UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

AVALIAÇÃO CITOTÓXICA E GENOTÓXICA *in vitro*, E FISIOLOGIA RESPIRATORIA *in vivo*, DOS EFEITOS DE PARTÍCULAS DE CARVÃO E CINZAS PROVENIENTES DE SANTA CATARINA

GRETHEL LEÓN MEJÍA

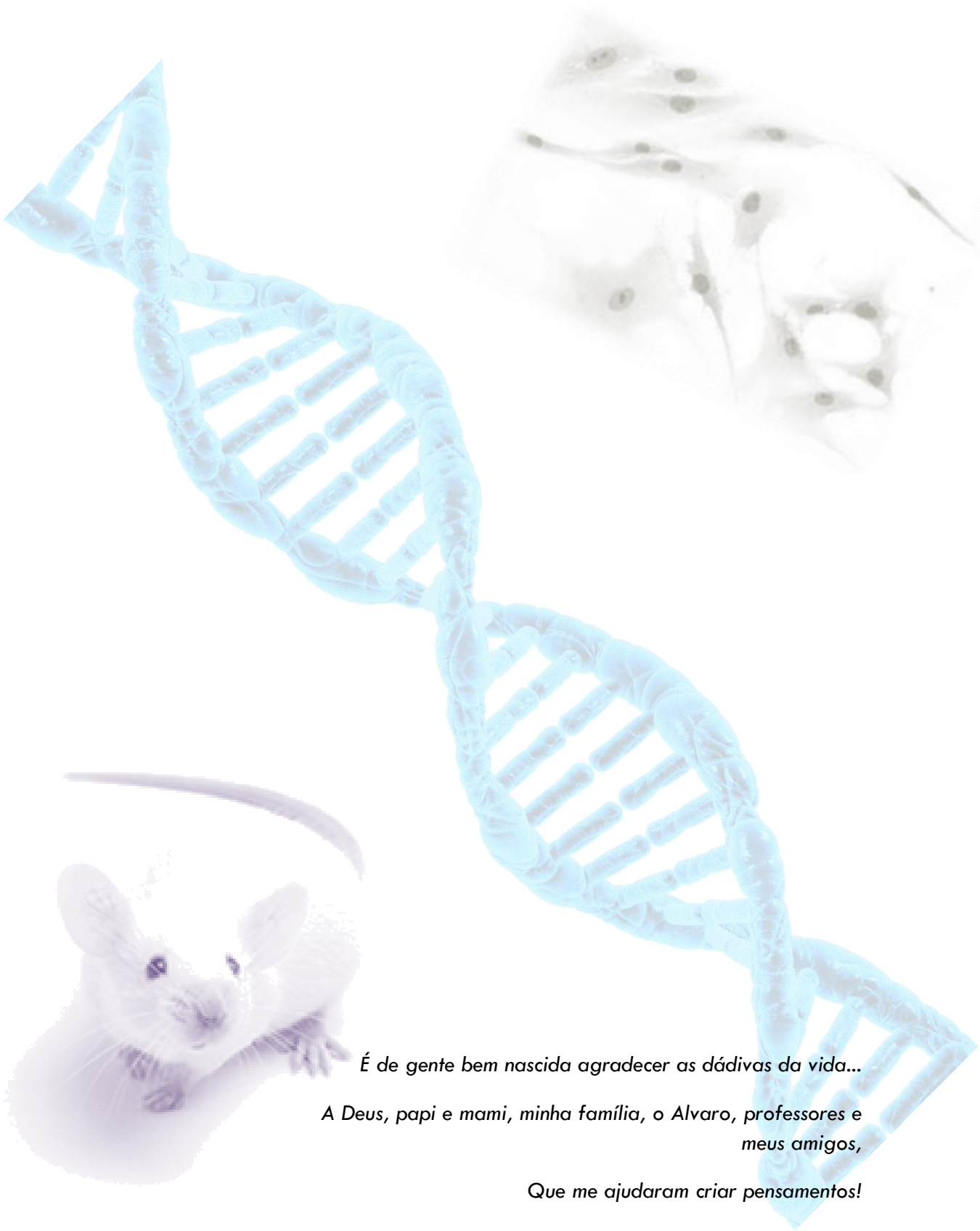
Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientador:

Prof. Dr. João Antonio Pêgas Henriques

Porto Alegre – Brasil

Março de 2016



É de gente bem nascida agradecer as dádivas da vida...

A Deus, papi e mami, minha família, o Alvaro, professores e meus amigos,

Que me ajudaram criar pensamentos!

"Si de verdad quieres algo, y trabajas muy duro, aprovechas las oportunidades, y nunca te rindes, vas a encontrar el camino… lo que haces marca la diferencia, y tienes que decidir qué tipo de diferencia quieres marcar".

Jane Goodall

INSTITUIÇÕES E FONTES FINANCIADORAS

Este trabalho foi desenvolvido nas instalações do Laboratório de Reparo de DNA em Eucariotos, situado no Departamento de Biofísica da Universidade Federal do Rio Grande do Sul. Parte das atividades experimentais foram realizadas durante estágio-sanduíche no Laboratório de Fisiologia da Respiração da Universidade Federal de Rio de Janeiro (UFRJ). Este trabalho foi subsidiado pela CAPES, a qual concedeu a bolsa de doutorado através do Programa Estudantes-Convênio de Pós-Graduação-PEC-PG. CAPES/CNPq – Brasil. Edital 14/2011.

Foi financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq – Brasil. Edital Universal Número 470833; Edital Produtividade Número 300902 e 303364. Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brasil. Edital Número PRONEM 11/2070-7).

AGRADECIMENTOS

São muitas as pessoas que contribuíram direta ou indiretamente para minha formação e poder atingir essa meta, portanto acredito que faltaram páginas neste trabalho para os agradecimentos. Agradeço:

- Ao Professor Dr. **João Antonio Pêgas Henriques,** pelo carinho, por acreditar em mim e me dar a oportunidade e honra de fazer o doutorado sob a sua orientação. Eu estarei eternamente grata!

- Ao Professor Dr. **Walter Zin,** por ser meu segundo orientador e me permitir ser sua aluna, por me acolher de braços abertos no seu laboratório, pelas importantes contribuições no meu trabalho e os ensinamentos durante o meu estagio no seu laboratório.

- À **Juliana da Silva,** pelos ensinamentos, pelo carinho, por sempre estar disposta a me ajudar durante todos estes anos no Brasil. Além disso, pelas boas contribuições no meu trabalho que ela sempre fez. Obrigada Ju!!

- Aos meus pais **Hilda** e **Luis,** sem eles não teria sido possível nenhum passo da minha vida e nenhuma conquista... Obrigada por sonhar junto comigo. Este trabalho é de vocês!

- Ao **Alvarito** pelo apoio, amor e a paciência que sempre teve durante os momentos difíceis e de ansiedade deste doutorado, você esteve comigo em cada conquista e cada fracasso... nesses momentos descobri que tudo fica mais fácil sabendo que tenho você ao meu lado. Gracias!

- Aos meus irmãos **Luis Gabriel, Oscar David, Jorge Mario, Cristian** e a **Gabriela,** aos meus tios (as) **Edelmira, Deyanira, Cielo, Yoni** e **Beatriz,** aos meus afilhados **Gustavo Andrés, Gustavito** e o **Esteban,** aos meus primos **Amy, Grey, Daisy, Yoni, Flor, Luisa** e **Natalia,** o meu muito obrigada!

- À **Miriana Machado,** pelo apoio constante, pelos ensinamentos e pelos bons momentos compartilhados.

- À **Izabel Villela,** pela amizade que nos une e pelas importantes sugestões na realização deste trabalho. Faltarão palavras para agradecer!

- À **Miriam Benicio,** obrigada pelo carinho, pelas importantes correções do "portunhol" e acertadas sugestões deste trabalho.

- À **Renata Okuro, Mariana Machado** e **Natalia Vasconcelos,** pela grande ajuda e colaboração no projeto, pela amizade que nos une e por compartilhar comigo

momentos inesquecíveis no Rio de Janeiro. Muito obrigada meninas, não tenho como agradecer!

- À **Carolina Uribe** e ao **Guillermo Mudry**, meus grandes amigos, anjos que a vida me deu para percorrer este caminho. Muchas gracias!

- Ao meu amigo **Cristiano Trindade** que me ajudou com sugestões e correções no meu trabalho e juntos compartilhamos bons momentos.

- Aos funcionários do Programa de Pós-Graduação em Biologia Celular e Molecular, principalmente à **Silvia Centeno** e ao **Luciano Saucedo**. Foram tantas risadas juntos que ainda lembro e posso sorrir. Obrigada pela ajuda a nós alunos quando mais precisamos.

- Ao Prof. **Luis Silva** por me doar as amostras para análises da minha tese, pelo apoio e acertadas sugestões na discussão dos meus resultados.

- Ao Prof. **Johnny Dias**, à **Liana Niekraszewicz** e **à Claudia Telles** do Instituto de Física da UFRGS, pela amabilidade e pela ajuda e críticas nas análises das amostras pelo Método PIXE.

- Aos Professores membros da minha Comissão de Acompanhamento: Prof. Dr. **Guido Lenz** e Profª. **Jenifer Saffi**, pelas acertadas sugestões no meu trabalho.

- A todos os professores do Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, pelos valiosos ensinamentos durante as suas disciplinas.

- À **família Dau Sanchez**, pelo apoio e carinho incondicional, principalmente ao **Juan Dau** e ao **Carlitos Dau**, pela valiosa amizade que nos une.

- Ao **Milton Quintana**, pela amizade, pela força, apoio profissional e compreensão durante a finalização do doutorado.

- À **Diana Narváez**, pela amizade que nos une, pelas boas conversas e conselhos no meu trabalho.

- Aos maravilhosos amigos que estão na Colômbia e que me esperam de braços abertos: **María Cecilia Sánchez, Javier Arrieta, Yeni Guzmán, Oscar Montiel, Marco Támara, Elena Rubio, Sandra Pérez, Isaura Torres, Sandra Ochoa, Karina Bravo, Jaime Luna, Antonio Acosta, Marco Anaya, Martha Ruiz, Karen Franco, Dayan Lozano, Nebys Navarro, Moisés Arquez** e ao **David Mestra**, que direta ou indiretamente estiveram comigo para me apoiar no meu trabalho.

- Aos amigos que conheci em Porto Alegre: **Dayana, Fabrício, Leidy, Nedy, Jose Vargas, Omaira, Fausto, Lila, Felix** e a **Renildes.**

- Aos meus amigos: **Andreia, Diana, André, Gabriel, Luisa, Michelle, Jose, Fer, Iuri, Leonardo, Márcia, Priscila, Tati, Mariana, Victoria, Larissa** e à **Nusha,** muito obrigada pelo carinho, muito obrigada pelas boas festas, conversas e pelos bons momentos.

- Ao pessoal do Laboratório de Fisiologia da Respiração (UFRJ): **Diego, Giovanna, Alexa, Aline, Tamiris, Igor** e ao **Neto.**

- Ao programa convênio Pós-Graduação **PEC-PG** e a **CAPES** pelo financiamento no meu doutorado.

- A todas aquelas pessoas cuja minha falta de memória não permitiu colocar os nomes no papel, mas que, em seu momento, foram uma grande ajuda para fazer este trabalho, muito obrigada!

ÍNDICE GERAL

LISTA DE FIGURAS

INTRODUÇÃO

CAPITULO I

CAPITULO II

DISCUSSÃO GERAL

LISTA DE TABELAS

INTRODUÇÃO

CAPITULO I

CAPITULO II

Supplementary results

ABREVIATURAS

8-OH-dG	8-hidroxi-2'-desoxiguanosina
AP-1	Proteína ativadora-1
APOP	Apoptose
ATI	Células alveolar tipo I
ATII	Células tipo II
BaP	Benzo[a]pireno
BN	Célula binucleada
CBMN-cyt	*Cytokinesis-block micronucleus cytome* (CBMN Cyt) *assay*
CCP	Produtos da combustão do carvão
CFA	Coal Fly ash (cinza volátil)
COAL	Carvão mineral
CWP	Pneumoconiose de trabalhadores de carvão
DSB	Quebras duplas de DNA
ECM	Matriz extracelular
EGF	Fator de crescimento epidermal
ENDOIII	Endonuclease III
ERN	Espécies reativas do nitrogênio
ERO	Espécies reativas do oxigênio
Est	Elastância estática
FPG	Formamidopirimidina DNA glicosilase
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
HAP	Hidrocarbonetos Aromáticos Policíclicos
HE	Hematoxilina e eosina
IARC	Agência Internacional de Pesquisa do Câncer
IL-1	Interleucina-1
IL-6	Interleucina-6
MCP-1	Proteína-1 quimiotáctica de monócitos

MMS	Metil metano sulfonato
Mn	Célula mononuclear
MN	Micronúcleo
NBUD	Broto
NDI	Índice de divisão nuclear
NECR	Necrose
NF-kB	Fator nuclear kappa B
NPB	Ponte nucleoplásmico
OGG1	8-oxoguanina DNA glicosilase
PAF	Fator ativador de plaquetas
PAH	Hidrocarbonetos Aromáticos Policíclicos
PDGF	Fator de crescimento derivado de plaquetas
PIXE	Emissão de raios-X induzidas por partículas
PM	Material particulado
PMF	Fibrose massiva progressiva
PMN	Célula polimorfonuclear
ppm	Partes por milhão
SH	Grupo sulfidrilo
SSB	*Single strand break*
TGF-β	Fator de crescimento transformante beta
TNF-α	Fator de necrose tumoral-α
UFPs	Partículas ultrafinas
V	Volts
XRD	Técnica da difracção de raios-X
ΔE	Componente viscoelástico da elastância
ΔP1	Pressão utilizada para vencer o componente viscoso
ΔP2	Pressão relativa ao componente viscoelástico e/ou inomogêneo

RESUMO

Durante as atividades de mineração de carvão, grandes quantidades de cinzas, metais, óxidos e hidrocarbonetos aromáticos policíclicos (HAP) são liberados ao ambiente. As partículas de carvão e cinzas são quimicamente complexas e têm a capacidade de interagir com mecanismos celulares e não celulares que desencadeiam a ativação de macrófagos, células epiteliais e fibroblastos, a liberação de espécies reativas de oxigênio (ERO) e expressão de citocinas. O objetivo do presente estudo foi avaliar a citotoxicidade e genotoxicidade *in vitro*, e fisiologia respiratoria *in vivo*, da exposição a partículas de carvão e cinzas provenientes de Santa Catarina-Brasil. Foram avaliadas quatro amostras, duas de carvão mineral (COAL11 e COAL16) e duas de cinzas produto da combustão de cada carvão (CFA11 e CFA16). Particularmente, a amostra CFA16 é produto da co-combustão com óleo combustível e óleo diesel. Na avaliação *in vitro*, COAL11, COAL16 e CFA16 foram mais citotóxicas do que a cinza CFA11 como avaliado no ensaio clonogênico. A exposição das células V79 às partículas de carvão e cinzas induziram lesões primárias no DNA detectadas no ensaio Cometa Alcalino. Partículas de COAL16 e CFA16, em maiores concentrações, foram capazes de induzir danos oxidativos no DNA em células V79, como demonstrado no ensaio Cometa Modificado (FPG e ENDOIII). Nos biomarcadores do ensaio CBMN-Cyt os resultados mostraram que as concentrações mais altas de carvão e cinzas induziram efeitos citotóxicos e instabilidade cromossômica. *In vivo*, camundongos BALB/c foram analisados 24 h após instilação intratraqueal com partículas de carvão e cinzas. Os camundongos expostos a partículas de carvão apresentaram rigidez pulmonar e obstruções das vias aéreas centrais quando comparado com o grupo controle, nos parâmetros da mecânica pulmonar. Quando expostos às partículas de carvão e cinzas, os animais mostraram recrutamento de células, principalmente as mononucleares, e expressão de citocinas, principalmente TNF-α e IL-1β, quando comparado com o grupo controle. Na histologia, não foram encontradas alterações significativas nos alvéolos, nem formação de fibras elásticas e colágenas. Resultados no ensaio Cometa Alcalino mostraram efeitos genotóxicos significativos em células de sangue periférico nos animais expostos a partículas de carvão e cinzas. As análises dos elementos inorgânicos usando o método PIXE, demostraram translocação extrapulmonar de metais ao fígado, baço e encéfalo. Comparativamente, de todas as amostras avaliadas, a CFA11 apresentou o maior diâmetro no tamanho das partículas como avaliado mediante análise de difração de laser. Os resultados demostraram que o processo de combustão altera a toxicidade das partículas de carvão, e o uso de combustíveis adicionais dentro do processo de queima, como foi o caso da amostra CFA16, muda as caraterísticas das partículas geradas quanto ao tamanho e toxicidade das mesmas. Estes resultados *in vitro* e *in vivo* estão relacionados com os compostos contidos na superfície das partículas, tais como óxidos, metais e HAP detectados nas amostras. Este conjunto de dados aportam ao estado do conhecimento sobre os riscos da exposição continua a este tipo de partículas.

ABSTRACT

During the coal mining activities, large amounts of fly ash, metals, oxides and polycyclic aromatic hydrocarbons (PAH) are released to the environment. Coal and fly ash particles are chemically complex and are capable of interacting with cellular and non-cellular mechanisms that trigger the release of reactive oxygen species (ROS) and expression of cytokines from activated macrophages, epithelial cells and fibroblasts. The aim of this study was to evaluate the cytotoxicity and genotoxicity *in vitro*, and respiratory physiology *in vivo*, of exposure to coal and coal fly ash particles from Santa Catarina-Brazil. Four samples were collected, two from coal (COAL11and COAL16) and two from coal fly ash released during combustion (CFA11 and CFA16). Particularly, the CFA16 sample was the product of co-firing with fuel oil and diesel oil. *In vitro* evaluation, COAL11, COAL16 and CFA16 were more cytotoxic than CFA11sample as assessed in clonogenic assay. The exposure of V79 cells to coal particles and coal fly ash induced primary DNA damage detected in Comet Alkaline test. COAL16 and CFA16 particles, at higher concentrations, were capable of inducing oxidative DNA damage in V79 cells, as demonstrated in the Modified Comet assay (FPG and ENDOIII). Results in the biomarkers CBMN-Cyt test showed that higher concentrations of coal and coal fly ash particles induced cytotoxic effects and chromosomal instability. *In vivo*, BALB/c mice, were analyzed 24 h after intratracheal instillation with coal and coal fly ash particles. The mice exposed to coal particles showed significant rigidity and obstruction of the central airways when compared with the control group. In addition, the animals showed recruitment of cells, mainly mononuclear cells, and expression of cytokines, particularly TNF-α and IL-1β as compared to the control group. Histologically, there were no significant alterations in the alveoli, or formation of elastic and collagen fibers. Alkaline Comet assay results showed significant genotoxic effects on peripheral blood cells in animals exposed to coal and coal fly ash particles. The analysis of inorganic elements using the PIXE method demonstrated the efficient metal extrapulmonary translocation into the bloodstream to the liver, spleen and brain. When comparing particle size, CFA11showed the largest diameter as assessed by analysis of laser diffraction. The results showed that the combustion process alters the toxicity of coal particles and the use of additional fuel in the firing process, modifies the toxicity and size of the particles, as was the case of the CFA16 sample. These results *in vitro* and *in vivo* are associated with the compounds contained in the surface of the particles, such as oxides, metals and PAH detected in these samples. This study contribute to the state of knowledge of the risks of continuous exposure of these particles.

ESTRUTURA DO TRABALHO

Este trabalho está dividido da seguinte forma: uma introdução geral, os objetivos (geral e objetivos específicos), um capítulo correspondente à primeira fase *in vitro*, desenvolvida no Laboratório de Reparação de DNA de Eucariotos da UFRGS, e outro capítulo onde são apresentados os resultados correspondentes à segunda fase, realizada *in vivo,* desenvolvida no Laboratório de Fisiologia da Respiração da UFRJ. A seguir, são apresentadas uma discussão geral, as conclusões (geral e específicas), as perspectivas, as referências bibliográficas e os anexos.

A introdução geral apresenta generalidades sobre o carvão, o carvão na geração de eletricidade, reservas de carvão ao redor do mundo e no Brasil. São também descritos os efeitos biológicos do carvão e das cinzas de carvão, as células envolvidas nas principais vias associadas com o desenvolvimento de transtornos pulmonares, as partículas de carvão-cinza e a formação de ERO e os agentes genotóxicos correlacionados ao carvão e cinzas.

O Capítulo 1 apresenta um trabalho manuscrito submetido para a revista "Environmental Science and Pollution Research", que abordou os efeitos genotóxicos das partículas de carvão e cinzas, usando o ensaio Cometa Alcalino e Modificado e o ensaio CBMN-Cyt na linhagem de fibroblastos de pulmão de hamster chinês V79.

O Capítulo 2 mostra resultados comparativos da inflamação pulmonar aguda, mecânica pulmonar, efeitos genotóxicos e translocação extrapulmonar em camundongos BALB/c expostos às partículas de carvão e cinzas.

Em seguida, uma discussão geral aborda os resultados desses dois capítulos, e a sua importância para a contribuição científica desse estudo. Por fim, são apresentados a conclusão final, as perspectivas do trabalho e os anexos.

Os Anexos 1 e 2 contêm publicações desenvolvidas durante o doutorado, mas que não estão diretamente relacionados ao presente trabalho, e que foram utilizados na discussão deste. O Anexo 1 contém o artigo cientifico intitulado "Genetic damage in coal miners evaluated by buccal micronucleus cytome assay", publicado na revista "Ecotoxicology and Environmental Safety", no qual foram avaliados os efeitos genotóxicos em células da mucosa oral e concentrações de elementos inorgânicos numa população exposta à resíduos de mineração na Guajira – Colômbia. O Anexo 2 é um capítulo de livro intitulado "Occupational Exposure to Coal, Genotoxicity and Cancer Risk", aceito pela revista "InTech - Environmental Health Risks", que apresenta uma ampla revisão sobre os mecanismos associados ao desenvolvimento de câncer e outras doenças respiratórias e a exposição ao carvão, incluindo diferentes abordagens e biomarcadores usados em outros estudos ao nível mundial.

INTRODUÇÃO

I. INTRODUÇÃO

1. O CARVÃO

1.1. GENERALIDADES

O carvão é uma rocha sedimentar que é composta de uma mistura de sustâncias químicas orgânicas constituídas por carbono, hidrogênio, nitrogênio, oxigênio e enxofre. É um mineral negro e brilhante, formado a partir da vegetação compactada entre os extratos da rocha que, ao ficar enterrada, foi alterada pelos efeitos combinados de pressão e calor durante milhões de anos. Isto gerou mudanças físicas e químicas na vegetação, transformando-a em turba e depois em carvão (Carbunión, 2009).

Tipos de carvão:

O índice de mudança sofrido por um carvão ao se transformar de turba até antracite, conhecido como carbonização, tem uma grande importância nas propriedades físicas e químicas, sendo denominado "nível do carvão". Os carvões de categoria baixa, como o lignite e os carvões sub-betuminosos, são normalmente mais macios e esmigalháveis, e têm um aspecto mate e terroso. Caracterizam-se por níveis de umidade altos e conteúdo em carbono baixo, e seu conteúdo energético também é baixo (Figura 1).

Figura 1. Tipos de carvões

Os carvões de nível alto são mais duros e resistentes, e normalmente têm uma cor mais negra e vítrea. Contém mais carbono, menos umidade e produzem mais energia. O antracite encontra-se no topo da escala, tem um conteúdo superior em carbono e energia, e um nível inferior de umidade (Carbunión, 2009; Cerrejón, 2013).

1.2. O CARVÃO NA GERAÇÃO DE ELETRICIDADE

O carvão é um recurso de grande utilidade em todo o mundo (Figura 2). O carvão térmico é utilizado nas centrais elétricas para gerar eletricidade e na atualidade fornece 41% da eletricidade de todo o mundo. Nas centrais elétricas, o carvão é moído antes da queima para que seja obtido um pó fino, o qual aumenta a área de superfície, fazendo que se queime mais rapidamente. Nestes sistemas de combustão de carvão esfarelado, o carvão em pó é insuflado na câmara de combustão de uma caldeira, onde o mesmo queima a uma alta temperatura. Os gases quentes e a energia calórica produzida convertem a água que passa por tubos que rodeiam a caldeira em vapor (Campbell et al., 2013).

O vapor de alta pressão é conduzido até a turbina, que contém milhares de pás tipo propulsor. O vapor pressiona estas pás, fazendo com que o eixo da turbina gire em grande velocidade. Há ainda um gerador montado num extremo do eixo da turbina, que consiste de várias bobinas de cabo. A eletricidade é gerada quando estas bobinas giram rapidamente num campo magnético forte. Depois de passar pela turbina, o vapor condensa e regressa à caldeira para voltar a ser aquecido (Figura 2).

A eletricidade gerada transforma-se em alta tensão (até 400.000 volts), que é utilizada para uma transmissão econômica e eficaz através das linhas de alta tensão (Carbunión, 2009; Planete-energies, 2015).

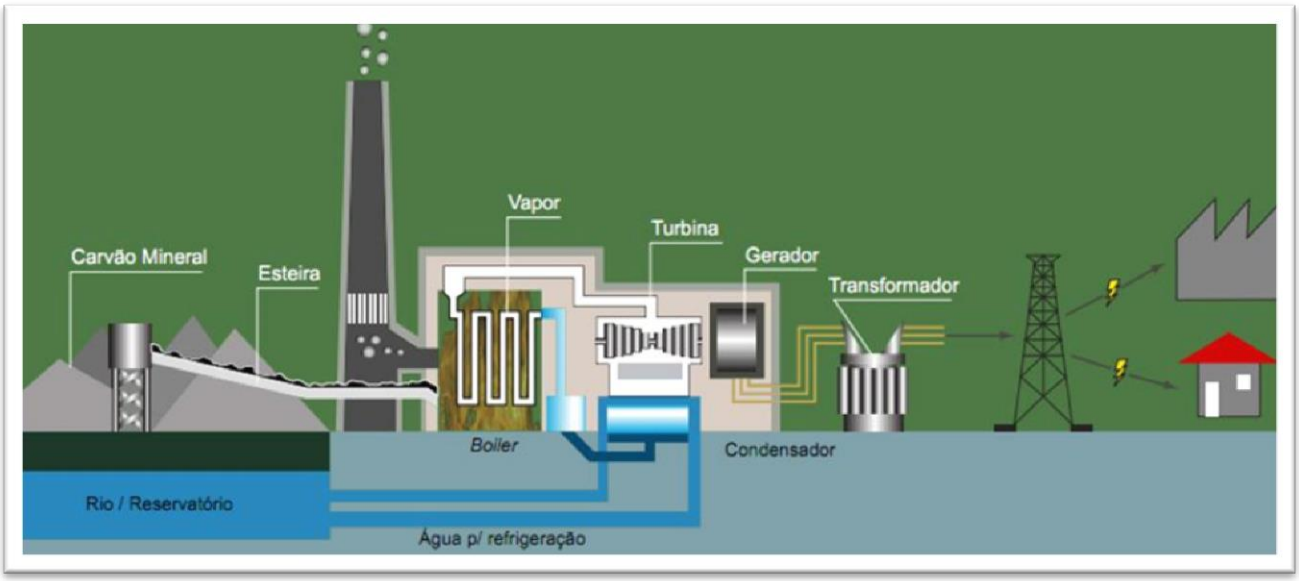


Figura 2. Conversão do carvão em eletricidade. Adaptado de Carbunión (2009).

Outros usos do carvão: o carvão é usado na produção de cimento, e é essencial para a produção de ferro e aço. Perto de 64% da produção de aço em todo o mundo provém do ferro fundido em altos fornos que utilizam carvão. Em vários países, o carvão é convertido em um combustível líquido cujo processo se denomina liquefação. O combustível líquido pode ser refinado para produzir combustível de transporte e outros produtos derivados do petróleo, como plásticos e solventes (Campbell et al., 2013).

O carvão também é utilizado em refinarias de alumínio, fábricas de papel e as indústrias farmacêuticas. Adicionalmente, os sub-produtos do carvão são utilizados como componentes para sabonetes, aspirinas, solventes, corantes, plásticos e fibras, como o raiom e o nylon (Cerrejón, 2013).

2. O CARVÃO NO MUNDO

As reservas mundiais totalizam 847,5 bilhões de toneladas, quantidade suficiente para atender a produção atual por 119 anos (Cerrejón, 2013). De acordo com dados da International Energy Agency (IEA), o carvão é a fonte mais utilizada para geração de energia elétrica no mundo por meio de usinas termelétricas. Em segundo lugar, está a aplicação industrial para a geração de calor (energia térmica) (Burt et al., 2013; International Energy Agency, 2016).

Como é mostrado na Figura 3, existem reservas de carvão em mais de 70 países, em todos os continentes, mas as maiores reservas estão localizadas na China, Estados Unidos, Rússia e Índia (BP, 2015).

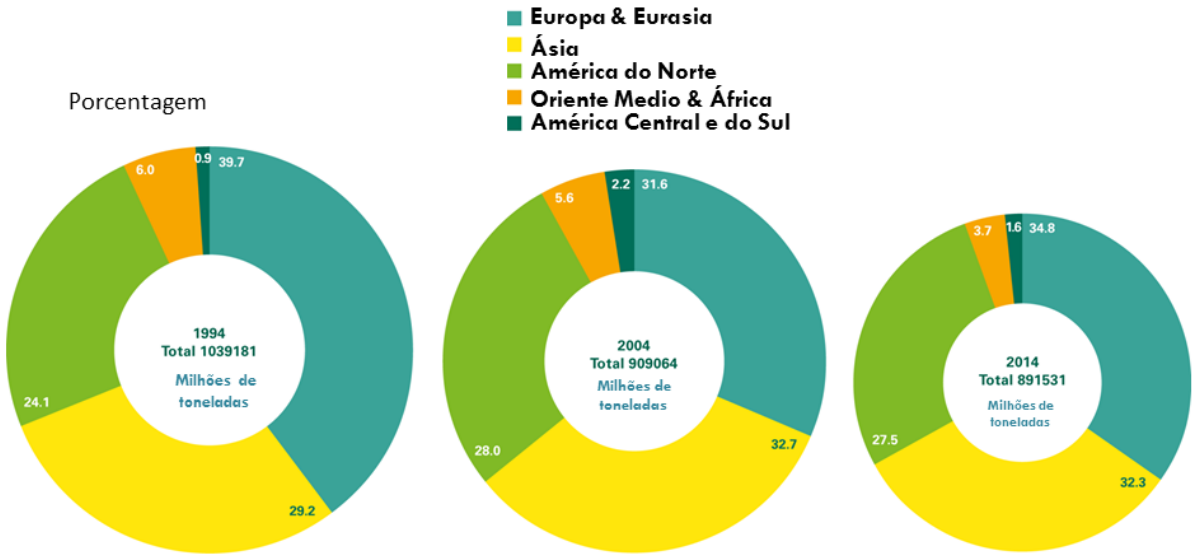


Figura 3. Distribuição das reservas de carvão em 1994, 2004 e 2014. Adaptado de BP: British Petroleum Statistical Review of World Energy (2015).

Segundo dados da *British Petroleum* (BP) (2015), o consumo mundial do carvão cresceu 0,4% em 2014. A Figura 4 mostra a distribuição do consumo mundial de carvão mineral, medida em tonelada equivalente de petróleo (tep), utilizada na mensuração do poder calorífico (BP, 2015).

Figura 4. Consumo mundial de carvão mineral – 2014 (em Mtep). Adaptado de BP. British petroleum (2015).

2.1. RESERVAS NO BRASIL

As reservas brasileiras são compostas pelo carvão dos tipos lignite e sub-betuminoso. No Brasil, o carvão é encontrado principalmente nos estados do Rio Grande do Sul, Santa Catarina, Paraná (ABCM, 2015). Do volume de reservas, o Rio Grande do Sul responde por 89,25%; Santa Catarina, 10,41%; Paraná, 0,32% e São Paulo, 0,02%, como mostrado na Figura 5.

A maioria do carvão rio-grandense é do tipo betuminoso alto volátil C, enquanto o carvão catarinense é do **tipo betuminoso alto volátil A**, considerado de melhor qualidade (Aneel, 2009; ABCM, 2012).

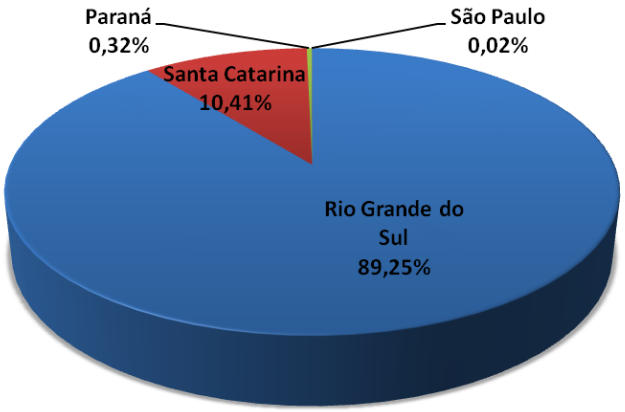


Figura 5. Volume de reservas no Brasil. Adaptado: Agência Nacional de Energia Elétrica (Aneel, 2009).

A abundância das reservas e o desenvolvimento de tecnologias de "limpeza" e combustão eficiente, conjugados à necessidade de expansão dos sistemas elétricos e restrições ao uso de outras fontes, indicam que o carvão mineral continuará sendo, por muitas décadas, uma das principais fontes de geração de energia elétrica no Brasil. Estima-se que 85% do carvão utilizado no Brasil é consumido na produção de termoeletricidade, 6% na

indústria cimenteira, 4% na indústria de papel celulose e os restantes 5% nas indústrias de cerâmica, de alimentos e secagem de grãos (Aneel, 2009).

2.1.1. Carvão de Santa Catarina

No sul do Brasil, os estados de Santa Catarina e Rio Grande do Sul possuem importantes jazidas de carvão. A bacia de Santa Catarina é uma bacia intracratônica, na qual 1.200 m de rochas sedimentares e vulcânicas foram depositadas em uma base gnáissico-granítico (Silva et al., 2009; ABCM, 2012).

Um constituinte importante do carvão de Santa Catarina é a **PIRITA**. Pirita é um mineral cobre-amarelado com um lustre metálico brilhante. Ela apresenta em sua composição química disulfeto de ferro (FeS_2) e é o mineral de sulfeto mais comum. Ocorre geralmente em pequenas quantidades em rochas ígneas, metamórficas e sedimentares (Geology, 2005). A pirita exposta ao meio ambiente durante o processo de mineração e escavação pode reagir com oxigênio e água, produzindo ácido sulfúrico e lixiviando o solo. Sua oxidação é exotérmica, podendo levar à combustão espontânea dos depósitos de rejeitos nas minas de carvão o qual polui o ar e leva à formação de chuva ácida (Hurlbut, 1985, Peterson, 2008).

A maioria do carvão de Santa Catarina é extraído de minas subterrâneas e é usado quase exclusivamente para geração de energia elétrica pelo **complexo termelétrico (Tractebel Suez) Jorge Lacerda** (Figura 6) (Silva and da Boit, 2011).

Este complexo termelétrico (Figura 6) é a maior planta de energia de carvão de América do Sul, gerando 875MW/h de eletricidade com um consumo de 200000 tons de carvão/mês.

Figura 6. Localização da área de mineração de Santa Catarina e o complexo termelétrico (Tractebel Suez) Jorge Lacerda. Adaptado de Quispe et al., (2012).

A usina citada acima produz 80.000 tons/mês de cinzas, das quais 80% são cinzas voláteis e 20% são cinzas pesadas. Essa usina é uma das maiores produtoras de cinzas do mundo (Silva et al., 2009). A usina queima carvões com conteúdo de enxofre menor que 2% do total de S, com 40% de cinzas voláteis e está equipado com queimadores low-NOx. Cerca de 98,5% da cinza volátil é capturada por meio de precipitadores eletrostáticos (Silva et al., 2010; Quispe et al., 2012).

2.2. IMPACTOS DO USO DO CARVÃO

O carvão é uma das formas de produção de energia mais agressivas ao meio ambiente. Ainda que sua extração e posterior utilização na produção de energia gerem benefícios econômicos (como empregos diretos e indiretos, aumento da demanda por bens e serviços na região e aumento da arrecadação tributária), o processo de produção, da extração até a combustão, provoca significativos impactos socioambientais (Aneel, 2009; Burt et al., 2013).

A ocupação do solo exigida pela exploração das jazidas, por exemplo, interfere na vida da população, nos recursos hídricos, na flora e fauna locais, ao provocar barulho, poeira e erosão. O efeito mais severo, porém, é o volume de emissão de gases como o nitrogênio (N) e dióxido de carbono (CO_2) provocado pela combustão. Estimativas apontam que o carvão é responsável por cerca de 30% do total de emissões de CO_2, sendo o principal agente do efeito estufa (Dai et al., 2012; World Coal Association, 2016).

No sul do Brasil existe uma considerável preocupação pela emissão de material particulado e a baixa eficiência dos sistemas de controle dessas partículas finas (Teixeira et al., 2009). As atividades de processamento do carvão tem sido causa de alterações na qualidade do ar de algumas áreas nos estados de Santa Catarina e Rio Grande do Sul. Além disso, o depósito desmesurado de cinzas a céu aberto pode expor as cinzas pesadas à lixiviação e liberar uma série de contaminantes ao meio ambiente (Silva and da Boit, 2011).

2.3. SUBPRODUTOS DO CARVÃO: CINZAS DE CARVÃO

Antes da combustão, o carvão é pulverizado em moinhos. Posteriormente, com ou sem combustíveis secundários, é injetado em forno mediante uma corrente de ar quente a alta velocidade, e, estando em suspensão, é queimado a uma temperatura de 1500 ± 2000 °C, que está acima do ponto de fusão da maioria dos minerais presentes (Heidrich et al., 2013).

A combustão do carvão produz resíduos classificados como **escórias, cinzas de fundo (pesadas) e cinzas voláteis (leves)**. As partículas mais finas (cinzas voláteis ou também chamadas de *coal fly ash*) são obtidas por precipitação mecânica ou eletrostática do pó em suspensão presente nos gases procedentes da combustão, enquanto que as mais grossas (pesadas) caem ao fundo das caldeiras por gravidade

(Martinez, 2012; EPA, 2015). As reações que se originam durante a combustão dependem do tipo de carvão (grau de carbonização, origem geológica), do tamanho do grão do pó, do tipo de caldeira usado e das condições do processo de combustão (Martinez, 2012).

As cinzas voláteis compõem-se principalmente por Sílica (SiO_2-S), Alumínio (Al_2O_3-A), óxido férrico (Fe_2O_3- F), cal (Ca O-C), carvão sem queimar e em menor proporção (cerca do 5% em peso): magnésio (MgO), óxido de enxofre (SO_3), alcalinos (Na_2O e K_2O e outros elementos traços) (Caballero & Médico, 2013).

3. EFEITOS BIOLÓGICOS DO CARVÃO E AS CINZAS VOLÁTEIS DE CARVÃO (COAL FLY ASH-CFA).

O material particulado (PM) que é gerado na mineração e na combustão do carvão é caracterizado pelo tamanho. Estas partículas são classificadas de acordo com o seu diâmetro aerodinâmico (em micras) em partículas grossas (PM10), finas (PM 2,5), ultrafinas (PM 0,1) e nanopartículas (Cassee et al., 2013; EPA, 2015). Em geral, as partículas PM10 depositam-se nas vias aéreas superiores e são eliminadas pelo *clearance mucociliar* ou por processos mecânicos (como tosse ou espirro) e as partículas PM 2,5, PM 0,1 e nanopartículas (partículas respiráveis) são mais propensas a atingir o parênquima pulmonar (Mazzoli-Rocha et al., 2010).

Estas partículas, após serem geradas, são quase totalmente espalhadas na atmosfera. Devido ao tamanho tão pequeno, estas frações permanecem voando por longos períodos de tempo, no qual podem ser inaladas pelos trabalhadores expostos ocupacionalmente (CDC-NIOSH, 2012). Dependendo da sua toxicidade, propriedades químicas e concentração no ar, as partículas de CFA podem constituir um risco para os trabalhadores expostos. Quando as partículas de CFA são inaladas e depositam-se no pulmão, podem gerar efeitos na saúde pela lixiviação de compostos genotóxicos e alterações dos mecanismos imunológicos, os quais afetam o parênquima pulmonar, levado a doenças como bronquite, asma e até câncer (Carbone et al., 2009).

A toxicidade das partículas de carvão e cinzas geralmente aumentam com a diminuição do tamanho da partícula e com a mistura de agentes orgânicos e inorgânicos que contribuem na composição das partículas, como os metais e hidrocarbonetos aromáticos policíclicos (HAP) (Bonner, 2004; Donaldson et al., 2005; Sambandam et al., 2015).

Especificamente, como a carga de partículas no pulmão aumenta, os macrófagos alveolares e células epiteliais são ativados e isso conduz à liberação de mediadores inflamatórios, ERO, enzimas (elastases, proteases, colagenases), citocinas (TNF-α, MIP-1) e fatores de crescimento (TGF-β) que controlam e estimulam a fibrose, os eventos genotóxicos e a morte celular (Borm, 1997; Gilmour et al., 2004; Sambandam et al., 2015).

4. AGENTES GENOTÓXICOS CORRELACIONADOS AO CARVÃO E CINZAS

4.1. Metais pesados

O carvão contém diversas quantidades de elementos traço e metais na sua composição global, uma vez que é formado pela matéria orgânica comprimida que contém praticamente todos os elementos da tabela periódica, incluindo **metais pesados.** Designa-se metal pesado ao grupo de elementos que ocorrem em sistemas naturais em pequenas concentrações, apresentam densidade igual ou acima de 5 g/cm^3 e são elementos que possuem número atômico maior do que 23 (Jaishankar et al., 2014).

O conteúdo de metais pesados no carvão varia segundo a jazida de carvão e a região geográfica. Uma variedade de produtos químicos (na sua maioria metais) estão associados com o carvão, seja no carvão diretamente ou nas camadas de rocha que estão por cima e entre suas jazidas (Goodell, 2006).

Muitos dos metais liberados na mineração e na queima de carvão são elementos biologicamente tóxicos. Alguns dos elementos químicos mais comumente encontrados nos resíduos de carvão e no carvão incluem: Alumínio, Antimônio, Arsênio, Bário, Berílio, Cadmio, Cálcio, Cromo, Cobalto, Cobre, Ferro, Chumbo, Magnésio, Manganês, Mercúrio, Molibdênio, Níquel, Potássio, Selênio, Prata, Sódio, Estrôncio, Estanho, Tório, Vanádio, Zinco (Goodell, 2006; Toppin, 2009).

MECANISMOS DE GENOTOXICIDADE DOS METAIS: Apesar das diversas propriedades físico-químicas dos metais, vários mecanismos predominantes sobressaem e parecem ser comuns a alguns metais: (1) inibição do sistema de reparo do DNA, resultando em instabilidade genômica e acúmulo de mutações; (2) inibição de defensas antioxidantes; (3) ativação de sinalização mitótica (4) modulação de expressão gênica (Figura 7). (Beyersmann and Hartwig, 2008; Leffa & Andrade, 2009).

A atividade biológica e a toxicidade de alguns metais também são influenciadas em grande parte por sua capacidade de mudar seu estado de oxidação pela oxidação (perda de elétrons) e redução (ganho de elétrons). Os metais de transição são eletronicamente estáveis em mais de um estado de oxidação. Como resultado desta propriedade, os metais de transição desempenham importantes papeis na catálise de reações de oxidação biológica. Metais de transição como Fe e Cu têm sido amplamente estudados devido ao seu envolvimento em muitos processos de doenças pulmonares e não pulmonares, em virtude da sua capacidade de aumentar a produção de ERO. A disponibilidade biológica e a absorção de metais são influenciadas em grande medida pela sua solubilidade em água e lipídeos, mas, ainda mais importante pela sua solubilidade em fluidos biológicos, que contêm uma variedade de ligantes orgânicos, os quais facilitam o seu transporte pela circulação sanguínea (Bresgen and Eckl, 2015).

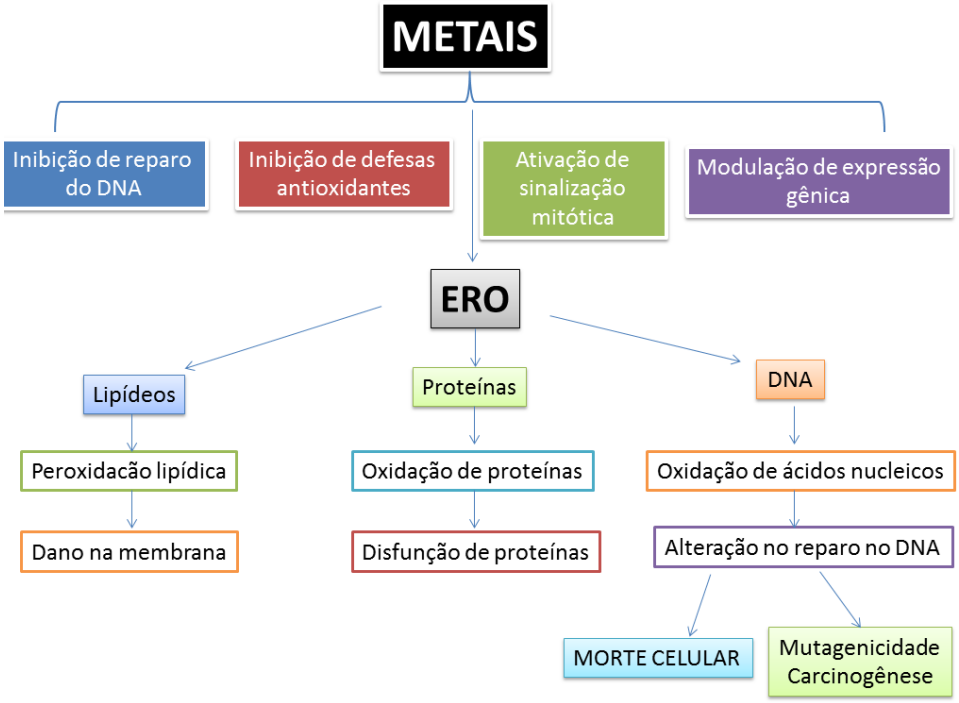


Figura 7. Principais mecanismos de carcinogenicidade de metais. Adaptado de Ercal et al., (2001); Jaishankar et al., (2014).

Sabe-se que os metais redox ativos como o Fe, Cu, Cr, Co e outros sofrem reações cíclicas redox e possuem a capacidade de produzir radicais ativos tais como o radical ânion superóxido e óxido nítrico em sistemas biológicos (Jomova & Valko, 2011).

Além disso, a reação oxidativa de Fenton depende da catálise de metais, principalmente de Fe, assim como também de Co, Cr e V. Via reação de Fenton, são produzidos radicais altamente reativos tais como o radical hidroxila (OH•), o qual surge como um produto da reação entre superóxido, e H_2O_2, catalisado principalmente por Fe2+ ou Fe3+ (Jomova & Valko, 2011; Sharma et al., 2012).

Reação de Fenton ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\bullet + HO^-$).

Por outro lado, os metais redox inativos, tais como Cd, As, Hg e Pb mostram seus efeitos tóxicos pela união de grupos sulfidrila das proteínas e a depleção da glutationa (Jomova & Valko, 2011). Como consequência da geração de oxidantes, acontece aumento da peroxidacão lipídica, dano no DNA, e homeostase alterada do cálcio e sulfidrila (Angelé-Martínez et al., 2014). Estudos relatam que a atividade da proteína de reparo O^6–alquilguanina-DNA alquiltransferase é inibida por um grande número de íons metálicos, que incluem Cd, Cu, Hg, Zn e Ag. Esta inibição é provavelmente devida à interação dos metais com os resíduos de cisteína na proteína (Hernández-Franco et al., 2009).

Pesquisas sugerem que a exposição ao Cd resulta em uma diminuição dos níveis de expressão da 8-oxoguanina DNA glicosilase (OGG1), como resultado da atenuação na união do fator de transcrição SP1 ao promotor de hOGG1 (Giaginis et al., 2006), enquanto que o Cr, dependendo da concentração, inibe a expressão de OGG1 em células humanas. Também se sabe que o Cr induz a inibição ou ativação da DNA polimerase como resultado da formação de adutos DNA-DNA (Hodges and Chipman, 2002). O Cd e o Ni suprimem a remoção do dano oxidativo no DNA que é reconhecido pela Formamidopirimidina-DNA glicosilase (Fpg), o que sugere a sua inibição (Hartwig et al., 2002).

Adicionalmente, as células fagocíticas podem ser outra fonte importante de ERO em resposta a íons metálicos. Vários estudos têm sugerido que a capacidade de gerar ERO por ciclo redox de quinonas e compostos relacionados, podem requerer íons metálicos. Os íons metálicos podem aumentar a produção do fator de necrose tumoral alfa (TNF-a) e ativar a proteina cinase C, assim como induzir a produção de proteínas de estresse (Knaapen et al., 2004; Bonner., 2007; Beyersmann and Hartwig, 2008). Por tanto, alguns dos mecanismos associados com a toxicidade de íons metálicos são muito similares aos efeitos produzidos por muitos xenobióticos orgânicos. As diferenças específicas na toxicidade de íons metálicos podem estar relacionadas com diferenças na solubilidade, capacidade de absorção, transporte, reatividade química e complexos que se formam dentro do organismo (Ercal et al., 2001; Jaishankar et al., 2014).

4.2. Hidrocarbonetos Aromáticos Policíclicos (HAP)

A combustão do carvão resulta num aumento na concentração de poluentes, tais como o dióxido de enxofre, óxidos de nitrogênio, mercúrio e HAP na atmosfera (Chen et al., 2004; Micic et al., 2007). As combustões espontâneas que acontecem nos centros de armazenamento do carvão convertem-se em uma das principais fontes de geração de HAP. Dentre os HAP relacionados com mineração do carvão, estão benz[a]antraceno, crisene, benzo[a]pireno, indeno[1,2,3-c,d]pireno e benzo[g,h,i]perileno (Achten and Hofmann, 2009). Muitos destes são liberados para a atmosfera, onde formam misturas complexas que são potencialmente perigosas se são levados em conta os efeitos aditivos e sinérgicos.

Os HAP constituem uma família de compostos caracterizada por possuírem dois ou mais anéis aromáticos condensados (Figura 8). Estas substâncias, bem como os seus derivados nitrados e oxigenados, têm ampla distribuição e são encontrados como constituintes de misturas complexas em todos os compartimentos ambientais (Achten & Hofmann, 2009).

Dezesseis dos HAP existentes são particularmente tóxicos e cancerígenos, e por isso são definidos pela Agencia de Proteção Ambiental de Estados Unidos (US EPA) como poluentes prioritários (Laumann et al., 2011). Os seres humanos encontram-se expostos pela inalação, ingestão e absorção dérmica. A ligação entre a exposição humana às misturas complexas de HAP e o desenvolvimento de doenças como o câncer, e doenças respiratórias e cardiovasculares têm sido descritas (ATSDR, 1995; Pope et al.,

2002; IARC, 2010). Apesar das similaridades estruturais, os HAP podem variar muito no seu potencial cancerígeno. A IARC classificou alguns destes compostos como carcinogênicos (grupo 1) ou provável carcinógeno (grupo 2A) para humanos, tendo como exemplo benzo[*a*]pireno e dibenzo[*a,h*]antraceno, respetivamente (IARC, 2010).

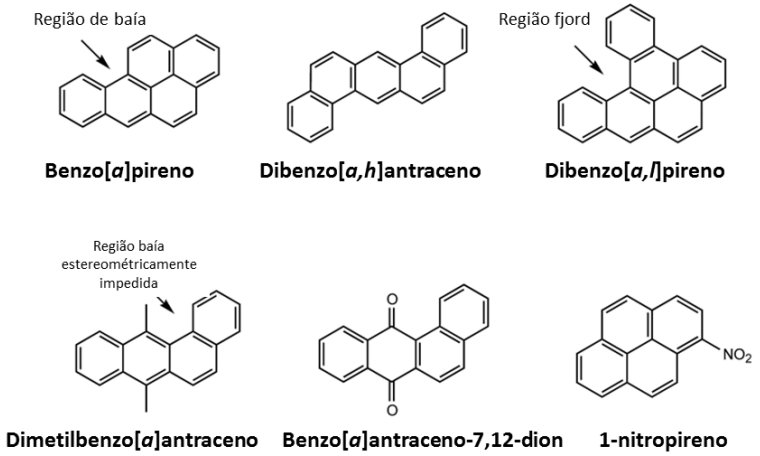


Figura 8. Exemplos de alguns HAP comumente estudados. Adaptado de Jarvis et al., (2014).

Quando os HAP são absorvidos no corpo, são metabolizados por vários órgãos (incluindo o fígado, rins, pulmões), são excretados na bílis, urina ou leite materno e armazenados em uma quantidade limitada no tecido adiposo. As principais vias de exposição são inalação, ingestão e contato dérmico. A característica lipofílica dos HAP permite a fácil penetração nas membranas celulares (Yu et al., 2011). O metabolismo posterior torna os HAP mais solúveis em água, tornando mais fácil que sejam eliminados pelo corpo. Porém, os HAP também podem ser convertidos em metabólitos mais tóxicos ou carcinogênicos (Jarvis et al., 2014).

Após a exposição, estas moléculas induzem a expressão de enzimas metabolizadoras da fase I e II, incluindo redutases aldo-cetona, citocromo P-450, catechol-O-methyltransferase, epóxido hidrolase, peroxidases, glutathiona S-transferases, N-acetyltransferases, sulfotransferases e outras enzimas que catalisam reações de conjugação (Jarvis et al., 2014).

HAP, MECANISMOS DE DANO NO DNA: os HAP sofrem ativação metabólica para diol-epóxidos, os quais ligam-se covalentemente ao DNA. Depois, eles formam adutos ou induzem estresse oxidativo que geram mutações. Se os mecanismos de reparo do DNA são alterados pela taxa de formação de adutos, o resultado é uma acumulação de mutações no DNA que podem levar a carcinogênese. Na figura 9 é mostrado um exemplo da formação de adutos com o DNA depois da exposição a benzo[a]pireno. Vários estudos indicam que o número de adutos formados está relacionado com o grau de exposição ao HAP. De fato, tem sido descrito que a

exposição a HAP ativa os genes envolvidos na apoptose, controle do ciclo celular e reparo do DNA (Castorena-Torres et al., 2008). Além disso, estudos moleculares têm revelado que adutos no DNA bloqueiam a atividade da polimerase na replicação, contribuindo para o aumento de danos no DNA pela redução da atividade de reparação (Hsu et al., 2005).

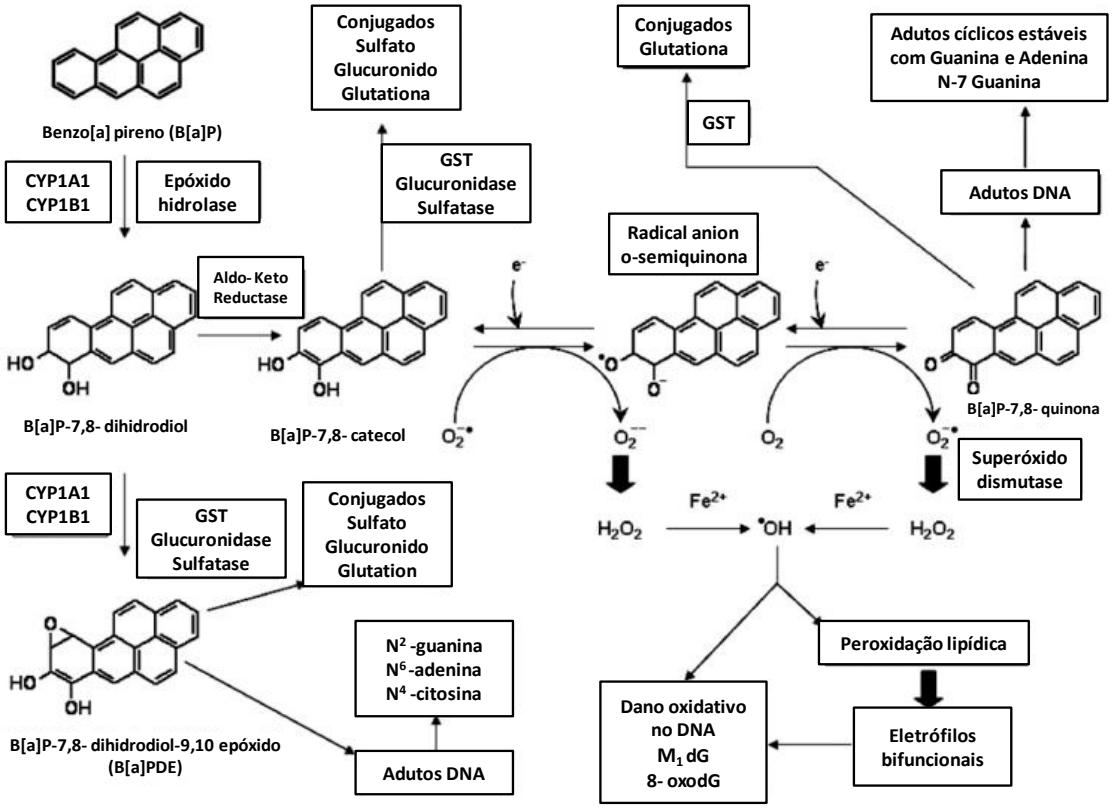


Figura 9. Vias do metabolismo, geração de ERO e formação de adutos com o DNA depois da exposição a benzo[a]pireno. Adaptado de Singh et al., (2007).

Tem sido relatado que derivados BaP possuem a capacidade para entrar nos ciclos redox e induzir a produção de ERO, causando o estresse oxidativo (An et al., 2011). Os radicais-cations BaP são precursores de 6-OH-de BAP. A auto-oxidação deste derivado pode resultar na formação de BAP quinonas, tais como 6, 12, e 1,6-e 3,6-BAP diol (Briedé et al., 2004; Singh et al., 2007) (Figura 17). Estes metabólitos podem sofrer ciclo redox para seus correspondentes BAP dióis e produzir ERO como o superóxido, que são então convertidos em radicais hidroxila através da reação de Haber-Weiss. Os radicais livres reagem com guanina e causam danos no DNA, incluindo a produção de 7-hidro-8-oxo-20-desoxiguanosina (8-oxo-dG) (Bonner et al., 2005; Singh et al., 2007).

A falha dos mecanismos de reparação e constante exposição à HAP podem induzir mutagênese nas células. Estas mutações estão presentes em vários genes, incluindo os que participam na sobrevivência celular. Em particular, mutações em p53 estão associadas com risco de carcinogênese em indivíduos expostos a HAP. Uma vez que a proteína p53 é um fator de transcrição que regula proliferação celular, diferenciação, apoptose e reparação do DNA, as mutações induzidas nesta importante

proteína podem levar a graves danos nas células e genes (Mordukhovich et al., 2010). Na Figura 10, estão resumidos os tipos de danos causadas por misturas complexas de HAP.

Carcinogênese dos HAP: a formação de adutos em genes relacionados com a reparação do DNA não é o único mecanismo por meio do qual os HAP podem induzir carcinogênese. Um perigo adicional da exposição aos HAP é a sua semelhança com hormônios esteróides, o qual permite aos HAP a capacidade de ativar os receptores de estrogênio (ER) e do metabolismo (Bekki et al., 2013).

No entanto, o mecanismo mais importante de carcinogênese é um sistema de reparo do DNA deficiente em genes chaves envolvidos no controle do ciclo celular. A exposição crônica à HAP está relacionada com uma alta taxa de mutagênese e é provável que o dano no DNA seja acumulativo. Vários estudos têm associado à exposição crônica ocupacional dos HAP a vários tipos de câncer, incluindo o câncer de bexiga, pulmão, rim, fígado e mama (Shen et al., 2006; Karami et al., 2011; Muñoz & Albores, 2011).

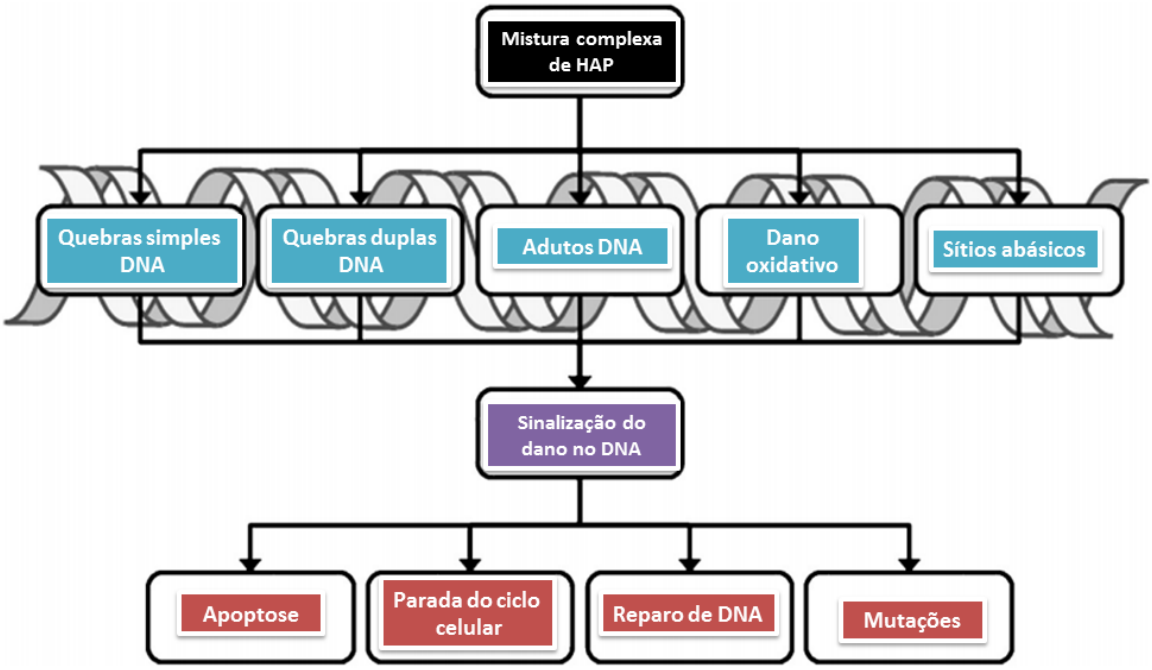


Figura 10. Danos genotóxicos causadas por misturas complexas de HAP. Uma grande variedade de danos de DNA, incluindo quebras simples, formação de adutos, dano oxidativo e sítios abásicos resultantes de depurinação podem ocorrer depois da exposição a misturas complexas de HAP. A resposta a este dano estimula a sinalização de dano ao DNA. O Dano no DNA tolerável pode levar a outras respostas, incluindo a parada do ciclo celular e reparação do DNA, enquanto que o dano ao DNA excessivo/irreparável pode levar à apoptose. Ambos os cenários são considerados respostas positivas, uma vez que impedem o processo cancerígeno. As mutações podem ocorrer como resultado de danos no DNA não reparados ou indevidamente reparados,

ou erros na transdução de sinal, e são um resultado negativo ao promover e acelerar o processo carcinogênico. Adaptado de Jarvis et al., (2014).

4.3. Sílica (Quartzo)

O silício (Si) é o segundo elemento mais abundante na terra, ao lado do carbono. Um átomo de Si combinado com dois átomos de oxigênios forma dióxido de silício ou sílice (SiO_2). O dióxido de silício é o componente principal da areia. O quartzo é uma das formas cristalinas do silício existente na natureza, que pode também ser encontrada em outras formas cristalinas, tais como a tridimita, a cristobalita e a trípoli, ou na forma amorfa como a sílica gel ou a sílica coloidal. A sílica livre cristalizada, cuja forma mais conhecida é o quartzo, é a sílica cristalina não combinada com nenhum elemento químico (Mason and Thompson, 2010).

É conhecido que o pó de carvão contém até 10% de quartzo. O quartzo faz parte de uma matriz de carbono que influencia claramente as propriedades intrínsecas tanto do carvão quanto das cinzas. A presença de quartzo nas cinzas de carvão pode ser atribuída aos resíduos de grãos de quartzo no carvão que não sofreram fusão durante o processo de combustão. A presença de quartzo nas cinzas é menor que no carvão mineral, sugerindo que algo do quartzo reagiu durante a combustão e foi incorporado dentro da matriz vítrea (Silva et al., 2010).

A inalação da forma cristalina da sílica tem sido historicamente associada com o desenvolvimento de uma severa doença respiratória, a silicose, a qual é uma pneumoconiose pulmonar caracterizada pela proteinose alveolar e fibrose difusa resultante na função pulmonar restritiva progressiva (Hamilton et al., 2008). Existe evidência que a exposição à sílica pode estar associada ao desenvolvimento de doenças autoimunes, tais como esclerodermia (esclerose sistêmica), artrite reumatoide, enfermidade renal crônica e lúpus, enquanto que algumas formas de sílica cristalina podem causar câncer de pulmão (IARC, 1997; Pelucchi et al., 2006). O mecanismo de ação molecular ainda não está claro e não está esclarecido se um único mecanismo constitui a base de todas as doenças antes mencionadas. Porém, a inflamação severa, produto da exposição crônica às partículas de sílica, parece ser um passo comum na patogênese delas (Hamilton et al., 2008).

5. CÉLULAS ENVOLVIDAS NAS PRINCIPAIS VIAS ASSOCIADAS COM O DESENVOLVIMENTO DE TRANSTORNOS PULMONARES

A importância do papel das partículas de carvão e cinzas em doenças do trato respiratório, assim como em outros órgãos alvo, e a sua relação com alguns tipos de câncer como, por exemplo, o câncer gastrointestinal, é objeto de debate permanente há décadas (Jenkins et al., 2013; Diabaté et al., 2011).

Dados sobre os efeitos dos pós e cinzas de carvão têm sido obtidos de estudos *in vitro* (Voelkel et al., 2003; Diabaté et al., 2011) e estudos em animais (Gilmour et al., 2004; Magnani et al., 2013). Porém, estudos epidemiológicos ou de biomonitoramento de trabalhadores de carvão constituem uma fonte de dados de extrema importância na avaliação do risco e dos efeitos da exposição ocupacional a partículas de carvão (Rohr et al., 2013; León-Mejia et al., 2014).

Os mecanismos para explicar o destino das partículas nas vias respiratórias têm sido revisados em várias pesquisas (Linak et al., 2007; Cohen et al., 2008). Fatores importantes incluem propriedades intrínsecas da partícula (químicas e morfológicas), assim como fatores no hospedeiro, que incluem o volume pulmonar, taxa de respiração e profundidade. Estudos em animais e também em biopsias humanas têm sido a base de um contínuo debate sobre os parâmetros cruciais da toxicidade da partícula, a qual inclui aspectos como a durabilidade da partícula e a lixiviação, deposição de partículas, translocação e remoção de partículas através da linfa mucociliar e intersticial (nodos linfáticos) (Knaapen et al., 2004; Schmid et al., 2009).

A deposição de partículas de carvão ou cinzas atingem duas células alvo principais: macrófagos e células epiteliais. Como consequência, outras células, como também componentes intersticiais, podem ser afetados (Schins and Borm, 1999; Knaapen et al., 2004) como é mostrado na Figura 11.

5.1. Macrófagos

Os macrófagos são células fagocíticas mononucleadas presentes no tecido conectivo dos vertebrados. São células extremamente versáteis devido ao papel que têm na apresentação e processamento de antígenos, na produção de moléculas com atividade biológica, por exemplo, citocinas e fatores de crescimento, e no metabolismo de lipídeos. Estas células caracterizam-se pela sua capacidade de fagocitar e degradar material particulado (Hirota and Ter, 2012).

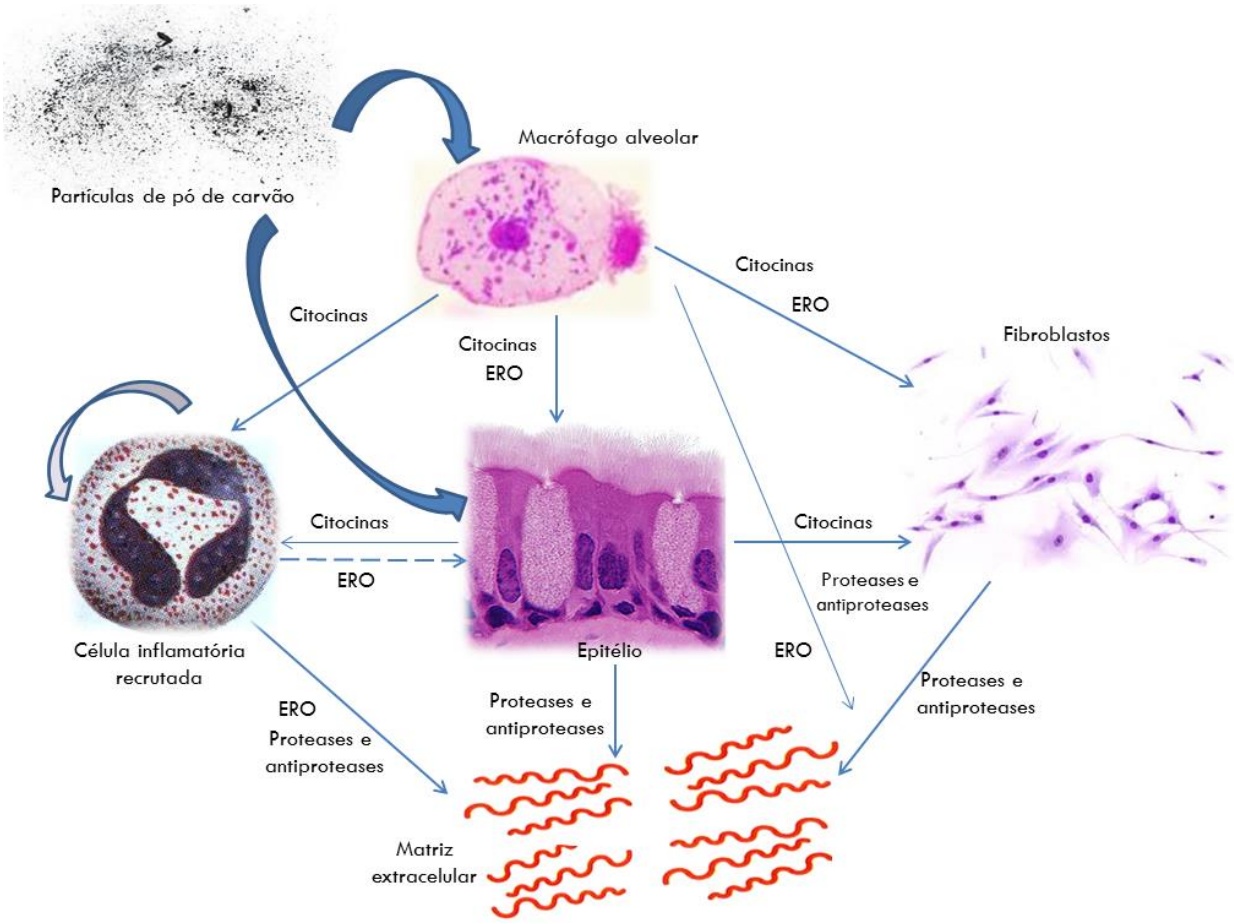


Figura 11. Principais vias associadas com o desenvolvimento de transtornos pulmonares induzidos por partículas de carvão. As células alvo primárias de partículas de pó de carvão inaladas são macrófagos e células epiteliais. Os macrófagos ativados (fagocitose, toxicidade) produzem quantidades excessivas de espécies reativas do oxigênio (ERO) e citocinas. As ERO podem ser adicionalmente geradas por mecanismos independentes à via celular devido a propriedades químicas intrínsecas de partículas de carvão (por exemplo presença de radicais na superfície ou de ferro). As células epiteliais e fibroblastos, os quais são os principais produtores de componentes da matriz extracelular (ECM), incluindo colágeno, proteoglicanos e fibras elásticas, são também conhecidos por produzir citocinas e ERO depois da estimulação. Adicionalmente, as células fagocíticas (neutrófilos, monócitos/ macrófagos) podem ser recrutados mediante quimiocinas produzidas pelos macrófagos alveolares ou também células epiteliais, e podem amplificar a produção local de ERO e citocinas. Ambas, ERO e citocinas, podem causar dano ou proliferação do tecido epitelial local e mesenquimal, o que pode ter consequências na morfologia do tecido pulmonar e deposição de componentes da ECM. A formação e degradação da ECM podem também ser afetadas por ERO e pelas proteases e antiproteases produzidas por vários tipos de células no tecido circundante. O tecido local também contém fatores antioxidantes para fazer frente a ERO, tal como no fluido de recobrimento epitelial e nas células alvo, e citocinas antagonistas presumivelmente atuando como mecanismos *feedback* anti-inflamatório. Adaptado de Schins & Borm, (1999).

Os macrófagos alveolares são considerados células chaves em relação às doenças pulmonares induzidas pelo pó mineral, tais como a pneumoconiose, silicose ou asbestose (Castranova and Vallyathan, 2000). O importante papel dos macrófagos alveolares (e intersticiais) na eliminação de partículas baseia-se em várias observações, como o fato do número de macrófagos alveolares nos pulmões aumentar em pessoas e animais cronicamente expostos a partículas minerais, a fagocitose resultar em um aumentado estado de ativação do macrófago e, durante a sua ativação, uma ampla gama de produtos incluindo oxidantes, lipídeos bioativos, citocinas, fatores de crescimento, proteases e antiproteases poderem ser liberados. Várias células pulmonares podem atuar como alvos para estes mediadores, incluindo fibroblastos, células epiteliais e células endoteliais, e, além disso, todos estes tipos de células podem liberar mediadores secundários (Castranova & Vallyathan, 2000) (Figura 7). Por último, a fagocitose de macrófagos representa a fase lenta de remoção de partículas, que pode ser saturada rapidamente, causando uma sobrecarga de partículas (Oberdörster, 1995) e afetando grandemente o parênquima pulmonar.

5.2. Células epiteliais

O epitélio das vias respiratórias é uma barreira mucosa pseudo-estratificada, que consiste em vários tipos de células situadas de forma adjacente, fortemente aderidas entre si, com escassa matriz extracelular e relacionadas com o tecido conjuntivo através da membrana basal (Fröhlich & Salar-Behzadi, 2014). O tecido epitelial constitui uma barreira de defesa contra as agressões ambientais e a infecção, proporcionando não somente uma barreira mecânica e física para impedir a entrada de partículas estranhas, mas também por sua capacidade para orquestrar as respostas imunes inatas e adaptativas (Camelo et al., 2014).

As células epiteliais também produzem vários componentes da matriz extracelular, tais como laminina, colágeno IV e fibronectina, os quais são importantes componentes da membrana basal, e também secreta vários produtos no lume do trato respiratório, incluindo mucos e proteínas surfactantes (Sziksz et al., 2015).

A superfície das vias respiratórias inferiores, onde tem lugar o intercambio de gases, está coberta principalmente por dois tipos de células do epitélio alveolar: **células epiteliais alveolar tipo I (ATI)**, as quais cobrem 90% da superfície das vias respiratórias devido ao seu fenótipo aplanado e cuja função principal é o intercambio de gases (Figura 12); e as **células epiteliais alveolares (ATII)**, que são as mais abundantes e cuja função é manter o espaço alveolar pela secreção de vários tipos de proteínas, agentes tensoativos e outros componentes da ECM (Serrano-Mollar, 2012).

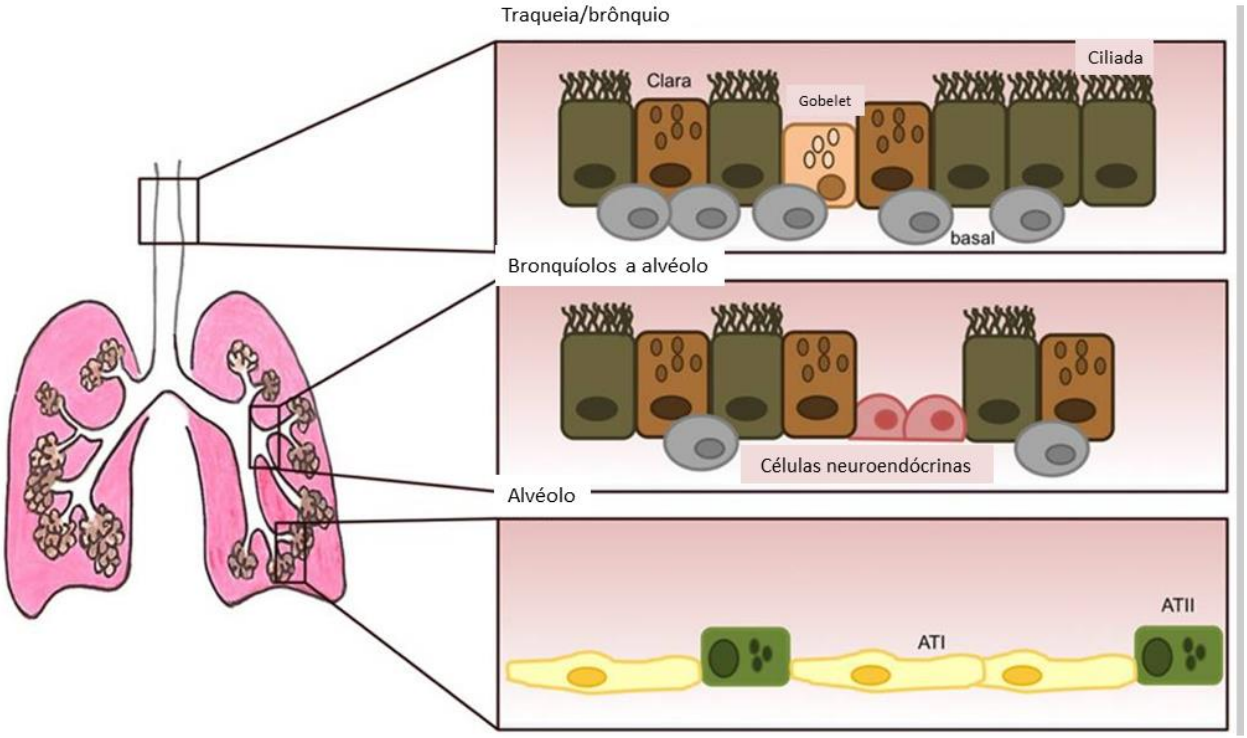


Figura 12. Células do epitélio. O epitélio de pulmão adulto é composto por diversos tipos de células. O epitélio traqueobronquial forma uma camada pseudoestratificada que consiste em **células ciliadas e células secretoras (Clara)** e **Células Gobelet**. Abaixo desta camada, as **células basais** humanas (que acredita-se que são as células progenitoras epiteliais) estão presentes em grande número. **Células neuroendócrinas** também podem estar presentes e tornam-se inervadas por células ganglionares. Acredita-se que possuem o papel de regulação da proliferação e diferenciação celular. Os bronquíolos respiratórios levam aos alvéolos, que na sua maioria estão alinhados por **células alveolar tipo I (ATI) e células tipo II (ATII).** Adaptado de Camelo et al., (2013).

Além das ações de fatores derivados dos macrófagos sobre o epitélio, as células epiteliais podem interagir diretamente com partículas depositadas e isto pode resultar num dano reversível ou irreversível no epitélio. Estas células representam a principal barreira para a entrada no corpo. Devido a sua localização e função, as células epiteliais são um dos principais alvos do PM, e o impacto sobre estas células inclui inflamação, citotoxicidade e genotoxicidade (Davies, 2014).

No caso específico dos efeitos biológicos produzidos por partículas de carvão, estes começam quando as partículas entram em contato com o epitélio respiratório, onde podem ser absorvidos e entrar na circulação sistêmica ou podem ser submetidos ao *clearance mucociliar*. O ***clearance mucociliar*** é o mecanismo pelo qual os cílios das células epiteliais bronquiais eliminam as partículas capturadas no muco das vias respiratórias (Figura 13). Alternativamente, as partículas podem ser ingeridas por macrófagos localizados na barreira ar-sangue ou podem ser metabolizadas. A magnitude destes processos é dependente do tamanho da partícula. Outros fatores importantes são a

agregação, forma, material, cristalinidade, carga superficial e a hidrofilia da superfície das partículas (Fröhlich & Salar-Behzadi, 2014).

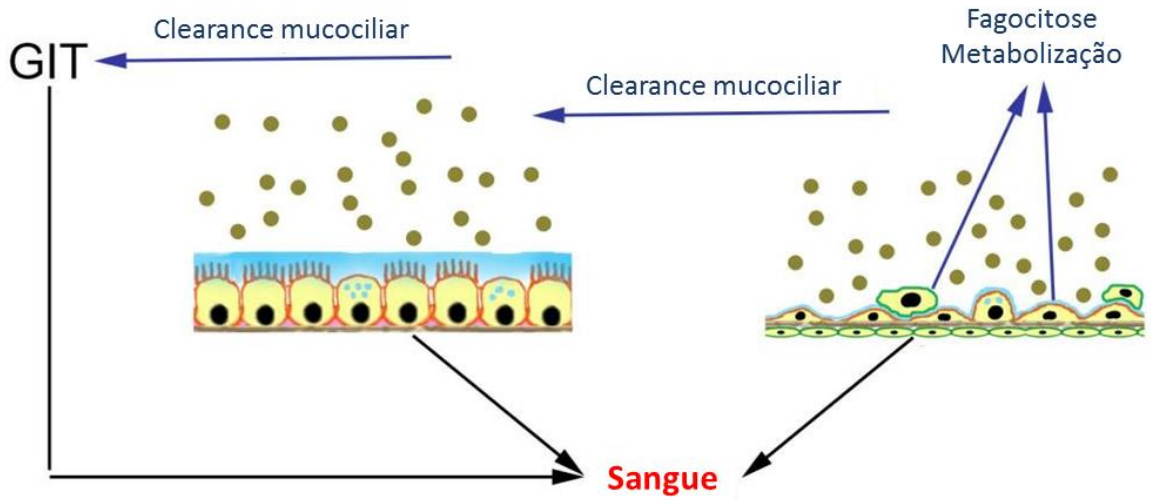


Figura 13. Destino das partículas inaladas. As partículas inaladas são conduzidas às vias respiratórias (epitélio bronquial) e os alvéolos, podendo ser absorvidas através do epitélio bronquial e entrar na circulação sistêmica ou removidas do epitélio bronquial pelo *clearance mucociliar* e ser depois absorvida no trato gastrointestinal (GIT). Via de absorção: **setas pretas**; vias de metabolização e excreção: **setas azuis.** Adaptado de Fröhlich & Salar-Behzadi, (2014).

Na bronquite, as células epiteliais bronquiais podem ser afetadas. As hiperplasias das células produtoras de muco e glândulas nas paredes dos brônquios podem levar ao aumento da produção de muco, assim como à disfunção das células ciliadas, resultando em uma remoção mucociliar prejudicada (Schins & Borm, 1999). Estudos deixam evidente que as células bronquiais e as células epiteliais alveolares possuem um papel muito ativo na toxicidade das partículas, uma vez que estas células têm importantes funções imunoinflamatórias. De fato, vários dos produtos tipicamente liberados pelas células inflamatórias, tais como ERO e citocinas, são também produzidas ou secretadas em certa medida pelas células epiteliais do pulmão (Sager et al., 2008).

5.3. Fibroblastos

Os fibroblastos não são uma população homogênea de células, e sim morfologicamente heterogêneos, com diferentes aparências, dependendo de sua localização e atividade. Os fibroblastos sintetizam glicosaminoglicanos e colágeno da matriz extracelular (ECM), e são responsáveis pela síntese de todas as fibras do tecido conjuntivo, incluindo a reticular, de colágeno e elásticas (Figura 14 e 15) (Kendall and Feghali-Bostwick, 2014).

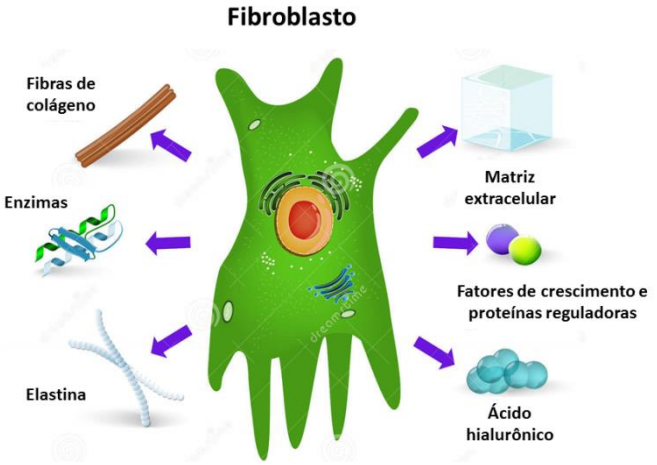


Figura 14. Funções dos fibroblastos

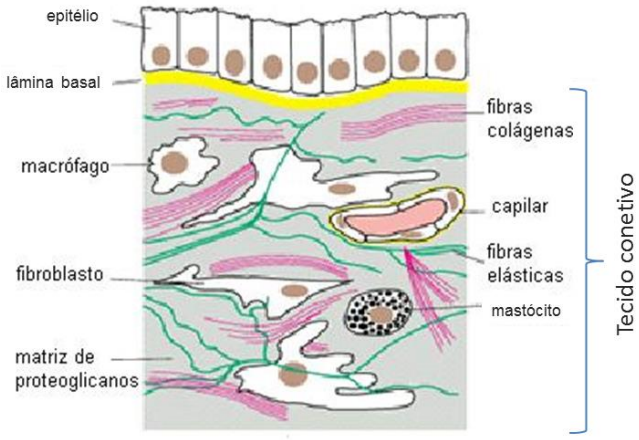


Figura 15. Estrutura do tecido conetivo

Os macrófagos ativados e outros tipos de células, incluindo neutrófilos e células epiteliais, liberam vários fatores que estimulam a quimiotaxia nos fibroblastos, crescimento, proliferação e/ou produção de colágeno. Na fibrose pulmonar, os fibroblastos intersticiais são considerados efetores celulares chaves. Isto também pode ser o caso da fibrose induzida pelo carvão e outros tipos de pó, uma vez que o desenvolvimento da fibrose caracteriza-se pela substituição do parênquima pulmonar normal pela matriz de colágeno, sendo os fibroblastos pulmonares a principal fonte de colágeno e outros componentes da matriz intersticial (Ancochea et al., 2004).

5.4. Células inflamatórias recrutadas

Quando existe deposição de partículas no pulmão, as células inflamatórias, tais como os **monócitos** periféricos sanguíneos ou **neutrófilos**, podem ser recrutados do sangue periférico, por fatores liberados mediante a ativação de macrófagos alveolares, ou também por outras células pulmonares (células epiteliais, fibroblastos, endotélio) (Grommes and Soehnlein, 2011).

O recrutamento de neutrófilos pode conduzir a um aumentado e persistente estado inflamatório na região alveolar, o qual é conhecido como alveolite (Trujillo et al., 2013). Além do dano direto por partículas, as células epiteliais alveolares podem chegar a ser significativamente prejudicadas por uma variedade de fatores produzidos por estas células inflamatórias, incluindo ERO e proteases (Grommes and Soehnlein, 2011). Seja pela ação direta das partículas ou pela ação indireta (por exemplo, proteases, ERO), o epitélio alveolar pode ser destruído e/ou remodelado. O consumo de oxigênio pode ser afetado pela perda de células alveolares funcionais de tipo I e sua substituição por células proliferativas de tipo II ou fibroblastos, levando à fibrose alveolar (Camelo et al., 2014).

6. MECANISMOS E MEDIADORES

Os mecanismos de toxicidade de partículas de carvão e cinzas podem ser arbitrariamente subdivididos em várias vias que envolvem a *produção de ERO* e *a proteção antioxidante*, a *expressão* e *liberação de citocinas, fatores de crescimento* e *produtos relacionados, tais como eicosanoides* e *receptores de citocinas*. Estas vias estão baseadas sobre conceitos chaves da ativação de macrófagos e inflamação pulmonar, e são considerados mediadores cruciais nos efeitos respiratórios que são observados na exposição crônica às poeiras minerais (Refsnes et al., 2006).

6.1. Redes de citocinas

As citocinas regulam a função de todas as células nucleadas, são multifuncionais e participam numa ampla gama de eventos biológicos, incluindo inflamação, metabolismo, crescimento celular e diferenciação, morfogênese, fibrogênese e homeostase (Zhang and An, 2007). Além das células do sistema imune, as principais fontes de citocinas no pulmão são células epiteliais, células endoteliais e fibroblastos.

No campo de pesquisa sobre partículas, as citocinas atualmente são consideradas importantes mediadores dos efeitos tóxicos e patogênicos observados em humanos expostos às poeiras minerais (Rimal et al., 2005; Bonner, 2007). Várias citocinas têm sido estudadas em relação às partículas de carvão e cinzas de carvão, incluindo Fator de necrose tumoral-α (TNFα), Fator de crescimento transformante beta (TGF-β), Interleukina-1 (IL-1), Interleukina-6 (IL-6) e Fator de crescimento derivado de plaquetas (PDGF) (Vallyathan et al., 1998; Schins & Borm, 1999; Ates et al., 2011). Além disso, vários outros fatores podem ser liberados das células inflamatórias, e também de outras células pulmonares, em resposta à exposição de poeiras minerais. Entre estes fatores estão eicosanoides, tais como prostaglandina-E_2 (PGE_2), tromboxano A_2 (TXA_2) e leucotrieno B_4 (LTB_4) e moléculas de adesão, tais como as Intercelular-I (ICAM-I) (Demers et al., 1993; Church and Robinson, 2012).

A classificação das citocinas levando em consideração doenças das vias respiratórias é mais adequada. Dentre destas, são consideradas as categorias citocinas pró inflamatórias, citocinas derivadas de células T, citocinas quimoatraentes (quimiocinas) para eosinófilos, neutrófilos, monócitos/macrófagos e células T, citocinas anti-inflamatórias e fatores de crescimento (Tabela 1) (Chung, 2001; McInnes, 2008).

TABELA 1. TIPOS DE CITOCINAS

Categoria	Citocinas
Linfoquinas	IL-4, IL-5, IL-6, IL-10, IL-13
Fatores quimotáticos para neutrófilos	Quimiocinas CXC (IL-8, GRO-α, ENA-78), IL-1, TNF, IL-17
Eosinófilos	Quimiocinas CC (eotaxina, RANTES, MCP-4), GM-CSF
Monócitos/macrófagos	MCP-1, MIP-1α, RANTES
Células T	IL-16 (CD4+), MIP-1α (CD8+), STCP-1 (Th2), RANTES (memoria), MCP-1
Proinflamatórias	IL-1β, TNF-α, IL-6
Antiinflamatórias	IL-10, IL-1RA, IFN-γ
Fatores de crescimento	TGF-β, PDGF, EGF, IGF

IL: interleucina; **GRO- α**: oncogene α relacionado com crescimento; **ENA-78:** ativador de neutrófilo 78 derivado de células epiteliais; **TNF:** fator de necrose tumoral; **RANTES:** ativação regulada, células T normais expressas e secretadas; **MCP:** proteínas quimotáticas de monócitos; **GM-CSF:** fator estimulante de colônias de macrófagos e granulócitos; **MIP-1 α:** proteína 1α inflamatória de macrófagos; **STCP-1:** proteína-1 quimoatraente estimulada por células T; **Th2:** células helper T-tipo 1; **IL-1RA:** receptor antagonista IL-1; **IFN-γ:** interferon gamma; **TGF-β:** fator de crescimento transformante β; **PDGF:** fator de crescimento derivado de plaquetas; **EGF:** fator de crescimento epidermal; **IGF:** fator de crescimento parecido a insulina. Adaptado de Chung (2001).

Fator de necrose tumoral-α e receptores (TNF-α)

TNFα é uma proteína de 17 kDa predominantemente liberada por macrófagos e monócitos, mas que também pode ser produzida por outras células, tais como os neutrófilos e linfócitos. A liberação de TNFα é aumentada por vários agentes, incluindo ERO e várias citocinas, tais como interferon-γ (INF γ) e IL-1. O gene TNF-α humano está localizado no cromossoma 6, unido ao gene que codifica para o Fator de Necrose Tumoral-β, próximo à região do Complexo Maior de Histocompatibilidade (MHC) (Khan, 2008). Efeitos importantes de TNF-α incluem a indução do crescimento de fibroblastos, liberação de colagenase e PGE_2, expressão de moléculas de aderência de neutrófilos, aumento da produção de ERO pelos neutrófilos, macrófagos e fibroblastos, e a indução da expressão de antioxidantes. TNF-α é um agente quimiotáctico para neutrófilos e também para monócitos (Bonner, 2007).

Existem dois tipos de receptores de TNF, TNF-R1 (CD120a ou P55) e TNF-R2, os quais estão envolvidos em processos inflamatórios e pertencem à superfamília de receptores de TNF. Estes receptores são proteínas transmembrana com domínios intracelulares que carecem de atividade enzimática intrínseca e, em consequência, requerem proteínas citoplásmicas que ajudam a iniciar as vias de sinalização. TNF-R1 possui um domínio de morte intracelular e TNF-R2 interage com moléculas da família do fator 2 associado ao receptor (TRAF). Após a união de TNF-α aos seus receptores, acontece a indução de duas vias de sinalização intracelular. Uma via conduz à transcrição de outros genes, e outra via conduz à morte celular e apoptose. Os dois principais fatores ativados por TNF-α são AP-1 e NF k-B (Khan, 2008).

O TNF-α liberado pelos macrófagos tem sido observado em resposta a vários tipos de poeiras minerais. Estudos demostram o papel do TNF-α e mediadores na indução de fibrose pela exposição ocupacional às partículas e cinzas de carvão, nos quais a liberação de TNF-α pelos macrófagos alveolares se mostra aumentada (Lee et al., 2010). Além do TNF-α ser considerado um fator crucial na pneumoconiose, vários autores têm demostrado a importância do TNF-α na gênese da doença nos diferentes estágios da doença (Lee et al., 2010; Ulker et al., 2008).

A grande e relativamente estável variação individual na liberação de TNF-α e a sua relação com o desenvolvimento de CWP têm, aparentemente, relação com os polimorfismos genéticos. A transcrição de TNFα é regulada por um promotor de 1100 pares de bases e bialelismo causado por mutações pontuais (G a A) nas posições -238

e -308, que provavelmente são contribuintes significativos para a expressão diferencial das citocinas na população humana (Kim et al., 2002; Ates et al., 2011).

Interleucina-1

As proteínas interleucina-1 IL-1α e IL-1β compartilham 26% de homologia, mas encontram-se unidas a um receptor IL-1 idêntico de 80 kDa. Os genes IL-1 estão localizados no cromossomo 2. Uma variedade de células ativadas produz IL-1, incluindo monócitos, macrófagos, linfócitos-B, células Natural Killer, neutrófilos e fibroblastos. As principais fontes de IL-1 são estimuladas pelos monócitos e macrófagos, os quais predominantemente liberam IL-1β (Khan, 2008).

A expressão de IL-1 nestas células pode ser aumentada por vários agentes, incluindo TNF-α. A aderência aos monócitos também induz a liberação de IL-1. A produção de IL-1 pode ser atenuada por PGE_2 e glucocorticosteróides (Bonner, 2007; Khan, 2008).

Várias poeiras minerais são conhecidas por estimular a liberação de IL-1 pelos monócitos/macrófagos, incluindo sílica e asbestos, evidenciando o papel chave de IL-1 no processo inflamatório (Cassel et al., 2008; Dostert et al., 2008).

Interleucina-6

A interleucina-6 (IL-6) é uma citocina pró inflamatória membro da família das citocinas denominadas "citocinas tipo IL-6". IL-6 é uma citocina multifuncional produzida pela maioria das células nucleadas, incluindo monócitos, macrófagos (alveolares), células endoteliais, fibroblastos e células B e T (Khan, 2008). O gene que codifica para IL-6 está localizado no cromossoma 7. As moléculas de IL-6 têm diferentes tamanhos (de 17 kD a 85 kD) e os monócitos liberam preferencialmente uma proteína de 24 kD. IL-6 possui uma variedade de efeitos que são mediados via receptores específicos de IL-6 (80kD). IL-6 é um regulador chave de células B e células T. Várias citocinas são conhecidas por estimular a liberação de IL-6, incluindo IL-1, TNF- α, PDGF e TGF-β (Scheller et al., 2011).

IL-6 contribui notavelmente na iniciação e na extensão do processo inflamatório, pelo que tem um importante papel na resposta da fase aguda e sepse (Scheller et al., 2011). Além disso, tem sido descrito que IL-6 desempenha um papel fundamental na patogênese de doenças pulmonares. De fato, vários estudos em animais e seres humanos têm demostrado uma possível associação entre a expressão de IL-6 e o desenvolvimento de fibrose (Pantelidis et al., 2001; Qiu et al., 2004).

7. PARTÍCULAS DE CARVÃO - CINZA E A MATRIZ EXTRACELULAR (ECM)

O tecido conectivo dos pulmões consiste de colágeno, fibras elásticas e proteoglicanos. Os colágenos são as moléculas mais abundantes da matriz extracelular. Componentes não colagenosos importantes da matriz extracelular são **elastina, fibronectina, laminina, proteoglicanos e hialuronano**. Vários componentes da matriz extracelular têm atividade quimiotática para as células inflamatórias e fibroblastos, tais como colágeno I, III e IV, laminina e fibronectina (Pelosi et al., 2007). Os componentes da matriz extracelular são produzidos por uma variedade de células pulmonares. Os colágenos intersticiais do pulmão são sintetizados pelos fibroblastos, células endoteliais, células mesoteliais pleurais e células epiteliais alveolares. Várias citocinas e compostos relacionados são capazes de estimular a produção de colágenos (Theocharis et al., 2016). A degradação de colágenos intersticiais está determinada por colagenases que são sintetizadas por células intersticiais, por macrófagos e neutrófilos. As colagenases podem ser ativadas por processos oxidativos e, por exemplo, têm sido detectadas em pacientes com fibrose pulmonar (Wilson and Wynn, 2009).

Outras proteases que degradam colágeno do pulmão incluem elastases de neutrófilos da matriz, catepsinas e gelatinase e as elastases de macrófagos (Yamashita et al., 2014). As sínteses de proteases podem ser aumentadas por citocinas, tais como TNFα, IL-1 e PDGF. Várias citocinas têm, por um lado, um papel no aumento do crescimento de fibroblastos e/ou produção de colágeno e, por outro lado, aumentam a degradação da matriz de colágeno. Fontes destas antiproteases incluem macrófagos e fibroblastos pulmonares e a sua produção pode ser estimulada por citocinas, tais como IL-1 e TGF-β (Greene and McElvaney, 2009).

8. PARTÍCULAS CARVÃO-CINZA E A FORMAÇÃO DE ERO

O papel das ERO está sendo estudado na patogênese de uma variedade de doenças pulmonares. Basicamente, têm sido propostos dois mecanismos pelos quais a exposição ao pó mineral provoca a formação de ERO *in vivo*:

1) Formação de ERO pelas propriedades intrínsecas das partículas, por exemplo, mecanismos não celulares; e
2) Excessiva formação de ERO pelos macrófagos e neutrófilos ativados durante a fagocitose de partículas e a inflamação persistente (Knaapen et al., 2004).

1) Geração não celular de ERO: vários mecanismos têm sido propostos sobre a toxicidade não celular de partículas minerais relacionados com as propriedades físico-químicas tais como tamanho, forma, presença de HAP, metais na superfície e conteúdo de ferro (Zhang et al., 2002; Dwivedi et al., 2012).

O ferro presente nas partículas pode ter um papel importante na toxicidade de partículas de carvão e cinzas de carvão. Altas concentrações de ferro no organismo podem levar à oxidação de biomoléculas (via reação de Fenton) e posteriormente às

lesões pulmonares. A formação de radicais hidroxila via reação de Fenton pode ser um fator determinante na prevalência de doenças pulmonares nos mineiros de carvão. Isto é devido provavelmente aos componentes que contém ferro do carvão e das cinzas (Cohn et al., 2006), o que estaria relacionado com a geração de ERO em fluidos biológicos, e poderia causar dano oxidativo direto, mediante mecanismos não celulares (Schins & Borm, 1999; Porter et al., 2002).

Outro fator importante que determina a capacidade de geração oxidante das partículas minerais são os metais presentes nessas partículas, os quais podem aumentar a geração de ERO via reações de Haber-Weiss (Knaapen et al., 2004; Bonner, 2007). Adicionalmente, os constituintes orgânicos como os HAP associados com a superfície da partícula podem ser fatores que também contribuem para a formação oxidante (Bonner, 2007). Durante sua ativação metabólica, os radicais semiquinona podem ser produzidos e podem sofrer ciclo redox, levando à formação de ERO (Donaldson et al., 2005, Møller et al., 2014).

2) Geração celular de ERO: dentro do pulmão, ERO e espécies reativas do nitrogênio (ERN) podem ser gerados endogenamente por vários tipos de células, incluindo células fagocíticas, endoteliais vasculares e células epiteliais pulmonares. Numerosos estudos demonstraram a capacidade de partículas oriundas do ambiente e fibras minerais, incluindo amianto, sílica cristalina, metais pesados contidos nos pós, cinza com óleo, cinza de carvão e material particulado, de induzir a produção de ERO (*in vivo*) em neutrófilos e em macrófagos, um processo que provavelmente envolve a ativação de NADPH oxidases (van der Vliet, 2008; Magnani et al., 2013; Joshi et al., 2015).

Os fagócitos são também uma fonte potente de ERN, incluindo óxido nítrico ($NO^{\cdot}$) e peroxinitrito ($ONOO^{-}$), o que desencadeia a atividade induzível da enzima óxido nítrico sintase (iNOS) (Fubini and Hubbard, 2003; Sugiura and Ichinose, 2011). Embora os neutrófilos sejam considerados os mais potentes fagócitos em respeito à geração de ERO relacionado com partículas, a principal fonte de ERN no pulmão são os macrófagos alveolares (Knaapen et al., 2004; Laskin et al., 2010).

8.1. TIPOS DE DANOS CAUSADOS PELAS ERO EM MACROMOLÉCULAS

Dano oxidativo

Os efeitos celulares mais importantes das ERO no pulmão podem ser:

- Dano nas membranas celulares mediante processos de peroxidacão lipídica.
- Oxidação de proteínas
- Dano no DNA de células alvo.

A **peroxidacão lipídica** é um processo de reação em cadeia nos ácidos graxos insaturados da membrana, resultando na formação de radicais lipídicos, e pode levar ao dano celular e remodelamento do tecido (Piljac Zegarac, 2015).

A peroxidacão de lipídeos da membrana conduz à perda da fluidez da membrana e da elasticidade, à deterioração da função celular e inclusive à ruptura celular. Os diferentes produtos diretos da peroxidacão lipídica, como o malondialdeído (MDA) e isoprostanas e 4-hydroxynonenal, são considerados entre os mais importantes biomarcadores de estresse oxidativo nos tecidos. O malondialdeído é um composto carbonílico reativo e, por sua vez, mutagênico e cancerígeno. Reage com o DNA formando adutos. De fato, a medição das sustâncias reativas do ácido tiobarbitúrico (TBARS) constitui um ensaio bem estabelecido, utilizado para detectar a peroxidacão de lipídeos em presença de malondialdeído (Piljac Zegarac, 2015).

As reações das ERO com **proteínas** podem conduzir à inativação de enzimas envolvidas no metabolismo celular ou na modificação estrutural de componentes intra ou extracelulares. A oxidação de proteínas pode causar a fragmentação de resíduos de aminoácidos, a formação de *cross-links* proteína-proteína e oxidação do esqueleto das proteínas, o que conduz, por fim, à perda da função. As proteínas danificadas afetam as vias intracelulares e são fatores que contribuem para diferentes transtornos e doenças. Se os mecanismos proteolíticos responsáveis pela degradação de proteínas não funcionam corretamente, as proteínas alteradas acumulam-se na célula e podem contribuir para o desenvolvimento de condições patológicas (Piljac Zegarac, 2015).

Vários ensaios têm sido desenvolvidos para detectar especificamente os biomarcadores de dano oxidativo de proteínas, como por exemplo, nitrotirosina, que é um produto proveniente da nitracão da tirosina e a formação de grupos carbonil, que se formam nas cadeias laterais das proteínas durante a oxidação (Piljac Zegarac, 2015).

O **dano oxidativo no DNA** pode ter várias consequências, que vão desde morte celular e destruição do tecido, até proliferação celular. Além destas interações diretas com o DNA, as ERO podem também atuar como reguladores de cascatas sinalizadoras a nível intracelular e fatores de transcrição de uma variedade de genes, incluindo aqueles de citocinas pró-inflamatórias, moléculas de adesão e proto-oncongenes (Schieber and Chandel, 2014).

O **7-hidro-8-oxo-2'-deoxiguanosina (8-oxodG), produto da oxidação do C8 da deoxiguanosina (dG)**, é uma das modificações oxidativas mais prevalentes que ocorrem no DNA em resposta às ERO. A proporção 8-oxodG/dG (Figura 16) pode ser considerada como um biomarcador de estresse oxidativo e tem sido estudado em relação à exposição às poeiras minerais *in vitro* e *in vivo* (Haghdoost et al., 2005).

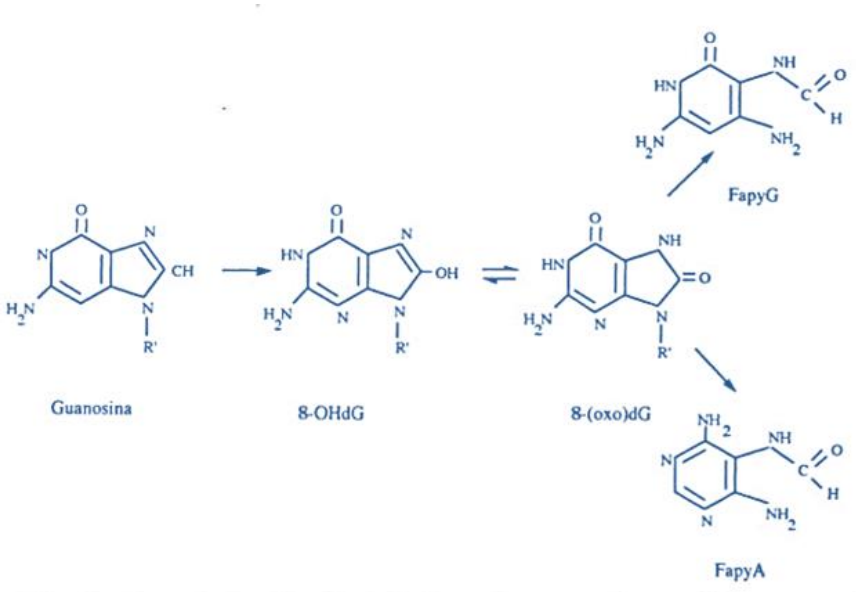


Figura 16. Produtos da oxidação da Guanosina gerados por distintos mecanismos de estresse oxidativo. R' desoxirribosa; 8-OH-dG: 8-hidroxi-2'-desoxiguanosina; 8-(oxo)dG: 8-oxo-2'-desoxiguanosina; FapyG: 2,6-diamino-4-hidroxi-5-formamido-pirimidina; FapyA: 5-formamido-4,6-diamino-pirimidina. Adaptado de Oliva et al., (2009).

Comparado aos compostos carcinogênicos e genotóxicos solúveis, as partículas formam um grupo mais específico, uma vez que o seu comportamento e características, tanto físicas quanto químicas, são totalmente diferentes e normalmente muito complexas. Isto se dá principalmente porque os mecanismos de genotoxicidade induzidos por partículas são pobremente entendidos. Para que os efeitos genotóxicos aconteçam, é necessário que as partículas fagocitadas pelas células alvo entrem e estejam em contato próximo com o DNA. Além disso, o aspecto de internalização de partículas permite também a avaliação dos mecanismos nos sistemas celulares, os quais são indiretamente ativados na produção de ERO/ERN, que contribuem para os efeitos genotóxicos nestas células (Figura 17) (Knaapen et al., 2004).

Levando em consideração o possível papel dos grupos reativos estáveis localizados na superfície das partículas, estes precisam de interação direta ou a proximidade das partículas com moléculas alvo, por exemplo, um receptor (sinalização celular) ou DNA genômico (genotoxicidade) (Moller et al., 2014). Além disso, tem sido descrito que as partículas podem também gerar resíduos de difusão livre em suspenção em soluções aquosas, o que dá às partículas a capacidade de "atuar a distância" (Knaapen et al., 2004).

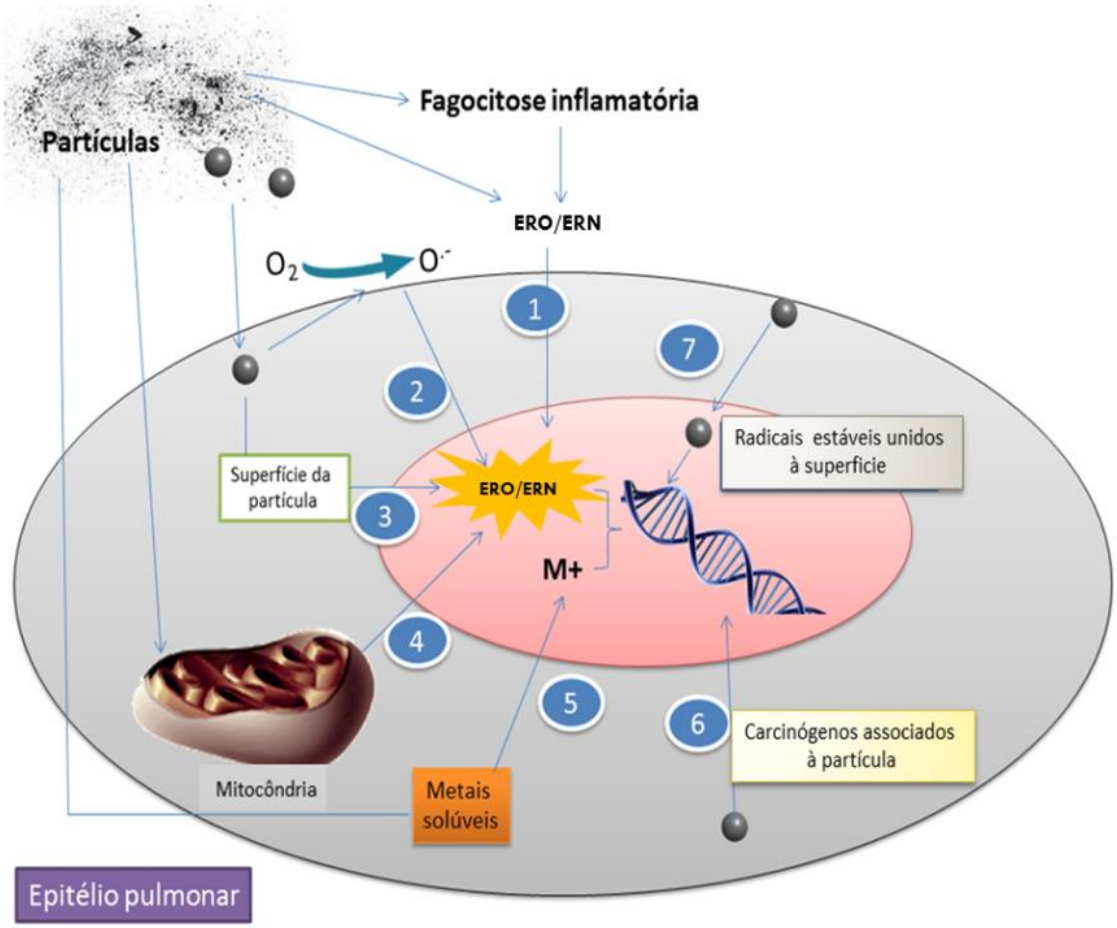


Figura 17. Possíveis mecanismos de dano no DNA induzido por partículas em geral. **1)** As partículas podem ativar a explosão oxidativa fagocítica (genotoxicidade secundária). **(2)** Alternativamente, as partículas podem também gerar diretamente ERO/ERN em células alvo (2-5). **3)** Por exemplo, via ativação similar a enzimas NAD(P)H através da superfície das partículas reativas. **4)** Pela ativação mitocondrial. **5)** Além disso, metais solúveis (transição) associados à partícula podem aumentar a geração de ERO por meio de reações Haber-Weiss. **6)** Compostos genotóxicos absorvidos (por exemplo, HAP) podem danificar diretamente o DNA. **7)** Finalmente, depois da translocação de partículas no núcleo, o dano pode ser induzido pelos radicais estáveis unidos à superfície. As vias 3 e 4 são ainda hipotéticas, é são necessárias maiores investigações para esclarecer o seu papel. Adaptado de Knaapen et al., (2004).

Adicionalmente, estudos *in vivo* realizados até o momento levam à pergunta: a inflamação pulmonar poderia ser relacionada com efeitos genotóxicos secundários? Este questionamento está baseado na dúvida se fenômenos como estresse oxidativo, inflamação e dano no DNA são independentes ou são gerados por uma causa comum (partículas), ou se existe uma sequência de eventos inter-relacionados, mas que são potencialmente diferentes. A Figura 18 ressalta três possíveis relações onde a oxidação do DNA é um evento secundário à produção de ERO ou inflamação. É possível que o estresse oxidativo estimule a inflamação (relação A) ou a inflamação mediada pelas partículas cause estresse oxidativo (relação B). Porém, também é possível que as

partículas causem ambos, tanto estresse oxidativo como inflamação por diferentes mecanismos de ação (relação C) (Moller et al., 2014).

Têm sido realizadas tentativas para decifrar a sequência de eventos em sistemas como condições acelulares, cultura de células e modelos animais, mas sem conclusões que possam ser aplicadas às condições de exposição humana (Moller et al., 2014).

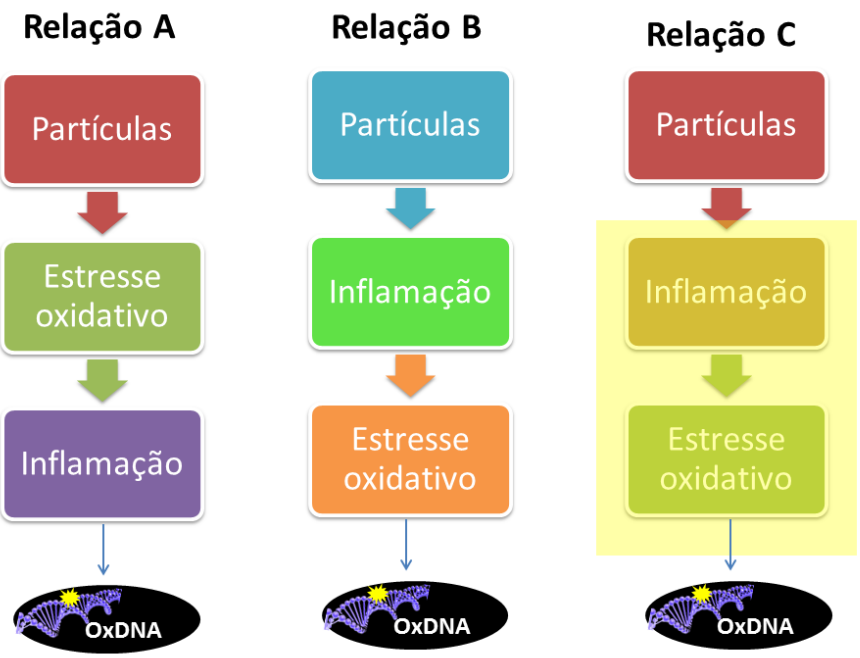


Figura 18. Relação entre a exposição a partículas e a geração de estresse oxidativo, inflamação e dano oxidativo no DNA (OxDNA). Adaptado de Moller et al., (2014).

8.2. O PAPEL DAS ERO NAS DOENÇAS PULMONARES INDUZIDAS POR PARTICULAS DE CARVÃO-CINZAS

As doenças pulmonares ocupacionais mais frequentes no mundo são as geradas pela inalação de pós minerais como o amianto, sílica e o pó de carvão. A inalação de partículas de carvão ou cinzas pode causar uma variedade de doenças pulmonares, incluindo pneumoconiose simples (CWP), fibrose maciça progressiva (PMF), bronquite crônica, perda da função pulmonar e enfisema (Schins and Borm, 1999; Santo Tomas, 2011; Burt et al., 2013)

Vários estudos têm proporcionado apoio adicional para o papel significativo das ERO em transtornos pulmonares associadas com a exposição às partículas e cinzas de carvão (Petsonk et al., 2013). As ERO podem desempenhar um papel-chave nos mecanismos de iniciação e progressão de várias das doenças citadas anteriormente após a exposição a estas partículas. Estímulos primários induzidos por ERO resultam no aumento da secreção de citocinas pró-inflamatórias e de outros mediadores, promovendo eventos que parecem ser importantes na progressão da lesão celular e doença pulmonar. Evidências sugerem que a inalação de partículas insolúveis, tais como

partículas de carvão ou cinzas, pode estar envolvida na facilitação de múltiplas vias para a geração persistente de ERO, o que pode levar a uma continua inflamação que conduz à progressão de doenças como **pneumoconiose** (Vallyathan et al., 1998; Porter et al., 2002). Além disso, considera-se que as ERO têm um papel muito importante na patogênese de **enfisema**, mediante o dano oxidativo a antiproteases, reduzindo a sua atividade. O enfisema surge da degradação da matriz extracelular do pulmão. Esta matriz esta composta por fibras elásticas formadas principalmente por colágeno tipo IV, proteoglicanos e elastina. A reduzida atividade de antiproteases pode causar uma destruição exagerada do tecido pulmonar pelas proteases (Raut et al., 2013), característica do enfisema típico observado em trabalhadores de carvão (Petsonk et al., 2013).

A **bronquite crónica** é considerada uma doença ocupacional comum em mineiros de carvão, a qual consiste em uma inflamação crônica, caracterizando-se por um fluido exudativo inflamatório e infiltrado celulares no epitélio que reveste as vias respiratórias centrais e as glândulas associadas. Neutrófilos ativados, macrófagos alveolares, eosinófilos e células epiteliais produzem ERO, o que leva a diferentes tipos de dano, como peroxidação lipídica da membrana. Essa produção de ERO conduz à ativação da transcrição de genes de citocinas pró-inflamatórias e quimiocinas, à regulação positiva de moléculas de adesão e aumento da liberação de mediadores pró-inflamatórios que regulam as respostas inflamatórias no pulmão. Os brônquios inflamados produzem uma secreção mucosa abundante, o que conduz a tosse e dificuldade para a entrada e saída de ar dos pulmões (Jang et al., 2016), característica comum em pessoas expostas cronicamente à mineração de carvão.

OBJETIVOS

II. OBJETIVOS

OBJETIVO GERAL:

- Avaliar a citotoxicidade e genotoxicidade na linhagem de fibroblastos de pulmão de hâmster chinês (células V79), e fisiologia respiratória em camundongos, de partículas de carvão e cinzas provenientes de Santa Catarina.

OBJETIVOS ESPECÍFICOS:

- Avaliar o potencial citotóxico de partículas de carvão e cinzas de carvão em células V79.
- Avaliar os efeitos genotóxicos de partículas de carvão e cinzas de carvão em células V79.
- Avaliar a capacidade das partículas de carvão e cinzas de carvão de induzir danos oxidativos no DNA em células V79.
- Avaliar o citoma (CBMN-Cyt) de células V79 após exposição às partículas de carvão e cinzas de carvão.
- Analisar a mecânica respiratoria de camundongos expostos às partículas de carvão e cinzas.
- Analisar a indução de citocinas (TNF-α, IL-1B e IL-6) em tecido pulmonar de camundongos expostos às partículas de carvão e cinzas.
- Avaliar os efeitos genotóxicos no sangue periférico de camundongos expostos às partículas de carvão e cinzas.
- Analisar a celularidade e morfometria do tecido pulmonar dos camundongos expostos às partículas de carvão e cinzas.
- Analisar a formação de fibras elásticas e de colágeno no tecido pulmonar dos camundongos expostos às partículas de carvão e cinzas.
- Analisar a translocação de metais a órgãos extrapulmonares de camundongos expostos às partículas de carvão e cinzas.

CAPÍTULO I

CYTOTOXICITY AND GENOTOXICITY INDUCED BY COAL AND COAL FLY ASH PARTICLES SAMPLES IN V79 CELLS

Manuscrito submetido para publicação na revista

"Environmental Science and Pollution Research".

APRESENTAÇÃO

CAPITULO I

O Capítulo I apresenta o trabalho que originou um manuscrito, o qual foi submetido à revista "Environmental Science and Pollution Research", em janeiro de 2016, intitulado "CYTOTOXICITY AND GENOTOXICITY INDUCED BY COAL AND COAL FLY ASH PARTICLES SAMPLES IN V79 CELLS".

Os resultados deste capítulo indicam que a exposição das células V79 a partículas de carvão e cinzas induz dano no DNA. Além disso, o uso de endonucleases específicas (ENDO III e FPG) indicou a presença de dano oxidativo no DNA. Também, os biomarcadores do ensaio CBMN-Cyt mostraram que a exposição a altas concentrações de partículas de carvão e cinzas induzem efeitos citotóxicos e instabilidade cromossômica. Estes resultados poderiam estar associados com o conteúdo de compostos como óxidos, HAP e metais presentes na superfície destas partículas.

CYTOTOXICITY AND GENOTOXICITY INDUCED BY COAL AND COAL FLY ASH PARTICLES SAMPLES IN V79 CELLS

Grethel León-Mejía[ab]*, Luis F.O. Silva[c], Matheus S. Civeira[c], Marcos L.S. Oliveira[c], Miriana Machado[d], Izabel Vianna Villela[d], Andreas Hartmann[e], Suziane Premoli[f], Dione Silva Corrêa[f], Juliana Da Silva[f] , João Antônio Pêgas Henriques[a]*

[a]Departamento de Biofísica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[b]Unidad de Investigación, Desarrollo e Innovación en Genética y Biología Molecular, Universidad Simón Bolívar, Barranquilla, Colombia.

[c]Laboratory of Environmental Researches and Nanotechnology Development, Centro Universitário La Salle, Mestrado em Avaliação de Impactos Ambientais em Mineração. Victor Barreto, 2288 Centro 92010-000, Canoas, RS, Brazil.

[d]InnVitro Pesquisa e Desenvolvimento, Porto Alegre, RS, Brazil.

[e]Novartis Pharma AG, Basel, Switzerland.

[f]Laboratório de Genética Toxicológica, Universidade Luterana do Brasil (ULBRA), Canoas-RS, Brazil.

***Corresponding authors: João Antônio Pêgas Henriques, Grethel León-Mejia.** Phone: +55 51 33166069; Fax: +55 5133167003. Departamento de Biofísica, Prédio 43422, Laboratório 210, Campus do Vale, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Bairro Agronomia. CEP 91501-970, Porto Alegre, RS, Brasil.

E-mail addresses: **pegas@cbiot.ufrgs.br (Pêgas Henriques)**

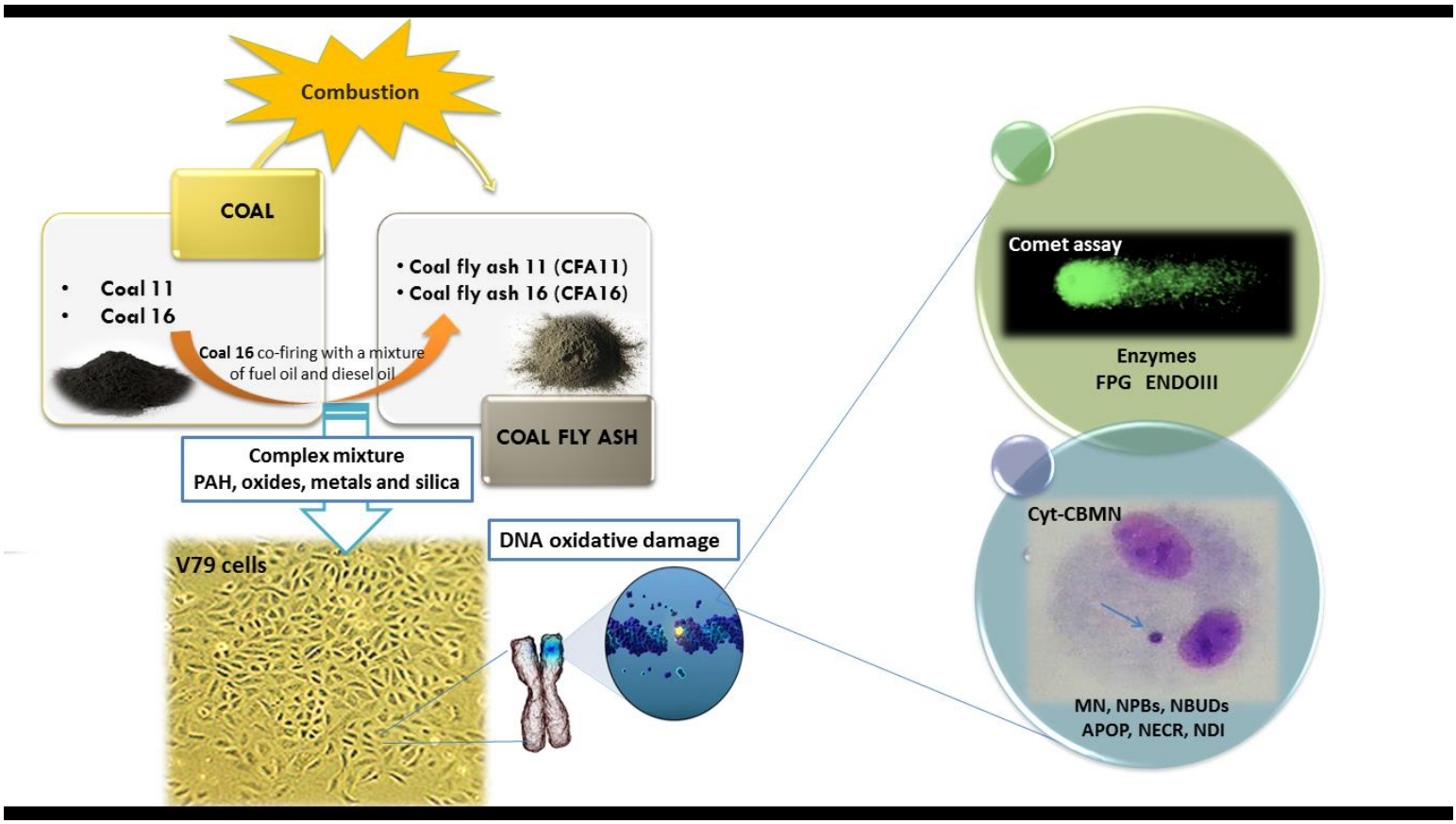
grethelleon@gmail.com (G.León-Mejía).

Abstract

Exposure to coal or coal combustion products can cause harmful effects in *in vitro* and *in vivo* systems, mainly by the induction of oxidative damage. The aim of this work was to assess cytotoxic and genotoxic effects using the V79 cell line treated with coal and coal fly ash particles derived from a coal power plant located in Santa Catarina-Brazil. Two coal samples (COAL11 and COAL16) and two coal fly ash samples (CFA11 and CFA16) were included in this study. COAL16 was co-firing with a mixture of fuel oil and diesel oil. The comet assay data showed that exposure of V79 cells to coal and coal fly ash particles induced primary DNA lesions. Application of lesion-specific endonucleases (FPG and ENDO III) demonstrated increased DNA effects indicating the presence of high amounts of oxidative DNA lesions. The cytokinesis-block micronucleus cytome assay analysis showed that exposure of V79 cells to high concentrations of coal and coal fly ash particles induced cytotoxic effects (apoptosis and necrosis) and chromosomal instability (nucleoplasmic bridges, nuclear buds and MN formation). These results may be associated with compounds contained in the surface of the particles as hazardous elements, oxides and PAH which were detected in the samples.

Key words: Coal, coal fly ash, DNA oxidative damage, comet assay, micronucleus assay.

Graphical abstract



1. Introduction

Coal is a heterogeneous mixture containing large quantities of organic and inorganic matter. The organic components of coal consists of chemical compounds including carbon, hydrogen, oxygen, sulfur, nitrogen and organometallic forms (Oliveira et al. 2011). The presence of mineral matter in coal may result in a number of environmental problems related to mining, preparation and combustion of the coals (Depoi et al. 2008; Ribeiro et al. 2011).

In Santa Catarina (Brazil), where the largest thermal plants is located, the coals used for power production tend to have high contents of ash and sulfur (Silva et al. 2009). Due to high concentrations of mineral matter such as sulfides, clays, and quartz, Brazilian coal must be pulverized and beneficiated, specifically with flotation, before its combustion. Then, the coal is burned inside a boiler, producing a solid combustion-by-product known as coal ash (Quispe et al.2012). Coal ash is composed of amorphous inorganic components, minerals such as silicates, oxides and hydroxides mainly of iron, sulfates, carbonates, phosphates and sulfides, and organic constituent or unburnt coal (char) (Vassilev and Vassileva 1996a,b). The combustion temperature is an important factor which determines the physical properties of the particles. High temperature (>1400°C) is used in conventional combustion, the major aluminosilicate melts and condenses to form small, spherical particles (Borm, 1997). Depending on the toxicity, the chemical properties and concentration in air, the particles of fly ash can constitute a risk to exposed workers due to inhalation (Donaldson et al. 2005).

When coal is being burnt a significant amount of toxic substances are formed and released into the environment including components such as asbestos, silica, selenium (Se), cadmium (Cd) arsenic (As), chromium (Cr), copper (Cu) and mercury

(Hg) (IARC 1997; NRC 2006; IARC 2012) and polycyclic aromatic hydrocarbons (Rohr et al. 2013). PAH are considered to pose potential health hazards because some PAH are known carcinogens (Liu et al. 2008).

The aim this work was to assess the cytotoxic and genotoxic potential effects of the coal and coal fly ash particles, from coal power plant located in Santa Catarina-Brazil, *in vitro* using the V79 *(Chinese hamster lung fibroblasts)* cell line. For an assessment of cytotoxicity the clonogenic assay was used. The comet assay was applied using the standard protocol for the detecting of primary DNA damage as well as a protocol using specific endonucleases for the detection of oxidative damage. The CBMN-Cyt assay was used as a measure of cytotoxic, cytostatic and genotoxic effects.

2. Materials and Methods

2.1. Reagents and chemicals

Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum (FBS), trypsin-EDTA, L-glutamine and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO) and cytochalasin B and methyl methanesulfonate, were purchased from Sigma (St. Louis, MO, USA). Low-melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (EndoIII) were obtained from New England BioLabs (Beverly, MA, USA). Hydrochloric acid (HCl; 5 M), potassium chloride (KCl), methanol, ethanol and acetic acid were obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2.Characteristics of coal and coal fly ash particles

Coal and Coal Fly Ash (CFA) particles samples used in this work were previously characterized and data are available in (Silva et al. 2009; Quispe et al. 2012; Silva et al. 2010; Oliveira et al.2012).

Briefly, samples were collected in Santa Catarina State- Brazil, where the Jorge Lacerda – Tractebel Suez Thermoelectric Complex is located. The Jorge Lacerda power plant has seven different pulverizing fuel units. The feed coals and coal fly ash were collected simultaneously from each of these units over a five-day period. The coal is, in general, high volatile C to high volatile A bituminous in rank, although individual units may be receiving different ratios of the blend components. Four samples, two coal (COAL11 and COAL16) and two coal fly ash (CFA11 and CFA16) were included in this study. Particularly, the coal fly ash sample 16 (CFA16) was collected from Unit 3 while co-firing with a mixture of fuel oil and diesel oil as part of the boiler start-up procedure. Representative samples of around 15–20 kg of coal fly ash and 2 kg of coal were collected from each unit. The coal fly ash samples were collected from the respective electrostatic precipitators. The samples were dried in a furnace (40 °C, 16 h), homogenized and sieved through a 450 μm screen. Subsamples of this material were ground to pass through a 20-mesh sieve.

The coal samples were ashed at 815 °C. The resultant ashes, in addition to the powdered portions of each fly ash sample, were calcined at 1050 °C and then fused into borosilicate disks following the methods described by Norrish and Hutton (1969). The loss on ignition at 1050 °C was also determined for each sample as part of the preparation process. The major element oxides in each ash sample were determined by X-ray fluorescence (XRF) spectrometry techniques using a Philips PW2400 spectrometer system.

The coal and coal fly ash samples were acid digested following a two-step method devised to retain volatile elements (Querol et al. 1997). The process involved a hot HNO_3 extraction, followed by HF-HNO_3 -$HClO_4$ digestion of the residue. The resulting solutions were analyzed by inductively coupled plasma atomic-emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) for a range of major and trace elements. Morphology and composition of particles and minerals were investigated using a Field Emission Scanning Electron Microscope (FE-SEM; Zeiss Model ULTRA plus) equipped with an energy dispersive X-ray spectrometer (EDS).

2.3. Quantification of polycyclic aromatic hydrocarbons (PAH)

The PAH present in the sample were quantified by using HPLC/ UV/Vis. Extraction and clean-up are essential parts in the analysis of organic compounds originated from solid matrices. The technique has been adapted, followed by studies bases Cavalcante et al. (2008) and Sun et al. (1998). 5 g of dry soil samples (30°C during 24 h) were used (in triplicate). The extraction by ultrasound technique was adapted and optimized using solvents with different polarities, hexane and acetone (1:1), the soil and the solvents were put into the ultrasound machine during 10 minutes, then they were centrifuged and the extract was removed. The extraction was repeated four times and the extracting solution was then vacuum filtered in order to eliminate non soluble materials and concentrated in a rotavapor. Adsorption chromatography with a glass column was used for the clean-up procedure. After adding the extract to the adsorption column, the compounds were eluted with different solvent systems, performed with some changes of Begas (2013).The elute volume was reduced to 1 mL and finally, each extract was injected into a chromatographic system (HPLC-UV) in duplicate. The chromatographic conditions were: 5 µm Kromasl reverse phase column

(250 x 4,6 mm), injection volume 20 µL, mobile phase acetonitrile (A): water MilliQ (B), gradient method t (A:B): 0 (1:1), 10 min (7:3), 20 min (8:2), 25 min (8:2), 28 min (1:1) and 30 min (1:1) and λ=254. Analytic curves were built by external standardization for quantification.

2.4. Cell culture and treatment

V79 is a Chinese hamster lung fibroblast cell line and was obtained from the Rio de Janeiro cell bank (Rio de Janeiro, Brazil). The choice of this cell line for this study was based on the fact that the V79 cells have stable karyotype and is a good experimental model system widely used in genotoxicity and cytotoxicity assays. Cells were grown as monolayers in continuous culture in cell culture flasks of 25 or 75 cm^2 under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37°C under a humidified 5% CO_2 atmosphere. For establishing cultures or passage of V79 cells, 0.15% trypsin–0.08% EDTA and phosphate-buffered saline solution (PBS) was used.

To prepare the particle suspensions for *in vitro* experiments, coal and CFA particles were suspended in DMEM without FBS and just before use the samples were twice sonicated for 10 min to obtain a uniform dispersion and to prevent particle aggregation. Starting from the concentration of 1 mg/mL serial dilutions were performed to prepare incubation culture medium DMEM with decreasing concentrations of samples. All experiments were independently repeated at least three times.

2.5. Cytotoxicity assay

The clonogenic assay allows cell viability assessment by colony formation. 200 cells were seeded in each well of 6 well-plates. After 24 hours, cells were exposed to five different concentrations of coal and coal fly ash and a negative control (FBS-free medium) during 24 h at 37 ºC. Then, the treatment-containing media was removed and the cells were washed with PBS and reincubated in complete medium at 37 ◦C in a humidified atmosphere containing 5% CO_2 for six days. After this period, the colonies were fixed with methanol, stained with 1% crystal violet and counted. The survival was expressed as a percentage relative to the negative control. We determined the IC_{50} values and selected five concentrations of each sample to be used in the next experiments.

2.6. Comet assay

2.6.1. Alkaline comet assay

The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly 3 X 10^5 V79 cells were seeded in each well of 12 well-plates. After 24 h, the cells were exposed to the coal and coal fly ashes and negative control FBS-free medium for 3 h and to positive control (150 μM H_2O_2) for 2 h. After treatment, the cells were trypsinized and re-suspended in complete medium. Twenty microliters of this cell suspension was mixed with 90 μl of 0.75% low-melting point agarose (LMP). This mixture was placed into a slide previously coated with 1.5% of normal melting point agarose processed at 60 °C. The agarose layers were covered with a cover slip and after gel solidifying the cover slips were removed. The slides were immersed overnight in lysis solution (2.5M NaCl, 100mM EDTA and 10mM Tris, pH 10.0–10.5, 1% with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C in dark.

Afterwards, the slides were placed for 30 min in alkaline buffer at 4 °C (300mM NaOH and 1mM EDTA, pH > 13) to unwind the DNA. The alkaline electrophoresis was carried out for 30 min at 25 V and 300 mA. The gels were neutralized with 0.4M Tris (pH 7.5) with 3 washes of 5 min each. The gels were dried at room temperature. Slides were then rinsed and stained with SYBR Gold fluorescent stain. Slide analyses were performed by means of an automated scoring PathFinder™ Screen Tox system (Imstar, France) (Sharma et al. 2012). Finally, for each sample, 400 cells (100 cells from each of two replicate slides per culture) were evaluated for genotoxicity by the analysis of the % Tail DNA parameter (the proportion of DNA in the comet tail) (Duez et al. 2003).

2.6.2. Modified comet assay

The modified alkaline comet assay enables identification of oxidative DNA damage through the use of specific enzymes that repair oxidative damage: (a) Formamidopyrimidine DNA glycosylase (FPG) recognizes the common oxidized purine 8-oxoGua, and also ring-opened purines, or formamidopyrimidines (Fapy); (b) Endonuclease III converts oxidized pyrimidines to strand breaks (Collins 2014; Azqueta et al. 2014).

The modified comet assay includes the same steps of the standard comet assay up to the step of cell lysis. After this step, the slides are removed of the lysis solution and washed three times with buffer enzymes (HEPES 40 mM, KCl 100 mM, EDTA 0,5 mM, bovine serum albumin 0,2 mg/mL, pH 8,0), drained, and incubated at 37 °C in enzyme buffer supplemented with 60 µl of FPG (1 µg/mL solution) for 45 min and EndoIII (1 µg/mL solution) for 30 min; the following steps were carried out as mentioned above.

2.7. Cytokinesis-block micronucleus cytome (CBMN-Cyt) assay

To verify the potential genotoxic effects of coal and coal fly ashes, 15×10^4 cells per well were seeded in 12-well plates. After 24 h, the cells were exposed to the particles (coal and coal fly ashes) for 24 h or 40 μM MMS for 2 h as a positive control. After treatment, the culture medium was discarded and replaced with fresh culture media. Cytochalasin B (4.5 μg/mL) was added and the cells were incubated for another 48 h. The cells were then harvested and spread onto clean microscope slides. The slides were then fixed in an ethanol:glacial acetic acid (3:1) solution and stained with 10% Giemsa for 10 min.

For the analysis 1000 binucleated (BN) cells were scored under a light microscope (magnification 1000X). Five hundred cells per slide were scored and classified to determine the ratios of mononucleate, binucleate, multinucleate cells in order to determine the nuclear division index (NDI). The NDI is a biomarker of cytostasis and provides a measure of the proliferative status of the viable cell fraction. It is calculated using the formula NDI = (M1 + 2M2 + 3M3 + 4M4)/*N*, where M1–M4 represent the number of cells with 1–4 nuclei, and *N* is the total number of viable cells scored (excluding necrotic and apoptotic cells). Cytotoxicity effects were assessed by the frequency of necrotic and apoptotic cells. When 500 cells were randomly scored, the number of apoptotic cells and necrotic cells were counted. Micronucleus frequency, NPBs and NBUDs per 1000 BN cells per slide were used as indicators of DNA damage and chromosomal instability.

2.8. Statistical analysis

Each treatment group was compared with the control samples (untreated) using one-way ANOVA followed by Tukey's *post hoc* test. Graph Pad Prism 5.0 software was

used for the statistical analysis (GraphPad Inc., San Diego, CA). Differences were considered significant when the P-value was less than 0.05.

3. Results

3.1. Characteristics of coal and coal fly ash particles

Characteristics of the frequency distribution of particle diameters of coal and coal fly ashes particles samples are shown in Figure 1. Coal11 shows a mean diameter of 29.74 µm and at least 40% of the sample showed particles less than 10 µm. CFA11 had an average diameter of 65.52 µm, but approximately 25% had particles smaller than 10 µm. Coal16 showed an average diameter of 37.34 µm and more than 30% had a size less than 10 µm. CFA16 all particles had a diameter less than 10 µm.

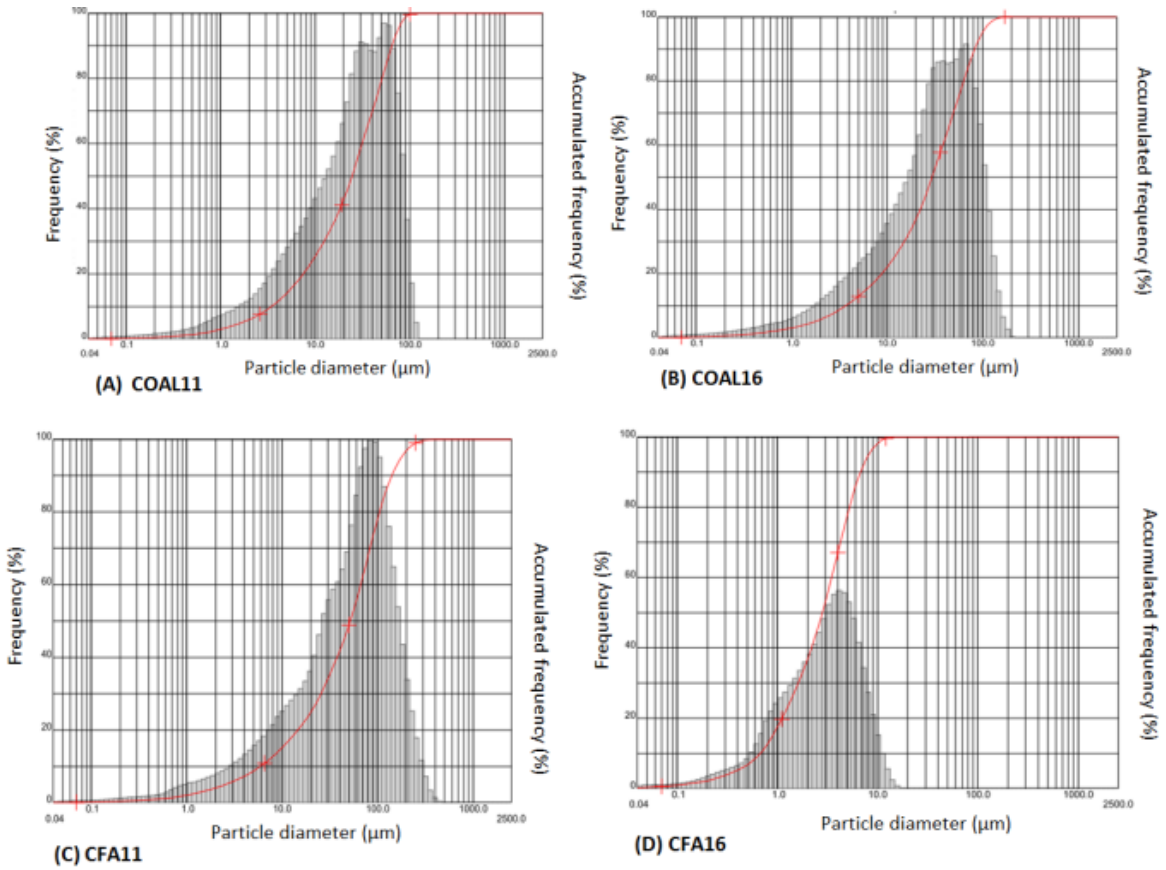


Figure 1. Histogram of the frequency distribution of particle diameters of (a) Coal 11 (COAL11), (b) coal 16 (COAL16), (c) coal fly ash 11 (CFA11) and (d) coal fly ash 16 (CFA16) particles samples indicated by analysis of laser diffraction.

Chemistry composition of coal and coal fly ashes samples (wt %) are presented in Table 1. The composition of oxides was similar in all samples. In particular, higher concentrations were observed for SiO_2 followed by Al_2O_3.

Table 1. Chemistry composition of coal (COAL11 and COAL16) and coal fly ashes (CFA11 and CFA16) samples (wt %) indicated by XRD data.

Compounds	COAL11	CFA11	COAL16	CFA16
SiO_2	63.32	61.42	63.11	56.23
TiO_2	0.43	1.22	1.82	1.46
Al_2O_3	28.37	24.98	27.53	29.29
Fe_2O_3	2.32	6.01	2.00	4.79
MgO	0.32	0.83	0.23	0.97
CaO	1.05	1.64	0.78	1.56
Na_2O	0.61	0.59	0.63	0.46
K_2O	2.98	2.85	3.01	3.32
P_2O_5	0.00	0.07	0.00	0.22
SO_3	0.60	0.33	0.89	1.66

In Table 2 trace element concentrations in coal and coal fly ashes samples (ppm) are shown (Silva et al., 2010). Concentrations of trace elements in coal samples were similar, but in general the coal fly ashes had higher concentrations of trace elements.

3.1.1. Quantification of polycyclic aromatic hydrocarbons (PAH)

Table 3 shows the results of quantification of PAH. Compounds found in higher concentrations in all sample particles are Anthracene, Fluoranthene, Benzo(a)anthracene. In particular, the amounts of PAH were higher in coal samples compared to coal fly ash samples. Specifically, in COAL11 contents of naphthalene, phenanthrene and benzo(a)anthracene are significant, whereas in COAL16 contents of acenaphthene, anthracene and fluoranthene were higher.

Table 2. Trace elements concentrations in coal (COAL11 and COAL16) and coal fly ashes (CFA11 and CFA16) samples (ppm) analyzed by coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS).

Trace elements	COAL11	CFA11	COAL16	CFA16
As	9.73	10.0	12.82	139.0
B	34.38	<1	60.16	<1
Ba	219.18	427.0	215.34	729.1
Be	4.27	7.9	4.11	12.6
Bi	<0.8	<1	<0.8	7.2
Cd	<0.8	<1	<0.8	3.4
Ce	83.51	168.1	85.86	194.0
Co	6.88	15.0	8.40	17.7
Cr	59.46	105.3	61.85	161.1
Cs	10.40	23.9	10.42	29.3
Cu	17.09	38.5	21.07	54.8
Dy	5.34	10.6	5.42	14.3
Er	2.82	5.6	2.79	7.7
Eu	1.01	1.9	1.02	2.3
Ga	16.38	30.4	16.78	110.9
Gd	7.22	14.2	7.25	17.1
Ge	7.67	14.7	6.50	85.2
Hf	5.74	10.7	5.71	14.5
Hg	0.18	0.00044	0.23	0.6817
Ho	1.06	2.2	1.06	3.0
La	31.37	63.6	32.42	75.0
Li	41.27	79.1	42.33	104.6
Lu	<0.8	1.1	<0.8	1.5
Mn	141.40	307.3	120.50	326.5
Mo	2.44	5.3	2.72	19.6
Nb	32.46	113.2	32.93	126.3
Nd	35.19	68.3	36.16	79.5
Ni	14.96	35.0	19.09	43.6
Pb	19.26	43.2	25.07	161.0
Pr	8.08	16.3	8.36	19.0
Rb	57.15	122.2	56.04	143.8
Sb	<0.7	2.1	0.62	7.1
Sc	14.11	25.7	14.77	36.8
Se	3.01	5.2	3.78	18.6
Sm	7.60	14.4	7.88	16.9
Sn	3.93	7.4	4.03	36.5
Sr	67.27	132.0	61.99	200.3
Ta	5.24	64.3	5.20	81.8
Tb	0.91	1.8	0.91	2.4
Th	15.15	33.5	15.42	41.3
Tl	1.42	4.2	1.82	9.7
Tm	<0.8	1.1	<0.8	1.5
U	6.92	14.5	6.12	24.8
V	103.48	197.9	108.46	351.3
W	4.01	23.1	4.10	71.0
Y	34.25	69.0	33.79	92.8
Yb	3.20	6.4	3.24	8.7
Zn	91.76	219.9	91.24	913.0
Zr	263.74	530.9	245.53	746.0

Table 3. PAH concentration per sample (± standard deviation) analyzed by HPLC/UV/Vis.

Compounds	COAL11 ($\mu g.kg^{-1}$)	CFA11 ($\mu g.kg^{-1}$)	COAL16 ($\mu g.kg^{-1}$)	CFA16 ($\mu g.kg^{-1}$)
Naphthalene	14532.0±867.5	2725.6±15.17	2456.0±202.5	16.4±0.053
Acenaphthene	15.2±0.543	13.9±2.72	26.9±0.766	5.2±0.127
Phenanthrene	994.3±45.2	194.4±19.5	251.4±10.3	23.2±1.7
Anthracene	3247.0±50.10	2837.0±30.17	4448.0±51.15	3052.0±6.88
Fluoranthene	2582.0±325.1	1431.0±8.69	7986.0±217.9	6356.0±371.0
Benzo(a)anthracene	3477.0±88.55	429.4±6.10	2218.0±46.40	1540.0±9.98
Benzo(g,h,i)perylene	1.0±6.49	1.0±5.53	1.0±1.20	0.9±4.98
Benzo(b)fluoranthene	0.8±0.016	0.2±0.003	0.6±0.001	0.2±0.080
Dibenzo(a,h)anthracene	15.2±0.164	16.3±0.561	15.6±0.624	13.2±0.006
Indeno(1,2,3-c,d)pyrene	9.6±0.015	ND	9.7±0.013	ND
Benzo(k)fluoranthene	0.8±0.002	ND	0.8±0.007	ND

3.2. Cytotoxicity

The clonogenic assay was employed to determine the colony-forming ability in V79 cells exposed to different concentrations of mineral coal dust and coal fly ash. As demonstrated in Figure 2, COAL11 and CFA11, COAL16, and CFA16 particle samples were able to decrease the cell viability in a dose-dependent manner.

COAL11 showed a higher cytotoxic effect when compared to CFA11. In addition, by nonlinear regression application, IC_{50} values of 0.4 mg/mL and 0.83 mg/mL for COAL11 and CFA11, respectively, were determined. CFA16 was slightly more cytotoxic than COAL16, with IC_{50} values of 0.59 mg/mL for COAL16 and 0.55 mg/mL for CFA16 (Figure 2).

Based on these results, concentration ranges that induced up to 50 % of decrease in the viability were chosen for genotoxicity analysis.

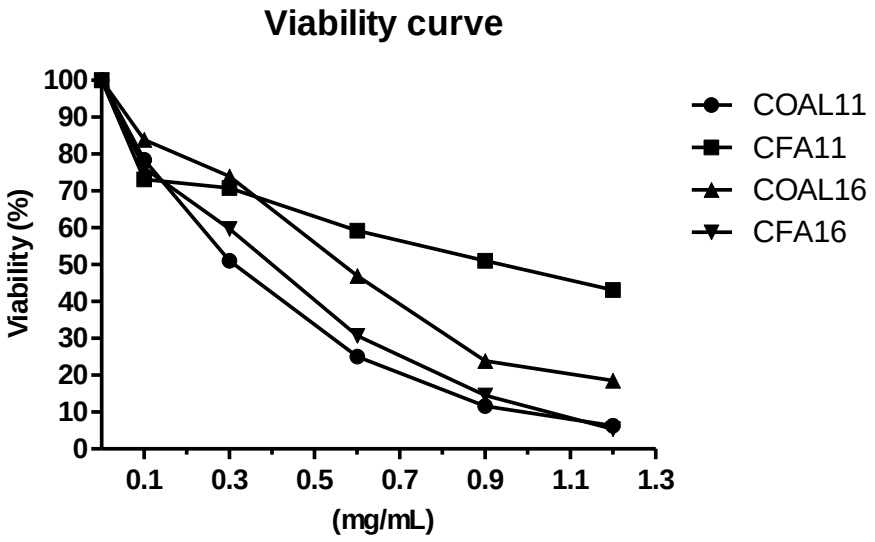


Figure 2. Cell viability of V79 exposed to COAL11, coal fly ash 11 (CFA11), COAL16, and coal fly ash 16 (CFA16) particles samples, evaluated using clonogenic assay after 24 h exposure time for V79 cell line. The concentration was considered cytotoxic when cell survival was 50%. The results are shown as the mean of three independent experiments.

3.3. Genotoxicity and oxidative DNA Damage in V79 cells

3.3.1. Alkaline comet assay

After verifying the cytotoxic profile, the DNA damage potential of the four samples was evaluated by the alkaline comet assay. For this purpose, cells were exposed to 0.025, 0.05, 0.1, 0.2 and 0.4 mg/mL COAL11; 0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL CFA11; and 0.0375, 0.075, 0.15, 0.3 and 0.6 mg/mL COAL16 or CFA16 for 3 h at 37 °C and 5 % CO_2.

In the results, for COAL11 and CFA11 samples, a dose-dependent relationship was observed for the % Tail DNA parameter (Figure 3a, 3b). Furthermore, a significant increase in % Tail DNA was detected in cells exposed to 0.2 and 0.4 mg/mL COAL11 and 0.1, 0.2, 0.4 and 0.8 mg/mL CFA11 when compared to the negative control ($P < 0.05$). As demonstrated in Figure 4, COAL16 and CFA16 samples induced a significant

increase in DNA damage when cells were exposed to 0.075, 0.15, 0.3 and 0.6 mg/mL concentrations, compared to the negative control (P <0.05).

3.3.2. Lesion-specific endonucleases-modified comet assay

The induction of oxidative damage was evaluated through the modified version of the comet assay where slides were treated with the FPG and EndoIII repair enzymes which introduce breaks at DNA sites with oxidative lesions. In COAL11 and CFA11 samples no significant increase in the % Tail DNA was observed when slides were incubated with ENDO III or FPG enzymes in comparison to the negative control (Figure 3a,3b).

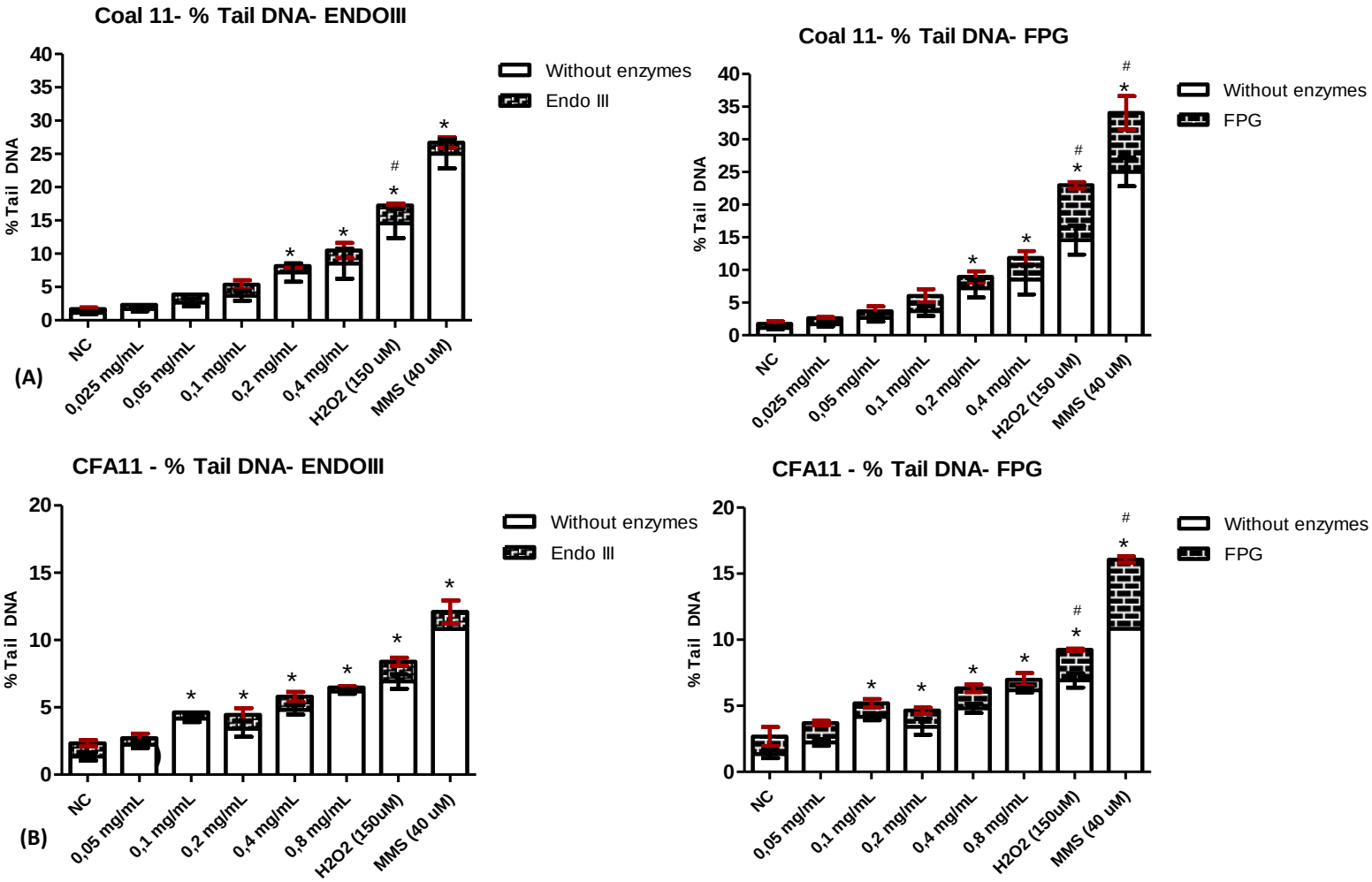


Figure 3. (A) Coal 11 (COAL11) and (B) coal fly ash 11 (CFA11). % Tail DNA in Alkaline Comet Assay (white) and oxidative damage % Tail DNA (dotted) in the modified comet assay in V79 cells after treatment with Endo III and FPG enzyme. *Statistically significant difference in relation to the negative control (NC) ($p < 0.05$). #Oxidative damage increase significant difference in relation to the negative control with ENDO III and FPG ($p < 0.05$). The results are shown as the mean ± standard error.

As shown in Figure 4, for COAL16, no increase in induction of DNA breaks was observed after ENDOIII incubation. However, using the FPG enzyme, a significant increase in oxidative damage was detected in cells exposed to 0.6 mg/mL COAL16 when compared to the related negative control (Figure 4a). For the CFA16 sample, a significant increase in oxidative damage was found in comparison to the related negative control using 0.3 mg/mL ENDOIII or 0.3 mg/mL and 0.6 mg/mL FPG, respectively (Figure 4b). Taken together, it was observed that for all samples, the ENDO III enzyme was able to produce a slight increase in the % Tail DNA parameter, however in some of the samples evaluated the increase was not statistically significant.

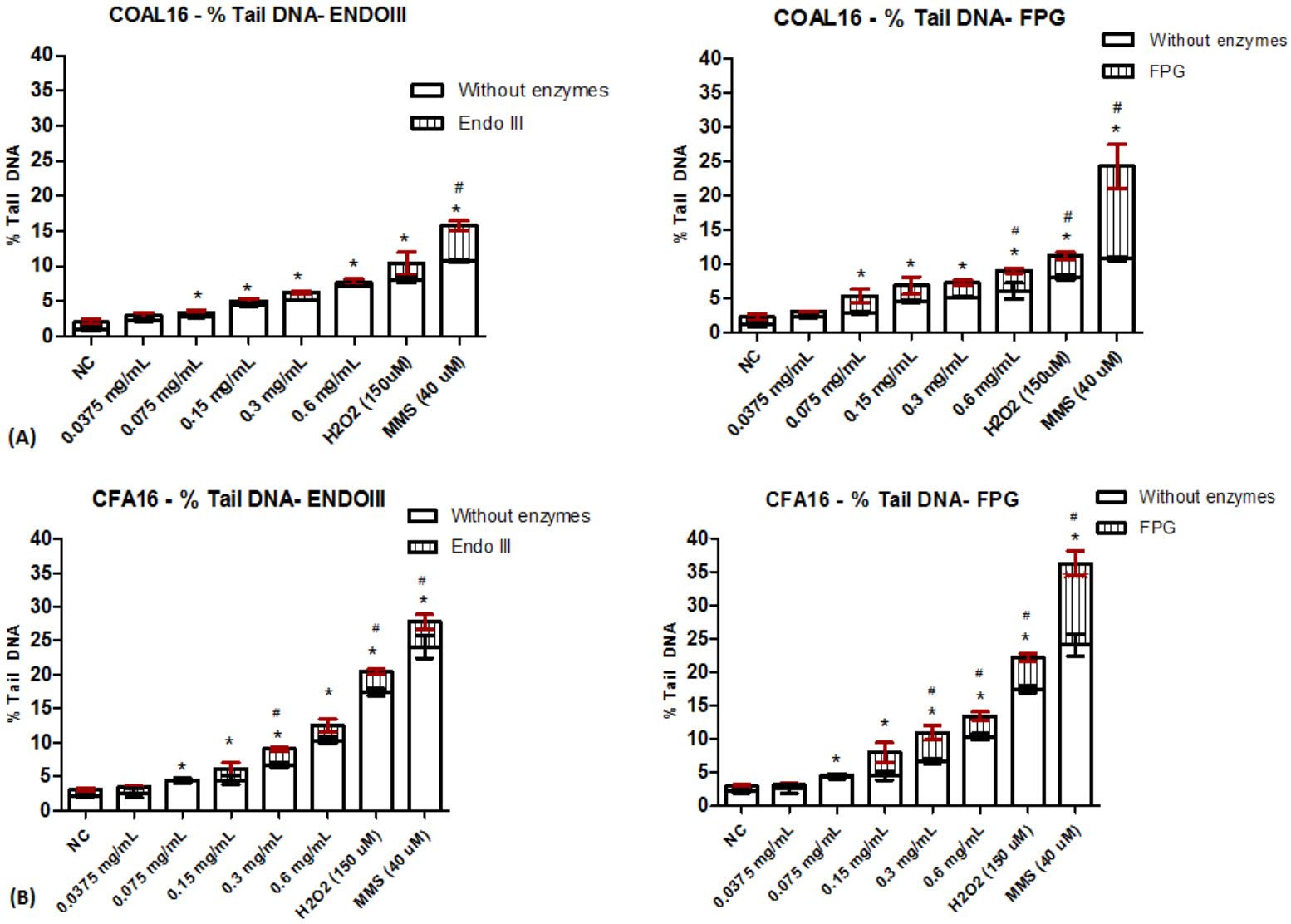


Figure 4. (A) Coal 16 (COAL16) and (B) coal fly ash 16 (CFA16). % Tail DNA in Alkaline Comet Assay (white) and oxidative damage % Tail DNA (dotted) in the modified comet assay in V79 cells after treatment with Endo III and FPG enzyme. *Statistically significant difference in relation to the negative control (NC) ($p < 0.05$). #Oxidative damage increase significant difference in relation to the negative control with ENDO III and FPG ($p < 0.05$). The results are shown as the mean ± standard error.

3.3.3. CBMN-Cyt assay in V79 cells

Cells were exposed to the same concentration ranges evaluated in the comet assay and analyzed by the CBMN-Cyt assay. Firstly, the nuclear division index was determined to detect cytostatic effects. No differences were observed among the cultures exposed to the negative control and to coal and coal fly ashes particles (Table 4).

The results for COAL11 (Table 4) show that the number of apoptotic and necrotic cells increased in a dose-dependent manner with mineral coal concentration, but the finding was only statistically significant at concentrations 0.2 mg/mL and 0.4 mg/mL ($P < 0.05$). The chromosomal damage or instability status was determined by MN frequency, NPBs and NBUDs in the BN cells. These biomarkers were significantly different in the highest concentrations when compared to the negative control group ($P < 0.05$).

For the CFA11 sample (Table 4), the increase in the number of apoptotic cells was only significant for a concentration of 0.8 mg/mL compared to the negative control. A dose-dependent trend was observed in MN frequency, NPBs and NBUDs but only the highest concentration (0.8 mg/mL) was significantly different comparing with negative control group ($P < 0.05$).

The results for COAL16 sample are shown in Table 4. The number of apoptotic and necrotic cells were statistically significantly increased at the highest concentrations (0.3 mg/mL and 0.6 mg/mL). The biomarkers MN, NPBs and NBUDs were statistically significantly increased at the highest concentration (0.6 mg/mL) compared to the negative control ($P < 0.05$).

Table 4 also shows the results of CFA16 sample. The number of apoptotic cells was only significantly increased in 0.6 mg/mL and the positive control ($P < 0.05$). The number of necrotic cells was significantly increased at concentrations of 0.3 and 0.6 mg/mL when compared to the negative control group ($P < 0.05$). The values of the biomarkers MN, NPBs and NBUD were significantly increased in the concentration 0.6 mg/mL compared to the negative control ($P < 0.05$). The positive control, MMS showed the expected effect demonstrating the sensitivity of the test system.

Table 4. CBMN cytome biomarkers in V79 cells exposed to different concentrations of coal and coal fly ash samples

Groups (mg/mL)	NDI	MN	NPBs	NBUDs	APOP (%)	NECR (%)
COAL11						
0	2.01±0.02	2.08±0.38	1.0±0.50	1.08±0.28	0.70±0.13	0.65±0.26
0.025	2.02±0.03	3.33±0.57	1.75±0.25	1.25±0.25	1.21±0.11	0.93±0.18
0.05	2.03±0.03	3.16±0.52	1.91±0.38	1.83±0.38	1.13±0.30	0.98±0.16
0.1	1.99±0.04	3.91±0.38	1.91±0.57	2.16±0.39	1.25±0.18	1.35±0.40
0.2	1.99±0.01	4.25±0.50*	2.41±0.28*	2.50±0.66*	1.55±0.25*	1.88±0.51*
0.4	1.99±0.03	4.16±0.52*	2.66±0.80*	2.58±0.87*	2.71±0.27*	2.48±0.20*
CFA11						
0	1.91±0.01	1.41±0.28	1.25±0.43	1.16±0.14	0.48±0.07	0.43±0.14
0.05	1.93±0.01	3.41±0.94	1.75±0.25	1.66±0.16	0.58±0.18	0.61±0.17
0.1	1.92±0.01	3.16±1.12	2.41±1.18	1.50±0.25	0.75±0.31	0.65±0.35
0.2	1.90±0.02	3.83±0.62	2.58±0.80	1.50±0.27	0.81±0.28	0.65±0.17
0.4	1.89±0.02	4.25±0.86	2.91±0.38	1.66±0.14	1.11±0.54	1.10±0.39
0.8	1.90±0.01	4.91±0.94*	4.00±0.43*	2.00±0.43*	1.46±0.44*	1.20±0.44
COAL16						
0	1.99±0.03	3.41±0.62	1.91±0.14	1.41±0.28	0.75±0.18	0.55±0.12
0.0375	2.00±0.06	4.41±1.50	3.58±1.23	1.91±0.38	1.13±0.16	0.86±0.18
0.075	1.96±0.01	4.83±0.62	4.33±1.15	2.91±0.38	1.46±0.16	1.30±0.39
0.15	2.00±0.04	5.41±0.80	4.50±2.70	2.75±0.25	1.50±0.38	1.38±0.69
0.3	1.99±0.03	5.16±0.72	5.25±0.86	3.25±0.66	1.56±0.51*	1.50±0.36*
0.6	2.00±0.02	6.91±0.38*	8.08±2.08*	3.91±2.00*	1.76±0.58*	1.66±0.33*
CFA16						
0	1.97±0.01	2.33±0.14	1.00±0.25	0.83±0.38	1.43±0.88	0.50±0.40
0.0375	1.97±0.02	4.16±1.04	2.25±0.66	1.08±0.76	1.71±0.51	0.90±0.35
0.075	2.01±0.03	4.58±1.66	2.41±0.38	1.83±0.87	2.46±0.79	1.43±0.18
0.15	2.02±0.05	4.58±0.52	1.91±1.04	1.58±0.80	2.03±0.80	0.95±0.22
0.3	2.04±0.03	4.75±0.50	2.25±0.25	2.16±0.14	2.10±0.68	1.76±0.46*
0.6	2.04±0.01	5.58±1.12*	3.00±1.25*	2.83±0.76*	3.45±1.38*	1.80±0.05*
40 µM MMS	1.98±0.05	13.20±1.75*	7.87±0.75*	5.33±0.80*	3.30±0.45*	2.68±0.50*

The biomarkers MN, NPBs, and NBUDs were counted by scoring 1000 binucleated cells per slide, whereas 500 cells were scored to calculate the Apop, Necr, and NDI. BN, binucleated cells; NDI, nuclear division index; Apop, apoptotic cells; Necr, necrotic cells; MN, micronuclei; NPBs, nucleoplasmic bridges; NBUDs, nuclear buds. Data are expressed as mean ± standard error of three independent experiments. *Significant difference compared with negative control group within a column, $p < 0.05$.

4. Discussion

It is known that extraction and burning of coal is an important pollution factor, which may represent a threat to human health and natural populations (León-Mejía et al. 2011; Rohr et al. 2013). In Santa Catarina (Brazil), where the largest of the thermal plants in Brazil is located, the coals used for power production tend to be high in ash and sulfur (Silva et al. 2009). In the present study the potential cytotoxicity and genotoxic effects of the coal and coal fly ash particles from Santa Catarina, was assessed *in vitro* using V79 (Chinese hamster lung fibroblasts) cells. Four samples were tested, two samples of coal particles and two samples of fly ash products of the coal burning process.

In our study we applied the comet assay using the standard protocol and specific endonuclease-modified comet assay to determine DNA strand breaks and oxidative damage, respectively. In this work the enzymes Endo III and FPG were used. The endonucleases are lesion-specific and convert DNA base lesions to breaks under the conditions of the alkaline comet assay (Collins, 2014). FPG and ENDOIII recognize different types of oxidative damage. While FPG is specific for oxidized purines and ring-opened purines, ENDOIII recognizes oxidized pyrimidines, including thymine glycol and uracil glycol (Dizdaroglu, 2005; Collins, 2014). For sample COAL16, a statistically significant increase in levels of DNA damage was observed when using 0.6 mg/mL FPG while for sample CFA16 increased DNA damage was observed when using 0.3 mg/mL Endo and both concentrations of FPG. These results demonstrate that the components of coal and coal fly ash are capable of inducing oxidative DNA damage likely caused by the components of these particles. The effects induced by coal exposure have been described in different cells such as murine alveolar type II epithelial cells (C10) (Albrecht et al. 2002) and in 7TD1 cells (Gosset et al. 1991). ROS

generation and oxidative damage by coal fly ash particles have been described in different cell lines, in human peripheral blood mononuclear cells (Dwivedi et al.2012), in rat alveolar macrophages (NR8383) (Diabaté et al. 2002), in BEAS-2B human lung epithelial cells (Diabaté et al. 2011) and in rat lung epithelial (RLE) cells (van Maanen et al. 1999).

Our data showed that 50% of the coal and coal fly ash particles have diameter less than 30 µm, in particular 10% of sample COAL11 contains particles of 3.44 µm, CFA11 contains 5.91 µm particles, COAL16 contains particles of 3.69 µm and CFA16 contains particles of 0.70 µm. Comparing the diameter of all samples, CFA16 contains smaller particles, it is possible the use of mixture of fuel oil and diesel oil in the combustion led to a more efficient burning and the formation of smaller particles.

Other components contained in coal particles were trace elements such as As, Co, Cr, Fe, Hg, Mn, Ni, Pb, V and Zn. In coal fly ash particles representative trace elements were As, Ba, Ce, Cr, Mn, Hg, Pb, Ni, Rb, Sr, V, Zn and Zr. High temperature combustion as used in coal combustion, can release metals such as Cd, Pb and Zn which become volatile in the cooling phase and are condensed on the surface of core aluminosilicate (Silva et al. 2010). It is known that redox active metals like Fe, Cu, Cr and Co and other metals undergo redox cycling reactions and possess the ability to produce reactive radicals such as superoxide anion radical and nitric oxide in biological systems (Jomova and Valko, 2011). Furthermore, Fenton oxidative reaction depends of catalysts of metal mainly Fe, but also Co, Cr, V, Co. Via Fenton reaction are produced radicals highly reactive such as the hydroxyl radical (OH·), which arises as a product of the reaction between superoxide and H_2O_2 catalyzed mainly by Fe2+ or Fe3+ (Sharma et al. 2012). On the other hand, the redox inactive metals, such as Cd, As, Hg and Pb show their toxic effects via bonding to sulphydryl groups of proteins and depletion of

glutathione (Jomova and Valko, 2011). DNA damage caused by ROS can be recognized by DNA glycosylases, apurinic/apyrimidinic endonucleases of the base excision repair mechanism and in some cases by the nucleotide excision repair machinery, leading to increase on DNA strand-breaks (Sander and Wilson, 2005) as was observed in this study.

The samples analyzed in this study contained oxides such as TiO_2, Al_2O_3, Fe_2O_3, CaO, MgO, MnO, Na_2O, K_2O, P_2O_5, SO_3 and SiO_2 also known as silica. These elements are most commonly found in coal and coal mining residues. Silica arranged in a three-dimensional network forms the quartz. Importantly, in all of the particles samples a high concentration of SiO_2 was found. IARC classified inhaled crystalline silica (quartz or cristobalite) from occupational sources as a Group 1 carcinogen (IARC, 1997). Silica can also initiate apoptosis in response to oxygen- and nitrogen-based free radicals, leading to mitochondrial dysfunction, increased gene expression of death receptors (Fubini and Hubbard, 2003).

Some effects may be associated with early events in carcinogenesis, eg micronucleus formation, which has been suggested as a predictive of cancer risk biomarker (Bonassi et al. 2011). Accordingly, our results in the CBMN-Cyt demonstrate increased chromosomal instability associated with exposure to coal and coal fly ashes. There is a growing concern whether the human populations remain at risk of exposure to carcinogenic coal products such as silica dust and organic compounds (Jenkins et al. 2013).

In the CBMN-Cyt assay analysis, DNA damage events are scored specifically in once-divided binucleated (BN) cells and include (a), micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss, (b), nucleoplasmic bridges

(NPBs), a biomarker of DNA misrepair and/or telomere end-fusions, and (c), nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes (Fenech, 2007). Accordingly, when we compared the CBMN-Cyt biomarkers to measure the chromosomal instability assessed by biomarkers MN, NPBs and NBUD, we found a statistically significant increases of the biomarkers in the highest concentrations of the coal and coal fly ash samples. Despite the genotoxic effects caused by exposure to the highest concentrations of these particles, none of these particle samples caused cytostatic effects evaluated using the nuclear division index (NDI), which provides a measure of the proliferative status of the viable cell fraction (Fenech, 2007).

In our results, cells that were exposed to COAL11 and COAL16 particles showed comparable genotoxic effects. Particularly the CFA16 sample showed stronger genotoxic effects in exposed cells than the sample CFA11. CFA16 contains higher concentrations of anthracene, fluoranthene, benzo(a)anthracene compared with CFA11. Despite the structural similarities, PAHs can vary greatly in their carcinogenic potential. The exposure to particle-bound PAHs can lead to DNA adduct formation and ROS generation (Singh et al. 2007). The response to this damage stimulates different signaling pathways. DNA damage tolerable can lead to responses such as cell cycle arrest and DNA repair, while the excessive / irreparable damage DNA can lead to the activation of the apoptosis program against damage caused by carcinogens (Castorena-Torres et al. 2008; Jarvis et al. 2014). This coincides with the results of CBMN-cyt that in highest concentrations caused cell death as it was shown in apoptosis and necrosis biomarkers.

Conclusions

In summary, our data show that the components contained in the particles may be related to ROS generation, DNA damage and formation of pro-mutagenic adducts, as evidenced by the biomarkers used in this study. The comet assay results showed that exposure of V79 cells to coal and coal fly ash particles induced primary DNA lesions. Besides, in the Cyt-CBMN analysis the V79 cells exposed to high concentrations of coal and coal fly ash particles were also able to induce cytotoxic effects (apoptosis and necrosis) and chromosomal instability (nucleoplasmic bridges, nuclear buds and MN formation).

Acknowledgments. This work was supported by grants from Conselho Nacional para o Desenvolvimento Científico e Tecnológico-CNPq – Brazil. Universal Grant Number 470833; Produtividade Grant Numbers 300902 and 303364. Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil. Grant Number PRONEM 11/2070-and PRONEX/FAPERGS/CNPq no. 10/0044-3). Grethel León-Mejía benefited from a scholarship financed by PEC-PG/CAPES. Marcos L.S. Oliveira and Luis F.O. Silva benefited from a scholarship financed by CNPq.

References

Albrecht C, Borm PJA, Adolf B, Timblin CR, Mossman BT (2002) In Vitro and in Vivo Activation of Extracellular Signal-Regulated Kinases by Coal Dusts and Quartz Silica. Toxicology and Applied Pharmacology 184: 37–45. doi: 10.1006/taap.2002.9500

Azqueta A, Slyskova J, Langie SA, O'Neill Gaivão I, Collins A (2014) Comet assay to measure DNA repair: approach and applications. Front Genet 25: 5-288. doi: 10.3389/fgene.2014.00288

Begas E, Papandreou C, Tsakalof A, Daliani D, Papatsiba G, Asprodini E (2013) Simple and Reliable HPLC Method for the Monitoring of Methotrexate in osteosarcoma Patients. Journal of Chromatographic Science 1–6. doi: 10.1093/chromsci/bmt08

Bonassi S, El-Zein R, Bolognesi C, Fenech M (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26: 93-100. doi: 10.1093/mutage/geq075.

Borm PJ (1997) Toxicity and occupational health hazards of coal fly ash (CFA). A review of data and comparison to coal mine dust. Ann Occup Hyg 41: 659-76. doi: 10.1093/annhyg/41.6.659

Castorena-Torres F, Bermudez de Leon M, Cisneros B, Zapata-Perez O, Salinas JE, Albores A (2008) Changes in gene expression induced by polycyclic aromatic hydrocarbons in the human cell lines HepG2 and A549. Toxicology In Vitro 22: 411-421.

Cavalcante RM, De Lima DM, Correia LM, Nascimento RF (2008) Técnicas de extrações e procedimentos de CLEAN-UP para a determinação de Hidrocarbonetos Policíclicos Aromáticos (HPA) em sedimento da costa do Ceará. Quimica Nova 31: 1371-1377. dx.doi.org/10.1590/S0100-40422008000600019

Collins AR (2014) Measuring oxidative damage to DNA and its repair with the comet assay. Biochim Biophys Acta 1840: 794-800. doi: 10.1016/j.bbagen.2013.04.022

Depoi FS, Pozebon D, Kalkreuth WD (2008) Chemical characterization of feed coals and combustion-by-products from Brazilian power plants. International Journal of Coal Geology 76: 227–236. doi:10.1016/j.coal.2008.07.013

Diabaté S, Mülhopt S, Paur HR, Wottrich R, Krug HF (2002) In vitro effects of incinerator fly ash on pulmonary macrophages and epithelial cells. Int J Hyg Environ Health 204: 323-6. doi:10.1078/1438-4639-00109

Diabaté S, Bergfeldt B, Plaumann D, Ubel C, Weiss C (2011) Anti-oxidative and inflammatory responses induced by fly ash particles and carbon black in lung epithelial cells. Anal Bioanal Chem 401: 3197-212. doi: 10.1007/s00216

Dizdaroglu M (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutat. Res 591: 45–59. doi:10.1016/j.mrfmmm.2005.01.033

Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, (2005) Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol 2:10. doi: 10.1186/1743-8977-2-10

Duez P, Dehon G, Kumps A, Dubois J (2003) Statistics of the Comet assay: a key to discriminate between genotoxic effects. Mutagenesis 18**:** 159-66. doi: 10.1093/mutage/18.2.159

Dwivedi S, Saquib Q, Al-Khedhairy AA, Ali AY, Musarrat J (2012). Characterization of coal fly ash nanoparticles and induced oxidative DNA damage in human peripheral blood mononuclear cells. Sci Total Environ 15: 331-8. doi: 10.1016/j.scitotenv.2012.08.004

Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2: 1084-1104. doi:10.1038/nprot.2007.77

Fubini B, Hubbard A (2003) Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic Biol Med 34: 1507-16. doi:10.1016/S0891-5849(03)00149-7

Gosset P, Lassalle P, Vanhée D, Wallaert B, Aerts C, Voisin C, Tonnel AB (1991) Production of tumor necrosis factor-alpha and interleukin-6 by human alveolar macrophages exposed in vitro to coal mine dust. Am J Respir Cell Mol Biol 5: 431-6. doi: 10.1165/ajrcmb/5.5.431

IARC (International Agency for Research on Cancer), Arsenic, metals, fibres, and dusts, A review of human carcinogens (2012) http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C.pdf 100C: 526.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Silica, some silicates, coal dust and para-aramid fibrils. Monogr. Eval. Carcinog. Risks Hum. (1997) http://monographs.iarc.fr/ENG/Monographs/vol68/mono68.pdf. 68:1– 475.

Jarvis IW, Dreij K, Mattsson Å, Jernström B, Stenius U (2014) Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. Toxicology 3: 27-39. doi: 10.1016/j.tox.2014.03.012.

Jenkins WD, Christian WJ, Mueller G, Robbins KT (2013) Population cancer risks associated with coal mining: a systematic review. PLoS One 8: 71312. doi: 10.1371/journal.pone.0071312

Jomova K, Valko M (2011) Advances in metal-induced oxidative stress and human disease. Toxicology 283: 65-87. doi: 10.1016/j.tox.2011.03.001

León-Mejía G, Espitia-Pérez L, Hoyos-Giraldo L.S, Da Silva J, Hartmann A, Henriques JA, Quintana M (2011) Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ 409: 686–691. doi: 10.1016/j.scitotenv.2010.10.049.

Liu G, Niu Z, Van Niekerk D, Xue J, Zheng L (2008) Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions, analysis, and toxicology. Rev Environ Contam Toxicol 192: 1-28.

National Research Council (NRC) (2006) Managing coal combustion residues in mines. National Academies Press.Washington, D.C.

Norrish K, Hutton JT (1969) An accurate X-ray spectrographic method for the analysis of a wide range of geological samples. Geochimica et Cosmochimica Acta 33: 431–453. doi:10.1016/0016-7037(69)90126-4

Oliveira MLS, Waanders F, Silva LFO, Jasper A, Sampaio CH, McHabe D, Hatch RS, Hower JC (2011) A Multi-analytical Approach to Understand the chemistry of Fe-minerals in Fee Coals and Ashes. Coal Combustion and Gasification Products 3: 51-62. doi: 10.4177/CCGP-D11-00006.1

Oliveira MLS, Ward CR, French D, Hower JC, Querol X, Silva LFO (2012) Mineralogy and leaching characteristics of beneficiated coal products from Santa Catarina, Brazil. International Journal of Coal Geology 94: 314–325. doi:10.1016/j.coal.2011.10.004

Querol X, Whateley MKG, Fernandez-Turiel JL, Tuncali E (1997) Geological controls on the mineralogy and geochemistry of the Beypazari lignite, central Anatolia, Turkey. International Journal of Coal Geology 33: 255–71. doi:10.1016/S0166-5162(96)00044-4

Quispe D, Pérez-López R, Silva LFO, Nieto JM (2012) Changes in mobility of hazardous elements during coal combustion in Santa Catarina power plant (Brazil). Fuel 94: 495–503. doi:10.1016/j.fuel.2011.09.034

Ribeiro J, Valentim B, Ward C, Flores D (2011) Comprehensive characterization of anthracite fly ash from a thermo-electric power plant and its potential environmental impact. Int. J. Coal Geol 86: 204–212. doi:10.1016/j.coal.2011.01.010

Rohr P, Kvitko K, da Silva FR, Menezes AP, Porto C, Sarmento M, Decker N, Reyes JM, Allgayer M. da C Furtado TC, Salvador M, Branco C, da Silva J (2013). Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat Res 758: 23-8. doi:10.1016/j.mrgentox.2013.08.006

Sander M, Wilson S (2005) Base Excision Repair, AP Endonucleases and DNA Glycosylases, John Wiley & Sons Ltd, Chichester. doi: 10.1038/npg.els.0003871

Sharma A, Soussaline F, Sallette J, Dybdahl M (2012) The influence of the number of cells scored on the sensitivity in the comet assay. Mutat Res 749: 70-5. doi: 10.1016/j.mrgentox.2012.07.003

Sharma P, Bhushan A, Rama J, Dubey S, Pessarakli M (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. 26. http://dx.doi.org/10.1155/2012/217037

Silva L, Ward CR, Hower J, Izquierdo M, Waanders F, Oliveira M, Li Z, Hatch R, Querol X (2010) Mineralogy and leaching characteristics of coal ash from a major Brazilian power plant. Coal Combustion and Gasification Products 2: 51–65. doi: 10.4177/CCGP-D-10-00005.1

Silva LFO, Moreno T, Querol X (2009) An introductory TEM study of Fe-nanominerals within coal fly ash. Sci. Total Environ 407: 4972–4974. doi: 10.1016/j.scitotenv.2009.05.044

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184–91. doi:10.1016/0014-4827(88)90265-0

Singh R, Sram RJ, Binkova B, Kalina I, Popov TA, Georgieva T, Garte S, Taioli E, Farmer PB (2007) The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat Res 620: 83-92. doi:10.1016/j.mrfmmm.2007.02.025

Sun F, Littlejohn D, Gibson MG (1998). Ultrasonication extraction and solid phase extraction clean-up for determination of US EPA 16 priority pollutant polycyclic aromatic hydrocarbons in soils by reversed-phase liquid chromatography with ultraviolet absorption detection. Analytica Chimica Acta 25: 1–11. doi:10.1016/S0003-2670(98)00186-X

van Maanen JM, Borm PJ, Knaapen A, van Herwijnen M, Schilderman PA, Smith KR, Aust AE, Tomatis M, Fubini B (1999) In vitro effects of coal fly ashes: hydroxyl radical generation, iron release, and DNA damage and toxicity in rat lung epithelial cells. Inhal Toxicol 11: 1123-41. doi: 10.1080/089583799196628

Vassilev SV, Vassileva CG (1996a) Mineralogy of combustion wastes from coal-fired power stations. Fuel Process Technol 47: 261–80. doi:10.1016/0378-3820(96)01016-8

Vassilev SV, Vassileva CG (1996b) Occurrence, abundance and origin of minerals in coals and coal ashes. Fuel Process Technol 48: 85–106. doi:10.1016/S0378-3820(96)01021-1

CAPÍTULO II

COMPARATIVE ACUTE LUNG INFLAMMATION, PULMONARY MECHANICS, GENOTOXIC EFFECTS AND EXTRAPULMONARY TRANSLOCATION METAL IN MICE EXPOSED TO COAL AND COAL FLY ASH PARTICLES

Manuscrito a ser submetido para publicação

APRESENTAÇÃO

CAPITULO II

O Capítulo II apresenta um manuscrito intitulado "COMPARATIVE ACUTE LUNG INFLAMMATION, PULMONARY MECHANICS, GENOTOXIC EFFECTS AND EXTRAPULMONARY TRANSLOCATION METAL IN MICE EXPOSED TO COAL AND COAL FLY ASH PARTICLES".

Este manuscrito relata os efeitos da exposição às partículas de carvão e cinzas, em camundongos BALB/c, após 24 h de instilação intratraqueal. Estes efeitos estão relacionados com a significativa rigidez pulmonar e obstrução das vias aéreas centrais detectadas na mecânica pulmonar, recrutamento de células, principalmente mononucleares, e liberação de citocinas pró-inflamatorias. Também são descritos os efeitos genotóxicos em células de sangue periférico dos camundongos e a translocação eficiente de metais como Cr, Fe e Ni do pulmão para o fígado, baço e encéfalo.

COMPARATIVE ACUTE LUNG INFLAMMATION, PULMONARY MECHANICS, GENOTOXIC EFFECTS AND EXTRAPULMONARY TRANSLOCATION METAL IN MICE EXPOSED TO COAL AND COAL FLY ASH PARTICLES

Grethel León-Mejía[ab*], Mariana Nascimento Machado[c], Renata Okuro[c], Luis Silva[d], Johnny Dias[e], Claudia Telles[e], Andreas Hartmann[f], Juliana Da Silva[g], João Antônio Pêgas Henriques[ah], Walter Araujo Zin[c*]

[a]Departamento de Biofísica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[b]Unidad de Investigación, Desarrollo e Innovación en Genética y Biología Molecular, Universidad Simón Bolívar, Barranquilla, Colombia.

[c]Universidade Federal do Rio de Janeiro, Instituto de Biofisica Carlos Chagas Filho, Rio de Janeiro, Brazil.

[d]Laboratory of Environmental Researches and Nanotechnology Development, Centro Universitário La Salle, Mestrado em Avaliação de Impactos Ambientais em Mineração. Victor Barreto, 2288 Centro 92010-000, Canoas, RS, Brazil.

[e]Laboratório de Implantação Iônica, Instituto de Física, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil.

[f]Novartis Pharma AG, Basel, Switzerland.

[g]Laboratório de Genética Toxicológica, Universidade Luterana do Brasil (ULBRA), Canoas-RS, Brazil.

[h]Instituto de Biotecnologia, Universidade de Caxias do Sul (UCS), Caxias do Sul, RS, Brazil.

***Corresponding author:** Laboratório de Fisiologia da Respiração, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av Carlos Chagas Filho 373, CCS, Rm G2-042, Ilha do Fundão, 21941-902-Rio de Janeiro, RJ, Brazil. Tel: +55 21 2562 6557; fax: +55 21 2280 8193.

E-mail addresses: wazin@biof.ufrj.br

walter_zin@hotmail.com **(W.A.Zin).**

grethelleon@gmail.com **(G. León-Mejía).**

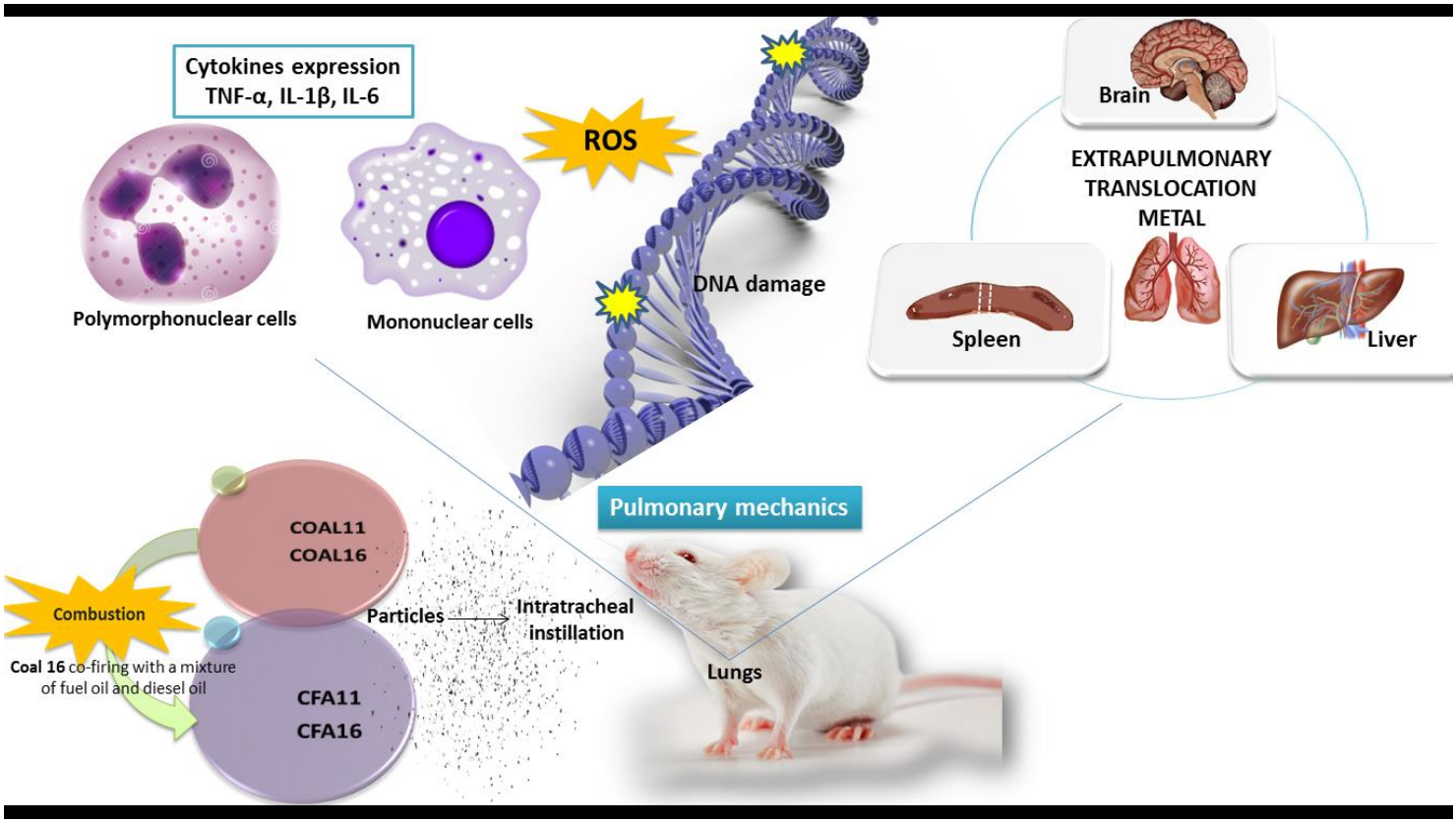
Abstract

Continuous exposure to particles of coal mining can cause a variety of diseases, such as Coal Workers Pneumoconiosis (CWP); silicosis; cancer and Chronic Obstructive Pulmonary Disease (COPD), as emphysema and chronic bronchitis. Particle load in the lung increases the alveolar macrophages and epithelial cells are activated, leading to the release of inflammatory mediators, Reactive Oxygen Species (ROS), enzymes, cytokines and growth factors that control and stimulates the fibrosis, genotoxic events and cell death. The aim of the present work was to compare the acute lung inflammation, pulmonary mechanics, genotoxic effects and extrapulmonary translocation metal in BALB/c mice exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles after 24 hours of intratracheally instilled BALB/c mice with 100 mg/kg of coal and CFA particles.

In results, only animals acutely exposed to coal particles showed significant rigidity and obstruction of the central airways. However, all animals exposed to coal and coal fly ash particles showed recruitment of cells, mainly mononuclear cells, and expression of cytokines, particularly TNF-α and IL-1β as compared to the control group. In histological analysis were not found significant alterations in the alveoli, or significant formation of elastic fibers and collagen. Comet assay alkaline data showed that exposure of animals to coal and coal fly ash particles induced significant primary DNA lesions. By Particle-induced X-ray emission technique (PIXE) was demonstrated the content of Cr, Fe and Ni in the liver, spleen and brain, showing the efficient translocation of metals from the bloodstream to extrapulmonary organs. These results are associated with the chemical heterogeneity of coal and coal fly ash particles such as the content of oxides, metals and PAH as was shown in this study.

Key words: Coal, coal fly ash, inflammation, ROS, DNA damage, metals translocation.

Graphical abstract



1. Introduction

There are numerous damaging environmental impacts of coal that occur through its mining, preparation, combustion, waste storage, and transport. Prior to its combustion, coal is pulverized and burned at temperatures of 1000 -1300 ºC (Silva et al., 2010). During this process, the inorganic particles do not have complete combustion and particulate coal fly ashes are produced (Martinez, 2012). Although coal fly ash particles are generally large and spherical in shape, there are usually irregularly-shaped particles present also, such as angular particles of quartz (Jones et al., 2009, Silva et al., 2010). The properties of fly ash generated during the coal combustion process will depend on the temperature used, the boiler type, the control devices used, such as electrostatic precipitators (Strand et al., 2002), and the type of coal which determines the properties of the coal fly ash due to its degree of carbonization and geological origin (Tsiridis et al., 2006, Oliveira et al., 2011, Mozgawa et al., 2014).

The chronic inhalation of coal dust or coal fly ash particles can cause a variety of lung diseases, including simple pneumoconiosis (CWP), progressive massive fibrosis (PMF), chronic bronchitis, loss of pulmonary function and emphysema (Schins & Borm, 1999, Santo Tomas, 2011). It is known that coal dust contains up to 10% of quartz. Quartz is part of a carbonaceus-matrix that clearly influences the intrinsic properties of coal dust (Jones et al., 2009). Paradoxically, crystalline silica (quartzo) is classified as carcinogenic to humans but the coal dust is not by the International Agency for Research on Cancer (IARC, 1997, Schins & Borm, 1999; Guidi et al., 2015).

When the coal and fly ashes particles are inhaled, they activate macrophages, neutrophils and epithelial cells. Activated macrophages and neutrophils produce excessive amounts of reactive oxygen species (ROS) and cytokines. Epithelial cells and fibroblasts which are the main producers of components of the extracellular matrix (including collagens, proteoglycans and elastic fibres) are also known to produce cytokines and ROS upon stimulation

(Schins & Borm, 1999; Sakai & Tager, 2013). ROS can also be generated by independent mechanisms in addition to the cellular pathway due to the inherent chemical properties of coal dust and components such as oxides (Vassilev and Vassileva 1996a,b), metals (Knaapen et al., 2004, Silva et al. 2010) and polycyclic aromatic hydrocarbons (PAH) (Liu et al., 2008; Rohr et al. 2013).

Experimental studies have led to the understanding of some of the biological mechanisms of pulmonary oxidative stress and inflammation induced by coal and coal fly ash particles (Smith et al., 2006, Kania et al., 2014). The alveolar wall has a large surface area and is composed of type I alveolar epithelial cells, endothelial cells, and the fused basement membrane between these two types of cells. This arrangement of cells forms a thin air–blood barrier and contributes to effective gas exchange. Inhaled ultrafine particles are able to migrate from the alveolar surface into the blood circulation (Furuyama et al., 2009). In fact, It has been described that inhaled insoluble particles penetrate into the microvasculature of the lung, entering the blood circulation and are then deposited in extrapulmonary organs in animals (Kreyling et al. 2002; Oberdörster 2002, Furuyama et al., 2009). The extrapulmonary toxicity probably depends upon the amount, water solubility and components of the particles. In this context, metals can be translocated to extrapulmonary organs, making them available to cause direct toxicity to other organ systems (Kreyling et al., 2002, Mani et al., 2007).

The aim of the present work was to compare the acute lung inflammation, pulmonary mechanics, genotoxic effects and extrapulmonary translocation in BALB/c mice exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles from Santa Catarina State-Brazil.

2. Materials and Methods

2.1. Animals

Male BALB/c mice (5–8 weeks old and 25-30 gr) were purchased from CEMIB-UNICAMP (Centro multidisciplinar para investigação biológica -Brazil). The mice were housed in plastic cages under controlled environmental conditions (controlled temperature of 24°C, 50% humidity controlled and artificial light (12-h light/12-h dark cycle). All animals had access to water and food *ad libitum*. This study was approved by Ethics Committee on the Use of Animals, Health Sciences Center of the University Federal de Rio de Janeiro (UFRJ). All de animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, USA.

2.2. Characteristics of coal and coal fly ash particles

Samples were collected in Santa Catarina State- Brazil, where is located the Jorge Lacerda – Tractebel Suez Thermoelectric Complex. The Jorge Lacerda power plant has seven different pulverized fuel units. The feed coals and coal fly ash were collected simultaneously from each of these units over a five-day period. The coal is, in general, high volatile C to high volatile A bituminous in rank, although individual units may be receiving different ratios of the blend components. In this study four samples were included, two coal (COAL11 and COAL16) and two coal fly ash (CFA11 and CFA16). The coal fly ash samples were collected from the respective electrostatic precipitators. Particularly, the coal fly ash sample 16 (CFA16) was collected from Unit 3 were taken while co-firing with a mixture of fuel oil and diesel oil as part of the boiler start-up procedure. More details about coal and coal fly ash particles samples used in this work are available in Silva et al. (2009), Silva et al. (2010), Oliveira et al.(2012), and Quispe et al. (2012).

To prepare the particle suspensions for animal experiments, prior to intratracheal instillation, COAL and CFA particles were suspended in sterile saline solution (0.9% NaCl) and

just before use in animals, the samples were twice sonicated for 10 min to obtain a uniform dispersion and to prevent particle aggregation.

2.3. Experimental design

Thirty BALB/c mice were included in this study. Mice received 100 mg/kg of COAL and CFA particles, as previously described in murine models of acute inflammation (Broeckaert et al., 1997). Were included four groups exposed to COAL and CFA particles and one control group (six animals in each experimental group). Animals were instilled intratracheally with 60 μL of this particles suspension. Control mice were instilled with 60 μL saline solution. Twenty four hours following a single instillation, blood samples from the inferior vena cava were obtained for analysis of genotoxicity by comet assay. In parallel to the collection of the animal blood samples additional samples of human blood by finger pricks were collected and processed under the same conditions. These samples were used as internal standards. The animals were tracheotomized and were analyzed the pulmonary mechanics parameters. After euthanized the animals, samples of spleen, brain, liver, and lungs were collected and used for analysis of metals by PIXE. The left lung was collected and immediately stored in buffer formalin and processed for histological analyses. Lung right was cryo-preserved and used for analysis of cytokines.

2.4. Pulmonary mechanics

Twenty-four hours after saline or particle suspension administration the animals were sedated (diazepam, 1 mg i.p.), anesthetized (pentobarbital sodium, 20 mg/kg body weight i.p.) and paralyzed (pancuronium bromide, 0.1 mg/kg). After placed in the supine position on a surgical table and a snugly fitting cannula (0.8 mm ID) was introduced into the trachea. Afterwards, the animals were mechanically ventilated (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a frequency of 100 breaths min^{-1} tidal volume of 0.2 mL, flow equal to 1 mL s−1, and positive endexpiratory pressure amounting to 2 cmH_2O. The anterior chest wall was surgically removed. A pneumotachograph (1.5-mm ID, length = 4.2 cm, distance

between side ports = 2.1 cm) was connected to the tracheal cannula for the measurement of airflow (*V'*). Changes in lung volume were obtained by flow signal digital integration. The pressure gradient across the pneumotachograph was determined by means of a Validyne MP45-2 differential pressure transducer (Engineering Corp, Northridge, CA, USA). Equipment resistive pressure (=Req.*V'*) was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. Transpulmonary pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp, Northridge, CA, USA). Briefly, we determined lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, static elastance (E_{st}), and viscoelastic component of elastance (ΔE) by the end-inflation occlusion method (Bates et al., 1985). ΔP1 selectively reflects airway resistance, and ΔP2 represents stress relaxation or viscoelastic properties and mechanical heterogeneities of the lung (Bates et al., 1989; Saldiva et al., 1992). Lung mechanics were measured 10–15 times in each animal.

2.5. Histological study

Heparin (1000 IU) was intravenously injected immediately after the determination of respiratory mechanics. The trachea was clamped at end expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly euthanized the mice. The lungs of mice control and mice instilled with COAL and CFA particles were quickly excised for histological analyses. Pulmonary left lobes were immediately formalin fixed and processed according to routine histological procedures. The right lungs were removed en *bloc* and quick-frozen by immersion in liquid nitrogen and fixed with Carnoy's solution (Nagase et al., 1992). After fixation, the tissue was embedded in paraffin. Four-µm-thick slices were cut and stained with hematoxylin-eosin (HE) or picrosirius red. Slides were qualitatively screened for histological lesions in the alveolar and the airway tissues.

Morphometric analysis was performed with an integrating eyepiece with a coherent system made of a 100-point and 50 lines (known length) grid coupled to a conventional light

microscope (Axioplan, Zeiss, Oberkochen, Germany). The volume fraction of collapsed and normal alveoli was determined in each sample by the point-counting technique (Gundersen et al., 1988) across 10 random non-overlapping microscopic fields at x400 magnification. The total amount of points also included those falling on tissue, airways and other non-alveolar structures.

The number of mononuclear (MN) and polymorphonuclear (PMN) cells in the pulmonary tissue was counted in each animal across 10 random non-overlapping microscopic fields at ×1000 magnification in a 10,000 $\mu m^{2.}$ In the same field the amount of points that fell on lung tissue was also counted, so that cellularity was expressed as percentage of lung tissue area (Gundersen et al., 1988; Capelozzi et al., 1997).

2.6. Cytokine analyses

The analyses of pro-inflammatory cytokines released were performed by ELISA kits (R&D Systems Europe, Abingdon, UK) for tumor necrosis factor-α (TNF-α), IL-1β and IL-6 according to the manufacturer's protocols.

2.7. Alkaline comet assay

The comet assay was carried out according to of the original methodology (alkaline version) described by Singh et al., 1988; with minor modifications. For the preparation of the samples, 10 uL of whole blood were mixed with heparin and 120 uL of low melting point agarose (LMA)(Invitrogen) at 37 ºC. This mixture was placed in a slide previously covered with 1.5% of normal melting point agarose (NMA) (Cambrex Bioscience Rockland) processed at 60 ºC. The mixture of LMA and blood on the slide was covered with a cover slip. After solidifying of the gel on the slides, the cover slip was removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, 1% with freshly added 1% Triton X-100 and 10% DMSO) at 4 ºC. Direct light exposure of the samples was avoided during the whole process. The slides were removed of lysis solution and placed for 30 min close to the anode of an

electrophoresis box containing alkaline buffer at 4 ºC (300 mM NaOH and 1 mM EDTA, pH > 13). The electrophoresis was carried out for 30 min at 25 V and 300 mA. Afterwards the alkalinity was neutralized with 0.4 M Tris (pH 7.5) with washes of 5 min for each slide. Finally, the DNA was stained with 30 ul Syber Green solution (2 µl/mL) and assessed using a fluorescence microscope equipped with a green filter of 540 nm.

For each sample images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. The cells were classified according to tail size into five classes ranging from undamaged (0) to maximally damaged (4), obtaining a measure of the individual damage for each animal and consequently for each analyzed group. The calculation of the damage index was carried out according to the visual classification system (Collins, 2004). The values for the damage index can range from 0 (100 cells class 0) up to 400 (100 cells class 4).

2.8. Particle-induced X-ray emission (PIXE)

To measure the total content of metals was used the particle induced X-ray emission (PIXE) technique (He et al., 1993; Johansson et al., 1995). This technique is multielemental character, high sensitivity, simplicity and high sample throughput (Mireles et al., 2004). Samples of spleen, liver, brain and lungs were collected. For the analyses, the samples were dried at 40 ºC for 96 h, then macerated using a mortar, and finally pressed into pellets which were positioned on the target of the reaction chamber. A 3 MV Tandetron accelerator provided 2.0 MeV proton beams with an average current of 5 nA at the target. The X-rays induced by the beam in the samples were detected by a Si(Li) detector with an energy resolution of about 155 eV at 5.9 keV. The spectra were analyzed with the GUPIXWIN software package (Maxwell et al., 1995; Campbell, 2000) and the final results are expressed in parts per million (ug g^{-1}). The chemical elements analyzed in the samples by the PIXE method were: sodium (Na),

magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), rubidium (Rb), chromium (Cr) and nickel (Ni). The organic matrix of the organs evaluated (the organic composition of the sample) was determined by the Rutherford Backscattering Spectrometry (RBS) technique.

2.9. Statistical analyses

For each histological parameter, pulmonary mechanics, cytokines and PIXE analyses measured in controls and treated mice, the mean values ± standard deviation (SD) were calculated. Each exposed group was compared with the control group using one-way ANOVA followed by Tukey's *post hoc* test. Student's t test (Welch correction) were used to compare the chemical elements analyzed by PIXE. Differences were considered to be significant at the 95% level ($p < 0.05$). All analyses were performed with the PRISMA 5.0 statistical software package (GraphPad Inc., San Diego, CA).

3. Results

3.1. Characteristics of coal and coal fly ash particles

Scanning electron microphotographs of CFA11 and CFA16 are shown in Figure 1. The particles are spherical in shape and form aggregates with particles of smaller sizes. Approximately 40% of COAL11 particles have a smaller diameter of 10 µm. In CFA11 and COAL16, a percentage of 25% and 30% of the particles had a diameter less than 10 µm respectively. In CFA16, 100% of the sample consisted of particles smaller than 10 µm (data not shown).

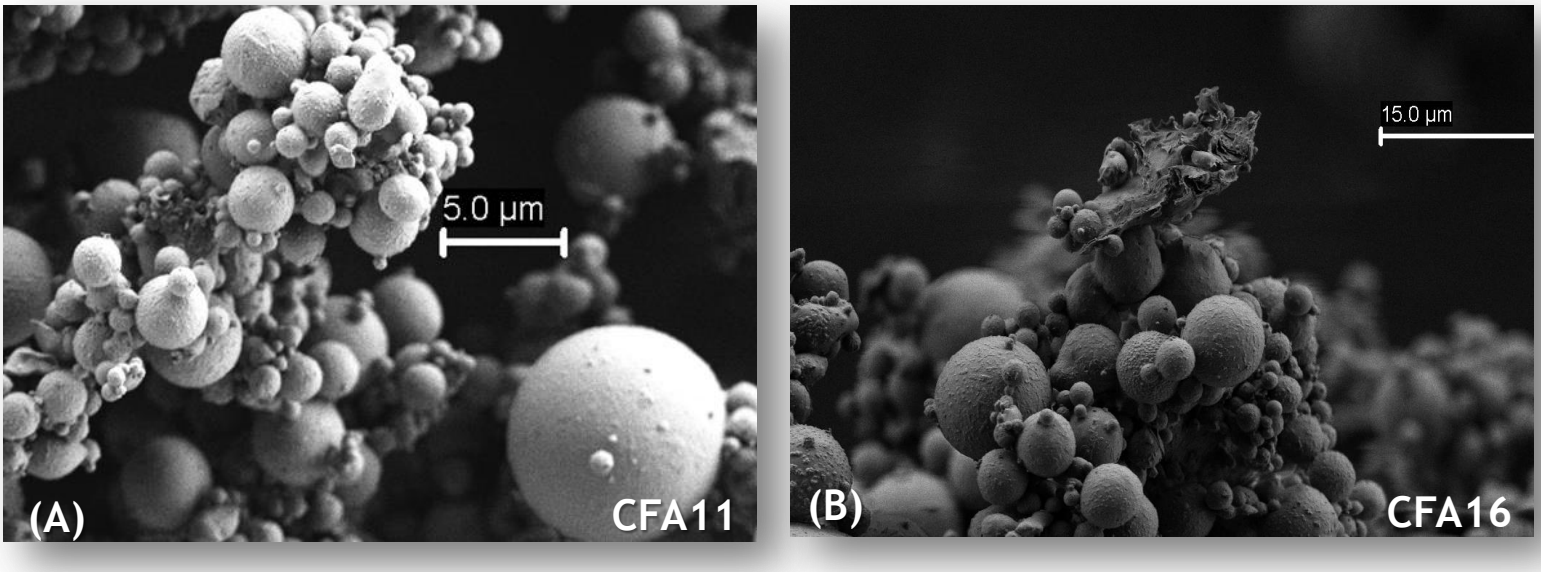


Figure 1. Electron scanning micrographs of (A) coal fly ash 11 (CFA11) and (B) coal fly ash 16 (CFA16) particles samples.

3.2. Pulmonary mechanics

In Table 1, pulmonary mechanics parameters of animals exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles are shown. In elastance and ΔP1 (Table 1) parameter analysis we observed significantly differences in animals exposed to coal particles (COAL11 and COAL16) in relation with control group. In ΔE viscoelastic component of elastance and ΔP2 (Table 1) parameter we found a significant increase only in animals exposed to COAL16 particles compared to the control group.

3.3. Histology

In analysis of histology was found a significant increased number of mononuclear cells in lung parenchyma of animals exposed to COAL11, CFA11, COAL16 and CFA16 particles compared with control group (Table 1). Significantly higher values of polymorphonuclear cells were found in the lung tissue of animals exposed to COAL16 particles compared with the control group (Table 1). In Figure 2 are showed representative photomicrographs of lung parenchyma of exposed group to coal and coal fly ash particles and control group. The COAL11 and COAL16 particles exposure caused significant inflammation in the lung parenchyma, evidenced by thickening of septa and alveolar edemas mainly in the COAL16 exposed group particles. In Table 1 is shown the percentage of normal and collapsed areas in pulmonary tissue. It was not found significant alterations in alveoli of animals exposed to coal and coal fly ash particles in relation to control group. In analysis of collagen and elastic fiber formation no significant difference was found between tissue lung of animals exposed to coal and coal fly ash particles and the control group (Table 1).

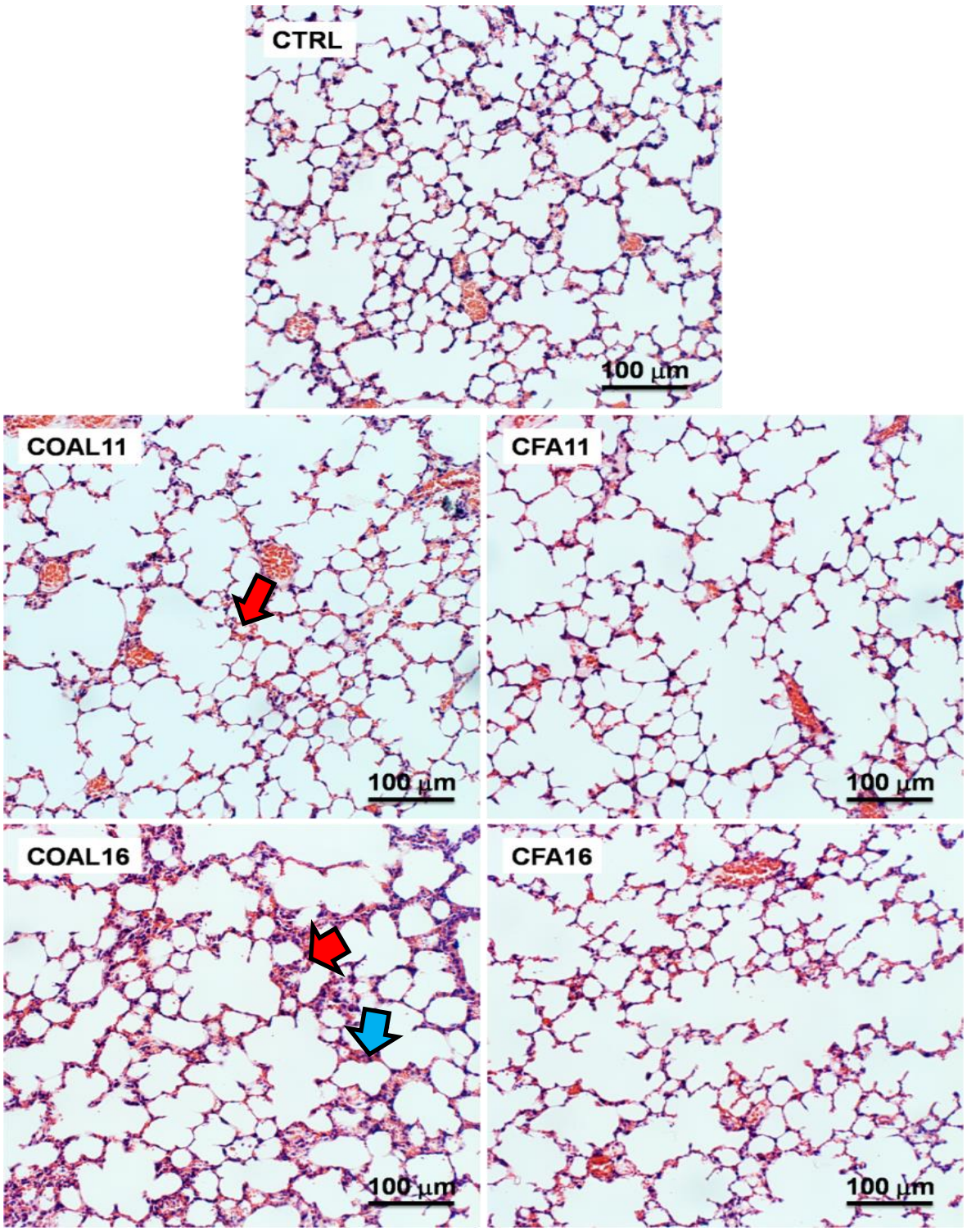


Figure 2. Representative photomicrographs of lung parenchyma stained with hematoxylin-eosin in control animals and in mice intratracheally instilled with a suspension of COAL11, CFA11, COAL16 and CFA16 (n = 6). Blue arrow indicates cell infiltrate along the alveolar septum. Red arrows indicate alveolar collapse. Twenty random non-coincident microscopic fields were evaluated per animal at a magnification of × 200. Bar equals 100 µm.

Table 1. Respiratory mechanics, histological outcomes and inflammatory markers in lung parenchyma.

	CTRL	COAL11	CFA11	COAL16	CFA16
Mechanics					
ΔP1 (cmH_2O)	0.03±0.02	0.85±0.65*	0.14±0.08	1.32±1.10*	0.48±0.23
ΔP2 (cmH_2O)	0.65±0.05	1.38±0.58	0.79±0.10	2.75±1.78*	1.26±0.44
Est (cmH_2O/mL)	18.50±2.72	47.56±20.20*	28.35±7.39	39.36±10.60*	28.66±6.90
ΔE (cmH_2O/mL)	3.24±0.25	6.95±3.03	3.86±0.60	10.75±5.71*	5.95±2.22
Histology					
Normal area (%)	39.69±8.46	40.01±4.39	38.37±3.50	33.75±5.77	38.98±2.15
Alveolar collapse (%)	45.51±8.39	45.20±6.25	44.45±7.79	55.92±8.96	51.35±2.46
PMN (cel x $10^{-3}/\mu m^2$)	31.21±7.05	48.04±11.49	40.05±5.87	59.13±19.61*	44.68±9.73
MN (cel x $10^{-3}/\mu m^2$)	29.10±6.58	37.77±4.83*	38.32±3.86*	48.55±5.13*	41.68±6.19*
Collagen fiber formation (tissue fibers %)	0.15±0.02	0.21±0.05	0.13±0.01	0.29±0.13	0.22±0.07
Elastic fiber formation (tissue fibers %)	0.20±0.12	0.25±0.08	0.16±0.06	0.22±0.07	0.23±0.14
Cytokines					
TNF-α (pg/mg of protein)	94.90±32.71	342.04±103.15*	264.10±81.11*	525.26±138.05*	280.21±107.44*
IL-1β (pg/mg of protein)	114.46±31.19	491.36±109.17*	277.81±150.58	546.06±124.52*	397.78±181.39*
IL-6 (pg/mg of protein)	79.39±22.35	282.88±59.92	182.54±67.44	960.16±794.02*	288.51±205.63

Values are mean ± SD. Control mice (CTRL, n=6) and exposed group (COAL11 =6, CFA11 =6, COAL16 =6, CFA11 =6); ΔP1 and ΔP2, resistive and viscoelastic/inhomogeneus pressures, respectively; Est, static elastance; ΔE, viscoelastic component of elastance; PMN and MN, polymorpho-and mononuclear cells, respectively; percentage of normal and collapsed areas in pulmonary tissue. *Significantly different from CTRL group (p<0.05).

3.4. Cytokines

Table 1 shown inflammation markers. Significant levels of TNF-α cytokine expression were observed in animals exposed to coal and coal fly ash particles compared with the control group. IL-1β was significantly higher in all exposed groups except CFA11 exposed group in relation to control group. Significant levels of IL-6 expression were only observed in animals exposed to COAL16 particles compared with control group.

3.5. Alkaline comet assay

In the Figure 3 is shown significant DNA damage index values in mice exposed to coal and coal fly ash particles compared with the control group.

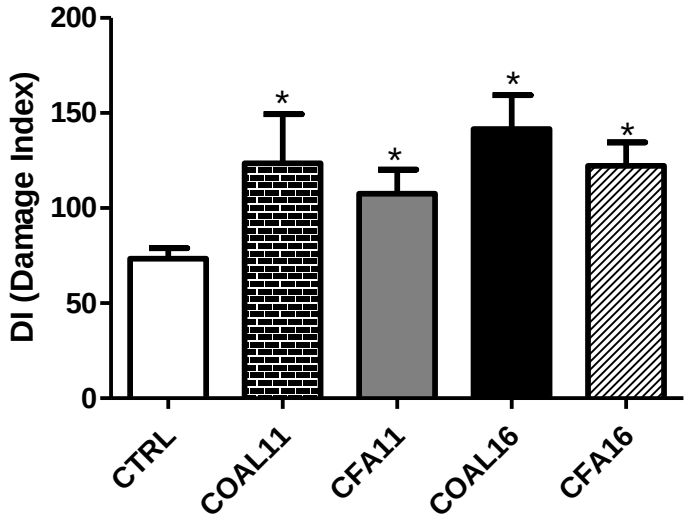


Figure 3. DNA damage index values for mice exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles comparing with control group. *Significantly different from CTRL group (p<0.05). The results are shown as mean ± standard deviation.

3.6. Particle-induced X-ray emission (PIXE)

The chemical elements present in significant concentrations in lung, liver, spleen and brain determinate by the PIXE method are presented in Figure 4. Additional results of the other inorganic elements are shown as supplementary results.

In lungs (Figure 4a), elements significantly higher in COAL11, CFA11 and COAL16 were Cr, Fe, Ni and Si. In CFA16 were significantly higher Cr and Si. In liver (Figure 4b), we found significant difference for animals exposed to COAL11 and CFA16 in elements such as Cr, Fe and Ni compared with the control group. In CFA11 exposed animals was statistically significant levels of Fe and Ni, and in COAL16 only Fe levels were significantly higher. In spleen (Figure 4c), the animals exposed to COAL11 showed significantly higher Fe, Cr and Ni. CFA11 exposed animals showed significant levels of Cr and Fe. In exposed group to COAL16 and CFA16 was found significant difference in Cr and Ni, and Fe respectively. As seen in Figure 4d, in brain no significant difference was observed in the concentrations of the elements of animals exposed to COAL11 and CFA11 particles. Only significant values were found in animals exposed to COAL16 in Fe and Ni and in CFA16 significant difference in Ni in relation to control group.

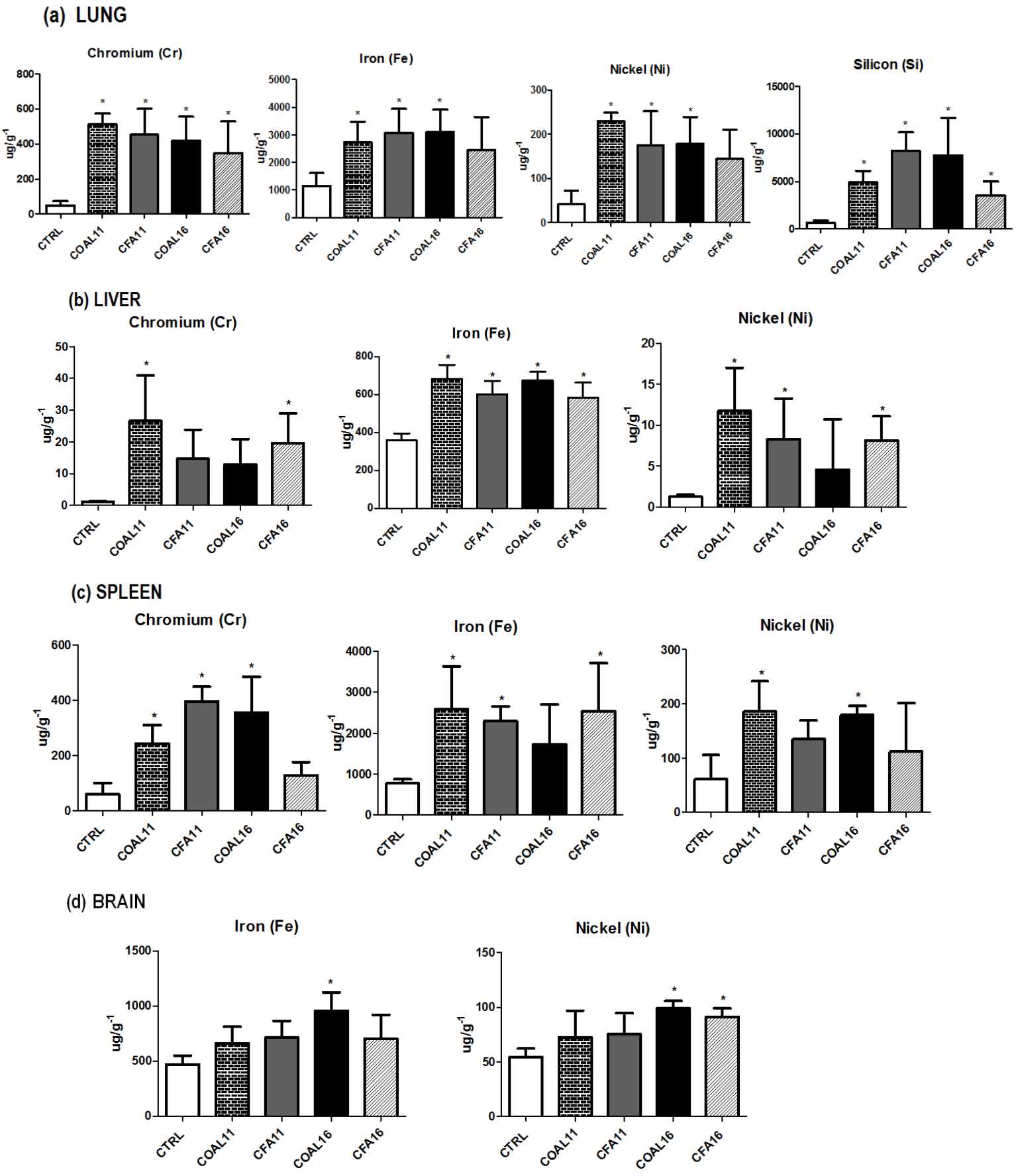


Figure 4. Significant concentration of inorganic elements in (a) lung, (b) liver, (c) spleen and (d) brain mice samples (ppm) of the control group and exposed group to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles by PIXE method (mean ± standard deviation).). *Significant increase in relation to the control group at P <0.05; Student's t test (Welch correction).

4. Discussion

The production, extraction and combustion process of coal generates significant environmental impacts and potential hazards for living organisms (Teixeira et al., 2009; León-Mejía et al., 2011). Several studies show that chronic inhalation of coal and coal fly ash particles can causes adverse pulmonary health effects such as bronchitis, asthma, progressive massive fibrosis, cancer and pneumoconiosis (Schins & Borm, 1999, Cohen et al., 2008). In this work we compared acute lung inflammation, pulmonary mechanics, genotoxic effects and extrapulmonary translocation in BALB/c mice exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles from Santa Catarina State-Brazil.

In our study we analyzed pulmonary mechanics parameters of animals exposed to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles. A statistically significant increase in pulmonary impedance was observed, the elastance and ΔP1parameters were statistically significant in the exposed group to the coal particles (COAL11 and COAL16) compared with control group. Which means that there were changes that made lungs more rigid and obstruction of central airways. When we analyzed the ΔE viscoelastic component of elastance and ΔP2 parameters, we found that only the exposed animals to particles COAL 16 showed significant changes, the lung became more rigid and the parenchyma more inhomogeneous compromising significantly also peripheral airways.

As was shown in the results, these particles induced influx of mononuclear inflammatory cells and significant increase in the release of cytokines such as TNF-α which was observed in animals exposed to both coal particles and coal fly ash particles. It is known that TNF-α has a primary role in coordinating the inflammatory response and activating the cascade of cytokines and the stimulation of neighboring cells which produce mediators involved in the recruitment of neutrophils, monocytes and lymphocytes (Driscoll et al., 1997; Khan, 2008). IL-1β acts synergistically with TNF-α to initiate the inflammatory cascade. IL-1β has similar biological effects

to TNF-α and together are known as cytokines of fast response and act on the amplification of inflammation through the activation of macrophages which secrete other pro-inflammatory cytokines such as IL-6 and IL-8, lipid mediators, ROS and RNS (Schins & Borm, 1999; Chung, 2006). The increase in IL-1β levels was statistically significant in all the exposed groups except in animals exposed to CFA11 in relation to the control group. This effect may be associated with determining factors in the inflammatory process, such as particle size and PAH concentrations in CFA11 sample.

A mixture of organic and inorganic agents contribute to the composition of coal and coal fly ash particles, including transition metals and PAH (Bonner, 2007; Masto et al., 2015), some of these particle constituents influence their characteristics such as solubility. There are particles that are poorly soluble and generates a persistent inflammation, which may explain the influx of pro-inflammatory cytokines, with a peak influx 24 h after particles intratracheal instillation. In this context, there are several factors that determine the toxicity of a particle and plays an important role in determining the toxic response such as dose size, deposition, durability and defense (removal of macrophages and inflammatory cells) (Borm et al., 2004). These particles of coal and coal fly ash, in addition to PAHs and metals also contain hazardous oxides as silica (SiO_2) and Al_2O_3. *In vitro* and *in vivo* studies showed that these oxides promote the formation of oxidants, oxidative stress and inflammatory processes persistent in lung (Fubini & Hubbard, 2003; Lin et al., 2006; Krewski et al., 2007; Alshatwi et al., 2013; Guidi et al., 2015).

These coal and coal fly ash particles are heterogeneous and vary in diameter. It is generally accepted that the particle size is an important factor in determining the toxic effects of particulate matter (Donaldson et al., 2005; Mazzoli-Rocha et al., 2010). In particular, these coal and coal fly ash samples are composed of fine and ultrafine particles. Approximately 40% of COAL11 particles consisted of particles smaller than 10 μm. In CFA11 and COAL16, a percentage of 25% and 30% of the particles had a diameter less than 10 μm respectively.

Particularly, in CFA16 all particles were smaller than 10 μm. Notably CFA16 sample is a product of co-firing with a mixture of fuel oil and diesel oil, which greatly influenced its characteristic. It is possible that the use of these fuel mixture in the combustion led to a more efficient burning and the formation of smaller particles.

The probability of deposition and toxicity of the particle in the respiratory tract increases abruptly in accordance with the size. Due to their small size, many ultrafine particles have a reactive surface that can react and inactivate many mediators and important cellular components (Borm et al., 2004). A significant influx of mononuclear cells in lung parenchyma of animals exposed to COAL11, CFA11, COAL 16 and CFA16 particles was observed. Interestingly, only recruitment of polymorphonuclear cells was found in the lung tissue of animals exposed to COAL16 particles. In fact, in the analysis of histology was observed thickening of alveolar septa, increased edema and cellular infiltrates in the lung parenchyma of animals exposed to COAL16 particles, indicating an active inflammatory process as was evidenced also in the increased expression of IL-6 in the lung of animals exposed to these particles.

When the particles are in the alveolar space they may react with the extracellular matrix. Immediately, phagocytic cells such as neutrophils, monocytes and macrophages are activated, trying to clean the particles. Depending on the particle surface characteristics, this removal process may succeed or not. In response, activated macrophages can also express transcription factors; followed by the release of ROS and RNS, quimotactics factors, lytic enzymes, cytokines and growth factors with eventual cell death (necrosis / apoptosis) (Shi et al., 2001). In addition, Both ROS and cytokines can cause damage or proliferation of local mesenchymal and epithelial tissue local and can have effects on lung tissue morphology, cell migration and the deposition of ECM (extracellular matrix) components (Schins & Borm, 1999, Fubini & Hubbard, 2003).

ECM formation and degradation can also be affected by ROS, but also proteases and antiproteases produced by several types of cells in the surrounding tissue. The local tissue factors also contain antioxidants such as the epithelial coating fluid and intracellularly in target cells, as well as cytokine antagonists presumably acting as anti-inflammatory feedback mechanisms (Schins & Borm, 1999). It has been described that the complexity of this response leads to the development of pulmonary disorders induced by exposure to mineral coal particles (Cui et al., 2015) and coal fly ash (Zierold & Sears, 2015). In fact, it has been reported that coal fly ash particles are cytotoxic (Kondo et al., 1993) and fibrogenic (Gursinsky et al., 2006). The prolonged exposure to coal fly ash or coal dust can contribute to severe interstitial fibrosis in miners exposed chronically (Jindal et al., 2001).

When macrophages are activated they release cytokines and / or growth factors which stimulate collagen production by fibroblasts (Zhao et al., 2015). The period of exposure of animals to coal and fly ash particles used in this study was 24 hours and did not lead to the formation of collagen and elastic fibers which are produced in longer periods of time. Neither a significant alteration in alveolar areas was observed in lung tissues of animals exposed. Therefore the observed results in pulmonary mechanics could be associated with inflammatory processes, mainly related with the activation of mononuclear phagocytic cells, but not to alterations on the alveoli.

The results from exposed animals to particles of coal and coal fly ash showed a significant increase in levels of DNA damage in relation to the control group. Among the possible mechanisms of DNA damage induced by particles, the activation of the phagocytic oxidative burst is considered (Knaapen et al., 2004). In addition, this response can be intensified by the metal content of these particles, because these metals are present in the form of easily water-soluble salts, they can increase ROS generation through the Haber-Weiss reactions, inducing a significantly greater oxidative and inflammogenic reactivity (Knaapen et al., 2004, Di Pietro et

al. 2009) and DNA strand breaks (Sander and Wilson 2005; Angelé-Martínez et al., 2014). In our study, significant concentrations of metals such as Cr, Fe and Ni were present in lung, liver, spleen and brain. Fe was a common metal in all organs analyzed, which is related to the composition of coal and important content of pyrite in coal Santa Catarina (Silva et al., 2010).These results show the efficient translocation of metals from the lung by the bloodstream to extrapulmonary organs. Mani, et al., 2007 demonstrated that the toxic metals inhaled with fly ash in rats may get translocated into extrapulmonary organs as liver; Wallenborn et al., 2007 showed that PM-associated metals deposited in the lung of rats may be released into systemic circulation and reach organs such as heart and liver at different rates depending on their solubility and can be deposited and hence may manifest their toxic effects on different tissues (Zeng et al., 2016).

Conclusions

In summary, our data shows that the coal particles induced changes in the lungs which became more rigid, leading to resistance to movement during the respiratory cycles and induced the obstruction of central airways. The most significant changes in pulmonary mechanics were observed in animals exposed to COAL16 particles samples that lead to the parenchyma more inhomogeneous compromising significantly also peripheral airways. The complex composition of these particles causes persistent inflammation, which can be associated with the results in pulmonary mechanics, the activation of mononuclear phagocytic cells and proinflammatory cytokine release. The comet assay results showed that animals exposure to coal and coal fly ash particles induced primary DNA lesions which can be associated with oxidative damage consequence of the activation of phagocytic cells and cell independent mechanisms due to intrinsic chemical properties of the coal and coal fly ashes as well the presence of surface radicals, oxides, PAH and metals as Cr, Fe and Ni which translocated efficiently through the bloodstream to the liver, spleen and brain as shown.

Acknowledgments

We thank very cordially to Alexia Nascimento for the help in this work. This work was supported by grants from Conselho Nacional para o Desenvolvimento Científico e Tecnológico-CNPq – Brazil. Universal Grant Number 470833; Produtividade Grant Numbers 300902 and 303364. Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil. Grant Number PRONEM 11/2070-and PRONEX/FAPERGS/CNPq no. 10/0044-3). Grethel León-Mejía benefited from a scholarship financed by PEC-PG/CAPES. Luis F.O. Silva benefited from a scholarship financed by CNPq.

REFERENCES

Alshatwi, A. A. Subbarayan, P. V. Ramesh, E. Al-Hazzani, A. A. Alsaif, M. A. and Alwarthan, A. A. Aluminium oxide nanoparticles induce mitochondrial-mediated oxidative stress and alter the expression of antioxidant enzymes in human mesenchymal stem cells," Food Additives and Contaminants A, vol. 30, no. 1, pp. 1–10. 2013.

Angelé-Martínez, C., Goodman, C., Brumaghim, J. Metal-mediated DNA damage and cell death: mechanisms, detection methods, and cellular consequences. Met. Integr. Biometal Sci. 6, 1358–1381. 2014.

Bates, J.H.T., Abe, T., Romero, P.V., Soto, J., Measurement of alveolar pressure in closed-chest dogs during flow interruption. J. Appl. Physiol. 67, 488–492. 1989.

Bates, J.H.T., Rossi, A., Milic-Emili, J., Analysis of the behavior of the respiratory system with constant inspiratory flow. J. Appl. Physiol. 58, 1840–1848. 1985.

Bonner, J.C. Lung fibrotic responses to particle exposure. Toxicol Pathol. Jan;35(1):148-53. 2007.

Borm PJ, Schins RP, Albrecht C. Inhaled particles and lung cancer, part B: paradigms and risk assessment. Int J Cancer. May 20;110(1):3-14. 2004

Broeckaert F, Buchet JP, Huaux F, Lardot C, Lison D, Yager JW. Reduction of the ex vivo production of tumor necrosis factor alpha by alveolar phagocytes after administration of coal fly ash and copper smelter dust. J Toxicol Environ Health. Jun 6;51(2):189-202. 1997

Campbell, J.L.The Guelph PIXE software package III. Nucl. Instrum. Methods Phys. Res. B 170, 193. 2000.

Capelozzi, V.L., Saldiva, P.H.N., Antonângelo, L., Carvalho, T.S., Logulo, A., Carvalho, C.R.R., Deheinzelin, D. Quantitation in inflammatory pleural disease to distinguish tuberculous and paramalignant from chronic non-specific pleuritis. J. Clin. Pathol. 50, 935–940. 1997.

Chung KF. Cytokines as targets in chronic obstructive pulmonary disease. Curr Drug Targets. Jun;7(6):675-81. 2006.

Cohen R.A, Patel A, Green F.H. Lung Disease Caused by Exposure to Coal Mine and Silica Dust. 29(6):651-661. 2008.

Collins, A.R. The Comet Assay for DNA Damage and Repair: Principles, Applications, and Limitations. Molecular Biotechnology. 26: 249-261. 2004.

Cui X, Xing J, Liu Y, Zhou Y, Luo X, Zhang Z, Han W, Wu T, Chen W. COPD and levels of Hsp70 (HSPA1A) and Hsp27 (HSPB1) in plasma and lymphocytes among coal workers: a case-control study. Cell Stress Chaperones. Jan 27. 2015

Di Pietro A, Visalli G, Munaò F, Baluce B, La Maestra S, Primerano P, Corigliano F, De Flora S. Oxidative damage in human epithelial alveolar cells exposed in vitro to oil fly ash transition metals. Int J Hyg Environ Health. Mar;212(2):196-208. 2009.

Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol 2:10. doi: 10.1186/1743-8977-2-10. 2005.

Driscoll K. E, Carter J. M, Hassenbein D .G, and Howard B. Cytokines and particle-induced inflammatory cell recruitment. Environ Health Perspect. Sep; 105(Suppl 5): 1159–1164. 1997.

Fubini B, Hubbard A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic Biol Med. Jun 15;34(12):1507-16. 2003.

Furuyama A, Kanno S, Kobayashi T, Hirano S. Extrapulmonary translocation of intratracheally instilled fine and ultrafine particles via direct and alveolar macrophage-associated routes. Arch Toxicol. May;83(5):429-37. doi: 10.1007/s00204-008-0371-1. 2009.

Guidi P, Nigro M, Bernardeschi M, Lucchesi P, Scarcelli V, Frenzilli G. Does the crystal habit modulate the genotoxic potential of silica particles? A cytogenetic evaluation in human and murine cell lines. Mutat Res Genet Toxicol Environ Mutagen. Oct;792:46-52. 2015.

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sorensen, S.B., Vesterby, A., West, M.J. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857–881. 1988.

Gursinsky T, Ruhs S, Friess U, Diabaté S, Krug HF, Silber RE, Simm A. Air pollution-associated fly ash particles induce fibrotic mechanisms in primary fibroblasts. Biol Chem. Oct-Nov;387(10-11):1411-20. 2006.

He, F., Buoso, M.C., Burattini, E., Fazinic, S., Galassini, S., Haque, A.M.I., Jaksic, M., Moschini, G. Target preparation for trace element determination of biological materials using techniques. Nucl. Instrum. Meth. Phys. Res. Sect. A 334, 238–245. 1993.

IARC, International Agency for Research on Cancer. 68:337, Cas 65996–9. 1997

Jindal SK, Aggarwal AN, Gupta D. Dust-induced interstitial lung disease in the tropics. Curr Opin Pulm Med. Sep;7(5):272-7. 2001.

Johansson, S.A.E., Campbell, J.L., Malmqvist, K.G. Paticle-induced X-ray Emission Spectrometry (PIXE). John Wiley & Sons, INC. 1995.

Jones T, Wlodarczyk A, Koshy L, Brown P, Shao L, BéruBé K. The geochemistry and bioreactivity of fly-ash from coal-burning power stations. Biomarkers. Jul;14 Suppl 1:45-8. 2009.

Kania N, Setiawan B, Widjadjanto E, Nurdiana N, Widodo MA, Kusuma HM. Subchronic inhalation of coal dust particulate matter 10 induces bronchoalveolar hyperplasia and decreases MUC5AC expression in male Wistar rats. Exp Toxicol Pathol. Oct;66(8):383-9. 2014.

Khan, M.M. Role of Cytokines, in: Immunopharmacology. Springer US, pp. 33–59. 2008.

Knaapen AM, Borm PJ, Albrecht C, Schins RP. Inhaled particles and lung cancer. Part A: Mechanisms. Int J Cancer. May 10;109(6):799-809. 2004.

Kondo T, Takahashi S, Sato H, Yamada M, Kikuchi T, Furuya K. Cytotoxicity of size-density fractionated coal fly ash in rat alveolar macrophages cultured in vitro. Toxicol In Vitro. Jan;7(1):61-7. 1993.

Krewski D, Yokel RA, Nieboer E, Borchelt D, Cohen J, Harry J, Kacew S, Lindsay J, Mahfouz AM, and Rondeau V. Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide. J Toxicol Environ Health B Crit Rev. 10(Suppl 1): 1–269. 2007.

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdörster G, Ziesenis A. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J Toxicol Environ Health A. Oct 25;65(20):1513-30. 2002.

León-Mejía, G; Espitia-Pérez, L; Hoyos-Giraldo, L.S; Da Silva, J; Hartmann, A; Henriques, J.A; Quintana, M. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci Total Environ. Jan 15;409(4); 686-91. 2011.

Lin W, Huang YW, Zhou XD, Ma Y. In vitro toxicity of silica nanoparticles in human lung cancer cells. Toxicol Appl Pharmacol. Dec 15;217(3):252-9. 2006.

Liu G, Niu Z, Van Niekerk D, Xue J, Zheng L. Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions, analysis, and toxicology. Rev Environ Contam Toxicol.;192:1-28. 2008.

Mani U, Prasad AK, Suresh Kumar V, Lal K, Kanojia RK, Chaudhari BP, Murthy RC. Effect of fly ash inhalation on biochemical and histomorphological changes in rat liver. Ecotoxicol Environ Saf. Sep;68(1):126-33. 2007.

Martinez, J. Cenizas volantes de carbón y cenizas de hogar o escoria. Ficha técnica, España, 2012.

Masto, RE; Sarkar, E; George J, Jyoti, K; Dutta, P; Ram, LC. PAHs and potentially toxic elements in the fly ash and bed ash of biomass fired power plants. Fuel Processing Technology. Volume 132. Pages 139–152. 2015.

Maxwell, J.A., Teesdale, W.J., Campbel, J.L.The Guelph PIXE software package II. Nucl. Instrum. Methods Phys. Res. Sect. 95, 407–421. 1995.

Mazzoli-Rocha F, Fernandes S, Einicker-Lamas M, Zin WA. Roles of oxidative stress in signaling and inflammation induced by particulate matter. Cell Biol Toxicol. Oct;26(5):481-98. 2010.

Mireles, A., Solís, C., Andrade, E., Lagunas-Solar, M., Pina, C., Flocchini, R.G. Heavy metal accumulation in plants and soil irrigated with wastewater from Mexico city. Nucl. Instrum. Methods Phys. Res. Sect. 220, 187–190. 2004.

Mozgawa W, Król M, Dyczek J, Deja J. Investigation of the coal fly ashes using IR spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc. 2014.

Nagase, T., Lei, M., Robatto, F.M., Eidelman, D.H., Ludwig, M.S. Tissue viscance during induced constriction in rabbit lung: morphological–physiological correlation. J. Appl. Physiol. 73, 1900–1907. 1992.

Oberdorster G, Utell MJ. Ultrafine particles in the urban air: to the respiratory tract—and beyond? Environ Health Perspect;110: A440–1. 2002.

Oliveira M.L.S., Ward C.R, French D , Hower J.C , Querol X , Silva L.F.O. Mineralogy and leaching characteristics of beneficiated coal products from Santa Catarina, Brazil. International Journal of Coal Geology 94. 314–325. 2012.

Oliveira, M.L.S., Waanders, F., Silva, L.F.O., Jasper, A., Sampaio, C.H., McHabe, D., Hatch, R.S., Hower, J.C. A Multi-analytical Approach to Understand the chemistry of Fe-minerals in Fee Coals and Ashes. Coal Combustion and Gasification Products 3, 51-62. 2011.

Quispe D., Pérez-López R, Silva LFO, Nieto JM. Changes in mobility of hazardous elements during coal combustion in Santa Catarina power plant (Brazil). Fuel 94, 495–503. 2012.

Rohr P, Kvitko K, da Silva FR, Menezes AP, Porto C, Sarmento M, Decker N, Reyes JM, Allgayer M. da C Furtado TC, Salvador M, Branco C, da Silva J. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat Res 758: 23-8. doi:10.1016/j.mrgentox.2013.08.006. 2013.

Sakai N., Tager A. Fibrosis of Two: Epithelial Cell-Fibroblast Interactions in Pulmonary Fibrosis. Biochim Biophys Acta. Jul; 1832(7): 911–921. 2013.

Saldiva, P.H.N., Zin, W.A., Santos, R.L.B., Eidelman, D.H., Milic-Emili, J. Alveolar pressure measurement in open-chest rats. J. Appl. Physiol. 72, 302– 306. 1992.

Sander M, Wilson S. Base Excision Repair, AP Endonucleases and DNA Glycosylases, John Wiley & Sons Ltd, Chichester. doi: 10.1038/npg.els.0003871. 2005.

Santo Tomas LH. Emphysema and chronic obstructive pulmonary disease in coal miners. Curr Opin Pulm Med. Mar;17(2):123-5. 2011.

Schins RP, Borm PJ. Mechanisms and mediators in coal dust induced toxicity: a review. Ann Occup Hyg. Jan;43(1):7-33. 1999.

Shi X, Ding M, Chen F, Wang L, Rojanasakul Y, Vallyathan V, Castranova V. Reactive oxygen species and molecular mechanism of silica-induced lung injury. J Environ Pathol Toxicol Oncol. 20 Suppl 1:85-93. 2001.

Silva L.F.O. Ward C.R. Hower J.C, Frans Waanders M.I, Oliveira M.L.S, Li Z., Hatch R.S, Querol X. Mineralogy and Leaching Characteristics of Coal Ash from a Major Brazilian Power Plant. I S S N 1 9 4 6 - 0 1 9 8. 2010.

Silva L.F.O., Moreno T. Querol X. An introductory TEM study of Fe-nanominerals within coal fly ash. Science of the Total Environment 407. 4972–4974. 2009.

Singh, N.P., MCcoy, M.T., Tice, R.R.. Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individuals cells, Exp. Cell Res. 175. 184–191. 1988.

Smith KR, Veranth JM, Kodavanti UP, Aust AE, Pinkerton KE. Acute pulmonary and systemic effects of inhaled coal fly ash in rats: comparison to ambient environmental particles. Toxicol Sci. Oct;93(2):390-9. 2006.

Strand M., Pagels J., Szpila A., Gudmundsson A., Swietlicki E., Bohgard M., Sanati M. Fly Ash Penetration through Electrostatic Precipitator and Flue Gas Condenser in a 6 MW Biomass Fired Boiler. Energy & Fuels. 16, 1499-1506. 2002.

Teixeira, E.C., Meira, L., Santana, E., Ramos, R., Wiegand, F. Chemical composition of PM10 and PM2.5 and seasonal variation in south Brazil. 2009.

Tsiridis V., Samaras P., Kungolos A., Sakellaropoulos G. P. Application of Leaching Tests for Toxicity Evaluation of Coal Fly Ash. Environmental Toxicology. Volume 21, Issue 4, 2006.

Vassilev SV, Vassileva CG. Mineralogy of combustion wastes from coal-fired power stations. Fuel Process Technol 47: 261–80. doi:10.1016/0378-3820(96)01016-8. 1996a.

Vassilev SV, Vassileva CG. Occurrence, abundance and origin of minerals in coals and coal ashes. Fuel Process Technol 48: 85–106. doi:10.1016/S0378-3820(96)01021-1. 1996b.

Wallenborn JG, McGee JK, Schladweiler MC, Ledbetter AD, Kodavanti UP. Systemic translocation of particulate matter-associated metals following a single intratracheal instillation in rats. Toxicol Sci. Jul;98(1):231-9. Epub 2007 Apr 13. 2007.

Zeng, X., Xu, X., Boezen, H.M., Huo, X., 2016. Children with health impairments by heavy metals in an e-waste recycling area. Chemosphere 148, 408–415. doi:10.1016/j.chemosphere.2015.10.078.

Zhao L, Wei Z, Yang F, Sun Y. Transforming growth factor-β1 induced cellular proliferation and collagen synthesis was mediated by reactive oxygen species in pulmonary fibroblasts. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi. Jan;33(1):15-9. 2015.

Zierold KM, Sears CG. Community views about the health and exposure of children living near a coal ash storage site. J Community Health. Apr;40(2):357-63. 2015.

Supplementary results

Table 1. Concentration of inorganic elements in lung, liver, spleen and brain mice samples (ppm) of the control group and exposed group to coal (COAL11 and COAL16) and coal fly ash (CFA11 and CFA16) particles by PIXE method.

		Inorganic elements (ppm)															
		Na	Mg	Al	Si	P	S	Cl	K	Ca	Mn	Fe	Cu	Zn	Rb	Cr	Ni
Lung (n=6)	Control	12420.3± 4433.1	696.1± 285.7	359.7± 199.2	630.2± 278.9	9502.6± 167.8	4688.3± 813.1	6943± 2569.6	11859± 2026.4	520.8± 261.3	20.9±3.0	1144.8± 463.4	15.3±3.9	47.9±22.7	80.0±33.9	49.7±24.4	41.8±29.8
	COAL11	24165±10476	734.7± 91.4	2729.5± 1447.1	4878± 1227.0*	8583± 509.8	6936± 1645.3	9340.6± 2600.7	9421.3± 1052.1	690± 466.3	52.9±13.7	2716.6± 756.3*	19.9±9.9	58.1±29.5	59.7±28.2	510.9± 63.3*	229.4± 18.8*
	CFA11	21234±1956.8	678.4± 55.3	4109.3± 2549.6	8197.9± 1959.7*	8342± 806.3	6602.6± 2063.3	8543.6± 2705.7	10382± 1283.2	765± 198.7	51.8±25.5	3070.3± 869.4*	24.7±5.1	88.7±29.3	68.4±24.4	453.6± 147.4*	175.1± 77.1*
	COAL16	30251±8665.5	700.3± 162.5	4880± 3542.4	8195.6± 3500.9*	8151± 1203.7	6298± 435.2	4828.1± 5893.6	10114.6± 831.7	1608.8± 1092.3	44.0±13.3	3103± 802.1*	15.1±2.8	102.9± 43.0	56.9±37.1	420.6± 135.7*	178± 60.5*
	CFA16	16711.3± 6112.6	430.1± 108.2	2345.3± 1124.9	3508.6± 1471.1*	7558± 78.7	6335± 387.1	12315± 3896	12143± 1022.6	1335.2± 755.9	36.1± 18.5	2446.6± 1199.1	12.7±5.3	66.6±8.1	59.9±20.7	349± 180.4*	143.9± 65.9
Liver (n=6)	Control	4571.3±560.5	1627± 79.6	383.8± 55.3	387.4± 227.4	6513.3± 2100.1	8017.6± 1364	4555.3± 685.8	12308± 544.3	236.9±75.3	6.6±2.6	357.3± 37.5	36.2±4.8	138.9± 24.3	62.6±15.3	1.1±0.08	1.2±0.04
	COAL11	5958.6±1009.2	1653.3± 187.5	380.7± 65.3	678.7± 146.5	7908± 230.3	10857± 396.5	4293.6± 468.4	11700.3± 551.8	185.8± 36.0	5.7±2.5	681.8± 74.5*	36.4±2.3	157.4±9.5	56.4±4.3	26.6± 14.3*	11.7±5.2*
	CFA11	6271.6±1231.1	1810.6± 374.8	422.6± 95.3	476.7± 155.2	8555.3± 874.7	12279.6± 1857.1	4547.3± 692.0	12541.3± 767.6	200.8± 19.6	3.2±1.0	602.3± 57.3*	35.1±1.0	162.4±3.2	60.6±2.9	14.7±7.4	8.2±4.0*
	COAL16	6837±508.9	1118.5± 337.2	195.9± 87.5	233.9± 217.4	8983± 1283	8757.3± 2047.5	4609.6± 747.5	10386.6± 529.6	215.9± 52.1	5.7±2.3	673.6± 45.5*	40.2±4.6	169.8± 63.4	70.5±36.0	12.8±8.0	4.5±6.1
	CFA16	5707±494.7	1343.3± 324.4	204.5± 140.7	362.1± 197.2	9691± 1415.8	8238± 2177.5	4196.6± 291.9	11871.6± 865.4	188.7±4.3	4.8±1.5	583.5± 80.0*	36.6±7.6	180.4± 50.0	79.0±5.5	19.6±9.3*	8.1±2.9*
Spleen (n=6)	Control	3816±886.2	1301.2± 348.2	440.4± 140.0	754.1± 94.3	4949.6± 1342.0	6378± 1059.5	7743.6± 1574.1	12196.9± 10462.6	240.6± 203.7	24.4±20.1	773.3± 101.7	10.2±5.1	109.2± 24.8	85.5±9.1	59.2±40.9	61.6±43.8
	COAL11	3868.3±386.6	1068.1± 268.1	528.1± 333.0	1091.9± 521.7	4898± 941.0	6855.6± 832.8	7069.6± 1181.1	12241.7± 8963.2	241.5± 106.5	41.7±29.4	2595.6± 1040.8*	188.0± 157.1	200.1± 90.7	54.4±5.3	243.0± 67.1*	185.8± 56.4*
	CFA11	2997.3±197.7	1085.7± 156.4	496.9± 104.7	1213.6± 540.3	5230± 1683.3	6371.6± 255.7	6423± 2089.4	10391.7± 8156.1	179.1± 57.2	50.6±27.7	2295.5± 356.1*	10.6±9.5	88.4±13.9	32.7±22.0	395.5± 53.5*	134.8± 35.2
	COAL 16	5562±2363.8	927± 164.5	189.2±27.7	731.8± 528.4	7623.6± 4528.6	5773.3± 752.1	5739.3± 628.2	10714.5± 8230.6	224.4± 124.2	26.1±10.0	1730.6± 975.2	9.6±1.5	97.4±18.7	66±17.8	355.4± 129.7*	179.6± 17.3*
	CFA16	4938.6±621.8	975.1± 146.2	245.5± 229.4	1731.2± 1997.5	9148± 3487.4	7299.6± 1826.1	9496± 2905.4	423.2± 109.6	347.2± 344.1	567.8± 552.6	2540.8± 1177.1*	17.3±5.1	106.1± 35.8	45.4±30.7	128.7± 47.6	111.6± 89.7

Brain (n=6)	Control	9263±188.6	862.1± 155.7	176.1±61.9	128.9± 61.9	10675.3± 855.7	7345± 1400.5	5392.3± 145.0	15698± 2093.8	489.1± 118.8	16.6± 2.4	469.3± 81.4	21.3±3.1	82.7±20.6	32.7± 9.4	108.5± 18.7	54.4± 7.8
	COAL11	8963.6±1071.9	750.5± 71.6	183±36.9	191.9± 89.5	11518.6± 206.7	6316± 210.3	5196.6± 267.8	16135.6± 273.5	1603.3± 2268.7	12.0± 4.0	660.3± 152.0	22.6±4.1	81.9±5.8	28.9± 7.3	145.7± 36.0	72.7±24.3
	CFA11	9360±947.0	749.8± 96.9	107.2± 100.5	152.2± 12.1	12629.6± 299.6	5848± 114.1	5348± 377.9	17155.3± 1905.2	2077.4± 3048.9	16.4± 2.9	713.9± 150.7	19.9±4.6	87.9±9.6	30.1± 5.4	202.1± 56.8	75.5± 19.1
	COAL16	10017.6±722.1	878.4± 140.8	136.9± 142.1	244.4± 137.8	11854± 671.8	6132.6± 174.8	5676± 143.1	16018± 1212.4	1612.8± 2085.0	12.3± 1.7	957.7± 166.3*	19.8±4.3	92.0±0.4	32.0± 11.0	137.2± 64.7	99.1± 6.6
	CFA16	9314±539.8	891.3± 64.8	134.1±45.6	91.2±82	11864.3± 835.9	5909± 122.9	5090± 252.5	16245± 809.0	330.4± 56.5	12.6± 3.9	703.6± 218.6	22.9±3.4	86.5±3.9	37.7± 15.5	120.5± 61.6	91.2± 7.9*

*Significant increase in relation to the control group at P <0.05; Student's t test (Welch correction).

DISCUSSÃO GERAL

V. DISCUSSAO GERAL

Nos últimos anos a mineração do carvão teve um notável aumento na sua demanda mundial. Porém, apesar das vantagens econômicas, a produção de energia elétrica a partir do carvão mineral é uma das formas mais agressivas ao ambiente (Silva et al., 2010). Sabe-se que durante as atividades de mineração de carvão, grandes quantidades de partículas de carvão, HAP, metais e óxidos são liberados ao ambiente (Burt et al., 2013; Rohr et al., 2013). Uma vez liberadas, estas substâncias constituem misturas complexas e, se os efeitos sinérgico, aditivo e potencializador da mistura resultante forem levados em consideração, este é um dos maiores riscos para a saúde e segurança das populações expostas.

De acordo com a *International Energy Agency* (IEA), o carvão mineral é responsável por 40% da produção de energia elétrica mundial (IEA, 2015). O Brasil conta com importantes reservas e termelétricas no sul do país, especificamente no estado de Santa Catarina (Silva et al., 2009). Durante o processo de combustão do carvão, os elementos nele contidos são volatizados e emitidos para a atmosfera juntamente com parte da matéria inorgânica, que é liberada sob a forma de partículas de cinzas voláteis. Estas partículas são coletadas em precipitadores eletrostáticos, mas na maioria das vezes esse método não é totalmente eficiente, e grande parte dessas partículas de cinzas são liberadas ao ambiente (Burt et al., 2013; Liu et al., 2015; Wang et al., 2015).

Estudos experimentais sobre os impactos negativos do carvão têm sido relatados na literatura, em animais (León et al., 2007; Zocche et al., 2010), em bactérias (Chakraborty & Mukherjee, 2009), plantas (Naidoo and Chirkoot, 2004) e humanos (Rohr et al., 2013, León-Mejía et al., 2011; León-Mejía et al., 2014; Minina et al., 2015). O ponto comum entre esses estudos é a avaliação do dano potencial para os organismos vivos ocasionado pela mineração de carvão.

Com a finalidade de compreender melhor os efeitos da exposição às partículas de carvão e cinzas, este trabalho avaliou duas amostras de carvão e das cinzas geradas a partir da queima dos mesmos, provenientes do estado de Santa Catarina. Esta avaliação foi realizada *in vitro* na linhagem de fibroblastos de pulmão de hâmster chinês (células V79) e *in vivo* em camundongos expostos mediante instilação intratraqueal.

Como demonstrado no **Capítulo I**, os resultados *in vitro* usando o ensaio Cometa Alcalino mostraram que a exposição das células V79 às partículas de carvão e cinzas induziram lesões primárias no DNA, e, no ensaio Cometa Modificado, foi evidenciado dano oxidativo. Adicionalmente, nos biomarcadores do ensaio Cyt-CBMN, as concentrações mais altas induziram citotoxicidade e instabilidade cromossômica.

No **Capítulo II** são demostrados os efeitos da exposição a partículas de carvão e cinzas em camundongos BALB/c após 24 horas de instilação intratraqueal. Estes efeitos foram relacionados com a mecânica pulmonar, recrutamento de células (principalmente as mononucleares) e liberação de citocinas pró-inflamatórias. Também,

os resultados obtidos mostraram efeitos genotóxicos em células de sangue periférico e a translocação de metais como Cr, Fe e Ni do pulmão para o fígado, baço e encéfalo.

Um ponto importante que deve ser salientado na discussão sobre os efeitos e as propriedades das partículas de carvão e cinzas avaliadas é o processo de combustão das mesmas. Antes da combustão, o carvão é pulverizado, sendo, posteriormente, injetado em presença ou não de combustíveis secundários, no forno e queimado a altas temperaturas (Heidrich et al., 2013). As reações que são originadas durante a combustão, e as propriedades das partículas derivadas, são dependentes do tipo de carvão, do tamanho do pó de carvão, do tipo de caldeira usada e das condições do processo de combustão, como, por exemplo, o uso de combustíveis adicionais (Martinez, 2012).

Como se pode observar na Figura 1 e nas Tabelas 1, 2 e 3 (Capítulo I), estas partículas são bastante heterogêneas, tanto em tamanho quanto na sua composição. Curiosamente, a amostra CFA16 foi queimada com uma mistura de óleo combustível e óleo diesel, que, evidentemente, influenciaram nas suas características químicas. Esta co-combustão pode explicar o fato de que elementos que normalmente volatilizam-se durante a combustão do carvão ficaram condensados na matriz das partículas (Silva et al., 2010), os quais possivelmente contribuíram para a heterogeneidade da composição e para a maior concentração destes elementos traço. Como observado na Figura 1, de todas as amostras, a CFA16 teve menor tamanho nas partículas, sendo portanto possível que o uso da mistura de combustível diesel e óleo empregados na queima COAL16 levassem a um processo de combustão mais eficiente e à formação de partículas menores.

De modo geral, em relação à concentração de óxidos nas amostras de carvão e cinzas (Tabela 1, Capítulo I), observa-se uma homogeneidade, exceto SiO_2 e Al_2O_3, que encontram-se em maior concentração. Também é importante salientar que estas partículas contêm quantidades significativas de dióxido de silício (SiO_2), também conhecido como sílica, e óxido de alumínio (Al_2O_3) na sua superfície. Estudos nos quais foram avaliadas *in vitro* a citotoxicidade (Sandberg et al., 2012; Zhang et al., 2015) e a genotoxicidade (Guidi et al., 2015) da sílica propõem, como ponto comum, a geração de estresse oxidativo. Com respeito à sílica, estudos *in vivo* descrevem que a geração de estresse oxidativo (formação de radicais hidroxila ou ERN) desencadeiam eventos de sinalização para NF-kB e AP-1, levando isto a um ponto chave na patogênese de diversas doenças como silicose, fibrose e câncer (Ding et al., 2002, Chen et al., 2002, Cox, 2011). Além disso, estudos *in vitro* (Achary et al., 2012; Rajiv et al., 2016) e *in vivo*, descrevem que nanopartículas de óxido de alumínio (Al_2O_3) promovem a formação de radicais, como o radical hidroxila, e induzem estresse oxidativo (Shrivastava et al., 2014). Em adição, outros estudos experimentais sugerem que a presença de Al está associada com processos inflamatórios no pulmão, os quais podem desencadear doenças respiratórias (Wagner et al., 2007) e processos cancerígenos (Spinelli et al., 2006; Exley et al., 2007).

É importante ressaltar que, além dos óxidos, outros fatores também influenciam nas propriedades destas partículas, como a presença de metais e componentes orgânicos como os HAP na superfície do carvão e nas cinzas (Tabelas 2 e 3, Capítulo I).

Esses componentes contribuem notavelmente na formação de ERO e danos no DNA, e influenciam nos mecanismos de reparação do DNA, na mutagênese e na proliferação celular. Além desse conjunto de observações, estes fatores poderiam também influenciar na capacidade das partículas de gerar processos inflamatórios (Knaapen et al., 2004, Moller et al., 2014).

Os resultados de genotoxicidade e dano oxidativo *in vitro*, descritos no Capítulo I, estão relacionados com os componentes destas partículas. Sugere-se que existe uma soma de efeitos de cada componente na mistura, isto é, óxidos, metais e HAP. Sabe-se que a presença de metais solúveis, tais como o Fe, Pb, Hg, Cd, Ag, Ni, V, Cr, Mn e Cu, associados às partículas são capazes de gerar ERO via reação de Haber-Weiss, seguido da indução de estresse oxidativo (Knaapen et al., 2004; Lodovici & Bigagli, 2011).

Dentre os compostos associados às partículas, os HAP possuem um papel importante na genotoxicidade das mesmas. Os HAP podem ser metabolicamente ativados e podem também induzir a formação de ERO, levando a estresse oxidativo e formação de adutos ou quebras simples no DNA (Perera et al., 2005; Mazzoli-Rocha et al., 2010; Valavanidis et al., 2013). Muitos dos HAP são considerados carcinogênicos, sendo associados a diversos tipos de câncer, como câncer de pulmão, de estômago e de pele (ATSDR, 2013; Jarvis et al., 2014). A principal via de ativação metabólica dos HAP é a geração de diol epóxidos catalisada pelo citocromo P450, dando lugar à formação de adutos no DNA, de radicais catiônicos e à formação de quinonas com atividade redox (Desler et al., 2009; Das et al., 2014). Particularmente, as amostras de carvão apresentaram maior conteúdo de HAP quando comparadas com as cinzas, o que provavelmente influenciou os efeitos inflamatórios significativos observados no pulmão dos animais expostos. Os HAP presentes, e em altas concentrações, em todas as amostras foram Antraceno, Fluranteno e Benzo(a)antraceno. O Antraceno e o Fluoranteno são considerados agentes mutagênicos, mas não carcinogênicos, e estão classificados no Grupo 3 pela IARC (IARC, 2010), embora existam estudos em modelos experimentais que demostrem as propriedades do Fluoranteno e seus efeitos carcinogênicos (Boström et al., 2002). O Benzo(a)antraceno é classificado pela IARC no grupo 2A, isto é, com "comprovação limitada em humanos e evidências suficientes de carcinogenicidade em animais" (IARC, 2010). Porém, o Benzo(a)antraceno é considerado um composto mutagênico para uma ampla variedade de organismos, incluindo os seres humanos (US Environmental Protection Agency, 1999; European Commission, 2002; Kido et al., 2013).

Comparando de forma geral a resposta das células *in vitro*, tanto no ensaio Cometa Alcalino e Modificado (Figura 3, Capítulo I) quanto nos biomarcadores do ensaio CBMN-Cyt (Tabela 4, Capítulo I), foi observado que os efeitos das partículas COAL11, COAL16 e CFA16 foram mais citotóxicos e genotóxicos e causaram maior dano oxidativo que os efeitos da cinza CFA11. É provável que a diferença dos efeitos observados entre CFA11 e CFA16 esteja associada com as concentrações mais altas dos compostos em CFA16, como anteriormente mencionado, derivados da combustão com combustíveis adicionais. Nesse sentido, a toxicidade e o dano oxidativo induzido por partículas de carvão e cinzas têm sido descritos em vários estudos usando diversas

linhagens celulares e usando diferentes biomarcadores (Dwivedi et al., 2012; Sambandam et al., 2015). Diabaté et al. (2011) evidenciaram que células epiteliais bronquiais BEAS-2B expostas às cinzas de carvão induziram a geração de ERO, aumento de glutationa total intracelular e da produção de OH-1, levando à conclusão de que, provavelmente, os metais de transição biodisponíveis da matriz das cinzas de carvão contribuem para estes efeitos. Estudos usando o teste de Salmonella/Microssoma (Teste de Ames) e o ensaio cometa Alcalino em células sanguíneas humanas indicam que os lixiviados de cinzas de carvão têm um potencial mutagênico e genotóxico (Chakraborty & Mukherjee, 2009). Adicionalmente, Gasparotto et al. (2013) demonstraram em macrófagos *in vitro* que as cinzas de carvão contêm partículas ultrafinas e componentes que induzem dano celular sub-crônico, efeitos inflamatórios e estresse oxidativo.

Considerando os prováveis efeitos que podem ser induzidos *in vivo* pela exposição às partículas de cinzas e carvão, a literatura aponta que alguns destes efeitos podem estar associados a eventos precoces de carcinogênese (por exemplo, a formação de micronúcleos), os quais têm sido sugeridos como um biomarcador preditivo de risco de câncer (Bonassi et al., 2011). Coerentemente, nossos resultados do ensaio CBMN-Cyt (Tabela 4, Capítulo I) mostraram uma maior instabilidade cromossômica associada com a exposição às partículas de carvão e cinzas. De fato, existe uma preocupação cada vez maior sobre o risco da exposição a produtos carcinogênicos derivados da mineração de carvão, tais como sílica, metais e compostos orgânicos (Jenkins et al., 2013).

Embora seja difícil comparar as análises *in vitro* e *in vivo* devido à complexidade que envolve um organismo vivo, algumas das respostas de estudos *in vitro* observadas no Capítulo I apoiam os resultados obtidos *in vivo* no Capítulo II.

De modo geral, observa-se que os pulmões de animais expostos agudamente às partículas de carvão apresentaram rigidez e obstruções das vias aéreas centrais (Tabela 1, Capítulo II). Nestes experimentos, os animais expostos às partículas de COAL16 também mostraram comprometimento das vias aéreas periféricas, como observado pelos parâmetros significativos do componente viscoelástico da elastância e $\Delta P2$, respectivamente. Este fato corrobora os efeitos observados para a mistura de concentrações significativas de compostos contidos na amostra COAL16, como SiO_2, alto conteúdo de antraceno, fluoranteno, acenafteno e benzo(a) antraceno. Como descrito por Hartwig (2002), as lesões pulmonares podem surgir após exposição às partículas que contêm compostos capazes de gerar dano oxidativo como os HAP. Estas lesões podem estar associadas à morte celular (Ghanem et al., 2006) e efeitos inflamatórios potenciais em modelos murinos (Happo et al., 2008).

Em relação à resposta inflamatória (Tabela 1, Capítulo II), observou-se um significativo influxo de células mononucleares nos animais expostos, tanto às partículas de carvão quanto às de cinzas. Porém, o recrutamento de células polimorfonucleares foi evidente somente nos animais expostos a COAL16. Essas respostas significativas nos permitem associar este recrutamento de células mononucleares com a liberação de citocinas durante o processo inflamatório. Todos os animais expostos a estas partículas

apresentaram aumentos significativos de TNF-α quando comparados com o grupo controle. A expressão de IL-1 β também foi evidente em todos os animais, porém níveis mais baixos foram encontrados nos expostos às partículas de CFA11. Na análise histológica pode se observar que a exposição a partículas de COAL11 e COAL16 provocaram uma importante inflamação no parênquima pulmonar, evidenciada pela presença de espessamento de septo e edema alveolar principalmente nos animais expostos a partículas de COAL16 (Figura 2, Capítulo II). De modo interessante, o grupo exposto a COAL16 apresentou maior infiltrado celular no parênquima pulmonar, indicando um processo inflamatório ativo como observado no significativo recrutamento de células mononucleares e polimorfonucleares. De fato, somente os animais expostos às partículas de COAL16 mostraram níveis altamente significativos de expressão de IL-6, quando comparados ao grupo controle. Vários autores descrevem que durante o início agudo da inflamação, após a ativação de células fagocíticas como os macrófagos, são liberadas citocinas como TNF-α e IL-1β as quais estimulam a liberação de IL-6 (Schins & Borm, 1999; Scheller et al., 2011; Li et al., 2015). IL-6 contribui notavelmente na extensão do processo inflamatório (Scheller et al., 2011; Allen & Kurdowska, 2014). Levando na conta isto, os animais expostos às partículas COAL16 apresentaram maior expressão de citocinas TNF-α e IL-1β fato que pode explicar a significativa liberação de IL-6 nesse grupo.

Para associar estes resultados, vale ressaltar que estas partículas são consideradas pobremente solúveis e quimicamente complexas. Partículas com estas características são capazes de gerar uma inflamação persistente e manter um pico de influxo de citocinas pró-inflamatórias, inclusive 24 horas após a instilação intratraqueal, como foi observado em nosso estudo. Diante destes achados, sugere-se uma associação entre a ativação da resposta inflamatória pulmonar e as alterações observadas na mecânica respiratória. Porém, como foi observado, não houve alterações significativas no percentual de alvéolos colapsados (Tabela 1, Capitulo II).

Tem sido descrito que após da ativação de macrófagos, ocorre a estimulação de células epiteliais e fibroblastos, os quais são os principais produtores de componentes da matriz extracelular, incluindo colágeno, proteoglicanos e fibras elásticas, e em consequência existe deposição de fibras colágenas, contribuindo para o processo de fibrose pulmonar (Schins & Borm, 1999). Apesar do papel importante da inflamação na fibrogênese, a mesma não está necessariamente relacionada à resposta fibrótica, como foi verificado neste estudo. Os animais foram expostos durante 24 horas a estas partículas, período muito curto para a formação e deposição de fibras de colágeno e elásticas, mas sim um período acertado para o pico de expressão de citocinas (Tabela 1, Capítulo II).

Sabe-se que quanto menor o diâmetro aerodinâmico da partícula, maior o nível de inflamação desencadeado por elas (Knaapen et al., 2004). Cerca de 40% das partículas da amostra de COAL11 apresentaram diâmetros menores de 10 µm. O diâmetro das partículas de CFA11 foi maior comparado às demais amostras. Entretanto, aproximadamente 25% dessa amostra teve diâmetro menor de 10 µm. Mais de 30% das partículas de COAL16 apresentaram diâmetro menor que 10 µm, e todas as partículas de CFA16 tiveram diâmetros menores de 10 µm.

Os estudos apontam que o diâmetro da partícula determina em grande parte sua toxicidade potencial (para revisão, ver: Donaldson et al., 2001; Donaldson & Stone, 2003). A probabilidade de deposição de partículas ultrafinas (UF) no trato respiratório aumenta abruptamente entre as menores partículas. Além disso, é conhecido que as partículas ultrafinas possuem um potencial inflamatório maior que as partículas finas (Donaldson et al., 2001; Liu et al., 2015). Elas têm a capacidade de depositar-se sobre estruturas epiteliais frágeis na região de trocas gasosas. Adicionalmente, o acúmulo excessivo de partículas afeta a função fagocítica dos macrófagos, associado com a resposta inflamatória, deposição intersticial de partículas e a proliferação de células epiteliais (Borm et al., 2004). Além disso, durante a ativação dos macrófagos, uma ampla gama de produtos incluindo oxidantes, lipídeos bioativos, citocinas, fatores de crescimento, proteases e antiproteases podem ser liberados (Schins & Borm, 1999; Hiraiwa et al., 2013). Este fato poderia explicar os significativos efeitos na mecânica pulmonar, o recrutamento de células mononucleares e a expressão de citocinas TNF-α e IL-1β, observados após exposição às partículas de COAL11, COAL16 e CFA16.

De todas as amostras estudadas, CFA11 apresentou maior diâmetro das partículas (65,52 µm), o que poderia justificar as análises de mecânica pulmonar serem similares ao grupo controle. Porém, de maneira interessante, estudos demonstram que partículas de diâmetro de 5 a 10 µm podem causar uma fagocitose incompleta ou "frustrada" (Champion and Mitragotri, 2006; Schinwald and Donaldson, 2012). Neste cenário, produz-se uma inflamação persistente, fato que poderia ter relação com o significativo recrutamento de células mononucleares e a expressão de TNF-α após exposição às partículas CFA11.

Os efeitos genotóxicos significativos evidenciados em células do sangue periférico dos camundongos expostos às diferentes partículas poderiam estar associados aos mecanismos celulares e não celulares de formação de ERO e, em consequência, ao dano oxidativo gerado pela exposição a estas partículas, como foi observado também em cultura células de fibroblastos de pulmão de hamster chinês (V79), descritas no primeiro capítulo deste trabalho. Neste contexto, estudos de genotoxicidade usando sangue periférico de camundongos demostram os efeitos genotóxicos potenciais da mineração de carvão (León et al., 2007; Caballero-Gallardo & Olivero-Verbel, 2016).

Muitos autores descrevem o papel importante dos metais na geração de dano oxidativo (Beyersmann and Hartwig, 2008; Jomova & Valko, 2011; Jaishankar et al., 2014). Assim, no presente estudo, foram analisadas as concentrações de elementos inorgânicos presentes em diferentes órgãos, além do pulmão. Diversos estudos descrevem que as partículas ultrafinas podem ter seu acesso a outras células no epitélio, ao interstício, assim como às paredes vasculares aumentado (Borm et al., 2004; Mo et al., 2009; Terzano et al., 2010) e, com isso, migrar para órgãos extrapulmonares (Oberdörster et al., 2004). Em adição, tem sido descrito que os metais presentes na superfície das partículas podem se translocar do pulmão para outros órgãos, manifestando seus efeitos tóxicos em diferentes tecidos (Mani et al., 2007; Kreyling et al., 2011).

É importante ressaltar que o sistema respiratório pode não ser o único alvo na toxicidade de metais, mesmo na sua forma inalatória (Zeng et al., 2016). Algumas partículas e outros compostos solúveis difundem-se pela corrente sanguínea, podendo se dissociar durante seu transporte. O sangue pode então transportar metais tóxicos, que podem dar lugar a efeitos sistêmicos, afetando não somente o pulmão, mas outros órgãos como o encéfalo, fígado, rins, entre outros, e levando a doenças neurológicas, danos renais e hepáticos (Borm et al., 2004; Zeng et al., 2016).

De modo geral, os metais que estiveram presentes em concentrações significativas no pulmão, fígado, baço e no encéfalo foram Cr, Fe e Ni (Figura 3, Capítulo II). Elementos como Cr e o Ni são catalogados pela IARC na categoria 1 como carcinógenos para os seres humanos (IARC, 2012). É importante ressaltar como elemento comum em todos os órgãos analisados, destaca-se o Fe, o qual está relacionado com a contituição do carvão como o importante conteúdo de pirita no carvão de Santa Catarina (Silva et al., 2010).

É interessante notar que metais solúveis têm sido descritos pela sua capacidade de se translocar pela circulação sanguínea a órgãos extrapulmonares (Kreyling et al., 2011). Mani et al. (2007) descreveram que ratos expostos às partículas de cinza de carvão mostraram aumento das concentrações de Cd nos pulmões, fígado e rins, indicando que após a absorção do Cd nos pulmões, este foi transportado a órgãos extrapulmonares.

O fígado pode ser o lugar primário de biotransformação de xenobióticos. É um órgão propenso a sofrer danos por receber substâncias tóxicas do sistema circulatório, além de ser o local da ativação de alguns destes componentes (Singh et al., 2016). Assim, muitos destes compostos são detoxificados e eliminados, principalmente como conjugados, alguns deles concentram-se até atingir níveis tóxicos e outros são bioativados, transformando-se em intermediários reativos que podem levar a danos no fígado e desencadear doenças, incluindo o câncer (Butterworth, 2010). Os metais pesados, por exemplo, têm afinidade pelos grupos sulfidrila livres do GSH, de aminoácidos e de proteínas, e podem também causar inibição não competitiva de muitas enzimas (Zenget al., 2016). A exposição a metais pode reduzir drasticamente a reserva de grupos SH disponíveis e produzir estresse oxidativo (Jomova & Valko, 2011). Sabe-se que o fígado é o principal local de armazenamento de ferro, sendo susceptível ao dano oxidativo. Ferro e outros metais pesados (Cu, V, Ni) têm a capacidade de gerar ERO e em consequência, aumentar a peroxidacão lipídica das membranas hepáticas e a disfunção mitocondrial (Butterworth, 2010).

No que diz respeito ao baço, sua função está relacionada à hematopoese e a destruição de células sanguíneas vermelhas velhas. Forma parte do sistema linfático e é o centro de atividade do sistema imune (Suttie, 2006). O baço é um sítio de toxicidade direta ou indireta e um alvo para alguns xenobióticos, o qual pode levar a doenças imunológicas e câncer (De Jong & Van Loveren, 2007). Whittaker et al. (1996) descreveram que, em ratos, a toxicidade de metais como Fe está associada com processos de morte celular em diferentes órgãos além do baço, sugerindo que o estresse oxidativo está envolvido na patogênese das lesões. Outros elementos metálicos como Cr

têm sido associados com alterações no baço e no sistema imunológico de camundongos (Ferreira et al., 2003).

Outro órgão bastante suscetível ao dano de radicais livres é o encéfalo. Este é um órgão que tem uma alta concentração de ácidos graxos poliinsaturados, associada a uma alta atividade metabólica e relativamente baixa capacidade antioxidante. O ferro é essencial no encéfalo, mas altas quantidades podem levar à geração de dano nas células cerebrais, uma vez que pode conduzir ao estresse oxidativo (Hagemeier et al., 2012). Além do Fe, outro metal que esteve em concentrações significativas no encéfalo foi o Ni. Embora os mecanismos moleculares de carcinogenicidade do Ni ainda não sejam claros, sugere-se que a exposição ao Ni induz estresse oxidativo, pela redução na expressão de enzimas antioxidantes e geração de quebras duplas no DNA (para revisão, ver Kim et al., 2015).

De forma similar ao descrito neste estudo, alguns autores relatam que a inalação contínua de poluentes ocupacionais como as partículas de carvão ou cinzas contribui significativamente para o surgimento e progressão de várias doenças respiratórias mediadas pela formação de ERO e estresse oxidativo (Pelucchi et al., 2006; Park et al., 2009; Centers for Disease Control and Prevention (CDC), 2012). Como é mostrado no Anexo 1, no trabalho intitulado "Genetic damage in coal miners evaluated by buccal micronucleus cytome assay", foram realizadas análises de elementos inorgânicos no sangue periférico de mineiros expostos cronicamente às partículas de carvão numa mina localizada em Guajira-Colômbia. Na análise das concentrações dos elementos, foram observadas quantidades significativamente maiores de Si e Al no grupo exposto. Estes elementos foram encontrados em quantidades consideráveis em forma de óxidos na composição do carvão em Guajira, caso similar à composição do carvão e cinzas do estado de Santa Catarina (Tabelas 1 e 2, Capítulo I).

É importante ressaltar que a exposição crônica às misturas complexas como as produzidas durante as atividades de mineração de carvão tem sido objeto de estudo ao redor do mundo, usando diferentes biomarcadores (Donbak et al., 2005; Santa Maria et al., 2007; Minina et al., 2015). Sendo assim, a Tabela 1 do capítulo do livro "Occupational Exposure to Coal, Genotoxicity and Cancer Risk" (Anexo 2) apresenta uma compilação de estudos acerca da exposição a resíduos de combustão e mineração de carvão em humanos.

Avaliar os riscos por meio de estudos de biomonitoramento em populações expostas às misturas complexas pode ser bastante complicado. É importante levar em consideração que os trabalhadores não estão expostos a um único componente, sendo necessário, portanto, considerar a toxicidade de cada componente na mistura, e a natureza imprescindível dos efeitos da interação, os quais podem ser aditivos ou sinérgicos (Pastor et al., 2002; Cogliano, 2016).

Em relação aos efeitos da exposição a subprodutos do carvão, estudos sobre a toxicidade das partículas e de biomonitoramento de trabalhadores expostos à mineração de carvão representam uma boa referência na discussão sobre os efeitos das cinzas no tecido pulmonar. Neste contexto, Celik et al. (2007) encontraram um dano citogenético significativo nos trabalhadores expostos às cinza e gases provenientes da

queima de carvão, atribuído aos efeitos acumulativos e relacionados à complexidade da mistura produzidos pela combustão.

Se considerarmos conjuntamente os resultados *in vitro* e *in vivo*, pode ser observado que, nos resultados *in vitro* (Capítulo I), a citotoxicidade e genotoxicidade foram notáveis nas células expostas às partículas CFA16. *In vivo* (Capítulo II) efeitos inflamatórios importantes e na mecânica respiratória foram observados nos animais expostos a partículas de carvão. Essas observações podem estar relacionadas com a complexidade e diferença entre os dois modelos. *In vitro* os diferentes microcomponentes destas partículas conseguem se lixiviar, interagir e causar dano no DNA, entretanto no modelo *in vivo* existe um processo de metabolização de compostos como os HAP, somado ao fato que estão presentes partículas de diferentes tamanhos que tentam ser fagocitadas pelos macrófagos desencadeando um processo inflamatório muito ativo associado à formação de ERO, dano nas macromoléculas e mudanças no tecido pulmonar.

Particularmente, se comparamos os efeitos entre as partículas de CFA11 e CFA16, pode se notar que as células expostas às partículas CFA16 apresentaram níveis de danos oxidativos similares às expostas a partículas de carvão. Nas análises *in vivo*, nos parâmetros da mecânica pulmonar e expressão de citocinas, os valores foram maiores nos animais expostos à CFA16, quando comparado com os expostos à CFA11. Essas observações nos levam a sugerir que, além do processo de combustão alterar a toxicidade das partículas de carvão, o uso de combustíveis adicionais no processo de queima altera as características das partículas geradas em relação ao tamanho e composição química e consequentemente aumenta os possíveis riscos das populações expostas.

Em resumo, a heterogeneidade química e o tamanho das partículas de cinzas e carvão podem explicar os efeitos citotóxicos, genotóxicos e pulmonares e os mecanismos "acelulares" e "celulares" relacionados com a formação de ERO (Figura 19). Dentre os mecanismos "acelulares" de geração de oxidantes, os metais e óxidos presentes nas partículas de carvão e cinzas, por diferentes vias, desempenham um papel crítico. Como consequência, importantes macromoléculas como lipídeos, proteínas e principalmente o DNA sofrem modificações oxidativas. Adicionalmente, os HAP contidos nas partículas também contribuem na toxicidade celular pela geração de ERO e formação de adutos com o DNA. Estes componentes antes mencionados podem não somente afetar o pulmão, mas também outros órgãos, devido à eficiente translocação pela circulação sanguínea. Uma segunda via indireta importante de formação de ERO está relacionada com mecanismos celulares, consequência da produção aumentada de oxidantes pelos macrófagos e neutrófilos durante a fagocitose de partículas de carvão e cinzas e da inflamação produzida.

Todo este cenário passa a ser alarmante, se forem consideradas as populações expostas à inalação contínua, às altas cargas de partículas em células fagocíticas no pulmão, e o desequilíbrio redox que estão ligados à origem de processos patológicos. Este trabalho contribui para conhecimento sobre os efeitos destas partículas e os riscos da exposição, além de contribuir para o estabelecimento de novas estratégias de controle e prevenção nas populações expostas.

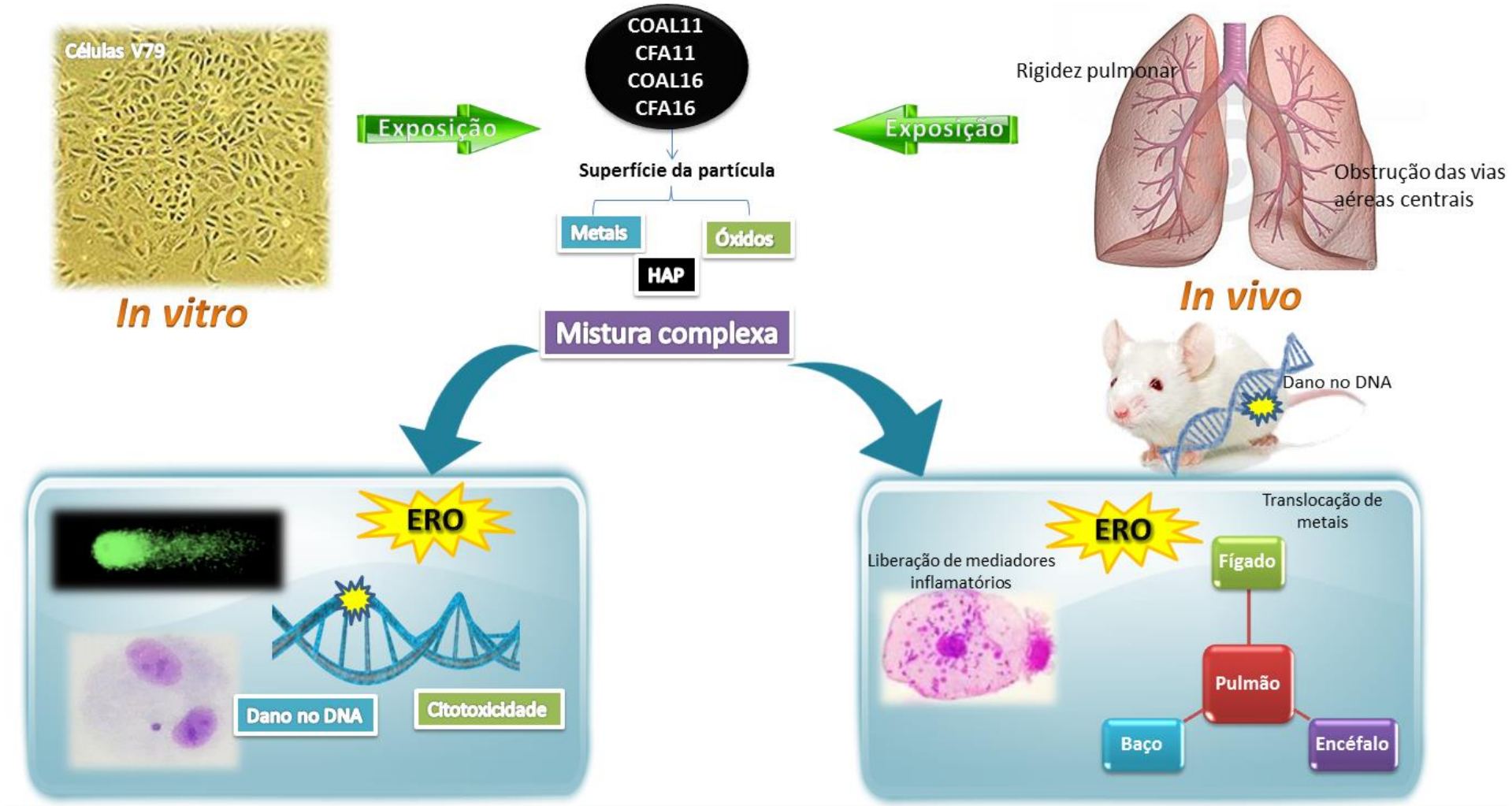


Figura 19. Resumo gráfico do trabalho: avaliação *in vitro* e *in vivo* dos efeitos de partículas de carvão e cinzas provenientes de Santa Catarina

CONCLUSÕES

VI. CONCLUSÕES

1. Conclusão Geral

O conjunto de resultados desta tese permite concluir que as amostras de partículas de carvão e cinzas provenientes de Santa Catarina causam lesões primárias no DNA e, nas concentrações mais altas, induzem citotoxicidade e instabilidade cromossômica associada ao dano oxidativo *in vitro*. Adicionalmente, foi possível demostrar que a exposição aguda às partículas de carvão e cinzas em camundongos afeta a mecânica pulmonar, induz o recrutamento de células principalmente mononucleares, a expressão de citocinas pró-inflamatórias no pulmão e efeitos genotóxicos em células do sangue periférico. Além disso, foi demonstrada a translocação eficiente de alguns metais pela corrente sanguínea, do pulmão ao fígado, baço e encéfalo. Estes resultados estão relacionados com compostos contidos na superfície das partículas, tais como óxidos, metais e HAP detectados nas amostras.

2. Conclusões específicas

- Os resultados no Ensaio Clonogênico permitiram evidenciar o potencial citotóxico das partículas em cultura de células de fibroblastos de pulmão de hâmster chinês (V79).

- As análises pelo ensaio Cometa Alcalino mostraram que a exposição das células V79 às partículas de carvão e cinzas induziram lesões primarias no DNA.

- As partículas de COAL16 e CFA16, nas concentrações mais altas, são capazes de induzir danos oxidativos no DNA em células V79.

- A análise dos biomarcadores do CBMN-Cyt evidenciou que as concentrações mais altas de partículas de carvão e cinzas foram capazes de induzir efeitos citotóxicos (apoptose e necrose) e instabilidade cromossômica (formação de ponte, broto e MN), porém nenhuma das amostras teve efeito significativo sobre a proliferação celular.

- Nas análises de mecânica pulmonar, os animais expostos às partículas de carvão apresentaram rigidez e obstruções das vias aéreas centrais, oferecendo maior resistência ao movimento durante os ciclos respiratórios.

- As partículas de carvão e cinzas induziram significativamente o recrutamento de células, principalmente células mononucleares no pulmão e, portanto, a liberação de citocinas pró-inflamatórias como TNF-α e IL-1β.

- Efeitos genotóxicos foram observados no sangue periférico de camundongos expostos a partículas de carvão e cinzas, os quais poderiam estar associados à geração de dano oxidativo.

- Na análise da morfometria, não foram evidenciadas alterações significativas no percentual de alvéolos colapsados após 24 horas de instilação intratraqueal com partículas de carvão e cinzas.

- Na análise da formação de fibras, não foi observada a formação de fibras elásticas e colágenas no pulmão dos camundongos expostos.

- Na avaliação da concentração dos elementos nos diferentes órgãos dos camundongos pelo método de PIXE, foram encontradas quantidades significativamente maiores de Cr, Fe e Ni, quando comparadas com o grupo controle.

- A detecção de metais no fígado, baço e encéfalo permite concluir que existe translocação eficiente de metais pela circulação sanguínea até órgãos extrapulmonares.

- Os efeitos observados *in vitro* e *in vivo* estão relacionados com a geração de ERO e formação de adutos pró-mutagênicos, consequência dos efeitos de componentes destas partículas como HAP, óxidos e metais.

PERSPECTIVAS

VII. PERSPECTIVAS

Para uma melhor compreensão dos efeitos gerados pela exposição a partículas de carvão e cinzas, seria de grande importância a avaliação dos seguintes aspectos:

- Avaliar os efeitos citotóxicos e genotóxicos em outras linhagens celulares como macrófagos alveolares murinos (RAW264.7), células epiteliais humanas (A549) e células do fígado (HEPG2);
- Avaliar a indução de estresse oxidativo *in vitro* (medida de SOD, CAT, GPx, GSH);
- Avaliar *in vivo* períodos de exposição subcrônica, e também períodos de exposição crônica às partículas de carvão e cinzas;
- Conjuntamente ao uso de períodos de exposição subcrônica, seria interessante incluir análise de TGF-β para reforçar a investigação histológica de formação de fibras de colágeno em camundongos expostos a partículas de carvão e cinzas;
- Incluir análises de marcadores de estresse oxidativo (medida de SOD, CAT, GSH) no pulmão de camundongos expostos a partículas de carvão e cinzas;
- Incluir a análise da expressão de quimiocinas no pulmão de camundongos expostos a partículas de carvão e cinzas, tais como IL-8 e MCP-1/CCL2;
- Incluir análises de expressão de RNAm para analisar a expressão de pró-colágeno 3 e pró-colágeno 9, os quais estão relacionados com períodos de remodelamento do tecido pulmonar em camundongos;
- Incluir técnicas de imunohistoquímica para visualizar a expressão da síntese de pró-colágeno 3 em camundongos expostos a partículas de carvão e cinzas;
- Analisar a expressão de diferentes marcadores de proteínas de estresse em camundongos, após exposição a partículas de carvão e cinzas, tais como caspase 8 e NF-kB usando western blotting;
- Incluir análises de histologia de fígado, baço, encéfalo, rins e coração de camundongos expostos a partículas de carvão e cinzas;
- Analisar a translocação de HAP do pulmão ao fígado de camundongos expostos a partículas de carvão e cinzas; e
- Utilizar ICP-MS (espectrometria de massas com plasma de acoplamento indutivo) para determinar as concentrações de elementos inorgânicos presentes em outros órgãos de camundongos, como rins e coração.

REFERÊNCIAS

VIII. REFERÊNCIAS

Achary, V.M.M., Parinandi, N.L., Panda, B.B., 2012. Aluminum induces oxidative burst, cell wall NADH peroxidase activity, and DNA damage in root cells of Allium cepa L. Environ. Mol. Mutagen. 53, 550–560. doi:10.1002/em.21719

Achten, C., Hofmann, T., 2009. Native polycyclic aromatic hydrocarbons (PAH) in coals - a hardly recognized source of environmental contamination. Sci. Total Environ. 407, 2461–2473. doi:10.1016/j.scitotenv.2008.12.008.

Agency for Toxic Substances & Disease Registry, ATSDR. 2012. Polycyclic Aromatic Hydrocarbons (PAHs) What Health Effects Are Associated With PAH Exposures. 1-68. On line http://www.atsdr.cdc.gov/csem/pah/docs/pah.pdf

Allen, T.C., Kurdowska, A., 2014. Interleukin 8 and acute lung injury. Arch. Pathol. Lab. Med. 138, 266–269. doi:10.5858/arpa.2013-0182-RA

An, J., Yin, L., Shang, Y., Zhong, Y., Zhang, X., Wu, M., Yu, Z., Sheng, G., Fu, J., Huang, Y., 2011. The combined effects of BDE47 and BaP on oxidatively generated DNA damage in L02 cells and the possible molecular mechanism. Mutat. Res. 721, 192–198. doi:10.1016/j.mrgentox.2011.02.002

Ancochea, J., Antón, E., Casanova, A., 2004. Nuevas estrategias terapéuticas en la fibrosis pulmonar idiopática. Arch. Bronconeumol. 40, 16–22.

Aneel- Agência Nacional de Energia Elétrica. Atlas de Energia Elétrica do Brasil. Capítulo 9- Carvão mineral. 2009. Disponível on line, www.aneel.gov.br/arquivos/pdf/atlas_par3_cap9.pdf

Angelé-Martínez, C., Goodman, C., Brumaghim, J., 2014. Metal-mediated DNA damage and cell death: mechanisms, detection methods, and cellular consequences. Met. Integr. Biometal Sci. 6, 1358–1381. doi:10.1039/c4mt00057a

Associação Brasileira do carvão Mineral, ABCM. História do Carvão no Brasil. 2012. Disponível on line, http://www.carvaomineral.com.br/interna_conteudo.php?i_subarea=8&i_area=4

Ates, I., Yucesoy, B., Yucel, A., Suzen, S.H., Karakas, Y., Karakaya, A., 2011. Possible effect of gene polymorphisms on the release of TNFα and IL1 cytokines in coal workers' pneumoconiosis. Exp. Toxicol. Pathol. Off. J. Ges. Für Toxikol. Pathol. 63, 175–179. doi:10.1016/j.etp.2009.11.006

ATSDR, 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs). U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA.

Bal, W., Protas, A.M., Kasprzak, K.S., 2011. Genotoxicity of metal ions: chemical insights. Met. Ions Life Sci. 8, 319–373.

Bekki, K., Toriba, A., Tang, N., Kameda, T., Hayakawa, K., 2013. Biological effects of polycyclic aromatic hydrocarbon derivatives. J. UOEH 35, 17–24.

Beyersmann, D., Hartwig, A., 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch. Toxicol. 82, 493–512. doi:10.1007/s00204-008-0313-y

Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26, 93–100. doi:10.1093/mutage/geq075

Bonner, J.C., 2002. The epidermal growth factor receptor at the crossroads of airway remodeling. Am. J. Physiol. - Lung Cell. Mol. Physiol. 283, L528–L530. doi:10.1152/ajplung.00126.2002

Bonner, J.C., 2007. Lung fibrotic responses to particle exposure. Toxicol. Pathol. 35, 148–153. doi:10.1080/01926230601060009

Bonner, M.R., Rothman, N., Mumford, J.L., He, X., Shen, M., Welch, R., Yeager, M., Chanock, S., Caporaso, N., Lan, Q., 2005. Green tea consumption, genetic susceptibility, PAH-rich smoky coal, and the risk of lung cancer. Mutat. Res. 582, 53–60. doi:10.1016/j.mrgentox.2004.12.008

Borm PJ. 1997. Toxicity and occupational health hazards of coal fly ash (CFA). A review of data and comparison to coal mine dust. Ann Occup Hyg 41: 659-76. doi: 10.1093/annhyg/41.6.659.

Borm, P.J.A., Schins, R.P.F., Albrecht, C., 2004. Inhaled particles and lung cancer, part B: paradigms and risk assessment. Int. J. Cancer J. Int. Cancer 110, 3–14. doi:10.1002/ijc.20064

Boström, C.-E., Gerde, P., Hanberg, A., Jernström, B., Johansson, C., Kyrklund, T., Rannug, A., Törnqvist, M., Victorin, K., Westerholm, R., 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environ. Health Perspect. 110 Suppl 3, 451–488.

BP. British Petroleum, BP Statistical Review of World Energy. Coal. 2015. Disponível on line, http://www.bp.com/content/dam/bp/pdf/Energy-economics/statistical-review-2014/BP-statistical-review-of-world-energy-2014-full-report.pdf Pag. 30-34.

Brazova, J., Sismova, K., Vavrova, V., Bartosova, J., Macek, M., Lauschman, H., Sediva, A., 2006. Polymorphisms of TGF-beta1 in cystic fibrosis patients. Clin. Immunol. Orlando Fla 121, 350–357. doi:10.1016/j.clim.2006.08.015

Bresgen, N., Eckl, P.M., 2015. Oxidative stress and the homeodynamics of iron metabolism. Biomolecules 5, 808–847. doi:10.3390/biom5020808

Briedé, J.J., Godschalk, R.W.L., Emans, M.T.G., De Kok, T.M.C.M., Van Agen, E., Van Maanen, J., Van Schooten, F.-J., Kleinjans, J.C.S., 2004. In vitro and in vivo studies on oxygen free radical and DNA adduct formation in rat lung and liver during benzo[a]pyrene metabolism. Free Radic. Res. 38, 995–1002. doi:10.1080/10715760400000976

Burt E; Orris P; Buchanan S. 2013. Scientific Evidence of Health Effects from Coal Use in Energy Generation. University of Illinois at Chicago School of Public Health Chicago, Illinois, USA.

Butterworth, R.F., 2010. Metal toxicity, liver disease and neurodegeneration. Neurotox. Res. 18, 100–105. doi:10.1007/s12640-010-9185-z

Caballero A. L., Médico O. A. 2013. Caracterización y posible uso de cenizas resultantes de la combustión del carbón, en la futura Termocentral de lecho fluidizado. Río Turbio (Argentina). Visir-Redisa. Universidad Nacional de la Patagonia Austral.

Caballero-Gallardo, K., Olivero-Verbel, J., 2016. Mice housed on coal dust-contaminated sand: A model to evaluate the impacts of coal mining on health. Toxicol. Appl. Pharmacol. 294, 11–20. doi:10.1016/j.taap.2016.01.009

Camelo, A., Dunmore, R., Sleeman, M.A., Clarke, D.L., 2014. The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. Front. Pharmacol. 4. doi:10.3389/fphar.2013.00173.

Campbell RJ. 2013. Increasing the Efficiency of Existing Coal-Fired Power Plants. Increasing the Efficiency of Existing Coal-Fired Power Plants. Congressional Research Service.

Carbone F., Pagliara R., Barone A.C., Beretta F., D'Anna A. 2009.Characterization of Nano-Ashes Generated During Pulverized Coal Combustion. Report Ricerca Sistema Elettrico.

Carbunión, usos del carbón. 2009. Pag. 1-7. Disponível on line, www.carbunion.com/panel/carbon/uploads/usos_carbon_4.pdf

Cassee, F.R., Héroux, M.-E., Gerlofs-Nijland, M.E., Kelly, F.J., 2013. Particulate matter beyond mass: recent health evidence on the role of fractions, chemical constituents and sources of emission. Inhal. Toxicol. 25, 802–812. doi:10.3109/08958378.2013.850127

Cassel, S.L., Eisenbarth, S.C., Iyer, S.S., Sadler, J.J., Colegio, O.R., Tephly, L.A., Carter, A.B., Rothman, P.B., Flavell, R.A., Sutterwala, F.S., 2008. The Nalp3 inflammasome is essential for the development of silicosis. Proc. Natl. Acad. Sci. U. S. A. 105, 9035–9040. doi:10.1073/pnas.0803933105

Castranova, V., Vallyathan, V., 2000. Silicosis and coal workers' pneumoconiosis. Environ. Health Perspect. 108, 675–684.

Celik, M., Donbak, L., Unal, F., Yüzbasioglu, D., Aksoy, H., Yilmaz, S., 2007. Cytogenetic damage in workers from a coal-fired power plant. Mutat. Res. 627, 158–163. doi:10.1016/j.mrgentox.2006.11.003

Centers for Disease Control and Prevention (CDC), 2012. Pneumoconiosis and advanced occupational lung disease among surface coal miners--16 states, 2010-2011. MMWR Morb. Mortal. Wkly. Rep. 61, 431–434.

Cerrejón-minería responsable. Usos del carbón. 2013. Disponível on line, http://www.cerrejon.com/site/mas-sobre-el-carbon/usos-del-carbon.aspx

Chakraborty, R., Mukherjee, A., 2009. Mutagenicity and genotoxicity of coal fly ash water leachate. Ecotoxicol. Environ. Saf. 72, 838–842. doi:10.1016/j.ecoenv.2008.09.023

Champion, J.A., Mitragotri, S., 2006. Role of target geometry in phagocytosis. Proc. Natl. Acad. Sci. U. S. A. 103, 4930–4934. doi:10.1073/pnas.0600997103

Chen, F., Shi, X., 2002. NF-kappaB, a pivotal transcription factor in silica-induced diseases. Mol. Cell. Biochem. 234-235, 169–176.

Chen, Y., Bi, X., Mai, B., Sheng, G., Fu, J., 2004. Emission characterization of particulate/gaseous phases and size association for polycyclic aromatic hydrocarbons from residential coal combustion. Fuel 83, 781–790. doi:10.1016/j.fuel.2003.11.003

Chung, K.F., 2001. Cytokines in chronic obstructive pulmonary disease. Eur. Respir. J. Suppl. 34, 50s–59s.

Church, M.K., Robinson, C.F., 2012. Eicosanoids in Inflammatory Conditions of the Lung, Skin and Joints. Springer Science & Business Media.

Cogliano, V.J., 2016. Lack of data drives uncertainty in PCB health risk assessments. Environ. Sci. Pollut. Res. Int. 23, 2212–2219. doi:10.1007/s11356-015-5157-4

Cohen RA, Patel A, Green FH. 2008. Lung disease caused by exposure to coal mine and silica dust. Semin Respir Crit Care Med. Dec;29(6):651-61.

Colobran, R., Pujol-Borrell, R., Armengol, M.P., Juan, M., 2007. The chemokine network. I. How the genomic organization of chemokines contains clues for deciphering their functional complexity. Clin. Exp. Immunol. 148, 208–217. doi:10.1111/j.1365-2249.2007.03344.x

Cox, L.A.T., 2011. An exposure-response threshold for lung diseases and lung cancer caused by crystalline silica. Risk Anal. Off. Publ. Soc. Risk Anal. 31, 1543–1560. doi:10.1111/j.1539-6924.2011.01610.x

Dai, S., Ren, D., Chou, C.-L., Finkelman, R.B., Seredin, V.V., Zhou, Y., 2012. Geochemistry of trace elements in Chinese coals: A review of abundances, genetic types, impacts on human health, and industrial utilization. Int. J. Coal Geol., Minerals and Trace Elements in Coal 94, 3–21. doi:10.1016/j.coal.2011.02.003

Das, D.N., Panda, P.K., Mukhopadhyay, S., Sinha, N., Mallick, B., Behera, B., Maiti, T.K., Bhutia, S.K., 2014. Prediction and validation of apoptosis through cytochrome P450 activation by benzo[a]pyrene. Chem. Biol. Interact. 208, 8–17. doi:10.1016/j.cbi.2013.11.005

Davies, D.E., 2014. Epithelial barrier function and immunity in asthma. Ann. Am. Thorac. Soc. 11 Suppl 5, S244–251. doi:10.1513/AnnalsATS.201407-304AW

De Jong, W.H., Van Loveren, H., 2007. Screening of xenobiotics for direct immunotoxicity in an animal study. Methods, Animal Models in Immunotoxicology 41, 3–8. doi:10.1016/j.ymeth.2006.09.003

Dellinger, B., Pryor, W.A., Cueto, R., Squadrito, G.L., Hegde, V., Deutsch, W.A., 2001. Role of free radicals in the toxicity of airborne fine particulate matter. Chem. Res. Toxicol. 14, 1371–1377.

Demers, L.M., Kuhn, D.C., Gaydos, L., Stauffer, J.L., 1993. Eicosanoid and Cytokine Production by the Pulmonary Alveolar Macrophage, in: Nigam, S., Honn, K.V., Marnett, L.J., Jr, T.L.W. (Eds.), Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury, Developments in Oncology. Springer US, pp. 497–502.

Desler, C., Johannessen, C., Rasmussen, L.J., 2009. Repair of DNA damage induced by anthanthrene, a polycyclic aromatic hydrocarbon (PAH) without bay or fjord regions. Chem. Biol. Interact. 177, 212–217. doi:10.1016/j.cbi.2008.10.056

Diabaté, S., Bergfeldt, B., Plaumann, D., Ubel, C., Weiss, C., 2011. Anti-oxidative and inflammatory responses induced by fly ash particles and carbon black in lung epithelial cells. Anal. Bioanal. Chem. 401, 3197–3212. doi:10.1007/s00216-011-5102-4

Ding, M., Chen, F., Shi, X., Yucesoy, B., Mossman, B., Vallyathan, V., 2002. Diseases caused by silica: mechanisms of injury and disease development. Int. Immunopharmacol. 2, 173–182.

Donaldson, K., Stone, V., 2003. Current hypotheses on the mechanisms of toxicity of ultrafine particles. Ann. Dellstituto Super. Sanità 39, 405–410.

Donaldson, K., Stone, V., Clouter, A., Renwick, L., MacNee, W., 2001. Ultrafine particles. Occup. Environ. Med. 58, 211–216. doi:10.1136/oem.58.3.211

Donaldson, K., Tran, L., Jimenez, L.A., Duffin, R., Newby, D.E., Mills, N., MacNee, W., Stone, V., 2005. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part. Fibre Toxicol. 2, 10. doi:10.1186/1743-8977-2-10

Donbak, L., Rencuzogullari, E., Yavuz, A., Topaktas, M., 2005. The genotoxic risk of underground coal miners from Turkey. Mutat. Res. 588, 82–87. doi:10.1016/j.mrgentox.2005.08.014

Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., Tschopp, J., 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320, 674–677. doi:10.1126/science.1156995

Driscoll, K.E., Howard, B.W., Carter, J.M., Janssen, Y.M., Mossman, B.T., Isfort, R.J., 2001. Mitochondrial-derived oxidants and quartz activation of chemokine gene expression. Adv. Exp. Med. Biol. 500, 489–496.

Dwivedi, S., Saquib, Q., Al-Khedhairy, A.A., Ali, A.-Y.S., Musarrat, J., 2012. Characterization of coal fly ash nanoparticles and induced oxidative DNA damage in human peripheral blood mononuclear cells. Sci. Total Environ. 437, 331–338. doi:10.1016/j.scitotenv.2012.08.004.

EPA, 2015. http://www3.epa.gov/warm/pdfs/Fly_Ash.pdf

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539.

European Commission 2002. Opinion of the scientific committee on food on the risks to human health of Polycyclic Aromatic Hydrocarbons in food. Series editor. Scientific Committee on food, URL: http://ec.europa.eu/food/fs/sc/scf/out153_en.pdf

Exley, C., Charles, L.M., Barr, L., Martin, C., Polwart, A., Darbre, P.D., 2007. Aluminium in human breast tissue. J. Inorg. Biochem. 101, 1344–1346. doi:10.1016/j.jinorgbio.2007.06.005

Ferreira, M.E., de Lourdes Pereira, M., Garcia e Costa, F., Sousa, J.P., de Carvalho, G.S., 2003. Comparative study of metallic biomaterials toxicity: a histochemical and immunohistochemical demonstration in mouse spleen. J. Trace Elem. Med. Biol. Organ Soc. Miner. Trace Elem. GMS 17, 45–49.

Finney-Hayward, T.K., Bahra, P., Li, S., Poll, C.T., Nicholson, A.G., Russell, R.E.K., Ford, P.A., Westwick, J., Fenwick, P.S., Barnes, P.J., Donnelly, L.E., 2009. Leukotriene B4 release by human lung macrophages via receptor- not voltage-operated Ca2+ channels. Eur. Respir. J. 33, 1105–1112. doi:10.1183/09031936.00062708

Fröhlich E, Salar-Behzadi S. 2014. Toxicological assessment of inhaled nanoparticles: role of in vivo, ex vivo, in vitro, and in silico studies. Int J Mol Sci. Mar 18;15(3):4795-822.

Fubini, B., Hubbard, A., 2003. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic. Biol. Med. 34, 1507–1516.

Gasparotto, J., Somensi, N., Caregnato, F.F., Rabelo, T.K., DaBoit, K., Oliveira, M.L.S., Moreira, J.C.F., Gelain, D.P., 2013. Coal and tire burning mixtures containing ultrafine and

nanoparticulate materials induce oxidative stress and inflammatory activation in macrophages. Sci. Total Environ. 463-464, 743–753. doi:10.1016/j.scitotenv.2013.06.086

Geoscience News and Information, Geology.com. 2005. On line: http://geology.com/minerals/pyrite.shtml

Ghanem, M.M., Battelli, L.A., Mercer, R.R., Scabilloni, J.F., Kashon, M.L., Ma, J.Y.C., Nath, J., Hubbs, A.F., 2006. Apoptosis and Bax expression are increased by coal dust in the polycyclic aromatic hydrocarbon-exposed lung. Environ. Health Perspect. 114, 1367–1373.

Giaginis, C., Gatzidou, E., Theocharis, S., 2006. DNA repair systems as targets of cadmium toxicity. Toxicol. Appl. Pharmacol. 213, 282–290. doi:10.1016/j.taap.2006.03.008

Guidi, P., Nigro, M., Bernardeschi, M., Lucchesi, P., Scarcelli, V., Frenzilli, G., 2015. Does the crystal habit modulate the genotoxic potential of silica particles? A cytogenetic evaluation in human and murine cell lines. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 792, 46–52. doi:10.1016/j.mrgentox.2015.07.005

Gilmour, M.I., O'Connor, S., Dick, C.A.J., Miller, C.A., Linak, W.P., 2004. Differential pulmonary inflammation and in vitro cytotoxicity of size-fractionated fly ash particles from pulverized coal combustion. J. Air Waste Manag. Assoc. 1995 54, 286–295.

Goodell J. 2006.Big Coal: The Dirty Secret Behind America's Energy Future. New York, N.Y.: Houghton-Mifflin.

Greene, C.M., McElvaney, N.G., 2009. Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. Br. J. Pharmacol. 158, 1048–1058. doi:10.1111/j.1476-5381.2009.00448.x.

Grommes, J., Soehnlein, O., 2011. Contribution of neutrophils to acute lung injury. Mol. Med. Camb. Mass 17, 293–307. doi:10.2119/molmed.2010.00138

Gulumian, M., Borm, P.J.A., Vallyathan, V., Castranova, V., Donaldson, K., Nelson, G., Murray, J., 2006. Mechanistically identified suitable biomarkers of exposure, effect, and susceptibility for silicosis and coal-worker's pneumoconiosis: a comprehensive review. J. Toxicol. Environ. Health B Crit. Rev. 9, 357–395. doi:10.1080/15287390500196537

Hagemeier, J., Geurts, J.J.G., Zivadinov, R., 2012. Brain iron accumulation in aging and neurodegenerative disorders. Expert Rev. Neurother. 12, 1467–1480. doi:10.1586/ern.12.128

Haghdoost, S., Czene, S., Näslund, I., Skog, S., Harms-Ringdahl, M., 2005. Extracellular 8-oxo-dG as a sensitive parameter for oxidative stress in vivo and in vitro. Free Radic. Res. 39, 153–162. doi:10.1080/10715760500043132

Hamilton, R.F., Thakur, S.A., Holian, A., 2008. Silica binding and toxicity in alveolar macrophages. Free Radic. Biol. Med. 44, 1246–1258. doi:10.1016/j.freeradbiomed.2007.12.027

Happo, M.S., Hirvonen, M.-R., Halinen, A.I., Jalava, P.I., Pennanen, A.S., Sillanpaa, M., Hillamo, R., Salonen, R.O., 2008. Chemical compositions responsible for inflammation and tissue damage in the mouse lung by coarse and fine particulate samples from contrasting air pollution in Europe. Inhal. Toxicol. 20, 1215–1231. doi:10.1080/08958370802147282

Hartwig, A., 2002. Role of DNA repair in particle- and fiber-induced lung injury. Inhal. Toxicol. 14, 91–100. doi:10.1080/089583701753338659

Hartwig, A., Asmuss, M., Ehleben, I., Herzer, U., Kostelac, D., Pelzer, A., Schwerdtle, T., Bürkle, A., 2002. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ. Health Perspect. 110 Suppl 5, 797–799.

Heidrich, C., Feuerborn, H.J., Weir, A. 2013. Coal combustion products: a global perspective. World of Coal ash (WOCA). 22-25.

Herbst, R.S., Sandler, A.B., 2004. Overview of the current status of human epidermal growth factor receptor inhibitors in lung cancer. Clin. Lung Cancer 6 Suppl 1, S7–S19.

Hernández-Franco, P., Valverde, M., Rojas, E., 2009. Los Metales Como Inhibidores Del Sistema De Reparación Del Adn [WWW Document]. Tip Rev. Espec. En Cienc. Quím.-Biológicas. URL http://www.redalyc.org/articulo.oa?id=43215406003 (accessed 2.15.16).

Hicks, A., Goodnow, R., Cavallo, G., Tannu, S.A., Ventre, J.D., Lavelle, D., Lora, J.M., Satjawatcharaphong, J., Brovarney, M., Dabbagh, K., Tare, N.S., Oh, H., Lamb, M., Sidduri, A., Dominique, R., Qiao, Q., Lou, J.P., Gillespie, P., Fotouhi, N., Kowalczyk, A., Kurylko, G., Hamid, R., Wright, M.B., Pamidimukkala, A., Egan, T., Gubler, U., Hoffman, A.F., Wei, X., Li, Y.L., O'Neil, J., Marcano, R., Pozzani, K., Molinaro, T., Santiago, J., Singer, L., Hargaden, M., Moore, D., Catala, A.R., Chao, L.C.F., Benson, J., March, T., Venkat, R., Mancebo, H., Renzetti, L.M., 2010. Effects of LTB4 receptor antagonism on pulmonary inflammation in rodents and non-human primates. Prostaglandins Other Lipid Mediat. 92, 33–43. doi:10.1016/j.prostaglandins.2010.02.003

Hiraiwa, K., van Eeden, S.F., Hiraiwa, K., van Eeden, S.F., 2013. Contribution of Lung Macrophages to the Inflammatory Responses Induced by Exposure to Air Pollutants, Contribution of Lung Macrophages to the Inflammatory Responses Induced by Exposure to Air Pollutants. Mediat. Inflamm. Mediat. Inflamm. 2013, 2013, e619523. doi:10.1155/2013/619523, 10.1155/2013/619523

Hirota, K., Ter, H., 2012. Endocytosis of Particle Formulations by Macrophages and Its Application to Clinical Treatment, in: Ceresa, B. (Ed.), Molecular Regulation of Endocytosis. InTech.

Hodges, N.J., Chipman, J.K., 2002. Down-regulation of the DNA-repair endonuclease 8-oxo-guanine DNA glycosylase 1 (hOGG1) by sodium dichromate in cultured human A549 lung carcinoma cells. Carcinogenesis 23, 55–60.

Hsu, G.W., Huang, X., Luneva, N.P., Geacintov, N.E., Beese, L.S., 2005. Structure of a high fidelity DNA polymerase bound to a benzo[a]pyrene adduct that blocks replication. J. Biol. Chem. 280, 3764–3770. doi:10.1074/jbc.M411276200.

Hurlbut, Cornelius S.; Klein, Cornelis, 1985, Manual de Mineralogia, 20th ed., John Wiley and Sons, New York, p 285-286.

IARC monographs on the evaluation of carcinogenic risk to human. 100C. Lyon: International Agency for Research on Cancer; 2012.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Silica, some silicates, coal dust and para-aramid fibrils. 1997. Monogr. Eval. Carcinog. Risks Hum. http://monographs.iarc.fr/ENG/Monographs/vol68/mono68.pdf. 68:1– 475.

IARC, 2010. Some Non-Heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures. IARC Monographs On the Evaluation Of Carcinogenic Risks to Humans, vol. 92.IARC Press.

IARC, International Agency for Research on Cancer, Agents Classified by the IARC Monographs, volume 92. 2010. On line http://monographs.iarc.fr/ENG/Monographs/vol92/mono92.pdf

International Energy Agency (IEA). 2016. On line, http://www.iea.org/

Ito, M., Hanaoka, M., Droma, Y., Hatayama, O., Sato, E., Katsuyama, Y., Fujimoto, K., Ota, M., 2008. The association of transforming growth factor beta 1 gene polymorphisms with the emphysema phenotype of COPD in Japanese. Intern. Med. Tokyo Jpn. 47, 1387–1394.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscip. Toxicol. 7, 60–72. doi:10.2478/intox-2014-0009

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscip. Toxicol. 7, 60–72. doi:10.2478/intox-2014-0009

Jang, A.-S., Jun, Y.J., Park, M.K., 2016. Effects of air pollutants on upper airway disease. Curr. Opin. Allergy Clin. Immunol. 16, 13–17. doi:10.1097/ACI.0000000000000235

Jarvis, I.W.H., Dreij, K., Mattsson, Å., Jernström, B., Stenius, U., 2014. Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. Toxicology 321, 27–39. doi:10.1016/j.tox.2014.03.012

Jenkins, W.D., Christian, W.J., Mueller, G., Robbins, K.T., 2013. Population cancer risks associated with coal mining: a systematic review. PloS One 8, e71312. doi:10.1371/journal.pone.0071312

Jomova K, Valko M. 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283: 65-87. doi: 10.1016/j.tox.2011.03.001.

José Zocche, J., Dimer Leffa, D., Paganini Damiani, A., Carvalho, F., Avila Mendonça, R., Dos Santos, C.E.I., Appel Boufleur, L., Ferraz Dias, J., de Andrade, V.M., 2010. Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. Environ. Res. 110, 684–691. doi:10.1016/j.envres.2010.06.003

Joshi, G.N., Goetjen, A.M., Knecht, D.A., 2015. Silica particles cause NADPH oxidase-independent ROS generation and transient phagolysosomal leakage. Mol. Biol. Cell 26, 3150–3164. doi:10.1091/mbc.E15-03-0126

Jundi, K., Greene, C.M., 2015. Transcription of Interleukin-8: How Altered Regulation Can Affect Cystic Fibrosis Lung Disease. Biomolecules 5, 1386–1398. doi:10.3390/biom5031386.

Karami, S., Boffetta, P., Brennan, P., Stewart, P.A., Zaridze, D., Matveev, V., Janout, V., Kollarova, H., Bencko, V., Navratilova, M., Szeszenia-Dabrowska, N., Mates, D., Gromiec, J.P., Sobotka, R., Chow, W.-H., Rothman, N., Moore, L.E., 2011. Renal cancer risk and occupational exposure to polycyclic aromatic hydrocarbons and plastics. J. Occup. Environ. Med. Am. Coll. Occup. Environ. Med. 53, 218–223. doi:10.1097/JOM.0b013e31820a40a3

Kasperska-Zajac, A., Brzoza, Z., Rogala, B., 2008. Platelet-activating factor (PAF): a review of its role in asthma and clinical efficacy of PAF antagonists in the disease therapy. Recent Pat. Inflamm. Allergy Drug Discov. 2, 72–76.

Kendall, R.T., Feghali-Bostwick, C.A., 2014. Fibroblasts in fibrosis: novel roles and mediators. Front. Pharmacol. 5, 123. doi:10.3389/fphar.2014.00123

Khan, M.M., 2008. Role of Cytokines, in: Immunopharmacology. Springer US, pp. 33–59.

Kido, T., Sakakibara, H., Ohura, T., Guruge, K.S., Kojima, M., Hasegawa, J., Iwamura, T., Yamanaka, N., Masuda, S., Sakaguchi, M., Amagai, T., Shimoi, K., 2013. Evaluation of chlorinated benz[a]anthracene on hepatic toxicity in rats and mutagenic activity in Salmonella typhimurium. Environ. Toxicol. 28, 21–30. doi:10.1002/tox.20693

Kim, H.S., Kim, Y.J., Seo, Y.R., 2015. An Overview of Carcinogenic Heavy Metal: Molecular Toxicity Mechanism and Prevention. J. Cancer Prev. 20, 232–240. doi:10.15430/JCP.2015.20.4.232

Knaapen, A.M; Borm, P.J; Albrecht, C; Schins, R.P. 2004. Inhaled particles and lung cancer. Part A: Mechanisms. Int J Cancer. 109; 799-809.

Koedrith, P., Seo, Y.R., 2011. Advances in carcinogenic metal toxicity and potential molecular markers. Int. J. Mol. Sci. 12, 9576–9595. doi:10.3390/ijms12129576

Kreyling, W.G., Möller, W., Schmid, O., Semmler-Behnke, M., Oberdörster, G., 2011. Translocation of Inhaled Nanoparticles, in: Cassee, F.R., Mills, N.L., Newby, D. (Eds.), Cardiovascular Effects of Inhaled Ultrafine and Nanosized Particles. John Wiley & Sons, Inc., pp. 125–143.

Kuhn, D.C., Stauffer, J.L., Gaydos, L.J., Demers, L.M., 1995. Inflammatory and fibrotic mediator release by alveolar macrophages from coal miners. J. Toxicol. Environ. Health 46, 9–21. doi:10.1080/15287399509532014

Lai, H.Y., Rogers, D.F., 2010. Mucus hypersecretion in asthma: intracellular signalling pathways as targets for pharmacotherapy. Curr. Opin. Allergy Clin. Immunol. 10, 67–76. doi:10.1097/ACI.0b013e328334643a

Laskin, D.L., Sunil, V.R., Fakhrzadeh, L., Groves, A., Gow, A.J., Laskin, J.D., 2010. Macrophages, reactive nitrogen species, and lung injury. Ann. N. Y. Acad. Sci. 1203, 60–65. doi:10.1111/j.1749-6632.2010.05607.x

Laumann, S., Micić, V., Kruge, M.A., Achten, C., Sachsenhofer, R.F., Schwarzbauer, J., Hofmann, T., 2011. Variations in concentrations and compositions of polycyclic aromatic hydrocarbons (PAHs) in coals related to the coal rank and origin. Environ. Pollut. Barking Essex 1987 159, 2690–2697. doi:10.1016/j.envpol.2011.05.032

Lee, J.S., Shin, J.H., Lee, J.O., Lee, K.M., Kim, J.H., Choi, B.-S., 2010. Serum Levels of Interleukin-8 and Tumor Necrosis Factor-alpha in Coal Workers' Pneumoconiosis: One-year Follow-up Study. Saf. Health Work 1, 69–79. doi:10.5491/SHAW.2010.1.1.69

Lee, J.S., Shin, J.H., Lee, K.M., Hwang, J.-H., Baek, J.E., Kim, J.H., Choi, B.-S., 2014. Serum levels of TGF-β1 and MCP-1 as biomarkers for progressive coal workers' pneumoconiosis in retired coal workers: a three-year follow-up study. Ind. Health 52, 129–136.

Leffa, D. D. , Andrade, V. M. 2009.Potencial genotóxico de metais em áreas mineradas de carvão. Universidade do Extremo Sul Catarinense (Unesc).

León, G., Pérez, L.E., Linares, J.C., Hartmann, A., Quintana, M., 2007. Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area. Mutat. Res. 630, 42–49. doi:10.1016/j.mrgentox.2007.02.007

León-Mejía, G., Espitia-Pérez, L., Hoyos-Giraldo, L.S., Da Silva, J., Hartmann, A., Henriques, J.A.P., Quintana, M., 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ. 409, 686–691. doi:10.1016/j.scitotenv.2010.10.049

León-Mejía, G., Quintana, M., Debastiani, R., Dias, J., Espitia-Pérez, L., Hartmann, A., Henriques, J.A.P., Da Silva, J., 2014. Genetic damage in coal miners evaluated by buccal micronucleus cytome assay. Ecotoxicol. Environ. Saf. 107, 133–139. doi:10.1016/j.ecoenv.2014.05.023

Li, J., Kartha, S., Iasvovskaia, S., Tan, A., Bhat, R.K., Manaligod, J.M., Page, K., Brasier, A.R., Hershenson, M.B., 2002. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L690–699. doi:10.1152/ajplung.00060.2002.

Li, R., Kou, X., Xie, L., Cheng, F., Geng, H., 2015. Effects of ambient PM2.5 on pathological injury, inflammation, oxidative stress, metabolic enzyme activity, and expression of c-fos and c-jun in lungs of rats. Environ. Sci. Pollut. Res. Int. 22, 20167–20176. doi:10.1007/s11356-015-5222-z

Linak, W.P., Yoo, J.-I., Wasson, S.J., Zhu, W., Wendt, J.O.L., Huggins, F.E., Chen, Y., Shah, N., Huffman, G.P., Gilmour, M.I., 2007. Ultrafine ash aerosols from coal combustion: Characterization and health effects. Proc. Combust. Inst. 31, 1929–1937. doi:10.1016/j.proci.2006.08.086.

Liu, L., Urch, B., Poon, R., Szyszkowicz, M., Speck, M., Gold, D.R., Wheeler, A.J., Scott, J.A., Brook, J.R., Thorne, P.S., Silverman, F.S., 2015. Effects of Ambient Coarse, Fine, and Ultrafine Particles and Their Biological Constituents on Systemic Biomarkers: A Controlled Human Exposure Study. Environ. Health Perspect. doi:10.1289/ehp.1408387

Lodovici, M., Bigagli, E., Lodovici, M., Bigagli, E., 2011. Oxidative Stress and Air Pollution Exposure, Oxidative Stress and Air Pollution Exposure. J. Toxicol. J. Toxicol. 2011, 2011, e487074. doi:10.1155/2011/487074, 10.1155/2011/487074

Magnani, N.D., Marchini, T., Vanasco, V., Tasat, D.R., Alvarez, S., Evelson, P., 2013. Reactive oxygen species produced by NADPH oxidase and mitochondrial dysfunction in lung after an acute exposure to residual oil fly ashes. Toxicol. Appl. Pharmacol. 270, 31–38. doi:10.1016/j.taap.2013.04.002

Mani, U., Prasad, A.K., Suresh Kumar, V., Lal, K., Kanojia, R.K., Chaudhari, B.P., Murthy, R.C., 2007. Effect of fly ash inhalation on biochemical and histomorphological changes in rat liver. Ecotoxicol. Environ. Saf. 68, 126–133. doi:10.1016/j.ecoenv.2006.10.013

Martinez, J. 2012.Cenizas volantes de carbón y cenizas de hogar o escoria. Ficha técnica, España.

Mason, E., Thompson, S.K., 2010. A brief overview of crystalline silica. J. Chem. Health Saf. 17, 6–8. doi:10.1016/j.jchas.2009.09.001

Mazzoli-Rocha, F., Fernandes, S., Einicker-Lamas, M., Zin, W.A., 2010. Roles of oxidative stress in signaling and inflammation induced by particulate matter. Cell Biol. Toxicol. 26, 481–498. doi:10.1007/s10565-010-9158-2.

Mclnnes IB. Cytokines. 2008. Effector mechanisms in autoimmunity and inflammation. Elsevier, 367-377.

Micic, V., Straub, K., Blum, P., Kappler, A., 2007. Natural attenuation of naphthalene and benzene at a former gasworks site. Water Sci. Technol. Water Supply 7, 145–153. doi:10.2166/ws.2007.077

Minina, V.I., Kulemin, I.E., Tolotchko, T.A., Meĭer, A.V., Savtchenko, I.A., Volobaev, V.P., Gafarov, N.I., Semenikhina, M.V., 2015. [Genotoxic effects of occupational environment in Kuzbass miners]. Med. Tr. Prom. Ekol. 4–8.

Mo, Y., Wan, R., Chien, S., Tollerud, D.J., Zhang, Q., 2009. Activation of endothelial cells after exposure to ambient ultrafine particles: the role of NADPH oxidase. Toxicol. Appl. Pharmacol. 236, 183–193. doi:10.1016/j.taap.2009.01.017

Møller, P., Danielsen, P.H., Karottki, D.G., Jantzen, K., Roursgaard, M., Klingberg, H., Jensen, D.M., Christophersen, D.V., Hemmingsen, J.G., Cao, Y., Loft, S., 2014. Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. Mutat. Res. Rev. Mutat. Res. 762, 133–166. doi:10.1016/j.mrrev.2014.09.001

Mordukhovich, I., Rossner, P., Terry, M.B., Santella, R., Zhang, Y.-J., Hibshoosh, H., Memeo, L., Mansukhani, M., Long, C.-M., Garbowski, G., Agrawal, M., Gaudet, M.M., Steck, S.E., Sagiv, S.K., Eng, S.M., Teitelbaum, S.L., Neugut, A.I., Conway-Dorsey, K., Gammon, M.D., 2010. Associations between polycyclic aromatic hydrocarbon-related exposures and p53 mutations in breast tumors. Environ. Health Perspect. 118, 511–518. doi:10.1289/ehp.0901233

Moustakas, A., Heldin, C.-H., 2009. The regulation of TGFbeta signal transduction. Dev. Camb. Engl. 136, 3699–3714. doi:10.1242/dev.030338.

Muñoz B., Albores A. 2011. DNA Damage Caused by Polycyclic Aromatic Hydrocarbons: Mechanisms and Markers. Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del I.P.N.

Naidoo, G., Chirkoot, D., 2004. The effects of coal dust on photosynthetic performance of the mangrove, Avicennia marina in Richards Bay, South Africa. Environ. Pollut. Barking Essex 1987 127, 359–366.

Noskovičová, N., Petřek, M., Eickelberg, O., Heinzelmann, K., 2015. Platelet-derived growth factor signaling in the lung. From lung development and disease to clinical studies. Am. J. Respir. Cell Mol. Biol. 52, 263–284. doi:10.1165/rcmb.2014-0294TR

Oberdörster, G., 1995. Lung particle overload: implications for occupational exposures to particles. Regul. Toxicol. Pharmacol. RTP 21, 123–135. doi:10.1006/rtph.1995.1017.

Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., Cox, C., 2004. Translocation of inhaled ultrafine particles to the brain. Inhal. Toxicol. 16, 437–445. doi:10.1080/08958370490439597

Oliva M.R, Muñiz P, Valls V, Iradi A, Catala M.D, Cañete C, Drehmer E, Saez G. 2009. Radicales libres y modificación oxidativa del DNA. Implicaciones en la carcinogeneses experimental y humana.

Pantelidis, P., Fanning, G.C., Wells, A.U., Welsh, K.I., Du Bois, R.M., 2001. Analysis of tumor necrosis factor-alpha, lymphotoxin-alpha, tumor necrosis factor receptor II, and interleukin-6 polymorphisms in patients with idiopathic pulmonary fibrosis. Am. J. Respir. Crit. Care Med. 163, 1432–1436. doi:10.1164/ajrccm.163.6.2006064

Park, H.S., Kim, S.R., Lee, Y.C., 2009. Impact of oxidative stress on lung diseases. Respirol. Carlton Vic 14, 27–38. doi:10.1111/j.1440-1843.2008.01447.x

Pastor, S., Lucero, L., Gutiérrez, S., Durbán, R., Gómez, C., Parrón, T., Creus, A., Marcos, R., 2002. A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. Mutagenesis 17, 79–82.

Pease, J.E., Sabroe, I., 2002. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. Am. J. Respir. Med. Drugs Devices Interv. 1, 19–25.

Pelosi, P., Rocco, P.R.M., Negrini, D., Passi, A., 2007. The extracellular matrix of the lung and its role in edema formation. An. Acad. Bras. Ciênc. 79, 285–297.

Pelucchi, C., Pira, E., Piolatto, G., Coggiola, M., Carta, P., La Vecchia, C., 2006. Occupational silica exposure and lung cancer risk: a review of epidemiological studies 1996-2005. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO 17, 1039–1050. doi:10.1093/annonc/mdj125

Pelucchi, C., Pira, E., Piolatto, G., Coggiola, M., Carta, P., La Vecchia, C., 2006. Occupational silica exposure and lung cancer risk: a review of epidemiological studies 1996-2005. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO 17, 1039–1050. doi:10.1093/annonc/mdj125

Perera, F., Tang, D., Whyatt, R., Lederman, S.A., Jedrychowski, W., 2005. DNA damage from polycyclic aromatic hydrocarbons measured by benzo[a]pyrene-DNA adducts in mothers and newborns from Northern Manhattan, the World Trade Center Area, Poland, and China. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 14, 709–714. doi:10.1158/1055-9965.EPI-04-0457

Peters-Golden, M., 2008. Expanding roles for leukotrienes in airway inflammation. Curr. Allergy Asthma Rep. 8, 367–373.

Peterson, M. 2008. Produção de sulfato ferroso a partir da pirita: desenvolvimento sustentável, (Tese de doutorado). Florianópolis, Disponível em: < http://www2.enq.ufsc.br/teses/d059.pdf>

Petsonk, E.L., Rose, C., Cohen, R., 2013. Coal mine dust lung disease. New lessons from old exposure. Am. J. Respir. Crit. Care Med. 187, 1178–1185. doi:10.1164/rccm.201301-0042CI.

Piljac Zegarac J. 2015. Oxidative Stress: Effects on Lipids, Proteins, and DNA. http://www.brunswicklabs.com/blog/default-blog/oxidative-stress-effects-on-lipids-proteins-and-dna

Planete-Energies, 2015, on line http://www.planete-energies.com/en/medias/close/coal-used-power-generation-iron-and-steel-and-petrochemical-industries

Porter, D.W., Millecchia, L., Robinson, V.A., Hubbs, A., Willard, P., Pack, D., Ramsey, D., McLaurin, J., Khan, A., Landsittel, D., Teass, A., Castranova, V., 2002. Enhanced nitric oxide and reactive

oxygen species production and damage after inhalation of silica. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L485–493. doi:10.1152/ajplung.00427.2001

Qiu, Z., Fujimura, M., Kurashima, K., Nakao, S., Mukaida, N., 2004. Enhanced airway inflammation and decreased subepithelial fibrosis in interleukin 6-deficient mice following chronic exposure to aerosolized antigen. Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol. 34, 1321–1328. doi:10.1111/j.1365-2222.2004.02013.x

Quispe, D., Pérez-López, R., Silva, L.F.O., Nieto, J.M., 2012. Changes in mobility of hazardous elements during coal combustion in Santa Catarina power plant (Brazil). Fuel 94, 495–503. doi:10.1016/j.fuel.2011.09.034

Rajiv, S., Jerobin, J., Saranya, V., Nainawat, M., Sharma, A., Makwana, P., Gayathri, C., Bharath, L., Singh, M., Kumar, M., Mukherjee, A., Chandrasekaran, N., 2016. Comparative cytotoxicity and genotoxicity of cobalt (II, III) oxide, iron (III) oxide, silicon dioxide, and aluminum oxide nanoparticles on human lymphocytes in vitro. Hum. Exp. Toxicol. 35, 170–183. doi:10.1177/0960327115579208.

Raut, A.M., An, S., D.Mhaisekar, S., Mhaisekar, D., 2013. Role of α-1-antitrypsin and oxidative stress in emphysema. Int. J. Med. Res. Health Sci. 2, 206–211.

Refsnes, M., Hetland, R.B., Øvrevik, J., Sundfør, I., Schwarze, P.E., Låg, M., 2006. Different particle determinants induce apoptosis and cytokine release in primary alveolar macrophage cultures. Part. Fibre Toxicol. 3, 10. doi:10.1186/1743-8977-3-10

Rimal, B., Greenberg, A.K., Rom, W.N., 2005. Basic pathogenetic mechanisms in silicosis: current understanding. Curr. Opin. Pulm. Med. 11, 169–173.

Rohr, P., Kvitko, K., da Silva, F.R., Menezes, A.P.S., Porto, C., Sarmento, M., Decker, N., Reyes, J.M., Allgayer, M. da C., Furtado, T.C., Salvador, M., Branco, C., da Silva, J., 2013. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat. Res. 758, 23–28. doi:10.1016/j.mrgentox.2013.08.006

Rohr, P; Kvitko, K; da Silva, F.R; Menezes, A.P; Porto, C; Sarmento, M; Decker, N; Reyes, J.M; Allgayer, Mda C; Furtado, T.C; Salvador, M; Branco, C; da Silva, J. 2013. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat Res. Dec 12;758(1-2); 23-8.

Sager, T.M., Kommineni, C., Castranova, V., 2008. Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area. Part. Fibre Toxicol. 5, 17. doi:10.1186/1743-8977-5-17

Sambandam, B., Devasena, T., Islam, V.I.H., Prakhya, B.M., 2015. Characterization of coal fly ash nanoparticles and their induced in vitro cellular toxicity and oxidative DNA damage in different cell lines. Indian J. Exp. Biol. 53, 585–593.

Sandberg, W.J., Låg, M., Holme, J.A., Friede, B., Gualtieri, M., Kruszewski, M., Schwarze, P.E., Skuland, T., Refsnes, M., 2012. Comparison of non-crystalline silica nanoparticles in IL-1β release from macrophages. Part. Fibre Toxicol. 9, 32. doi:10.1186/1743-8977-9-32

Santa Maria, S.R; Arana, M; Ramirez, O. 2007. Chromosomal aberrations in peripheral lymphocytes from male native miners working in the Peruvian Andes. Genetics and Molecular Biology. 30; 1135-1138.

Santo Tomas, L.H., 2011. Emphysema and chronic obstructive pulmonary disease in coal miners. Curr. Opin. Pulm. Med. 17, 123–125. doi:10.1097/MCP.0b013e3283431674

Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim. Biophys. Acta 1813, 878–888. doi:10.1016/j.bbamcr.2011.01.034

Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. Curr. Biol. CB 24, R453–462. doi:10.1016/j.cub.2014.03.034

Schins, R.P., Borm, P.J., 1999. Mechanisms and mediators in coal dust induced toxicity: a review. Ann. Occup. Hyg. 43, 7–33.

Schinwald, A., Donaldson, K., 2012. Use of back-scatter electron signals to visualise cell/nanowires interactions in vitro and in vivo; frustrated phagocytosis of long fibres in macrophages and compartmentalisation in mesothelial cells in vivo. Part. Fibre Toxicol. 9, 34. doi:10.1186/1743-8977-9-34

Schmid, O., Möller, W., Semmler-Behnke, M., Ferron, G.A., Karg, E., Lipka, J., Schulz, H., Kreyling, W.G., Stoeger, T., 2009. Dosimetry and toxicology of inhaled ultrafine particles. Biomark. Biochem. Indic. Expo. Response Susceptibility Chem. 14 Suppl 1, 67–73. doi:10.1080/13547500902965617

Seong Lee, Shin JH, and Choi BS. 2015. Serum Levels of IL-8 and ICAM-1 as Biomarkers for Progressive Massive Fibrosis in Coal Workers' Pneumoconiosis. J Korean Med Sci. Feb; 30(2): 140–144.

Serhan, C.N., Chiang, N., Dalli, J., Levy, B.D., 2015. Lipid mediators in the resolution of inflammation. Cold Spring Harb. Perspect. Biol. 7, a016311. doi:10.1101/cshperspect.a016311

Serrano-Mollar, A., 2012. La célula epitelial como factor etiopatogénico de la fibrosis pulmonar. Arch. Bronconeumol. 48, 2–6. doi:10.1016/S0300-2896(12)70044-3

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. J. Bot. 2012, 1–26. doi:10.1155/2012/217037

Sharma, S.V., Bell, D.W., Settleman, J., Haber, D.A., 2007. Epidermal growth factor receptor mutations in lung cancer. Nat. Rev. Cancer 7, 169–181. doi:10.1038/nrc2088

Shen, J., Desai, M., Agrawal, M., Kennedy, D.O., Senie, R.T., Santella, R.M., Terry, M.B., 2006. Polymorphisms in nucleotide excision repair genes and DNA repair capacity phenotype in sisters discordant for breast cancer. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 15, 1614–1619. doi:10.1158/1055-9965.EPI-06-0218.

Shrivastava, R., Raza, S., Yadav, A., Kushwaha, P., Flora, S.J.S., 2014. Effects of sub-acute exposure to TiO2, ZnO and Al2O3 nanoparticles on oxidative stress and histological changes in mouse liver and brain. Drug Chem. Toxicol. 37, 336–347. doi:10.3109/01480545.2013.866134

Silva, L., Ward, C.R., Hower, J., Izquierdo, M., Waanders, F., Oliveira, M., Li, Z., Hatch, R., Querol, X., 2010. Mineralogy and leaching characteristics of coal ash from a major Brazilian power plant. Coal Combustion and Gasification Products. 2, 51–65.

Silva, L.F.O., da Boit, K.M., 2011. Nanominerals and nanoparticles in feed coal and bottom ash: implications for human health effects. Environ. Monit. Assess. 174, 187–197. doi:10.1007/s10661-010-1449-9

Silva, L.F.O., Oliveira, M.L.S., da Boit, K.M., Finkelman, R.B., 2009. Characterization of Santa Catarina (Brazil) coal with respect to human health and environmental concerns. Environ. Geochem. Health 31, 475–485. doi:10.1007/s10653-008-9200-y

Silverman, E.S., Palmer, L.J., Subramaniam, V., Hallock, A., Mathew, S., Vallone, J., Faffe, D.S., Shikanai, T., Raby, B.A., Weiss, S.T., Shore, S.A., 2004. Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am. J. Respir. Crit. Care Med. 169, 214–219. doi:10.1164/rccm.200307-973OC

Singh, D., Cho, W.C., Upadhyay, G., 2015. Drug-Induced Liver Toxicity and Prevention by Herbal Antioxidants: An Overview. Front. Physiol. 6, 363. doi:10.3389/fphys.2015.00363

Singh, R., Sram, R.J., Binkova, B., Kalina, I., Popov, T.A., Georgieva, T., Garte, S., Taioli, E., Farmer, P.B., 2007. The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat. Res. 620, 83–92. doi:10.1016/j.mrfmmm.2007.02.025

Spinelli, J.J., Demers, P.A., Le, N.D., Friesen, M.D., Lorenzi, M.F., Fang, R., Gallagher, R.P., 2006. Cancer risk in aluminum reduction plant workers (Canada). Cancer Causes Control CCC 17, 939–948. doi:10.1007/s10552-006-0031-9

Sugiura, H., Ichinose, M., 2011. Nitrative stress in inflammatory lung diseases. Nitric Oxide Biol. Chem. Off. J. Nitric Oxide Soc. 25, 138–144. doi:10.1016/j.niox.2011.03.079

Suttie, A.W., 2006. Histopathology of the Spleen. Toxicol. Pathol. 34, 466–503. doi:10.1080/01926230600867750

Sziksz E, Pap D, Lippai R, Béres NJ, Fekete A, Szabó AJ, and Vannay A. 2015. Fibrosis Related Inflammatory Mediators: Role of the IL-10 Cytokine Family, Fibrosis Related Inflammatory Mediators: Role of the IL-10 Cytokine Family. Mediat. Inflamm. Mediat. Inflamm.

Teixeira, E.C., Meira, L., Santana, E., Ramos, R., Wiegand, F., 2009. Chemical composition of PM10 and PM2.5 and seasonal variation in south Brazil.

Terzano, C., Di Stefano, F., Conti, V., Graziani, E., Petroianni, A., 2010. Air pollution ultrafine particles: toxicity beyond the lung. Eur. Rev. Med. Pharmacol. Sci. 14, 809–821.

Theocharis, A.D., Skandalis, S.S., Gialeli, C., Karamanos, N.K., 2016. Extracellular matrix structure. Adv. Drug Deliv. Rev. 97, 4–27. doi:10.1016/j.addr.2015.11.001

Toppin Ording E. 2009. Heavy Metals and Coal: Carbon Footprint Aside, Coal is not Environmentally Friendly. 101.

Trujillo, G., Habiel, D.M., Ge, L., Ramadass, M., Cooke, N.E., Kew, R.R., 2013. Neutrophil recruitment to the lung in both C5a- and CXCL1-induced alveolitis is impaired in vitamin D-binding protein-deficient mice. J. Immunol. Baltim. Md 1950 191, 848–856. doi:10.4049/jimmunol.1202941

Tzouvelekis, A., Ntolios, P., Karameris, A., Vilaras, G., Boglou, P., Koulelidis, A., Archontogeorgis, K., Kaltsas, K., Zacharis, G., Sarikloglou, E., Steiropoulos, P., Mikroulis, D., Koutsopoulos, A., Froudarakis, M., Bouros, D., 2013. Increased expression of epidermal growth factor receptor (EGF-R) in patients with different forms of lung fibrosis. BioMed Res. Int. 2013, 654354. doi:10.1155/2013/654354

U.S. Environmental Protection Agency. 1999. Compendium Method TO-13A, determination of polycyclic aromatic hydrocarbons (PAHs) in ambient air using gas chromatography/mass spectrometry (GC/MS), URL: http://www.epa.gov/ttn/amtic/ files/ambient/airtox/to-13arr.pdf.

Ulker, O., Yucesoy, B., Demir, O., Tekin, I., Karakaya, A., 2008. Serum and BAL cytokine and antioxidant enzyme levels at different stages of pneumoconiosis in coal workers. Hum. Exp. Toxicol. 27, 871–877. doi:10.1177/0960327108098332

Valavanidis, A., Vlachogianni, T., Fiotakis, K., Loridas, S., 2013. Pulmonary Oxidative Stress, Inflammation and Cancer: Respirable Particulate Matter, Fibrous Dusts and Ozone as Major Causes of Lung Carcinogenesis through Reactive Oxygen Species Mechanisms. Int. J. Environ. Res. Public. Health 10, 3886–3907. doi:10.3390/ijerph10093886

Vallyathan, V., Goins, M., Lapp, L.N., Pack, D., Leonard, S., Shi, X., Castranova, V., 2000. Changes in bronchoalveolar lavage indices associated with radiographic classification in coal miners. Am. J. Respir. Crit. Care Med. 162, 958–965. doi:10.1164/ajrccm.162.3.9909074

Vallyathan, V., Shi, X., Castranova, V., 1998. Reactive oxygen species: their relation to pneumoconiosis and carcinogenesis. Environ. Health Perspect. 106 Suppl 5, 1151–1155.

van der Vliet, A., 2008. NADPH oxidases in lung biology and pathology. Host defense enzymes, and more. Free Radic. Biol. Med. 44, 938–955. doi:10.1016/j.freeradbiomed.11.016.

Wagner, A.J., Bleckmann, C.A., Murdock, R.C., Schrand, A.M., Schlager, J.J., Hussain, S.M., 2007. Cellular interaction of different forms of aluminum nanoparticles in rat alveolar macrophages. J. Phys. Chem. B 111, 7353–7359. doi:10.1021/jp068938n

Wang, R., Liu, G., Zhang, J., 2015. Variations of emission characterization of PAHs emitted from different utility boilers of coal-fired power plants and risk assessment related to atmospheric PAHs. Sci. Total Environ. 538, 180–190. doi:10.1016/j.scitotenv.2015.08.043

Water, Air & Soil Pollution 199, 261–275. CDC-The National Institute for Occupational Safety and Health (NIOSH). 2012. http://www.cdc.gov/niosh/topics/surveillance/ords/coalminingrelatedrespiratorydiseases.html

Whittaker, P., Hines, F.A., Robl, M.G., Dunkel, V.C., 1996. Histopathological evaluation of liver, pancreas, spleen, and heart from iron-overloaded Sprague-Dawley rats. Toxicol. Pathol. 24, 558–563.

Wilson, M.S., Wynn, T.A., 2009. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol. 2, 103–121. doi:10.1038/mi.2008.85

World Coal Association, Disponível on line. https://www.worldcoal.org/

Xaubet, A., Marin-Arguedas, A., Lario, S., Ancochea, J., Morell, F., Ruiz-Manzano, J., Rodriguez-Becerra, E., Rodriguez-Arias, J.M., Inigo, P., Sanz, S., Campistol, J.M., Mullol, J., Picado, C., 2003. Transforming growth factor-beta1 gene polymorphisms are associated with disease progression

in idiopathic pulmonary fibrosis. Am. J. Respir. Crit. Care Med. 168, 431–435. doi:10.1164/rccm.200210-1165OC

Yamashita, C.M., Radisky, D.C., Aschner, Y., Downey, G.P., 2014. The importance of matrix metalloproteinase-3 in respiratory disorders. Expert Rev. Respir. Med. 8, 411–421. doi:10.1586/17476348.2014.909288

Yu, M.-H., Tsunoda, H., Tsunoda, M., 2011. Environmental Toxicology: Biological and Health Effects of Pollutants, Third Edition. CRC Press.

Zeng, X., Xu, X., Boezen, H.M., Huo, X., 2016. Children with health impairments by heavy metals in an e-waste recycling area. Chemosphere 148, 408–415. doi:10.1016/j.chemosphere.2015.10.078

Zhang, J.-M., An, J., 2007. Cytokines, Inflammation and Pain. Int. Anesthesiol. Clin. 45, 27–37. doi:10.1097/AIA.0b013e318034194e

Zhang, Q., Dai, J., Ali, A., Chen, L., Huang, X., 2002. Roles of bioavailable iron and calcium in coal dust-induced oxidative stress: possible implications in coal workers' lung disease. Free Radic. Res. 36, 285–294.

Zhang, Z., Rong, Y., Cui, X., Shen, Y., Zhou, Y., Xiao, L., Chen, W., 2015. [Oxidative stress and mitochondrion-related cell apoptosis in human bronchial epithelial 16HBE cells induced by silica dust]. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi Zhonghua Laodong Weisheng Zhiyebing Zazhi Chin. J. Ind. Hyg. Occup. Dis. 33, 801–805.

ANEXO I

"Genetic damage in coal miners evaluated by buccal micronucleus cytome assay"

Ecotoxicol Environ Saf. 2014 Sep;107:133-9. doi: 10.1016/j.ecoenv.2014.05.023

Ecotoxicology and Environmental Safety 107 (2014) 133–139

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Genetic damage in coal miners evaluated by buccal micronucleus cytome assay

Grethel León-Mejía [a,*], Milton Quintana [b], Rafaela Debastiani [c], Johnny Dias [c], Lyda Espitia-Pérez [d], Andreas Hartmann [e], João Antônio Pêgas Henriques [a,f,*], Juliana Da Silva [g]

[a] Departamento de Biofísica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil
[b] Unidad de Investigación, Desarrollo e Innovación en Genética y Biología Molecular, Universidad Simón Bolívar, Barranquilla, Colombia
[c] Laboratório de Implantação Iônica, Instituto de Física, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil
[d] Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Colombia
[e] Novartis Pharma AG, Basel, Switzerland
[f] Instituto de Biotecnologia, Universidade de Caxias do Sul (UCS), Caxias do Sul, RS, Brasil
[g] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil (ULBRA), Canoas-RS, Brasil

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form
30 April 2014
Accepted 19 May 2014

Keywords:
Coal
Coal miners
Buccal micronucleus cytome assay
Inorganic elements
Occupational exposure

ABSTRACT

During coal mining activities, large quantities of coal dust, ashes, polycyclic aromatic hydrocarbons and metals are released into the environment. This complex mixture presents one of the most important occupational hazards for health of workers. The aim of the present study was to evaluate the genetic damage together with the presence of inorganic elements, in an exposed workers population to coal mining residues of Guajira-Colombia. Thus, 100 exposed workers and 100 non-exposed control individuals were included in this study. To determine genetic damage we assessed the micronucleus (MN) frequencies and nuclear buds in buccal mucosa samples (BMCyt) assay, which were significantly higher in the exposed group than non-exposed control group. In addition, karyorrhectic and karyolytic cells were also significantly higher in the exposed group (cell death). No significant difference was observed between the exposed groups engaged in different mining activities. No correlation between age, alcohol consumption, time of service and MN assay data were found in this study. However, the content of inorganic elements in blood samples analyzed by a Particle-induced X-ray emission technique (PIXE) showed higher values of silicon (Si) and aluminum (Al) in the exposed group. In this study we discuss the possibility of DNA damage observed in the mine workers cells be a consequence of oxidative damage.

1. Introduction

It is known that coal mining activities are a major source of environmental contamination. Mining activities release large amounts of substances that can form complex mixtures containing CO_x, NO_x, SO_x, aluminum silicon crystals, quartz, metals (arsenic, boron, cadmium, chromium, lead, copper, selenium, iron, and zinc), and polycyclic aromatic hydrocarbons (PAH) into the environment (Zhou et al., 2005).

The main route of coal mining exposure to these potentially hazardous residues is by inhalation of coal dust particles from the extraction and manipulation activities. Currently, it is known that chronic inhalation of coal dust particles can result in lung disorders including simple pneumoconiosis, progressive massive fibrosis, bronchitis, lung function loss, emphysema and cancer. Studies were able to establish that some of these disorders could have their origin in genetic damage generated by the inhalation of mineral particles. In particular interaction of particles with macrophages, epithelial cells and other cells could lead to generation of reactive oxygen species (ROS) (Schins and Borm, 1999; Cooke et al., 2003).

The effects of coal exposure have been studied using bacteria (Nakajima et al., 2008), bats (Zocche et al., 2010), rodents (Da Silva et al., 2000, León et al., 2007) and human cells (Celik et al., 2007; Rohr et al., 2013a, 2013b). Some studies in workers exposed to coal mining residues assessed by chromosomal aberrations (Santa Maria et al., 2007), sister chromatid exchange, and micronuclei

* Corresponding authors at: Departamento de Biofísica, Prédio 43422, Laboratório 210, Campus do Vale, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Bairro Agronomia, CEP 91501-970, Porto Alegre, RS, Brasil. Fax: +55 5133167003.
E-mail addresses: grethelleon@gmail.com, grethy40@hotmail.com (G. León-Mejía), pegas@cbiot.ufrgs.br (J.A.P. Henriques).

http://dx.doi.org/10.1016/j.ecoenv.2014.05.023
0147-6513/

(MN) in peripheral blood lymphocytes (Donbak et al., 2005; León-Mejía et al., 2011) demonstrated that occupational exposure to coal dust can lead to a significant induction of cytogenetic damage. In a previous study, we found elevated DNA damage in coal mining workers from Guajira-Colombia, assessed by the Comet assay and MN test in lymphocytes (León-Mejía et al., 2011). Despite these findings, coal dust remains classified as "not classifiable as to its carcinogenicity to humans" (Group 3) by the International Agency for Research on Cancer (IARC, 1997).

The fact that a very high percentage of cancers have an epithelial origin suggests that micronuclei in epithelial cells are an important biomarker that can be used for epidemiological studies. Micronuclei that are detected in exfoliated buccal cells reflect genotoxic events that occurred in basal cells, and these events can be observed in exfoliated cells over an approximately three week period (Holland et al., 2008). The buccal micronucleus cytome assay (BMCyt assay) is considered a fast and simple method for *in situ* biomonitoring of human populations exposed to environmental genotoxicans (Majer et al., 2001; Bonassi et al., 2011).

The aim of the present study was to evaluate the genotoxic effects in exfoliated buccal cells and concentrations of inorganic elements in a population exposed to coal residues in the open-cast mine "El Cerrejón" in Guajira-Colombia using the buccal micronucleus cytome assay (exfoliated buccal cells; BMCyt assay) and the particle-induced X-ray emission (PIXE) in blood samples. The MN data in buccal were compared to MN data in lymphocytes from our previous study (León-Mejía et al., 2011) to assess whether buccal cells can be used as a non-invasive source to investigate biomarkers of genetic damage in exposed individuals.

2. Materials and methods

2.1. Individuals and sampling

This study was approved by the Committee on Research Ethics at University of Sinú Ethic and details of the study through the informed consent were obtained from each individual before the research began.

This study involved a total of 200 individuals, who live in the same region in order to ensure a comparable genetic background and life habits. The exposed group were 100 workers occupationally exposed to coal with a minimum time of service of 5 years in "El Cerrejón" open-cast coal mine, in the Guajira Department in the north coast of Colombia, South America. The non-exposed control group consisted of 100 individuals with no known exposure to genotoxic agents including coal, radiation, chemicals or cigarettes. Both study populations (exposed and non-exposed groups) lived in the same region; it was considered that the two populations should have presented the same genetic background and the same life habits.

The workers were involved in different activities in the mine: (i) *transport* of *extracted coal* ($n=50$), in which the workers are involved in coal transport up to arrival in the storing centers; (ii) *equipment field maintenance* ($n=18$), these workers drive trucks to spread water onto the roads where large quantities of coal dust are generated, and also maintain the coal extraction equipment; (iii) *coal stripping* ($n=17$), these workers are engaged in coal stripping activities and the accumulation of the material for the transport in trucks, they also extinguish fires generated by spontaneous combustion of coal; (iv) *coal embarking* ($n=15$), these workers are involved in shipping of coal in containers to be exported to other countries. All workers were exposed to large quantity of coal dust, but was perceived that the coal stripping group was the most exposed to coal mining residues.

All individuals in the study were required to answer a questionnaire and participate in a face-to-face interview, which included determination of standard demographic data and questions concerning medical issues (exposure to X-rays, vaccinations, medication, *etc.*), life style (smoking, alcohol consumption, diet, *etc.*), cancer history, other chronic diseases and occupation (number of working hours per day, protective measures adopted). All individuals included in the study were non-smokers and have time of service ≥ 5 years. Buccal cell and blood samples were obtained from all individuals.

2.2. Buccal micronucleus cytome assay (BMCyt assay)

After informed consent was obtained from each individual, buccal mucosa samples from all 200 individuals were collected. The subjects were asked to rinse their mouth with water before sampling. The exfoliated buccal mucosa cells were collected using a cytobrush to gently scrape the mucosa of the inner lining of both cheeks. All buccal sample tubes were coded and kept in upright position at room temperature.

The cells were washed three times in 0.9 percent phosphate saline buffer, the smears were made from the pellet and fixed in methanol:acetic acid (3:1). For microscopic analysis, the slides were incubated at 37 °C overnight and then stained with Giemsa (Stich and Rosin, 1984; Acar et al., 2001). The frequency of MN was determined in 2000 cells for each person following recommendations of Thomas et al. (2009). All slides were scored by one reader blinded to the exposure status of the individuals.

MN and other nuclear abnormalities were classified according to Tolbert et al. (1992) and Thomas et al. (2009). Nuclear anomalies, such as karyorrhectic and karyolytic cells (different forms of cell death), and nuclear buds (indicative of gene amplification) were assessed in 2000 cells/individual and recorded separately.

2.3. Particle-induced X-ray emission (PIXE)

Peripheral blood samples from all 200 individuals were collected by venipuncture. Thus, 5 mL of blood were drawn into heparin tubes (Becton Dickenson, vacutainer) for the particle-induced X-ray emission (PIXE) analysis. All blood samples tubes were coded and kept at room temperature. Blood samples were analyzed for the total content of metals by the particle induced X-ray emission (PIXE) technique (He et al., 1993; Johansson et al., 1995). This technique has been successfully employed to detect trace elements in plants and animals because of its multielemental character, high sensitivity, simplicity and high sample throughput (Mireles et al., 2004).

For the analyses, the blood samples were dried at 40 °C for 72 h, then macerated using a mortar, and finally pressed into pellets which were positioned on the target of the reaction chamber. A 3 MV Tandetron accelerator provided 2.0 MeV proton beams with an average current of 5 nA at the target. The X-rays induced by the beam in the samples were detected by a Si(Li) detector with an energy resolution of about 155 eV at 5.9 keV. The spectra were analyzed with the GUPIXWIN software package (Maxwell et al., 1995; Campbell, 2000) and the final results are expressed in parts per million (ug g^{-1}). The chemical elements analyzed in the samples by the PIXE method were: sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn), bromine (Br) and rubidium (Rb). The organic matrix of the blood (the organic composition of the sample) was determined by the Rutherford Backscattering Spectrometry (RBS) technique.

2.4. Statistical analysis

The normality of the variables was evaluated using the Kolmogorov–Smirnov test; χ^2 and *t*-tests were used to compare the demographic characteristics of study populations and chemical elements analyzed by PIXE. The statistical analysis of differences in MN frequency between the exposed and control group were carried out using the non-parametric Mann–Whitney *U*-test, and statistical differences between the five groups (non-exposed control, extracted coal transport, equipment field maintenance, coal stripping, and coal embarking) were analyzed using the non-parametric two-tailed Kruskal–Wallis test with the Dunn correction. Correlations between MN frequency in lymphocytes obtained in our previous study (León-Mejía et al., 2011) and MN frequencies in buccal cells of the present study in control and exposed individuals were determined by Spearman rank correlation test. The critical level for rejection of the null hypothesis was considered to be $P<0.05$. All analyses were performed with the PRISMA 5.0 statistical software package.

3. Results

The mean age and standard deviation of exposed group was 44.0 ± 7.5 years (range, 24–60 years), and non-exposed control group was 43.7 ± 7.8 years (range, 27–60 years). The mean time of service of the exposed group was 17.7 ± 6.9 years (range, 5–30 years). The percentage of alcohol consumption for non-exposed group was 45 percent and for exposed group was 55 percent, considering as alcohol consumer to drink alcohol in excess of once/week.

Table 1 summarizes the values of the MN frequencies for both study groups, exposed and control groups, with exposed group differentiated by the mining area activities. There was no statistically significant difference between the different mining area activities ($P>0.05$; Kruskal–Wallis test), however the micronuclei frequencies observed to each individual subgroup exposed to coal mining were significantly increased compared to control group values ($P<0.05$; Kruskal–Wallis test).

Table 1
Parameters of genetic damage (micronuclei), gene amplification (nuclear bud) and cell death (karyorrhexis and karyolitic cells) observed in exfoliated buccal of the control and exposed group divided by mining area activities (mean ± standard deviation).

Groups	Micronucleus test parameters			
	Micronucleus	Nuclear bud	Karyorrhexis	Karyolytic
Control ($n=100$)	1.0 ± 2.2	1.0 ± 1.1	35.8 ± 30.5	25.3 ± 17.3
Exposed ($n=100$)	8.8 ± 12.8[a]	2.7 ± 4.2[b]	64.8 ± 54.9[b]	36.1 ± 40.1[b]
Exposed per mining area				
Transport of extracted coal ($n=50$)	8.9 ± 11.7[c]	2.3 ± 2.9[c]	63.1 ± 49.0[c]	30.3 ± 38.4
Equipment field maintenance($n=18$)	8.7 ± 14.9[c]	1.6 ± 2.3	64.4 ± 56.0[c]	36.3 ± 37.6[c]
Coal stripping ($n=17$)	11.3 ± 17.7[c]	4.1 ± 6.5[c]	85.7 ± 78.3[c]	38.2 ± 68.8[c]
Coal embarking ($n=15$)	5.7 ± 6.1[c]	3.0 ± 4.3[c]	52.0 ± 33.7[c]	29.0 ± 16.1

[a] $P<0.001$, Mann–Whitney U-test.
[b] Significant difference compared to the control group at $P<0.05$.
[c] Significant difference compared to the control group at $P<0.05$, Kruskal Wallis test.

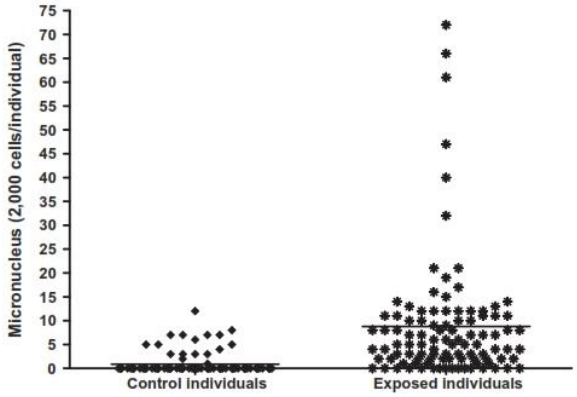


Fig. 1. Scatterplot comparing MN frequencies (BMCyt assay) of control individuals and exposed individuals. Horizontal lines represent the group mean MN frequencies.

The results obtained for MN frequency in exfoliated buccal cells showed that the values in the exposed group to coal mine residues are significantly higher compared with the control group, which were evaluated by Mann–Whitney U-test ($P<0.001$). In addition, Table 1 lists additional markers of nuclear abnormalities in exfoliated buccal cells: nuclear buds, karyorrhectic and karyolytic cells. The mean values of the exposed group of these biomarkers were statistically significant compared to control group ($P<0.05$).

The Spearman correlation coefficient of MN frequency with respect to age for the exposed ($P=0.7159$; $r=-0.03685$) *versus* control group ($P=0.1475$; $r=0.5272$) were not significant ($P>0.05$). A high inter-individual variation between MN frequencies was only observed in the exposed group and ranged from 0 percent to 3.6 percent (0–72 MN/2000 cells) (Fig. 1). Fig. 2 shows the scatterplot comparing MN frequencies of the control group and exposed group divided by mining area activities. However, there were no significant correlations between the MN frequency and the service time for the different exposed groups ($P>0.05$): extracted coal transport ($P=0.9116$; $r=-0.0500$); equipment field maintenance ($P=0.1618$; $r=0.5126$); coal stripping ($P=0.9359$; $r=-0.02112$); and coal embarking ($P=0.0760$; $r=-0.6360$). The MN data in buccal cells obtained in this study were compared with MN data in lymphocytes obtained in our previous study (León-Mejía et al., 2011). Fig. 3 shows a significant and positive correlation between MN frequency in lymphocytes and buccal cells of control and exposed individuals ($P<0.001$; $r=0.573$).

The chemical elements present in the samples determinate by the PIXE method are presented in Table 2. The organic matrix of the blood (the organic composition of the sample) was 72.50 percent carbon, 7.50 percent oxygen, 13.50 percent nitrogen and 6.50 percent fluorine. There was no individual differences in the ppm concentrations of metals in the blood of workers measured by PIXE, in relation to the function performed in coal mining area as assessed by Kruskal–Wallis ANOVA and Dunn's post-test. Thus, for the evaluation of different correlations with regards to chemical present in blood samples, the whole study population of the exposed individuals was considered as the "exposed group". In the analysis of the difference between exposed and control group, the exposed group showed significantly higher ppm levels of aluminum (Al) and silicon (Si) using t-Student test with Welch correction ($P<0.05$). The ppm amounts of metals showed no correlation with age or exposure time (Spearman correlation).

4. Discussion

Coal mining is an activity with a high potential for environmental pollution. In the case of exposure to coal mining residues, the studies that used biomarkers of biological effects, susceptibility and exposure as epidemiological tools are still scarce and most studies assessed underground mining activities (Agostini et al., 1996; Moriske et al., 1996; Santa Maria et al., 2007; Donbak et al., 2005). However, potential genotoxic effects caused by coal opencast mining activities on human health remain poorly explored.

In the present study, MN formation in exfoliated buccal cells of workers exposed to open coal mining was used as a biomarker for genotoxic exposure. This study did not show any effect of alcohol consumption, age and time of service on the MN frequency of the populations investigated. In concordance, Holland et al. (2008) cite that most occupational studies conducted with the buccal micronucleus cytome assay do not find a statistically significant influence of age and lifestyles in the MN frequency of study populations.

When we compared the MN frequencies in the group exposed to coal mining residues we observed a significantly higher frequency compared to the matched control group. No significant difference was observed in the extent of MN formation among the four different mining activities (transport of extracted coal, equipment field maintenance, coal stripping, and coal embarking). This observation indicates that the workers did show a genotoxic response to a complex mixture independent of the working area. Several individuals in the exposed group showed a higher MN frequency and high inter-individual variability. There was no clear difference in the exposed subgroups of the different working areas; therefore it can be assumed that there was no specific factor that would induce a particular high MN frequency. In a recent study using lymphocytes from coal mine workers from

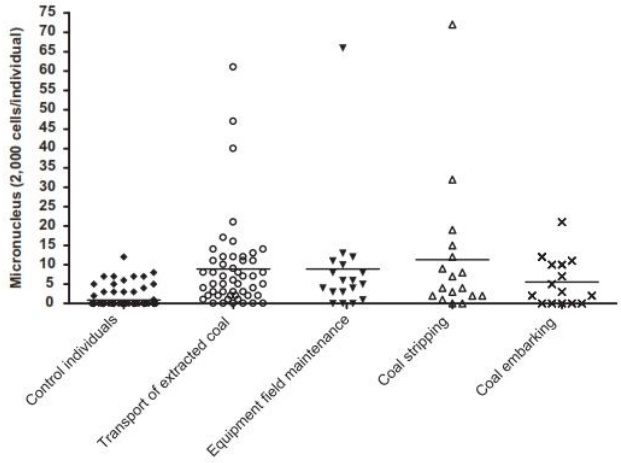


Fig. 2. Scatterplot comparing MN frequencies (BMCyt assay) of the control group and exposed group divided by mining area activities. Horizontal lines represent the group mean MN frequencies.

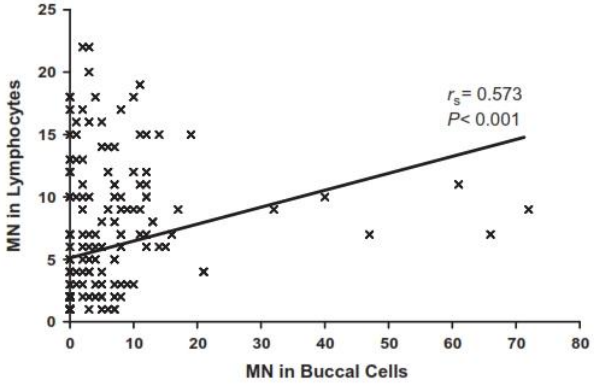


Fig. 3. Nonparametric Spearman correlation analysis between MN frequency in lymphocytes and buccal cells of control and exposed individuals (n=200).

Guajira-Colombia, we found comparable results with regards to occupational hazard effects using the Comet assay and MN test in lymphocytes (León-Mejía et al., 2011). These previous results demonstrated that the group exposed to coal mining residuals exhibited a significantly higher extent of DNA damage in peripheral lymphocytes in the Comet assay. In the exposed group, MN frequency was 2.9-fold and DNA damage index was 6.6-fold higher compared to the control group (León-Mejía et al., 2011), in this study we observed that MN frequency in buccal cells was 8.8-fold higher than control group. While the comet assay detects primary DNA damage with high sensitivity (Collins et al., 2008), the assessment of the MN frequency in isolated lymphocytes has become a reliable biomarker of chromosome breakage and/or whole chromosome loss (Fenech et al., 2003; Fenech, 2006). It has been demonstrated that high frequencies of MN in peripheral blood lymphocytes are predictive of cancer risk and that high levels of MN formation are associated with early events in carcinogenesis (Bonassi et al., 2007; Kirsch-Volders et al., 2014).

In our previous study (León-Mejía et al., 2011), MN frequencies were analyzed in lymphocytes from the same sample groups as those used in the current study, and these data were compared with data on MN in buccal cells from the current study. There was a significant and positive correlation between MN frequencies in the lymphocytes and buccal cells of the control and exposed individuals ($P<0.001$; $r=0.573$; Fig. 3). Similarly Ceppi et al.

Table 2
Concentration of inorganic elements in the blood samples (ppm) of the control group and exposed group divided by mining area activities by PIXE method (mean ± standard deviation).

Groups	Inorganic elements (ppm)													
	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe	Cu	Zn	Br	Rb
Control (n=100)	8942 ± 1657	209 ± 81	109 ± 50	44 ± 30	1629 ± 284	5717 ± 892	14,927 ± 2623	9285 ± 1481	312 ± 116	2945 ± 431	5 ± 2	39 ± 10	18 ± 9	17 ± 9
Exposed (n=100)	8049 ± 2019	210 ± 170	**127 ± 60***	**81 ± 95***	1529 ± 422	5396 ± 1051	13,572 ± 3366	8731 ± 2109	292 ± 106	2777 ± 538	5 ± 2	37 ± 10	17 ± 8	19 ± 11
Exposed per mining area														
Transport of extracted coal (n=50)	8042 ± 1900	219 ± 202	117 ± 63	71 ± 91	1525 ± 467	5401 ± 878	13,531 ± 2794	8675 ± 1786	291 ± 120	2783 ± 482	5 ± 2	37 ± 9	17 ± 8	18 ± 11
Equipment field maintenance (n=18)	8450 ± 2235	172 ± 89	150 ± 61	97 ± 123	1517 ± 360	5354 ± 1491	14,473 ± 3834	9001 ± 2315	298 ± 103	2854 ± 704	5 ± 2	37 ± 13	17 ± 7	18 ± 12
Coal stripping (n=17)	7855 ± 2398	205 ± 121	127 ± 46	91 ± 65	1526 ± 474	5388 ± 1234	13,003 ± 3916	8524 ± 2544	276 ± 81	2775 ± 635	4 ± 2	34 ± 11	15 ± 7	19 ± 9
Coal embarking (n=15)	7788 ± 1784	232 ± 183	133 ± 63	73 ± 101	1565 ± 290	5443 ± 779	13,216 ± 3973	8811 ± 2499	306 ± 91	2658 ± 363	5 ± 2	38 ± 8	18 ± 6	21 ± 10

* Significant increase in relation to the control group at $P<0.05$; Student's t test (Welch correction).

(2010) prepared a compilation of 19 studies which measured the MN frequency in buccal cells and lymphocytes showed a high correlation between both tissues, revealing that the MN evaluation in buccal cells has a similar potential to demonstrate the effects of exposure to genotoxic agents. The formation of micronuclei in both lymphocytes and epithelial cells has been proposed as a useful biomarker to assess the cytogenetic damage in biomonitoring studies (Diler and Celik, 2011; Rohr et al., 2013b; Kirsch-Volders et al., 2014). The formation of micronuclei in both lymphocytes and epithelial cells has been proposed as a useful biomarker to assess the damage cytogenetic in biomonitoring studies (Diler and Celik, 2011).

Our results further support that the mechanism of MN formation in buccal exfoliated cells is consistent with the model proposed for lymphocytes (recently reviewed by Ceppi et al. (2010)).

Besides the formation of MN, cell death indicators (karyorrhectic and karyolytic cells) and other nuclear anomalies such as nuclear buds (indicative of gene amplification) (Diler and Celik, 2011) were also evaluated in buccal cells. The results showed that the number of karyorrhectic and karyolytic cells were significantly higher in the exposed group compared with the control group. These markers of genetic damage found in our study suggest that these events could be a consequence of exposure to some genotoxic agents related to coal mining residues forming a complex mixture of agents present at low concentrations can interact additively or synergistically (Kalantzi et al., 2004), similar to what had been demonstrated by Rohr et al. (2013a, 2013b).

The main route of exposure of coal mine workers to potentially hazardous coal residues is by inhalation of particles. Today it is known that chronic inhalation of this cocktail (which may contain a mixture of substances such as inorganic elements and PAH) can produce pulmonary disorders (Schins and Borm, 1999; Beckman and Ames, 1997; Cooke et al., 2003). Some characteristics of coal from "El Cerrejon" are moisture (~10 percent), volatile (~30 percent), ash (~8 percent), sulfur (~1 percent), carbon (70 percent), hydrogen (~6 percent), oxygen (~5 percent), nitrogen (~1 percent) and different metals (ETSU and Department of Trade and Industry, 2000). In our study we included the assessment of several elements (Na, Mg, Al, Si, P, S, Cl, K, Ca, Fe, Cu, Zn, Br and Rb). The elements assessed in peripheral blood samples showed no difference when comparing the four mining area activities and no correlation with age or time of service was observed. In the analysis of the levels of the different elements in the study population we observed significantly higher amounts of silicon (Si) and aluminum (Al) in the exposed compared to the control group. In the composition of the Cerrejón-Guajira coal these elements are found in substantial quantities in the form of oxides (ETSU and Department of Trade and Industry, 2000), and presence of Al and Si in coal fly ash is recognized in coal fly ash (Prahalad et al., 2000). The abundance of different mineral elements in Cerrejón coal determined by Scanning Electron Microscopy Computer-Controlled shows that more than 80 percent of weight of the mineral material is composed of clay and quartz minerals (aluminum silicate, aluminum silicate, and silica). Analysis of the product reveals that the combustion ashes are formed mainly of aluminum silicates, iron oxide and quartz particles (Irons and Quick, 2000). It is known that inhalation of particulate material typically contains high levels of Al. Experimental studies indicate that the presence of excessive Al is associated with inflammatory processes in the lung which can trigger respiratory diseases (Clarke et al., 2000; Wagner et al., 2007) and carcinogenic processes (Spinelli et al., 2006; Exley et al., 2007; Neumann et al., 2011). The interaction of inorganic elements with living matter is complex, but it is possible that common mechanisms for the majority of inorganic compounds include oxidative stress, DNA repair modulation and disturbance of signal transduction pathway (Beyersmann and Hartwig, 2008).

PAH are also associated with the generation of oxidative stress. The spontaneous combustion of coal is very common in centers of open mining storage systems, and is a major cause of the production of PAH. Many PAH have mutagenic and carcinogenic effects (Cherng et al., 1996; IARC, 1997; Da Silva et al., 2000). Exposure to PAH has been associated with increased DNA damage by cytokinesis-block micronucleus cytome and oxidative stress in occupational exposed populations (Duan et al., 2009, Guo et al., 2014). Several studies have showed buccal MN induction in exposed populations (Giri et al., 2012, Karahalil et al., 1999) and *in vitro* studies suggest that PAH-quinones induce genotoxic effects by modulating the metabolic machinery inside the cells by a combined effect of oxidative stress (Gurbani et al., 2013, Ekstrand-Hammarstrom et al., 2013). Mixtures of DNA-reactive procarcinogens compounds such as PAH at environmentally relevant low-dose concentrations give rise to markedly elevated DNA damage (Hewitt et al., 2007). One of the proposed mechanisms of generation of DNA damage by exposure to PAH is associated with oxidation–reduction processes occurring during the metabolism of these compounds, which result in the formation of quinones. These quinones can undergo redox cycling and produce reactive oxygen species (ROS) (Singh et al., 2007). Another way of ROS generation by exposure to coal mine residues is related to the inhalation of coal dust particles which triggers an inflammatory cell response in macrophages and lung epithelial cells producing large amounts of ROS and cytokines. There is evidence of oxidative damage in coal mining workers, as higher levels of SOD (superoxide dismutase) in individuals exposed to coal (due to an enzymatic response) comparing with non-exposed individuals (Rohr et al., 2013a, 2013b). ROS may also be generated independently of the cellular pathway due to the intrinsic chemical properties of coal dust such as iron content and the radicals on the surface (Schins and Borm, 1999). It is known that ROS are capable of causing oxidative damage to DNA such as single strand breaks and base and nucleotide modifications, particularly in guanosine. The oxidative modifications induce a broad response in the repair characterized by excision of modified bases and nucleotides (Bennett, 2001; Klaunig et al., 2011).

5. Conclusions

In summary, increased levels of micronuclei in the BMCyt assay were observed in coal mining workers. The data of the present study are in agreement with the results of a previous study assessing DNA damage in lymphocytes using the comet assay and MN assay (León-Mejía et al., 2011). The increased MN frequencies observed in the mine workers may be a consequence of oxidative damage resulting from their exposure to coal residues mixtures, including inorganic elements, as Al and Si. However, there are several additional compounds that are released during the processes of exploration and extraction of coal and therefore, due to the complex mixture, it is difficult to relate the genotoxic effects found to the actions of a single compound. Therefore, our study demonstrates that buccal cells present a suitable and non-invasive source to investigate biomarkers of genetic damage in exposed individuals.

Acknowledgments

We thank very cordially Jaime Luna for the help during this work. We also want to thank the help of SINTRACARBON-"El Cerrejón" study population and to the non-exposed controls for

their cooperation during the sampling. This work was supported by grants from COLCIENCIAS 1283-408-20487/2008 (Colombia) Conselho Nacional para o Desenvolvimento Científico e Tecnológico CAPES/CNPq – Brazil. Universal Grant Number 470833; Produtividade Grant Numbers 300902 and 303364. Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil. Grant Number PRONEM 11/2070-7).

References

Acar, H., Caliskan, U., Demirel, S., Largaespada, D.A., 2001. Micronucleus incidence and their chromosomal origin related to therapy in acute lymphoblastic leukemia (ALL) patients: detection by micronucleus and FISH techniques. Teratog. Carcinog. Mutagen. 21, 341–347.

Agostini, J., Otto, P., Wajntal, A., 1996. Chromosome damage in underground coal miners: detection by conventional cytogenetic techniques and by submitting lymphocytes of unexposed individuals to plasma from at-risk groups. Braz. J. Genet. 19, 641–646.

Beckman, K.B., Ames, B.N., 1997. Oxidative decay of DNA. J. Biol. Chem. 272, 19633–19636.

Bennett, M.R., 2001. Reactive oxygen species and death: oxidative DNA damage in atherosclerosis. Circ. Res. 88 (7), 648–650.

Beyersmann, D., Hartwig, A., 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch. Toxicol. 82, 493–512.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28, 625–631.

Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., Kirsh-Volders, M., Knasmueller, S., Zeiger, E., Carnesoltas, D., Cavallo, D., da Silva, J., de Andrade, V.M., Demircigil, G.C., Dominguez, Odio A., Donmez-Altuntas, H., Gattas, G., Giri, A., Giri, S., Gomez-Meda, B., Gomez-Arroyo, S., Hadjidekova, V., Haveric, A., Kamboj, M., Kurteshi, K., Martino-Roth, M.G., Montero Montoya, R., Nersesyan, A., Pastor-Benito, S., Favero Salvadori, D.M., Shaposhnikova, A., Stopper, H., Thomas, P., Torres-Bugarin, O., Yadav, A.S., Zuniga Gonzalez, G., Fenech, M., 2011. The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutat. Res. 728, 88–97.

Campbell, J.L., 2000. The Guelph PIXE software package III. Nucl. Instrum. Methods Phys. Res. B 170, 193.

Celik, M., Donbak, L., Unal, F., Yuzbasioglu, D., Aksoy, H., Yilmaz, S., 2007. Cytogenetic damage in workers from a coal-fired power plant. Mutat. Res. 627, 158–163.

Ceppi, M., Biasotti, B., Fenech, M., Bonassi, S., 2010. Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues. Mutat. Res. 705 (1), 11–19.

Cherng, S.H., Lin, S.T., Lee, H., 1996. Modulatory effects of polycyclic aromatic hydrocarbons on the mutagenicity of 1-nitropyrene: a structure-activity relationship study. Mutat. Res. 367 (4), 177–185.

Clarke, R.W., Coull, B., Reinisch, U., Catalano, P., Killingsworth, C.R., Koutrakis, P., Kavouras, I., Murthy, G.G., Lawrence, J., Lovett, E., Wolfson, J.M., Verrier, R.L., Godleski, J.J., 2000. Inhaled concentrated ambient particles are associated with hematologic and bronchoalveolar lavage changes in canines. Environ. Health Perspect. 108 (12), 179–187.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. 17, 1195–1214.

Da Silva, J., De Freitas, T.R.O., Heuser, V., Marinho, J., Erdtmann, B., 2000. Genotoxicity biomonitoring in coal regions using wild rodent *Ctenomys torquatus* by Comet assay and micronucleus test. Environ. Mol. Mutagen. 35, 270–278.

Diler, B.S., Celik, A., 2011. Cytogenetic biomonitoring of carpet fabric workers using micronucleus frequency, nuclear changes, and the calculation of risk assessment by repair index in exfoliated mucosa cells. DNA Cell Biol., 821–827.

Donbak, L., Rencuzogullari, E., Yavuz, A., Topaktas, M., 2005. The genotoxic risk of underground coal miners from Turkey. Mutat. Res. 588, 82–87.

Duan, H., Leng, S., Pan, Z., Dai, Y., Niu, Y., Huang, C., Bin, P., Wang, Y., Liu, Q., Chen, W., Zheng, Y., 2009. Biomarkers measured by cytokinesis-block micronucleus cytome assay for evaluating genetic damages induced by polycyclic aromatic hydrocarbons. Mutat. Res. 677 (1–2), 93–99.

Ekstrand-Hammarstrom, B., Magnusson, R., Osterlund, C., Andersson, B.M., Bucht, A., Wingfors, H., 2013. Oxidative stress and cytokine expression in respiratory epithelial cells exposed to well-characterized aerosols from Kabul, Afghanistan. Toxicol. In Vitro 27 (2), 825–833.

ETSU and Department of Trade and Industry, 2000. The Fate of Trace Elements in PF Combustion Systems. Report No. COAL R193 – DTI/Pub URN 00/947, ⟨http://webarchive.ationalarchives.gov.uk/tna/+/⟩⟨http://www.dti.gov.uk/files/file18569.pdf/⟩.

Exley, C., Charles, L.M., Barr, L., Martin, C., Polwart, A., Darbre, P.D., 2007. Aluminium in human breast tissue. J. Inorg. Biochem. 101 (9), 1344–1346.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. Human MicronNucleus project, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res. 534, 65–75.

Fenech, M., 2006. Cytokinesis-block micronucleus assay evolves into a cytome assay of chromosomal instability, mitotic dysfunction and cell death. Mutat. Res. 600, 58–66.

Giri, S.K., Yadav, A., Kumar, A., Dev, K., Gulati, S., Gupta, R., Aggarwa, N., Gautam, S.K., 2012. CYP1A1 gene polymorphisms: modulator of genetic damage in coal-tar workers. Asian Pac. J. Cancer Prev. 13 (7), 3409–3416.

Guo, H., Huang, K., Zhang, X., Zhang, W., Guan, L., Kuang, D., Deng, Q., Deng, H., Zhang, X., He, M., Christiani, D., Wu, T., 2014. Women are more susceptible than men to oxidative stress and chromosome damage caused by polycyclic aromatic hydrocarbons exposure. Environ. Mol. Mutagen., 10.1002/em.21866, in press.

Gurbani, D., Bharti, S.K., Kumar, A., Pandey, A.K., Ana, G.R., Verma, A., Khan, A.H., Patel, D.K., Mudiam, M.K., Jain, S.K., Roy, R., Dhawan, A., 2013. Polycyclic aromatic hydrocarbons and their quinones modulate the metabolic profile and induce DNA damage in human alveolar and bronchiolar cells. Int. J. Hyg. Environ. Health 216 (5), 553–565.

He, F., Buoso, M.C., Burattini, E., Fazinic, S., Galassini, S., Haque, A.M.I., Jaksic, M., Moschini, G., 1993. Target preparation for trace element determination of biological materials using techniques. Nucl. Instrum. Meth. Phys. Res. Sect. A 334, 238–245.

Hewitt, R., Forero, A., Luncsford, P.J., Martin, F.L., 2007. Enhanced micronucleus formation and modulation of BCL-2:BAX in MCF-7 cells after exposure to binary mixtures. Environ. Health Perspect. 115 (Suppl. 1), S129–S136.

Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat. Res. 659 (1–2), 93–108.

IARC, International Agency for Research on Cancer, 1997. Agents Classified by the IARC Monographs, vols. 1–105, Cas N 000050-41-9, p. 68.

Irons, R.M.A., Quick, W., 2000. The Fate of Trace Elements in Pulverised Fuel (PF) Combustion Systems. PowerGen UK plc, CRE Group Ltd, University of Nottingham p. R193.

Johansson, S.A.E., Campbell, J.L., Malmqvist, K.G., 1995. Paticle-induced X-ray Emission Spectrometry (PIXE). John Wiley & Sons, INC..

Kalantzi, O.I., Hewitt, R., Ford, K.J., Alcock, R.E., Thomas, G.O., Morris, J.A., 2004. Inter-individual differences in the ability of human milk-fat extracts to enhance the genotoxic potential of the procarcinogen benzo[a]pyrene in MCF-7 breast cells. Environ. Sci. Technol. 38, 3614–3622.

Karahalil, B., Karakaya, A.E., Burgaz, S., 1999. The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. Mutat. Res. 442 (1), 29–35.

Klaunig, J.E., Wang, Z., Pu, X., Zhou, S., 2011. Oxidative stress and oxidative damage in chemical carcinogenesis. Toxicol. Appl. Pharmacol., 86–99.

Kirsch-Volders, M., Bonassi, S., Knasmueller, S., Holland, N., Bolognesi, C., Fenech, M.F., 2014. Commentary: critical questions, misconceptions and a road map for improving the use of the lymphocyte cytokinesis-block micronucleus assay for in vivo biomonitoring of human exposure to genotoxic chemicals–A HUMN project perspective. Mutat. Res. 759, 49–58.

León, G., Perez, L.E., Linares, J.C., Hartmann, A., Quintana, M., 2007. Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. Mutat. Res. 630, 42–49.

León-Mejía, G., Espitia-Pérez, L., Hoyos-Giraldo, L.S., Da Silva, J., Hartmann, A., Henriques, J.A., Quintana, M., 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ. 409 (4), 686–691.

Majer, B.J., Laky, B., Knasmüller, S., Kassie, F., 2001. Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. Mutat. Res. 489, 147–172.

Maxwell, J.A., Teesdale, W.J., Campbel, J.L., 1995. The Guelph PIXE software package II. Nucl. Instrum. Methods Phys. Res. Sect. 95, 407–421.

Mireles, A., Solís, C., Andrade, E., Lagunas-Solar, M., Pina, C., Flocchini, R.G., 2004. Heavy metal accumulation in plants and soil irrigated with wastewater from Mexico city. Nucl. Instrum. Methods Phys. Res. Sect. 220, 187–190.

Moriske, H.J., Drews, M., Ebert, G., Menk, G., Scheller, C., Schondube, M., Konieczny, L., 1996. Indoor air pollution by different heating systems: coal burning, open fireplace and central heating. Toxicol. Lett. 88, 349–354.

Nakajima, T., Hasegawa, H., Nakamata, S., Takanashi, H., Ohki, A., 2008. Mutagenicity of eluent by hot water extraction of various coals: effect of chlorination. Fuel 87, 3132–3136.

Neumann, V., Schulz, F., Theile, A., Loseke, S., Püschel, K., Tannapfel, A., 2011. Case report of a rare occupational disease: a during life non-recognised occupational disease – talcosis. Pneumologie 65 (8), 471–476.

Prahalad, A.K., Inmon, J., Ghio, A.J., Gallagher, J.E., 2000. Enhancement of 2′-deoxyguanosine hydroxylation and DNA damage by coal and oil fly ash in relation to particulate metal content and availability. Chem. Res. Toxicol. 13 (10), 1011–1019.

Rohr, P., da Silva, J., da Silva, F.R., Sarmento, M., Porto, C., Debastiani, R., dos Santos, C.E.I., Dias, J.F., Kvitko, K., 2013a. Evaluation of genetic damage in open-cast coal

mine workers using the buccal micronucleus cytome assay. Environ. Mol. Mutagen. 54 (1), 65–71.
Rohr, P., Kvitko, K., da Silva, F.R., Menezes, A.P., Porto, C., Sarmento, M., Decker, N., Reyes, J.M., Allgayer, Mda C., Furtado, T.C., Salvador, M., Branco, C., da Silva, J., 2013b. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat. Res. 758 (1–2), 23–28.
Santa Maria, S.R., Arana, M., Ramirez, O., 2007. Chromosomal aberrations in peripheral lymphocytes from male native miners working in the Peruvian Andes. Genet. Mol. Biol. 30, 1135–1138.
Schins, R., Borm, P., 1999. Mechanisms and mediators in coal dust induced toxicity: a review. Ann. Occup. Hyg. 32 (1), 7–33.
Singh, R., Sram, R.J., Binkova, B., Kalina, I., Popov, T.A., Georgieva, T., Garte, S., Taioli, E., Farmer, P.B., 2007. The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat. Res./Fundam. Mol. Mech. Mutagen. 620, 83–92.
Spinelli, J.J., Demers, P.A., Le, N.D., Friesen, M.D., Lorenzi, M.F., Fang, R., Gallagher, R.P., 2006. Cancer risk in aluminum reduction plant workers (Canada). Cancer Causes Control 17 (7), 939–948.
Stich, H.F., Rosin, M.P., 1984. Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. Cancer Lett. 22, 241–253.
Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2009. Buccal micronucleus cytome assay. Nat. Protoc. 4 (6), 825–837.
Tolbert, P.E., Shy, C.M., Allen, J.W., 1992. Micronuclei and other nuclear anomalies in buccal smears: methods development. Mutat. Res. 271, 69–77.
Wagner, A.J., Bleckmann, C.A., Murdock, R.C., Schrand, A.M., Schlager, J.J., Hussain, S.M., 2007. Cellular interaction of different forms of aluminum nanoparticles in rat alveolar macrophages. J. Phys. Chem. B. 111 (25), 7353–7359.
Zhou, H.C., Zhong, Z.P., Jin, B.S., Huang, Y.J., Xiao, R., 2005. Experimental study on the removal of PAHs using in-duct activated carbon injection. Chemosphere 59 (6), 861–869.
Zocche, J.J., Leffa, D.D., Damiani, A.P., Carvalho, F., Mendonca, R.A., Iochims, dos Santos, C.E., Boufleur, L.A., Dias, J.F., de Andrade, V.M., 2010. Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. Environ. Res. 110, 684–691.

ANEXO II

Capítulo de Livro intitulado "Occupational Exposure to Coal, Genotoxicity and Cancer Risk"

Será publicado na revista "InTech - Environmental Health Risks"

ISBN 978-953-51-4706-0

Provisional chapter

Occupational Exposure to Coal, Genotoxicity, and Cancer Risk

Grethel León-Mejía , Milton Quintana Sosa ,
Paula Rohr , Katia Kvitko,
João Antonio Pêgas Henriques and Juliana da Silva

Additional information is available at the end of the chapter

Abstract

Coal is a heterogeneous mixture containing large quantities of organic and inorganic matter, including carbon, hydrogen, oxygen, sulfur, nitrogen, and organometallic forms. The presence of mineral matter in coal may result in a number of environmental and human health problems related to its mining, preparation, and combustion. During coal mining activities, large quantities of coal dust, ashes, polycyclic aromatic hydrocarbons (PAHs), and heavy metals are released into the environment, forming a complex mixture. This mixture becomes one of the most important occupational risks for the health and safety of workers due to its synergistic, additive, and enhancing effects. Once inside the organism, this cocktail-like mixture can interact with cellular mechanisms related to the production of reactive oxygen species (ROS) and can cause damage in important macromolecules such as DNA, lipids, and proteins. In this review, human populations exposed to coal and coal burning were analyzed. Data from different studies were evaluated in relation to the effect of complex mixture exposition on DNA damage and mechanisms, and the background factors, such as gender, age, or smoking habit. The high temperatures that occur in combustion processes affect the characteristics of the resulting particles. The coal fly ash is released by combustion and its composition varies depending on the coal type and the method of collection used such as electrostatic precipitators. Compounds such as PAH once activated by the organisms have been shown to have mutagenic and carcinogenic activity due to its ability to form adducts with purines. Moreover, metals that commonly are evaporated during the cooling process increase its toxicity. The particles when inhaled can pass from the alveoli into the bloodstream and affect extrapulmonary organs. Several studies have described the inflammatory cascade that triggers exposure to coal and coal fly ash particles; they have a complex composition capable of generating a persistent inflammatory process, resulting in diseases widely described as emphysema, bronchitis, pneumoconiosis, asthma, and cancer. Several human biomonitoring studies have been conducted evaluating the inflammatory process and

the release of cytokines, polymorphisms involved in detoxification mechanisms, different biomarkers associated with occupational exposure, DNA damage, and the influence of oxidative stress in disease development. The relationship between chronic exposure to coal and coal ash particles and cancer is still widely debated. This review gave us a broad assessment about the associated mechanisms between cancer and exposure to coal and different findings around the world.

Keywords: coal, biomonitoring, DNA damage, ROS, PAH, diseases

1. Introduction

In the last decades, the human population genetics integrity has been compromised by the great industrial activity, which exposes people to a variety of chemicals and genotoxic agents. As a result, it is important to determine what is considered as an "acceptable" level of genetic damage in a concrete population, carry out assay genotoxicity as a routine and also monitor those who, by their occupation or lifestyle, are more exposed or with a bigger risk of having alterations on their genetics stability [1].

One method to quantify the exposition to those substances, as well as its possible impact on the organism, is the use of biological monitoring procedures, or biomonitoring, through biomarkers. Biomonitoring studies try to establish a connection between the environmental factors and the diseases. They detect first alterations in nonmalignant phases, so as to prevent health problems by recognizing the environmental cause of them.

The biological markers, or biomarkers, are the measurable changes (biochemical, physiological, or morphological) that associate to a toxic exposure or any early biochemical alteration, whose study on the biological fluids, tissues, or exhaled air that allow to assess the health risk exposure intensity. The identification of genotoxicity markers believed to cause genome damage is useful, since it can define a prepathogenesis state, such as cancer. It is of vital importance for different diseases prevention, which is the final goal of biomonitoring. In order to achieve it, there must be two stages: 1) detecting human exposure to environment carcinogenic agents; 2) determining genotoxic effects *in vivo* [2].

The combined use of genetic biomarkers and classic epidemiology tools (clinic history and questionnaires) has enabled the identification of early effects to the occupational exposure to distinct pollutant around the world [2–4]. Many biomarkers are used to assess genotoxic effects on human populations exposed to complex mixtures of chemicals. Although there are different possibilities, micronuclei (MN) frequency, chromosomal aberrations (CAs), and comet assay are the most commonly chosen biomarkers. MN originates from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division [5, 6]. MN induction reflects clastogenic and aneugenic damage and is a predictive biomarker of cancer risk [7]. Comet assay detects DNA lesions in individual cells obtained under a variety of experimental conditions; the technique can also be used to evaluate DNA repair [8, 9].

The large inter-individual variability in the capacity to activate or inactivate potential genotoxic and carcinogenic compounds is probably influenced by polymorphisms of the genes encoding the metabolizing enzymes. Genes and proteins involved in metabolization/detoxification of xenobiotics, as well as those involved in DNA repair, are usually used as potential markers of susceptibility for the development of several diseases in which the etiology is related to exposure to environmental hazards. Polymorphisms in such genes have been linked with an increased risk of cancer in several case-control studies [10].

Biomonitoring studies in populations exposed to complex mixtures of chemicals considering individual susceptibility are quite complicated due to inadequate toxicity data, and the unpredictable nature of interaction effects that may be synergistic, additive, or enhancers.

2. Occupational exposure to coal

The coal reserves in a worldwide level is up to 847.5 billion of tons, enough amount to serve the current production for 119 years. This prediction is different from the ones related to oil and gas, which have available supplies for less time [11]. According to data from the International Energy Agency (IEA), coal is the most used resource for energy generation in the world, responsible for 41% of the total production. Nowadays, the main application of mineral coal is to generate energy through thermal power plants. These reserves are considered to have a 109-year lifespan and their coalfields are located in 75 countries. The main world coal producers are China, the United States, India, Australia, Indonesia, Russia, South Africa, Germany, Poland, and Kazakhstan, which are responsible for 91% of the world's production [12]. If those projections are right, the consequences of coal mining and combustion will have large effects in the environment. Thus, the exposed populations monitoring is fundamental with the aim of contributing to the state of knowledge about the health risk and motivate the establishment of control, hygiene, and prevention strategies.

It is well known that coal mining activities are one of the biggest resources of contamination due to the large quantity of substances liberated in the environment. The content of the coal dust and ashes produced by burning are not always homogeneous and this depends on the source and rank of the coal [13, 14]. Coal dust is constituted from carbon, hydrogen, oxygen, nitrogen, quartz (crystalline silica), and inorganic minerals, such as beryllium, cadmium, cobalt, chromium, iron, boron, copper, nickel, antimony, zinc, aluminum (Al), titanium, magnesium, manganese, mercury, and lead [15]. As observed, coal is a mixture of a variety of chemicals, including hydrocarbons, which may raise polycyclic aromatic hydrocarbons (PAHs). All technological processes associated with open fire or temperatures between 400 and 600°C, that may lead to PAH, should be considered potentially hazardous [16, 17].

In relation to coal mining residues exposure, studies in which biomarkers of effect, susceptibility, and exposure are used as epidemiological tools remain rare and a big part of them come from studies on underground coal mining [18, 19]. The effects generated by open coal mining are little explored, though. In open coal mining, the residues pass directly to the atmosphere, where complex mixtures are formed, and the coal exposure to environmental factors such as

sunlight facilitates the processes of spontaneous combustion and, therefore, the release of PAH [20].

Studies about the coal exposure and its harmful effects have been conducted around the world [21–23]. The main way for exposure of the coal mining workers to the potentially dangerous residues is through the inhaling of coal dust particles from mining and manipulation. It is a known fact that the coal mining continuous exposure can cause a variety of diseases, such as coal workers pneumoconiosis (CWP), silicosis, cancer, and chronic obstructive pulmonary disease (COPD), as emphysema and chronic bronchitis [24].

Many studies have established that some of those diseases could have been originated from the genotoxic damage generated by the inhalation of those mineral particles, able to interact with macrophages, epithelial cells, and other cells generating the production of large amount of reactive oxygen species (ROS) [24–26]. The continuous inhalation of coal dust and fly ashes particles is an important cell and non-cell source of ROS in the lung. This may be associated to the damage of target cells of that tissue and other cell lines, after spreading through the bloodstream [27].

Coal-induced DNA damage is related to macrophage activation and the recruitment of polymorphonuclear cells. This cell activation induces the release of inflammatory mediators, such as cytokines, ROS and reactive nitrogen species (RNS). The proinflammatory properties of ROS and RNS include endothelial cell damage, lipid peroxidation and oxidation, the formation of chemostatic factors, the recruitment of neutrophils, and DNA damage [26, 28]. Interaction of ROS with DNA can result in DNA structural and transcriptional errors [29, 30]. Damage caused by ROS is recognized by DNA glycosylases, apurinic/apyrimidinic endonucleases of the base excision repair (BER) mechanism, and in some cases, by the nucleotide excision repair (NER) machinery, leading to DNA strand-breaks [31, 32].

Although chronic exposure may continue to damage the DNA, it has been suggested that inorganic elements can induce DNA single-strand breaks, possibly via the generation of ROS and that this type of damage is soon repaired. Metals are also known to modulate gene expression of enzymes [33]. In addition, PAH can induce DNA lesions as single-strand breaks via DNA repair mechanisms, related with increased adduct formation and electrophilic metabolites [34–36]. Electrophilic metabolites covalently interact with the DNA [37, 38], and adducts are formed with purines, especially guanine, after metabolic activation by enzymatic complex P450 [39]. The International Agency for Research on Cancer (IARC) classified quartz, main constituent of coal, into IARC Group 1 (carcinogen), due to sufficient evidence for carcinogenicity in experimental animals and in humans [40, 41]. The other factor that could lead to different results in coal dust exposure, with positive and negative results, might be explained by the possible differences in composition, in which the proportion of the metals, PAH, and silica (quartz) content may have an influence on the genotoxicity. Despite those findings, coal dust remains classified as non-carcinogen for human (Group 3) in IARC [40, 41]. The importance of coal as an energy source makes its characterization and estimation of risks of extreme importance to the safety of those individuals and the environment.

Several factors may explain conflicting results among different studies with human exposed to coal, e.g. cigarettes smoked, age, gender, nutritional status, and individual polymorphisms [6, 42]. Susceptibility is critical to an understanding of coal diseases, including cancer, and many xenobiotic agents act to alter susceptibility. Unknown individual susceptibility, inadequate toxicity data, and the unpredictable nature of interaction effects make the implementation of a human biomonitoring assessment for complex mixtures of chemicals extremely complicated.

3. Oxidative stress and genotoxic damage related with coal exposure

One important aspect to consider about the coal exposure is the amount of products generated during the coal combustion. The burning of coal, in order to generate electricity, produces flue gasses and particulate materials like coal fly ashes and residues as scoria and bottom ash. The finer particles (coal fly ash) are obtained by mechanical or electrostatic precipitation of the dust in suspension in the gases produced by combustion, while the coarser particles fall to the bottom by gravity and are removed at the bottom of the boiler [43, 44].

The combustion temperature is an important factor that determines the physical properties of the particles. In the combustion of conventional high temperature (>1400°C), the main aluminosilicate melts and condenses to form spherical particles. The coal fly ash particles produced are mostly irregularly shaped and contain a complex mixture consisting of unburned carbon; oxides; quartz; elements such as Al, silicon (Si), iron, and calcium; elements trace as nickel, arsenic, chromium, copper, lead, cadmium, zinc [45, 46], and PAH [47].

The coal fly ash has a relatively low toxicity as compared with coal or quartz [45]. Studies have determined the role of coal fly ash particle size and the release of iron, which leads to generation of radicals and oxidative stress. In this context, it was demonstrated the ability of coal fly ash release of bioavailable iron, which triggers processes and redox oxidant production [48]. In addition, it was shown that interleukin 8 (IL-8) levels in human lung epithelial cells are increased in response to coal fly ash and vary with the bioavailability of iron, as a function of source of coal and particle size [49]. The smaller size fraction had more stimulatory activity, which may be related to the fact that iron is more concentrated in this fraction. Particle size is a critical factor because a larger surface area allows more significant transport of metal and other adsorbed components, increasing the pulmonary toxicity of particulate matter (PM) [50].

The particles are classified according to their aerodynamic diameter (in micrometer) in coarse (PM 10), fine (PM 2.5), ultrafine (PM 0.1) [51]. The smaller particles are more harmful with respect to health effects because of their very high alveolar deposition fraction, large surface area, chemical composition, ability to induce inflammation, and potential to translocate to the circulation to extrapulmonary organs [52–54]. These particles could trigger persistent lung inflammation compared to the coarse particles in addition to the exposure to genotoxic compounds, which are contained in the particles [26, 55].

Depending on the toxicity, the chemical properties, and the concentration in air, coal and coal fly ash particles can constitute a risk to exposed workers. When these particles are inhaled and

deposited in the lungs, they can lead to health risks due to the leaching of genotoxic compounds and altered immunological mechanisms affecting the lung parenchyma causing diseases [56]. These nanometric particles are very small, which allows them to penetrate the biological organs and affect its normal function. More specifically, as the particle load in the lung increases the alveolar macrophages and epithelial cells are activated, leading to the release of inflammatory mediators, ROS, enzymes (elastases, proteases, collagenases), cytokines [tumor necrosis factor alpha (TNF-α), interleukins], and growth factors (TGF-β) that control and stimulates the fibrosis, genotoxic events, and cell death [45, 57, 58].

Persistent inflammatory processes have been accepted as a crucial factor in the pathogenesis. In [59], it was investigated whether systemic TNF-α, soluble TNF-α receptors (p55, p75), IL-6, and soluble IL-6 receptor could be markers of biological activities of Chinese CWP. Interestingly, those results suggest that serum levels of TNF receptors and IL-6 are associated with the fibrotic process of CWP and serum cytokine levels may be correlated with the severity of CWP. In the pathogenesis of these respiratory diseases related with coal exposure, oxidative damage plays a key role. Either acting in association or independently, the chemical and physical characteristics can lead to the generation of ROS and oxidative stress [60, 61].

These particles are chemically heterogeneous and can be a source of oxidants by themselves ("acellular" mechanisms), due to their composition, such as oxides, metals, and PAH [26]. Soluble metals (transition) associated to the particle can increase the generation of ROS by Haber-Weiss reactions. PAH may be metabolically activated and induce ROS and oxidative stress, also forming bulky adducts or strand breaks on DNA [50, 62, 63].

Another way of generating oxidants is via cellular. Once in the lungs, alveolar macrophages are activated and generate large amounts of ROS, and chemoattractant factors of other inflammatory cells such as monocytes and neutrophils are released, which amplify this response generating more oxidants [64]. The particle size is a critical factor, because very large particles are difficult to phagocytose, leading to the process of incomplete or "frustrated" phagocytosis aggravating the response [65, 66].

Considering three different scenarios with respect to exposure to particles, the generation of oxidative stress, inflammation, and oxidative DNA damage, several authors questioned whether the lung inflammation may be related to secondary genotoxic effects. They also questioned if phenomena of oxidative stress, inflammation and DNA damage are independent or interrelated, whether oxidative stress stimulates inflammatory processes, or inflammation mediated by particles cause oxidative stress, or even if it is possible that particles may cause both phenomena of oxidative stress and inflammation but for different mechanisms of action [26, 61].

In normal physiological conditions, there is a balance between ROS generation and antioxidant defenses. However, the continuous inhalation of particles may interfere in this equilibrium leading to oxidative stress process in the lung. Consequently, a high loading of particles alters the oxidant-antioxidant balance, leading to oxidative damage and the beginning of pathological processes [67]. The most important effects of ROS in the lung include damage to cell

membranes by lipid peroxidation process, protein oxidation, and DNA damage in target cells [27].

As seen in **Figure 1**, oxidative DNA damage can have many consequences, from cell death and tissue destruction to cell proliferation. Furthermore, ROS can also act as regulators in signaling pathways intracellularly and transcription factors of a variety of genes including those of proinflammatory cytokines, adhesion molecules, and proto-oncogenes [68].

In vitro effects induced by coal exposure have been described in different cells such as murine alveolar type II epithelial cells (C10) [69] and in 7TD1 cells [70]. ROS generation and oxidative damage by coal fly ash particles have been described in different cell lines, in human peripheral blood mononuclear cells [71], in rat alveolar macrophages (NR8383) [72], in BEAS-2B human lung epithelial cells [73], and in rat lung epithelial (RLE) cells [74].

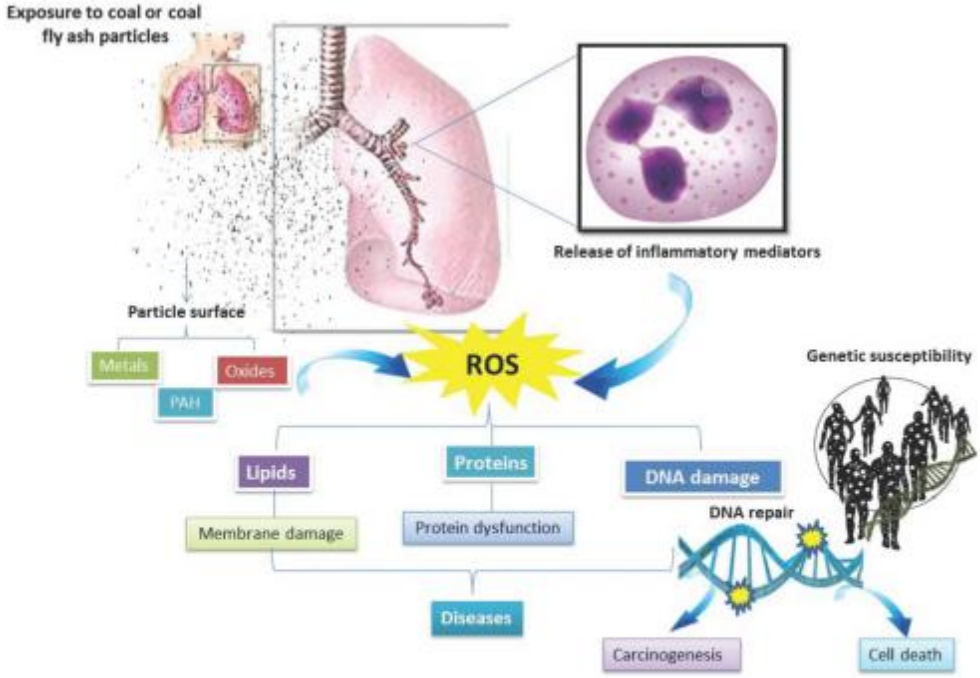


Figure 1. Main pathways associated with the generation of oxidative damage and the development of diseases induced by coal and coal fly ash particles.

ROS induce point mutations and CAs in cells. Many inhaled toxic substances contained in the particles contribute to oxidative modification that has as target of attack specific components of the cytoplasm and the nucleus. Such changes include DNA breakage, DNA oxidative modification, base modifications, alterations in the DNA sequence, poly-ADP ribosylation, activation of kinases, activation of proto-oncogenes, and inactivation of tumor suppressor genes. Persistent generation of ROS generated by mineral particles indestructible or engulfed incompletely leads to damage to organelles keys [59, 61, 75]. The oxidation of C8 deoxyguanosine (dG), resulting in 7-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), is the most common oxidative lesion generated by ROS. The proportion of 8-oxodG/dG has been considered as a

biomarker of oxidative stress and has been studied in relation to exposure to mineral particles *in vitro* and *in vivo* [76].

Human biomonitoring studies about the effects of exposure to coal and residues using different biomarkers have been conducted around the world. In this context, our group has obtained interesting findings in workers exposed to coal mining in Colombia and Brazil. In [77], it was found that Brazilian workers with occupational exposure to coal had significantly increased genetic damage in peripheral blood lymphocytes compared with unexposed individuals. Exposed workers presented lower average levels of thiobarbituric acid reactive substances (TBARSs) and catalase activity (CAT). In addition, DNA damage evaluated by human buccal micronucleus cytome (BMCyt) assay was observed in mine workers, which could be a consequence of oxidative damage resulting from exposure to coal residue mixtures [78].

In Colombia, DNA damage in lymphocytes of coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay were observed [79]. Also, in buccal mucosa samples, the micronucleus frequencies and nuclear buds were significantly higher in the exposed group than in non-exposed control group. Interestingly, blood samples of Colombian mining workers analyzed showed higher values of Si and Al, characteristic elements of coal particles, compared with the control group [80]. All these studies converge to a point: the compounds contained in the particles may be related to ROS generation, DNA damage, and formation of pro-mutagenic adducts.

These are important findings if we consider that oxidative DNA damage can lead to long-term risk of cancer and other diseases caused by air pollution by these particles. In **Table 1**, can be observed an overview of key studies on the genotoxicity in human population exposed to coal and coal combustion products. These studies demonstrated DNA damage using different methods, related with inorganic elements and oxidative stress.

References	Country	Exposure(s)	Biomarker	Outcome(s)
[16]	Brazil	Underground coal mining	Mitotic index in lymphocyte cultures and micronuclei in buccal mucosa cells.	A decreased mitotic index, an excess of micronuclei and a higher frequency of chromosome abnormalities (fragments, polyploidy and overall chromosome alterations) were observed in the miners when compared with the controls.
[81]	Slovenia	_	Sister-chromatid exchanges (SCE), unstable chromosome and chromatid aberrations and micronuclei in blood lymphocytes	Significantly higher levels of chromosomal aberrations, SCE and micronuclei in exposed group compared with the control group.
[82]	Brazil	Underground workers directly exposed,	Oxidative stress biomarkers (TBARS, GSH/GSSG, α-tocopherol, GST, GR, GPx, SOD,	The results showed that subjects directly and indirectly exposed to coal dust face an oxidative stress condition. They also indicate

References	Country	Exposure(s)	Biomarker	Outcome(s)
		surface workers indirectly exposed, residents living near the mines.	CAT).	that people living in the vicinity of the mine plant are in health risk regarding coal mining-related diseases.
[22]	Turkey	Coal combustion products	Chromosomal aberrations (CAs), polyploidy, sister-chromatid exchanges (SCEs), and micronuclei (MN) in blood cells.	Significantly higher levels of CA, polyploidy, SCE, and MN in peripheral blood lymphocytes of workers compared with controls.
[83]	Turkey	Underground coal mining	SCE, CA, and micronuclei frequencies in peripheral lymphocytes.	Increase in sister chromatid exchanges, chromosomal aberrations, and micronucleus frequencies found in underground coal miners as compared to control group.
[21]	China	Indoor smoky coal emissions that contain high levels of polycyclic aromatic hydrocarbons (PAHs)	GSTM1 and GSTT1 genotypes. Expression of p53 protein in sputum samples.	The GSTM1 null genotype may enhance susceptibility to lung cancer due to these indoor coal combustion emissions. Smoky coal use was strongly associated with overexpression of p53 in tumor cells among highly exposed women.
[79]	Colombia	Open cast mining	(MN) frequency and DNA damage (comet assay) in lymphocytes.	The biomarkers evaluated showed statistically significant higher values in the exposed group compared to the non-exposed control group.
[80]	Colombia	Open cast mining	Micronucleus (MN) frequencies, nuclear buds, karyorrhectic and karyolytic cells in buccal mucosa samples and content of inorganic elements in blood samples by PIXE.	MN frequencies and nuclear buds in buccal mucosa samples were significantly higher in the exposed group than in the non-exposed control group. In addition, karyorrhectic and karyolytic cells were also significantly higher in the exposed group (cell death). Blood samples showed higher values of silicon (Si) and aluminum (Al) in the exposed group.
[84]	Russian	Underground	Chromosomal and	A higher frequency of chromosomal

References	Country	Exposure(s)	Biomarker	Outcome(s)
		coal mining	chromatid type aberrations in blood lymphocytes	aberrations in the exposed group compared with the control group.
[77]	Brazil	Open coal mining	MN and nucleoplasmic bridge frequencies in peripheral lymphocytes, damage index and damage frequency (comet assay).	Increased MN and nucleoplasmic bridge frequencies in peripheral lymphocytes, increased damage index and damage frequency (comet assay). Lower average levels of TBARS and catalase activity (CAT), while the mean superoxide dismutase activity (SOD) levels were higher in the exposed group.
[78]	Brazil	Open coal mining	Buccal micronucleus cytome (BMCyt) DNA damage, cell death, and basal cell frequency in buccal cells.	The exposed group presented a significantly higher frequency of basal cells, micronuclei in basal and differentiated cells, and binucleated cells compared to the non-exposed group. No correlation between DNA damage and metal concentration in the blood of mine workers.
[19]	Peru	Underground coal mining	Chromosomal aberrations in peripheral lymphocytes	Miners occupationally exposed to underground mining activity have an increased frequency of chromosomal aberrations compared with the controls.
[85]	_	Coal fly ash particles	SCE frequencies in peripheral blood lymphocytes.	No increased SCE frequencies were found in PBLs of workers potentially exposed to coal fly ash when compared to the control group. No differences were observed between the exposed and control groups for frequencies of gene mutations at the HPRT locus in PBLs, for micronucleus frequencies using the cytokinesis block method, or for urinary mutagen excretion measured with *Salmonella typhimurium* tester strains TA98 and TA97.
[86]	Germany	Underground coal mining	Structural chromosomal aberrations in peripheral lymphocytes	Coal miners had significantly higher frequencies of chromosomal aberrations compared with controls.

References	Country	Exposure(s)	Biomarker	Outcome(s)
[87]	Turkey	Underground coal mining	Sister chromatid exchange (SCE) and micronucleus (MN) frequency in lymphocytes of Turkish CWP patients.	SCE and MN frequencies in CWP patients were found significantly higher than in coal workers and unexposed groups.

Table 1. Overview of key studies on the genotoxicity in human population exposed to coal and coal combustion products.

4. Conclusions

The coal mining activities generate different types of compounds that are released into the environment. Once into the atmosphere, these compounds form a complex mixture that consists of metals, oxides, and PAH. These compounds can interact with "acellular" and cellular mechanisms related with ROS production. The metals found in the coal fly ash and coal particles by different ways lead to the ROS formation. Important macromolecules as DNA, proteins, and lipids can suffer oxidative modifications. The PAHs contained in the particles also influence the particles toxicity. A second indirect way for excessive ROS formation is related to cellular mechanisms, which is consequence of oxidative burst of macrophages and neutrophils during phagocytosis of particles and inflammation produced.

If we think in exposed populations, we cannot ignore the social and environmental impact associated with coal mining. The continuous inhalation, the high load of particles in phagocytic cells, the oxidant-antioxidant imbalance which are linked to the origin of pathological processes; this whole scenario is worrisome to biologic level for these populations. In addition, in recent years, coal mining had a remarkable increase in demand; international mining companies have increased their investments in exploration around the world. For this reason, human biomonitoring studies in exposed populations become really necessary to contribute to knowledge state about the risk for those people in order to motivate the design of control, hygiene, and prevention strategies, besides epidemiological surveillance.

Author details

Grethel León-Mejía [1,2], Milton Quintana Sosa [2], Paula Rohr [3], Katia Kvitko[4], João Antonio Pêgas Henriques [1,5*] and Juliana da Silva [6*]

*Address all correspondence to: pegas.henriques@gmail.com

*Address all correspondence to: juliana.silva@ulbra.br

1 Laboratory of Molecular Radiobiology, Center of Biotechnology, Postgraduate Program in Cell and Molecular Biology, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil

2 Simon Bolivar University, Faculty of Basic Sciences, Unit of Research, Development and Innovation in Genetics and Molecular Biology, Barranquilla, Colombia

3 Laboratory of Celular and Molecular Biology, Academic Unit of Health Sciences, Universidade do Extremo Sul Catarinense (UNESC) – Criciúma SC), Brazil

4 Postgraduate Program in Genetics and Molecular Biology (PPGBM), Federal University of Rio Grande do Sul (UFRGS) Porto Alegre, Rio Grande do Sul, Brazil

5 Laboratory of Molecular Radiobiology Center of Biotechnology, Federal University of Rio Grande do Sul (UFRGS) Porto Alegre, Rio Grande do Sul, Brazil

6 Laboratory of Genetic Toxicology, Postgraduate Program in Molecular and Cell Biology Applied to Health (PPGBioSaúde), Lutheran University of Brazil (ULBRA), Canoas, Rio Grande do Sul, Brazil

References

[1] Zalacain, M; Sierrasesumaga, L; Patino, A. (2005). The cytogenetic assay as a measure of genetic instability induced by genotoxic agents. An Sist Sanit Navar. 28(2);227–36.

[2] Pastor, S; Lucero, L; Gutierrez, S; Durban, R; Gomez, C; Parron, T; et al. (2002). A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. Mutagenesis. 17(1);79–82.

[3] Martino-Roth, M.G; Viegas, J; Roth, D.M. (2003). Occupational genotoxicity risk evaluation through the comet assay and the micronucleus test. Genet Mol Res. 2(4); 410–7.

[4] Cassini, C; Calloni, C; Bortolini, G; Garcia, SC; Dornelles, MA; Henriques, JÁ; Erdtmann B; Salvador M. (2011). Occupational risk assessment of oxidative stress and genotoxicity in workers exposed to paints during a working week. Int J Occup Med Environ Health. 24(3);308–19.

[5] Fenech, M; Morley, A. (1985). Solutions to the kinetic problem in the micronucleus assay. Cytobios. 43;233–46.

[6] Mateuca, R; Lombaert, N; Aka, P.V; Decordier, I; Kirsch-Volders, M. (2006). Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie. 88(11);1515–31.

[7] Bonassi, S; Znaor, A; Ceppi, M; Lando, C; Chang, W.P; Holland, N; et al. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis. 28(3);625–31.

[8] Tice, R.R; Agurell, E; Anderson, D; Burlinson, B; Hartmann, A; Kobayashi, H; et al. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 35(3);206–21.

[9] Collins, A.R. (2014). Measuring oxidative damage to DNA and its repair with the comet assay. Biochim Biophys Acta 1840;794–800. doi: 10.1016/j.bbagen.2013.04.022.

[10] International Agency for Research on Cancer (IARC), Monographs on the Evaluation of Carcinogenic Risk to Humans, Reevaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide Agency for Research on Cancer, Toluene, World Health Organization. (1999). Lyon 71;829–864. Online: http://monographs.iarc.fr/ENG/Monographs/vol71/mono71.pdf

[11] Cerrejón-minería responsable. Usos del carbón. (2013). Online: http://www.cerrejon.com/site/mas-sobre-el-carbon/usos-del-carbon.aspx

[12] International Energy Agency (IEA). (2016). Online: http://www.iea.org/

[13] Aneel-Agência Nacional de Energia Elétrica. Atlas de Energia Elétrica do Brasil. Capítulo 9- Carvão mineral. (2009). Online: www.aneel.gov.br/arquivos/pdf/atlas_par3_cap9.pdf

[14] Caballero, A.L; Médico, O.A. (2013). Caracterización y posible uso de cenizas resultantes de la combustión del carbón, en la futura Termocentral de lecho fluidizado. Río Turbio (Argentina). Visir-Redisa. Universidad Nacional de la Patagonia Austral. Online: http://www.redisa.uji.es/artSim2013/CaracterizacionDeResiduosSolidos/Caracterizacion%20Cenizas%20Combustion%20Carbon.pdf

[15] Kalkreuth, W; Holz, M; Kern, M; Machado, G; Mexias, A; Silva, M.B; Willett, J; Finkelman, R; Burger, H. (2006). Petrology and chemistry of permian coals from the Paraná basin: Santa Terezinha, Leão-Butiá and Candiota Coal fields, Rio Grande do Sul, Brazil. Intern. J. Coal Geol. 68;79–116.

[16] Kvitko, K; Bandinelli, E; Henriques, J; Heuser, V; Rohr, P; Da Silva, F; Schneider, N; Fernandes, S; Ancines, C; Da Silva, J. (2012). Susceptibility to DNA damage in workers occupationally exposed to pesticides, to tannery chemicals and to coal dust during mining. Genet. Mol. Biol. 35;1060–68.

[17] Jarvis, I.W; Dreij, K; Mattsson, Å; Jernström, B; Stenius, U. (2014). Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. Toxicology. 3(321);27–39.

[18] Agostini, J; Otto P; Wajntal A. (1996). Chromosome damage in underground coal miners: detection by conventional cytogenetic techniques and by submitting lymphocytes of unexposed individuals to plasma from at-risk groups. Braz J Genet. 19;641–6.

AQ1

AQ2

AO3

[19] Santa Maria, S.R; Arana, M; ,Ramirez O. (2007). Chromosomal aberrations in peripheral lymphocytes from male native miners working in the Peruvian Andes. Genet Mol Biol. 30;1135–8.

[20] León, G; Perez, L.E; Linares, J.C; Hartmann, A; ,Quintana M. (2007). Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. Mutat Res. 630;42–9.

[21] Lan, Q; ,He X. (2004). Molecular epidemiological studies on the relationship between indoor coal burning and lung cancer in Xuan Wei, China. Toxicology. 198;301–5.

[22] Celik, M; Donbak, L; Unal, F; Yüzbasioglu, D; Aksoy, H; Yilmaz, S. (2007). Cytogenetic damage in workers from a coal-fired power plant. Mutat Res. 627(2);158–63.

[23] Karami, S; Boffetta, P; Brennan, P; Stewart, P.A; Zaridze, D; Matveev, V; Janout, V; Kollarova, H; Bencko, V; Navratilova, M; Szeszenia-Dabrowska, N; Mates, D; Gromiec, J.P; Sobotka, R; Chow, W.H; Rothman, N; Moore, L.E. (2011). Renal cancer risk and occupational exposure to polycyclic aromatic hydrocarbons and plastics. J Occup Environ Med. 53(2);218–223; 1076–2752.

[24] Petsonk, E.L; Rose, C; ,Cohen R. (2013). Coal mine dust lung disease. New lessons from old exposure. Am J Respir Crit Care Med. 187(11);1178–85.

[25] Vallyathan, V; Shi, X; Castranova, V. (1998). Reactive oxygen species: their relation to pneumoconiosis and carcinogenesis. Environ Health Perspect. 106(Suppl. 5);1151–5.

[26] Knaapen, A.M; Borm, P.J; Albrecht, C; ,Schins R.P. (2004). Inhaled particles and lung cancer. Part A: Mechanisms. Int J Cancer 109;799–809.

[27] Schins, R.P; ,Borm P.J. (1999). Mechanisms and mediators in coal dust induced toxicity: a review. Ann Occup Hyg. 43(1);7–33.

[28] Ba, X; Aguilera-Aguirre, L; Rashid, QT; Bacsi, A; Radak, Z; Sur, S; Hosoki, K; Hegde, ML; Boldogh I. (2014). The role of 8-oxoguanine DNA glycosylase-1 in inflammation. Int J Mol Sci. 15(9);16975–97.

[29] Schottenfield, D; Bebe-Dimmer, J. (2006). Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. CA: Cancer J. Clin. 56;69–83.

[30] Svilar, D; Goellner, EM; Almeida, KH; Sobol, RW. (2011). Base excision repair and lesion-dependent subpathways for repair of oxidative DNA damage. Antioxid Redox Signal. 14(12);2491–507.

[31] Sander, M; ,Wilson S. (2005). Base Excision Repair, AP Endonucleases and DNA Glycosylases, John Wiley & Sons Ltd, Chichester.

[32] Storr, S.J; Woolston, C.M; Zhang, Y; ,Martin S.G. (2013). Redox environment, free radical, and oxidative DNA damage. Antioxid Redox Signal. 18(18);2399–408.

[33] Jomova, K; ,Valko M. (2011). Advances in metal-induced oxidative stress and human disease. Toxicology. 283;65–87.

[34] Brescia, G; Celotti, L; Clonfero, E; Neumann, G.H; Forni, A; Foa, V; Pisoni, M; Ferri, G.M; Assennato, G. (1999). The influence of cytochrome P450 1A1 and glutathioneS-transferase M1 genotypes on biomarker levels in coke-oven workers. Arch. Toxicol. 73;431–9.

[35] Rojas, M; Cascorbi, I; Alexandrov, K; Kriek, E; Auburtin, G; Mayer, L; Kopp-Schneider, A; Roots, I; Bartsch, H. (2000). Modulation of benzo[a]pyrene diolepoxide-DNA adduct levels in human white blood cells by CYP1A1, GSTM1 and GSTT1 polymorphism. Carcinogenesis 21;35–41.

[36] Pavanello, S; Pulliero, A; Lai, A; Gaiardo, A; Mastrangelo, G; Clonfero, E. (2005). Anti-B[a]PDE-DNA formation in lymphomonocytes of humans environmentally exposed to polycyclic aromatic hydrocarbons, G. Ital. Med. Lav. Ergon. 27;312–4.

[37] Pereira-Neto, A.D; Moreira, J.C; Dias A.E.X.O; Arbilla G; Ferreira L.F.V; Oliveira A.S; Barek J. (2000). Avaliação da Contaminação Humana por Hidrocarbonetos Policíclicos Aromáticos (HPAS) e seus Derivados Nitrados (NHPAS): Uma Revisão Metodológica. Quim. Nova 23;9.

[38] Singh, R; Sram, R.J; ,Binkova B; Kalina, I; Popov, T.A; ,Georgieva T; ,Garte S; Taioli, E; ,Farmer P.B. (2007). The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat. Res. 620;83–92.

[39] Baird, W.M; Hooven, L.A; ,Mahadevan B. (2005). Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ. Mol. Mutagen. 45;106–14.

[40] International Agency for Research on Cancer (IARC). (2012). Arsenic, metals, fibres, and dusts: a review of human carcinogens. Lyon. 100C;526. Online: http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C.pdf

[41] International Agency for Research on Cancer (IARC). (1997). Silica, some silicates, coal dust and para-aramid fibrils, IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans. Lyon. 68;528. Online: http://monographs.iarc.fr/ENG/Monographs/vol68/mono68.pdf

[42] Bonassi, S; Coskun, E; Ceppi, M; Lando, C; Bolognesi, C; Burgaz, S; et al. (2011). The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutat Res. 728(3);88–97.

AQ4

[43] Silva, L; Ward, C.R; ,Hower J; ,Izquierdo M; ,Waanders F; ,Oliveira M; ,Li Z; Hatch, R; ,Querol X. (2010). Mineralogy and leaching characteristics of coal ash from a major Brazilian power plant. Coal Combust Gasificat Prod. 2;51–65.

[44] Martinez, J. (2012). Cenizas volantes de carbón y cenizas de hogar o escoria. Ficha técnica, España.

[45] Borm, P.J. (1997). Toxicity and occupational health hazards of coal fly ash (CFA). A review of data and comparison to coal mine dust. Ann Occup Hyg. 41;659–76.

[46] Swaine, D.J; ,Goodarzi F. (1997). Environmental Aspects of Trace Elements in Coal. Kluwer, Dordrecht.

[47] Ruwei, W; Jiamei, Z; Jingjing, L; Liu, G. (2013). Levels and patterns of polycyclic aromatic hydrocarbons in coal-fired power plant bottom ash and fly ash from Huainan, China. Arch Environ Contam Toxicol. 65(2);193–202.

[48] Ball, B.R; Smith, K.R; Veranth, J.M; ,Aust A.E. (2000). Bioavailability of iron from coal fly ash: mechanisms of mobilization and of biological effects. Inhal Toxicol. 12(Suppl. 4);209–25.

[49] Smith, K.R; Veranth, J.M; Hu, A.A; Lighty, J.S; ,Aust A.E. (2000). Interleukin-8 levels in human lung epithelial cells are increased in response to coal fly ash and vary with the bioavailability of iron, as a function of particle size and source of coal. Chem Res Toxicol. 13(2):118–25.

[50] Mazzoli-Rocha, F; Fernandes, S; Einicker-Lamas, M; Zin, W.A. (2010). Roles of oxidative stress in signaling and inflammation induced by particulate matter. Cell Biol Toxicol. 26(5);481–98.

[51] Donaldson, K; ,Stone V. (2003). Current hypotheses on the mechanisms of toxicity of ultrafine particles. Ann Ist Super Sanità;. 39;405–10.

[52] Oberdörster, G; Sharp, Z; Atudorei, V; Elder, A; Gelein, R; Kreyling, W; Cox, C. (2004). Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol. 16(6–7);437–45.

[53] Schins, R.P; Lightbody, J.H; Borm, P.J; ,Shi T; Donaldson, K; ,Stone V. (2004). Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents. Toxicol. Appl. Pharmacol. 195;1–11.

[54] Mani, U; Prasad, A.K; ,Suresh Kumar V; Lal, K; Kanojia, R.K; Chaudhari, B.P; Murthy R.C. (2007). Effect of fly ash inhalation on biochemical and histomorphological changes in rat liver. Ecotoxicol Environ Saf. 68(1);126–33.

[55] Donaldson, K; Tran, L; Jimenez, L.A; Duffin, R; Newby, D.E; Mills, N. (2005). Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol. 2;10.

[56] Carbone, F; Pagliara, R; Barone, A.C; Beretta, F; D'Anna, A. (2009). Characterization of nano-ashes generated during pulverized coal combustion. Report Ricerca Sistema

AQ5

Elettrico. Online: http://www.enea.it/it/Ricerca_sviluppo/documenti/ricerca-di-sistema-elettrico/centrali-carbone-rendimenti/rse109.pdf.

[57] Gilmour, M.I; O'Connor, S; Dick, C; Miller, C.A; ,Linak W.P. (2004). Differential pulmonary inflammation and in vitro cytotoxicity of size-fractionated fly ash particles from pulverized coal combustion. J Air Waste Manag Assoc. 54;286–95.

[58] Sambandam, B; Devasena, T; Islam, V.I; Prakhya, B.M. (2015). Characterization of coal fly ash nanoparticles and their induced in vitro cellular toxicity and oxidative DNA damage in different cell lines. Indian J Exp Biol. 53(9);585–93.

[59] Zhai, R; Liu, G; Ge, X; Bao, W; Wu, C; Yang, C; Liang, D. (2002). Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin 6 (IL-6), and their soluble receptors in coal workers' pneumoconiosis. Respir Med. 96(10);829–34.

[60] Bonner, J.C. (2007). Lung fibrotic responses to particle exposure. Toxicol Pathol. 35(1); 148–53.

[61] Møller, P; Danielsen, P.H; Karottki, D.G; ,Jantzen K; ,Roursgaard M; Klingberg, H; Jensen, D.M; Christophersen, D.V; Hemmingsen, J.G; Cao, Y; ,Loft S. (2014). Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. Mutat Res Rev Mutat Res. 762;133–66.

[62] Risom, L; Møller, P; Loft, S. (2005). Oxidative stress-induced DNA damage by particulate air pollution. Mutat. Res. 592;119–37.

[63] Valavanidis, A; Vlachogianni, T; Fiotakis, K; Loridas, S. (2013). Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health. 10;3886–907.

[64] Fubini, B; ,Hubbard A. (2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic Biol Med. 34;1507–16.

[65] Champion, J.A; ,Mitragotri S. (2006). Role of target geometry in phagocytosis. Proc Natl Acad Sci U S A. 103(13);4930–4.

[66] Schinwald, A; ,Donaldson K. (2012). Use of back-scatter electron signals to visualise cell/nanowires interactions in vitro and in vivo; frustrated phagocytosis of long fibres in macrophages and compartmentalisation in mesothelial cells in vivo. Particl Fibre Toxicol. 9;34.

[67] Porter, D.W; Leonard, S.S; ,Castranova V. (2006). Particles and Cellular Oxidative and Nitrosative Stress. Chapter 6. National Institute for Occupational Safety and Health. pp. 119–138.

AQ6

[68] Schieber, M; ,Chandel N.S. (2014). ROS function in redox signaling and oxidative stress. Curr Biol. 24(10);453–62.

[69] Albrecht, C; Borm, P.J.A; Adolf, B; Timblin, C.R; ,Mossman B.T. (2002). In vitro and in vivo activation of extracellular signal-regulated kinases by coal dusts and quartz silica. Toxicol Appl Pharmacol. 184;37–45.

[70] Gosset, P; Lassalle, P; Vanhée, D; Wallaert, B; Aerts, C; Voisin, C; Tonnel, A.B. (1991). Production of tumor necrosis factor-alpha and interleukin-6 by human alveolar macrophages exposed in vitro to coal mine dust. Am J Respir Cell Mol Biol. 5;431–6.

[71] Dwivedi, S; Saquib, Q; Al-Khedhairy, A.A; Ali, A.Y; Musarrat, J. (2012). Characterization of coal fly ash nanoparticles and induced oxidative DNA damage in human peripheral blood mononuclear cells. Sci Total Environ. 15;331–8.

[72] Diabaté, S; Mülhopt, S; Paur, H.R; Wottrich, R; Krug, H.F. (2002). In vitro effects of incinerator fly ash on pulmonary macrophages and epithelial cells. Int J Hyg Environ Health. 204;323–6.

[73] Diabaté, S; Bergfeldt, B; Plaumann, D; Ubel, C; Weiss, C. (2011). Anti-oxidative and inflammatory responses induced by fly ash particles and carbon black in lung epithelial cells. Anal Bioanal Chem. 401(10);3197–212.

[74] van Maanen, J.M; Borm, P.J; ,Knaapen A; van Herwijnen, M; Schilderman, P.A; Smith, K.R; Aust, A.E; Tomatis, M; Fubini, B. (1999). In vitro effects of coal fly ashes: hydroxyl radical generation, iron release, and DNA damage and toxicity in rat lung epithelial cells. Inhal Toxicol. 11;1123–41.

[75] Liou, G.Y; ,Storz P. (2010). Reactive oxygen species in cancer. Free Radic Res. 44(5);479–96.

[76] Haghdoost S, Czene S, Näslund I, Skog S, Harms-Ringdahl M. (2005). Extracellular 8-oxo-dG as a sensitive parameter for oxidative stress in vivo and in vitro. Free Radic Res. 39(2);153–62.

[77] Rohr, P; Kvitko, K; da Silva, F.R; Menezes, A.P; Porto, C; Sarmento, M; Decker, N; Reyes, J.M; Allgayer, Mda C; Furtado, T.C; Salvador, M; Branco, C; da Silva, J. (2013a). Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat Res. 758(1–2); 23–8.

[78] Rohr, P; da Silva, J; da Silva, F.R; Sarmento, M; Porto, C; Debastiani, R; Dos Santos, C.E; Dias, J.F; Kvitko, K. (2013b). Evaluation of genetic damage in open-cast coal mine workers using the buccal micronucleus cytome assay. Environ Mol Mutagen. 54(1);65–71.

[79] León-Mejía, G; Espitia-Pérez, L; Hoyos-Giraldo, L.S; Da Silva, J; Hartmann, A; Henriques, J.A; Quintana, M. (2011). Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci Total Environ. 409(4);686–91.

[80] León-Mejía, G; Quintana, M; Debastiani, R; Dias, J; Espitia-Pérez, L; Hartmann, A; Henriques, J.A; Da Silva, J. (2014). Genetic damage in coal miners evaluated by buccal micronucleus cytome assay. Ecotoxicol Environ Saf. 107;133–9.

[81] Al-Sabti, K; Lloyd, D.C; Edwards, A; ,Stegnar P. (1992). A survey of lymphocyte chromosomal damage in Slovenian workers exposed to occupational clastogens. Mutat. Res. 280;215–23.

[82] Ávila Júnior, S; Possamai, F.P; Budni, P; Backes, P; Parisotto, E.B; Rizelio, V.M; Torres, M; Celik, M; Donbak, L; Unal, F; Yuzbasioglu, D; Aksoy, H; Yilmaz, S. (2009). Cytogenetic damage in workers from a coal-fired power plant. Mutat Res. 627;158–63.

[83] Donbak, L; Rencuzogullari, E; Yavuz, A; Topaktas, M. (2005). The genotoxic risk of underground coal miners from Turkey. Mutat Res. 588(2);82–7.

[84] Minina, V.I; Kulemin, I.E; Tolotchko, T.A; Meĭer, A.V; Savtchenko, I.A; Volobaev, V.P; Gafarov, N.I; ,Semenikhina M.V. (2015). Genotoxic effects of occupational environment in Kuzbass miners. Med Tr Prom Ekol. (5);4–8.

[85] Stierum, R; Hageman, G; Welle, I; Albering, H; Schreurs, J; Kleinjans, J. (1993). Evaluation of exposure reducing measures on parameters of genetic risk in a population occupationally exposed to coal fly ash. Mutat Res. 319;245–55.

[86] Wolf, G; Arndt, D; Kotschy-Lang, N; Obe, G. (2004). Chromosomal aberrations in uranium and coal miners. Int J Radiat Biol. 80(2);147–53.

[87] Ulker, O; Ustundag, A; Duydu, Y; Yucesoy, B; Karakaya, A. (2008). Cytogenetic monitoring of coal workers and patients with coal workers' pneumoconiosis in Turkey. Environ Mol Mutagen. 49;232–7.

AQ7

ANEXO III

Curriculum vitae

GRETHEL LEÓN MEJIA

Curriculum vitae

Nome: **Grethel**

Sobrenome: **Léon Mejía**

Carteira de identidade: **25.800.386 de Montería.**

Nascimento: **16 de Abril de 1983.**

Lugar de Nascimiento: **Montería – Córdoba- Colômbia.**

Correio electrónico: grethelleon@gmail.com

ESTUDOS REALIZADOS

Formação acadêmica /titulação

2012-2016 Doutorado em Biología Celular e Molecular

Universidade Federal Do Rio Grande do Sul.

Título: AVALIAÇÃO CITOTÓXICA E GENOTÓXICA *in vitro*, E FISIOLOGIA RESPIRATORIA *in vivo*, DOS EFEITOS DE PARTÍCULAS DE CARVÃO E CINZAS PROVENIENTES DE SANTA CATARINA.

Orientador: João Antonio Pêgas Henriques.

2009-2011 Mestrado em Biología Celular e Molecular.

Universidade Federal de Rio Grande do Sul

Título: AVALIAÇÃO DOS EFEITOS GENOTÓXICOS E CITOGENÉTICOS NA POPULAÇÃO DE TRABALHADORES DE MINERAÇÃO DE CARVÃO DE CERREJÓN (GUAJIRA COLÔMBIA) UTILIZANDO DIFERENTES BIOMARCADORES.

Orientador: João Antonio Pêgas Henriques.

2000-2006 Graduação em Biología

Universidad de Córdoba- Colômbia.

Título: Estudio exploratorio de genotoxicidad por carbón en roedores mediante la técnica del ensayo cometa en la zona de influencia minera de Puerto Libertador – Córdoba.

Orientador: Milton Quintana Sosa.

1994-1999 Ensino medio

Colegio INEM "Lorenzo María Lleras". Montería – Córdoba.

ATIVIDADES COMPLEMENTARES

Participação em eventos

- Taller de Actualización en Vigilancia en Salud Pública. Secretaría de Salud y Seguridad Social. Colômbia. (2002).
- XII Seminario de Ciencias del Mar: Investigación y Desarrollo de Territorios Promisorios. INVEMAR – Universidad Nacional de Colômbia. (2003).
- IV Encuentro Regional Costa Caribe de Semilleros de Investigación. Universidad de Córdoba. Colômbia. (2004).
- Primer simposio Internacional Era Postgenómica y Avances en Medicina. Corporación Universitaria del Sinú. Colômbia. (2004).
- Seminario "Con Licencia para Soñar" ¿Cómo hacerme un Líder efectivo con Proyección Social? Universidad de Córdoba. Colômbia. (2004).
- I Simposio de Biología. Universidad de Córdoba. Colômbia. (2004).
- Primer Simposio Internacional de Resistencia Bacteriana e Infecciones Intrahospitalarias. Universidad del Sinú. Colômbia. (2005).
- IV Congreso Internacional y VII Congreso Colombiano de Genética. Universidad Industrial de Santander. Colômbia. (2006).
- I Encuentro Departamental de Semilleros de Investigación. Nodo Córdoba. Universidad de Córdoba. Colômbia. (2006).
- Diplomado en Docencia Universitaria en Competencias Laborales. Universidad Pontificia Bolivariana. Colômbia. (2007).
- VII congreso Latinoamericano de mutagénesis, carcinogénesis y teratogénesis ambiental y I Congreso Colombiano de mutagenesis y carcinogénesis ambiental - Genes, ambiente, cáncer, prevención y salud –Cartagena. Colômbia. (2007).
- IV COURSE OF THE LATIN AMERICAN SCHOOL OF HUMAN AND MEDICAL GENETICS. Caxias do Sul-Brasil. (2008).
- Curso "Epidemiología Molecular, Susceptibilidad Genética y Riesgo de Cáncer", Universidad del Cauca. Colômbia. (2008).

- ORGANIZACIÓN DEL I CURSO TEORICO EN EPIDEMIOLOGIA MOLECULAR, EPIGENETICA Y EXPOSICIÓN OCUPACIONAL A GENOTÓXICOS. Universidad del Sinú. Colômbia. (2009).

- Curso: Estrategia eficiente de búsqueda, organización, citación y generación de bibliografía para un documento: Manejo de la base de datos ISI Web of Knowledge y el software EndNote X2. Universidad de Córdoba. Colômbia. (2009).

- 30 Semana Científica do Hospital de Clínicas de Porto Alegre – Seminário em Genética Médica Populacional. Porto Alegre-Brasil. (2010).

- 30 Semana Científica do Hospital de Clínicas de Porto Alegre - Seminário da Magia á Ciência Experimental. Porto Alegre-Brasil. (2010).

- 30 Semana Científica do Hospital de Clínicas de Porto Alegre- Seminário em do Delineamento á Publicação. Porto Alegre-Brasil. (2010).

- 30 Semana Científica do Hospital de Clínicas de Porto Alegre – Seminário em Nanotecnologia Aplicada âs Ciências da Saúde. Porto Alegre-Brasil. (2010).

- Curso: VIII Course of Latin American School of Human and Medical Genetics. Caxias do Sul, Brasil. (2012).

- XV congreso Latinoamericano de Genética. XLI Congreso Argentino de Genética. XLIV Congreso de la Sociedad de Genética de Chile. Presentación de Póster "Evaluación de los efectos genotóxicos y citogenéticos en mineros de carbón usando biomarcadores". Argentina. (2012).

- Curso de Férias, quinta edição, verão 2013- "você conhece a célula?" Universidade Federal do Rio Grande do Sul. Brasil. (2013).

- 11th ICEM-International Conference on Environmental Mutagens, presentación del pôster: "Cytotoxic and genotoxic assessment of coal dust and coal fly ash samples in V79 cells". Foz de Iguaçu-Brasil. (2013).

- Workshop HUMN and HUMNxl-Lymphocyte micronucleus and buccal micronucleus cytome assays for Human an Environmental Monitoring. Canoas, Brasil. (2013).

- Curso de introdução a Microscopia Confocal de Fluorescência. Universidade Federal de Rio Grande do Sul, Brasil. (2013).

- Escola de Altos Estudos em Toxicologia: "Avanços Tecnológicos voltados à Toxicologia e a Saúde". Presentación del pôster: "Cytotoxic and genotoxic potential of coal dust and coal fly ash in V79 cells". Porto Alegre. Brasil. (2014).

- Curso Escola de Altos Estudos em Toxicologia: "Avanços Tecnológicos voltados à Toxicologia e a Saúde". Porto Alegre. Brasil. (2014).

- XII Congresso Brasileiro da MutaGen, SP, Brasil. (2016).

EXPERIÊNCIA LABORAL

Projeto PAB Secretaria de Saúde. Colômbia. 2004.

Pesquisadora na Universidade do Sinú - Projetos: 1) "Estudio exploratorio de genotoxicidad por carbón en roedores mediante la técnica Ensayo Cometa (SCGE) en la zona de Explotación Minera de Puerto Libertador, Córdoba" – Colômbia. 2005-2006.

2) "Evaluación de la exposición, efectos genotóxicos y susceptibilidad genética en una población ocupacionalmente expuesta a residuos de minería de carbón". Colômbia. 2008-2010.

Docente-Pesquisador na Universidade Simón Bolívar, 2015 - até data atual. - Projetos: 1) "Evaluación del riesgo ocupacional de soldadores mediante el análisis de daño en el ADN, de metales y de la influencia de polimorfismos en genes del metabolismo y reparación del ADN".

2) "Efecto de las emisiones diesel y su asociación con los genes polimórficos del metabolismo (CYP1A1, GSTM1, GSTT1) y los genes de reparación (OGG1, XRCC1), en mecánicos ocupacionalmente expuestos". Colômbia.

ARTIGOS PUBLICADOS:

GRETHEL LEON MEJIA, MILTON QUINTANA SOSA, LYDA ESPITIA, JUAN CARLOS LINARES, "Estudio exploratorio de genotoxicidad por carbón en Roedores Mediante la Tecnica de Ensayo Cometa en la zona de Influencia Minera de Puerto Libertador-Córdoba". Salud UIS ISSN: 0121-0807 ed: Universidad Industrial de Santander. v.38 fasc.1 p.45 – 47. 2006.

GRETHEL LEON MEJIA, MILTON QUINTANA SOSA, LYDA MARCELA ESPITIA PEREZ, JUAN CARLOS LINARES ARIAS, ANDREAS HARTMANN, "Genotoxic Effects in wild rodents (Rattus rattus and Mus musculus) in an Open Coal Mining Area". Mutation Research-Genetic Toxicology and Environmental Mutagenesis. ISSN: 1383-5718 ed: Elsevier v.630 fasc.1 p.42 - 49 , 2007.

León-Mejía G, Espitia-Pérez L, Hoyos-Giraldo LS, Da Silva J, Hartmann A, Henriques JA, Quintana M. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci Total Environ. Jan 15;409(4):686-91. 2011.

León-Mejía G, Quintana M, Debastiani R, Dias J, Espitia-Pérez L, Hartmann A, Henriques JA, Da Silva J.Genetic damage in coal miners evaluated by buccal micronucleus cytome assay. Ecotoxicol Environ Saf. 107:133-9. doi: 10.1016/j.ecoenv.2014.05.023. 2014.

Capítulo de livro: "Occupational Exposure to Coal, Genotoxicity and Cancer Risk". revista "InTech - Environmental Health Risks". ISBN 978-953-51-4706-0. Estado: Aceito para Publicação no 2016.