UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DOS EFEITOS TOXICOLÓGICOS *IN SILICO, IN VITRO* E *IN VIVO* DO CARVÃO BETUMINOSO E SUB-BETUMINOSO DA COLÔMBIA

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor.

Orientador

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*"Soy de los que piensan que **la ciencia tiene una gran belleza**. Un científico en su laboratorio no es sólo un técnico: **es también un niño colocado ante fenómenos naturales** que le impresionan como un cuento de hadas"*

Marie Curie

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ABREVIATURAS

ATM *Ataxia-Telangiectasia Mutated* ou proteína mutada na ataxia telangectasia

ATR *Ataxia Telangiectasia and Rad3-related protein* ou Cinase ataxia telangiectasia e RAD3 relacionada

BN Células binucleadas

CBMN-cyt *Cytokinesis-block micronucleus cytome assay* ou Ensaio do micronúcleo citoma com bloqueio da citocinese.

CC Células com cromatina condensada

FPG Formamidopirimidina DNA glicosilase

IARC Agência Internacional de Pesquisa do Câncer

KHC Células cariorréticas

KYL Células cariolíticas

MN Micronúcleos

MNBN Micronúcleo em células binucleadas

MNMONO Micronúcleo em células mononucleadas

NBUDS *Nuclear buds* ou Brotos nucleares

PAH / HAP *Polycyclic Aromatic Hydrocarbons* ou Hidrocarbonetos Aromáticos Policíclicos

PIXE *Particle-induced X-ray emission* ou Emissão de raios-X induzidas por partículas

MP/PM Material particulado

ROS / ERO *Reactive Oxygen Species* ou Espécies Reativas de Oxigênio

%Tail DNA Percentagem de DNA na cauda

RESUMO

A Colômbia é o país com as maiores reservas de carvão da América Latina, localizadas principalmente no norte do país, na região da Costa Atlântica. Nos departamentos de La Guajira, Cesar e Córdoba são encontrados 90% do carvão térmico. Nestas regiões, a mineração de carvão acontece a céu aberto, ocorrendo a liberação de material particulado com uma mistura de compostos químicos no ambiente. Esta mistura complexa está associada à indução de efeitos genotóxicos em diferentes organismos. Ainda assim, é pouca a informação da composição química e dos efeitos biológicos gerados pelo carvão da Colômbia. O objetivo deste trabalho foi caracterizar os principais compostos químicos do carvão betuminoso da mina El Cerrejón e sub-betuminoso da mina La Guacamaya, Colômbia, e avaliar seus efeitos biológicos utilizando modelos *in sílico, in vitro* e *in vivo.* A caracterização das amostras de carvão identificou os principais óxidos inorgânicos, elementos inorgânicos e hidrocarbonetos aromáticos policíclicos. Alguns destes compostos podem induzir a geração de Espécies Radicais de Oxigênio (ERO), que leva a estresse oxidativo, e provoca a interrupção do ciclo celular como reação aos danos no DNA. Além disso, os carvões betuminoso e sub-betuminoso da Colômbia induzem citotoxicidade, lesões primárias no DNA e instabilidade cromossômica em níveis similares, associadas ao dano oxidativo detectado *in vitro.* Da mesma forma, foi demostrado in vivo que a inalação subaguda de carvão sub betuminoso, produz acumulação de pó de carvão no tecido do pulmonar, resultando na indução de ERO e lesões primárias no DNA em células do sangue periférico e pulmão, além de provocar um aumento nos níveis das defesas antioxidantes e a liberação de citocinas pró-inflamatório. Em conclusão, os efeitos observados in sílico, in

vitro e in vivo estão relacionados com a capacidade de alguns compostos químicos do carvão da Colômbia de induzir a geração de estresse oxidativo celular e tecidual.

ABSTRACT

Colombia has the largest coal reserves in Latin America. Mineral reserves are located mainly in the north (Caribbean region), where 90% of the thermal coal is located in the departments of La Guajira, Cesar, and Córdoba. In these regions, coal mining is open-pit, which is related to the release of particulate material into the environment with a mixture of chemical compounds. This complex mixture is associated with thegenotoxic effects in different organisms. However, the information about chemical composition and biological effects generated by coal in Colombia is scarce. Our objective was to characterize the main chemical compounds of the bituminous coal from El Cerrejón and sub-bituminous mine from La Guacamaya mine in Colombia and to evaluate the biological effects using *in vitro*, *in vitro,* and *in vivo* models. Characterization of the carbon samples identified the main inorganic oxides, inorganic elements, and polycyclic aromatic hydrocarbons. Some of these compounds may induce the generation of ROS, which leads to oxidative stress and cause cell cycle disruption as a reaction to DNA damage. In addition, bituminous and sub-bituminous coals induce cytotoxicity, primary DNA damage, and chromosomal instability at similar levels associated with oxidative damage detected *in vitro*. Likewise, it has been shown that subacute inhalation of sub-bituminous carbon, produces accumulation of coal dust in lung tissue *in vivo*. This leads to the induction of ROS and, consequently, causes oxidative stress, primary lesions in the DNA in cells of the peripheral blood and lung. In addition, it causes increased levels of antioxidant defenses and markers of oxidative damage, as well as the release of proinflammatory cytokines. In conclusion, the effects observed in vitro, in vitro and in vivo are related to the ability of some chemical compounds of coal in Colombia to induce oxidative stress.

ESTRUTURA DO TRABALHO

O presente trabalho está estruturado em introdução, objetivos (gerais e específicos), três capítulos escritos em formato de artigos científicos, discussão geral, conclusões, perspectivas, referências bibliográficas e anexos.

A introdução contém informações gerais sobre o carvão, classificação dos carvões, usos, produção mundial e na Colômbia, mineração a céu aberto, efeitos da mineração, efeitos biológicos dos compostos químicos do carvão e usos e aplicação da biologia de sistemas.

O capítulo I apresenta um manuscrito que aborda a caracterização química dos principais elementos do carvão betuminoso e sub-betuminoso e os possíveis efeitos na saúde humana focado nas alterações do ciclo celular, empregando uma abordagem de biologia de sistemas.

No capítulo II é apresentado os efeitos citotóxicos, genotóxicos e mutagênicos da exposição ao carvão betuminoso e sub-betuminoso com o uso de células V79 (fibroblastos de pulmão de hamster chinês).

O capítulo III expõe os efeitos toxicológicos *In Vivo* da exposição inalada de pó carvão sub-betuminoso, em ratos Wistar machos.

A seguir, é apresentada a discussão geral, que integra os resultados dos três capítulos, as possíveis hipóteses que poderiam explicar os resultados obtidos e sua contribuição. Para finalizar, são apresentadas as conclusões, perspectivas e anexos do presente trabalho.

Nos anexos 1 e 2 são apresentados o certificado do Comitê de Ética para Uso de Animais,

da Universidade Luterana do Brasil – ULBRA, e o Atestado Sanitário do Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL), da Universidade Federal do Rio Grande do Sul – UFRGS. Por fim, o anexo 3 apresenta o currículo

1. INTRODUÇÃO

1.1. Carvão

O carvão é um mineral usado como combustível fóssil formado em um processo natural, que pode ocorrer em ambientes anaeróbios, onde a matéria orgânica é enriquecida com carbono pela perda progressiva de hidrogênio, nitrogênio e oxigênio ao longo de milhões de anos (El Cerrejon S.A., 2011; International Energy Agency, 2016). Os principais compostos do carvão são o carbono, o hidrogênio, o oxigênio, o nitrogênio e o enxofre, que podem estar associados com outros elementos rochosos, como arenito, siltito, folhelhos e diamictitos e/ou minerais como a pirita (Chadwick et al. 2013).

1.2. Classificação do carvão

A *American Society for Testing and Materials* classificou o carvão por sua composição vegetal e pelas condições de pressão e temperatura durante o processo de formação. A classificação divide os carvões em quatro tipos: (1) antracito, (2) betuminoso, (3) sub-betuminoso e (4) lignito (UPME 2005).

1.2.1. Carvão antracito

O carvão antracito é caracterizado por apresentar coloração negra e brilho semimetálico, uma maior porcentagem de carbono (86% - 98%), uma baixa porcentagem de matéria volátil (1%) e uma baixa humidade. Além disso, seu poder calorífico chega a mais de 32,6 MJ/kg. Essas características tornam um carvão de melhor qualidade e pouco contaminante (UPME 2005).

1.2.2. Carvão betuminoso

Este tipo de carvão, chamado de hulha, também é conhecido como carvão de coque. Possui um teor de carbono entre 45 – 86%, uma maior porcentagem de umidade, e poder calorífico que varia de 24,5 - 32,6 MJ/kg (UPME 2005).

1.2.3. Carvão sub-betuminoso

Carvão com menor poder calorífico, que varia de 8,2 a 24,5 MJ/kg. Seu teor de carbono oscila entre 35 e 45%, e a porcentagem de umidade 20 – 30%, além de um alto teor de matéria volátil. Alguns destes carvões apresentam poder metalúrico (UPME 2005).

1.2.4. Carvão lignito

Carvão formado pela compressão da turfa, caracterizado pela presença de alta umidade (> 30%), baixo teor de carbono (25 – 35%) e alto teor de cinzas junto com materiais voláteis (96%). Portanto, possui um poder calorífico baixo (9,3 - 18,2 MJ/kg) (UPME 2005).

1.3. Usos do carvão

Dependendo da classificação do tipo de carvão, que inclui a composição e poder calorífico, este pode ser usado para diferentes finalidades. O carvão do tipo **antracito** é usado para a geração de calor ou vapor nas indústrias de aço e ferro, e na fabricação de borracha sintética, corantes e nos filtros de purificação de água para consumo humano. O carvão do tipo b**etuminoso** é usado nos processos de obtenção de aço e na produção de vapor para geração de energia elétrica. O carvão do tipo s**ub-betuminoso** é utilizado na geração de energia elétrica e em alguns processos industriais. Os carvões de tipo l**ignito s**ão usados para geração de calor (sistemas de aquecimento em casas) e em alguns processos

industriais, para gerar vapor. Além disso, o carvão também é utilizado na produção de benzeno, óleos, alcatrão e, por meio da liquefação, como substituto do petróleo (UPME 2005).

1.4. Reservas de carvão no mundo

As reservas de carvão no mundo (Fig. 1) somam aproximadamente 847,5 bilhões de toneladas, sendo que as maiores porcentagens (52,7%) são de carvão dos tipos antracito e betuminosos, e os 47,3% restantes são de carvão sub-betuminoso e lignito (UPME 2005). Essas reservas são suficientes para atender às necessidades atuais e por mais 130 anos (British Petroleum 2017).

As reservas de carvão estão distribuídas pelo mundo em quase todos os continentes e as maiores quantidades estão localizadas no hemisfério norte. Assim, porcentagens de reservas que equivalem a mais de 60% do volume total de carvão no mundo são encontradas em países como Estados Unidos (28,6%), Rússia (18,5%) e China (13,5%) (British Petroleum 2017).

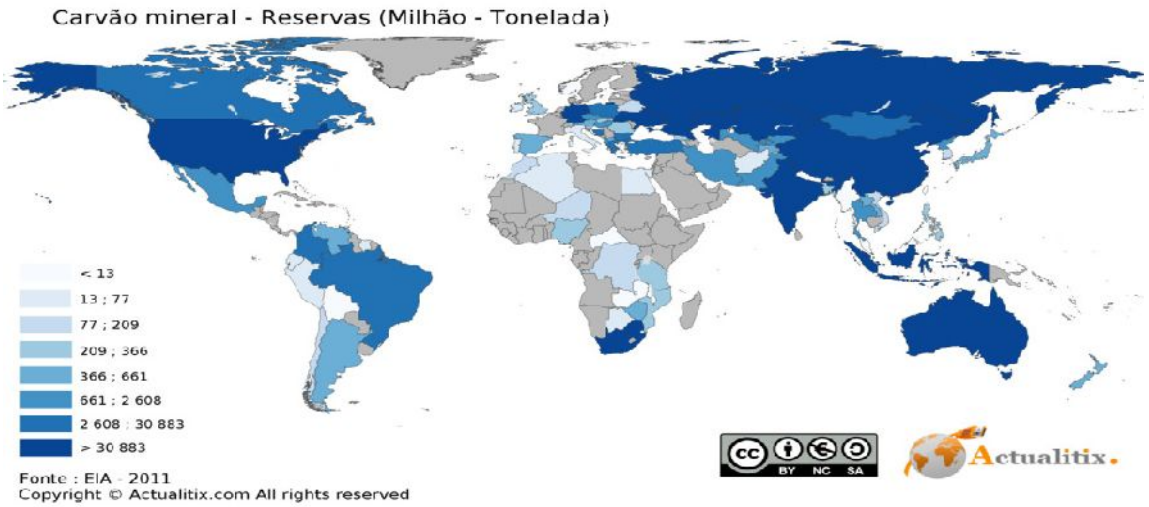

Figura 1. Distribuição das reservas de carvão mineral no mundo em milhões de toneladas.
Fonte: https://es.actualitix.com/pais/wld/carbon-reserva.php

1.5. Reservas de carvão na Colômbia

A Colômbia é o país com as maiores reservas de carvão da América Latina e é considerado um dos países exportadores de carvão mais jovens no mundo (Rudas 2014). Possui recursos potenciais de 16.992 milhões de toneladas (Mt), e exportações de 85 Mt por ano (Agencia Nacional de Mineria 2013; Jähnig and Volkmann 2009). A maior parte da atividade mineradora do país está focada na produção de carvão mineral (47%), que equivale a 2 % do produto interno bruto (PIB) (Agencia Nacional de Mineria 2013; Energética. 2007).

As reservas do mineral estão localizadas principalmente no norte da Colômbia, na região da Costa Atlântica, onde são encontrados 90% das reservas de carvão da melhor qualidade, que equivalem a 98% do carvão nacional (UPME 2012). Estas reservas estão situadas nos Departamentos de Guajira, Cesar, Córdoba, Norte de Santander, Cundinamarca, Boyacá, Antioquia, Valle del Cauca e Cauca (Fig. 2).

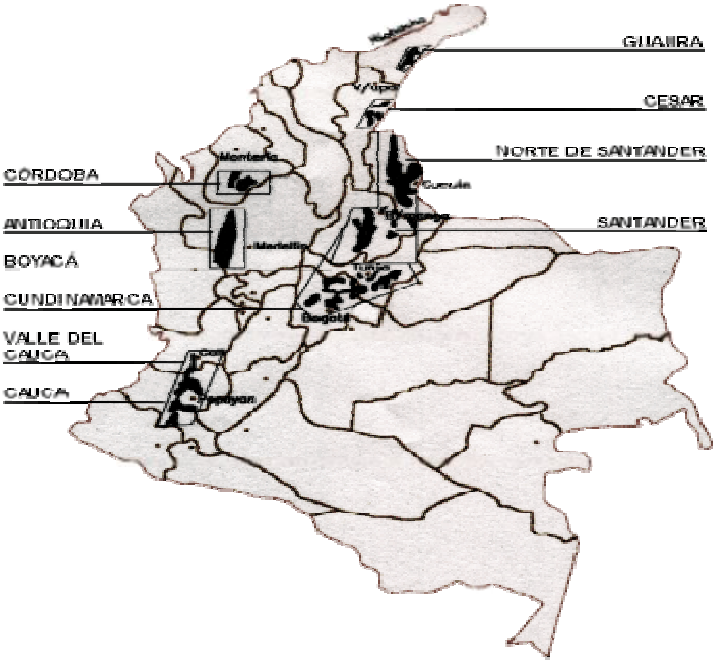

Figura 2. Zonas carboníferas da Colômbia.
Fonte: http://www.upme.gov.co/guia_ambiental/carbon/areas/zonas/indice.htm.

1.5.1. Mina de Carvão El Cerrejón

A mina de carvão El Cerrejón é a maior mina de carvão a céu aberto do mundo e está localizada no extremo nordeste do país, ao sul do Departamento de La Guajira. Ela possui reservas estimadas em 2.193 Mt de carvão numa extensão de terra de 69 mil hectares, distribuídas em cinco áreas: Zona Norte, Patilla, Oreganal, Zona Central e Zona Sul. El Cerrejón possui carvão do tipo betuminoso, com alto poder calorífico, baixo teor de cinzas e baixa concentração de elementos-traço (UPME 2012). Algumas análises químicas feitas neste carvão indicam que mais de 80% do peso do material mineral do carvão de EL Cerrejón está composto por argila e quartzo (silicato de alumínio - Alsil, e sílica – Si) (Irons 2000). A produção deste tipo de carvão com altos teores de sílica tem sido relacionada com altos níveis de poluição da água potável das comunidades perto da mina (Pulido 2007).

1.5.2. Mina de carvão La Guacamaya

A mina La Guacamaya é uma mina de carvão a céu aberto localizada no Departamento de Córdoba entre os vales dos rios San Jorge, San Pedro e Uré. Possui reservas calculadas em 381 Mt, distribuídas em quatro camadas, com espessuras entre 0,8 a 3,5 metros. Também possui carvão Sub-betuminoso de tipo A o qual apresenta um teor total de enxofre de 2,3%, e alto teor de material volátil (Prada et al. 2016). A mineração deste tipo de carvão tem sido relacionada à genotoxicidade ambiental em algumas espécies de roedores das áreas de exploração (Leon et al. 2007).

1.6. Mineração do carvão a céu aberto

As minas a céu aberto são chamadas assim porque suas atividades se desenvolvem na superfície da terra. Para sua exploração, é necessário fazer a escavação da mina, que acontece de duas formas: com meios mecânicos, ou seja, com o uso de máquinas ou com o uso de explosivos. O processo de mineração inicia com a retirada da cobertura de solo até que os mantos de carvão sejam desvendados. Os resíduos adicionais de rocha são recolhidos e armazenados na área de rejeitos até serem recolocados na mina, quando o processo de mineração finaliza (Aguiar et al. 2008).

Depois deste processo inicial de retirada da cobertura, ocorre uma operação sequencial que envolve os processos de perfuração e detonação da rocha e do carvão para dividi-los e facilitar sua extração. Após, são carregadas e transportadas as rochas e resíduos de carvão em caminhões de alta capacidade, que transportam o material para as áreas de eliminação ou de manipulação do carvão. A seguir, inicia-se a preparação do carvão, na qual uma parte desse é lavado para que ocorra a diminuição do teor de cinzas. Finalmente, o carvão é transportado até o local de armazenamento (Aguiar et al. 2008).

1.6.1. Impactos da mineração de carvão a céu aberto

A mineração de carvão a céu aberto está relacionada com a liberação de material particulado (MP) no ambiente. Este MP está associado com o desenvolvimento de efeitos genotóxicos em populações com exposição ocupacional ou ambiental. As partículas que são liberadas no ambiente incluem uma mistura de elementos químicos, tais como hidrocarbonetos aromáticos policíclicos (HAP) e elementos inorgânicos (EI) (Espitia-Pérez et al. 2018).

Alguns estudos mostraram que o material gerado pela exploração do carvão pode levar ao aumento da mortalidade causada por câncer colorretal e câncer de pulmão (Fernandez-Navarro et al. 2012). Além disso, tem relação com a alta mortalidade por distúrbios cardíacos, respiratórios e renais (Hendryx 2009), a pneumoconiose dos mineiros de carvão (Finkelman et al. 2002; Love et al. 1997), o incremento na frequência de doenças respiratórias e casos graves de asma (Pless-Mulloli et al. 2000).

1.7. Material particulado do carvão

O MP é um conjunto de partículas sólidas ou líquidas suspensas e dispersas no ar (WHO 2004) que variam em composição química, morfologia (tamanho/forma), parâmetros ópticos (cor/espalhamento da luz) e características elétricas (carga/resistência). Esse material pode ter origem natural ou artificial. As partículas naturais incluem aquelas formadas pela evaporação do mar sob a forma de gotículas, os pólens, as poeiras, as erupções vulcânicas e outros fenômenos geotérmicos. As partículas artificiais são aquelas geradas por motores de veículos, caldeiras industriais e fumo do cigarro, entre outras (Olsson et al. 2010). Dependendo da fonte e da forma como estas partículas interagem com outros compostos químicos presentes na atmosfera, sua composição química pode variar, tendo, inclusive, influência sobre os impactos causados na saúde humana (Armstrong et al. 2004). As queimadas também são importantes fontes de poluição do ar com MP e está poluição está associada com o desenvolvimento de problemas respiratórios nas populações que inalam este material (Queiroz et al. 2007).

O MP gerado pelas minas de carvão a céu aberto é caracterizado por misturas complexas (Pulido 2007) de partículas que variam em tamanho, morfologia, características químicas e físicas, dependendo do tipo de carvão extraído (López and Ward 2008; Valentim

et al. 2009). A produção do MP acontece desde o início das atividades de mineração, com a retirada do material "estéril" e a sua acumulação na área de rejeito, e vai aumentado durante as atividades de mineração associadas, tais como perfuração, detonação, carregamento e descarga do carvão e transporte em estradas de chão batido (Chadwick et al. 2013). Além disso, o carvão é extraído e armazenado em áreas de temperaturas altas e sem proteção contra o sol, sendo capaz de gerar combustão incompleta e espontânea, que contribui com a formação de hidrocarbonetos aromáticos policíclicos (HAP) (Leon-Mejia et al. 2011). As atividades de mineração do carvão também são responsáveis por 60% das emissões de dióxido de enxofre, um dos precursores da chuva ácida, sendo uma das causas associadas com a geração de doenças respiratórias (International Energy Agency 2016b).

1.7.1. Composição do MP do carvão

Alguns estudos têm mostrado a presença no MP do entorno de minas de carvão de compostos como óxidos de enxofre (SOx), óxidos de nitrogênio (NOx) e monóxido de carbono (CO) (International Energy Agency 2016b), elementos inorgânicos e HAP (Espitia-Pérez et al. 2018; León-Mejía et al. 2016).

1.7.1.1. Óxidos

Os óxidos são formados pela reação de um composto químico com o oxigênio, e os diferentes elementos químicos que constituem o carvão contribuem na formação óxidos (He et al. 2014). O enxofre é encontrado no carvão em diferentes teores conforme o tipo de carvão extraído. Quando o carvão é usado para a geração de energia ou em alguns processos industriais, o enxofre pode transformar-se em óxido de enxofre (SO_X), que é liberado na atmosfera. Um exemplo em particular é o dióxido de enxofre (SO_2), que está

associado a efeitos adversos na saúde e no ambiente (Uren et al. 2014). Outros óxidos que também podem ser liberados no ambiente são óxido de nitrogênio (NO) e dióxido de nitrogênio (NO_2). Os mesmos são produzidos pela combustão de carvão em altas temperaturas na geração de energia e/ou a partir da oxidação de NO a NO_2 na atmosfera. O NO_2 é um gás tóxico que ajuda na formação do ozônio (He et al. 2014). Além desses, outro óxido encontrado é o monóxido de carbono (CO), um gás tóxico incolor e inodoro, resultado da combustão incompleta de carvão ou madeira (International Energy Agency 2016b).

1.7.1.2. Hidrocarbonetos aromáticos policíclicos (HAPs)

Os HAPs são substâncias orgânicas constituídas por átomos de carbono e hidrogênio agrupados em pelo menos duas estruturas de anéis aromáticos condensados ou fundidos (Canadian Council of Ministers of the Environment 2010). Esses compostos apresentam propriedades lipofílicas que facilitam sua penetração através das membranas celulares (Yu et al. 2011). Os HAPs são divididos em duas categorias: os de baixo peso molecular, compostos com menos de cinco anéis, e os de elevado peso molecular, constituídos por cinco ou mais anéis. Na Figura 3 são apresentadas as estruturas dos HAPs mais comumente estudados.

Em geral, as populações são expostas aos HAPs pela inalação de ar contaminado, ingestão de alimentos contaminados, hábitos como o tabagismo, ou pela respiração de fumaças como as produzidas pelas lareiras (ACGIH 2005). No ar, os HAPs têm meia vida curta, mas esta pode aumentar quando estes misturam-se aos elementos químicos no MP do ambiente (Mumtaz et al. 1996). Os HAPs presentes no ambiente são principalmente gerados pela combustão incompleta de combustíveis que possuem carbono, e são oriundos

de fontes naturais, industriais, comerciais, veiculares e residenciais. Tem sido demostrada a presença destes elementos nas áreas de mineração de carvão do El Cerrejón, como produto da combustão espontânea (Stracher et al. 2010).

A exposição ocupacional aos HAPs acontece durante a respiração dos gases de escape gerados pelos veículos usados nas minas e envolve também todos os trabalhadores do processo de mineração, metalurgia, ou refino do petróleo (Armstrong et al. 2004; Wei See et al. 2006). Em todos estes ambientes, as pessoas estão expostas a ingestão, inalação e contato dérmico com HPAs (Ravindra et al. 2008).

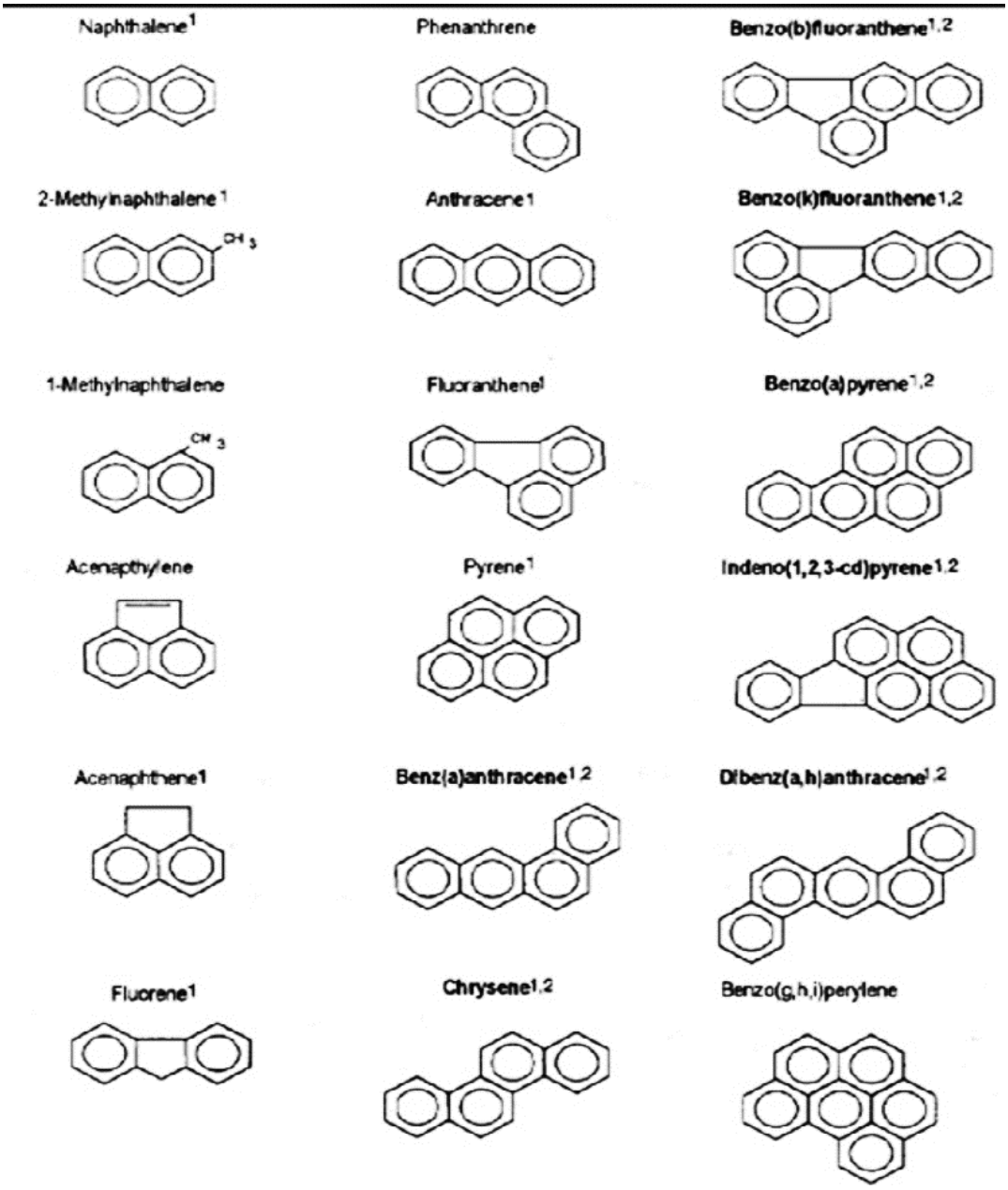

Figura 3. Hidrocarbonetos aromáticos policíclicos (HAPs) mais comumente analisados. **Fonte:** https://www.researchgate.net/figure/The-most-commonly-analyzed-polycyclic-aromatic-hydrocarbons-PAHs-9_fig1_288258362

A exposição aos HAP por curtos períodos de tempo (exposição aguda) tem sido relacionada com efeitos na saúde como, por exemplo, redução da função pulmonar em

asmáticos e alguns efeitos trombóticos em pessoas com doença cardíaca coronariana (ACGIH 2005).

Também é bem conhecido que as misturas de poluentes que contêm níveis elevados de HAP levam a irritação ocular, náuseas, vómitos, diarreia, entre outros sintomas (Unwin et al. 2006). Existem estudos que mostram que trabalhadores expostos às misturas que contenham HAPs (assim como outros produtos químicos) apresentam uma série de problemas na saúde, além do aumento do risco de câncer de pele, pulmão, bexiga e gastrointestinal (Bach et al. 2003; Boffetta et al. 1997; Olsson et al. 2010). Em tempo prolongado de exposição aos HAPs (exposição crônica), os efeitos sobre a saúde são maiores e incluem a redução da função imunológica, catarata, danos no rim e fígado (ATSDR 1995), problemas respiratórios e alterações da função pulmonar (Srogi 2007, Rengarajan et al. 2015).

1.7.1.3. Elementos Químicos Inorgânicos

Os elementos químicos inorgânicos são um tipo de matéria constituída por átomos da mesma classe. Os metais pesados são elementos químicos que se originam de forma natural e são encontrados em toda a crosta terrestre (Oliveira et al. 2013). Estes elementos são capazes de gerar contaminação ambiental quando removidos do local de formação por ação humana, como a mineração, fundição, produção industrial e uso doméstico e agrícola de compostos com metais (He et al. 2005; Herawati et al. 2000). Alguns estudos que avaliam os impactos ambientais da mineração de carvão e a liberação de metais têm

demostrado alterações na acidez do solo e liberação de metais tóxicos (Adriano 2001), além de danos na vegetação (Madejón et al. 2002).

Altas concentrações de elementos-traço no meio ambiente podem gerar efeitos nocivos no organismo, que estariam associados à alta toxicidade desses elementos e à capacidade de bioacumulação. Neste cenário, estes elementos podem induzir danos ao DNA (Sunjog et al. 2016) e câncer em humanos (Moran-Martinez et al. 2013) e/ou animais (Lanctot et al. 2016; Lourenco et al. 2013).

Nos processos de extração de carvão são liberados ao ambiente alguns metais pesados como cobre (Cu), chumbo (Pb), cádmio (Cd), níquel (Ni), vanádio (V), zinco (Zn) e enxofre (S), os quais aparecem associados a problemas da toxicidade do carvão (Tchounwou et al. 2012).

1.8. Efeitos Toxicológicos da Exposição a Carvão

As principais doenças ocupacionais geradas pela exposição ao MP gerado pelas atividades de mineração de carvão a céu aberto são a pneumoconiose simples dos trabalhadores de carvão, a fibrose massiva progressiva e a doença pulmonar obstrutiva crônica (Donoghue 2004). Além disso, alguns estudos realizados em populações com exposição ao pó de carvão sugerem que os efeitos observados na saúde estão relacionados não só a eventos ocasionais de aumento nos níveis de MP de carvão, mas também quando estes níveis estão abaixo do permitido pelos padrões de qualidade do ar estabelecidos pelas agencias mundiais (WHO 2004).

A agência de proteção ao ambiente dos Estados Unidos (USEPA) demostrou que a poluição gerada pelo MP tem relação com alterações na função pulmonar, com o desenvolvimento de doenças respiratórias e também com o aumento da mortalidade a curto

prazo (Yu et al. 2011). Outras alterações ocasionadas pelo material particulado no meio ambiente incluem as mudanças climáticas pela absorção e reflexão da radiação do sol (EPA 1996).

1.8.1. Efeitos da exposição a hidrocarbonetos aromáticos policíclicos

Os efeitos genotóxicos gerados pela maioria dos HAPs têm sido demostrados em roedores e em algumas linhagens celulares de mamífero (ACGIH 2005). Quando os HAPs entram no organismo, sofrem ativações enzimáticas pelo complexo citocromo *P450* que produz metabólitos altamente eletrofílicos que podem interagir com o DNA e RNA e levar ao surgimento de tumores (Bernardo et al. 2016). No caso do benzo(a)pireno, a oxidação enzimática com a P450 é seguida pela hidrólise, levando à formação de benzo(a)pireno-7,8-diol-9,10- epóxido (Fig. 4), que apresenta caráter eletrofílico, favorecendo a união entre HPA e o DNA (Bernardo et al. 2016). Esta união pode induzir mutações que levam a ativação de oncogenes ou inativação de genes supressores de tumores, fazendo parte do mecanismo carcinogênico (Jarvis et al. 2014).

Estas mutações ocasionadas pela exposição aos HAPs podem levar ao descontrole do ciclo celular e aumento nos danos do DNA e que, finalmente, conduzem a carcinogênese. A exposição a HAPs foi previamente associada com mutações de p53 em câncer de pulmão e mama em humanos (Mordukhovich et al. 2010).

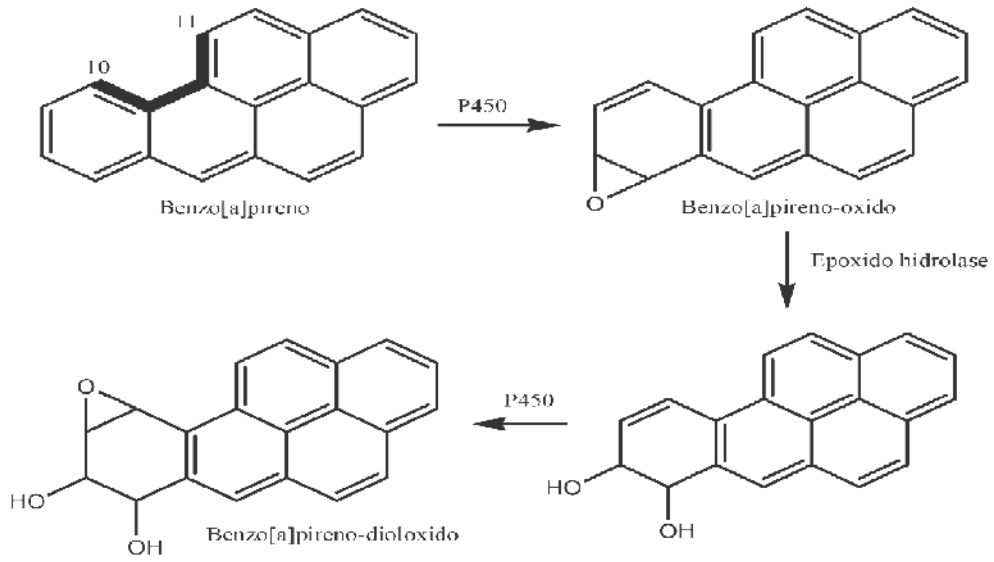

Figura 4. Ativação metabólica do benzo[a]pireno com formação de diolepóxido. Fonte: (Bernardo et al. 2016).

A mineração de carvão a céu aberto dá origem a misturas complexas que incluem a presença de HAPs com potencial carcinogênico fraco, mas que ao serem misturadas com alguns elementos do ambiente, potencializam da sua toxicidade (USEPA 2010).

1.8.2. Efeitos Toxicológicos da Exposição a metais pesados

Os metais pesados podem ocasionar genotoxicidade via indução de estresse oxidativo (D Beyersmann and Hartwig 2008), modulação dos mecanismos de reparo do DNA (A, H. 2007) e desregulação da proliferação celular.

Os metais podem ser classificados, pela toxicidade e abundância no meio-ambiente, em: não críticos, como Na, K, Ca, Mg, Fe e Al; tóxicos muito raros ou insolúveis, como W, Zr, Ba e Ti; e muito tóxicos e relativamente acessíveis, como Ni, Cu, Zn, As, Cd, Hg e Pb (Förstner and Wittmann 1981). Esta toxicidade dos metais está

relacionada com os mecanismos de captação pelas membranas celulares, distribuição intracelular e biodisponibilidade (D Beyersmann and Hartwig 2008). Metais como Fe, Cu, Cd, Cr, Ni, entre outros, têm a capacidade de gerar espécies reativas de oxigênio (ERO), que podem induzir peroxidação lipídica, dano ao DNA, depleção de grupamentos tióis, alterar vias de transdução de sinais e a homeostase do cálcio (Stohs and Bagchi 1995).

As ERO formados na reação oxidativa de Fenton, ou pela indução de um processo inflamatório ou pela formação intermediaria de tioradicais, geram danos no DNA que podem ocorrer de forma direta ou indiretamente, e a maior parte destes danos são reparados pelos mecanismos celulares, sendo que apenas uma fração deste dano resulta numa mutação (Cadenas and Davies 2000).

O aumento nos níveis de ERO podem provocar alterações no equilíbrio redox intracelular, o que afeta as vias de transdução de sinais, que ativam ou inativam múltiplos fatores de transcrição relevantes para a funcionamento celular, que podem levar ao desenvolvimento do câncer (Cadenas and Davies 2000).

1.9. Efeitos Toxicológicos Gerados pela Exposição ao Carvão

1.9.1. Aumento de Espécies Reativas de Oxigênio e Estresse Oxidativo

Todos os organismos aeróbios utilizam o oxigênio como aceptor final de elétrons na sua cadeia transportadora para a geração de energia ATP. A redução do oxigênio à água, entretanto, pode formar subprodutos altamente reativos, conhecidos como ERO (Da Silva and Gonçalves 2010). A produção de ERO, e as espécies reativas de nitrogênio (ERN), fazem parte do metabolismo humano e é notada, por exemplo, em processos como a fagocitose, onde são produzidas para eliminar o agente agressor (Wulf 2002). Exemplos

destas espécies reativas são o ânion superóxido ($O2^{\bullet -}$), o ânion peroxinitrito ($ONOO^-$), o peróxido de hidrogênio (H_2O_2), o óxido nítrico ($NO^{\bullet}$) e o radical hidroxila ($OH^{\bullet}$) (Wulf 2002). O estresse oxidativo é conhecido como a perda do equilíbrio fisiológico entre os níveis de radicais livres e suas defesas antioxidantes na célula, tecido ou órgão (Pradeep et al. 2013). Porém, a produção elevada de espécies reativas pode levar a morte celular por necrose ou apoptose, por meio de interação com lipídeos, proteínas e DNA (Da Silva and Gonçalves 2010; Valko et al. 2006). Os mecanismos dos sistemas celulares permitem a internalização das partículas do MP, sendo logo ativados indiretamente para a produção de ERO/ERN (Fig. 5) (Knaapen et al. 2004).

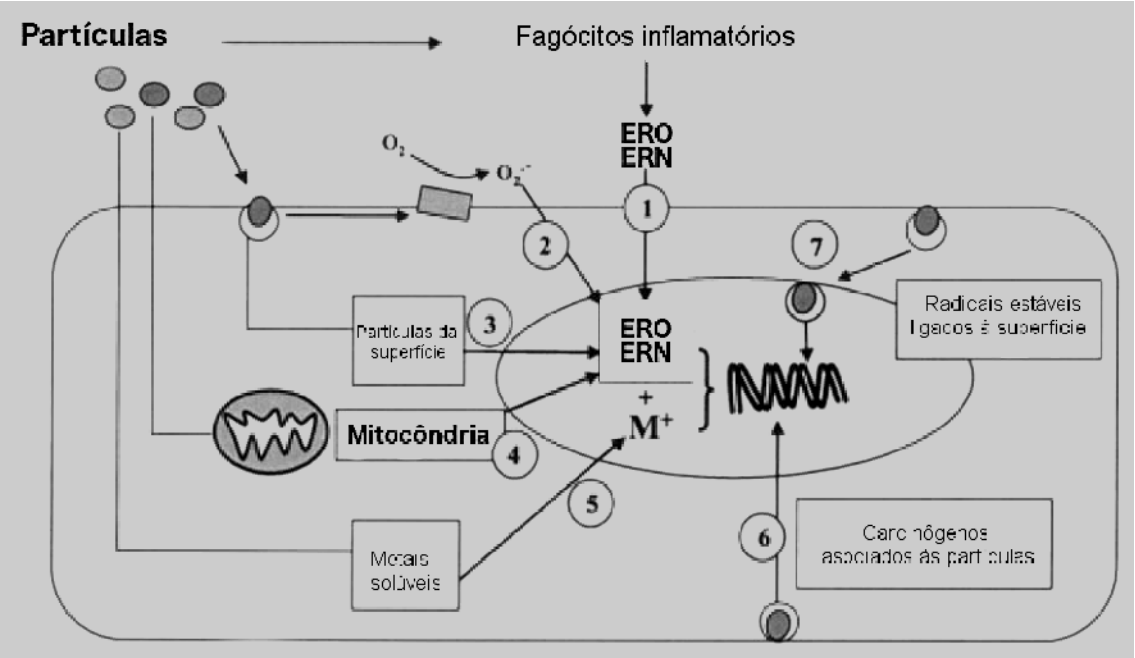

Figura 5. Possíveis mecanismos de dano ao DNA induzido pela exposição ao MP: **1.** Explosão oxidativa fagocítica (genotoxicidade secundária), **2.** Geração direta de ERO/ERN em células alvo (2-5). **3.** Via de ativação similar a enzimas NAD(P)H, **4.** Ativação mitocondrial. **5.** Associação de metais solúveis, que podem aumentar a geração de ERO (via da reação Haber-Weiss), **6.** Alguns compostos absorvidos podem danificar diretamente o DNA (genotoxicidade), e **7.** Translocação de partículas no núcleo. **Adaptado** de (Knaapen et al. 2004).

A exposição aos compostos químicos presentes nas partículas de pó do carvão pode levar à formação de ERO pelas vias celular e não celular (Knaapen et al. 2004). A via não celular de geração de ERO está relacionada com características físico-químicas (tamanho e forma), presença de compostos tóxicos, além de elevadas concentrações de ferro (Dwivedi et al. 2012; Zhang et al. 2002). Alguns compostos metálicos presentes no MP do carvão podem aumentar a produção de ERO pela via da reação de Haber-Weiss (Bonner 2007; Knaapen et al. 2004), induzindo a peroxidação lipídica e causando danos ao DNA, depleção de grupamentos tióis, alterando assim vias de transdução de sinais e a homeostase do cálcio (Stohs and Bagchi 1995). Também tem sido descrito que a presença de elevadas concentrações de ferro no organismo leva à oxidação de biomoléculas (via reação de Fenton) e, posteriormente, ao desenvolvimento de lesões pulmonares (Jomova and Valko 2011; Prousek 2007). Na reação de Fenton, é formado a radical hidroxila, que pode estar relacionado com a prevalência de doenças pulmonares nos trabalhadores das minas de carvão (Cohn et al. 2006).

Pela via celular, as ERO e os ERN são gerados em células fagocíticas, endoteliais vasculares e epiteliais pulmonares. Vários estudos relatam que elementos como sílica cristalina, metais pesados contidos nos pós, cinza com óleo, cinza de carvão podem induzir a geração de ERO em neutrófilos e em macrófagos, os quais envolvem as NADPH oxidases na ativação destas moléculas (Joshi et al. 2015; Magnani et al. 2013; van der Vliet 2008).
Os macrófagos alveolares são os principais geradores de ERN como óxido nítrico ($NO^{\cdot}$) e peroxinitrito ($ONOO^{-}$), ambos induzidos pela ação da enzima óxido nítrico sintase (Fubini and Hubbard 2003; Sugiura and Ichinose 2011). A produção destes relaciona-se com a inalação de MP (Knaapen et al. 2004; Laskin et al. 2013). O dano oxidativo pulmonar gerado por ERO pode se refletir em danos nas membranas celulares, que favorecem o

desenvolvimento de alterações da permeabilidade do fluxo iônico e do fluxo de outras substâncias. Como resultado, percebe-se a perda da seletividade para entrada e/ou saída de nutrientes e substâncias tóxicas, alterações do DNA, oxidação de lipoproteínas e o comprometimento de componentes da matriz extracelular (proteoglicanos, colágeno e elastina) (Fubini and Hubbard 2003; Schieber and Chandel 2014).

1.9.1.1. Danos oxidativos em lipídios

As ERO podem contribuir na peroxidacão lipídica, levando ao dano celular e remodelamento do tecido, o que resulta da formação de radicais lipídicos, gerados pela reação em cadeia nos ácidos graxos insaturados (Piljac Zegarac 2015). A peroxidacão de lipídeos, também pode levar à perda da fluidez da membrana e da elasticidade, perda da função celular e ruptura celular. Nesta reação com os lipídeos são gerados importantes biomarcadores de estresse oxidativo nos tecidos como malondialdeído (MDA), isoprostanas e 4-hidroxinonenal. O MDA é um composto mutagênico e cancerígeno que reage com o DNA formando adutos (Piljac Zegarac 2015).

1.9.1.2. Danos oxidativos em proteínas

A oxidação de proteínas é outro processo que pode ocorrer pela ação das ERO. Neste, as ERO reagem com proteínas e levam à inativação de enzimas envolvidas no metabolismo celular ou na transformação de componentes intra ou extracelulares (Schieber and Chandel 2014). Esta reação com as proteínas também é capaz de provocar quebra nos resíduos de aminoácidos, que podem levar à formação de pontes (*cross-link*) entre proteína-proteína e oxidações estruturais das proteínas, que levam à perda da função das mesmas

(Tice et al. 2000). A oxidação proteica contribui para diferentes transtornos e doenças, caso os mecanismos de degradação proteica não sejam executados corretamente (Piljac Zegarac 2015).

1.9.1.3. Danos oxidativos no DNA

O ataque dos radicais livres ao DNA leva a mudanças na proliferação celular, que podem ser causadas por modificações das bases de DNA, geração de sites AP, rompimento duma ou as duas cadeia de DNA, mutações, ativação de oncogenes e inativação de genes supressores, ou ainda pela geração de danos endoteliais que favorecem a metástase (Jarvis et al. 2014). Isso ocorre como consequência da quebra da estrutura do açúcar fosfato em uma ou nas duas cadeias de DNA, realizando a modificação das bases nitrogenadas e a formação de ligações cruzadas DNA-DNA ou proteína-DNA (Schieber and Chandel 2014).

1.9.2. Alterações Cromossômicas

Determinados compostos químicos têm a capacidade de induzir efeitos genotóxicos que provocam danos no DNA. Com isso, sucede o desenvolvimento de lesões celulares, como as anomalias cromossômicas de número, que podem ocorrer pela inibição da polimerização de proteínas como tubulina e actina, necessárias na formação das estruturas do citoesqueleto (microtúbulos e microfilamentos) essenciais para a mitose, ocasionando, também, danos nos cinetócoros ou coesinas, que são necessários na segregação das cromátides na anáfase (Parker et al. 2014); e adicionalmente, os compostos químicos conseguem causar segregações cromossômicas incorretas e aberrações

cromossômicas numéricas, as quais alteram a formação do fuso mitótico, ocasionando alterações nos centrômeros e cinetócoros pela inativação dos processos de alinhamento das cromátides no eixo, ou quando estas são separadas na anáfase (Kirsch-Volders et al. 2011).

Estas alterações nos cromossomos foram avaliadas em culturas de células V79 *in vitro* (León-Mejía et al. 2016), nas células da mucosa bucal (pelo fácil acesso e o alto grau de divisões celulares) (Thomas et al. 2008), e também em linfócitos. Porém, no último caso, há necessidade de cultura celular *in vitro* - ensaio BMNCyt - que considera outras alterações celulares como a formação de brotos nucleares, células binucleadas (relacionadas a defeito citocinético) e anormalidades nucleares, que representam morte celular reconhecidas pela presença de cromatina condensada, células cariorréticas e picnóticas (Tolbert, Shy, and Allen 1992).

Alguns estudos em cultura de células (Morris et al. 1989; Zhai et al. 2012) realizados em populações de animais (Cabarcas-Montalvo et al. 2012; da Silva et al. 2000) e humanas com exposição aos resíduos de mineração do carvão (León-Mejía et al. 2011; Rohr et al. 2013) estabeleceram que a presença de misturas complexas geradas pelas atividades de exploração do mesmo têm a capacidade de ocasionar lesões celulares como a instabilidade genômica. Outros danos reportados incluem: presença de micronúcleos (MN), pontes nucleoplásmicas (NPB) e brotos nucleares (NBUDS) em linfócitos e em células da mucosa oral.

Os MN em células binucleadas são cromossomos inteiros ou fragmentos de cromossomos, enquanto que as NPB são cromossomos dicêntricos ou cromátides, não separadas durante a anáfase/telófase, na qual a quebra de um NPB ou de um NBUD também pode levar à formação de um MN (Chadwick et al. 2013; Gautam et al. 2016). Os MN também são encontrados em células mononucleadas (MNMONO), quando estas são

incapazes de se dividir *ex vivo* por danos ao DNA que ativaram paradas do ciclo celular (*checkpoints*), impedindo a progressão da divisão da célula. Podem também ocorrer em células que terminaram a sua replicação do DNA, mas que, porém, não são capazes de se dividir porque já saíram da mitose, ou a partir de MN provenientes de NBUDS produzidos na fase S pela eliminação do DNA amplificado os complexos de reparação de DNA (Park et al. 2001; Yu et al. 2011).

1.9.3. Alterações do Ciclo Celular

A exposição ao MP tem sido associada com alterações em várias etapas do ciclo celular (Deng et al. 2007; Gualtieri et al. 2011). Os compostos químicos deste podem induzir uma alteração marcante do ciclo celular, que se reflete no aumento do número de células com parada transitória em G2. Este efeito foi associado à fosforilação aumentada de Chk2 e está acompanhado por alterações graves no ciclo celular, as quais resultam no aumento da frequência de células com dois núcleos e MN, e está também relacionado com a ativação metabólica dos químicos orgânicos, a qual pode causar danos no DNA e ao fuso (Longhin et al. 2013).

No ciclo celular, as proteínas ATM (Ataxia-telangiectasia mutada) – ATR (ATM e Rad3 relacionadas) ajudam na resposta ao dano no DNA e geram a ativação das cinases do check-point, o que poderia resultar na parada do ciclo celular via p53 dependente (Pearce and Humphrey 2001). Nessa via, que depende de p53, Chk1/2 atua fosforilando p53 e, pela ativação transcricional de mediadores p21 e 14-3-3, inibe a Cdk1/ciclinaB1 (Fig. 6) (Branzei and Foiani 2008).

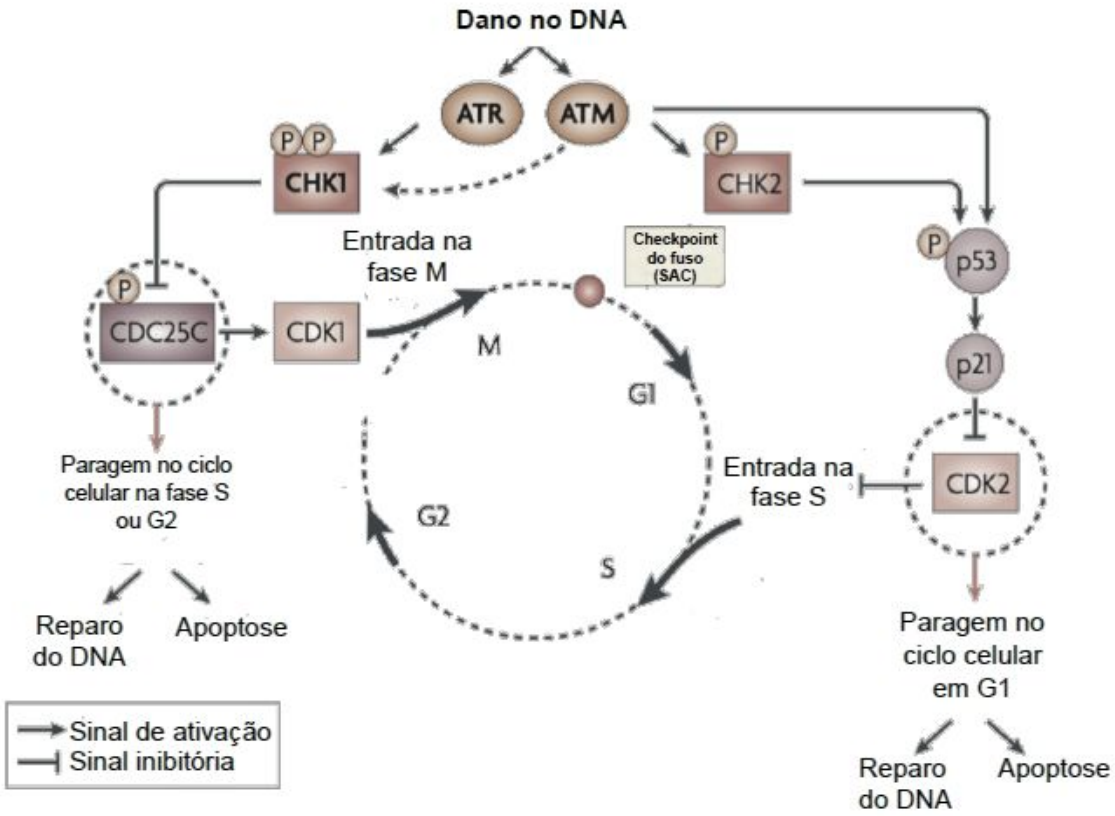

Figura 6. Alterações apresentadas no ciclo celular induzidas pela via de sinalização ATM-ATR. Adaptado de Lapenna and Giordano (2009).

Os efeitos produzidos pelo MP no ciclo celular sugerem que estas partículas também são capazes de induzir parada na progressão da mitose, provavelmente devido ao distúrbio na estrutura dos microtúbulos e na montagem do fuso mitótico (Gualtieri et al. 2011). Podem também gerar outros tipos de alterações do aparelho mitótico, tais como fusos tripolares, multipolares e incompletos (Lapenna and Giordano 2009). Algumas alterações que atuam de modo semelhante no fuso mitótico foram relatadas em estudos feitos com células V79 expostas a MP (Glowala et al. 2001) e em células BEAS-2B de epitélio bronquial humano, também expostas a MP (Longhin et al. 2013). Esses estudos

indicam que a exposição ao MP pode impactar na dinâmica dos microtúbulos e na parada do fuso, sendo este o principal mecanismo relacionado à parada da transição M/A. De acordo com relatos, o aumento no número de polos do fuso e a amplificação dos centrossomas podem contribuir para o atraso do início da anáfase pela ativação do Spindle Assembly Checkpoint (SAC) (Acilan and Saunders 2008).

1.10. Introdução à Biologia de sistemas

A biologia sofreu uma mudança progressiva na era pós-genômica pelo aumento gradual dos dados "ômicos" (genômica, proteômica, trascritômica, metabolômica, entre outros), o que levou ao desenvolvimento de ferramentas para a análise desta informação. Iniciou também uma nova forma de análise - ou desenho de redes - que incluía diferentes dados biológicos tais como gene-proteína, proteína-metabólito, transcrito-proteína, entre outros. Essas redes, ou grafos, possuem propriedades matemáticas definidas, o que facilita a compressão dos dados (Bader and Hogue 2003) e estão constituídas por dois elementos principias:

1. vértices ou nós (nodes) (Fig. 7), que constituem as partes que interagem num sistema (Barabási and Oltvai 2004; Newman 2008), e estão formadas por proteínas, genes, metabólitos, ácidos nucleicos, RNAs, carboidratos ou qualquer outro componente celular, moléculas sintéticas (por exemplo, fármacos), elementos inorgânicos (isto é, íons metálicos) ou quaisquer moléculas de importância biológica; e
2. conectores (edges) (Fig. 7), que mostram o vínculo entre os nós (Barabási and Oltvai 2004; Newman 2008).

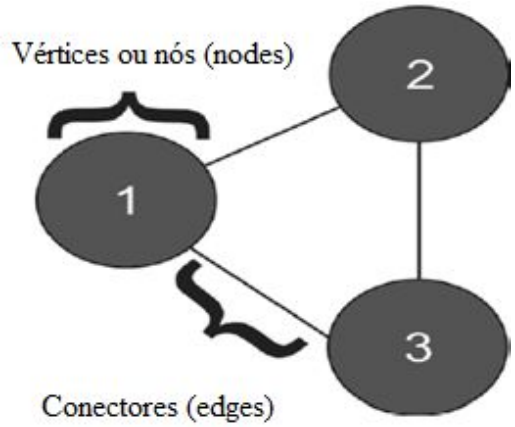

Figura 7. Principias elementos da rede.

Para o entendimento das redes de interação é necessário definir os tipos de conectores existentes. Dependendo da interação, a rede pode ser dirigida ou bidirecionada. Numa rede dirigida, a informação é difundida no sentido dos conectores e, nas redes bidirecionais, a informação se difunde nas duas direções, o que faz com que este tipo de rede seja propenso a sofrer modificações, quando alguma interferência negativa acontece.

Além disso, existem três modelos de redes de interação: **Redes de livre escala,** definida pela lei de potenciação. É uma rede com uma dinâmica que possibilita o crescimento pela adição de novos nós, o que faz que este tipo de rede inicie com um pequeno grupo de nós, e consiga aumentar seu tamanho exponencialmente. Alguns nós terão conectividade acima da média de conectividade da rede (menor quantidade), e nós com baixa conectividade (menor quantidade). **Redes aleatoriamente conectada (randômica)** são aquelas onde os nós têm a mesma probabilidade de ter iguais quantidades conexões (não é regida por uma lei de potenciação). As **Redes hierárquica** são onde a

maior parte dos nós têm conectividade semelhante e na sua disposição mostra os graus de hierarquia de forma evidente (Barabási and Oltvai 2004).

As redes randômicas e hierárquicas não são usadas para modelar conexões em um sistema biológico, uma vez que as interações proteínas-proteínas e genes-proteínas em sistemas vivos não são aleatórias, e a organização no nível molecular da célula não é hierárquica (Barabási and Oltvai 2004; Newman 2008). Neste sentido, é importante a clusterização dos elementos centrais para melhor entendimento devido à complexidade das análise de redes

1.10.1. Clusterização

Também chamada de modularização é o processo que inclui uma variedade de procedimentos que ajudam a gerar uma classificação, baseado no princípio de agrupamento de partes individuais em um sistema, e analisa as regiões altamente conectadas (Wagner, Pavlicev, and Cheverud 2007).

A modularização pode ser usada na organização de itens por funcionalidade (Scardoni, Petterlini, and Laudanna 2009). As ciências foram baseadas em categorizações que estruturaram os domínios para a sua investigação. Um exemplo disto é a classificação usada nos elementos químicos, a qual é fundamental para a química inorgânica e a teoria atômica da matéria.

Quando falamos de evolução, percebemos que os organismos possuem moléculas que estão organizadas, e geram interações com biomoléculas e em alguns casos com outros organismos e o seu ambiente (Wagner et al. 2007). Os sistemas também podem ser chamados de clusters ou módulos e conseguem influenciar diretamente a formação de redes

moleculares e/ou a dinâmica evolutiva da espécie (Wagner et al. 2007). A modularização em um interatoma possibilita a observação de grupos de elementos altamente conectados e também pode ajudar a identificar novas funções para algumas biomoléculas, assim como facilitar o estudo de novas rotas bioquímicas, que podem ser fundamentais para algum processo.

1.10.2. Análises de Centralidade

A centralidade em análise de rede avalia a importância de um vértice em um grafo usando Grau de nó, Betweenness e Closeness.

1.10.2.1. Grau de nó (*node degree*)

É uma medida usado para o entendimento de análises topológicas e calcula o número de nós conectadas diretamente com outro nó (Scardoni and Laudanna 2012). Este parâmetro considera os conectores diretos que incidem e saem de um nó determinado. Em termos biológicos, os hubs podem ser proteínas que possuem um grande número de biomoléculas parceiras diretas como, por exemplo, fatores de transcrição, que podem interagir com uma ampla gama de proteínas (Ferecatu et al. 2009; Scardoni and Laudanna 2012) e mediar a ativação de distintos bioprocessos.

1.10.2.2. Centralidade de interseção (Betweenness)

É uma medida de centralidade usada como parâmetro para avaliar a circulação de informação em um determinado nó (Scardoni and Laudanna 2012) e considera os caminhos

mais curtos entre os nós. Quanto maior é o valor da centralidade betweenness num nó, maior será o poder que ele terá para controlar ou modificar a mensagem dentro da rede. São chamados de gargalos ou *bottlenecks*, e não necessariamente possuem muitas conexões com outros nós; além disso, também podem ser chamados de *hubs* (grau de nó acima da média dos nós da rede). Os *hub*-gargalos (HGs) são nós com uma maior possibilidade de importância topológica numa rede de interação, porque combinam as características de ter muitas conexões do hub, e podem possuir capacidade de ser um nó comunicante em múltiplos processos, que é característica do gargalo (Yu et al. 2004).

Um exemplo de *betweenness* em uma rede é uma proteína capaz de interagir em diferentes processos biológicos (proteína p53, participa em ciclo celular, reparo de DNA e apoptose) (Ferecatu et al. 2009).

1.10.2.3. Centralidade de proximidade (Closeness)

É um parâmetro que analisa as vias mais curtas entre um determinado vértice e todos os outros vértices da rede, e classifica os parâmetros de isolamento ou aproximação em um nó (Scardoni et al. 2009). Valores altos de closeness indicam proximidade, e baixos valores indicam que uma proteína é afastada da maior parte dos outros vértices de um sistema (isolada) (Scardoni et al. 2009).

Um exemplo deste parâmetro é a proteína p53, que depois de ser ativada coordena vários processos e proteínas ao mesmo tempo ou em momentos diferentes (Ferecatu et al. 2009), e possui um impacto direto em diversos processos biológicos.

2. OBJETIVOS

2.1. OBJETIVO GERAL:

✓ Caracterizar a composição química do carvão betuminoso da mina El Cerrejón e sub-betuminoso da mina La Guacamaya, da Colômbia, e avaliar os seus efeitos biológicos utilizando modelos *in silico, in vitro* e *in vivo.*

2.2. OBJETIVOS ESPECÍFICOS:

✓ Determinar a concentração de elementos inorgânicos, hidrocarbonetos aromáticos policíclicos e óxidos inorgânicos do carvão betuminoso e sub-betuminoso da Colômbia.

✓ Analisar as possíveis alterações *in silico* geradas pelos compostos químicos do carvão da Colômbia no ciclo celular por biologia de sistemas.

✓ Avaliar *in vitro* os efeitos citotóxicos, genotóxicos e mutagênicos em células de fibroblastos de hamster chineses-V79, submetidas a tratamento com carvão betuminoso e sub-betuminoso de Colômbia.

✓ Analisar alterações histológicas em órgãos de ratos machos adultos da linhagem Wistar expostos à inalação de pó de carvão sub-betuminoso da mina La Guacamaya, da Colômbia.

✓ Avaliar os efeitos genotóxicos, os parâmetros estresse oxidativo e indução de citocinas (TNF-α e IL-1B) em ratos machos adultos da linhagem Wistar, expostos à inalação de pó de carvão sub-betuminoso da mina La Guacamaya da Colômbia.

3. CAPÍTULO I

Chemical characterization and systems chemo-biology analysis of major chemical constituents of Colombian coal and its implication for human health

Manuscrito submetido para publicação na Enviromental Pollution

CHEMICAL CHARACTERIZATION AND SYSTEMS CHEMO-BIOLOGY ANALYSIS OF THE MAJOR CHEMICAL CONSTITUENTS OF COLOMBIAN COAL AND ITS IMPLICATION ON HUMAN HEALTH

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ABSTRACT

One of the main characteristics of cancer is the alteration in the cell cycle. Consequently, the study of cell-cycle regulators has emerged as an important topic in cancer research, particularly in terms of environmental exposure. Particulate matter and coal dust around coal mines have the potential to induce cell-cycle alterations. Therefore, in the present study, we performed chemical analyses to identify the main compounds present in two mineral coal samples from Colombian mines and performed systems chemo-biology analysis to elucidate the interactions between these chemical compounds and proteins associated with the cell cycle. Our results highlight the role of oxidative stress generated by the exposure to the residues of coal extraction, such as major inorganic oxides (MIOs), inorganic elements (IEs) and polycyclic aromatic hydrocarbons (PAH) on DNA damage and alterations in the progression of the cycle cellular, (blockage and / or delay), as well as structural dysfunction in several proteins. Particularly, IEs such as Cr, Ni and S and PAHs such as benzo[a]pyrene may have an essential role in the regulation of the cell cycle through both DNA damage and DNA damage inhibition. In this process, cyclins; cyclin-dependent kinases; zinc finger proteins, such as P53, and protein kinases may play a central role.

Keywords: coal, Colombia, cell cycle, systems chemo-biology

Capsule

Using a system biology approach, we identified the main effects generated by MIOs, IEs and PAHs in humans and we analyze the main biological processes affected by the exposure of harmful chemical mixtures.

3.1. INTRODUCTION

One of the most lagest open-pit coal mines in the world is located in northern Colombia (Energética, 2007). According to the 2015 BP Statistical Energy Survey, Colombia aims to increase its coal production by 35% to 115,000 tons per year by 2015 from 85,000 tons in 2011. Almost 50% of this increase is to be produced from open-pit mines (BP, 2014). According to Chaulya, 2004 and Huertas et al., 2012b, during surface coal mining, activities associated with coal extraction, such as topsoil removal, drilling, blasting, overburden loading and unloading, coal transport over unpaved roads and wind erosion of exposed surfaces, release major air pollutants into the atmosphere as particulate matter (PM) and coal dust. In addition to coal, which is the main component, PM and coal dust around coal mines can also contain O, N, H, trace species and several inorganic minerals. The trace species may include SiO_2, Cu, Al, Ni, Cd, B, Sb, Fe, Pb and Zn (Huertas et al., 2012a). In mining, the excess of occupational exposure to metals is the main cause of metal-related cancers (Gloscow, 2007). Additionally, in open-cast coal mines, the coal is stored at elevated ambient temperatures, where coal combustion may lead to the emission of polycyclic aromatic hydrocarbons (PAHs) (Liu et al., 2008), most of which exhibit mutagenic and carcinogenic activities (Celik et al., 2007).

There is a growing body of evidence that links long-term exposure to coal-mining residues with increased risks of cardiovascular mortality (Brook et al., 2010; Pope and Dockery, 2006), premature mortality (Callén et al., 2009) and cancer (Pope III et al., 2002; Pope III et al., 2011). However, the mechanistic understanding of how these substances exert their various adverse effects is still incomplete. In vitro toxicological studies have found that PM causes damaging effects on cells, including genotoxicity (Billet et al., 2008;

de Kok et al., 2005), cell death (Alfaro-Moreno et al., 2002; Hsiao et al., 2000), cell-cycle alterations (Poma et al., 2006) and stimulation of pro-inflammatory cytokine production (Schins and Borm, 1999). Some of the mechanisms proposed for these effects include the occurrence of oxidative damage through the production of reactive oxygen species (ROS) (Valko et al., 2006); the release of growth factors, such as TGF-β (Borm, 1997; Sambandam et al., 2015), and reduced proliferation associated with cell-cycle arrest in response to genotoxic stresses and structural dysfunction of proteins (Gualtieri et al., 2011; Kocbach et al., 2008). Furthermore, a recent study (Espitia-Perez et al., 2018) revealed a highly significant correlation between $PM_{2.5}$ levels around the coal-mining areas of northern Colombia and incidences of mitotic arrest, centromere damage, kinetochore malfunction and disruption of the mitotic spindle in local populations.

It has been shown that oxidative stress can override the spindle checkpoint (D'Angiolella et al., 2007), inducing microtubule depolymerisation (Parker et al., 2014) and alterations in the spindle structure (Choi et al., 2007). This observation supports prior results showing that the organic components of $PM_{2.5}$, particularly PAHs, have deleterious effects on the cell cycle and cause DNA damage (Longhin et al., 2013). DNA-integrity checkpoints G1/S and G2/M and metaphase–anaphase (M/A) transitions are particularly implicated in cell-cycle delay (Branzei and Foiani, 2008).

Considering that one of the main characteristics of cancer is the alteration in the cell cycle (Otto and Sicinski, 2017), the study of cell-cycle regulators has emerged as important in cancer research, particularly in terms of environmental exposure (Puente et al., 2014). Populations are rarely exposed to single air pollutants; therefore, experimental investigations focused on single-pollutant effects do not accurately assess real-world exposure risks. Specifically, it has been suggested that air quality management must be

developed in a multi-pollutant perspective, instead of a single-pollutant view (Huang et al., 2012). Furthermore, although several recent studies have investigated the combined toxicity of complex mixtures of chemicals (Labranche et al., 2012), detailed investigations into synergistic toxicity and possible mechanisms involved in biological responses to complex exposures remain scarce (Ku et al., 2017). Therefore, in the present study, we performed a chemical analysis of mineral coals from two different Colombian mines to identify the main compounds present therein. We then performed systems chemo-biology analyses to reveal the interactions between these compounds and proteins associated with the cell cycle, elucidating their underlying regulatory mechanisms.

3.2. MATERIAL AND METHODS

3.2.1. Coal samples collection

To construct a chemo-biology interactome network for the proteins associated with the cell cycle and the major chemical constituents present in the coal samples, we performed chemical characterization of a bituminous and sub-bituminous coal sample, each collected from a different open-pit mine in Colombia. The coal samples used for chemical characterization were collected from coalfaces at the 'El Cerrejón' (La Guajira, Colombia) and 'Guacamaya' (Puerto Libertador, Córdoba, Colombia) coal mines in December 2013 (Figure 1). The samples were collected from six random sampling points at each mine and prepared as a homogeneous pool. Coals from El Cerrejón are typically bituminous with a volatile content of 37.4% and an ash content of 6.8% (dry basis) (Feng et al., 2003). Coals from Guacamaya are sub-bituminous with a high S content (2.30% total S with 1.06% as pyritic, 1.10% as organic and 0.14% from sulphates) and a high volatile content (Prada et al., 2016). While detailed chemical characterizations of El Cerrejón coal have been reported

elsewhere (Nathan et al., 1999), other Colombian coals, such as those obtained from the Guacamaya mine, have not been sufficiently characterized.

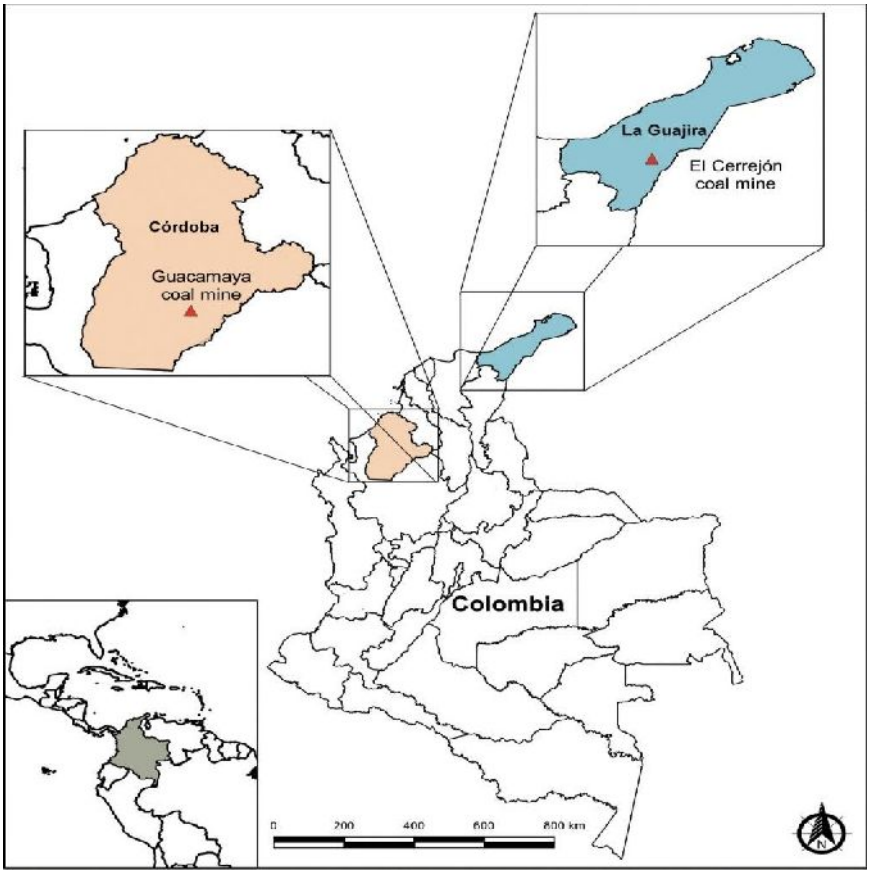

Figure 1. Coal sample collection sites in Colombia: 'El Cerrejón' (La Guajira, Colombia) and 'Guacamaya' (Puerto Libertador, Córdoba, Colombia) coal mines

3.2.1.1. Analytical methods

Chemical analysis of the coal samples comprised an identification of the major inorganic oxides (MIOs) in the coal ashes, inorganic element (IE) determination and quantification of PAHs.

3.2.1.2. Analysis of MIOs in coal ashes

We obtained a fraction of bituminous and sub-bituminous coal samples from Colombia which was incinerated at 815 °C separately. The ashes obtained were processed according to the methods described by Norrish and Hutton (1969). Finally, the detection of MIOs was performed using X-ray fluorescence spectrometry (XRF) in a Philips PW2400 spectrometer system equipped with SuperQ software.

3.2.1.3. IE measurements by particle-induced X-ray emission (PIXE) assay

The elemental composition of each coal sample was measured by the conventional in vacuo PIXE assay, as described by Johansson et al., (1995). Individual portions of each coal sample were homogenized using a mortar and pressed into pellets, and then placed in the reaction chamber at ~ 10-5 mbar, into 3-MV Tandetron accelerator, equipped with an energy resolution of ~ 155 eV to 5.9 keV for obtaining the spectra. The spectra were analized using GUPIXWIN software (Campbell et al., 2010), and expressed in parts per million. Each sample was evaluated three times in independent experiments to obtain the mean and standard deviation.

3.2.1.4. Measurement and quantification of PAHs

The PAH contents of the coal samples were quantified using the HPLC-UV/Vis method according to Sun et al. (1998) and Cavalcante et al. (2008). Briefly, 5 g of each coal sample was dried at 30°C for 24 h (in duplicate) and used for extraction purposes. The extraction was performed by ultrasonification in 5 mL acetone/hexane (1:1, *v/v*) for 15 min. The filtrate was then concentrated on a rotary evaporator and then further under a stream of

nitrogen gas to ~2 mL. A clean glass column was used for adsorption chromatography. The concentrated extracts were fractionated using a 20 × 1.5-cm column containing pre-cleaned silica gel (20 h at 110°C). The column was first eluted with 20 mL hexane/dichloromethane (9:1, *v/v*), then further with 30 mL hexane/dichloromethane (4:1, *v/v*) and finally with 10 mL dichloromethane/methanol (9:1, *v/v*). The eluted volumes were reduced to 1 mL, and finally, each extract was injected into an HPLC-UV system. The chromatographic conditions were as follows: 5 µm Kromasil C18 reverse-phase column (250 × 4.6 mm); injection volume: 20 µL; mobile phase (A): acetonitrile; mobile phase (B): MilliQ water; gradient method: 0 min (1:1), 10 min (7:3), 20 min (8:2), 25 min (8:2), 28 min (1:1), 30 min (1:1) and = 254 nm. Analytical curves were created using external standardization for quantification. In our study, we detected 11 PAHs in the samples. The PAHs detected and their limits of detection were naphthalene (1.7976 g L^{-1}), acenaphthalene (0.0041 g L^{-1}), phenanthrene (0.1758 g L^{-1}), anthracene (0.0339 g L^{-1}), fluoranthene (0.3787 g L^{-1}), benzo[a]anthracene (0.3411 g L^{-1}), benzo[b]fluoranthene (0.0691 g L^{-1}), dibenzo[a,h]anthracene (1.1110 g L^{-1}), benzo[k]fluoranthene (2.2221 g L^{-1}), indene[1,2,3-cd]pyrene (3.5788 g L^{-1}) and benzo[g,h,i]perylene (0.0005 g L^{-1}). All chromatographic operations were performed in duplicate at ambient temperature.

3.2.2. Interactome data mining and design of the chemo-biology network

To design the interactome network among the main chemical substances present in the coal samples and their potential interactions with *Homo sapiens* proteins involved in the cell cycle, we used STITCH search engine version 5.0 [http://stitch.embl.de/] and STRING 10.0 [http://http://string-db.org/newstring_cgi/show_input_page.pl/] (Jensen et al., 2008;

Snel et al., 2000). A total of 36 chemical elements were detected in the chemical analysis of both coal samples with XRF, PIXE and HPLC/UV/Vis methodologies, and these were used for the prospection of networks with the STITCH metasearch engine. While STITCH allows visualization of the physical interactions between chemical elements and proteins, STRING metasearch engine generates protein-protein interactions (PPIs) (Feltes et al., 2013). Each chemical–protein interaction (CPI) and PPI has a confidence level between 0 and 1.0 (where 1.0 indicates the highest confidence). Parameters used by the STITCH and STRING metasearch engines were as follows: all predictive methods enabled except text mining; interactions: 50; degree of confidence: 0.7 and network depth: 1. The results were combined and analysed using Cytoscape 3.4.0 (Shannon et al., 2003) and the search engine GeneCards (Rebhan et al., 1997; Safran et al., 2010) using the default parameters.

The chemical elements with no interaction with the STITCH use were excluded. Then, using Cytoscape 3.4.0., we created the interactome that fused the small CPI and PPI networks (not shown) that were generated by STITCH and STRING, respectively.

3.2.2.1. Centrality analysis

To evaluate the node degree, betweenness, and identify in the interactome the 'central' nodes (chemical compounds/proteins), a centrality analysis of the interactome was performed with CentiScaPe 2.1 installed in Cytoscape (Scardoni et al., 2009).

3.2.2.2. Modular analysis of the major CPI-PPI network

In the interactome or CPI-PPI network, we analyzed clusters or highly connected regions which are indicative of functional protein complexes. These regions were identified

using the Molecular Complex Detection application (MCODE) (Bader and Hogue, 2003; Scott, 2017). The MCODE application is included within the Cytoscape program and was used with the following parameters: loops; grade limit: 2; cluster expansion by a neighbor shell allowed; removal of a single connected node from the clusters; cut off node density: 0.1; node score limit: 0.2; score: 2 and maximum network depth: 100.

3.2.2.3. Gene ontology (GO) analysis

The genetic ontology analysis was performed using Biological Networks Gene Ontology tool (BiNGO 3.0.3) (Maere et al., 2005), which is an application installed in Cytoscape. The clusters obtained with MCODE were analyzed to determine the main bioprocesses associated with each cluster. The degree of functional enrichment was evaluated quantitatively using the hypergeometric distribution by group and category (p-value). The correction of the multiple tests was also evaluated with the application of the false discovery rate algorithm (Benjamini and Hochberg, 1995), implemented in BINGO, with a significance of $p < 0.05$.

3.3. RESULTS AND DISCUSSION

3.3.1. Chemical characterization, interactome data mining and design of the chemo-biology network

The chemical characterizations of the El Cerrejón and Guacamaya coal samples are shown in Tables S1–S3 in the Supplementary Information. Chemical analysis by XRF revealed a similar oxide composition for each coal ash (Table S1). A total of 10 different oxides were determined. As expected, samples from El Cerrejón showed a bulk chemical composition containing several metal oxides in the order $SiO_2 > Al_2O_3 > Fe_2O_3 > K_2O >$

MgO. Ashes from the sub-bituminous coal samples from Guacamaya showed higher concentrations of CaO, MgO and SO_3 and lower concentrations of SiO_2 and Al_2O_3 than those reported in similar studies on bituminous and sub-bituminous coals (Blissett and Rowson, 2012).

Table S1. Major inorganic oxide components in coal ashes (%wt) as identified by XRF

Elements	Guacamaya (%)	El Cerrejon (%)
SiO_2	13.01	62.21
TiO_2	0.33	0.92
Al_2O_3	7.52	19.62
Fe_2O_3	9.7	8.42
MgO	13.66	2.01
CaO	29.64	1.62
Na_2O	10.58	1.11
K_2O	0.44	2.17
P_2O_5	0.04	0.17
SO_3	15.08	1.75

%wt: percentage by weight

Table S2. Inorganic element concentrations in coal samples as revealed by PIXE assay (mean ± standard deviation)

Inorganic elements	**Guacamaya (PPM)**	**El Cerrejón (PPM)**
Sodium (Na)	**9212 ± 1050.73*****	3283 ± 285.97
Magnesium (Mg)	**4779.33 ± 332.27*****	2166.33 ± 167.63
Aluminium (Al)	1634.33 ± 75.22	**21311 ± 2110.55*****
Silicon (Si)	1341.67 ± 91.09	**37252.33 ± 3907.90*****
Sulphur (S)	4084 ± 312.64	**6807.67 ± 239.43*****
Chlorine (Cl)	52.94 ± 10.28	**243.77 ± 40.05****
Potassium (K)	137.33 ± 2.76	**1873.33 ± 180.79*****
Calcium (Ca)	**7838 ± 241.01*****	1675 ± 595.25
Titanium (Ti)	115.37 ± 14.79	**879.97 ± 241.27****
Chromium (Cr)	4.638 ± 3.591	20.98 ± 0.97
Manganese (Mn)	9.56 ± 1.38	**85.46 ± 18.21***
Iron (Fe)	3078.67 ± 76.63	**6113.33 ± 1399.06***
Nickel (Ni)	17.61 ± 21.44	241.77 ± 163.14
Zinc (Zn)	12.40 ± 1.71	**31.63 ± 6.89***
Strontium (Sr)	180.7 ± 8.54	ND

Unpaired T Test/ **Bold** for significantly higher values ***P < 0.001, **P < 001, *P < 0.05

ND = Not detected

Table S3. Polycyclic aromatic hydrocarbon concentrations per sample (mean ± standard deviation) as revealed by HPLC/UV/Vis

PAH	Guacamaya ($\mu g.kg^{-1}$)	El Cerrejón ($\mu g.kg^{-1}$)
Naphthalene	465 ± 16.34	864.1 ± 90.88
Acenaphthene	2.4 ± 0.03	3.8 ± 0.89
Phenanthrene	386.6 ± 1.46	846.2 ± 47.75
Anthracene	309.9 ± 13.37	1285.1 ± 110.57
Fluoranthene	1159.9 ± 59.17	1437.5 ± 245.52
Benzo[a]anthracene	676.5 ± 38.95	5045.4 ± 413.45
Benzo[g,h,i]pyrene	0.91 ± 7.60	1.3 ± 3.56
Benzo[b]fluoranthene	0.06 ± 0.02	0.6 ± 0.04
Dibenzo[a, h]anthracene	13.2 ± 0.001	28.9 ± 3.85
Indeno[1,2,3-cd]pyrene	9.5 ± 0.006	12.3 ± 0.005
Benzo[k]fluoranthene	0.9 ± 0.06	30.8 ± 1.10

As shown in Table S2, 15 IEs were identified by PIXE. Typically, bituminous samples from El Cerrejon showed higher concentrations of Si, Al, S and Fe than those of the sub-bituminous samples from Guacamaya. Conversely, relatively high concentrations of Na, Ca and Mg were present in the Guacamaya samples. Sr was detected only in the Guacamaya samples. Finally, concentration data for the 11 PAHs identified by HPLC/UV/Vis are shown in Table S3. For both samples, the most abundant PAHs detected were naphthalene, phenanthrene, anthracene, fluoranthene and benzo[a]anthracene. In general, however, higher concentrations of all PAHs were found in the El Cerrejón samples.

Chemical characterization of the bituminous and sub-bituminous coal samples revealed no significant differences in their chemical compositions. To design the chemo-biology interactome, a primary analysis was established with these 36 compounds (i.e. 10 MIOs detected in coal ash, 15 IEs and 11 PAHs).

From the initial list, all compounds not connected were excluded, yielding 24 protein-interacting compounds (Table 1) and the generation of 48 small CPI-PPI networks using the STRING and STITCH metasearch engines. All the small networks were combined, resulting in a large CPI-PPI network with 2,057 nodes and 24,957 edges (Figure 2). This large CPI-PPI network was then analysed using CentiScaPe 2.1 to identify the nodes (proteins) with a central position in the network architecture. In this scenario, nodes known as hub-bottlenecks (HBs) are the most important and combine hub (high degree) and bottleneck (high betweenness) characteristics (Azevedo and Moreira-Filho, 2015). Through centrality analysis, we observed three HB nodes (UBC, UBA52 and RPS27A) and 15 bottlenecks (HSP90AA1, CAD, SRC, JUN, MAPK14, APP, CREBBP, AKT1, K, Na, Ni, Mg, Fe, benzo[a]pyrene and Cr).

Table 1. Chemical constituents of coal samples found in the major CPI-PPI network

Compound	Classification	Chemical classification
Acenaphthene	Organic	Polycyclic Aromatic Hydrocarbon
Anthracene	Organic	Polycyclic Aromatic Hydrocarbon
Benzo(a)pyrene	Organic	Polycyclic Aromatic Hydrocarbon
Benzo(b)fluoranthene	Organic	Polycyclic Aromatic Hydrocarbon
Fluoranthene	Organic	Polycyclic Aromatic Hydrocarbon
Naphthalene	Organic	Polycyclic Aromatic Hydrocarbon
Phenanthrene	Organic	Polycyclic Aromatic Hydrocarbon
SiO_2	Inorganic	Oxide
TiO_2	Inorganic	Oxide
Fe_2O_3	Inorganic	Oxide
Al	Inorganic	Metal
Ca	Inorganic	Alkaline earth metal
Cl	Inorganic	Halogen
Cr	Inorganic	Transition metal
Fe	Inorganic	Transition metal
K	Inorganic	Alkali metal
Mg	Inorganic	Alkaline earth metal
Mn	Inorganic	Transition metal
Na	Inorganic	Alkali metal
Ni	Inorganic	Transition metal
S	Inorganic	Non-metal
Sr	Inorganic	Alkaline earth metal
Ti	Inorganic	Transition metal
Zn	Inorganic	Transition metal

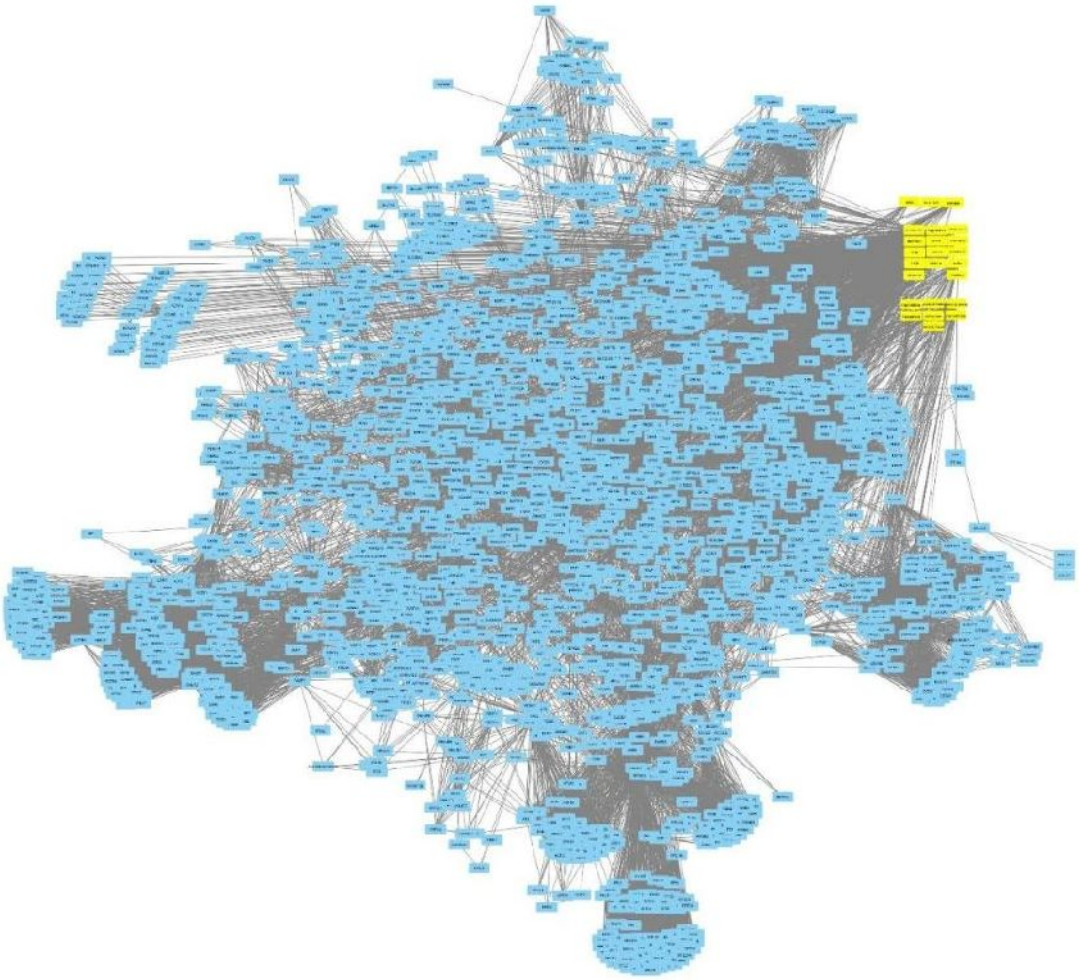

Figure 2. Main CPI-PPI network generated by the Cytoscape 3.4.0 program. Final network shows 24 chemical elements detected in coal samples after chemical characterization (yellow), 2057 nodes (24 substances, 2,033 proteins) and 24,957 edges (connections).

Ubiquitin (UBC) and two ubiquitin-coding genes (UBA52 and RPS27A) presented the highest node degree and betweenness values, thus representing highly central proteins inside the network (Feltes et al., 2013). UBC is a small 76-amino acid protein that is involved in several different pathways within the cell, including the clearing of damaged/misfolded proteins during proteotoxic stress (Bianchi et al., 2015). UBC genes are upregulated under oxidative stress response (Lee and Ryu, 2017) to increase cellular UBC above threshold levels, thus conferring resistance against oxidative stress.

To understand how coal chemical constituents interact with cell-cycle processes, we identified the modules in the main CPI-PPI network using the MCODE program. From these analyses, we obtained eight significant modules related to cell-cycle processes (Figures 3–4). Clusters 6, 11, 13 and 14 are associated with MIOs, IEs and PAHs (Figure 3), clusters 9 and 12 appear to be associated with IEs and PAHs (Figures 4A and 4B) and clusters 2 and 4 are associated with IEs (Figures 4C and 4D). The analysis revealed 15 common proteins associated with different cell-cycle processes (Table 4).

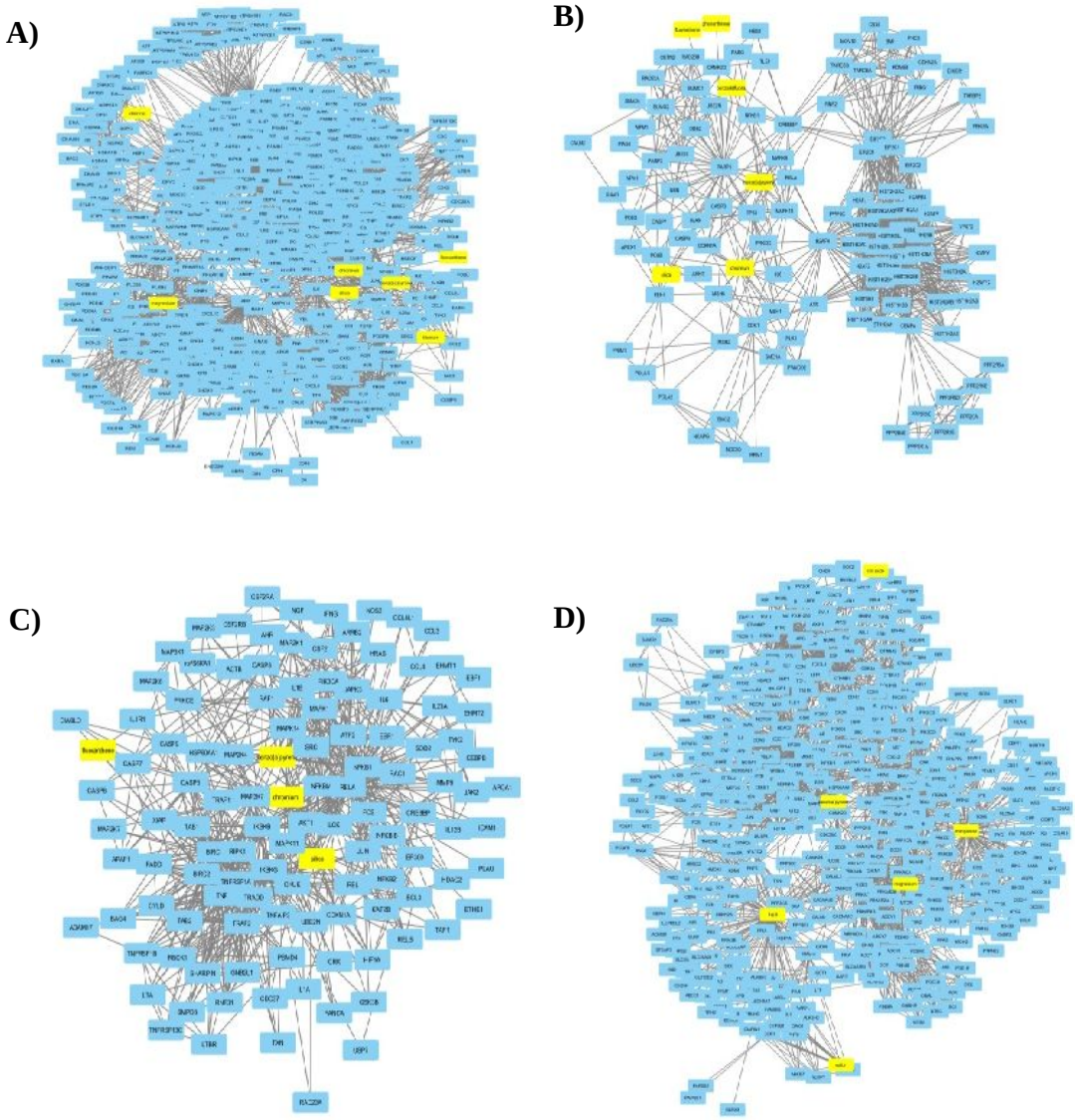

Figure 3. Cluster analysis of the major CPI-PPI network showing clusters 6, 11, 13 and 14 associated with MIOs, IEs and PAHs (yellow)**. A)** Cluster 6, composed of 487 nodes and 5,545 edges, with C*i* = 22,725. The associated constituents are SiO_2, Ti, Mg, Cr, Cl, fluoranthene and benzo[a]pyrene. **B)** Cluster 11, composed of 117 nodes and 867 edges, with C*i* = 14,695. The associated constituents are SiO_2, Cr, benzo[b]fluoranthene, fluoranthene, phenanthrene and benzo[a]pyrene. **C)** Cluster 13, composed of 118 nodes and 732 edges, with C*i* = 12,303. The associated constituents include SiO_2, Cr, fluoranthene and benzo[a]pyrene. **D)** Cluster 14, composed of 432 nodes and 2,520 edges, with C*i* = 1,164. The associated constituents are S, Mn, Mg, Fe, Fe_2O_3 and benzo[a]pyrene.

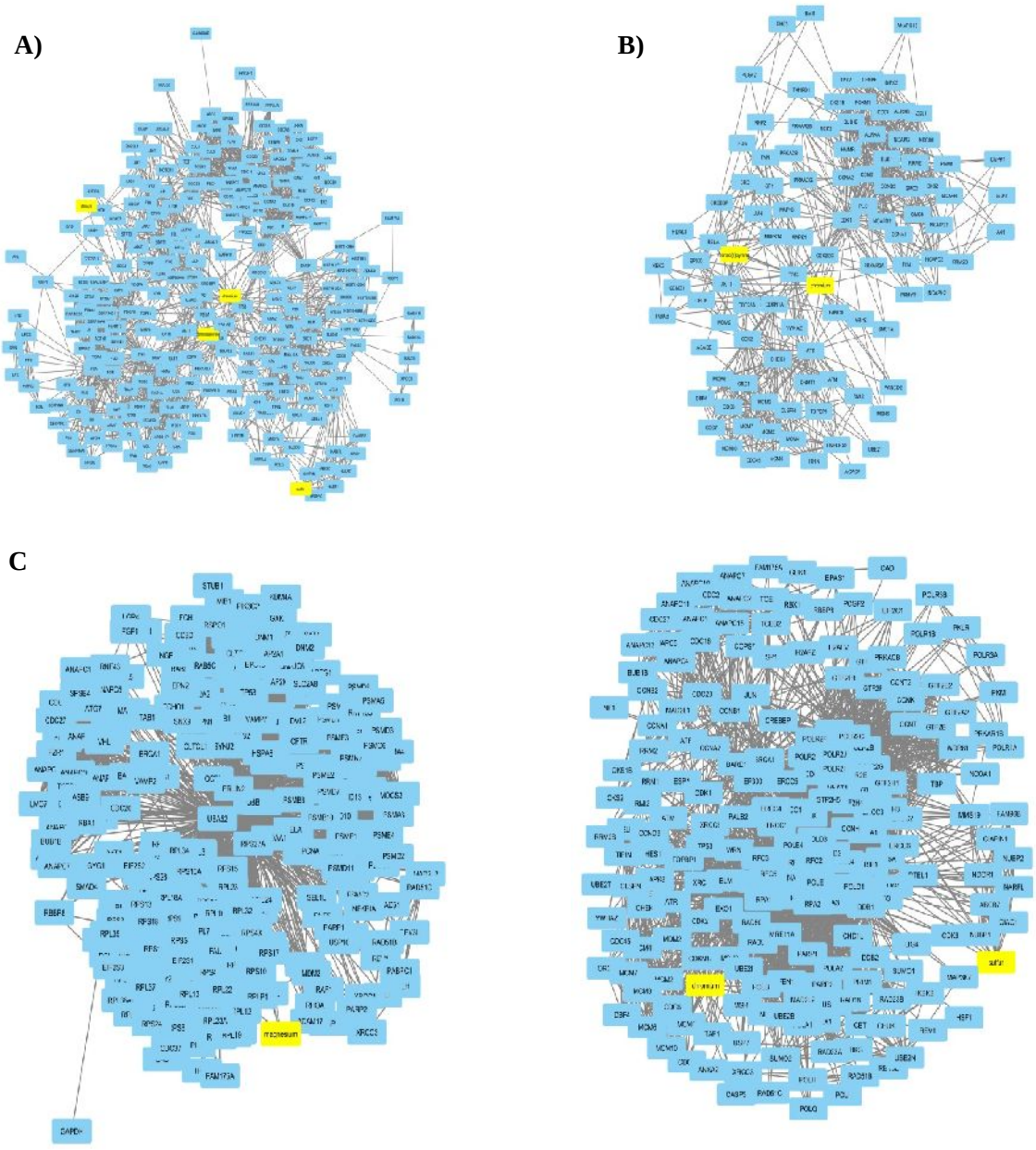

Figure 4. Cluster analysis of the major CPI-PPI network showing clusters 9 and 12 associated with IEs and PAHs (yellow) and **clusters 2 and 4** associated with IEs (yellow)**. A)** Cluster 9, composed of 249 nodes and 2,180 edges, with C*i* = 17,44. The associated compounds are S, Cr, Ti and benzo[a]pyrene. **B)** Cluster 12, composed of 102 nodes and 741 edges, with C*i* = 14,388. The associated compounds are Cr and benzo[a]pyrene. **C)** Cluster 2, composed of 250 nodes and 5,976 edges, with C*i* = 47,618, associated with Mg. **D)** Cluster 4, composed of 208 nodes and 3,134 edges, with C*i* = 2,999, associated with Cr and S.

3.3.2. Systemic effects of MIOs, IEs and PAHs in the cell cycle and DNA damage

Tables 2–4 show the results of the GO analysis of each cluster and the cell-cycle processes category. The main biological processes linked to clusters 6, 11, 13 and 14 included the following: *(i) cell-cycle process, (ii) mitotic cell cycle, (iii) cell cycle, (iv) cell-cycle checkpoint, (v) regulation of cell cycle and (vi) cell-cycle arrest* (Table 2).

Interestingly, DNA repair bioprocesses were found in this module only in co-occurrence with MIOs, IEs and PAHs. The particular combination of these compounds is associated with increased DNA damage in cell systems *in vitro* (Leon-Mejia et al., 2016) and human populations in coal mining environments (Leon-Mejia et al., 2011). The main mechanism proposed for these effects involves oxidative damage through the production of ROS (Valko et al., 2006). In this regard, within the same module, proteins regulated by oxidative stress inside the cell were identified as bottlenecks (AKT, APP, JUN and CREBBP). While AKT has been reported to be regulated by oxidative stress for cell survival (Wang et al., 2000), several studies have indicated that oxidative stress participates in events that enhance amyloidogenic APP processing in neurons (Lin and Beal, 2006; Mouton-Liger et al., 2012) and that affect cerebrovascular endothelial APP processing (Muche et al., 2017). ROS-facilitated protein phosphorylation can also lead to kinase-mediated activation of transcription factors, such as the JUN group (Nathan and Cunningham-Bussel, 2013), affecting cell-cycle progression by their ability to regulate the expression and function of cell-cycle regulators, such as cyclins (Chiba et al., 2017; Schreiber et al., 1999), and apoptosis (Meixner et al., 2010). Together with JUN, CREBBP is also involved in cell division and cell proliferation, and it has been found to be upregulated by the oxidative stress response in retinoblastoma cells (Meixner et al., 2010).

Table 2. Major cell cycle bioprocesses in **clusters 6, 11, 13 and 14** associated with MIOs, IEs and PAHs

GO ID	p-va	corr p-'	k*	n#	Description	Genes in test set
22402	1,64E-22	9,74E-21	77	582	cell cycle process	APP|CDKN1A|CETN2|CLTC|UBE2D1|PSMD8|PSMD9|PPP3CA|PSMD7|PSMD4|PSMD2|PSMD3|PSMD1|AKT1|IL12B|NBN|POLE|APC2|H2AFX|CDC25A|DNM2|PSMA5|PSMA6|DNAJC2|PSMA3|ADAM17|PSMA4|PSMA1|PSME3|PSME1|PSME2|TP53|PSMD10|PSMD12|PSMD11|RGS14|PSMD13|CUL2|THBS1|EGFR|PSMB10|PSMB6|PSMB7|PSMB4|C6|PSMB5|PSMB2|PSMB3|POLD1|PSMB1|CLTCL1|APBB1|UBE2I|TGFB1|SMAD3|VDR|RPA1|MRE11A|CDC6|HSPA2|PSMB8|MAPK12|PSMB9|PPP5C|RAD50|PSMC6|PSMC3|APC|IL8|PSMC4|PSMC1|PSMC2|CDK2|MDM2|CTNNB1|CALR|SUGT1
278	9,54E-21	5,08E-19	60	380	mitotic cell cycle	APP|CDKN1A|CETN2|CLTC|UBE2D1|PSMD8|PSMD9|PPP3CA|PSMD7|PSMD4|PSMD2|PSMD3|PSMD1|AKT1|POLE|APC2|CDC25A|DNM2|PSMA5|PSMA6|DNAJC2|PSMA3|ADAM17|PSMA4|PSMA1|PSME3|PSME1|PSME2|PSMD10|PSMD12|PSMD11|RGS14|PSMD13|CUL2|EGFR|PSMB10|PSMB6|PSMB7|PSMB4|C6|PSMB5|PSMB2|PSMB3|POLD1|PSMB1|CLTCL1|UBE2I|CDC6|PSMB8|PSMB9|PPP5C|PSMC6|PSMC3|APC|PSMC4|PSMC1|PSMC2|CDK2|MDM2|SUGT1
7049	3,12E-18	1,43E-16	84	794	cell cycle	APP|CDKN1A|STEAP3|CCNH|CETN2|CLTC|UBE2D1|PSMD8|PSMD9|PPP3CA|PSMD7|PSMD4|PSMD2|PSMD3|PSMD1|AKT1|IL12B|EP300|NBN|POLE|APC2|ANXA1|H2AFX|CDC25A|DNM2|PSMA5|GAK|PSMA6|DNAJC2|PSMA3|ADAM17|PSMA4|PSMA1|PSME3|PSME1|PSME2|TP53|PSMD10|PSMD12|PSMD11|RGS14|PSMD13|CUL2|THBS1|EGFR|PSMB10|PSMB6|PSMB7|PSMB4|C6|PSMB5|PSMB2|PSMB3|POLD1|PSMB1|CLTCL1|APBB1|UBE2I|TGFB1|SMAD3|VDR|RPA1|MRE11A|CDC6|HSPA2|PSMB8|MAPK12|PSMB9|CYLD|PPP5C|RAD50|PSMC6|PSMC3|APC|IL8|PSMC4|PSMC1|PSMC2|CDK2|MDM2|CTNNB1|REN|CALR|SUGT1
22402	4,62E-06	1,46E-04	22	582	cell cycle process	CDKN1A|NPM1|UBE2I|CDKN2A|CETN2|PLK1|H2AFX|NCAPG|SMC1A|MLH1|CENPA|NDC80|SMC2|MSH6|PPP2CA|POLA1|PPP5C|MSH2|FANCD2|CDK1|NBN|TP53
75	5,61E-06	1,73E-04	11	107	cell cycle checkpoint	CDKN1A|MSH2|CDKN2A|PLK1|H2AFX|CDK1|NBN|PPP2R5C|SMC1A|TP53|ATR
6281	7,00E-12	4,71E-10	22	298	DNA repair	POLQ|FEN1|PARP1|PRKDC|PARP2|H2AFX|RAD23A|SMC1A|MLH1|RAD23B|DDB2|MSH6|POLB|POLA1|MSH2|SUMO1|FANCD2|APEX1|UBE2N|NBN|TP53|ATR
51726	1,37E-05	1,78E-04	19	446	regulation of cell cycle	MAP2K1|JUN|CREBBP|CDKN1A|HDAC2|NGF|TNF|CYLD|KAT2B|IL1A|ADAM17|IFNG|CDC37|CASP3|IL1B|IL12B|AKT1|HRAS|MAP2K6
51726	5,25E-11	1,98E-09	48	447	regulation of cell cycle	CDS1|CDKN1A|HDAC2|TRRAP|HDAC1|CITED2|CUL1|ILK|FOXO4|ETS1|EGFR|SOX2|CCND3|CCND1|CDH1|AKT1|IL12B|SFN|PRKACA|BTRC|JUNB|HRAS|MEN1|APC2|TCF7L2|JUN|CREBBP|MAP2K1|TIPIN|SMAD3|CDKN2A|GSS|INSR|PTPN11|CDC25C|CDC25A|SMARCA4|FOSL1|KAT2B|COPS5|APC|PKIA|MDM2|TIMELESS|ATM|TCF4|TCF3|TP53
22402	9,37E-06	1,83E-04	46	583	cell cycle process	CAMK2B|CDKN1A|NCAPG2|CUL1|UBE2D1|ILK|FOXO4|EGFR|SOX2|PPP2CA|PPP3CA|CCND1|CDH1|RUVBL1|ABL1|AKT1|IL12B|BTRC|HRAS|MEN1|SKP1|APC2|TCF7L2|MAP2K1|TIPIN|UBE2I|SMAD3|CSNK1A1|CDKN2A|GSS|CDC25C|CDC25A|KAT2B|PPP5C|APC|MDM2|TIMELESS|CTNNB1|NCAPD2|MDM4|ATM|TCF4|NCAPD3|TCF3|TP53|TAF1
7049	2,41E-05	4,31E-04	55	795	cell cycle	CDKN1A|STEAP3|NCAPG2|UBE2D1|ILK|CDC73|SOX2|PPP3CA|CCND1|CDH1|RUVBL1|AKT1|IL12B|EP300|BTRC|HRAS|MEN1|SKP1|APC2|MAP2K1|TIPIN|ANXA1|DUSP1|FBXW11|CDC25C|CDC25A|KAT2B|TIMELESS|TP53|CDS1|CAMK2B|CUL1|FOXO4|EGFR|RNF2|PPP2CA|ABL1|TCF7L2|UBE2I|SMAD3|CSNK1A1|CDKN2A|GSS|PPP1CA|PPP5C|APC|MDM2|CTNNB1|NCAPD2|MDM4|ATM|TCF4|NCAPD3|TCF3|TAF1
7050	2,63E-05	4,64E-04	18	109	cell cycle arrest	TCF7L2|CDKN1A|MAP2K1|SMAD3|CDKN2A|GSS|CUL1|ILK|FOXO4|SOX2|KAT2B|APC|IL12B|ATM|TCF4|TP53|HRAS|MEN1

#: total number of nodes in the gene ontology (GO) annotation; *: number of nodes related to a given GO in the network.

Table 3. Major cell cycle bioprocesses in **clusters 9 and 12** associated with IEs and PAHs

GO ID	p-value	corr p-	k*	n#	Description	Genes in test set
22402	2,71E-34	3,52E-31	67	582	cell cycle process	UBE2D1|BUB1B|CDC20|PPP3CA|CDC23|EXO1|CHEK1|CDC27|IL12B|AKT1|NEK2|NBN|HRAS|TIPIN|ANAPC7|H2AFX|CDC25C|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|MSH2|IFNG|CKS2|TIMELESS|ANAPC4|BIRC5|ANAPC5|TP53|ANAPC1|ANAPC2|ANAPC13|BLM|CUL5|CUL2|NCAPG|CDCA8|PKMYT1|CENPA|THBS1|ANAPC10|EGFR|AURKB|ANAPC11|AURKA|CCNB2|CCNB1|FZR1|BUB1|BARD1|UBE2I|TGFB1|PLK1|MRE11A|CDC6|MLH1|NDC80|TPX2|CENPE|RAD50|RAD51|CDC16|CDK2|CDK1|ATM|MAD2L1
7049	3,93E-33	3,40E-30	75	794	cell cycle	UBE2D1|BUB1B|FOXM1|CKS1B|CDC20|PPP3CA|CDC23|EXO1|CHEK1|CDC27|IL12B|AKT1|EP300|NEK2|NBN|HRAS|TIPIN|ANAPC7|H2AFX|CDC25C|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|MSH2|IFNG|CKS2|TIMELESS|ANAPC4|BIRC5|ANAPC5|TP53|ANAPC1|ANAPC2|ANAPC13|BLM|CUL5|CUL2|NCAPG|CDCA8|PKMYT1|CENPA|THBS1|ANAPC10|EGFR|AURKB|ANAPC11|AURKA|CCNB2|CCNB1|FZR1|CDC45|MAPK1|CLSPN|BUB1|MAPK3|BARD1|UBE2I|TGFB1|PLK1|MRE11A|CDC6|MLH1|NDC80|TPX2|CENPE|RAD50|RAD51|CDC16|CDK2|CDK1|ATM|ATR|MAD2L1
22403	2,04E-31	1,06E-28	57	435	cell cycle phase	BUB1B|CDC20|PPP3CA|CDC23|EXO1|CHEK1|CDC27|AKT1|NEK2|NBN|TIPIN|ANAPC7|H2AFX|CDC25C|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|CKS2|TIMELESS|ANAPC4|BIRC5|ANAPC5|ANAPC1|ANAPC2|ANAPC13|BLM|CUL5|CUL2|NCAPG|CDCA8|PKMYT1|ANAPC10|EGFR|AURKB|ANAPC11|AURKA|CCNB2|CCNB1|FZR1|BUB1|UBE2I|PLK1|MRE11A|CDC6|MLH1|NDC80|TPX2|CENPE|RAD50|RAD51|CDC16|CDK2|CDK1|ATM|MAD2L1
51726	1,03E-27	3,84E-25	54	446	regulation of cell cycle	BUB1B|FOXM1|CKS1B|CDC23|CHEK1|IL12B|AKT1|NEK2|NBN|PRKACA|HRAS|TIPIN|H2AFX|CDC25C|CCNA2|MSH2|IFNG|CDC37|CKS2|TIMELESS|BIRC5|TP53|ANAPC2|BLM|CUL5|CUL2|PKMYT1|THBS1|ANAPC10|EGFR|BRIP1|CCNB1|FZR1|CDC45|RBBP8|CLSPN|BUB1|BARD1|JUN|CREBBP|TGFB1|PLK1|MRE11A|CDC6|TPX2|CENPE|COPS5|CDC16|FAM175A|CDK2|CDK1|ATM|ATR|MAD2L1
278	6,00E-23	8,21E-21	46	380	mitotic cell cycle	ANAPC13|BLM|CUL5|CUL2|NCAPG|CDCA8|UBE2D1|BUB1B|PKMYT1|CENPA|ANAPC10|EGFR|AURKB|ANAPC11|AURKA|CDC20|PPP3CA|CCNB2|CCNB1|FZR1|CDC23|CDC27|AKT1|NEK2|BUB1|TIPIN|UBE2I|ANAPC7|PLK1|CDC6|CDC25C|NDC80|CCNA2|TPX2|CENPE|CCNA1|CDC16|CDK2|TIMELESS|CDK1|ANAPC4|BIRC5|ANAPC5|ANAPC1|MAD2L1|ANAPC2
87	1,10E-22	1,36E-20	38	239	M phase of mitotic cell cycle	ANAPC13|NCAPG|CDCA8|BUB1B|PKMYT1|ANAPC10|AURKB|ANAPC11|AURKA|CDC20|CCNB2|CCNB1|FZR1|CDC23|CDC27|NEK2|BUB1|TIPIN|UBE2I|ANAPC7|PLK1|CDC6|CDC25C|NDC80|CCNA2|TPX2|CENPE|CCNA1|CDC16|CDK2|TIMELESS|CDK1|ANAPC4|BIRC5|ANAPC5|ANAPC1|MAD2L1|ANAPC2
75	8,32E-21	6,55E-19	27	107	cell cycle checkpoint	BLM|BUB1B|BRIP1|CCNB1|FZR1|CDC45|CHEK1|RBBP8|NBN|CLSPN|HRAS|BUB1|TIPIN|TGFB1|PLK1|H2AFX|CDC6|CCNA2|CENPE|MSH2|FAM175A|CDK1|BIRC5|ATM|TP53|ATR|MAD2L1
7346	1,07E-11	3,66E-10	24	174	regulation of mitotic cell cycle	TGFB1|PLK1|BUB1B|CDC6|CDC25C|PKMYT1|ANAPC10|EGFR|CCNA2|TPX2|CENPE|CCNB1|CDC23|CDC16|CDK2|CDK1|BIRC5|ATM|NEK2|NBN|TP53|HRAS|BUB1|MAD2L1
10564	1,19E-08	2,53E-07	19	138	regulation of cell cycle process	TIPIN|CREBBP|TGFB1|MRE11A|CDC25C|PKMYT1|FOXM1|ANAPC10|TPX2|CENPE|CCNB1|FZR1|CDC23|CDC16|TIMELESS|BIRC5|ATM|NEK2|BUB1
7093	3,57E-07	5,87E-06	12	52	mitotic cell cycle checkpoint	CCNA2|CENPE|CCNB1|TGFB1|CDK1|BUB1B|ATM|NBN|TP53|HRAS|BUB1|MAD2L1
51327	1,15E-06	1,75E-05	15	102	M phase of meiotic cell cycle	H2AFX|MRE11A|MLH1|RAD51B|MSH6|CCNA1|RAD50|RAD51C|RAD51|EXO1|CHEK1|CKS2|ATM|NEK2|NBN
51329	1,15E-06	1,75E-05	15	102	interphase of mitotic cell cycle	BLM|CUL5|CUL2|CDC6|CDC25C|ANAPC10|EGFR|PPP3CA|CCNB1|CDC23|CDK2|AKT1|ANAPC4|BIRC5|ANAPC5
51321	1,33E-06	1,99E-05	15	103	meiotic cell cycle	H2AFX|MRE11A|MLH1|RAD51B|MSH6|CCNA1|RAD50|RAD51C|RAD51|EXO1|CHEK1|CKS2|ATM|NEK2|NBN
7049	4,53E-45	6,63E-42	59	794	cell cycle	CDKN1A|MCM7|NCAPG2|BUB1B|FOXM1|SMC4|SMC2|CKS1B|CHEK1|EP300|AKT1|NEK2|TIPIN|CDC25C|SMC1A|CDC25A|MSH6|CCNA2|CCNA1|DBF4|MSH2|FANCD2|TIMELESS|MCM3|CKS2|BIRC5|MCM6|TP53|MCM2|NCAPG|CDCA8|PKMYT1|NCAPH|RNF2|AURKB|AURKA|CCNB2|CCNB1|CDC45|MAPK1|CLSPN|BUB1|MAPK3|PLK1|FANCA|CDC7|CDC6|NDC80|TPX2|CENPE|CDK2|CCNG1|MDM2|CDK1|ATM|NCAPD2|NCAPD3|ATR|MAD2L1
22403	7,21E-37	5,27E-34	44	435	cell cycle phase	CDKN1A|NCAPG2|BUB1B|NCAPG|CDCA8|PKMYT1|SMC4|NCAPH|AURKB|SMC2|AURKA|CCNB2|CCNB1|CHEK1|AKT1|NEK2|BUB1|TIPIN|PLK1|FANCA|CDC7|CDC6|CDC25C|SMC1A|CDC25A|NDC80|MSH6|CCNA2|TPX2|CCNA1|CENPE|DBF4|FANCD2|CDK2|CCNG1|TIMELESS|MDM2|CDK1|CKS2|BIRC5|ATM|NCAPD2|NCAPD3|MAD2L1
22402	5,73E-34	2,79E-31	46	582	cell cycle process	CDKN1A|NCAPG2|BUB1B|NCAPG|CDCA8|PKMYT1|SMC4|NCAPH|AURKB|SMC2|AURKA|CCNB2|CCNB1|CHEK1|AKT1|NEK2|BUB1|TIPIN|PLK1|FANCA|CDC7|CDC6|CDC25C|SMC1A|CDC25A|NDC80|MSH6|CCNA2|TPX2|CCNA1|CENPE|DBF4|MSH2|FANCD2|CDK2|CCNG1|TIMELESS|MDM2|CDK1|CKS2|BIRC5|ATM|NCAPD2|NCAPD3|TP53|MAD2L1
278	4,13E-32	1,21E-29	39	380	mitotic cell cycle	CDKN1A|NCAPG2|BUB1B|NCAPG|CDCA8|PKMYT1|SMC4|NCAPH|RNF2|AURKB|SMC2|AURKA|CCNB2|CCNB1|AKT1|NEK2|BUB1|TIPIN|PLK1|CDC7|CDC6|CDC25C|SMC1A|CDC25A|NDC80|CCNA2|TPX2|CCNA1|CENPE|DBF4|CDK2|CCNG1|TIMELESS|MDM2|CDK1|BIRC5|NCAPD2|NCAPD3|MAD2L1
87	1,23E-30	3,01E-28	33	239	M phase of mitotic cell cycle	NCAPG2|BUB1B|NCAPG|CDCA8|PKMYT1|SMC4|NCAPH|AURKB|SMC2|AURKA|CCNB2|CCNB1|NEK2|BUB1|TIPIN|PLK1|CDC6|CDC25C|SMC1A|CDC25A|NDC80|CCNA2|TPX2|CCNA1|CENPE|CDK2|CCNG1|TIMELESS|CDK1|BIRC5|NCAPD2|NCAPD3|MAD2L1
51726	1,02E-26	1,36E-24	37	446	regulation of cell cycle	CDKN1A|HDAC1|BUB1B|PKMYT1|FOXM1|CKS1B|CCNB1|CDC45|CHEK1|AKT1|NEK2|CLSPN|BUB1|TIPIN|JUN|CREBBP|PLK1|CDC7|CDC6|CDC25C|SMC1A|CDC25A|CCNA2|TPX2|CENPE|MSH2|CDK2|CCNG1|TIMELESS|MDM2|CDK1|CKS2|BIRC5|ATM|TP53|ATR|MAD2L1
75	2,29E-21	2,57E-19	21	107	cell cycle checkpoint	TIPIN|CDKN1A|PLK1|BUB1B|CDC6|SMC1A|CCNA2|CENPE|CCNB1|CDC45|MSH2|CHEK1|CCNG1|CDK1|BIRC5|ATM|CLSPN|TP53|BUB1|ATR|MAD2L1
7346	2,52E-15	2,30E-13	20	174	regulation of mitotic cell cycle	CDKN1A|PLK1|BUB1B|CDC6|CDC25C|PKMYT1|CCNA2|TPX2|CENPE|CCNB1|CDK2|CCNG1|MDM2|CDK1|BIRC5|NEK2|ATM|TP53|BUB1|MAD2L1
10564	4,70E-13	3,44E-11	17	138	regulation of cell cycle process	TIPIN|CREBBP|CDKN1A|CDC7|CDC25C|PKMYT1|FOXM1|SMC1A|TPX2|CENPE|CCNB1|TIMELESS|MDM2|BIRC5|NEK2|ATM|BUB1
7093	4,22E-10	1,81E-08	11	52	mitotic cell cycle checkpoint	CCNA2|CENPE|CCNB1|CDKN1A|CCNG1|CDK1|BUB1B|ATM|TP53|BUB1|MAD2L1
51329	9,39E-07	2,41E-05	11	102	interphase of mitotic cell cycle	CCNB1|CDKN1A|DBF4|CDK2|MDM2|AKT1|BIRC5|CDC7|CDC6|CDC25C|CDC25A

#: total number of nodes in the gene ontology (GO) annotation; *: number of nodes related to a given GO in the network.

Table 4. Major cell cycle bioprocesses in **clusters 2 and 4** associated with IEs

GO ID	p-value	corr p-	k*	n#	Description	Genes in test set
278	6,09E-38	2,63E-36	60	380	mitotic cell cycle	CLTC|BUB1B|PSMD8|CDC20|PSMD9|PSMD7|CDC23|PSMD2|PSMD3|CDC27|PSMD1|AKT1|ANAPC7|RPS6|DNM2|PSMA5|PSMA6|PSMA3|ADAM17|PSMA4|PSMA1|PSME3|PSME1|RPL24|PSME2|ANAPC4|ANAPC5|ANAPC1|ANAPC2|PSMD10|ANAPC13|PSMD12|PSMD11|CUL5|PSMD13|CUL2|ANAPC10|PSMB10|ANAPC11|PSMB6|PSMB7|PSMB4|CCNB1|FZR1|PSMB5|PSMB2|PSMB3|PSMB1|CLTCL1|PSMB8|PSMB9|MAD2L2|PSMC6|PSMC3|PSMC4|PSMC1|CDC16|PSMC2|MDM2|MAD2L1
22402	4,93E-32	1,84E-30	65	582	cell cycle process	CLTC|BUB1B|PSMD8|CDC20|PSMD9|PSMD7|CDC23|PSMD2|PSMD3|CDC27|PSMD1|AKT1|ANAPC7|RPS6|DNM2|RAD51B|PSMA5|PSMA6|PSMA3|ADAM17|RAD51C|PSMA4|PSMA1|PSME3|PSME1|RPL24|PSME2|ANAPC4|ANAPC5|TP53|ANAPC1|ANAPC2|PSMD10|ANAPC13|PSMD12|PSMD11|CUL5|PSMD13|CUL2|ANAPC10|PSMB10|ANAPC11|PSMB6|PSMB7|PSMB4|CCNB1|FZR1|PSMB5|PSMB2|PSMB3|PSMB1|CLTCL1|BARD1|PSMB8|PSMB9|MAD2L2|PSMC6|RAD51|PSMC3|PSMC4|PSMC1|CDC16|PSMC2|MDM2|MAD2L1
7049	1,12E-25	3,45E-24	67	794	cell cycle	CLTC|BUB1B|BRCA1|PSMD8|CDC20|PSMD9|PSMD7|CDC23|PSMD2|PSMD3|CDC27|PSMD1|AKT1|ANAPC7|RPS6|DNM2|RAD51B|PSMA5|GAK|PSMA6|PSMA3|ADAM17|RAD51C|PSMA4|PSMA1|PSME3|PSME1|RPL24|PSME2|ANAPC4|ANAPC5|TP53|ANAPC1|ANAPC2|PSMD10|ANAPC13|PSMD12|PSMD11|CUL5|PSMD13|CUL2|ANAPC10|PSMB10|ANAPC11|PSMB6|PSMB7|PSMB4|CCNB1|FZR1|PSMB5|PSMB2|PSMB3|PSMB1|CLTCL1|BARD1|PSMB8|PSMB9|MAD2L2|PSMC6|RAD51|PSMC3|PSMC4|PSMC1|CDC16|PSMC2|MDM2|MAD2L1
22403	3,84E-07	8,30E-06	30	435	cell cycle phase	ANAPC13|CUL5|CLTC|CUL2|BUB1B|ANAPC10|ANAPC11|CDC20|CCNB1|FZR1|CDC23|CDC27|CLTCL1|AKT1|ANAPC7|RPS6|DNM2|RAD51B|MAD2L2|ADAM17|RAD51C|RAD51|CDC16|MDM2|RPL24|ANAPC4|ANAPC5|ANAPC1|MAD2L1|ANAPC2
87	5,19E-06	1,03E-04	21	239	M phase of mitotic cell cycle	ANAPC13|ANAPC7|CLTC|RPS6|BUB1B|ANAPC10|ANAPC11|CDC20|MAD2L2|CCNB1|FZR1|CDC23|CDC27|CDC16|CLTCL1|RPL24|ANAPC4|ANAPC5|ANAPC1|MAD2L1|ANAPC2
7049	5,73E-35	4,37E-33	71	794	cell cycle	CCNK|CDKN1A|CCNT2|CCNT1|MCM7|CCNH|CETN2|BUB1B|BRCA1|CKS1B|CDC20|CDC23|EXO1|CHEK1|CDC27|EP300|NBN|POLK|POLE|TIPIN|LIG1|ANAPC7|LIG4|LIG3|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|DBF4|MSH2|CKS2|TIMELESS|MCM3|ANAPC4|ANAPC5|MCM6|TP53|ANAPC1|ANAPC2|MCM2|ANAPC13|BLM|ANAPC10|ANAPC11|CCNB2|CCNB1|CDC45|POLD1|CLSPN|BARD1|UBE2I|UBE2B|RPA1|MRE11A|CDC7|CDC6|MAD2L2|POLA1|RAD50|CDK6|RAD51|CDC16|CDK2|MDM2|CDK1|ATM|MNAT1|ATR|MAD2L1|TAF1
22403	2,64E-31	1,79E-29	53	435	cell cycle phase	CCNK|CDKN1A|CETN2|BUB1B|CDC20|CDC23|EXO1|CHEK1|CDC27|NBN|POLK|POLE|TIPIN|ANAPC7|LIG3|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|DBF4|CKS2|TIMELESS|ANAPC4|ANAPC5|ANAPC1|ANAPC2|ANAPC13|BLM|ANAPC10|ANAPC11|CCNB2|CCNB1|POLD1|UBE2I|UBE2B|RPA1|MRE11A|CDC7|CDC6|MAD2L2|POLA1|RAD50|CDK6|RAD51|CDC16|CDK2|MDM2|CDK1|ATM|MNAT1|MAD2L1|TAF1
22402	7,25E-28	4,15E-26	56	582	cell cycle process	CCNK|CDKN1A|CETN2|BUB1B|CDC20|CDC23|EXO1|CHEK1|CDC27|NBN|POLK|POLE|TIPIN|ANAPC7|LIG3|RAD51B|MSH6|CCNA2|CCNA1|RAD51C|DBF4|MSH2|CKS2|TIMELESS|ANAPC4|ANAPC5|TP53|ANAPC1|ANAPC2|ANAPC13|BLM|ANAPC10|ANAPC11|CCNB2|CCNB1|POLD1|BARD1|UBE2I|UBE2B|RPA1|MRE11A|CDC7|CDC6|MAD2L2|POLA1|RAD50|CDK6|RAD51|CDC16|CDK2|MDM2|CDK1|ATM|MNAT1|MAD2L1|TAF1
51726	1,75E-27	9,46E-26	50	446	regulation of cell cycle	CCNK|CDKN1A|CCNT2|CCNT1|BUB1B|BRCA1|CKS1B|CCND3|CDC23|CASP3|CHEK1|NBN|TIPIN|DDB1|CCNA2|MSH2|CKS2|TIMELESS|TP53|ANAPC2|BLM|ANAPC10|BRIP1|CCNB1|CDC45|RBBP8|CLSPN|BARD1|JUN|CREBBP|UBE2B|MRE11A|CDC7|GTF2H1|CDC6|MAD2L2|CDK7|COPS5|CDK6|ERCC3|CDC16|FAM175A|CDK2|ERCC2|MDM2|CDK1|ATM|MNAT1|ATR|MAD2L1
75	5,24E-20	2,13E-18	25	107	cell cycle checkpoint	CDKN1A|BLM|BUB1B|BRCA1|BRIP1|CCNB1|CDC45|CHEK1|RBBP8|NBN|CLSPN|TIPIN|CDC6|DDB1|MAD2L2|CCNA2|MSH2|ERCC3|FAM175A|ERCC2|CDK1|ATM|TP53|ATR|MAD2L1
278	4,71E-19	1,88E-17	39	380	mitotic cell cycle	ANAPC13|CCNK|CDKN1A|BLM|CETN2|BUB1B|ANAPC10|ANAPC11|CDC20|CCNB2|CCNB1|CDC23|POLD1|CDC27|POLK|POLE|TIPIN|UBE2I|ANAPC7|CDC7|CDC6|MAD2L2|CCNA2|POLA1|CCNA1|CDK6|DBF4|CDC16|CDK2|TIMELESS|MDM2|CDK1|ANAPC4|ANAPC5|MNAT1|ANAPC1|MAD2L1|ANAPC2|TAF1
87	3,12E-14	1,00E-12	28	239	M phase of mitotic cell cycle	ANAPC13|CCNK|CETN2|BUB1B|ANAPC10|ANAPC11|CDC20|CCNB2|CCNB1|CDC23|CDC27|POLK|TIPIN|UBE2I|ANAPC7|CDC6|MAD2L2|CCNA2|CCNA1|CDC16|CDK2|TIMELESS|CDK1|ANAPC4|ANAPC5|ANAPC1|MAD2L1|ANAPC2
51329	2,90E-11	6,99E-10	18	102	interphase of mitotic cell cycle	CDKN1A|BLM|CDC7|CDC6|ANAPC10|POLA1|CCNB1|CDC23|CDK6|DBF4|POLD1|CDK2|MDM2|ANAPC4|ANAPC5|MNAT1|POLE|TAF1
51327	1,02E-07	1,44E-06	15	102	M phase of meiotic cell cycle	UBE2B|RPA1|MRE11A|LIG3|RAD51B|MSH6|CCNA1|RAD50|RAD51C|RAD51|EXO1|CHEK1|CKS2|ATM|NBN
51321	1,18E-07	1,65E-06	15	103	meiotic cell cycle	UBE2B|RPA1|MRE11A|LIG3|RAD51B|MSH6|CCNA1|RAD50|RAD51C|RAD51|EXO1|CHEK1|CKS2|ATM|NBN
10564	8,19E-06	9,75E-05	15	138	regulation of cell cycle process	TIPIN|CREBBP|CDKN1A|UBE2B|MRE11A|CDC7|BRCA1|ANAPC10|MAD2L2|CCNB1|CDC23|CDC16|TIMELESS|MDM2|ATM
7093	2,08E-05	2,38E-04	10	52	mitotic cell cycle checkpoint	MAD2L2|CCNA2|CDKN1A|CCNB1|CDK1|BUB1B|ATM|NBN|TP53|MAD2L1
7346	2,63E-05	2,95E-04	16	174	regulation of mitotic cell cycle	CDKN1A|BUB1B|CDC6|ANAPC10|MAD2L2|CCNA2|CCNB1|CDC23|CDC16|CDK2|MDM2|CDK1|ATM|NBN|TP53|MAD2L1
86	9,00E-05	9,82E-04	7	21	G2/M transition of mitotic cell cycle	CDKN1A|CCNB1|CDK2|ANAPC4|ANAPC5|ANAPC10|TAF1

#: total number of nodes in the gene ontology (GO) annotation; *: number of nodes related to a given GO in the networ

The oxidative stress can generate alterations in the progression of the cycle cellular, (blockage and/or delay), as well as structural dysfunction in several proteins. DNA-integrity checkpoints G1/S and G2/M and M/A transitions determine cell-cycle delays (Rieder, 2011) depending on the cyclin-dependent kinase (Cdk)/cyclin system, such as Cdk1/cyclin B1, which drives the progression from G2 to the mitotic phase (Pearce and Humphrey, 2001). The protein kinases ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) promote DNA damage response and stimulates the checkpoint protein kinases Chk1/2, that can influence cell-cycle arrest. CDK1 and other important proteins related to cell-cycle checkpoints, such as CDC25C and CDC25A, as well as DNA damage were found to be the key proteins inside this cluster. Oxidative stress often induces cell-cycle arrest (Klein and Ackerman, 2003; Pyo et al., 2013), in part through the degradation of the CDC25C protein through a Chk1 protein kinase-dependent pathway (Savitsky and Finkel, 2002).

Cell-cycle arrest associated with complex mixtures of PAHs, metals and other organic compounds upon exposure to coal mining residues has been observed *in vitro* (Tucker and Ong, 1985) and *in vivo* (Espitia-Perez et al., 2018). More recently, exposure to benzo[a]pyrene (also present in the cluster) has been reported to induce cell-cycle arrest and apoptosis in human choriocarcinoma cancer cells through the generation of ROS (Kim et al., 2017).

3.3.3. Systemic effects of IEs and PAHs in the cell cycle

As shown in Table 3, the GO analysis of clusters 9 and 12 revealed 14 main process annotations associated with the cell cycle and particularly Cr and benzo[a]pyrene. The main biological processes found in these clusters included the following: *i) regulation of mitotic*

cell cycle, ii) cell-cycle checkpoint and *iii) interphase of mitotic cell cycle*. Several reports have demonstrated that more-than-additive mortality is common for IE/PAH mixtures. The PAH toxicity in individual aspects suggests that they may modify the accumulation of IEs and improve element-derived reactive ROS. Redox-active elements (e.g. Cu and Ni) are also capable of enhancing the redox cycling of PAHs (Gauthier et al., 2015). Several reports have implicated IEs as modifiers of P450 function and regulation, which implies that such elements could alter P450-mediated PAH mutagenicity and carcinogenicity (Peng et al., 2015). Cr is typically used in coal mining processes (Pandey et al., 2014) and is particularly associated with the fine fractions of PM (Kothai et al., 2009). The genotoxic effects of Cr are predominantly represented by the formation of oxidative adducts and apurinic/apyrimidinic lesions, eventually resulting in DNA breakage (Vasylkiv et al., 2010). Additionally, Cr(VI) has been shown to be aneugenic, as revealed by both chromosome assays and centromere-positive micronuclei assays (Wise and Wise, 2010). However, the combined toxicity of Cr and benzo[a]pyrene has rarely been studied.

Interestingly, *in vitro* cell-cycle analysis has demonstrated that mixtures of benzo[a]pyrene and metals reduce the cell population in the G1 phase and increase cell arrest or accumulation in the G2/M phase (Muthusamy et al., 2018). Once more, the mechanisms suggested include oxidative stress (Fischer et al., 2005), DNA-repair alteration (Tran et al., 2002) and suppressor protein p53 inhibition (Chiang and Tsou, 2009). Particularly, *in vitro* exposure to a combination of benzo[a]pyrene with As, Cr and Pb increase the ROS-mediated oxidative stress in HepG2 cells (Muthusamy et al., 2018). In this regard, within the same module, proteins regulated by oxidative stress and DNA damage inside the cell were also identified as bottlenecks (AKT1, JUN and CREBBP) together with benzo[a]pyrene. Other trace species found in our IE analysis, such as SiO_2, have also been

found to cause DNA damage, oxidative stress, cell-cycle arrest at the G2/M checkpoint and apoptosis synergistically in co-exposure with benzo[a]pyrene (Asweto et al., 2017).

3.3.4. Systemic effects of IEs in the cell cycle

This cluster addresses a particular area of interest in relation to whether metal ions and IEs interfere with other cellular responses to DNA damage, such as cell-cycle progression and control. In clusters 2 and 4, AKT1, JUN and CREBBP and the TP53, CCNB1, CCNA2, CDK6, CDK2, CDK1, ATM, ATR and CDK7 proteins were found to be bottlenecks together with Cr and S. The biological processes linked to this cluster and its respective proteins are presented in Table 4.

Among all the chemical species present in coal mining environments, IEs in particular, are capable of causing the most oxidative damage through the generation of ROS (Valko et al., 2006). IEs can enter to the body through the inhalation or consumption of contaminated meals and then accumulate in the bloodstream (Schweinsberg and Von Karsa, 1990). These elements are deposited in tissues by various mechanisms (Bridges and Zalups, 2005) and may cause DNA damage. In this cluster, together with proteins regulated by oxidative stress and DNA damage, we also found proteins such as cyclins and cyclin-dependent kinases that have been reported to be down-regulated in response to ROS and are implicated in the induction of cell-cycle arrest as one of the immediate defence mechanisms against genotoxic damage from oxidative stress (Burch and Heintz, 2005). Particularly, CCNB1 seems to be depleted in response to oxidative stress, causing the regulation of G2/M transit via the Chk1-Cdc2 DNA damage checkpoint pathway (Pyo et al., 2013). Conversely, because altered cell cycle progression and/or cell cycle control and DNA repair inhibition have been observed under low, non-cytotoxic concentrations of the metal compounds, some authors have suggested that inhibition could also be a result of the ability of metal ions to

modify zinc finger proteins involved in cell-cycle control and DNA repair (Hartwig et al., 2002). Interestingly, as a response to DNA damage caused by Cd(II), some authors have reported the suppression of TP53-mediated cell-cycle arrest in human breast cancer MCF7 (Méplan et al., 1999). Other IEs involved in the modification of zinc finger proteins include Ni and Co (Hartwig and Schwerdtle, 2002). However, no similar implications have been reported for Cr and S.

As discussed in the previous section, Cr(VI) has been demonstrated to be consistently mutagenic in bacterial and mammalian model systems, and its carcinogenic activity is thought to be due to the induction of DNA damage generated by reactive intermediates, eventually resulting in DNA breakage (Vasylkiv et al., 2010). Free radicals from SO_2 metabolisms, such as $SO_3^{\cdot-}$, $SO_4^{\cdot-}$, $SO_5^{\cdot-}$ may also induce DNA strand breaks (Meng et al., 2005), and recent studies have confirmed that SO_2 derivatives (bisulfite and sulfite) cause mitotic delay in cultured human blood lymphocytes in a dose-dependent manner (Uren et al., 2014).

3.4. CONCLUSIONS

Using a systems chemo-biology approach, we examined how some of the major chemical constituents of coal dust and PM derived from coal mining activities interact with specific biological processes related to the cell cycle. Thus, the main proteins and compounds present in the network were taken into account for the design of a molecular model about the effect of major coal residues on the cell cycle (Figure 5). The analysis performed in the present study suggests that coal residue MIOs (SiO_2), IEs (Ti, Mg, Cr, Cl and S) and PAHs (benzo[a]pyrene, fluoranthene, benzo[b]fluoranthene and phenanthrene) can induce ROS generation. The resultant oxidative stress can induce cell cycle arrest

through the upregulation of proteins such as AKT, APP, JUN and CREBBP, leading to DNA damage response activation by ATM/ATR and Chk1/Chk2 or by CDC25C or CCNB1 degradation. The model also considered that protein p53 can be activated by Chk1/Chk2 and induce cell-cycle arrest, senescence or apoptosis.

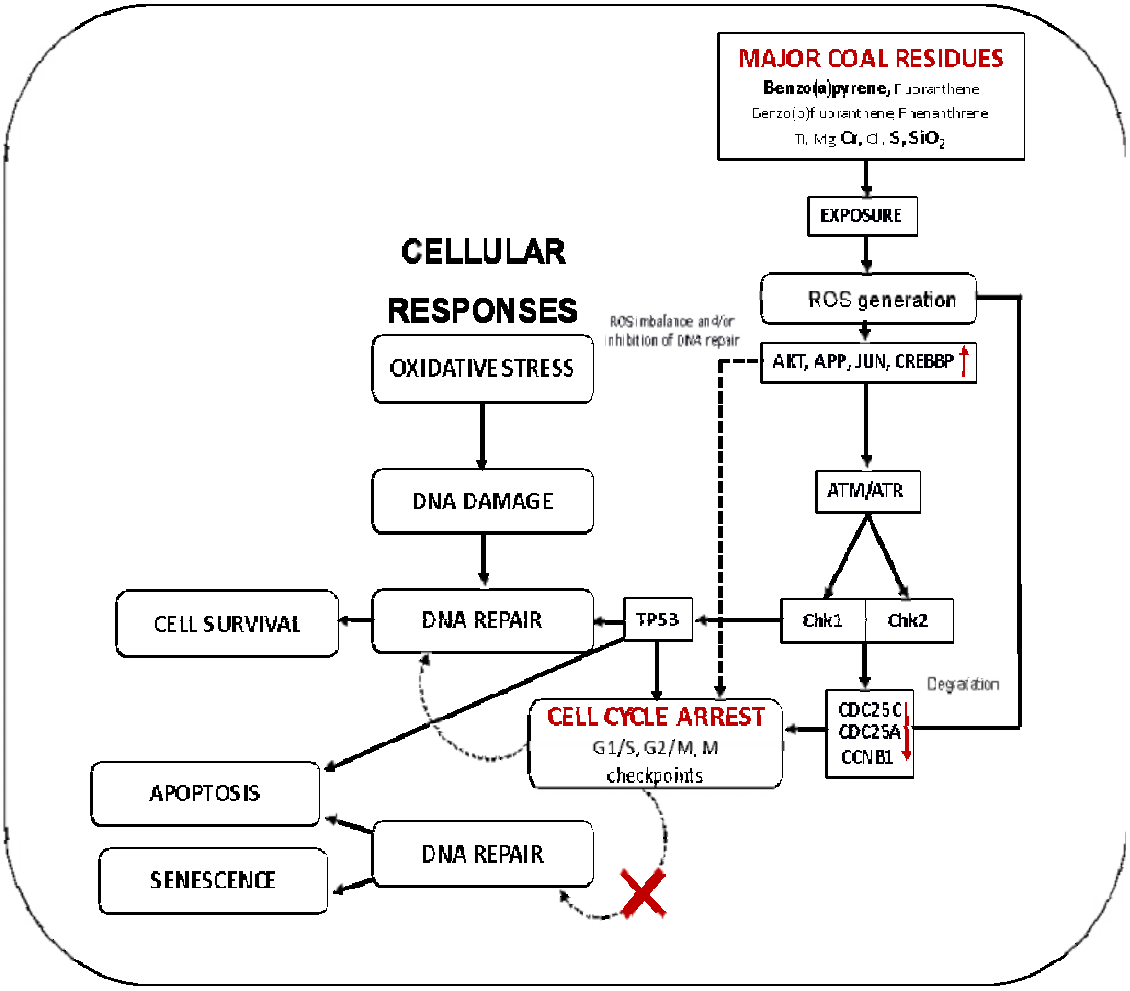

Figure 5. Molecular model illustrating how major coal residues potentially affect cell cycle progression: Exposure to major coal residues, such as benzo[a]pyrene, fluoranthene, benzo[b]fluoranthene, phenanthrene, Ti, Mg, Cr, Cl, S and SiO_2, is able to generate reactive oxygen species (ROS) via several pathways (e.g. Fenton-like reactions). The ROS imbalance and/or inhibition of the DNA repair process can lead to oxidative stress and the upregulation of several proteins associated with the oxidative response (AKT, APP, JUN and CREBBP) that are also involved in the control of the cell cycle. DNA and protein damage caused by oxidative attack trigger repair pathways, i.e. the DNA damage response, including the ATM/ATR pathway, and activate the checkpoint protein kinases Chk1/2, which may result in cell cycle arrest. Oxidative stress can also induce cell cycle arrest through the degradation of CDC25C via the Chk1 protein kinase-dependent pathway. As a response to DNA damage, the activation of TP53 activates the expression of various genes related to cell death, cell cycle arrest, senescence, DNA repair and various other processes.

Conflict of interest

The authors declare no conflict of interest

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4. CAPÍTULO II

In vitro effects of bituminous and subbituminous coal samples: purine oxidation and DNA damage in V79 cells

Manuscrito a ser submetido na Mutation Research

In vitro effects of bituminous and sub-bituminous coal samples: purine oxidation and DNA damage in V79 cells

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ABSTRACT

In recent years, an increasing body of evidence has demonstrated that occupational and environmental exposure to open-cast coal mining residues such as Polycyclic Aromatic Hydrocarbons (PAHs) and inorganic elements (IE) might cause a wide range of chronic diseases through the generation of DNA damage and genomic instability. Coal is a fossil fuel of economic importance in Colombia. The main goal of our study is to evaluate the cytotoxic and genotoxic effects induced in V79 cells by the exposure to bituminous and sub-bituminous coal samples from two open-pit coal mines located in La Guajira (bituminous coal from El Cerrejon mine (ECCS)) and Cordoba (sub-bituminous coal from La Guacamaya mine (LGCS)) from Colombia. The IC_{50} values obtained after 3 and 24 h showed significantly increased inhibition of clonogenic cell survival induced by bituminous samples from El Cerrejón mine compared to sub-bituminous samples from La Guacamaya mine. The comet assay showed that exposure to coal of ECCS and LGCS, induced primary DNA lesions after 3 and 24 h. A significant dose-depend increase in % Tail DNA in the alkaline comet assay was observed in cells exposed to ECCS for 3 h. When the lesion-specific endonuclease Formamidopyrimidine-DNA Glycosylase (FPG) was applied, % tail DNA damage increased in cells exposed to LGCS. Results of the enzyme-modified comet assay suggest oxidation of guanine in DNA generated by coal exposition. The cytokinesis-block micronucleus cytome assay (CBMN cyt) showed that exposure to bituminous coal induces the formation of micronuclei (MN) in V79 cells line from 3 h of exposure, while sub-bituminous coal only induces MN formation in 24 h. DNA damage could be a consequence of the ability of the complex mixture in bituminous and sub-bituminous coal from Colombia, including PHA (benzo[a]pyrene, fluoranthene, benzo[b]fluoranthene and phenanthrene) and IE (Ti, Mg, Cr, Cl and S), to cause oxidative damage.

Keywords: bituminous coal; sub-bituminous coal; DNA damage; purine oxidation; in vitro; V79 cells.

4.1. Introduction

Colombia has significant coal reserves in Latin America. Production is estimated approximately 16,992 million tonnes (Mt), which is ranked as the fifth largest exporter of coal worldwide, equivalent to 50 Mt for year (1). Most of the reserves are located in the Caribbean region (departments of La Guajira, Cesar, and Córdoba), where technified and large-scale open-pit mining predominates with 90% of the thermal coal. (1,2). The coal in this region is characterized by a calorific value of 11,600 BTU/lb, approximately, low humidity, ash, and sulfur. Coal produced in Colombia is used in industry and for the generation of heat, vapor, and electricity, and is easily transported overseas thanks to the proximity of coastal ports (3).

According to American Society for Testing and Materials, coal is classified by vegetal composition, temperature and pressure conditions in the formation process in anthracite, bituminous, sub-bituminous and lignite (2). Anthracite-type coal is used in the steel and iron industries for heat or steam generation, and in the manufacture of synthetic rubber, colorants, and water purification filters. Bituminous coal is used in processes for obtaining steel and in the production of steam for the generation of electricity energy. Sub-bituminous coal is used in the generation of electricity and some industrial processes. Lignite type coals are used for heat generation (heating systems) and industrial processes to generate steam. Likewise, coal is used for production of benzene, oils, tar, and liquefaction, as a substitute for petroleum (2).

Coal from “El Cerrejón” is typically bituminous with a volatile content of 37.4% and ash content of 6.8% (dry basis), 76.2%, hydrogen 4.8%, oxygen 11.1%, nitrogen 1.7%,

and sulfur 0.7% (4). Coal exploited in “La Guacamaya” are sub-bituminous with a high sulfur content (2.30% total sulfur: 1.06% as pyritic, 1.10% as organic and 0.14% from sulfates) and a high volatile content (5). Although, a chemical characterization has been reported with some elements present in El Cerrejón coal (6). Other Colombian coals like those exploited in the La Guacamaya mine have not been sufficiently characterized. In this sense, previously our group conducted a study concerning the characterization of the main chemical compounds in the bituminous coal of El Cerrejón and sub-bituminous coal of La Guacamaya of Colombia (6). Results from this study reported different amounts of major inorganic oxides (MIOs) (SiO_2, TiO_2, Al_2O_3, Fe_2O_3, MgO, CaO, Na_2O, K_2O, P_2O_5, and SO_3), inorganic elements (IE) (Na, Mg, Al, Si, S, Cl, K, Ca, Ti, Cr, Mn, Fe, Ni, Zn, and Sr), and polycyclic aromatic hydrocarbons (PAH) (naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene, benzo[a]anthracene, benzo[g,h,i]pyrene, benzo[b]fluoranthene, dibenzo[a, h]anthracene, indeno[1,2,3-cd]pyrene and benzo[k]fluoranthene) (7).

El Cerrejón and La Guacamaya mines are open pits coal mining (3). Open-pit coal mining is considered the largest generator of particulate matter (PM). The PM is classified by aerodynamic diameter in microns into particles: gross (PM_{10}), fine ($PM_{2.5}$), ultrafine ($PM_{0.1}$), and nanoparticles (less than 0.1) (8). In general, PM10 particles deposit in the upper respiratory airways and are eliminated by mucociliary clearance or by mechanical processes (such as coughing or sneezing) and PM2.5, PM0.1 and nanoparticles, due to their small size, can reach the pulmonary parenchyma (9). Depending on size these particles are dispersed by the air in the atmosphere, remain in the environment for long time, and inhaled by occupationally exposed workers (10,11), and human settlements and fauna nearby (12).

Studies from Leon-Mejia et al. (13,14) and Espitia-Perez et al. (15,16) demonstrated DNA damage induced by coal, in the areas of influence of the El Cerrejón and La Guacamaya mines in Colombia, using alkaline comet assay and micronucleus assay in workers, habitants and native animals. Suggesting the possible genotoxic effect, generated by the inhalation of the MP from the coals and their components (13,16–18). Other toxicological investigations using PM with some of the chemical compounds present in the coal (*in vitro*), showed that these elements could induce several types of adverse cellular effects, including genotoxicity (19,20), cell death (21,22), alterations of the cell cycle (23,24), and stimulation of the production of proinflammatory cytokines (25). In silico Torres-Avila et al.,. (7) showed that some chemical components of coal could induce ROS generation resulting in oxidative stress that can cause cell cycle arrest through the positive regulation of proteins (7).

Our study evaluated the potential cytotoxic, genotoxic and mutagenic generated by bituminous coal from El Cerrejón and sub-bituminous coal from La Guacamaya of Colombia in V79 cell line (lung fibroblasts, Chinese hamster), for determine the effect caused by the exposure for two Colombian coal types and eliminate the possible effects that could cause other toxic molecules in the environment.

4.2. Materials and methods

4.2.1. Coal samples

Coal samples used for in vitro treatments were collected from coalfaces from “El Cerrejón” (La Guajira, Colombia) (ECCS) and “La Guacamaya” (Puerto Libertador, Córdoba, Colombia) (LGCS) (Figure 1). Samples were collected for coal mines in December of 2013, from six random sampling points from each mine and prepared as a

homogeneous pool and pulverized. Coals from “El Cerrejón” are typically bituminous (4). Coals from “La Guacamaya” are sub-bituminous (5). Detailed chemical characterization of both coal samples used in the in vitro test is available at Torres-Avila et al. (7).

4.2.2. Cell culture and treatment

The cell line V79 (Chinese hamster lung fibroblasts) was obtained in the cell bank from Rio de Janeiro (Rio de Janeiro, Brazil), characterized by a stable karyotype and widely used in genotoxicity and cytotoxicity assays. The cells were cultured with Dulbecco's Eagle modified medium (DMEM) high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% penicillin/streptomycin 10,000 Units/mL /10,000 µg/mL and 0.1% fungizone at 37°C in a humidified atmosphere with 5% CO2 and following the protocol used by Leon-Mejia et al. (18).

Initially, dilutions of 1 mg/mL were prepared with ECCS and LGCS individually in DMEM medium without FBS, then sonicated for 10 min 2 times and kept under agitation at 180 rpm overnight at room temperature to obtain a uniform dispersion of the particles of coal. From the initial concentration, serial dilutions were made using DMEM, until reaching the desired concentrations. These dilutions were prepared each time an experiment was started. All the experiments were carried out three times (18).

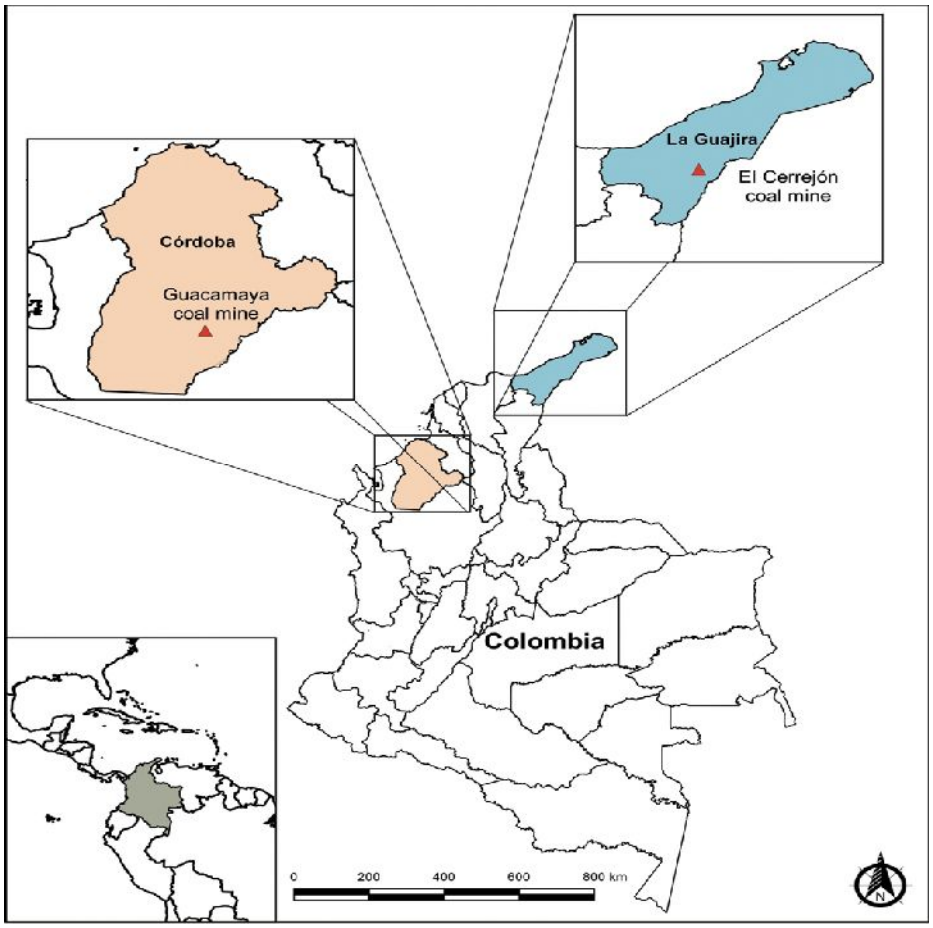

Figure 1. Coal samples collection sites in Colombia: “El Cerrejón” (La Guajira, Colombia) and “Guacamaya” (Puerto Libertador, Córdoba, Colombia) coal mines.

4.2.3. Cytotoxicity assay

The clonogenic assay allows the evaluation of cell viability through the formation of colonies. Initially, 200 cells per well were seeded in 6-well cell culture plate and incubated for 24 h. After , the cells cultures was treated with coal dilution (0.15, 0.25, 0.5, 0.75 and 1 mg/mL) of ECCS and LGCS for 3 and 24 h. The negative control was incubated with DMEM medium (free FBS). The treatment was removed, and incubated with DMEM medium for seven days. Each experiment was performed three times individually.

Subsequently, the colonies obtained were fixed with methanol and colored with violet crystal (1%). The percentage of clonogenic survival was expressed relative to the negative control, and IC50 values were calculated (22, 23, 24).

4.2.4. High-Throughput Comet Assay

The alkaline comet assay was carried out according to Singh et al. (28) and Tice et al. (29) with several modifications for a high-throughput comet assay version, which allows the processing of multiple samples (29). The high-throughput "96-mini gel format" is an 8x12 multi-array on GelBond® film (Lonza, Rockland Inc. ME, USA) (30). A detailed experimental protocol has been described by Kiskinis (31). For this assay 6 x 10^4 V79 cells per well were seeded in 12-well cell culture plate and incubated for 24 h. After, the cells cultures was treated with 0.14, 0.29 and 0.57 mg/mL coal dilution of ECCS and 0.17, 0.34 and 0.68 mg/mL coal dilution of LGCS for 3 h and 24 h. The negative control was incubated with DMEM medium (free FBS) and the positive control was treated with 150 µM of H_2O_2 for 3 h. Cells were then collected using 0.15% trypsin-0.08% EDTA and resuspended in complete medium. An aliquot of 10-µL of V79 cells suspension was drawn for cell viability assessment using trypan blue dye exclusion assay (cell viability ≥ 90%). Cells were gently resuspended, concentrated (800rpm for 10 min), transferred to a 96-well microplate and mixed in the ratio of 1:10 with a previously prepared 0.5% low melting point agarose (LMA-Invitrogen) in PBS (pH 7.4) without calcium and magnesium. 5 µL aliquots of LMA-cell suspension were added onto the hydrophilic side of pre-cut GelBond® sheets positioned on an ice-cold metal plate. One mini gel of the 8X12 array was prepared from V79 cell suspension harvested from each well. Minigels were immersed overnight 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°C in the dark and rinsed the next day using cold PBS. For DNA unwinding, films were immersed in a cold electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH≥13) for 40 min. The electrophoresis was carried out at 0.8-1.0 V/cm for 40 min (32). The minigels were neutralized with cold Tris buffer (0.4M Tris, pH 7.5) for 3×5 min, fixed with 96% ethanol for 1.5 h and kept in the dark in a dust-free area. Finally, GelBond® films were cut into arrays containing 3x4 mini gels, stained with 10 mL of ethidium bromide (1 µg/mL), rehydrated with cold deionized water. Preparations were stored in the dark at 4°C until scoring the same or the next day (33). For semi-automated scoring, stained cells were analyzed using an Olympus BX51 fluorescence microscope (Olympus, Japan) and examined at 40X magnification under a green filter of 540 nm. Direct light exposure of the samples was avoided during the whole process. Individually, we analyzed 100 randomly selected 50 cells from each of two replicate slides. To avoid selection bias, overlapping comets were excluded (33). % Tail DNA was scored using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK).

The alkaline comet assay using lesion-specific enzyme Formamidopyrimidine DNA glycosylase (FPG) (New England Biolabs, MA, USA) was used to detect oxidized purines (34). The protocol was used as previously described with minor modifications for the high-throughput comet assay (35). After the lysis mini gels were washed with the enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA and 0.2 mg/mL bovine serum albumin), and incubated with (i) FPG (1:1000, 45 min), and (ii) with enzyme buffer (control) at 37°C for 30 min. FPG recognizes oxidized purines, specifically 8-oxo-guanine (35). DNA unwinding, electrophoresis, neutralization, staining and scoring procedures were made

according to the same high-throughput comet assay protocol, as described above. Significant differences between 7-11% in registered values of DNA in the tail were identified for each treatment (FPG) over control (buffer without enzyme) (36). Different dilutions of FPG enzymes were tested, and calibration curves were established using H_2O_2 (0.1 M) an oxidative genotoxic agent**.**

4.2.5. Cytokinesis-block micronucleus cytome assay

The cytokinesis-block micronucleus cytome (CBMN cyt) assay was used to measure DNA damage, evaluating: (i) micro-nuclei (MN), as a biomarker of chromosomal breakage and/or complete chromosomal loss; (ii) nucleoplasmic bridges (NPB), a defective DNA repair biomarker and telomere terminal fusions; and (iii) nuclear buds formation (NBUD), a biomarker for the elimination of amplified DNA and/or DNA repair complexes. The Cytostatic effects was evaluated by estimating the nuclear division index (NDI) calculated by the proportion of mono, bi and multinucleated cells and finally the cytotoxicity establishing the proportion of necrotic and / or apoptotic cells in V79 cells that were exposed to the same concentrations and time intervals used in the alkaline and modified comet assay(37)

To calculate the potential genotoxic effects of Colombian coal, 15 x 10^4 V79 cells per well plate were seeded in 6 well cell culture plates and incubated for 24 h. The cells cultures was treated with 0.14, 0.29 and 0.57 mg/mL coal dilution of ECCS and 0.17, 0.34 and 0.68 mg/mL coal dilution of LGCS for 3 and 24 h. The negative control was incubated with DMEM medium (free FBS), and the positive control was treated with Methyl methanesulfonate (MMS) 40 µM for 2 h. After the treatment, the wells were washed to

remove residues of the treatment and placed DMEM with cytochalasin B (3 µg/mL) and were incubated for 24 h. Then the cultures were washed with PBS 1X, and the cells were collected using 0.15% trypsin-0.08% EDTA which was inactivated with SBF and transferred in 15 mL falcon and centrifuged for 800 rpm for 5 min. The supernatant was discarded, the pellet was homogenized, and cold KCL 75mM was added and incubated for 3 min. After, 500 µl of fixative solution (ethanol: glacial acetic acid (3:1)) was added and centrifuged at 800 rpm for 5 min. The supernatant is discarded, then 5 mL of fixative solution is added to the pellet and carefully mixed and centrifuged (2 times), then the samples are homogenized and kept at 4 ° C overnight. The next day, the samples are centrifuged, to concentrate the cells, the pellet is homogenized gently and finally, it is dripped on slides to be colored with 10% Giemsa for 10 min (37).

For the analysis of cytome assay, 1000 binucleated cells (BN) were counted under an optical microscope (100X). To estimating the nuclear division index (NDI), 500 cells per slide were counted, and the proportions of mononuclear, binucleated and multinucleated cells were determined. The calculation was performed according to the procedure used by Fenech (2000). The frequency of micronuclei, NPB and NBUD per 1000 BN cells (500 cells per slide) were used as indicators of DNA damage and chromosomal instability (37).

4.2.6. Statistical analysis

The data from clonogenic assay in 3 and 24 hrs were used to calculate IC_{50} values using nonlinear regression. The data from the alkaline comet assay, alkaline comet assay modified and CBMN cyt in 3 and 24 hrs of exposition were evaluated for normality using Shapiro-Wilk test. The treatments with normality were compared with the negative control

(untreated) using one-way ANOVA followed by Tukey's post hoc test. The treatments non-normally distributed were subjected to Kruskal-Wallis test with a Dunn's multiple comparison tests. We used a significant P value < 0.05. Statistical analyses were carried out using software Graph Pad Prism 7.0 software (GraphPad Inc., San Diego, CA).

4.3. Results

4.3.1. Cytotoxicity induced by ECCS and LGCS exposure

The clonogenic assay was used to determine the colony formation capacity of V79 cells exposed to different concentrations of pulverized coal samples of ECCS and LGCS (Figure 2) in two different exposure times (3 and 24 h). ECCS and LGCS were able to decrease the cell viability in a dose-dependent manner. However, ECCS showed a higher cytotoxic effect compared to LGCS. The IC_{50} values of bituminous coal from ECCS is 0.57 mg/mL in 3 h and 0.23 mg/mL in 24 h. In sub-bituminous coal of LGCS the IC_{50} values was little increased in 3 h (0.68 mg/mL) and 24 h (0.34 mg/mL). Decreased of IC_{50} values was observed in 24 h in the two samples.

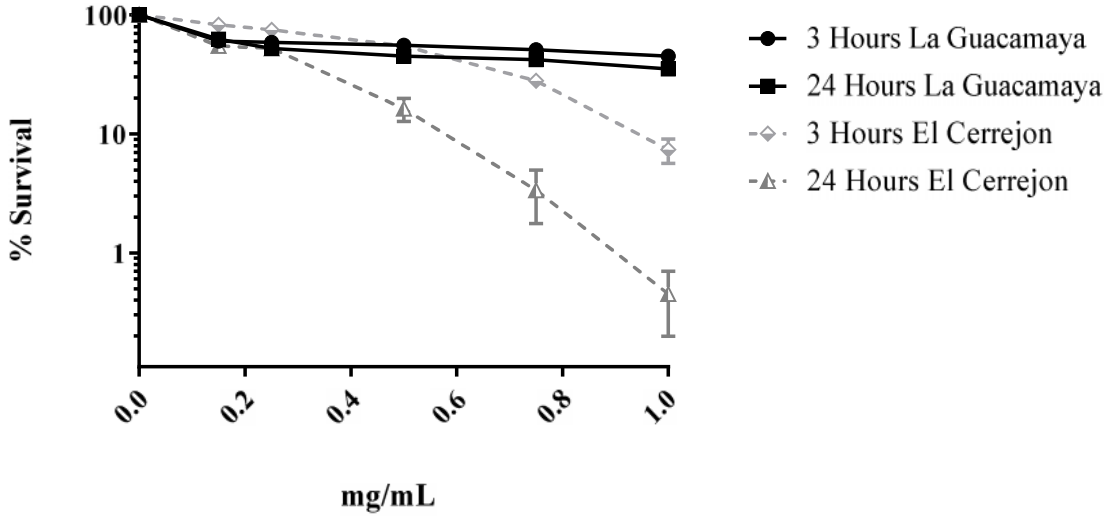

Figure 2. Cell viability of V79 exposed to ECCS and LGCS evaluated using clonogenic assay after 3-and 24 h of exposure for the V79 cell line. The concentration was considered cytotoxic when cell survival was 50 %.

4.3.2. Effect of ECCS and LGCS exposure on alkaline and FPG high-throughput Comet assay

DNA damage induced by ECCS and LGCS was determined by the alkaline high-throughput comet assay evaluating the % Tail DNA for each treatment. Cells were exposed to 0.14, 0.29 and 0.57 mg/mL of ECCS and 0.17, 0.34 and 0.68 mg/mL of LGCS for 3 and 24 h. Compared to the NC, all treatments showed statistically significant differences ($P < 0.05$) in the alkaline and modified comet assay (Figure 3). However, a dose-depend relationship was observed only for % Tail DNA in the alkaline version in 3 h with ECCS. Under FPG conditions, % Tail DNA increased significantly in cells exposed to 0.14 mg/mL of ECCS and 0.17 mg/mL of LGCS and remained almost unchanged at higher doses. The

positive control, H_2O_2 (150 µM) showed expected effect, demonstrating the sensitivity of the test.

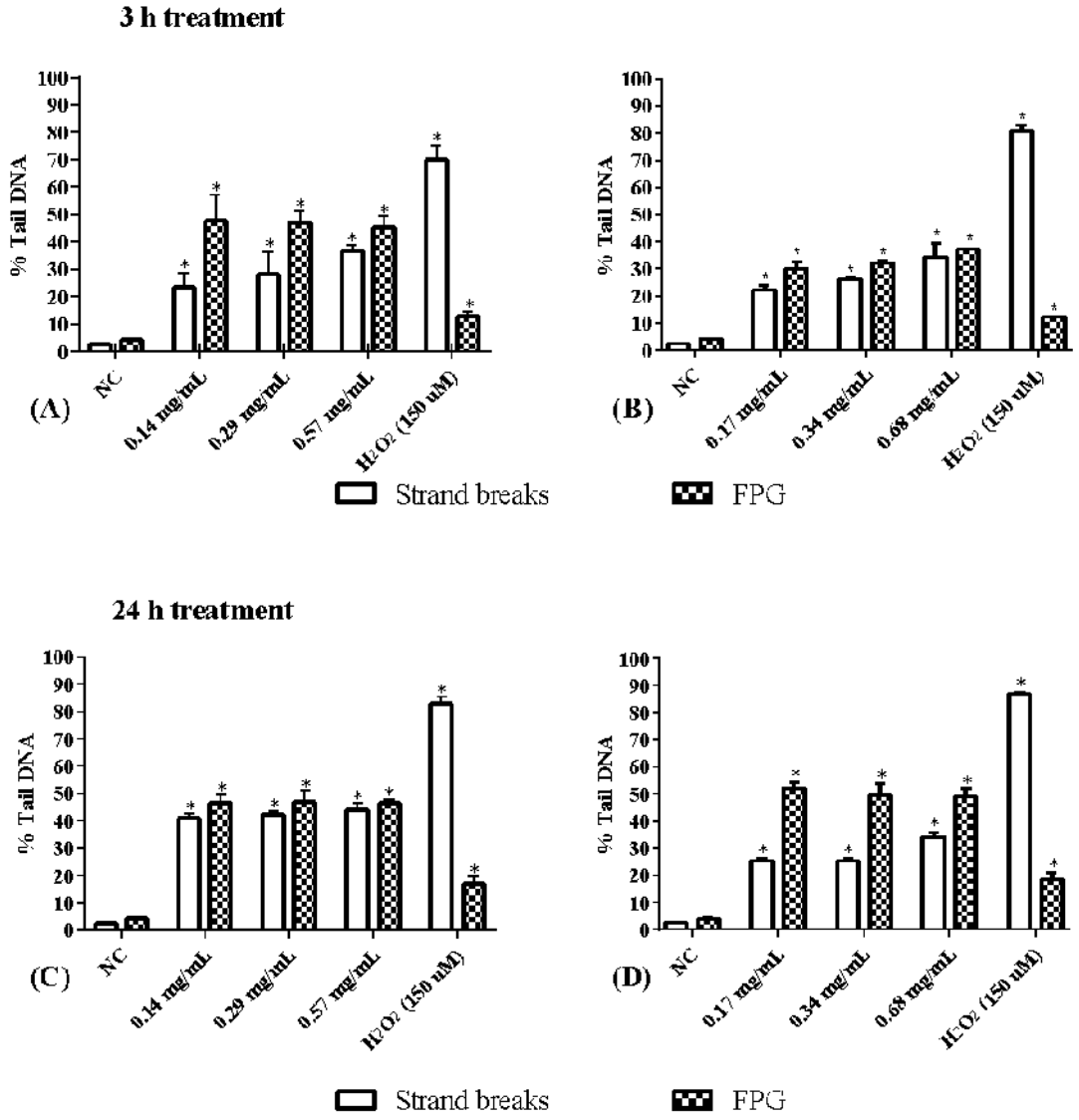

Figure 3. % Tail DNA in the alkaline comet assay (strand breaks in white) and oxidized purines (grid) in modified comet assay with FPG in V79 cells. **(A)** 3 h treatment with ECCS, **(B)** 3 h treatment with LGCS, **(C)** 24 h treatment with ECCS and **(D)** 24 h treatment with LGCS. *Statistically significant difference about negative control (NC) $P < 0.05$. The results are shown as the mean ± SEM

4.3.3. Effect of ECCS and LGCS exposure on the CBMN Cyt assay

The frequencies of the CBMN-cyt assay parameters presented in Table 1 were significantly higher ($P < 0.05$). When we evaluated the biomarker MN, the exposure at 0.57 mg/mL for 3 h with ECCS showed a significant increase ($P < 0.05$). When the time exposition was increased to 24 h, the concentrations of 0.29 and 0.57 mg/mL of ECCS and 0.68 mg/mL of LGCS showed significance ($P < 0.05$) for the biomarker of MN. The NBUD biomarker, only showed a significant increase ($P < 0.05$) when used sub-bituminous coal (LGCS) for 24 h and dilution of 0.68 mg/mL. The other parameters evaluated for CBMN-cyt assay, show increase in number, but not shown statistically significant levels when was compared with negative control.

The nuclear division index did not show significant differences in 3 and 24 h of exposure to the different concentrations of coal used from ECCS and LGCS (Table 1). Similar results were obtained when the cytotoxicity index was analyzed, which did not show significant differences in the number of apoptotic and necrotic cells in 3 h and 24 h of exposure (Table 1) compared to the negative control. For the positive control was used Methyl methanesulfonate (MMS) 40 μM for demonstrating the sensitivity of the assay.

Table 1. CBMN cytome biomarkers in V79 cells exposed for 3 and 24 h and different concentrations of El Cerrejón and La Guacamaya coal.

Exposure time	NDI	MN	NPBs	NBUDs	APOP (%)	NECRO (%)
3 h						
El Cerrejón Coal						
NC	1.86±0.04	3.00±1.15	1.50±0.57	1.25±0.50	0.25±0.50	0.00±0.00
0.14 mg/mL	1.79±0.03	4.50±1.29	3.50±1.00	1.75±0.95	0.25±0.50	0.25±0.50
0.29 mg/mL	1.82±0.03	6.50±3.10	3.75±1.89	1.00±0.00	0.50±0.57	0.75±0.95
0.57 mg/mL	1.80±0.03	**9.75±1.70***	4.75±2.87	3.25±2.50	0.75±0.50	1.00±1.41
MMS 40 µM	1.81±0.05	**9.25±1.70***	**6.75±4.19***	**4.75±0.95***	**3.25±0.95***	**2.00±0.81***
La Guacamaya Coal						
NC	1.86±0.04	3.00±1.15	1.50±0.53	1.00±0.75	0.75±1.03	0.37±0.51
0.17 mg/mL	1.84±0.04	5.00±2.94	3.00±2.94	1.50±1.91	1.00±0.81	0.50±1.00
0.34 mg/mL	1.84±0.05	4.25±2.50	3.00±1.41	1.25±1.25	0.25±0.50	1.00±1.15
0.68 mg/mL	1.84±0.05	5.25±2.21	2.00±2.16	0.75±0.95	0.50±0.57	0.50±1.00
MMS 40 µM	1.81±0.05	**8.75±1.25***	**5.75±3.49***	**3.62±1.50***	**2.62±1.06***	**2.00±0.75***
24 h						
El Cerrejón Coal						
NC	1.87±0.01	2.25±0.88	1.37±1.18	1.12±0.35	0.25±0.46	0.50±0.53
0.14 mg/mL	1.84±0.04	7.00±3.36	4.50±3.10	1.50±1.73	0.25±0.50	0.25±0.50
0.29 mg/mL	1.85±0.01	**9.50±6.40***	5.50±1.29	2.75±1.89	0.50±0.57	0.25±0.50
0.57mg/mL	1.84±0.01	**9.25±4.42***	5.25±2.75	2.00±1.15	0.75±0.50	0.50±0.57
MMS 40 µM	1.82±0.05	**11.0±5.03***	**10.88±3.56***	**3.25±0.70***	**2.25±0.95***	**1.37±0.74***
La Guacamaya Coal						
NC	1.87±0.01	2.25±0.95	0.50±0.57	0.50±0.53	0.25±0.46	0.50±0.53
0.17 mg/mL	1.81±0.03	8.00±0.81	9.00±4.08	3.00±1.15	0.50±1.00	1.00±1.41
0.34 mg/mL	1.80±0.04	10.25±2.98	6.75±3.09	3.00±2.16	0.50±0.57	0.00±0.00
0.68 mg/mL	1.80±0.02	**13.25±2.98***	7.00±3.16	**4.00±1.82***	0.25±0.50	1.00±0.81
MMS 40 µM	1.82±0.05	**11.00±5.03***	**10.75±2.06***	**3.00±0.75***	**2.25±0.95***	**1.75±0.46***

Data are expressed as mean ± SEM of three independent experiments *Bold for significant difference compared with negative control (NC) group within a column, $p < 0.05$.

4.4. Discussion

In recent years the genotoxic potential of the bituminous coal of El Cerrejón mine was estimated in neighboring populations and workers (Espitia-Pérez et al., 2016 and 2018 Leon-Mejia et al., 2011; Espitia-Pérez et al., 2018). While, genotoxic potential of sub-bituminous coal of La Guacamaya mine was evaluated in native animals (León et al., 2007). The potentially harmful effect related to the exposition of coal mining waste could increase by exposure to another type of contaminant or xenobiotics in the environment. Therefore, we evaluated the cytotoxic and genotoxic potential caused by exposure of bituminous coal from ECCS and sub-bituminous coal from LGCS in V79 cells line (Chinese hamster lung fibroblasts) to determine the potential generated from each coal sample without the presence of other possible contaminants.

After exposure to ECCS and LGCS for 3 and 24 h in the different concentrations, the V79 cells showed evidence of cytotoxicity (Fig. 2). We observed a variation in the concentration of IC50 related to time and type of coal, indicating that the cytotoxic effects of ECCS (bituminous coal) are 16.17% higher in 3 h and 32.36% higher in 24 h when compared with sub-bituminous coal of LGCS. These results demonstrate higher cytotoxicity in ECCS bituminous coal, which could be related to the levels of chemical compounds in this coal reported by Torres-Avila et al. (7). Bituminous coal of ECCS has a high concentration of (silicon, aluminum, sulfur, iron, sodium, manganese, nickel, chlorine, zinc, benzo (a) anthracene, fluoranthene, anthracene, naphthalene, phenanthrene, and benzo (g, h, i) pyrene, compared to sub-bituminous coal of LGCS (7), which can be influence our results.

We used the alkaline high-performance comet assay and modified to evaluate the genotoxic activity of ECCS and LGCS in 3 and 24 h. In the alkaline comet assay modified was used the FPG enzyme to detect oxidized purines and open ring purines (38,39). Our results of the alkaline high-performance comet assay showed the presence of primary lesions (%DNA tail increased) in V79 cells exposed for 3 and 24 h with ECCS and LGCS. Additionally, our results of comet assay modified showed that the cultures exposed with ECCS maintain the levels of % tail DNA concerning dose and time of exposure, while the cultures exposed with LGCS presented an increase in % tail DNA when the time increased for 24 hrs. These results could indicate that the oxidative damage presented by ECCS could be starting the process of DNA repair by the damage levels do not increase concerning time, while the damage caused by LGCS is exceeding the antioxidant capacity, as it increased level oxidative lesions. A work conducted by Leon-Mejia et al., with coal and its products related DNA damage and oxidative stress generated by the presence of IE and PAH (18). Another study reported the risk of environmental and/or occupational exposure to miscellaneous chemical products (40). The response of cells exposed to LGCS could be associated with the production of ROS in more significant proportion, and this exceeds the antioxidant protection and cellular mechanisms repair only a part of the DNA as demonstrated by Rohr et al., (41) and Sambandam et al., (42). These results may also be associated with those obtained by Torres-Avila et al. (7) that reported the different levels of inorganic elements (heavy metals) in bituminous coal from ECCS and sub-bituminous coal from LGCS and the coal from LGCS presented high levels of chromium. Valko et al., (43) reported that some inorganic elements (heavy metals) could generate greater oxidative damage by generating ROS.

Our results about the CBMN-Cyt test indicate that the bituminous coal from ECCS produces an increase in the frequency of MN in 3 h with 0.57 mg/mL. The cells exposed to 0.29 mg/mL for 24 h to ECCS produced chromosomal instability (increased MN frequency). While the cells exposed to sub-bituminous coal about the LGCS needs exposition to a dose of 0.68 mg/mL for 24 h to initiating elevating MN frequency significantly. These data are support of results of Torres-Avila et al., (7), that characterized the IE, PAH and major inorganic oxides (MIO) of the coal samples used in our study and that report higher levels in the ECCS and where they associate this combination of elements with the generation of oxidative stress and DNA damage and alterations in the progression of the cell cycle, (blockage and/or delay), as well as structural dysfunction in several proteins (7). Other studies have reported the combination of IEs, PAHs, and MIOs with increased DNA damage in vitro cellular systems (11,18) and in human populations in environments with coal mining (44).

Besides, cell cultures exposed to sub-bituminous coal for LGCS presented nucleoplasmic bridges that indicate a higher level of damage, which could be associated with the higher levels of chromium reported in LGCS(7). Since the genotoxic effects of Cr are represented predominantly due to the formation of oxidative adducts and apurinic/apyrimidinic lesions and finally results in DNA breaks (45). Also, Cr (VI) has been shown to be aneugenic, measured both by chromosomal assays and by positive centromeres of micronuclei (46).

4.5. Conclusions

In summary, our results showed that the bituminous coal of ECCS can induce an increase in MN frequency and oxidative damage from 3 h of exposition with a low dose, whereas the

sub-bituminous coal of LGCS only increased MN frequency with the highest dose in 24 h. Additionally, induced nucleoplasmic bridges a defective DNA repair biomarker and telomere terminal fusions and increase in levels of oxidative lesions. These differences could be related to chemical components in different proportions in bituminous coal of ECCS and sub-bituminous coal of LGCS could be associated with the generation of reactive oxygen species (ROS) in different levels, and consequently lead to DNA damage and the formation of pro mutagenic adducts.

Conflict of interest

The authors declare no conflict of interest

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5. CAPÍTULO III

Genotoxic, histopathological, oxidative stress, and inflammatory effects in Wistar rats exposed by the inhalation of sub-bituminous coal

Manuscrito a ser submetido na Journal of Exposure Science and Environmental Epidemiology

Genotoxic, histopathological, oxidative stress, and inflammatory effects in Wistar rats exposed by the inhalation of sub-bituminous coal

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Abstract

Coal dust inhalation is associated with lung disorders such as simple pneumoconiosis, progressive massive fibrosis, bronchitis, loss of lung function, emphysema, and cancer in coal miners. Our objective was to evaluate the effects (genotoxic, histopathological, oxidative stress, and inflammatory) of subacute inhalation of sub-bituminous coal from Colombia on adults male of Wistar rats. Our results showed that subacute inhalation of sub-bituminous coal for 14 days increased the levels of DNA damage index in blood and lung, histopathological lesions in lungs compatible with anthracosis, and accumulation of coal residues in lung tissues. The biochemical analysis showed an increase in antioxidant defenses expressed by elevation of levels of glutathione S-transferase (GST) in lung and superoxide dismutase (SOD) in livers. The markers of oxidative damage (levels of thiobarbituric acid reactive species (TBARS) and total reduced thiol content (SH) were increased in lungs. Also, we observed the expression of tumor necrosis factor alpha (TNF-α). Our data showed the relation between inhalation of sub-bituminous coal dust and generation of genotoxic, histopathological, biochemical alterations, and expression of inflammation factor in lungs. These reactions may be also associated with the generation and evolution of diseases in lungs caused by the inhalation of particulate matter from coal mining. This work provides a state of knowledge about the effects of inhalation of sub-bituminous coal from Colombia in terms of DNA damage, histopathological alterations, oxidative esters and related inflammatory response, and invites further studies to know in depth the evolution of the initial damage produced by the inhalation of coal.

Keywords: genotoxicity; oxidative stress; inhalation subacute; sub-bituminous coal; Colombia**.**

5.1. Introduction

Inhalation of coal dust is the main cause of pneumoconiosis and chronic obstructive pulmonary disease in workers of coal mines (1). In addition, several studies have suggested that health effects of this pollutant are not only restricted to occupationally exposed populations but by the levels of circulating particles. (2). The US Environmental Protection Agency, showed particulate matter (PM) in the air has a direct relationship in lung function changes and respiratory diseases in human populations; in addition, mortality rate induced by pollution varies from 2 to 8% per 50 μg/m3 of PM (3).

The PM generated by coal extraction in drilling, blasting, loading, unloading, and transport is composed by a mixture of particles that vary in size, shape, and chemical composition that depends on the extracted coal type (4–6). Some compounds found in PM of coal are inorganic oxides such as sulfur dioxide (SO_2), associated with the generation of adverse effects on health and the environment. Other oxides also reported in the carbon PM are nitrogen oxide (NO) and nitrogen dioxide (NO_2) that can be converted into nitric acid and contributing to the production of acid rain and carbon monoxide (CO) have harmful effects on health (7),

Other chemical compounds that have been reported in the carbon PM are polycyclic aromatic hydrocarbons (PHA), and inorganic elements such as heavy metals (8,9). The PAHs are organic substances (10) with lipophilic characteristics that allow easy penetration into cell membranes (11). PAHs with low molecular weight are considered less toxic, but they are capable of reacting with other pollutants such as ozone, sulfur oxide, and sulfur dioxide to produce compounds with a high level of toxicity as (diol, nitro, and dinitro-PAHs) (11). Other work of Zocche et al. (12) showed that high concentrations of inorganic

elements (including heavy metals) in the environment, may generate genotoxic effects. In addition, the high toxicity and bioaccumulation capacity induce damages to genetic material (13), and cancer in humans (14) and animals (15).

Coal exploration activities in Colombia can generate changes related to increased genomic instability in lymphocytes such as in oral cells of human populations (16), animals (17,18) that live in the vicinity of mines, as well as in workers (19,20). La Guacamaya coal mine is an open-pit coal mine, located in Cordoba department (Colombia), and is a sub-bituminous coal type A, with high sulfur content (2.30% of total sulfur: 1.06% as pyritic, 1.10 % as organic and 0.14% sulfates) and a high volatile content (21,22). Sub-bituminous coal is used for electricity production due to the calorific potential. A previous study characterized the inorganic oxides (principals), inorganic elements, and PAH of coal sample used to subacute inhaled exposure in the present study (8). Also, another study reported levels of cytocity and DNA damage in V79 cells (Chinese hamster fibroblasts) caused by the exposition of cells to sub-bituminous coal from La Guacamaya in different concentrations (23).

The goal of this study is to evaluate the effects (genotoxic, histopathological, oxidative stress, and inflammatory) of sub-acute inhalation of sub-bituminous coal from Colombia in adults of male Wistar rats. The effects produced by the inhalation of sub-bituminous coal in low doses in a controlled environment simulate the initial impact on populations exposed to particulate matter (PM) from open-pit coal mining in Colombia.

5.2. Material and methods

The Ethics Committee for the Use of Animals of the Lutheran University of Brazil reviewed and approved the project (2017/259). The experimental procedures of this project were carried out taking into account the recommendations of the Brazilian Society of Laboratory Animal Sciences (SBCAL-COBEA), National Council for the Control of Animal Experimentation (CONCEA) and the National Institute of Health of the United States (24).

5.2.1. Animals and experimental design

Male of Wistar rats (*Rattus novergicus*, 16 weeks old) were used as a reliable experimental model for human populations. The experimental animals were acquired from the Center for Reproduction and Experimentation of Laboratory Animals (CREAL) in the Federal University of Rio Grande do Sul (UFRGS) and maintained in the Bioterium of Lutheran University of Brazil (ULBRA). The animals were managed in groups of 4 animals in polypropylene boxes of 50 × 60 × 22 cm with wire bar lids and fragments of pine wood as bedding (Maravalha Rossa Ltda., Porto Uniao, SC, Brazil). The experimental animals were received in the bioterium of ULBRA with 14 weeks and with the habituation phase of two weeks. The maintenance conditions were 22 ± 2°C, humidity of 55 ± 10%, light control (12h daylight/12h darkness), free access to water and standard food (CR1 lab chow, Nuvilab Ltda., Curitiba, Brazil).

5.2.2. Grouping and treatments

After complying with the period of habituation, the animals were randomized in four experimental groups (12 animals per group). The animals were exposed in groups of 4

animals (one hour per day) in an inhalation chamber of 0.087 m^3 with constant air flow and sub-bituminous coal from La Guacamaya mine of Colombia in doses of 6.25, 12.5 and 25 mg/m^3/day. One group of animals was used as a control and exposed to inhalation of filtered air. The animals were weighed three times during the experimentation (day 1, 7 and 14). Then, 24h after the last inhaled dose of coal dust, the animals were euthanized for decapitation with guillotine. Appropriate procedures were taken to minimize suffering and discomfort of animals.

5.2.3. Alkaline comet assay

The alkaline comet assay was performed as described by Singh et al. (25) with minor modifications following the guide by Tice et al. (26). We collected 2 mL of blood in a tube containing heparin and a fraction of lung immediately after euthanasia. The lung was washed with cold PBS and transferred to Eppendorf with 500 µL of cold PBS and homogenized. The blood and lung samples were protected from direct light and cooled until processed. After processing the samples were colored with silver nitrate. Finally, the analysis of the samples was carried using an optical microscope analyzing 50 cells were per plate (2 per sample) and differentiating the damage levels (0,1, 2, 3 and 4), for the calculation of the damage index was made according to the visual classification system (27). Additionally, positive control (1 M H_2O_2) was used to demonstrate the sensitivity of alkaline comet assay.

5.2.4. Histological examination

Tissue sections of lung, liver, kidney, heart, and brain were fixed in formalin 10% and then embedded in paraffin. A portion of 5 µm was cut in a microtome and placed in a glass slide, colored with hematoxylin and, eosin (28), the readings of the samples were made by a veterinarian pathologist in an optical microscope and without identification of the treatments.

5.2.5. Oxidative stress

A generous fraction of liver and lung of each animal was separated by dissection and immediately placed on ice and stored at -20 °C. For the subsequent analysis, the tissues were homogenized in 50 mM PBS at pH 7.4, centrifuged to remove cell debris (10,000 x g, 10 minutes) and finally, the supernatant was collected and stored at -20 °C. Samples were normalized for total proteins contents using bovine serum albumin (BSA) as standard (29).

5.2.5.1. Evaluation of enzymatic antioxidant defenses

The activities of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were measured in liver and lung. The SOD activity was determined by evaluating the inhibition rate induced by each sample on the superoxidation of superoxide-dependent adrenaline and measured at 480 nm for 10 min of kinetics with readings each 30 seconds (30). The CAT activity was evaluating the decrease in the absorbance of hydrogen peroxide (H2O2) in kinetics at 240 nm for 5 min every 30 seconds (31). GPx activity was determined by a decrease in the absorbance of tert-butyl hydroperoxide, measured in the kinetics of 6 min with readings each 30 seconds at 340 nm (32). The GST activity was determined by the rate of increase of

conjugated glutathione (GSH) in absorbance of 1-chloro-2,4-dinitrobenzene for 5 min of kinetics and readings each 30 seconds at 340 nm (33). The results were expressed as U SOD/mg protein, U CAT/mg protein, U GPx/mg protein, and U GST/mg protein. Additionally, the relationship between SOD and the sum of CAT, GPx, and GST (PER) activities were analyzed for understanding of the effects induced by coal dust exposure on the enzymatic antioxidant system, taking into account that all enzymes related, work in enzymatic pathways to convert superoxide anion into water (34).

5.2.5.2. Analyses of redox status in biomolecules (proteins and lipids)

We determined the redox state assessment of the general cellular environment by evaluation of the content of total reduced thiol (SH) (35), where the samples were mixed with 10 mM 5,5-dithiobis-2-nitrobenzoic acid prepared in ethanol. The SH content was determined after 60 min by the absorbance at 412 nm and the results were expressed in µmol SH/mg protein. The formation of reactive species of thiobarbituric acid (TBARS) was evaluated as the index of lipid peroxidation (36), where 10% trichloroacetic acid was added to each sample, then centrifuged at 10,000 RCF at 4ºC. The supernatant was heated with 0.67% thiobarbituric acid for 20 minutes. The level of TBARS was determined by measuring the absorbance at 532 nm and the results were expressed in nmol of TBARS/mg of protein.

5.2.6. Expression of interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α)

Systemic concentrations of interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) with commercial reagents (ELISA Ready-SET-Go, eBioscience, USA). The intra-assay coefficient variation was of <4.5% for all cytokines. Subsequently, 96-wells were incubated overnight with captured antibody anti-IL-1β or TNF-α diluted in PBS 1X. After blocking for 1 h to avoid non-specific binding, we added 100 µL of standard anti-IL-1β or TNF-α and plasma samples. The cytokines were detected by the use of horseradish peroxidase-labeled monoclonal antibody to each target after the addition of 100 µL anti-rat anti-IL-1β or TNF-α biotinylated. The antibodies were placed in each well and incubated for 2 h at room temperature. The microplate was gently washed to remove unbound enzyme-labeled antibodies. The horseradish peroxidase in each well was determined by the addition of 100 µL sulfuric acid 1 M to reaction stop. All readings were performed at 405 nm in plate reader (EZ Reader 400, EUA). The interpolation from the calibration curve determined cytokines concentrations and the results were presented in pg/mL.

5.2.7. Statistical analysis

We checked normality of all the data obtained, using the Shapiro-Wilk test, normal data were tested using one-way ANOVA with post hoc Tukey test. In the absence of normality, data were tested with Kruskal-Wallis test with Dunns comparisons. The criterion of significance was set at $p<0.05$. The statistical analyses were carried out using Graph Pad Prism 7.0 software (GraphPad Inc., San Diego, CA).

5.3. Results

5.3.1. Damage index in blood and lung using comet assay

Fig. 1 shows statistically significant higher DNA damage indexes of male Wistar rat exposed to sub-bituminous coal in whole blood when was exposed with 12.5 and 25 mg/m^3/day, while in lung tissue was only observed a significant increase with the inhalation of 25 mg/m^3/day.

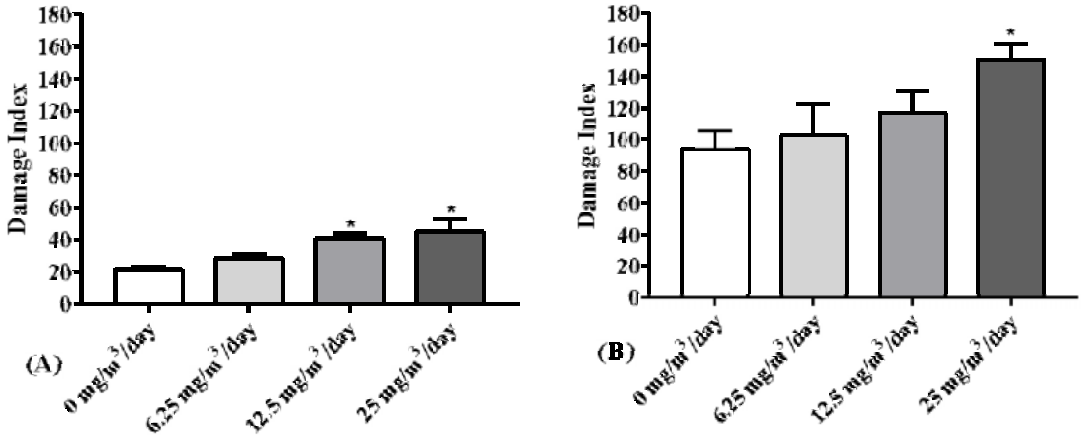

Figure 1. DNA Damage index (DI) in whole blood and lung tissue of control rats (0 mg/m^3/day) and rats exposed to inhalation of sub-bituminous coal of La Guacamaya mine. (A) DNA Damage index in whole blood, and (B) DNA Damage index in lung tissue. Results are shown as mean ± SEM. ***Statistically significant from the control group (p <0.05).**

5.3.2. Histopathological effects studied and Bodyweight alterations.

Histopathological alterations were detected only in the lung tissue. The histological alterations reported in the lung were the multifocal presence of black pigments inside the macrophages in different levels depending on exposition dose used (Figure 2).

In Table 1, we reported histopathological alterations with different levels of multifocal black pigments inside the macrophages in the lung tissue of male Wistar rats exposed to the inhalation of sub-bituminous coal from La Guacamaya mine.

Subacute inhalation of sub-bituminous coal from La Guacamaya mine did not induce toxic effects in weight of adult male of Wistar rats. The animals showed no clinical signs of toxicity related to the inhalation of coal along the experiments or abnormal macroscopic lesions in the necropsy examination.

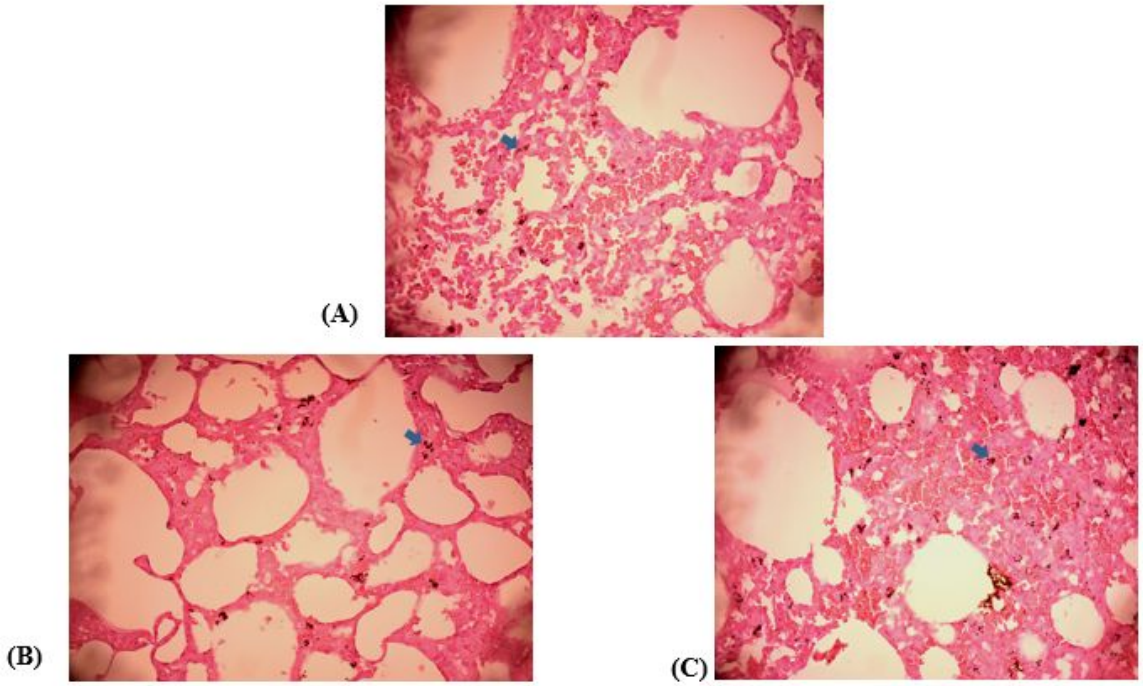

Figure 2. Different levels of multifocal black pigments inside macrophages (blue) in the lung tissue of male Wistar rats exposed to inhalation of sub-bituminous coal from La Guacamaya mine (A) discrete (+) (B) moderate (++) and (C) severe (+++).

Table 1. Numbers of animals and percentage of multifocal black pigments inside macrophages in different levels discrete (+)moderate (++) and several (+++) in the lung tissue of control rats (0 mg/m^3/day) and rats exposed to inhalation of sub-bituminous coal from La Guacamaya mine.

Alteration	0 mg/m^3/day	6 mg/m^3/day	12,5 mg/m^3/day	25 mg/m^3/day
Not Alterations (n; %)*	12 (100%)	8 (66.66%)	2 (16.66%)	3 (25%)
Discrete (+) (n; %)*	0	3 (25%)	2 (16.66%)	2 (16.66%)
Moderate (++) (n; %)*	0	1 (8.33%)	8 (66.66%)	5 (41.66%)
Severe (+++) (n; %)*	0	0	0	2 (16.66%)

*Columns contain information of quantity of animal (n) and percentage (%) per alteration reported.

5.3.3. Enzymatic levels of antioxidant defenses

Table 2 shows the enzymatic levels of antioxidant defenses in lung and liver of rats exposed to subacute inhalation of sub-bituminous coal dust. It was detected a significant increase in glutathione S-transferase (GST) activity in lungs on animals exposed to 12.5 and 25 mg/ m^3/day, while in liver, it was detected a significant increase in superoxide dismutase (SOD) activity in rats exposed to 25 mg/m^3/day (Table 2).

Table 2. Antioxidant enzyme activity in lung and liver of control rats (0 mg/m^3/day) and rats exposed to inhalation of sub-bituminous coal from La Guacamaya mine.

Enzymatic antioxidant defenses	**0 mg/m^3/day**	**6 mg/m^3/day**	**12.5 mg/m^3/day**	**25 mg/m^3/day**
Lung				
SOD activity (U SOD/mg protein)	27.48±0.9	25.74±1.91	26.67±1.81	25.48±1.31
CAT activity (U CAT/mg protein)	54.92±8.92	39.1±8.83	40.5±9.31	84.89±10.64
GPx activity (U GPx/mg protein)	0.65±0.02	0.76±0.05	0.67±0.02	0.68±0.04
GST activity (U GST/mg protein)	10.67±2.63	17.72±2.74	**20.67±3.55***	**30.95±8.32***
SOD/PER ratio	0.72±0.17	1.00±0.28	0.89±0.15	0.39±0.06
Liver				
SOD activity (U SOD/mg protein)	77.56±3.11	77.27±5.76	84.68±6.82	**115.4±8.13***
CAT activity (U CAT/mg protein)	207.1±36.89	181.7±79.28	163.3±47.73	156.0±32.76
GPx activity (U GPx/mg protein)	1.26±0.06	1.20±0.07	1.28±0.08	1.50±0.29
GST activity (U GST/mg protein)	0.049±0.01	0.047±0.01	0.057±0.01	0.058±0.009
SOD/PER ratio	0.50±0.08	0.60±0.25	1.18±0.27	1.32±0.42

Results are expressed in mean ± SEM. ***Bold indicates significant changes (p <0.05).**

5.3.4. Levels of markers of oxidative damage

The markers of oxidative damage showed significantly increased levels of thiobarbituric acid species reactives (TBARS) and a significant decrease in the levels of reduced thiol content (SH) in the lung of animals exposed to 25 mg/m3/day. However, we did not detect changes in markers of oxidative damage in the liver (Figure 2).

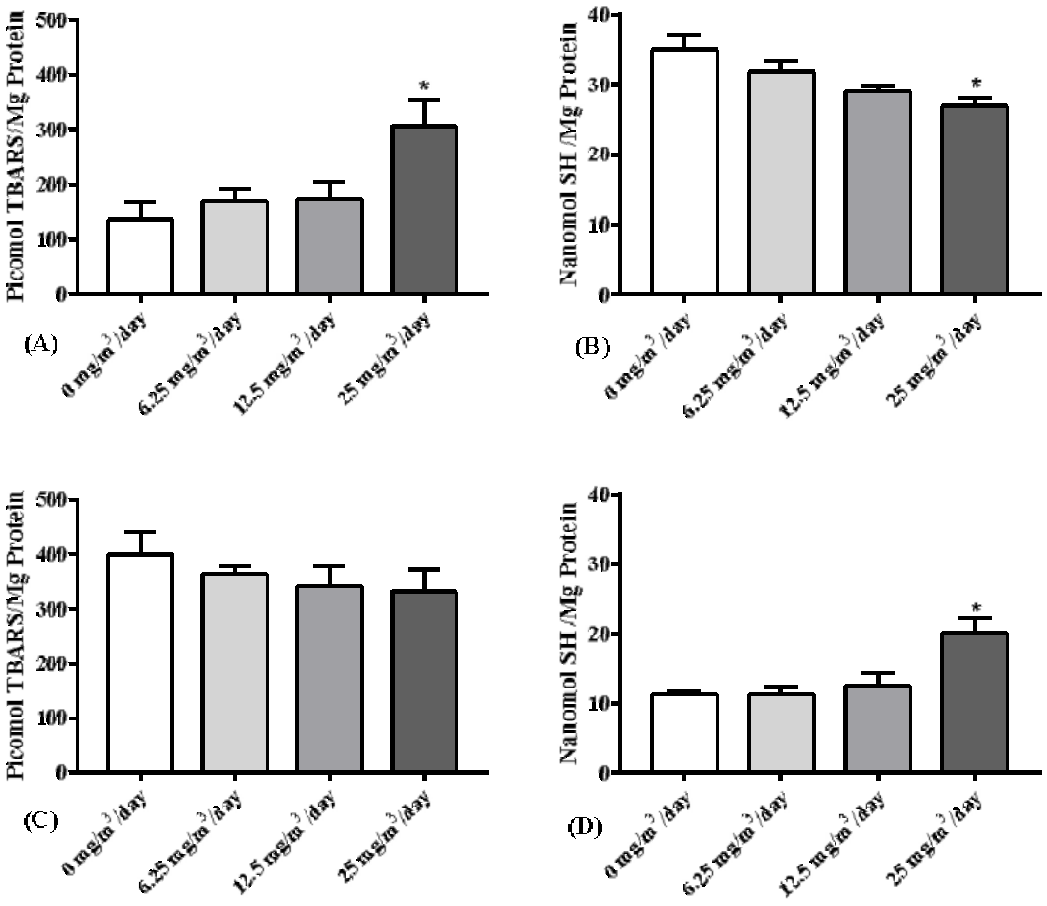

Figure 3. Oxidative damage markers levels in lung an liver of control rats (0 mg/m^3/day) and rats exposed to inhalation of sub-bituminous coal from La Guacamaya. (A) levels of TBARS in lung, (B) levels of SH in lung, (C) levels of TBARS in liver and (D) levels of SH in liver. Results are expressed as the mean ± SEM. *** indicates significant changes (p <0.05).**

5.3.5. Systemic concentrations of inflammatory factors (TNF-α and IL-1β) in plasma

Figure 4 showed a significant increase in levels of tumor necrosis factor alpha (TNF-α) in Male of Wistar rats exposed to 25 mg/m^3/day. However, the levels of interleukin-1β (IL-1β) did not shown significant changes.

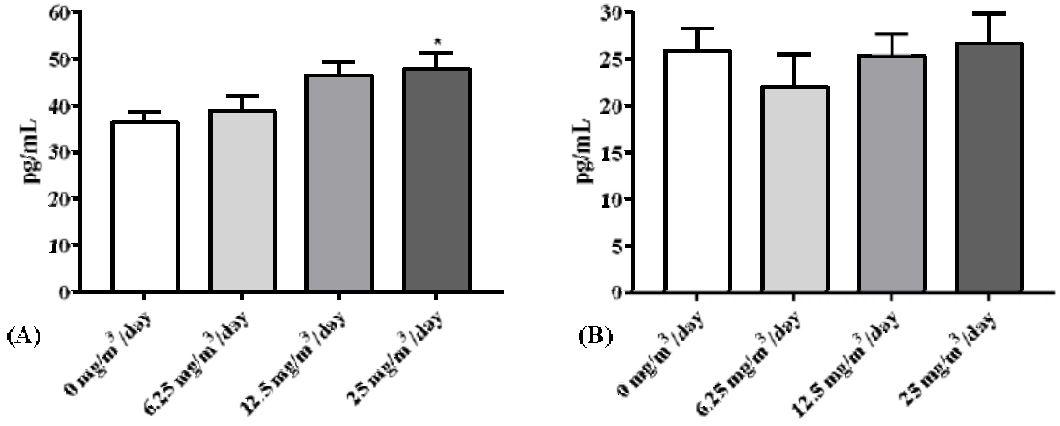

Figure 4. Systemic concentrations of inflammatory factors of control rats (0 mg/m^3) and rats exposed to inhalation of sub-bituminous coal from La Guacamaya. **(A)** Levels of TNF-α and **(B)** Levels of IL-1β. Results expressed as the mean ± SEM. ***Statistically significant difference (p <0.05).**

5.4. Discussion

Particulate matter released to the environment by open-pit coal mining generates health complications in coal mine workers (37,38). This complications also have demonstrated in human populations (39) and wildlife in the vicinity of coal mining areas (28,40). In the world, research has been conducted on the effects of coal on exposed populations, despite these researches few environmental measures are taken to protect affected communities. Studies in Colombia that evaluate the impact of sub-bituminous coal are scarce. In this study, it was exposed to subacute inhalation of sub-bituminous coal from Colombia, male

Wistar rats in a controlled ambient, to generate an approximation of the affected populations and reveal the effect caused by exposure to sub-bituminous coal in a short time.

The alkaline comet assay has been used to biomonitoring the health status of different biological species (18,41–43). Our results showed an increased DNA damage index in whole blood and lung tissue of male Wistar rats exposed to sub-bituminous coal dust for 14 days. These results are supported by the results obtained in the biomonitoring of coal mining areas in Colombia (16,19) and Brazil (44), where the evidence of genotoxic damage was demonstrated in workers and inhabitants of the zones. Additionally, a work of Torres-Avila et al,. reported different levels of inorganic elements, polycyclic aromatic hydrocarbons, and inorganic oxides in sub-bituminous coal from La Guacamaya mine with the ability to induce oxidative stress leading to DNA damage, blockage and/or delay in the progression of the cell cycle, as well as structural dysfunction in several proteins (8). Other studies also supported that the exposure to the residues of coal extraction produces the same consequences and, additionally, inflammatory activity (45–47). In addition, other work of Torres-Avila et al. reported that exposition to sub-bituminous coal from La Guacamaya increased the primary lesion levels and oxidative lesion in DNA in V79 cell line (23).

The lung tissue analysis of male of Wistar rat exposed to subacute inhalation of sub-bituminous coal showed multifocal black pigments into macrophages, which was increasing with the elevation of exposition dose. The presence of black pigments inside the macrophages in the lung tissue could be related to the accumulation of sub-bituminous coal dust. These results are compatible with the results showed in coal work pneumoconiosis (CWP) (48,49). Additionally, inhalation of coal dust can lead to the development of several diseases, including CWP, bronchitis, emphysema, Caplan syndrome and silicosis (48,49),

lung tumors (50,51), and induce bronchoalveolar reactive hyperplasia and reordering of epithelial cells (52).

In our study, the evaluation of total antioxidant defenses in the lung showed an increase in the detoxification system in GST levels, indicating that male of Winstar rats exposed to sub-bituminous carbon induces the response of the detoxification system in the lung that would associate with the increase in ROS generation stimulated by chemical compounds of coal accumulated in the tissue. These results are consistent with the results of several studies that show that the increase in ROS generation can lead to oxidative stress (9,44,53). Additionally, Da Silva et al. (54) reported the risk of environmental and/or occupational exposure to miscellaneous chemical products that can lead to oxidative stress.

Torres-Avila et al. (8) reported that the sub-bituminous coal from La Guacamaya mine has a variable composition of chemical compounds associated with the generation of toxicologic effects in the humans. This work also reports the presence of different concentrations of inorganic elements such as Na, Mg, Al, Si, S, Cl, K, Ca, Ti, Cr, Mn, Fe, Ni, Zn and Sr, polycyclic aromatic hydrocarbons such as Naphthalene, Acenaphthene, Phenanthrene, Anthracene, Fluoranthene, Benzo (a) anthracene, Benzo (g,h,i) pyrene, Benzo (b) fluoranthene, Dibenzo (a,h) anthracene, Indeno (1,2,3-cd) pyrene, and Benzo (k) fluoranthene and major inorganic oxides such as SiO_2, TiO_2, Al_2O_3, Fe_2O_3, MgO, CaO, Na_2O, K_2O, P_2O_5 and SO_3) (8). In the same work Torres-Avila et al. (8) proposed a model where the residues of benzo [a] pyrene, fluoranthene, benzo [b] fluoranthene, phenanthrene, Ti, Mg, Cr, Cl, S, and SiO2 presented in coal was capable of generating reactive oxygen species (ROS) for several routes (similar to Fenton reactions). The ROS can affect the progression of the cell cycle (8).

Subacute inhalation of sub-bituminous coal produces alterations in the level of markers of oxidative damage in lipids and proteins. The increase in lipid peroxidation levels could affect the normal functioning of biological membranes after the transformation of polyunsaturated lipids into polar lipid hydroperoxides (34,55). Additionally, thiols are part of total antioxidant defenses in the body and play a role against reactive oxygen species (56). The decrease of sulfhydryl groups could lead to the excessive generation of bonds disulfide, misfolding, aggregation, degradation of proteins, and cell death because it is known that SH groups proteinaceous or nonproteinaceous are highly susceptible to oxidation by ROS induced (57–59).

Finally, ROS generation induced by the exposure to residues of coal mining has also been reported to cause an increase in inflammatory activity (45–47). In this work, was analyzed the levels of rapid response cytokines (IL-1β and TNF-α) with similar biological functions and act together in the initiation of the inflammatory processes (60, 61). In our study, the inhalation of the sub-bituminous coal-induced liberation of TNF-α, which has an important role in the initiation of the inflammatory response in lungs and other organs. This initial reaction results in the activation of the cytokine cascade and the stimulation of neighboring cells, which produce mediators involved in the recruitment of neutrophils, monocytes, and lymphocytes (62, 63). It is also known that coal particles are poorly soluble and tend to generate inflammatory processes (64). This reaction could be associated with accumulation of coal dust in the lung tissue detected in the histopathological analysis.

In conclusion, our work demonstrates that sub-acute inhalation of sub-bituminous coal from La Guacamaya mine from Colombia, increased the genotoxicity, produced

histopathological lesions, induced primary inflammatory response, and the mainly mechanism suggested was oxidative stress.

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Conflicts of interest

None declared

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6. DISCUSSÃO GERAL

A Colômbia é o país com as maiores reservas de carvão da América Latina e é considerado um dos países exportadores de carvão mais jovens no mundo (Rudas 2014). O país possui recursos potenciais de 16.992 milhões de toneladas (Mt) de carvão, e exportações de 85 Mt por ano (Agencia Nacional de Mineria 2013; Jähnig and Volkmann 2009). A maior parte da atividade mineradora do país está focada na produção de carvão mineral (47%) (Agencia Nacional de Mineria 2013).

As principais reservas do mineral estão localizadas no norte da Colômbia, na região da Costa Atlântica, nos departamentos de La Guajira, Cesar e Córdoba, onde a mineração do carvão acontece a céu aberto (UPME 2012). O carvão nesta região é caracterizado por possuir um poder calorífico de aproximadamente 11.600 BTU/lb, baixa umidade, cinza e enxofre. O carvão produzido na Colômbia é usado na indústria e na geração de calor, vapor e eletricidade, e é facilmente transportado para o exterior graças à proximidade dos portos costeiros (UPME 2012).

A mina de carvão El Cerrejón é a maior mina de carvão a céu aberto do mundo e possui reservas estimadas em 2.193 Mt de carvão do tipo betuminoso, com alto poder calorífico, baixo teor de cinzas e baixa concentração de elementos-traço (UPME 2012). A produção deste tipo de carvão com altos teores de sílica tem sido relacionada com altos níveis de poluição da água potável das comunidades perto da mina. Além disso, as populações destas regiões começaram a apresentar algumas doenças e sintomas como dores de cabeça, erupções cutâneas e doenças do pulmão (Pulido 2007).

A mina La Guacamaya é também uma mina de carvão a céu aberto, possuindo reservas calculadas em 381 Mt com carvão Sub-betuminoso de tipo A, o qual apresenta um

teor total de enxofre de 2,3%, e alto teor de material volátil (Prada Fonseca et al. 2016). A mineração deste tipo de carvão tem sido relacionada à genotoxicidade ambiental em algumas espécies de roedores das áreas de exploração (Leon et al. 2007).

Para compreender melhor os efeitos produzidos pela exposição ao carvão betuminoso e sub betuminoso da Colômbia, este trabalho caracterizou e avaliou amostras destes carvões. A caraterização das amostras identificou os principais compostos nos carvões da Colômbia e, após esta etapa, foi feita a avaliação *in sílico* e analisado o efeito dos compostos em proteínas de *Homo sapiens* com o uso de ferramentas bioinformáticas. A avaliação *in vitro* foi feita em linhagens de células V79 (fibroblastos de pulmão de Hâmster chinês) e a avaliação *in vivo* feita em ratos Wistar machos adultos expostos a inalação subaguda de carvão sub-betuminoso.

No **Capítulo I,** foram identificados os principais elementos químicos do carvão betuminoso e sub betuminoso da Colômbia, com o objetivo de conhecer os diferentes níveis dos principais óxidos inorgânicos, elementos inorgânicos e hidrocarbonetos aromáticos policíclicos. Adicionalmente, neste capítulo, foi feita a análise de químio-biologia de sistemas entre os compostos detectados e as proteínas de *Homo sapiens*, para a compreensão dos mecanismos de atuação destes compostos do carvão da Colômbia no ciclo celular.

Como pode ser observado nas Tabelas S1, S2 e S3 do **Capítulo I**, as partículas de carvão betuminoso e sub-betuminoso da Colômbia apresentam diferentes concentrações de elementos inorgânicos, hidrocarbonetos aromáticos policíclicos e óxidos inorgânicos em diferentes concentrações. O carvão betuminoso possui maiores concentrações de Si, Al, S, Fe, Ti, K, Cl, Ni, Mn, Zn, Benzo[a]anthracene, Fluoranthene, Anthracene, Naphthalene,

Phenanthrene, Benzo[g,h,i]pyrene, Benzo[b]fluoranthene, Dibenzo[a,h]anthracene, Indeno[1,2,3-cd]pyrene, Benzo[k] fluoranthene, SiO_2, Al_2O_3, TiO_2, K_2O e P_2O_5, enquanto que o carvão sub betuminoso possui maiores concentrações de Na, Ca, Mg, Cr, Sr, Acenaphthene, CaO, SO_3, MgO, Fe_2O_3 e Na_2O.

É importante ressaltar a presença de óxidos inorgânicos (Tabela S1, **Capítulo I**) como o SiO_2, conhecido como sílica, e Al_2O_3, assim como a presença elementos inorgânicos e hidrocarbonetos aromáticos policíclicos (Tabelas S2 e S3, **Capítulo I**) nas partículas de carvão betuminoso e sub-betuminoso da Colômbia. Estudos *in vitro* têm demostrado que a sílica possui a capacidade para a geração de estresse oxidativo, levando à geração de citotoxicidade e genotoxicidade (Guidi et al. 2015; Sandberg et al. 2012; Zhang et al. 2015). Complementarmente, estudos *in vivo* reportam a geração de estresse oxidativo pela formação de radicais hidroxila ou ERN, que provocam episódios de sinalização da NF-kB e AP-1, claves na patogênese das doenças pulmonares como silicose, fibrose e câncer (Chen and Shi 2002; Cox 2011; Ding et al. 2002). Similarmente, foi demostrada a formação da radical hidroxila e a geração de estresse oxidativo causada pelo óxido de alumínio (Al_2O_3) (Shrivastava et al., 2014) *in vitro* (Achary et al., 2012; Rajiv et al., 2015) e *in vivo*.

Também é importante ressaltar a presença de elementos inorgânicos do tipo metais e componentes orgânicos como os HAP presentes no carvão da Colômbia. Estes compostos podem influenciar consideravelmente a formação de ERO, que levam a danos no DNA e à indução de processos inflamatórios (Knaapen, et al., 2004; Møller et al., 2014).

As análises de químio-biologia de sistemas do **Capítulo I** mostram os possíveis efeitos dos compostos químicos do carvão da Colômbia nos processos do ciclo celular. Curiosamente, os bioprocessos de reparo de DNA foram encontrados em associação com

óxidos, elementos inorgânicos e hidrocarbonetos aromáticos policíclicos. Ao que parece, esta combinação particular está associada ao aumento do dano ao DNA em sistemas celulares *in vitro*, como foi reportado por (León-Mejía et al. 2016) em populações humanas em ambientes de mineração de carvão. Adicionalmente, Valko et al. (2006) informou que o principal mecanismo proposto para esses efeitos envolve dano oxidativo pela produção de ROS. O estresse oxidativo gerado pelos ROS pode causar alterações na progressão do ciclo celular (bloqueio e/ou atraso), bem como disfunção estrutural em diversas proteínas. Além disso, é sabido que os pontos de verificação de integridade do DNA (G1/S e G2/M e M/A) determinam atrasos no ciclo celular (Rieder 2011). A parada do ciclo celular associada a misturas complexas de HAPs, metais e outros compostos orgânicos após exposição a resíduos de mineração de carvão foi observada *in vitro* por (Tucker and Ong 1985), e *in vivo* por Espitia-Perez et al. (2018). Mais recentemente, a exposição ao benzo [a] pireno (também presente no cluster) tem sido relatada como indutora de parada do ciclo celular e apoptose em células de câncer de coriocarcinoma humano pela geração de ROS (Kim et al,. 2017).

No **Capítulo II,** os resultados de citotoxicidade e genotoxicidade *in vitro* descritos estão relacionados com os mesmos do Capítulo I, no qual é indicada a composição das amostras de carvão betuminoso e sub-betuminoso. Esta relação é sugerida pela composição do carvão da Colômbia (óxidos, elementos inorgânicos e hidrocarbonetos aromáticos policíclicos). É sabido que a presença de elementos inorgânicos de tipo metais solúveis, como o Fe, Pb, Hg, Cd, Ag, Ni, V, Cr, Mn e Cu tem a capacidade de gerar ERO pela via da reação de Haber-Weiss e, como consequência, levar a estresse oxidativo (Knaapen et al. 2004; Lodovici and Bigagli 2011). Os HAP também têm um papel importante na geração de genotoxicidade nas partículas de pó de carvão, as quais, ao serem ativadas

metabolicamente, conseguem induzir a formação de ERO, levando a produção de estresse oxidativo e formação de adutos ou quebras simples no DNA (Mazzoli-Rocha et al., 2010; Perera et al., 2005 & Valavanidis et al., 2013). A principal via para a ativação metabólica dos HAP é catalisada pela citocromo P450 para a formação de diol epóxido, que leva a formação de adutos no DNA, radicais catiônicos e formação de quinonas com atividade redox (Das et al. 2014; Desler, Johannessen, and Rasmussen 2009). É destacado que o Antraceno e o Fluoranteno presentes nas amostras do carvão da Colômbia estão classificados no Grupo 3 pela IARC como mutagênicos (IARC 2010), apesar de existem estudos que demostrem efeitos carcinogênicos do Fluoranteno (Boström et al. 2002). O Benzo(a)antraceno é classificado no grupo 2A pela IARC (IARC 2010), como mutagênico para vários organismos, incluindo humanos (Kido et al. 2011; Scientific Committee on Food 2002; USEPA 1999)

No **Capítulo III**, podemos observar que pulmões de ratos expostos à inalação subaguda com partículas de carvão sub betuminoso mostraram presença multifocal de pigmentos de coloração preta no interior de macrófagos (Figura 2 Tabela 1, **Capítulo III**). Nestes experimentos, os animais expostos a partículas de carvão sub-betuminoso mostraram lesões compatíveis com pneumoconioses, que vão aumentando conforme maior dose inalada. A pneumoconiose de trabalhadores de carvão é uma doença pulmonar crônica causada pela inalação de poeira, que desencadeia a inflamação dos alvéolos, resultando em danos irreversíveis ao pulmão e varia em gravidade do simples ao avançado (CDC 2012).

Os efeitos genotóxicos evidenciados em células do sangue e pulmão de ratos expostos a diferentes concentrações de carvão sub betuminosos poderiam estar associados com a acumulação de resíduos de carvão observada no estudo histopatológico, tendo em

conta que as partículas de carvão são consideradas pouco solúveis e quimicamente complexas. Elas são compostas por uma variedade de elementos químicos que, com a ajuda dos mecanismos celulares e não celulares, contribuem para a formação de ERO. Como consequência, geram dano oxidativo, como foi observado também em culturas de células V79 (fibroblastos de pulmão de Hâmster chinês), em resultados descritos no **Capítulo II** deste trabalho e no **Capítulo I.** Neste, foi sugerido que o estresse oxidativo produzido pelos ERO pode gerar alterações na progressão do ciclo celular (bloqueio e/ou atraso) e disfunção estrutural em diversas proteínas. Esta hipótese também se apoia em alguns estudos de genotoxicidade feitos por Caballero-Gallardo & Olivero-Verbel (2016) e León et al. (2007), nos quais foi determinado o potencial genotóxico dos resíduos da mineração de carvão usando sangue periférico de camundongos silvestres.

Assim, no presente estudo, foi analisada a atividade enzimática antioxidante no pulmão e no fígado de ratos. No pulmão, as duas concentrações mais altas são as que apresentam elevados níveis na atividade da enzima GST, que também poderiam estar relacionados com a acumulação de pó de carvão. esta acumulação foi observada na histopatologia em maiores níveis nas concentrações mais altas, o que indicaria que os pulmões estão reagindo e depurando os produtos de estresse oxidativo, que estaria sendo gerado pela acumulação de resíduos de carvão com capacidade de induzir a produção de ERO, já que tem sido descrito por vários autores o rol dos elementos inorgânicos do tipo metais na geração de dano oxidativo (Detmar and Hartwig 2008; Jaishankar et al. 2014; Jomova and Valko 2011). Dados que são complementados com estudos que relatam a existência de vários polimorfismos funcionais da GST, que provavelmente contribuem na resposta contra os xenobióticos e depuração de produtos de estresse oxidativo. Portanto,

podem determinar a suscetibilidade a várias patologias inflamatórias, incluindo câncer e doenças cardiovasculares e respiratórias (Hayes et al. 2005; McIlwain et al. 2006).

No fígado, a atividade elevada da SOD poderia estar relacionada com a capacidade de translocação de alguns metais solúveis. Tem sido descrito por Winfried et al. (2011) que alguns metais solúveis possuem a capacidade de transloucar-se pela circulação sanguínea a órgãos extrapulmonares e, complementarmente, Mani et al. (2007) demostraram que ratos expostos à cinza de carvão expressam concentrações de Cd nos pulmões, fígado e rins, indicando que o Cd foi levado para órgãos extrapulmonares. Além disso, sabe-se que o fígado é um lugar importante para biotransformação de xenobióticos sendo, portanto, um órgão predisposto a sofrer danos por receber as substâncias tóxicas do sistema circulatório e, adicionalmente, também pode ser o local da ativação de alguns destes componentes (Singh, Cho, and Upadhyay 2016). Com o aumento dos níveis da SOD no fígado, é indicado que as defesas antioxidantes estão sendo ativadas, conforme descrito por Fukaya & Yamaguchi (2004) que a superóxido dismutase (SOD) desempenha um papel importante na remoção de radicais superóxido que induzem morte celular e apoptose.

A inalação subaguda de carvão sub-betuminoso também produz alterações no nível de marcadores de dano oxidativo em lipídios e proteínas. A peroxidação lipídica é um marcador sensível devido à alta probabilidade de lipídios sofrerem oxidação e, portanto, é um marcador de estresse oxidativo altamente utilizado. O aumento dos níveis de peroxidação lipídica detectados no pulmão poderiam indicar que os resíduos de carvão acumulados neste órgão podem afetar o funcionamento normal das membranas biológicas das células no pulmão. Avery (2011) e Halliwell & Gutteridge (2006) reportaram que o aumento dos níveis de peroxidação lipídica pode afetar o funcionamento das membranas biológicas após a transformação de lipídios poli-insaturados em hidro peróxidos lipídicos

polares. Adicionalmente, a diminuição dos grupos sulfidrila detectada no pulmão poderia levar à geração excessiva de ligações disulfeto, desdobramento, agregação e degradação de proteínas, e até mesmo à morte celular. É possível que isso aconteça, uma vez que é sabido que os grupos SH proteináceous ou não proteináceous são altamente suscetíveis à oxidação induzida pelos ERO (Cumming et al. 2004; Sitia and Molteni 2004; Winterbourn and Metodiewa 1999).

Em relação à resposta inflamatória (Figura 4, **Capítulo III**), observou-se um significativo de TNF-α em ratos expostos a altas concentrações de carvão. É descrito por vários autores que, após a ativação de células fagocíticas como os macrófagos, são liberadas citocinas como TNF-α e IL-1β (início agudo da inflamação) (Li et al. 2015; Scheller et al. 2011; Schins 1999).

Considerando os efeitos induzidos *in vivo* pela exposição às partículas de carvão sub betuminoso, cabe ressaltar que estes efeitos poderiam estar associados com a geração de eventos prévios a carcinogênese. Coerentemente, nossos resultados do ensaio CBMN-Cyt (Tabela 4, **Capítulo II)** mostraram uma maior instabilidade cromossômica, associada com a exposição às partículas de carvão sub-betuminoso, e tem sido sugerida a utilização de MN como um biomarcador preditivo de risco de câncer (Bonassi et al. 2011). Assim, aparece a preocupação do risco causado pela exposição a produtos derivados da mineração de carvão, tais como sílica, metais e compostos orgânicos (Jenkins et al. 2013).

Se considerarmos conjuntamente os resultados dos **Capítulos I, II** e **III** observamos a complementação dos dados dos três capítulos, onde a diversidade química achada nos carvões betuminoso e sub betuminoso pode explicar a geração de ERO que leva a estresse oxidativo e dano no DNA demostrado *in sílico*, *in vitro* e *in vivo.* É importante considerar os riscos que sofrem as populações humanas, vegetais e animais por estarem em exposição

aos resíduos de mineração de carvão na Colômbia. Também, os desequilíbrios causados pela inalação de partículas aumentam os possíveis riscos para o desenvolvimento de doenças respiratórias e de câncer.

7. CONCLUSÕES

7.1. Conclusão Geral

O conjunto de resultados desta tese permitiu concluir que as amostras de carvão betuminoso e sub-betuminoso da Colômbia apresentam citotoxicidade, genotoxicidade e mutagenicidade ocasionadas pela mistura complexa de suas constituições, tanto *in vitro* como *in vivo*. Os mecanismos de danos celulares demonstrados neste estudo envolvem estresse oxidativo, processo inflamatório e bloqueio de ciclo celular.

7.2. Conclusões específicas

- As análises de químico biologia de sistemas a partir da caracterização química das amostras de carvão mostraram que a mistura complexa de óxidos, metais e HPA estão associados com a formação de ERO, aumento do estresse oxidativo e induzem, consequentemente, parada do ciclo celular, senescência ou apoptose.
- Os resultados do ensaio clonogênico permitiram evidenciar o potencial citotóxico dos carvões betuminoso e sub-betuminoso em culturas de células V79 (fibroblastos de pulmão de Hâmster Chinês), sendo o carvão betuminoso levemente mais citotóxico.
- As análises pelo ensaio cometa alcalino e modificado com o uso da endonuclease FPG demostraram que a exposição das de células V79 aos dois tipos de carvão induziram níveis similares de lesões primárias e danos oxidativos em bases púricas no DNA.
- Nas análises dos biomarcadores do CBMN-Cyt foi observado que em altas concentrações de carvão betuminoso ocorre a indução de instabilidade

cromossômica com formação de micronúcleos (MN) tanto em 3 horas como em 24 horas de exposição, enquanto que nessa concentração o carvão sub-betuminoso induz formação de broto e MN apenas em 24 horas de exposição.

- Nas análises histopatológicas dos órgãos de ratos Wistar expostos a inalação subaguda de carvão sub-betuminoso foram encontradas alterações histopatológicas somente no tecido pulmonar.
- As análises do ensaio cometa alcalino de células do sangue periférico e tecido pulmonar de ratos Wistar expostos a inalação de carvão sub-betuminoso apresentaram aumento significativo dos níveis de lesões primárias no DNA.
- A inalação de carvão sub-betuminoso em ratos Wistar provoca alterações significativos dos níveis enzimáticos de defesas antioxidantes, dos marcadores de dano oxidativo de lipídios e proteínas do pulmão e fígado, assim como a liberação da citosina pró-inflamatória (TNF-α).

8. PERSPECTIVAS

Para aprofundar os conhecimentos sobre os efeitos da exposição ambiental aos resíduos da mineração do carvão betuminoso e sub-betuminoso, poderiam ser implementadas as avaliações dos aspectos relacionados a seguir.

✓ Determinar concentrações de elementos inorgânicos presentes nos órgãos como cérebro, coração, pulmão, fígado e rim de ratos Wistar expostos à inalação de partículas de carvão da Colômbia.

✓ Analisar concentrações de HAP no pulmão de ratos Wistar expostos à inalação de partículas de carvão da Colômbia.

✓ Avaliar os efeitos genotóxicos na *medula óssea* de ratos Wistar expostos à inalação das partículas de carvão.

✓ Confirmar as concentrações de elementos inorgânicos usando técnicas mais sensíveis, como a espectrometria de massas com plasma de acoplamento indutivo.

✓ Avaliar *in vivo* períodos de exposição crônica nas partículas de carvão de Colômbia.

✓ Analisar a expressão de diferentes marcadores de proteínas de estresse em ratos, após exposição à inalação de partículas de carvão da Colômbia, usando western blotting.

✓ Avaliar os efeitos citotóxicos e genotóxicos em outras linhagens celulares e diferentes tempos, com a exposição das partículas de carvão de Colômbia.

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Anexos

Anexos 1. Certificado da Comissão de ética de Uso de Animais da Universidade Luterana do Brasil.

ULBRA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação dos parâmetros histológicos, genotóxicos, mutagênicos e bioquímicos em ratos Wistar expostos ao pó de carvão das minas Cerrejon e Guacamaya da Colômbia", registrada com o nº2017/259, sob a responsabilidade de Juliana da Silva - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da UNIVERSIDADE LUTERANA DO BRASIL, em reunião de 12/06/2017.

O(a) responsável fica ciente que nenhuma alteração poderá ser feita na condução do projeto, sem a prévia autorização por escrito desta Comissão.

Após dez dias do término das atividades, ou quando esta Comissão julgar necessário, o(a) responsável deverá apresentar relatório.

Finalidade			( ) Ensino (x) Pesquisa Científica				
Vigência da autorização: 12/06/2017 à 01/07/2018							
Espécie animal	Linhagem/Raça	Idade	Peso aproximado	Quantidade			Origem
				M	F	M+F	
Rato heterogênico	Wistar	16 Semanas	170 g	96*	***	96**	Biotério - Universidade Federal de Pelotas /UFPEL

Canoas, 13 de junho de 2017.

Prof. Dr. Paulo Taday Campos Lopes
Coordenador
Comissão de Ética no Uso de Animais (CEUA)
Portaria nº 122/14 - 27/08/2014 - ULBRA

Anexos 2. Atestado sanitário do Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL) da UFRGS.

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
CENTRO DE REPRODUÇÃO E EXPERIMENTAÇÃO DE ANIMAIS DE LABORATÓRIO

CREAL
UFRGS

ATESTADO SANITÁRIO

Data de emissão: 04 de outubro de 2017.

Identificação animal

Espécie	Linhagem	Total
Ratos (*rattus norvegicus*)	Wistar	48 machos

Com classificação genética correspondente a heterogênico e classificação sanitária correspondente a convencional monitorada, provenientes da sala de produção – IVCs (Sistema de reprodução tipo harém), do Centro de Reprodução e Experimentação de Animais de Laboratório da Universidade Federal do Rio Grande do Sul (CREAL/UFRGS), alimentados com ração Nuvilab-Nuvital CR1. Os animais com idade aproximada de 14 semanas (DN: 25 e 26/06/2017).

Destino dos animais

Os animais serão transportados pelo Sr. Joel Amadeo Machado, em carro oficial da UFRGS, para Universidade Luterana do Brasil (ULBRA), Laboratório de Genética Toxicológica, Av. Farroupilha, 8001, Prédio 22, 4º andar, sala 22, São José, Canoas - RS,sob a responsabilidade da professora Juliana Silva.

Declaração do médico veterinário

Declaro que os animais acima identificados não manifestam sinais clínicos de doenças aparentes e estão clinicamente sadios.

*Este atestado é valido por 10 dias

Dra. Fernanda Bastos de Mello
Médica veterinária CREAL/UFRGS

Fernanda Bastos de Mello
Médica Veterinária
CREAL - UFRGS

Av. Bento Gonçalves, 9500, prédio 43.300, Porto Alegre/ RS, Tel/Fax: (51) 3308-9045/9043
www.ufrgs.br/creal - creal@ufrgs.br

Anexo 3. Curriculum Vitae

JOSÉ FERNANDO TORRES AVILA

Nacionalidade: Colombiano

Estado Civil: Solteiro

Data de Nascimento: 30/04/1979

E-mail: fernandot85@hotmail.com

FORMAÇÃO

Doutorado em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul - UFRGS. Tese: Caracterização química e efeitos biológicos de duas amostras de carvão das minas Cerrejon e Guacamaya na Colômbia usando modelos in silico, in vitro e in vivo. Orientador: João Antônio Pêgas Henriques. Em andamento, com conclusão prevista para abril de 2018.

Mestrado em Ciências Microbiologia. 2008. Universidade Nacional da Colômbia, Bogotá. Dissertação: Estudo da farmacorresistência e genotipagem do Mycobacterium leprae colombiano no período 2000-2004. Orientadora: Martha Inírida Guerrero.

Graduação em Microbiologia. 2002. Universidade Libre de Barranquilla – Colômbia. TCC: Produção de ácido glucónico por Acetobacter diazotrophicus PAL5. Orientadora: Paulina Lara Calderon.

PUBLICAÇÕES E PALESTRAS

¿Is M. leprae's drug resistance a real cause for concern? First approach to molecular monitoring from multibacillary Colombian patients with and without previous leprosy treatment. Artigo publicado na Revista Biomédica, ISSN: 0120-4157 V.34 Fasc.1 p.1 - 6 ,2014

Genotyping of Colombian Mycobacterium leprae for determining patterns of disease transmission Colombia. Artigo publicado na Revista de Saúde Pública, ISSN:0124-0064, V: 11 Fasc.1, 2009

Limitaciones Para el Serodiagnóstico del Virus del Oeste del Nilo en Zonas Endémicas con Co-circulación de Flavivirus en el Caribe Colombiano. Artigo publicado na Revista Colombia Médica. ISSN: 1657-9537.V:36: N° 3, 2005.

Presencia de Salmonella spp en Alimentos en el Caribe Colombiano. Artigo curto publicado na Revista Infectio ISSN0123-9392. V: 8, N:2 2004

Detección de Anticuerpos contra Ehrlichia, Bartonella y Coxiella en Habitantes Rurales del Caribe Colombiano. Artigo curto publicado na Revista Infectio, ISSN0123-9392.V:8 N:2 2004.

Problemática Ocupacional y Ambiental del Uso de Mercurio en la Minería de Oro, I Palestrante convidado no Simposio Internacional de Genotixidad y Salud Ocupacional, Barranquilla Noviembre 24 y 25 de 2012.

Genotoxicidad del Carbón. Palestrante convidado no Congreso de Salud Ambiental “Adaptación al Cambio Climático y Salud Ambiental” Bogotá D.C. 1-3 de Agosto de 2012.

Presencia de Salmonella spp en Alimentos en el Caribe Colombiano. Cartel apresentado no IV Encuentro de Investigación en Enfermedades Infecciosas, 10 al 12 de junio de 2004. Paipa-Boyacá-Colombia.

Tras el Descubrimiento de un Virus Emergente en Córdoba: Avance sobre la Circulación del Virus del Oeste del Nilo. Palestrante convidado no 1º Simposio de la Biología "Reconcíliate con la Ciencia", Octubre 19, 20 y 21 de 2004. Montería, Córdoba, Colombia.

IDIOMAS

Espanhol: idioma nativo

Português: fala fluentemente e lê/escreve

Inglês: lê e escreve com competência básica

EXPERIÊNCIA

PROFESSOR E PESQUISADOR – UNIVERSIDADE SIMON BOLIVAR DE BARRANQUILLA, COLÔMBIA. (Desde Janeiro de 2012) – Atuação como Pesquisador na Unidade de Investigação Científica, Desenvolvimento e Inovação em Genética e Biologia Molecular, Professor de Fundações biológicas do comportamento em Psicologia 2012-1, Genética Microbiana em Microbiologia 2012-2, Biologia em Enfermagem e Fisioterapia 2013 -1 e 2.

PROFESSOR – UNIVERSIDADE LIBRE DE BARRANQUILLA, COLÔMBIA. (Desde Agosto de 2012 – Dezembro 2012) – Atuação como Professor de Biologia Molecular especializada no Mestrado em Microbiologia Molecular

COORDENADOR DE PESQUISA FORMATIVA - FUNDAÇÃO UNIVERSITÁRIA DO ÁREA ANDINA, BOGOTÁ – COLÔMBIA. (De fevereiro de 2008 – Novembro 2011) – Atuação como coordenador metodológico e de investigação do Centro de Pesquisa e Desenvolvimento.

PROFESSOR- FUNDAÇÃO UNIVERSITÁRIA DO ÁREA ANDINA, BOGOTÁ – COLÔMBIA. (De Maio de 2007 – Janeiro de 2008) – Atuação como Professor de metodologia e orientação metodológica de TCC.

PESQUISADOR - CENTRO DERMATOLÓGICO FEDERICO LLERAS ACOSTA BOGOTÁ– COLÔMBIA. (De Julho de 2006 – Julho de 2007) – Atuação como pesquisador no projeto "Estudo da variabilidade genômica e resistência a drogas de Mycobacterium leprae".

PROFESSOR E PESQUISADOR – UNIVERSIDADE DEL SINU – MONTERIA – COLÔMBIA**.** (De Janeiro de 2005 – Junho de 2005) – Atuação como professor no curso de Biologia Celular e Molecular para Medicina e pesquisador em projetos de pesquisa em HIV e HTLV, Teste de Citogenética Humana, amostragens para a obtenção de sangue.

PROFESSOR– UNIVERSIDADE DE CORDOBA – MONTERIA – COLÔMBIA. (De Fevereiro de 2004 – Dezembro de 2004) – Atuação como professor no curso de Mecanismos de Doenças Infecciosas Medicina Veterinária.

PESQUISADOR – INSTITUTO DE PESQUISA BIOLÓGICA DO TRÓPICOS UNIVERSIDADE DEL SINU – MONTERIA – COLÔMBIA. (De Julho de 2003 – Outubro de 2004) – Atuação como pesquisador em projetos de Microbiologia (isolamento,

identificação, sorotipagem, manutenção, extração de DNA bacteriano, eletroforéses, PCR, imunofluorescência, ELISA).