CLÁUDIA ROBERTA BRUNNQUELL SCZEPANSKI

PAPEL DO ESTRESSE OXIDATIVO NA ATROFIA E PROTEÓLISE DO MÚSCULO ESQUELÉTICO NO ENFISEMA PULMONAR INDUZIDO PELA PAPAÍNA

Londrina
2014

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Tese apresentada ao Programa de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de Doutor.

Orientador: Prof. Dr. Rubens Cecchini

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BRUNNQUELL, Cláudia Roberta. **Papel do estresse oxidativo na atrofia e proteólise do músculo esquelético no enfisema pulmonar induzido pela papaína** 2014, 97f. Tese. Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

O objetivo deste estudo foi estabelecer um modelo experimental adequado de enfisema pulmonar associado às modificações musculoesqueléticas em estágios precoces da doença, assim como comparar as consequências musculares do enfisema pulmonar induzido por papaína e elastase. Foram utilizados hamsters Syrian Golden machos (100-140g) distribuídos em diferentes grupos de acordo com o tempo experimental (tempo de sacrifício após a instilação de elastase aos 10, 20, 40 ou 70 dias, E10, E20, E40 ou E70/EE, respectivamente) e papaína (70 dias, EP); e enzima administrada (40mg/ml de papaína em salina ou 5,2 UI de elastase pancreática suína em salina) com seus respectivos controles (C10, C20, C40, C70/CS) sendo instilados 0,3 ml da solução por animal. Após o tempo experimental, os animais foram pesados para avaliação do índice de caquexia (IC) e sacrificados. O lobo médio do pulmão direito foi coletado para análise histológica e quantificação do enfisema pulmonar. O gastrocnêmio foi retirado para análise morfométrica da fibra muscular, massa muscular, relação da massa muscular com a massa corporal total (MM/MT), lipoperoxidação por quimiluminescência (QL), proteínas carboniladas (PC), glutationa total (GST) e oxidada (GSSG), substâncias reativas ao ácido tiobarbitúrico (TBARS) e atividade proteolítica quimiotripsina-like e calpaína-like. Ao compararmos os grupos 70 dias (CS, EE e EP) foi verificado diminuição dos interceptos alveolares no EE e EP em relação ao CS (CS: 70.64 ± 0.58; EE: 48.17 ± 1.16; EP: 51.56 ± 0.49; $p<0,05$). Quando comparado ao CS, houve uma redução da massa muscular no grupo EP (18,34%) e aumento no grupo de EE (8,37%), bem como da relação MM/MT. Além disso, a reação de QL, PC e atividade quimotripsina-like foram elevadas no grupo EP, assim como a GST diminui no EE. O grupo EE mostrou crescente número de fibras com aumento das áreas transversais e da atividade calpaína-like. Na comparação com seus respectivos controles, os grupos com enfisema pulmonar (E10, E20, E40 E E70) apresentaram diminuição significativa do número de interceptos alveolares. O IC foi de 9,70 ± 1,64% e 10,81 ± 1,55% nos grupos E10 e E20. A frequência de distribuição das áreas transversais das fibras se mostrou diferente entre os grupos 20, 40 e 70 dias. Além disso, TBARS, relação entre a GST e GSSG, reação de QL e PC não mostraram diferenças significativas. A atividade calpaína-like foi significativamente reduzida no grupo E40. Estes dados nos permitem concluir que a elastase e a papaína, quando utilizadas para induzir modelos experimentais de enfisema, levam há diferentes tempos e velocidades de adaptação muscular, fornecendo informações sobre a escolha de um modelo experimental adequado. Em adição, o modelo por elastase leva a adaptação muscular precoce, com ausência de atrofia e sem envolvimento direto do estresse oxidativo neste processo.

Palavras-chave: Elastase. Papaína. Enfisema pulmonar. Músculo esquelético. Estresse oxidativo.

BRUNNQUELL, Cláudia Roberta. **Role of oxidative stress in atrophy and proteolysis of skeletal muscle in the pulmonary emphysema induced by papain.** 2014, 97p. Tesis. Londrina State University. Londrina, 2014.

ABSTRACT

The objective of the present study was to establish a suitable experimental model of pulmonary emphysema associated with musculoskeletal changes at early stages of the disease, as well as compare the muscular effects of pulmonary emphysema induced by papain and elastase. Syrian Golden hamsters (100-140g) were distributed in different groups according to the experimental time (time of sacrifice after instillation of elastase at 10, 20, 40 or 70 days, E10, E20, E40 or E70/EE, respectively) and papain (70 days, EP); and administered enzyme (40mg/ml of papain in saline or 5.2 IU of porcine pancreatic elastase in saline) with their respective controls (C10, C20, C40, C70 or CS), being instilled 0, 3 ml of the solution per animal. After experimental time, animals were weighed for cachexia index (CI) and sacrificed. Middle lobe of the right lung was collected for histological analysis and quantification of pulmonary emphysema. Gastrocnemius was removed for morphometric analysis of muscle fiber, ratio of muscle mass to total body mass (MM / MT), lipid peroxidation chemiluminescence (CL), carbonyls protein (CP), total (GST) and oxidized glutathione (GSSG), thiobarbituric acid reactive substances (TBARS) and chymotrypsin-like and calpain-like proteolytic activities. When compared the groups euthanized at 70 days (CS, EE and EP), was observed decreased of alveolar intercepts in EE and EP relative to CS (CS: 70.64 ± 0.58; EE: 48.17 ± 1.16; EP: 51.56 ± 0.49; $p<0,05$) was observed. In comparision with control group, there was reduction on muscle weight in the EP group (18.34%) and increased in the EE group (8.37%) as well as relation between MM/MT. Additionally, CL reaction, CP and chymotrypsin-like activity were elevated in the EP group, as GST was decreased in the EE group. The EE group showed increasing of number of fibers with increased cross-sectional areas with increased calpain-like activity. When compared to their respective control groups, emphysemic groups (E10, E20, E40 and E70) had significantly decreased in the number of crossed alveolar intercepts. The CI showed 9.70±1.64% and 10.81±1.55% on E10 and E20 groups. The frequency distribution of fibre cross-sectional areas showed differences between groups at 20, 40 and 70 days. Additionally, TBARS, relationship between GST and GSSG, CL reaction, and CP showed not significant differences. Calpain-like activity was significantly decreased in E40 group. These data show that elastase and papain, when used to induce experimental models of emphysema, lead to different speeds and types of muscular adaptation, providing information on choosing a suitable experimental model. In addition, elastase model lead to early muscle adaptation, without atrpophy or direct involved of oxidative stress in the process.

Key-words: Elastase. Papain. Pulmonary emphysema. Skeletal muscle. Oxidative stress.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AP-1	Ativador Protéico 1
ATP	Adenosina Trifisfato
Ca^{2+}	Cálcio
CAT	Catalase
DPOC	Doença Pulmonar Obstrutiva Crônica
ERK	Sinalizador Extracelular Regulado por Quinases
ERRO	Espécies Reativas de Oxigênio
GPx	Glutationa Peroxidase
GR	Glutationa Redutase
GSH	Glutationa Reduzida
GSSG	Glutationa Oxidada
GT	Glutationa Total
H_2O_2	Peróxido de Hidogênio
HNE	Hidroxinonenal
HSP	Proteínas de Choque Térmico
IMC	Índice de Massa Corporal
JNK	Quinase Terminal Jun
IL-6	Interleucina 6
MDA	Malonaldeído
Mn-SOD	Superóxido Dismutase manganês
NF-Kb	Fator de Transcrição NF-κB
NO	Óxido Nítrico
O_2^-	Ânion Superóxido
OMS	Organização Mundial da Saúde
p38	Proteína Quinase Ativada por Mitógeno
PTEN	Fosfatase
SOD	Superóxido Dismutase
TNF-α	Fator de Necrose Tumoral Alfa
VEF1	Volume Expiratório Forçado no primeiro segundo

SUMÁRIO

1 INTRODUÇÃO

1.1 DOENÇA PULMONAR OBSTRUTIVA CRÔNICA

A Doença Pulmonar Obstrutiva Crônica (DPOC) é uma condição progressiva e lenta, caracterizada pela limitação, não totalmente reversível, do fluxo aéreo com estreitamento das pequenas vias aéreas e destruição do parênquima pulmonar, associada à resposta inflamatória anormal dos pulmões a partículas ou gases nocivos (Pauwels *et al.* 2001; Macnee 2005; Larsson 2007; Global Initiative for Chronic Obstructive Lung Disease [GOLD] 2014).

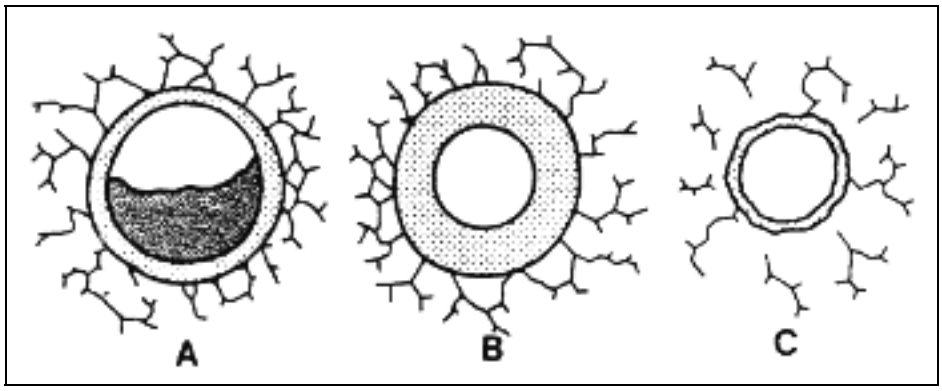
A obstrução das vias aéreas apresenta componentes reversíveis, como broncoconstrição e inflamação das vias aéreas, com exsudação de plasma, aumento das células inflamatórias e produção de muco e, irreversíveis, como a destruição do parênquima pulmonar e o remodelamento das vias aéreas com fibrose peribronquiolar e aumento da colapsabilidade decorrente da destruição das fibras elásticas do tecido pulmonar (Fletcher *et al.* 1976; MacNee 2005; Anto *et al.* 2001).

As bases anatomopatológicas da DPOC são identificadas como alterações da estrutura e função das vias aéreas centrais (bronquite), vias aéreas pequenas (bronquiolite), vasculatura pulmonar (hipertensão pulmonar) e do parênquima pulmonar (enfisema), podendo ser caracterizadas por aumento das glândulas mucosas e infiltração crônica de células inflamatórias na parede brônquica e aumento permanente dos espaços aéreos distais aos bronquíolos terminais com destruição das paredes alveolares (Global Initiative for Chronic Obstructive Lung Disease [GOLD] 2014).

Sendo assim, a DPOC representa um grupo de lesões pulmonares, tanto de vias aéreas, como na bronquite crônica, quanto do parênquima pulmonar, como no enfisema (Huertas e Palange, 2011). Dentre os pacientes com DPOC, aproximadamente 20% apresentam enfisema, enquanto os outros 80% têm bronquite crônica ou, ainda, uma combinação destas duas doenças (Datasus, 2004).

A obstrução das vias aéreas está associada aos mecanismos responsáveis pelo bloqueio da luz brônquica (Figura 1).

Figura 1 – Mecanismos de obstrução das vias aéreas.



(A) A luz está parcialmente bloqueada, por exemplo, por secreções excessivas. (B) A parede das vias aéreas está espessada, por exemplo, por edema ou hipertrofia muscular, (C) A anormalidade é fora das vias aéreas; neste exemplo, o parênquima pulmonar está parcialmente destruído e a via aérea estreitou-se em virtude da perda de tração radial, como é o caso do enfisema. Fonte: West, 1996.

A bronquite crônica é uma inflamação com produção excessiva de muco pela árvore brônquica, com tosse produtiva crônica ou recorrente durante pelo menos três meses por ano, por dois anos consecutivos. (Reid 1954; Hogg 2004; Ministério da Saúde 2010).

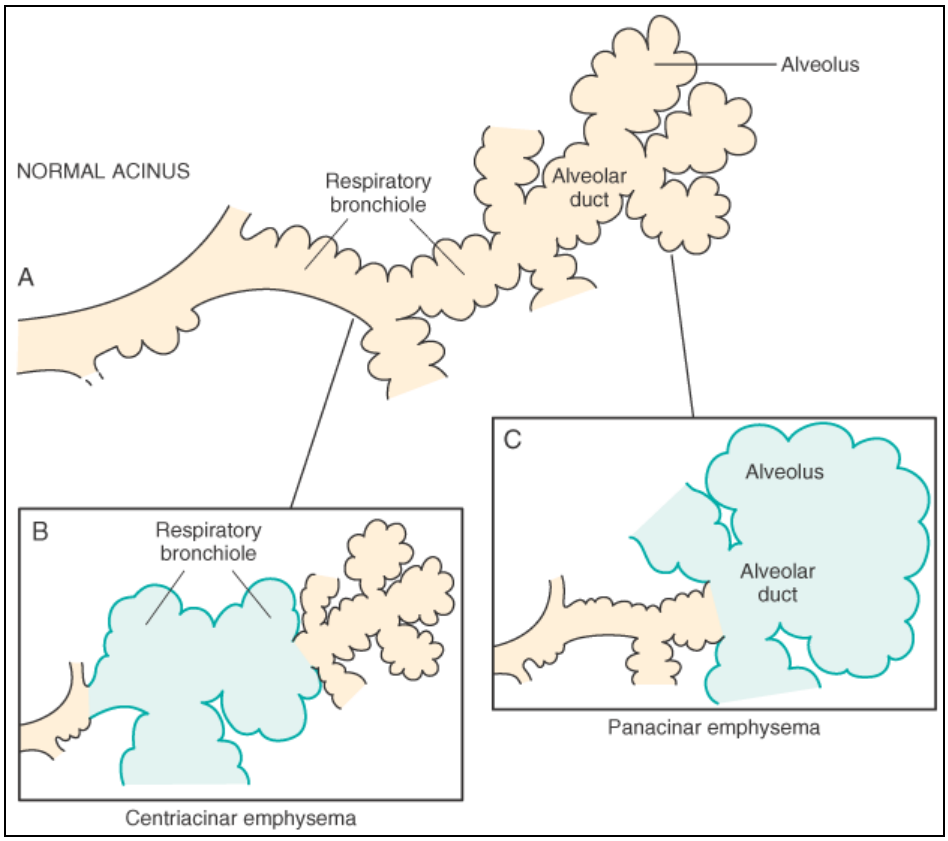
O enfisema pulmonar pode ser definido de acordo com o acometimento da anatomia dos ácinos pulmonares, indicando a etiologia, comportamento fisiopatológico da apresentação da doença e classificação em subtipos. Entre os subtipos estão o centroacinar, pan-acinar (Figura 2) e o pericicatricial (Palombini *et al.* 2001; Bethlem 2002; Rubin *et al.* 2006; Ministério da Saúde 2010).

O enfisema centroacinar é comum no lobo superior, associado à bronquite e à presença de bolhas. O pan-acinar predomina em bases com destruição de pequenos vasos e capilares pulmonares e de parede alveolar. No pericicatricial, a cicatriz se origina em outras doenças, atraindo os tecidos vizinhos, gerando hiperdistensão (Bethlem 2002; Goodman e Snider 2002).

Macroscopicamente, o enfisema pan-acinar pode gerar pulmões volumosos que, em geral, se superpõem ao coração. Já no centroacinar, os pulmões são pálidos e volumosos em estágios avançados da doença. Microscopicamente, observam-se alvéolos alargados, separados por septos finos, com fibrose centroacinar focal e desaparecimento das fixações dos alvéolos à parede externa das pequenas vias aéreas. No enfisema pan-acinar, há alargamento difuso dos ácinos, habitualmente estendendo-se desde a região hilar até a periferia dos

pulmões, com perda das paredes alveolares e destruição de parte do leito capilar (Kim *et al*. 1991; Palombini *et al.* 2001; Kumar *et al.* 2008).

Figura 2 –Enfisema centroacinar e panacinar.



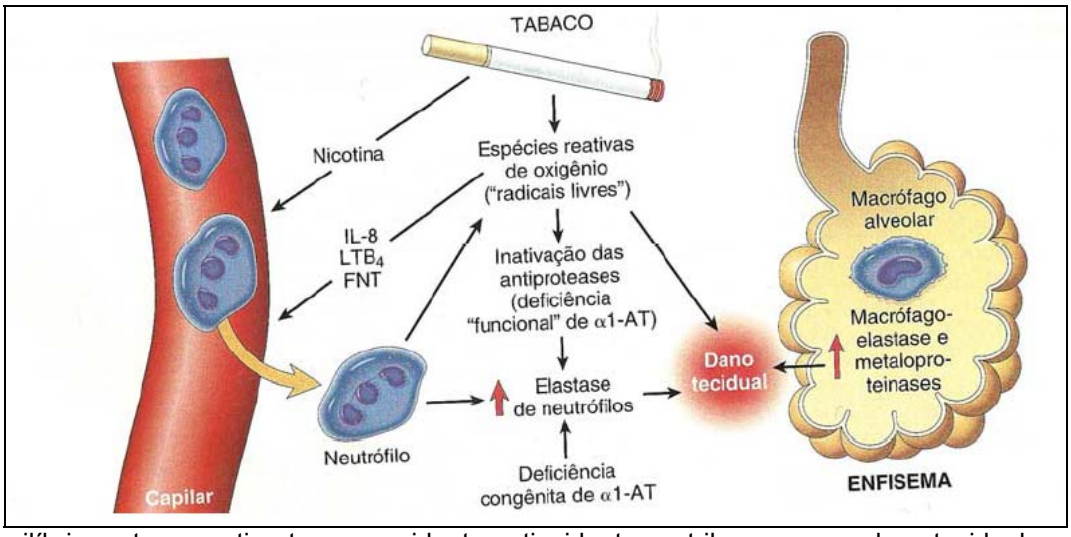
(A) Estruturas normais do ácino pulmonar. (B) Enfisema centroacinar com dilatação dos bronquíolos respiratórios. (C) Enfisema pan-acinar com distensão dos ductos alveolares e alvéolos.
Fonte: Robbins *et al*., 2005.

A DPOC é o resultado da interação entre fatores genéticos e diferentes estímulos ambientais, que incluem o tabagismo e a queima de biomassa. O tabagismo é o principal fator causal, respondendo por 80-90% dos casos. Exposições a poluentes ambientais e ocupacionais, fatores genéticos como a deficiência de alfa-1-antitripsina, hiperresponsividade das vias aéreas, determinados eventos perinatais e doenças da infância, infecções broncopulmonares recorrentes e fatores dietéticos também são fatores de risco. (Fletcher e Peto 1977; GOLD *et al.* 1996; Cheng *et al.* 2004; Campos 2006; Arbex *et al.* 2012).

As células inflamatórias são fonte de proteases e oxidantes, causando lesão tecidual e inativação de antiproteases. Também liberam radicais livres que amplificam o desequilíbrio oxidante-antioxidante (Figura 3).

De maneira geral, quatro mecanismos são responsáveis pelas alterações funcionais na DPOC: estresse oxidativo, inflamação, desequilíbrio do sistema protease-antiprotease e apoptose. A contribuição relativa destes mecanismos é variável, explicando, possivelmente, as diferentes formas de apresentação da doença. (Mac Nee 2005; Sharafkhaneh *et al.* 2008).

Figura 3 – Patogênese do enfisema pelo tabaco



Desequilíbrio protease-antiprotease e oxidante-antioxidante contribuem para o dano tecidual.
Fonte: Kumar *et al*., 2008.

1.1.1 Epidemiologia

A DPOC é uma das principais causas de morbimortalidade em todo o mundo, sendo um sério e crescente problema de Saúde Pública, responsável por incapacitação, problemas pessoais e sociais, mortes, elevados custos financeiro e social. É caracterizada pela deterioração progressiva e irreversível da função pulmonar, fazendo com que cerca de 40 a 70% dos doentes morram em até cinco anos após o diagnóstico (Mannino 2002).

Ao contrário de diversas doenças crônicas, como câncer, doença coronariana e acidente vascular encefálico, que estão em declínio, a progressão da DPOC é alarmante, exibindo números crescentes (Pauwels *et al.* 2001; Menezes *et al.* 2005).

Segundo estimativas da Organização Mundial da Saúde (OMS), ela será a terceira causa de morte em todo o mundo em 2030. Mais que três milhões de pessoas morreram em 2005, correspondendo a 5% de todas as mortes. No Brasil, a

DPOC é a sétima causa de mortalidade, responsável por mais de 37.000 óbitos por ano (Datasus 2013; Word Health Organization [WHO]. 2014).

1.1.2 Manifestações Sistêmicas

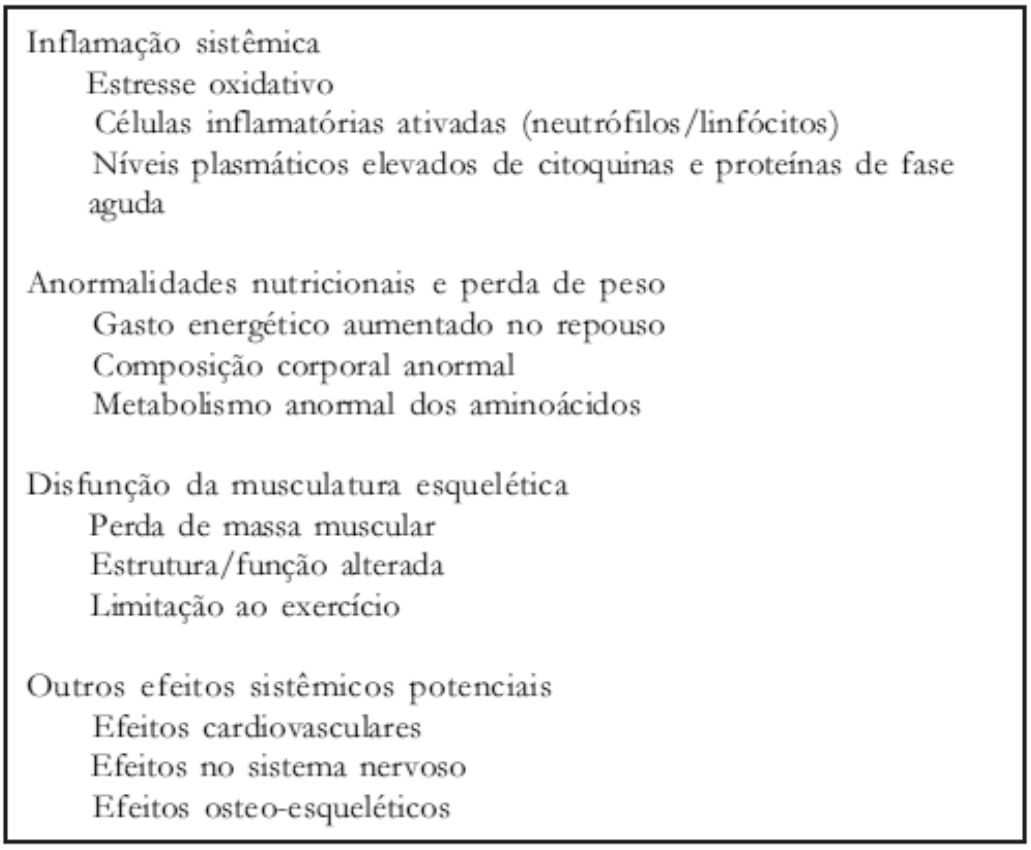
As manifestações sistêmicas da DPOC têm sido descritas há anos. No entanto, ainda é incerto se elas representam consequências dos distúrbios pulmonares, inflamação e remodelamento estrutural, ou se ela deve ser considerada uma doença sistêmica. (Decramer *el al.* 2005; Huertas e Palange 2011). A história natural da doença revela numerosas manifestações extrapulmonares e comorbidades, fatores que pioram o prognóstico e qualidade de vida dos pacientes (Agusti e Soriano 2008; Barnes e Celli 2009).

Além das anormalidades pulmonares, há efeitos em órgãos distantes dos pulmões (Agustí *et al.* 2003; Gan *et al.* 2004). Há décadas, muitos efeitos extrapulmonares têm sido descritos, como anormalidades renais e hormonais, perda de massa muscular, anemia e redução de células progenitoras na medula óssea (Palange 1998; Remels *et al.* 2007; Bolton *et al.* 2004; John *et al.* 2005; Palange *et al.* 2006).

A DPOC compromete os sistemas cardiovascular, musculoesquelético, endócrino e nervoso central, além de ser fator de risco para outras doenças graves. Mesmo pequenas reduções na função pulmonar determinadas pela DPOC aumentam o risco de câncer de pulmão, de arritmias ventriculares e de eventos coronarianos, e dobram a mortalidade por problemas cardiovasculares, independentemente do tabagismo (Hole *et al*.1996; Wasswa-Kintu *et al*. 2005; Sin *et al.* 2005).

De maneira geral, os efeitos extrapulmonares (Figura 4) incluem inflamação sistêmica, anormalidades nutricionais, perda de peso não intencional, disfunção músculoesquelética, doença cardíaca associada, osteoporose, ansiedade, depressão e efeitos adicionais em alguns órgãos (Decramer *et al.* 2005).

Figura 4 – Efeitos sistêmicos da DPOC.



Fonte: Agustí *et al*., 2003.

1.1.2.1 Inflamação sistêmica

A inflamação sistêmica relaciona-se com a maioria dos efeitos sistêmicos da DPOC (Langen *et al.* 2001; Wouters 2002b; Biskobing 2002; Sin e Man 2003; Agusti *et al.* 2003).

Muitos mecanismos foram propostos com o objetivo de explicar estes eventos. Mediadores inflamatórios nos pulmões, como a interleucina 8 (IL-8) e o fator de necrose tumoral α (TNF-α), quando em níveis elevados, tenderiam a atravessar a membrana alvéolo-capilar e atingir a circulação periférica, estimulando células inflamatórias circulantes (Oudijk *et al*. 2003; Agusti *et al*. 2003). No entanto, alguns estudos não conseguiram demonstrar uma relação entre o TNF-α e IL-8 no escarro e plasma, sugerindo que a inflamação sistêmica na DPOC não está relacionada com a inflamação pulmonar nestes pacientes (Vernooy *et al.* 2002; Hurst *et al.* 2005). Além disso, a reação inflamatória poderia ser secundária à hipóxia tecidual, reações induzidas por polissacarídeos, fumaça do cigarro, hiperinsuflação

pulmonar, disfunção músculoesquelética e envolvimento da medula óssea (Barbu *et al.* 2011) .

1.1.2.2 Anormalidades nutricionais e perda de peso

A perda de peso resulta do desequilíbrio entre ingestão e consumo calórico. A base fisiopatológica desta perda ainda não é muito bem entendida, podendo ser explicada pela alteração no metabolismo dos aminoácidos e maior atividade metabólica pela inflamação sistêmica, hipóxia tecidual e medicamentos (Schols *et al.* 1993; Amoroso *et al.* 1993; Sridhar 1995; Schols *et al.* 1996). Aproximadamente 10-15% dos pacientes com doença leve e moderada e 50% dos pacientes com doença grave apresentam perda de peso, principalmente pela perda de musculatura esquelética, decorrente do sedentarismo e distúrbios nutricionais que podem levar à caquexia, hipóxia tecidual, inflamação sistêmica, apoptose da musculatura estriada esquelética, estresse oxidativo, regulação alterada do óxido nítrico e alterações hormonais e eletrolíticas (Amoroso *et al.* 1993; Creutzberg *et al.* 1998; Pouw *et al.* 1998; Sridhar 1999; Schols 2000; Decramer *et al.* 2005).

1.1.2.3 Disfunção da musculatura esquelética

A disfunção muscular esquelética é comum em pacientes com DPOC, sendo caracterizada por alterações anatômicas específicas, como mudanças no tipo e composição de fibras e atrofia e, as alterações funcionais, como força e resistência que contribuem para a redução da capacidade funcional e qualidade de vida, contribuindo significativamente para um pior prognóstico (American Thoracic Society 1999; Decramer *et al.* 2005).

Os músculos respiratórios, particularmente o diafragma, parecem comportar-se de forma bastante diferente dos músculos esqueléticos. A diferença é, provavelmente, devido às diferentes condições sob a qual ambos trabalham nestes pacientes. Os músculos esqueléticos são geralmente subutilizados, enquanto o diafragma está constantemente trabalhando contra um aumento de carga (Levine *et al.* 1997; Sauleda *et al*. 1998).

Os mecanismos da disfunção muscular esquelética são incertos, mas destacam-se: o sedentarismo, com consequente dispneia de esforço pela

incapacidade física que acelera a perda de massa muscular, reduz a capacidade de gerar força e a resistência muscular; a hipóxia tecidual, que suprime a síntese de proteínas, causando perda de aminoácidos e reduzindo a expressão das isoformas da cadeia pesada da miosina; a inflamação sistêmica, pela ativação do fator de transcrição NF-κB (NF-κB), degradando as cadeias pesadas da miosina resultando em apoptose e estresse oxidativo (EO) que causa fadiga muscular e acelera a proteólise (Rennie *et al*. 1983; Reid *et al.* 1992b; Li *et al.* 1998; Agustí *et al.* 2002; Agustí *et al.* 2004, Campos 2006). A perda da massa muscular é caracterizada por diminuição involuntária do peso corporal em um curto período de tempo devido à degradação acelerada e redução da síntese de proteínas (Castaneda 2002).

A caquexia é uma síndrome associada à perda de massa muscular, reduzida ingestão de alimento, diminuição de atividade física e acelerada degradação de proteínas (Langstein e Norton, 1991; Walsmith e Roubenoff 2002). Pode ser decorrente da DPOC e caracteriza-se pela perda de 10% da massa corporal dentro de seis meses ou 5% em um mês (Argilés 2005; Delano e Moldawer 2006).

A disfunção muscular esquelética limita significativamente a capacidade de exercício, decorrente, em parte, da redução da massa muscular e disfunção da musculatura remanescente, ocasionada pelas alterações de trocas gasosas pulmonares e aumento dos níveis séricos de TNF-α, sinalizando para a apoptose (Godoy *et al.* 1996; Agustí *et al.* 2003).

Portanto, vários são os fatores que contribuem para o aparecimento da fadiga e intolerância ao esforço: fraqueza e perda de massa muscular decorrente do desequilíbrio entre a síntese e degradação de proteínas, diminuição de testosterona e hipóxia crônica (Rennie *et al.* 1983; Aasebo *et al.* 1993; Casaburi 1998); alteração das propriedades contráteis e tipo de composição das fibras, como resultado da perda de força, com substituição das fibras de contração lenta pelas fibras de contração rápida e proporção aumentada de fibras híbridas (Satta *et al.* 1997); diminuição do metabolismo energético pelos baixos níveis de glicogênio, ATP e creatina fosfato e descapilarização pelo baixo aporte de oxigênio (Jakobsson *et al.* 1990; Wust e Degens 2007); e fadiga muscular pela descapilarização e diminuição da capacidade oxidativa (Degens e Veerkamp 1994).

1.1.2.4 Outros efeitos sistêmicos

Os mecanismos envolvidos no desenvolvimento da osteoporose nos pacientes com DPOC grave não são claros, mas envolvem diversos fatores, como o uso de glicocorticóides, deficiência de vitamina D, tabagismo, baixo Índice de Massa Corporal (IMC), inflamação sistêmica e sedentarismo, levando a diminuição da massa muscular (Ionescu e Schoon 2003; Fernades e Bezerra 2006).

A DPOC aumenta o risco de doença cardiovascular. Estudos mostram que a função endotelial em ambos os pulmões e a circulação renal são anormais. (Dinh-Xuan *et al.* 1991; Baudouin *et al.* 1992; Howes *et al.* 1995; Sin e Man 2003).

Os mecanismos para a instalação da doença cardiovascular não são claros, porém, sabe-se do envolvimento da inflamação sistêmica e estresse oxidativo neste processo (Sin e Man 2003; Tonon *et al.* 2013b)

A DPOC afeta os vasos sanguíneos pulmonares, o ventrículo direito, assim como o ventrículo esquerdo, levando ao desenvolvimento de hipertensão pulmonar, hipertrofia cardíaca direita, o *cor pulmonale*, e disfunção ventricular direita e esquerda. Evidências apontam também para regurgitação tricúspide e disfunções valvares múltiplas (Gupta *et al*., 2011).

Indivíduos com DPOC sofrem, frequentemente, de ansiedade e ataques de pânico, associados com episódios de dispnéia. São propensos à depressão, que pode ser agravada pelo isolamento e limitação da capacidade funcional. A ansiedade tem sido identificada em pacientes com taxas variando de 21% a 96% dos casos. Distúrbios depressivos estão presentes em 27% a 79% desses indivíduos. Estas manifestações podem ter sua origem na baixa qualidade de vida dos pacientes. (Lacasse *et al.* 2001; Godoy e Godoy 2002; Laurin *et al.* 2007).

1.2 ESTRESSE OXIDATIVO MUSCULAR

O EO desempenha um papel importante na DPOC, especialmente nos pulmões. A formação de radicais está associada com a inflamação local, a fumaça do cigarro e a outras substâncias que podem resultar na inativação de antiproteases, dano epitelial do espaço aéreo, hipersecreção de muco, influxo

aumentado de neutrófilos para o tecido pulmonar, e a expressão de mediadores pró-inflamatórios (Rahman *et al.* 1996; Repine *et al*. 1997; MacNee 2005; Stevenson *et al.* 2006; Campos 2006; Takahashi *et al.* 2008)

Entretanto, além do comprometimento pulmonar, perda de massa muscular esquelética e disfunção muscular são características da DPOC (Gosker et al. 2000; Mador e Bozkanat 2001).

Do mesmo modo, o EO também pode estar envolvido nas complicações sistêmicas da DPOC. O EO ocorre quando há desequilíbrio entre o balanço de oxidantes, como as espécies reativas de oxigênio (ERO), e antioxidantes, podendo levar à modificação de biomoléculas (Rahman *et al* 1996; Halliwell e Gutteridge 2007).

Experiências *in vitro* demonstraram que os miócitos esqueléticos são capazes de se adaptar ao EO pelo aumento da expressão de enzimas antioxidantes, como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). No entanto, quando este aumento não é mais capaz de impedir a oxidação provocada por radicais, a função muscular pode ser afetada (Figura 5), tanto quanto em seu estado trófico, quanto na contratilidade (Franco *et al.* 1999).

Figura 5 – Alteração no estado trófico e contratilidade muscular em consequência do estresse oxidativo.

Músculo Esquelético	
Estado Trófico	Contratilidade e Fadigabilidade
- Diminuição da síntese protéica muscular	- Inibição da propagação do potencial de ação
- Aumento da degradação de proteína muscular	- Modificação da função do retículo sarcoplasmático
- Comprometimento da regeneração muscular	- Inibição de enzimas metabólicas envolvidas na produção de ATP
- Aumento da apoptose de núcleos de células musculares	- Inibição de proteínas contráteis

Fonte: Adaptado de Langen *et al*., 2003.

Em pacientes com DPOC, anormalidades da musculatura esquelética levam à redução da capacidade de exercício, má qualidade de vida e aumento da mortalidade (Swallow *et al*. 2007), sendo que o EO sistêmico e local podem contribuir para estas alterações musculares. Assim, o EO tem sido

investigado como um potencial mecanismo na disfunção muscular na DPOC (Schols *et al.* 1996; Barreiro *et al.* 2003; Boots *et al*. 2003; Barnes *et al.* 2003 Barreiro *et al.* 2005b; Barreiro *et al.* 2005a; Van Helvoort *et al.* 2006; Marin-Corral *et al.* 2009;. Barreiro *et al.* 2010).

Rabinovich e colaboradores (2001) e Couillard e colaboradores (2003) relataram que os níveis de glutationa reduzida (GSH) e oxidada (GSSG) e o Malonaldeído (MDA), um marcador de lipoperoxidação, não apresentaram nenhuma diferença significativa, quando medidos no vasto lateral de indivíduos com DPOC e saudáveis, no repouso.

Entretanto, Allaire e colaboradores (2002) e Koechlin e colaboradores (2005) encontraram acúmulo de lipofuscina, um marcador de dano oxidativo, em biópsias de músculo vasto lateral de pacientes com DPOC. Gosker e colaboradores (2005) mostraram que a capacidade antioxidante total e os níveis de ácido úrico, do músculo vasto lateral de pacientes com DPOC, estavam aumentados, e os níveis de MDA normais, sugerindo que o sistema antioxidante é acionado, posteriormente, por níveis elevados de espécies reativas de oxigênio.

Em um estudo, Barreiro e colaboradores (2008) observaram carbonilação protéica no músculo vasto lateral de pacientes com DPOC, assim como Fermoselle e colaboradores (2012) que observaram, além do aumento na carbonilação de proteínas, aumento na produção do ânion superóxido (O_2^-), da atividade da superóxido dismutase (SOD), da ubiquitinação de proteínas e da atividade de atrogina-1, e diminuição do tamanho das fibras de contração rápida no músculo quadríceps de homens caucasianos com DPOC.

Além disso, no músculo esquelético de indivíduos com DPOC, as mitocôndrias e as proteínas mitocondriais foram mais suscetíveis a danos oxidativos do que outros componentes celulares, sugerindo que os danos protéicos podem provocar aumento do metabolismo oxidativo (Haycock et al. 1996; Gosker *et al*. 2000).

Mattson e colaboradores (2002) avaliaram os níveis de MDA e glutationa total (GT), e a atividade da GPx e de glutationa redutase (GR) no vastolateral e gastrocnêmio de hamsters Syrian Golden com enfisema pulmonar, induzidos, experimentalmente, por administração intratraqueal de elastase (25 Unidades Internacionais (UI)/100 g de peso corporal), e observaram, após seis meses, que os níveis de MDA, GT e a atividade da GR aumentaram no

gastrocnêmio dos animais com enfisema., enquanto que a atividade da GPx diminui no vasto lateral. Zhang e colaboradores (2010) mostraram, após cinco meses, aumento da lipofuscina, capacidade antioxidante total, e das atividades da SOD e CAT no gastrocnêmio de ratos Sprague-Dawley com enfisema pulmonar induzidos por administração intratraqueal de elastase (40 UI/100 g de peso corporal).

Fermoselle e colaboradores (2011), em um modelo experimental de camundongos com enfisema induzido por instilação de elastase (20UI/100g de peso corporal), não detectaram aumento dos níveis de MDA, atividade da SOD e da GPx, após 34 semanas.

Barreiro e colaboradores (2012) utilizaram camundongos AKR/J (camundongos amplamente utilizados na pesquisa do câncer por sua elevada incidência de leucemia e em imunologia como fonte de antígeno Thy1 e que expressam o retrovírus endógeno AKV, causador de linfoma no timo, em todos os tecidos, além de depleção lipídica adrenocortical) em um modelo de enfisema pulmonar induzido por inalação de fumaça do cigarro não filtrado (24 horas/dia) por seis meses e observaram aumento na carbonilação de proteínas, níveis de MDA e Hidroxinonenal (HNE), nitrotirosina e da superóxido dismutase manganês (Mn-SOD) no gastrocnêmio.

Em 2013, Tonon e colaboradores, ao induzirem enfisema pulmonar experimentalmente por meio da instilação intratraqueal de papaína (40mg/mL), em hamsters, mostraram aumento nos níveis de MDA, da lipoperoxidação por quimiluminescência, e da atividade proteolítica no músculo gastrocnêmio de hamsters, sugerindo que a atrofia muscular pode ser mediada pela atividade proteolítica, com possível envolvimento do EO.

Foi demonstrado em estudos em animais, que o estresse oxidativo induzido *in vivo* causa modificação nas miofibrilas, as quais são rapidamente degradadas por proteases, levando a atrofia, e que a sobrecarga de cálcio e a deficiência de vitamina E relacionam-se com a perda de massa muscular e necrose (Soares *et al.* 1993; Thomas *et al.* 1993; Nagasawa 1997).

As proteínas podem ser lesadas pelo ataque direto de radicais livres, ou por meio de ligações entre os produtos finais da cadeia de lipoperoxidação, como o 2,4-hidroxinonenal, ou por glicação (Halliwell e Gutteridge 2007). Além disso, vários estudos apontam para o estresse oxidativo como modulador de vias

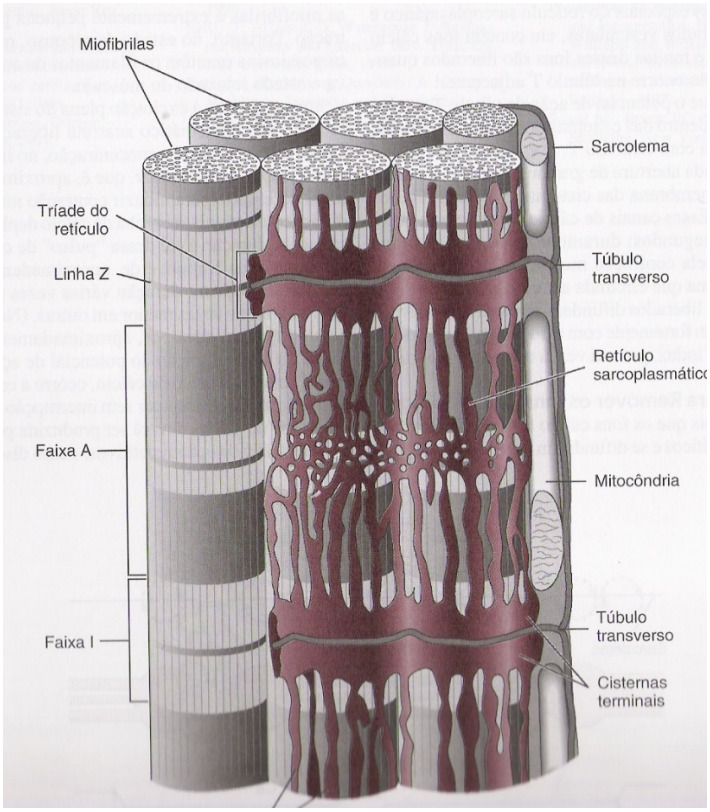
proteolíticas dependentes de cálcio (Goll *et al.* 2003; Purintrapiban *et al.* 2003; Du *et al*. 2004) e independentes de cálcio (Gomes *et al.* 2001; Li *et al*. 2003).

Tonon e colaboradores (2013) observaram uma forte associação entre a atividade proteolítica e marcadores da peroxidação lipídica, bem como dano muscular, em um modelo de enfisema pulmonar induzido por papaína (40 mg/ml). Estudos demonstraram, no músculo esquelético, menor liberação de cálcio do retículo sarcoplasmático e redução da sensibilidade ao cálcio após exposição a peróxido de hidrogênio (H_2O_2), sugerindo que as ERO poderiam prejudicar a contratilidade muscular (Brotto e Nosek 1996; Andrade *et al.* 1998).

Vários sistemas proteolíticos contribuem para a degradação de proteínas musculares Os mais importantes são: proteólise mediada por proteases lisossomais, por calpaína, por caspases e pelo sistema proteassomal. Evidências apontam que estes sistemas desempenham papel importante na perda protéica durante a atrofia (Ikemoto *et al.* 2001; Du *et al.,* 2004; Powers *et al*. 2010; Tonon *et al.* 2013). Alguns trabalhos indicam que as calpaínas e a caspase-3 são capazes de produzir dissociação dos complexos de actina e miosina, sinalizando para a degradação protéica de miofilamentos durante a perda de massa muscular (Goll *et al.* 2003; Du *et al.* 2004).

Segundo Guyton (2011), o músculo estriado esquelético é formado por um conjunto de fibras, compostas por miofibrilas, dispostas em sarcômeros em série. O sarcômero é a região entre duas linhas Z e, em sua maioria, formado por complexos protéicos de actina e miosina (Figura 6). Sendo assim, a partir do dano a estas proteínas, mediado pelo estresse oxidativo, as proteases lisossomais seriam capazes de degradá-las. Da mesma forma, actina e miosina dissociadas são oxidadadas e eficientemente degradadas pelo sistema proteassomal (Tidball e Spender 2002).

Figura 6 – Estrutura da fibra muscular, miofibrilas, sarcômeros e sistema tubular.



Fonte: Guyton *et al.* 2011.

1.3 EVIDÊNCIAS SOBRE ADAPTAÇÕES MUSCULARES

O músculo esquelético é um tecido altamente adaptável, que responde a mudanças em relação ao tipo de atividade ou de tensões mecânicas e ambientais aos quais é submetido. As vias de sinalização envolvidas nessas múltiplas adaptações têm sido descritas, mas há uma carência de informações sobre os fatores responsáveis por iniciar estes processos. ERO são produzidas em vários locais no músculo esquelético e, há cada vez mais evidências de que estas espécies desempenhem papéis na modulação das vias de sinalização redox-sensíveis, que são importantes para a adaptação muscular (Jackson 2009).

Durante os anos de 1980 e 1990, estudos revelavam que as ERO induzidas por atividade ou inatividade muscular seriam citotóxicas para as fibras do músculo estriado esquelético. Entretanto, pesquisas recentes mostram que o aumento na produção de ERO desempenha papel importante na regulação de vias de sinalização necessárias para promover adaptação da fibra muscular em resposta ao exercício e a inatividade (Powers *et al*. 2010).

Nas células musculares, quando as ERO são produzidas em baixas concentrações, exercem importantes papéis fisiológicos, como aumento da permeabilidade ao Ca^{2+}, aumento da força de contração e regulação da expressão gênica. As contrações musculares podem aumentar a formação de NO, o qual tem sido apontado como responsável por importantes papéis fisiológicos, como regulação metabólica, regulação do fluxo sangüíneo, expressão gênica e modulação no processo de contração muscular (Reid *et al*. 1992; Schreck *et al.* 1992; Kobzik *et al.* 1994; Andrade *et al*. 1998; Bredt 1999; Reid 2001).

A excessiva produção de ERO e NO contribuem, significativamente, para a redução da força de contração, aumenta a fadiga, consequentemente, para o aumento das lesões musculares. Embora essas espécies sejam requeridas para manutenção de alguns importantes processos fisiológicos, o aumento, pode ser potencialmente tóxico, alterando o equilíbrio redox intracelular para um estado mais oxidado (Alessio e Goldfarb 1988; Reid *et al*. 1992; Galler *et al.* 1997; Andrade *et al*. 1998; Clanton *et al.* 1999).

Portanto, enquanto altas concentrações de radicais livres podem lesar componentes celulares, níveis baixos a moderados podem desempenhar múltiplas funções regulatórias em vias de sinalização celular, como o controle da expressão gênica e a modulação da função muscular. A sinalização da expressão por ERO contribui para a adaptação de fibras musculares em resposta ao aumento ou diminuição da atividade muscular. Estas diferenças quanto à função das ERO devem-se tanto à magnitude, quanto ao padrão temporal da produção de radicais livres (Powers 2010).

Sendo assim, pesquisas com modelos experimentais são realizadas com o intuito de compreender as possíveis adaptações musculares esqueléticas na DPOC e o envolvimento do estresse oxidativo neste processo.

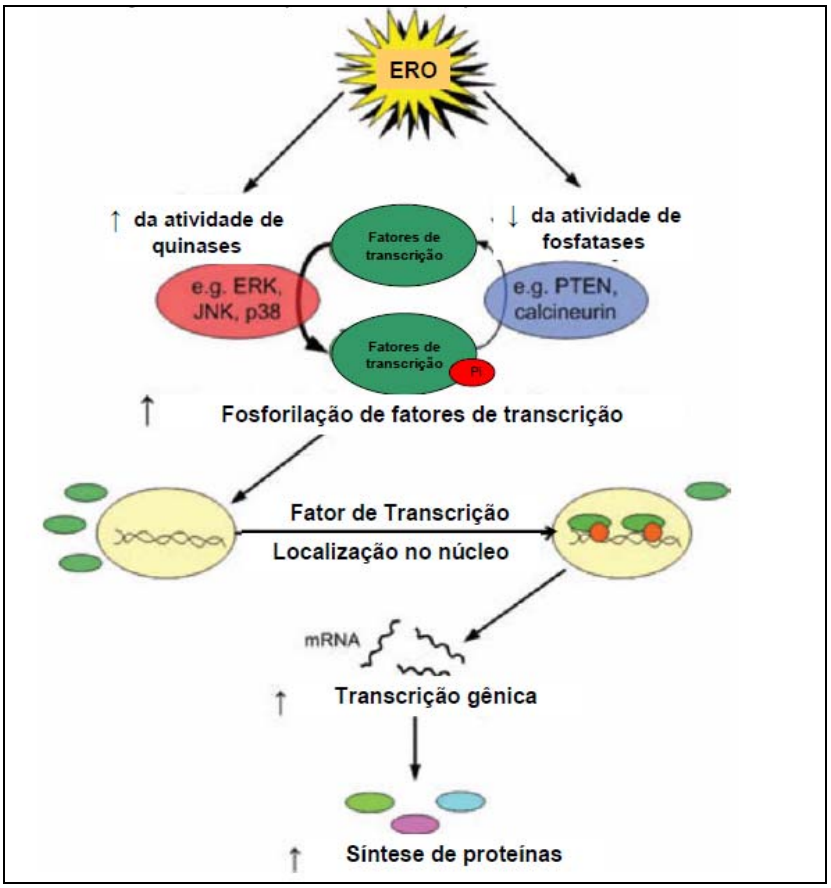
Jackson e McArdle (2011) relataram, em estudos prévios, que um único período de atividade contrátil no músculo esquelético de ratos pode aumentar a atividade de enzimas antioxidantes, como a SOD e CAT em conjunto com as proteínas de choque térmico, HSP60 e HSP70, e que estas alterações se reproduzem no músculo humano. (McArdle *et al.* 2001; Khassaf *et al*. 2001; McArdle *et al.* 2004).

Além disso, outros estudos mostram a sinalização redox em diversos processos no músculo esquelético, como a manutenção da produção de força

durante as contrações, a captação de glicose e a sinalização da insulina (Jackson 2009).

Assim, o EO provoca modificação no potencial redox da célula que conduz a mudanças nos padrões de regulação da sinalização da expressão gênica. O principal mecanismo pela qual a sinalização redox controlaria a expressão gênica seria por meio das quinases e fosfatases pela fosforilação e desfosforilação de proteínas responsáveis pelo aumento ou a ativação de fatores de transcrição (Figura 7) (Chiarugi e Cirri 2003; Torres e Forman 2003, Powers 2010).

Figura 7 – Interação das quinases e fosfatases na regulação de fatores de transcrição e subsequente síntese de proteínas



Fonte: Powers, 2010.

As ERO também podem ser reconhecidas como mediadores de algumas respostas adaptativas do músculo esquelético através da ativação de fatores de transcrição, como o NF-κB, o ativador protéico 1(AP-1) e fator de choque térmico 1 (Cotto e Morimoto 1999; Jackson *et al.* 2002; Ji *et al.* 2004; Ristow *et al.* 2009).

Evidências indicam que o TNF-α regula a expressão de mais de 150 genes, entre eles, genes associados a miogênese, e isso parece ser fundamental no entendimento da adaptação celular do músculo estriado, tanto em estados fisiológicos, quanto patológicos, principalmente no que diz respeito ao músculo estriado esquelético (Kandarian e Jackman, 2006; Kramer e Goodyear 2007; Bakkar *et al*. 2008).

Apesar das ERO poderem promover a ativação do NF-κB, a capacidade de ligação ao DNA do NF-κB oxidado é diminuída, sugerindo que as ERO também podem inibir a sua atividade transcricional (Kabe *et al.* 2005). Portanto, pode-se perceber que o estresse oxidativo pode tanto promover, quanto inibir a ativação transcricional do NF-κB, o que tem levado a um debate com relação ao seu controle redox e sinalização (Pantano *et al*. 2006).

Indivíduos com DPOC apresentam alterações na composição e estrutura do músculo esquelético, incluino diminuição do número de fibras do tipo I e aumento das fibras do tipo II, expressão da cadeia pesada da miosina e atrofia das fibras tipo I e II (Hughes *et al.* 1983; Sato *et al* 1997; Satta *et al.* 1997; Jobin *et al.* 1998; Whittom *et al.* 1998; Maltais *et al.* 1999; Gosker *et al*. 2002).

Mattson e colboradores (2004) demonstraram, após seis meses, atrofia das fibras de contração rápida do tipo IIA e IIB no músculo esquelético de hamsters com enfisema induzido por elastase (25UI/100g de peso corporal) Especificamente, há atrofia e aumento do número das fibras IIB e decréscimo do tipo IIA.

A redução das fibras do tipo IIA, mais oxidativas, pode contribuir para a diminuição da resistência, enquanto o aumento das fibras do tipo IIB pode ser responsável pela preservação da produção de força máxima e ausência de atrofia muscular global (Mattson *et al.* 2002a).

Em contrapartida, Lamb e Westerblad (2011) relataram que as fibras de contração rápida são altamente sensíveis às ERO, afetando o desempenho muscular pela alteração da sensibilidade ao Ca^{2+}. Ao contrário, as fibras de contração lenta são bastante insensíveis ao aumento de ERO e isso pode contribuir para a manutenção da força de contração destas fibras.

Em 2012, Rinaldi e colaboradores realizaram um estudo sobre a progressão do enfisema em camundongos expostos à fumaça de cigarro e

concluíram que a exposição de camundongos durante seis meses à fumaça de cigarro levou à substituição de fibras do tipo IIA por IIB no músculo sóleo.

Levine e colaboradores (2003) relatam que há remodelamento do diafragma de indivíduos com DPOC, consistindo na transformação das fibras de contração rápida em lenta, bem como nas adaptações dentro de cada tipo de fibra. Os mesmos autores ainda relacionam estas mudanças à variação do volume expiratório forçado no primeiro segundo (VEF1) e diminuição de força muscular.

O músculo vasto lateral de indivíduos com DPOC grave apresenta alteração de fibra do tipo I para II (Bustamante *et al.* 2008; Fermoselle *et al*. 2012; Levine *et al.* 2013).

No entanto, no diafragma, acontece o inverso, transformação de fibras de contração rápida para lenta (Levine *et al.* 1997; Levine *et al.* 2002; Levine *et al.* 2003; Barreiro *et al.* 2005b; Levine *et al.* 2008; Marin-Corral *et al*. 2009; Levine *et al.* 2013).

Clanton e Levine (2009), em um estudo de revisão em modelos de enfisema pulmonar, descreveram que o diafragma e outros músculos respiratórios podem sofrer remodelamento a partir das alterações das características de resistência e aumento da capacidade oxidativa do músculo. Em modelos animais há perda dos sarcômeros em série. Em humanos, as adaptações incluem aumento do número das fibras de contração lenta, tipo I, com diminuição das fibras do tipo II, e atrofia de ambos os tipos de fibra.

Embora existam relatos de diminuição do teor de cadeias pesadas da miosina, tornando os músculos predominantemente oxidativos e mais eficientes, estudos reportam redução da sensibilidade ao Ca^{2+} e redução do recolhimento elástico miofibrilar. (Ottenheijm *et al*. 2005; van Hees *et al*. 2009; Clanton e Levine, 2009).

Considerando que esta mudança do tipo de fibras no diafragma é benéfica, tornando o diafragma mais resistente à fadiga, a redução da geração da força relacionada à perda do conteúdo de miosina, provavelmente contribui para a fraqueza. Além disso, o diafragma é exposto ao estresse oxidativo e lesão de sarcômeros, que podem ativar vias proteolíticas, levando a perda protéica (Ottenheijm *et al.* 2008).

Gagnon e colaboradores (2014), ao analisarem o quadríceps de indivíduos com DPOC, não encontraram alterações no número de capilares em contato com as fibras do tipo I e II e na área de superfície destas fibras.

As modificações oxidativas induzidas por ERO em proteínas musculares parecem ser a chave para as alterações na estrutura, metabolismo e contração muscular de indivíduos e modelos experimentais de DPOC (Barreiro 2014).

Em geral, os músculos dos membros inferiores são mais afetados que os músculos inspiratórios, provavelmente como resultado do desuso ou descondicionamento (Mador e Bozkanat , 2001).

1.4 MODELOS EXPERIMENTAIS DE DPOC

Modelos animais têm sido fundamentais, nos últimos anos, para o entendimento da DPOC. A utilização destes modelos apresenta um grande número de vantagens e algumas desvantagens, nas circunstâncias experimentais. A seleção deve ser realizada de acordo com o modelo que é mais adequado para a hipótese a ser testada. Alterações intrapulmonares com lesão de proteínas e outros componentes são utilizadas na indução da doença em animais (Mahadeva e Shapiro 2002; Wright e Churg 2002).

Com o intuito de promover as lesões, substâncias exógenas como a elastase pancreática suína, papaína, elastase neutrofílica humana, proteinases 3, dióxido de nitrogênio, ozônio e a fumaça do cigarro podem ser administradas por instilação intratraqueal ou inalação. Também são descritos modelos com animais *knockou*t e autoimunes (Snider *et al.* 1986; Wright e Churg 1990; Chitano *et al.* 1995; Keil *et al.* 1996; Hautamaki 1997; Taraseviciene-Stewart *et al.* 2005).

Os animais mais utilizados para a indução da DPOC são camundongos, hamsters, ratos, cães e porcos, por meio da administração de proteases e inalação de fumaça de cigarro (Hayes *et al.* 1975; Martorana *et al*. 1982; Wright e Churg 2002; Monteiro *et al*. 2004; Vidal *et al.* 2011).

1.4.1 Indução pela Inalação de Fumaça de Cigarro

A exposição crônica de animais à fumaça de cigarro é capaz de reproduzir as alterações anatômicas e fisiopatológicas de humanos com DPOC (Wright e Churg 2010).

O enfisema pulmonar induzido pela fumaça do cigarro já foi estudado em diversas condições experimentais (Churg *et al.* 2008; Valenca e Porto 2008; Wright *et al.* 2008; Zheng *et al.* 2009; Huh *et al.* 2011; Toledo *et al.* 2012). A partir destes estudos conseguiu-se estabelecer critérios para a utilização deste modelo. Variáveis como o número de cigarros e o tempo de exposição diário (superior a seis meses), métodos de exposição, como o animal entrar em contato direto com a fumaça, e a espécie do animal, ainda variam muito entre os trabalhos (Bracke *et al.* 2006; Wright *et al.* 2008; Zheng *et al*. 2009; Jardim *et al.* 2010; Zhang *et al*. 2011;Rinaldi *et al.* 2012).

1.4.2 Modelos Genéticos

A utilização de modelos genéticos é importante para o estudo da DPOC, uma vez que pode reproduzir aspectos relacionados com a doença humana, principalmente em relação à deficiência de α-1-antitripsina. Várias linhagens de camundongos são conhecidas por terem mutações naturais ou induzidas em laboratório que geram condições anormais no desenvolvimento do animal e resultam na DPOC, como a Tight skin (Tsk -/+), Pallid mice (pa/pa) e Beige mice (bg) (March *et al.* 2000; Shapiro 2000).

1.4.3 Indução pela Administração de Proteases

Há aproximadamente 50 anos foi descrito pela primeira vez que a administração intratraqueal de papaína desencadeava enfisema pulmonar. Este fato, combinado aos achados clínicos de pacientes com deficiência da α-1-antitripsina, culminou na base para a hipótese protease-antiprotease para o desenvolvimento da doença. Esta metodologia é simples e facilmente reprodutível, com desenvolvimento de características morfológicas semelhantes às de humanos. Além disso, pode ser considerado um modelo rápido, com alterações da arquitetura pulmonar e

estabilização em algumas semanas (Martorana *et al*. 1982; March *et al.* 2000; Mahadeva e Shapiro 2002; Monteiro *et al.* 2004; Anciães *et al.* 2011).

As enzimas proteolíticas como a elastase pancreática suína e a papaína podem levar à destruição do parênquima pulmonar, com lesão estrutural e inflamação sistêmica, desenvolvendo enfisema panacinar semelhante a de humanos. Modelos experimentais de enfisema pulmonar, com administração destas enzimas, têm sido utilizados (Pushpakom *et al.* 1970; Hyatt *et al.* 2000; Fusco *et al.* 2002; Antunes e Rocco 2011).

A papaína é reconhecida por apresentar uma potente atividade elastolítica, degradando não apenas a elastina, como também o colágeno dos tecidos (Turino *et al.* 2002). Fusco *et al.* (2002) relataram que a destruição do parênquima pulmonar tem início com a agressão provocada pela papaína, com alterações da arquitetura pulmonar, progredindo até estabilizar-se em 40 dias.

Estudos mostram que a instilação intratraqueal de papaína (20mg/kg, dissolvida em 3,5ml/kg de solução fisiológica a 0,9%) pode desenvolver enfisema panacinar, semelhante ao de humanos (Pushpakom *et al.* 1970; Fusco *et al.* 2002; Fló *et al.* 2006; Anciães *et al.* 2011). Em 2011, Anciães e colaboradores instilaram papaína em ratos e detectaram alterações nos parâmetros mecânicos e morfométricos, 28 dias após a instilação, os quais se mantiveram até 40 dias.

A elastase pancreática suína é a mais utilizada entre as proteases, pois é uma enzima elastina específica, que tem induzido o mais reproduzível e consistente alargamento dos espaços alveolares em roedores, resultando em recrutamento agudo de neutrófilos, e acúmulo subagudo de macrófagos nos pulmões, com hemorragia. O alargamento dos espaços alveolares que ocorre pela degradação de elastina, com consequente deposição de fibras elásticas desorganizadas, pode se estabilizar após 28 dias da instilação. (Cantor *et al*. 2000; Shapiro 2000; Kawakami *et al.* 2008; Vidal *et al.* 2011).

Estudos demonstram que a gravidade do enfisema depende da dose de elastase administrada (0,05-0,5mg/100g de peso corporal, com atividade específica da enzima de 26 a 28 UI/mg). No entanto, antes da instilação, deve-se levar em consideração a sobrevida do animal, a qual em doses acima de 0,3 mg/100g de peso corporal, aproxima-se de 50 % (Hayes *et al.* 1974; Kawakami *et al.* 2008; Antunes e Rocco 2011).

Analisando os modelos experimentais da DPOC por fumaça de cigarro, genéticos e por proteases pode-se traçar vantagens e desvantagens (Figura 7) destes métodos (Ribeiro-Paes *et al.* 2012).

Figura 8 – Vantagens e desvantangens dos modelos animais de enfisema pulmonar

Modelo Experimental de DPOC	**Vantagens**	**Desvantagens**	**Referências**
Enfisema induzido por proteases	- Método simples, de fácil aplicação, resultados rápidos e alta reprodutibilidade - Características Morfológicas semelhantes a doença em humanos	- Falta de constituintes inflamatórios - Processo fisiológico diferente de humanos	March *et al*. (2000)
Modelos genéticos	- Reprodução da patologia humana, principalmente em relação a deficiência da α-1-antitripsina - Demonstra o papel das proteases no desenvolvimento da doença	- Os aspectos da patologia são reproduzidos somente individualmente - Necessidade de mais estudos em relação aos aspectos inflamatórios	Shapiro (2000) Fugita e Nakanishi (2007)
Fumaça de Cigarro	- Reprodução dos aspectos relacionados ao processo inflamatório na DPOC - Alterações nas vias aéreas semelhantes a humanos	- Longo tempo para início dos sintomas - Tempo de exposição altamente variável	Zheng *et al.* (2009)

Fonte: Adaptado de Ribeiro-Paes *et al.* 2012.

Como descrito acima, a ação lesiva da elastase e da papaína são consideravelmente diferentes tanto quantitativa quanto qualitativamente levando a diferentes respostas sistêmicas e em tecidos à distância. Considerando este fato, a hipótese de trabalho formulada para este estudo foi que a indução experimental de

enfisema pulmonar por elastase e papaína leva a diferentes modificações musculoesqueléticas em termos de geração de estresse oxidativo, atividade proteolítica e atrofia.

1.5 JUSTIFICATIVA

Considerando que:

1. A DPOC será a terceira causa de morte em todo o mundo em 2030 e mais de três milhões de pessoas morreram em 2005, correspondendo a 5% de todas as mortes. E que, no Brasil, a DPOC é a sétima causa de mortalidade, responsável por mais de 37.000 óbitos por ano.

2. A história natural da doença revela numerosas manifestações extrapulmonares e comorbidades, fatores que pioram o prognóstico e qualidade de vida dos pacientes.

3. A disfunção muscular esquelética é comum em pacientes com DPOC, sendo caracterizada por alterações anatômicas específicas, como mudanças no tipo e composição de fibras e atrofia e, alterações funcionais, como diminuição da força e resistência, contribuindo para a redução da capacidade funcional e qualidade de vida e aumento da mortalidade em humanos.

4. O EO, tanto local quanto sistêmico, pode contribuir para as alterações musculares, causando modificação nas miofibrilas, as quais são rapidamente degradadas por proteases, levando a atrofia.

5. As proteínas podem ser lesadas pelo ataque direto de radicais livres, ou por meio de ligações entre os produtos finais da cadeia de lipoperoxidação, atuando como modulador de vias proteolíticas dependentes e independentes de cálcio em longos tempos de exposição da lesão pulmonar.

6. O músculo esquelético é um tecido altamente adaptável, que responde a mudanças em relação ao tipo de atividade ou de tensões mecânicas e ambientais aos quais é submetido e, que há cada vez mais evidências de que as ERO desempenhem papéis na modulação das vias de sinalização.

7. Modelos animais de enfisema pulmonar induzidos por elastase pancreática suína e papaína têm sido fundamentais para o entendimento da DPOC, e que sua utilização apresenta um grande número de vantagens.

8. Estudos sobre a caracterização do enfisema pulmonar em diferentes modelos experimentais, associados a manifestações sistêmicas como perda de massa corporal e massa muscular, do ponto de vista do estresse oxidativo e adaptações musculares, são escassos.

Pretende-se, com este estudo, avaliar dois modelos experimentais de indução do enfisema pulmonar no que diz respeito à alteração da musculatura esquelética, em estágios precoces da lesão pulmonar.

2 OBJETIVO

2.1 OBJETIVO GERAL

Estabelecer um modelo experimental adequado de enfisema pulmonar associado às modificações musculoesqueléticas em estágios precoces da doença, assim como comparar as consequências musculares do enfisema pulmonar induzido por papaína e elastase.

2.2 OBJETIVOS ESPECÍFICOS

a) Avaliar, no músculo esquelético de hamsters, 70 dias após a indução de enfisema pulmonar por elastase ou papaína, o estresse oxidativo e sua relação com a diminuição de massa muscular, com a proteólise e com as adaptações musculares esqueléticas;
b) Caracterizar a evolução das manifestações musculoesqueléticas em um modelo de enfisema pulmonar em hamsters, induzido por elastase em estágio precoce do desenvolvimento da doença.

ARTIGO CIENTÍFICO 1

Adaptive modifications in peripheral skeletal muscle of hamsters at the early stages of elastase-induced pulmonary emphysema

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Abstract

No studies have demonstrated if there is progression in skeletal muscle adaptation as it happens on pulmonary adaptation on the experimental emphysema using elastase instillation in the early stages of emphysema progression. Here we characterized the evolution of muscle adaptation in a model of pulmonary emphysema induced by elastase, related to peripheral muscle mass adaptation, oxidative stress and protein degradation in the early stages of disease progression. For this purpose, we evaluated pulmonary lesions, body weight, muscle loss, oxidative stress (thiobarbituric acid reactive substances, ratio total and oxidized glutathiones, chemiluminescence stimulated by tert-butyl hydroperoxide, and carbonyl proteins), chymotrypsin-like and calpain-like proteolytic activities in the gastrocnemius muscles of hamsters. Emphysema (E) groups received intrathracheal induction of elastase (5.2 IU/100g). Control (C) groups received intrathracheal instillation of sterile NaCl 0,9% (300 μL/100g). Both groups were euthanized at 10, 20, 40 and 70 days after instillation (*n*=10/group). Compared to their respective controls, all elastase groups had a significantly decrease in the number of crossed alveolar intercepts. The cachexia index showed 9.70±1.64% and 10.81±1.55% on E10 and E20. The frequency distribution of fiber cross-sectional areas showed differences between groups at 20, 40 and 70 days. Additionally, thiobarbituric acid reactive substances, ratio total and oxidized glutathiones, chemiluminescence stimulated by tert-butyl hydroperoxide, and carbonyl proteins showed no differences. Calpain-like activity was significantly decreased in E40. The evaluated parameters lead to the conclusion that elastase lead to early muscle adaptation, but oxidative stress is not already directly involved in the process.

Key-words: Elastase. Emphysema. Skeletal muscle. Proteolysis. Oxidative stress.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation and irreversible airflow obstruction, involving abnormalities of airway, bronchitis, and lung parenchyma, characterized by emphysema (Huertas and Palange 2011; Balkissoon et al. 2011). COPD is one of the leading causes of morbidity and mortality, and represents a huge and growing economic and social burden. In the year 2020, it is projected to be the third leading cause of death worldwide (Global initiative for chronic obstructive lung disease [GOLD] 2013).

Experimental models using different animals allows deeper investigation of different factors that can influence emphysema establishment. Until the present moment, there are many experimental models of COPD, each of them presenting different advantages and disadvantages. However, none of them constitutes a model that exactly reproduces all phases of development and clinical features of emphysema (Vidal et al., 2012). Since 1963 the hamster constitutes the more important biological model for investigation of physiological function and pathological dysfunction in the emphysema (Karlinsky and Snider 1978; Mattson et al. 2002a). The experimental model of pulmonary emphysema that uses intratracheal instillation of porcine pancreatic elastase is used for more than three decades and it has been well characterized. Until this moment it is considered the most consistent and impressive model of airway enlargement. This model is suitable to investigate the pulmonary structure and function and emphysema progression, as well as factors associated to pulmonary disturbances and collagen remodeling failure related to this pathology (Vidal et al., 2012). In addition, the model results in morphological and histological pulmonary alterations equivalent to those in humans (Fusco et al. 2002; Antunes and Rocco 2011), and airspace enlargement continue over the first month after instillation and then stabilizes (Shapiro, 2000). Studies have demonstrated that from 16 days after elastase instillation, pulmonary structure is already destroyed and pulmonary function disabled (Ishizawa et al., 2004; Luthje et al., 2009; Kawakami et al., 2008; Hayes et al., 1975).

One important clinic aspect of COPD is that it is often accompanied by important systemic consequences such as limb muscle dysfunction with reduced muscle strength and endurance. These systemic manifestations have a considerable impact on the exercise tolerance and quality of life of the patients, and are associated with increased mortality (Marquis et al. 2002; Couillard and Prefaut 2005; Swallow et al. 2007; Debigaré and Maltais 2008). Systemic inflammation and both systemic and local oxidative stress are counted among the most widely

studied molecular mechanisms clearly contributing to muscle alterations in COPD patients (Barreiro et al. 2003; Barreiro et al. 2005a; Barreiro et al. 2005b; Barreiro et al. 2010; Marin-Corral et al., 2009). Howevwr, the use of human biopsies to study molecular mechanisms of skeletal muscle adaptations has limitations related to the size of samples and access to populations, making necessary the use of animal models.

In line of this, the hamster has also been used to explore the physiological function of the skeletal muscle in pulmonary emphysema (Lossnitzer and Kelly 1968). Mattson et al. (2004) demonstrated, in hamsters with 180 days of pulmonary emphysema induced by elastase instillation, that emphysema induces fiber atrophy in locomotory muscles. The atrophic response is not specific to locomotory muscles composed of a given fibre type or oxidative capacity, nor is it limited to a specific fiber population. The results of Mattson and Martin (2005) provided evidence that, in hamsters with about 240 days of pulmonary emphysema induced by elastase instillation, emphysema induces increased fatigability and slowed recovery of locomotory skeletal muscle. However, Mador et al. (2010), after about 300 days, in the same model, demonstraded that emphysema did not reduce maximal exercise capacity. As seen, results are controversial and, although pulmonary modifications has demonstrated to be modified very early in the disease, no studies demonstrate if skeletal muscle suffer differences on adaptative process during the same period.

Oxidative stress occurs when the reactive oxygen species (ROS) formation overcome the antioxidants capacity. This happen when increased ROS production or waste of antioxidant levels or both ocuur. This condition leads to increased lipid peroxidation that is believed to play an important role in altering peripheral muscle function in patients with COPD (Rahman et al. 1996; Zhang et al. 2010). It is reported that muscle atrophy may be developed by an imbalance between protein breakdown and synthesis, in favor of the former and that this imbalance is strongly regulated by oxidative stress (Doucet et al. 2007; Powers et al., 2010). Human studies demonstrated that emphysema is also associated with reduced oxidative enzyme and/or antioxidant activitiy (Maltais et al. 1996). Mattson and Poole (1998) demonstrated that emphysema decreased oxidative enzyme capacity in limb muscle of hamsters. Mattson et al (2002b) showed that emphysema led oxidative stress in peripheral skeletal muscle of hamsters induced by elastase after 180 days. Xiao-lei (2010) have demonstrated that, oxidative stress was present in peripheral skeletal muscle five months after elastase instillation. As mentioned, these oxidative modifications have a great impact on muscle function, structure and protein balance, leading to degradation and consequent atrophy (Barreiro 2014; Powers et al. 2010). However, we are aware of any study to date which has

demonstrated if there is progression in skeletal muscle adaptation as it happens on pulmonary adaptation in the experimental emphysema using elastase instillation in the early stages of emphysema progression. In the present study, we characterized the evolution of muscle adaptation in a model of pulmonary emphysema induced by elastase, in aspects related to peripheral muscle mass adaptation, oxidative stress and protein degradation in the early stage of disease progression.

Materials and Methods

Animals

Adult male Syrian Golden hamsters, weighing 100–140g were used (n = 10/group). The animals were given water and commercial food (Nuvilab CR1; Nuvital Nutrients Ltd.) ad libitum, and the environment was controlled on a 12-h light/dark cycle. The protocols conformed to the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 86–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892], and the study was approved by Ethics Committee on Animal Experimentation from the Universidade Estadual de Londrina, Brazil (ref.8288). The animals were randomly divided into 8 groups, according to the time of sacrifice after instillation (10, 20, 40 and 70 days) used to induce pulmonary emphysema with their respective controls. Under deep ketamine/xylazine anesthesia (150/30 mg/kg im), either sterile NaCL 0,9% (0.3 mL/100 g body weight) or porcine elastase (5,2 IU/100 g body weight [Alfa Aesar, Shrewsbury, MA, USA] in 0.3 mL of normal saline) was instilled intratracheally using a 27-gauge hypodermic needle, according to the procedure described by Mattson et al. (2002b) with modifications. The procedure consists on making a short cut on the skin of the tracheal region, seclusion of muscle layer and trachea, insertion of the needle between tracheal rings, instillation and final suture of the skin. To ensure a uniform distribution of saline or elastase throughout the lungs, each animal was submitted to a gentle manual negative pressure maneuver. Briefly, just after instillation, in the moment of final expiration, thorax was momently restrained in order to avoid lung expansion. After active inspiration, a negative pressure is generated and thorax released. This maneuver, through acute differences of pressure, allows complete spread of instilled solution until distal airways. The 8 groups were labeled as follows: control + saline (C), animals instilled with only approximately 0.3 mL of sterile saline; and emphysema with porcine elastase (E), animals instilled with approximately 0.3 mL of 5.2 IU elastase in sterile

saline in their respective times (C10, E10, C20, E20, C40, E40, C70, E70). After procedure, the animals were returned to their cages; their appearance and body weights were monitored once a week until euthanasia.

Tissue collection and preparation

Ten, twenty, forty and seventy days after intratracheal instilation, the hamsters were weighed and euthanized. The right gastrocnemius muscle was excised, weighed, frozen in liquid nitrogen, and stored at −86°C until use. The left gastrocnemius was divided. One portion was destined to histological procedures (described below), the other portion was pulverized and frozen in liquid nitrogen and stored at −86°C until use. The gastrocnemius muscle has previously been demonstrated as a good indicator of alterations in the skeletal muscle of emphysemic hamsters (Tonon et al. 2013), since there is no obvious gross division of fibers in this muscle and therefore, the whole muscle is typically analyzed (Mattson et al. 2002a). In addition, the middle lobe of the left lung was fixed in 10% formalin for morphometric evaluations.

For the oxidative stress analysis, muscles from C and E hamsters were prepared as described above. Tissues were placed on ice and homogenized for 60-s periods at 60-s intervals in an Ultraturrax homogenizer containing 10 mg/mL, 50 mg/ mL or 60 mg/ml of tissue in 50 mM or 10 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. The total homogenate (10 mg/mL) was used for the tert-butyl hydroperoxide-stimulated chemiluminescence (CL) and thiobarbituric acid reactive substances (TBARS) assays. For total protein carbonylation determination, tissues (60 mg/ml) were treated according to the method of Reznick and Packer (1994), with adaptations as described below. For total and oxidized glutathione assays, the supernatant from total homogenate was obtained by centrifugation at 10,000xg for 15 minutes at 4°C from a homogenate 50 mg/ml. Additionally, the cachexia index was determined (considering initial and final body weight of the emphysemic animals and body weight gain in the CS group) in order to identify a pattern of general wasting (Guarnier et al. 2010).

Morphometric analysis of lungs and gastrocnemius

After the middle lobe of the right lung was removed, samples were fixed in paraformaldehyde for 48 h and embedded in paraffin for histological studies. Paraffin embedded tissues were

semi-serialy sectioned into cuts of 5 μm (5 cuts/slide per lung, interval of 50μm/cut). After this procedure, cuts were stained in hematoxylin and eosin. To verify emphysema establishment and severity, alveolar destruction was determined by the number of times that a predetermined group of coherent lines (1.25 mm^2 of area and 1.50 mm of total length) crossed the parenchymal structures. The group identifications were covered and lung images were captured (3 non-coincident fields/section) using a light microscope (100× magnification). Images were obtained using a high resolution camera coupled to the microscope. The base lines were then superposed onto the images; the lesser the structures were crossed, the more extensive was the lesion (adapted from Fusco et al. 2002). Additionally, in order to verify if the number of captured fields was enough, a coefficient of variation was calculated, stablishing as acceptable values below 20%. All sets of measurements by animal were considered acceptable. Then, the 30 values produced by animal had its mean obtained, and this mean was used to represent an animal on the group comparison. The number produced was named crossed alveolar intercepts (CAI).

In order to establish a histological paramenter of muscle adaptation, the measurement of cross-sectional area of gastrocnemius fibers was performed. Firtsly, muscle samples were collected and fixed in methanol and glacial acetic acid (3:1 v/v) for seventy minutes and embedded in paraffin. Then, semi-serial sections of 5 μm were peformed (5 cuts/slide, interval of 50 μm/cut) in each muscle collected. Sections were stained with hematoxylin and eosin. To perform cross-sectional area measurements, the slides identifications were covered, and muscle images were captured (5 fields/cut/animal), totaling aproximatelly 600 measured fibers/animal, using a light microscope (200× magnification). Images were obtained using a high resolution camera coupled to the microscope. An image analysis system (Image-Pro-Plus 4.0; Media Cybernetics, Silver Spring, MD, USA) was used to determine fiber area in μm^2. Due to natural heterogeneity on fibers size distribution on all groups, then, the values were grouped by procedure (C or E), frequency distribution was performed based on area intervals (30 classes) from 600 to 15000 μm^2, and absolute frequence by class was calculated.

Oxidative stress parameters

Thiobarbituric Acid Reactive Substances (TBARS) Assay. Lipid peroxidation is a direct consequence of oxidative stress in tissues. The chain reaction produces the famous end product malondyaldehyde (MDA), that can form adducts modifying protein structures. Thus, the extent of lipid peroxidation of the muscle homogenates from each group was determined

by the thiobarbituric acid reactive substances (TBARS) assay. MDA formed during peroxidation reacts with thiobarbituric acid (TBA) to generate a colored product, a $(TBA)_2$–MDA adduct. In an acid solution, $(TBA)_2$–MDA absorbs light at 532 nm and is readily extractable by an organic solvent such as n-butanol. MDA levels were measured, and the results were expressed in nmol/g tissue (Oliveira and Cecchini 2000).

Carbonyl protein content. Carbonyl proteins are modification promoted by proteins exposure to oxidative stress. The carbonyl protein content was measured as described by Reznick and Parker (1994), with modifications. 60 mg/mL of gastrocnemius muscle were placed in glass homogenization tubes containing homogenizing buffer (50 mM phosphate buffer, 1 mM Ethylenediamine tetraacetic acid, pH 7.4). Tissue samples were homogenized and incubated for 15 min in an ice bath. The samples were centrifuged at 3,000 × g for 10 minutes at room temperature (RT), and 1 mL of each protein extract was placed in glass tubes. A volume of 4 mL of 2,4-dinitrophenylhydrazine (DNPH) solution prepared in 2.5 N HCl was added to each tube, and the reaction mixtures were incubated for 1 h at RT, with vortexing every 15 minutes. Next, the samples were washed with 5 mL of 20% trichloroacetic acid (TCA) (w/v) and centrifuged for 10 minutes to collect the protein precipitates. Another wash was performed using 10% TCA, and protein pellets were dispersed mechanically. Finally, the pellets were washed 3 times with 4 mL of ethanol-ethylacetate (1:1, v/v) to remove free DNPH and lipid contaminants. The final precipitates were dissolved in 2 mL of 6 M guanidine hydrochloride, and any insoluble materials were removed by additional centrifugation. The carbonyl content was calculated by reading the peak absorbance at 355–390 nm of the DNPH-treated samples, versus samples treated with only 2.5 M HCl. The following formula was used to calculate the concentration of carbonyls: C = Abs (355–390nm) × 45.45 nmol/mL, where C is the concentration of DNPH/mL, and 45.45 is its absorption coefficient. The procedures were performed in an ice bath until the TCA wash step. The carbonyl content was expressed as nmol/mg total protein.

Tert-butyl hydroperoxides-initiated chemiluminescence (CL). With the objective of determine direct oxidative attack to muscle cell membranes, reaction mixtures were placed in 2-mL luminescence tubes containing the following: total muscle homogenate (8.75 mg/mL), 10 mM KH2PO4/K2HPO4 buffer (with 120 mM KCl, pH 7.4), and 6 mM tert-butyl hydroperoxide, in a final volume of 1 mL. The tert-butyl hydroperoxideinitiated CL reaction was assessed using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), with a

response range of 300–650 nm. The tubes were kept in the dark until the assay was carried out in a room at 33°±1°C (Gonzalez-Flecha et al. 1991; Oliveira and Cecchini 2000). For each animal, a 60-min curve, in which each point represented the differential smoothing of 600 readings, was obtained by interpolation; after that, area under de CL curve was extracted by integral calculus of each animal curve. This value, expressed as area under de CL curve in relative light units and corrected by g of tissue (RLU/g tissue), together with total analysis of the CL curve, was used to determine the amount of lipid hydroperoxides present in the sample.

Total (GST) and oxidized (GSSG) glutathiones. Redox imbalance was determined by the levels of total (GST) and oxidized (GSSG) glutathione on homogenate supernatant, extracted as described above. The reactions were based on titration with 5,5-dithio-bis (2-nitrobenzoic acid), evidenced by a yellow color formation. Oxidized glutathione (GSSG) was determined by previous incubation with 4-vinylpyridine for 60 minutos at room temperature, according to the method described by Tietze (1969). Volumes of supernatant were adjusted for the assay in order to contain 50 mg tissue/mL of KH_2PO_4. The results were expressed by relationship between GST and GSSG.

Muscle Proteolytic activity

Chymotrypsin-like proteolytic activity was quantifyed using a Proteasome Glo™ Chymiotrypsin-like Cell Based Assay kit (Promega, Madison, WI, USA). This kit estimates the activity of the proteasome; the final objective is evaluate if proteloytic activity is present on skeletal muscle. The assay involves the use of a specific luminogenic substrate (succinyl-leucine-valine-tyrosine-aminoluciferin) to determine chymotrypsin-like activity. The proteasome cleavage of the substrate produces a luminescent signal by the luciferase contained in the reaction medium. The 3 major proteolytic activities (chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolytic or caspase-like activity), occurring within the 20S core of the 26S proteasome complex are responsible for most of the protein degradation, which includes degradation of damaged cellular proteins. Therefore, this coupled-enzyme system, with simultaneous proteasome cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that can be considered proportional to the amount of proteasome activity in the muscle tissue. Thus, during muscle excision, a segment of the gastrocnemius muscle of each animal was frozen in

liquid nitrogen, pulverized, and frozen at −86°C. For the assay, 25 mg of the muscle powder was added to 1 mL of 10mM KH2PO4, pH 7.4, in 0.9% NaCl and gently homogenized. Fifty microliters of the resulting muscle homogenate was pipetted in duplicate onto 96-well microplates, and the final reagent mixture with luminescent substrate was added to the medium. After 5 minutes, under light protection, the luminescent signal at the plate was detected with a Glo-Runner microplate reader luminometer (Turner Designs), and the results were expressed as RLU/mg total protein.

In the same manner, the estimation of activity of a calcium-dependent protease was also measured by a CL assay using a Calpain-Glo™ Protease Assay kit (Promega; Madison, WI, USA). Homogenates were prepared at a concentration of 6.25 mg/mL of 10 mM KH_2PO_4 buffer, pH 7.4, in 0.9% NaCl. The luminescent signal is proportional to the calcium-dependent proteolytic activity in samples, which is also based on the specific reaction of the labelled luciferin substrate by the enzyme luciferase. The results are expressed as RLU/mg tissue.

Protein concentration

The protein concentration was determined by the method of Lowry et al. (1951) modified by Miller (1959). This method involved the use of bovine serum albumin (BSA) as a standard.

Statistical analysis

When distribution was considered normal, the results are presented as mean ± standard error of the mean (SEM) for 10 animals and values were compared using two-way analysis of variance followed by Bonferroni's multiple comparison test, with $p < 0.05$ considered significant. Curves of CL-induced by t-butyl hydroperoxides were compared by two-way ANOVA. When distribution did not pass on normality test, data were presented as median followed by 25 and 75 percentils and values were compared by Freadman test followed by Dunn's multiple comparison test. Muscle morphometry had values of fibers crossed sectional areas distributed in frequencies and their histograms were constructed based on them.

Results

Emphysema condition

The extention of lung damage on groups was evaluated by the number of crossed alveolar intercepts (CAI, as previously mentioned). A previous pilot study demonstrated on lung damage on saline-treated group (CS) relative with control (nor saline, papain or elastase instillation – data not shown). The E (10, 20, 40 and 70 days) group had significantly decreased CAI values (45.30 ± 1.25, 34.53±0.93, 61.29±1.04, 40.55±1.17, respectively) when compared to the C (10, 20, 40 and 70 days) group (81.79 ± 0.38, 66.36 ± 0.65, 68.78±0.6, 72.33±0.69, $p<0.0001$ for all comparisons). The cachexia index (CI, expressed as a percentage), that reflects not only the loss of total body weight but also the absence of weight gain (Tonon et al. 2013), using gain of body mass of control group as reference, was calculated and showed 9.70±1.64% and 10.81±1.55% on E10 and E20 groups. The value of 3.26±1.18% and 0.39 ± 3.12% showed by E40 and E70 groups was not considered cachexia (Argilés, 2005). The relationship between gastrocnemius mass (MM) and total mass (TM), called MM/TM ratio, expressed as percentage, was not significantly differences in the C and E comparison (Table 1).

The frequency distribution of fibre cross-sectional areas showed differences between groups at 20, 40 and 70 days. The 50th percentile of fibre area occurred in the fourth class (2000–2499 μm^2) for the C20, C40 and C70 group, in the third class (1500–1999 μm^2) for the E20 group, and the fifth class (2500–2999 μm^2) for the E40 and E70 group (Figure 1).

Oxidative Stress

Tert-butyl hydroperoxide-initiated chemiluminescence was used to analyze the levels of lipid hydroperoxides in muscle homogenate of animals. This assay indicates that the increase in CL is closely related to the oxidative stress previously suffered by the tissue. It induces the consumption of antioxidants and augments the formation of lipid hydroperoxides; which results in increased photon emission (Oliveira and Cecchini 2000; Barbosa et al. 2003; Mattson and Martin 2005). Figure 2A, B, C, D represent CL curves from C and E (10, 20, 40, 70 days) groups at 60 min, obtained by interpolation. The two-way ANOVA did not show significant differences in the curve comparison. The values of area under the CL curve and peak of the curve, obtained by interpolation, also showed not significant differences (Figure

2E). In addition, table 2 did not show differences in TBARS, PC, GST/GSSG between groups.

Analyses of chymotrypsin and calpain-like proteolytic activities

The estimative of two different proteolytic systems operation were evaluated. Chymotrypsin-like proteolytic activity was not different when C (226841 [165099-379822]; 313612 [109606-441105]; 370936 [225779-519799]; 20503 [10243-81822] RLU/mg protein) and E group (409011 [256709-464217]; 51417 [21355-128359]; 69163 [58664-86523]; 53450 [41679-67436] RLU/mg protein) were compared at 10, 20, 40 and 70 times, respectively (Figure 3A). Calpain-like proteolytic activity was not different in C (76660 [51082-114083]; 55381 [36848-77313]; 21553 [12928-28272] RLU/mg protein) and E group (105621 [86005-161687]; 34670 [19805-46251]; 44916 [22489-52239] RLU/mg protein) at 10, 20 and 70 times. However, the comparison between C and E at 40 days (51412 [50863-54664] and 20512 [17564-21919] RLU/mg protein, respectively) was significantly decreased for Calpain-like activity (Figure 3B).

Discussion

Since 1975, studies have demonstrated that intratracheal instillation of porcine pancreatic elastase can result in an increase of linear mean intercept (Hayes et al. 1975), decreased of pulmonary function (Raub et al 1982) and exercise capacity (Vidal et al. 2012), inducing the pulmonary emphysema, resulting in histologic, morphologic and physiologic alterations of the lungs equivalent to human (Hayes et al. 1975; March et al. 2000; Mahadeva and Shapiro 2002). This model is based in the imbalanced production of proteases and antiproteases in the lung (Rufino and Silva 2006). The administration of porcine pancreatic elastase has produced the most consistent and impressive airspace enlargement in rodents, guinea pigs, dogs, and primates (Kuhn et al. 1976; Snider et al. 1986). However, different doses and times have been used to induce emphysema. Ishizawa et al. (2004) applied elastase (2U/100g body weight) by intranasal instillation to induce the experimental emphysema in mice and after three weeks, observed pulmonary abnormalities and an increase of mean linear intercept. Vidal et al. (2012) also observed this increase after 28 days by intratracheal instillation. After intranasal administration of elastase (1.2U/100g weight body), neutrophil accumulation was observed in the first day and inflammation was resolved by one week. At 2 weeks, a significant increase

in total lung volume was demonstrated and the lung destruction continued to 3 weeks (Kawakami et al. 2008). In 2009, emphysematous disturbances were induced by intratracheal instillation of porcine pancreatic elastase in two experimental groups (5 U/100 g body weight and 3.3 U/100 g body weight) and was observed high absolute mortality (Lüthje et al. 2009). However, a study with hamsters demonstrated that an elastase dose of 0.2 mg (5.2U/100g body weight) by intratracheal instillation, was enough to produce a lesion of moderate severity with minimal mortility after 16 days (Hayes et al. 1975). Therefore, porcine pancreatic elastase is the most used protease because elastin is a specific enzyme, which has induced consistent and reproducible enlargement of alveolar spaces in rodents, resulting in neutrophil recruitment acute, subacute and accumulation of macrophages in the lungs, with hemorrhage. Alveolar spaces enlargement occurs by elastin degradation and disorganized elastic fibers can stabilize 28 days after instillation (Cantor et al. 2000; Shapiro 2000; Vidal et al. 2012).

In the present study, the four times used to evaluate lung injury promoted by elastase induction (10, 20, 40 and 70 days), with a single dose (5.2U/100g weight body), promoted emphysema with significant decrease on crossed alveolar intercepts values in comparison with respectives control groups, developing pulmonary injury in a level enough to promote emphysema, demonstrating pattern of panacinar emphysema with part of lobe histoarchitecture preserved, but alveolar septs ruptured. The dose of induction used was very close to doses capable to induce increase in animal mortality just after induction. Studies in the literature have demonstrating that this dose (Hayes et al. 1975, Raub et al. 1982) and times (Kawakami et al. 2008; Vidal et al. 2012) can promote the histologic and functional characteristics of emphysema with low mortality.

Many extrapulmonary effects of COPD have been described over the last decades, including muscle wasting (Remels et al. 2007). In limb muscles, underloading leads to less muscle mass (Bernard et al. 1998). In humans, the chief repercussion of this condition is a reduction in exercise capacity and quality of life (Swallow et al. 2007). These processes have been credited to adaptative responses from muscle mass to new oxygen uptake and distribution (Wust and Degens 2007). However, mechanisms of muscle mass loss are uncertain and experimental studies with human biopsies are difficult. So, to study molecular and biochemic aspects, we used animals models. Most systemic manifestations of emphysema have been reproduced in mice using repeated elastase challenges, and persisted during prolonged periods after the lesion is induced (Lüthje et al. 2009). Hamsters has been used to explore the physiological function of the skeletal muscle (Lossnitzer and Kelly 1968).

In the present study, when early systemic adaptation was evaluated by body and skeletal muscle losses, it was not found differences between MM/TM in the times of induction. Elastase induction promoted body mass loss at 10 and 20 days when compared with control group, 9.7% and 10.81%, respectively. These data were reflected on morphometry, that decreased the fiber area at 20 days and increased the fiber area at 40 and 70 days, respectively. Additionally, calpain-like activity decreased the activity at 40 days. Mattson and Martin (2005) demonstrated that emphysema can increase fatigability and impair the speed of recovery of locomotory skeletal muscle contractile function with no effect on maximal force in a model of elastase after eigth months. This same group revealed that maximal muscle force may be preserved in the face of emphysema-induced fiber atrophy after 8 months (Mattson et al. 2008). Elastase-induced emphysema did not reduce maximal exercise capacity in the hamster after 24 months (Mador et al. 2010). However, studies using mice models observed lower total body weight and lower weight of the gastrocnemius after 34 weeks (Fermoselle et al. 2011) and lower exercise capacity after 28 days (Vidal et al. 2012). Thus, data suggest that muscle mass loss occurs at 20 days, which is completely reversed at 70 days, which could be explained by muscular adaptation, with fiber type modification. Mattson et al. (2002a) found increased type IIX fibers on gastrocnemius of hamsters. The same group observed that fiber cross-sectional area of fast-twitch types IIA, IIX and/or IIB fibres was reduced in the caudal biceps femoris, vastus lateralis, tibialis anterior, gastrocnemius and plantaris muscles of emphysema hamsters. In line of this, it is clear that elastase model is not suitable for studying muscle mass loss as systemic manifestation of emphysema.

In skeletal muscle fibers, ROS are normally synthesized at low levels and are absolutely required for normal force production. However, when levels of ROS are either reduced by the action of free radical scavengers or excessively produced under inflammatory conditions muscle force generation may be considerably impaired, leading to further muscle dysfunction and fatigue (Reid et al. 1992; Jackson et al. 2007). Therefore, oxidative modifications induced by oxidants on key muscle proteins involved in muscle structure, metabolism, and contraction of patients with chronic conditions and experimental models of disease have a great impact on their function and structure (Barreiro 2014). Specifically, protein carbonylation was shown to alter the function of enzymes and structural proteins involved in muscle contractile performance (Tonon et al. 2013; Barreiro 2014). COPD human studies showed that oxidative stress correlated negatively with muscle strength, suggesting a link between oxidative stress and loss of muscle mass (Van Helvoort et al. 2007; Barreiro et al. 2009). Cachetic COPD patients exhibited greater muscle oxidative stress associated with increased of ubiquitin-

proteassome system activity, protein ubiquitination and atrophy of type II muscle fibers (Fermoselle et al. 2012). However, in experimental models, Mattson et al (2002b) demonstrated increase in MDA and GST levels and activity of glutathione reductase (GR) in gastrocnemius of emphysemic hamsters after six months. Zhang et al. (2010) observed lipofuscin inclusions higher in the gastrocnemius muscle, as well as increase of antioxidant enzymes activity in emphysemic rats induced by elastase after five months. In contrast, Fermoselle et al. (2011) did not find differences in total carbonylated protein, MDA–protein adducts levels and glutathione peroxidase (GPx) activity in mice after 34 weeks.

The present study showed no difference between groups in relation to CL curves, area under the curve, peak of the curve, TBARS, PC, GSH, GSSG. When a tissue is challenged with *tert*-butyl hydroperoxide, the obtained CL curves should reflect both the attack suffered by the membrane and the tissue antioxidant consumption mediating the damage caused by this attack. Thus, this model was not able to trigger lipid peroxidation and protein carbonylation. Yet, other models used muscles from hamsters with emphysema induced by papain showed TBARS, CL and PC elevated after 70 days (Tonon et al. 2013). Putting these results together, the evaluated parameters lead to the conclusion that elastase lead to early muscle adaptation, but oxidative stress is not already directly involved in the process. This seems a phenomenon that become installed and contributes to accelerated muscle loss in advanced stages of the disease, in this experimental model.

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Conflict of Interest

All the authors declare any conflict of interest.

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Table 1 – Animal and skeletal muscle adaptation characteristics.

Time after instillation (days)	Crossed Alveolar Intercept		CI (%)	MM/TM	
	C	E	E	C	E
10	81.79 ± 0.38	45.30 ± 1.25*	9.70±1.64	0.17 [0.16-0.22]	0.21 [0.20-0.22]
20	66.36 ± 0.65	34.53±0.93*	10.81±1.55	0.12 [0.12-0.12]	0.14 [0.14-0.14]
40	68.78±0.61	61.29±1.04*	3.26±1.18	0.13 [0.12-0.14]	0.15 [0.15-0.17]
70	72.33±0.69	40.55±1.17*	0.39±0.12	0.14 [0.13-0.15]	0.17 [0.16-0.18]

C– Group instilled with 300 µL of NaCl 0.9%; E– Group instilled with 300 µL of elastase 5.2 IU in NaCl 0.9% at 10, 20, 40 and 70 days after instillation. CI – Cachexia index = (initial body weight – final body weight + body mass gain of CS group) / (initial body weight – body mass gain of CS group) x 100. MM/TM- muscle mass/total mass. Values are expressed as means ± SEM or as median [25 - 75 percentiles]. Groups were composed by 10 animals / group.
*$p<0.05$ when compared E with C by Two way ANOVA followed by Bonferroni's multiple comparison test for normal date.

Table 2 – Effect of emphysema on skeletal muscle in terms of TBARS, PC and GST/GSSG in hamsters.

Time after instillation (days)	TBARS (nmol/g tissue)		CP (nmols carbonyl/mg total protein)		GST/GSSG	
	C	E	C	E	C	E
10	1.05[0.83-1.32]	0.90[0.86-1.21]	3.585[3.081-4.039]	4.410[3.099-5.676]	4.356 [3.517-8.170]	5.801[5.623-8.169]
20	1.37[1.03-1.41]	1.22[1.04-1.47]	4.118[2.518-5.492]	2.830[2.532-6.028]	8.563[7.703-9.843]	8.855[8.562-20.97]
40	0.91[0.72-1.33]	1.09[0.81-1.21]	3.736[2.937-4.761]	3.591[3.456-4.017]	10.47[8.421-14.89]	10.66[8.516-11.60]
70	0.40[0.35-0.69]	0.49[0.41-0.59]	7.272[6.514-7.883]	7.352[6.583-7.829]	11.03[9.738-14.17]	12.07[10.86-19.44]

C– Group instilled with 300 μL of NaCl 0.9%; E– Group instilled with 300 μL of elastase 5.2 IU in NaCl 0.9% at 10, 20, 40 and 70 days after instillation. TBARS - thiobarbituric acid reactive substances. CP- carbonyl protein. GST/GSSG- relationship GST (total glutathione) and GSSH (oxidized glutathione). Values are expressed as median [25 - 75 percentiles]. Groups were composed by 10 animals / group.

Figure 1 – Histrogram of frequency distribution of fibers crossed sectional areas (μm^2). Muscles were sectioned and 5 semi-serial cuts / slide (50 um of interval) were positioned on 2 slides. 3 fields by cut were captured and 20 fibers measured/field/animal, totalizing 600 fibers measurements by animal. The values were grouped by procedure (C or E) and frequence distribution determined. C- animals treated with 0.3 mL of sterile saline only; E- animals treated with 0.3 mL of 5.2 IU elastase in sterile saline. Animals were euthanized after 10 days (A), 20 days (B), 40 days (C) and 70 days (D).

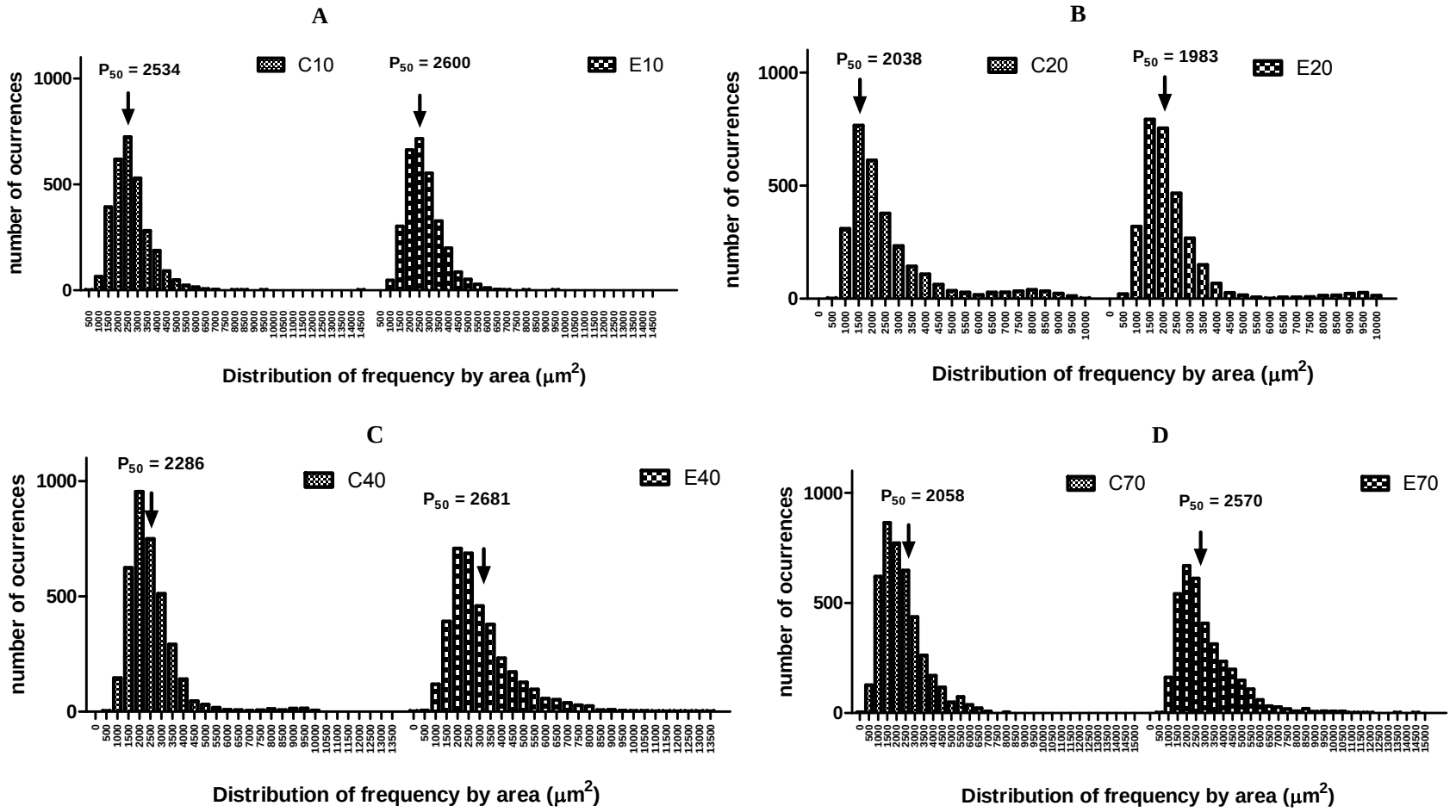
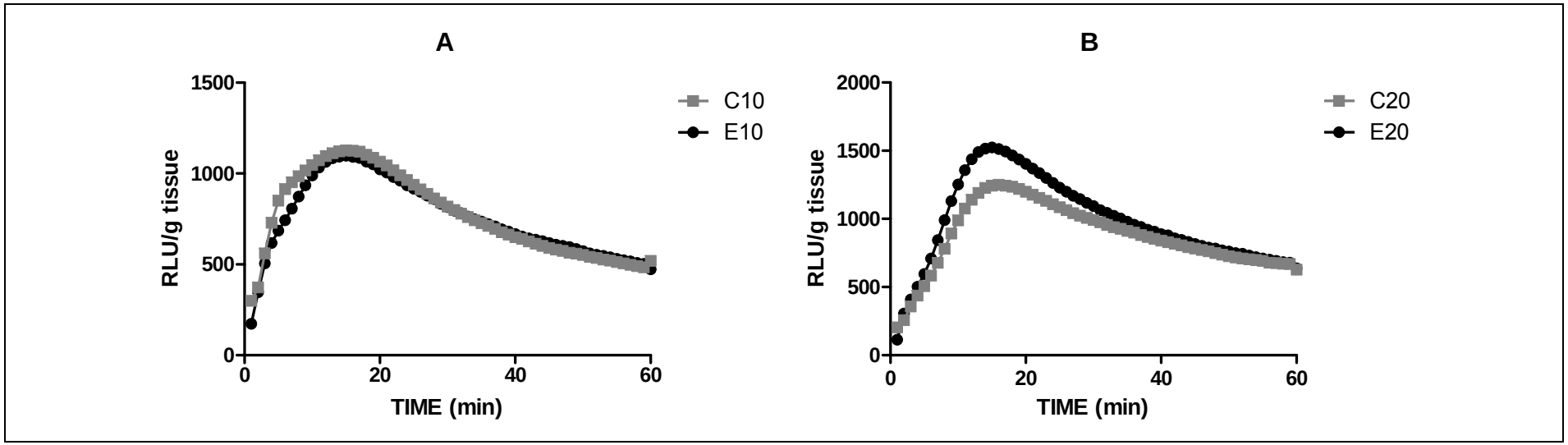
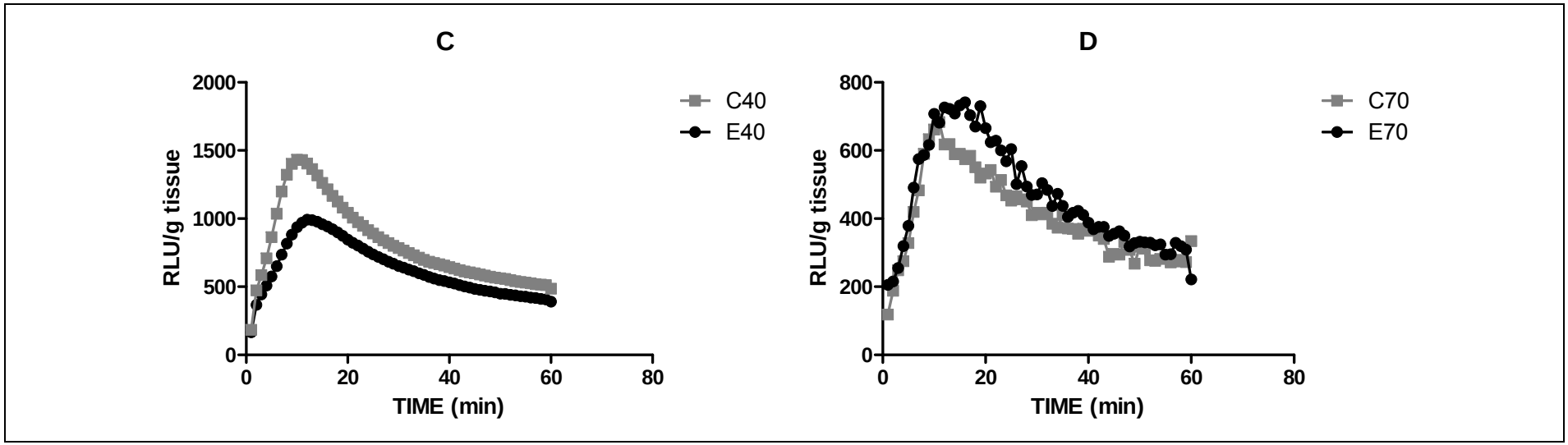


Figure 2 – Effect of emphysema induced by elastase on skeletal muscle of hamsters in terms of lipid hydroperoxide. C– Group instilled with 300 µL of NaCl 0.9%; E– Group instilled with 300 µL of elastase 5.2 IU in NaCl 0.9%. (A), (B), (C), (D) The tert-butyl hydroperoxide-initiated chemiluminescence was monitored continuously for 60 min in gastrocnemius homogenates at 10, 20, 40 and 70 days after instillation, respectively. Each curve represented mean of 10 animals curves, corrected by g of tissue. The entire curves were employed to perform statistical comparison by two-way ANOVA. (E) Area under the curve and Peak of the curve of each animal curve was determined to compare groups. The difference was indicated by Two way ANOVA followed by Bonferroni's multiple ($^{*}p<0.05$) or Friedman test followed by Dunn's multiple comparison test ($^{\#}p<0.05$). Values are expressed as means ± SEM or median [25 - 75 percentiles]. Groups were composed by 10 animals / group.

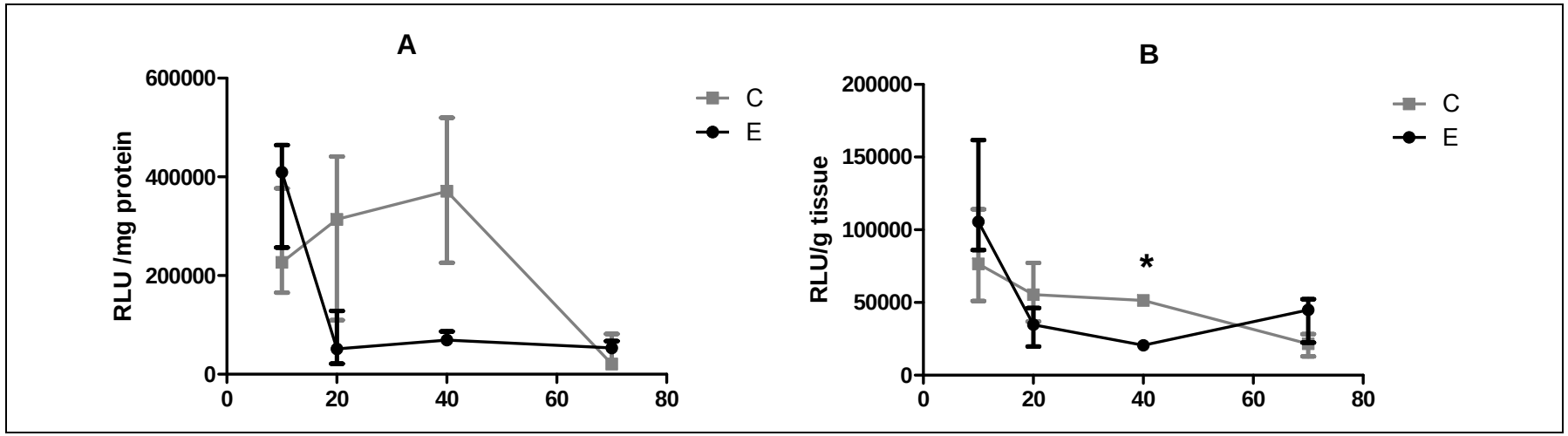




E

Time after instillation (days)	AREA UNDER THE CURVE		PEAK OF THE CURVE	
	C	E	C	E
10	21727±3549	24393±2324	1257[813.5-1639]	1109[969.7-1240]
20	27206±5483	36056±4755	1072[785.1-1975]	1555[1302-1638]
40	28577±4438	20997±3662	1709[989-1924]	1227[568.5-1354]
70	10775±1564	15106±2469	751.1[461.3-1207]	732.3[559.3-913.5]

Figura 3 – Quantification of estimative of proteolytic activity in gastrocnemius of hamsters with emphysema induced by elastase quantified by a luminescent kit. at 10, 20, 40 and 70 days after instillation (A) Chymotrypsin-like activity (B) Calpain-like activity. C – Group instilled with 300 µL of NaCl 0.9%; E–Group instilled with 300 µL of elastase 5.2 IU in NaCl 0.9%.The difference was indicated by Friedman test followed by Dunn's multiple comparison test ($^{*}p<0.05$). Values are expressed median [25 - 75 percentiles]. Groups were composed by 10 animals / group.



ARTIGO CIENTÍFICO 2

Oxidative and proteolysis-related parameters of skeletal muscle from hamsters with experimental pulmonary emphysema: a comparison between papain and elastase induction

Running title: muscle loss on papain and elastase emphysema

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Abstract

The objective of this study was to investigate if emphysema induced by elastase or papain triggers the same effects on skeletal muscle in what is related with oxidative stress and proteolysis in hamsters. For this purpose, we evaluated pulmonary lesions, body weight, muscle loss, oxidative stress (thiobarbituric acid reactive substances, total and oxidized glutathiones, chemiluminescence stimulated by tert-butyl hydroperoxide, and carbonyl proteins), chymotrypsin-like and calpain-like proteolytic activities, and muscle fibre cross-sectional area in the gastrocnemius muscles of emphysemic hamsters. Two groups of animals received different intrathracheal inductions of experimental emphysema: by 40 mg/mL papain (EP) or 5.2 IU/100 g animal (EE) elastase (n = 10 animals/group). The control group received intrathracheal instillation of 300 µL sterile NaCl 0.9%. Compared with the control group, the EP group had reduced muscle weight (18.34%) and the EE group had increased muscle weight (8.37%). Additionally, tert-butyl hydroperoxide-initiated chemiluminescence, carbonylated proteins, and chymotrypsin-like proteolytic activity were elevated in the EP group compared to CS group, while total glutathione was decreased compared to EE group. The EE group showed more fibres with increased cross-sectional areas and increased calpain-like activity. Together, these data show that elastase and papain, when used to induce experimental models of emphysema, lead to different speeds and types of adaptation. These findings provide more information on choosing a suitable experimental model for studying skeletal muscle adaptations in emphysema.

Key-words: Elastase. Papain. Emphysema. Skeletal muscle. Proteolysis. Oxidative stress.

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Introduction

Chronic obstructive pulmonary disease (COPD) comprises a group of pulmonary abnormalities of the airways or lung parenchyma, bronchitis, or emphysema, and is a major cause of morbidity and death throughout the world (Petty 2006; Huertas & Palange 2011). In addition to these pulmonary abnormalities, COPD is associated with significant effects in distant organs outside the lungs (Agustí *et al.* 2003; Gan *et al.* 2004). Peripheral skeletal muscle dysfunction is characterized by reduced muscle strength and endurance, and considered one of the main systemic effects of emphysema (Couillard & Prefaut 2005; Swallow *et al.* 2007). Therefore, it is important to understand how muscle function is affected to identify the factors that contribute to the muscle dysfunction and the mechanism of muscle wasting in COPD, and so improve the management of the disease (Wust & Degens 2007). The literature contains numerous animal models that develop emphysema, resulting in destruction of lung parenchyma structure with morphological and histological alterations equivalent to those in humans (Mahadeva & Shapiro 2002; Wright & Churg 2002).

Experimental models using proteolytic enzymes such as papain or elastase, instilled into the airways of animals, are based on unbalanced protective and aggressive substances in pulmonary tissue (Fusco *et al.* 2002; Antunes & Rocco 2011). In general, animal models presents advantages as homogeneity, usually good reproducibility, no difference with different therapies and a wide range of unpredictable factors affecting the development of the disease in clinical conditions (Holecek 2012). Since the prevalence of cachexia in patients with COPD is up to 45% (Schols *et al.* 1993), its aetiology in the disease is multifactorial, and the number of papers examining muscle loss in models of COPD is extremely small, it would be beneficial if animal models could reproduce the human cachectic disorders as closely as possible. Mattson *et al.* (2004) demonstrated that emphysema induces fibre atrophy and the response is not specific to locomotory muscles composed of a given fibre type or oxidative capacity, in hamsters after 180 days of pulmonary emphysema induced by elastase instillation (25IU/100g body weight). Rinaldi *et al.* (2012) described muscular changes and dysfunction, particularly in muscles with a mixed fibre type composition, on an experimental model of six months of cigarette smoke exposure, and showed that this model led to systemic problems resembling those seen in humans with emphysema. In addition, Tonon *et al*. (2013) demonstrated that muscle atrophy, observed in a model of 60 days of emphysema induced by papain instillation (40mg/ml) in rats, is mediated by increased muscle proteolysis, with involvement of oxidative stress.

Oxidative stress, resulting from an inability of the antioxidant systems to cope with elevated oxidant production, plays an important role in altering peripheral muscle function in patients with COPD (Zhang *et al.* 2010). Skeletal muscle is a highly malleable tissue that responds to changes in its pattern of activity or the mechanical and environmental stresses placed upon it. The signalling pathways involved in these multiple adaptations have been described. Reactive oxygen species are produced at various sites in skeletal muscle, and there is increasing evidence that these species play targeted roles in modulating redox-sensitive signalling pathways that are important to the muscle for making adaptations (Jackson 2009; Powers *et al*. 2010). The oxidative modifications induced by oxidants on key muscle proteins involved in muscle structure, metabolism, and contraction of experimental models of disease have a great impact on their function, structure, and protein balance, leading to degradation and consequent atrophy (Barreiro 2013; Powers *et al*. 2010).

Our study was motivated by three points:

(1) Different changes are observed at different experimental times in different experimental models of emphysema.

(2) Redox and proteolytic modifications can be considered representative of skeletal muscle loss as a systemic manifestation of the disease.

(3) Thus far, no studies have compared oxidative stress, muscle mass loss, and muscle adaptation on different models of pulmonary emphysema.

Considering these points, we investigated if emphysema induced by elastase or papain triggers the same systemic effects on skeletal muscle in what is related with oxidative stress and proteolysis.

Materials and Methods

Animals

Adult male Syrian Golden hamsters, weighing 100–140 g, were used (n = 10/group). The animals were given water and commercial food (Nuvilab CR1; Nuvital Nutrients Ltd) *ad libitum*, and the environment was controlled on a 12-h light/dark cycle.

The protocols conformed to the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 86–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892], and the study was approved by the Ethics Committee on Animal Experimentation from the Universidade Estadual de Londrina, Brazil (ref.8288).

The animals were randomly divided into three groups, according to the instillation procedure used to induce emphysema. Under deep ketamine/xylazine anaesthesia (150/30 mg/kg i.m.), saline (0.3 mL/100 g body weight; control+saline [CS] group), papain (40 mg/100 g body weight in 0.3 mL normal saline; emphysema with papain [EP] group) (Tonon *et al.* 2013), or porcine elastase (5.2 IU/100 g body weight [Alfa Aesar, Shrewsbury, MA, USA] in 0.3 mL normal saline; emphysema with porcine elastase [EE] group) (Hayes *et al.* 1975) was instilled intratracheally through a 27-gauge hypodermic needle, according to the procedure described by Mattson *et al.* (2002b) with modifications. The procedure involves making a short incision in the skin of the tracheal region, seclusion of the muscle layer and trachea, insertion of the needle between tracheal rings, instillation, and final suturing of the skin. To ensure a uniform distribution of saline, elastase or papain throughout the lungs, each animal was submitted to a gentle manual negative pressure manoeuvre. Briefly, just after instillation, in the moment of final expiration, the thorax was momentarily restrained to prevent lung expansion. After active inspiration, a negative pressure is generated and the thorax released. This manoeuvre, through acute differences of pressure, allows complete spread of the instilled solution to the distal airways.

After the procedure, the animals were returned to their cages. Their appearance and body weights were monitored daily for the first two weeks, and then once a week for 70 days (Tonon *et al.* 2013). The cachexia index was determined ([(initial body mass - final body mass + body mass gain of control) / (initial body mass + body mass gain of control)] x 100%), considering initial and final body weight of the emphysemic animals and body weight gain in the CS group to identify a pattern of general wasting. (Guarnier *et al.* 2010). This index reflects both loss of total body weight and absence of weight gain (Tonon *et al.* 2013).

Tissue collection and preparation

Seventy days after intratracheal instillation, the hamsters were weighed and euthanized. The right gastrocnemius muscle was excised, weighed, frozen in liquid nitrogen, and stored at −86°C until use for analysis of oxidative parameters. The left gastrocnemius was divided: one portion was saved for morphometric histological procedures, and the other portion was pulverized, frozen in liquid nitrogen, and stored at −86°C until used for proteolysis assays.The gastrocnemius muscle has previously been demonstrated as a good indicator of alterations in the skeletal muscle of emphysemic hamsters (Mattson *et al* 2004), including in aspects related to oxidative parameters (Mattson *et al* 2002b, Tonon *et al*, 2013). In hamsters, there is no

gross division of fibers in this muscle (Mattson *et al* 2002a), and therefore, the whole muscle, and consequently both fiber types, are typically analysed. In addition, the middle lobe of the right lung was fixed in 10% formalin for morphometric evaluations.

For the oxidative stress analysis, muscles from CS, EP, and EE hamsters were prepared as described above. Tissues were placed on ice and homogenized for 60 s periods at 60 s intervals in an Ultraturrax homogenizer containing 10 mg/mL, 50 mg/ mL, or 60 mg/mL tissue in 50 mM or 10 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. The total homogenate (10 mg/mL) was used for the tert-butyl hydroperoxide-stimulated chemiluminescence and thiobarbituric acid reactive substances (TBARS) assays. For total protein carbonylation determination, tissues (60 mg/mL) were treated according to the method of Reznick and Packer (1994), with adaptations as described below. For total and oxidized glutathione assays, the supernatant from total homogenate was obtained by centrifugation at 10,000×*g* for 15 minutes at 4°C from a homogenate 50 mg/mL.

Morphometric analysis of lungs and gastrocnemius

After the middle lobe of the right lung had been removed, samples were fixed in paraformaldehyde for 48 h and embedded in paraffin for histological studies. Paraffin-embedded tissues were semi-serially sectioned into cuts of 5 μm (5 cuts/slide per lung, interval of 50 μm/cut). After this procedure, cuts were stained in haematoxylin and eosin.

To verify emphysema establishment and severity, alveolar destruction was determined by the number of times that a predetermined group of coherent lines (1.25 mm^2 area and 1.50 mm total length) crossed the parenchymal structures. The group identifications were covered and lung images were captured (three non-coincident fields/section) using an optical microscope (100× magnification). Images were obtained using a high-resolution camera coupled to the microscope. The base lines were then superposed onto the images; the less the structures were crossed, the more extensive was the lesion (adapted from Fusco *et al.* 2002). Additionally, to verify if the number of captured fields was enough, a coefficient of variation was calculated, with acceptable values being below 20%. All sets of measurements by animal were considered acceptable. The mean of the 30 values from each animal was calculated. These values, named the crossed alveolar intercepts (CAI), were used to represent each animal in the group comparison.

To establish a histological parameter of muscle adaptation, the cross-sectional area of gastrocnemius fibres was measured. Muscle samples were collected and fixed in methanol

and glacial acetic acid (3:1 v/v) for 70 minutes, and then embedded in paraffin. Semi-serial sections of 5 μm were made (5 cuts/slide, interval of 50 μm/cut) from each muscle. The sections were stained with haematoxylin and eosin. To measure the cross-sectional area, the slide identifications were covered, and muscle images were captured (5 fields/cut per animal), totalling approximately 600 measured fibres/animal, using an optical microscope (200× magnification). Images were obtained using a high-resolution camera coupled to the microscope. An image analysis system (Image-Pro-Plus 4.0; Media Cybernetics, Silver Spring, MD, USA) was used to determine fibre area in square micrometres. Because of natural heterogeneity in the fibre size distribution within the groups, the values were grouped by procedure (CS, EE, or EP), the frequency distribution was estimated based on area intervals (30 classes) from 600 to 15000 μm^2.

Oxidative stress parameters

Thiobarbituric Acid Reactive Substances (TBARS) Assay. Lipid peroxidation is a direct consequence of oxidative stress in tissues. The chain reaction produces malondyaldehyde (MDA), which can form adducts modifying protein structures. Thus, the extent of lipid peroxidation of the muscle homogenates from each group was determined by the TBARS assay. MDA formed during peroxidation reacts with thiobarbituric acid (TBA) to generate a coloured product, a $(TBA)_2$–MDA adduct. In an acid solution, $(TBA)_2$–MDA absorbs light at 532 nm and is readily extractable by an organic solvent such as n-butanol. MDA levels were measured, and the results were expressed in nanomoles per gram of tissue (Oliveira & Cecchini 2000).

Carbonyl protein content. Carbonyl proteins are a modification caused by exposure of proteins to oxidative stress. The carbonyl protein content was measured as described by Reznick and Parker (1994), with modifications. Gastrocnemius muscle (60 mg/mL) was placed in glass homogenization tubes containing homogenizing buffer (50 mM phosphate buffer, 1 mM ethylenediamine tetraacetic acid, pH 7.4). The tissue samples were homogenized and incubated for 15 min in an ice bath. The samples were centrifuged at 3,000 × g for 10 min at room temperature, and 1 mL each protein extract was placed in glass tubes. A volume of 4 mL 2,4-dinitrophenylhydrazine (DNPH) solution prepared in 2.5 N HCl was added to each tube, and the reaction mixtures were incubated for 1 h at room temperature, with vortexing every 15 min. Next, the samples were washed with 5 mL 20% trichloroacetic acid (TCA) (w/v) and centrifuged for 10 minutes to collect the protein precipitates. Another

wash was performed using 10% TCA, and the protein pellets were dispersed mechanically. Finally, the pellets were washed three times with 4 mL ethanol–ethylacetate (1:1, v/v) to remove free DNPH and lipid contaminants. The final precipitates were dissolved in 2 mL 6 M guanidine hydrochloride, and any insoluble materials were removed by additional centrifugation. The carbonyl content was calculated by comparing the peak absorbance at 355–390 nm of the DNPH-treated samples with that of samples treated with only 2.5 M HCl. The following formula was used to calculate the concentration of carbonyls: C = Abs(355–390 nm) × 45.45 nmol/mL, where C is the concentration of DNPH per millilitre, and 45.45 is its absorption coefficient. The procedures were performed in an ice bath until the TCA wash step. The carbonyl content was expressed as nanomoles per milligram of total protein.

Tert-butyl hydroperoxide-initiated chemiluminescence. The tert-butyl hydroperoxide-initiated chemiluminescence assay was used to analyse the levels of lipid hydroperoxides in muscle homogenate of animals. In this assay, an increase in chemiluminescence is closely related to the oxidative stress previously suffered by the tissue. The assay induces the consumption of antioxidants and augments the formation of lipid hydroperoxides, which results in increased photon emission (Oliveira & Cecchini 2000; Barbosa *et al.* 2003; Mattson & Martin 2005).

Reaction mixtures in 2 mL luminescence tubes consisted of: total muscle homogenate (8.75 mg/mL), 10 mM KH_2PO_4/K_2HPO_4 buffer (with 120 mM KCl, pH 7.4), and 6 mM tert-butyl hydroperoxide, in a final volume of 1 mL. The tert-butyl hydroperoxide-initiated chemiluminescence reaction was assessed using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), with a response range of 300–650 nm. The tubes were kept in the dark until the assay was carried out in a room at 33 ± 1°C (Gonzalez-Flecha et al. 1991; Oliveira and Cecchini 2000). For each animal, a 60 min curve, in which each point represented the differential smoothing of 600 readings, was obtained by interpolation; after that, the area under the chemiluminescence curve was extracted by integral calculus of each animal curve. This value, expressed as area under the chemiluminescence curve in relative light units and corrected by gram of tissue (RLU/g tissue), together with total analysis of the chemiluminescence curve, was used to determine the amount of lipid hydroperoxides present in the sample.

Total (GST) and oxidized (GSSG) glutathiones. Redox state was determined by the levels of total (GST) and oxidized (GSSG) glutathione in homogenate supernatant, extracted as described above. The reactions were based on titration with 5,5-dithio-bis(2-nitrobenzoic acid), which results in formation of a yellow colour. Oxidized glutathione (GSSG) was

determined by previous incubation with 4-vinylpyridine for 60 min at room temperature, according to the method described by Tietze (1969). Supernatant volumes were adjusted for the assay to contain 50 mg tissue per millilitre of KH_2PO_4. The results are expressed in micromoles per milligram of protein.

Muscle proteolytic activity

Chymotrypsin-like proteolytic activity was quantified using a Proteasome Glo™ Chymotrypsin-like Cell Based Assay kit (Promega, Madison, WI, USA). This kit estimates the activity of the proteasome; the final objective is to evaluate whether proteolytic activity is present in skeletal muscle. The assay involves the use of a specific luminogenic substrate (succinyl-leucine-valine-tyrosine-aminoluciferin) to determine chymotrypsin-like activity. Proteasome cleavage of the substrate produces a luminescent signal from the luciferase contained in the reaction medium. The three major proteolytic activities (chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolytic or caspase-like activity) occurring within the 20S core of the 26S proteasome complex are responsible for most of the protein degradation, which includes degradation of damaged cellular proteins. Therefore, this coupled-enzyme system, with simultaneous proteasome cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that can be considered proportional to the amount of proteasome activity in the muscle tissue.

For the assay, 25 mg muscle powder was added to 1 mL 10 mM KH_2PO_4, pH 7.4, in 0.9% NaCl and gently homogenized. Fifty microlitres of the resulting muscle homogenate was pipetted in duplicate onto 96-well microplates, and the final reagent mixture with luminescent substrate was added to the medium. After 5 min, under light protection, the luminescent signal at the plate was detected with a Glo-Runner microplate reader luminometer (Turner Designs), and the results were expressed as RLU per milligram total protein.

In a similar manner, the activity of a calcium-dependent protease was measured by a chemiluminescence assay using a Calpain-Glo™ Protease Assay kit (Promega; Madison, WI, USA). Homogenates were prepared at a concentration of 6.25 mg/mL 10 mM KH_2PO_4 buffer, pH 7.4, in 0.9% NaCl. The luminescent signal is proportional to the calcium-dependent proteolytic activity in samples, which is also based on the specific reaction of the labelled luciferin substrate by the enzyme luciferase. The results are expressed as RLU per milligram tissue.

Protein concentration

The protein concentration was determined by the method of Lowry *et al.* (1951) modified by Miller (1959). This method involved the use of bovine serum albumin (BSA) as a standard.

Statistical analysis

When the distribution was considered normal, the results are presented as mean ± standard error of the mean (SEM) for 10 animals and values were compared using one-way analysis of variance followed by Bonferroni's multiple comparison test, with $P < 0.05$ considered significant. Curves of chemiluminescence induced by tert-butyl hydroperoxide were compared by two-way ANOVA. When the distribution did not pass a normality test, data are presented as median and interquartile range, and values were compared by Kruskall–Wallis test followed by Dunn's multiple comparison test. For the muscle morphometry data, histograms of the fibre cross-sectional areas were prepared and absolute frequencies calculated.

Results

Emphysema condition

The extent of lung damage in each group was evaluated by the number of CAI. A previous pilot study demonstrated no lung damage in the saline-treated group (CS) relative to a control (no saline, papain, or elastase instillation – data not shown). The EE and EP groups had significantly lower CAI values (48.17 ± 1.16 and 51.56 ± 0.49, respectively) than the CS group (70.64 ± 0.58, $P < 0.0001$ in both comparisons). The EE and EP groups also had significant differences in pulmonary emphysema ($P < 0.05$). Representative images of lung injury are presented in Figure 1 and CAI values are presented in Table 1. The cachexia index, using gain of body mass in the control group as the reference, was 4.6% ± 0.48% in the EP group. The EE group did not show cachexia (0.76% ± 1.17%). In addition, gereral body weight showed by CS, EE and EP during the course of 70 days is demonstrated on supplemented Figure 1. The ratio of gastrocnemius mass (MM) / total mass (MT) was significantly increased in the EE (0.16 ± 0.003%) when compared to CS (0.14 ± 0.004%). In contrast, when the CS and EP groups were compared, the relationship showed a significantly

decrease (0.12 ± 0.004%). These results represented 8.37% muscle gain in the EE group and 18.34% muscle loss in the EP group compared with the gastrocnemius muscle weight of the CS animals.

The frequency distribution of fibre cross-sectional areas showed differences between groups. Representative images of gastrocnemius muscles are presented in Figure 2. The 50th percentile of fibre area occurred in the fourth class (2000–2499 μm^2) for the CS group, the fifth class (2500–2999 μm^2) for the EE group, and the third class (1500–1999 μm^2) for the EP group. The cumulative frequency to the class containing the 50th percentile was 1401 occurrences in the CS group, compared with 1811 occurrences for the EE group, and 2195 for the EP group. The complete histograms of the frequency distributions are presented in Figure 2D.

Oxidative stress

Figure 3A presents chemiluminescence curves from the CS, EE, and EP groups at 60 min, obtained by interpolation. The two-way ANOVA showed significant differences in all comparisons (CS × EE, CS × EP, and EE × EP, $P < 0.05$). The area under the chemiluminescence curve, obtained by interpolation, was significantly higher in the EP group (18960 ± 2598 RLU/g tissue) compared with the CS group (10775 ± 1564 RLU/g tissue, $P < 0.05$), but the EE group (15106 ± 2469 RLU/g tissue) was not significantly different. Figure 3C shows carbonyl protein levels in the CS, EE, and EP groups. Only the EP group (80.71 ± 3.39 nmol carbonyl/mg total proteins) showed higher carbonyl protein levels relative to the CS group (69.16 ± 3.55 nmol carbonyl/mg total proteins, $P < 0.05$).

TBARS and GSSG did not show any significant differences between the groups. GST levels were not significantly different between the CS group (median, 73.5 [IQR, 67.13–83.7] μM/mg total proteins) and the EP group (median, 62.15 [IQR, 50.07–70.68] μM/mg total proteins) or the EE group (median, 82.10 [IQR, 65.83–86.26] μM/mg total proteins). However, the GST level in the EP group was significantly lower than in the EE group ($P < 0.05$).

Analyses of chymotrypsin and calpain-like proteolytic activities

Chymotrypsin-like proteolytic activity did not differ between the CS group (24259 ± 9445 RLU/mg protein) and the EE group (55665 ± 12299 RLU/mg tissue). However, the EP group

(66265 ± 7905 RLU/mg protein) had a significantly higher chymotrypsin-like proteolytic activity (Figure 4A). On the other hand, calpain-like activity was significantly higher in the EE group (45490 ± 4585 RLU/mg total protein) than the CS group (21330 ± 3382 RLU/mg total protein), and the EP group (20735 ± 1668 RLU/mg total protein), but the CS and EP groups did not differ (Figure 4B).

Discussion

Emphysema has been shown to reduce the contractile function of locomotory skeletal muscle (Bernard *et al.* 1998), and lead to skeletal muscle loss in humans (Gosselink *et al.* 1996). These processes have been credited to muscle mass adapting to modified oxygen uptake and distribution (Wust and Degens, 2007). In addition, oxidative stress is involved in the process of skeletal muscle changes in patients with COPD (Fermoselle *et al.* 2011). However, as gaining access to human biopsy samples for experiments is difficult, most studies regarding to molecular and biochemical aspects of COPD use animals, with different ways of inducing emphysema. Most experimental models use proteolytic enzymes, such as papain and elastase, instilled or nebulized into the airways. These models are based on theory regarding the unbalanced production of aggressive and protective substances in the lung (Fusco *et al.* 2002). They result in histological, morphological, and physiological alterations to the lungs equivalent to the changes found in human emphysema (Hayes *et al.* 1975). In addition, few studies consider time used by induction influence, or even different inductions, on systemic manifestations (Mattson *et al.* 2008).

In the present study, both elastase and papain caused pulmonary injury at a level sufficient to promote emphysema. Papain and elastase produced a pattern of panacinar emphysema with part of the lobe histoarchitecture preserved, but rupture of the alveolar septs. However, injuries in the ruptured extremities were more extensive with elastase compared with papain induction. In addition, quantitative evaluation of emphysema showed a significant decrease in the CAI values compared with saline instillation. The induction doses used were very close to the doses that increase animal mortality immediately after induction; despite this, CAI were different between the groups. Classic studies in the literature compared the two models (Schulz *et al.* 1989; Karlinski and Snider 1978), and demonstrated that, although they induce slightly different patterns of lesion, they both establish the histological and functional characteristics of emphysema. However, at present, no studies have demonstrated differences in the skeletal muscle adaptations between the two models.

In emphysema, the main factors that contribute to muscle wasting dysfunction are muscle weakness and loss of muscle mass (Wust and Degens 2007), which both have a direct impact on exercise tolerance. In the present study, when systemic adaptation was evaluated by general and skeletal muscle losses, gross morphological and morphometric differences were found between the two forms of induction. The gastrocnemius muscle has previously been demonstrated as a good indicator of alterations in the skeletal muscle of emphysemic hamsters, where there is no obvious gross division of fibers ans consequently the total muscle is analysed (Tonos *et al.* 2013).

In the present study, elastase induction did not promote general loss, unlike in the papain group, which showed 4.6% weight loss compared with control group 70 days after induction. These data were reflected in skeletal muscle loss and in morphometry in some extension. In principle, these data could be explained by differences in food restriction caused by hypoxia. Some studies reported the action of nicotine in modulating the leptin secretion and consequently appetite. This pathway obviously has the influence of hypoxia and seems to be particularly important on cigarette-smoke induced emphysema. The decrease in oxygen consumption and increase in cytokine levels are present in very early stages after experimental emphysema induction and have their origin in pulmonary lesion. We monitored plasma TNF-alpha levels at 10, 20, 40 and 70 days after instillation and found no significant differences between CS, EE and EP groups (data not shown). In addition, we monitored food consumption during the first 15 days after induction and also found no differences between groups although the surgery procedure could affect swallow. Although the anorexic effects of hypoxia may contribute to muscle wasting as well, reductions in muscle mass independent of hypophagia have also been reported (Langen *et al.* 2013).

Studies in humans and animals demonstrated that skeletal muscle in COPD demonstrated increased oxidative stress parameters, what could have a relationship with hypoxia through increased generation of superoxide anion in the respiratory electric chain (Mattson *et al.* 2002b). Mattson *et al.* (2002a) found that fibre cross-sectional area and distribution also varied within and among muscles in their studies of hamsters with emphysema induced by elastase. These information are reinforced by correlation with citrate synthase activity, a marker of muscle oxidative capacity. However, based on our results, models of inducing emphysema by elastase or papain seems to promote different systemic consequences at the same time point.

The difference in the distribution of fibre cross-sectional area in this study showed that papain induction can produce at least initial skeletal muscle adaptation at 70 days after induction.

Although no fiber type was assayed, it is reasonable to assume that fiber type modification would be present, as observed in previous studies. Together, these data indicate that skeletal muscle adaptation is different between the two models.

Among several factors, oxidative stress is a major player in the aetiology of skeletal muscle dysfunction. Although low levels of oxidants are required for normal cell adaptation, moderate to high levels of reactive oxygen species alter the function and structure of proteins (Fermoselle *et al.* 2011; Grune *et al.* 2003). Langen *et al.* (2013) point to oxidative stress as an important key factor in proteolysis regulation affecting the atrophy associated with COPD. Specifically, protein carbonylation, a common variety of protein oxidation, alters the function of key enzymes and structural proteins involved in muscle contraction (Fermoselle *et al.* 2011; Tonon *et al.* 2013). Thus, it is reasonable to evaluate if both emphysema models developed oxidative modifications, since other studies have shown that MDA is elevated in skeletal muscle of humans with COPD, in hamsters 180 days after induction, and in mice 34 weeks after induction of emphysema with elastase (Wijnhoven *et al.* 2006; Fermoselle *et al.* 2011; Mattson *et al.* 2002b). In this study, TBARS, the classic method for evaluating oxidative stress, showed no difference between groups. In contrast, when chemiluminescence stimulated by t-BOOH was assayed, the gastrocnemius muscle of animals with emphysema induced by papain showed higher membrane lipid peroxides. Chemiluminescence is a very sensitive method that takes into account the kinetic analysis of the ascending part of the curve in addition to its peak. The technique is based on the concept that increased variation in these two parts of the curve represents higher levels of pre-existing membrane lipoperoxides. Thus, when a tissue is challenged with tert-butyl hydroperoxide, the chemiluminescence curves should reflect both the attack suffered by the membrane and the tissue antioxidant consumption mediating the damage caused by this attack. Thus, the change in membrane permeability due to oxidative imbalance can be observed based on analysis of the chemiluminescence curve and confirmed through the area under the chemiluminescence curve (Barbosa *et al.* 2003). In addition, MDA can react with proteins leading to carbonylation (Guarnier *et al.* 2010), which could diminish its adducts at the time point of this study. Here, the EP group showed an increase in the overall height of the chemiluminescence curve in both experimental periods, with a maximum height achieved. This result was in agreement with the peak of carbonyl proteins content and area under the chemiluminescence curve. Yet, muscles from hamsters with emphysema induced by papain showed decreased GST compared to elastase, a parameter of redox balance that is frequently diminished in skeletal muscle atrophy, with no increasing in GSSG levels (Guarnier *et al.* 2010; Bernardes *et al.* 2014).

Perhaps the thiolation group or trypeptide may have other destinations, such as thilolation or glutathiolation. Since we have evidence to support this conclusion, we propose that the ratio GST/GSSG may not reliably reflect the redox status when muscle loss occurs at least in models of emphysema. But the evaluated parameters still lead to the conclusion that skeletal muscle adaptation differs between elastase and papain induction, including events related to oxidative stress.

Oxidative stress may differentially regulate protein loss within peripheral muscles of in patients with severe COPD who exhibit different body composition (Fermoselle *et al.* 2012). This seems to be the trigger factor to the muscle mass loss. A model that is unable to generate oxidative stress in the muscle, do not trigger skeletal muscle loss. At our knowledge, the only model that demonstrated muscle loss in the papain-induced emphysema was the study published by Tonon *et al.* (2013), that showed increased chymotrypsin-like activity in skeletal muscle of hamsters with emphysema 60 days after induction. No studies involving elastase-induced emphysema were performed in this sense. Fermoselle *et al.* (2011) demonstrated induction of several components of atrophy pathways in the elastase induction model after 34 weeks. Interestingly, no studies had evaluated calpain or calpain-like activity. Proteasome and calpains are two of the four proteolytic systems in skeletal muscle. They are extensively involved in metabolic protein turnover and have different effects on atrophy processes (Goll *et al.* 2007). Proteasome is involved in and has its activity induced by oxidative stress (Powers *et al.* 2010). It can accelerate degradation of oxidatively modified proteins, leading to atrophy (Grune *et al.* 2003b). The calpains seem to be involved in turnover of myofibrillar proteins (Goll *et al.* 2007). When activated, calpains can degrade myofilament stabilizers, exposing heavy or light chain myosin to degradation by proteasome (Costelli *et al.* 2005). We can speculate that this may be made possible by changes in fibre size as demonstrated in the EE group, which showed a concomitant increase in calpain-like activity.

In summary, elastase and papain can induce pulmonary emphysema through practically the same extension of pulmonary lesions. However, by 70 days after emphysema induction, the papain model of induction led to skeletal muscle atrophy and oxidative stress activation concomitant to chymotrypsin-like proteolytic activity; elastase induction led to increased muscle weight and fibres of higher sizes concomitant to calpain-like activity participation in the adaptation. These data, when put together, indicate that elastase and papain, when used to induce experimental models of emphysema, lead to different types of adaptation. These findings provide more information for choosing a suitable experimental model when studying skeletal muscle adaptations to emphysema.

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TABLES

Table 1 – Animal and skeletal muscle adaptation characteristics.

	CS	EE	EP
Crossed Alveolar Intercept	70.64 ± 0.58	48.17 ± 1.16*	51.56 ± 0.49*#
Cachexia Index (%)	-	0.76±0.17	4.6±0.48
MM/MT (%)	0.14 ± 0.004	0.16 ± 0.003*	0.12 ± 0.004*#
% of variation on gastrocnemius (compared with CS)	-	+ 8.37	−18.34

CS – Group instilled with 300 μL NaCl 0.9%; EE – Group instilled with 300 μL papain 40 mg/mL in NaCl 0.9%; EP – Group instilled with 300 μL elastase 5.2 IU in NaCl 0.9%. Cachexia index = (initial body weight − final body weight + body mass gain of CS group) / (initial body weight − body mass gain of CS group) × 100 (Tonon et al. 2013). MM – gastrocnemius weight; MT – Total animal weight; MM/MT – ratio between values × 100, expressed as percentage. Values are expressed as means ± SEM (CS: $n = 10$; EE: $n = 10$; EP: $n = 10$). *$P < 0.05$ compared with CS and #$P < 0.05$ comparing EP with EE by one way ANOVA followed by Bonferroni's multiple comparison test.

Table 2 – Effect of emphysema on skeletal muscle in terms of TBARS, GST, GSSG in hamsters.

	CS	EE	EP
TBARS (nmol/g tissue)	0.42[0.35–0.67]	0.49[0.41–0.59]	0.42[0.28–0.49]
GST (μM/mg total proteins)	73.5[67.13–83.7]	82.1[65.83–86.26]	62.15[50.07–70.68#
GSSG (μM/mg total proteins)	6.15[5.6–7.78]	6.82[2.75–7.65]	4.13[2.42–5.85]

CS – Group instilled with 300 μL NaCl 0.9%; EE – Group instilled with 300 μL papain 40 mg/mL in NaCl 0.9%; EP – Group instilled with 300 μL elastase 5.2 IU in NaCl 0.9%. TBARS – thiobarbituric acid reactive substances. GST – total glutathione. GSSH – oxidized glutathione. Values are expressed as median [25–75 percentile]. Groups were composed of 10 animals. #$P < 0.05$ when EP with EE were compared by Kruskall–Wallis test followed by Dunn's multiple comparison test.

FIGURE CAPTIONS

Figure 1 – Histological images from lungs of hamsters treated with elastase, papain, or saline. (**A**) control + saline (CS): animals treated with 0.3 mL sterile saline only; (**B**) emphysema + elastase (EE): animals treated with 0.3 mL 5.2 IU elastase in sterile saline; and (**C**) emphysema + papain (EP): animals treated with 0.3 mL 40 mg/mL papain in sterile saline. Alveolar destruction was determined by the number of times that a predetermined group of coherent lines (1.25 mm^2 total area and 1.50 mm total length) crossed the parenchymal structures, named crossed alveolar intercepts. The less these structures are crossed, the more extensive is the lesion.

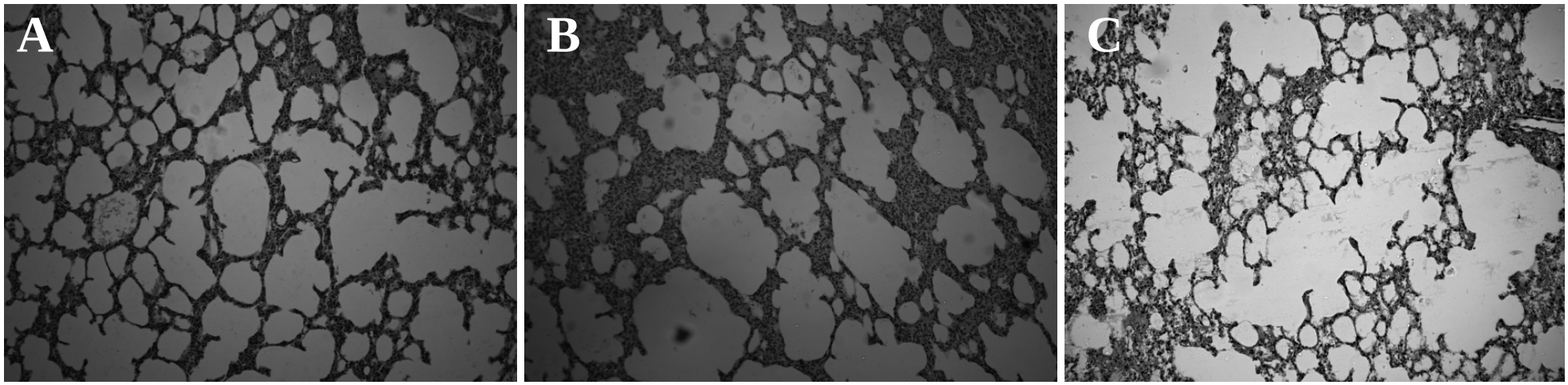
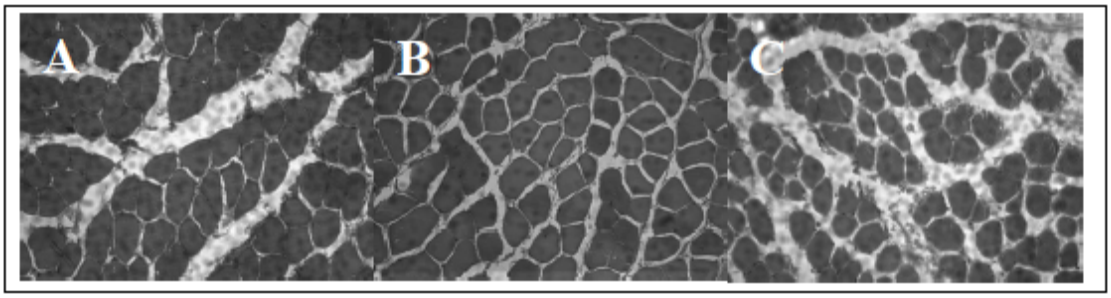


Figure 2 – Histological and morphometric differences in skeletal muscles of hamsters with emphysema induced by papain or elastase. (**A**) control + saline (CS): animals treated with 0.3 mL sterile saline only; (**B**) emphysema + elastase (EE): animals treated with 0.3 mL 5.2 IU elastase in sterile saline; and (**C**) emphysema + papain (EP): animals treated with 0.3 mL 40 mg/mL papain in sterile saline. Animals were euthanized after 70 days and lungs stained with haematoxylin and eosin. Original magnification 200×. (**D**) Histogram of frequency distribution of fibre cross-sectional areas (μm^2). Muscles were sectioned and five semi-serial cuts per slide (50 µm interval) were positioned on two slides. Three fields per cut were captured and 20 fibres measured per field per animal, totalling 600 fibre measurements per animal. The values were grouped by procedure (CS, EE, or EP) and frequency distribution determined. Values of median [25 – 75 percentiles], minimal and maximal values were inserted in the graph to illustrate changes in the distribution between groups.



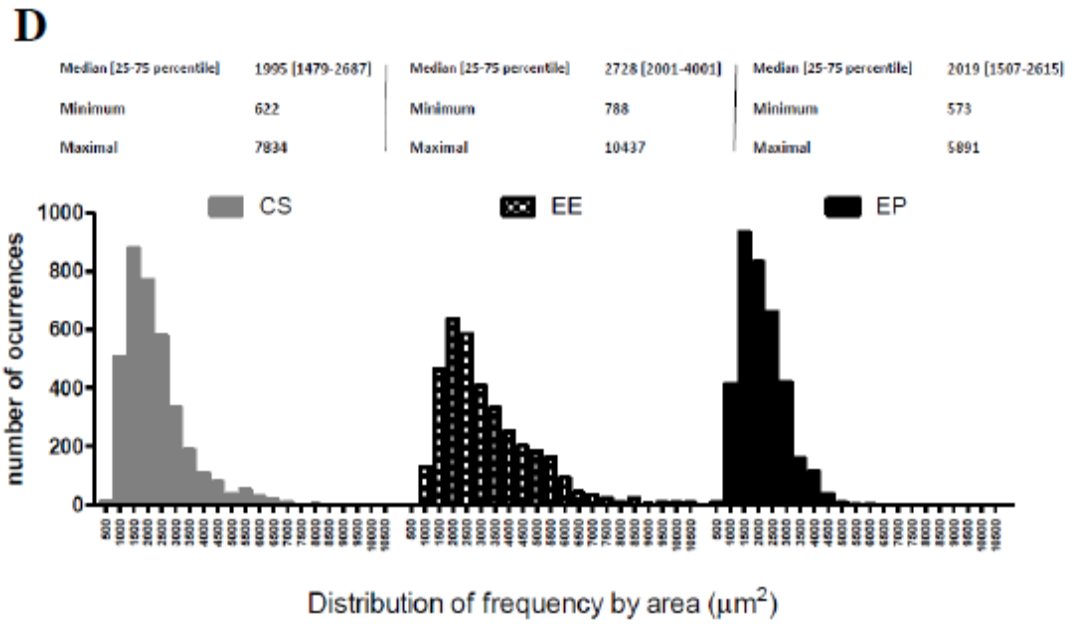


Figure 3 – Effect of emphysema induced by papain or elastase on skeletal muscle of hamsters in terms of lipid hydroperoxide and carbonyl proteins. (**A**) The tert-butyl hydroperoxide-initiated chemiluminescence was monitored continuously for 60 min in gastrocnemius homogenates. Each curve represents the mean of 10 animals' curves, corrected by grams of tissue. The entire curves were used to perform statistical comparison by two-way ANOVA. (**B**) Area under the curve of each animal curve was determined to compare groups. (**C**) Levels of protein carbonylation in muscle homogenates of control, emphysema induced by elastase, and emphysema induced by papain animals. The difference was assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. Values are expressed as means ± SEM (n = 10 per group).*$P < 0.05$ compared with CS and $^{\#}P < 0.05$ comparing EP with EE.

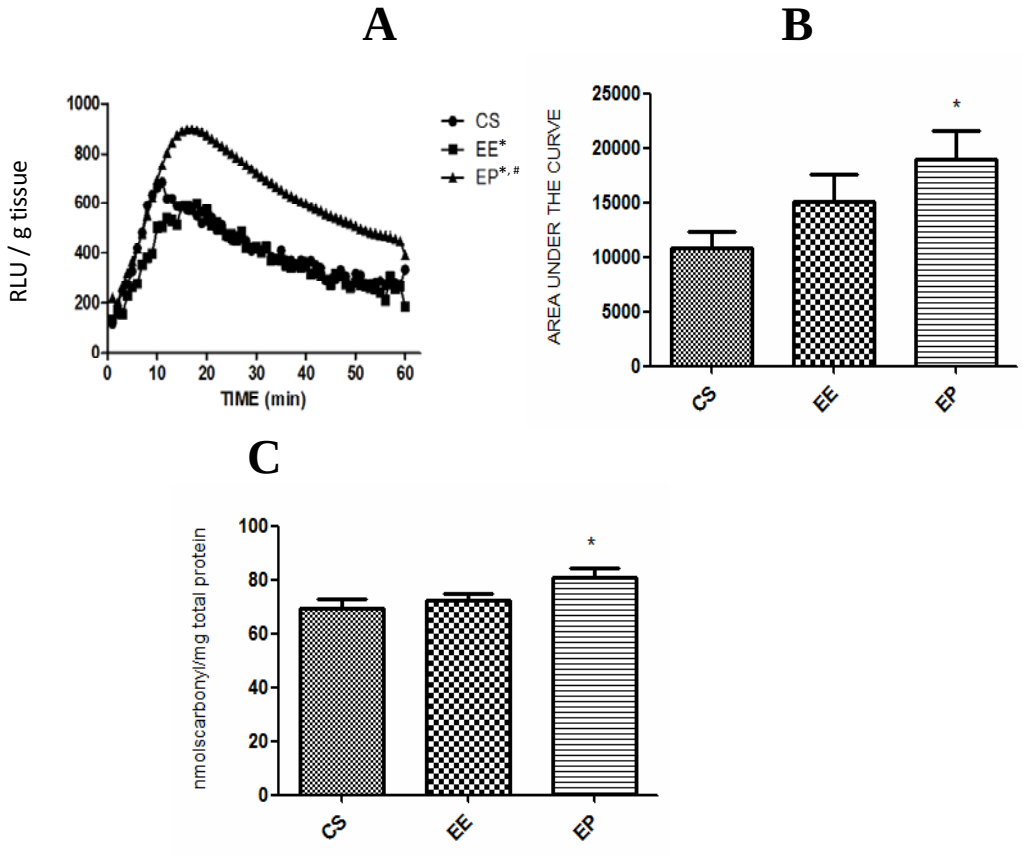
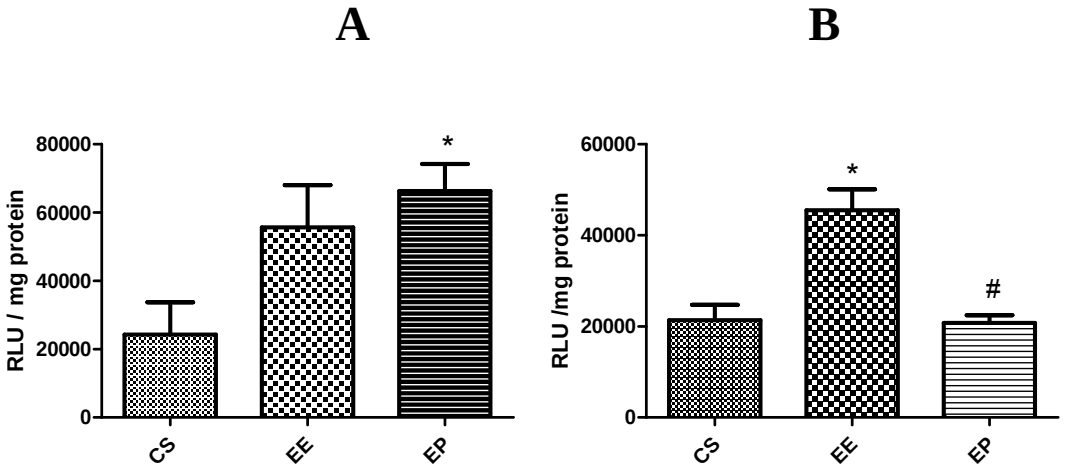


Figure 4 – Quantification of proteolytic activity in gastrocnemius of hamsters with emphysema induced by papain or elastase quantified by a luminescent kit. (A) Chymotrypsin-like activity (B) Calpain-like activity. Control and experimental groups were compared by one-way ANOVA followed by Bonferroni's test. **P* < 0.05 compared with CS. #*P* < 0.05 compared with EE. CS – Group instilled with 300 µL NaCl 0.9%; EE – Group instilled with 300 µL papain 40 mg/mL in NaCl 0.9%; EP – Group instilled with 300 µL elastase 5.2 IU in NaCl 0.9%.



CONCLUSÃO

Este estudo mostra evidências que, a elastase e a papaína, quando utilizadas para induzir modelos experimentais de enfisema pulmonar, levam há diferentes tempos e velocidades de adaptação muscular, fornecendo informações sobre a escolha de um modelo experimental adequado.

O modelo de enfisema pulmonar induzido por papaína leva a atrofia muscular com envolvimento do estresse oxidativo e atividade proteolítica neste processo. Em adição, o modelo por elastase pode levar a uma adaptação muscular precoce, com ausência de atrofia e participação do estresse oxidativo. No entanto, este parece ser um fenômeno que permanece instalado, podendo contribuir para a perda de massa muscular em estágios avançados da doença.

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