**Universidade Federal de Ouro Preto**

Núcleo de Pesquisa em Ciências Biológicas

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Tese

# Avaliação do potencial terapêutico da quercetina sobre o desequilíbrio redox e inflamação em células e camundongos expostos à fumaça de cigarro.

*Natália Pereira da Silva Araújo*

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**Ministério da Educação**
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# AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DA QUERCETINA SOBRE O DESEQUILÍBRIO REDOX E INFLAMAÇÃO EM CÉLULAS E CAMUNDONGOS EXPOSTOS À FUMAÇA DE CIGARRO

**Natália Pereira da Silva Araújo**

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Tese apresentada ao programa de Pós-graduação em Ciências Biológicas da Universidade Federal de Ouro Preto, como parte integrante dos requisitos para obtenção do título de Doutor em Ciências Biológicas, área de concentração: Bioquímica Metabólica e Fisiológica

Orientador: Prof. Dr. Frank Silva Bezerra

Co-orientador: Prof. Dr. Rodrigo Cunha de Alvim Menezes

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## RESUMO

A fumaça de cigarro é altamente tóxica e a sua inalação promove o aumento da produção de espécies reativas, bem como uma resposta inflamatória pulmonar e sistêmica em seja em modelos experimentais ou em humanos. A quercetina é um potente antioxidante dietético que exibe atividades antiinflamatórias. O objetivo deste estudo foi avaliar os efeitos da quercetina na redução do desequilíbrio redox e da inflamação induzidos pela exposição, *in vitro* e *in vivo*, em curto e longo prazo, à fumaça do cigarro. *In vitro*, a quercetina nas concentrações de 25 e 50 μM, atenuou os efeitos do extrato da fumaça do cigarro diminuindo a geração de espécies reativas de oxigênio e óxido nítrico em macrófagos alveolares J774A.1. *In vivo*, camundongos machos C57BL/6 foram divididos em cinco grupos: controle (CG), veículo (VG), quercetina (QG), fumaça de cigarro (CSG), quercetina e fumaça de cigarro (QCSG). CSG e QCSG foram expostos à fumaça de cigarro por cinco e sessenta dias consecutivos. Ao término do protocolo experimental, os animais foram eutanasiados e foram retirados o lavado broncoalveolar, o sangue, o tecido pulmonar e hepático. Os camundongos que receberam 10 mg/kg/dia de quercetina diluída em uma solução de propilenoglicol/Salina (50%) via gavagem orogástrica, uma hora antes da primeira exposição à fumaça de cigarro por cinco dias, apresentaram uma diminuição no influxo de leucócitos no lavado broncoalveolar, reestabelecendo o desequilíbrio redox, com isso, preservando o padrão histológico do parênquima pulmonar e a função pulmonar em comparação com o grupo exposto à fumaça de cigarro. No segundo estudo, os camundongos que receberam a mesma dose de QC antes da exposição à fumaça de cigarro por 60 dias apresentaram um menor influxo de células inflamatórias, estresse oxidativo, reação inflamatória e alterações histopatológicas no fígado em comparação aos animais expostos a fumaça de cigarro. Esses resultados sugerem que a quercetina atuou de forma preventiva em células e tecidos de diferentes modelos experimentais de exposição a fumaça de cigarro.



**Palavras-chave:** Fumaça de cigarro, desequilíbrio redox, inflamação, quercetina, pulmão, fígado.

## ABSTRACT

Cigarette smoke is highly toxic and its inhalation promotes an increase in the production of reactive species, as well as a pulmonary and systemic inflammatory response, both in experimental models or humans. Quercetin is a potent dietary antioxidant that exhibits anti-inflammatory activities. This study aimed to evaluate the effects of quercetin in reducing redox imbalance and inflammation induced by exposure, in vitro, and in vivo, in the short and long term, to cigarette smoke. In vitro, quercetin in 25 and 50 μM concentrations, attenuated the effects of cigarette smoke extract, thus increasing the generation of reactive oxygen and nitric oxide species in alveolar macrophages J774A.1. In vivo, male C57BL/6 mice were divided into five groups: control (CG), vehicle (VG), quercetin (QG), cigarette smoke (CSG), quercetin and cigarette smoke (QCSG). CSG and QCSG were exposed to cigarette smoke for five and sixty consecutive days. At the end of the experimental protocol, the animals were euthanized and the bronchoalveolar lavage, blood, lung, and liver tissue were removed. Mice receiving 10 mg/kg/day of quercetin via orogastric gavage before exposure to cigarette smoke for five days presented a decrease in the influx of leukocytes in the bronchoalveolar lavage, re-establishing the redox imbalance, thereby, preserving the histological pattern of the parenchyma and lung function compared to the group exposed to cigarette smoke. In the second study, the mice that received the same dose of quercetin before exposure to cigarette smoke for 60 days dissipated a lower influx of inflammatory cells, oxidative stress, inflammation, and histopathological changes in the liver compared to animals exposed to cigarette smoke. These results suggest that quercetin acted preventively in cells and tissues of different experimental models exposed to cigarette smoke.


**Key-words:** Cigarette smoke; redox imbalance; inflammation; quercetin; lung; liver.

# LISTA DE ABREVIAÇÕES

$^1O_2$ – Oxigênio singleto

ADP – Adenosina difosfato

AMP – Adenosina monofosfato

AP-1 – Proteína ativadora 1

ARE – Elemento de resposta antioxidante

ATP – Adenosina trifosfato

CAT – Catalase

CCL-2 – Quimiocina ligante 2 (motivo C-C)

CEUA - Comitê de ética em pesquisa no uso de animais

CO – Monóxido de carbono

COX – Ciclooxigenase

Cu – Cobre

DPOC – Doença Pulmonar Obstrutiva Crônica

DNA – Ácido desoxirribonucléico

ERO – Espécies reativas de oxigênio

FADH - Flavina adenina dinucleotídeo

FC – Fumaça de cigarro

GPx – Glutationa peroxidase

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

GR- Glutationa redutase

$H_2O$ – Água

$H_2O_2$ – Peróxido de hidrogênio

HbCO - Carboxihemoglobina

HO-1 – Heme oxigenase-1

IL-10 – Interleucina 10

IL-1β – Interleucina 1-beta

IL-6 – Interleucina 6

IL-8 – Interleucina 8

INFγ – Interferon gamma

IκB – Inibidor do kappa B

MAPK – Proteína quinase ativada por mitógenos

MMP – Metaloproteinase de matriz

NADH – Nicotinamida Adenina Dinucleotídio

NFκB – Fator nuclear kappa B oxidado

NOX – NADPH

OMS- Organização mundial de saúde

$O_2$ – Oxigênio molecular

$O_2^{\cdot-}$ – Radical superóxido

$OH^{\cdot}$ – Radical hidroxila

$ONOO^-$ – Peroxinitrito

$ONOOH^{\cdot}$ – Ácido peroxinitroso

PKC – Proteína quinase C

QC – Quercetina

SOD – Superóxido dismutase

TNF-α – Fator de necrose tumoral - alfa

XO – Xantina oxidase

Zn – Zinco

# LISTA DE ILUSTRAÇÕES

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# 1. INTRODUÇÃO

## 1.1.Fumaça de cigarro

O tabagismo é um importante fator de risco para doenças não transmissíveis, especialmente aquelas relacionadas ao sistema respiratório. No século passado, o tabagismo foi responsável por mais de 100 milhões de mortes em todo mundo e estimativas apontam que até 2030 cerca de 8 milhões de pessoas morrerão a cada ano em decorrência de alguma doença relacionada ao tabagismo (Asma, Song et al. 2014, Organization 2017, Jafari, Rajabi et al. 2021).

A fumaça de cigarro (FC) é altamente tóxica, contém uma variedade de material particulado, várias substâncias químicas e espécies reativas de oxigênio (EROs) capazes de induzir estímulos inflamatórios envolvidos no processo de iniciação e progressão de diversas doenças tanto pulmonares quanto hepáticas (Altamirano and Bataller 2010, Barbosa, Costa et al. 2010, Westra, Schlage et al. 2013, Dalrymple, Ordonez et al. 2015, Lin, Taggart et al. 2021).

A FC pode ser dividida em duas fases, a fase de alcatrão ou de partículas e a fase gasosa, que juntas são fonte de mais de 2000 espécies reativas por volume de FC inalado (Smith and Fischer 2001, Cantin and Richter 2012). Os produtos da combustão do cigarro são fontes de vários metais, incluindo cobre e ferro que podem reagir com $H_2O_2$ na reação de Fenton/Haber-Weiss gerando o radical hidroxila (-OH•) (Berg, Youdim et al. 2004, Cantin and Richter 2012). A fase gasosa também é fonte de aldeídos como acetaldeído, acroleína e crotonaldeído que têm o potencial de induzir o estresse oxidativo e a inflamação (van der Toorn, Slebos et al. 2013, Lin, Taggart et al. 2021).

O organismo é continuamente exposto a processos oxidativos, seja devido à alta irrigação de seus tecidos ou pela constante presença de oxigênio e poluentes tóxicos no ar inspirado. Além destas fontes exógenas de substâncias pró-oxidantes, também estão presentes nestes órgãos as espécies reativas geradas a partir de processos metabólicos vitais, como na cadeia respiratória mitocondrial (Park, Kim et al. 2009, Biswas, Hwang et al. 2013, Domej, Oettl et al. 2014).

A inalação da FC pode danificar diretamente células e tecidos, desativar mecanismos de defesa, iniciar o processo inflamatório e perturbar o equilíbrio redox seja pelas EROs presentes na FC ou por aquelas resultantes da resposta inflamatória associada ao contato com FC (Vaamonde-Garcia, Riveiro-Naveira et al. 2012, Hoffmann, Zarrintan et al. 2013, Xiong, Wu et al. 2021).

O estímulo para a produção das EROs via exposição à FC ocorre principalmente por ativação da enzima xantina oxidase e do complexo enzimático NADPH oxidase (NOX). O aumento na concentração das EROs leva a ativação de vias centrais de sinalização redox-sensíveis, tais como, proteínas quinases (PKC)**,** o fator nuclear kappa B (NF-kB) e proteína ativadora-1 (AP-1), que ativam múltiplos genes inflamatórios e contribuem para o desequilíbrio redox (Drost, Skwarski et al. 2005, Boutten, Goven et al. 2010, Wu, Lin et al. 2014).

### 1.2. Doença Pulmonar Obstrutiva Crônica (DPOC)

Doença Pulmonar Obstrutiva Crônica (DPOC) é o termo usado para um grupo de doenças pulmonares caracterizadas por sintomas respiratórios crônicos, anormalidades na estrutura pulmonar e declínio progressivo da função pulmonar, acontecendo, na maioria das vezes, de forma irreversível (Agustí and Hogg 2019). Nesse grupo, duas doenças se destacam, o enfisema pulmonar e a bronquite crônica. O enfisema é caracterizado pela destruição do septo alveolar com diminuição da área de superfície e a bronquite pelo espessamento dos brônquios e bronquíolos com hipersecreção de muco (Vestbo, Hurd et al. 2013, Vogelmeier, Criner et al. 2017). Portadores de DPOC apresentam um processo inflamatório crônico, concomitantemente à uma hiper-reatividade das vias aéreas, falta de ar, tosse crônica, disfunção da musculatura esquelética, intolerância ao exercício e piora do estado nutricional (Barnes and Celli 2009, Cavailles, Brinchault-Rabin et al. 2013, Liu, Pleasants et al. 2015).

A DPOC impõe uma carga socioeconômica significativa principalmente devido à sua alta prevalência, morbidade e mortalidade, sendo um problema mundial de saúde pública. A DPOC é uma doença prevenível e tratável que pode apresentar efeitos extrapulmonares significativos, interferindo na qualidade de vida e na sobrevida dos indivíduos acometidos (Vestbo 2014, Burney, Patel et al. 2015, Lopez-Campos, Tan et al. 2016).

Os principais fatores de risco para desenvolvimento da DPOC são os genéticos como a deficiência da alfa-1 antitripsina, e os ambientais, tais como a poluição atmosférica por resíduos de petróleo, a exposição ocupacional a poeiras como a sílica e o tabagismo, sendo esse último, o mais importante e evitável (Salvi 2014, Postma, Bush et al. 2015, Alter, Baker et al. 2020).

A patogênese da DPOC envolve processos característicos: inflamação crônica e persistente, desequilíbrio entre proteases e antiproteases, o desequilíbrio redox, além de destruição de tecido e reparo inadequado, que ocorrem de modo concomitante e podem ser

desencadeados pela inalação de partículas ou gases nocivos (Macnee 2007, Seifart and Plagens 2007, Fischer, Pavlisko et al. 2011, Lugg, Scott et al. 2021, Zhigang Wang 2021).

O processo inflamatório pulmonar é marcado por um maior infiltrado de células inflamatórias principalmente, neutrófilos, macrófagos ativados e linfócitos T CD8+ que atuam através de mediadores como, citocinas pró-inflamatórias e espécies reativas de oxigênio (ERO) (Karadag, Karul et al. 2008, Tudorache, Oancea et al. 2015, Ding L 2021).

Na inflamação neutrófila, há a produção de diversas proteases como elastases, metaloproteinases e catepsinas que degradam a matriz extracelular e geram fragmentos de fibras elásticas ou derivados de fibras colágenas capazes de atrair monócitos que são precursores de macrófagos. Macrófagos ativados liberam mediadores inflamatórios que promovem o recrutamento de neutrófilos para o foco inflamatório, os neutrófilos perpetuam a inflamação e esse processo leva à destruição do parênquima (Hunninghake, Davidson et al. 1981, He, Turino et al. 2010). Na homeostase, a atividade das proteases é prevenida pela ação de antiproteases como a alfa-1 antitripsina e inibidores de metaloproteinases de matriz (TIMP), sendo assim, em situações que favoreçam a produção de proteases como na inflamação, há um desequilíbrio entre proteases e antiproteases o que favorece o dano pulmonar (Almeida-Reis, Theodoro-Junior et al. 2017, Horio, Ichiyasu et al. 2017, Sun, Bao et al. 2017, Ding L 2021).

Outro fator importante na patogênese da DPOC é o desequilíbrio redox, caracterizado pelo desequilíbrio entre oxidante/antioxidante em favor dos oxidantes, pode levar ao dano direto ao parênquima pulmonar como também amplificar os demais mecanismos apontados anteriormente (Singh, Verma et al. 2017).

### 1.3. Doença Hepática

O hábito de fumar, além dos efeitos deletérios às vias aéreas, está associado à inflamação sistêmica, ao estresse oxidativo e à resistência à insulina, fatores que favorecem danos extrapulmonares como o dano hepático (Altamirano and Bataller 2010, Martins, Campos et al. 2017).

O fígado é um órgão multifuncional responsável pelo metabolismo de nutrientes e xenobióticos como drogas, álcool e outras toxinas, assim, doenças hepáticas representam importante foco de atenção. Dentre as doenças hepáticas, a doença hepática gordurosa não-alcoólica é a mais comum em países industrializados, ela está relacionada ao estilo de vida e o tabagismo é um importante fator de risco para seu desenvolvimento. A doença hepática

gordurosa não alcoólica, quando não tratada, pode progredir para hepatite, cirrose e insuficiência hepática (Estes, Anstee et al. 2018, Okamoto, Miyake et al. 2018).

No fígado, a fumaça de cigarro causa danos diretos e indiretos por induzir o desequilíbrio redox que dispara uma cascata de eventos que podem resultar em inflamação, deposição de de fibras colágenas e apoptose que juntos levam ao carcinoma hepatocelular (Altamirano and Bataller 2010).

O desequilíbrio redox induzido pela exposição à FC em longo prazo produz toxinas com efeitos imunológicos e oncogênicos no fígado (El-Zayadi 2006). A FC aumenta a severidade das lesões hepáticas relacionadas à hepatite C e à hepatite B (Yu, Pai et al. 2000, Pessione, Ramond et al. 2001). A FC também pode levar à hipóxia tecidual por elevar os níveis de carboxihemoglobina com diminuição da capacidade de transporte de oxigênio pelas hemácias, e aumentar o risco de desenvolvimento de câncer hepático, devido aos múltiplos carcinógenos encontrados em sua composição, como hidrocarbonetos, nitrosamina, alcatrão e cloreto de vinila (Helen and Vijayammal 1997, Altamirano and Bataller 2010).

Além disso, a maior parte da nicotina (90 a 95%) absorvida pelo organismo é metabolizada no fígado onde inibe a proliferação e diferenciação de linfócitos, resultando na supressão da formação de anticorpos (Moszczyński, Zabiński et al. 2001) .

### 1.4. Desequilíbrio Redox

O desequilíbrio redox acontece sempre que os níveis das EROs excedem a capacidade antioxidante do organismo, seja de forma transitória ou persistente, podendo interferir na homeostase celular e vias de sinalização redox-sensíveis ocasionando danos oxidativos que podem culminar em morte celular (Lushchak 2015). Entretanto, níveis baixos ou moderados das EROs são necessários ao metabolismo, uma vez que atuam como sinalizadores intracelulares, transdutores de sinais mediadores inflamatórios (Valko, Leibfritz et al. 2007).

#### 1.4.1. Espécies Reativas de Oxigênio (ERO)

As EROs podem ser geradas a partir de fontes exógenas ou endógenas. Dentre as exógenas podemos citar a exposição à radiação ionizante, à luz ultravioleta, às toxinas do ambiente, quimioterápicos e também à fumaça de cigarro (Rahal, Kumar et al. 2014).

Dentro das células, a maior parte das EROs é produzida pelas mitocôndrias através do escape de elétrons da cadeia transportadora de elétrons, principalmente nos complexos I e III

com elétrons que derivam de NADH ou FADH (Georgieva, Ivanova et al. 2017). Entre outras fontes intracelulares, podemos citar os produtos do metabolismo do citocromo p450, a beta oxidação via peroxissomos, a enzima xantina oxidase e o complexo enzimático NADPH oxidase (NOX), ambos estimulados pela FC (Burtenshaw, Hakimjavadi et al. 2017, Kellner, Noonepalle et al. 2017).

A xantina oxidase (XO) catalisa a oxidação da hipoxantina e xantina, na via de formação do ácido úrico usando o oxigênio como aceptor de elétrons e gerando $O_2^-$ como produto intermediário (Malik, Dhiman et al. 2018). Essa via é ativada em situações de hipóxia como a induzida pela FC. A fase gasosa da FC é rica em monóxido de carbono (CO), após sua inalação, o CO atinge a corrente sanguínea e combina-se com a hemoglobina formando um complexo denominado carboxihemoglobina (HbCO) evitando assim, a ligação da hemoglobina com o oxigênio. Como a HbCO circula mais rápido que a oxihemoglobina, a concentração sérica de $O_2$ diminui drasticamente causando a hipóxia. Na ausência de $O_2$, ocorre uma maior conversão de ATP em ADP, AMP, iosina e, finalmente, a hipoxantina, um substrato para a XO, favorecendo a geração das EROs (Heunks, Vina et al. 1999, Kayyali, Budhiraja et al. 2003, Miranda, Paulo et al. 2004) (Figura 1).

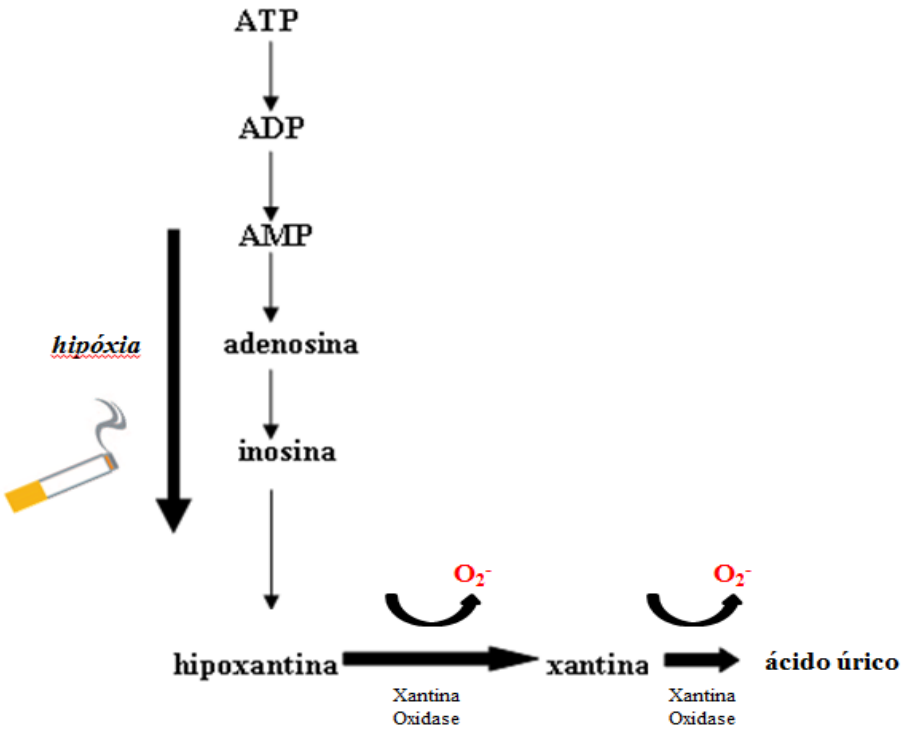



**Figura 1**: Mecanismo de formação das EROS via ação da enzima Xantina Oxidase. ATP: adenosina trifosfato; ADP: adenosina difosfato; AMP: adenosina monofosfato; $O_2^-$: ânion superóxido. (Adaptado de (Miranda, Paulo et al. 2004).

O complexo enzimático NADPH oxidase (NOX) se encontra acoplado à membrana plasmática e catalisa a transferência de elétrons do NADPH para o oxigênio molecular formando o ânion superóxido ($O_2^-$) (Figura 2), na homeostase, a NOX apresenta baixo nível de atividade e seus componentes proteicos são segregados em citoplasmáticos e membranares (Kim, Han et al. 2014, Chang, Park et al. 2017). A família NOX é composta por sete membros (NOX 1-5 e DUOX 1-2) com subunidades catalíticas distintas e diferentes mecanismos de ativação. Nessa família, acredita-se que a NOX 1 e/ou NOX 2 estejam envolvidas nos mecanismos citotóxicos relacionados a exposição à FC (Asano, Horinouchi et al. 2012). A ativação de NOX 1 e NOX 2 é dependente da fosforilação da proteína quinase C (PKC) que promoverá a translocação dos componentes citossólicos de NOX para a membrana plasmática favorecendo a geração das EROs (Rastogi, Geng et al. 2016, Ganguly, Kaur et al. 2021). Aldeídos e cetonas e outros compostos estáveis presentes na FC são capazes de reagir com grupos tióis e atuarem na regulação da atividade de enzimas como a PKC (Jaimes, DeMaster et al. 2004, Asano, Horinouchi et al. 2012).

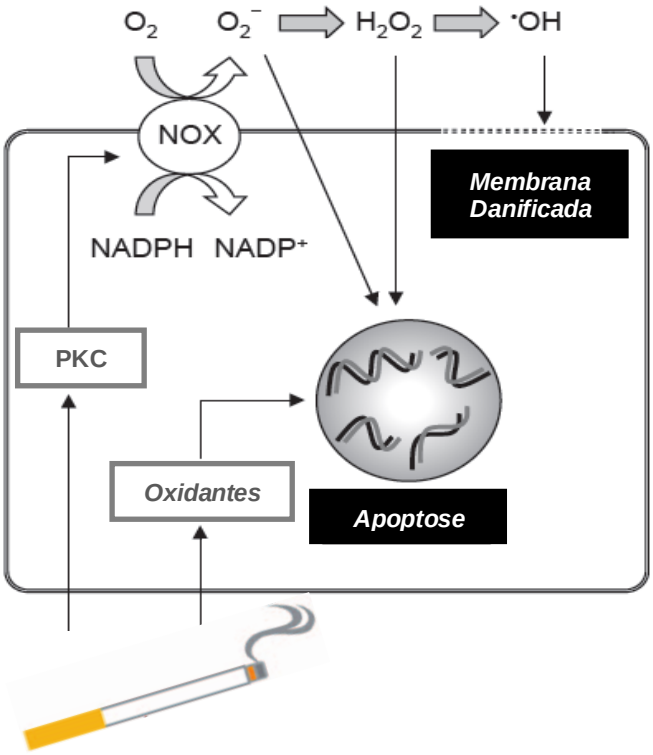



**Figura 2**: Mecanismo de formação das EROs via ação do complexo enzimático NADPH oxidase. $O_2$: oxigênio; $O_2^-$: ânion superóxido; $H_2O_2$: peróxido de hidrogênio; $^{\bullet}OH$: radical hidroxila; NOX: NADPH oxidade; PKC: proteína quinase C. (Adaptado de (Asano, Horinouchi et al. 2012).

A NOX pode ser considerada uma importante ligação entre o desequilíbrio redox e a inflamação. Estudos sugerem um papel quimiotático de algumas de suas subunidades, podendo então, ocasionar um maior recrutamento de neutrófilos e macrófagos. Outros sugerem que a ativação deste complexo enzimático está relacionada à ativação de outras vias pró-inflamatórias como a do fator nuclear kappa B (NF-κB) e proteínas quinases ativadas por mitógenos (MAPK), ambas envolvendo a exposição à FC (Yao, Yang et al. 2007, Lorenzen, Mullen et al. 2017).

Esses processos enzimáticos geram principalmente o $O_2^-$ que é um precursor de outras EROs, portanto, sua produção é considerada o primeiro ponto para a iniciação e propagação do desequilíbrio redox, podendo funcionar como um regulador da cadeia de reações oxidativas. Uma vez que o $O_2^-$ é formado, sua dismutação é feita pela enzima superóxido dismutase levando a formação de peróxido de hidrogênio ($H_2O_2$). O $H_2O_2$ é uma espécie reativa não radicalar que apresenta maior estabilidade que outras EROs e a capacidade de atravessar membranas celulares, apresentando, portanto, grande poder de difusão pelos compartimentos celulares sendo altamente tóxico. Na presença de metais como ferro e cobre, a toxicidade do $H_2O_2$ aumenta consideravelmente, pois a interação entre esses compostos pode levar a formação do radical hidroxila ($OH^{\bullet}$) através das reações de Fenton/Haber-Weiss. Por apresentar maior força reativa e ser muito instável o $OH^{\bullet}$ é a ERO que mais gera dano oxidativo (McCord, Keele et al. 1971, Halliwell 1978, Kehrer 2000, Birben, Sahiner et al. 2012, Lorenzen, Mullen et al. 2017).

### 1.4.2. Defesa Antioxidante

Organismos aeróbios possuem um sistema antioxidante integrado que é capaz de bloquear os efeitos prejudiciais das EROs. Este sistema é caracterizado como enzimático quando atua através da atividade de enzimas antioxidantes ou não enzimático, quando a atividade antioxidante é exercida por substâncias com efeito antioxidante que podem ser tanto endógenos quanto exógenos, esses últimos adquiridos através da dieta (Halliwell and Whiteman 2004, Barreiros, David et al. 2006, Birben, Sahiner et al. 2012).

O sistema enzimático é baseado, principalmente, na ação de três importantes enzimas, sendo elas a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) (Figura 3).

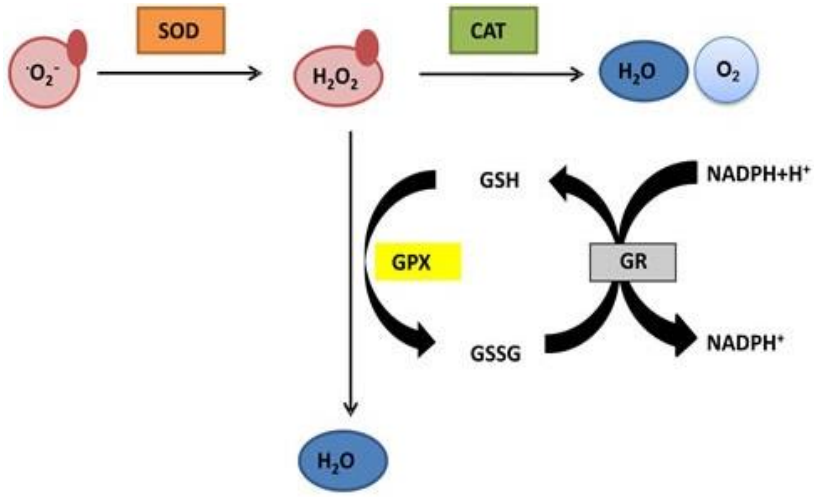



**Figura 3**: Esquema da integração do sistema de defesa antioxidante enzimático. $O_2^{\cdot-}$: ânion superóxido; $H_2O_2$: peróxido de hidrogênio; SOD: superóxido dismutase; CAT: catalase; GPx: glutationa peroxidase; GSH: glutationa reduzida; GSSH: glutationa oxidada. (Adaptado de (Sznarkowska, Kostecka et al. 2017).

A SOD é considerada a primeira linha de defesa do organismo, promove a remoção do $O_2^{\cdot-}$ e gera $O_2$ e $H_2O_2$ como produtos. Nos organismos aeróbicos, três tipos de SOD são expressos, a SOD1 (Cu/Zn-SOD) expressa no citoplasma e compartimentos nucleares, a SOD2 (Mn-SOD) expressa na mitocôndria e a SOD3 (EC-SOD) que é expressa no espaço extracelular (Rahman 2007, Gaurav, Varasteh et al. 2017).

O $H_2O_2$ formado é convertido em $H_2O$ por ação das enzimas GPx ou CAT. A GPx está presente nos compartimentos citoplasmático e mitocondrial e sua atividade catalítica depende da presença de seu co-substrato, a glutationa oxidada. Na conversão de $H_2O_2$ em $H_2O$, a GPx utiliza elétrons providos pela glutationa oxidada (GSSG) que passa para seu estado reduzido sendo denominada glutationa reduzida (GSH). A manutenção dos níveis de GSH é mantida pela ação da enzima glutationa redutase (GR) que reduz GSSG em GSH através oxidação do NADPH. Sendo assim, a razão GSH/GSSG pode ser considerado um indicador do estado redox celular (Townsend, Tew et al. 2003, Ballatori, Krance et al. 2009, Birben, Sahiner et al. 2012). A catalase é uma heme-proteína que, na maioria das células, encontra-se nos peroxissomos e sua principal função é fazer a conversão do $H_2O_2$ em $H_2O$, assim como a GPx. Desta maneira, GPx e CAT dividem a mesma função, entretanto, a GPx apresenta melhor desempenho na presença de baixos níveis de $H_2O_2$, enquanto que a ação da CAT é favorecida em altas concentrações desse substrato, essa diferença de eficiência das duas enzimas em concentrações mais elevadas de $H_2O_2$ pode ser devido à necessidade de reciclagem da GSH no sistema glutationa, sendo um fator limitante para a atividade da GPx (Winterbourn 2013, Glorieux and

Calderon 2017). Essas enzimas compõem a principal linha de defesa enzimática dos pulmões, sendo assim, no intuito de preservar a funcionalidade pulmonar em resposta aos oxidantes provenientes da exposição à FC, a manutenção do estado redox é de grande importância, e nesse caso, as respostas antioxidantes dependem da ativação da via do fator de transcrição eritróide 2 (Nrf2)(Kinnula 2005, Birben, Sahiner et al. 2012).

O Nrf2 atua através de sua ligação ao elemento de resposta antioxidante (ARE), presente no núcleo, e responsável pela transcrição de genes citoprotetivos. Na homeostase, o Nrf2, encontra-se no citoplasma ligado a uma proteína inibitória denominada Keap1, entretanto, em resposta ao estresse oxidativo, o Nrf2 se dissocia da proteína Keap1 e segue em direção ao núcleo induzindo a expressão de genes para enzimas antioxidantes (figura 4) (Boutten, Goven et al. 2010).

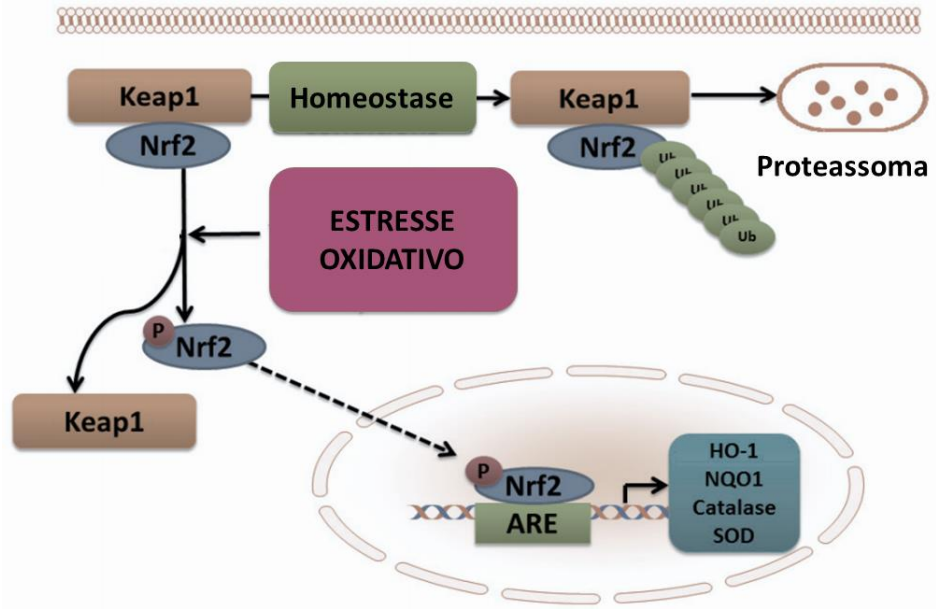



**Figura 4**: Mecanismo de ativação da via antioxidante Nrf2 pelo estresse oxidativo. Nrf2: fator nuclear eritroide 2 relacionado ao fator 2; Keap1: Kelch-like ECHassociated protein 1; ARE: elemento de resposta antioxidante; HO-1: heme oxigenase 1; NQO1: NADPH quinona oxiredutase; SOD: superóxido dismutase. (Adaptado de (Asano, Horinouchi et al. 2012).

Assim como as enzimas antioxidantes, que são capazes de prevenir os danos causados pelas EROs, existem substâncias que atuam como antioxidantes e que fazem parte do sistema de defesa antioxidante não enzimático. Essas substâncias são, em sua maioria, exógenas aos organismos, devendo então, ser adquiridas através da alimentação. São exemplos de antioxidantes não enzimáticos o ácido ascórbico (vitamina C), o α-tocoferol (vitamina E), carotenoides e compostos fenólicos como a quercetina (Rahman 2007, D'Andrea 2015).

### 1.4.3. Quercetina

A quercetina, um flavonoide de ocorrência natural, pode ser encontrada em alimentos de origem vegetal como cebola, maçã, grãos e sementes, entre outros. Como todos os flavonóis, a QC apresenta uma estrutura química básica contendo um esqueleto de difenil propano e dois anéis benzênicos ligados a um anel pirano e vários radicais hidroxila, o que lhe permite uma série de propriedades bioquímicas com efeito antioxidante e anti-inflamatório (Ganesan, Faris et al. 2010, Xu, Hu et al. 2019, Mehrbod, Hudy et al. 2020).

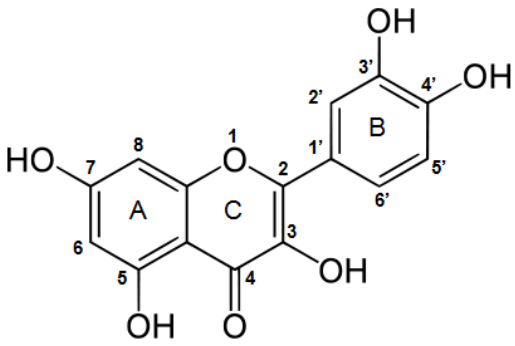



**Figura 5:** Fórmula estrutural da Quercetina. Fonte: (D'Andrea 2015).

A figura 5 mostra a estrutura química da quercetina, denominada aglicona, entretanto, a maior parte da ingestão dietética de quercetina consiste em seus derivados glicosilados, ou seja, com uma ou duas moléculas de açúcar ligadas à sua estrutura. Essa conjugação com moléculas de açúcar pode afetar tanto sua biodisponibilidade, bem como a bioatividade.

A quercetina apresenta baixa biodisponibilidade, principalmente por sua baixa solubilidade e por sofrer extenso metabolismo antes de atingir a corrente sanguínea (Cai, Fang et al. 2013, Guo and Bruno 2015). Li *et. al.* (2016) sugerem uma taxa de absorção da forma aglicona de aproximadamente 2%. Estudos têm mostrado diferentes mecanismos de absorção para as formas aglicona e glicosilada da quercetina. Embora o mecanismo ainda não esteja claro, há uma forte evidência de que parte da aglicona possa ser absorvida já no estômago, uma vez que é estável ao ácido gástrico, entretanto, a absorção é mais efetiva na parte superior do intestino delgado e ocorre por difusão passiva (Crespy, Morand et al. 1999, Nait Chabane, Al Ahmad et al. 2009, Guo and Bruno 2015, Li, Yao et al. 2016).

Em relação às formas glicosiladas, o mecanismo de absorção pode ser diferente dependendo do tipo de açúcar ligado, entretanto, nenhum glicosídeo pode ser absorvido no estomago, sendo assim, totalmente absorvida via intestino delgado. A absorção das formas

glicosiladas depende de uma prévia hidrólise da molécula de açúcar liberando então a aglicona, essa hidrólise é catalisada principalmente pela Lactase Florizina Hidrolase (LPH) presente na membrana do enterócito, ou, em menor escala, os glicosídeos podem ser absorvidos intactos pelos enterócitos através do transportador de glicose acoplado ao sódio (SGLT1) onde então serão hidrolisados à forma aglicona por uma β-glucosidase (Day, Gee et al. 2003, Lotito, Zhang et al. 2011, Orfali, Duarte et al. 2016). No enterócito, a quercetina aglicona sofrerá reações de glucoronização e sulfatação formando metabólitos que serão exportados da corrente sanguínea ao fígado via sistema porta, no fígado ocorrerá uma metabolização adicional formando principalmente os metabólitos 3´-O-sulfo-quercetina-3-O-glucoronídeo e 3´-O-metil-quercetina-3-O-glucoronídeo que poderão ser detectados no plasma e na urina (Figura 6). (Murota, Shimizu et al. 2000, Sesink, O'Leary et al. 2001, Kaldas, Walle et al. 2005, Lotito, Zhang et al. 2011).

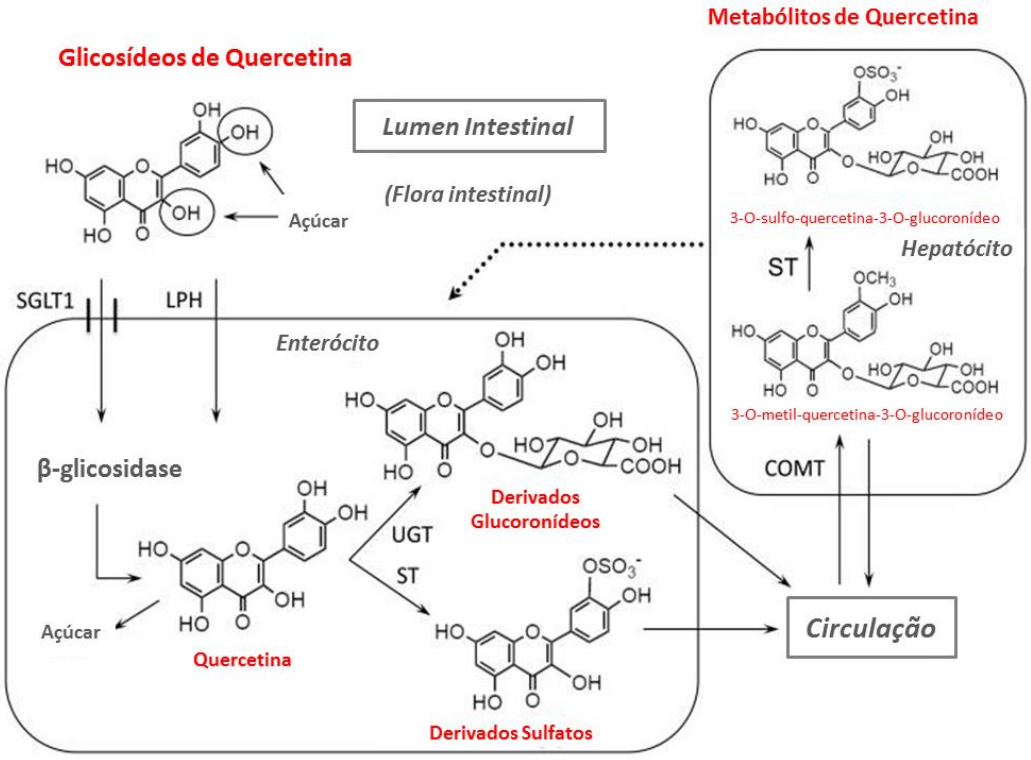



**Figura 6:** Metabolismo da quercetina. SGLT1: transportador de glicose acoplado ao sódio; LPH: Lactase Florizina Hidrolase; UGT: UDP-glucuronosiltransferase; ST: sulfato transferase; COMT: catecol-O-metiltransferase. Fonte: Adaptado de (Lotito, Zhang et al. 2011).

A ingestão diária de quercetina na dieta varia entre 5-40 mg/dia, entretanto, esses valores podem aumentar para 200-500 mg/dia em indivíduos que consomem grandes quantidades de alimentos ricos neste nutriente. Entre os americanos consumo diário de quercetina é de 25 mg

e a média de consumo na dieta ocidental é de cerca de 16 mg/dia (Sak 2014, Carrasco-Pozo, Castillo et al. 2016).

Quanto à toxicidade, a maioria dos estudos em humanos demonstraram segurança no uso da quercetina em doses de até 1g/dia, embora recentemente Han e colaboradores (2020) mostraram que indivíduos com DPOC toleraram, com segurança, uma dose de quercetina de até 2.000 mg/dia por curto prazo (Mohammadi-Sartang, Mazloom et al. 2017, Andres, Pevny et al. 2018, Han, Barreto et al. 2020, Huang, Liao et al. 2020, Dagher, Mury et al. 2021). Por outro lado, estudos *in vitro* demonstraram que altas concentrações de quercetina podem ter efeito pró-oxidante, principalmente devido a seu produto de oxidação que é o quercetina-quinona (Boots, Li et al. 2007, Sak 2014).

O poder antioxidante da quercetina é atribuído principalmente à presença de dois grupos em sua molécula, um catecol no anel B e uma hidroxila na posição 3 do anel C, isso faz com que a quercetina apresente uma configuração ideal para a eliminação de radicais livres tornando-a o flavonóide com maior potencial antioxidante (Lemanska, van der Woude et al. 2004, Di Meo, Lemaur et al. 2013). O efeito antioxidante da quercetina se dá por vários mecanismos como sua capacidade de reagir com espécies reativas e formar radicais fenoxi menos reativos, também possui atividade redutora de íons cobre e ferro impedindo a reação com $H_2O_2$ na reação de Fenton/Haber-Weiss, atua na prevenção da peroxidação lipídica através da neutralização de várias espécies reativas de oxigênio e pode também atuar inibindo várias proteínas quinases, além de estar envolvida na ativação da via do Nrf2 ocasionando um aumento no nível de glutationa intracelular melhorando assim a atividade das enzimas antioxidantes e inibição da via do NF-κB (Davies, Reddy et al. 2000, Kahraman, Erkasap et al. 2003, Tanigawa, Fujii et al. 2007, Chirumbolo 2010, Mitani, Azam et al. 2017, Zhang, Zhang et al. 2020, Yin, Liu et al. 2021). Sendo assim, ao eliminar as EROs, a quercetina também pode reduzir a inflamação.

A quercetina também atua na modulação de metaloproteinases de matriz (MMP). As MMPs são enzimas capazes de clivar proteínas levando à degradação da matriz extracelular e remodelamento tecidual, são ativadas por vários fatores incluindo o desequilíbrio redox e inflamação, desta forma, a quercetina diminui a atividade das MMPs por sua ação antioxidante e também por atuar como inibidor competitivo, como no caso da MMP-9 (Zhao and Zhang 2015, Chuang, Yeh et al. 2016).

A ação antioxidante e anti-inflamatória exercida pela quercetina em doenças do trato respiratório e hepáticas tem sido estudada por diversos autores citados a seguir.

Li e colaboradores (2012), utilizando células epiteliais humanas das vias aéreas, demonstraram que a quercetina diminuiu a expressão de uma mucina secretada nas vias respiratórias (MUC5AC), por meio da via de transdução de sinal PKC/EGFR/ERK, sugerindo sua utilização no tratamento de doenças inflamatórias crônicas das vias aéreas (Li, Li et al. 2012). De outra forma, o estudo in vivo utilizando camundongos C57BL6 tratados com elastase e lipopolissacarídeo (LPS), a quercetina reduziu o estresse oxidativo através da diminuição da peroxidação lipídica, avaliada pelo teste de substâncias reativas ao ácido tiobarbitúrico (TBARS), a inflamação pulmonar através da redução da expressão de citocinas pró-inflamatórias e MUC5AC, e também a expressão e a atividade das metaloproteinases MMP9 e MMP12, sugerindo um importante papel na modulação do estresse oxidativo e na inflamação pelo tratamento com a quercetina, podendo atuar de forma benéfica diminuindo a progressão do enfisema pulmonar (Ganesan, Faris et al. 2010).

O tratamento com quercertina em modelo de inflamação das vias aéreas induzida pela fumaça de cigarro em ratos, suprimiu a síntese de proteínas mucinas através da inibição da via NF-kB e fosforilação do fator de crescimento epidérmico (EGFR), diminuindo o estresse oxidativo e a resposta inflamatória, o que sugere um potencial terapêutico da quercetina no tratamento de doenças pulmonares crônicas (Yang, Luo et al. 2012).

Mitani e colaboradores (2017) demonstraram que a administração da quercetina em células mononucleares de sangue periférico de pacientes com DPOC intermediária e severa e em linhagem celular monocítica humana U937 exposta ao extrato de fumaça de cigarro, foi capaz de aumentar a expressão de Nrf2 e reverter a resistência das células a corticosteroides, sugerindo seu potencial no tratamento da DPOC (Mitani, Azam et al. 2017).

Recentemente, Saeedi-Boroujeni e Mahmoudian-Sani mostraram que a quercetina, por sua ação antioxidante e antiinflamatória, tem potencial para ser usada no tratamento da COVID-19, principalmente em relação à inflamação grave, condição de maior risco de vida em pacientes acometidos pela doença (Saeedi-Boroujeni and Mahmoudian-Sani 2021).

Em relação a doenças hepáticas diversos autores (Ma, Li et al. 2015, Kabirifar, Ghoreshi et al. 2017, Peng, Gong et al. 2017, Zhao, Gong et al. 2021) têm demonstrado o efeito hepatoprotetor da quercetina em diferentes modelos experimentais, entretanto nenhum deles demonstrou esse efeito quanto à hepatotoxicidade induzida por FC.

Ma et al. (2015) demonstraram, em camundongos ICR machos com inflamação induzida por CCl4, que a QC apresentou função hepatoprotetora ao inibir níveis de iNOS, COX-2, IL-1β e NO através da supressão das vias de sinalização TLR2/4 e MAPK/NF-κB. Os

autores sugerem que o efeito antiinflamatório apresentado se deve à atividade antioxidante da QC (Ma, Li et al. 2015).

Em ratos Wistar machos, a ligação do ducto biliar ocasionou aumento nos níveis de expressão de Rac1, Rac1-GTP e NOX1 assim como nos níveis de marcadores de fibrose induzindo lesão hepática. Nesse estudo, a administração de QC suprimiu a expressão de Rac1, Rac1-GTP e NOX1e aumentou a atividade das enzimas antioxidantes superóxido dismutase e catalase (Kabirifar, Ghoreshi et al. 2017).

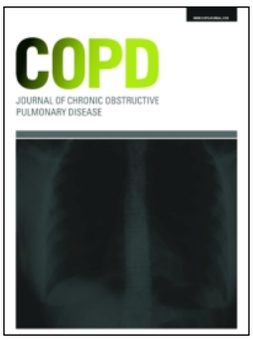
A administração de QC promoveu, em camundongos BALB/c com lesão hepática aguda induzida por LPS/d-GalN, uma melhor taxa de sobrevivência, redução nas alterações histopatológicas, diminuição na expressão de marcadores de estresse oxidativo e apoptose, além de diminuir os níveis das citocinas inflamatórias TNF-α, IL-6 e IL-1β ao inibir a ativação das vias de sinalização de NF-κB e MAPK, demonstrando seu potencial terapêutico em relação às lesões hepáticas (Peng, Gong et al. 2017).

Em camundongos C57BL/6, a quercetina foi capaz de prevenir a lesão hepática causada por toosendamina, principal composto ativo de dois medicamentos chineses, através da ativação da via antioxidante Nrf2 / glutamato-cisteína ligase / GSH (Jin, Huang et al. 2019).

Recentemente Zhao e colaboradores (2021) demostraram o efeito hepatoprotetor da quercetina em um modelo experimental de peixe-zebra com esteatose hepática induzida por etanol, nesse estudo, a QC exerceu sua ação antioxidante através da regulação da via PI3K / Keap1 / Nrf2 mediada por P2X7R (Zhao, Gong et al. 2021).

Quando analisados em conjunto, esses achados sugerem que a quercetina pode atuar de forma benéfica em várias patologias pulmonares e hepáticas por exercer ação moduladora sobre a inflamação e o desequilíbrio redox, através da interação com um amplo espectro de alvos moleculares centrais para a sinalização celular. Portanto, este estudo teve como objetivo avaliar se a administração da quercetina foi capaz de influenciar nos processos oxidativos e inflamatórios induzidos pela exposição à fumaça de cigarro em células e em camundongos expostos durante cinco e sessenta dias.

## 2. QUERCETIN ATTENUATES ACUTE LUNG INJURY CAUSED BY CIGARETTE SOMOKE BOTH *IN VITRO AND IN VIVO*



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# Quercetin Attenuates Acute Lung Injury Caused by Cigarette Smoke Both In Vitro and In Vivo

Natália Pereira da Silva Araújo[a], Natália Alves de Matos[a], Suianne Leticia Antunes Mota[b], Ana Beatriz Farias de Souza[a], Sílvia Dantas Cangussú[a], Rodrigo Cunha Alvim de Menezes[c], and Frank Silva Bezerra[a]

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**ABSTRACT**

Cigarette smoke is highly toxic and is a major risk factor for airway inflammation, oxidative stress, and decline in lung function—the starting points for chronic obstructive pulmonary disease. Quercetin is a potent dietary antioxidant that displays anti-inflammatory activities. The goal of this study was to evaluate the effects of quercetin on reducing the redox imbalance and inflammation induced by short-term cigarette smoke exposure. *In vitro*, 25 and 50 $\mu$M quercetin attenuated the effects of cigarette smoke extract (increased generation of reactive oxygen species and nitric oxide) on J774A.1 cells (macrophages). We further examined the effects of quercetin *in vivo*. Male C57Bl/6 mice that received 10 mg/kg/day of quercetin *via* orogastric gavage before exposure to five days of cigarette smoke demonstrated reduced levels of leukocyte, oxidative stress, histological pattern changes of pulmonary parenchyma, and lung function alterations compared to the group that did not receive quercetin. These results suggest that quercetin may be an effective adjuvant for treating the effects of cigarette smoke exposure.

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**KEYWORDS**
Quercetin; cigarette smoke; oxidative stress; mice; short-term exposure

## Introduction

In the last century, approximately 100 million people were killed by smoking, and more than 8 million people a year will die from diseases related to cigarette smoke [1,2]. Cigarette smoke is highly toxic, containing a variety of particulate matter, chemicals, and reactive oxygen species (ROS) that are capable of inducing inflammation throughout the pulmonary epithelium [3,4]. Chronic smoking is a major risk factor for the development of chronic obstructive pulmonary disease [5–9]. Although pulmonary changes caused by short-term cigarette smoke exposure do not show all the features of chronic obstructive pulmonary disease (COPD) compared to long-term use, both models exhibit similar pathophysiological mechanisms, including inflammation and redox imbalance [10–12].

The airway epithelium acts as a protective barrier between the lungs and environmental toxins; thus, the changes caused by the inhalation of cigarette smoke can be first observed here [13]. The initial cellular activation—epithelial, endothelial, and resident macrophages—caused by ROS and other toxins present in cigarette smoke induces cytokine and chemokine secretion and subsequent recruitment of immune cells, mainly macrophages and neutrophils [4, 14]. These processes stimulate further production of cytokines, as well as proteases and ROS, which amplify the inflammatory response and redox imbalance. These processes collectively cause tissue destruction and is considered the starting point for COPD pathogenesis [15,16].

Redox imbalance occurs whenever ROS levels exceed the body's antioxidant system, either transiently or persistently. High ROS levels can interfere with cellular homeostasis and redox-sensitive signaling pathways, causing oxidative damage that can culminate in cell death [17].ROS are produced by living organisms during normal cellular metabolism or by activated inflammatory cells. They are also present in environmental pollutants. Due to this constant contact with ROS, organisms have gradually developed antioxidant defense systems, including antioxidant enzymes and non-enzymatic substances with antioxidant effects, to prevent damage to proteins, lipids and DNA [18]. The enzymatic system is primarily dependent on the action of three key players: superoxide dismutase, which converts superoxide anions to hydrogen peroxide; glutathione peroxidase, which reduces peroxides; and catalase, which converts hydrogen peroxide into water and molecular oxygen [17]. Non-enzymatic ROS scavengers include minerals, phenolic compounds, and vitamins. Of the phenolic compounds, flavonoids have rich antioxidant properties, with quercetin (3,5,7,3′,4′-pentahydroxyl flavone) being the most abundant flavanol and present in a

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variety of foodstuffs. Moreover, it has strong antioxidant capacity due to its ability to remove $O_2$-, $OH^-$, singlet oxygen [19–22]. It interacts with a broad spectrum of cell targets reducing the expression of inflammatory cytokines and growth factors, reducing secretion of mucin proteins and metalloproteinases activities [23–25].Thus, quercetin has been explored as a treatment option for pulmonary diseases, specifically, its ability to modulate inflammation [22] and redox imbalance [26]. However, the ability of quercetin to counteract the deleterious effects of cigarette smoke have not been elucidated. The goal of this study was to determine whether treatment with quercetin can correct the redox imbalance induced by cigarette smoke exposure using cellular (alveolar macrophages) and *in vivo* approaches (C57BL/6 mice).

## Materials and methods

### *In vitro experiments*

Murine J774A.1 macrophages were cultured in 75 $cm^2$ growth flask (Sardesdt AG& Co., Germany) at 37 °C in a 5% $CO_2$ atmosphere and grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen Co. Ltd., Carlsbad, CA, USA), 1% (v/v) penicillin, 3.7% (v/v) sodium bicarbonate, 1% (v/v) glutamine (200 mM), and 2.5 mL of HEPES (1 M) (final concentration 200 g/mL). When the cells reached 80% confluency, they were collected with a cell scraper and used for experiments.

### *Preparation of cigarette smoke extract (CSE)*

Before experimentation, CSE was prepared from filtered Marlboro Red cigarettes (10 mg of tar, 0.9 mg of nicotine, and 10 mg of carbon monoxide per cigarette) as previously described [27]. The cigarette smoke was bubbled through 10 mL of supplemented culture medium in a modified syringe-driven apparatus. The resulting suspension was titrated to pH 7.4 and sterilized with a 0.22 $\mu$m Millipore filter. This solution was considered to be 100% CSE. For experiments, the CSE was diluted with culture medium to 0.5% CSE. Control medium was prepared by bubbling air through 10 mL of culture medium in the same conditions [11].

### *Cytotoxicity of cigarette smoke extract and quercetin*

The toxicity of cigarette smoke and quercetin (Sigma Aldrich) was determined using a colorimetric assay based on the reduction of resazurin, basically in the presence of viable cells, resazurin suffers a colorimetric change from blue to red [28,29]. J774A.1 cells (1 × 106 cells/mL in 200 µL of culture medium) were seeded in 96-well plates and exposed to decreasing concentrations of CSE: 20; 10; 5; 2.5; 1.25; 0.6; 0.3; 0.15% in culture medium or quercetin (QC): 100; 50;25; 12.5; 6.25; 3.12; 1.6; 0.8 µM in culture medium for 24 h. Next, 20 µL of resazurin (1 mM) was added to each well, and the absorbances were measured with a Microplate reader ($\lambda_{ex}$ = 570 nm; $\lambda_{em}$ = 600 nm) to determine cell viability [30]. These data were used to calculate the 50% cytotoxic concentrations (CC50) for quercetin and CSE.

### *Reactive oxygen species (ROS) production in J774A.1 cells*

Intracellular ROS levels were detected using the 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) assay as previously described [11]. J774A.1 cells (2.5 × 104 cells/well) were seeded in 96-well plates containing DMEM supplemented with 10% fetal bovine serum and cultured for 45 min at 37 °C in humidified air supplemented with 5% $CO_2$. The supernatant was then removed and the cells were resuspended in DMEM containing 10, 25, and 50 $\mu$M of quercetin for 6 h (37 °C and 5% $CO_2$ in the dark). Next, the cells were washed with Hanks' balanced salt solution (HBSS) and exposed to medium containing 0.5% CSE and incubated in the dark with carboxy-H2DCFDA for 24 h at 37 °C in humidified air supplemented with 5% $CO_2$. Cells were washed with HBSS, medium without red phenol was added, and fluorescence intensity was determined using an automated plate reader model ($\lambda_{ex}$ = 485 nm; $\lambda_{em}$ = 535 nm; VICTOR X3 equipped with the PerkinElmer 2030 workstation and Workout 2.5). Mean fluorescence intensity was reported as the percent (%) increase over control (culture medium without quercetin or CSE.

### *Nitric oxide (NO) production in J774A.1 cell*

NO production by J774.A1 cells was determined based on the accumulation of nitrite in cell supernatant using the Griess method [31]. Cells (1 × 106 cells/well of RPMI supplemented with 10% of fetal bovine serum) were added to 48-well microplates and maintained for 2 h at 37 °C in humidified air supplemented with 5% $CO_2$ to allow for cell adhesion. The wells were washed with 200 $\mu$L of sterile PBS to remove non-adhered cells, and 200 $\mu$L of RPMI supplemented with 10% of fetal bovine serum was added. Next, 200 $\mu$L of RPMI with 10% of SFB containing quercetin (10, 25, and 50 $\mu$M) or quercetin + 0.5% CSE was added to the wells. After 24 h, 50 $\mu$L of the supernatant was collected and mixed with 100 $\mu$L of Griess reagent (a 1:1 mixture of 1% sulfanilamide in 2.5% phosphoric acid and 0.1% dihydrochloride N-[1-naftil]-ethylenediamine in 2.5% phosphoric acid). The plate was incubated in the dark for 10 min at 37 °C. The absorbance reading was measured using an ELISA reader ($\lambda$ = 570 nm). The nitrite concentration was calculated by linear regression using a standard curve of 1 mM sodium nitrite in 10% RPMI of fetal bovine serum ($R^2 > 0.99$).

### *In vivo experiment*

All *in vivo* experiments were approved by the ethics committee of Federal University of Ouro Preto (2015/20). Thirty Male C57BL/6 mice (10-12 weeks old) obtained from the Animal Science Center of the Federal University of Ouro Preto (CCA–UFOP) were housed under controlled conditions (12 h light/dark cycle, 21 °C ± 2 °C, 50% ± 10% humidity) and provided food and water *ad libitum*. Mice were

divided into 5 groups ($n = 5$ per group): a control group exposed to ambient air (CG); a group that received 200 µL of vehicle solution containing 50% propylene glycol (Sigma Aldrich) and 50% saline (VG) by orogastric gavage; a group that received 10 mg/kg/day of quercetin diluted in propylene glycol solution (QG); a group exposed to cigarette smoke (CSG); a group treated with quercetin and exposed to cigarette smoke (QCSG). The administration of quercetin was performed *via* orogastric gavage 1 h before exposure to cigarette smoke.

### *In vivo cigarette smoke exposure protocol*

In an inhalation chamber, mice were exposed to 12 commercial full-flavor filtered Virginia cigarettes (tar: 10 mg; nicotine: 0.9 mg and carbon monoxide: 10 mg) per day divided into three times daily (morning, afternoon, and night) for 5 consecutive days as previously described [11, 32]. The cigarette smoke (CS) and QCSG animals were initially placed in an inhalation chamber (40 cm × 30 cm × 25 cm) inside an exhaustion chapel. Each cigarette was coupled to a 60-mL plastic syringe through which the smoke was injected inside the inhalation chamber. The cigarette smoke and QCSG groups were maintained in the smoke atmosphere for 6 min; after, the cover was removed from the inhalation chamber for 1 min to allow the air to completely exhaust. The procedure was then repeated with the remaining cigarettes. The control group, VG, and QG groups were subjected to the same conditions except without the cigarette exposure.

### *Lung function*

Lung function analysis was performed 24 h after the last exposure. The mice were individually anesthetized *via* intraperitoneal administration with ketamine (100 mg/kg) and xylazine (20 mg/kg). A small neck incision was made, and the trachea was cannulated with an 18 G catheter coupled to a 2-cm long tube, which connected the trachea to the flow head (MLT1L). Breathing was monitored using a differential pressure transducer (Spirometer, ADInstruments, Bella Vista, NSW, Australia). The lung function of each mouse was recorded for 60 s with three repetitions each; this signal was amplified (Octal Bridge Amp model ML228; Powerlab 4/35 model ML870; ADInstruments) and recorded with LabChart v8.1.11 using a standard desktop PC. The evaluated parameters were tidal volume, respiratory rate, and minute ventilation.

### *Blood collection*

After recording lung function, the animals received an overdose of ketamine (130 mg/kg) and xylazine (0.3 mg/kg). Their blood was collected by cardiac puncture and stored in polypropylene tubes (2 mL) with 15 µL of heparin. Erythrocyte, hematocrit, and hemoglobin counts were evaluated using an electronic counting device (Mindray® Bio-Medical Electronics Co. Ltda, Shenzhen, China).

### *Assessment and analysis of the bronchoalveolar lavage fluid (BALF)*

Immediately after blood collection, the chest of each animal was opened to collect the bronchoalveolar lavage fluid (BALF). Briefly, the thorax of each animal was opened, the left main bronchus was clamped, the trachea was cannulated, and the right lung was washed with 1.5 mL of saline solution (3 × 500 µL). The samples were kept on ice to avoid cell lysis. A Neubauer chamber was used to determine the total leukocyte counts. For the differential cell count in the BALF, 250 µL of each sample was centrifuged in a cytocentrifuge (INBRAS, São Paulo, BR). Cells were stained with a fast panoptic coloration kit (Laborclin, Pinhais, Parana), and 100 cells were counted per slide [11, 33].

### *Homogenized tissue*

After BALF collection, the right ventricle of each animal was perfused with saline to remove blood from the lungs. The right lung was removed and homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at 15,521 g for 10 min. The supernatant was collected and stored at -80 °C for biochemical analyses.

### *Antioxidant defense and oxidative stress biomarkers in lung homogenates*

Lung homogenates were used to measure antioxidants enzyme activity and oxidative damage. Superoxide dismutase (SOD) activity was measured using ELISA ($\lambda = 570$ nm) as previously described [34], which is based on the enzyme's ability to inhibit the autoxidation of pyrogallol. Catalase activity (conversion of hydrogen peroxide ($H_2O_2$) to water and molecular $O_2$ per minute)was measured spectrophotometrically ($\lambda = 240$ nm) as previously described [35]. Glutathione analysis was determined using an assay adapted from a commercial kit (CS0260, Sigma, St. Louis, MO, USA); the method is based on the ability to reduce the 5.5′-Dithio Acid -bis- (2-nitrobenzoic) to thio-2-nitrobenzoic acid according to the Griffith assay [36]. To calculate the concentration of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) a standard serial dilution curve is prepared, and the concentration of reduced form (GSH) is calculated from the subtraction of the total glutathione value by the value of oxidized glutathione. Oxidative damage was determined based on lipid peroxidation of thiobarbituric acid reactive substances as previously described [37] and by measuring the levels of oxidatively modified proteins *via* the carbonyl protein assay [38]. Total protein was measured according to the Bradford method [39].

### *Stereological analyses*

The left lung was fixed, processed, and stained with hematoxylin and eosin. Twenty random images obtained from the histological slides of the lungs were scanned with a 40× objective lens. The volume density analyses of the alveolar septum (Vv[sa]) and the alveolar spaces (Vv[a]) were performed in a test system composed of sixteen points and a

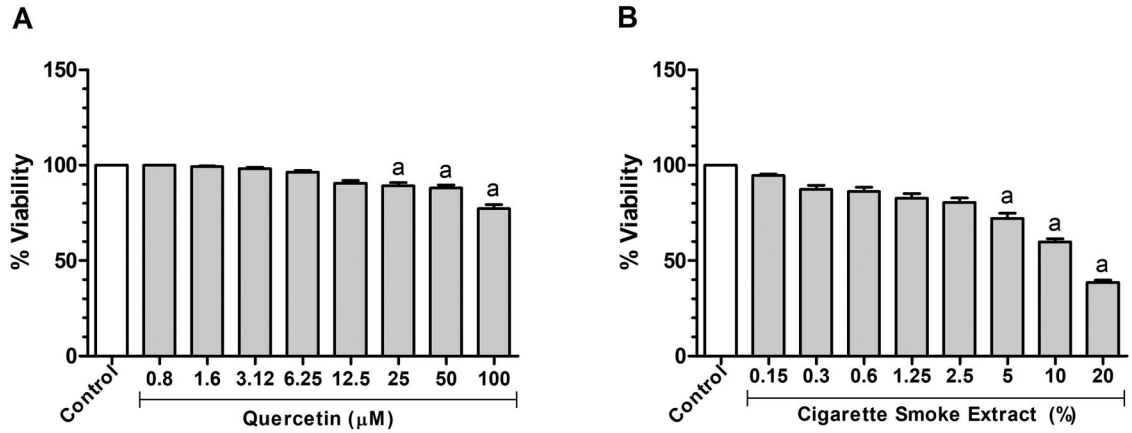


**Figure 1.** Effect of quercetin and cigarette smoke extract on J774A.1 cell viability within 24 h. (A) Analysis of quercetin administration (0.8, 1.6, 3.12, 6.25, 12.5, 25, 50 and 100 $\mu$M) on cell viability of J774. (B) Analysis of cigarette smoke extract administration (0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20%) on cell viability of J774. Data were expressed in median, minimum and maximum value and were analyzed by Kruskal-Wallis followed by Dunn's post-test ($p < 0.05$). The letter (a) represents a significant difference when compared to control. This test was performed in sextuplicate from two independent experiments.

test area delineated by the prohibited line to avoid overestimation of the number of structures. The test system was connected to a monitor attached to a microscope. The number of points (*PP*) that reached the alveolar septa was determined according to the total number of test points (*PT*) in the system using the equation $VV = PP/PT$. To ensure uniformity and proportionality in the pulmonary samples, we analyzed 18 random fields in a cycloid test system attached to a monitor. The reference volume was estimated by point counting using the test point system. A total area of 1.94 $mm^2$ was analyzed to determine Vv[sa] and Vv[a] [40].

### Statistical analysis

The normal distribution of each variable was assessed using the Kolmogorov-Smirnov test; data are presented as the mean and standard error of the mean (SEM). For comparison among groups, a one-way ANOVA followed by Tukey's post-test was used. For non-parametric data, we used Kruskal Wallis test followed by Dunn's post-test, and the data are expressed as the median, minimum, and maximum values. In both cases, the difference was considered significant when the *P* value was $< 0.05$. The statistical analyses were performed using Prism v.5 (Windows 7, GraphPad Software, San Diego, CA USA).

## Results

### Cytotoxicity of cigarette smoke extract and quercetin

To determine the nontoxic working concentrations of CSE and quercetin, their cytotoxicity was measured using J774.A1 cells by the resazurin assay. Both CSE (Kruskal-Wallis, $p < 0.0001$) and quercetin (Kruskal-Wallis, $p < 0.0001$) decreased the number of viable cells in dose-dependent manners (Figure 1). Basically, a decrease in cell viability was observed after 24 h exposure to 25 ($p < 0.05$), 50 ($p < 0.05$) and 100 $\mu$M ($p = 0.001$) quercetin when compared to that in the CG (untreated cells). In cells exposed to CSE, cell viability decreased after 24 h exposure to 5% ($p = 0.01$), 10% ($p = 0.001$) and 20% ($p = 0.001$) when compared to control. To obtain assurance that CSE and quercetin were not toxic to the cell, the half cytotoxic concentrations ($CC_{50}$) were determined, specifically 14.8% for CSE and 221.5 µM for quercetin at 24 h. Thus, 10, 25, and 50 $\mu$M quercetin [41] and 0.5% CSE [11] were used for the subsequent experiments.

### Effect of CSE on ROS and nitric oxide production in J774A.1 cell

ROS production increased significantly when the cells were exposed to CSE for 24 h (ANOVA, $p < 0.0001$) in relation to control ($p = 0.01$). When the cells were pretreated with quercetin at 25 ($p = 0.001$) and 50 $\mu$M ($p = 0.0001$) and then exposed to CSE for 24 h, we observed a reduction in ROS production compared to CSE treatment alone (Figure 2A).

We next measured NO production, as it is an important inflammatory mediator. Relative to the control condition (no CSE), NO concentration increased significantly (ANOVA, $p = 0.0011$) when the cells were exposed to CSE for 24 h in relation to control ($p = 0.001$). However, pretreatment with quercetin at all of the tested concentrations 10 ($p = 0.01$) and 25 $\mu$M ($p < 0.05$) prevented this increase in relation to cells exposed to cigarette smoke extract (Figure 2B).

### Lung function in C57BL/6 mice

We found that cigarette smoke (CSG) induced changes in breathing patterns, including respiratory rate (ANOVA, $p = 0.0002$) and the tidal volume (ANOVA, $p < 0.0001$) compared to the control group (CG) exposed to ambient air ($p = 0.001$). Surprisingly, quercetin (QCSG) prevented these variations in tidal volume ($p = 0.001$) compared to CSG (Table 1).

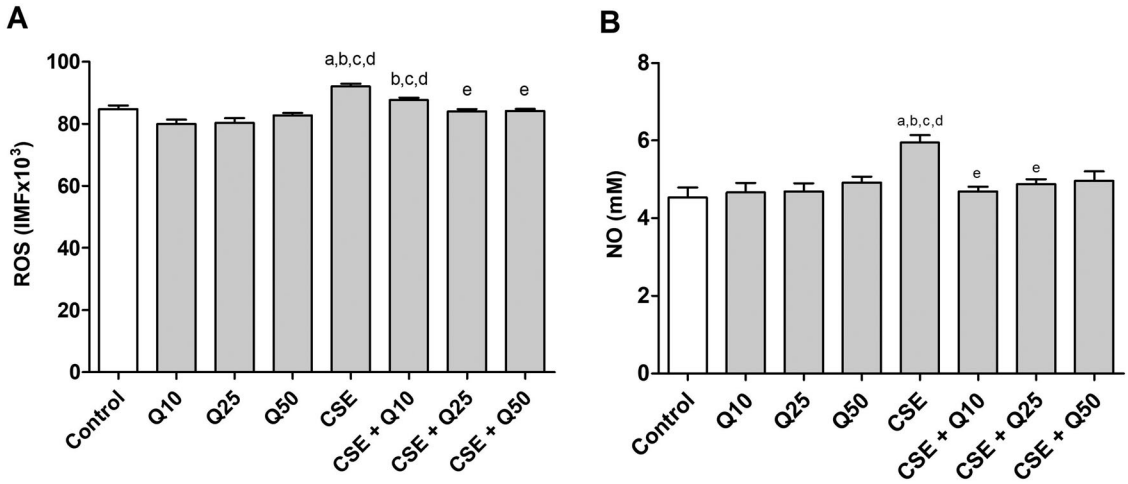


**Figure 2.** Effect of cigarette smoke extract and quercetin on ROS and NO production in J774A.1 cell. (A) Quantitative analysis of ROS production in J774 cells administered with Quercetin (10, 25 and 50 $\mu$M) and/or CSE (0.5%). (B) NO production measured as nitrite content J774 cells administered with Quercetin (10, 25 and 50 $\mu$M) and/or CSE (0.5%). Data are expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test ($n = 5$-8, $p < 0.05$). The letter (a) represents a significant difference when compared to control. The letter (b) represents a significant difference when compared to Q10. The letter (c) represents a significant difference when compared to Q25. The letter (d) represents a significant difference when compared to Q50. The letter (e) represents a significant difference when compared to CSE.

**Table 1.** Assessment of lung function in mice.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| **RR (breaths/min)** | 172.2 ± 6.42 | 153.6 ± 9.90 | 159.4 ± 10.40 | 91.8 ± 15.82$^{a,b,c}$ | 134.2 ± 6.76 |
| **VT (mL)** | 0.26 ± 0.01 | 0.28 ± 0.02 | 0.26 ± 0.02 | 0.57 ± 0.05$^{a,b,c}$ | 0.32 ± 0.04$^{d}$ |
| **MV (mL/min)** | 44.74 ± 3.54 | 43.88 ± 5.19 | 41.77 ± 5.28 | 54.75 ± 12.76 | 43.22 ± 7.11 |
| **Bodymass (g)** | 28.70 ± 0.73 | 31.31 ± 1.12 | 31.47 ± 0.87 | 29.81 ± 0.44 | 29.47 ± 0.61 |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group; RR: respiratory rate; VT: tidal volume; MV: minute ventilation. The letter (a) represents a significant difference between CSG when compared to CG. The letter (b) represents a significant difference between CSG when compared to VG. The letter (c) represents a significant difference between CSG when compared to QG. The letter (d) represents a significant difference between QCSG when compared to CSG. Data are expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test, $n = 5$ animals per group ($p < 0.05$).

**Table 2.** Hematological parameters of experimental groups.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| **Erithrocytes (x10$^6$/mm$^3$)** | 7.6 ± 0.23 | 7.2 ± 0.16 | 7.2 ± 0.09 | 8.5 ± 0.08$^{a,b,c}$ | 8.2 ± 0.08$^{b,c}$ |
| **Hemoglobin (g/dL)** | 12.4 ± 0.31 | 12.2 ± 0.14 | 12.2 ± 0.36 | 14.3 ± 0.18$^{a,b,c}$ | 13.4 ± 0.20$^{b,c}$ |
| **Hematocrit (%)** | 37.4 ± 0.95$^{a}$ | 36.6 ± 0.53$^{a}$ | 36.6 ± 1.08$^{a}$ | 42.9 ± 0.54$^{a,b,c}$ | 40.1 ± 0.62$^{b,c}$ |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. The letter (a) represents a significant difference between CSG when compared to CG. The letter (b) represents a significant difference between CSG or QCSG when compared to VG. The letter (c) represents a significant difference between CSG or QCSG when compared to QG. Data are expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test, $n = 5$ animals per group ($p < 0.05$).

### *Action of quercetin on hematological parameters in C57BL/6 mice*

The exposure to cigarette smoke (CSG) promoted increases in erythrocytes (ANOVA, $p < 0.0001$), hemoglobin (ANOVA, $p < 0.0001$) and hematocrit (ANOVA, $p < 0.0001$) when compared to the control group exposed to ambient air ($p = 0.001$). However, pretreatment with quercetin (QCSG) did not promote changes in these parameters compared to CSG (Table 2).

### *Recruitment of total and differential cell count in BALF*

Exposure to cigarette smoke for five days promoted an increase in influx of total leukocytes (ANOVA, $p < 0.0001$), macrophages (ANOVA, $p < 0.0001$), lymphocytes (ANOVA, $p < 0.0001$), and neutrophils (ANOVA, $p < 0.0001$) into the BALF compared to CG ($p = 0.001$)(Figure 3). Interestingly, pretreatment with quercetin before cigarette smoke exposure resulted in decreased numbers of total leukocytes ($p = 0.001$), macrophages ($p = 0.001$) and lymphocytes ($p = 0.001$) when compared to exposed group (CSG).

### *Antioxidant defense and oxidative stress biomarkers in lung homogenates*

Cigarette smoke exposure promoted a decrease of SOD (ANOVA, $p = 0.0002$) and catalase activity (ANOVA, $p = 0.0025$) in the CSG compared to the control group (CG) ($p < 0.05$), which was attenuated by quercetin pretreatment ($p = 0.01$) (Table 3). The GSH/GSSG ratio was lower (Kruskal-Wallis, $p = 0.0027$) in the CSG than to the control group ($p < 0.05$), VG and QG, and pretreatment with quercetin had no effect on this reduction. In contrast, the

damage to lung parenchyma was evaluated based on levels of carbonylated proteins (ANOVA, $p = 0.0028$) and TBARS (ANOVA, $p = 0.0001$), which both increased in the CSG compared to the control group (ambient air) ($p = 0.01$). In addition, the QCSG showed a reduction in carbonylated proteins ($p < 0.05$) and TBARS ($p = 0.001$) concentration compared to the CSG.

### *Stereological analyses of the lung tissue*

In the stereological analyses (Figure 4), the volume densities of the alveolar air space (Vv[a]) were increased in the CSG (Kruskal-Wallis, $p = 0.001$) compared to the control group ($p < 0.05$) (Figure 4 A), and the volume densities of alveolar septa (Vv[sa]) were decrease in CSG (Kruskal-Wallis, $p = 0.0011$) compared to control group ($p = 0.01$) (Figure 4 B). However, quercetin administration promotes a decrease of Vv[a] ($p < 0.05$) and an increase of Vv[sa] ($p < 0.05$) compared to the CSG.

## Discussion

The initial changes caused by cigarette smoke exposure in relation to the pathogenesis of COPD and other lung diseases typically involve inflammation and redox imbalance. Thus, studies of natural products with antioxidant and anti-inflammatory proprieties that could be used to counteract the deleterious effects of cigarette smoke have been encouraged. In this study, we showed that the antioxidant effect of quercetin was effective against the oxidative stress and changes in lung morphology and function induced by cigarette smoke. To our knowledge, this is the first study that examines the initial changes in lung function caused by exposure to cigarette smoke.

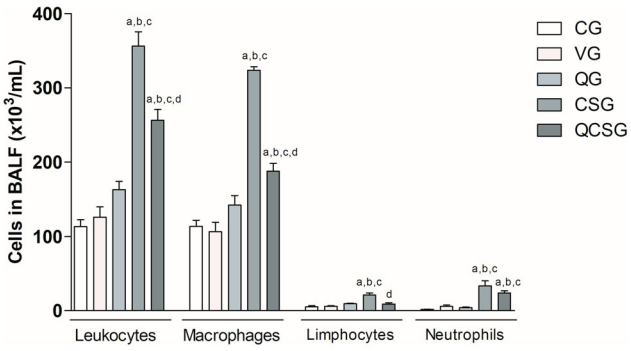


**Figure 3.** Total and differential count of bronchoalveolar lavage fluid. CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. The letter (a) represents a significant difference between groups when compared to CG. The letter (b) represents a significant difference between groups when compared to VG. The letter (c) represents a significant difference between groups when compared to QG. The letter (d) represents a significant difference between groups when compared to CSG. Data are expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test, $n = 5$ animals per group, $p < 0.05$.

First, we evaluated the effects of quercetin on J774A.1 alveolar macrophage exposed to cigarette smoke extract. Alveolar macrophages reside in the pulmonary alveoli and act as the first line of defense in the lungs by orchestrating airway inflammatory events in response to the toxins present in cigarette smoke. These cells secrete multiple inflammatory mediators and ROS [42–46]. In the present study, ROS and NO production were measured in J774.A1 alveolar macrophages exposed to cigarette smoke extract with or without quercetin pretreatment. We found that exposure to cigarette smoke extract increased ROS and NO production, which contributes to an increase in oxidative stress. This result is consistent with the fact that NO is an important modulator of macrophage activity. Pretreatment of quercetin at all doses reduced ROS and NO to baseline levels. This dramatic result may be explained by the ability of quercetin to diffuse at high rates through cell membranes and on the surfaces of lipid bilayers and also in the aqueous phase [47].

After demonstrating the antioxidant effects of quercetin *in vitro*, we next tested the *in vivo* effects using a short-term cigarette smoke exposure model. We observed significant hematological alterations in response to cigarette smoke exposure. Specifically, erythrocyte, hemoglobin, and hematocrit levels were all increased. This result is unsurprising given that the gas phase of the cigarette smoke is rich in carbon monoxide (CO). After inhalation, CO reaches the bloodstream and combines with hemoglobin to form carboxyhemoglobin (HbCO), which blocks oxygen binding. Because HbCO circulates faster than oxyhemoglobin, the serum $O_2$ concentration decreases dramatically, causing hypoxia [48,49]. Hypoxia then activates the xanthine oxidase (XO) pathway, resulting in increased conversion of ATP to ADP, AMP, iosin, and hypoxanthine (the substrate for XO), whose metabolism favors the generation of ROS [50,51]. Because quercetin does not interact with CO, it was unsurprising that quercetin pretreatment did not prevent the hematological alterations. Thus, our results suggest that quercetin acts by neutralizing the ROS generated by XO.

**Table 3.** Antioxidant defense and oxidative stress biomarkers in lung homogenates.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| **SOD (U/mg protein)** | 30.34 ± 1.74 | 28.05 ± 1.08 | 28.40 ± 2.05 | 21.21 ± 1.09$^{a,b,c}$ | 30.03 ± 1.49$^{d}$ |
| **CAT (U/mg protein)** | 1.4 ± 0.04 | 1.6 ± 0.14 | 1.5 ± 0.16 | 1.01 ± 0.07$^{a,b,c}$ | 1.67 ± 0.15$^{d}$ |
| **GSH/GSSG ratio** | 21.8 (11.26; 36.63) | 16.3 (10.82; 29.42) | 15.1 (12.32; 23.94) | 6.4 (5.73; 9.85)$^{a,b,c}$ | 10.6 (9.51; 14.67) |
| **TBARS(nmol/mg protein)** | 1.85 ± 0.20 | 2.74 ± 0.43 | 2.73 ± 0.27 | 4.01 ± 0.23$^{a,b,c}$ | 1.84 ± 0.17$^{d}$ |
| **Protein carbonyl (nmol/ mg protein)** | 12.22 ± 2.16 | 17.03 ± 2.13 | 18.14 ± 0.99 | 26.14 ± 3.7$^{a,b,c}$ | 11.44 ± 2.42$^{d}$ |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. The letter (a) represents a significant difference between groups when compared to CG. The letter (b) represents a significant difference between groups when compared to VG. The letter (c) represents a significant difference between CSG when compared to QG. The letter (d) represents a significant difference between QCSG when compared to CSG. SOD, CAT, TBARS and protein carbonyl were expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's post-test, $n = 5$ animals per group ($p < 0.05$). In regards to GSH/GSSG ratio, the data were expressed in median,minimum and maximum value and were analyzed by Kruskal-Wallis followed by the Dunn's post-test, $n = 5$ animals per group ($p < 0.05$).

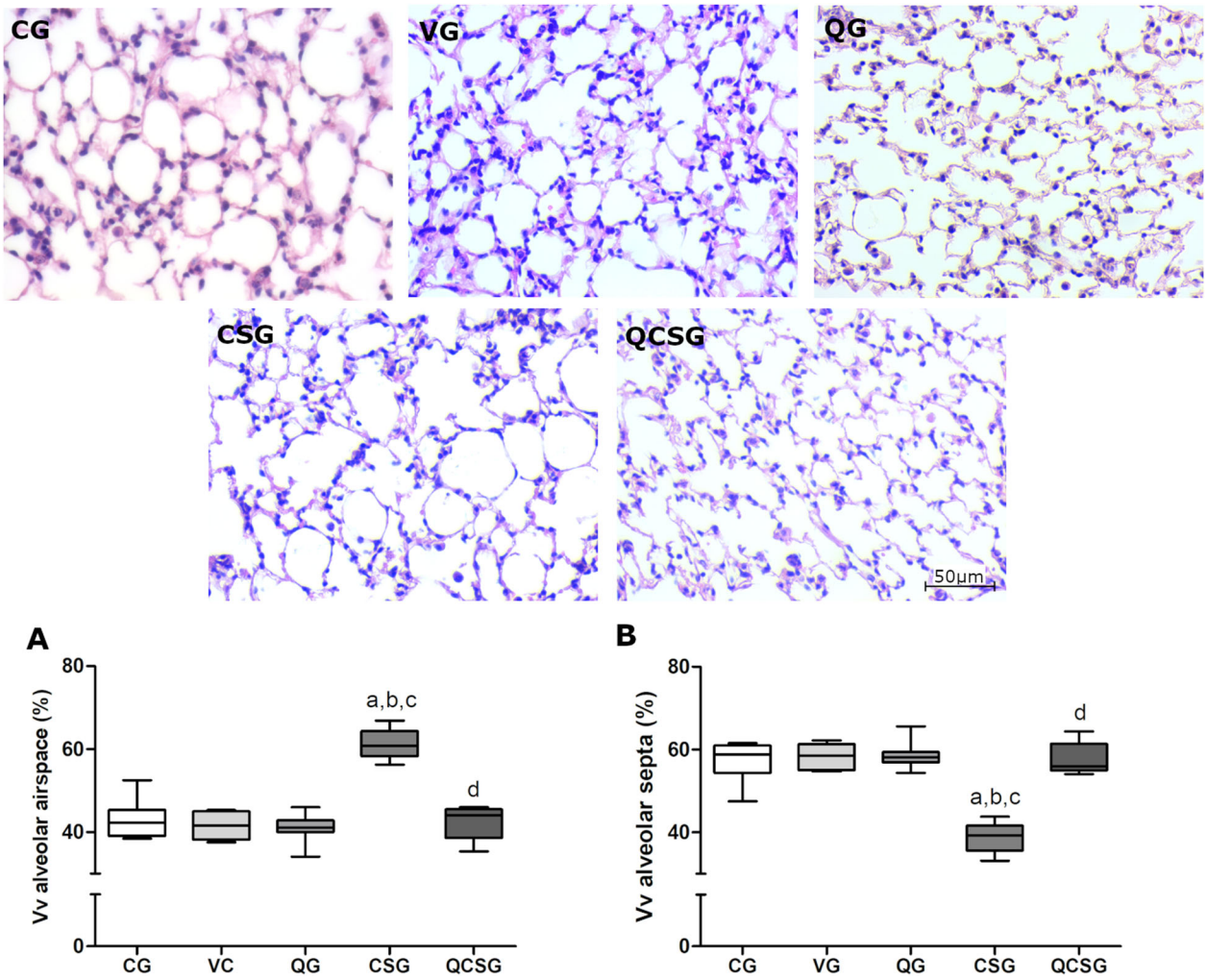


**Figure 4.** Morphometric evaluations of the lung parenchyma. Photomicrographs of lung sections stained with hematoxylin and eosin. Bar = 50 µm, 400x magnification. CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. (A) Volume density of alveolar septa. (B) Volume density of alveolar airspace. The letter (a) represents a significant difference between CSG when compared to CG. The letter (b) represents a significant difference between CSG when compared to VG. The letter (c) represents a significant difference between CSG when compared to QG. The letter (d) represents a significant difference between QCSG when compared to CSG. For A and B, data were expressed in median, minimum and maximum value and were analyzed by Kruskal-Wallis followed by Dunn's post-test, $n = 5$ animals per group, $p < 0.05$.

Exposure to cigarette smoke promoted increased influx of inflammatory cells into the airways, corroborating our previous studies [11, 33, 52]. Specifically, we showed that oxidants present in cigarette smoke can stimulate alveolar macrophages to release ROS and chemotactic factors that attract neutrophils and other inflammatory cells. Activated macrophages and neutrophils also generate ROS *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [53,54]. In our study, cigarette smoke exposure led to a greater recruitment of macrophages and neutrophils in CSG compared to the control group, suggesting a greater generation of ROS in CSG. We also found that pretreatment with quercetin reduced the influx of inflammatory cells, particularly macrophages, to the BALF in animals exposed to cigarette smoke. A similar result was shown by Ganesan and colleagues (2010) using a model of lung injury induced by elastase/LPS that was pretreated with quercetin at 10 mg/kg/day for 10 days [24].

The oxidant load in the lungs due to cigarette smoke exposure leads to a redox imbalance that culminates in oxidative damage to macromolecules, such as lipids and proteins. Previous data showed that changes in antioxidant enzyme activity and oxidative damage occur from the third day of CS exposure [40]. Here, we evaluated the redox imbalance by measuring oxidative damage. Our results showed that contact with cigarette smoke significantly decreased the activity of the antioxidants, enzymes SOD and catalase. Pretreatment with quercetin restored their activity to control levels. Quercetin—one of the most potent antioxidants in the flavonoid family—has been shown to be effective against redox imbalance in several models and is a strong superoxide scavenger [55]. Antioxidant enzymes, in particular SOD, lose part of their activity when there is an excess of their substrate. Thus, the $O_2^-$ present in the cigarette smoke as well as the increase in $O_2^-$ generation by NADPH oxidases resulting from cigarette smoke exposure likely contributed to the loss of SOD activity observed herein [56]. Because the product of $O_2^-$ dismutation by SOD is $H_2O_2$, which is the substrate for catalase, a reduction in SOD activity would also result in loss of catalase activity.

Thus, it is likely that the effect of quercetin in restoring SOD and catalase activity is due, at least in part, to its effect on reducing $O_2^-$ levels. Another marker of redox imbalance is the ratio GSH/GSSH. Several authors have correlated the decrease in GSH/GSSH ratio to oxidative stress and the emergence of various diseases [57–59]. Because the lung contains high levels of glutathione [60] and COPD is a disease related to oxidative stress, the progression of COPD is substantially affected by the status of glutathione. In this study cigarette smoke exposure caused a decrease in the GSH/GSSH ratio and pretreatment with quercetin had no effect. These results suggest that pretreatment with quercetin before cigarette smoke exposure promotes the endogenous antioxidant defense mechanisms—i.e. by increasing the activity of antioxidant enzymes, such as SOD and catalase. The decreased SOD and catalase activities and decreased GSH/GSSG ratio reflect the susceptibility of the lung to cigarette smoke-induced oxidative stress. These results are corroborated by our experiments showing higher levels of lipid peroxidation and protein carbonylation in the lung parenchyma of mice exposed to cigarette smoke. Interestingly, quercetin supplementation was prevented this damage. Despite the low administered dose of quercetin, its antioxidant effects were clearly demonstrated in our experimental model. This may be due to the ability of quercetin to sit between lipid bilayers; the increase in its local concentration would allow quercetin to exert its antioxidant activity more efficiently [19, 61].

Redox imbalance causes structural damage to essential lung components and is responsible for irreversible damage to the lung parenchyma [62,63]. Our results demonstrate that exposure to cigarette smoke for a short time caused alterations in the alveolar structure as shown by an increase in alveolar volume density and a decrease in alveolar septa volume density. Pretreatment with quercetin was able to prevent these alterations. These results corroborate our previous studies that showed similar results using other antioxidants [11, 52]. The stereological changes in the pulmonary parenchyma caused by short-term exposure to cigarette smoke are well-established in our group [33, 40, 52, 64], but this is the first time that we have related these changes to lung function parameters.

Changes in pulmonary architecture caused by short-term exposure to cigarette smoke reflected a change in the breath control balance. The group exposed to cigarette smoke presented a higher level of tidal volume compared to the other groups, and pretreatment with quercetin attenuated this effect. The CSG also presented a lower respiratory rate index when compared to CG. We believe that these changes occurred due to as a physiological response to short exposure to cigarette smoke in the attempt to reestablish respiratory balance, as there was no significant change in minute volume between groups.

Quercetin has low bioavailability, mainly due to its low solubility and to undergo extensive metabolism before reaching the bloodstream [65,66]. Li and colleagues (2016) suggest an absorption rate of the aglycone form of approximately 2% [25]. A strength of our study is the low dose of quercetin that was used (10 mg/kg/day), which is equivalent to a dose of 48.65 mg/day for a human of 60 kg [67]. This amount can be easily achieved by increasing the intake of foods rich in this flavonoid, such as capers (180.77 mg/100g), purple onions (19.93 mg/100g) and apple with the peel (4.4 mg/100g) [68] without the need for supplementation.

## Conclusions

Quercetin is a highly potent antioxidant both in alveolar macrophages and in the pulmonary parenchyma of mice. Importantly, it significantly prevented the oxidative damage and consequently the morphological and functional pulmonary alterations produced by short-term exposure to cigarette smoke, in this study. Thus, we propose that an increase in consumption of this flavonoid would be an effective adjuvant in the treatment of pulmonary lesions induced by cigarette smoke and can act to prevent or even decrease the rate of lesion progression.

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## Declaration of interest statement

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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## 3. PROTECTIVE EFFECTS OF QUERCETIN ON LIVERS FROM MICE EXPOSED TO LONG-TERM CIGARETTE SMOKE

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*Research Article*

# Protective Effects of Quercetin on Livers from Mice Exposed to Long-Term Cigarette Smoke

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Cigarette smoke is highly toxic, and it can promote increased production of reactive species and inflammatory response and leads to liver diseases. Quercetin is a flavonoid that displays antioxidant and anti-inflammatory activities in liver diseases. This study aimed at evaluating the protective effects of quercetin on livers from mice exposed to long-term cigarette smoke exposure. Male C57BL/6 mice were divided into five groups: control (CG), vehicle (VG), quercetin (QG), cigarette smoke (CSG), quercetin, and cigarette smoke (QCSG). CSG and QCSG were exposed to cigarette smoke for sixty consecutive days; at the end of the exposures, all animals were euthanized. Mice that received quercetin daily and were exposed to cigarette smoke showed a reduced influx of inflammatory cells, oxidative stress, inflammatory reaction, and histopathological changes in the liver, compared to CSG. These results suggest that quercetin may be an effective adjuvant for treating damage to the liver due to cigarette smoke exposure.

## 1. Introduction

Cigarette smoking is considered one of the leading preventable causes of morbidity and mortality representing a risk factor for several diseases such as cardiovascular, chronic obstructive pulmonary, and liver diseases [1, 2]. Smoking kills about 6 million people a year, and it is estimated that this number will reach 8 million by 2030. Cigarette smoke (CS) is a highly toxic complex mixture of components containing more than 5000 compounds that can promote damage to biological tissues [3].

The CS produces reactive oxygen species (ROS) that, at high concentrations, brings about changes in the redox process and induction of inflammatory responses, triggering several comorbidities [4–6]. The exposure to cigarette smoke causes damage to cells and tissues by developing oxidative processes. The installation of oxidative stress can occur directly through the production of ROS or by the activation of the inflammatory process, which, in turn, results in an increased level of radical species [7, 8].

The liver is the biggest gland of the body, which is responsible for processing drugs, alcohol, and other toxins. As an immunologically complex organ, the liver contains multiple types of tissue-resident inflammatory cells, like lymphocytes [9] and Kupffer cells [10]. Therefore, liver diseases represent an important public health problem, and understanding the mechanisms of the pathophysiological process is crucial. The redox imbalance induced by chronic exposure to cigarette smoke produces toxins with direct or indirect toxic effects in the liver, promoting an increase in the number

of inflammatory cells, collagen deposition, and apoptosis. Moreover, constituents of cigarette smoke exert immunoregulatory actions and hepatic carcinogens [11].

Previous studies showed that anti-inflammatory and antioxidant activities in natural compounds prevented diseases. In this context, it is worth to highlight the role of quercetin, which is the most abundant flavonol, and it is present in a variety of foodstuffs like fruits and vegetables. Quercetin is present in plants, and it presents many different glycosidic forms (formed by attaching a glycosyl group), which directly reflects on the bioavailability and bioactivity profile of this substance. However, a part of the absorbed quercetin can circulate in the blood as aglycone (the lacking sugar moieties form). Quercetin can act beneficially in several pathologies, especially those that affect the lungs [12–14]. A recent study by our research group showed that quercetin acts as a potent agent in preventing oxidative damage and pulmonary changes [15]. Some studies also extend the effects of quercetin to liver pathologies, exerting a modulating action on inflammation, fibrosis, and redox imbalance through the interaction with a wide spectrum of central molecular targets for cell signaling machines, thus playing a promising role in the treatment of chronic inflammatory liver diseases [16, 17]. However, the ability of quercetin to counteract the deleterious effects of cigarette smoke in the liver has not been elucidated. The goal of this study was to evaluate the effects of quercetin on reducing the redox imbalance and liver inflammation induced by long-term cigarette smoke exposure.

## 2. Material and Methods

*2.1. Animals.* The ethics committee of the Federal University of Ouro Preto (2015/20) approved all animal experiments, and the experiments were performed according to the rules of animal protection and the ethical principles of the Brazilian Society of the Science in Laboratory Animals (SBCAL).

Forty male C57BL/6 mice (12 weeks old) obtained from the Laboratory of Experimental Nutrition (LABNEX) at the Federal University of Ouro Preto (UFOP) were housed under controlled conditions (12 h light/dark, 21°C ± 2°C, 50% ± 10 % humidity) with access to food and water ad libitum. The mice were divided into 5 groups ($n = 8$ per group): a control group exposed to ambient air (CG), a group that received 200 $\mu$L of vehicle solution containing 50% propylene glycol (Sigma Aldrich, Missouri, USA) and 50% saline (VG) by orogastric gavage, a group that received 10 mg/kg/day of quercetin (Sigma Aldrich, Missouri, USA) diluted in 200 $\mu$L of propylene glycol solution (QG), a group exposed to cigarette smoke (CSG), and a group administered with quercetin and exposed to cigarette smoke (QCSG). The administration of quercetin was performed via orogastric gavage 1 h before exposure to cigarette smoke [12, 13].

*2.2. Cigarette Smoke Exposure Protocol.* In a smoking chamber, the mice were exposed to 12 commercial full-flavor filtered Virginia cigarettes (tar: 10 mg, nicotine: 0.9 mg, and carbon monoxide: 10 mg) per day divided into three times daily (morning, afternoon, and night) for 60 consecutive days as previously described [18]. The cigarette smoke (CS) and QCSG animals were initially placed in an inhalation chamber (40 cm × 30 cm × 25 cm) inside an exhaustion chapel. Each cigarette was coupled to a 60 mL plastic syringe through which the smoke was injected inside the inhalation chamber. CSG and QCSG were maintained in the smoke atmosphere for 6 min; then, the cover was removed from the inhalation chamber for 1 min to allow the air to completely exhaust. The procedure was then repeated with the remaining cigarettes. The CG, VG, and QG were subjected to the same conditions, but without exposure to cigarette smoke. At the end of the exposition, the animals were also weighed on a digital scale (Mark®; Série M/Bel Analytical Equipment LTDA, São Paulo, Brazil).

*2.3. Blood and Liver Collection.* The animals were euthanized twenty-four hours after the last exposure to CS by an overdose of ketamine (130 mg/kg) and xylazine (0.3 mg/kg). The blood was collected through the cardiac puncture in polypropylene tubes containing 15 $\mu$L of anticoagulants. Subsequently, it was centrifuged at 10.000 rpm for 15 minutes, and the plasma was collected and stored in a freezer (-80°C). Immediately after euthanasia, the right ventricle was perfused with saline to remove blood from the liver; subsequently, two aliquots of liver were separated. The first sample was fixed in 4% neutral buffered formalin, dehydrated, cleared, embedded in paraffin, cut into 4–5 $\mu$m sections, and stained with hematoxylin and eosin (H&E) for histopathological analyses. Afterwards, 100 mg of the tissue was placed in polypropylene tubes with 1 mL of phosphate buffer (pH 7.8), and it was homogenized in a tissue homogenizer. Then, the samples were centrifuged at 4°C for 10 minutes and 13.000 rpm (MIKRO 200R; laboratory technology Hettich, Tuttlingen, Germany), and the supernatant was collected and stored at -80°C [15].

*2.4. Biochemical Measurements.* The samples containing 300 $\mu$L of plasma were sent to the Pilot Laboratory of Clinical Analysis (Lapac) in Ouro Preto-MG, in order to determine the concentrations of liver function markers, including aspartate aminotransferase (AST; K048-6), alanine aminotransferase (ALT; K049-6), lactate dehydrogenase (LDH; K014-2,) and gamma-glutamyltransferase (GGT-1; K080-2), which were determined by an automatic spectrophotometry in the clinical analyzer Randon Acess Clinical Analyzer, Wiener Lab, model CM-200 (WIENER LAB, Rosario, Argentina) by the enzymatic-colorimetric method using specific kits that were purchased from Bioclin®, Quibasa (MG, Brazil).

*2.5. Histopathological Analysis.* The histopathological analysis was carried out using a light microscope (Leica DFC 300 FX) with a 40x microscopic objective. For histological and morphometric analysis, we obtained twenty randomly chosen images of histological slides that were prepared from the liver sections. These slides were scanned using the Leica Application software. Histological screening focused on the detection and grading of the following abnormalities: inflammatory reaction, sinusoidal congestion, degenerative hepatocyte lesions (hydropic and steatotic), sinusoidal congestion,

hyperemia, bleeding focus, intralobular and portal granulomas, fibrosis, and necrosis. The changes in the experimental histopathological parameters present were semiquantified according to a scoring method as (0) absent, (1) mild, when present in approximately less than 33% in the total area of the tissue, (2) moderate, when present between approximately 33% and 66% in the total area of the tissue, and (3) intense, when present in approximately more than 66% in the total area of the tissue [19, 20]. The counting of the number of inflammatory cells present in the hepatic lobes included the resident Kupffer cells. Slides were analyzed using the Leica Q-Win Plus software (Leica Microsystems, Inc., Buffalo Grove, Illinois, USA).

*2.6. Analysis of Antioxidant Defense and Biomarkers of Oxidative Stress.* A hepatic homogenate was used for the analysis of antioxidant defense and oxidative damage. The superoxide dismutase (SOD) activity was measured according to the method described by Marklund and Marklund, based on the enzyme ability to inhibit the autoxidation of pyrogallol [21]. The catalase (CAT) activity was measured using a spectrophotometer according to the method described by Aebi, which is based on the decrease in $H_2O_2$ at an absorbance of 240 nm [22]. The glutathione analysis was determined using an assay adapted from a commercial kit (CS0260, Sigma, St. Louis, MO, USA), and the method is based on the ability to reduce the 5.5′-Dithio Acid-bis-(2-nitrobenzoic) to thio-2-nitrobenzoic acid according to the Griffith assay [23]. In order to calculate the concentration of total glutathione (GSH+GSSG) and oxidized glutathione (GSSG), a standard serial dilution curve was prepared, and the concentration of reduced form (GSH) was calculated from the subtraction of the total glutathione value by the value of oxidized glutathione. In order to measure lipid peroxidation, we used the method described by Buege and Aust [24], which thiobarbituric acid reacts with oxidized lipids and the concentration of TBARS was provided using the molar extinction coefficient, following the law of Lambert Beer. For the determination of the carbonylated protein, a protocol adapted from the methodology described by Reznick and Packer was used [25]. The total protein was measured according to the Lowry method [26].

*2.7. Immunoenzymatic Assay for Inflammatory Mediators.* Interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemotactic protein 1 (CCL2), and interferon-$\gamma$ (INF-$\gamma$) were performed in liver homogenate by the enzyme-linked immunosorbent assay (ELISA) method. The assays were performed using commercial kits (PeproTech, Ribeirão Preto, Brazil), and the antibodies and reagents were prepared according to the instructions from the manufacturer. Immunoenzymatic assays were performed in 96-well plates, on which 100 $\mu$L of monoclonal antibody was added to the protein (or peptide) of interest, and the sample were diluted in phosphate-buffered saline (PBS) containing 0.1% fetal bovine serum. After incubation for 12 hours at room temperature, the non-adsorbed antibodies were discarded, and blocking was performed with a solution containing PBS with 1% fetal bovine serum for one hour. Samples of the liver tissue supernatant, which were previously processed and stored at -80°C, were applied in a volume of 50 $\mu$L for each well. The reading of the staining intensity was performed in an ELISA reader using a wavelength of 490 nm. The quantification of chemokines and interleukins present in the samples was determined based on the optical density obtained with the peptide standard curve, analyzed by the SOFT Max PRO 4.0 software [27, 28], and the result obtained was a cytokine picogram per milliliter (pg/mL).

*2.8. Statistical Analysis.* The statistical analyses were performed using Prism version 5.00 for Windows 7 (GraphPad Software, San Diego, CA USA). The normal distribution of each variable was assessed using the Kolmogorov-Smirnov test. The normally distributed data were analyzed with one-way ANOVA followed by Tukey's posttest, and the data are expressed as mean ± standard error of the mean (SEM). For nonparametric data, we used the Kruskal Wallis test followed by Dunn's posttest, and the data are expressed as the median, minimum, and maximum values. In both cases, the difference was considered significant when the $p$ value was $<0.05$.

## 3. Results

*3.1. Hepatic Function Markers and Body Weight.* The animals from the five experimental groups were evaluated to investigate the effects of cigarette smoke on liver damage biomarkers. It can be seen from Table 1 that a higher level of ALT (ANOVA, $p = 0.0071$) in the animals was exposed to long-term cigarette smoke (CSG) when compared with the control groups ($p < 0.05$). However, there were no significant differences between the CSG and QCSG regarding the markers of the liver function (Table 1).

There was a decrease of the final body weight (ANOVA, $p < 0.0001$) of the CSG compared to CG and VG ($p = 0.001$). However, there was no difference with respect to the final body weight between CSG and QCSG ($p > 0.05$) (Table 1).

*3.2. Histopathological Aspects.* The semiquantitative analyzes were performed for the presence and intensity of hepatic lesions of mice from the experimental groups. The histopathological evaluation revealed that the control group (CG) had almost completely preserved liver histology (Figure 1(a), Tables 2 and 3). Exposure to cigarette smoke (CSG) resulted in changes in the liver parenchyma such as inflammatory reaction, sinusoidal congestion, hyperemia, granulomatous reaction, and bleeding focus (Figures 1(b)–(e), Tables 2 and 3). The quercetin administration (QCSG) was able to improve the effects of long-term cigarette smoke exposure, with decreasing or the disappearing of injuries (Figure 1(f), Tables 2 and 3). Hydropic degeneration was observed in all groups; however, there were no significant differences (Figure 1, Tables 2 and 3).

The semiquantitative analysis showed higher frequency of sinusoidal congestion/hyperemia in livers of mice exposed to long-term cigarette smoke (75%) than all groups (CG-0%, VG-12.5%, QG-12.5%, QCSG-12.5%) (Table 2). Moreover, the quercetin administration associated to CS (QCSG) provoked an increase in the severity of this injury (12.5%)

Table 1: Serum biochemical parameters and body weight of C57BL/6 mice.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| ALT (U/L) | 12.41 ± 1.17 | 12.56 ± 1.90 | 11.56 ± 1.72 | 24.50 ± 4.75[a,b,c] | 18.80 ± 2.27 |
| AST (U/L) | 88.36 ± 5.45 | 96.85 ± 10.4 | 88.26 ± 5.49 | 83.80 ± 6.93 | 83.80 ± 4.40 |
| GGT-1 (U/L) | 2.46 ± 0.82 | 2.81 ± 0.61 | 4.87 ± 0.42 | 3.38 ± 0.56 | 2.98 ± 0.50 |
| LDH (U/L) | 386.2 ± 31.1 | 412.2 ± 23.5 | 356.4 ± 18.8 | 383.5 ± 26.6 | 389.4 ± 45.0 |
| Body weight (g) | 27.78 ± 0.38 | 26.83 ± 0.31 | 25.60 ± 0.97 | 24.11 ± 0.65[a,b] | 23.47 ± 0.34[a,b] |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group, ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT-1: gamma-glutamyltransferase; LDH: lactate dehydrogenase. [a] represents a significant difference between groups when compared to CG; [b] represents a significant difference between groups when compared to VG; [c] represents a significant difference between groups when compared to QG. ALT, AST, GGT-1, LDH, and body weight were expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's posttest, $n = 5 - 8$ animals per group ($p < 0.05$).

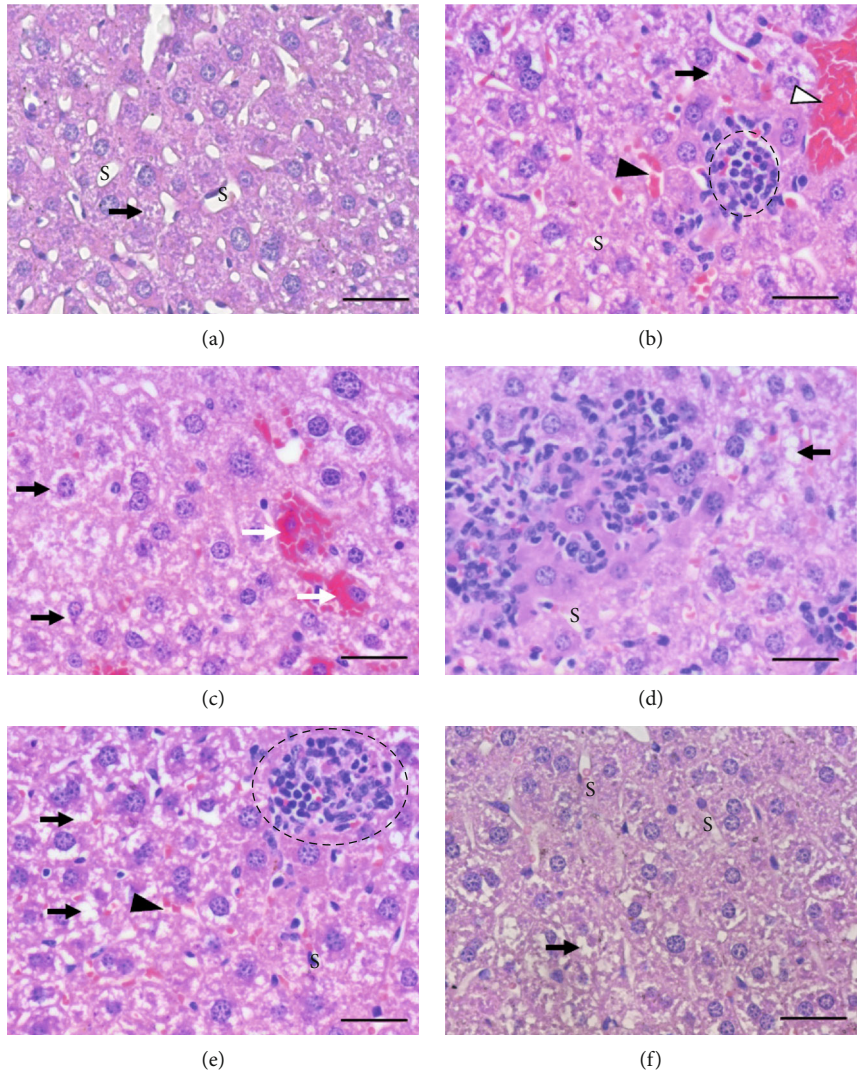


Figure 1: Histopathological aspects of the liver. Photomicrographs of liver sections stained with hematoxylin and eosin. Bar = 50 $\mu$m, 400x magnification. (a) Representative image of groups exposed to ambient air (CG, VG, QG). (b)–(e) Representative images of the lesions found in the group exposed to cigarette smoke (CSG). (f) Representative image of the group pretreated with quercetin and exposed to cigarette smoke (QCSG). (a) Preserved liver parenchyma, presence of hydropic degeneration (black arrow), and preserved sinusoid capillaries (S). (b) Hydropic degeneration (black arrow), congestion of sinusoid (black arrowhead), presence of granuloma (dotted circle), and hyperplasia (white arrowhead). (c) Hydropic degeneration (black arrow) and bleeding focus (white arrow). (d) Inflammatory cell infiltration and hydropic degeneration (black arrow). (e) Hydropic degeneration (black arrow), congestion of sinusoid (black arrowhead), and presence of granuloma (dotted circle). (f) Preserved hepatic parenchyma notes the hydropic degeneration (black arrow) and well-preserved sinusoid capillaries (S).

Table 2: Frequency of histopathological changes in the liver of C57BL/6 mice in the experimental groups.

| Lesions | CG $N = 8$ (%) | VG $N = 8$ (%) | QG $N = 8$ (%) | CSG $N = 8$ (%) | QCSG $N = 8$ (%) |
|---|---|---|---|---|---|
| Hydropic degeneration | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) |
| Sinusoidal congestion/hyperemia | 0 (0.0) | 1 (12.5) | 1 (12.5) | 6 (75.0) | 1 (12.5) |
| Bleeding focus | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (25.0) | 0 (0.0) |
| Granulomas | 0 (0.0) | 0 (0.0) | 0 (0.0) | 6 (75.0) | 0 (0.0) |
| Inflammatory reaction | 0 (0.0) | 2 (25.0) | 1 (12.5) | 7 (87.5) | 1 (12.5) |
| Fibrosis | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Necrosis | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group.

Table 3: The semiquantitative score values (score minimum-score maximum) of the main histological lesions found in the livers of experimental animals.

| Lesions | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| Hydropic degeneration | 1 (1-2) | 1 (1-2) | 1 (1-2) | 3 (1-3) | 1 (1-3) |
| Sinusoidal congestion/hyperemia | 0 | 0 (0-1) | 0 (0-1) | 2 (0-3)$^{abc}$ | 0 (0-1)$^{d}$ |
| Inflammatory reaction | 0 | 0 (0-1) | 0 (0-1) | 1 (0-3)$^{abc}$ | 0 (0-1)$^{d}$ |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. [a] represents a significant difference between CSG when compared to CG; [b] represents a significant difference between CSG when compared to VG; [c] represents a significant difference between CSG when compared to QG; [d] represents a significant difference between groups when compared to CSG. Sinusoidal congestion/hyperemia ($p = 0.0008$). Inflammation reaction ($p = 0.0003$). The data were analyzed by Kruskal-Wallis followed by Dunn's posttest ($n = 8$ animals per group).

compared to CSG ($p = 0.0008$) (Table 3). In the CSG, this lesion ranged from absent (25%), moderate (25%), to intense (50%) in severity (Table 3). While in the other groups, the sinusoidal congestion/hyperemia ranged only from absent (CG: 100%, VG: 87.5%, QG: 87.5%, QCSG: 87.5%) to mild intensity (VG: 12.5%, QG: 12.5%, QCSG: 12.5%) (Table 3).

The bleeding focus and granulomatous reactions were observed only in animals exposed to long-term cigarette smoke (CSG) (25% and 75%, respectively) (Figures 1(b), 1(c), and 1(e) and Table 2).

The inflammatory reaction was more frequent in the group exposed to CS (CSG) (87.5%) than VG (25%) and QG (12.5%) (Figures 1(b), 1(d), and 1(e) and Table 2). There was not any inflammatory reaction in the control group (CG: 0%); in these animals, only the resident inflammatory cells were observed (Figure 1(a), Table 2). The quercetin administration associated to cigarette smoke (QCSG) decreased the frequency of the inflammatory reaction to 12.5% of the animals ($p = 0.0003$) (Table 2), i.e., the inflammatory reaction reduced to values similar to those of the VG and QG. Moreover, the inflammatory reaction was more intense in CSG, presenting a mild range from (37.5%) to moderate (37.5%) to intense, while the other groups presented only mild intensity, when observed (VG: 25%, QG: 12.5%, QCSG: 12.5%) (Table 3).

Moreover, hepatic fibrosis and necrosis were not observed in the animals studied (Table 2).

The number of inflammatory cells to the liver was greater (ANOVA, $p < 0.0001$) in animals exposed to cigarette smoke (CSG) ($29.43 \pm 2.24$) compared to control groups (CG: $16.51 \pm 0.11$, VG: $18.39 \pm 0.41$, QG: $16.83 \pm 0.49$) ($p = 0.001$ ). Pretreatment with quercetin ($14.82 \pm 0.52$) resulted in a lower number of inflammatory cells in the liver when compared to CSG ($p = 0.001$) and presented values similar to those of the control group (Figure 2).

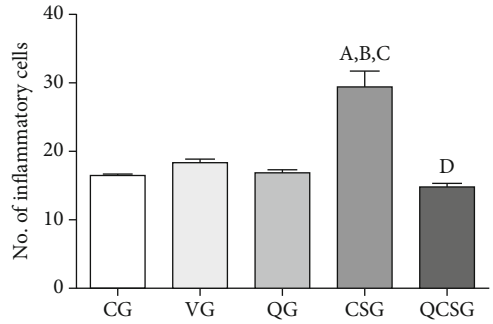


Figure 2: Number of inflammatory nucleus into the hepatic parenchyma of C57BL/6 mice. CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group. [a] represents a significant difference between groups when compared to CG. [b] represents a significant difference between groups when compared to VG. [c] represents a significant difference between groups when compared to QG. [d] represents a significant difference between groups when compared to CSG. Data were expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's posttest, $n = 8$ animals per group ($p < 0.05$).

*3.3. Antioxidant Defense and Oxidative Stress Biomarkers in the Liver.* Cigarette smoke exposure promoted an increase of the SOD activity (ANOVA, $p = 0.0009$) compared to CG, VG, and PG ($p < 0.05$), which was attenuated by pretreatment with quercetin ($p = 0.001$). The catalase activity was lower (ANOVA, $p = 0.0019$) in the CSG and QCSG groups

Table 4: Antioxidant defense and oxidative stress biomarkers in liver homogenate.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| SOD (U/mg protein) | 90.74 ± 4.72 | 88.01 ± 5.0 | 93.26 ± 5.42 | 115.4 ± 5.85[a,b,c] | 81.42 ± 5.36[d] |
| CAT (U/mg protein) | 0.28 ± 0.02 | 0.20 ± 0.03 | 0.22 ± 0.01 | 0.13 ± 0.03[a] | 0.14 ± 0.02[a] |
| GSH/GSSG ratio | 7.21 (2.59; 13.30) | 5.84 (4.11; 6.85) | 6.05 (5.24; 9.04) | 0.41 (0.34; 1.40)[c] | 6.61 (3.19; 10.64)[d] |
| TBARS (nmol/mg protein) | 0.34 ± 0.02 | 0.24 ± 0.02 | 0.31 ± 0.02 | 2.07 ± 0.50[a,b,c] | 0.27 ± 0.04[d] |
| Protein carbonyl (nmol/ng protein) | 12.73 ± 1.28 | 13.13 ± 1.90 | 16.07 ± 2.05 | 16.18 ± 0.93 | 14.12 ± 1.58 |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group, SOD: superoxide dismutase; CAT: catalase; GSH: glutathione sulfide; GSSG: oxidized glutathione; TBARS: thiobarbituric acid reactive substances. [a] represents a significant difference between groups when compared to CG; [b] represents a significant difference between groups when compared to VG; [c] represents a significant difference between groups when compared to QG; [d] represents a significant difference between groups when compared to CSG. SOD, CAT, TBARS, and protein carbonyl were expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's posttest, $n = 5 - 8$ animals per group ($p < 0.05$). The GSH/GSSG ratio data were expressed in median, minimum, and maximum value and were analyzed by Kruskal-Wallis followed by Dunn's posttest that was used, $n = 5 - 8$ animals per group ($p < 0.05$).

Table 5: Inflammatory markers in the liver of experimental groups.

| | CG | VG | QG | CSG | QCSG |
|---|---|---|---|---|---|
| CCL2 (pg/mL) | 4025.3 ± 137.6 | 3279.4 ± 250.9 | 3153.2 ± 134.4 | 1852.4 ± 303.7[a,b,c] | 3693.6 ± 175.9[d] |
| INF-$\gamma$ (pg/mL) | 537.56 ± 11.85 | 513.25 ± 42.14 | 559.05 ± 42.42 | 324.62 ± 21.59[a,b,c] | 519.28 ± 24.11[d] |
| IL-10 (pg/mL) | 1466.3 ± 99.79 | 1529.2 ± 149.5 | 1471.6 ± 43.6 | 2342.3 ± 218.1[a,b,c] | 1698.6 ± 57.5[d] |
| IL-6 (pg/mL) | 1711.2 ± 57.7 | 1712.0 ± 49.6 | 1688.1 ± 48.1 | 1780.6 ± 57.3 | 1668.7 ± 56.1 |

CG: control group; VG: vehicle group; QG: quercetin group; CSG: cigarette smoke group; QCSG: quercetin and cigarette smoke group, CCL2 or MCP1: monocyte chemotactic protein 1; INF-$\gamma$: interferon-$\gamma$; IL-10: interleukin 10; IL-6: interleukin 6. [a] represents a significant difference between groups when compared to CG; [b] represents a significant difference between groups when compared to VG; [c] represents a significant difference between groups when compared to QG; [d] represents a significant difference between groups when compared to CSG. Data were expressed as mean ± standard error of the mean and were analyzed by one-way ANOVA followed by Tukey's posttest, $n = 8$ animals per group ($p < 0.05$).

compared to the control ($p = 0.01$). The GSH/GSSG ratio was lower (Kruskal Wallis, $p = 0.0158$) in the CSG than QC ($p < 0.05$), and the administration of quercetin increased the GSH/GSSG ratio compared to CSG ($p < 0.05$). The damage to liver parenchyma was evaluated based on levels of TBARS (ANOVA, $p < 0.0001$); as a result, the lipid oxidation was higher in the group exposed to cigarette smoke compared to CG, VG, and QG ($p = 0.001$), and the lipid oxidation was also attenuated by pretreatment with quercetin ($p = 0.001$) (Table 4).

*3.4. Inflammatory Mediators' Levels in the Liver.* Inflammatory markers IL-6, IL-10, INF-$\gamma$, and CCL2 were measured in order to assess the inflammatory status of the liver. The animals exposed to cigarette smoke for sixty days showed higher levels of INF-$\gamma$ (ANOVA, $p = 0.0002$) and IL-10 (ANOVA, $p = 0.0005$) in liver homogenate when compared with CG, VG, and QG ($p = 0.01$). For both markers, pretreatment with quercetin led to a decrease in cytokine levels compared to CSG ($p < 0.05$). The levels of CCL2 chemokine (ANOVA, $p < 0.0001$) in the liver of the group exposed to smoke were lower than the control group ($p = 0.001$), and the administration of quercetin promoted an increase in the levels of this marker compared to CSG ($p = 0.001$). For the analysis of IL-6, no statistical difference was observed between the experimental groups (ANOVA, $p = 0.65$) (Table 5).

## 4. Discussion

In this study, we investigated the antioxidant effect of quercetin against the oxidative stress, inflammation, and changes in liver morphology induced by cigarette smoke. We observed morphological changes and redox imbalance in the livers of mice exposed to long term-cigarette smoke, strengthening the deleterious effect of cigarette smoke on the liver parenchyma, corroborating previous studies [29–31]. A study conducted by Ogenyi et al. revealed an adverse effect of passive cigarette smoke on the liver morphology and biochemistry of Wistar rats [31]. Moreover, a study conducted by Adedayo et al. reported that the smoke extract of tobacco nicotine has adverse and compromising effects on the liver of male Sprague-Dawley rats [30].

Liver diseases represent an important focus of attention in view of the multiple functions of the liver in the body. Use of herbal drugs in the treatment of liver diseases has a long tradition [17]. In this context, quercetin, the most abundant flavonoid in nature, has been found to exhibit a potent antioxidant capacity and the ability to counteract several insults to the liver. Kalantari et al. demonstrated that quercetin is effective for the prevention of tert-butyl hydroperoxide-induced hepatic damage in mice [32]. Similarly, Lee et al. reported that quercetin prevents liver inflammatory injury, induced in rats by ethanol extracts from tartary buckwheat [33]. However, to our knowledge, this is the first study that

examines the antioxidant effect of quercetin against deleterious changes in liver morphology and in the oxidative stress induced by cigarette smoke.

We observed that there was a higher level of ALT in the animals exposed to long-term cigarette smoke when compared with the control group. ALT is one of the most used biomarkers for measuring liver injury, and several studies with cigarette smoke use this biomarker to estimate the harmful effects of smoke on the liver tissue [9, 34, 35]. Studies describe the role of quercetin in reducing liver damage, in face of an injury-causing agent, by determining ALT levels [36–38]. However, despite the findings of the current study showing that quercetin combats ROS, inflammation, and helps with antioxidant defense, there was no statistical difference between the ALT levels between CSG and QCSG.

Previous studies have demonstrated a reduction in the body weight in animals exposed to cigarette smoke [1, 18, 39]. A study by our research group correlated this reduction in the body weight with inflammation and subsequent secretion of TNF-$\alpha$, in addition to food satiety in animals exposed to cigarette smoke [18]. Here, our results corroborate with these studies, where a decrease in the body weight was observed in the animals exposed to CS, perhaps as a consequence of the inflammatory process in the liver tissue. Pretreatment with quercetin was not able to reverse this parameter. Our results are in agreement with other studies that did not observe changes in the body weight with low concentration of quercetin [40–42].

We observed that the quercetin administration was able to improve the effects of long-term cigarette smoke exposure, with injury decreases resulting from this exposure, such as inflammatory reaction, sinusoidal congestion, hyperemia, granulomatous reaction, and bleeding focus. Moreover, we observed a significant increase in the number of inflammatory cells into the liver in response to cigarette smoke exposure that may be directly related to the time of exposure to cigarette smoke, which was long. Several studies showed a relationship between cigarette smoke and a greater influx of total leukocytes, either by the formation of reactive oxygen species or by the presence of nicotine [43–45]. The quercetin reduced the number of inflammatory cells to the liver in animals exposed to long-term cigarette smoke, supporting the cytoprotective and anti-inflammatory effects of quercetin [46]. A similar result showed by Barros et al. found evidence of the hepatoprotective quercetin activity in acute paracetamol toxicity, as shown by a reduction in necrosis and inflammation [47].

Previous data showed that the cigarette smoke exposure promoted changes in the antioxidant enzyme activity and oxidative damage in the liver of mice and rats [48, 49]. We evaluated the redox imbalance by measuring oxidative damage, and our results showed that the cigarette smoke significantly increased the activity of the SOD, and the quercetin administration restored their activity to control levels. These results prove the ability of quercetin to combat and neutralize reactive oxygen species (ROS), preventing oxidative stress according to Dias et al. [50]. These authors found that quercetin, by scavenging ROS, prevents the elevation of the SOD activity in the liver of streptozotocin-induced diabetic rats. The increase in the SOD activity in the liver tissue caused by cigarette smoke has also been described [51, 52] in rats.

GSH is a key player in the maintenance of the cellular redox status and exists primarily in two redox forms, reduced (GSH) and oxidized (GSSG) [53]. The GSH/GSSG ratio can serve as a good indicator of the cellular redox state [54]. Our results indicated that CS reduced the GSH/GSSG ratio, while quercetin, in turn, was able to restore the GSH/GSSG ratio. This result possibly indicates a greater activity of GPx in the group exposed to cigarette smoke and a lower activity of this enzyme in group pretreated with quercetin and exposed to cigarette smoke. Therefore, we believe that quercetin will neutralize ROS instead of GPx, thus maintaining the redox status of the cells. Our data corroborate with the results from a previous study by Olayinka et al. and Chen, 2010, which reported protective effects of quercetin, regarding the ability of quercetin to increase the GSH/GSSG ratio in liver injury induced by procarbazine and ethanol, respectively [55, 56]. Therefore, an increase in the antioxidant system and consequent reduction of ROS induced by the quercetin administration observed in the present study may be a main factor for protection of the liver against injury caused by CS exposure.

In our experimental model, the CAT activity decreased in CSG compared to that in control groups, and the quercetin administration was unable to regulate the activity of this enzyme. Similarly, a series of studies in the lung and liver reported a decrease of the catalase activity in models that promote an increase in ROS [18, 57–59]. A depletion of catalase reserves promoted by a persistent increase in production of reactive oxygen species may have occurred. In the other hand, the liver has one of the highest organ contents of GSH [60, 61]. The glutathione system is expected to be more active in neutralizing ROS production after exposure to cigarette smoke when compared to other components of the antioxidant system, such as the CAT activity. Despite the decreased CAT activity, quercetin showed a strong antioxidant activity by maintaining oxidative balance. According to Xu et al., the antioxidant activity of quercetin is mainly manifested through its effect on glutathione [62].

In this study, the long-term cigarette smoke significantly increased the TBARS levels that were restored by the quercetin administration. These results are in accordance with previous studies that evaluated the effect of cigarette smoke on the liver [34, 52, 63]. Some studies in liver damage have also demonstrated that quercetin modulates lipid and lipoprotein metabolism damage caused by oxidative stress inducing agents other than cigarette smoke [32]. Our results demonstrate that quercetin is effective in decreasing lipid peroxidation and in preventing oxidative damage in the liver parenchyma of mice exposed to long-term cigarette smoke.

We observed a decrease of CCL2 and IFN-$\gamma$, as well as an increase of IL-10 in the C57BL/6 mice exposed to long-term CS. Regarding the levels of IL-6 in the hepatic homogenate, there were no changes observed between the experimental groups. As the primary injury of cigarette smoke occurs in the lung, it is expected that these inflammatory markers are more active in this organ, compared to other organs such as the liver [18]. The IL-10 increase in the liver tissue of mice

exposed to CS may be a response to the influx of inflammatory cells to that organ, and the concomitant decrease in proinflammatory mediators, such as CCL2 and INF-$\gamma$, may be a consequence of the IL-10 which regulates the proinflammatory activity of these mediators, and it has been described in immunologic studies in several organs [44, 64, 65]. Studies describe the regulation of IL-10 over other cytokines and chemokines, and, depending on the inflammatory profile of the region with injury caused by oxidant agents, it exerts a modulation on INF-$\gamma$ and CCL2 [66, 67].

Our data demonstrated that the pretreatment with quercetin restored the levels of inflammatory markers to the levels of the control group and are in agreement with those of Chen, who demonstrated the role of quercetin in restoring cytokine and chemokine levels in the liver tissue exposed to ethanol [56]. Other works have also described in several organs, the role of quercetin in modulating inflammatory response through the induction or repression of pro or anti-inflammatory cytokines [13, 18, 33, 46, 68]. However, this is the first study that examines the antioxidative and anti-inflammatory properties of quercetin against deleterious changes in liver morphology and in the oxidative damage induced by cigarette smoke.

In summary, these results suggest that quercetin has a promising role as an auxiliary pharmacological tool in the treatment of damage to the liver due to the exposure to cigarette smoke.

## Data Availability

I declare that data related to the manuscript entitled "Protective effects of quercetin on livers from mice exposed to long-term cigarette smoke" are available for publicy.

## Conflicts of Interest

The author(s) declared no potential conflicts of interest.

## Authors' Contributions

Pedro A. Machado-Junior and Natália P. S. Araújo contributed equally to this work.

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## 4. CONSIDERAÇÕES FINAIS

Baseado nos resultados apresentados neste estudo podemos concluir que:

A quercetina exerceu efeito antioxidante através da diminuição da produção intracelular de espécies reativas, além de aumentar a atividade de enzimas antioxidantes e prevenir danos oxidativos a nível tecidual.

A quercetina apresentou efeito anti-inflamatório através da redução do influxo celular e dos níveis de citocinas e quimiocinas.

A quercetina diminuiu alterações do padrão histológico do parênquima pulmonar e hepático.

A quercetina reduziu alterações da função pulmonar.

Em resumo, esses resultados sugerem que a quercetina pode ser um adjuvante eficaz no tratamento de doenças decorrentes da exposição à fumaça do cigarro.

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## 6. APÊNDICES

6.1.Artigos publicados durante o doutoramento

*Article*

Toxicology and Industrial Health

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# Extrapulmonary effects of temporal exposure to cigarette smoke

**Thais Lourenço Martins, Keila Karine Duarte Campos, Natália Pereira da Silva Araújo, Dafne Fernandes Machado and Frank Silva Bezerra**

**Abstract**

This study aimed to evaluate the extrapulmonary effects of exposure to cigarette smoke (CS) through the analysis of blood components and histopathological examinations of the trachea and diaphragm muscle (DM) in C57BL/6 mice. Thirty-six animals were exposed to six cigarettes per day for 5 days. The mice were divided into a control group (CG) and groups exposed to CS for 1 (CS1D), 2 (CS2D), 3 (CS3D), 4 (CS4D), and 5 (CS5D) days. The trachea, DM, and blood were collected for morphometric and biochemical analyses. In comparison with the CG, CS4D and CS5D mice showed an increased influx of inflammatory cells into the DM and trachea. Increased glycogen deposits in the tracheal tissue of CS3D mice were observed, compared with that in CG, CS1D, and CS2D mice. In the blood serum, the number of inflammatory cells and the concentration of cholesterol increased in CS1D mice, compared with the CG. Alanine aminotransferase (ALT) levels were elevated in CS5D mice, compared with those in CS3D and CS4D mice. Aspartate aminotransferase (AST) levels were elevated in CS3D and CS5D mice, compared with those in the CG. Urea levels were significantly increased in CS5D mice, compared with CS1D mice. Our results showed extrapulmonary effects of short-term exposure to CS in adult mice.

**Keywords**

Cigarette smoke, diaphragm, trachea, inflammatory cells, mice

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## Introduction

Tobacco use is the main isolated cause of illness and death in the world, including lung cancer, pulmonary emphysema, chronic bronchitis, and heart disease (Dalrymple et al., 2015). Tobacco currently kills about 6 million people each year and this number could increase to more than 10 million by the year 2030 (Petyaev, 2016).

Cigarette smoke (CS) contains approximately 6000 chemical components (Dalrymple et al., 2015). Direct and indirect exposure to CS leads to an excessive production of proteases that activate an inflammatory cascade, culminating in a systemic response to tissue damage, inflammation induction, and redox imbalance (Cantin and Richter, 2012). Exposure to CS leads to an increase in the number of macrophages, neutrophils, and lymphocytes in the lung. Macrophages secrete pro-inflammatory substances such as cytokines and metalloproteinases that induce local inflammation (Barnes et al., 2003; Bosken et al., 1992), leading to an increase in the number of leukocytes in the blood, particularly neutrophils. The main consequences of CS inhalation are related to damage the respiratory tract, which is the primary risk factor for the development of chronic obstructive pulmonary

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# The administration of surfactant decreased oxidative stress in lungs of mice exposed to cigarette smoke

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ARTICLE INFO

*Keywords:*
Cigarette smoke
Exogeneous surfactant
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Lung inflammation
Anti-oxidant

ABSTRACT

The alveolar surfactant, which composition consists of a unique and complex mixture of lipids and proteins, has immunomodulatory action. This study aimed to evaluate the effects of exogenous surfactant on pulmonary inflammatory response in mice exposed to cigarette smoke (CS). Twenty-four mice C57BL/6 were divided into four groups: control group exposed to ambient air (CG); surfactant treated group (SG); CS exposed group (CSG) and CS exposed group treated with surfactant (CSSG). For five days, CSG and CSSG were exposed to 12 commercial cigarettes/day and SG and CSSG received the surfactant by intranasal instillation. At the end of the experiment, the animals were euthanatized for the collection of bronchoalveolar lavage fluid (BALF) and lungs. The total number of leukocytes in BALF increased in CSG compared to CG, however, there was a decrease in CSSG compared to CSG. There was an increase in lipid peroxidation in SG and CSG compared to CG while there was a decrease in CSSG compared to CSG. Regarding the antioxidant enzymes, the catalase (CAT) activity increased in all groups compared to CG and the superoxide dismutase (SOD) activity decreased in CSG compared to the CG and SG. There was an increase in TNF in SG, CSG and CSSG compared to CG. There was an increase in IL-17 in CSSG compared to CG. There was an increase in CCL5 in SG and CSSG compared to CG. Therefore, our results demonstrated that the administration of exogenous surfactant was able to decrease the oxidative processes in the lungs of mice induced by short-term exposure to CS.

## 1. Introduction

Smoking is the most common and avoidable risk factor associated with the development of chronic obstructive pulmonary disease (COPD) [1]. COPD affects > 200 million people worldwide and is expected to become the third leading cause of death globally by 2020 [2–4]. Cigarette smoke (CS) is the major etiological factor in the pathogenesis of COPD and contains > $10^{15–17}$ oxidants/free radical molecules per puff, increasing oxidative burden in the lungs in smokers [5]. CS is highly toxic containing about six thousand chemical components that in contact with the body are able to trigger processes related to redox imbalance and inflammation, which can lead to damage to proteins, cell membranes and DNA [6–9].

The surface of the epithelium of the airways and airspaces is the first line of defense of the lungs against toxic inhalants. The events that occur in these compartments of the respiratory tract are relevant for the pathogenesis of respiratory diseases caused by smoking, in particular, COPD [10]. Persistent airway inflammation in patients with COPD is characterized by high numbers of neutrophils as well as the presence of activated macrophages and T lymphocytes, particularly $CD8^+$ [11].

The redox imbalance has been pointed out as one of the main factors related to the pathogenesis and progression of COPD [12], which is characterized by chronic inflammation of the airways, airflow limitation and pulmonary emphysema [13]. As the components of CS are able to activate leucocytes and produce reactive oxygen species [14], it is necessary to study substances such as pulmonary surfactant that have properties which decrease redox imbalance.

The redox imbalance is established when there is a transient or persistent increase in normal levels of ROS that is capable of causing a disorder in the cell nucleus and signaling pathways, leading to the oxidative modification of cellular constituents, which, if not counterbalanced, may culminate in cell death through necrosis or apoptosis

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Intensive Care Medicine Experimental

RESEARCH Open Access

# Exogenous surfactant prevents hyperoxia-induced lung injury in adult mice

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## Abstract

**Background:** In addition to the risk of developing ventilator-induced lung injury, patients with ARDS are at risk of developing hyperoxic injury due the supra-physiological oxygen supplementation clinically required to reverse hypoxemia. Alterations of endogenous surfactant system participate in the pulmonary dysfunction observed in ARDS. Administration of exogenous surfactant could have protective effects during hyperoxia.

**Methods:** Male BALB/c mice (8–10 weeks), a strain highly sensitive to hyperoxia, received the exogenous surfactant-containing protein SP-B and SP-C by intranasal instillation 12 h before starting 24 h of exposure to hyperoxia in an inhalation chamber and were compared to mice receiving hyperoxia alone and to controls subjected to normoxia.

**Results:** Compared to the hyperoxia group, the administration of exogenous surfactant was able to reduce lung inflammation through a reduction in the influx of neutrophils and inflammatory biomarkers such as TNF, IL-17, and HMGB1 expression. The antioxidant activity prevented oxidative damage by reducing lipid peroxidation and protein carbonylation and increasing superoxide dismutase activity when compared to the hyperoxia group.

**Conclusion:** Our results offer new perspectives on the effects and the mechanism of exogenous surfactant in protecting the airway and lungs, in oxygen-rich lung microenvironment, against oxidative damage and aggravation of acute inflammation induced by hyperoxia.

**Keywords:** ARDS, BALB/c mice, Exogenous surfactant, Hyperoxia, Lung injury, Oxidative stress

## Background

Studies have shown that alterations of the endogenous surfactant system contribute to pulmonary dysfunction and atelectasis in ARDS [1]. These alterations include decrease of pool size and functional modifications of endogenous surfactant. Mechanical ventilation by itself can lead to the oxidation of surfactant components [1]. A meta-analysis of studies in adult patients with ARDS showed that exogenous surfactant (ES) administration may improve oxygenation in the first 24 h but does not mitigate mortality and long-term oxygenation (> 120 h) [2]. Important concerns have been raised, however, regarding the practical administration of surfactant and its ability to reach the alveoli [3].

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## 7. ANEXOS

7.1.Anexo 1 – Protocolo de aprovação CEUA/UFOP

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**OFÍCIO CEUA Nº. 094/2015, de 16 de novembro de 2015**

**Ilustríssimo(a) Senhor(a)**
**Professor(a) Doutor(a) Frank Silva Bezerra**
**DECBI/UFOP**

Senhor(a) Pesquisador(a),

Estamos enviando o certificado de aprovação do protocolo nº. 2015/20, "AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DA QUERCETINA SOBRE O DESEQUILÍBRIO REDOX E INFLAMAÇÃO EM CAMUNDONGOS C57BL/6 EXPOSTOS À FUMAÇA DE CIGARRO".

2. Atendendo ao art. 6º, IX, da Resolução Normativa CONCEA nº 1, de 09.07.2010, solicitamos que encaminhe a CEUA/UFOP o relatório final do projeto, após executá-lo, conforme modelo do *site* da CEUA.

Atenciosamente,

**Prof.ª Dr.ª Lenice Kappes Becker**
**Coordenadora da CEUA/UFOP.**