UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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Fumo passivo e os efeitos sobre mucosa laringotraqueal em ratos *Wistar* machos, durante crescimento: Estudo Experimental.

Porto Alegre
2015

Catalogação na Publicação

Leão, Henrique Zaquia Leão
Fumo passivo e os efeitos sobre mucosa laringotraqueal em ratos Wistar machos, durante o crescimento : Estudo experimental / Henrique Zaquia Leão Leão. -- 2015.
75 f. : il., graf., tab. ; 30 cm.

Tese (doutorado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2015.

Orientador(a): Prof. Dr. Geraldo Pereira Jotz Jotz ; coorientador(a): Prof. Dr. Claudio Galleano Zettler Zettler, Profa. Dra. Paula Rigon da Luz Soster Soster.

1. Cigarro. 2. Laringe. 3. Traqueia. 4. Ratos jovens. 5. Histopatologia. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor em Ciências da Saúde

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Profa. Dra. Paula Rigon da Luz Soster

Porto Alegre
2015

À Odila,

sempre na memória

de minhas conquistas

pessoais e profissionais.

Agradecimentos

Doutor Geraldo Jotz.

Tenho que agradecer por todas “as estradas” de Pós-Graduação, começando pelaUNIFESP. Agradeço, ainda, por sua inesgotável energia, sensitiva e motora, mantendo o “tônus” do trabalho.

Doutor Cláudio Zetler.

Homem com quem você senta para um café e saboreia conhecimentos de alto nível.

Doutora Paula Soster.

Onde encontrei apoio e disposição permanente para ajudar.

Doutor Marcelo Lammers.

Estatura ética e conhecimento que cursa desde uma técnica adequada para tratar o animal até as sofisticadas técnicas de pesquisa molecular.

Doutor Eduardo Cambruzzi.

Quando me deparei com o complexo “mundo da leitura patológica”, recebi a segurança, conhecimento e o bom humor de quem domina os movimentos do charriot.

Doutor Deivis de Campos.

Trouxe inestimável contribuição na confecção final do artigo.

Doutora Cláudia Rhoden.

Exemplo de competência científica e cuidado acadêmico que garante continuidade qualificada dos nossos trabalhos.

Doutores Professores do Programa de Pós-Graduação da UFCSPA.

Descobri, no ciclo acadêmico desta Universidade, uma equipe de competências acadêmico-científicas com alto nível formador.

Biólogo Antonio Severino.

Parceiro incansável que motiva e é motivado pelos desafios científicos.

Resumo

Introdução: Os cigarros contêm substâncias tóxicas e carcinogênicas. Neste contexto, o consumo de cigarros e assemelhados tem sido associado a um alto índice de patologias, sendo considerado fator de risco de até 10% do número total de óbitos entre adultos. Evidências recentes sugerem que a exposição de crianças ao fumo passivo, pode ocorrer logo nos primórdios do seu desenvolvimento, ocasionando assim, inúmeras patologias. **Objetivo:** O objetivo deste estudo foi analisar o possível efeito histopatológico na mucosa laríngea e traqueal de ratos *Wistar* jovens submetidos ao modelo experimental de inalação crônica de fumaça de cigarro (*fumante passivo*), por meio de microscopia óptica. **Material e Método:** Foram estudados vinte e quatro ratos jovens da raça *Wistar* por um período de 120 dias. Os animais foram divididos em dois grupos: fumante passivo (n = 16) e controle (n = 8). O nível de exposição ao cigarro foi avaliado a partir do nível urinário de cotinina. **Resultados:** Embora sem lesões cancerígenas, a análise histopatológica na mucosa laríngea e traqueal de todos os ratos do experimento demonstrou que a proporção de inflamação leve e focal foi maior nos animais que foram expostos à inalação crônica de fumaça de cigarro (*P=0,041*). **Conclusão:** A análise histopatológica demonstrou lesão inflamatória leve e focal na região mucosa infraglótica dos animais expostos, sem lesão displásica ou neoplásica na mucosa laríngea e traqueal.

Palavras –Chave: Cigarro, Laringe, Traqueia, Ratos jovens, Histopatologia

Abstract

Introduction: Cigarettes contain toxic and carcinogenic substances. In this context, cigarette smoking and similars have been associated with a high incidence of pathologies, being considered a risk factor for up to 10% of the total number of deaths in adults. Recent evidence suggests that children's exposure to smoking may occur soon, in the early days of its development, causing so many diseases.
Objective: The objective of this study was to analyze the possible histopathological effects on laryngeal and tracheal mucosa of Wistar young undergoing experimental model of chronic inhalation of cigarette smoke (passive smoking), by optic microscopy. **Methods:** Twenty-four young rats Wistar were studied for a period of 120 days. The animals were divided into two groups: passive smoking (n = 16) and control (n = 8). The level of exposure to cigarette smoke was evaluated from urinary cotinine level. **Results:** Although they not present cancerous lesions, histopathological analysis of laryngeal and tracheal mucosa in all animals of the experimental showed that the proportion of moderate and focal inflammation was higher in animals exposed to chronic inhalation of cigarette smoke (P = 0.041).
Conclusion: Histopathologic analysis revealed moderate and focal inflammatory lesion in the region of infraglottic mucosa of animals exposed without dysplastic or neoplastic lesion in laryngeal and tracheal mucosa.
Key-words: Cigarette; Larynx; Trachea; Young rats; Histopathology.

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LISTA DE ABREVIATURAS

ACTH hormônio adrenocorticotrófico

C controle ou *control*

CFP corrente de fumaça primária

CFS corrente de fumaça secundária

DNA ácido desoxirribonucleico

F fumantes

FA fumaça ambiente

FLC fumaça lateral do cigarro

HE hematoxilina eosina

HPLC high performance liquid chromatography ou cromatografia líquida de alto desempenho

NNK nitrosamina-4-(metilnitrosamino)-1-(3-pridil)-1-(butanona)

PM 2,5 filtrado aerosol com 2,5µm

PS passive smoking ou fumante passivo

Sumário

1. Introdução

A fumaça liberada pela queima do cigarro libera metais-traço tóxicos para a saúde humana e ambiental[1]. A fumaça do cigarro pode ser classificada em duas categorias: uma via principal da fumaça de cigarro (*mainstream*), geralmente inalada por fumantes ativos e a segunda e uma via secundária da fumaça de cigarro (*sidestream*), ou fumaça lateral do cigarro (FLC), emitida pelo cigarro e inalada pelos assim chamados “fumantes passivos”. É sabido que a exposição à FLC contém uma variedade de oxidantes e outros compostos nocivos em quantidade maior do que aqueles contidos na fumaça da via principal. Os fumantes passivos são, dessa forma, expostos quase às mesmas substâncias químicas na fumaça do cigarro que os fumantes ativos. Sendo assim, o fumo passivo aumenta o risco de doenças relacionadas ao tabaco em não-fumantes[1].

A fumaça ambiente (FA) liberada pela queima do tabaco é uma mistura da fumaça exalada diretamente pelos fumantes (corrente de fumaça primária - CFP) e uma porção liberada pela queima da porção distal do cigarro (corrente de fumaça secundária – CFS ou fumaça lateral do cigarro - FLC). Esta FA pode causar doenças nos consumidores diretos e também pode gerar efeitos danosos nas pessoas que a aspiram indiretamente. As pessoas expostas à FA são denominadas fumantes passivos[2,3,4]

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1.1 Epidemiologia do consumo de tabaco

De acordo com a Organização Mundial da Saúde (OMS), em torno de 200.000 fumantes passivos morrem anualmente, devido ao consumo indireto de fumígeros[2]. Acredita-se que em torno da metade das crianças do mundo respiram ar contaminado pela fumaça de cigarro. Adicionalmente, recentes evidências sugerem que a exposição de crianças ao cigarro pode ocorrer logo nos primórdios do seu desenvolvimento fetal[7].

Seong et al[6] demonstraram a presença de nicotina no cabelo de crianças recém-nascidas, enquanto Chang et al[7] relataram que crianças, cujo pai era fumante no período pré-concepção e que continuavam expostas ao fumo passivo pós-nascimento, estariam mais suscetíveis ao desenvolvimento de leucemia infantil.

A prevalência de tabagismo no Brasil foi investigada em 2004 pelo Ministério da Saúde através do Instituto Nacional do Câncer (INCA)[8] em 16 capitais, por meio de um inquérito epidemiológico que entrevistou 23.457 indivíduos, sendo 10.175 do gênero masculino e 13.282 do sexo feminino. A prevalência de tabagismo encontrada variou de 12,9% a 25,2% (em homens: de 16,9% a 28,2%; em mulheres: de 10,0% a 22,9%) (Brasil, 2004a)[8]. Esses índices são bastante preocupantes, porém

inferiores aos apontados em inquéritos anteriores, como o de 1998, realizado pelo Instituto Brasileiro de Geografia e Estatística (IBGE) cuja prevalência de tabagismo foi de 39,3% para os homens e de 25,2% para as mulheres. (IBGE, 1998)[8]. O hábito de fumar foi mais frequente entre os homens, numa proporção de 2:1, porém nas regiões Sudeste e Sul tal proporção aproximou-se da unidade. O hábito mostrou-se mais frequente entre os adultos, porém nas regiões mais urbanizadas foi também frequente entre jovens de 15 a 24 anos. Foi achado especialmente entre os indivíduos com menor escolaridade e, consequentemente, de menor renda. O tabagismo foi também detectado em elevada porcentagem de adolescentes de 13 a 15 anos, especialmente nas regiões de Porto Alegre (48,3%) e Curitiba (46,3%). Um dado promissor em nosso país foi o elevado índice de cessação de uso de tabaco, em torno de 50% em todas as capitais pesquisadas, índice superior ao de outros países, como por exemplo, dos EUA que é de 40%. O inquérito também apontou uma elevação na taxa de mortalidade para o câncer de pulmão nas mulheres, em todas as regiões do país, o que pode estar associado ao aumento da prevalência do tabagismo nesse gênero. Isto difere do que vem sendo observado entre os homens, que apresentam tendências estáveis ou mesmo de queda a partir da metade da década de 90[8].

1.2 Caracterização do tabaco

A disseminação do tabaco se iniciou com a chegada de Colombo às Américas, no final do século XV. Levado à Europa, o produto teve grande aceitação e se difundiu pelo mundo. Essa grande aceitação é explicada pelo alto poder viciante da nicotina, alcaloide presente nas folhas da planta. A nicotina é o principal componente psicoativo do tabaco. No século XVIII a nicotina era oferecida no tabaco em pó, e no século XIX, na forma de charutos . Em 1881 foi inventada a máquina de manufatura do cigarro, tornando-se o principal meio de provimento de nicotina. Estima-se que atualmente haja cerca de 1,3 bilhão de fumantes no mundo. A maioria dos usuários é do gênero masculino, com uma tendência para se tornar uma doença predominantemente feminina nas sociedades ocidentais. Hoje, é comum a iniciação ao tabagismo na adolescência, com o uso esporádico[9].

1.3 Toxicidade do tabaco

O ato de fumar cigarro resulta em absorção de nicotina pelo epitélio respiratório, sendo transformada em mais de vinte metabólitos excretados na urina. A cotinina é o principal metabólito da nicotina que se acumula no organismo com o uso regular do cigarro, sendo detectada em diferentes fluidos do organismo como a urina, a saliva e o soro sanguíneo, e tem sido usada como um biomarcador para o consumo ativo e passivo de tabaco[10,11,12,13,14]. Entre os vários componentes nocivos do cigarro, as nitrosaminas (Nitrosamina-4-(metilnitrosamino)-1-(3-piridil)-1-(butanona) (NNK) podem se ligar ao DNA e induzir mutações pontuais[15,16]. NNKs são metabolizadas, em cascata, através das enzimas citocromo P450 e enzimas da família das cicloxigenases e lipoxigenases, originando produtos que se ligam ao DNA[17,18]. Já foi observado que em ratos, a administração de NNKs induz mutações em genes da família RAS, sendo estes animais mais suscetíveis à proliferação celular e menos responsivos aos inibidores de proliferação, podendo promover o desenvolvimento de adenocarcinomas[19]. A administração subcutânea de NNK em ratas grávidas aumentou a incidência de adenocarcinomas pulmonares na prole, enquanto a administração intrabronquial de NNK nas ratas grávidas na véspera do nascimento aumentou em 65% o risco de desenvolver adenocarcinomas pancreáticos na prole[20].

1.4 Doenças associadas ao consumo de cigarro

As agressões causadas pela inalação crônica da fumaça do cigarro incluem diferentes tipos de cânceres, afetando laringe, faringe, estômago, fígado, pâncreas, bexiga, rim e ureter (*International Agency for Research on Cancer*, 2009)[21]. Além disso, mesmo a exposição em níveis relativamente pequenos de poluição atmosférica ou fumo passivo pode ser suficiente para induzir alterações biológicas do sistema cardiovascular[21].

Entre as doenças mais frequentes associadas ao consumo de cigarro em adultos, destaca-se a elevada frequência de câncer de boca, orofaringe e esôfago[22], sendo o carcinoma epidermoide a forma histológica mais comum. A fumaça de cigarro é considerada indutora de tumores de laringe em hamsters[23] e pelo menos um estudo demonstrou um aumento de 1% a 9% na incidência de tumores pulmonares adenomatosos[24].

1.5 Modelos experimentais de exposição à fumaça do cigarro

Apesar de todas as evidências epidemiológicas dos danos causados pelo consumo ativo e passivo de cigarro em humanos, o uso de um modelo animal é imprescindível para controlar as variáveis inerentes ao ser humano (por exemplo, gênero, faixa etária, etnia, faixa social, hábitos alimentares, higiene pessoal), bem como servir de base para ensaios metodológicos que sirvam de base para o desenvolvimento de agentes preventivos. As dificuldades de estabelecimento de um modelo animal vêm exatamente da necessidade de exposição crônica dos efeitos deletérios da inalação de fumaça, o que demandaria trabalhos de longa duração e de difícil acompanhamento.

Mais recentemente, alguns autores têm desenvolvido modelos animais de indução de patologias frente a exposição aguda à fumaça de cigarro. Chiba et al[25] demonstraram que ratos expostos durante 14 dias à fumaça do cigarro apresentavam maior presença de neutrófilos no trato respiratório e uma maior responsividade dos músculos dos brônquios à ação de ACTH. Já Izzatti et al[26] demonstraram que células pulmonares de camundongos expostos a fumaça de cigarro durante 1 mês apresentavam uma diminuição na expressão de um grande número de microRNAs, os quais são pequenos RNAs não codificantes envolvidos na regulação de processos biológicos como crescimento celular e apoptose.

Modelos de exposição crônica à fumaça de cigarro também têm apresentado resultados satisfatórios na indução de doenças. Balansky et al[5] demonstraram que o cigarro pode ser um potente carcinógeno em camundongos, quando administrado logo após o nascimento, pelo período de 120 dias, sendo detectada a presença de diferentes tumores no sistema respiratório a partir dos 75 dias de administração. Wittel et al[27] demonstraram que ratos expostos a inalação de fumaça do cigarro durante 90 dias apresentavam uma diminuição da função pancreática relacionada à alterações morfológicas, como uma maior deposição de matriz extracelular sem a presença de inflamação. Já D'agostini et al[28] demonstraram que a exposição diária durante 120 dias de camundongos à fumaça induz degeneração do parênquima no fígado.

Os modelos experimentais de exposição crônica à fumaça do cigarro tem utilizado diferentes animais com tempos diferentes de exposição por períodos que variam de semans a meses[5, 20, 23, 25, 26, 27, 28.] Estas variações trazem dificuldades para estabelecermos parâmetros comparativos nos achados experimentais.

Embora tenhamos respostas do efeito lesivo do tabaco sobre a mucosa respiratória em vários estudos experimentais e clínicos, nos falta reconhecer o efeito da exposição moderada à fumaça ambiente sobre a mucosa laringotraqueal no desenvolvimento do estágio infantil até a maturidade reprodutiva, assim como carecemos de estudos que mostrem a resposta tecidual à fumaça ambiente, da mucosa laríngea comparada com a mucosa traqueal no mesmo indivíduo.

1.6 Anatomia da laringe

A laringe é estruturada por um esqueleto osteocartilaginoso com três cartilagens únicas suspensas pelo osso hioide e três cartilagens pares. Seguindo abaixo do osso hioide encontraremos a cartilagem tireoide. No ângulo interno da cartilagem tireoide, está fixada a cartilagem epiglótica que se estende por trás e acima do osso hioide. A cartilagem cricoide faz a base da laringe onde se articulam a cartilagem tireoide e um par de pequenas cartilagens que fixam as pregas vocais: as cartilagens aritenoides. A cricoide se junta com o primeiro anel cartilaginoso da traqueia. Os movimentos são controlados por músculos intrínsecos. Nos espaços internos da laringe, encontraremos uma cobertura mucosa que possui um epitélio respiratório nos espaços ventilatórios (supraglótico e infraglótico) e um epitélio estratificado escamoso não queratinizado na superfície vibratória das pregas vocais, que reconhecemos como região glótica.

É na glote que a voz é produzida. A adução das pregas e o “aprisionamento” do ar no espaço infraglótico, gera uma pressão aérea que, quando liberada, provoca a vibração da mucosa vocal e a vocalização. Assim, é de se reconhecer que as áreas supraglótica e infraglótica, sofrem menor atrito e a região glótica sofre mais.

A laringe pode ser dividida em três espaços internos que mostram diferentes funções: supraglótico, separando a via digestiva da via aérea; glótico, com função fonatória e infraglótico que dá entrada na traqueia e é comparável com a luz traqueal adjacente[29].

1.6.1 Organização histológica da glote

Histologicamente, as pregas vocais apresentam cinco camadas[30, 31, 32]

1- A camada mais superficial é formada por epitélio de revestimento estratificado pavimentoso não queratinizado;

2- Sob o epitélio, encontramos a membrana basal com tecido conjuntivo frouxo, baixa densidade de fibras elásticas e baixa celularidade. Essa composição confere pouca resistência à vibração e permite a mobilidade do revestimento mucoso;

3- A camada intermediária da lâmina própria é composta de fibras elásticas e colágenas;

4- A camada profunda da lâmina própria é formada também por fibras elásticas e colágenas, entretanto as fibras colágenas estão proporcionalmente mais abundantes do que as elásticas, quando comparadas às fibras que compõem a camada intermediária. Juntas, as camadas intermediária e profunda compõem o ligamento vocal, com maior a concentração de fibras colágenas e menor a concentração de fibras elásticas;

5- A camada mais profunda da prega vocal é composta pelo músculo vocal.

O tecido conjuntivo que compõe a lâmina própria das pregas vocais tem propriedades peculiares. Nas camadas intermediária e profunda, as fibras se dispõem paralelas ao plano epitelial de forma organizada. Já na camada superficial, essas fibras são pequenas e imaturas e se dispõem de forma menos regular, mas ainda percebe-se o paralelismo. As fibras colágenas e elásticas conferem, respectivamente, resistência e elasticidade às pregas vocais, além de fornecerem arcabouço para sustentação e contenção dos demais componentes histológicos[30, 31, 32, 33, 34]

De acordo com Kurita et al[35], há diferenças na porção membranosa e nas densidades das pregas vocais de humanos e ratos. Porém, a lâmina própria de ambos é dividida em três porções, isto é, uma camada de superfície com componentes fibrosos escassos, uma camada intermediária com mais componentes e uma camada profunda com mais colágenos e fibras elásticas. A divisão entre a superfície e a porção intermediária não é muito óbvia, contudo a transição da intermediária para a camada profunda é bastante clara.

Além da matriz extracelular fibrosa, a matriz extracelular amorfa representa importante componente da lâmina própria sem a qual as pregas vocais não exerceriam adequadamente a função fonatória. Essa matriz, na forma de gel, é constituída por substâncias dispostas entres as fibras conjuntivas e as células e tem a capacidade de reter água e controlar a viscosidade do tecido. O ácido hialurônico é a principal glicosaminoglicana da lâmina própria. Tem a função de regular a hidratação e viscosidade do tecido, além de conferir estabilidade e elasticidade à matriz extracelular e amortecer o choque da vibração durante a fonação. O ácido hialurônico também tem papel importante na proliferação, regeneração e no reparo tecidual das pregas vocais, incluindo a fibrogênese, e na proteção à degradação das proteoglicanas. Também lhe foi atribuída a capacidade de carrear substâncias pela matriz extracelular como os fatores de crescimento, estimulando as mitoses dos fibroblatos da lâmina própria[30, 33, 36, 37,38].

Chan et al.[39] submeteram laringes frescas de cadáveres humanos à ação da hialuronidase para remoção do ácido hialurônico dos tecidos vocais e mediram as propriedades viscoelásticas de cisalhamento linear antes e após a aplicação da hialuronidase. Os autores identificaram que a rigidez da cobertura das pregas vocais diminuiu cerca de 35%, enquanto que a viscosidade aumentou 70% em frequências de vibração mais altas, quando o ácido hialurônico foi digerido pela hialuronidase.

Isso sugere que o ácido hialurônico é importante na determinação das propriedades biomecânicas da lâmina própria das pregas vocais, contribuindo com a viscosidade e rigidez ideais do tecido, importantes para o controle de frequência fundamental.

Em fetos, altos níveis de ácido hialurônico estão associados à regeneração de feridas, enquanto que a queda induzida de seus níveis pela hialuronidase leva ao aumento da fibroplasia e depósito de colágeno[40, 41].

A estrutura das pregas vocais sofre alterações com a idade e é um pouco diferente entre os gêneros. Hartnick et al[42] estudaram as lâminas próprias das pregas vocais de 34 laringes pediátricas e observaram que a lâmina própria, ao nascimento, não exibe a clássica divisão em três camadas. A lâmina própria das pregas vocais dos recém-nascidos se caracterizava por uma única camada hipercelular e pobre em fibras. As células, nesta camada, apresentavam alta atividade e aparentemente sem diferenciação. Não havia fibras elásticas formadas, mas sim vestígios de elastina. Aos dois meses começava a haver diferenciação celular e notava-se a divisão em duas camadas, uma mais superficial, pobre em células e mucina e outra mais profunda e com maior celularidade e mucina. Aos 11 meses, 20% dos espécimes exibiam uma divisão em três camadas da lâmina própria, porém não com as características das camadas clássicas do adulto. Havia duas camadas hipocelulares, uma superficial, próxima ao epitélio e outra profunda, próxima ao músculo vocal, e entre elas uma terceira camada hipercelular. Aos sete anos, todos os espécimes mostravam uma divisão trilaminar, como exposto anteriormente. Entretanto, a camada média apesar de já apresentar uma faixa com maior densidade de colágeno e fibras elásticas, ainda não configurava o ligamento vocal. Os espécimes entre 11 e 12 anos já apresentavam o padrão clássico trilaminar da lâmina própria do adulto.

Ramos et al.[43] estudaram a variação da concentração do ácido hialurônico em ratas, segundo a idade. Foram selecionadas oito ratas jovens e cinco idosas. Suas pregas vocais foram removidas e tratadas para medição do ácido hialurônico por método fluorimétrico. Como resultado, identificaram que ratas idosas apresentam menos da metade da concentração de ácido hialurônico nas pregas vocais quando comparadas às jovens.

Chen e Thibeault[44] analisaram culturas de fibroblastos de três amostras de biópsias de pregas vocais humanas de idades diferentes (21, 59 e 79 anos) e observaram que a idade interferiu negativamente na proliferação e diferenciação dos fibroblastos e na sua capacidade de síntese da matriz extracelular. Entretanto, mantinham-se os níveis de expressão gênica das substâncias da matriz extracelular.

Hammond et al[45], analisando laringes frescas de cadáveres humanos, notaram aumento significativo das quantidades de fibras elásticas de acordo com a idade. Os espécimes foram divididos em três faixas etárias: crianças (1 a 5 meses), adultos (30 a 39 anos) e idosos (65 a 82 anos). Verificaram que entre as crianças e os adultos houve acréscimo significativo de 23% de elastina e entre os adultos e os idosos esse acréscimo era de 879%. Não foram observadas diferenças entre os gêneros.

Imunohistologicamente, a laringe representa um órgão “transicional” que conecta a faringe com a área ventilatória. Segundo Jecker et al[29], ocorre um aumento crânio-caudal do número de células imunológicas da laringe, ajustando com a população celular da mucosa traqueal, mas, por razões ainda desconhecidas, a prega vocal está excluída da escala de crescimento de células imunológicas, mostrando um baixo número delas. O poder de defesa antigênica, na prega vocal, é dependente de estruturas como epitélio escamoso e membrana basal, muito espessa neste espaço.

A literatura consultada mostra que, durante o desenvolvimento, a mucosa dos diferentes espaços glóticos e a mucosa da traqueia, mostram respostas diferentes à agressão promovida pela fumaça do cigarro.

2. Objetivos

Objetivo Geral

O objetivo deste estudo é analisar, qualitativamente, o efeito histopatológico da inalalção crônica de fumaça de cigarro sobre a mucosa laringotraqueal de ratos *Wistar* jovens, submetidos ao modelo experimental.

Objetivos específicos

1. Aferir a massa ponderal dos animais submetidos à inalação crônica de fumaça de cigarro, durante o seu desenvolvimento.
2. Aferir a concentração de cotinina na urina, após exposição crônica dos animais à fumaça de cigarro.
3. Determinar a concentração de material particulado fino (PM 2,5μm), durante a queima passiva de cigarro, durante a exposição.
4. Analisar as lesões histopatológicas na mucosa laríngea supraglótica, glótica e infraglótica, e na mucosa traqueal dos animais expostos à fumaça de cigarro.

3. Artigo submetido para publicação no

<u>American Journal of Otolaryngology</u>

Elsevier Editorial System(tm) for American Journal of Otolaryngology

Manuscript Draft

Manuscript Number: YAJOT-D-15-00366

Title: Passive smoking and the effects on laryngeal and tracheal mucosa in male wistar rats during growth: an experimental study

Article Type: Original Contribution

Keywords: Cigarette; Larynx; Trachea; Young rats; Histopathology.

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May 10, 2015

Editor-in-Chief

American Journal of Otolaryngology

Dear Editor,

Please find attached the article entitled “Passive smoking and the effects on laryngeal and tracheal mucosa in male wistar rats during growth: an experimental study” whose authors are: Henrique Zaquia Leão, Claudio Galleano Zettler, Eduardo Cambruzzi, Marcelo Lammers, Paula Rigon da Luz Soster, Fernanda Bastos de Mello, Guilherme Reghelin Goulart, Deivis de Campos, Geraldo Pereira Jotz. We would greatly appreciate IF it could be submitted for publication in American Journal of Otolaryngology.

This work has not been previously published, nor is it submitted elsewhere for publication. All authors have read, approved the manuscript and have agreed to have their names listed as authors.

Thank you for your attention

We are looking forward to hearing from you.

Sincerely yours,

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Passive smoking and the effects on laryngeal and tracheal mucosa in male wistar rats during growth: an experimental study.

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1. Introduction

Epidemiology of tobacco use

The ambient smoke (AS) released by burning tobacco is a mixture of smoke exhaled by smokers directly (primary smoke stream - PSS) and released by burning a portion of the distal portion of the cigarette (secondary smoke stream - SSS). This AS can cause diseases in direct consumers and can also generate harmful effects on people who aspire indirectly. People exposed to AS are called passive smokers [1, 2, 3].

According to the World Health Organization (WHO), around 200.000 passive smokers die each year due to indirect consumption of tobacco's derived products [1]. It is believed that around half the world's children breathe air polluted by tobacco smoke. In addition, recent evidence suggests that children's exposure to smoking may occur in the early days of their fetal development. Seong et al [4] demonstrated the presence of nicotine in the hair of newborn children, while Chang et al [5] showed that children whose father was a smoker in the preconception period and still exposed to secondhand smoke after birth may be more susceptible to the development of childhood leukemia.

Tobacco toxicity

The cigarette smoking results in absorption of nicotine through the respiratory epithelium and is converted into more than twenty metabolites excreted in urine. Cotinine is the major metabolite of nicotine which accumulates in the body of the cigarette with regular use, being detected in different body fluids such as urine, saliva and blood plasma, and has been used as a biomarker for the active and passive consumption tobacco [6-10]. Among the many harmful constituents of cigarettes, nitrosamines (Nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (NNK) can bind to DNA and cause point mutations [11, 12]. NNKs are metabolized in cascade, through the cytochrome P450 family of enzymes and the enzymes cyclooxygenase and lipoxygenase, leading to products that bind to DNA [13, 14]. It has been observed that in mice, the administration NNKs induces mutations in the ras family, since these animals are more susceptible to cell proliferation and less responsive to proliferation inhibitors, and can promote the development of adenocarcinomas [15]. NNK subcutaneous administration to pregnant hamsters increased the incidence of pulmonar adenocarcinomas in the spawn, while intrabronchial administration of NNK in pregnant hamsters at birth eve increased by 65% the risk of developing pancreatic adenocarcinomas in the offspring [16].

Diseases associated with cigarette smoking

The aggression caused by chronic inhalation of cigarette smoke include different types of cancers, affecting the larynx, pharynx, stomach, liver, pancreas, bladder, kidney and ureter (*International Agency for Research on Cancer*, 2009) [17]. Moreover, even in relatively small exposure levels of air pollution or passive smoking can be sufficient to induce biological changes in the cardiovascular system [17]. Among the most frequent diseases inferred by cigarette smoking in adults, there is the high frequency of oral cancer, oropharynx and esophagus [18], squamous cell carcinoma is the most common histological form. Cigarette smoke is considered inducing laryngeal tumors in rats [19] and at least one study has shown an increase of 1% to 9% in the incidence of adenomatous lung tumors [20].

The larynx is divided in three internal spaces that show different functions: supraglottic, separating the gastrointestinal tract of air, glottal, with voice function and infraglottic that enters the trachea and is comparable with the adjacent tracheal lumen [21]. Thus, in the immunohistological analysis, the larynx is a "transitional" organ that connects the pharynx with the ventilatory area. According to Jecker et al [21], there is

an increase in the larynx craniocaudal immune cells, adjusting the cell population to tracheal mucosa, but for unknown reasons, the vocal fold is excluded from the growth range of immune cells, showing a low number them. The power of antigenic defense on the vocal folds is dependent of structures such as stratified squamous epithelium and basal membrane, wich are very thick in this space.

Our hypothesis is that, during development, the mucosa of different glottal spaces and the mucosa of the trachea, show different responses to aggression promoted by cigarette smoke. Thereby, this study aims to qualitatively analyze the possible histopathological effects on laryngeal and tracheal mucosa of young Wistar rats submitted to experimental model of chronic inhalation of cigarette smoke.

2. Metodology

Experimental design

This study was approved by the Ethics Committee on Animal Use (CEUA) with nº 19.127 protocol of the Universidade Federal do Rio Grande do Sul and Protocol

084/12 of the Ethics Committee on Animal Use (CEUA) of the Universidade Federal de Ciências da Saúde de Porto Alegre, Brazil.

Twenty-four male Wistar rats (*Rattus norvegicus*), with 12 days of life, were obtained from the research sector in animals of the Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA, RS, Brazil. The animals were randomly divided into two groups: Group PS - passive smoking (n = 16) and group C - control (n = 8). The number of animals for this type of experiment has been statistically validated [22]. The induction of animals to passive smokers above was adjusted in accordance with previous experimental protocols [19, 23-25], as is as follows:

Exposure to smoke

The animals of group PS were placed in plastic boxes with wire mesh platform to food and water, measuring approximately 30x40x16 cm^3 with four animals in each box. The boxes containing the animals were transferred to a laminar flow for "CQ 800", Union equipment®, laboratorial line measuring 60 cm x 80 cm, ending in a pyramid upper exhaust pipe base 20 cm x 20 cm. The procedures regarding the concentration of particles in the exhaust hood were adjusted according to previous protocols [23, 25] (concentration between 100-300 ppm – PM25). The airflow in the chapel had a single top output; passive, with an average temperature of 22 °C and average humidity of 60%.

The concentration of the smoke particles was measured with a nephelometer DustTrakII®, model 8532, serial number 8,53E+09, with channel for aerosol filter and

2.5 µg/m^3, every minute five minutes after initial exposure, every minute, until completing the cycle.

Starting 12 days after birth until the period of 120 days, the animals were exposed daily to the smoke generated by burning cigarettes (16 cigarettes per day), with a frequency of four cigarettes simultaneous, four times a day (at 8h , 12h, 16h and 20h) with a duration of 20 minutes for each exposure. The cigarette combustion persisted an average of 10 minutes. In combustion intervals, the animals that had not yet been weaned were placed together with her mother.

The administration of smoke was carried out without the mother's presence until the day of weaning (21 days), avoiding contamination in breast milk. During exposure to smoke environment, the boxes of group PS were transferred to safety cabin with four cigarettes in combustion. The ambient smoke (in the chapel) dissipated through the top of passively chapel, followed by pipe designed out of the trial room. We used commercial filter cigarettes having the following composition: 10 mg of tar, 0.8 mg nicotine and 10 mg carbon monoxide.

The animals from group C were located in two open boxes in the same room as the animals were exposed to secondhand smoke, no exposure to cigarette smoke at a distance of 5 meters from the chapel which was near the window (closed), where same (chapel) was sealed. The animals of both groups had free access to water and feed (*ad libitum*). After 120 days of exposure, in day after, following all the protocols required by Resolution nº 1000 of the Federal Council of Veterinary Medicine, all animals were sacrificed through the appropriate dose use of ketamine (90 mg / kg) and xylazine (10 mg / kg), and the larynx and trachea removed in bloc.

Analysis of the levels of cotinine

Exposure to cigarette in animals was assessed by urinary concentrations of cotinine (a biomarker for tobacco passive consumption). Urine samples were collected at day of sacrifice of animals measured at the end of the exposure time (120 days), while they were still alive under anesthesia, the bladder via the suprapubic puncture and subjected to quantification of cotinine levels using high performance liquid chromatography (HPLC) according to the protocol established by Ceppa et al [26].

Histological processing

After sacrificing the animals, the organs (*in bloc*) were fixed in 4% paraformaldehyde (16 hours at 4 °C), dehydrated in ethanol, diaphanized in xylene, embedded in Paraplast® and sectioned in microtome. Were obtained 5 µm semi-serial sections were covered beforehand blades extended in poly-lysine (Sigma). The cuts were made in sagittal, stained with hematoxylin and eosin (HE) for morphological analysis.

Pathological analysis

The histological slides were given special numbering and analyzed pathologically by experienced pathologists, in double-blind study. The changes were classified according to the severity observed (qualitative analysis). We evaluated the presence or absence of inflammation in mucosal dysplasia and carcinoma, as well as different degrees of differentiation of these last two lesions.

Statistical analysis

The statistical analysis of this study was performed with GraphPad Prism 5.0 software (GraphPad Software, Inc., USA). A *t* test for independent samples was used to compare differences in final weight of the animals and cotinine urine concentrations between the two groups ($P < 0.05$). In addition, the Chi-square test was used to detect differences in the proportion of light and focal inflammation between group PS and group C.

3. Results

At the end of the experiment, the analysis of animal weight, showed no significant differences between the PS group (376.4 + 52.8 g) and group C (363.2 + 35.8 g) ($P = 0.532$) (Table 1). Moreover, analysis of the cotinine concentration in urine of the rats of the PS group (4.72 + 0.89 ng / ml) was significantly higher than the concentration in the C group (0.65 ± 0.12 ng / ml) ($P = 0.0001$) (Figure 1).
Exposure to the particulate smoke, measured by the nephelometer, showed an average exposure of 98.4 $\mu g/m^3$, between the sixth and tenth minute of exposure, 86.6 $\mu g/m^3$, between the eleventh and fifteenth minute of exposure drastically falling to 2.8 $\mu g/m^3$ in the last five minutes of exposure (Figure 2).

Among all animals of group C, 75% (n= 6) showed the laryngeal and tracheal mucosa within normal limits 12.5% (n= 1) showed acute tracheitis and moderate and focal inflammation. For animals of the PS group, 37.5% (n= 6) showed mucous within the normal range and 62.5% (n= 10) had moderate and focal inflammation (Table 2). In this context, according to the chi-square test there is a statistically significant association between the groups and the classification of laryngeal and tracheal mucosa. By analysis, it appears that the proportion of moderate and focal inflammation is higher in group PS than in group C (P = 0.041) (Table 2).

No animal showed any degree of dysplastic or neoplastic lesion. In addition, the removal of the tracheal block, macroscopic examination showed no alteration that would biopsies in other areas (Figure 3A-D).

4. Discussion

Passive smoking is an ongoing public health problem that discusses the changes in habits and behaviors in populations worldwide as new laws increase of increasingly restrictive, especially indoors.

There are some techniques described in the literature to simulate secondhand smoke animals and better study their effects on the aero mucous digestive. In this context, calls are made "smoking machines" where cigarettes are connected, with suctora pump, smoke invades the experiment environment [14, 20, 27, 28]. Our study was carried out with the combustion of cigarettes in a closed chapel with passive diffusion of smoke, using the same model performed in other studies to assess the effects of passive smoke in rats [19, 24].

In relation to humans, there is a great individual variation in the urinary excretion rates of nicotine and cotinine [29]. Moreover, use of animal model minimizes distortions as mode of use, exposed area, concentration and frequency of exposure to smoke. In this sense, the choice of animal models also exclude the possible diferences that we may have to gender and race.

The average level of cotinine found that the experimental group was 7.25 times higher than that found in the control group, showing a significant exposure to environmental smoke in the urine. Cotinine is a marker of high specificity and high sensitivity showing a post-exposure time of 3 to 4 days and is measurable in urine [7, 27].

The levels of urinary cotinine in passive smokers has shown a positive correlation with health complication risks in children and is clearly the best biomarker for passive exposure to nicotine [7]. With these results, we prove the animal's exposure to the more than 4.700 products produced by the combustion of cigarettes, with different effects in animals.

The exposure of rats for 120 days corresponds to the development period, compared to humans, extending from childhood to adolescence [30]. This ensures a limited period of exposure to animal development period. The active exposure to smoking in young adolescents, has been identified as an independent mechanism to generate carcinogens that promote genetic changes [27]. The tumorigenesis in mice exposed to secondhand smoke after birth and continuously in its early life, was recognized for Balansky et al [27], lungs, liver and urinary system.

In the present study, ensuring passive exposure at the age of development, was demonstrated mild and focal inflammatory lesions, without detecting dysplasia or subsequent injuries. Although the inflammatory process is an important aspect in the pathophysiology of laryngeal disorders, the main objective of our study was to evaluate the carcinogenesis of the effects of passive smoking in the laryngotracheal structures.

After macroscopic and microscopic analysis of the specimens was not observed the presence of dysplastic lesions or carcinoma in the larynx and trachea samples.

The localized inflammatory response in infraglottic larynx, corroborates the findings of Jecker et al [31], who described a growing population of immune cells in endolaryngeal mucosa, with a thinning of the same in the glottic region. Similarly, other studies [32] have shown that long-term passive smoke directly affects the auditory tube and middle ear mucosa.

We were surprised to not detect any tumor, since, according to the estimate of animal life already described, we could expect more aggressive results as tumor lesions in the tracheal mucosa. Haussmann et al [33], claim that the larynx is the most sensitive part to attacks by cigarette smoke and thus affecting the answers were within our expectations. In the study by Haussmann et al [33] were found lesions as diffuse squamous metaplasia of the pseudostratified epithelium and squamous hyperplasia on the base of the epiglottis. However, the authors did not observe dysplastic lesions and/or carcinogens in the study group.

The methodology of smoke exposure was carefully studied as what has been in the literature [19, 20, 23-28], and we choose to place the animals in a chapel, left the air more focused environment with the burning of cigarettes with higher tar and nicotine contente in the market.

All of systems exposure to cigarette smoke have similar characteristics and consist of a cigarette smoke generator and exposure chamber where animals are confined and exposed to smoke. So we decided not to put in a generator, but rather under passive cigarette combustion, to be exposed animals by flashing nose or across the body [34].

The study by Chen et al [34] showed small differences regarding biological effect of three types of exposures. It is known that the evaluation of the effect of conventional cigarettes or models used for animal use is complex due to the inadequacy of live models representing the disorders that affect humans [35].

Passive smoking takes place through the exposure to smoke from burning tobacco and the transformation in more than 4.722 products, where 44 of these products are proven carcinogens [36]. Furthermore, studies show that a greater number of products formed by the combustion of the cigarette; on the order of 5000 parts thrown to the environment where these, 98 are considered carcinogenic [37].

It is observed that the particulate smoke have a greater exposure to animals between 8 and 12 minutes, peaking at 10 minutes, as shown in Figure 2, it can be inferred that the period of greatest harmful action to mucosal tissues of animals would be near the peak of greater exposure to particulate smoke. Furthermore, it is observed that exposure is minimal after 15 minutes of exposure to simultaneously burn of 4 cigarettes, with this objectionable if the exposure environment in smokers smoke peaks remains constant or if there is a variation as shown in this study with a Gauss curve, limited in this way, the harmful effects of smoke with secondhand smoke indoors.

Inflammatory lesions observed and anatomopathological examination in animals in our study are consistent with those found in humans. It should also be noted that despite the daily passive smoking, we had no loss of animals during the experiment, indicating that susceptibility to the development of diseases have a close relationship with the immune system of animals and compared, human beings, being able to suggest that not all passive smokers develop any kind of diseases, which depend on the comorbidities associated with the animals and humans exposed to cigarette smoke, as well described in the literature [38, 39].

Some studies report that smoking contributes to weight loss and that cigarette smoke can alter the hypothalamic appetite regulation in the central nervous system [40- 42], a fact not observed in our study. However, another recent study [19] shows that animals of smoking group had a significant decrease in weight compared to the control group [43]. Conversely, other authors found no significant effect of cigarette exposure on body weight. We believe this may be explained by methodological differences, since the time the animals are exposed to smoke can affect several biological parameters.

Despite the knowledge gathered over the past 60 years of research, even today we suffer the effects of passive exposure to cigarette smoke, especially children and the elderly. Semple et al [42] have shown that individuals who do not smoke and live with smokers are exposed to up to 10 times more pollution than those who live in homes where there are smokers, and 3 times more than recommended by WHO. It should be noted also that the opposite is not true, ie, that people not exposed to cigarette smoke can not develop laryngeal cancer and tracheal, demonstrating that the immune system has close relationship with the development or not of certain diseases, associated with comorbid factors [27].

5. Conclusion

Histopathological analysis showed no degree of dysplastic or neoplastic lesion in any region of the mucosa of the larynx and trachea of young Wistar rats submitted to chronic inhalation of cigarette smoke. Observed a higher moderate and focal inflammation in the smoking group, with obvious damage to the infraglottic region.

6. Acknowledgements

The authors thank Antonio Generoso Severino, the Clovis Tadeu Bevilacqua Son and academic Bruno Mastella, Cilomar Martins de Oliveira Filho, Pedro Henrique Duarte Marks and Victor Hugo Vargas Bittencourt throughout commitment during the experiment.

7. Conflict of interest

There are no conflicts of interest on the part of the authors.

8. Financial support

Financial support by Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA and Universidade Federal do Rio Grande do Sul - UFRGS.

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10. Table and figures legends

Table 1 - Initial and final weight of the control group (C) and passive smoking group (PS).

Table 2. Proportion of moderate and focal inflammation in the smoker group and the control group. Chi-square test (P = 0.041).

Figure 1. Cotinine levels (ng / ml of urine) in the control and passive smoking groups.

Figure 2. Exposure to the particulate matter (PM2,5).

Figure 3. **A)** Supraglottic larynx region - normal histological aspects: mucosa covered by pseudostratified ciliated epithelium with goblet cells (arrow), lamina propria consists of connective tissue and cartilage tissue of hyaline type of thyroid cartilage. **B)** Supraglottic region of the Larynx - normal histological aspects: Lamina propria consists of seromucous glands and connective tissue (arrow). The mucosa exhibits a ciliated pseudostratified epithelium with goblet cells. The photomicrograph includes hyaline cartilage of the epiglottis. **C)** Infraglottic of the Larynx: Presence of moderate chronic inflammatory infiltrate in the lamina (arrow). **D)** Infraglottic Laryngeal Region:moderate lymphocytic infiltrate permeating seromucous glands of the lamina própria (arrow). Hematoxylin-eosin, 100X.

Table 01.

	Initial Average (g)	Final Average (g)
C	**20,1**	**363,24**
PS	**21,9**	**376,42**

Table 02.

Laryngeal and Tracheal Mucosa	Control Group (n=8)	Smoking Group (n=16)
Within the normal range	*6*	*6*
Moderate and focal inflamation	*1*	*10*
Tracheitis Acute	*1*	*-*

Figure 01.

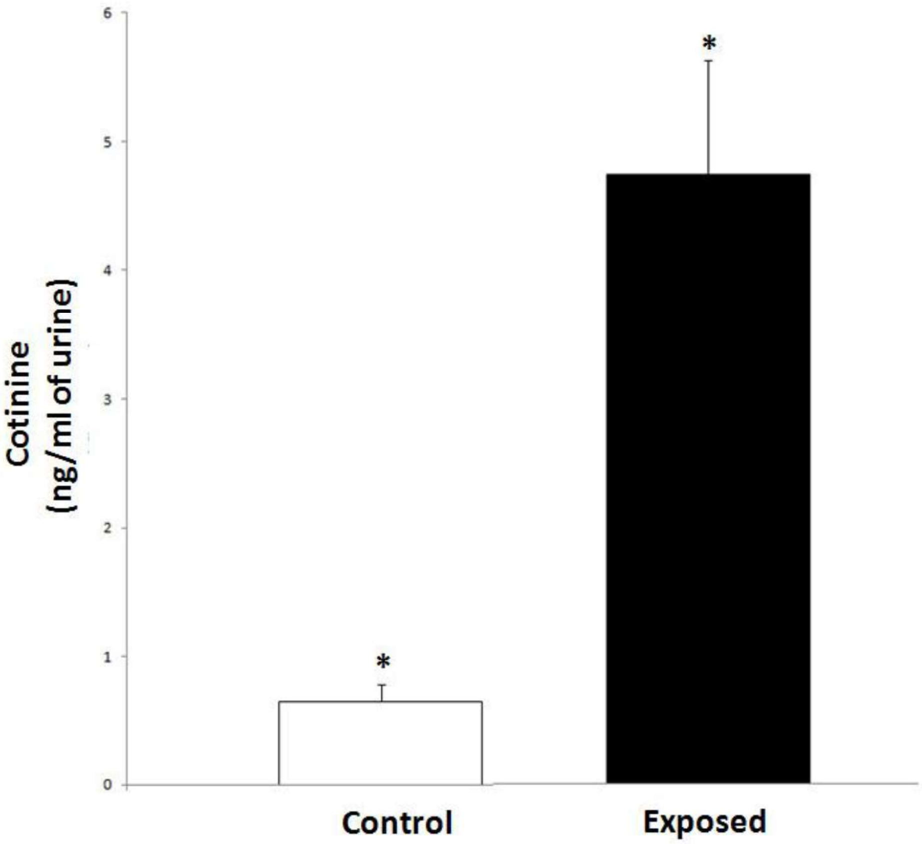
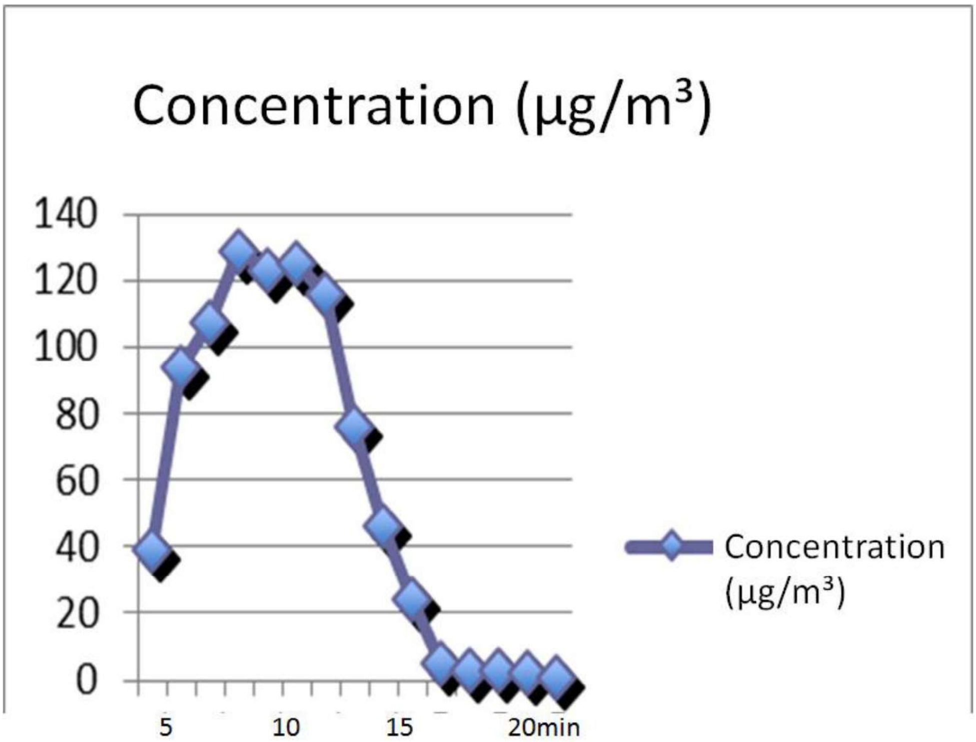


Figure 02.


Concentration (µg/m³)
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120
100
80
60
40
20
0
5
10
15
20min
Concentration (µg/m³)

Figure 03 A.

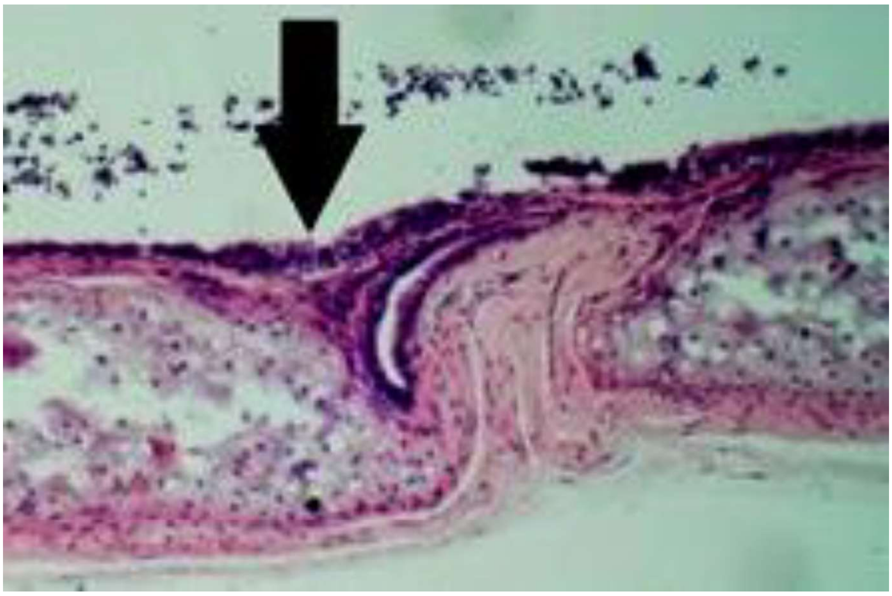


Figure 03 B.

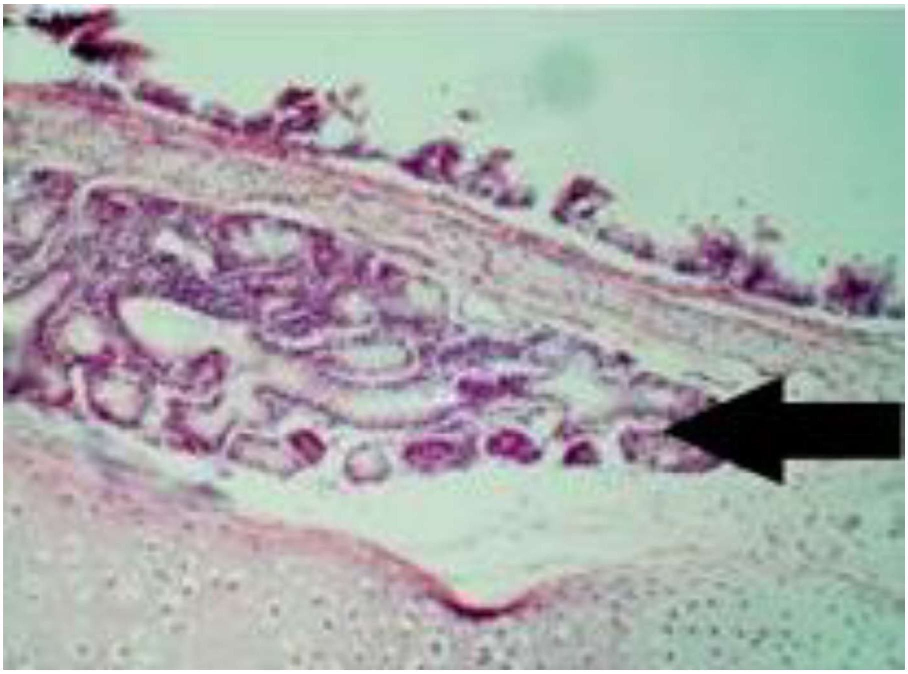


Figure 03 C.

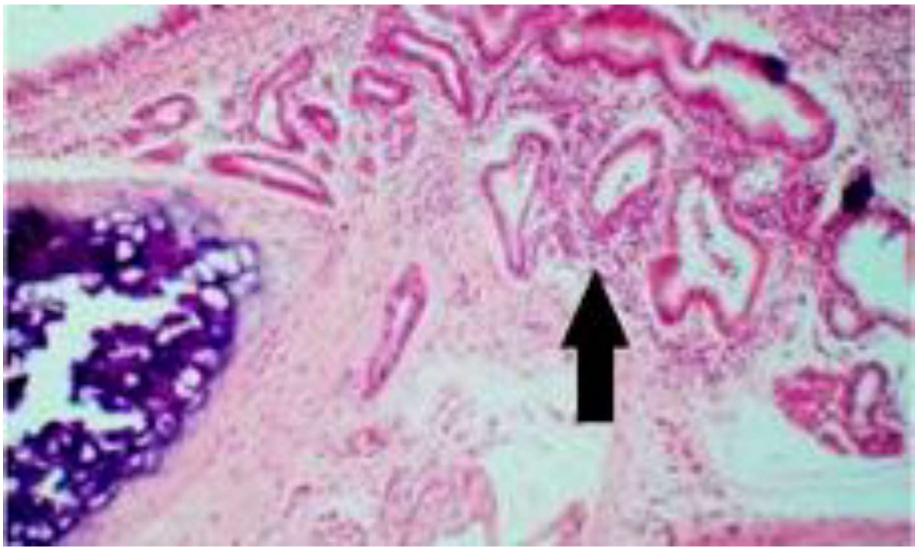
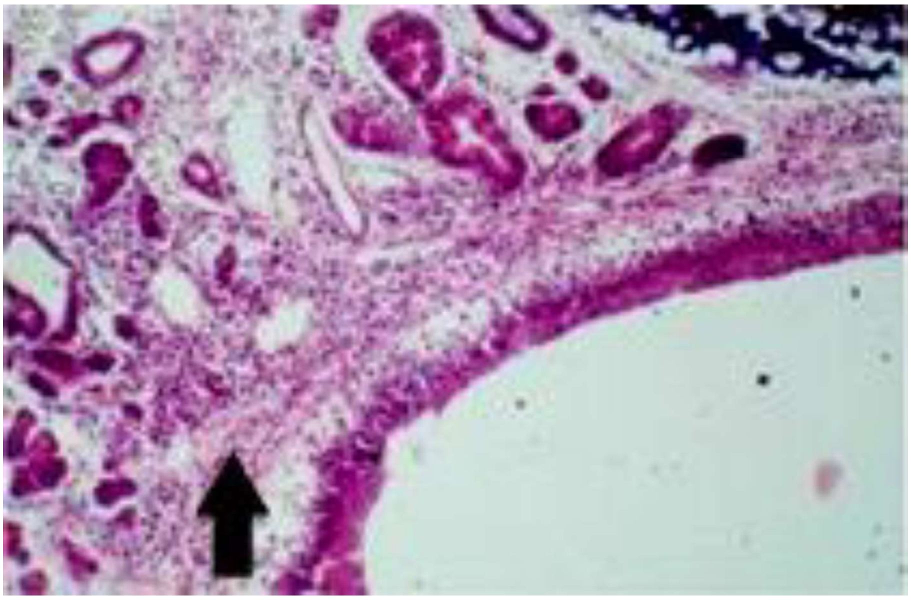


Figure 03 D.



4. Conclusão

Ao final do experimento, a análise da massa ponderal dos animais expostos ao particulado da fumaça e dos animais controle, não mostrou diferença significativa (P = 0.532).

A concentração de cotinina urinária nos animais expostos ao particulado da fumaça foi significativamente mais alta que a concentração de cotinina urinária nos animais do grupo controle (P = 0.0001).

A concentração de material particulado fino (PM 2,5μm) durante a queima passiva do cigarro atingiu média de 92.5 ng/m^3, entre seis e quinze minutos do período de exposição.

Os animais expostos ao particulado da fumaça apresentaram inflamação moderada e focal na lâmina própria da região infraglótica e infiltrado linfocítico permeando as glândulas seromucosas da lâmina própria.

A análise histopatológica não demonstrou qualquer grau de lesão displásica ou neoplásica na mucosa laríngea e traqueal de ratos Wistar jovens submetidos à inalação crônica de fumaça de cigarro. Ficou demonstrada uma inflamação laríngea leve e focal no grupo fumante, com lesão evidente da região infraglótica

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7. Anexos

Anexo 01 – Medidas das concentrações de cotinina nos grupos experimentais

Animais controle	medida 1	medida 2	medida 3	media das leituras (em ng/ml)	media da duplicata
c1	0,58	0,62	0,57	0,59	0,643333
c1	0,63	0,62	0,84	0,696667	
c2	0,71	0,69	0,67	0,69	0,705
c2	0,67	0,68	0,81	0,72	
c3	0,63	0,65	0,65	0,643333	0,643333
c4	0,59	0,62	0,63	0,613333	0,613333
c5	0,91	0,94	0,91	0,92	0,921667
c5	0,96	0,89	0,92	0,923333	
c6	0,58	0,45	0,51	0,513333	0,508333
c6	0,51	0,48	0,52	0,503333	
c7	0,65	0,71	0,72	0,693333	0,698333
c7	0,71	0,72	0,68	0,703333	
c8	0,48	0,49	0,45	0,473333	0,481667
c8	0,51	0,49	0,47	0,49	

Tabela . Medidas das concentrações de cotinina no grupo controle. Resultou uma média de 0,651875 com desvio padrão de 0,126972.

Animais expostos	medida 1	medida 2	medida 3	media das leituras (em ng/ml)	media da duplicata
f1	3,45	3,85	3,98	3,76	3,626667
f1	3,56	3,47	3,45	3,493333	
f2	3,91	4,32	4,25	4,16	4,195
f2	4,35	4,19	4,15	4,23	
f3	5,18	5,32	5,28	5,26	5,513333
f3	5,9	5,74	5,66	5,766667	
f4	3,58	3,45	3,89	3,64	3,658333
f4	3,75	3,55	3,73	3,676667	
f6	5,78	6,12	6,33	6,076667	6,076667
f7	4,22	4,68	4,92	4,606667	4,606667
f8	5,8	5,21	5,33	5,446667	5,631667
f8	5,67	5,87	5,91	5,816667	
f10	4,21	4,28	4,05	4,18	4,125
f10	3,98	4,15	4,08	4,07	
f11	3,58	3,64	3,95	3,723333	3,723333
f12	3,85	3,78	3,92	3,85	3,995
f12	4,16	4,05	4,21	4,14	
f13	6,58	6,15	6,29	6,34	6,215
f13	6,04	5,97	6,26	6,09	
f15	4,89	4,52	4,74	4,716667	4,775
f15	4,91	4,78	4,81	4,833333	
f16	5,37	5,41	5,67	5,483333	5,343333
f16	5,17	5,11	5,33	5,203333	

Tabela . Medidas das concentrações de cotinina no grupo exposto. Resultou uma média de 4,729615 com desvio padrão de 0,894876.

ANEXO 2 – Aprovação do Comitê de Ética da UFCSPA

UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
UFCSPA

A Comissão de Ética no uso de Animais, analisou o Projeto:

Projeto: 12-084 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
GERALDO PEREIRA JOTZ
HENRIQUE ZAQUIA LEÃO

Título: FUMO PASSIVO E LESÃO EM MUCOSA LARINGOTRAQUEAL EM RATOS WISTAR MACHOS, DURANTE O CRESCIMENTO: ESTUDO EXPERIMENTAL.

Este projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim com eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 16 de outubro de 2012.

Pedro R. T. Romão
Coordenador CEUA/UFCSPA

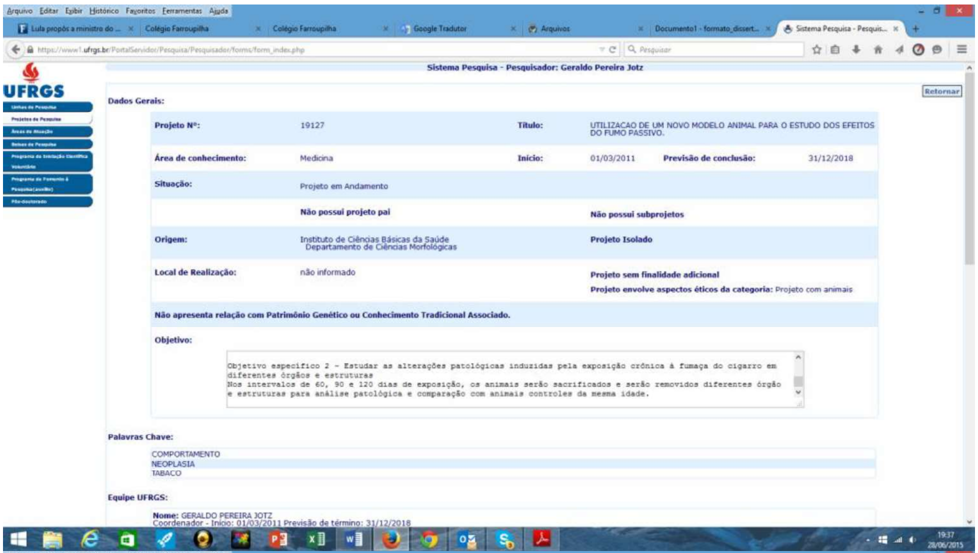
ANEXO 3 – Aprovação do Comitê de Ética da UFRGS

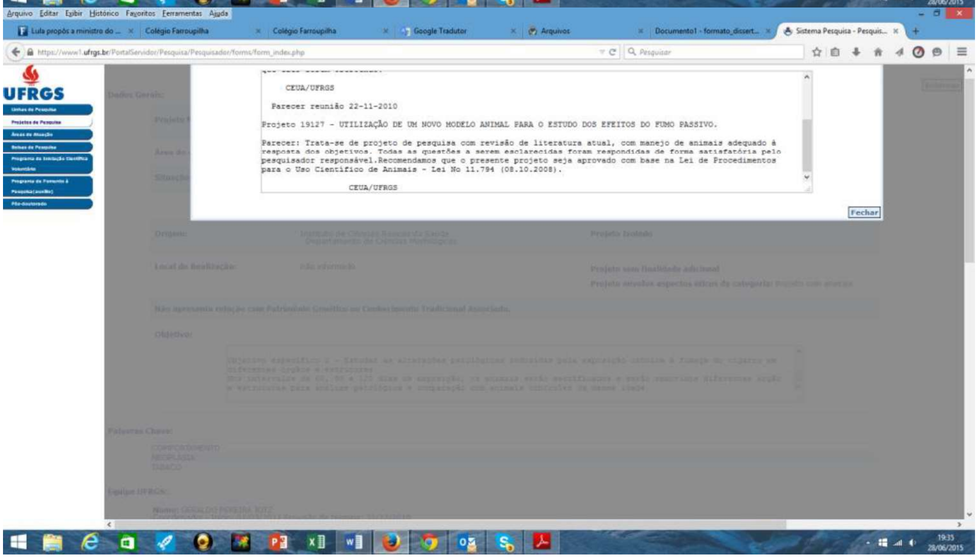
Parecer reunião 22-11-2010

Projeto 19127 - UTILIZAÇÃO DE UM NOVO MODELO ANIMAL PARA O ESTUDO DOS EFEITOS DO FUMO PASSIVO.

Parecer: Trata-se de projeto de pesquisa com revisão de literatura atual, com manejo de animais adequado à resposta dos objetivos. Todas as questões a serem esclarecidas foram respondidas de forma satisfatória pelo pesquisador responsável.Recomendamos que o presente projeto seja aprovado com base na Lei de Procedimentos para o Uso Científico de Animais - Lei No 11.794 (08.10.2008).

CEUA/UFRGS





ANEXO 4 – Normas da Revista – *American Journal of Otolaryngology*

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Código de campo alterado

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Structured abstract

A structured abstract, by means of appropriate headings, should provide the context or background for the research and should state its purpose, basic procedures (selection of study subjects or laboratory animals, observational and analytical methods), main findings (giving specific effect sizes and their statistical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

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Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
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