Eleonora de Paula Amaral

Análise histopatológica do tecido epitelial e da musculatura da língua de ratos submetidos à inalação da fumaça do tabaco

Histopathological analysis of the epithelial tissue and tongue musculature of rats submitted to inhalation of tobacco smoke

Uberaba

2018

Universidade de Uberaba
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ELEONORA DE PAULA AMARAL

"ANÁLISE HISTOPATOLÓGICA DO TECIDO EPITELIAL E DA MUSCULATURA DA LÍNGUA DE RATOS SUBMETIDOS À INALAÇÃO DA FUMAÇA DO TABACO"

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Resumo

Introdução: O tabagismo é um fator de risco para sérios problemas de saúde que podem acometer também os fumantes passivos. Além disso, está associado à várias alterações nos tecidos da cavidade oral. **Objetivo:** Avaliar a presença de displasia epitelial, de inflamação, a porcentagem de colágeno, a densidade de mastócitos e a intensidade de células imunomarcadas por anti-HIF-1α na musculatura lingual de ratas expostas à fumaça do cigarro. **Metodologia:** Vinte e sete ratos albinos fêmeas *Wistar* foram divididos em três grupos: ratos não expostos à inalação da fumaça do tabaco (Grupo controle) (n=7); ratos expostos à inalação da fumaça do tabaco durante 30 dias (TAB 30) (n=10); e ratos expostos à inalação da fumaça do tabaco durante 45 dias (TAB 45) (n=10). Com o auxílio de um equipamento, os animais foram expostos à fumaça de quatro cigarros por dia no interior de câmaras cilíndricas. Os intervalos entre o tempo de exposição à fumaça e ao ar ambiente eram controlados por uma bomba peristáltica. Semanalmente o peso dos animais e o percentual de monóxido de carbono de cada câmara cilíndrica eram avaliados. Ao final do experimento os animais foram submetidos à eutanásia com dose excessiva de Tiopentato de sódio via intraperitoneal com posterior coleta de sangue para dosagem de cotinina plasmática. Posteriormente as línguas dos animais foram coletadas, processadas histopatologicamente e coradas pelos corantes Hematoxilina e eosina, Picrossírius e Azul de Toluidina. Os cortes adicionais foram utilizados para processamento imunohistoquímico para HIF-1α. Todas as análises foram realizadas isoladamente nos três terços da língua com auxílio de microscopia de luz comum. As análises estatísticas foram realizadas utilizando os testes estatísticos ANOVA e Kruskal-Wallis e pós testes de Tukey e de Dunn. Foram utilizados ainda, os testes de correlação de Pearson e de Spearman. Todas as análises foram realizadas utilizando o nível de significância de 5%. **Resultados:** Nos grupos expostos à fumaça do cigarro foi observada menor porcentagem de colágeno, maior densidade de mastócitos e maior intensidade de células imunomarcadas por anti-HIF-1α. Não foi observada displasia epitelial. A inflamação foi observada em apenas dois casos. Houve também correlação positiva e significativa entre a porcentagem de colágeno e densidade de mastócitos. **Conclusão:** Sabendo da importância da musculatura lingual na deglutição, mastigação e fala o presente estudo chama a atenção para prevenção contra a exposição passiva ao tabaco.

Palavras-Chave: fumaça lateral, fumantes passivos, língua, ratos, tabaco.

Abstract:

Introduction: Smoking is a risk factor for serious health problems that can also affect passive smokers. Furthermore, it is associated with several changes in the tissues of the oral cavity. **Objective:** To evaluate the presence of epithelial dysplasia, inflammation, the percentage of collagen, the mast cell density and the anti-HIF-1α-immunoregulated cell intensity in the lingual musculature of rats. **Methods**: Twenty-seven Wistar female albino rats were divided into three groups: rats not exposed to inhalation of tobacco smoke (control group) (n = 7); rats exposed to inhalation of tobacco smoke for 30 days (TAB 30) (n = 10); and rats exposed to inhalation of tobacco smoke for 45 days (TAB 45) (n = 10). With the aid of equipment, the animals are exposed to smoke of four cigarette per day inside cylindrical chambers, the smoke puffs and the intervals between the time of exposure to smoke and the time of exposure to ambient air were controlled by a peristaltic pump. Weekly the weight of the animals and the percentage of carbon monoxide of each cylindrical chamber were evaluated. At the end of the experiment, the animals were submitted to euthanasia with excessive dose of sodium thiopentate via intraperitoneal and blood samples were collected for dosage of plasma cotinine. Subsequently the tongue of the animals with collected, histopathologically processed and stained by dyes Hematoxylin and eosin, Picrossírius and Toluidine Blue. Additional cuts were used for the immunohistochemical processing for HIF-1α. All analyzes were performed separately in the different thirds of the tongue and with the aid of light microscopy. Statistical analyzes were performed using the statistical tests ANOVA and Kruskal-Wallis and post tests of Tukey and Dunn. Pearson and Spearman correlation tests were also used. All analyzes were performed using the significance level of 5%. **Results:** In the groups exposed to cigarette smoke, a lower percentage of collagen, a higher density of mast cells and a higher intensity of anti-HIF-1α-immunolabelled cells were observed. No epithelial dysplasia was observed. Inflammation was observed in only two cases. There was also a positive and significant correlation between a percentage of collagen and mast cell density. **Conclusion:** Knowing the importance of lingual musculature in swallowing, mastication and speech of the present study calls attention to prevention against passive exposure to tobacco.

Keywords: passive smoker, rats, second hand smoke, tobacco, tongue.

Lista de Figuras

Lista de Abreviaturas

pH – Potencial hidrogeniônico

MMC – Mastócitos de mucosa

CTMC – Mastócitos de tecido conjuntivo

SpO2 – Saturação de Oxigênio

HIF-1 – Fator de Transcrição 1 Induzido por hipóxia

HIF-1α – Fator de Transcrição 1 Induzido por hipóxia alfa

HIF-1β – Fator de Transcrição 1 Induzido por hipóxia beta

EPO – Eritropoietina

VEGF - Fator de crescimento endotelial vascular

ALDA – Aldolase A

ENO1 – Enolase 1

LDHA – Lactato desidrogenase A

PFKL - Fosfofructoquinase L

PGK1 - Fosfogliceratoquinase 1

ATP - Adenosina trifosfato

REDD1 – Proteína regulada em danos ao DNA e desenvolvimento 1

MMP-9 – Metaloproteinase de Matriz 9

HO-1 – Heme oxigenasse

TAB 30 – Ratos Expostos à fumaça do Tabaco por 30 dias

TAB 45 - Ratos Expostos à fumaça do Tabaco por 45 dias

mg – Miligrama

g – gramas

kg - Kilograma

mL – Mililitro

ng – Nanograma

µm – Micrometro

µm^2 – Micrometro quadrado

Índice

Introdução Geral

Introdução

De acordo com a Organização Mundial de Saúde (OMS) o tabagismo é considerado uma doença crônica decorrente da dependência da nicotina (WHO/Tobacco, 2015), estando inserido no CID (Classificação Internacional de Doenças) desde 1997 dentro dos grupos de transtornos mentais e de comportamentos decorrentes do uso de fumo (CID10 F17) e dos problemas relacionados com o uso do tabaco (CID10 Z72.0) (DATASUS, 2008). Esse hábito representa o maior fator de risco modificável associado a sérios problemas de saúde como câncer, doenças cardiovasculares, doenças respiratórias e infertilidade (COLOMBO et al., 2014).

A OMS estima que exista aproximadamente um bilhão de fumantes no mundo e cinco milhões de mortes atribuídas direta ou indiretamente ao uso do tabaco. Caso não haja mudança no modelo de consumo de tabaco, o número de fumantes passará de 1,6 bilhões e o número de mortes anuais chegará a 10 milhões nos próximos três anos. Portanto, o hábito de fumar cigarro é uma importante ameaça à saúde pública em todo o mundo e uma das principais causas evitáveis de mortes (WHO/Tobacco, 2015).

Dados de 2004 apontaram que do total mundial de não fumantes expostos à fumaça proveniente do cigarro de terceiros, 40% eram crianças, 33% homens e 35% mulheres (OBERG et al., 2011). Além disso, 10% das mortes relacionadas ao tabaco são de não fumantes expostos à fumaça de cigarro (WHO | Tobacco, 2015).

Em 2008 no Brasil, o Instituto Brasileiro de Geografia e Estatística (IBGE) divulgou os dados da Pesquisa Especial de Tabagismo, os quais apontaram que 17,2% da população brasileira com mais de 15 anos de idade fumavam derivados de tabaco. A pesquisa revelou ainda que 27,9% dos brasileiros não fumantes eram expostos à fumaça produzida por cigarro de terceiros, principalmente no ambiente familiar (IBGE, 2008).

Ainda no Brasil, em 2013, a Pesquisa Nacional de Saúde (PNS) divulgou que 15% da população com mais de 18 anos era usuária de derivados de tabaco. Além disso foi demonstrado nessa pesquisa que 10,7% da população era de não fumantes expostos à fumaça no ambiente familiar e 13,5% de não fumantes expostos à fumaça no ambiente de trabalho (PNS, 2013).

A fumaça do cigarro é classificada em duas categorias, a fumaça central ou principal e a fumaça periférica ou lateral. A fumaça central é gerada durante as tragadas e produzida em altas temperaturas (acima de 950ºC), sendo a principal fonte de exposição dos próprios usuários do

cigarro, ou seja, dos fumantes ativos. A fumaça lateral, por sua vez, é gerada durante a queima lenta do cigarro que ocorre no intervalo entre as tragadas com temperatura mais baixa em relação à fumaça central (cerca de 350ºC), sendo considerada como a fonte de exposição dos indivíduos que involuntariamente inalam a fumaça presente no ambiente, também conhecidos como fumantes passivos (SOPORI ; KOZAK, 1998; MELLO et al., 2005; VALENTI et al., 2011).

A fumaça é constituída por várias substâncias químicas diferentes e em proporções inexatas, as quais estão distribuídas entre elementos particulados, que ficam retidos no filtro do cigarro e elementos gasosos, que atravessam o filtro (HOFFMANN et al., 2001; PERFETTI; RODGMAN, 2011).

Segundo estimativa, a fumaça do cigarro contém aproximadamente 5600 componentes individuais (PERFETTI; RODGMAN, 2011), dos quais, pelo menos 158 apresentam propriedades carcinogênicas ou tóxicas, dentre elas a nicotina, o acetaldeído, o benzeno, o formaldeído, os metais, policíclicos aromáticos, a acroleína, o monóxido de carbono e o dióxido de carbono (FOWLES; DYBING, 2003; THORNE; ADAMSON, 2013).

Os elementos tóxicos presentes na fumaça do cigarro, além de representar ameaças aos fumantes ativos, devido à sua inalação direta, também aumentam os riscos para a saúde dos fumantes passivos, isso porque a fumaça lateral apresenta quase a mesma quantidade de elementos totais que a fumaça central. Portanto, o risco de doenças relacionadas com o tabagismo também aumenta nesses indivíduos (BEHERA, et al., 2014). Um estudo de revisão relatou uma série de pesquisas que demonstraram que a fumaça lateral é mais tóxica e mais tumorigênica quando comparada à fumaça principal (SCHICK, S; GLANTZ, 2005).

A fumaça lateral pode levar ao aumento da agregação plaquetária, à disfunção endotelial, ao aumento da rigidez arterial e do estresse oxidativo, ao mesmo tempo em que leva à diminuição de antioxidantes endógenos, além de causar inflamação, diminuir a produção de energia e a vazão parassimpática para o coração. Estes mecanismos desencadeiam efeitos negativos no sistema cardiovascular de indivíduos expostos à essa fumaça, como lesão endotelial, trombose e aterosclerose (BARNOYA; GLANTZ, 2005).

Imediatamente à inalação da fumaça resultante da combustão do cigarro, os indivíduos expostos já começam a sentir os efeitos deletérios como irritação dos olhos, nariz, garganta e pulmões, dor de cabeça, vertigem, elevação da pressão arterial e angina (COELHO et al., 2012).

Vários metabólitos do cigarro como tiocianato, monóxido de carbono, nicotina, cotinina, além de carboxiemoglobina são utilizados como biomarcadores para avaliar o grau de tabagismo e a exposição à fumaça em fumantes ativos e passivos, entretanto, muitos deles apresentam baixa especificidade (JARVIS et al., 1987). A nicotina por exemplo, apresenta a meia vida relativamente curta, de 2 horas aproximadamente, o que dificulta a sua detecção nos fluidos corporais e quantificação da exposição ao tabaco (FEYERABEND et al., 1986; MELLO et al., 2005). A carboxihemoglobina, sofre influência do monóxido de carbono presente no ambiente, comprometendo assim sua fidedignidade (MELLO et al., 2005). O monóxido de carbono e o tiocianato, por sua vez, não são marcadores específicos para a fumaça do cigarro (FEYERABEND et al., 1986).

Dessa forma, a cotinina tem sido o principal marcador biológico utilizado para avaliar o consumo de tabaco, bem como para o controle de sua abstinência (JARVIS et al., 1987). A cotinina é o principal metabólito da nicotina, o qual é oxidado no fígado pela enzima CYP2A6 (Citocromo P450, família 2, subfamília A, polipeptídeo 6) e posteriormente distribuídos para os vários fluidos humanos (YOSHIDA et al., 2002; JUNG et al., 2012; RAJA et al., 2016). Das amostras utilizadas para avaliar as taxas de cotinina, os níveis na saliva, no soro, no plasma e na urina são os mais apropriados pois estão correlacionados com a nicotina total absorvida (PETERSEN et al., 2010).

A escolha da cotinina como biomarcador se dá em virtude de sua especificidade para a exposição ao tabaco, por possuir restrita distribuição nos fluidos corporais, além de apresentar baixa taxa metabólica e meia-vida na circulação 10 vezes mais longa, quando comparado à nicotina. Além disso a cotinina pode ser facilmente coletada de forma não invasiva caso sejam utilizadas amostras de saliva e urina (MELLO et al., 2005; JUNG, S. et al., 2012). Além de que, nos tabagistas, a concentração sérica de cotinina se mantém constante durante todo o dia (MELLO et al., 2005).

Mesmo após a interrupção do uso do cigarro, a fumaça secundária e consequentemente a nicotina e outras substâncias tóxicas e carcinogênicas continuam presentes por seis meses nos dedos, na urina e nos ambientes habitados pelos usuários. Dessa forma, os fumantes passivos também continuam expostos à essas substâncias prejudiciais por igual período de tempo (MATT et al., 2016).

A cavidade oral é a porta de entrada do corpo humano, sendo diretamente exposta à fumaça proveniente do tabaco. Dessa forma, o tabagismo é um importante fator de risco para muitas doenças e condições adversas orais como câncer oral, lesões pré-cancerosas, estomatite nicotínica, melanoses, candidose oral, hiperqueratose, gengivite, periodontite, manchas em dentes e restaurações, perda de implantes dentários, peri-implantite e halitose além de contribuir para um mal prognóstico nos tratamentos odontológicos (GELSKEY, 1999; SHAM et al., 2003; SUSIN et al., 2004; CORRAINI et al., 2008; REIBEL, 2003; FURUKAWA et al., 2013; SHAMMARI et al., 2016).

Essas alterações orais provocadas pelo tabagismo provavelmente resultam das substâncias irritantes, tóxicas e carcinogênicas provenientes da queima do cigarro, entretanto também podem estar associadas ao ressecamento da mucosa, em razão da alta temperatura intra-oral, à mudança de pH e à alteração na resposta imune (SHAM et al., 2003). Existem evidências de melhoras no quadro de saúde bucal, como cáries, recessão gengival, perda dental, perda de implantes dentários e desordens da mucosa após a interrupção do uso do cigarro em diferentes idades (SHAM et al., 2003; WARNAKULASURIYA et al., 2010; FURUKAWA et al., 2013).

Todas as alterações orais provenientes do uso do tabaco devem ser levadas em consideração, entretanto, as lesões pré-cancerosas e o câncer bucal são as mais investigadas no âmbito das pesquisas na área de patologia bucal (SHAMMARI et al., 2016). O câncer de células escamosas orais representa cerca de 90% de todos os cânceres de boca (SHAM et al., 2003). Apesar das melhorias na prevenção primária, terapia, morbidade e mortalidade, as taxas de câncer bucal permanecem altas (LI et al., 2015). A língua é um dos sítios mais acometidos pelo carcinoma de células escamosas, principalmente na região das bordas laterais e base (MOORE et al., 2000). Os tumores de língua são considerados como um dos piores sítios no que diz respeito à sobrevivência do indivíduo, isso se dá em função de sua natureza altamente invasiva, o que leva à graves alterações nas funções deste órgão (LI et al., 2015).

A língua é um órgão muscular localizado no assoalho bucal, composto de um complexo organizado de músculos estriados classificados em extrínsecos, que possuem inserção óssea e intrínsecos, que se inserem na própria língua. Os músculos extrínsecos estão associados com os movimentos de posição da língua, enquanto que os intrínsecos estão relacionados com a mudança de forma do órgão (SANDERS; MU, 2013).

A língua se destaca por possuir papel crucial na fonética, limpeza, mastigação e deglutição dos alimentos, uma vez que é responsável por misturar, controlar e impulsionar o bolo alimentar em direção à orofaringe (PARADA et al., 2012). Alterações nas funções linguais podem levar à disfagia e aos distúrbios obstrutivos do sono (SANDERS; MU, 2013).

Morfologicamente a língua se divide em três terços, o terço apical, o terço médio e o terço posterior. O terço apical compreende a região que vai do ápice lingual ao frênulo e o terço médio corresponde à região que vai do frênulo à papila circunvalada, sendo que ambos se encontram localizados na cavidade oral e são móveis. O terço posterior encontra-se posteriormente à papila circunvalada, estando localizado na faringe e é relativamente imóvel (PARADA et al., 2012; SANDERS; MU, 2013).

O complexo muscular da língua é revestido por uma superfície de epitélio escamoso estratificado queratinizado contendo em sua superfície dorsal, sensores de gustação, de dor, táteis e de temperatura (PARADA et al., 2012). Além dos tecidos glandulares, vasculares e nervosos, as células musculares estriadas intrínsecas da língua formam feixes que são separados por tecido conjuntivo e células adiposas (GARTNER et al., 1997).

As principais estruturas de tecido conjuntivo na língua são o septo médio, que é uma espessa camada de tecido conjuntivo na linha média da língua; o septo paramediano que é uma fascia que separa o músculo genioglosso do músculo inferior longitudinal e o septo lateral, que é o tecido conjuntivo que separa o músculo inferior longitudinal (SANDERS; MU, 2013)

O tecido conjuntivo lingual mantém o volume do órgão constante durante a contração muscular (SANDERS; MU, 2013). A distribuição regional das fibras colágenas é discutida em termos de compreensão da biomecânica lingual em estados normais e patológicos, por exemplo, a maior concentração de fibras na região anterior da língua é justificada por se tratar de uma região onde a resistência estrutural e a resiliência são importantes para falar e engolir (MILLER et al., 2002).

Devido às ações diretas do tabaco e seus derivados, as toxinas presentes levam à destruição do tecido conjuntivo (WEI et al., 2005). As concentrações de nicotina encontradas em fumantes passivos são capazes de aumentar a expressão da classe metaloproteinase de matriz denominada colagenase I, que leva à degradação de fibras colágenas (BARNOYA; GLANTZ, 2005).

As metaloproteinases de matriz também apresentam significante função na progressão tumoral através do aumento da angiogênese, a qual é induzida pela presença do tumor, além de destruir a arquitetura tecidual local, permitindo assim, a invasão tumoral (KERKELÄ; SAARIALHO-KERE, U, 2003; FUN et al., 2016).

Outro componente do tecido conjuntivo lingual são os mastócitos. Primeiramente descritos apenas como células com funções efetoras nos processos inflamatórios alérgicos, os mastócitos atualmente são considerados como células imunes multifuncionais envolvidas em vários estados tanto patológicos como fisiológicos (da SILVA et al., 2014). Os mastócitos são derivados de células progenitoras hematopoiéticas que migram para tecidos vascularizados onde completam sua maturação e vivem por muito tempo. Mastócitos maturados geralmente residem próximos ao epitélio, vasos sanguíneos, nervos, células musculares e glândulas mucosas (GALLI, 2005; METCALFE, 2008).

Os mastócitos são células metacromáticas que apresentam grânulos em seu citoplasma e numerosos receptores em sua superfície. Quando estimuladas através de seus receptores, geram respostas que levam à liberação de uma variedade de mediadores biologicamente ativos, os quais são armazenados no interior de seus grânulos, esse processo é conhecido como degranulação (GALLI, 2005; da SILVA et al., 2014).

Entre os mediadores biologicamente ativos liberados, estão as proteases, proteoglicanas, citocinas, quimiocinas, fatores de crescimento, entre outros que vão desencadear diferentes funções em circunstâncias diversas, o que é influenciado pelo subtipo de mastócito e o microambiente, o qual é encontrado (GORDON, 1990; da SILVA et al., 2014). Em humanos, os mastócitos maturados são classificados em duas subcategorias de acordo com as proteases, as quais armazenam em: mastócito triptase/quimase, que armazenam quimase, triptase e carboxipeptidade A3 em seus grânulos e mastócito triptase, que armazenam apenas triptase em seus grânulos (IRANI et al., 1986; SCHWARTZ, 2006; JEONG et al., 2013). Recentemente foi descrito um terceiro subtipo de mastócito encontrado em amostras de epitélio de vias aéreas em pacientes com asma e esôfago de pacientes com esofagite eosinofilica, esses mastócitos expressam triptase e carpoxipeptidase A3 e não expressam quimase (ABONIA et al., 2010; DOUGHERTY et al., 2010).

Em ratos, os mastócitos são classificados em dois subtipos baseados nas diferenças histológicas, funções, e composição, como: mastócitos de mucosa (MMC) e mastócitos de tecido

conjuntivo (CTMC). Os mastócitos de mucosa (MMC) expressam predominantemente quimases do tipo mMCP-1 e mMCP-2 e são encontrados em mucosa epitelial do trato gastrointestinal e do pulmão, enquanto que, os mastócitos de tecido conjuntivo (CTMC), expressam quimase do tipos mMCP-4, triptases do tipo mMCP-5emMCP-6, além de carboxipeptidade A e são encontrados em submucosa intestinal, cavidade peritonial, pele e língua (MATSSON, 1992; IRANI et al., 1986; JEONG et al, 2013).

Além da participação nos processos de homeostase e remodelação tecidual, os quais são bastante conhecidos, essas células também têm sido bastante citadas por possuírem importantes funções como: estabelecimento de interação neuroimune (SUZUKI, A. et al., 2004), no processo de angiogênese (CRIVELLATO et al., 2004; da SILVA et al., 2014), na resposta imune inata, adaptativa e imuno tolerância. Quando ocorre regulação inadequada de suas funções, os mastócitos podem participar também de quadros patológicos como nos casos de alergias (da SILVA et al., 2014), doença de Crohn (GELBMANN et al., 1999), doenças autoimunes (BROWN; HATFIELD, 2012), mastocitose (METCALFE, 2008), doença cardiovascular (JENNE; TSCHOPP, 1991) e câncer (da SILVA et al., 2014).

A exposição à fumaça do tabaco ativa a cascata inflamatória e mastócitos, resultando na liberação dos mediadores pró-inflamatórios, contribuindo para o prolongamento desse processo (MORTAZ et al., 2008). O processo inflamatório contribui para a diminuição do suprimento sanguíneo e consequentemente à níveis baixos de oxigênio, o que leva a hipóxia (TAYLOR; COLGAN, 2017). A hipóxia portanto, ocorre quando a necessidade de oxigênio de uma célula ultrapassa o fornecimento vascular e, caso esse quadro seja mantido, a hipóxia pode ser letal para as células (TAYLOR; COLGAN, 2017).

Além da inflamação, a hipóxia tecidual também pode ser provocada pela nicotina presente no cigarro, pois estimula o organismo a secretar hormônios adrenocorticais, os quais agirão promovendo contração vascular. Além disso, o monóxido de carbono contribui para a redução na saturação de oxigênio (SpO2), uma vez que possui alta afinidade por hemácias, o que prejudica a ligação das hemácias às moléculas de oxigênio ao mesmo tempo que forma o complexo carboxihemoglobina, levando ao quadro de hipóxia (MIYANG et al., 2014; LEONE, 2015).

O fator de transcrição 1 induzido por hipóxia (HIF-1) é uma proteína básica envolvida em importantes respostas adaptativas para hipóxia, promovendo a sobrevivência celular sob essas

condições (JEONG et al., 2013). É composta de duas subunidades: HIF-1α e HIF-1β, sendo a subunidade α expressa na dependência da disponibilidade de oxigênio (SEMENZA et al., 2004).

Em condições de normóxia, o HIF-1α é sintetizada, porém sofre rápida degradação por hidroxilases, contudo, quando a disponibilidade de oxigênio é reduzida, as hidroxilases são inibidas e consequentemente a degradação do HIF-1α não ocorre, permanecendo assim, estável no citoplasma. O acúmulo de HIF-1α no citoplasma permite sua translocação para o núcleo, onde se une com a subunidade β. A subunidade α e β formam juntas o fator de transcrição HIF-1, os quais ativam a transcrição de genes como: eritropoietina (EPO), responsável por regular a eritropoiese e importante determinante da capacidade de transporte de oxigênio no sangue; o fator de crescimento endotelial vascular (VEGF), primeiro regulador de angiogênese; enzimas glicolíticas como aldolase A (ALDA), enolase 1 (ENO1), lactato desidrogenase A (LDHA), fosfofructoquinase L (PFKL), fosfogliceratoquinase 1 (PGK1), os quais forncem um atalho metabólico para a geração de ATP na ausência de oxigênio, entre outros (SEMENZA et al., 2004; BERCHNER-PFANNSCHMIDT et al., 2008; TAYLOR; COLGAN, 2017).

Estudos anteriores relataram que a fumaça do cigarro induz a ativação de HIF-1α, o qual vai regular a expressão de níveis de VEGF, REDD1, MMP-9 e HO-1, os quais estão envolvidos na doença pulmonar obstrutiva crônica (YU et al., 2012; DAIJO et al., 2016). Outros estudos demonstraram que no microambiente tumoral, a histamina liberada através da degranulação de mastócitos induz a expressão de HIF-1α que por sua vez, induz a migração de mais mastócitos através da produção de VEGF (JEONG et al., 2013).

Alguns estudos semelhantes avaliaram língua de ratos expostos à fumaça do tabaco e encontraram alterações como desordens nas papilas filiformes (MARTINS et al., 2014), superexpressão de bcl-2, um proto-oncogene e inibidor de apoptose, em queratinócitos da língua, tornando-os passíveis de mutações e consequentemente, de progressões tumorais (ASSIS et al., 2005). Outro estudo realizado também na língua de ratos demonstrou hiperplasia epitelial, aumento da camada de queratina e displasia média na mucosa lingual, além de evidente expressão positiva de ki-67, um importante marcador de proliferação celular, usado para avaliar frações de crescimento em neoplasias humanas (de OLIVEIRA SEMENZATI et al., 2012).

Embora alguns autores já demonstraram alterações epiteliais das línguas de ratos expostos à fumaça do tabaco (ASSIS et al., 2005; MARTINS et al., 2014), não foram encontrados estudos que descreveram alterações no tecido conjuntivo lingual frente à inalação passiva do tabaco.

Dessa forma, levantamos a hipótese de que não somente o epitélio, mas o tecido conjuntivo lingual também poderia sofrer alterações teciduais decorrentes da fumaça lateral do cigarro. Sabendo da importância da língua na deglutição, mastigação e fala, justifica-se a realização do presente estudo que teve como objetivo realizar avaliação histopatológica do epitélio e da musculatura das línguas de ratos expostos à fumaça lateral do cigarro.

Capítulo I: artigo submetido à revista *Inhalation Toxicology*

Histopathological analysis of the epithelial tissue and tongue musculature of rats submitted to inhalation of tobacco smoke

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Abstract:

Introduction: Smoking is a risk factor for serious health problems that can also affect passive smokers. Furthermore, it is associated with several changes in the tissues of the oral cavity. **Objective:** To evaluate the presence of epithelial dysplasia and inflammation, collagen percentage, mast cells density and intensity of immunolabelled cells to anti-HIF-1α in the rats tongue musculature. **Methods:** Twenty seven female Wistar albino rats were divided into three groups: rats not exposed to tobacco smoke inhalation (Control group) (n = 7); rats exposed to smoke inhalation for 30 days (TAB 30) (n = 10); and rats exposed to inhalation of tobacco smoke for 45 days (TAB 45) (n = 10). Subsequently, the animals were submitted to euthanasia with subsequent removal of the tongue for histological and immunohistochemistry processing. **Results:** In the groups exposed to cigarette smoke, a lower percentage of collagen, a higher density of mast cells and a greater intensity of anti-HIF-1α immunolabelled cells were observed. There was also a positive and significant correlation between the percentage of collagen and mast cell density. **Conclusion:** Exposure of rats to smoke of tobacco causes decrease in perimysial collagen fibers, increase in the number of mast cells, and increase in immunostaining for HIF-1α in lingual muscle cells. Knowing the importance of lingual musculature in swallowing, chewing and speaking, new studies should be carried out to better understand the pathogenesis of lingual changes in passive inhalation of tobacco smoke.

Keywords: passive smoker, rats, second hand smoke, tobacco, tongue.

Introduction

Smoking is the major modifiable risk factor associated with severe health problems such as cancer, cardiovascular disorders, respiratory diseases, infertility and one of the leading causes of preventable death (Colombo et al., 2014). Cigarette smoke contains approximately 5600 constituents (Perfetti and Rodgman 2011), of which at least 158 present carcinogenic or toxic properties (Fowles and Dybing 2003; Thorne and Adamson 2013). Thus, passive smoking also increases the risk of developing diseases caused by the toxins of cigarette components (Behera et al., 2014), and accounts for 10% of tobacco-related deaths in these individuals (WHO / Tobacco 2015).

Smoking and exposure to tobacco smoke are important risk factors for several changes in the oral mucosa, such as neoplasia and epithelial dysplasia (Reibel 2003). Oral epithelial dysplasia is histopathologically characterized by cellular and structure alterations of the lining epithelium (Jaber 2010). Only one study that demonstrated dysplasia in the lingual epithelium of rats exposed to tobacco smoke was found (de Oliveira Semenzati et al., 2012).

In addition to the epithelium, the toxic substances of tobacco lead to the destruction of collagen fibers in the connective tissue (Wei et al., 2005), by increasing the expression of metalloproteinases (Carty CS et al., 1996; Barnoya and Glantz 2005). Connective tissue mast cells also suffer from the action of tobacco toxic substances, such as acrolein, which promotes mastocyte degranulation with a consequent inflammatory reaction (Hochman et al., 2008). In addition, exposure to long-term tobacco smoke increases the number of mast cells that produce important growth factors in angiogenesis (Kispert et al., 2017) and fibrosis (da Silva et al., 2014).

Tissue hypoxia, induced by smoking and exposure to tobacco smoke, can also cause tissue damage (Taylor and Colgan 2017). Hypoxia in these cases can be triggered by the action of nicotine which stimulates the secretion of adrenocortical hormones that promote vascular contraction and reduction of local blood flow (Miyang et al., 2014). Additionally, carbon monoxide contributes to the reduction in oxygen saturation (SpO2) and the formation of carboxyhemoglobin which also contributes in tissue hypoxia (Miyang et al., 2014; Leone 2015).

One way to assess whether the tissue is in hypoxia is to investigate the presence of hypoxia-induced transcription factor 1 (HIF-1) (Semenza 2004). HIF-1 is a heterodimeric protein composed of two subunits: HIF-1α and HIF-1β, the α subunit being expressed in dependence on

the availability of oxygen (Semenza 2004). In hypoxia conditions the HIF-1 complex translocates to the nucleus and induces the transcription of genes related to hypoxia-adaptive mechanisms such as angiogenesis, oxygen transport, generation of ATP in the absence of oxygen, apoptosis and metastasis (Semenza 2004), which will assist in cell survival under these conditions (Jeong et al., 2013).

Although some authors have shown epithelial changes in the tongues of rats exposed to tobacco smoke (Assis et al., 2005; Martins et al., 2004), no studies have been found to describe changes in the lingual musculature due to passive inhalation of tobacco. Thus, we hypothesized that not only the epithelium, but also the lingual musculature could present tissue alterations resulting from side stream smoke. Knowing the importance of tongues in swallowing, chewing and speaking, it is justified to carry out the present study that aimed to perform histopathological evaluation of the epithelium and musculature of the tongues of rats exposed to side stream smoke.

Methods

The present study was elaborated according to the Guide for Care and Use of Laboratory Animals (NRC 2011) and approved by the Ethics Committee on Animal Use of the University of Uberaba (CEUA), protocol nº 070/2017 (Annex I).

Animals and groups

Twenty-seven female albino rats (Rattus norvegicus) of the Wistar lineage, with a mean initial body mass of 150 g (± 10 g) supplied by the Biotério Central of the City Hall of Ribeirão Preto Campus of the University of São Paulo were used. The sample calculation was performed considering the loss rate of approximately 30% (Santiago et al., 2017).

The animals were housed in standard cages at the Bioengineering Laboratory of the Medical School of Ribeirão Preto (FMRP / USP), where they remained during the whole experimental period under ambient temperature of 20° C to 24° C, with light cycles of 12 hours

clear and 12 hours dark, with relative air humidity at 55% (± 10%) and without restriction to food or water.

The animals were randomly assigned to three experimental groups: female rats not exposed to the inhalation of tobacco smoke (control group) (n = 7); female rats exposed to smoke inhalation for 30 days (TAB 30) (n = 10); and female rats exposed to inhalation of tobacco smoke for 45 days (TAB 45) (n = 10).

Exposure of rats to tobacco smoke

The exposure of rats to tobacco smoke was performed according to methods previously described (Wang et al., 1999; Gentner and Weber 2012, Santiago et al., 2017).

The animals were placed inside a rectangular acrylic box composed by four cylindrical transparent acrylic tubes. In each tube was placed an animal. Each tube has one end with a controllable physical restrictor to prevent the animals from retreating and another tapered end that connects with a container that distributes the smoke interspersed with ambient air to the four tubes. The cigarette smoke "puffs" inside the box were removed by suction through a peristaltic pump (Provitec - model: AWG 5,000 AX-D), which has a timer that controls the time of injection of ambient air or smoke within the tubes in 30 second cycles of exposure to ambient air and 15 seconds of exposure to smoke. All equipment, except the pump, was placed inside the transparent acrylic box, connected to an exhaust hood that promotes the cleaning of ambient air. Four animals were simultaneously exposed to cigarette smoke (Annex II, figure 1).

The cigarette used was Marlboro (Phillips Morris), with each unit containing: 0.8 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide (values measured by Laboratory Labstat - Canada, according to product label).

The animals went through the set-up phase in the equipment for a period of 20 minutes without smoke, twice a day, with six- hour interval, in the morning and afternoon for seven consecutive days. The 20 minutes corresponded to the time of exposure to two cigarettes (cycles of 30 seconds of exposure to ambient air and 15 seconds of exposure to smoke).

Later, the animals were submitted to adaptation to tobacco smoke for seven consecutive days by burning a cigarette twice a day (morning and afternoon), with a six-hour interval between exposures. After the adaptation period, the rats were exposed to the smoke of two cigarettes in

the morning and two more cigarettes in the afternoon, with a 6 hour interval, for 30 or 45 days according to the experimental groups. In order to guarantee similar exposure to tobacco smoke, once every seven days the percentage of carbon monoxide in each camera of the equipment was measured using a portable carbon monoxide meter (Instrutemp ITMCO-1500, SP, and Brazil)

Evaluation of weight, euthanasia and blood sampling

The animals were weighed weekly with a semi-analytical balance (Digital Electronic Scale from 0 to 3kg, TOLEDO, mod. 9094) with a capacity of up to 5200g, with 0.1g precision resolution.

At the end of the experiment the animals were submitted to euthanasia with excessive dose (3mL/100g) of Thiopental (sodium thiopentate, 1.25% solution) administered intraperitoneally. Then the blood samples were collected by means of open cardiac puncture and kept in boxes with ice. Subsequently, within two hours after the collection, these samples were centrifuged for separation of the plasma using a centrifuge (HETTICH, mod. EBA 280). Soon after, the plasma was stored at -20° C until plasma cotinine was assessed in order to verify if plasma cotinine levels were the same as those found in passive smokers.

Dosage of cotinine

Plasma cotinine dosage was performed by means of gas chromatography analysis, according to the routine methodology of the Laboratory of Forensic Toxicological Analysis of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP / USP). Standard: cotinine in 1.0 mg / mL solution in methanol (Sigma-AldrichTM - Missouri, USA). Gas chromatography has specificity of 97.4% and sensitivity of 96.3%. To be considered as a passive smoker, plasma cotinine levels should be between 2.1 and 17.5 ng / mL (Connor et al., 2009).

After the dosage of cotinine, the heads of animals were placed in 10% formaldehyde and then transported to the Human Anatomy Laboratory of the Department of Structural Biology of the Federal University of Triângulo Mineiro (DBE / UFTM), where the fragments of tongues were collected.

Collection of tongues samples, histochemical and immunohistochemical processing

The tongue were collected by means of a medial incision in the region of the retromolar trigone and anterior region of the neck, followed by avulsion of the skin, resection of the musculature of the floor of the mouth and disarticulation of the temporomandibular joint. An incision was made in the region of the epiglottic vallecula with removal of the tongue and mandible, which were fixed in 10% formaldehyde for later lingual resection.

Fragments of the tongue were then collected by a longitudinal cut along the medial lingual groove, from the base to the apex. These fragments were processed histopathologically obtaining 5-μm-thick sections. Then, the fragments were stained with Hematoxylin and Eosin for histopathological evaluation of epithelial dysplasia and inflammation; with Toluidine blue for determination of mast cell density; and Picrossirius for quantification of collagen percentage. Additional sections were used for immunohistochemical processing for HIF-1α.

Analysis of dysplasia and inflammation

The analysis of dysplasia and inflammation was performed using a light microscope (Nikon Eclipse 80i) and a 40x objective lens. The analyses were performed separately in the apical, middle and posterior thirds.

A semi-quantitative evaluation of the intensity of epithelial dysplasia and inflammatory infiltrate in the different thirds of the tongue was performed in all fields, using the following scores: 0 - absent or 1 - present (Semberova et al., 2013).

Determination of the percentage of collagen

For morphometric quantification of collagen, picrossirius-stained slides were analyzed on a standard Axio 4.1 light microscope (Zeiss, Berlin, Germany) using a 20x objective and a polarizing filter. The analyses were performed separately in the apical, middle and posterior thirds. All fields of the slides containing muscle tissue were transmitted to the computer monitor with the aid of an Axiocam image capture camera (Zeiss, Berlin, Germany). Axiovision 4.8 software (Zeiss, Berlin, Germany) was used for morphometric analysis of the collagen

percentage. In the polarized image the collagen presented birefringence with yellowish, reddish or greenish coloration, being quantified semi-automatically (Figures 2A and 2B).

Determination of mast cell density

To determine the mast cell density, the blades stained by toluidine blue were analyzed using the 40x objective. The analyses were performed separately in the apical, middle and posterior thirds. With the aid of a micrometer blade, the area of each field (0.14 mm^2) was calculated, being multiplied by the total number of fields analyzed to obtain the total area. With the total number of mast cells and the total area analyzed, the mast cell density was calculated and expressed in mast cells per μm^2.

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Immunohistochemical processing and analysis of anti-HIF-1α-immunolabelled cells:

The anti-HIF-1α mouse monoclonal antibody (Sigma-Aldrich, dilution 1: 500, Saint Louis, MO, USA) diluted in bovine serum albumin dissolved in PBS (Sigma-Aldrich, Missouri, USA) was used for the immunohistochemical reaction, according to the manufacturer's instructions. For detection of the antibody, the polymer detection system (Spring, Pleasanton, CA, USA) was used as a biotin-free system. The immunohistochemical reaction of each antibody was visualized using chromogenic diaminobenzidine-DAB solution (Sigma Chemical CO, Missouri, USA).

The immunohistochemical evaluation of the immunomarker cells was performed semi-quantitatively using a microscope (Nikon Eclipse 80i) and a 40x objective. For this evaluation, the following scores were used: 1 - weak expression (up to 33% of the immunolabellated cells), 2 - strong expression (> 33% of the immunolabellated cells) (de Araújo et al. 2017).

Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.0 and Bioestat 5.0 software. For the evaluation of the final weight of the animals, cotinine dosage and mast cell

density, ANOVA and Tukey post-test were used, since the data presented normal distribution. However, for the evaluation of the collagen percentage and the immunostaining for HIF-1α, the Kruskal-Wallis tests and the Dunn post-test were applied, since the data presented a non-normal distribution. To correlate the mast cell density and collagen percentage, the Pearson correlation test was used for the data that presented normal distribution and the Spearman correlation test for data that presented a non-normal distribution. All analyses were performed assuming a significance level of 5%.

Results

During the adaptation period, approximately 8% of the animals were lost, being two animals in TAB- 45 group.

The mean levels of carbon monoxide were similar at all measurements (338.79 ± 1.16 ppm). Animals from all groups presented significant weight gain when comparing initial body weight and final body weight ($p < 0.0001$). When the final weight of the animals was compared between the groups, it was observed that the groups exposed to tobacco had significantly lower final weight in relation to the control group (Figure 3).

The mean values and standard deviation of the cotinine dosage were: control group (0.005 ± 0.009); TAB 30 (7.66 ± 0.96) and TAB 45 (9.13 ± 0.95). Thus, mean cotinine levels were significantly higher in the groups exposed to smoke than in the control group, and the TAB 45 group had higher cotinine levels when compared to the TAB 30 group ($p < 0.0001$).

No epithelial dysplasia was observed in any of the analyzed cases (Figure 4A). Chronic inflammation was observed in the apical third of the tongue of two cases, one animal of control group and one animal of TAB 45 group (Figure 4B).

The percentage of collagen in the apical and posterior third of the tongue was significantly lower in the TAB 30 and TAB 45 groups than in the control group ($p < 0.0001$). In the middle third, there was no significant difference between the groups in relation to the percentage of collagen (Figure 5).

The mast cell density was significantly higher in TAB 30 and TAB 45 groups than in the control group, observed in the apical third ($p = 0.0608$), in the middle third ($p = 0.0163$) as well

as in the posterior third of the tongue ($p = 0.0007$) (Figure 6 and 7).

There was a positive and significant correlation between collagen percentage and mast cell density when evaluating the tongue as a whole, both in TAB 30 and TAB 45 groups ($p < 0.0001$). However, there was no significant correlation when the three thirds were evaluated singly (Figure 8A and 8B).

Immunostaining for HIF-1α was observed only in the lingual muscle cells and was significantly higher in the TAB 45 group than in the control group: in the apical third ($p = 0.0036$), in the middle third ($p = 0.0029$), and in the posterior third ($p = 0.0007$). TAB 30 group showed significantly higher immunostaining for HIF-1α than the control group only in the posterior third of the tongue ($p = 0.0007$) (Figure 9 and 10).

No significant correlation was observed between mast cell density and immunolabelled intensity for HIF-1α (data not shown).

Discussion

The present study was the first to evaluate the percentage of collagen, mast cell density and immunolabelled for HIF-1α in rat tongues exposed to secondary tobacco smoke.

The loss of 8% of the animals was probably due to intoxication from tobacco smoke, which was already foreseen in the exposure protocol (Santiago et al., 2017).

Although animals of all groups showed weight gain, the final weight of the animals in the groups exposed to tobacco was significantly lower in comparison to the final weight of the control group animals. This finding corroborates another study that also observed lower body weight in rats exposed to secondary tobacco smoke (Chaichalotornkul et al., 2015). The decrease in weight gain in animals exposed to cigarette smoke may have been caused by the action of nicotine in the smoke that leads to reduced appetite and altered eating patterns, as already demonstrated in other studies (Jo et al., 2002; Chaichalotornkul et al., 2015).

Epithelial dysplasia has been described in the tongue and pharynx of rats exposed to smoke (de Oliveira Semenzati et al., 2012). However, we did not find any cases with dysplasia in the present study, perhaps due to the amount of cigarettes and the time of exposure, which may not have been enough to cause this alteration. In addition, only two cases of chronic inflammation

were observed. Since this inflammation was present in the apical third, we believe that this process is a result of bite trauma.

Although we have not found studies that evaluated the percentage of collagen in the tongues of both humans and animals exposed to tobacco smoke, some authors have already shown an increase in the amount of collagen in the salivary glands (Ferragut et al., 2011) and in mammary tissue (Kispert et al., 2017) from rats and in the heart of hamsters (Wu et al., 2014) as a consequence of passive inhalation of tobacco. In the present study, the percentage of collagen in the apical third and the posterior third of the tongue was significantly lower in TAB 30 and TAB 45 groups than in the control group. Our findings corroborate with other studies that also found a reduction in the amount of collagen in the lungs of mice (Valença and Porto 2008) and in the skin of Wistar rats (Chaichalotornkul et al., 2015) exposed to cigarette smoke.

The reduction in the percentage of collagen in the tongue of rats exposed to tobacco smoke may have been caused by nicotine and cotinine, leading to an increase in the expression of metalloproteinases, such as collagenases (Barnoya and Glantz 2005; Kispert et al., 2017), that induce the destruction of collagen fibers type I, II and III (Kerkelä and Saarialho-Kere 2003). Moreover, the inhalation of tobacco smoke leads to an increase in the production of reactive oxygen species, such as superoxide (Barnoya and Glantz 2005; Domej et al., 2006; Barbosa et al., 2010) and reactive nitrogen species, such as nitric oxide (Domej et al., 2006), which also cause destruction of the extracellular matrix (Domej et al., 2006; Valença and Porto 2008).

As there was a lower percentage of collagen found only in the apical third and the posterior third of TAB 30 and TAB 45 groups, we believe that these regions would be more exposed to the action of tobacco smoke. This is because during exposure to smoke, animals constantly open their mouths, which allows greater contact of the smoke with the apical third of the tongue. In addition, during inhalation the smoke penetrates the nasal region and passes through the oropharynx directly reaching the posterior region of the tongue, which could also justify the lower percentage of collagen in this region.

Studies have shown that there is an increase in the number of mast cells in the ducts and lymph nodes of mammary tissue (Kispert et al., 2017) and in the airways (Mortaz et al., 2012) of rats exposed to tobacco smoke. These findings corroborate with the present study because we found significantly higher mast cell density in the apical, middle and posterior thirds of TAB 30 and TAB 45 groups in relation to the control group.

Mast cells are metachromatic cells that display granules in their cytoplasm and numerous surface receptors (Galli et al., 2005, da Silva et al., 2014), which when stimulated release biologically active mediators such as proteases, proteoglycans, cytokines, chemokines and growth factors (da Silva et al., 2014). As already described, resident mast cells of the airways are exposed to side stream smoke (Mortaz et al., 2012). Furthermore, it is known that toxic substances present in tobacco smoke, such as acrolein, cause mast cell degranulation (Hochman et al., 2008) and that some substances released during this degranulation cause recruitment of progenitor mast cells to the tissue, where they are finally matured (Collington et al., 2011). Thus, the increase in mast cell density in the groups exposed to tobacco smoke in the present study may have occurred as a result of chemical mediators released by these cells, which would be participating in the recruitment of progenitor mast cells from the bone marrow, contributing to the increase of mast tissue population in the lingual tissue.

Mast cells have been prominent because they are involved in several physiological and pathological processes, but one of the most well-known functions of these cells is the participation in the process of collagen synthesis through fibroblastic activation (da Silva et al., 2014). Studies have shown a positive correlation between fibrosis and mast cell density in tongue of chronic chagasic patients (de Lima Pereira et al., 2007) and atherosclerotic aortas (Ramalho et al., 2013) of necropsied patients. These studies corroborate our findings because we observed a positive and significant correlation between the percentage of collagen and the density of mast cells in the tongue. Thus, in the present study, the passive inhalation of tobacco smoke would be causing an increase in number and mast cell degranulation with consequent release of fibrogenic factors. However, as there was a lower percentage of collagen in the groups exposed to tobacco smoke when compared to control group, we believe that the collagen destruction caused by the action of the smoke is overcoming the deposition process.

HIF-1 is a basic protein composed of two subunits: HIF-1α and HIF-1β. The α subunit is expressed in dependence on the availability of oxygen. This protein is involved in important adaptive responses to hypoxia, promoting cell survival under these conditions (Semenza 2004). Although studies have reported a decrease in HIF-1α in lung tissue of adult rats (Wagner et al., 2015) and progeny of rats (Singh et al., 2015) exposed to tobacco smoke, our findings demonstrated that the immunolabeling anti-HIF- 1α was significantly higher in the TAB 45 groups when compared to the control group, in the three thirds analyzed. In the posterior third,

TAB 30 group presented significantly higher anti-HIF-1α immunolabeling than the control group. Our findings corroborate other studies that also found an increase in HIF-1α levels in cultures of human lung cancer cells exposed to nicotine (Zhang et al., 2007), in the nasal tissue of rat models with eosinophilic rhinosinusitis exposed to the smoke of tobacco (Lee et al., 2004) and in orbital fibroblasts exposed to tobacco smoke extract (Görtzg et al., 2016).

In the present study, the increase in HIF-1α expression in the groups exposed to tobacco smoke probably occurred due to the action of nicotine and carbon monoxide present in the smoke, which would cause both vasoconstriction (Miyang et al., 2014) and complex formation carboxyhemoglobin, which prevent the binding of the oxygen molecule to hemoglobin, difficulting the transport and supply oxygen to the cells (Miyang et al., 2014; Leone 2015). As TAB 45 group showed the highest immunolabeling for HIF-1α in relation to the control group in the three thirds analyzed, we believe that tissue hypoxia occurs with a longer exposure to tobacco smoke. However, as the posterior third of TAB 30 group also presented greater immunolabeling for HIF-1α when compared to the control group, we believe that this region was more exposed to the local action of the smoke inhalation.

Therefore, the exposure of rats to cigarette smoke causes decrease in perimysial collagen fibers, increase in the number of mast cells and increase in immunolabeling for HIF-1α in lingual muscle cells. Knowing the importance of lingual musculature in swallowing, chewing and speaking, new studies should be carried out to better understand the pathogenesis of lingual changes in passive inhalation of tobacco smoke.

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Declaration of conflict of interest

There is no conflict of interest to declare.

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Conclusão

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Portanto, a exposição de ratos à fumaça do cigarro provoca diminuição das fibras colágenas perimisiais, aumento do número de mastócitos e aumento da imunomarcação para HIF-1α nas células musculares linguais, pela primeira vez descritas. Sabendo da importância da musculatura lingual na deglutição, mastigação e fala o presente estudo chama a atenção para prevenção contra a exposição passiva ao tabaco.

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Anexos e Apêndice

Anexo I

UNIUBE Educação e Responsabilidade Social — Comitê de Ética em Experimentação Animal

Ofício CEEA–070/2017 — Uberaba, 1 de dezembro de 2017

Ilma Profa.
Sanívia Aparecida de Lima Pereira

Assunto: Encaminha processo nº 024/2017, sobre o protocolo de pesquisa *"Análise hitopatológica do tecido epitelial e da musculatura da língua de ratas, submetidas a inalação da fumaça do tabaco"*.

Prezado(a) Professor(a),

Em resposta a sua solicitação, informo que o protocolo acima referido foi submetido avaliação do CEEA-UNIUBE, na reunião do dia 20/09/2017, sendo considerado **aprovado.**

Atenciosamente,

Profa. Joely F. Figueiredo Bittar
Coordenadora do CEEA-UNIUBE

Campus Aeroporto – Av. Nenê Sabino, 1801, Bloco R – B. Universitário – 38055-500 – Uberaba, MG – Fone: (34) 3319-8959 – Fax: (34) 3314-8910
e-mail: cep@uniube.br

Anexo II

Figura 1

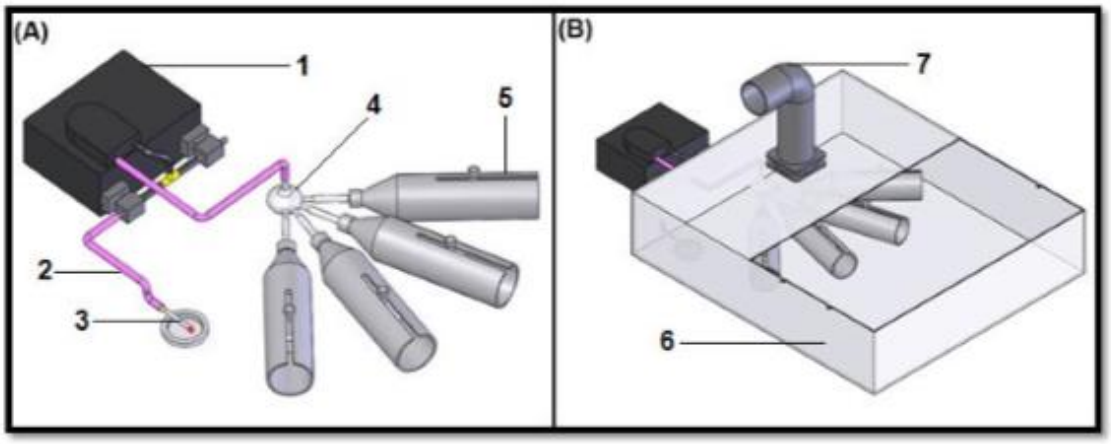

Figura 1. (A) Desenho esquemático do sistema usado para expor os animais à fumaça do cigarro. Bomba peristáltica [1], tubulação [2], cigarro [3], recipiente esférico para distribuição da fumaça [4] e os tubos cilíndricos [5], onde os animais ficaram contidos. (B) Desenho esquemático da caixa externa isolante [6] e exaustor para o meio externo [7] (imagem fornecida pelo Laboratório de Bioengenharia da Faculdade de Medicina de Ribeirão Preto [2015]).

Anexo III

Journal: Inhalation Toxicology
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Updated April 2016

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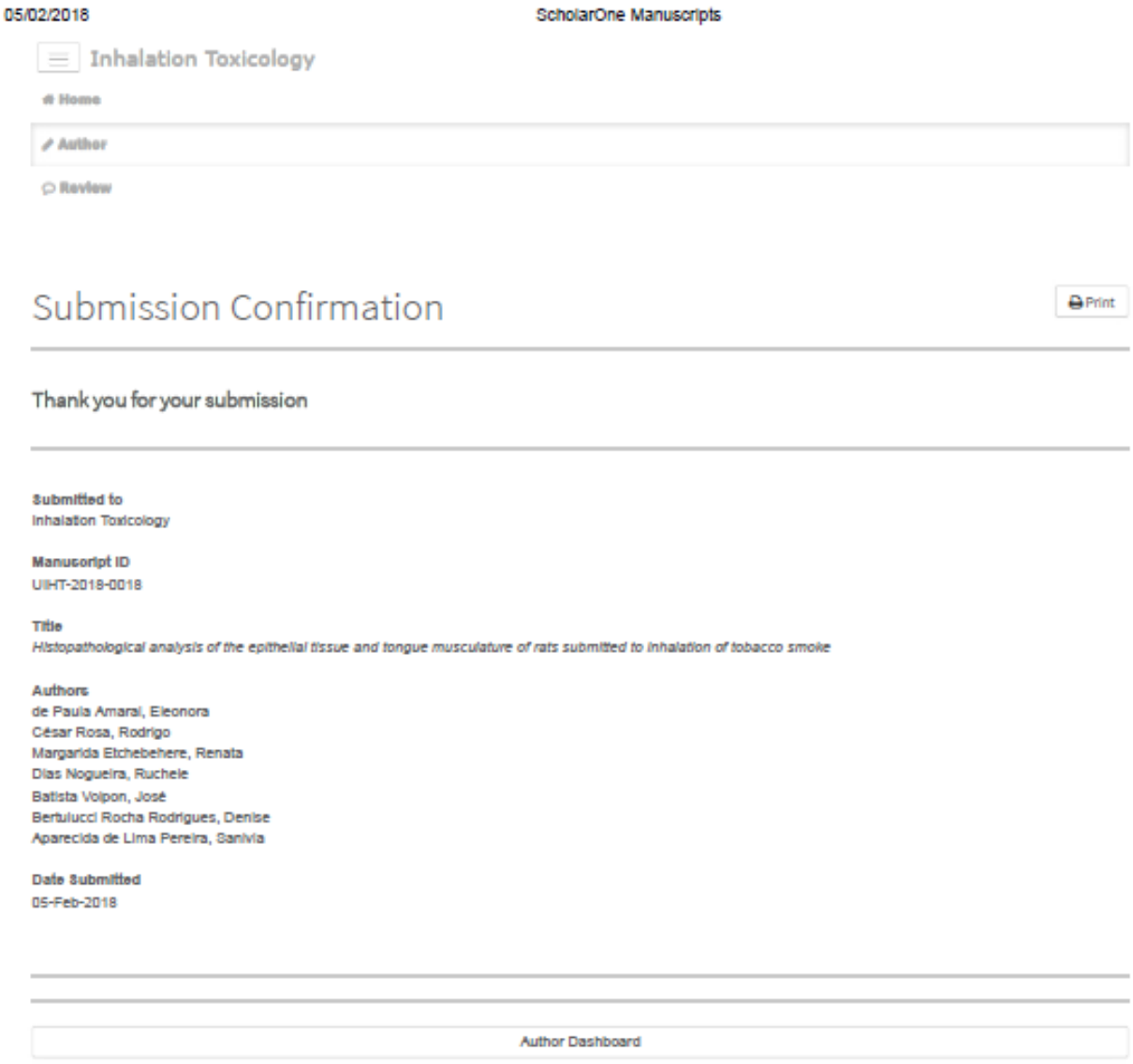
Anexo IV

Inhalation Toxicology

Histopathological analysis of the epithelial tissue and tongue musculature of rats submitted to inhalation of tobacco smoke

Journal:	*Inhalation Toxicology*
Manuscript ID	Draft
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	de Paula Amaral, Eleonora; Universidade de Uberaba César Rosa, Rodrigo; Universidade Federal do Triangulo Mineiro Margarida Etchebehere, Renata; Universidade Federal do Triangulo Mineiro Dias Nogueira, Ruchele; Universidade de Uberaba Batista Volpon, José ; Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto Bertulucci Rocha Rodrigues, Denise; Universidade de Uberaba Aparecida de Lima Pereira, Sanivia; sanivia.pereira@uniube.br.

Anexo V



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Title
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Authors
de Paula Amaral, Eleonora
César Rosa, Rodrigo
Margarida Etchebehere, Renata
Dias Nogueira, Ruchele
Batista Volpon, José
Bertulucci Rocha Rodrigues, Denise
Aparecida de Lima Pereira, Sanivia

Date Submitted
05-Feb-2018

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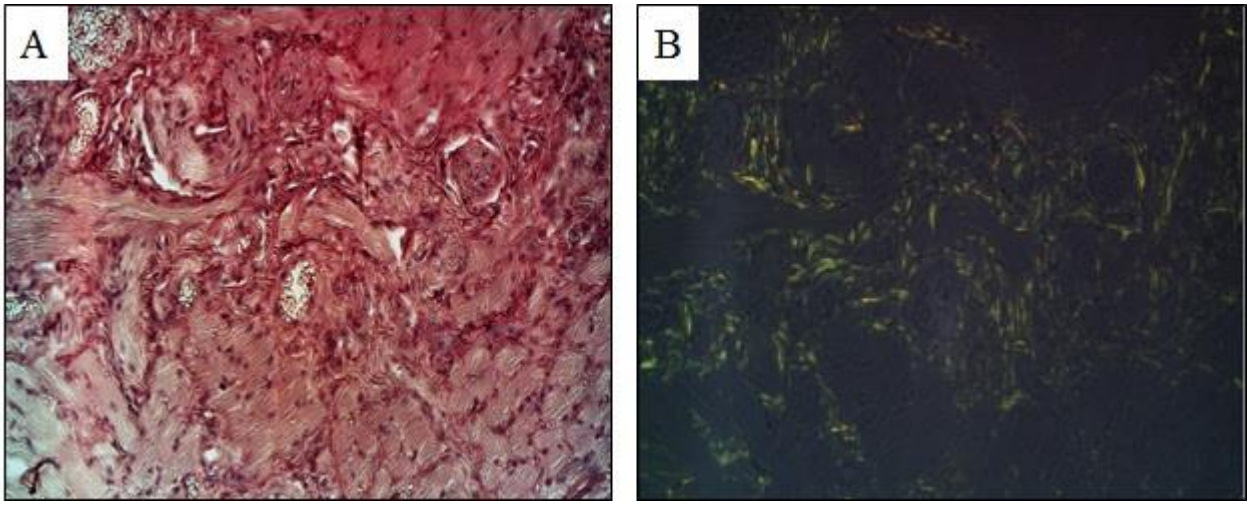
Apêndice

Figura 2A e 2B



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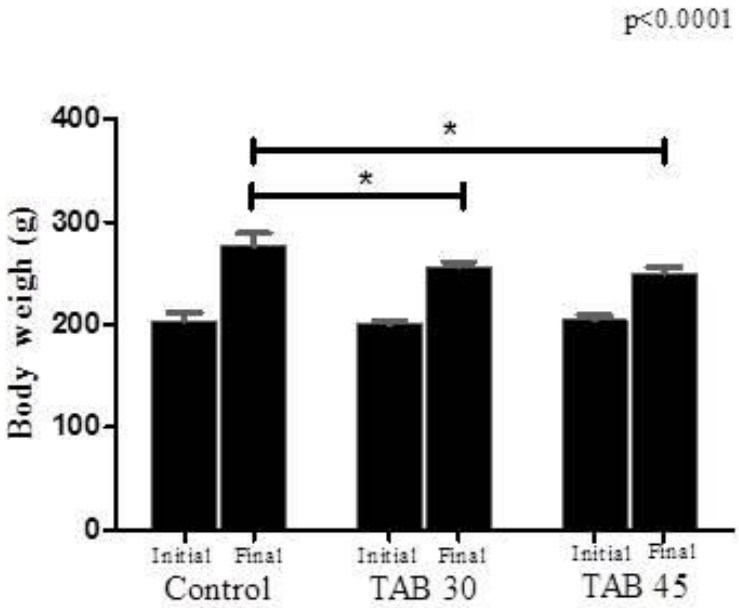

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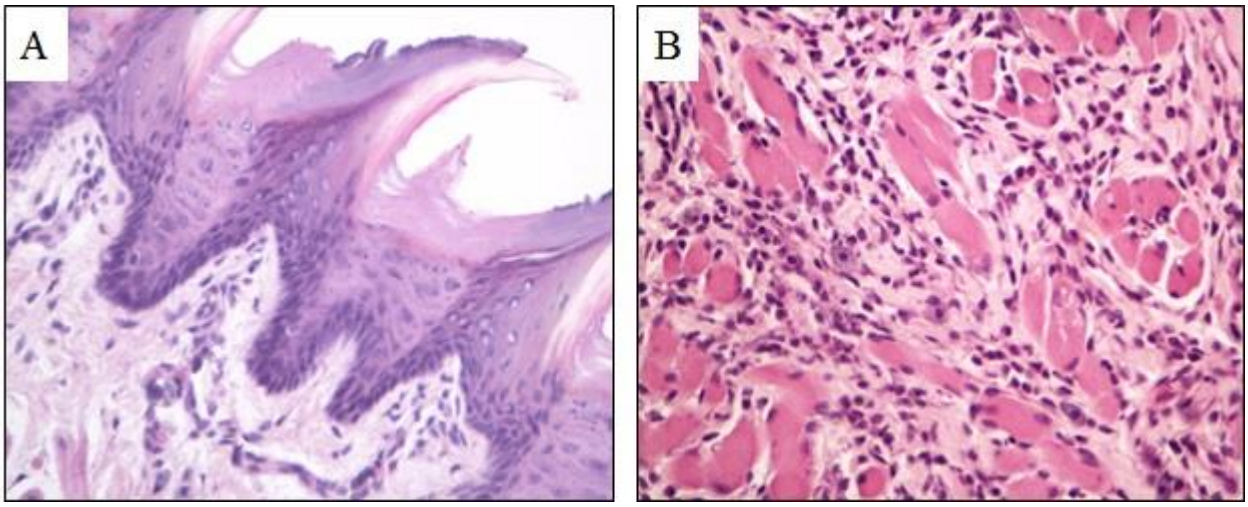

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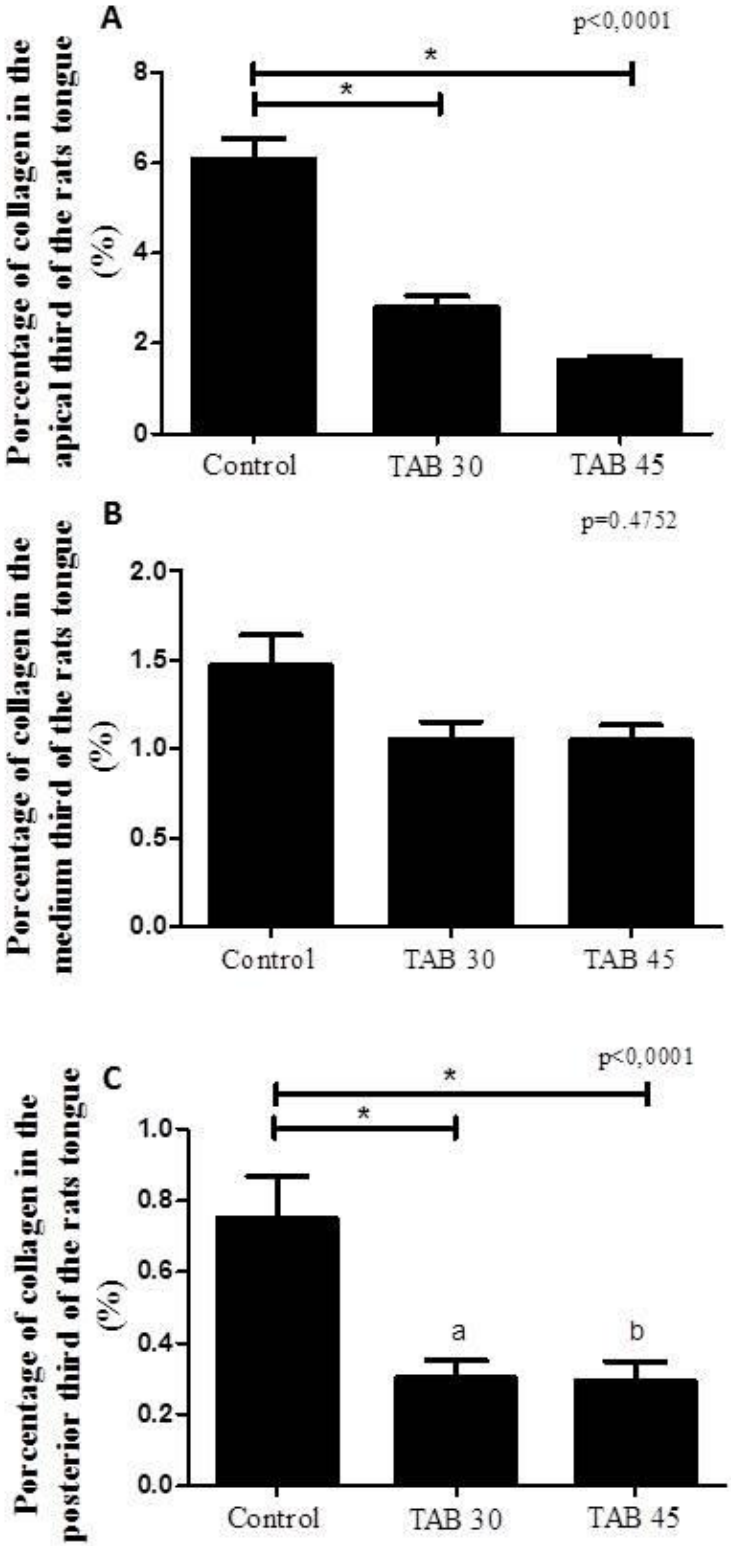
A
p<0,0001
*
*
Porcentage of collagen in the apical third of the rats tongue (%)
8
6
4
2
0
Control
TAB 30
TAB 45
B
p=0.4752
Porcentage of collagen in the medium third of the rats tongue (%)
2.0
1.5
1.0
0.5
0.0
Control
TAB 30
TAB 45
C
p<0,0001
*
*
Porcentage of collagen in the posterior third of the rats tongue (%)
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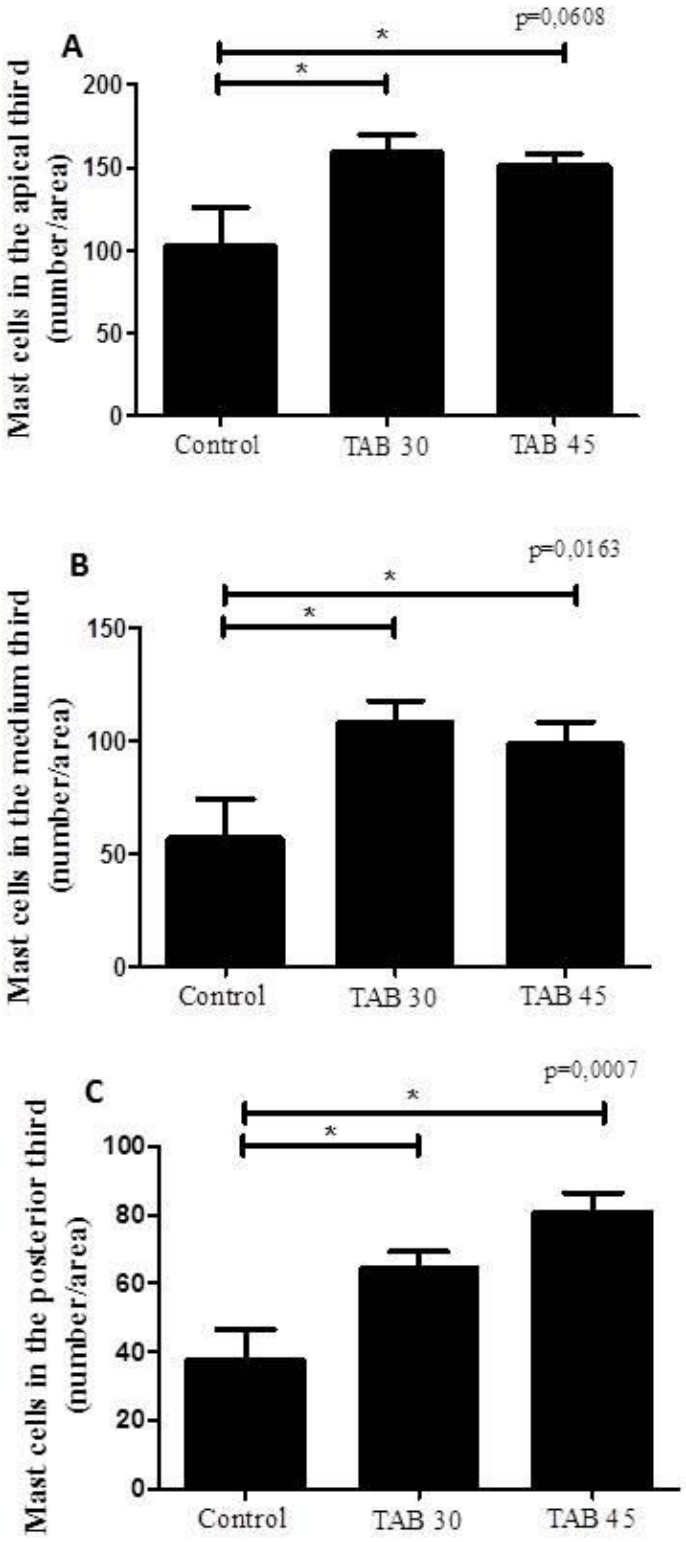
0.8
0.6
0.4
0.2
0.0
a
b
Control
TAB 30
TAB 45

Figura 6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*
Mast cells in the apical third (number/area)
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150
100
50
0
Control
TAB 30
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*
*
Mast cells in the medium third (number/area)
150
100
50
0
Control
TAB 30
TAB 45
C
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Figura 7

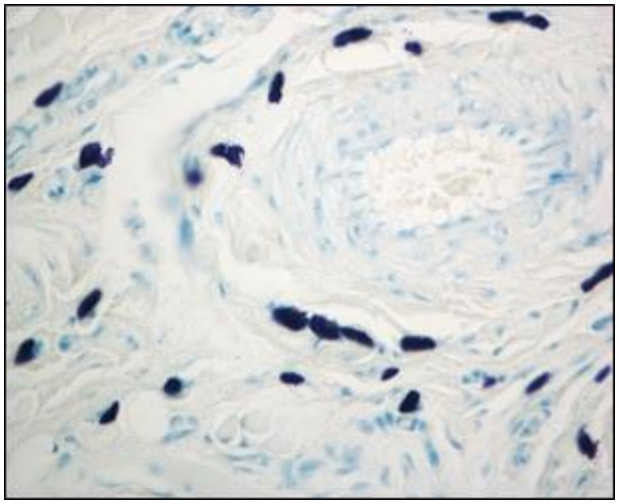


Figura 8A e 8B

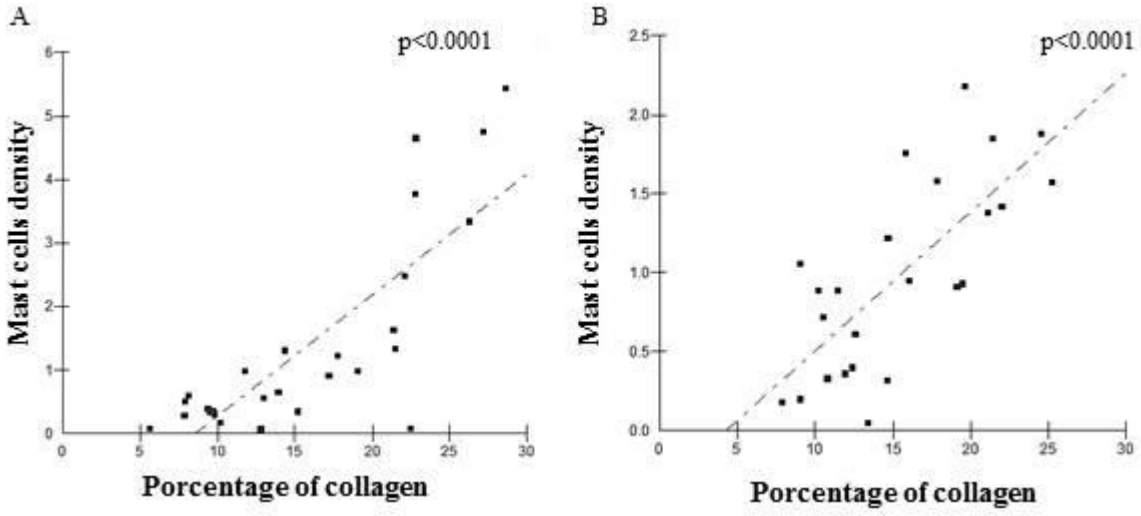
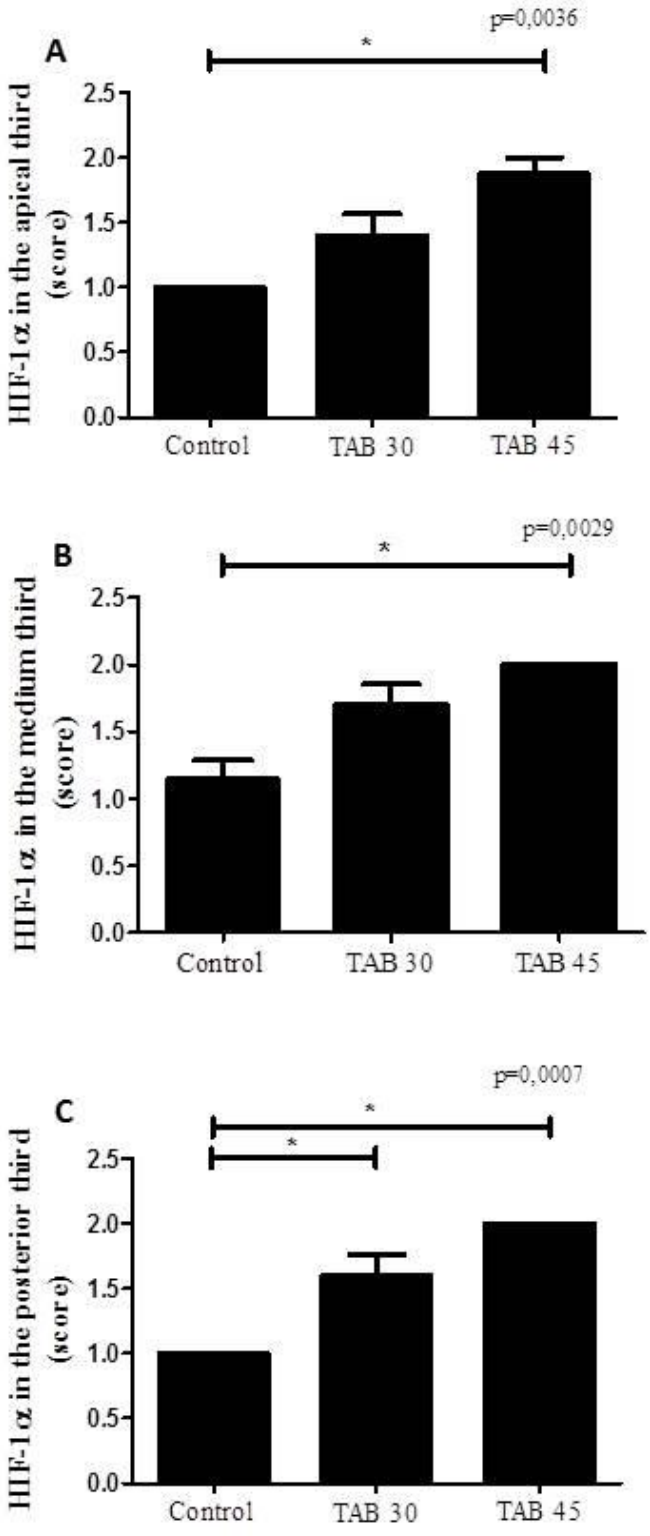


Figura 9


A
p=0,0036
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2.5
2.0
1.5
1.0
0.5
0.0
Control
TAB 30
TAB 45
B
p=0,0029
*
HIF-1α in the medium third (score)
2.5
2.0
1.5
1.0
0.5
0.0
Control
TAB 30
TAB 45
C
p=0,0007
*
*
HIF-1α in the posterior third (score)
2.5
2.0
1.5
1.0
0.5
0.0
Control
TAB 30
TAB 45

Figure 10