Universidade do Minho
Escola de Ciências da Saúde

Manuel José Lima da Costa Rodrigues

Terminais Nervosos na Laringe: morfologia, aspectos patológicos e perspectivas terapêuticas

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Trabalho efectuado sob a orientação do
Professor Doutor Armando Almeida
e co orientação do
Professor Doutor Rui Nunes

Maio de 2008

"Observa o teu culto, a família e cumpre teus deveres para com teu pai, tua mãe e todos os teus próximos. Educa as crianças e não precisarás de castigar os homens."

Pitágoras, filósofo e matemático grego séc. VI a.C.

À minha Mãe...

Ao meu Pai

À Fátima

À Margarida e ao Rodrigo

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"Existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."
(Fernando Pessoa)

Resumo

Os conhecimentos actuais sobre a morfologia e a topografia dos terminais axonais sensitivos da laringe que medeiam a interacção entre irritantes no lume respiratório e o sistema nervoso e dos terminais motores responsáveis pela actividade dos músculos intrínsecos são escassos e, em alguns estudos, contraditórios. Além das funções sensitiva e motora, desconhece-se o papel destas terminações nervosas na etiologia de algumas patologias da laringe cuja base pode ser neurogénica bem como de terapêuticas dirigidas a este tipo de distúrbios. Os objectivos deste trabalho consistiram, por um lado, no estudo da morfologia das fibras sensitivas da mucosa da laringe que contêm os neuropeptídeos Substancia P (SP) e Peptídeo Relacionado com o Gene da Calcitonina (CGRP), do possível papel destas moléculas na etiologia de patologias da laringe e na avaliação de novas potencialidades terapêuticas daí resultantes; adicionalmente, ao nível dos terminais motores, a sua morfologia e distribuição foi estudada não só no Homem mas também noutros mamíferos e em espécies de outras classes de vertebrados com diferentes capacidades de vocalização, com o objectivo de explorar novas hipóteses de abordagem terapêutica na etiologia de patologias das cordas vocais.

Neste trabalho mostra-se pela primeira vez que as fibras sensitivas intra-epiteliais da laringe projectam para o lume da via respiratória, terminando ao nível da camada mucociliar. Foi desenvolvido um modelo experimental de laringite neurogénica baseado na entubação nasogástrica (ENG). Neste modelo, verificou-se um aumento da presença de infiltrado celular inflamatório na laringe, acompanhado pela libertação de CGRP e SP das fibras sensitivas terminais, aumento da expressão da enzima Cicloxigenase-2 (COX-2) e de citocinas pró-inflamatórias e redução na expressão de citocinas anti-inflamatórias. Adicionalmente, demonstramos que a administração por períodos curtos do inibidor específico da COX-2 Etoricoxibe produzia actividade anti-inflamatória no modelo ENG, reduzindo a expressão de COX-2 e da citocina pró-inflamatória factor de necrose tumoral-alfa (TNF-α); este facto sugere que fármacos inibidores específicos da COX-2 poderão ser utilizados no tratamento da laringite, já que apresentam menos efeitos secundários aerodigestivos do que os fármacos utilizados actualmente, nomeadamente os anti-inflamatórios não esteróides clássicos (AINEs), inibidores não selectivos das isoformas COX-1 e COX-2. A indução prolongada da laringite neurogénica também revelou uma diminuição da expressão dos supressores tumorais p53 e p16, o que faz

supor um ambiente mais favorável ao desenvolvimento de eventuais lesões pré-neoplásicas em laringes expostas a agentes cancerígenos conhecidos como, por exemplo, o fumo do tabaco e o álcool.

O estudo da distribuição das placas motoras (PMs) nos músculos intrínsecos da laringe, através da detecção histoquímica da enzima acetilcolinesterase, revelou dois padrões distintos: um em que as PMs se distribuem de modo difuso, é encontrado em espécies com maiores capacidades de vocalização como a humana (PMs mais concentradas no terço médio), certas aves e em alguns batráquios; pelo contrário, em mamíferos como o coelho e o rato, espécies que revelam capacidades limitadas para variar a emissão de sons, a distribuição limitava-se a uma região em "banda" situada a meio da distância entre as duas extremidades de inserção dos músculos. Este aspecto revelou-se interessante, dado que a anatomia macroscópica da laringe e do próprio sistema nervoso central nestes mamíferos é mais parecida com a humana do que a das aves ou batráquios.

As conclusões obtidas pelos trabalhos que constituem esta tese foram: (i) as fibras sensitivas na mucosa laríngea do rato podem atravessar o epitélio e atingir o nível da camada mucociliar, justificando a importância da hidratação para que estas se encontrem menos sujeitas a irritação externa; (ii) os neuropeptídeos presentes nestas fibras medeiam a laringite neurogénica desencadeada por um modelo experimental desenvolvido no presente trabalho; (iii) a laringite pode ser em parte revertida por um fármaco (Etoricoxibe) com menos efeitos adversos aerodigestivos relativamente aos AINEs tradicionais; (iv) durante a indução prolongada de laringite verifica-se uma diminuição da expressão de supressores tumorais, o que pode facilitar o desenvolvimento de lesões potencialmente neoplásicas; (v) a distribuição das placas neuromusculares pelos músculos vocais de diferentes espécies de mamíferos e classes de vertebrados indica que uma maior versatilidade na capacidade vocal parece estar associada a uma maior distribuição das placas motoras ao longo das fibras musculares; (vi) a maior concentração de placas na região central dos músculos vocais no Homem poderá permitir a aplicação terapêutica mais específica de fármacos em patologias das cordas vocais. Estudos futuros, a desenvolver a partir dos dados recolhidos pelos trabalhos incluídos nesta tese, deverão explorar as potencialidades terapêuticas entreabertas pelos modelos animais aqui desenvolvidos.

Abstract

The present knowledge on the morphology and topography of laryngeal axonal terminals of sensitive fibres relaying the interaction between airway lumen irritants and the nervous system, and on the motor terminals that mediate the activity of the laryngeal intrinsic muscles are scarce and contradictory. Besides their sensitive and motor functions, the role of these nervous endings in the aetiology of some pathologies of the larynx that can be neurogenic is unknown, as well as the therapeutics driven to this type of pathologies. The aims of this work were the morphological analysis of the main sensitive fibres of the laryngeal mucosa (C-fibres), which contain the neuropeptides Substance P (SP) and Calcitonin Gene Related Peptide (CGRP) as main mediators, and the possible function of these molecules in the aetiology of laryngeal pathologies. Consequently, we evaluate new therapeutic approaches. Concerning the study of laryngeal motor terminals (neuromuscular junctions), their distribution and morphology were analysed in humans, in other mammals (rat and rabbit) and in other classes of vertebrates with different vocalization capacities, also to evaluate new hypothesis on therapeutic approaches for the ethiology of vocal cord pathologies.

We demonstrate for the first time that intraepithelial laryngeal sensitive fibres project to the lumen of the larynx at the level of the mucociliar layer. We have also created a new model of neurogenic laryngitis based on nasogastric intubation (NGI). In this model we show a decrease in the levels of fibres immunoreactive for CGRP and SP, possibly due to peptide release, accompanied by a strong increase in the laryngeal expression of Cycloxygenase-2 (COX-2) and pro-inflammatory cytokines, as well as a decrease in the expression of anti-inflammatory cytokines. On the other hand, we show that the COX-2 inhibitor Etoricoxib has an anti-inflammatory effect in this laryngitis by reducing the expression of COX-2 and the putative pro-inflammatory marker TNF-α. This suggests that selective COX-2 inhibitory drugs may be studied in the future as an alternative treatment of laryngitis, due to their smaller adverse side effects at the aero-digestive level, when compared with common non-specific non-steroidal anti-inflammatory drugs (NSAIDs). The persistent induction of neurogenic laryngitis also reveals a decrease in the expression of the most important molecules with tumoral suppressor functions (p53 and p16). These data suggest the development of local tissue conditions for the induction of

pre-neoplasic and eventually neoplasic lesions in larynxes exposed to factors known to be cancerous as, for example, tobacco smoke or alcohol.

The study of the distribution of motor plates in the intrinsic muscles of the larynx reveals two different patterns: a diffuse one in species with larger vocalization capacities, as in humans, some birds and batrachians, although with a higher concentration in the middle third in the case of humans; and another with a distribution of motor end plates limited to a "band" located at the middle distance between the two insertion points of muscle fibres, in species that reveal limited capacities to emit different frequencies, as in mammals like the rabbit and the mouse. This aspect was interesting, given that the macroscopic anatomy of the larynx and brain in these mammals is more similar to humans than to birds or batrachians. In terms of clinical relevance, the concentration of motor end plates in the middle third of vocal cords suggests that the selective application of drugs at this level in the laryngeal muscle may improve the treatment of some motor pathologies of the larynx.

The studies included in the present thesis allowed the following conclusions: (i) the sensitive fibres along the rat laryngeal mucosa can terminate at the mucociliar level, which highlights the need for mucosa hydration to avoid stimulation by external irritants; (ii) the neuropeptides present in these fibres mediate the neurogenic laryngitis induced by the NGI model; (iii) this laryngitis can be partly reverted by a drug with less aero-digestive side effects (Etoricoxib); (iv) a prolonged laryngeal inflammatory process induces a decrease in the expression of tumoral suppressors, which may facilitate the development of potentially neoplasic conditions; (v) the distribution of motor end plates along the vocal muscles of different mammal and vertebrate species show that a larger vocal capacity seems to be associated with a diffuse distribution pattern of motor plates along muscle fibres; (vi) the larger concentration of motor plates in the middle third of human vocal cord muscles may underlay several pathologies of the cords. Overall, future studies obtained from the basic experimental models developed in this thesis should explore the clinical potential targets suggested by these animal studies.

Índice:

LISTA DE ABREVIATURAS

AINEs – Anti-inflamatórios não esteróides

AMP – Adenosina monofosfato

CGRP – Peptídeo relacionado com o gene da calcitonina

COX – Cicloxigenase

EGP – Factor de crescimento epitelial

ENG – Entubação nasogástrica

EPr – Receptor da prostaglandina E

IL – Interleucina

NK – Neurocinina

PAR – Receptor da proteinase

PG – Prostaglandinas

PM – Placas motoras

RFL – Refluxo faringolaríngeo

RGE – Refluxo gastroesofágico

RNAm – Ácido ribonucleico mensageiro

RT-PCR – Reacção em cadeia da polimerase em tempo real

SP – Substância P

TNF-α – Factor de necrose tumoral - alfa

Capítulo 1

Introdução

1. INTRODUÇÃO

1.1. Enquadramento conceptual

A morfologia e o papel dos terminais nervosos sensitivos nos mecanismos fisiopatológicos e terapêuticos da laringe são ainda pouco conhecidos. A estimulação dos receptores presentes nestes terminais pode por vezes desencadear respostas reflexas graves, que podem resultar inclusivamente em paragem cardiorrespiratória (Widdicombe e Lee, 2001; Mort, 2004). Por outro lado, o aumento da mortalidade nas unidades de cuidados intensivos está muitas vezes relacionado com a aspiração de secreções (Gomes et al, 2003), desempenhando neste caso a enervação laríngea um papel fundamental de defesa na sobrevivência do organismo.

Um fenómeno essencial em que as fibras sensitivas periféricas estão implicadas é na neuroinflamação, cujo processo patológico foi já estudado em vários tecidos (Craft et al, 2005; Ransohoff et al, 2007). Mais recentemente, a relação entre fenómenos inflamatórios prolongados e o cancro tem sido demonstrada por um número crescente de estudos em diversos órgãos, nomeadamente nos pulmões e no esófago (Lagorce et al, 2003; Blanco et al, 2007). Assim, é possível que os terminais nervosos das fibras sensitivas periféricas da laringe sejam não só imprescindíveis para a função laríngea como possam, também, estar na base de diversas patologias. Deste modo, o estudo aprofundado da enervação sensitiva poderá constituir um foco de investigação de alternativas terapêuticas ao nível laríngeo. No entanto, no que se refere à laringe propriamente dita, o papel dos terminais sensitivos no desenvolvimento da inflamação ou do cancro é, até ao momento, desconhecido.

Relativamente aos terminais motores periféricos, a sua activação desencadeia todos os movimentos laríngeos, nomeadamente a deglutição, a respiração e a fonação. Conhecer a sua distribuição nos músculos da laringe reveste-se de especial importância, não só para a compreensão destas funções como também das patologias subjacentes ao seu mau funcionamento (Blitzer et al, 1986). Estes conhecimentos poderão ser importantes para a análise de novas aplicações terapêuticas, nomeadamente através da administração mais adequada de fármacos em patologias que estejam directamente relacionadas com a distribuição destes terminais nervosos nos músculos laríngeos.

1.2. ENERVAÇÃO SENSITIVA DA LARINGE

1.2.1. Troncos nervosos principais

O principal nervo responsável pela enervação sensitiva da laringe é o nervo vago, embora algumas fibras dos nervos glossofaríngeo e trigémio possam também estar envolvidas (Widdicombe et al, 1986). Os ramos do nervo vago que transportam fibras aferentes laríngeas são, principalmente, o ramo interno do nervo laríngeo superior e, em menor percentagem, o nervo recorrente (Canning et al, 2004; Gray, 2005). As fibras do nervo laríngeo interno enervam a mucosa laríngea até ao nível das cordas vocais através do seu ramo superior e apresentam os corpos celulares do 1º neurónio ao nível do gânglio superior (jugular). Abaixo da região glótica e da traqueia, as aferências sensitivas são conduzidas por fibras resultantes de anastomoses de fibras do ramo inferior do nervo laríngeo superior interno com fibras do nervo recorrente, as quais possuem os pericários situados no gânglio inferior (nodoso) (Canning et al, 2004; Gray, 2005). Todas estas aferências laríngeas projectam centralmente para o núcleo do tracto solitário do tronco cerebral (Lucier et al, 1986; Patrickson et al, 1991; Mrini e Jean, 1995; Gestreau et al, 1997; Ambalavanar et al, 2004). Foi demonstrada ainda a

presença de algumas aferências da mucosa laríngea para pequenos grupos de neurónios localizados no núcleo dorsal do vago (Hayakawa et al, 2001). Nos mecanismos reflexos, as fibras do tracto solitário estabelecem conexões com neurónios motores do núcleo ambíguo do bolbo raquidiano, nomeadamente durante a deglutição (Jean, 2001).

1.2.2. Fibras e terminais periféricos

No que diz respeito aos receptores sensitivos, sabe-se que a sua localização é intra-epitelial, correspondendo a terminações nervosas livres das fibras periféricas e a gomos gustativos (Widdicombe, 1986; Yoshida et al, 2000; Nishijima e Atoji, 2004). Desta forma, exceptuando os gomos gustativos, a activação destes receptores, dada a sua localização, exige a difusão de moléculas através da membrana citoplasmática das células epiteliais e/ou dos complexos juncionais que unem as porções apicais das membranas laterais das células epiteliais.

Nas vias respiratórias altas, as fibras sensitivas periféricas são amielínicas tipo C ou mielínicas tipo Aδ (Widdicombe, 1995; Tsuda et al, 1998; Undem et al, 2004; Mazzone, 2005). As principais fibras envolvidas na nocicepção são as fibras C amielínicas. Estas contêm como principais moléculas dois neuropeptídeos, o Peptídeo Relacionado com o Gene da Calcitonina (CGRP) (Tanaka et al, 1993; Hauser-Kronberger et al, 1997) e a Substância P (SP) (Hisa et al, 1985). As neurocininas A e B também podem também estar presentes, mas assumem menor importância (Bauman et al, 1998). A SP e CGRP estão implicadas na indução e desenvolvimento de processos inflamatórios neurogénicos noutros tecidos (Kopp et al, 2002). No entanto, nenhum modelo experimental foi desenvolvido na laringe para avaliar se algum mecanismo neurogénico estaria envolvido na resposta inflamatória. É interessante verificar que, apesar do elevado número de casos clínicos de laringite

aguda ou crónica, não existem na literatura modelos animais experimentais que permitam estudar estas patologias em detalhe.

1.2.3. Inflamação neurogénica

Um modelo experimental capaz de induzir laringite neurogénica deverá originar:

a) Libertação dos neuropeptídeos CGRP e SP da periferia de terminações nervosas livres das fibras sensitivas para o tecido circundante, após estimulação nóxica mecânica ou química transduzida num sinal eléctrico por receptores localizados na membrana citoplasmática das fibras (Figura 1; Irwin et al, 2000).

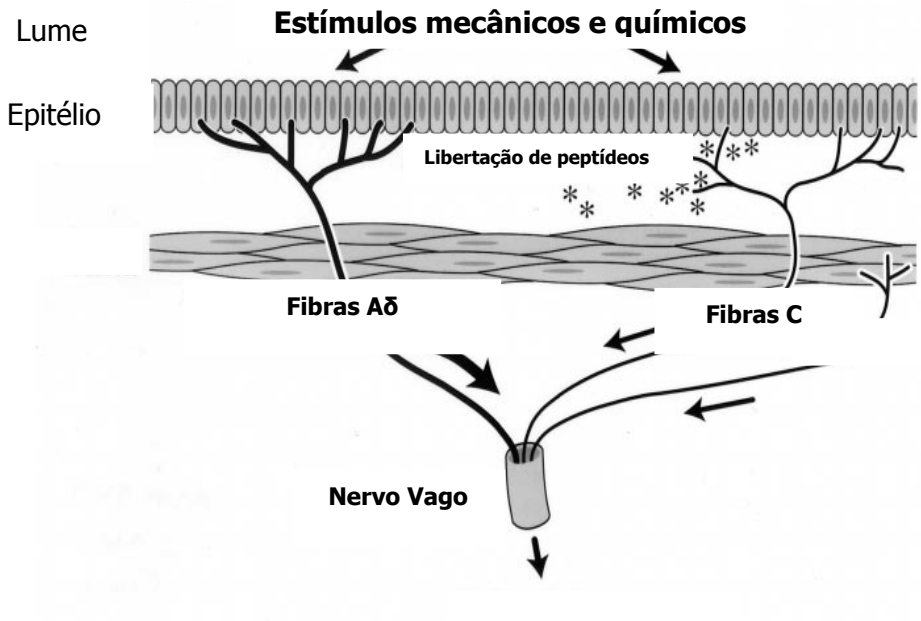

Figura 1. As fibras sensitivas de localização intraepitelial são amielínicas (fibras C) e mielínicas Aδ, mas os peptídeos libertados localmente no processo de inflamação neurogénica (SP e CGRP) localizam-se nas fibras C (adaptado de *Irwin et al*, *2000*)

A SP libertada nos tecidos periféricos causa extravasamento plasmático mediado pelos receptores Neurocinina-1 (NK-1), enquanto a CGRP é um poderoso vasodilatador que contribui também para o edema tecidular desencadeado pela SP. Deste modo, a SP e CGRP contribuem para o reforço da cascata inflamatória dos tecidos inflamados (Vera-Portocarrero e Westlund, 2004) e para a sensitização periférica implicada em fenómenos de cronificação dolorosa (Figura 2; Mantyh e Yaksh, 2001). Deve ser salientado que, para além da acção neurogénica periférica, a libertação de CGRP e SP está também implicada nos mecanismos de sensitização central, já que os neurónios sensitivos aferentes primários responsáveis pelas ramificações axonais na periferia emitem também um prolongamento para o sistema nervoso central (Mantyh e Yaksh, 2001) (Figura 2).

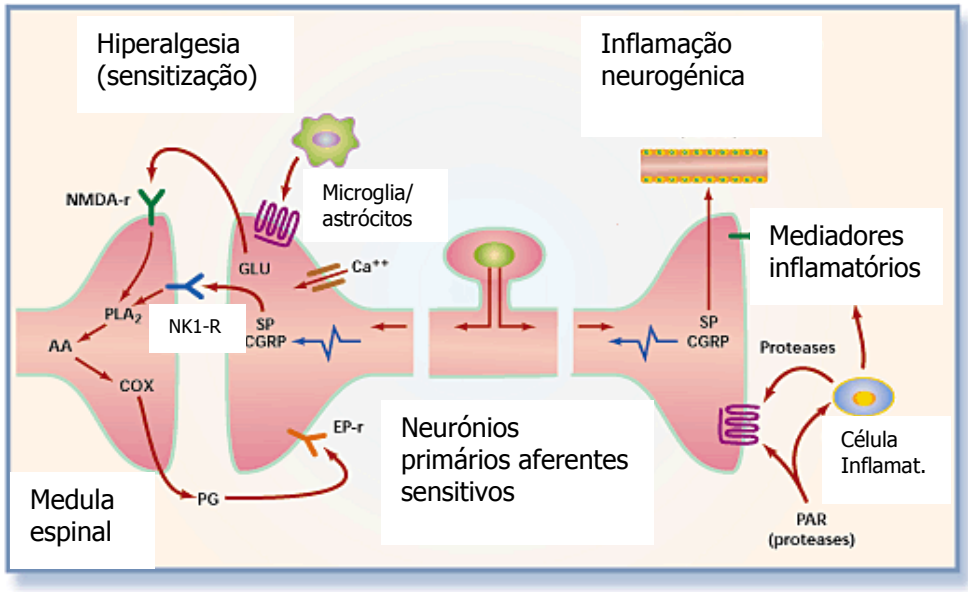

Figura 2. Após estimularem os respectivos receptores, os neuropeptídeos CGRP e SP activam a enzima cicloxigenase (COX) induzindo a produção de prostaglandinas (PG) quer na periferia quer ao nível da sinapse central. Em ambas as terminações axonais do neurónio aferente primário, as PG induzem a libertação de mais peptídeos (sensitização) (adaptado de *Mantyh e Yaksh, 2001*)

b) Os peptídeos (SP e CGRP) estimulam os respectivos receptores (NK-1 e CGRP-R), activam as enzimas cicloxigenases (COX) responsáveis pela produção de prostaglandinas (PG), as quais por sua vez potenciam a libertação de SP e CGRP através de mecanismos como a activação da

via adenilcíclase-AMPcíclico e o aumento do influxo de Ca^{2+} através de canais de cálcio dependentes da voltagem (Vanegas e Schaible, 2001; Geppetti et al, 2005) (Figura 3).

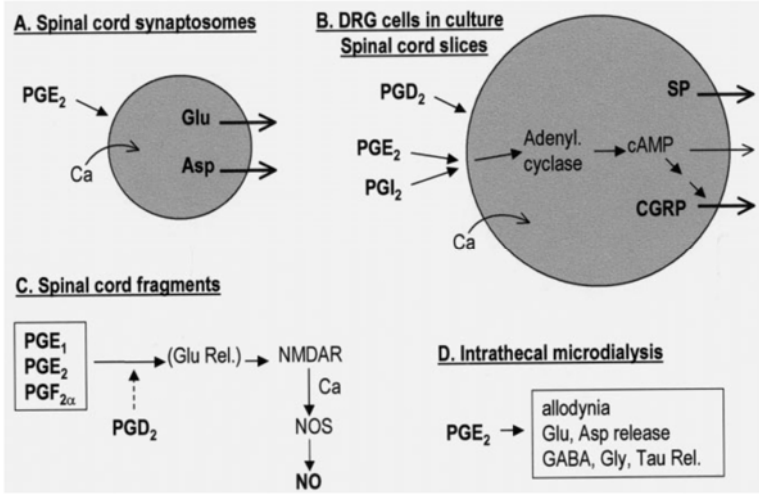

Figura 3. Esquema representando diversos mecanismos pelos quais as prostaglandinas, através da activação dos seus receptores na membrana citoplasmática dos terminais axonais sensitivos periféricos, podem contribuir para o processo inflamatório neurogénico. Aumenta a entrada de Ca^{2+} (A, B) e a activação da via adenilcíclase-AMPc (B), facilita a libertação de CGRP e SP (B), além de facilitar a libertação de Óxido Nítrico (C) e de diversos neurotransmissores (D) (adaptado de *Vanegas e Schaible, 2001*)

c) Os neuropeptídeos CGRP e a SP podem modificar a libertação de outros mediadores da inflamação, nomeadamente citocinas pró-inflamatórias [interleucina 1 (IL-1 β e o Factor de Necrose Tumoral-alfa (TNF-α)] ou anti-inflamatórias (IL-10) (Peters et al, 2006) (Figura 4). Por outro lado, as citocinas pró-inflamatórias IL-1β, IL-6 e TNF-α facilitam a libertação de SP e da CGRP dos terminais das fibras sensitivas periféricas (Nicole et al, 1997; Inoue et al, 1999) e induzem as células a libertarem Prostaglandinas (Coutaux et al, 2005), contribuindo para o mecanismo de retro-alimentação positivo do ciclo inflamatório neurogénico (Figura 4).

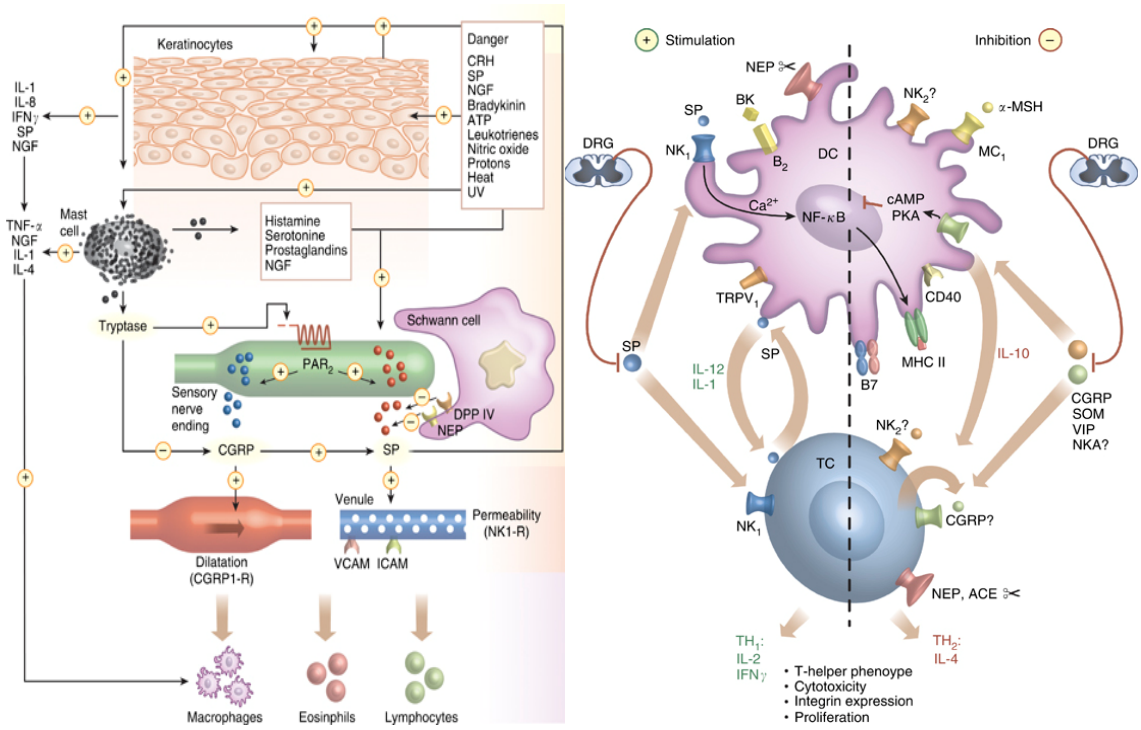

Figura 4. Os peptídeos CGRP e SP interferem na produção de várias citocinas, quer nas que exercem uma acção pró-inflamatória como as interleucinas IL-1 e TNF, quer anti-inflamatória (IL-10), completando o ciclo da inflamação neurogénica (adaptado de *Peters et al., 2006*)

Não foi encontrado descrito na literatura um único modelo experimental animal especialmente desenhado para induzir laringite neurogénica, mesmo utilizando substâncias como o fumo do tabaco ou o álcool; no entanto, são bem conhecidos clinicamente os efeitos destas substâncias sobre a laringe. Por outro lado, a entubação nasogástrica (ENG) é usada na generalidade da prática clínica médica e cirúrgica, inclusivamente nos cuidados intensivos adultos ou pediátricos (Gomes et al, 2003). Embora clinicamente a ENG provoque laringite (Friedman et al, 1981), provavelmente devido ao refluxo faringo-laríngeo ou ao traumatismo indirecto da mucosa laríngea, não existe até ao momento nenhum modelo animal baseado nesta técnica que permita avaliar os possíveis efeitos secundários resultantes da sua utilização; entre estes, o papel do refluxo faringo-

laríngeo, do tipo de laringite associada e da eventual etiologia neurogénica ou ainda a possível relação entre o refluxo e o cancro da laringe (Koufman, 1991; Cammarota et al, 2004; Galli et al, 2006).

1.2.4. Terapêutica da inflamação

Os fármacos utilizados actualmente em patologias inflamatórias da laringe (Laringite) são os corticosteróides e os anti-inflamatórios não esteróides (AINEs) inibidores não selectivos das duas isoformas da enzima Cicloxigenase (COX-1 e COX-2). Entre estes últimos, os mais utilizados incluem o Ibuprofeno e o Ácido Acetilsalicílico. Estes AINEs, assim como os corticóides, apesar de serem frequentemente utilizados na terapêutica da laringite, acabam por agravar a médio prazo a patologia devido aos efeitos secundários adversos a nível aerodigestivo.

Entre os efeitos colaterais mais frequentes e graves associados com os AINEs convencionais encontram-se as lesões agudas gastrointestinais, nomeadamente o risco de ulceração e perfuração gástrica, cuja incidência aumenta 3 a 4 vezes nos utilizadores habituais (Buttgereit et al, 2001). Sabe-se que, após a estimulação do receptor NK-1, a SP induz a produção de prostaglandinas inflamatórias (PGE_2) através do aumento da enzima COX e a CGRP potencia este fenómeno reforçando o processo inflamatório (Meggs, 1993; Schmelz e Petersen, 2001; Richardson e Vasko, 2002; Lacroix, 2003; O`Conner et al, 2004). No que concerne às duas isoformas da COX, COX-1 está intimamente implicada na prevenção de erosões e úlceras devido ao facto de ser constitutiva e exercer um papel fundamental na manutenção da arquitectura glandular do estômago (Lipsky et al, 2000). Esta acção desenvolve-se através da produção de prostaglandinas citoprotectoras, nomeadamente a PGE_2 e a PGI_2, as quais são responsáveis pela manutenção da integridade da mucosa gástrica e pela redução da produção de ácido, aumentando a produção de bicarbonato e melhorando o fluxo sanguíneo na mucosa digestiva (Eckmann et al, 1997; Buttgereit et al, 2001). Ao

contrário da COX-1, a COX-2 encontra-se expressa principalmente no local da lesão, onde se desenvolve um processo inflamatório (Koki et al, 2002). Assim, os AINEs convencionais, não selectivos das COX (inibem a COX-1 e a COX-2), ao inibirem em parte a acção da COX-1, apresentam efeitos secundários aerodigestivos; os inibidores específicos da COX-2 (coxibes), ao inibirem a COX-2, tratam a patologia em causa não inibindo a produção das prostaglandinas constitutivas do tubo digestivo. Dois grandes ensaios clínicos, envolvendo cerca de 8000 doentes (VIGOR e CLASS), concluíram que os inibidores selectivos da COX-2 reduziam entre 40 a 60% as complicações gastrointestinais, embora também se tenha verificado que o efeito "poupador" da COX-2 não era completo quando se administravam altas dosagens de coxibes (Fitzgerald e Patrono, 2001).

Em relação ao uso de corticóides, verifica-se também o mesmo tipo de complicações a nível digestivo que os AINEs comuns, neste caso devido ao aumento da secreção ácida induzida pela estimulação da bomba de protões K^+/H^+ (Ng et al, 1991; O´Neil et al, 1992; Wang et al, 1996). No que diz respeito aos efeitos secundários a outros níveis, os inibidores da COX-1 e COX-2 não apresentam diferenças significativas relativamente à insuficiência renal; no entanto, no que concerne a problemas cardiovasculares, alguns estudos revelaram-se contraditórios, pelo que é discutível a existência de um eventual efeito mais nefasto sugerido por algum destes inibidores específicos das COX. (Koki et al, 2002; Curtis et al, 2006; Gislason et al, 2006; Joshi et al, 2007; Spalding et al, 2007).

Apesar da evidência acumulada de que os AINEs inibidores específicos da COX-2 apresentam menos efeitos secundários aerodigestivos, o potencial terapêutico associado a esta característica nunca foi explorado nas diversas patologias do foro respiratório. Para que este interesse potencial se revele é necessário que a COX-2 seja expressa nas laringites e, simultaneamente, não se verifique aumento da expressão da COX-1. No entanto, que seja do nosso conhecimento, a expressão da COX-2 quer na mucosa normal quer em casos de laringite nunca foi avaliada em qualquer modelo

experimental, tal como nunca foi estudada a utilização de fármacos inibidores selectivos da COX-2 nestas patologias.

1.2.5. Inflamação crónica e cancro

A relação patogénica entre a inflamação crónica e o cancro tem sido estabelecida em diversos órgãos ou sistemas. Estudos recentes relacionaram a inflamação crónica em vias aerodigestivas com o carcinoma do estômago, do esófago ou do pulmão (Philip et al, 2004; Schottenfeld e Beebe-Dimmer, 2006; Ben-Baruch, 2006). Há cerca de uma década que se sabe que os tumores esofágicos, gástricos e colo-rectais expressam altos níveis de COX-2, ao contrário das mucosas normais que apresentam níveis baixos ou indetectáveis desta isoforma da Cicloxigenase (Warner e Mitchell, 2004). Níveis elevados de COX-2 induzem proliferação celular através do aumento da expressão do Factor de Crescimento Epitelial (EGF) e de determinadas Cinases da Ciclina (Warner e Mitchell, 2004; Ohtani et al, 2004), Outro dos mecanismos pró-neoplásicos exercidos pela COX-2 consiste na indução da adesão da matriz extracelular das células tumorais, levando a uma maior resistência à apoptose das células malignas e ao aumento da viabilidade tumoral, condição que mostrou ser reversível após a associação de inibidores da COX-2 (Dubois et al, 1998). Efectivamente, os inibidores da COX-2 revelaram recentemente um efeito potencial no tratamento de vários cancros como o do pulmão, cólon, próstata e mama (Harris et al, 2007). Estes dados levantam a hipótese do envolvimento da COX-2 na progressão e disseminação neoplásica em diversos órgãos. Nestes tumores, a COX-2 é responsável pelos altos níveis de PGE_2, a qual parece promover o desenvolvimento tumoral inibindo a actividade supressora neoplásica e estimulando a proliferação de células epiteliais (Sjödahl et al, 2001). No caso do tumor da próstata, a produção aumentada de PGE_2 favorece a angiogénese associada às células tumorais e, consequentemente, a progressão do cancro

(Myers et al, 2001). Também no caso da Polipose Adenomatosa Familiar, um inibidor específico da COX-2 (Celecoxib) foi aprovado pela FDA há cerca de sete anos como adjuvante no tratamento da doença (Steinbach et al, 2000). Além da COX-2, outras moléculas, nomeadamente o Factor de Necrose Tumoral (TNF) como promotor tumoral (Arnott et al, 2002; Szlosarek et al, 2006), bem como os inibidores do ciclo celular P16, P21 e P53 como supressores tumorais (Koch et al, 1996; Mitchell et al, 2003; Perez-Ordoñez et al, 2006), alteram a sua expressão nas lesões pré-neoplásicas e neoplásicas. A diminuição da expressão dos supressores tumorais pode contribuir para o desenvolvimento de tumores em vários órgãos ou sistemas (Serrrano, 1997; Warnakulasuriya et al, 1998; Polsky et al, 2001; Voorhoeve e Agami, 2003; Ohtani et al, 2004; Furth et al, 2006). Tal acontece porque, na ausência do supressor, a expressão de certas Cinases da Ciclina aumenta e, consequentemente, a fosforilação do gene do retinoblastoma ocorre a mais facilmente induzindo as células a entrarem em mitose (Ohtani et al, 2004) (Figura 5).

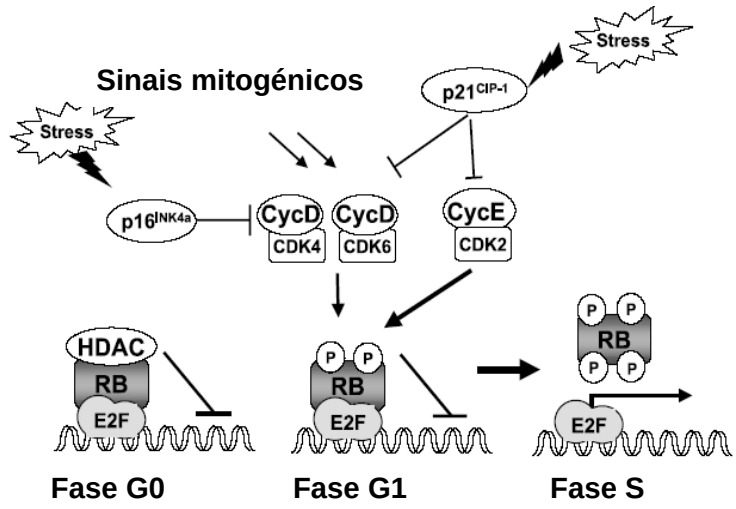

Figura 5. Os supressores tumorais p16 e p21 são fundamentais para evitar que as cínases da ciclina (CDK2, CDK4, CDK6) iniciem o processo de mitose nas células em G0 quando estas são submetidas a stress tecidular (adaptado de *Ohtani et al, 2004*)

No que concerne ao cancro da laringe, embora a relação com factores como a exposição ao fumo do tabaco, o consumo de bebidas alcoólicas e a determinados vírus e agentes tóxicos esteja perfeitamente estabelecida, não existe consenso relativamente a outros factores inflamatórios crónicos, nomeadamente os resultantes do refluxo gastroesofágico (Galli et al, 2006). Relativamente ao cancro laríngeo e à terapêutica com coxibes, os estudos realizados até ao momento foram realizados apenas *in vitro* (Ding et al, 2005) e, no que diz respeito à relação com a expressão da COX-2, os estudos são recentes e escassos (Kourelis et al, 2007). Para estabelecer a possível ponte entre laringite e cancro laríngeo, além de avaliar alterações da expressão de marcadores como a COX-2 e o TNF-α, é importante conhecer também a variação da expressão de moléculas supressoras tumorais como o p16 e o p53. No entanto, os estudos em modelos experimentais a este nível, no que respeita à laringe, são inexistentes.

1.3. ENERVAÇÃO MOTORA DA LARINGE

1.3.1 Troncos nervosos principais

Para funções reflexas como a tosse, a deglutição e a respiração, as junções neuromusculares ou placas motoras (PM) presentes nos músculos da laringe correspondem aos terminais motores de fibras provenientes de motoneurónios localizados no núcleo motor do vago do tronco cerebral ou na parte caudal do núcleo ambíguo (Gray, 2005). Para funções voluntárias como a vocalização, os neurónios situam-se mais superiormente na substância cinzenta periaquedutal do mesencéfalo e no núcleo retroambíguo, conforme demonstrado em modelos experimentais (Zang et al, 1995). No homem, este circuito mediado pelo tronco cerebral parece estar relacionado com emoções como o choro ou o riso, mas terá menos importância na regulação de outras expressões voluntárias como a voz e o discurso. Em estudos realizados no macaco, verificou-se a existência de

conexões neuronais que dependem do controlo cortical, principalmente do córtex motor laríngeo (porção terminal inferolateral do córtex primário adjacente à fissura de Sylvius) (Simonyan e Jürgens, 2002; 2005b). Estes neurónios estabelecem ligações com diferentes núcleos subcorticais (Simonyan e Jürgens, 2003; 2005a), o que revela um elevado grau de complexidade no comando motor da laringe. Os axónios destes neurónios subcorticais percorrem por sua vez os nervos laríngeos recorrentes e enervam todos os músculos intrínsecos da laringe excepto o cricotiroideu, o qual é enervado pelo ramo externo do nervo laríngeo superior. Para a função laríngea normal é necessário que exista uma perfeita interacção entre os sistemas cortical e subcortical (Ludlow, 2005). No entanto, alguns estudos indicam a presença de projecções directas de neurónios do córtex, através de vias corticobulbares, para os músculos laríngeos através dos nervos recorrentes (Cracco et al, 1990; Maccabee et al, 1991; Ludlow, 2005).

1.3.2. Placas motoras

Para a compreensão das funções habituais da laringe e dos mecanismos implicados na vocalização é importante conhecer a distribuição dos terminais nervosos, correspondentes às junções neuromusculares, nos músculos intrínsecos da laringe (Figura 6). A junção neuromuscular corresponde à sinapse entre a porção terminal de uma ramificação axonal do neurónio motor e a PM da célula muscular que é estimulada (figura 6) e a unidade motora é constituída por todas as fibras musculares (células musculares) enervadas pelas ramificações axonais de um neurónio motor (Hirsch, 2007). A estimulação do neurónio de uma unidade motora resulta na contracção de todas as células musculares que este enerva. Deste modo, músculos delicados que exigem uma grande variedade de movimentos têm unidades motoras com menos fibras por neurónio (por exemplo, os músculos da mímica facial têm menos de 10 fibras musculares enervadas por cada neurónio motor),

enquanto que em alguns músculos dos membros inferiores mais de 2000 fibras podem ser enervadas por cada motoneurónio (Gray, 2005). Cada fibra muscular, por sua vez, pode ser enervada por um número variável de PMs: pode ocorrer apenas uma PM localizada na região média da fibra muscular (uni-motora) – o que corresponde à generalidade das fibras musculares dos mamíferos; ou podem ser várias PMs distribuídas difusamente ao longo do comprimento da fibra (multi-motoras) – situação menos frequente, presente no ser humano em músculos específicos como os músculos extrínsecos do olho ou intrínsecos da laringe (Rosen et al, 1983; Périé et al, 1997; Khanna et al, 2003; Sheppert et al, 2003). Deste modo, quanto maior o número de placas e de neurónios a estimular uma fibra, maior será a variabilidade e a capacidade de controlo da contracção dessa mesma fibra.

Músculos da Laringe
ct - cricotiroideu (rato), g - glótico (galo)

PM - Placas motoras
(terminais nas fibras musculares)

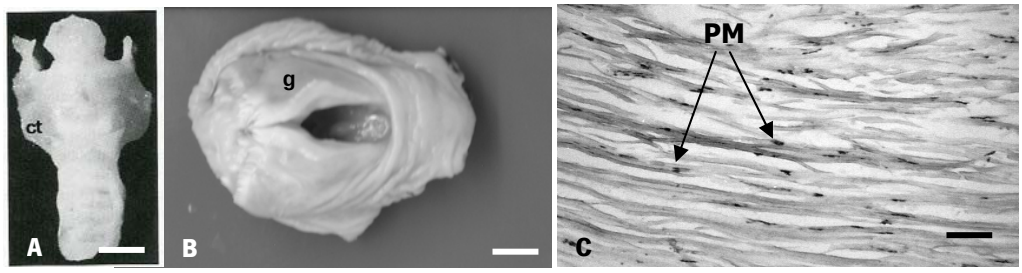

Figura 6. Laringe e respectivos músculos cricotiroideu (ct) do rato (A) e glótico (g) do galo (B) e os terminais motores neuromusculares que correspondem às placas motoras (PM) de um músculo esquelético (C) (Barra de ampliação: A- 2mm, B- 6mm, C- 20µm) (A, C - imagens adaptadas de *Pais-Clemente e Lima-Rodrigues, 1996*).

1.3.3. Distribuição das placas motoras nos músculos vocais dos vertebrados

Relativamente à vocalização e à origem da voz, sabe-se que mamíferos como os primatas, com uma anatomia mais semelhante à humana não são capazes de produzir ou "imitar" palavras, ao contrário de outros vertebrados como algumas aves (ex: papagaio), apesar de estes

possuírem uma anatomia cerebral e laríngea claramente distinta do ser humano. Além disso, sabe-se que o órgão e os músculos responsáveis pela produção dos sons diferem nas diferentes classes de vertebrados; no caso dos mamíferos, répteis e anfíbios é a laringe, através das cordas vocais, o órgão responsável por esta função, enquanto nas aves a vocalização é desempenhada por uma estrutura subtraqueal especializada, a siringe e os músculos associados (George e Berger, 1966). Por último, a cartilagem tiróide está presente na laringe dos mamíferos mas ausente noutros vertebrados, sendo o músculo correspondente ao tiroaritenoideu nestes designado por cricoaritenoideu (George e Berger, 1966; Storer et al, 1979; Kardong, 2002). Actualmente, o conhecimento da produção vocal nas diferentes classes animais e da enervação fina dos músculos vocais está ainda muito pouco desenvolvido (Fitch, 2000) sendo necessários estudos comparativos entre os diferentes grupos de espécies ao nível do aparelho vocal.

Como as PM transmitem o estímulo para a contracção das fibras musculares e a contracção de cada fibra e do músculo vai depender do número de PM envolvidas, a análise da sua distribuição ao longo dos músculos vocais, não só no ser humano como noutras espécies de mamíferos e em outros vertebrados, é importante para compreender a enervação motora periférica da laringe. De facto, considerando as funções habituais desempenhadas pelos músculos tiroaritenoideu (esfíncter glótico e fonação nos mamíferos), cricoaritenoideu (esfíncter glótico e fonação nos anfíbios, fonação nos mamíferos e esfíncter glótico nas aves) e pelos músculos siríngeos (fonação nas aves), a distribuição das PM nas fibras destes deverá elucidar sobre as diferentes capacidades de vocalização nestas classes de vertebrados. Contudo, existem muito poucos estudos comparativos sobre a distribuição das placas motoras nos músculos vocais dos vertebrados. No que diz respeito aos mamíferos, além do homem, somente dois estudos se debruçaram sobre a distribuição da enervação nas fibras dos músculos laríngeos no rato (Pais-Clemente e Lima-Rodrigues, 1996; Inagi et al, 1998); além destes, não se conheciam estudos sobre a anatomia do

controlo nervoso das fibras musculares da laringe ou da siríngе de outros mamíferos ou outros vertebrados, nomeadamente aves ou anfíbios. Apesar dos músculos cricoaritenoideu e tiroarinoideu serem muito importantes na produção dos sons através do encerramento glótico (Greene, 1989; Gray, 2005), a distribuição das PM nestes músculos no Homem ainda é motivo de discussão; alguns autores referem uma distribuição difusa ao longo dos músculos, sem nenhuma organização especial (Rosen et al, 1983; Périé et al, 1997), outros descrevem que as PM ocupam praticamente apenas os dois terços centrais das cordas vocais (Rossi e Cortesina, 1965a; 1965b), enquanto um 3º grupo de estudos apresenta uma distribuição das PM difusa por todo o músculo, mas com maior densidade no terço médio (Pais-Clemente e Lima-Rodrigues, 1996; Sheppert et al, 2003).

Uma razão clínica potencial para se efectuar a análise cuidada da enervação motora periférica da laringe no ser humano, é no bloqueio da transmissão neuromuscular para o tratamento de diversas disfonias, nomeadamente na disfonia espástica através, por exemplo, da aplicação selectiva de injecções de toxina botulínica (Blitzer et al, 1986; Castellanos et al, 1994; Zalvan e Blitzer, 2004). De facto, actualmente estas injecções não têm em consideração as possíveis variações na distribuição das PMs ao longo dos respectivos músculos, nomeadamente os músculos vocais, muitas vezes por desconhecimento da real densidade e distribuição das mesmas. Finalmente, outro aspecto clinicamente relevante diz respeito à possível relação entre a distribuição das PMs e determinadas patologias, como os nódulos das cordas vocais devido à possível correlação entre a variabilidade na concentração de placas motoras e a zona da corda vocal onde são mais frequentes os nódulos. Em resumo, o melhor conhecimento da real distribuição das PMs poderá permitir o tratamento mais selectivo de patologias do foro motor no aparelho vocal.

Capítulo 2

Objectivos e Metodologia

2. OBJECTIVOS E METODOLOGIA

Tendo em consideração que os estudos existentes sobre enervação periférica (sensitiva e motora) fina da laringe são escassos, o objectivo geral da presente dissertação foi estudar a morfologia e distribuição dos terminais axonais sensitivos e das placas motoras na laringe e desenvolver modelos experimentais que permitissem não só aprofundar o conhecimento de patologias laríngeas como lançar as bases de novas terapêuticas potenciais. O trabalho compreende 4 estudos completos e um estudo preliminar e utilizou o Rato como animal de experiência, tendo o estudo nº 5 utilizado outras espécies de vertebrados para efeitos comparativos.

1. Para determinar o padrão da enervação sensitiva periférica da laringe as fibras da mucosa laríngea foram identificadas com técnicas imunocitoquímicas utilizando anticorpos primários contra os peptídeos CGRP (Peptídeo Relacionado com o Gene da Calcitonina) e SP (Substância P), os dois principais neuromediadores utilizados pelos nervos sensitivos finos. A distribuição das fibras sensitivas marcadas imunorreactivas para a CGRP e SP e dos respectivos botões axonais e *en passant* foi analisada ao longo do epitélio e lâmina própria das diferentes zonas da mucosa laríngea após tratamento histológico e corte seriado das laringes (Capítulo 4.1).

2. Para avaliar o papel dos terminais sensitivos na génese de inflamação (laringite neurogénica) desenvolveu-se no rato um modelo experimental baseado na entubação nasogástrica (ENG). O objectivo geral deste estudo foi caracterizar o processo inflamatório ao nível da mucosa laríngea e estabelecer uma correlação entre o período de laringite e as alterações induzidas (i) na enervação sensitiva periférica e (ii) no padrão de expressão de diferentes marcadores

pró e anti-inflamatórios. Grupos de animais foram entubados sob anestesia com a sonda nasogástrica de menor diâmetro utilizada na clínica e disponível no mercado. A sonda colocada através do nariz tinha um comprimento previsto para se alojar no estômago, sendo a extremidade externa da sonda fixada através de um ponto de sutura à fossa nasal do animal durante períodos de tempo variáveis (grupos de animais submetidos a 1, 2 e 5 semanas de ENG). Para a caracterização do modelo inflamatório e avaliação do processo patológico procedeu-se à análise (e comparação com o grupo controlo – não entubados), na mucosa laríngea (i) da expressão dos neuropeptídeos inflamatórios CGRP e SP nas fibras sensitivas periféricas, (ii) do número de células COX-2-positivas através de técnicas de imunocitoquímica e (iii) da expressão dos níveis de RNAm, por RT-PCR em Tempo Real, de COX-1 e COX-2 e de outros mediadores pró-inflamatórios como as citoquinas IL-1β, IL-6 e TNF-α, e ainda da citoquina anti-inflamatória IL-10. (Capítulo 4.2).

3. Ao contrário dos AINEs não selectivos das enzimas COX-1 e COX-2 e dos corticóides, nenhum outro tipo de fármaco anti-inflamatório foi até ao momento usado com sucesso no controlo clínico da laringite. Com o objectivo de avaliar novas formas terapêuticas no tratamento da laringite crónica, foi testada no Rato a acção de um fármaco inibidor selectivo da COX-2 (Etoricoxibe) sobre a inflamação neurogénica da laringe que se desenvolve no modelo experimental de ENG (*ver item 2*). Foram analisadas as alterações induzidas em vários mediadores inflamatórios, nomeadamente (i) na expressão dos neuropeptídeos inflamatórios CGRP e SP nas fibras sensitivas periféricas, (ii) no número de células COX-2-positivas na mucosa laríngea e (iii) na expressão dos níveis de RNAm de COX-1 e COX-2, das citoquinas pró-inflamatórias IL-1β, IL-6 e TNF-α e da citoquina anti-inflamatória IL-10 (Capítulo 4.3).

4. Para avaliar se o processo inflamatório associado a uma laringite prolongada pode induzir condições pré-malignas na mucosa laríngea, além da análise histopatológica das laringes foi analisada a expressão dos supressores tumorais p16 e p53, através de técnicas de RT-PCR em tempo real, nos animais submetidos a diferentes períodos de laringite crónica induzida pelo modelo experimental de ENG. O objectivo deste estudo foi determinar a ocorrência de correlação entre o período de laringite e o padrão de expressão dos supressores tumorais (Capítulo 4.4).

5. Para caracterizar a distribuição dos terminais motores no aparelho vocal de diferentes classes de vertebrados, analisamos a disposição das placas motoras (PM) nos músculos vocais de mamíferos (Homem, Coelho e Rato), batráquios (Rã) e aves (Pombo e Galo) através do método histoquímico da detecção da enzima colinesterase. O objectivo principal deste trabalho foi esclarecer a distribuição das placas motoras nas cordas vocais humanas de modo a compreender melhor a etiologia de várias disfonias do tipo motor e sugerir uma melhor aplicabilidade clínica de fármacos já existentes. Por outro lado, a análise da distribuição das PM entre os diferentes vertebrados permitiu comparar a enervação dos músculos vocais entre as diferentes espécies e levantar hipóteses de correlação com as diferentes capacidades de vocalização (Capítulo 4.5).

Capítulo 3

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Capítulo 4

Resultados (trabalho experimental)

Capítulo 4.1

Publicação (I)

Lima-Rodrigues M, Nunes R & Almeida A.

"Intraepithelial nerve fibers project into the lumen of the larynx"

Laryngoscope, 114: 1074-1077

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Intraepithelial Nerve Fibers Project Into the Lumen of the Larynx

Manuel Lima-Rodrigues, MD; Rui Nunes, MD, PhD; Armando Almeida, PhD

Objectives/Hypothesis: **Studies on the morphology and location of the sensory receptors in the laryngeal mucosa have resulted in insufficient and sometimes conflicting data. In the present study the authors analyzed the distribution and morphology of sensory nerve plexuses and terminal fibers in the laryngeal mucosa of the rat.** ***Study Design:*** **Two groups of Male Wistar rats were used in this laboratory study; the larynx of the first group were used to analyse the sensitive innervation of its epithelium, whereas the larynx of the second group (controls) were tested for the specificity of the antibodies used.** ***Methods:*** **The larynges of the animals were entirely removed after perfusion, and coronal or horizontal sections were immunoprocessed for further randomized analysis of the mucosa. Primary afferents were detected by immunoreaction to two widely recognized markers of sensory nerves, calcitonin gene-related peptide and substance P, and visualized using diaminobenzidine as a chromogen.** ***Results:*** **The nerve plexuses were more densely distributed in the dorsal half of the vocal folds and in the laryngeal aspect of the epiglottis. Dense networks of fine fibers with many varicosities en passant, immunoreactive for both calcitonin gene-related peptide and substance P, occurred in the lamina propria and along the epithelial thickness. Calcitonin gene-related peptide–immunoreactive and substance P–immunoreactive fibers extended across the epithelium and projected to the laryngeal lumen itself, reaching the space between the cilia.** ***Conclusion:*** **The projection of intraepithelial nerve fibers into the lumen of the larynx indicates that in the absence of mucus, nerve endings may be exposed and thus receive direct stimulation from airborne substances. Furthermore, it suggests that the laryngeal mucosa of the rat may constitute an experimental model for studying the direct activation or manipulation of primary afferents at the periphery and neurogenic inflammation.** ***Key Words:*** **Substance P, calcitonin gene-related peptide, primary afferents, laryngeal mucosa, light microscopy, intraepithelial.**
Laryngoscope, **114:1074–1077, 2004**

INTRODUCTION

The laryngeal sensory system can initiate a wide range of reflexes with significant physiological effects related to body functions, namely, bronchial-pulmonary defense. This system interacts with components of the autonomic nervous system, as shown by the laryngeal reflex occurring in response to mechanical stimulation of the mucosa. The main innervation of the mucosa of the pharynx and larynx results from the superior laryngeal branch of the vagus nerve, although other cranial nerves, such as the trigeminal and glossopharyngeal nerves, may also be involved.[1] Although the central origin of the laryngeal innervation and the local distribution of major nerve trunks are well documented, few and conflicting data are known about the fine innervation of the laryngeal wall in different animal species.[2]

The sensory innervation of the larynx has long been known to be located in the mucosa.[3] Myelinated and unmyelinated fibers reaching the epithelium derive from a plexus in the submucosa, have numerous varicosities along their extension, and terminate as free axonal endings.[4] This nervous plexus was shown to be rich in substance P (SP)[5] and calcitonin gene-related peptide (CGRP),[6,7] two neuropeptides implicated in sensory innervation,[8–10] because of their key role in the transmission of nociceptive information to the central nervous system.[11]

The larynx sensory fibers do not reach the surface of the epithelium in humans, which suggests that mucosal irritants do not stimulate directly the primary fibers of the laryngeal mucosa.[12] This anatomical feature was not recorded in other species either,[2] including in the rat,[13] where fibers were described (using fluorescence microscopy) as only reaching the epithelium. Interestingly, a few sensory fibers were shown to reach the lumen of human nasal mucosa.[12] The objective of the present study was to review the morphology and location of the network of intraepithelial primary afferents immunoreactive to CGRP and SP in the laryngeal mucosa of the rat using bright-field light microscopy.

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MATERIALS AND METHODS

Five female rats (weight, 60–100g; age range, 20–40 d) were obtained from the Wistar Han colony of Charles Rivers Company (Barcelona, Spain). Animals were perfused under anesthesia (35% chloral hydrate intraperitoneally) through the ascending aorta with 100 mL 0.1 mol/L phosphate-buffered saline (PBS) followed by 1000 mL Zamboni fixative (2% paraformaldehyde and 1.5% picric acid).

The larynx was removed and immersed in the same fixative for 4 hours at 4°C, followed by an overnight sucrose bath (30% sucrose in 0.1 mol/L phosphate-buffered saline). Coronal and horizontal frozen sections were cut in a cryostat at 50 μm and processed for immunocytochemical study. Alternate sections were incubated overnight at 4°C with rabbit anti-CGRP (1:4000) (Amersham) or rabbit anti-SP (1:40000) (Peninsula Laboratories) anti-sera in PBS with 0.3% triton X-100 (PBST). After rinsing in PBST, the sections were incubated with biotinylated goat anti-rabbit (1:200) (Vector Laboratories) in PBST for 1 hour, washed in PBST, and incubated with avidin-biotin complex (ABC) (1:200) (Vector) in PBS for 1 hour. After rinsing in PBS and 0.1 mol/L tris-HCl buffer (pH 7.4) the antigen-antibody reaction was visualized in a solution containing 10 mg diaminobenzidine (DAB) (Sigma Chemical Company) and 4 μL H_2O_2 in 20 mL tris-HCl buffer. The immunostained sections were dehydrated, cleared in xylene, mounted in Entellan (Merck) and analyzed by means of bright-field light microscopy.

RESULTS

An extremely dense plexus of CGRP-immunoreactive nerve fibers was present in the laryngeal epithelial surface of the rat (Figs. 1–3). The nerve plexus was particularly abundant along the vocal folds, with a greater fiber density in the dorsal half, which decreased progressively in the ventral direction (Fig. 1). The CGRP innervation was also rich in the laryngeal aspect of the epiglottis (Fig. 3C).

The intraepithelial network is derived from nerve trunks abutting the epithelial deep surface from the underlying connective tissue of the lamina propria (Fig. 2A). The plexus is evenly distributed across the epithelial thickness with the immunoreactive fibers exhibiting a typical dotted appearance because of successive round varicosities appearing en passant along the fibers (Figs. 2B and 3A). It is important that the fiber endings crossed the most superficial epithelial cell layer and protruded from the upper epithelial surface to the respiratory lumen itself, reaching the free space among the cilia (Figs. 2 and 3A).

Immunostaining for SP depicted an intraepithelial nerve plexus with an overall location similar to that of CGRP fibers, but with a lower density and with fewer varicosities along the fibers (Fig. 4). It is important that the superficial axonal endings also protruded through the epithelium surface and reached the laryngeal lumen (Fig. 4).

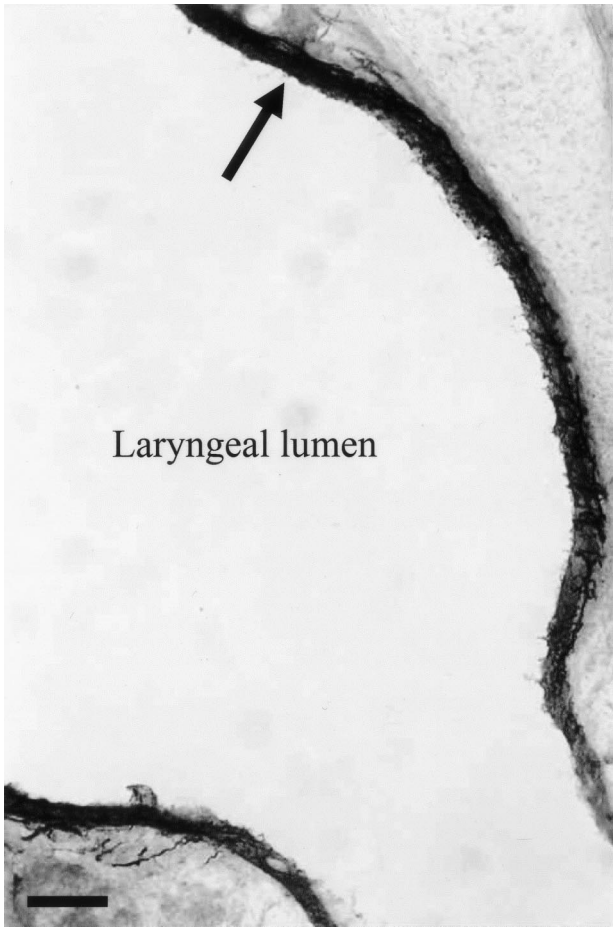

Fig. 1. Low-magnification microphotograph of the vocal fold showing the epithelial surface with the calcitonin gene-related peptide–immunoreactive plexus (arrow). Scale bar: 500 μm.

DISCUSSION

Innervation of Laryngeal Epithelium

It is known that in the rat there is a plexus of nonmyelinated sensory nerve fibers in the airway epithelium, especially at the basal level, with projections distributed toward the tight junctions near the lumen but not reaching it.[13,14] However, in our study, a great number of CGRP fibers and SP fibers showing a dotted appearance because of successive round en passant varicosities were shown clearly to protrude to the airway lumen among the cilia of the apical membrane of the cells lining the laryngeal epithelium. To the best of our knowledge, this is a new anatomical observation on the sensitive innervation of the larynx, which has not been described in the literature for the rat, human, or other species. In comparison with another study performed in the rat,[13] our results may reflect any combination of the following: 1) the use of much younger animals (weight, 60–100 vs. 250–400 g), 2) differences in the animal strain used (Wistar vs. Sprague-Dawley), or 3) differences in the experimental protocol followed (visualization of sensory fibers by bright field vs. fluorescent light microscopy).

The present data may help in understanding how the airborne substances can stimulate the mucosa. They suggest that in the absence of mucus the fibers are exposed and easily stimulated by irritating stimuli, for example, those caused by local inflammatory agents or exogenous irritants, such as tobacco smoke. Thus, the rat larynx may constitute a good model for the study of the action of inflammatory, degenerative, and neoplastic factors on the sensory innervation of the respiratory system. An electron microscopic study to show the relation between the apical membrane of lining epithelial cells and these free nerve endings should be undertaken in the rat. Electron microscope studies performed in humans[15] and dogs[16] showed that SP-immunoreactive and CGRP-immunoreactive nerve fibers did not reach the respiratory lumen but terminated

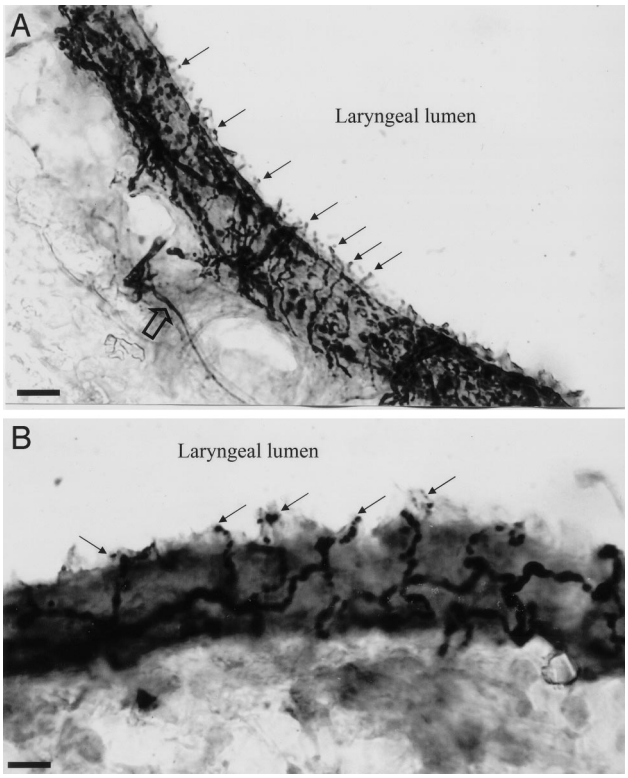

Fig. 2. Intermediate (**A**) and high (**B**) magnifications showing the intraepithelial vocal fold network of calcitonin gene-related peptide–immunoreactive (CGRP-IR) nerves from, respectively, the posterior and anterior epithelia. (**A**) The nerve trunks entering the plexus from the underlying lamina propria (open arrow) are evident. (**A** and **B**) The CGRP-IR terminal fibers with round en passant varicosities reaching the apical surface of the epithelium and protruding into the laryngeal lumen itself (small arrows). Scale bars: 7.5 (**A**) and 4 (**B**) μm.

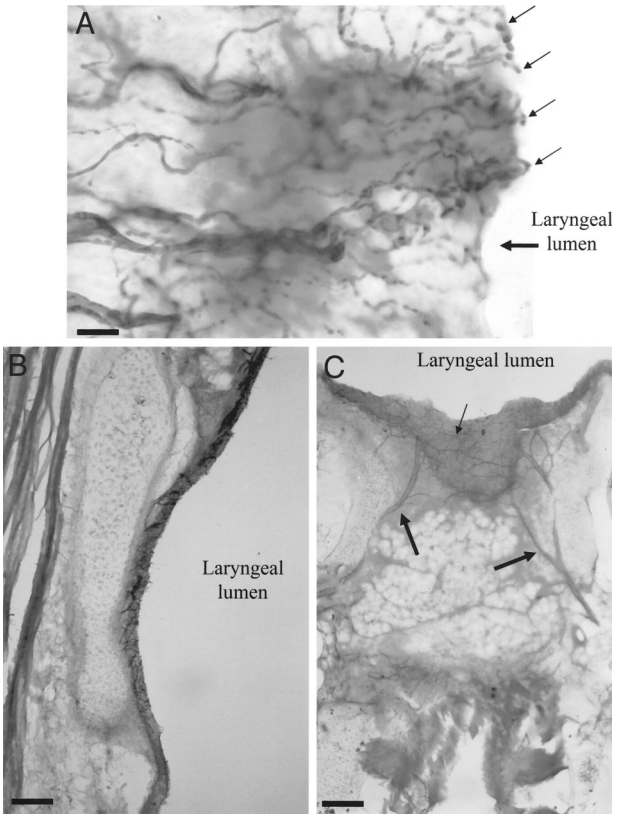

Fig. 3. The laryngeal intraepithelial calcitonin gene-related peptide–immunoreactive (CGRP-IR) nerve plexus. (**A**) Detail of the apical surface of the epithelium showing fibers and axonal buttons protruding into the laryngeal lumen (arrows). (**B**) Decreasing density of the CGRP-IR fiber network from the dorsal to ventral surface of the vocal fold epithelium. (**C**) Laryngeal aspect of the epiglottis intraepithelial plexus (small arrow). The bilateral CGRP-IR terminal fiber trunks of the superior laryngeal nerve (large arrows) are evident. Scale bars: 1.25 (**A**), 500 (**B**), and 800 (**C**) μm.

just under the epithelial junctional complexes that fuse the apical portion of the lateral cellular membranes of superficial epithelial cells.

In humans, a few fibers were shown to reach the surface of the epithelium in the nasal mucosa and the subepithelial excretory ducts of laryngeal mucous glands, but not the laryngeal lumen itself.[12] Neuroepithelial bodies, groups of neuroendocrine cells, but not the fibers that enervate them, were shown to be located frequently at airway openings and protrude into it.[17] Moreover, in the larynx the fibers enervating neuroepithelial bodies were not CGRP-positive or SP-positive.[12]

Calcitonin Gene-Related Peptide–Immunoreactive and Substance P–Immunoreactive Sensitive Fibers

The visceral sensory neurons of the vagus and glossopharyngeal nerves that enervate the laryngeal mucosa have cell bodies located in the nodose and petrosal ganglia, respectively.[2,18] The innervation of the larynx has been studied with the aid of immunocytochemistry, and several regulatory peptides were shown in the primary afferents of the laryngeal and tracheal mucosa: CGRP and SP, tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) in cats[6,19,20]; SP and CGRP in dogs[21]; NPY, VIP, SP, and CGRP in rats[13,22]; and VIP, NPY, SP, and CGRP in the human larynx.[7] However, CGRP and SP are considered to be the main putative neurotransmitters in the laryngeal primary afferent system. In the human mucosa the characteristic corpuscular fibers running underneath and within the epithelium contained only SP and CGRP.[7] In the canine nodose ganglion, the percentages of CGRP-immunoreactive (81,5%) and SP-immunoreactive (24,5%) neurons were much higher than those immunoreactive to other peptides.[9,21] A comparative study using immunocytochemical and tract-tracing techniques concluded that the density of the laryngeal sensory nerve fibers in the human and the dog was similar to that in the cat, and the regional distribution and density of SP-immunoreactive and CGRP-immunoreactive fibers showed almost the same distribution pattern as the sensory fibers showed with the use of neuroanatomical tracing techniques.[2]

Although the distribution pattern of CGRP-immunoreactive and SP-immunoreactive fibers was similar, suggesting an overlapping distribution, we found that SP-immunoreactive fibers were fewer in number than CGRP-immunoreactive fibers. This finding suggests that CGRP is the most important peptide that occurs in the

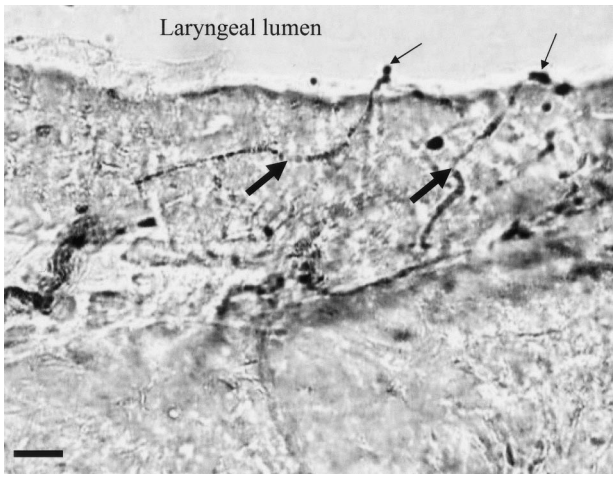

Fig. 4. Substance P–immunoreactive fibers in the laryngeal mucosa. The terminal buttons of these fibers reach the apical surface of the epithelium and protrude into the laryngeal lumen itself (arrows). Scale bar: 7.5 μm.

laryngeal epithelium, which is in accordance with results obtained previously in the dog.[21] Our study in the rat showed a more extended area occupied by the CGRP-stained fibers, which indicates that a fiber component containing exclusively CGRP may occur in the rat mucosa. However, a similar density and distribution of CGRP-immunoreactive and SP-immunoreactive fibers in the laryngeal epithelium were referred in the rat[13] and humans.[7] Once more, this result, which differs from that obtained in the study of Domeij et al.[13] in the rat, might reflect differences in the strain and in the age of the animals used or in the experimental protocol followed, or both (as mentioned earlier in the "Discussion" section).

As in other areas of the periphery, the high concentration of SP-immunoreactive and CGRP-immunoreactive fibers in the laryngeal epithelial lining indicates that they may consist of nociceptive sensory endings.[2] In fact, both neuropeptides were shown to be present in part of the C-fibers and Aδ-fibers of primary afferents mediating the transmission of nociceptive information from the periphery to the spinal or trigeminal dorsal horns of the central nervous system.[11] In the rat, denervated (vagotomy) animals and capsaicin-treated animals (capsaicin destroys most C-fiber primary afferents) have shown a strong or complete decrease in the number of CGRP fibers and SP fibers.[4,13] In accordance with data obtained for other species,[2] the dorsal half of the vocal folds and the laryngeal aspect of the epiglottis are more densely innervated, which suggests that these areas are more important for sensory reception in the rat larynx.

CONCLUSION

The anatomical data observed in the present study indicate that in the larynx of the rat the nociceptive endings of SP-immunoreactive and CGRP-immunoreactive primary afferents project directly to the lumen of the respiratory tract and thus may be directly activated by noxious stimulation of the larynx and stimulate cough reflex or neurogenic inflammation.

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Capítulo 4.2

Publicação (II)

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"A New Model of Laryngitis: neuropeptide, cyclooxygenase, and cytokine profile"

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A New Model of Laryngitis: Neuropeptide, Cyclooxygenase, and Cytokine Profile

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Objectives/Hypothesis: To develop and characterize a new model of laryngeal inflammation by analyzing the presence of neurogenic peptides and expression of cyclooxygenases (COX) and cytokines in the mucosa.

Study Design: Laryngitis induced by nasogastric intubation (NGI) was evaluated by histopathologic changes of the mucosa, alterations in calcitonin gene related peptide (CGRP) and substance P (SP) neuropeptides in sensory fibers, and COX-1,2, and cytokine (interleukin [IL]-1, IL-6, IL-10, tumor necrosis factor [TNF]-α) expression in the laryngeal mucosa.

Methods: Rats submitted to NGI for 1 to 5 weeks were compared with controls. Laryngeal sections were immunostained for stereologic analysis of SP and CGRP fiber density and number of mucosal cells expressing COX-2. Alterations in inflammatory mediators were evaluated by quantitative reverse-transcriptase polymerase chain reaction.

Results: NGI induced metaplasia of the epithelium and narrowing of the laryngeal lumen because of hypertrophy of laryngeal glandules and edema. An initial decrease in CGRP- and SP-immunoreactive fibers in the laryngeal mucosa (1–3 wk) was reverted with time (5 wk). COX-2 expression in mucosal cells increased progressively, reaching a maximum level at 5 weeks, and was observed in mononuclear immune cells, which is indicative of a chronic inflammatory process. In regard to mRNA expression levels of inflammatory mediators, TNF-α was increased during the 5 week NGI, and IL-10 decreased during the 5 weeks,whereas IL-1β, IL-6, and COX-2 increased in the first 1 to 2 weeks and returned to baseline at 5 weeks.

Conclusions: This NGI model results in laryngeal chronic inflammation without direct mechanical aggression of the mucosa and may contribute to the study of future therapeutic approaches to this pathology.

Key Words: Laryngeal inflammation, neuropeptides, cyclooxygenase-2, cytokines, nasogastric intubation, immunocytochemistry, mRNA expression, reverse-transcriptase polymerase chain reaction analysis, animal model.

***Laryngoscope*, 118:78–86, 2008**

INTRODUCTION

Nasogastric intubation (NGI) is largely used in clinical practice.[1] However, several complications such as laryngitis[2] and increased mortality because of aspiration pneumonia can be induced by this procedure. The experimental studies determining the mechanisms of laryngeal injury associated with nasogastric tubes are scarce.[2] Neurogenic mechanisms are implicated in many inflammatory diseases, and three group of factors are involved: 1) hydrogen ions (H^+) and adenosine triphosphate released by damaged tissue; 2) inflammatory mediators, including prostaglandins (PGs) and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and anti-inflammatory cytokines (IL-10); 3) and neurogenic peptides substance P (SP) and calcitonin gene related peptide (CGRP) present in the nerve fibers of the mucosa.[3] We previously demonstrated that neuropeptides SP and CGRP are present in the laryngeal sensory fibers that reach the epithelium surface and project to the respiratory lumen.[4] Because these fibers are accessible to direct stimulation, the rat laryngeal mucosa may constitute a good experimental model for studying the activation of primary afferents during the development of neurogenic inflammation.[4] SP and CGRP are important peptides in laryngeal sensory reflexes of cough and bronchial-pulmonary defense and can induce inflammatory responses in the respiratory tract.[5] As concerns the larynx, it is possible that these peptides can also induce laryngitis, but to the best of our knowledge, there are no experimental studies analyzing the expression of peptides and ILs such as IL-1β, IL-6, IL-10, and TNF-α in laryngeal inflammation.

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Acute and chronic inflammation induce the expression of cyclooxygenase (COX)-2 and release of PGs from arachidonic acid, which is cleaved from cell membrane phospholipids of damaged tissue.[3] PGs activate receptors in fiber membranes, triggering the cyclic adenosine monophosphate protein kinase A transduction pathway that increases intracellular calcium and releases SP from inflamed peripheral nerves, which further activates fibers by stimulating neurokin-1 receptors. CGRP receptors are colocalized with PG receptors at thin nerve terminals, and CGRP-receptor activation potentiates the effect of SP by retarding its release. Pro-inflammatory cytokines (IL-1, TNF-α, and IL-6) produced after tissue damage induce COX-2 synthesis and cells to release PGs.[3] The expression of COX-2 and COX-1 (constitutive) and their relation to neurogenic peptides and cytokines in chronic laryngitis is unknown. We designed an experimental rat model of NGI to obtain chronic inflammation of the larynx in which we evaluated potential changes in the 1) normal histology of the mucosa, 2) SP- and CGRP-immunoreactivity in laryngeal sensory fibers, 3) COX-2 expression in inflammatory cells of the lamina propria, and 4) COX-1, COX-2, IL-1β, IL-6, IL-10, and TNF-α mRNA expression in the mucosa.

MATERIALS AND METHODS

Animals

This study was performed in 54 male rats weighing 350 to 450 g obtained from the Wistar Han colony of Charles Rivers Company (Barcelona, Spain). Animals were lightly anesthetized with inhaled isoflurane to allow swallowing reflex and were submitted to the NGI procedure. A 10 to 12 cm narrow-bore nasogastric aspiration tube used for NGI in premature newborns (cat. No 533.04; Vigon Laboratoires Pharmaceutiques, Ecouen, France) was inserted through the nasopharynx until it reached the stomach. The external tip was sutured to the nasal lateral cartilages. Animals recovered and returned to their cages, being submitted to NGI for 1 ($n_{imuno} = 6$; $n_{PCR} = 7$), 2 ($n_{imuno} = 6$; $n_{PCR} = 6$), 3 ($n_{imuno} = 6$), and 5 ($n_{imuno} = 6$; $n_{PCR} = 6$) weeks. Twelve ($n_{imuno} = 6$; $n_{PCR} = 6$) nonintubated rats were used as controls (CONT). At the end of the intubation period, animals for immunohistochemical experiments were perfused under anesthesia (isoflurane and 35% chloral hydrate intraperitoneally) through the ascending aorta with 4% paraformaldehyde in phosphate-buffered saline (PBS) 0.01 mol/L. After confirmation that the NGI tube was still inserted in the stomach, the larynx was removed and immersed in the same fixative followed by 30% sucrose in 0.1 mol/L PBS overnight. Animals used for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) studies were anesthetized with isoflurane and sacrificed with 35% chloral hydrate intraperitoneally. Larynxes were excised immediately, macerated, immersed in 0.8 mL of TRIzol (Invitrogen, Carlsbad, CA), and stored at –80°C. The experiments were carried out in accordance with regulation of local authorities for handling laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Community Council Directive 86/609/EEC.

pHmetry

Measurements of pH were performed using a MI-414-4 cm pH combination microelectrode probe (Microelectrodes, Inc., Bedford, NH) attached to an Inolab pH Level 1 potentiometer (Wissenschaftlich Technische Werkstätten, Weillem, Germany). First, the pharyngeal pH and then the esophageal pH were recorded for each animal immediately before sacrifice (5 s for each pH measurement).

Histology and Immunocytochemistry

Laryngeal coronal frozen sections (20 μm) obtained with a cryostat were processed for immunohistochemistry. Alternate sections were incubated overnight at room temperature with rabbit antiCGRP (1:6,000; Bachem, Sao Carlos, CA) or rabbit antiSP (1:6,000; Bachem) antibodies in PBS with 0.3% triton X-100 (PBST). After rinsing in PBST, the sections were then incubated with biotinylated goat anti-rabbit (1:200; Vector Laboratories Inc, Burlingame, CA) in PBST for 1 hour, washed in PBST, and then incubated with avidin-biotin complex (1:200; Vector Laboratories Inc, Burlingame, CA) in PBS for 1 hour. After rinsing in PBS and 0.1 mol/L tris-HCl buffer (pH 7.4), the

TABLE I.
Sequences of Primers Used for Real-Time Polymerase Chain Reaction.

Target	Oligo	Sequence	Gene Bank ACC
β-Actin	Forward primer	5′ – GATTTGGCACCACACTTTCTACA – 3′	NM_031144
	Reverse primer	5′ – ATCTGGGTCATCTTTTCACGGTTGG – 3′	
IL-1β	Forward primer	5′ – GAAACAGCAATGGTCGGGAC – 3′	M98820
	Reverse primer	5′ – GAGACCTGACTTGGCAGAGG – 3′	
TNF-α	Forward primer	5′ – CCAACAAGGAGGAGAAGTTC – 3′	NM_012675
	Reverse primer	5′ – CCTGGTGGTTTGCTACGAC – 3′	
IL-6	Forward primer	5′ – CAAGAGACTTCCAGCCAG – 3′	NM_012589
	Reverse primer	5′ – CTCCGACTTGTGAAGTGGT – 3′	
IL-10	Forward primer	5′ – GCCAAGCCTTGTCAGAAATGA – 3′	NM_012854
	Reverse primer	5′ – TTTCTGGGCCATGGTTCTCT – 3′	
COX-1	Forward primer	5′ – GGCGTTGCTCATCCATCTACTC – 3′	S67721
	Reverse primer	5′ – AGCATCTGTGAGCAGTACCGG – 3′	
COX-2	Forward primer	5′ – TTTGTTGAGTCATTCACCAGACAGAT – 3′	S67722
	Reverse primer	5′ – ACGATGTGTAAGGTTTCAGGGAGAAG – 3′	

IL = interleukin; TNF = tumor necrosis factor; COX = cyclooxygenase.

antigen-antibody reaction was visualized with diaminobenzidine (DAB, Sigma, St. Louis, MO).

COX-2 immunohistochemistry was carried out according to the streptavidin-biotin-peroxidase complex technique (Ultravision Detection System Antipolyvalent, HRP, Lab Vision Corporation, Fremont, CA) using a primary antibody raised against COX-2 protein (1:400; rabbit monoclonal antibody, clone SP21; Neomarkers, Fremont, CA). Slides were sequentially washed in PBS/0.02% Tween 20 and incubated with 3% H_2O_2 in methanol for 10 minutes. This was followed by incubation with blocking solution for 10 minutes and the primary antibody solution for 2 hours at room temperature. Sections were then sequentially washed in PBS/0.02% Tween 20 and incubated with biotinylated goat antipolyvalent antibody for 10 minutes and streptavidin peroxidase for 10 minutes. DAB was used as chromogen. Slides were counterstained with Mayer hematoxylin (Merck, Darmstadt, Germany). Negative controls were performed by omission of the primary antibody. A colon carcinoma sample was used as positive control.

Stereologic Procedures

Sections were analyzed with an Axioskop 2 plus light microscope (Carl Zeiss, Göttingen, Germany), and images of laryngeal histologic data were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss). Cell and fiber numbers were estimated using the optical fractionator method.[6] This consists of virtual 3–dimensional-box (150 × 150 × 30 μm), equally spaced grids that were superimposed on every eighth coronal laryngeal section after drawing the limits of the area under study in the laryngeal mucosa. The number of COX-2 immunoreactive cells and SP- and CGRP-immunoreactive fibers that crossed the gridlines in every randomized site (chosen by the software) was counted according to well-defined stereologic rules. The estimated numbers were calculated from the ratio between the total number of counted cells and fibers crossing the grid site and the number of grid sites per area. The coefficients of error were automatically computed by the software according to the formulas of Gundersen for cell numbers.[6]

mRNA Extraction and Real-Time RT-PCR

Total mRNA in the larynx was extracted by adding 160 μL $CHCl_3$ followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The supernatant was carefully collected, and total mRNA was precipitated using isopropanol (2-propanol) followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The mRNA pellet was washed using 70% ethanol recentrifuged at 9,000 rpm for 5 minutes at 4°C, dried, and suspended with 50 μL of DNAse/RNAse free distilled water (Gibco, Carlsbad, CA). Quantification of total mRNA was performed by spectrophotometry (NanoDrop, NanoDrop Technologies, Inc., Wilmington, NC). Samples of total mRNA with the same concentration (2 ng/10 μL) were then reverse transcribed in a thermocyclator My Cycler Thermal Cycler (Bio-Rad, Hercules, CA) using a Superscript Kit II (Invitrogen) and Oligo dT (Invitrogen), according to the manufacturer's

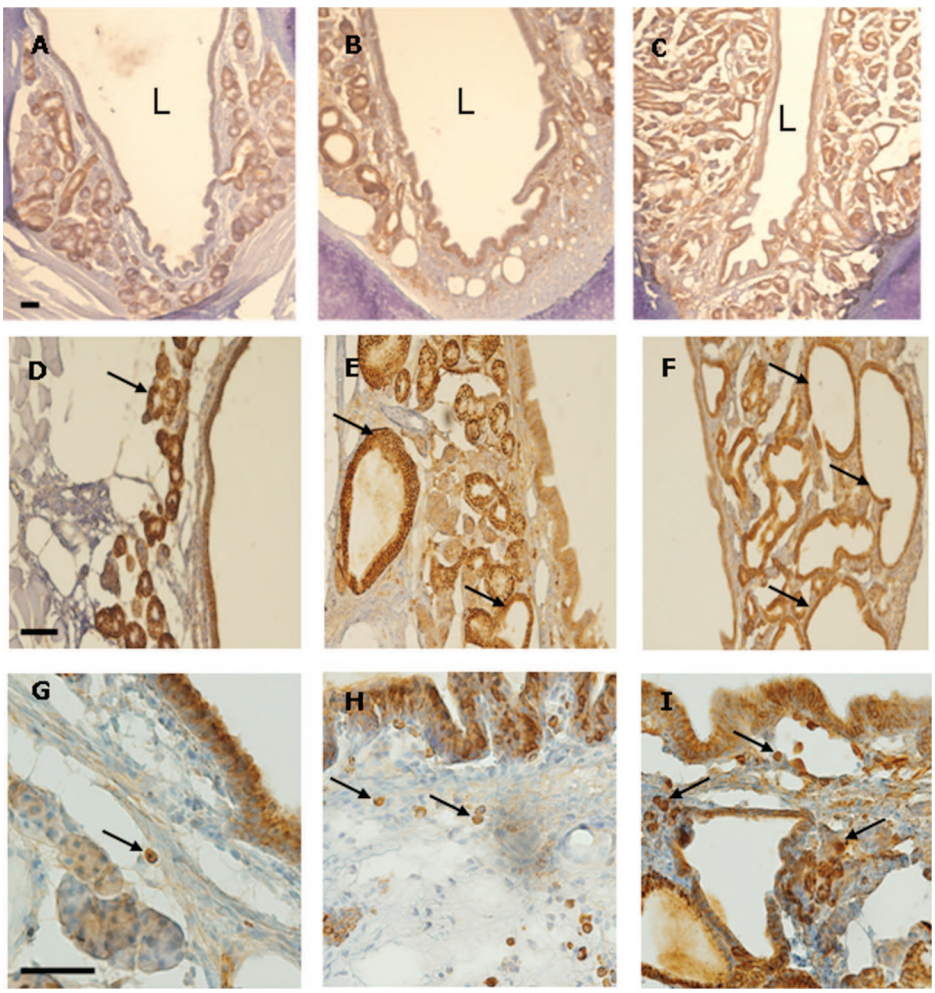

Fig. 1. Changes in histology and cyclooxygenase (COX)-2 expression of the laryngeal mucosa after no nasogastric intubation (NGI) (A, D, G) and 2 (B, E, H) and 5 (C, F, I) weeks of induction of the NGI model. A large increase of the glandular tissue was observed with increasing periods of laryngeal inflammation (A to C; arrows, D to F), with a consequent decrease in the laryngeal lumen (A to C). This was accompanied by large proliferation of mononuclear immune cells immunoreactive to COX-2 (arrows, G to I). L = laryngeal lumen. Magnification bar = 100 μm.

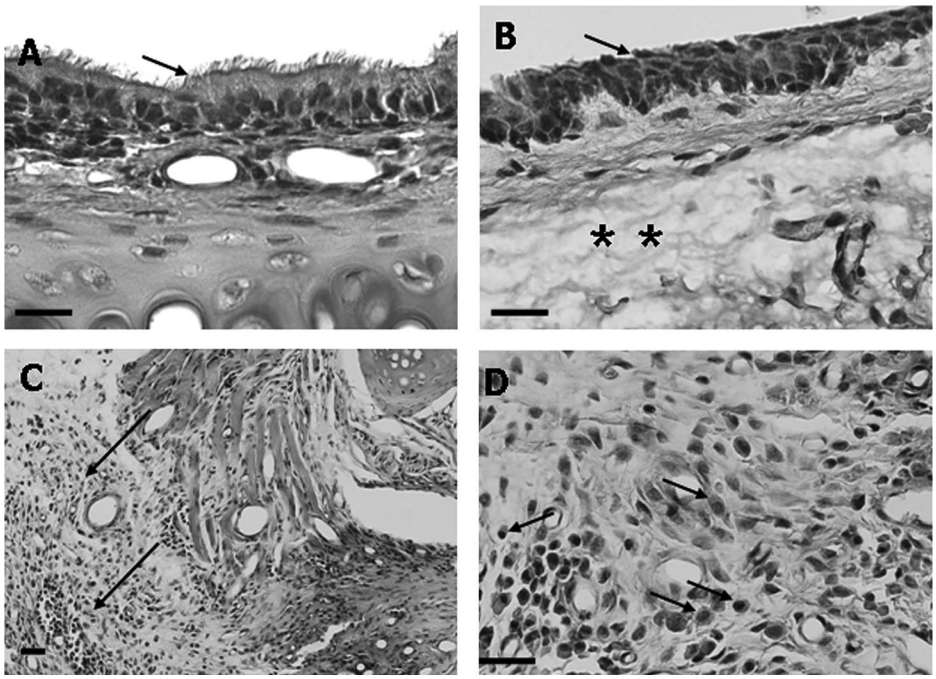

Fig. 2. Histologic changes in the connective tissue of the laryngeal mucosa induced by the nasogastric intubation (NGI) experimental model. Note replacement of typical ciliated pseudostratified respiratory epithelium (arrow, A) by another differentiated squamous stratified (nonkeratinized) epithelium (metaplasia) after 5 weeks of NGI (arrow, B). In addition, presence of chronic inflammation signs is evident, namely edema (asterisks, B) and large proliferation of mononuclear immune cells in the lamina propria (arrows, C), including plasmocytes (arrows, D). Magnification bar = 100 μm.

instructions. The cDNA was then subjected to real-time RT-PCR reactions for quantification of mRNA levels of β-Actin, IL-1β, TNF-α, IL-6, IL-10, COX-1, and COX-2, using the LightCycler (Roche, Basel, Switzerland), and an SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) was used according to the manufacturer instructions. Primer sequences used to amplify various cDNAs are shown in Table I. A typical real-time RT-PCR protocol was performed under the following conditions: 15 minutes at 95°C, followed by 40 cycles (94°C denaturing for 15 s; 58°C annealing for 20 s; 72°C extension for 15 s), melting at 60°C until 95°C for 90 seconds, and finally cooling to 35°C. The specificity of the SYBR Green assays was confirmed by melting point analysis. Gene expression of the housekeeping gene β-Actin was used for normalization of the results.

Data Analysis

Results were analyzed using Graph Pad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA). Means were compared using one-way analysis of variance (ANOVA) statistical evaluation followed by Tukey honestly significant difference post hoc test, and differences were considered to be significant at $P < .05$.

RESULTS

Histological and pHmetry Changes Induced by NGI

NGI induces a visible reduction of the laryngeal lumen (Fig. 1A–C), a consequence of a progressively increasing hyperplasia and hypertrophy of the glandular tissue (Fig. 1D–F). After 5 weeks of NGI, some areas of the epithelium showed a replacement of the differentiated pseudostratified respiratory epithelium of the larynx (Fig. 2A) by a squamous-stratified epithelium (metaplasia) and edema in the connective tissue (Fig. 2B). In addition, a large proliferation of plasmocytes and a few lymphocytes were present in the connective tissue of the mucosa (chronic inflammation) (Fig. 2C and D).

NGI resulted in significant changes in the pH recorded at the pharynx and esophagus (ANOVA, $P < .001$). Control rats showed a different pH between the pharyngeal and esophageal mucosa ($CONT_P \times CONT_E$, $P < .001$) (Fig. 3). No differences were observed after 1 week of NGI ($NGI_{P1w} \times NGI_{E1w}$, $P > .05$) because of a significant decrease in pharyngeal pH ($NGI_{P1w} \times CONT_P$, $P < .01$) (Fig. 3). After 2 weeks NGI, pH differences between

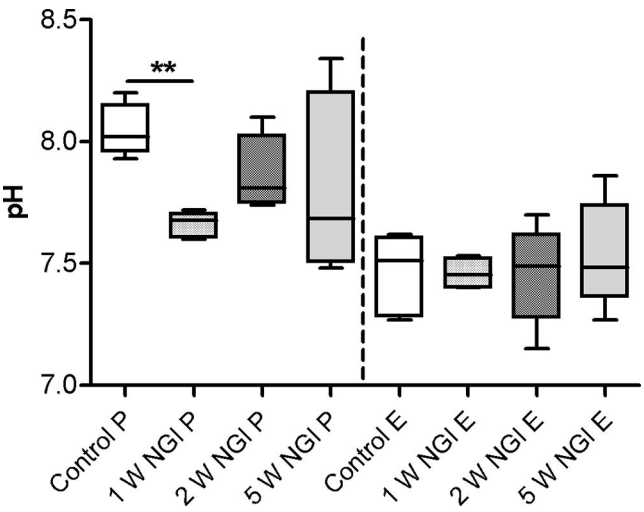

Fig. 3. Changes in the pH of the pharyngeal and esophageal mucosa measured at the end of the experimental period. Values for pharyngeal pH are placed at the left of the dashed line, whereas esophageal pH are recorded at the right. Control P and 1- to 5-week (W) nasogastric intubation (NGI) P = pharyngeal pH values for control and NGI animals; control E and 1 to 5 W NGI E = esophagic pH values for control and NGI animals. Only significant differences between pH values obtained in the same location are recorded in the graph. *$P < .05$, **$P < .01$, and ***$P < .001$.

mucosae were again significant ($NGI_{P2w} \times NGI_{E2w}$, $P < .001$), a trend still observed after 5 weeks NGI (although not reaching significance) (Fig. 3). No differences were observed in the esophageal pH along the NGI period.

Changes in Laryngeal Peripheral Sensory Enervation

CGRP-immunoreactive fibers were present in the epithelium and connective tissue of the lamina propria of the laryngeal mucosa (Figs. 4A and 5A–C). Laryngeal immunostaining of SP depicted an intraepithelial nerve plexus with an overall location similar to that of CGRP fibers but with a lower density of fibers both in control and intubated animals (Figs. 4B and 5D–F). NGI induced changes in the expression of neurogenic peptides (ANOVA, $P < .05$), with the number of CGRP-and SP-immunoreactive fibers decreasing already during the first week of NGI (CGRP: $NGI_{1w} \times CONT$, $P < .01$) and remaining decreased at the third week (CGRP: $NGI_{3w} \times CONT$, $P < .001$; SP: $NGI_{3w} \times CONT$, $P < .001$) (Figs. 4 and 5). Between 3 and 5 weeks, there was an increase in the number of fibers immunoreactive to CGRP ($NGI_{3–5w} \times CONT$, $P \leq .05$) and SP ($NGI_{3–5w} \times CONT$, $P \leq .01$) (Fig. 5), a recovery that reached control values in SP fiber density ($NGI_{5w} \times CONT$, $P > .05$) (Fig. 4B) and was near control values in terms of CGRP fiber density ($NGI_{5w} \times CONT$, $P < .05$) (Fig. 4A).

COX-2 Expression in Laryngeal Mucosa

The NGI model also induced changes in the expression of COX-2 in the laryngeal mucosa (ANOVA, $P < .05$). The number of cells expressing COX-2 enzyme in the laryngeal mucosa was higher after 2 weeks of NGI and increased significantly until the fifth week ($NGI_{5w} \times CONT$, $P \leq .05$) (Fig. 6). This was caused by a progressive increase in the number of mononuclear inflammatory cells expressing COX-2 in the lamina propria (Fig. 1G–I), lymphocytes, and plasma cells (Fig. 2), which is characteristic of chronic inflammation. However, in regard to the relative expression of COX-2 mRNA levels in the laryngeal mucosa (ANOVA, $P = .01$), the progressive increase until the second week ($NGI_{2w} \times CONT$, $P < .01$) was followed by a reduction to control values at 5 weeks ($NGI_{2w} \times NGI_{5w}$, $P < .05$) (Fig. 7A). On the contrary, no changes were observed in mRNA laryngeal levels of COX-1 constitutive enzyme (ANOVA, $P = .27$) (Fig. 7B). Thus, the ratio COX-2/COX-1 mRNA expression was changed after NGI (ANOVA, $P < .05$), with an increase until the second week (NGI2w $\times$ CONT, $P < .05$), followed by a return to control values at the end of the experimental period (Fig. 7C).

Laryngeal Levels of Pro-Inflammatory and Anti-Inflammatory Cytokines

The NGI model induced drastic changes of inflammatory mediators. The relative expression of IL-1β mRNA changed along the experimental period (ANOVA, $P < .0001$), increasing continuously after 1 week ($NGI_{1w} \times CONT$, $P < .01$) and 2 weeks ($NGI_{2w} \times CONT$, $P < .001$; $NGI_{1w} \times NGI_{2w}$, $P < .01$) but returning to control values at 5 weeks NGI ($NGI_{2–5w}$, $P < .001$) (Fig. 8A). NGI affected the level of expression of TNF-α mRNA (ANOVA,

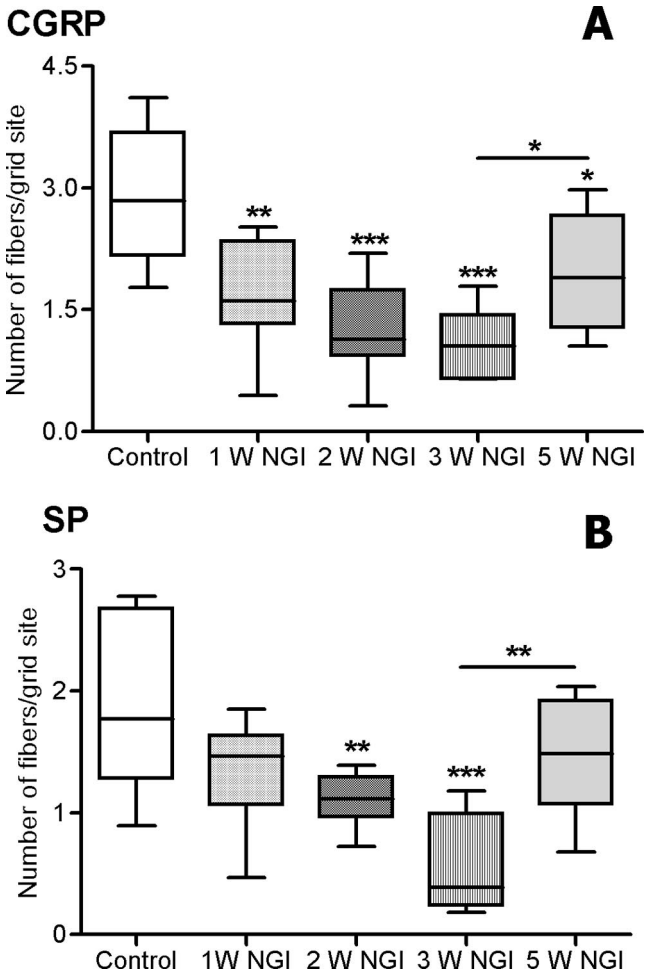

Fig. 4. Density of calcitonin gene-related peptide (CGRP)- (A) and substance P (SP)- (B) immunoreactive fibers in the laryngeal mucosa of animals without nasogastric tube (control group) and after different periods of nasogastric intubation (NGI). Note the significant decrease in the presence of these neurogenic peptides in laryngeal sensory fibers until the third week of NGI and the total (SP) or partial (CGRP) recovery observed at the end of the experimental period (5 wk). Asterisks over each week are comparisons with control values, whereas asterisks over each bar represent comparisons between values of groups submitted to NGI. *$P < .05$, **$P < .01$, and ***$P < .001$.

$P < .001$), which increased after 1 week ($NGI_{1w} \times CONT$, $P < .001$) and maintained a significant increased expression until the end of the experiment ($NGI_{2w} \times CONT$, $P < .01$; $NGI_{5w} \times CONT$, $P < .01$) (Fig. 8B). IL-6 mRNA changed significantly (ANOVA, $P < .0001$) in the laryngeal mucosa, showing an increase 1 week after NGI ($NGI_{1w} \times CONT$, $P < .001$) and then a progressive decrease until control values at 5 weeks ($NGI_{2w} \times CONT$, $P < .01$; $NGI_{1–5w}$, $P < .001$; $NGI_{2–5w}$, $P < .05$) (Fig. 8C). Changes in mRNA levels of the anti-inflammatory cytokine IL-10 (ANOVA, $P < .001$) showed a strong decrease 1 week after NGI ($NGI_{1w} \times CONT$, $P < .001$), which was still observed 5 weeks later ($NGI_{2W} \times CONT$, $P < .001$; $NGI_{5w} \times CONT$, $P < .001$) (Fig. 8D).

As concerns the analysis of the ratio between pro-inflammatory and anti-inflammatory cytokines, pro-inflammatory changes prevailed over anti-inflammatory

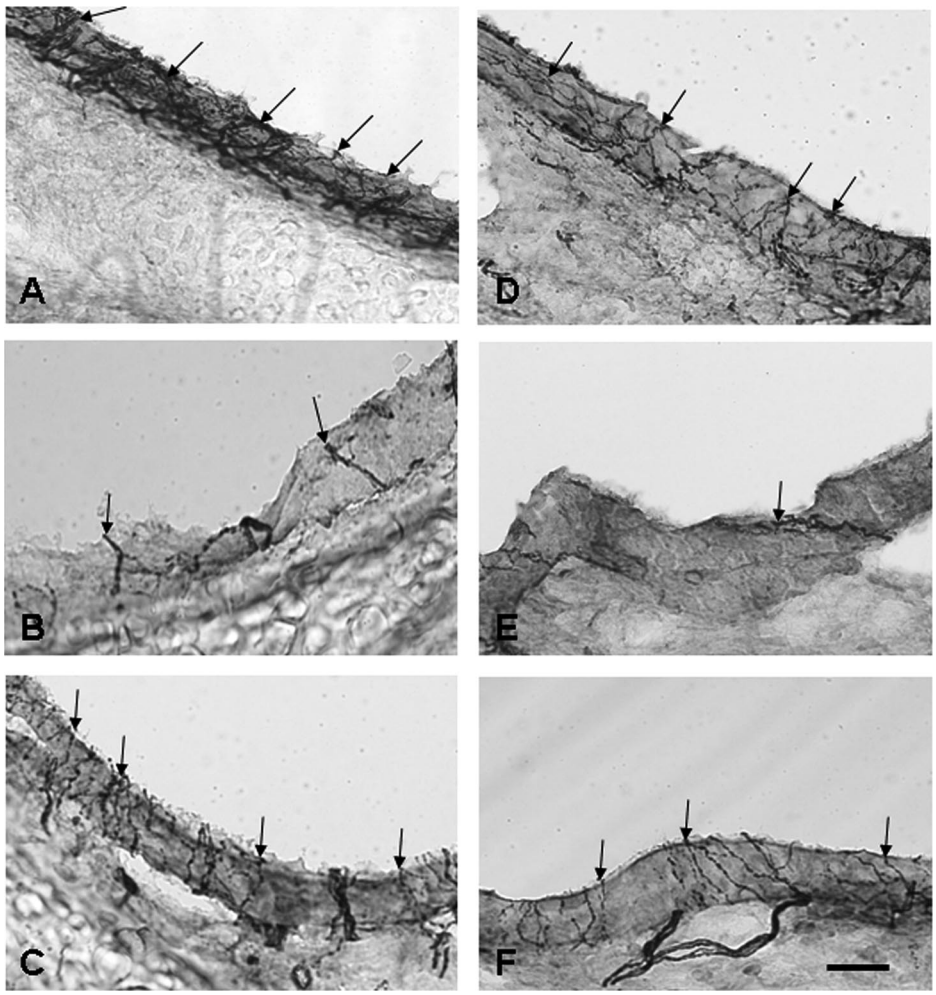

Fig. 5. Photomicrographs of calcitonin gene-related peptide (CGRP)- (A to C) and substance P (SP)- (D to F) immunoreactive fibers in the laryngeal epithelium of animals without nasogastric tube (controls, A and D) and after 3 (B and E) and 5 (C and F) weeks of nasogastric intubation (NGI). Note the strong decrease in CGRP and SP immunoreactive fibers (arrows) from nonintubation (A and D) to 3 weeks of laryngeal inflammation (B and E) and the recovery in the density of nociceptive primary afferent fibers containing CGRP and SP after 5 weeks of NGI (C and F). Magnification bar = 50 μm.

alterations. Accordingly, the ratio between IL-1β and IL-10 mRNA expression changed with time after NGI (ANOVA, $P < .0001$), with a progressive increase until the second week ($NGI_{1w} \times CONT$, $P < .05$; $NGI_{2w} \times CONT$, $P < .001$), followed by a return to control values (Fig. 8E). Interestingly, no changes were obtained for the ratio between TNF-α and IL-10 (Fig. 8F).

DISCUSSION

In the present study, we developed an NGI experimental model to induce laryngeal inflammation without direct mechanical injury to preserve the epithelium and lamina propria of the larynx. For model characterization, we showed that CGRP and SP present in nociceptive peripheral fibers decreased initially and then returned to control values. The inducible COX-2 enzyme shows the inverse pattern, increasing initially and then returning to baseline. However, the number of cells expressing COX-2 increased in inflammatory cells with increasing periods of NGI. Finally, IL-1β and IL-6 cytokines showed a pattern of mRNA expression along the 5 week NGI that was similar to COX-2 and inverse to CGRP and SP, whereas TNF-α was always increased and levels of IL-10 were always decreased along NGI.

Neurogenic inflammation is a well-defined process by which inflammation is triggered by the nervous system. As a pathway distinct from antigen-driven immune-mediated inflammation, it may play a key role in the understanding of a broad class of environmental health problems. It is a common pathway for disease in many organs and systems,[7–9] and a growing amount of evidence implicates the involvement of neurogenic etiology in disorders such as asthma, rhinitis, contact dermatitis, migraine headache, and rheumatoid arthritis.[10,11] In the respiratory tract, recent progresses in understanding the morphology of nerve fibers showed that intraepithelial nociceptors containing SP and CGRP neurogenic peptides project into the lumen of the larynx and can be directly stimulated by irritant substances.[4] The role of primary sensory neurons in arteriolar vasodilatation, increased vascular permeability, and leukocyte infiltration is mediated by peripheral release of peptides such as SP and CGRP from nerve terminals of depolarized peripheral nerve fibers.[12] SP binds to the neurokinin-1 receptor on target cells such as immune cells and vascular endothelial cells[11,13] and is the main mediator of vascular permeability and leukocyte infiltration, whereas CGRP is the prime mediator of neurogenic vasodilatation.[8,9] Moreover, SP

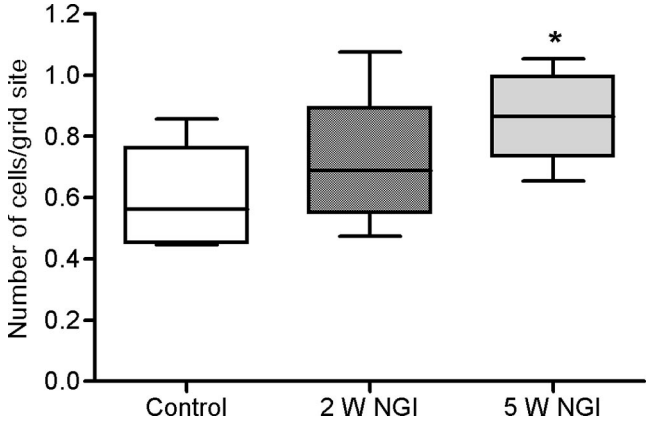

Fig. 6. Density of cyclooxygenase (COX)-2 immunoreactive cells in the laryngeal mucosa of animals without nasogastric tube and after 2 and 5 weeks of nasogastric intubation (NGI). Note the continuous increase in COX-2 containing cells in the laryngeal mucosa, which reaches significance at 5 weeks. *$P < .05$, **$P < .01$, and ***$P < .001$.

can increase production and release of PGE2[14] and release of IL-1.[15] Thus, the here observed initial decrease in CGRP and SP content in laryngeal sensory fibers (first 3 wk) probably resulted from the release of these peptides from mucosal nociceptors and induction of neurogenic inflammation. This is supported by the fact that PGE2, one important member of the E series of PGs, facilitates the release of neurogenic peptides SP and CGRP from primary sensory neurons.[16]

In regard to the partial recovery of CGRP and SP innervation between the third and fifth week of NGI, this may reflect a progressive refilling of laryngeal nociceptors with these peptides after their synthesis in perikarya of primary sensory neurons. Accordingly, the decreased expression of COX-2 mRNA to control values may account for a decrease in the release of SP and CGRP from primary afferents, which results in an increase of SP and CGRP immunoreactivity in peripheral fibers after 5 weeks NGI. On the other hand, there was an apparent disagreement between the continuous increase of COX-2 positive cells present in the laryngeal mucosa along the 5 weeks of NGI and the peak of COX-2 mRNA expression after 2 weeks NGI followed by a return to control values at 5 weeks. The progressive increase of the number of COX-2 immunoreactive cells until the fifth week of laryngeal inflammation should reflect not mRNA expression for de novo production of the enzyme but rather the recruitment of immune cells containing COX-2 to the inflamed larynx. Interestingly, prolonged over-production of PGs by COX-2 can increase SP and CGRP immunoreactivities in primary sensory neurons.[17] Thus, the increased number of COX-2 immunoreactive cells in the laryngeal mucosa after 5 weeks (but not 3 wk) of laryngeal inflammation may also contribute to the recovery of laryngeal SP and CGRP content after 5 weeks of NGI.

In the present study, the inflammation observed with NGI may have two origins. First is the presence of the nasogastric tube passing through the pharynx to the esophagus, which can indirectly have a mechanical effect

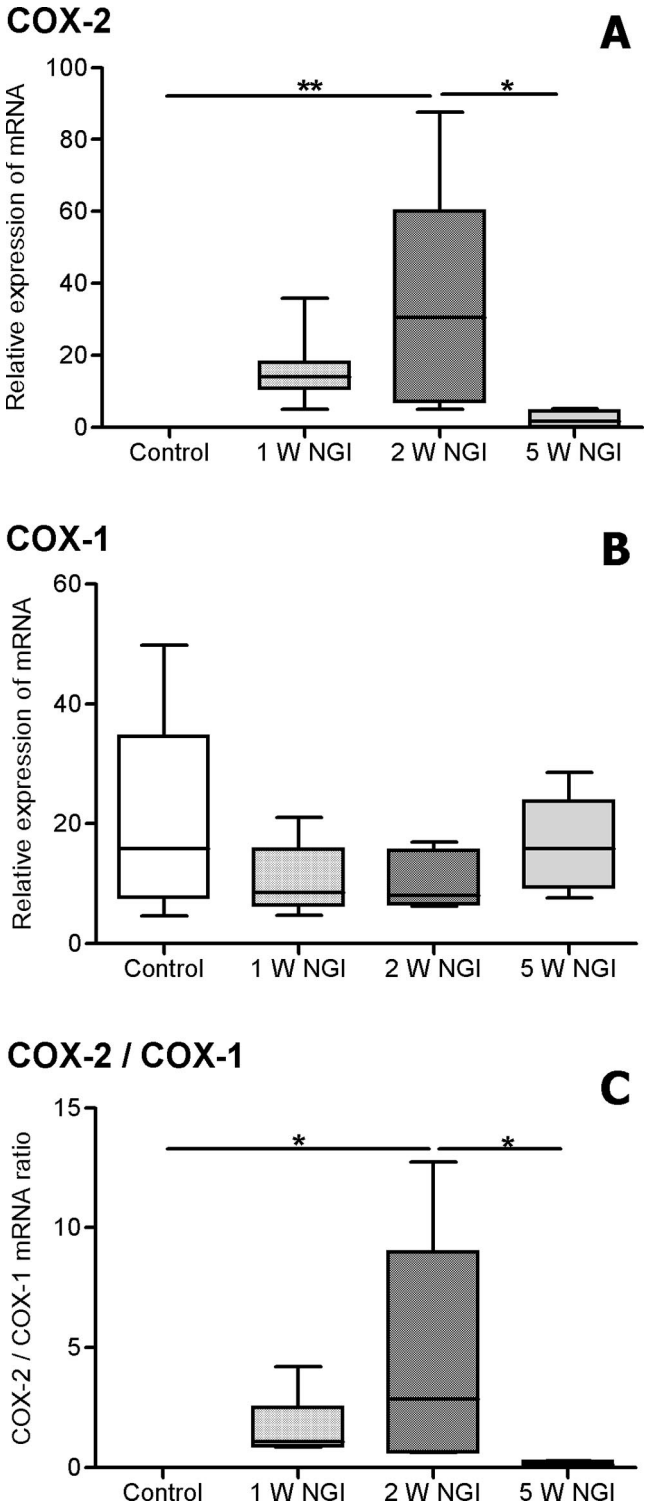

Fig. 7. Relative expression of cyclooxygenase (COX)-2 and COX-1 mRNA levels obtained by reverse-transcription polymerase chain reaction in the laryngeal mucosa of animals with or without induction of the nasogastric intubation (NGI) model. Note the rise of COX-2 mRNA expression until the second week and the return to basal values at the end of the experimental period (A). On the contrary, no changes were observed on the levels of the constitutive COX-1 enzyme (B). In regard to COX-2/COX-1 ratio of mRNA expression, the sequence of alterations was similar to that described for COX-2 (C). *$P < .05$, **$P < .01$, and ***$P < .001$.

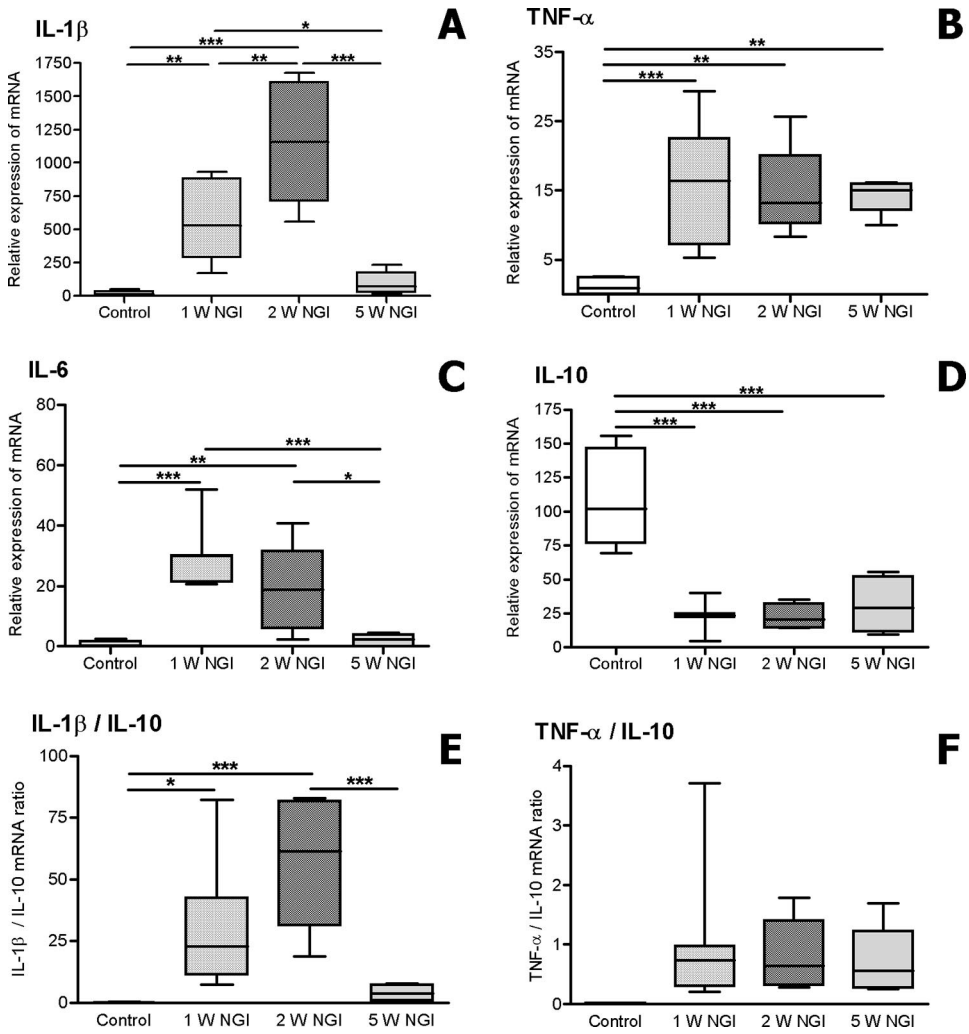

Fig. 8. Relative expression of mRNA levels obtained for pro-inflammatory and anti-inflammatory cytokines in the laryngeal mucosa of animals with or without induction of the laryngitis inflammatory model. Note the different evolution of expression of interleukin (IL)-1β (A), tumor necrosis factor (TNF)-α (B), IL-6 (C), and IL-10 (D). Although IL-1β and IL-6 showed a peak of expression (1–2 wk nasogastric intubation [NGI]) followed by a return to baseline values (5 wk NGI, A and C), TNF-α was increased along the entire period of NGI (B), and IL-10 was decreased along the 5 weeks of NGI (D). In regard to the IL-1β/IL-10 (E) and TNF-α/IL-10 (F) ratios of mRNA expression comparing pro- and anti-inflammatory cytokines, the sequence of alterations on the former was similar to that described for Il-1β, whereas the latter showed no alterations during experimental period. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

on the mucosa of the adjacent larynx. Second, the presence of acid from gastroesophagic reflux can directly activate nociceptors because H+ ions have receptors in the cell membrane of these fibers.[7] Accordingly, protons can induce CGRP release after activation of their receptors,[16] as observed here during the first weeks of NGI. The activation of nociceptors by noxious stimulation induces release of SP and CGRP, which increase blood vessel permeability and plasma extravasation.[7] Resident mast cells and other immune cells attracted to the inflamed laryngeal mucosa release cytokines and contribute to recruitment of more leukocytes and macrophages that release PGs and additional cytokines.[18] Cytokines IL-1β, IL-6, and TNF-α are well-known mediators of inflammation[18] because they cause PG release after production of arachidonic acid. Thus, cytokines form a link between tissue damage and inflammatory responses.[7] Binding of IL-1β to receptors on cell membrane initiates signaling cascades that upregulate transcription of genes such as COX-2, TNF-α, and IL-6. Moreover, IL-1β facilitates release of CGRP from nociceptors, probably by direct sensitization of nociceptors, whereas TNF-α sensitizes and induces ectopic activity in these primary afferent fibers.[18] These data strongly support the here observed matching opposite evolution of mRNA expression levels of IL-1β, IL-6, and COX-2 when compared with neurogenic peptides CGRP and SP present in nociceptive laryngeal fibers (decreasing during the first weeks and returning to control values at 5 wk). In regard to pro-inflammatory TNF-α and anti-inflammatory IL-10, their mRNA levels of expression in the laryngeal mucosa was, respectively, increased or decreased along the 5 weeks of NGI. This matching opposite evolution between the two cytokines is supported by other studies showing a similar close interaction.[18]

Although many functions have been suggested for COX-2, and the anti-inflammatory COX-2 inhibitors are known to have less aerodigestive adverse side effects,[16] the precise distribution and role of this enzyme is still unclear, and few studies on its role in chronic inflammation have been performed in the respiratory tract. In our study, the large increase in COX–2-immunoreactive inflammatory cells in the laryngeal mucosa after chronic NGI was noticed in mononuclear immune cells. This is clearly suggestive of the involvement of COX-2 in this form of laryngitis, and the location in mononuclear cells demonstrates a chronic inflammatory process. Selective COX-2 nonsteroidal anti-inflammatory drugs (NSAIDs) have less side effects than traditional (both antiCOX-1

and COX-2) NSAIDs on the digestive and respiratory systems because protective PGs do not decrease, whereas pro-inflammatory leukotrienes do not increase.[16] Accordingly, COX-1 inhibitors such as ibuprofen are effective in acute respiratory diseases but not in chronic pathologies because of their adverse side effects. The present model can be used in the future for evaluating the potential therapeutic value of selective COX-2 drugs in the treatment of chronic laryngitis.

CONCLUSIONS

A new animal model of chronic laryngitis was described, which results from NGI, a common technical procedure used in clinical practice. The resulting laryngitis develops an inflammatory process that presents a neurogenic component. COX-2 is also implicated in the inflammatory mechanism because its expression increases with time of intubation in mononuclear immune cells. Different patterns of mRNA expression of pro-inflammatory and anti-inflammatory mediators further characterized the inflammatory changes along time. This experimental NGI model of chronic laryngitis may be useful for future studies 1) in analyzing the molecular development of chronic inflammatory syndromes of the larynx, 2) in determining the clinical long-term consequences for chronic NGI patients, and 3) in evaluating the effect of new therapeutic approaches for clinical treatment of prolonged laryngitis.

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Capítulo 4.3

Publicação (III)

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"Role of Etoricoxib in the treatment of neurogenic laryngitis"

Artigo submetido

(2008)

ROLE OF ETORICOXIB IN THE TREATMENT OF NEUROGENIC LARYNGITIS

By

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Running title: Treatment of laryngitis with Etoricoxib

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ABSTRACT

Treatment of neurogenic laryngitis is unsatisfactory due to side effects of non-selective non-steroid anti-inflammatory drugs. Although specific cyclooxygenase-2 (COX-2) inhibitors are known to have less aerodigestive adverse side effects, there are no reports of their use in this pathology. Groups of rats were submitted to the nasogastric intubation model (NGI) of laryngitis during one and two weeks. One NGI group was submitted to a therapeutic trial with the specific COX-2 inhibitor Etoricoxib once daily (NGI+ETORI), another was submitted only to NGI by a daily administration of vehicle (NGI+VEHIC) and a third group was not intubated (CONT). Laryngeal sections were immunostained for analysis of the density of substance P (SP) and calcitonin gene-related peptide (CGRP) sensitive fibres and quantification of COX-2 positive cells through stereological analysis. Additionally, the expression of COX-2 and interleukins IL-1β, IL-6, IL-10 and tumour necrosis factor-α (TNF-α) were determined by Real Time RT-PCR.

In NGI+VEHIC animals, we observed a depletion of laryngeal epithelial SP- and CGRP- immunoreactive (IR) fibres, which was associated with decreased levels of IL-10 and with an over-expression of COX-2, IL-1β, IL-6, and TNF-α mRNA levels and COX-2-IR cells after 1 and 2 weeks of NGI. Additionally, histology revealed an increased number of mucosal inflammatory mononuclear cells and glandular hypertrophy and hyperplasia. Treatment with Etoricoxib during one week (NGI+ETORI) attenuated the CGRP-IR fibre depletion, the COX-2-IR increased cell number and TNF-α and COX-2 mRNA increased levels induced by NGI. Two weeks of treatment had no therapeutic effect. Data show that Etoricoxib is effective in neurogenic laryngitis for limited periods of administration and suggest that alternative treatments using specific COX-2 inhibitors may be considered in the future due to their reduced aerodigestive side effects.

INTRODUCTION

The aetiology of certain forms of laryngitis is unknown and therapeutics may fail due to the adverse side effects of the anti-inflammatory drugs and corticosteroids that are usually used (Warner et al, 2004). Neurogenic inflammation results from interaction between immune and nervous systems (Chrousos and Gold, 1992; Maier and Watkins, 1998; Black, 2002). Neuropeptides like substance P (SP) or calcitonin gene related peptide (CGRP) (Black, 2002; O'Conner et al, 2004), which are contained in peripheral sensitive nerve fibres, are involved in inflammatory actions induced by several stimuli (Weinstock, 1992; Foreman, 1987). Some studies showed a neurogenic component in some pathologies like asthma, rhinitis and rheumatoid arthritis (Richardson and Vasko, 2000). Recently, we demonstrated a neurogenic factor in a new model of experimental laryngitis (Lima-Rodrigues et al, 2007). The intraepithelial nerve fibres of the larynx are important for bronchopulmonar defence (Lima-Rodrigues et al, 2004). These fibres project into the laryngeal lumen (Lima-Rodrigues et al, 2004) and are rich in SP and CGRP (Hisa et al, 1985; Tanaka et al, 1993; Hisa et al, 1994). The release of these neuropeptides can induce neurogenic laryngitis as shown by the increased expression of inflammatory cytokines like interleukin-1β (IL-1β), IL-6 and tumour necrosis factor α (TNF-α), and the decreased expression of the anti-inflammatory cytokine IL-10 (Lima Rodrigues et al, 2007).

Nasogastric intubation (NGI) is largely used in clinical practice and can induce important respiratory disorders like aspiration pneumonia or laryngitis (Friedman and Baim, 1981; Gomes et al, 2003; Lima-Rodrigues et al, 2007). We previously demonstrated that NGI is a good model to study laryngitis in the rat since no direct lesion of the laryngeal epithelium is induced. Additionally, we observed an over-expression of COX-2 enzyme in the laryngeal mucosa (Lima-Rodrigues et al 2007), which represents the inducible form of COX enzyme following pathological stimulation in apposition to the constitutive COX-1 type (Warner and Mitchell, 2004). This indicates that selective COX-2 inhibitors may be useful in the treatment of respiratory pathology (Fitzgerald and Patrono, 2001), as in other pathologies the block of COX-2 activity by the administration of these non steroid anti-inflammatory drugs (NSAIDs) was shown to present an anti-inflammatory action (Emery

et al, 1999; Katory and Majima, 2000; McQuay and Moore, 2005).

Classic non-selective NSAIDs and corticosteroids used to treat laryngitis have well known aero-digestive adverse side effects when compared to the specific COX-2 inhibitors (Warner and Mitchell, 2004; Bruton et al, 2006; Poplawski and Sosnowski, 2006). Recent studies have demonstrated the utility and sensitivity of the latter drugs in throat pain (Shachtel et al, 2007) but, to the best of our knowledge, there are no experimental studies evaluating the use of selective COX-2 inhibitory NSAIDs in laryngitis. In order to analyse the potential effect of selective COX-2 inhibitors in neurogenic laryngitis, we studied the effect of Etoricoxib administrated from the beginning of nasogastric intubation upon the inflammatory process, through immunohistochemical techniques and molecular biology markers of inflammation, one and two weeks after NGI.

MATHERIAL AND METHODS

Animals

This study was performed in 60 male rats weighing 350–450 gr, obtained from the Wistar Han colony of Charles River Company (Barcelona, Spain). The experiments were carried out in accordance with National regulation for handling of laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Union Council Directive 86/609/EEC. Animals were lightly anesthetized with inhaled isoflurane in order to allow swallowing reflex, and submitted to NGI procedure (Lima-Rodrigues et al, 2007). A 10-12 cm small bore nasogastric aspiration tube used for NGI in premature newborns (cat. No 533.04; Vigon Laboratoires Pharmaceutiques, Ecouen, France) was inserted in the rat oesophagus until the stomach, with the external tip being sutured to the nasal lateral cartilages. Animals recovered and returned to their cages.

Animals were distributed along five groups, each one with 12 animals (n=6 for imunohistochemical processing; n=6, for molecular biology analysis):

- NGI+ETORI: submitted to NGI and treated with Etoricoxib since the first day of intubation during 1 week;
- NGI+ETORI: submitted to NGI and treated with Etoricoxib since the first day of intubation during 2 weeks;
- NGI+VEHIC: animals submitted to NGI were given only saline with 5% glucose for 1 week;
- NGI+VEHIC: animals submitted to NGI were given only saline with 5% glucose for 2 weeks;
- CONT: non-intubated rats were used as naïve control larynxes.

After completion of the intubation period, animals for immunohistochemical experiments were perfused under anaesthesia (isoflurane and 35% chloral hydrate, intraperitoneally) through the ascending aorta with 4% paraformaldehyde in PBS 0,01M. After confirmation that the NGI tube was still inserted until the stomach, the larynx was removed and immersed in the same fixative followed by 30% sucrose in 0.1 M PBS overnight. Animals used for Real Time RT-PCR studies were anesthetized with isoflurane and sacrificed with 35% chloral hydrate i.p.. Larynxes were excised immediately, macerated and immersed in 0,8 ml of TRIzol® (Invitrogen®, California, USA) and stored at -80ºC.

Drug therapy

Etoricoxib (Exxiv® 60 mg, Bial, Bial-Portela & Ca., S.A., Portugal) was suspended in 0.9% sterile saline with 5% glucose and administered to each animal in the dose of 6mg/Kg/day, by oral gavage, once a day.

Histology and Immunocytochemistry

Laryngeal coronal frozen sections (20 µm) obtained with a cryostat were processed for immunohistochemistry. Alternate sections were incubated overnight at room temperature with rabbit anti-CGRP (1:6000; Bachem, San Carlos, CA, USA) or rabbit anti-SP (1:6000; Bachem) antibodies in a PBS solution 0,1 M at pH 7,2 containing 0.3% triton X-100 (PBST). After washing with PBST, sections were then incubated with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA, USA) in PBST for 1 hour, washed in PBST and then incubated with avidin-biotin complex (ABC) (1:200; Vector Laboratories, Burlingame,

CA, USA) in PBS for 1 hour. After rinsing in PBS and 0.1 M tris-HCl buffer (pH 7.4) the antigen-antibody reaction was visualised with a diaminobenzidine (DAB) (Sigma Chemical Company, USA) solution.

COX-2 immunohistochemistry was carried out according to the streptavidin-biotin-peroxidase complex technique (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, USA), using a primary antibody raised against COX-2 protein (Rabbit monoclonal antibody, clone SP21; Neomarkers, Freemont, CA, USA) diluted 1:400. Slides were sequentially washed in PBST 0.02 % Tween 20 and incubated with 3% H_2O_2 in methanol for 10 min. This was followed by incubation with blocking solution for 10 min and then the primary antibody solution for 2 h, at room temperature. Sections were then sequentially washed in PBS/0.02% Tween 20 and then incubated with biotinylated goat anti-polyvalent antibody for 10 min and streptavidin peroxidase for 10 min. Immunoreaction was revealed using DAB as chromogen, as above. Slides were counterstained with Mayer haematoxylin (Merck, Darmstadt, Germany). Negative controls were performed by omission of the primary antibody.

Stereological procedures

Sections were analyzed in an Axioskop 2 plus light microscope and images of laryngeal histological data were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss, Germany). Cell and fibre numbers were estimated using the optical fractionator method (West et al, 1991). Briefly, this consists of virtual 3D-boxes (150µm x 150µm x 30µm) equally spaced grids that were superimposed by the software on every 8th coronal laryngeal section after drawing the limits of the area under study in the laryngeal mucosa. The number of COX-2 immunoreactive cells and SP and CGRP immunoreactive fibres that crossed the gridlines in every randomized site was counted. The estimated numbers were calculated from the ratio between the total number of counted cells and fibres crossing the grid site and the number of grid sites per area. The coefficients of error were automatically computed by the software according to the formulas of Gundersen for cell numbers (West et al, 1991; Gundersen et al, 1999).

mRNA extraction and RT-PCR

Total mRNA in the larynx was extracted by adding 160 µl $CHCl_3$ (Sigma-Aldrich, USA) followed by centrifugation at 13000 rpm for 15 min at 4°C. The supernatant was carefully collected and total mRNA was precipitated using iso-propanol (2-propanol, molecular biology, min. 99%; Sigma) followed by centrifugation at 13000 rpm for 15 min at 4°C. Ethanol 70% was used to wash the mRNA pellet, which was then, re-centrifuged at 9000 rpm for 5 min at 4°C. The supernatant was carefully discarded and the pellet allowed to dry at 4°C. Finally, the pellet was ressuspended with 50 µl of DNAse/RNAse free distilled water (Gibco, USA). Total mRNA was quantified by spectrophotometry using the NanoDrop® equipment (NanoDrop Technologies, Wilmington, USA). Subsequently, samples of total mRNA with the same concentration (2ng/10 µl) were reverse transcribed in a thermocyclator My Cycler Thermal Cycler® (Bio-Rad®, California, USA) using a Superscript Kit II (Invitrogen®, California, USA), and Oligo dT (Invitrogen®, California, USA). Reverse transcription was performed at 42°C for 60 minutes followed by RT inactivation at 70°C for 15 minutes. The cDNA was then subjected to Reverse Transcription Polymerase Chain Reaction (Real Time RT-PCR) procedures for quantification of laryngeal mRNA levels of β-Actin, IL-1β, TNF-α, IL-6, IL-10 and COX-2, using the LightCycler® (Roche®, USA) and a SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Primer sequences used to amplify various cDNAs were already determined and shown in a previous study of the group (Lima-Rodrigues et al, 2008). A typical RT-PCR protocol was performed under the following conditions: a 15 min hot start at 95°C, followed by 40 three-temperature cycles (94° C denaturing for 15 s; 58° C annealing for 20 s; and 72° C extension for 15 s), melting at 60° C until 95° C for 90 s and finally cooling to 35° C. Melting point analysis was used to confirm the specificity of the SYBR Green assays. Gene expression of the housekeeping gene β-Actin was used for normalization of the results.

Data analysis

Results are expressed after statistic analysis using Graph Pad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA). Means were compared using one-way ANOVA statistical evaluation

followed by Tukey HSD Post-hoc test, and differences were considered to be significant when $p < 0.05$.

RESULTS

Etoricoxib blocks the decrease of CGRP-IR fibers induced by one-week NGI

The experimental NGI model of laryngitis and therapeutical protocol using Etoricoxib induced different changes in the expression of neurogenic peptides at the end of both first and second week (ANOVA, CGRP $p<0,05$; SP $p<0,05$) **(Figs. 1-3)**.

After the first week, there was a significant decrease in the number of CGRP-IR **(Figs. 1A; 2A, B)** and SP-IR **(Fig. 1B; 2D, E)** fibres in NGI group, when compared with naïve animals (NGI+VEHIC$_{1w}$ x CONT, CGRP $p<0,001$; SP $p<0,001$, Tukey test). The one-week therapy with Etoricoxib reverted the decrease in the number of CGRP-IR fibres to control levels (NGI+ETORI$_{1w}$ x NGI+VEHIC$_{1w}$, $p<0,01$; NGI+ETORI$_{1w}$ x CONT, $p>0,05$, Tukey tests) **(Figs. 1A; 2C)**. However it did not affect the decrease of SP-IR fibres observed in the NGI plus vehicle group (NGI+ETORI$_{1w}$ x NGI+VEHIC$_{1w}$, $p>0,05$; NGI+ETORI$_{1w}$ x CONT, $p<0,05$, Tukey tests) **(Figs. 1B; 2F)**.

At the end of second week, there was a decrease in the number of CGRP-IR fibres either in NGI plus vehicle (NGI+VEHIC$_{2w}$ x CONT, $p<0,001$, Tukey test) **(Figs 1A; 3A, B)** or NGI animals treated with Etoricoxib (NGI+ETORI$_{2w}$ x CONT, $p<0,001$, Tukey test) **(Figs. 1A; 3A, C)**. Regarding SP-IR fibres, the same results were observed in both groups (NGI+VEHIC$_{2w}$ x CONT, $p<0,01$, Tukey test) **(Figs. 1B; 3D, E)** (NGI+ETORI$_{2w}$ x CONT, $p<0,001$, Tukey test) **(Figs. 1 B; 3D, F)**.

Etoricoxib blocks the increase of TNF-α mRNA expression induced by one-week NGI

The NGI experimental model induced significant changes in inflammatory mediators at the laryngeal mucosa. In what concerns TNF-α (ANOVA, $p<0,001$), mRNA levels increased significantly after one and two weeks of NGI (NGI+VEHIC$_{1w}$ x CONT, $p< 0,001$; NGI+VEHIC$_{2w}$x CONT, $p<0,001$; Tukey tests) **(Fig. 4)**. After an

one-week treatment with Etoricoxib (NGI+ETORI), the specific COX-2 inhibitor blocked the increase of TNF-α expression induced by NGI, reducing it to values similar to those obtained by controls (CONT) after one week of NGI (NGI+ETORI$_{1w}$ x NGI+VEHIC$_{1w}$, $p<0,001$; NGI+ETORI$_{1w}$ x CONT, $p>0,05$, Tukey tests). However no reduction of TNF-α values were observed after two weeks of treatment with Etoricoxib **(Fig. 4)**.

Concerning IL-1β expression (ANOVA, $p<0,01$), the treatment with Etoricoxib did not affect the level of expression after the first week, but even increased mRNA values (NGI+ETORI$_{2w}$ x CONT, $p<0,01$, Tukey test) after two weeks **(Fig. 5A)**. The IL-6 levels increased in the larynx of both groups of intubated rats (vehicle and Etoricoxib after one or two weeks) (ANOVA $p<0,001$) (NGI+ETORI$_{1w}$ x CONT, $p<0,05$; NGI+ETORI$_{2w}$ x CONT, $p<0,001$, Tukey tests) **(Fig. 5B)**. In the case of IL-10 (ANOVA, $p<0,05$), NGI induced increased expression after two weeks (NGI+VEHIC$_{2w}$ x CONT, $p<0,05$), which was reverted with the treatment with Etoricoxib (NGI+ETORI$_{2w}$ x NGI+VEHIC$_{2w}$, $p<0,05$, Tukey test) **(Fig. 5C)**.

Etoricoxib blocks the increase of COX-2-IR and mRNA expression induced by one-week NGI

Changes in the expression of COX-2 were detected through the increase in both the mRNA levels recorded by RT-PCR (ANOVA, $p<0,001$) **(Fig. 6A)** and in the number of COX-2 cells by immunohistochemistry (ANOVA, $p<0,01$) **(Fig. 6B; 7)**. In what concerns laryngeal mRNA levels of COX-2, no expression was detected in the CONT group. After one week of NGI, the values increased significantly (NGI+VEHIC$_{1w}$ x CONT, $p<0,05$, Tukey test), with the treatment with Etoricoxib being effective in the reduction of mRNA levels (NGI+ETORI$_{1w}$ x NGI+VEHIC$_{1w}$, $p<0,05$, Tukey test). After two weeks of treatment there was no reduction of the increased COX-2 mRNA levels observed in the NGI+VEHI group **(Fig. 6A)**.

Concerning the presence of laryngeal cells expressing COX-2, there was an increased number after the first week of NGI when compared to naïve animals (NGI+VEHIC$_{1w}$ x CONT, $p<0,01$, Tukey test) **(Fig. 7A,B)** which was also reverted at the end of first week of treatment with Etoricoxib **(Fig. 7B, C)**, but not after the second week (NGI+ETORI$_{1w}$ x NGI+VEHIC$_{1w}$, $p<0,001$; NGI+ETORI$_{2w}$ x NGI+VEHIC$_{2w}$, $p> 0,05$; Tukey tests) **(Fig. 6B; 7E, F).**

DISCUSSION

Classic anti-inflammatory drugs have aero-digestive adverse side effects due to their block of the constitutive COX-1 enzyme, contrary to specific cyclooxygenase-2 (COX-2) inhibitors, which block selectively the activity of the inducible COX-2 (Emery et al, 1999; Kiefer and Dannhardt, 2004). However, to the best of our knowledge, there are no indications in clinical practice to the use of the latter drugs in laryngitis, probably because the expression of COX-2 in the larynx and the potential therapeutic effects of these drugs have not been explored in detail. In clinics and in experimental studies, NGI induced laryngitis (Friedman et al, 1981; Lima-Rodrigues et al, 2007) and COX-2 over-expression was shown in inflammatory laryngeal cells (Lima-Rodrigues et al, 2007). In the present study, by showing decreased levels of COX-2 and TNF-α and reversion of depletion of the neurogenic CGRP peptide at the end of the first week, NGI animals treated with a specific COX-2 inhibitor revealed an anti-inflammatory effect during the first week of laryngitis.

Animals submitted to one week of nasogastric intubation (NGI+VEHIC$_{1w}$ group) showed increased COX-2, TNF-α and IL-6 mRNA levels, as well as an increased number of laryngeal cells immunoreactive to COX-2. These data are in accordance with studies including other inflammatory conditions, where these molecules are also significantly increased (Oprée and Kress, 2000; Slogoff et al, 2004). TNF-α and IL-6 induce COX-2 expression (Wendum et al, 2004) and are known to release the pro-inflammatory neuropeptides SP and CGRP (Kopp et al, 2000; Oprée and Kress, 2000) in different tissues, including the laryngeal mucosa (Lima Rodrigues et al, 2007). In the group submitted to NGI and treated simultaneously with Etoricoxib (NGI+ETORI$_{1w}$), COX-2 and TNF-α mRNA levels decreased, as well as the number of laryngeal cells immunoreactive to COX-2; this is indicative of an anti-inflammatory action of the COX-2 selective anti-inflammatory drug Etoricoxib in the larynx of intubated rats. However, no changes were observed in the IL-6, IL-1β and IL-10 mRNA levels. This could be conflicting data because Etoricoxib inhibits the κB nuclear factor (NF-κB) (Mack Strong et al, 2001; Pruthi et al, 2004; Kiefer and Dannhardt, 2004; Slogoff et al, 2004; Waes, 2007), an important mediator in many inflammatory conditions, and thus the levels of these interleukins were

expected to be altered. These results may be explained by the occurrence of alternative pathways of cytokine production to that of NF-κB, for example through alterations of the activity of cellular kinases such as IKKbeta, Erk, p38 MAPK, or Cdks (Fiebich et al, 2000; Tegeder et al, 2001). Another explanation may have been a counterbalanced production of interleukins, as other selective COX-2 inhibitors have the capacity to induce IL-6 production and not its reduction (Härtel et al; 2004; Cuzzocrea et al, 2002; Kiefer et al, 2004).

Concerning two weeks of NGI, comparatively to controls, there was an increased level of IL-6, TNF-α, IL-10 and COX-2 mRNA expression in the NGI+$VEHI_{2w}$ group, confirming the presence of a marked inflammatory process. The higher levels of the anti-inflammatory interleukin IL-10 can be viewed as an attempt of the immunologic system in order to auto-regulate the inflammatory process (Moore et al, 2001). Changes in mRNA expression and density of COX-2 cells are not equivalent because the area of inflammatory mucosa increased along the time of intubation and those are normalized values. Along the time of intubation the oedema of mucosa increases and although the absolute number of COX-2 positive cells increases, in the stereological analysis these values are diluted by the augmenting oedema. The treatment with Etoricoxib after two weeks of intubation(NGI+$ETORI_{2w}$) induced an increase of IL-1β and IL-6 mRNA levels and a decrease of IL-10 expression that did not occur in the NGI+$VEHIC_{2w}$ group. The increased levels of IL-10 mRNA in the NGI+VEHI group can be an immunologic anti-inflammatory response (Kourea et al, 2007; Toebak et al, 2007) that is aborted by Etoricoxib, whereas the increase of IL-6 can be explained by the capacity of COX-2 inhibitors to induce IL-6 production (Härtel et al; 2004; Cuzzocrea et al, 2002; Kiefer et al, 2004). It is possible that COX-2 inhibitors can also induce IL-1β production by cellular kinases as well as IL-6 but, to the best of our knowledge, there are no experimental data supporting this hypothesis. Concerning the TNF-α and COX-2 expression, which were decreased by Etoricoxib after one week of NGI, the values obtained following two weeks of treatment were similar to the NGI group, which seems to indicate a loss of therapeutic effect of the drug after long periods of administration (Katory and Majima, 2000; Tegeder et al, 2001).

Concerning neuropeptides, in this model of laryngitis Etoricoxib attenuated the depletion of CGRP but not SP at the end of the first week of NGI. In our previous studies, we found that SP-IR fibres were fewer in

number than CGRP-IR fibres in the larynx. This finding suggests that CGRP is the most important peptide that occurs in the laryngeal epithelium (Lima-Rodrigues et al, 2004; 2008). Accordingly, the most important neurogenic anti-inflammatory action of COX-2 inhibitors, was shown in the present work to occur through a CGRP-mediated mechanism. CGRP receptors are co-localized with prostaglandin receptor (EP4 receptor-like immunoreactivity) at thin nerve terminals and Prostaglandin E2 (PGE2) enhances CGRP release (Goodis et al, 2000). It is known that activation of mechanosensory nerves by COX-2 results from increasing levels of Prostaglandin E2 (PGE2) (Kopp et al, 2000). Thus, in the larynx, COX-2 inhibitors may block predominantly PGE2 production and CGRP depletion. At the end of the second week the drug becomes ineffective in avoiding CGRP (and SP) depletion, which is in accordance with the lost of anti-inflammatory effect seen already in terms of cytokine and COX-2 expression.

After this experimental therapeutic trial, we conclude that NGI induces neurogenic laryngeal inflammation through the release of neuropeptides SP and CGRP by laryngeal sensitive fibres and, simultaneously, the overexpression of other inflammatory markers. The attenuation of laryngitis induced by the selective COX-2 inhibitor Etoricoxib at first week of treatment indicates that this drug can be administrated during limited periods of time. Moreover, data suggest that Etoricoxib should be evaluated in the future as an alternative to the treatment of laryngitis due to reduced side effects comparatively to non-selective NSAIDs currently used in clinical practice. In support to this hypothesis, recent clinical studies demonstrate efficacy of other specific COX-2 inhibitor (Valdecoxib) in a throat pain clinical model of tonsillo-pharyngitis and no significant adverse side effects were observed (Schachtel et al, 2007).

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FIGURE LEGENDS

Figure 1- Effect of Etoricoxib treatment upon CGRP-IR **(A)** and SP-IR **(B)** fibre immunoreactivity after one and two weeks of NGI. Note that the decrease in CGRP-IR fibres was reverted by the treatment with Etoricoxib after the first, but not after the second week of NGI. The decrease induced in SP-IR fibres was not reverted by the treatment. *p<0,05; **p<0,01; ***p<0,001

Figure 2- Photomicrographs of CGRP-IR **(A, C)** and SP-IR **(D-F)** sensitive fibres in the laryngeal mucosa in naïve (control) animals **(A, D)**and following one week of NGI plus vehicle **(B, E)** and NGI plus treatment with the selective COX-2 inhibitor Etoricoxib **(C, F)**. It is clear the reduction in the presence of CGRP and SP immunoreactive fibres in the mucosa induced by NGI **(B-E)** and the recovery of CGRP-IR to control levels allowed by the treatment with Etoricoxib **(C)**. Arrows-fibres

Figure 3- Photomicrographs of CGRP-IR **(A, C)** and SP-IR **(D-F)** laryngeal fibres in control **(A, D)** animals and following two weeks of NGI plus vehicle **(B, E)** and NGI plus Etoricoxib treatment **(C, F).** Note, that in this case the treatment with selective COX-2 inhibitor did not revert **(C, F)** the reduction of laryngeal neuropeptidergic fibres induced by nasogastric intubation **(B, E).** Arrows-fibres

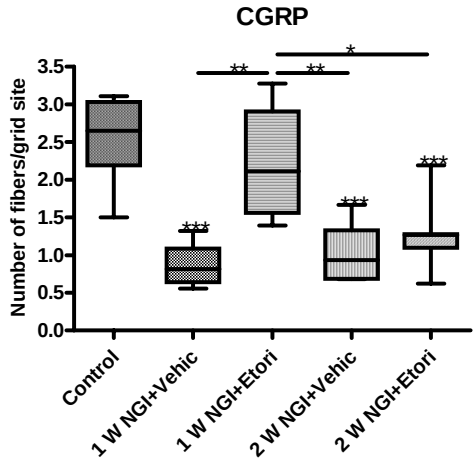
Figure 4- Effect of Etoricoxib treatment upon the relative mRNA expression of TNF-α after one and two weeks of NGI. The selective COX-2 inhibition reverted to the control levels the increase in TNF-α expression induced by NGI after one week of treatment, but not after two weeks. *p<0,05, **p<0,001; ***p<0,001

Figure 5- Effect of Etoricoxib treatment upon the relative mRNA expression of IL-1β **(A)**, IL-6 **(B)** and IL-10 **(C)** in the laryngeal mucosa, after one and two weeks of NGI. Animals treated with Etoricoxib showed higher levels of interleukins than NGI alone after two weeks of nasogastric intubation. *p<0,05, **p<0,001; ***p<0,001

Figure 6- Effect of Etoricoxib treatment upon laryngeal inflammation measured by COX-2 levels of mRNA **(A)** and cells **(B)** present in the mucosa after one and two weeks of NGI. Note that the drug was effective only at the first week by reducing both the levels of mRNA and the number of COX-2-IR cells to control levels. *p<0,05, **p<0,001; ***p<0,001

Figure 7- COX-2 immunoreactive (IR) cells in the laryngeal mucosa in naïve (control) animals **(A, D)** and following one week of NGI plus vehicle **(B, E)** and NGI plus treatment with the selective COX-2 inhibitor Etoricoxib **(C, F)**. The reduction of COX-2-IR cells in the mucosa induced by NGI **(B-E)** after one week **(C)** is more evident than after two weeks **(F)** of treatment with Etoricoxib. *Arrows* - COX-2-IR cells.

A


CGRP
Number of fibers/grid site
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
***
***
***
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

B

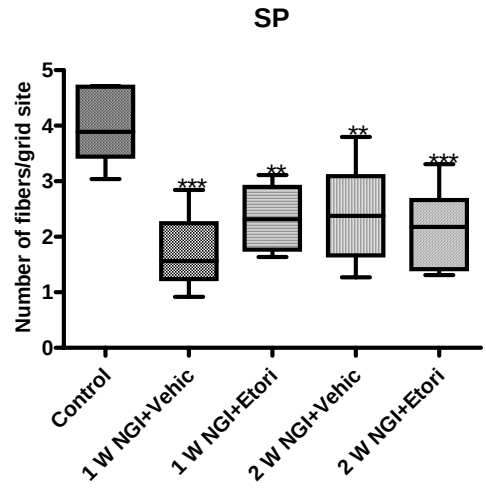

SP
Number of fibers/grid site
5
4
3
2
1
0
***
**
**
***
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

FIGURE 1

One Week

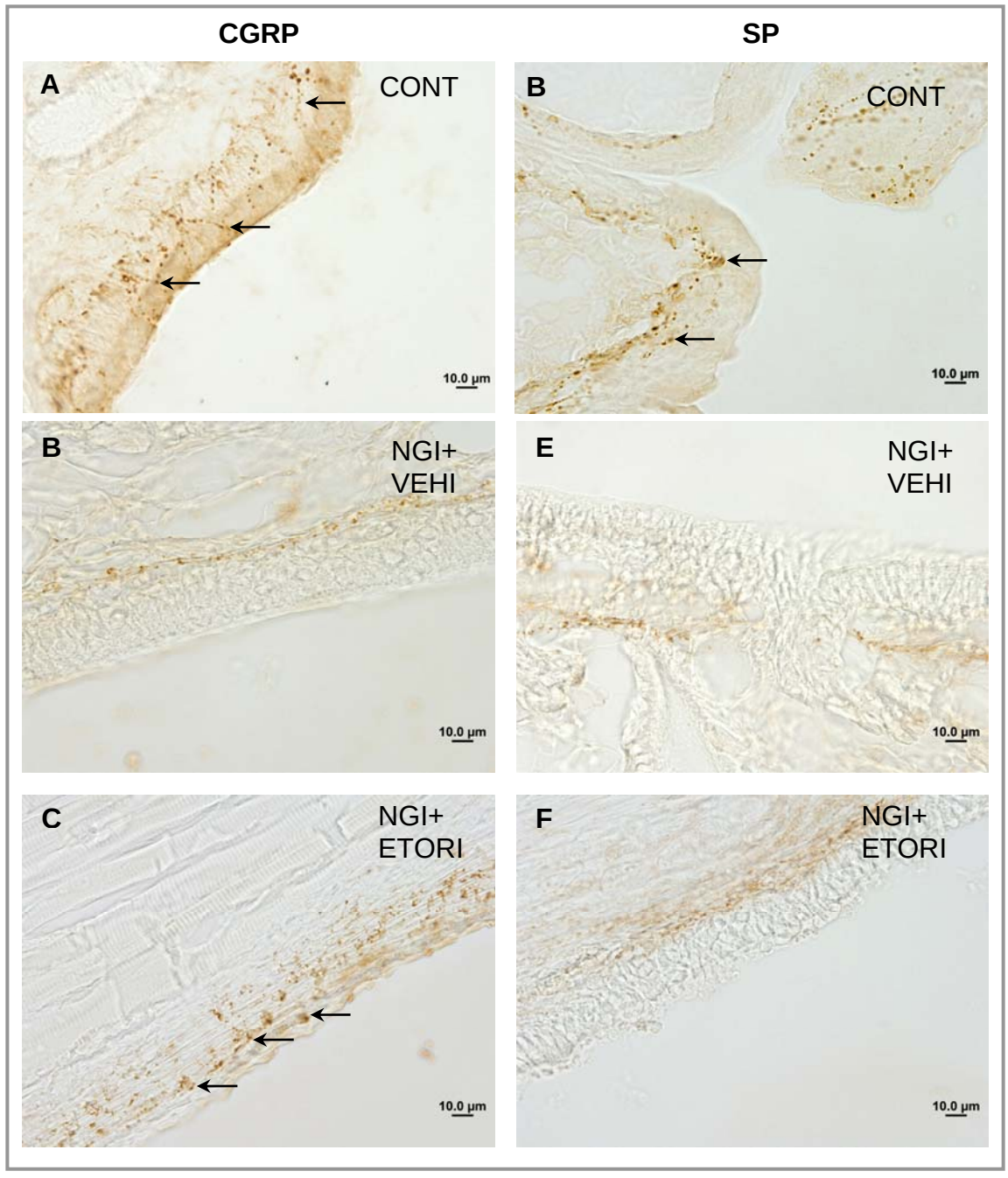


FIGURE 2

Two Weeks

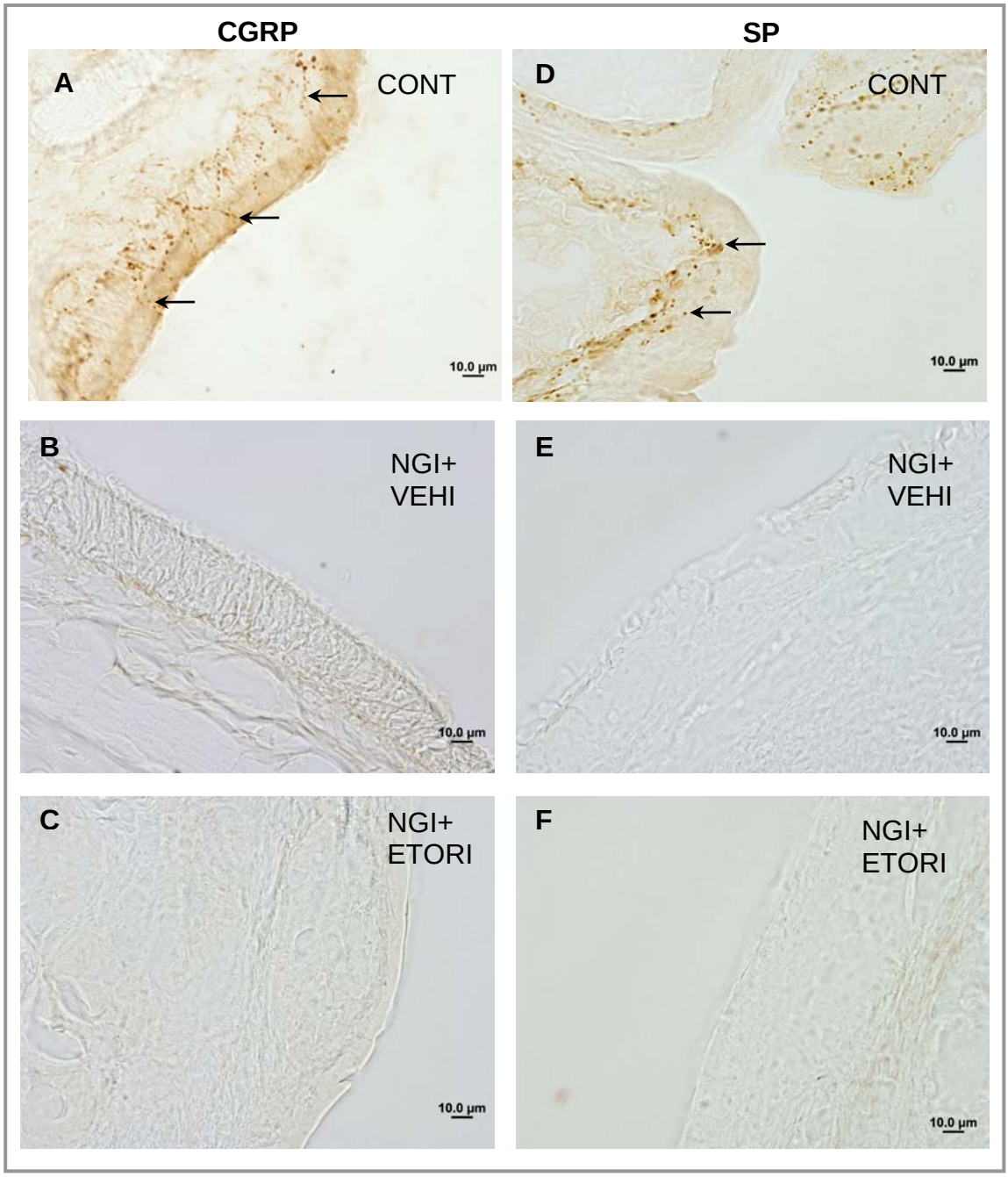


FIGURE 3

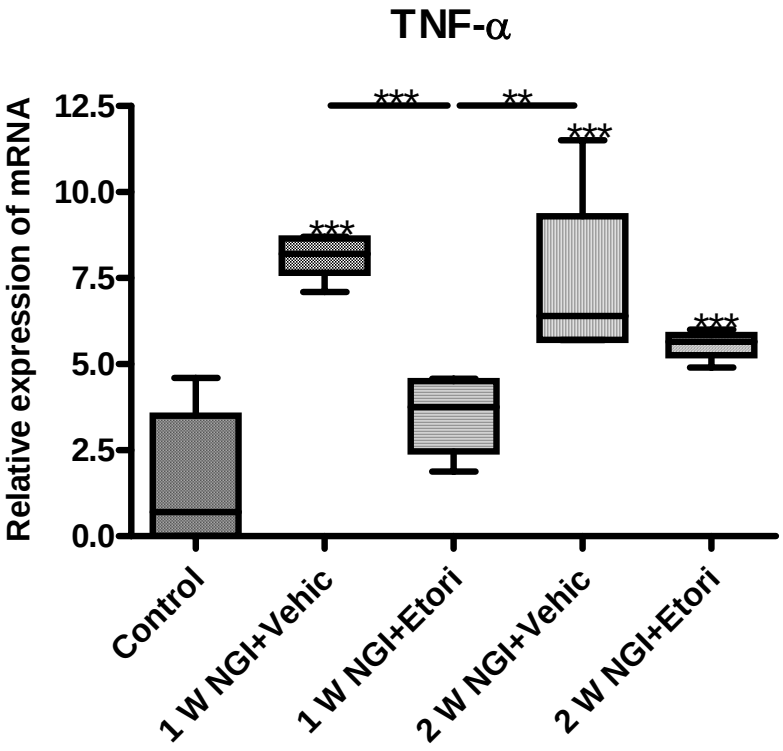
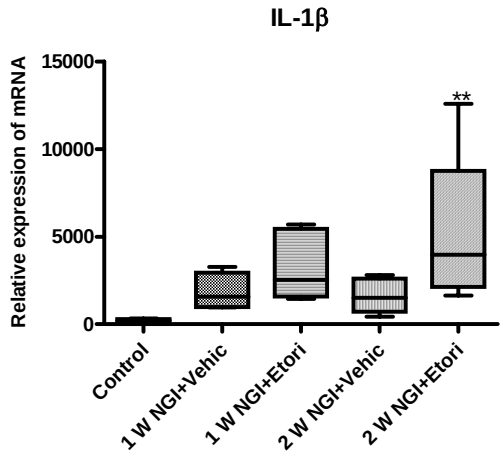
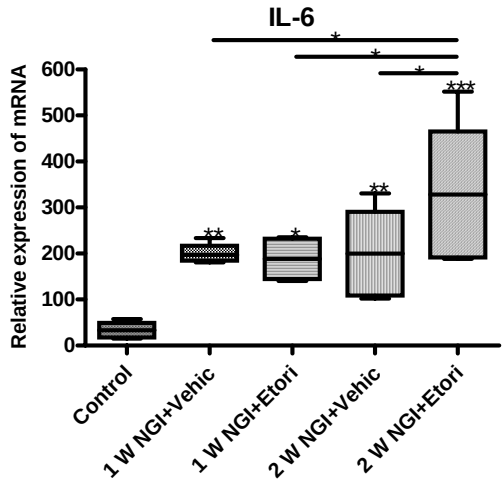

TNF-α
Relative expression of mRNA
12.5
10.0
7.5
5.0
2.5
0.0
***
**
***
***
***
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

FIGURE 4

A


IL-1β
Relative expression of mRNA
15000
10000
5000
0
**
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

B


IL-6
*
*
*
***
Relative expression of mRNA
600
500
400
300
200
100
0
**
*
**
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

C

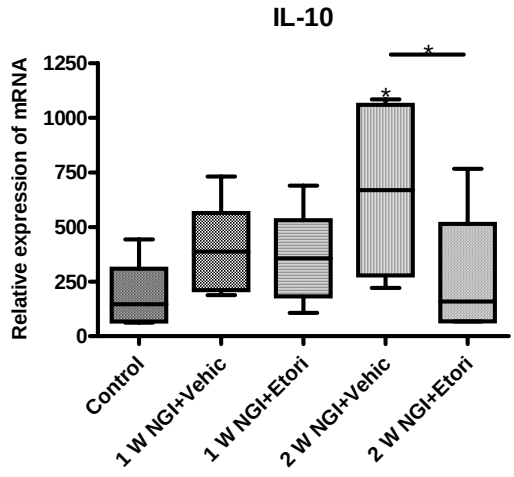
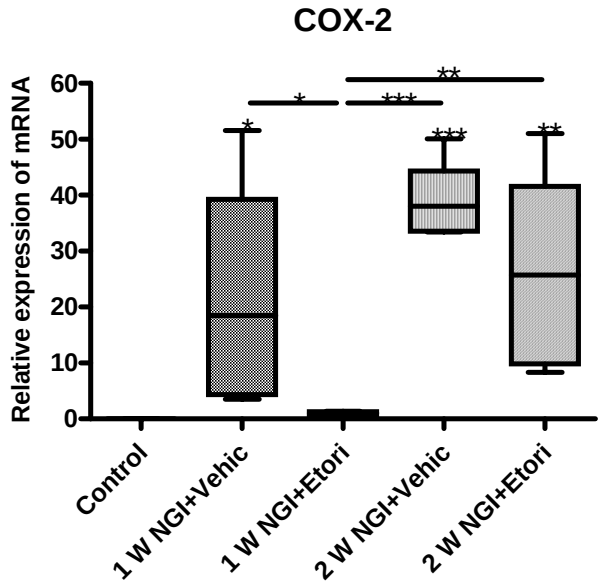

IL-10
*
*
Relative expression of mRNA
1250
1000
750
500
250
0
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

FIGURE 5

A


COX-2
Relative expression of mRNA
60
50
40
30
20
10
0
*
**
***
Control
1 W NGI+Vehic
1 W NGI+Etori
2 W NGI+Vehic
2 W NGI+Etori

B

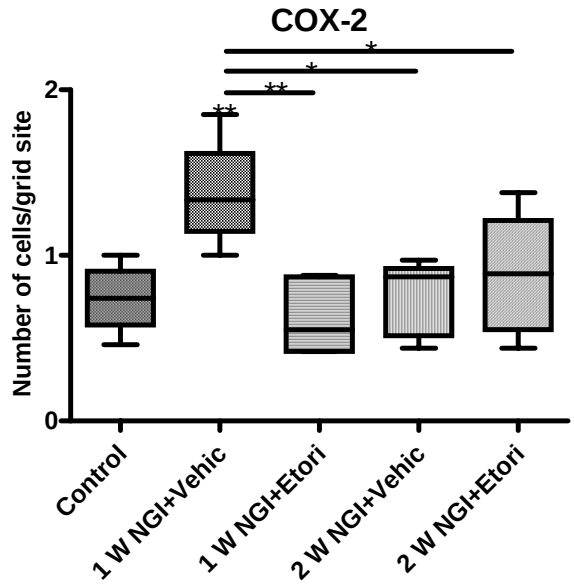

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FIGURE 6

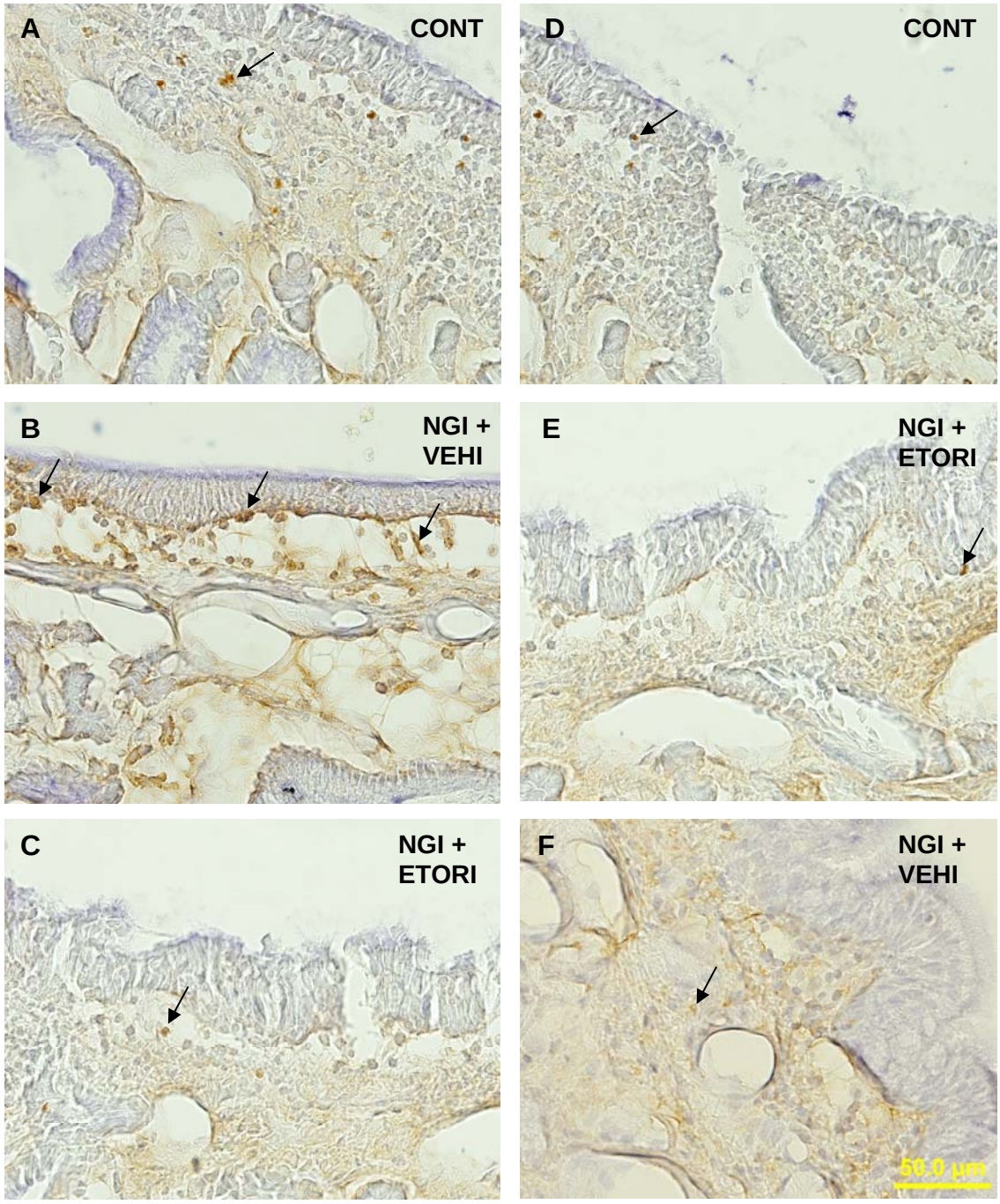

FIGURE 7

Capítulo 4.4

Publicação (IV)

Lima-Rodrigues M, Vieira A, Lamas N, Valle-Fernandes A, Cruz A, Baltazar F, Castro AG, Reis RM, Almeida A.

"Neurogenic laryngitis results in decreased expression of tumoral suppressors"

Abstract – Soc Neurosci Abstr (2008), in press

(2008)

NEUROGENIC LARYNGITIS RESULTS IN DECREASED EXPRESSION OF TUMORAL SUPPRESSORS

Lima-Rodrigues M, Vieira A, Lamas N, Valle- Fernandes A, Cruz A, Baltazar F, Castro AG, Reis RM, Almeida A

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p16 and p53 are two important tumoral suppressor molecules involved in the process of laryngeal tumoral transformation. Genetic changes at the sites of these two important tumour suppressor molecules are detected in pre-malignant laryngeal lesions and are used as potential markers for cancer risk assessment. We have developed recently an animal model of neurogenic laryngitis induced by nasogastric intubation (NGI)[1]. Here we evaluated the hypothesis of a possible correlation between chronic inflammation and the induction of premalignant lesions of the laryngeal mucosa by analyzing the expression of the mRNA of p16 and p53 tumoral markers in the larynx.

Groups of rats submitted to one, two and five weeks of NGI were compared with a control group (not intubated). Alterations in p16 and p53 were evaluated by quantitative real time RT-PCR in larynxes removed from these animals after sacrifice with a lethal dose of anaesthesia.

Concerning mRNA expression levels of these tumoral suppressors, p16 was decreased after 5 weeks of NGI whereas p53 decreased already after one week NGI and remained decreased during the 5 weeks of intubation.

Increasing periods of neurogenic laryngitis induced by NGI results in a decrease of mRNA levels of p16 and p53 tumoral suppressors in the laryngeal mucosa. Data suggest that chronic laryngitis may result in an increased risk of developing precancerous conditions and, eventually, laryngeal cancer.

Study supported by Calouste Gulbenkian Foundation Project nº 74551.

1. Lima-Rodrigues M et al. A New Model of Laryngitis: neuropeptide, COX and cytokine profile. Laryngoscope, 118:78-86 (2008).

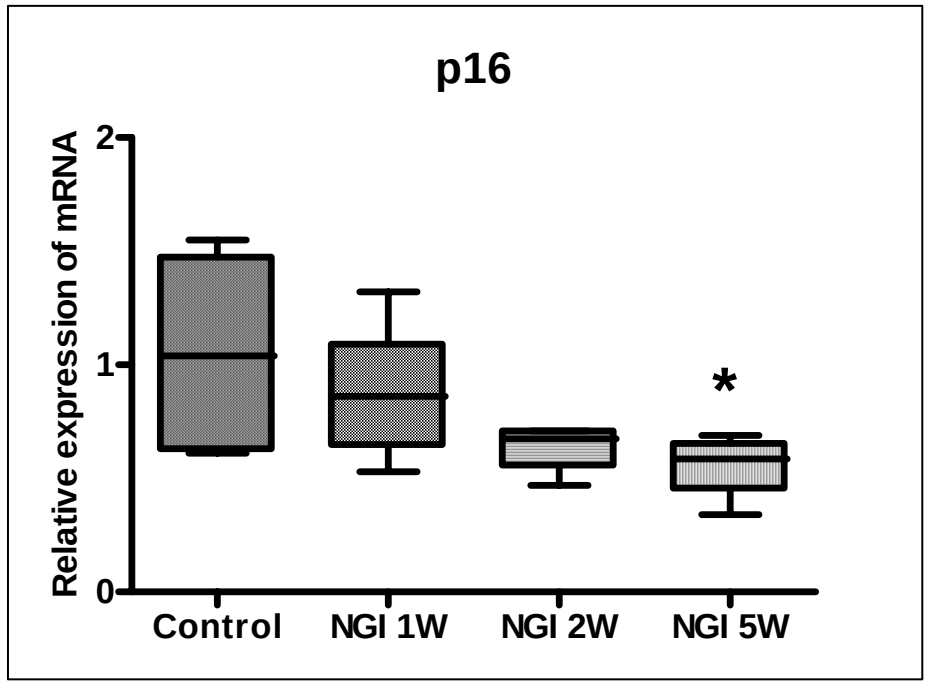

Figure 1- p16 relative expression of mRNA levels in the larynx of controls and animals submitted to different periods (W-weeks) of nasogastric intubation (NGI). Note the continuous decrease of p16 expression, reaching a significant difference from controls after 5 weeks of laryngitis. *p<0,05.

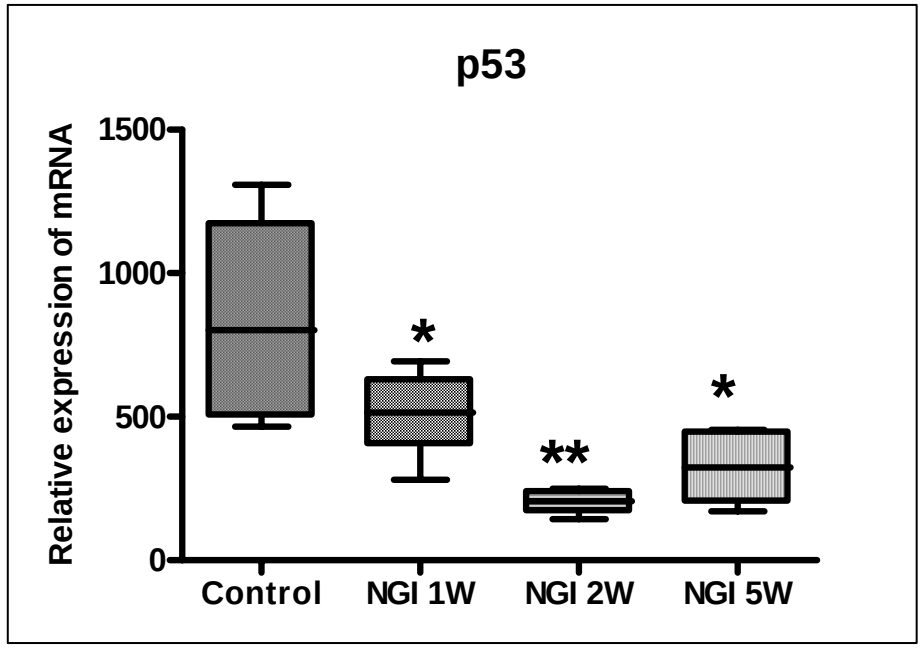

Figure 2- p53 relative expression of mRNA levels in the larynx of controls and animals submitted to different periods (W-weeks) of nasogastric intubation (NGI). Note de decrease of p16 expression in all the three groups of intubated rats, although being more evident after two weeks of NGI. *p<0,05; **p<0,01

Capítulo 4.5

Publicação (V)

Lima-Rodrigues M, Valle-Fernandes A, Nunes R, & Almeida A.

"Distribution of neuromuscular junctions in laryngeal and syringeal muscles in vertebrates"

Anatomical Record, 288A: 543-551

(2006)

THE ANATOMICAL RECORD PART A 288A:543–551 (2006)

Distribution of Neuromuscular Junctions in Laryngeal and Syringeal Muscles in Vertebrates

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ABSTRACT

Vertebrates are capable of producing a variable sound spectrum. In mammals, lissamphibia, and reptiles, the larynx is the vocal organ responsible for sound production, whereas in birds it is produced by the syrinx, an avian organ located at the base of trachea. The distribution of neuromuscular junctions responsible for the fine control of laryngeal muscle (LM) and syringeal muscle (SM), although studied with some detail in human LM, remains mostly unknown in other vertebrates. In the present study, we analyzed the distribution of motor end plates (MEPs) in LM/SM of different vertebrate classes using the histochemical detection of acetylcholinesterase: the thyroarytenoid and cricoarytenoid LM of mammal (human, rat, and rabbit) and cricoarytenoid LM of nonmammalian (frog and avian) species and the tracheobronchial SM of rooster and pigeon. In humans and frogs/avians, MEPs were distributed diffusely along, respectively, the thyroarytenoid-cricoarytenoid and the cricoarytenoid LM fibers, whereas in rats and rabbits, MEPs were concentrated in a transverse band located in the middle of thyroarytenoid and cricoarytenoid muscle fibers. In roosters and pigeons, MEPs were distributed diffusely along SM fibers. The highly diffuse MEP distribution along human thyroarytenoid and cricoarytenoid fibers indicates that these muscles can markedly change their degree of contraction, which may contribute for the large range of different sounds produced by human vocal folds. The same rationale was applied to discuss the possible functional significance of the morphological distribution of MEPs along the LM/SM of the other vertebrates analyzed. Anat Rec Part A 288A:543–551, © 2006 Wiley-Liss, Inc.

Key Words: motor end plates distribution; syringeal muscle; thyroarytenoid muscle; cricoarytenoid muscle; vertebrates

It is well known that vocalization varies significantly among vertebrates (Kardong, 2002). Although most aspects of vocal production are essentially similar between the vocal tracts of humans and other animals, a few key differences underlie vocal specificity along vertebrates: the importance of resonance capacity of the higher portion of the vocal tract, the position of the larynx in the throat, the capacity of vocal imitation, and the sophistication of nervous motor control over vocal articulates (Fitch, 2000; Fitch and Hauser, 2002). Even between mammals, two gross morphological differences are particularly prominent in nonhuman mammals and do not exist in humans: air sacs and vocal membranes. The former are present in bats and primates, whereas the latter are present in several primates, including apes (Mergell et al., 1999). Moreover, the organ responsible for sound production is not the same along vertebrates with the larynx and vocal folds being responsible for sound production in mammals, reptiles, and lissamphibians (Kardong, 2002), whereas in birds, this role is played by a special subtracheal structure, the syrinx. Finally, the thyroid cartilage is present in the larynx of mammals but is absent in the other verte-

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brate classes. This fact implicates that the role of the thyroarytenoid laryngeal muscle in phonation and as anatomical glottal sphincter in mammals is played by the cricoarytenoid muscle in lissamphibia (phonation and anatomical sphincter) and avians (just as glottal sphincter) (George and Berger, 1966; Storer et al., 1979; Kardong, 2002).

In anuran lissamphibians, vocal communication is crucial in their social behavior and thus they can have a complex vocalization pattern, mainly in males (Storer et al., 1979; Boyd et al., 1999; Kelley, 2004). The elaborated song production in songbirds is thought to parallel human speech in several aspects, namely, dependence on learning (Marler, 1970), gradual motor development (Marler and Peters, 1982; Podos et al., 1995), lateralized brain specialization areas for production and perception (Nottebohm, 1971; Vicario, 1993; Wild, 1993), and importance of vocal tract movements in many aspects of song production (Hoese et al., 2000). In contrast, very little is known about reptile vocalization (Young et al., 1995; Hartdegen et al., 2001; Sacchi et al., 2004), and little or nothing is known about vocal production in most nonpasserine birds and most mammalian orders (Fitch and Hauser, 2002).

The understanding of animal vocal production and its motor innervation is still largely unknown (Fitch, 2000). However, taking into account that the contraction of muscle fibers is mediated by motor units and their neuromuscular junctions, it is possible that the degree of vocal variability depends, at least in part, on the number and distribution of motor end plates (MEPs) along laryngeal muscle (LM) and syringeal muscle (SM). However, very few studies have focused on the fine anatomy of motor units in vocal muscles. In what concerns mammals other than humans, only two other studies analyzed the distribution of the motor innervation of the rat larynx (Pais-Clemente and Lima-Rodrigues, 1996; Inagi et al., 1998). To the best of our knowledge, no studies have been performed on the anatomy of laryngeal/syringeal fine muscle motor control in other mammals and other vertebrate taxa, including lissamphibia and birds.

In humans, the lateral cricoarytenoid and thyroarytenoid muscles are very important in sound production since they are essential in closing the glottis (by rotating the arytenoid cartilages medially) and in pitch control (Greene, 1989; Williams et al., 1999). The increasing clinical importance of botulinum toxin therapy to block thyroarytenoid and crycoarytenoid MEP in laryngeal distonia (Blitzer et al., 1986; Castellanos et al., 1994; Bielamowicz et al., 2002; Tisch et al., 2003; Maronian et al., 2004) requires a deeper knowledge of human laryngeal motor innervation in order to better understand the nature of this disease. However, the pattern of motor innervation of the thyroarytenoid and crycoarytenoid muscles is still a matter of discussion. MEPs distributed diffusely along LM with no recognized band or any cluster arrangement (Rosen et al., 1983; Périé et al., 1997), covering two-thirds of the vocal folds (Rossi and Cortesina, 1965a, 1965b), or with a clear higher density in LM middle third (Pais-Clemente and Lima-Rodrigues, 1996; Sheppert et al., 2003) have been described.

Taking into account the relevant role of thyroarytenoid muscles in mammals (phonation and glottal sphincter), the cricoarytenoid muscles in mammals (phonation), lissamphibia (phonation and glottal sphincter), and avians (glottal sphincter) and of the syringeal muscles in avians (phonation), we evaluate the pattern of fine motor innervation of these muscles in vertebrates. The present study analyzes the general distribution and morphology of MEPs in the thyroarytenoid and/or cricoarytenoid LM of three mammalian (human, rat, and rabbit), two avian (rooster and pigeon), and one lissamphibian (frog) species and in the tracheobronchial SM of the rooster and pigeon.

MATERIALS AND METHODS

Six laryngeal thyroarytenoid muscles from male adult rat (Wistar strain, obtained from Charles Rivers, Barcelona, Spain), rabbit (*Oryctolagus cuniculus*), and frog (*Rana perezi*) larynxes, six cricoarytenoid muscles from the rat, rabbit, frog, rooster (*Gallus gallus*), and male pigeon (*Columba livia*) larynxes, and six syringeal (bronchotracheal) muscles from the rooster and pigeon syrinxes were obtained after anesthetizing the animals with ether. Human vocal folds were obtained from six autopsy specimens. LM and SM were removed and immediately immersed in buffered 10% formalin, at pH 7.4, for 24 hr at room temperature. In order to obtain serial longitudinal sections of the muscle fibers, LM and SM were oriented appropriately and cut into 50 μm sections in a cryostat.

For identification of MEPs, we performed a histochemical detection of acetylcholinesterase activity by adapting the method described by Koelle and Friedenwald (1949). Briefly, sections were incubated in Koelle's medium for 2 hr with final staining in a 5% ammonium sulfide solution for 15 min. Sections were then placed in polylisine slides and mounted in entellan. The maintenance of proper pH of the reaction mixture and the addition of a selective pseudocholinesterase inhibitor (Iso-OMPA), combined with control sections where the reaction was performed without substrate (acetyltiocholine iodide), allowed the identification of a specific staining for acetylcholinesterase activity. All LM and SM serial sections were then analyzed in a light microscope Axioskop 2 plus (Carl Zeiss, Germany) and appropriate images of MEP distribution in the different species studied were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss).

RESULTS

In humans, both the thyroarytenoid (Fig. 1B–D) and cricoarytenoid laryngeal muscles presented a diffuse pattern of MEP distribution along their muscle fibers, with the middle zone (Fig. 1C) showing a higher density of MEPs, followed by the posterior (Fig. 1D) and the anterior (Fig. 1B) parts of the muscles. By contrast, in the rabbit (Fig. 1A) and the rat (Fig. 2A and B), both the thyroarytenoid (Figs. 1A and 2A) and crycoarytenoid (Fig. 2B) muscles presented their MEPs concentrated in a transverse band located in the middle of the muscle fibers. As in humans, the frog (Fig. 2C), the rooster (Fig. 3B), and the pigeon (Fig. 3D) showed MEPs diffusely distributed along the crycoarytenoid muscles. In what concerns the syrinx of the rooster (Fig. 4A) and pigeon (Fig. 4C), the distribution of MEPs along tracheobronchial syringeal muscles showed, in both cases (Fig. 4B and D, respectively), a scattered pattern along their entire extension.

In what concerns the morphology of laryngeal MEPs, they were round in the rat (Fig. 5A), rabbit, and human (Fig. 5D), whereas in the rooster (Fig. 5B), pigeon, and frog (Fig. 5C), they were elongated, reaching frequently a long fusiform profile. Syringeal MEPs were elongated in

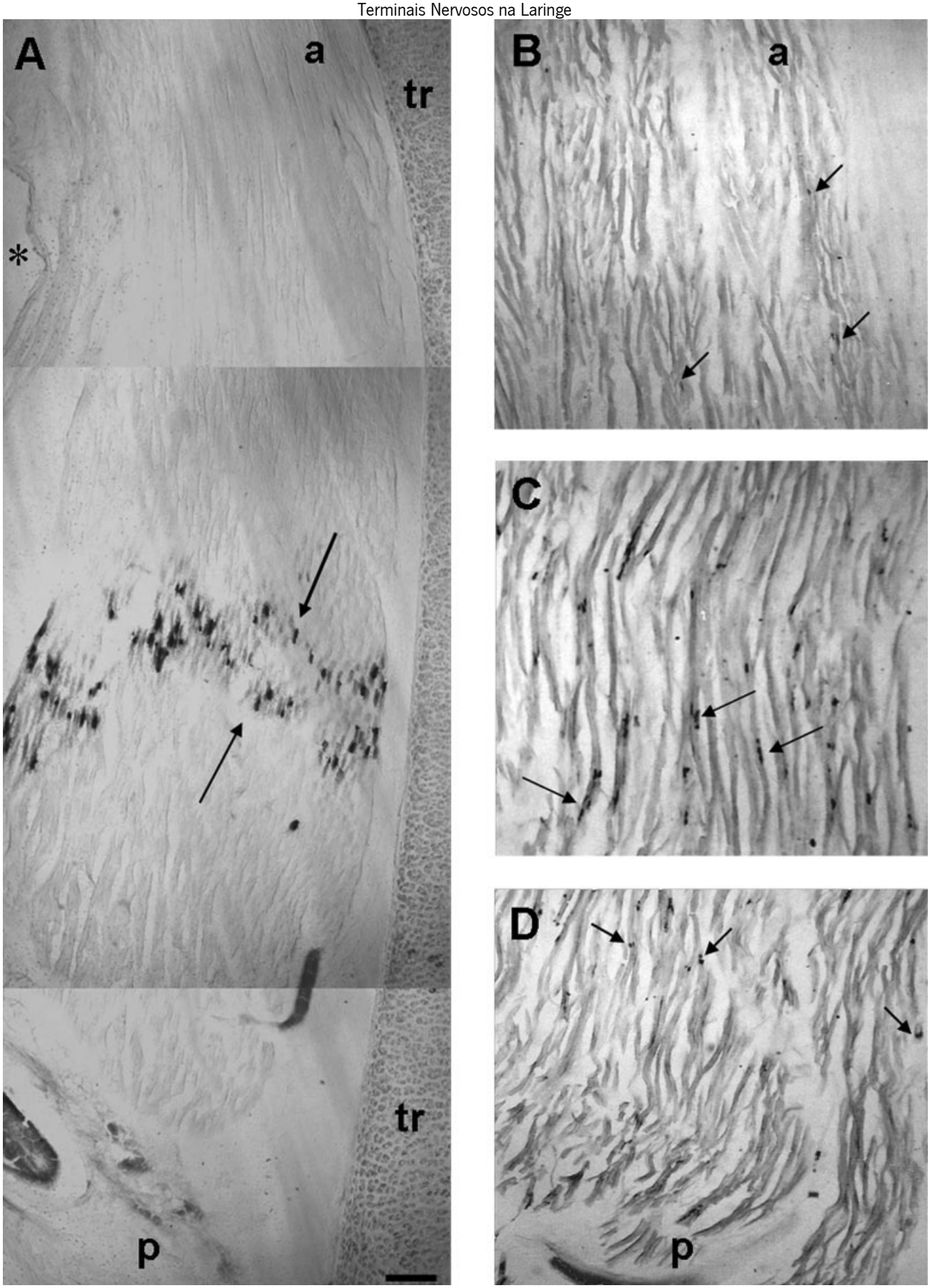

Fig. 1. Distribution pattern of motor end plates in the thyroarytenoid muscles of rabbit (**A**) and human (**B–D**). Note in the rabbit the concentration of MEPs in a transverse middle band (large arrows) of the thyroarytnoid muscle, whereas in human they are diffusely distributed along different areas of this glottal muscle, namely, the anterior (B), middle (C), and posterior (D) portion, although with a higher density in the middle portion. In the human larynx, small arrows indicate a few MEPs in the anterior and posterior areas, whereas in the medial zone, large arrows indicate multimotor end plates. Asterisk, laryngeal tract; tr, thyroid cartilage; p, posterior; a, anterior. Scale bar = 75 μm (A); 200 μm (B–D).

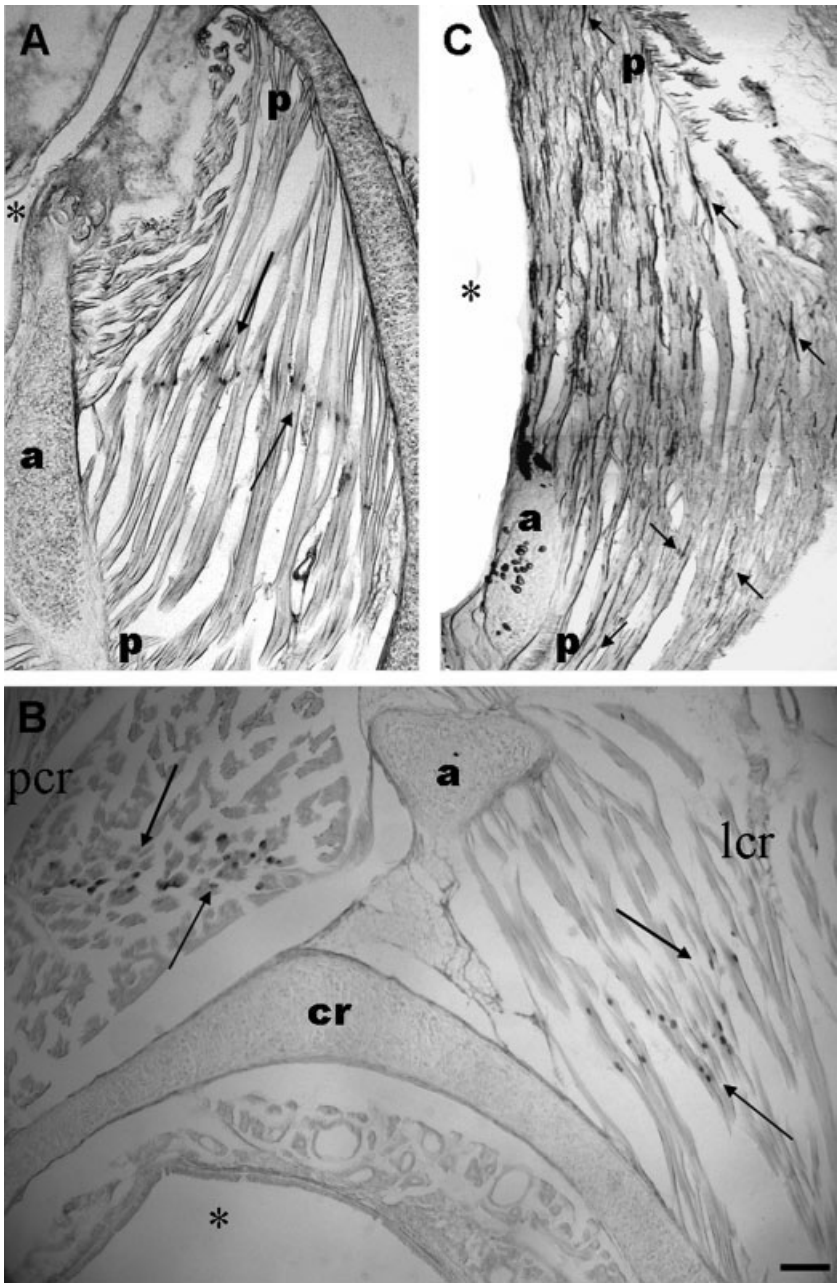

Fig. 2. Distribution pattern of motor end plates in the thyroarytenoid (**A**) and lateral/posterior cricoarytenoid (**B**) muscles of the rat and the cricoarytenoid muscles of the frog (**C**). In both laryngeal muscles analyzed in the rat, MEPs are concentrated in a transverse middle band (large arrows), whereas in the frog cricoarytenoid muscle, they are scattered along the muscle fibers (small arrows). Asterisk, laryngeal tract; a, arytenoid cartilage; cr, cricoid cartilage; lcr, lateral cricoarytenoid muscle; pcr, posterior cricoarytenoid muscle; p, muscle insertion points. Scale bar = 100 μm.

both the rooster (Fig. 5E) and pigeon (Fig. 5F). In humans, MEPs were aggregated in groups in the same fiber, forming multimotor end plates (Fig. 5D). In the frog vocal muscles (Fig. 4B) and in SM (Fig. 3B and D) and LM (Fig. 2B and D) of the rooster and pigeon, the fibers seem also to have several MEPs along their extension.

DISCUSSION

The data obtained in the present study indicate that the fine motor innervation of the LM and SM analyzed, which have important functions in vocalization, varies within different mammals and vertebrate taxa. Interestingly, the distribution pattern of neuromuscular junctions along the extension of LM in anuran lisamphibia and SM in birds is more similar to that present in human vocal folds than to the other mammals studied (rat and rabbit).

The sound source in mammals is the larynx (Fitch and Hauser, 2002), with the thyroarytenoid and cricoarytenoid muscles being relevant muscles supporting phonation in humans (Greene, 1989; Williams et al., 1999) and other

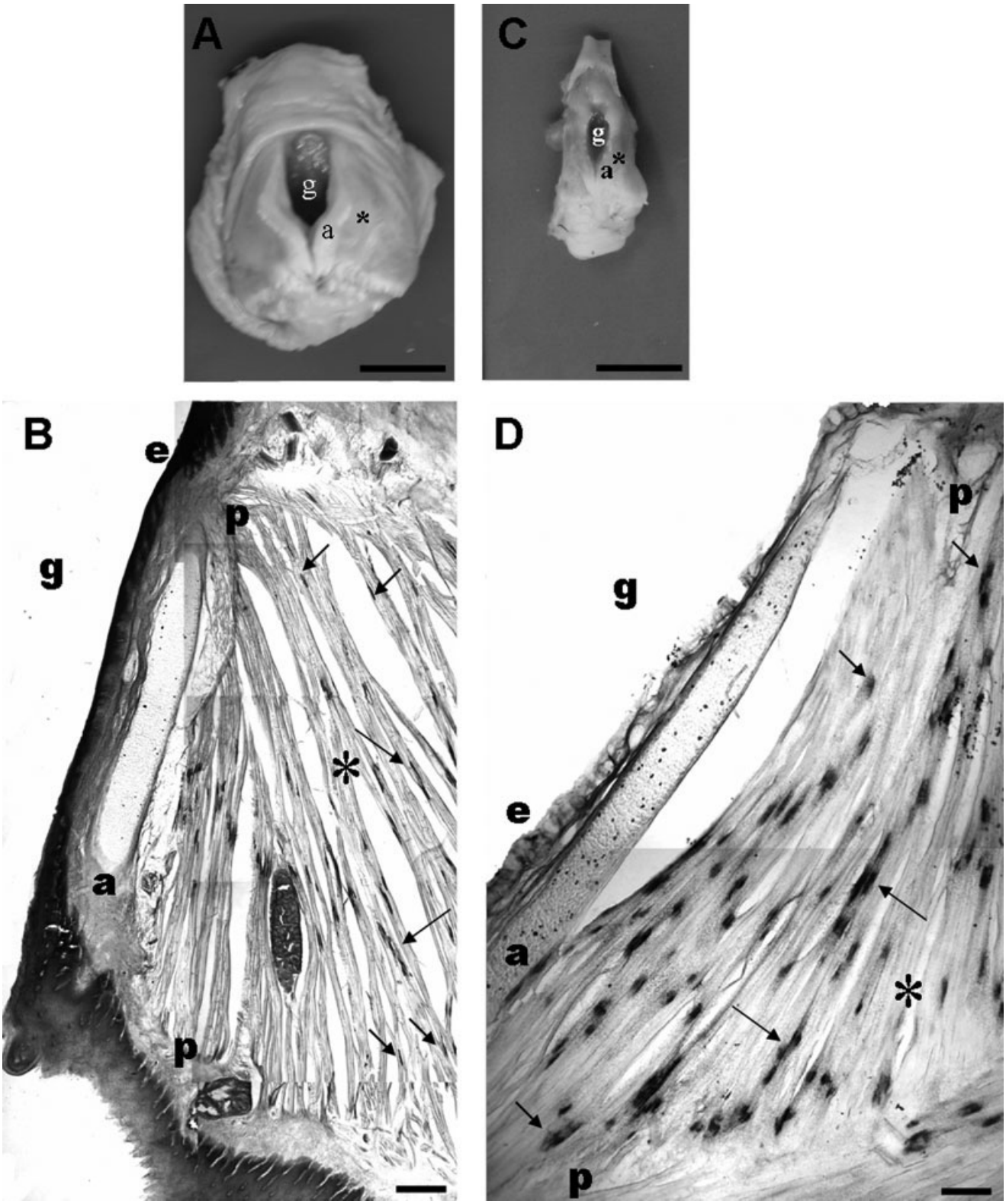

Fig. 3. Distribution pattern of motor end plates in the cricoarytenoid muscles of the rooster (**A** and **B**) and pigeon (**C** and **D**). In the external macroscopic morphology of the rooster (A) and pigeon (C) larynxes, it is possible to identify the crycoarytenoid cartilage (a), the crycoarytenoid muscle (asterisk), and the glottal aperture (g), which are shown at the microscopic level in B (rooster) and D (pigeon). In both avians, MEPs (arrows) are diffusely distributed (B, D) along the muscles, which may present several MEPs innervating the same muscle fiber (large arrows). e, laryngeal epithelium; p, muscle insertion points. Scale bar = 1 cm (A and C); 300 μm (B); 100 μm (D).

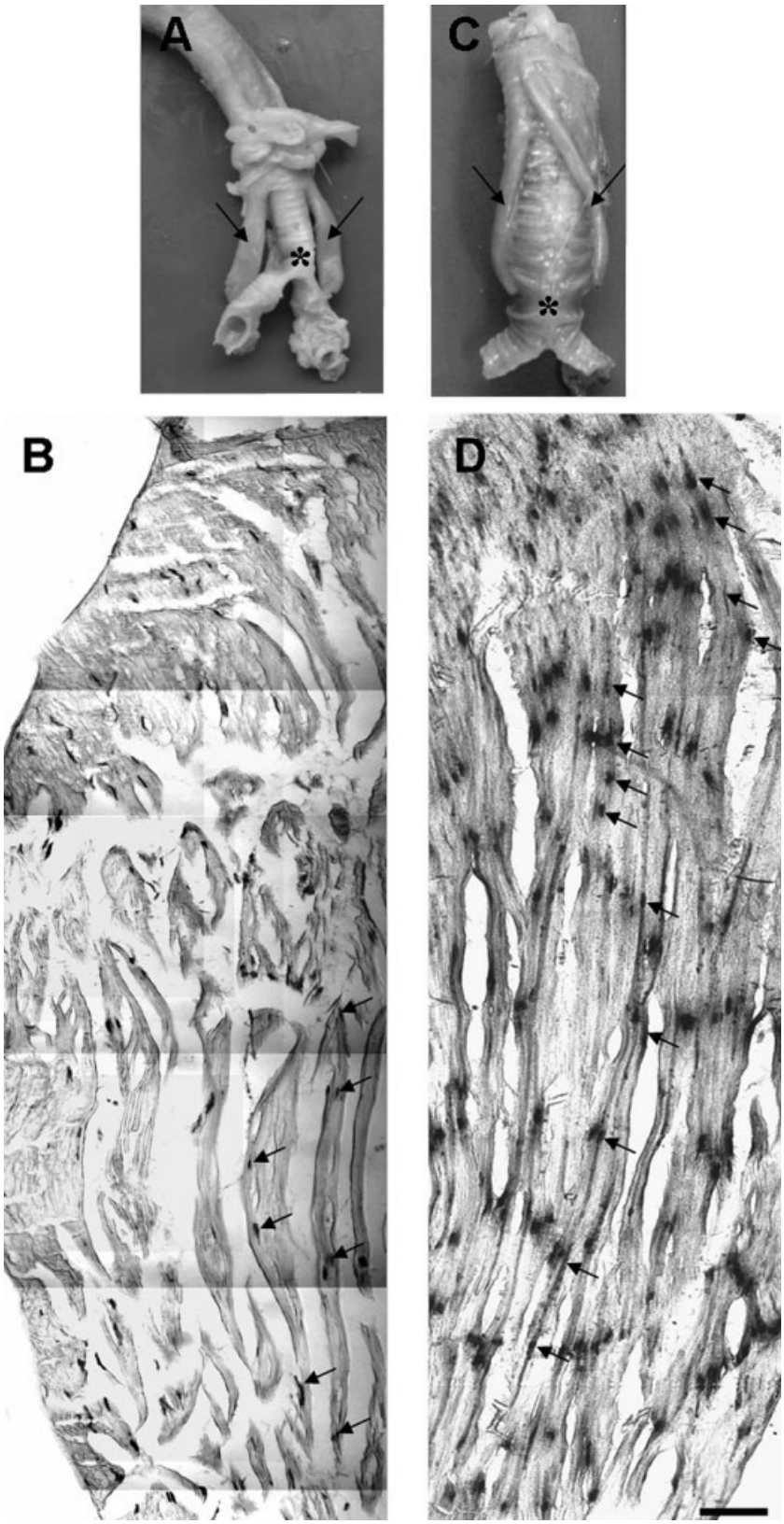

Fig. 4. Distribution pattern of motor end plates in the syringeal muscles of the rooster (**A** and **B**) and pigeon (**C** and **D**). Note in the external morphology of the rooster (A) and pigeon (C) the tracheobronchial syrinxes (asterisks) and syringeal muscles (arrows). In both avians, MEPs are diffusely distributed (B and D) along the extension of the syringeal muscles, with arrows indicating different series of neuromuscular junctions located apparently along the same fibers. Scale bar = 1.2 cm (A); 220 μm (B); 0.4 cm (C); 150 μm (D).

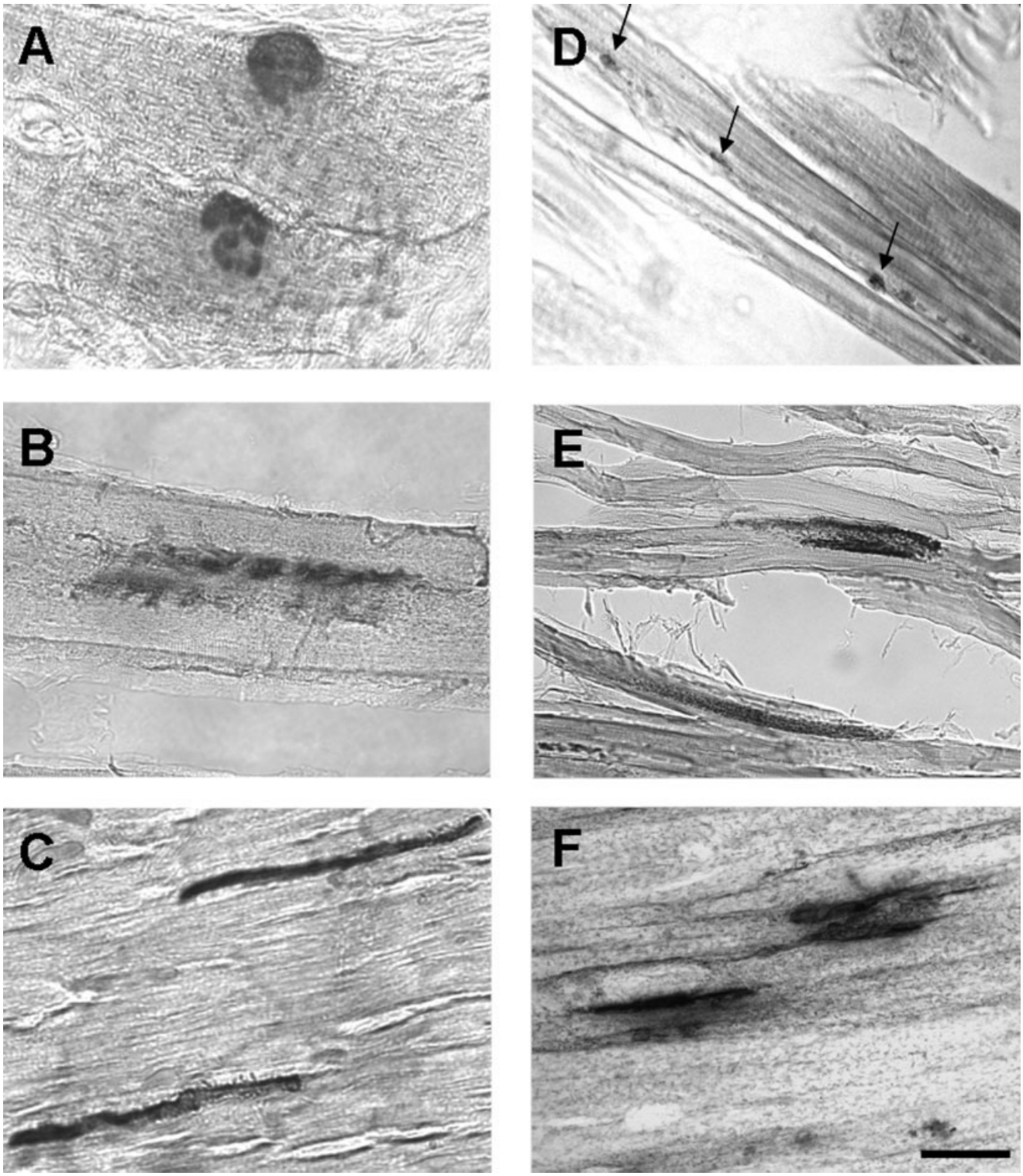

Fig. 5. Morphology of MEPs in the laryngeal muscles of mammalian [rat (**A**) and human (**D**)] and nonmammalian [rooster (**B**) and frog (**C**)] vertebrate classes and in syringeal muscles of the rooster (**E**) and pigeon (**F**). A similar round configuration of MEPs in laryngeal muscles of mammals (A and D) is in contrast with an elongated/fusiform morphology of MEPs in birds (B) and lissamphibia (C). An elongated morphology is also a characteristic of MEPs in avian syringial muscles (E and F). Note in the human vocal muscles several MEPs in a row are present in the same muscle fiber (multimotor end plates; arrows). Scale bar = 10 μm (A); 80 μm (B); 10 μm (C); 100 μm (D).

mammals. The differences between the human and rat vocal folds in what concerns MEP distribution confirmed our and other previous studies in humans (Pais-Clemente and Lima-Rodrigues, 1996; Sheppert et al., 2003) and rat (Pais-Clemente and Lima-Rodrigues, 1996; Inagi et al., 1998). Human vocal folds showed a clear scattered distribution of MEPs along LM, with a higher density in the middle third of the muscle, whereas in the rat (and rabbit), MEPs were concentrated along a narrow band at the midbelly of the vocal muscles. Although several factors are important for vocalization (see Introduction), the different MEP distribution between human and rat/rabbit vocal folds suggests a different motor innervation of laryngeal intrinsic muscles in humans when compared to the rat and rabbit. Vocal fold muscles in humans present an elaborated motor control since different muscle fibers are innervated at different rostrocaudal positions, thus allowing a complex pattern of possibilities for muscle contraction. By contrast, the fact that all muscle fibers in the rat and rabbit vocal folds are innervated approximately at the same rostrocaudal location suggests a more restricted mode of contraction of the entire LM muscles.

It is well known that some anuran lissamphibia and avian species can produce a complex pattern of different sounds, respectively, from the larynx and the syrinx (Storer et al., 1979). Applying the same rationale used for mammals, the diffuse distribution of MEPs (as in human vocal muscles) in lissamphibia cricoarytenoid muscles and avian SM may contribute to their vocal versatility. In what concerns the avian larynx, it does not appear to have the capacity for producing sound (McClelland, 1989), but may be used instead to modify sound originated from the syrinx (Harris et al., 1968; White, 1968). The diffuse distribution of MEPs in the rooster glottal muscles suggests some elaboration on the functions played by the avian cricoarytenoid muscles.

Given the present results, it is possible that the diffuse distribution of MEPs in the thyroarytenoid and cricoarytenoid in humans, in the latter muscle in anuran lissamphibia, and in the SM in birds may contribute to the fact that humans can talk and produce voice, as some lissamphibia and birds can produce very complex sounds. This difference in the fine motor innervation of vertebrate vocal muscles does not seem to be correlated with the dimension of muscle extension. In fact, animals with large larynxes can have a scattered (human, rooster) or centered (rabbit) MEP distribution in the cricoarytenoid muscles, whereas larynxes from smaller species can also have a scattered (frog, pigeon) or centered (rat) MEP distribution in the same muscles. Thus, in what concerns LM/SM motor innervation, humans are members of a group including birds and lissamphibia and excluding other mammals. Interestingly, several nonprimate species are able to mimic human speech to a remarkable degree, as highly trained parrots can have a large vocabulary of sounds that they use with a communication objective (Pepperberg, 1991). This indicates that in terms of speech by vocal imitation, humans are also members of an apparently uncharacteristic group that includes birds (and aquatic mammals such as dolphins) but excludes nonhuman primates (Fitch, 2000). However, physiological studies are needed to elucidate on a possible correlation between MEP distribution in vocal muscles and vocalization.

The morphological analysis of MEPs in the LM/SM of animal species studied also revealed clear differences between species: in mammalians (rat, rabbit, and human), the motor end plates had round configuration, whereas in birds (rooster, pigeon) and lissamphibia (frog), MEPs were elongated and, frequently, fusiform. This suggests that, contrary to the distribution of MEPs, the morphological pattern of these structures is similar between species of the same vertebrate taxa. Studies are needed in order to elucidate the physiological significance of the change in laryngeal MEP morphology along different vertebrate classes.

The higher concentration of MEPs in the middle third of the human vocal folds implicates a stronger tension of contraction in that particular area. This may contribute to the higher incidence of vocal cord nodes (kissing nodes) in the correspondent region of the vocal fold epithelium (Pontes et al., 2002). This observation suggests that the intramuscular injection of botulinum toxin in the middle third of the thyroarytenoid and cricoarytenoid LM may be of clinical importance not only for the treatment of spasmodic disphonia (Blitzer et al., 1986; Castellanos et al., 1994; Bielamowicz et al., 2002; Tisch et al., 2003; Maronian et al., 2004), but also for recovering from kissing nodes. However, other phonetic parameters such as aerodynamics, subglottal pressure, and amount/mode of phonation are important etiological factors that should also be taken into account for the treatment of vocal cord nodes (Gunter, 2004).

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Capítulo 5

Considerações finais

5. CONSIDERAÇÕES FINAIS

O sistema nervoso pode constituir a base etiopatogénica de patologias da laringe, em casos de inflamação neurogénica ou de distúrbios motores funcionais. Nas patologias respiratórias não infecciosas e não alérgicas, as terapêuticas são limitadas e os mecanismos pouco conhecidos, sendo os fármacos mais eficazes os corticosteróides e os anti-inflamatórios não esteróides com propriedades de inibição não selectiva das enzimas COX-1 e COX-2. O trabalho realizado nesta tese procurou não só conhecer em detalhe a morfologia das fibras sensitivas terminais da mucosa laríngea, mas também desenvolver um modelo experimental de laringite neurogénica onde as bases para futuras aplicações terapêuticas possam ser testadas; por outro lado, a avaliação detalhada da enervação motora dos músculos vocais permitiu fazer uma análise do controlo muscular implicado na vocalização dos vertebrados e perspectivar a aplicação mais específica e localizada de terapias centradas em patologias do foro motor da laringe.

5.1. Enervação sensitiva da laringe: morfologia, patologia e aspectos terapêuticos

No que concerne à enervação sensitiva da laringe, os estudos incluídos nesta tese permitiram conhecer em detalhe a rede nervosa e a distribuição fina dos terminais axonais na mucosa (PUBLICAÇÃO I; Capítulo 4.1). Foi também desenvolvido o primeiro modelo de laringite neurogénica descrito na literatura, o qual foi baseado na indução por entubação nasogástrica (ENG) (PUBLICAÇÃO II; Capítulo 4.2). Este modelo experimental permitiu determinar pela primeira vez que um fármaco inibidor selectivo da COX-2 (Etoricoxibe) tem uma acção eficaz na redução da laringite, abrindo novas perspectivas terapêuticas relacionadas com esta patologia a serem exploradas num futuro próximo, em alternativa ou complemento ao receituário tradicional (PUBLICAÇÃO III; Capítulo

4.3). O modelo de ENG revelou-se ainda um método viável para investigar uma possível relação entre a inflamação crónica e lesões que podem evoluir para neoplasia (metaplasia epitelial) e permitiu verificar que o prolongamento no tempo do período de laringite diminuía a expressão dos níveis de RNAm dos supressores tumorais p16 e p53, daí podendo resultar que as células da mucosa possam entrar mais facilmente em mitose e evoluir eventualmente para cancro da laringe (PUBLICAÇÃO IV; Capítulo 4.4).

Tal como na pele, os neuropeptídeos mais importantes presentes nas fibras sensitivas amielínicas C da mucosa da laringe são a CGRP (Tanaka et al 1993; Hauser-Kronberger et al, 1997) e a substância P (SP) (Hisa et al, 1985). No entanto, era difícil compreender como é que no lume respiratório as partículas em suspensão (irritantes inalados) podem desencadear a estimulação destas fibras, já que a localização intraepitelial não permitia a sua estimulação directa imediata. Demonstramos pela primeira vez que as fibras nervosas intraepiteliais projectam para o lume da laringe no Rato, sendo deste modo possível a sua estimulação directa imediata por irritantes inalados, principalmente na ausência de muco (PUBLICAÇÃO I; Capítulo 4.1). Foi também observada uma distribuição diferente das fibras e dos terminais nervosos ao nível da mucosa da laringe, concentrando-se em maior número ao nível da face laríngea da epiglote e da metade posterior das cordas vocais (PUBLICAÇÃO I; Capítulo 4.1). Este conhecimento sugere, quer do ponto de vista anestésico quer cirúrgico, que se deverá evitar a estimulação destas zonas mais enervadas da laringe de forma a diminuir a ocorrência de reacções vagais potencialmente perigosas.

No que diz respeito à avaliação do papel dos neuropeptídeos na inflamação laríngea e no seu tratamento, foi necessário desenvolver um modelo experimental de laringite neurogénica. Um conjunto de quatro aspectos levou-nos a considerar a hipótese de desenvolver um método original baseado num modelo de entubação nasogástrica (ENG): em primeiro lugar, a fiabilidade metodológica e a sua reprodutibilidade; em segundo, alguns estudos mostraram que o refluxo

gastroesofágico (RGE) ou o refluxo faringolaríngeo (RFL) constituem um importante factor etiológico no desenvolvimento de patologia inflamatória e neoplásica da laringe (Koufman 1991; Galli et al 2002; Ward e Hansan, 1988; Lewin et al, 2003; El-Serag et al, 2001); em terceiro, a ENG constitui um importante factor de incremento do RGE e das suas consequências patológicas (Novoski et al, 1999), tendo sido possível demonstrar no nosso modelo uma diminuição acentuada do pH, mais evidente inclusive a nível faríngeo do que a nível esofágico (PUBLICAÇÃO II; Capítulo 4.2). Finalmente, os estímulos nóxicos em áreas enervadas por fibras com neuropeptídeos como a Substância P (SP) e o Peptídeo Relacionado com o Gene da Calcitonina (CGRP) levam à sua libertação e resultam em aumento da permeabilidade vascular, edema e inflamação neurogénica (Kunkel, 1997). Após a construção e aplicação do modelo, constatamos que a densidade de fibras imunoreactivas para SP e CGRP na mucosa da laringe diminuíu ao longo das primeiras semanas de entubação, tendo recuperado parcialmente à quinta semana. Simultaneamente, verificou-se um aumento da expressão do RNAm das principais interleucinas pró-inflamatórias, nomeadamente das Interleucinas 1 e 6 (IL-1β e IL-6) e do Factor de Necrose Tumoral (TNF-α), bem como uma diminuição da citoquina anti-inflamatória IL-10. A par deste fenómeno, verificou-se ao nível da mucosa o aparecimento de um infiltrado de células inflamatórias mononucleares e de uma intensa hipertrofia glandular, demonstrativos de um processo inflamatório crónico (laringite). Adicionalmente, observamos que com o aumento do tempo de entubação nasogástrica o número de células inflamatórias que expressavam COX-2 foi também aumentando progressivamente e que a produção a nível local de RNAm de COX-2 também aumentou durante as duas primeiras semanas (PUBLICAÇÂO II; Capítulo 4.2). Estes resultados em conjunto permitiram classificar o modelo ENG desenvolvido nesta tese como representativo de um modelo de inflamação neurogénica, o qual poderá ser utilizado em estudos futuros sobre mecanismos e/ou terapêutica anti-inflamatória.

A laringite não tem um tratamento médico totalmente satisfatório, baseando-se as terapêuticas mais eficazes nos corticosteróides. No que diz respeito aos anti-inflamatórios não esteróides (AINEs), particularmente os fármacos clássicos inibidores não selectivos da ciclooxigenase (COX-1 e COX-2), a sua eficácia é ainda mais limitada devido ao facto de induzirem uma diminuição das prostaglandinas protectoras gástricas e um aumento dos níveis de leucotrienos, contribuindo para a manutenção e eventual cronificação do processo inflamatório (Warner e Mitchell, 2004). Por outro lado, sabe-se actualmente que os inibidores selectivos da ciclooxigenase-2 (COX-2) não apresentam o mesmo grau de efeitos secundários sobre as vias aerodigestivas que os AINEs clássicos. No entanto, não existiam estudos sobre a expressão da enzima COX-2 na laringite crónica em geral e em particular naquela provocada pela ENG. Num estudo prévio, verificamos que a par da libertação dos peptídeos CGRP e SP pelas fibras sensitivas periféricas e do aumento nítido da inflamação laríngea, se observava também um aumento da expressão da actividade COX-2, das Interleucinas IL-1β e IL-6 e de TNF-α (todas moléculas pró-inflamatórias), assim como uma diminuição da expressão da interleucina IL-10 (anti-inflamatória) (PUBLICAÇÃO II; Capítulo 4.2). O facto de as prostaglandinas aumentarem a libertação de CGRP e SP e os inibidores da COX-2 impedirem a formação de prostaglandinas (Konturek et al, 2005) reforçou a hipótese de os inibidores selectivos da COX-2 poderem constituir uma alternativa terapêutica importante na interrupção do mecanismo inflamatório neurogénico. De facto, o tratamento com o fármaco Etoricoxibe induziu uma (i) atenuação na libertação de neuropeptídeos implicados na inflamação neurogénica, (ii) diminuição significativa do número de células que expressam COX-2 na mucosa laríngea, (iii) dos níveis de expressão de RNAm de COX-2 e de TNF-α na laringe após a primeira semana de entubação nasogástrica, sem afectar as outras interleucinas analisadas (IL-1β, IL-6, IL-10), o que faz supor um efeito terapêutico sem efeitos adversos de paricular relevância (PUBLICAÇÃO III; Capítulo 4.3). Estes dados sugerem um possível interesse na aplicação dos AINEs inibidores selectivos da COX-2 no

tratamento da laringite, explorando as vantagens terapêuticas conhecidas e os reduzidos efeitos secundários adversos ao nível dos sistemas orgânicos respiratório e digestivo.

A inflamação crónica está correlacionada com cancro e um nível elevado de COX-2 pode ser também um marcador de lesão tumoral (Konturek et al, 2005). Na hipótese da laringite crónica poder estar relacionada com o cancro da laringe, foi avaliada também a expressão de outros marcadores neoplásicos na mucosa da laringe, nomeadamente as moléculas supressoras tumorais p16 e p53 (Serrano, 1997; Warnakulasuriya et al, 1998; Polsky et al, 2001; Voorhoeve e Agami, 2003; Ohtani e tal, 2004; Krishnamurthy, 2004; Furth et al, 2006). Verificamos que em ratos submetidos a entubação nasogástrica a produção de RNAm de p16 e p53 diminuiu progressivamente, e de modo significativo, durante as cinco semanas de entubação (PUBLICAÇÃO IV; Capítulo 4.4). Estas são moléculas supressoras tumorais porque impedem que a célula entre mais facilmente na fase S da mitose e num processo de divisão descontrolada, evitando deste modo que as células se tornem neoplásicas. Estes resultados fazem supor que a ENG provoca um ambiente mais favorável a desenvolver o cancro nos casos de exposição simultânea a outras substâncias como o álcool ou o fumo do tabaco, podendo ajudar a compreender a forma como o refluxo faringo-laríngeo pode contribuir para as lesões neoplásicas da laringe.

5.2. Enervação motora da laringe: morfologia e aspectos patológicos e potencialidades terapêuticas

Como as placas motoras são as responsáveis pela transmissão do estímulo nervoso dos neurónios às fibras musculares (Hirsch, 2007), o conhecimento da sua localização é essencial para determinar as capacidades específicas de contracção de cada músculo. Revela-se desta forma de

especial interesse a investigação do padrão de distribuição dos terminais axonais motores nos músculos laríngeos. Verificamos que espécies de mamíferos com capacidades de produzir sons limitados como o rato e o coelho apresentam as PM localizadas apenas numa determinada região dos músculos vocais, enquanto que espécies com capacidade de produzir maior variabilidade de sons, como o galo, o pombo ou a rã possuem uma distribuição difusa das PM ao longo de várias regiões dos músculos (PUBLICAÇÃO V; Capítulo 4.5). Atendendo ao facto da contracção muscular estar relacionada com o número de fibras activadas e a força de cada fibra com o número de placas por fibra, os resultados sugerem que espécies com uma distribuição mais difusa das PM tenham uma maior capacidade de controlar e variar a forma e a força de contracção dos músculos intrínsecos da laringe, a qual pode estar relacionada com a capacidade de produzir sons diferentes (PUBLICAÇÃO V; Capítulo 4.5).

Por outro lado, sabe-se que a toxina botulínica inibe a transmissão sináptica ao nível das PM e é utilizada para o tratamento de determinadas disfonias, nomeadamente a disfonia espástica, pelo que o conhecimento da localização das placas permitirá a aplicação de uma terapêutica mais dirigida (Castellanos et al, 1994). Outra situação clínica em que a utilização de toxina botulínica é defendida por alguns autores diz respeito aos granulomas laríngeos, com o objectivo de diminuir a tensão da corda e reduzir a probabilidade de traumatismo da corda vocal e de recidiva (Nasri et al, 1995; Orloff e Brin, 1999; Zalvan e Blitzer, 2004). Neste contexto, como os nódulos das cordas vocais se observam principalmente no terço médio devido a uma maior tensão e traumatismo da corda a este nível (Pontes et al, 2002), é possível que esta patologia possa estar relacionada com a maior concentração das PM nesta localização. Neste caso, tal como no caso dos granulomas, é provável que se possa usar o mesmo raciocínio e aplicar também a toxina botulínica como alternativa ou complemento terapêutico à cirurgia e à terapia da fala para o tratamento de casos de nódulos das cordas vocais (PUBLICAÇÂO V; Capítulo 4.5).

5.3

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Capítulo 6

Conclusões e perspectivas futuras

6. CONCLUSÕES E PERSPECTIVAS FUTURAS

1. A descoberta de terminais axonais livres de fibras sensitivas ao nível da camada mucociliar externa ao epitélio respiratório permitiu identificar a base anatómica para certas formas de laringite cuja base etiopatogénica resulta da activação directa das fibras e libertação dos neuropeptídeos CGRP e SP, os quais promovem inflamação neurogénica.

2. Tendo em consideração que a inflamação laríngea podia ter uma origem neurogénica, desenvolveu-se um modelo experimental de entubação nasogástrica (ENG) e descreveram-se os mecanismos patológicos e moleculares da inflamação laríngea desencadeada com o tempo de ENG. Assim, foi demonstrada pela primeira vez a laringite neurogénica como uma nova entidade clínica patológica de laringite, além das formas alérgica e infecciosa já descritas clinicamente.

3. Na laringite neurogénica, as terapêuticas existentes são limitadas, já que os corticosteróides e os anti-inflamatórios não esteróides (AINEs) inibidores não selectivos das enzimas COX-1 e COX-2 apresentam efeitos laterais adversos aerodigestivos que acabam por contribuir, posteriormente, para a manutenção da patologia, pelo que se tornam ineficazes. Tendo em conta os menores efeitos adversos a este nível dos inibidores selectivos da COX-2, foi possível demonstrar a actividade anti-inflamatória de um fármaco deste tipo (Etoricoxibe), abrindo perspectivas futuras de pesquisa destes fármacos no tratamento de patologias inflamatórias do aparelho respiratório superior.

4. Tendo sido demonstrado, nomeadamente na esofagite ou na bronquite, uma relação entre inflamação crónica e o cancro, a avaliação de diferentes marcadores de lesões pré-neoplásicas no modelo de laringite por ENG implicou a análise da expressão de supressores tumorais como as moléculas p16 e p53. Verificamos uma diminuição da expressão do RNAm quer do p16 quer do p53, ao longo das 5 semanas de entubação nasogástrica. Sabendo que estas moléculas são supressores tumorais, estes resultados fazem supor uma diminuição progressiva da capacidade da célula para compensar possíveis estímulos carcinogénicos indutores de mitose descontrolada.

5. No que diz respeito às patologias relacionadas com a enervação motora periférica da laringe, a análise dos terminais motores nos músculos vocais de variadas espécies de vertebrados permitiu compreender melhor a capacidade vocal de diferentes classes animais. Verificou-se que quanto mais disperso e difuso era o padrão de distribuição das PM nos músculos vocais (laríngeos ou siríngeos), maior a capacidade da variabilidade sonora aparente da espécie analisada. Além disso, a avaliação do padrão de distribuição dos terminais motores nos músculos laríngeos permitiu compreender melhor determinadas disfonias, como os nódulos das cordas vocais, nas quais os únicos tratamentos se baseiam na cirurgia ou na fisioterapia. Sendo possível utilizar fármacos inibidores da transmissão neuromuscular, como a toxina botulínica na forma tópica, na disfonia espástica, o melhor conhecimento da distribuição das PM na laringe do ser humano permitirá aplicar mais especificamente os fármacos, não só nesta disfonia mas também em outras patologias, como por exemplo como nos nódulos das cordas vocais.

Em termos de antevisão futura, este conjunto de conhecimentos permitirá realizar uma nova série de estudos, agora numa perspectiva que investigue novas possibilidades de aplicação clínica, num espaço temporal não muito longo. A utilização de fármacos inibidores selectivos da COX-2 em patologias da laringe poderá ser explorada, tendo em conta o efeito anti-inflamatório demonstrado no modelo de laringite neurogénica baseado na ENG desenvolvido neste trabalho. Por outro lado, a aplicação mais extensa e selectiva de fármacos com acção ao nível do bloqueio da transmissão eléctrica na junção neuromuscular pode ser avaliada em patologias que resultam de um uso inadequado das cordas vocais. Finalmente, a relação entre laringite crónica e o possível desenvolvimento de lesões pré-malignas terá que ser explorado ainda no modelo animal de inflamação crónica, de modo a: (i) completar a análise do conjunto de marcadores tumorais conhecido; (ii) verificar se a presença de alterações histopatológicas indicativas de lesões pré-neoplásicas ou neoplásicas necessita de períodos de inflamação mais prolongados.