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# Efeitos da exposição subcrônica de *Residual Oil Fly Ash* (ROFA) associada à administração de resveratrol

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

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## DEDICATÓRIA

Às pessoas que me incentivam:
Pai, Mãe, Márcia e Mariane

## RESUMO

*Residual oil fly ash* (ROFA) é um poluente do ar comum em áreas em que há queima de óleo. Assim como o material particulado (MP), o ROFA apresenta partículas de diferentes diâmetros que podem ser inaladas pelos seres humanos e causar dano principalmente ao sistema respiratório. O resveratrol (RSV), um polifenol natural, tem recebido cada vez mais atenção devido sua ampla bioatividade, incluindo inibição de tumorgênese, modificação de lipídios e restrição-calórica. O objetivo deste estudo foi investigar a exposição subcrônica ao ROFA e os efeitos da ingestão de RSV em pulmão de ratos. Para isso, trinta e três ratos Wistar machos foram distribuídos nos seguintes grupos: controle (n = 9, CTL), resveratrol (n = 8, RSV), *residual oil fly ash* (n = 8, ROFA) e ROFA tratados com RVS (n = 8, ROFA + RSV). Os ratos foram expostos ao ROFA por instilação intranasal e foram tratados com RVS (20mg/kg/dia) por gavagem durante 14 semanas. Após vinte e quatro horas, os pulmões foram coletados para a determinação de marcador de dano oxidativo (substâncias reativas ao ácido tiobarbitúrico – TBARS), estado antioxidante (atividade da superóxido dismutase – SOD e da catalase – CAT), dano ao DNA, concentração de metais e de interleucinas. Os resultados demonstram que não houve diferença estatisticamente significativa no TBARS e na atividade de SOD e CAT, na concentração de metais e de interleucinas entre os grupos. O grupo ROFA apresentou maior dano ao DNA quando comparado aos demais grupos. Em conclusão, o RSV exerceu efeito protetor evitando o dano ao DNA após a exposição subcrônica ao ROFA.



**Palavras-chave:** poluição do ar, antioxidantes, dano ao DNA, estresse oxidativo, inflamação, material particulado.

## ABSTRACT

Residual oil fly ash (ROFA) is a common pollutant in areas where there is oil burning. As the particulate matter (PM), the ROFA contains particles from various diameters that can be inhaled by humans and cause damage mainly to the respiratory system. Resveratrol (RSV), a natural polyphenol, has received increasing attention due its varied bioactivities, including the inhibition of tumorigenesis, lipid modification and calorie-restriction. The aim of this study was to investigate the subchronic exposure to ROFA and the effects of RSV intake on rat lungs. For this, thirty-three male Wistar rats were distributed into the following groups: control (n = 9, CTL), resveratrol (n = 8, RSV), residual oil fly ash (n = 8, ROFA) and ROFA plus treatment with RVS (n = 8, ROFA + RSV). The rats were exposed to ROFA by intranasal instillation and were treated with RVS (20mg/kg/day) by gavage for 14 weeks. After twenty four hours, lung tissues were collected for determination of oxidative damage marker (thiobarbituric acid reactive substances - TBARS), antioxidant status (superoxide dismutase – SOD and catalase - CAT activity), DNA damage, cytokines and metals levels. The results show no statistically significant difference in TBARS, SOD and CAT activity, cytokines and metals levels among groups. The ROFA group showed higher DNA damage when compared to the other groups. In conclusion, the present study demonstrated that RSV avoided DNA damage after subchronic ROFA exposure.



**Keywords**: resveratrol, air pollution, DNA damage, oxidative stress, inflammation, ROFA.

**Sumário**

## Lista de Abreviaturas

AMPK - 5’ adenosina monofosfato quinase ativada

CAT- catalase

DEP – partículas de exaustão do diesel

DNA – d*esoxirribonucleic acid* / ácido desoxirribonucleico

ERN - espécies reativas de nitrogênio

ERO - espécies reativas de oxigênio

ESCAPE - *European Study of Cohorts for Air Pollution Effects* / Estudo Europeu de Coorte dos Efeitos da Poluição Atmosférica

$H_2O_2$ – peróxido de hidrogênio

HPAs – hidrocarbonetos policíclicos aromáticos

IL-6 – interleucina 6

IL-8 – interleucina 8

IARC – *International Agency for Research on Cancer* / Agência Internacional de Pesquisa em Câncer

MP – material particulado

MP10 – material particulado com diâmetro inferior a 10 µm

MP2,5 – material particulado com diâmetro inferior a 2,5 µm

NAD+ - nicotinamida adenina nucleotídeo

NF-κB – *factor nuclear kappa B* / fator nuclear kappa B

NO – óxido nítrico

$O\bullet^-_2$ - radical superóxido

•OH - radical hidroxil

$ONOO^-$ - peroxinitrito

PUF – partícula ultrafina

ROFA - *residual oil fly ash*

RSV - resveratrol

SIRT 1 – sirtuina 1

SOD - superóxido dismutase

TNF-α – fator de necrose tumoral

# 1 INTRODUÇÃO

## 1.1 Poluição Atmosférica

Poluição atmosférica é um problema de saúde pública que abrange todo o mundo. Estudos epidemiológicos têm demonstrado associação entre a exposição à poluição atmosférica e o aumento de morbidade e mortalidade devido às doenças cardiovasculares e pulmonares, incluindo câncer de pulmão, doença pulmonar obstrutiva crônica e infarto do miocárdio, principalmente em indivíduos suscetíveis (1-5).

Os efeitos da poluição sobre a saúde continuam sendo objeto de interesse da comunidade científica e regulatória, além da própria sociedade. Recentemente, a Agência Internacional de Pesquisa em Câncer (IARC - *International Agency for Research on Cancer*) classificou a poluição atmosférica e o material particulado (MP), proveniente da poluição, como cancerígenos para seres humanos (grupo 1) (6).

Apesar de as últimas décadas serem marcadas por avanços tecnológicos importantes que contribuíram para a redução na concentração de emissões de poluentes, este avanço não ocorreu de forma uniforme. Infelizmente, os países em desenvolvimento ainda são os que emitem as maiores concentrações de poluentes à atmosfera (7).

Há diferentes fontes de poluição que variam de região para região e, consequentemente, emitem diferentes poluentes. Nos centros urbanos a que prevalece é aquela advinda principalmente dos veículos automotores: da queima de combustíveis fósseis e da biomassa. Assim, estudos têm sido desenvolvidos com o intuito de investigar os mecanismos de ação tóxica dos diferentes compostos presentes na poluição como diesel, biodiesel, MP, hidrocarbonetos policíclicos aromáticos (HPAs), entre outros e seus potenciais efeitos adversos à saúde do ser humano.

Diante da complexidade de seus constituintes e mecanismos de ação tóxica que a poluição atmosférica engloba, ainda existem *gaps* na literatura que precisam

ser elucidados referentes à maneira pela qual a poluição atmosférica contribui para os problemas de saúde ambiental e humana.

### 1.1.1 Definição e dados epidemiológicos

A poluição atmosférica é definida como sendo uma mistura física e quimicamente diversificada de partículas e gases provenientes de várias fontes como a queima de combustíveis fósseis, fontes naturais e também pela transformação atmosférica secundária (8). Ela é constituída por materiais carbonáceos (orgânico e elementar), sulfatos, nitratos, carbonatos, metais, peróxidos, quartzo, silicatos (argilas e amianto), óxidos minerais, bactérias, vírus, pólen, detritos animal e vegetal, e MP (9). Além disso, pode apresentar uma variedade de substâncias orgânicas como HPAs, e nitro-HPAs, aldeídos, cetonas, nitro compostos, e quinonas (10).

Estudos epidemiológicos demonstram prejuízos à saúde causados pela exposição à poluição do ar em diversos países. Pesquisadores de Harvard observaram, com base em um estudo em seis cidades dos Estados Unidos, que a mortalidade para todas as causas aumenta 14% a cada aumento de 10 $\mu g/m^3$ na concentração de $MP_{2.5.}$ Além disso, os autores evidenciaram um aumento de 26% em mortes decorrentes de problemas cardiovasculares e de 37% devido a câncer pulmonar para o mesmo aumento de $MP_{2.5}$. Contrariamente, a diminuição de 2,5 $\mu g/m^3$ de $MP_{2.5}$ com duração mínima de um ano foi associada a uma diminuição de 3,5% na mortalidade (11).

Na Europa, o projeto Estudo Europeu de Coorte dos Efeitos da Poluição Atmosférica (ESCAPE - *European Study of Cohorts for Air Pollution Effects)* tem correlacionado a exposição a longo prazo à poluição e efeitos adversos à saúde. De acordo com Raaschou-Nielsen *et al.* (2013), a exposição ao MP está associada ao risco de câncer de pulmão (4). Além disso, o mesmo grupo de pesquisadores relatou que a exposição de longo prazo às partículas finas está associada a causas naturais de mortes mesmo quando os valores de poluição se encontram abaixo do limite ambiental permitido na Europa (12).

No Brasil, os níveis ambientais elevados de poluição atmosférica têm sido associados ao aumento de visitas a emergências por causas respiratórias, a internações, e até morte em idosos e crianças (13-16).

Dentre os constituintes da poluição atmosférica, o MP tem sido largamente investigado e é caracterizado como uma complexa mistura de componentes orgânicos e inorgânicos dentre os quais destacam-se hidrocarbonetos, metais, sais e ainda microorganismos (17). De acordo principalmente com a fonte emissora e as condições climáticas, o MP pode variar de composição, tamanho e concentração (18). As partículas são classificadas de acordo com seu tamanho aerodinâmico como grossas (2,5–10 μm; $PM_{10}$), finas (0,1–2,5 μm; $PM_{2.5}$), e ultrafinas (<100 nm; PUF) (19, 20).

O tamanho da partícula é muito importante em termos de saúde, porque se relaciona aos efeitos biológicos no organismo – partículas com maior tamanho aerodinâmico depositam-se quase que exclusivamente no trato respiratório superior enquanto que partículas finas e ultrafinas podem atingir os alvéolos e, consequentemente, adentrar na circulação sistêmica (21, 22). As PUF são potencialmente mais perigosas devido ao seu pequeno tamanho e grande área de superfície, a qual determina o número de grupos reativos na superfície da partícula (20).

A poluição atmosférica tem sido correlacionada aos efeitos adversos à saúde. Por sua vez, esses efeitos não se restringem ao sistema respiratório, apesar deste ser o primeiro a entrar em contato. Estudos experimentais relatam que a exposição às partículas presentes na atmosfera pode causar danos sistêmicos: cardiovasculares (2,3), no sistema nervoso (23, 24) e reprodutivo (25).

Com ênfase ao sistema respiratório, estudos epidemiológicos demonstraram uma forte associação entre MP e desenvolvimento de doenças pulmonares como asma e doença pulmonar obstrutiva crônica (26). Além disso, o aumento do risco de câncer de pulmão foi observado até mesmo em áreas onde a concentração de $MP_{2.5}$ está abaixo da recomendada pelos padrões internacionais mais rígidos (12).

### 1.1.2 Mecanismos fisiopatológicos

Nas últimas décadas, os mecanismos fisiopatológicos pelos quais a poluição causa efeitos adversos à saúde humana começaram a ser elucidados. Tanto estudos *in vitro* quanto *in vivo* têm relatado efeitos deletérios causados pelos diferentes compostos da poluição, e entre os mecanismos envolvidos na injúria celular destacam-se o estresse oxidativo, a inflamação e o dano ao ácido desexirribonucleico (DNA).

As partículas finas, como mencionado, possuem a capacidade de penetrar nas vias respiratórias e de se depositar principalmente nos bronquíolos e alvéolos. Devido à diversidade de componentes presentes nessas partículas como metais de transição, HPAs e compostos orgânicos voláteis entre outros, há a formação de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN). Entre as ERO e ERN destacam-se o peróxido de hidrogênio ($H_2O_2$), o radical superóxido ($O_2^{\cdot -}$), o oxigênio singlet ($^1O_2$), o radical hidroxil ($^{\cdot}OH$), óxido nítrico (NO), peroxinitrito ($ONOO^-$), os quais são capazes de interagir com lipídios, proteínas e DNA e, consequentemente, causar dano e morte celular (27-30).

Os estudos de exposição aguda aos diferentes constituintes de poluição atmosférica têm relacionado principalmente o estresse oxidativo e a inflamação das vias aéreas como uma primeira resposta à exposição. O estresse oxidativo é caracterizado por um desequilíbrio entre substâncias antioxidantes e pró-oxidantes, onde há um deslocamento para o estado pró-oxidante. De maneira geral, as ERO e ERN são produtos naturais do metabolismo e são importantes para determinados eventos celulares como, por exemplo, transdução de sinal, ativação enzimática, expressão gênica, entre outros (31, 32). As células possuem um sistema enzimático de defesa antioxidante endógeno capaz de protegê-las contras as ERO, sendo que as principais são a superóxido dismutase (SOD), a catalase, (CAT) e o sistema redox da glutationa (glutationa peroxidase e glutationa S-tranferase). Em condições fisiológicas, essas enzimas são importantes na

proteção do dano oxidativo, como lipoperoxidação, oxidação de proteínas e dano ao DNA, pois são capazes de interceptar, neutralizar e reagir com intermediários gerados em excesso pelas ERO e ERN (33-35). Entretanto, pode haver uma exacerbação de ERO devido a um estímulo não fisiológico, como a exposição a poluentes, culminando no estresse oxidativo.

O processo de inflamação, após a exposição a xenobióticos, resulta em uma complexa sequência de eventos que objetivam remover a fonte de inflamação e resolver a reação inflamatória. Esse processo é regulado por mediadores que são secretados pelos tecidos e células inflamatórias e variam de acordo com o tipo e as propriedades da partícula, como solubilidade e superfície reativa (36). Estudos experimentais revelaram que após a exposição aos diferentes constituintes da poluição atmosférica há aumento dos biomarcadores de inflamação, tais como a ativação do fator nuclear kappa B (NF-κB), liberação de citocinas, além do aumento da produção de óxido nítrico (NO).

Em um trabalho de revisão, Moller *et al*. (2015), observaram que há diferenças na resposta inflamatória causada pela exposição dependendo do local do estudo (37). Os autores explicam que isso está associado à variação – de tempo, espaço e condições climáticas - dos constituintes presentes na atmosfera no período de realização dos estudos além da diversidade dos modelos de exposição utilizados que podem variar quanto ao tempo de exposição e tipo de poluente.

Os resultados de estudos *in vitro* e *in vivo* são controversos quando relacionam o tamanho da partícula ao potencial efeito inflamatório. A relação de que quanto maior a partícula maior será a resposta inflamatória é evidenciada em alguns estudos, contrária em outros, e outros ainda afirmam que há uma resposta inflamatória similar entre os diversos tamanhos de partículas (38-41).

Além do tamanho e composição, deve-se ter em consideração a solubilidade dos compostos presentes na partícula, pois pode ser um fator importante em relação aos efeitos inflamatórios. A fração solúvel em água de $MP_{10}$, de partículas ambientais e *residual oil fly ash* (ROFA) gera níveis mais elevados de secreção de citocinas do que a fração insolúvel (42).

As diferenças de resposta inflamatória podem ser explicadas pelo estresse oxidativo gerado pelos metais de transição presentes nas partículas. Estudos que utilizaram tratamentos com quelantes de metais de transição apresentaram diminuição da secreção de citocinas – interleucina 6 (IL-6), interleucina 8, (IL-8) e fator de necrose tumoral (TNF-α) (43, 44).

A instilação aguda de partículas provenientes da queima do diesel causa inflamação pulmonar, que pode ser caracterizada pelo influxo de células inflamatórias, aumento de proteínas totais, e estresse oxidativo (45-47). Já partículas de $MP_{10}$ podem induzir uma resposta inflamatória sistêmica caracterizada pelo aumento de mediadores pró-inflamatórios sistêmicos como IL-6 (48).

Além do estresse oxidativo e da inflamação, se tornou evidente a relação entre exposição à poluição atmosférica e danos ao DNA. O potencial carcinogênico das partículas pode estar vinculado a duas principais vias genotóxicas: a via primária e a via secundária (36, 49, 50). A via secundária pode ser proveniente de um dano genético resultante de excessiva e persistente formação de ERO/ERN geradas durante o processo de inflamação provocado por fagócitos ativados ou pela reação de Fenton devido à presença de metais (49, 51). Nessa via, as ERO/ERN podem causar dano ao DNA por mutações, deleções, ou inserções.

A via primária, por sua vez, é caracterizada como um dano genético causado pelas partículas na ausência de inflamação. Esse dano ocorre por mecanismos diretos e indiretos que também variam de acordo com as propriedades físicas e químicas das partículas. De forma direta, os componentes presentes nas partículas podem interagir com o DNA genômico, e de forma indireta os metais e/ou constituintes orgânicos presentes podem formar adutos de DNA (36).

A literatura é vasta a respeito da associação entre exposição à poluição do ar e dano ao DNA. Tanto estudos *in vitro* quanto *in vivo* demonstram aumento de 8-dihidro-2-deoxiguanosina (8-oxodG), formado pela oxidação da guanina ou incorporação durante a replicação ou reparo como nucleotídeo oxidado, após a

exposição a MP, partículas de exaustão do diesel (DEP), ROFA (52-56). Estudos em humanos também demonstram essa associação (57).

Entretanto, é importante ressaltar que a célula possui mecanismos de reparo que podem reverter o dano causado pela exposição a compostos mutagênicos como reparo por excisão de base (BER), reparo por excisão de nucleotídeo (NER), entre outros (58). Além desses mecanismos, o organismo tem a capacidade de se manter em homeostase sendo capaz de, quando necessário, eliminar uma célula não desejada ou danificada através do processo de apoptose (59).

Portanto, devido à elevada complexidade e variação dos componentes da poluição não é uma tarefa fácil se distinguir qual dos componentes provocaria o dano ao DNA. Diante disso e da complexidade envolvida na carcinogênese (o dano ao DNA representa apenas um de todos os complexos processos envolvidos na carcinogênese) é importante que a interpretação e extrapolação dos resultados sejam realizados cuidadosamente.

### 1.1.3 *Residual Oil Fly Ash* (ROFA)

O ROFA tem sido utilizado em estudos como um substituto para partículas de poluição atmosférica, uma vez que ele é rico em metais e sua constituição pode ser precisamente determinada. ROFA é o termo utilizado para se referir aos resíduos principalmente inorgânicos que permanecem após a oxidação incompleta de compostos de carbono (60). Geralmente as partículas de ROFA possuem tamanho menor do que 2,5 µm e são quimicamente consideradas complexas quando comparadas às demais partículas de poluição atmosférica; pode apresentar sulfatos, silicatos, carbono e nitrogênio contendo outros componentes, contaminantes fósseis e outros aditivos. Além dos elementos citados, também apresenta uma grande quantidade de metais que estão naturalmente presentes em combustíveis (petróleo, parafina, e óleo diesel) e permanecem quando a fração volátil é destilada.

Apesar de o ROFA não mimetizar a poluição atmosférica como um todo, este MP contém elevadas concentrações de poluentes que são encontrados na poluição do ar. Devido ao fato de o ROFA ser rico em metais, muitos trabalhos têm utilizado ele como um substituto de MP ambiental para avaliar os efeitos biológicos mediados pelos metais presentes nos poluentes atmosféricos.

O ROFA possui frações solúveis e não solúveis em água. Estudos relatam que os efeitos causados por ambas as frações são similares, sendo que os componentes solúveis causam um dano mínimo no pulmão em ratos expostos ao composto (61). Já Roberts *et al*. (2003), observou que a fração solúvel do ROFA aumenta a susceptibilidade à infecção pulmonar em ratos que receberam uma dose aguda das diferentes frações de ROFA (62). Essas variações na resposta podem ser explicadas pelas diferenças de composição, concentração e tempo de exposição.

A exposição aguda a este poluente tem demonstrado que ele é capaz de causar dano pulmonar em modelos experimentais. Muitos trabalhos têm associado o dano pulmonar à presença de metais na constituição do ROFA (44, 63, 64). Os primeiros estudos revelam que os metais inalados catalisam reações químicas de Fenton e, consequentemente, há produção de ERO (60). As ERO são capazes de induzir a expressão de citocinas pró-inflamatórias IL-6, IL-8 e TNFα em cultura de célula do epitélio respiratório humano, e também de antioxidantes (44).

Recentemente, alguns trabalhos têm sugerido uma relação tempo dependente capaz de explicar as diferentes respostas celulares após a instilação aguda ao ROFA. Magnani *et al.* (2011), observaram que no pulmão, inicialmente, ocorre oxidação fosfolipídica e o dano às proteínas se dá após transcorrer um período maior de tempo após a exposição (65). O mesmo grupo de pesquisadores verificou resposta semelhante em outros órgãos, o que demonstra efeitos sistêmicos à instilação de ROFA (66-68). Além disso, também foi observada uma resposta semelhante em relação às citocinas pró-inflamatórias. No pulmão, o TNF-α e a IL-6 estão presentes em uma maior concentração 3 horas após a exposição e em menor concentração após cinco horas (67). De maneira semelhante, Carvalho *et al.* (2014), observaram um aumento de colapso alveolar, influxo de

células polimorfonucleares, alterações ultraestruturais no parênquima e aumento de neutrófilos sanguíneos nas primeiras 6, 24, 48, 72 e 96 horas, sendo que estes desfechos retornaram aos valores normais após 120 horas da exposição ao ROFA (69). Corroborando com os dados, pesquisadores verificaram que a exposição aguda a este poluente causa lipoperoxidação no pulmão, mas esse perfil não foi observado na exposição crônica (70, 71). Esses dados sugerem adaptação tecidual ao estresse.

Com o intuito de avaliar os efeitos protetores na resposta celular em organismos expostos ao ROFA algumas substâncias antioxidantes têm sido utilizadas. Rhoden *et al.* (2004), demonstrou que a utilização de N-acetilcisteína, um antioxidante, impediu o desenvolvimento de processo inflamatório e de estresse oxidativo em modelo experimental de poluição do ar (72). De maneira semelhante, a administração sistêmica de dimetiluréia, um *scavenger* de uma variedade de espécies de oxigênio, demonstrou redução na citotoxicidade e na inflamação pulmonar além de aumento na atividade antioxidante causada pela exposição ao ROFA (62, 73). Isso sugere que o estresse oxidativo desempenha um papel importante no mecanismo de dano pulmonar produzido por este tipo de partícula.

Diante disso, os mecanismos responsáveis por desencadear dano pulmonar não são totalmente compreendidos e podem variar de acordo com as propriedades físico-químicas do ROFA, do tempo de exposição e de outros fatores. Além disso, substâncias antioxidantes podem interferir e, consequentemente, minimizar e/ou prevenir possíveis danos celulares.

## 1.2 Resveratrol

### 1.2.1 Origem e propriedades físico-químicas

Na década de 90, o intitulado “Paradoxo Francês” chamou atenção para a até então, pouco conhecida, substância resveratrol (RSV). Esse paradoxo se

estabeleceu a partir de estudo epidemiológico, o qual evidenciou que os franceses apresentavam baixa incidência de doenças coronárias apesar de possuírem uma dieta com elevado índice de gordura saturada (74). Isso foi associado ao consumo diário de vinho população. Em 2009, a Organização Mundial da Saúde divulgou dados que corroboram essa evidência: a proporção de morte relacionada à doença coronária na França foi de duas a três vezes menor do que a de outros países, tais como Estados Unidos, Reino Unido e Suécia (75). A partir disso, milhares de estudos começaram a ser desenvolvidos com o objetivo de avaliar os diferentes efeitos desta substância.

O RSV apresenta-se naturalmente em duas formas isoméricas: *cis* e *trans*-resveratrol (Figura 1), sendo que o *trans*-resveratrol (3,5'4-trihidroxiestilbeno) é a forma mais estável. É produzido por uma grande variedade de plantas incluindo uvas, amendoim, *berries,* e nozes como um mecanismo de defesa em reposta ao estresse, a injúria ou ao ataque de microrganismos (76, 77). Como outros polifenóis, ele tem sido associado a vários processos biológicos como, por exemplo, sendo um potente antioxidante, cardioprotetor, anticancerígeno e capaz de modular a atividade anti-inflamatória (78).

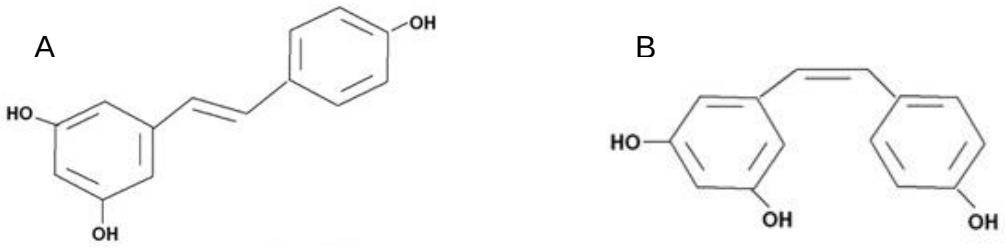


Figura 1. Estrutura química dos isômeros trans-resveratrol (A) e cis-resveratrol (B).

A maioria desses efeitos tem sido demonstrada em estudos realizados *in vitro*. Estudos realizados em modelos *in vivo,* tanto em animais como em humanos, demonstram que esses efeitos são difíceis de serem reproduzidos. Uma

plausível explicação para essa discrepância se deve, principalmente, a baixa biodisponibilidade do RSV quando administrado oralmente *in vivo* (79, 80).

Após a administração, o RSV é rapidamente metabolizado em conjugados glicurônicos e sulfatos. Em humanos, o pico de concentração ocorre após 30 minutos da ingestão, enquanto que em animais o tempo de meia vida se dá entre 12-15 minutos após a administração oral (81, 82). Entretanto, devido ao perfil lipofílico, a concentração nos tecidos deve ser maior do que a do plasma (77). Muitos dos efeitos relatados são devido à ação dos metabólitos do RSV já que a concentração e tempo de meia vida são maiores nos metabólitos do que a própria substância (83).

### 1.2.2 Mecanismo de ação

Muitos estudos têm sido desenvolvidos a fim de elucidar o mecanismo de ação do RSV. De maneira geral, a literatura demonstra que essa substância regula a função biológica por meio de proteínas-alvo específicas: as sirtuinas, particularmente a sirtiuna 1 (SIRT1), e a 5’ adenosina monofosfato quinase ativada (AMPK).

As sirtuinas, pertencentes a uma família de genes altamente conservada, removem grupos de acetil das proteínas e os transferem para a nicotinamida adenina nucleotídeo (NAD+), e são reconhecidas por estarem envolvidas em múltiplos processos biológicos associados ao metabolismo energético e a respostas ao estresse como, por exemplo, isquemia (84). Existem relatos controversos referentes à ativação da SIRT1 pelo RSV: enquanto que alguns estudos demonstram que as sirtuinas são ativadas pelo RSV (85) outros não relatam essa dependência (86).

Já o AMPK pode ser um alvo direto do RSV e sua atividade é modulada pelo aumento da razão de AMP/ATP, a qual reflete o estado de energia da célula. (87, 88). Geralmente, o AMPK é ativado em condições fisiológicas de estresse energético como hipóxia, exercício e jejum (89, 90). Estudos demonstram que o

AMPK é essencial para os efeitos metabólicos do RSV e é ativado independentemente de SIRT1 (91). Entretanto, também há estudos que demonstram diminuição da atividade de AMPK quando há inibição da atividade de SIRT1 (92).

Além das sirtuinas e do AMPK, sabe-se que o RSV tem a capacidade de interagir com um grande número de receptores, quinases e outras enzimas, os quais contribuem para distintos efeitos biológicos. Estudos têm sugerido que o RSV pode induzir a expressão de enzimas antioxidantes (77), promover a produção de óxido nítrico, inibir a agregação plaquetária, aumentar o colesterol HDL (93, 94), além de diminuir a inflamação e ser capaz de prolongar a vida de mamíferos metabolicamente comprometidos (88). Ainda, estudos demonstram que ele interfere na resistência à insulina e diabetes e é capaz de prevenir a obesidade (95, 96).

Além dos efeitos apresentados, a literatura tem demonstrado que no câncer o RSV age através de múltiplos mecanismos, entre eles, proapoptóticos, antiproliferativos, anti-inflamatórios, antiangiogênicos (97). Os efeitos antiproliferativos e inibitórios tem sido atribuídos à habilidade de bloquear a síntese de DNA e interferir em vários estágios do ciclo celular. Essa substância é capaz de estimular o reparo no DNA aumentando a atividade da p53 (98) ou estimulando a eficiência de reparo do DNA nas células com dano (99). Além disso, é capaz de induzir apoptose em muitas linhagens celulares de tumor por meio da ativação de vias intrínsecas e extrínsecas dos mecanismos envolvidos na morte celular (100-102).

Apesar de o mecanismo terapêutico não estar completamente elucidado, estudos têm demonstrado efeitos benéficos dessa substância em diferentes sistemas. De maneira geral, devido as características apresentadas o RSV é capaz de prevenir uma grande variedade de doenças, incluindo doenças cardiovasculares (103), câncer (104), dano isquêmico (94, 105), entre outras.

### 1.2.3 Potencial terapêutico

O RSV é uma substância natural que tem sido extensivamente estudada desde a sua descoberta. Estudos realizados *in vivo* e *in vitro* tem demonstrado que ele exerce diferentes efeitos, os quais podem prevenir ou retardar a progressão de muitas condições patológicas.

O RSV é neuroprotetor em modelo de injúria cerebral apresentando atividade antiapoptótica (106, 107) antioxidante e anti-inflamatória (107-109). Além disso, apresenta efeitos benéficos em doenças neurodegenerativas e no dano cognitivo (110-113). Além dos efeitos cerebrais, essa substância também tem sido vinculada a cardioproteção. O RSV foi capaz de prevenir a produção de ERO e de estresse oxidativo, além de preservar a atividade das enzimas antioxidantes, aumentar a resistência vascular e apresentar efeitos antiapoptóticos em células cardíacas e em modelos de isquemia e reperfusão (114, 115).

Estudos *in vitro* demonstram que o RSV pode agir como anticancerígeno e antimutagênico. Estudos têm relatado o potencial protetor do RSV contra câncer de mama (63), gástrico (116), coloretal (117) e outros tipos de cânceres, como o de pulmão e hepático (118, 119). A inibição da proliferação celular anormal via modulação da progressão do ciclo celular é uma das mais importantes estratégias para a quimioproteção e quimioterapia (120).

No pulmão, estudos têm demostrado que substâncias com características antioxidantes são capazes de modular mecanismos de defesa. A quercitina, um flavonóide polifenólico com propriedade antioxidante, antiapoptótica entre outras, demonstrou ser efetiva no tratamento ao dano pulmonar causado pela administração de ácido, o qual causa lesão pulmonar química aguda. A quercitina foi capaz de diminuir a infiltração de células inflamatórias e a expressão de óxido nítrico sintase e aumentar a atividade da SOD (121). De maneira semelhante, o eugenol, o qual também possui ações anti-inflamatórias e antioxidantes, diminuiu os danos pulmonares advindos da exposição às partículas de diesel sendo capaz de diminuir as células polimorfonucleares além de reduzir a morte celular, mas não o estresse oxidativo (122).

Por outro lado, estudos investigaram o efeito do RSV sobre o dano causado pela exposição ao cigarro. O cigarro é constituído por mais de 4000 compostos dentre os quais se destacam os aldeídos, a nicotina, o monóxido de carbono, o material particulado, entre outros (123). Dessa forma, o cigarro é considerado um composto potencialmente nocivo à saúde que pode causar doenças principalmente no sistema respiratório.

O RSV foi capaz de atenuar o dano oxidativo causado pelo cigarro por meio da diminuição da secreção de IL-6 e TNF-α e de malondealdeído, além de aumento da atividade de CAT, SOD, GSH-Px nos camundongos expostos. Isso pode ser explicado devido a inibição da ativação de NF-kB (124). Corrobar com as evidências, Kurus *et al*. (2009), verificou que essa substância tratou e preveniu as alterações histopatológicas na traqueia de ratos expostos à fumaça de cigarro (125). Apesar de o RSV apresentar um efeito positivo da função do ventrículo esquerdo, não foi capaz de prevenir os efeitos deletérios no plasma e no lavado broncoalveolar de porcos causados devido à exposição à fumaça do cigarro (126).

Estudo conduzido por Zhang *et al.* (2014), demonstrou que o RSV foi capaz de proteger o tecido pulmonar em camundongos expostos ao lipossacarídeo, substância capaz de causar endotoxemia. O RSV reverteu o estresse oxidativo evidenciado pela diminuição de biomarcadores pró-oxidantes (MDA e $H_2O_2$) e aumento de biomarcadores antioxidantes (CAT, SOD, razão GSH/GSSG), além de inibir a produção de NO e expressão de óxido nítrico sintase em camundongos que receberam lipossacarídeo (127).

Diante disso, se evidencia que um significante progresso na compreensão dos mecanismos celulares modulados pelo RSV foi alcançado. Contudo, ainda há necessidade de se elucidar as vias específicas de ativação e manutenção das funções biológicas, das defesas antioxidantes celulares e também, dos mecanismos envolvidos no reparo macromolecular.

# 2 JUSTIFICATIVA

Nas últimas décadas é crescente a preocupação acerca dos efeitos adversos da poluição atmosférica à saúde humana. A população das grandes cidades não tem como evitar a exposição à poluição atmosférica, uma vez que ela está constantemente presente na atmosfera, e tão pouco existe antídoto capaz de reverter ou minimizar as consequências advindas da exposição à mesma.

Em contrapartida, sabe-se que uma alimentação saudável e balanceada é essencial para a manutenção da vida. Uma das maneiras de se reduzir o extresse oxidativo é reduzir a ingestão calórica selecionando-se alimentos apropriados, principalmente porque os nutrientes compreendem um aspecto importante no sistema de defesa antioxidante. O resveratrol, por exemplo, é uma substância que apresenta propriedades antioxidantes, entre outras, e é encontrado em alimentos de fácil acesso como, por exemplo, a uva.

Até o presente momento não há estudos que correlacionam os efeitos do resveratrol com os efeitos deletérios consequentes a exposição à poluição atmosférica. Diante disso, verifica-se a importância de se analisar as consequências da exposição à poluição atmosférica e o potencial efeito terapêutico/protetor desse antioxidante.

# 3 OBJETIVOS

## 3.1 Objetivo Geral

Avaliar parâmetros de estresse oxidativo, de inflamação e de dano ao DNA no pulmão de ratos Wistar machos após a exposição subcrônica ao ROFA e os efeitos da associação com o resveratrol.

## 3.2 Objetivos Específicos

- Determinar os danos oxidativos no pulmão através da determinação de Espécies Reativas ao Ácido Tiobarbitúrico (TBA-RS);
- Determinar a atividade das enzimas antioxidantes Catalase (CAT) e Superóxido dismutase (SOD) no pulmão;
- Determinar concentração de citocinas pro-inflamatórias IL-6, TNF-α no pulmão através da imumohistoquímica;
- Determinar a concentração de metias no pulmão através de espectrometria de massa com plasma acoplado indutivamente (ICP-MS);
- Avaliar os danos ao DNA no tecido pulmonar através do ensaio cometa.

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## 5 ARTIGO

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**Title: Resveratrol prevents pulmonary DNA damage induced by subchronic exposure to residual oil flay ash (ROFA)**

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## ABSTRACT

Residual oil fly ash (ROFA) is a common pollutant in areas where there is oil burning. This particulate matter (PM) with wide distribution of particle diameters can be inhaled by human beings and therefore can cause damage to the respiratory system. Resveratrol (RSV), a natural polyphenol, has received increasing attention due its wide bioactivities, including the inhibition of tumorigenesis, lipid modification and calorie-restriction. The aim of this study was to investigate the subchronic exposure to ROFA and the effects of RSV intake on rat lungs. For this, thirty-three male Wistar rats were distributed into the following groups: control (n = 9, CTL), resveratrol (n = 8, RSV), residual oil fly ash (n = 8, ROFA) and ROFA with RVS treatment (n = 8, ROFA + RSV). The rats were exposed to ROFA by intranasal instillation and were treated with RVS (20mg/kg/day) by gavage for 14 weeks. After twenty four hours, lung tissues were collected for determination of oxidative damage markers (thiobarbituric acid reactive substances - TBARS), antioxidant status (superoxide dismutase - SOD and catalase - CAT activity), DNA damage, cytokines and metals levels. The results show no statistically significant difference in TBARS, SOD and CAT activity, cytokines and metals levels between groups. The ROFA group showed higher DNA damage when compared to the other groups. In conclusion, the present study demonstrated that RSV avoided DNA damage after subchronic ROFA exposure.


**Keywords:** resveratrol, air polluiton, ROFA, DNA damage, oxidative stress, inflammation, rat lung.

## INTRODUCTION

Ambient air in polluted areas contains thousands of chemical compounds known to possess mutagenic and carcinogenic properties. Many studies associate urban air pollution with significant health effects on exposed population, including morbidity and mortality due to cardiopulmonary diseases or lung cancer (Dominici *et al*., 2006; Fajersztajn *et al*., 2013).

Among the air pollutants, particulate matter (PM) seems to be of major concern from the health perspective (WHO, 2005). It is constituted by organic and inorganic compounds. The inorganic residues, such as fly ashes, are the result of an incomplete oxidation of carbonaceous materials and this pollutant significantly contributes to the ambient air particulate burden. Because of the unique composition of the Residual Oil Fly Ashes (ROFA), especially considering metals, it has been useful as surrogate for ambient air PM exposure in many biological studies (Ghio *et al*., 2002). Previous data suggests that ROFA administration via intratracheal/intranasal instillation and aerosol inhalation disclose functional and structural alterations such as acute lung injury, alveolar septal thickening, increased cellularity and lung inflammation (Mangnani *et al*., 2011 Ghio *et al*., 2002).

Resveratrol (3,5,4’-trihydroxystilbene; RSV) is a natural polyphenol found in some plants, including grapes and their derivatives, berries and nuts. Recently, resveratrol has received increasing attention because it has varied bioactivities, including the inhibition of tumorigenesis, lipid modification, anti-inflammation, antioxidation and calorie-restriction (reviewed by Park *et al.,* 2015). Moreover, previous experiments have demonstrated that resveratrol ameliorates trachea histological changes, lung inflammation and oxidative stress in animal models of cigarette smoke (Kurus *et al.,* 2009; Liu *et al*., 2014).

A comprehensive analysis of the time course of the changes in lung markers of oxidative stress, inflammation and DNA damage after chronic ROFA exposure as well as the potential protective effects of RSV have not yet been performed. Thus, we aimed to investigate the subchronic exposure to ROFA and the effects of RSV intake on rat lungs.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats were obtained from Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). Animals were maintained at controlled temperature (21±2ºC) with 12/12- hour light/dark cycle and food and water *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (NIH-USA). The study was approved by the Ethics Committee of UFCSPA (Protocol 13-109) and a total of 33 rats were utilized.

### *Experimental design*

*ROFA suspension.* ROFA was employed as a recognized ambient PM. ROFA particles were collected from electrostatic precipitator installed in one of the chimneys of a large steel plant in São Paulo, Brazil. Particles were prepared by suspending 20 μg of ROFA particles in 10 μl sterile saline solution, and sonicated for 20 min in an ultrasonic water bath.

*RSV solution.* RSV (≥ 98%, Pharma Nostra) was dissolved in saline with 0.05% of Tween 80.

Thirty-day-old male Wistar rats (200-283 g) were randomly distributed into four groups: control (n=9; CTL); resveratrol (n=8; RSV); residual oil fly ash (n=8; ROFA); and

ROFA with RVS treatment (n=8; RSV+ROFA). Rats received daily 20 mg/kg of resveratrol by oral gavage and 20 μg ROFA by intranasal instillation. The control groups received only the vehicle.

Animals were euthanized after 14 weeks of treatment and lung was dissected. The upper lobes and the inferior part of the right lung were used for oxidative stress and metal analyses, respectively, and stored in – 80ºC. The inferior part of left lung was fixed in formaldehyde for 48 hours and then embedded in paraffin.

*Biochemical analyses*

*Tissue preparation*

Lung tissue samples were homogenized in ice-cold 20 mM sodium phosphate buffer, pH 7.4 (1:5, w/v), containing 120 mM KCl, and protein inhibitors (1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.5 mM PMSF) with a Potter-Elvehjem glass homogenizer. The obtained suspension was centrifuged at 600 g for 10 min at 4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as tissue homogenate and kept at −80 °C until analysis.

*Protein content*

Protein concentration of lung homogenates was measured by Bradford's method (Bradford, 1976) using bovine serum albumin as standard.

*Thiobarbituric acid reactive substances assay*

Oxidative damage was determined as thiobarbituric acid reactive substances (TBARS) using a fluorometric assay (Buege and Aust, 1978). Briefly, lung homogenates

were mixed with 10% (w/v) trichloroacetic acid and centrifuged to precipitate proteins. The supernatants were added to thiobarbituric acid 0.67% (w/v) and incubated for 30 min at 100ºC. TBARS were extracted using butanol (1:1;v/v) and measured at 535 nm. The amount of TBARS was expressed in nmol/mg of protein using $\varepsilon = 1.56\ 10^5\ M^{-1}cm^{-1}$.

*Superoxide dismutase activity*

The superoxide dismutase (SOD) activity assay is based on the capacity of pyrogallol to autoxidize (Marklund and Marklund 1984). The inhibition of auto-oxidation of this compound occurs in the presence of SOD, whose activity was then indirectly assayed at 420 nm. One unit of SOD is defined as the enzyme quantity capable of inhibiting 50% of the reaction. A calibration curve was performed with purified SOD as standard. The results were expressed as USOD/mg protein.

*Catalase activity*

The catalase (CAT) activity was performed according to Aebi (1984). The reaction mixture contained 33 mM $H_2O_2$ in 50 mM phosphate buffer (pH 7.0) and lung homogeneized. The decomposition of hydrogen peroxide by CAT was determined at 25°C at 240 nm for 120 sec. The results were expressed in pmol/mg protein.

*Metal analysis*

The analyses of total amount of chemical elements were carried out based on a previously published method (Batista *et al,.* 2009). Prior to analysis, samples were solubilized in 1 ml tetramethylammonium hydroxide (TMAH) 50% (v/v) at room temperature for 12 h. Then, the volume was made up to 10 ml with a diluent containing

0.5% (v/v) $HNO_3$ and 0.01% (v/v) Triton X-100. In all experiments 10 µg/L of the internal standard rhodium (Rh) was used. Samples were directly analyzed by an inductively coupled plasma mass spectrometer (DRC-ICP-MS ELAN DRCII, PerkinElmer, SCIEX, Norwalk, USA) operating with high purity argon (99.999%, Praxaair, Brazil). In order to check the accuracy of the analysis, certified reference materials SRM 1577c and 1577b (bovine liver) from the National Institute of Standards and Technology (NIST) and SRM TORT-2 (Lobster Hepatopancreas) from European Virtual Institute for Speciation Analysis (EVISA) were analyzed in each batch of ordinary sample analysis.

*Comet Assay*

The alkaline comet assay in lung cells was performed as described in the literature with minor changes (Singh *et al*., 1988). Briefly, it was made a cell suspension of the lung in PBS buffer (pH = 7.40) and 20 µL of cell suspensions were then rapidly embedded in 90 µL of 0.75% low-melting point agarose at 37 °C. After solidification, the slides were immersed in iced-cold lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0; containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulfoxide (DMSO) at 4 °C in dark for a minimum of 1 h. Afterwards, to allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) at 4 ºC for 5 min in a horizontal electrophoresis box. The alkaline electrophoresis was carried out for 15 min at 25 V and 300 mA. After electrophoresis, slides were washed three times in a neutralization buffer (0.4 M Tris; pH 7.5) for 5 min and left to dry overnight at room temperature. Then, the slides were fixed for 10 min in trichloroacetic acid 15% (w/v), zinc sulfate 5% (w/v), glycerol 5% (v/v). Finally, the slides were stained with silver nitrate (sodium carbonate 5% (w/v), ammonium nitrate 0.05%

(w/v), silver nitrate 0.05% (w/v), tungstosilicic acid 0.125% (w/v), formaldehyde 0.075% (w/v), freshly prepared in the dark) (Garcia *et al.*, 2007). The images (50-60 cells/slide) were captured with high performance Nikon camera. The quantification of the DNA strand breaks of the stored images was done using the CASP software (CASPLab®) by which percentage of tail DNA, tail moment and olive tail moment were measurement (Końca *et al*., 2003).

*Immunohistochemistry*

For immunohistochemical analysis, the lung sections were deparaffinized and hydrated, endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide and antigen was retrieved with trypsin or high temperature. After, slides were incubated with primary antibodies anti-IL-6 (goat polyclonal antibody, 1:7000 dilution, Cat # sc-1265, Santa Cruz, CA, USA) and anti-TNFα (goat polyclonal antibody, 1:7000 dilution, Cat # sc-1350, Santa Cruz, CA, USA) for 1 h at 37°C in a moist chamber. Then, the slides were washed in PBS and incubated with Vectastain ABC kit (Vector Elite, Vector Laboratories, Inc.Ingold Road Burlingame, CA). After this step, the slides were washed in PBS and followed by the revelation 3.3 Diaminobenzidine (DAB) chromogen (Sigma Chemical Co., St. Louis, MO, USA). Finally, the slides were counterstaining with Harry's hematoxylin and mounted with cover slips. The software Image-Pro® Plus versão 4.5 (Media Cybernetics – Silver Spring MD, USA) was used to measure the total area of analyzed lung tissue and the positively marked tissue. The results are expressed in proportion of positively stained tissue area per total lung tissue area.

169

170 *Statistical analyses*

171 Data are presented as mean ± standard deviation (SD). All analyses were performed
172 using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). The normality of the
173 data was tested by Kolmogorov-Smirnov. A one-way analysis of variance (ANOVA) and
174 Kruskal-Wallis was used to parametric and non-parametric data, respectively, followed by
175 post-hoc Bonferroni test. The significance level was set at 5%.

176

177 **RESULTS**

178 *Metals concentrations*

179 The concentration of chemical elements such as of copper (Cu), cadmium (Cd),
180 nickel (Ni), zinc (Zn), aluminium (Al), iron (Fe) in ROFA are described in Table 1. No
181 differences were observed among groups considering the levels of chemical elements in the
182 rat lungs (Table 2).

183

**Table 1.** Concentrations of chemical elements in ROFA.

| **Metal** | µg/g **(mean ± SD)** |
|---|---|
| Pb | 3.1 ± 0.09 |
| Al | 789.9 ± 23.28 |
| Zn | 20.3 ± 0.04 |
| Cd | 0.04 ± 0.002 |
| Ba | 30.2 ± 0.31 |
| Cu | 9.7 ± 0.15 |
| Ni | 287.0 ± 10.8 |
| As | 4.1 ± 0.05 |
| Se | 7.5 ± 0.20 |
| Mn | 48.3 ± 0.98 |
| Sr | 8.4 ± 0.16 |
| Sb | 2.3 ± 0.57 |
| Fe | 20,397.2 ± 283.3 |
| Mg | 372.5 ± 1.93 |
| P | 388.5 ± 255.8 |
| Cr | 7.6 ± 0.23 |

ROFA, residual oil fly ash. Values are mean ± SD of three determinations.

**Table 2.** Concentrations of chemical elements in the lungs from rats after 14 weeks of exposure.

| Metal | CTL | RSV | ROFA | ROFA+RSV |
|---|---|---|---|---|
| Cu | 6.92 ± 0.36 | 6.93 ± 0.20 | 7.33 ± 0.82 | 6.88 ± 0.68 |
| Se | 2.46 ± 0.20 | 2.63 ± 0.22 | 2.51 ± 0.24 | 2.50 ± 0.13 |
| Zn | 60.34 ± 3.67 | 59.46 ± 3.65 | 62.24 ± 4.19 | 58.91 ± 7.52 |
| Mn | 1.73 ± 0.57 | 1.55 ± 0.32 | 1.60 ± 0.58 | 1.64 ± 0.38 |
| Fe | 1,196.36 ± 177.39 | 1,106.59 ± 160.26 | 1,157.85 ± 177.94 | 1,152.27 ± 189.30 |
| Mg | 703.48 ± 61.70 | 701.85 ± 68.87 | 753.60 ± 53.98 | 671.85 ± 92.97 |
| P | 11,358.37 ± 867.40 | 11,410.72 ± 1089.90 | 12,162.04 ± 846.17 | 10,880.63 ± 1604.43 |
| Cr | 4.99 ± 0.20 | 5.52 ± 0.28 | 5.11 ± 0.43 | 4.82 ± 0.23 |
| Pb | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.02 | 0.04 ± 0.02 |
| Al | 0.80 ± 0.34 | 0.67± 0.27 | 0.61 ± 0.11 | 0.65 ± 0.12 |
| Cd | 0.01 ± 0.001 | 0.01 ± 0.003 | 0.01 ± 0.002 | 0.01 ± 0.004 |
| Ni | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.06 ± 0.02 | 0.05 ± 0.01 |
| As | 4.48 ± 0.95 | 5.42 ± 1.69 | 3.81 ± 0.74 | 3.88 ± 0.67 |
| Sr | 0.46 ± 0.10 | 0.45 ± 0.13 | 0.49 ± 0.07 | 0.48 ± 0.06 |
| Sb | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.02 |

Data are expressed as mean ± SD. All values are in µg/g.
CTL, control; RSV, resveratrol; ROFA, Residual oil fly ash.
P > 0.05 (One-Way ANOVA).

*Oxidative markers*

No changes were observed in TBARS content (P=0.209) and in antioxidant status of CAT (P=0.421) and SOD (P=0.964) (Table 3).

**Table 3.** Measurement of oxidative stress in lungs from rats after 14 weeks of exposure.

| | CTL | RSV | ROFA | ROFA+RSV |
|---|---|---|---|---|
| TBARS (nmol/mg protein) | 5.49 ± 1.63 | 5.21 ± 2.52 | 5.14 ± 3.47 | 6.36 ± 1.54 |
| CAT (pmol/mg protein) | 3.92 ± 0.67 | 3.20 ± 1.19 | 3.04 ± 1.59 | 3.88 ± 1.20 |
| SOD (USOD/mg protein) | 0.25 ± 0.04 | 0.24 ± 0.47 | 0.24 ± 0.11 | 0.26 ± 0.04 |

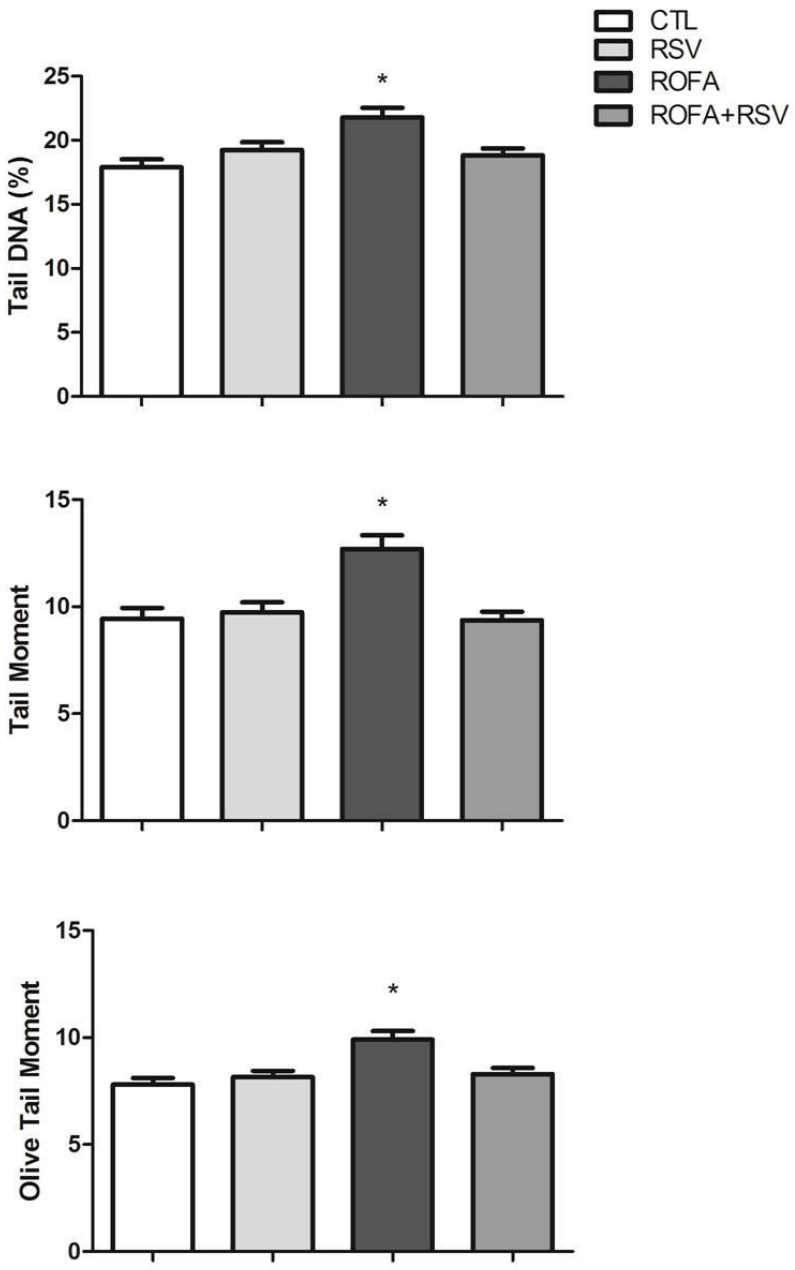
Values are presented in mean ± SD. CTL, control; RSV, resveratrol; ROFA, residual oil fly ash.
P > 0.05 (one way ANOVA).

*Immunohistochemistry*

There were no statistically significant differences in IL-6 and TNF-α levels in the lung among groups (P=0.483; P=0.106, respectively) (data not shown).

*DNA damage of lung cells*

After 14 weeks of ROFA exposure and RSV treatment, comet assay was performed to detect the degree of DNA damage in pulmonary cells. As shown in figure 1, tail DNA%, tail length and Olive tail moment of lung cells in the ROFA exposed group were higher than the others groups (P<0.001).




214

215 Figure 1. DNA damage in lung cells of rats (n= 8-9 per group) to ROFA and RSV
216 treatment for 14 weeks. CTL, control; RSV, resveratrol; ROFA, Residual oil fly ash. The
217 data are expressed as mean ± SD. *P < 0.001; One-Way ANOVA followed by Bonferroni
218 test.

219

## DISCUSSION

In the present study, we employed *in vivo* subchronic exposure to better evaluate the role of RSV against ROFA-induced pulmonary injury. For such purpose, oxidative stress, inflammatory markers and DNA damage in lungs were used as markers. After subchronic treatment we did not identify significant difference on oxidative and antioxidant markers, inflammation and metal content among the groups. However, RSV prevented DNA damage induced by ROFA.

ROFA is a suspension of the material produced after oil burning that has been used in some experimental models aiming to elucidate the adverse health effects of air pollution exposure (Arantes-Costa *et al.*, 2008; Ghio *et al*., 2002; Marchini *et al*., 2014). Although the use of this surrogate does not exactly mimic the overall exposure to air pollution, this PM contains many components of air pollution such as metals. The ROFA used in this work contains predominantly iron and aluminum as shown in table 1.

Different studies evaluated the role of oxidative stress, apoptosis, antioxidants, inflammation and defects of lung repair mechanisms leading to tissue damage following air pollution exposure (Mazzoli-Rocha *at al.,* 2014; Magnani *et al.*, 2011; Zin *et al.*, 2012). Time course studies have shown that functional and histological impairment of lung induced by ROFA returned to control values 120 h after exposure (Carvalho *et al*., 2014). According to Magnani *et al*., (2011), the macromolecular damage in lungs after acute ROFA exposure occur at different induction times. This evidence is consistent with different susceptibility to oxidative damage as related by previous study (Gurgueira *et al*., 2002).

To our knowledge, this is the first study that evaluated the presence of chemical elements in lungs after subchronic ROFA exposure. Wallenborn e*t al*., (2007), observed that PM-associate metals can translocate to systemic circulation and extrapulmonary organs

following a single intratracheal instillation based on their solubility; the less water-soluble metals are retained in the lung longer. This suggests a mechanism of rapid removal via either pulmonary capillaries or lung associated lymph nodes into the blood stream along with mucociliary clearance.

The same research group demonstrated that soluble zinc introduced through the lungs not only reaches but also accumulates in the heart following pulmonary exposure (Wallenborn *et al.,* 2009). More specifically, soluble components present in PM, including metals, may translocate outside of the lung and reach extrapulmonary organs after a single dose exposure. Oberdörster *et al*., (2004) have shown that particles can reach the central nervous system (CNS) by nasal uptake but unlike lungs, the CNS does not have efficient mechanisms of clearance. Based on our results - that have shown no difference in metal levels in lungs - we suggest that metals could have been accumulated on extrapulmonary organs after chronic exposure, although specific organs analyses were not done.

Oxidative stress and inflammation has been reported as a consequence to short-term inhalation exposure to concentrated ambient particles (Rhoden *et al.,* 2004). ROFA produced oxidative stress in rat lungs after acute exposure (data not published), however this mechanism of damage was not reported regarding chronic exposure (Damiani *et al.,* 2012) using the same exposure time but a different range of ROFA concentrations.

The majority of the studies have analyzed the adverse effects in acute exposures while few have investigating effects in subchronic and chronic exposure to air pollution. Responses to ROFA may vary depending on the composition of soluble metals, their interaction with each other and with the cells, the concentration used as well as the time of exposure.

RSV was employed in this study as an antioxidant with potential benefits. We choose the oral administration since their route usually occur considering food containing the compound. RSV has been reported to exert potent anti-oxidant activities not only directly by scavenging hydroxyl, superoxide and metal-induced radicals (Bradamante *et al.*, 2004), but also indirectly by enhancing the activity of anti-oxidant enzymes such as catalase and glutathione peroxidase (Spanier *et al.*, 2009) and decreasing $H_2O_2$ and MDA levels (Zhang *et al*., 2014). For instance, RSV has been reported to protect against cigarette smoke-mediated oxidative stress in human lung epithelial cells (Kode *et al*., 2008) and endotoxemia associated lung tissue injury in mice (Zhang *et al*., 2014).

Other compounds such as PM, organic extracts from PM, diesel exhausted particles, coal fly ash, has been associated with DNA damage in cells (Topinka *et al*., 2012; Sharma *et al.*, 2007; Gabelová *et al*., 2007). Different studies have investigated the effects of air pollution exposure on DNA damage *in vivo*. Meng *et al.*, (2007) observed an increase of DNA damage in rat lung cells in a dose–response manner due $PM_{2.5}$ exposure. Additionally, DEP caused oxidative DNA damage in guinea pigs lungs (Moller *et al.*, 2003).

In order to investigate the overall genotoxicity produced by the different chemical components of ROFA we used the alkaline version of the Comet assay, which is sensitive to detect DNA strand breaks, oxidative DNA lesions, and alkali-labile sites and has been proposed as a useful tool for assessing the genotoxicity of particles (Singh *et al.*, 1988; Schins 2002). Interesting findings are reported concerning the genotoxic properties like the role of organic extractable mutagens such as PAHs (Hsiao *et al.*, 2000) and the genotoxic effects of transition metals due its ability to generate ROS (Prahalad *et al.*, 2001; Knaapen *et al.*, 2002). In our study we did not find differences in oxidative stress and inflammation,

what suggest that DNA damage were caused directly by the particles and the RVT treatment avoided the damage induced by ROFA (Fig. 1). Besides, it is important to emphasize that cells have DNA protection systems in tumor prevention, such nucleotide-excision repair (NER), base-excision repair (BER), which could avoid irreversible mutations that contribute to oncogenesis (Hoeijmakers, 2001).

Many *in vitro* and *in vivo* animal models have demonstrated the potent protection conferred by RSV against inflammation, oxidative stress, and cancer (Baur and Sinclair, 2006). RSV can promote cell cycle arrest leading to apoptosis of tumor cells, prevent tumor-derived nitric oxide synthase expression to block tumor growth and migration, as well as act as an antioxidant to prevent DNA damage that can lead to tumor formation (Clément *et al.* 1998; Spanier *et al.*, 2009; Park *et al.*, 2015).

This study presents limitations. Ideally if the animals were exposed to environmental air rather than to intranasal suspensions of ROFA more precise outcomes determined by the ambient air could be found. Secondly, we did not measure the content of PAHs and other constituents of ROFA, which could explain some findings.

In conclusion, this work provides new insights for understanding the mechanisms involved in lung damage due to subchronic exposure to ambient particles for 14 weeks and the beneficial effects of RSV treatment.

## CONCLUSIONS

This is the first study to demonstrate that RSV could protect DNA damage due ROFA exposure. However, we emphasize further studies are needed to clarify whether the metals accumulates in others organs as well as which ROFA components are associated with the outcomes.

315

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## 6 CONSIDERAÇÕES FINAIS

Segundo os resultados obtidos neste trabalho, podemos concluir que a exposição subcrônica de ratos ao ROFA e ao RSV:

1) Não altera os danos oxidativos no pulmão;
2) Não altera a atividade das enzimas antioxidantes CAT e SOD no pulmão;
3) Não altera a concentração de citocinas pro-inflamatórias IL-6, TNF-α no tecido pulmonar;
4) Não altera a concentração de metais no pulmão;
5) Altera o dano ao DNA das células pulmonares, sendo que o ROFA danifica e o RSV previne esse tipo de dano.

Sendo assim, o RSV foi capaz de prevenir o dano ao DNA causado pela exposição ao ROFA, entretanto não foi observada alteração de resposta pulmonar nos demais parâmetros analisados.

Esse estudo sugere que o pulmão é capaz de se adaptar as condições ambientais após exposição subcrônica ao ROFA e, por isso, não observamos modificações quanto aos parâmetros analisados de estresse oxidativo, assim como a concentração de interleucinas pró-inflamatórias e de elementos químicos. Contudo, verificou-se que o RSV foi capaz de prevenir o dano ao DNA das células pulmonares causado pela exposição ao ROFA.

Devido à complexidade dos mecanismos de defesa celular e ao protocolo empregado, não é possível afirmar que tais danos ao DNA sejam capazes de desencadear carcinogênese. Por isso, mais estudos são necessários para investigar os mecanismos de defesa pulmonar as partículas ambientais a longo prazo, além dos mecanismos de proteção contra o dano ao DNA estabelecido pelo RVS.

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

**Projeto:** 13-109 **Versão do Projeto:** **Versão do TCLE:**

**Pesquisadores:**
CLÁUDIA RAMOS RHODEN
MARLISE DI DOMENICO

**Título:** EFEITOS DA ADMINISTRAÇÃO DE RESVERATROL SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO E INFLAMAÇÃO DE RATOS EXPOSTOS AO RESIDUAL OIL FLY ASH (ROFA).

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

Porto Alegre, 15 de julho de 2013.

Katya V. Rigatto
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g gram
ip intraperitoneal
mg milligram iv intravenous
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po oral
ng nanogram
LD50 medial lethal dose
ml milliliter
LC50 medial lethal concentration
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Hz hertz mol mole
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