable of inducing such effects on epithelial cells, that was probably not related to 1-OHPG or, indirectly, to benzopyrene. Also, the very low values of 1-OHPG in saliva corroborate this idea and suggest saliva is not an important reservoir for this metabolite. In fact, 1-OHPG is a benzopyrene metabolite found in urine after metabolism in the liver. Nevertheless, it was demonstrated that PAHs can be

locally activated by extra-hepatic cytocrome P450 enzymes in the aerodigestive tract [57,58], without systemic absorption and passage through the liver [8]. Therefore, it would be reasonable to look for this metabolite in saliva. Moreover, from the current literature, it is not clear whether 1-OHPG is a harmful substance in itself or whether the adverse effects depend on the parent compound (i.e., pyrene or the PAHs) [59]. Anyway, within the *chimarrão* group, we found a positive correlation between nuclear and cytoplasmic areas and salivary 1-OHPG. Regarding this result, it is important to recall that HPLC, the method used to measure 1-OHPG, can detect the smallest traces of PAHs but it lacks high specificity, which may compromise the accuracy when a substance is found in very low concentrations [59]. Eventually, we have to consider that there are environmental sources of benzopyrene other than *chimarrão* to which people are routinely exposed, including food [55,56], and also 1-OHPG levels can vary inter-individually according to CYP polymorphisms [12]. All these factors could have influenced our results. On the other hand, comparing the urinary 1-OHPG values between the groups and considering their *P* value, it is possible that a larger sample size would have made the differences statistically significant.

Our study cannot support the involvement of *chimarrão* drinking in dysplastic alterations of oral mucosa epithelial cells. Accordingly, Stefani *et al*.[17] found that cancers of the mouth, pharynx, colon, rectum, and female breast were not associated with *maté* consumption. Previous studies have attributed the carcinogenicity of *maté* to thermal injury, chemical components, or both. Also, tobacco smoking could be a powerful confounder of *maté* consumption [8]. We tried to avoid the biases represented by tobacco and alcohol. However, clinical studies are difficult to control intervening variables. Not only the amount and temperature of *chimarrão* but also different *yerba-maté* brands differing in origin, with wide variability of technology applied in cultivation

and manufacturing process, could also interfere with the properties of *yerba-maté* and consequently with the results obtained.

In conclusion, according to our results, *chimarrão* was associated with neither cytomorphometric alterations in epithelial cells in palate smears nor urinary and salivary 1-OHPG levels. Buccal smears showed higher nuclear and cytoplasmic area values in the *chimarrão* group, but this result did not support an association with dysplasia. Further studies investigating *chimarrão*/cell interactions at the molecular level are needed.

ACKNOWLEDGMENTS

This work was supported by grants from FINEP Research Grant *Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS* (PUCRSINFRA) # 01.11.0014-00. We also thank Dr. A. Leyva (USA) for English editing of the manuscript.

CONFLICT OF INTEREST

None declared.

The authors declare there is no conflict of interest related to this work.

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Discussão Geral

4 DISCUSSÃO GERAL

O câncer constitui tema desafiador à ciência e mobiliza pesquisas nas mais diversas áreas em busca de esclarecimentos sobre sua etiopatogênese, com vistas à otimização de alternativas preventivas, diagnósticas e terapêuticas. Nesse contexto, a identificação e eliminação de fatores de risco ambientais associados à doença é um dos caminhos possíveis. Os hidrocarbonetos aromáticos policíclicos (HAPs) são contaminantes onipresentes no ambiente, muitos deles classificados como carcinogênicos, provavelmente carcinogênicos, ou possivelmente carcinogênicos (IARC, 2010). Esses compostos são facilmente absorvidos pelo trato respiratório, gastrointestinal e pele e têm sido associados ao desenvolvimento de câncer em diversos sítios anatômicos (Evanoff *et al*., 1993; Roth *et al*., 2001; Burstyn *et al*., 2007; Cioroiu *et al*., 2013). Em função de exibir HAPs em sua composição, especialmente o composto benzopireno, a erva-mate, empregada no preparo do chimarrão, tem sido apontada como um fator de risco ao câncer de esôfago (Castellságue *et al*., 2000; Fagundes *et al*., 2006; Abnet *et al*., 2007; Kamangar *et al*., 2008), orofaringe e boca (Oreggia *et al*., 1991; Goldenberg *et al*., 2004). Entretanto, o tema é controverso, e a literatura prescinde de estudos que investiguem o papel de bebidas à base de erva-mate na carcinogênese oral. Tais fatos motivaram a realização da presente pesquisa, que investigou alterações citomorfométricas da mucosa oral e sua relação com os níveis de 1-hidroxipireno glucoronídeo em tomadores de chimarrão.

Os resultados obtidos evidenciaram ocorrência de área nuclear e citoplasmática significativamente maiores nas células epiteliais da mucosa jugal no grupo chimarrão do que no controle, entretanto sem alterações significativas da proporção núcleo-citoplasma. Nas amostras do palato, não foram observadas quaisquer diferenças

significativas entre os grupos, embora o grupo chimarrão tivesse exibido os maiores valores para as áreas de núcleo e citoplasma também neste sítio anatômico. As maiores áreas nuclear e citoplasmática no grupo chimarrão sugerem que a bebida exerça algum efeito sobre a estrutura celular. Por outro lado, a natureza do mesmo (inócua, dano ou reparo celular) e sua relação com componentes químicos específicos da erva-mate, benéficos (Gugliucci; Stahl, 1995; Filip *et al*., 2001; Bastos *et al*., 2007; Miranda *et al*., 2008) ou carcinogênicos (Zuin *et al*., 2005; Vieira *et al*., 2010), ou ainda com a elevada temperatura da bebida (Islami *et al*., 2009) não podem ser determinadas pelo presente estudo. A avaliação citomorfométrica constitui ferramenta para a identificação precoce de atipias celulares, em que o aumento volumétrico do núcleo pode representar tendência à displasia celular (Wilton, 1997; Ramaesh *et al.*, 1998; Webster *et al*., 2009). Porém, no presente estudo, o aumento da área nuclear foi acompanhado de aumento citoplasmático, o que manteve a proporção núcleo/citoplasma. Tal achado também põe em dúvida o caráter displásico das alterações observadas.

As concentrações de 1-OHPG não exibiram correlação com as alterações citométricas, enquanto área nuclear e área citoplasmática estiveram positivamente correlacionadas entre si, bem como os níveis de 1-OHPG salivar e urinário. Tais achados não permitem inferir que o metabólito esteja associado às alterações citométricas do epitélio oral e, considerando-se a possibilidade de algum efeito carcinogênico, outros compostos como o benzopireno-diol-epóxido (BPDE) deveriam ser investigados. Por outro lado, há que se ponderar que fatores como tamanho reduzido da amostra e variáveis intervenientes como dieta dos indivíduos, perfil genético, características específicas de diferentes procedências e processamentos da erva-mate, bem como limitações técnicas podem ter influenciado os resultados obtidos.

Embora sem significância estatística, um achado intrigante que contrariou as expectativas no presente estudo foi o fato de o grupo chimarrão/tabagista ter exibido os menores valores de área nuclear e citoplasmática. Uma vez que o tabaco é um agente carcinogênico reconhecido (Du *et al*., 2007; Benowitz *et al*., 2012; Krishna *et al*., 2014), esperava-se encontrar um efeito sinérgico decorrente de sua associação com o chimarrão e, consequentemente, alterações celulares relevantes. Tal achado abre margem à discussão proposta por alguns autores de que o chimarrão teria efeitos benéficos provenientes dos componentes bioativos da erva-mate como os flavonoides, as xantinas, as saponinas, os ácidos fenólicos e a cafeína, que funcionariam como antioxidantes, antimutagênicos e protetores celulares (Gugliucci; Stahl, 1995; Filip *et al*., 2001; Bastos *et al*., 2007; Miranda *et al*., 2008). Outro achado inesperado foi o fato de que as células provenientes do grupo tabagista não demonstraram alteração quando comparadas às células dos grupos chimarrão e controle. A justificativa que parece pertinente para tal resultado recai na criteriosa seleção da amostra, que excluiu da pesquisa indivíduos que tivessem hábito de ingerir bebida alcoólica ou que usassem colutórios à base de álcool. O achado, por sua vez, corrobora os relatos da literatura de que a ação carcinogênica do tabaco é favorecida por efeitos do álcool como aumento de permeabilidade da mucosa e aumento da solubilidade dos carcinógenos (Du *et al*., 2000; Figuero Ruiz *et al*., 2004).

A ingestão e a inalação habituais de substâncias químicas por parte do indivíduo, seja por meio de produtos alimentícios ou por hábitos culturais como o tabaco, bebidas ou drogas, repercute em sua constituição orgânica. Tal repercussão dependerá das propriedades de tais substâncias e das interações que estabelecerão com o organismo, inclusive com a estrutura celular. Os achados citométricos significativos verificados na mucosa jugal do grupo chimarrão sugerem que este seja capaz de determinar alterações no epitélio com maior probabilidade de ocorrência na mucosa jugal do que no palato.

Entretanto, a qualidade e o grau dessas mudanças na estrutura da célula epitelial ainda devem ser determinados. Seguindo na linha metodológica da citologia exfoliativa, a atividade nuclear poderia ser investigada nessas amostras por meio da técnica de AgNORs (*argyrophilic nucleolar organizer region*), e a aplicação da imunocitoquímica com o marcador anti-BPDE poderia identificar HAPs diretamente na estrutura celular. Novas pesquisas em nível molecular também se fazem necessárias para descrever as interações e os efeitos do chimarrão sobre as células epiteliais da mucosa oral e definir se o mesmo exerce efetivamente papel carcinogênico nesse sítio.

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Anexos

ANEXO A

Comprovante de submissão do manuscrito **Relevant topics on the relationship between polycyclic aromatic hydrocarbons and carcinogenesis** ao periódico *Quality of Life Research.*

Submission Confirmation

em.qure.0.3f8fff.365875b8@editorialmanager.com em nome de Carolien van der Gaag [em@editorialmanager.com]
Enviado: quarta-feira, 3 de dezembro de 2014 7:41
Para: Karen Cherubini

Dear Dr. Cherubini,

Thank you for submitting your manuscript,
"Relevant topics on the relationship between polycyclic aromatic hydrocarbons and carcinogenesis", to Quality of Life Research

During the review process, you can keep track of the status of your manuscript by accessing the following web site:

http://qure.edmgr.com/

If your manuscript is accepted for publication in Quality of Life Research, you may elect to submit it to the Open Choice program. For information about the Open Choice program, please access the following URL:
http://www.springer.com/openchoice

With kind regards,

Carolien van der Gaag
Editorial Office Quality of Life Research
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P.O. Box 990
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Telephone: +31 78 6576901

Now that your article will undergo the editorial and peer review process, it is the right time to think about publishing your article as open access. With open access your article will become freely available to anyone worldwide and you will easily comply with open access mandates. Springer's open access offering for this journal is called Open Choice (find more information on www.springer.com/openchoice). Once your article is accepted, you will be offered the option to publish through open access. So you might want to talk to your institution and funder now to see how payment could be organized; for an overview of available open access funding please go to www.springer.com/oafunding. Although for now you don't have to do anything, we would like to let you know about your upcoming options.

ANEXO B

Normas para submissão de manuscritos ao periódico *Quality of Life Research*

http://www.springer.com/medicine/journal/11136

ANEXO C

Comprovante de submissão do manuscrito **Cytomorphometry of oral mucosa epithelial cells and salivary and urinary 1-hydroxypyrene-glucuronide levels in *chimarrão* drinkers** ao periódico *Oral Oncology*.

Submission Confirmation

ees.oo.0.2dc19f.6083b3b4@eesmail.elsevier.com em nome de O Oncology Editorial Office [ooncology@elsevier.com]
Enviado: sábado, 6 de dezembro de 2014 21:52
Para: Karen Cherubini; karencherubibi66@gmail.com; Karen Cherubini

Re: "Cytomorphometry of epithelial cells of oral mucosa and salivary and urinary 1-hydroxypyrene-glucuronide levels in chimarrão drinkers"
Lisiane Candido, MSc; Carlos E Leite, PhD; Maria M Campos, PhD; Maria A Figueiredo, PhD; Fernanda G Salum, PhD; Vinicius D Silva, PhD; Tiago G Lopes, AS; Karen Cherubini, Ph.D.
Original Research article

Dear Prof. Cherubini,

Your submission entitled "Cytomorphometry of epithelial cells of oral mucosa and salivary and urinary 1-hydroxypyrene-glucuronide levels in chimarrão drinkers" has been received by journal Oral Oncology

You will be able to check on the progress of your paper by logging on to Elsevier Editorial Systems as an author. The URL is http://ees.elsevier.com/oo/.

Your username is: karen.cherubini@pucrs.br
If you need to retrieve password details please go to:
http://ees.elsevier.com/oo/automail_query.asp

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Elsevier Editorial System
Oral Oncology

ANEXO D

Normas para submissão de manuscritos ao periódico *Oral Oncology*.

http://www.elsevier.com/journals/oral-oncology/1368-8375/guide-for-authors

ANEXO E

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 30 de Novembro de 2011

O Projeto de: Tese

Protocolado sob nº: 0073/11

Intitulado: Análise citológica da mucosa oral e concentrações salivares e urinárias de 1 - hidroxipireno glucoronídeo em tomadores de chimarrão.

Pesquisador Responsável: Profa. Dra. Karen Cherubini

Pesquisadores Associados: Lisiane Cândido; Dra. Maria Martha Campos; Carlos Eduardo Leite

Nível: Tese / Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *30 de Novembro de 2011.*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS.

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 210
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900
Fone/Fax: (51) 3320-3538
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ANEXO F

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF. CEP-1974/11

Porto Alegre, 26 de dezembro de 2011.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 11/05695 intitulado **"Avaliação citológica da mucosa oral e concentrações salivares e urinárias de 1-hidroxipireno glucoronídeo em tomadores de chimarrão"**.

Salientamos que seu estudo pode ser iniciado a partir desta data.

Os relatórios parciais e final deverão ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. Rodolfo Herberto Schneider
Coordenador do CEP-PUCRS

Ilma. Sra.
Profa. Karen Cherubini
FO
Nesta Universidade

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www.pucrs.br/prppg/cep

ANEXO G

GRUPO: (1)CHIMARRÃO (2)CHIMARRÃO+FUMANTES

(3)FUMANTES (4)CONTROLE

FICHA PADRÃO DE IDENTIFICAÇÃO PACIENTE NÚMERO: _____

NOME:_____

DATA DE NASCIMENTO: _____ IDADE: _____ SEXO: ( ) FEMININO ( ) MASCULINO

ENDEREÇO: _____

BAIRRO: _____ CIDADE: _____UF____

TELEFONES: _____ RG/CPF: _____

PROCEDÊNCIA: _____

PROFISSÃO: _____

CHIMARRÃO? ( ) NÃO ( ) SIM –TEMPO (anos) _____ x/DIA _____ QUANTIDADE (L) _____

CIGARRO? ( ) NÃO ( ) SIM – TEMPO (anos) _____ x/DIA _____ ( )SEM FILTRO ( )COM FILTRO

ÁLCOOL ETÍLICO? ( ) NÃO ( ) SIM – TEMPO (anos)____ x/DIA _____ QUANTIDADE (mL) _____

DESTILADO ( ) FERMENTADO ( )

COLUTÓRIO BUCAL? ( )NÃO ( ) SIM ( ) JÁ USOU – QUAL? _____ x/DIA _____ TEMPO _____ QUANDO PAROU? _____

USUÁRIO DE DROGAS ILÍCITAS? ( ) NÃO ( ) SIM ( ) JÁ USOU – QUAL? _____ x/DIA _____ TEMPO _____ QUANDO PAROU? _____

TRATAMENTO PARA O CÂNCER? ( ) NÃO ( ) SIM – QUAL? _____

( ) QUIMIOTERAPIA ( ) RADIOTERAPIA ( ) OUTRO _____

TRANSPLANTADO? ( ) NÃO ( ) SIM DROGAS IMUNOSSUPRESSORAS? ( ) NÃO ( ) SIM

PACIENTE IMUNOSSUPRIMIDO? ( ) NÃO ( ) SIM

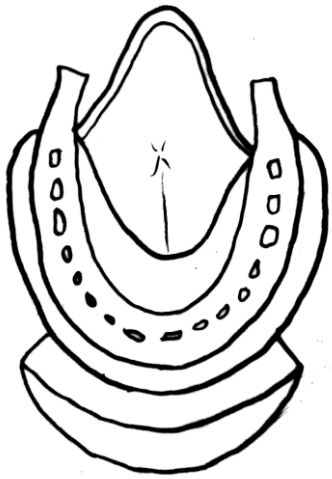
MEDICAMENTO TÓPICO ORAL? ( ) NÃO ( ) SIM ( ) JÁ USOU - QUAL?_____ x/DIA? _____ TEMPO _____ QUANDO PAROU? _____

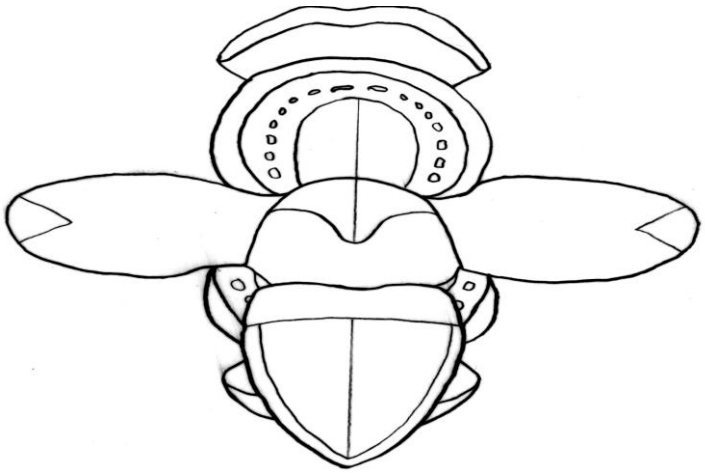
MEDICAMENTOS QUE FAZ USO REGULARMENTE:_____

QUEIXA BUCAL? ( ) NÃO ( )SIM – QUAL? _____

LESÕES BUCAIS? ( ) NÃO ( ) SIM - QUAL? _____

CASO OCORRA LESÃO BUCAL, LOCALIZÁ-LA NO DESENHO ESQUEMÁTICO





SÍTIO DE COLETA (1): PALATO MOLE CLASSIFICAÇÃO PAPANICOLAOU? CLASSE _____
PROCESSO INFLAMATÓRIO: (1) PRESENTE (2) AUSENTE
CELULARIDADE: (1) SUPERFICIAIS (2) INTERMEDIÁRIAS (3) PROFUNDAS
MÉDIAS: ÁREA DO NÚCLEO _____ ÁREA CITOPLASMA _____ N/C _____

SÍTIO DE COLETA (2): MUCOSA JUGAL CLASSIFICAÇÃO PAPANICOLAOU? CLASSE_____
PROCESSO INFLAMATÓRIO: (1) PRESENTE (2) AUSENTE
CELULARIDADE: (1) SUPERFICIAIS (2) INTERMEDIÁRIAS (3) PROFUNDAS
MÉDIAS: ÁREA DO NÚCLEO _____ ÁREA CITOPLASMA _____ N/C _____

CONCENTRAÇÕES DE HAPs SALIVARES: _____

CONCENTRAÇÕES DE HAPs URINA: _____

PORTO ALEGRE, _____