# UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
## DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO NACIONAL DE CANCEROLOGÍA

**LAS PARTÍCULAS SUSPENDIDAS ($PM_{10}$) INHIBEN LA VÍA DE REPARACIÓN POR ESCISIÓN DE NUCLEÓTIDOS EN CÉLULAS EPITELIALES DE PULMÓN**

## TESIS

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Índice

Resumen........7
Abstract........9
1. Introducción........10
1.1 Contaminación del aire por partículas suspendidas........10
1.2 Impacto de la contaminación del aire por PM en la salud........13
1.2.1 Evidencia epidemiológica........13
1.2.2 Evidencia toxicológica........14
1.3 Generación de Daño en el DNA........15
1.4 Respuesta ante el daño en el DNA y reparación........15
1.5 Generación y reparación de los aductos voluminosos en el DNA........18
1.5.1 Reparación por Escisión de Nucleótidos (NER)........19
1.5.2 Proteína XPA........22
2. Antecedentes de alteraciones de la vía de NER por PM........23
3. Justificación:........24
4. Hipótesis........25
5. Objetivos........25
5.1 Objetivo General........25
5.2 Objetivos Particulares........25
6. Material y métodos........26
6.1 Muestreo y recolección de $PM_{10}$........26
6.2 Cultivo celular y exposición a $PM_{10}$........26
6.3 Determinación de aductos de Benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) en el DNA 26
6.4 Determinación de los niveles de proteínas........28
6.4.1 Extracción de proteínas totales........28
6.4.2 Extracción de proteínas nucleares........28
6.4.3 Cuantificación de proteínas........29
6.4.4 Electroforesis de proteínas........29
6.5 Ensayo de Co-inmunoprecipitación........30
6.6 Actividad de la vía de NER mediante el ensayo de síntesis de DNA no programada. 31
6.7 Análisis estadístico........32
7. Resultados........33

7.1 Las $PM_{10}$ indujeron la formación de aductos de BPDE en el DNA ........................ 33
7.2 Las $PM_{10}$ desregularon los niveles de proteínas de diferentes puntos de la vía de NER. 34
7.3 Las $PM_{10}$ indujeron la dimetilación de la lisina 20 en la histona 4 en el núcleo. .. 37
7.4 Las $PM_{10}$ disminuyeron la fosforilación de XPA e incrementaron los niveles de la fosfatasa WIP1 ........................ 38
7.5 Las $PM_{10}$ inhibieron la interacción entre XPA y RPA ........................ 40
7.6 La actividad de la vía de NER se inhibió en las células expuestas a $PM_{10}$ .......... 41
8. Discusión: ........................ 44
9. Conclusión: ........................ 50
10. Perspectivas: ........................ 51
11. Referencias bibliográficas: ........................ 52
12. Anexo Artículos publicados ........................ 62

**Resumen**

El cáncer de pulmón es una enfermedad multifactorial generada tanto por factores genéticos como ambientales, destacando las aeropartículas contaminantes, mejor conocidas como partículas suspendidas, entre las que se encuentran las $PM_{10}$ (con un diámetro aerodinámico ≤ 10 micrómetros). Las $PM_{10}$ son un carcinógeno constituido por una mezcla heterogénea de componentes entre los que se encuentran endotoxinas, metales e hidrocarburos aromáticos policíclicos (HAP), por lo que inducen múltiples alteraciones en las células, entre ellos efectos genotóxicos, incluyendo la formación de aductos de HAP en el DNA. Sin embargo, no se ha dilucidado si las células expuestas a $PM_{10}$ eliminan correctamente el daño presente en el DNA. La vía de reparación por escisión de nucleótidos (NER) se encarga de remover los aductos de HAP a través de los pasos de reconocimiento, verificación y eliminación del daño. En este estudio, se evaluó el impacto de las $PM_{10}$ sobre la vía de NER en células epiteliales de pulmón (A549) expuestas a 10 microgramos por $cm^2$ de $PM_{10}$. Se realizó la exposición por 6, 12, 24 y 48 h y se utilizó Benzo(a)pireno como genotóxico inductor de la vía de NER (control positivo de reparación del DNA). Los niveles de aductos de Benzo(a)pireno diol epóxido (BPDE), como representante de los HAP, se midieron mediante un ensayo de ELISA. Los niveles de proteínas de cada paso de la vía se midieron por Western blot y la formación de complejos proteicos se evaluó mediante co-inmunoprecipitación de proteínas. La actividad de la vía de NER se determinó a través del ensayo de síntesis de DNA no programada. La exposición a $PM_{10}$ generó la formación de aductos de HAP-DNA, así como un aumento en los niveles de las proteínas RAD23 y XPD, responsables del reconocimiento de daño y apertura de la hebra de DNA, respectivamente; además, se observó un incremento en la dimetilación de la lisina 20 de la histona 4, que actúa como señal de reclutamiento para XPA, la principal proteína de andamio. Por el contrario, se encontró diminución tanto en los niveles de la proteína XPA como en la fosforilación de su residuo de serina 196, que es una modificación requerida para la correcta interacción de XPA con otras proteínas. La desfosforilación de XPA se asoció con un aumento en los niveles de la fosfatasa WIP1. Además, se observó que el complejo entre XPA y RPA que está encargado de reclutar a las proteínas efectoras de reparación, no se forma en las células expuestas a $PM_{10}$. Importantemente, se determinó que la exposición a $PM_{10}$ inhibe la actividad de la vía de NER a pesar de que se reconoce el daño, indicando que las alteraciones inducidas por las $PM_{10}$ en XPA conllevan a

deficiencias de la vía de reparación. Estos resultados sugieren que la exposición a $PM_{10}$ induce la acumulación de daño en el DNA lo que podría conducir a inestabilidad genómica explicando parte de los mecanismos de carcinogenicidad asociados a la exposición a las $PM_{10}$.

**Abstract**

Lung cancer is a multifactorial disease influenced by genetic and environmental factors, such as airborne particulate matter with a diameter size ≤10 µm ($PM_{10}$). $PM_{10}$ is a carcinogen that contains different elements such as endotoxins, metals, and polycyclic aromatic hydrocarbons (PAH) that induces multiple genotoxic effects, including the formation of PAH-DNA adducts. However, the way DNA repair pathways manage these adducts has not been elucidated. Nucleotide excision repair (NER) is an essential pathway that removes bulky DNA adducts through the recognition, verification, and elimination of the damage. In this study, we evaluated the effect of $PM_{10}$ on NER in lung epithelial cells (A549 cells) exposed to 10 µg/$cm^2$ of $PM_{10}$. The exposure was carried out for 6, 12, 24, and 48 h and Benzo(a)pyrene was used as a positive control of the NER pathway. As a representative of PAH, the levels of benzo(a)pyrene diol epoxide (BPDE) adduct were measured by ELISA assay. Protein levels of each step of the pathway were measured by Western blot and protein complex formation was assessed by protein co-immunoprecipitation. The activity of the NER pathway was determined through the unscheduled DNA synthesis assay. Our results showed that $PM_{10}$ induced DNA adducts, as well as an increase in RAD23 and XPD protein levels (acting as first responders in NER). Also, $PM_{10}$ increased the levels of H4K20me2, a recruitment signal for the NER scaffold factor XPA the main scaffold protein. However, we observed a decrease in total and phosphorylated XPA levels (Ser196), which is required for the correct interaction of XPA with other proteins. The dephosphorylation was associated with the increase of phosphatase WIP1. Additionally, the absence of the XPA-RPA protein complex required for DNA damage removal was determined. Importantly, the NER activity assay demonstrated disruption of NER functionality in A549 cells exposed to $PM_{10}$, indicating that XPA alterations lead to deficiencies in this DNA repair pathway. These results suggests that $PM_{10}$ exposure induces accumulation of DNA damage associated with NER inhibition, which could lead to genomic instability, highlighting the role of $PM_{10}$ inhalation as an important factor during lung cancer generation.

# 1. Introducción

## 1.1 Contaminación del aire por partículas suspendidas

La contaminación del aire se caracteriza por ser una mezcla compleja de gases, líquidos y sólidos cuya composición varía de acuerdo con factores geográficos, la época del año y las fuentes de emisión (naturales y antropogénicas) y generalmente se define como un fenómeno que causa daño tanto a los organismos como al ambiente. Entre las principales fuentes de contaminación atmosférica se encuentran los vehículos de combustión interna, las industrias, quema de residuos, la agricultura y la generación de energía (Li et al. 2021; Song et al. 2021). Actualmente la Organización Mundial de la Salud (OMS) reporta que la contaminación del aire extramuros ocasiona alrededor de 4.2 millones de muertes prematuras al año y provoca la pérdida de años de vida saludable en millones de individuos, especialmente porque 9 de cada 10 personas habitan zonas en las que los niveles de contaminantes sobrepasan los límites recomendables en las directrices de dicha organización. Los niveles elevados de contaminación atmosférica se relacionan directamente con la creciente incidencia y mortalidad de enfermedades como las cardiopatías, eventos cerebrovasculares, afecciones respiratorias y el cáncer, por lo tanto, la morbilidad atribuible a la contaminación del aire se sitúa en el mismo nivel que otros riesgos para la salud mundialmente conocidos como lo es el tabaquismo (Almetwally et al. 2020; Manisalidis et al. 2020; OMS 2021a, b).

Entre los principales contaminantes del aire extramuros se encuentran las partículas suspendidas mejor conocidas como PM por sus siglas en inglés (Particulate Matter), las cuales se clasifican con base en su diámetro aerodinámico en partículas suspendidas totales (≤100 µm), $PM_{10}$ (≤10 µm), $PM_{2.5}$ (≤2.5 µm) y en $PM_{0.1}$ (≤0.1 µm), esta característica determina el transporte y la remoción en el aire, así como la trayectoria de las partículas dentro de las vías respiratorias (Kuempel et al. 2015). En este sentido, las $PM_{10}$ y las fracciones menores han sido identificadas como partículas inhalables debido a que pueden ingresar a las vías respiratorias altas y alcanzar regiones profundas como los alvéolos, provocando efectos a nivel local y sistémico (Cassee et al. 2013; Falcon-Rodriguez et al. 2016) (Figura 1). Además de la inhalación, se conoce que las PM también pueden ingresar al organismo por absorción dérmica e ingestión lo cual refiere al consumo directo de las PM a través de bebidas o comidas contaminadas, así como durante el aclaramiento de las PM

removidas desde la vía aérea por el transporte mucociliar (Goettems Fiorin 2021; Thompson 2018).

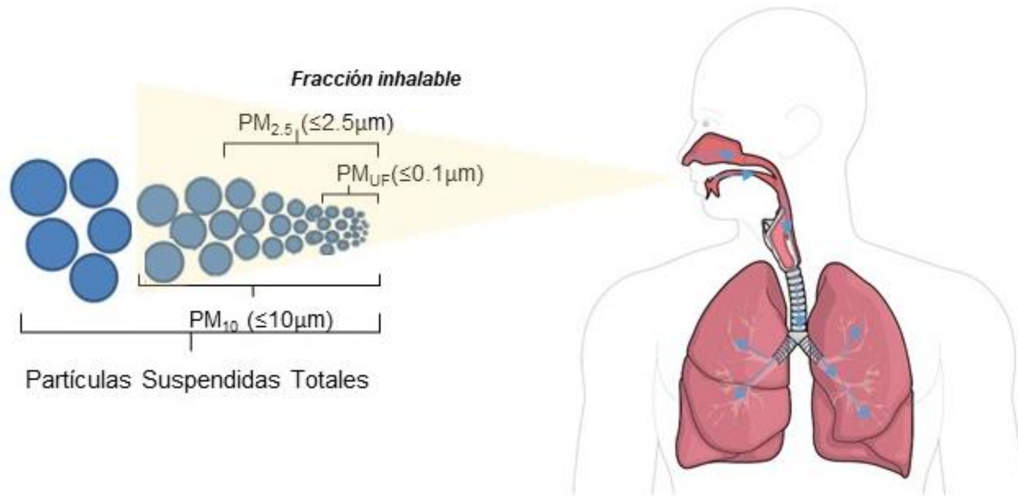


***Figura 1. Clasificación de las PM de acuerdo con su diámetro aerodinámico.*** Las PM se clasifican en partículas suspendidas totales, en partículas con diámetro ≤ 10 µm ($PM_{10}$), partículas con diámetro ≤ 2.5 µm ($PM_{2.5}$) y partículas con diámetro ≤ 0.1 µm ($PM_{UF}$). En la imagen se representa que cada fracción de PM contiene a las de menor diámetro en un porcentaje de aproximadamente 40 %. A las $PM_{10}$ y fracciones menores se les reconoce como fracción inhalable debido a que pueden ingresar a las vías respiratorias e incluso alcanzar zonas profundas como los alvéolos.

Durante un episodio de contaminación de veinticuatro horas se ha calculado que cada acino pulmonar puede recibir en promedio 30 millones de partículas y cada alvéolo un aproximado de 1500 partículas de las cuales hasta el 50% quedan depositadas en este sitio, por lo tanto las PM representan uno de los mayores factores de riesgo para la salud de los humanos (Goettems Fiorin 2021; Raaschou-Nielsen et al. 2016; Thompson 2018; Valavanidis et al. 2008). La exposición continua a $PM_{10\text{-}2.5}$ se relaciona estrechamente con distintas enfermedades crónico-degenerativas, principalmente se ha señalado que las PM son un factor de riesgo para el cáncer de pulmón (Chen et al. 2016a; Consonni et al. 2018; Fasola et al. 2020; Pope et al. 2002b; Zhou et al. 2017b). La composición de las PM es otro de los factores determinantes de sus efectos, debido a que contiene una mezcla heterogénea de materiales sólidos y líquidos generalmente asociados a un núcleo de carbón. Entre los principales componentes se encuentran elementos biológicos como: polen, endotoxinas bacterianas, virus, hongos y bacterias; compuestos inorgánicos como el carbono elemental, nitratos, sulfatos, y metales como el hierro, cobre, magnesio, zinc, níquel, además de

elementos orgánicos como las aminas aromáticas heterocíclícas, compuesto orgánicos volátiles y los hidrocarburos aromáticos policíclicos (HAP) entre los que destacan el Benzo(g)perileno, Benzo(b)fluoranteno y el carcinógeno Benzo(a)pireno (BaP) (Abayalath et al. 2022; Morakinyo et al. 2016).

Con base en la evidencia fundamentalmente epidemiológica la Agencia Internacional para la Investigación del Cáncer (IARC), clasificó a la contaminación del aire exterior, así como a las PM como un carcinógeno para los humanos del grupo I (en el que existe evidencia suficiente en humanos) (IARC 2015; Loomis et al. 2014). Se ha demostrado que existe una correlación cuantitativa entre la exposición a altas concentraciones de PM y el aumento de la mortalidad o morbilidad diaria y a largo plazo. Por lo tanto, con el propósito de proteger la salud humana, la OMS ha establecido en las guías de calidad del aire los límites permisibles internacionales de concentración de contaminantes en el aire incluido las PM, los cuales señalan para $PM_{10}$ una media anual de 15 $\mu g/m^3$ y 45 $\mu g/m^3$ como media de 24 horas, mientras que para $PM_{2.5}$ la media anual es de 5 $\mu g/m^3$ y 15 $\mu g/m^3$ como media de 24 horas (OMS 2021a). En el caso de México, los valores permisibles se encuentran publicados en la Norma Oficial Mexicana-NOM-025-SSA1-2021, estableciendo para $PM_{10}$ una media anual de 36 $\mu g/m^3$ y 70 $\mu g/m^3$ como media de 24 horas, y para $PM_{2.5}$ una media anual de 10 $\mu g/m^3$ y 41 $\mu g/m^3$ como media de 24 horas (NOM 2021; OMS 2021b). El establecimiento de los limites permisibles se basa en una serie de factores (políticos, sociales, económicos) que permiten una adecuada gestión de la calidad del aire en México acorde a las necesidades específicas del país. Sin embargo, se reconoce que los valores establecidos por esta norma siguen siendo considerablemente mayores a las guías de la OMS, por lo tanto, se tiene como objetivo alcanzar gradualmente los parámetros recomendados (CEMDA 2013).

A pesar del establecimiento de dichos parámetros, determinaciones realizadas mediante los sistemas de monitoreo de la calidad del aire reportan que tanto a nivel mundial como en México las concentraciones de $PM_{10}$ y $PM_{2.5}$ superan considerablemente los límites permisibles continuamente, principalmente en las zonas urbanas. En específico en la Ciudad de México durante el año 2019 se registraron días con valores de $PM_{10}$ de hasta 139 $\mu g/m^3$ promedio de 24 h y de 68 $\mu g/m^3$ como medida anual, mientras que para $PM_{2.5}$ se alcanzaron 133 $\mu g/m^3$ y 28 $\mu g/m^3$ respectivamente. Es relevante destacar que la exposición a PM produce efectos en la salud, principalmente a nivel cardio-respiratorio, aun en

concentraciones consideradas como bajas (señaladas por debajo de los límites establecidos por la OMS), por lo que no se ha identificado ningún umbral por debajo del cual no se produzcan daños (INECC 2020; OMS 2021a; Papadogeorgou et al. 2019).

## 1.2 Impacto de la contaminación del aire por PM en la salud

### 1.2.1 Evidencia epidemiológica

Los estudios epidemiológicos demuestran que por cada aumento de 10 $\mu g/m^3$ en la concentración de $PM_{10-2.5}$ en el aire se registra un incremento en padecimientos agudos como: las cefaleas, irritación ocular e infecciones respiratorias; además, aumentan los ingresos hospitalarios principalmente por padecimientos respiratorios e incrementa la mortalidad por problemas cardiovasculares (Badyda et al. 2016; Pope et al. 2002a; Tian et al. 2019; Turner et al. 2020). En relación con su clasificación como carcinógeno otorgada por la IARC (IARC 2015) un creciente número de estudios de cohorte han descrito que la exposición prolongada a PM incrementa la mortalidad por cáncer de pulmón hasta en un 8 % (Chen et al. 2016b; Pope et al. 2002a). Adicionalmente, la evidencia sobre la asociación de PM y la incidencia de esta neoplasia ha incrementado. Trabajos como el estudio ESCAPE (cohorte europea para los efectos de la contaminación del aire), el estudio AHSMOG-2 (Adventist Health and Smog Study-2), el UEBMI (Urban Employee Basic Medical Insurance), entre otros, muestran una asociación significativa entre el riesgo de cáncer de pulmón y la exposición a $PM_{10}$ o $PM_{2.5}$; específicamente por cada incremento de 10 $\mu g/m^3$ en la concentración en el ambiente, concluyendo que las PM contribuyen a la incidencia de esta enfermedad (Gharibvand et al. 2017; Raaschou-Nielsen et al. 2013; Zhou et al. 2017a).

Por otra parte, se ha demostrado que la exposición a $PM_{10}$ se asocia específicamente con el adenocarcinoma, un tipo de cáncer de pulmón (Moon et al. 2020). Evidencias más recientes señalan que otras neoplasias como son el cáncer de mama, el cáncer gástrico y el cáncer colorrectal podrían estar relacionadas con la exposición a PM; sin embargo, aún se requieren más estudios que confirmen dichas asociaciones (Yu et al. 2021). Debido a todos los efectos causados por las PM, se propone que la disminución de dicho contaminante en el ambiente proporcionaría un beneficio para la salud pública ya que se reduciría la carga de morbilidad derivada de las enfermedades inducidas por la exposición. Datos puntuales estiman que la reducción de 35 $\mu g/m^3$ en la concentración de $PM_{2.5}$

correspondería a una disminución de hasta el 14% de los casos de cáncer de pulmón (Hoek and Raaschou-Nielsen 2014; Zhang et al. 2020).

### 1.2.2 Evidencia toxicológica

Los estudios toxicológicos han permito describir distintas alteraciones a nivel celular y molecular producidas por la exposición a PM. Entre los principales efectos se encuentra la producción de mediadores de inflamación (interleucinas) (Jia et al. 2021), inhibición de apoptosis (Reyes-Zarate et al. 2016), cambios en componentes del citoesqueleto perturbando la arquitectura de las células (Calcabrini et al. 2004; Sánchez-Pérez et al. 2014), generación de especies reactivas de oxígeno (Chirino et al. 2010; Valavanidis et al. 2005) e incremento de la capacidad de invasión (Chen et al. 2018; Garcia-Cuellar et al. 2021). En relación con el potencial genotóxico de este contaminante, cuya evidencia fue fundamental para su clasificación como carcinógeno por la IARC, se sabe que las PM producen oxidación de bases nitrogenadas en el DNA (8-oxoguanina) (Chirino et al. 2010; de Oliveira Alves et al. 2020), daños de cadena sencilla y rompimientos de doble cadena de DNA (Rossner et al. 2014; Sanchez-Perez et al. 2009; Zhou et al. 2014), además, inducen la formación de aductos voluminosos de HAP (Abbas et al. 2013; Lepers et al. 2014b; Salcido-Neyoy et al. 2015a).

A pesar de la evidencia sobre la capacidad genotóxica de las PM aún se desconoce si el daño inducido es reparado de manera eficiente (Quezada-Maldonado et al. 2021). Se ha descrito que después de la exposición a PM incrementan proteínas como ATM y p53 que participan en la vía de respuesta global ante daño a DNA (Sanchez-Perez et al. 2009; Zhou et al. 2014); sin embargo, los estudios también han señalado que las células expuesta a las PM presentan inestabilidad genómica (Saint-Georges et al. 2009; Santibanez-Andrade et al. 2019), lo cual sugiere que los mecanismos de reparación de daño en el DNA podrían estar alterados en estas células. De manera importante, las deficiencias en la reparación del DNA pueden predisponer a la adquisición de mutaciones, principalmente cuando el daño es persistente y se acumula en las células (Hanahan and Weinberg 2011), por lo que su estudio es un área a explorar para conocer con mayor profundidad el potencial carcinogénico del material particulado. A continuación, se resumen algunos de los mecanismos de daño; así como los mecanismos que reparan este daño que podrían ser

abordados, enfatizando en aquellos involucrados en la generación y remoción de aductos voluminosos de HAP.

### 1.3 Generación de Daño en el DNA

El DNA es una molécula intrínsecamente reactiva y susceptible a diversas modificaciones, generadas tanto por alteraciones químicas como por cambios estructurales que en conjunto modifican la funcionalidad de los genes codificados. El daño en el DNA puede dividirse de manera general en daño endógeno y exógeno. El daño endógeno generalmente es provocado durante la replicación del DNA, además puede derivar de procesos metabólicos como la respiración celular y peroxidación de lípidos o de reacciones hidrolíticas y metilaciones no enzimáticas que generan aberraciones en las bases. Adicionalmente, se pueden generar rompimiento de cadena sencilla (SSB) o rompimientos de doble cadena (DSB) por errores de la topoisomerasa I y II (Jackson and Bartek 2009).

Por otra parte, entre los principales agentes que causan daño de tipo exógeno (químicos, físicos) destacan los metales, las aminas aromáticas, los HAP, así como la radiación ionizante y la luz UV, e incluso compuestos biológicos derivados de bacterias u hongos (endotoxinas y micotoxinas) por mencionar algunos. Específicamente, los metales, las endotoxinas o los HAP pueden generar especies reactivas de oxígeno (ROS) que a su vez producen cambios químicos en las bases del DNA alterando el emparejamiento correcto entre las bases. Adicionalmente, agentes como la luz UV o los metabolitos de diversos compuestos como los HAP o micotoxinas pueden unirse directamente al DNA provocando una variedad de aductos voluminosos. Tanto las lesiones formadas por ROS como los aductos del DNA pueden derivar en SSB, e incluso cuando estos SSB surgen en regiones proximidad de la cadena del DNA o no se reparan adecuadamente se generan DSB (Carol Bernstein 2013; Chatterjee and Walker 2017; Glukhov et al. 2008; Hoeijmakers 2009; Wang and Groopman 1999).

### 1.4 Respuesta ante el daño en el DNA y reparación

Debido a que el DNA sufre múltiples tipos de daño de forma constante, las células cuentan con el mecanismo de respuesta ante en el DNA (DDR), el cual detecta las lesiones en el DNA, señala su presencia y promueve su reparación a través de las distintas vías de

reparación, lo cual es esencial para mantener la estabilidad genómica al prevenir cambios en la secuencia del DNA. Además de las vías de reparación la DDR incluye procesos de tolerancia al daño y vías de puntos de control del ciclo celular (Giglia-Mari et al. 2011). Entre los componentes clave de la señalización DRR se encuentran las proteínas sensoras incluyendo a la histona H2Ax fosforilada en su residuo 139 (ɣH2AX) y a RPA, posteriormente se activan los transductores ATM y ATR, seguido de mediadores como 53BP1, BRCA1, MDC1, Claspina y TopBP1, las cuales a su vez activan a la proteína efectora p53 encargada de dictaminar el siguiente paso de respuesta; es decir, generar la activación de las vías de reparación, detención del ciclo celular o si el daño supera los mecanismos anteriores, inducir la muerte celular (apoptosis) o la senescencia. Las proteínas mencionadas anteriormente activan respuesta celular mediante la inducción de la transcripción o cambios postraduccionales (modulando su fosforilación, acetilación, ubiquitinación) de diversas proteínas involucradas en estos procesos (Jackson and Bartek 2009; Yoshiyama et al. 2013).

En el caso de la reparación del DNA cada una de estas vías elimina un tipo de lesión específica, de hecho, los mecanismos de reparación se encuentran altamente conservados a lo largo de la evolución (Chatterjee and Walker 2017). Existe un mecanismo de reparación directo del DNA, el cual elimina el daño inmediatamente después de ser producido. En la reparación directa participa una sola tipo de enzima llamada alquiltransferasa, como es la metilguanina-DNA metiltransferasa, encargada de remover grupos metilo presentes en la guanina ($O^6$-metilguanina) mediante su transferencia a un sitio activo de cisteína de dicha enzima lo que restaura la estructura original de la base, sin alterar el esqueleto del ADN (Sancar et al. 2004).

Por otra parte, las demás vías de reparación del DNA modifican la estructura primaria del DNA y constan de un conjunto de proteínas que actúan en una secuencia de tres pasos: (1) el reconocimiento de daño, (2) la activación de la maquinaria de reparación y (3) la eliminación de lesiones (Chatterjee and Walker 2017; Hakem 2008; Hoeijmakers 2001). Estas vías se pueden dividir de manera general en vías de escisión de bases nitrogenádas, encargadas de eliminar daños presentes en una sola cadena como el daño oxidante o los aductos. Incluye a la vía de reparación por escisión de bases (BER) y vía de reparación por escisión de nucleótidos (NER). Por otra parte, las vías de reparación de doble cadena, que están divididas a su vez en vía de recombinación homóloga (HR) y vía de recombinación

no homóloga (NHEJ). Adicionalmente se encuentra la vía de reparación de bases mal apareadas (MMR), encargada principalmente de la reparación de errores que surgen durante la replicación y recombinación del DNA (Figura 2).

La reparación del DNA es un proceso que previene la entrada de las células en la carcinogénesis, debido a que, si los daños en el DNA de las células en proliferación no se eliminan, los errores se acumulan en el genoma induciendo mutaciones que aumentan el riesgo de cáncer. La alteración en la reparación puede deberse tanto a cambios en la expresión de un gen o proteína que participa en la reparación del DNA, a cambios epigenéticos, así como a polimorfismos o mutaciones germinales de dichos genes (Carol Bernstein 2013; Dixon and Kopras 2004). Debido a que las $PM_{10}$ inducen la formación de aductos de HAP, y que estos son removidos a través de la vía de NER, ambos puntos se describen con mayor detalle.

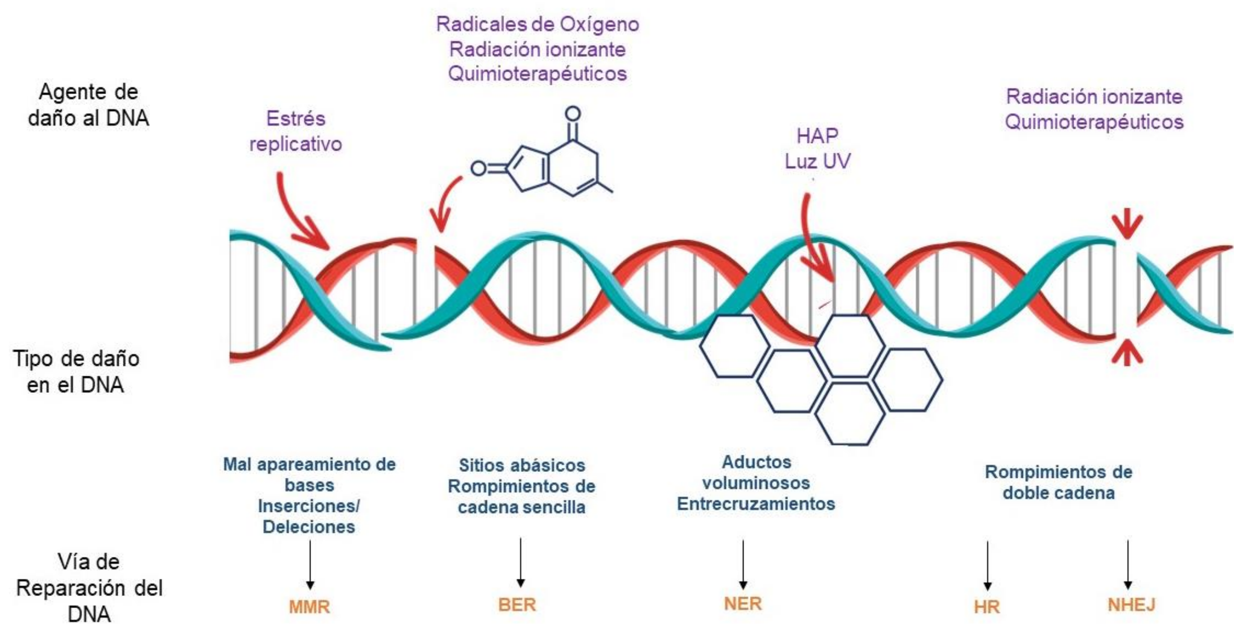


***Figura 2. Tipos de daño en DNA y vías de reparación***. Las células se enfrentan de manera constante a diversos agentes endógenos y exógenos que de manera directa o indirecta ocasionan distintos tipos de daño en el DNA, por lo que existen las vías de reparación del DNA, especializadas en eliminar cada tipo de lesión. La reparación del DNA evita la acumulación de daño, por lo tanto, previenen la generación de inestabilidad genómica e incluso la aparición de mutaciones. Vía de reparación de bases mal apareadas (MMR), vía de reparación por escisión de bases (BER), vía de reparación por escisión de nucleótidos (NER), vía de recombinación homóloga (HR) y vía de recombinación no homóloga (NHEJ).

## 1.5 Generación y reparación de los aductos voluminosos en el DNA

Entre los diferentes tipos de daño producidos en el DNA, destaca la formación de aductos voluminosos, los cuales se definen como la unión covalente entre un compuesto químico y una base del DNA. Estas lesiones son utilizadas frecuentemente como biomarcadores de exposición a diversos compuestos. Los aductos voluminosos más comunes son aquellos producidos por los HAP (Bai et al. 2017; Rajalakshmi et al. 2015). Los HAP son compuestos de carbono con dos o más anillos aromáticos, no polares, generalmente inertes que se distribuyen ampliamente en el ambiente, incluyendo a las PM (Abayalath et al. 2022).

Cuando ingresan a las células, los HAP son procesados metabólicamente por el sistema enzimático de los citocromos P450 (CYP450). El BaP es uno de los HAP más comunes en el ambiente. Cuando ingresa a las células es procesado por el CYP450, a partir de lo cual se genera el metabolito carcinogénico llamado benzo(a)pireno diol-epóxido (BPDE), dicho intermediario puede unirse a las bases del DNA formando así los aductos, comúnmente en la posición N2 de la guanina, así como en N7 y N3 debido a que son los sitios más nucleofílicos (Figura 3). Además, se pueden unir en N1 y O6 de la guanina o en la N7, N6, N3 y N1 de la adenina, N3, N4 y O2 de citosina y N3, O2 y O4 de la timina. Sin embargo, el BaP también puede formar aductos en el grupo fosfato del DNA. Otros compuestos como las aminas aromáticas, los compuestos nitro-aromáticos y múltiples agentes terapéuticos también inducen la formación de aductos voluminosos (Chatterjee and Walker 2017; Gaskell et al. 2007).

Cuando los aductos no se eliminan del DNA, estos pueden interferir con los procesos de transcripción y replicación, provocar inestabilidad genómica e incluso mutaciones. En relación con la carcinogénesis los aductos pueden alterar múltiples regiones del DNA, sin embargo, su impacto es mayor cuando se modifican secuencias del DNA en donde se codifican oncogenes o los genes supresores de tumores, por lo que se pueden presentar mutaciones conductoras. De hecho, se ha propuesto que diversos tipos de cáncer en los que están involucradas exposiciones ambientales inician con la formación y acumulación de aductos voluminosos. En específico se ha descrito que los aductos derivados de BaP inducen mutaciones de tipo GC a TA, GC a AT y GC a CG, además de mutaciones de desplazamiento del marco de lectura (Bai et al. 2017; Hwa Yun et al. 2020; Shukla et al.

1999). Por tal motivo, en la etiología del cáncer, es importante identificar la formación, así como la eliminación de aductos de DNA ante la exposición a un compuesto.

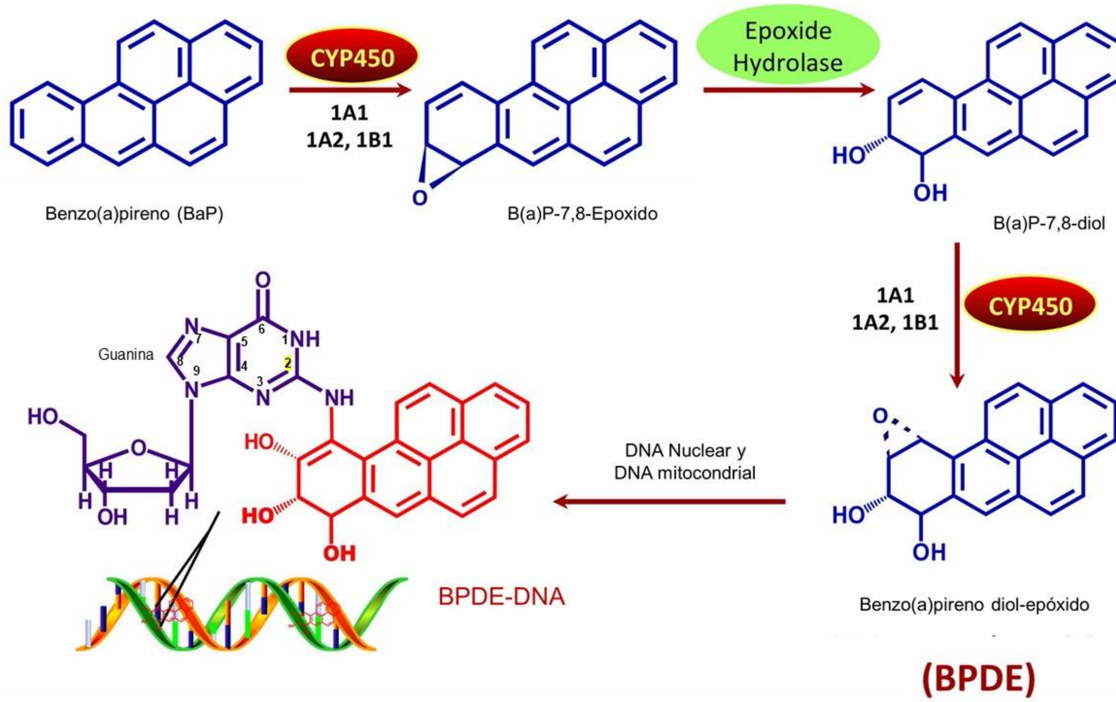


***Figura 3. Formación de Aductos de Benzo(a)pireno en el DNA.*** Dentro de las células el BaP es metabolizado en 3 pasos a través del sistema del citocromo P450 y la enzima epóxido hidrolasa lo que genera a la formación de la especie reactiva BPDE el cual se une covalentemente con las bases del DNA generando un aducto voluminoso preferentemente en residuos de guanina (principalmente en la posición N2, como se señala en la figura). Figura modificada de Barnes, et al., 2018.

### 1.5.1 Reparación por Escisión de Nucleótidos (NER)

La reparación por escisión de nucleótidos (NER) es la vía más versátil de reparación del DNA, porque elimina distintas lesiones de tipo voluminoso que distorsionan la cadena de DNA, como son las producidas por UV (fotoproductos) radicales libres y carcinógenos incluyendo los HAP (aductos) (Chatterjee and Walker 2017; Hoeijmakers 2001; Leibeling et al. 2006). La vía de NER se divide en dos tipos, la vía de reparación acoplada a la transcripción (TCR) y la vía de reparación del genoma global (GGR), las cuales difieren en las proteínas de reconocimiento de daño al DNA (Scharer 2013; Spivak 2015). La vía de NER consiste en tres pasos en los que participan al menos 30 proteínas en un orden definido incluyendo los factores XPA a XPG (xeroderma pigmentoso A-G) y ERCC1 (excision repair cross complementation group 1) (Figura 4).

- En el paso inicial de GGR las proteínas XPC, RAD23 y centrina 2 reconocen el daño mediante la detección de un cambio estructural de las cadenas del DNA, lo que permite el reclutamiento de los siguientes componentes.

- El segundo paso se conoce como la etapa de verificación de daño, en el cual se genera la apertura de la doble hélice del DNA alrededor del sitio de la lesión (20-25 nucleótidos de distancia al sitio de daño) mediante la participación de las helicasas XPD y XPB (5´-3´ y 3´-5´ respectivamente); además, se reclutan la proteína XPA en el sitio de DNA, la proteínas RPA, el complejo TFIIH formado por las proteínas con actividad enzimática p62, p52, p44 y por el complejo CAK (formado por Cdk7 y ciclina H).

- En el tercer paso se genera la escisión del daño mediante el corte de la hebra de DNA dañada a través de las endonucleasas XPG y XPF unida a ERCC1 (corta de 5 a 6 nucleótidos río abajo de la lesión y 20-22 nucleótidos rio arriba respectivamente). XPG interactúa directamente con RPA lo cual indica la posición exacta del sitio de corte. Posteriormente se ensambla la nueva cadena sintetizándola a partir de la hebra complementaria, para lo cual participa la DNA polimerasa δ ó ε, además de la ligasa I (D'Souza et al. 2022; Shuck et al. 2008; Spivak 2015).

Se ha descrito que, en la mayoría de los modelos celulares, la vía de NER se activa a las 4 h posteriores a la exposición a una sustancia genotóxica que induce la formación de una lesión voluminosa y para las 48 h post-exposición más de la mitad de los aductos formados han sido eliminados, lo cual concuerda con el incremento en los niveles de las proteínas XPA-XPG durante este tiempo de exposición (Shi et al. 2017; Yang et al. 2007)

La importancia de la vía de NER queda demostrada en síndromes como Xeroderma pigmentoso (XP) y síndrome de Cockayne, que son trastornos hereditarios caracterizados por mutaciones en genes de dicha vía. Particularmente el XP ocasiona fotosensibilidad, incrementa el riesgo de cáncer de piel (hasta en 10 000 veces), así como el riesgo de cáncer de órganos internos asociados con la deficiencia de reparación de lesiones voluminosas que conllevan a daño acumulativo e irreversible (Bradford et al. 2011; Mareddy et al. 2013).

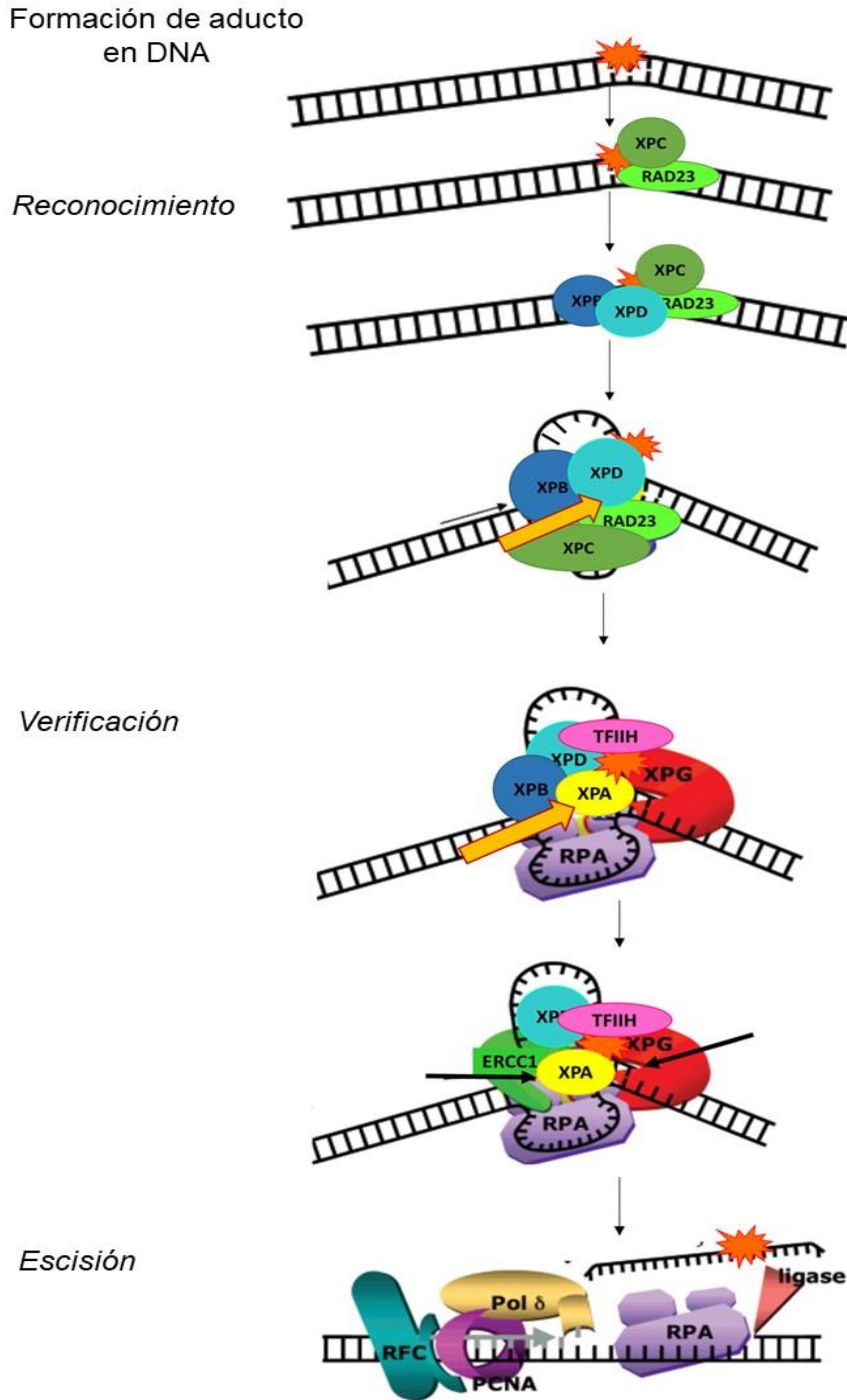


***Figura 4. Vía de reparación por Escisión de Nucleótidos (NER).*** La vía de NER se encarga de eliminar los daños que causan distorsión en la cadena de DNA, como lo son los aductos voluminosos formados por los HAP. Esta vía se divide en 3 grandes pasos que son el reconocimiento del daño, la verificación de la lesión y la escisión del daño. Las principales proteínas de la vía de NER son los factores Xeroderma Pigmentoso nombrados desde la A hasta la G. Esquema modificado de Gillet, et al., 2006.

### 1.5.2 Proteína XPA

Si bien cada una de las proteínas de la vía NER cumple con una función durante el proceso de reparación, la proteína XPA es uno de los principales factores limitantes del funcionamiento de la vía debido a que XPA es una metaloproteinasa de zinc que se une directamente a los nucleótidos dañados facilitando la verificación de la lesión voluminosa en DNA y actuando como andamio para el ensamblaje de proteínas del tercer paso de reparación (Camenisch et al. 2006; Koberle et al. 2006; Leibeling et al. 2006). El posicionamiento adecuado de XPA en el sitio de la lesión voluminosa está mediado por la histona 4 (H4), la cual en presencia de daño es dimetilada en su residuo de lisina 20 (H4K20me2) (Chitale and Richly 2018a, b). Adicionalmente, se ha demostrado que, durante la reparación, XPA interactúa mediante el residuo N-terminal y el dominio central con la proteína RPA formando un complejo proteico mediante el cual se reclutan las proteínas efectoras de la reparación como es el complejo XPF-ERCC1 (He et al. 1995; Krasikova et al. 2018; Li et al. 1995; Yang et al. 2002).

La proteína ATR lleva a cabo la fosforilación de XPA en su residuo Serina 196 ($pXPA^{S196}$), lo cual es una de las principales modificaciones de esta proteína durante el daño y reparación. Este proceso previene la degradación mediada por proteosoma y favorecer su interacción con otras proteínas del sistema de reparación. Por el contrario, si XPA es desfosforilada por acción de la fosfatasa WIP1, se inactiva y su capacidad de interacción disminuye (Lee et al. 2014; Nguyen et al. 2010b). Las alteración de XPA se asocian fuertemente con la susceptibilidad a la generación y acumulación de lesiones voluminosas, así como con una disminución en la capacidad de reparación de DNA por la vía de NER (Fan and Luo 2010; Lee et al. 2012; Nguyen et al. 2010a; Shell et al. 2009a). Un ejemplo de susceptibilidad al daño genético se demostró con los ratones deficientes de XPA que desarrollaron 4 veces más aductos, posterior al tratamiento con cisplatino comparado con los ratones que contenían XPA íntegra (Dzagnidze et al. 2007).

## 2. Antecedentes de alteraciones de la vía de NER por PM

Diversos estudios tanto de personas expuestas a altos niveles de $PM_{10-2.5}$, como estudios toxicológicos en distintos tipos de células señalan que las PM inducen la formación de aductos voluminosos, principalmente formados por los HAP, entre los que se encuentran los aductos de BPDE (benzo(a)pireno diol epóxido) (Garcia-Suastegui et al. 2011; Lepers et al. 2014a; Salcido-Neyoy et al. 2015b; Topinka et al. 2011). Por otra parte, se ha observado que la exposición a PM puede alterar los niveles de genes o proteínas de distintas vías de reparación, incluyendo la vía de NER.

Existen pocos trabajos que han abordado el impacto de las PM sobre la vía de NER; entre ellos se encuentra el trabajo de Calderon-Garciduenas, 2013, en el que se demostró que habitantes de la Ciudad de México, expuestos a niveles de $PM_{2.5}$ por encima de los estándares establecidos presentaban un incremento de los niveles de expresión de *XPA* en la corteza frontal asociado con la formación de aductos. Por otra parte, en un estudio in vitro, se identificó la desregulación de los genes *RAD23* y *ERCC1* en macrófagos humanos expuestos a $PM_{10}$ (Bastonini et al. 2011; Calderon-Garciduenas et al. 2013). Adicionalmente, Mehta y colaboradores, 2008 reportaron que la exposición a $PM_{2.5}$ inhibe la reparación del daño en DNA generado por la luz ultravioleta (UV) y por BaP en las células de pulmón A549, lo cual se asoció con una mayor frecuencia de mutaciones espontáneas y asociadas a la exposición a UV. Estudios posteriores en fibroblastos de pulmón embrionario humano señalaron que los compuestos orgánicos extraídos de $PM_{2.5}$ inducen un incremento de la proteína XPC, sin embargo disminuyen la capacidad de reparación de lesiones del DNA por la vía NER (Mehta et al. 2008; Rossner et al. 2013).

A pesar de los trabajos que sugieren que el funcionamiento de la vía de NER se altera a causa de las $PM_{2.5}$, se conoce poco sobre los efectos que tienen las partículas de mayor tamaño ($PM_{10}$) sobre las proteínas y sobre la actividad de dicho mecanismo de reparación del DNA. Además, se desconoce si el daño generado, es reparado adecuadamente en las células expuestas a $PM_{10}$. Por lo tanto, esclarecer estos mecanismos es relevante debido a que, si el DNA no es reparado adecuadamente, se puede generar un estado de inestabilidad genómica, que, a largo plazo, puede conducir al desarrollo de cáncer.

## 3. Justificación:

Las $PM_{10}$ inducen la formación de aductos de HAP en el DNA, y la acumulación de estas lesiones puede ocasionar errores en la replicación, generar inestabilidad genómica y predisponer a mutaciones. Es importante establecer si la exposición a $PM_{10}$ altera a las proteínas que participan en la vía de reparación por escisión de nucleótidos que está encargada de eliminar a los aductos voluminosos, así como determinar el funcionamiento de la vía de NER en las células expuestas a $PM_{10}$. Así mismo se debe considerar que la inhibición de este mecanismo puede favorecer alteraciones genómicas que conducen a un proceso carcinogénico. Con este abordaje, contribuiríamos a la descripción de las vías que se alteran en las células expuestas a $PM_{10}$, lo que tendrá un impacto en el conocimiento de los mecanismos de carcinogenicidad asociados con este contaminante ambiental.

## 4. Hipótesis

Las $PM_{10}$ disminuirán la eficiencia de reparación de la vía NER a través de la reducción de los niveles y marcas postraduccionales de las proteínas que participan en el paso de verificación de daño en DNA en células epiteliales de pulmón.

## 5. Objetivos

### 5.1 Objetivo General

Analizar los efectos de la exposición de células epiteliales de pulmón (A549) a las aeropartículas contaminantes $PM_{10}$ sobre la vía de reparación por escisión de nucleótidos.

### 5.2 Objetivos Particulares

En un curso temporal de exposición a $PM_{10}$:

- Cuantificar los niveles de aductos de BPDE en el DNA.
- Analizar los cambios en los niveles de proteínas de la vía de NER que participan en cada paso de la reparación del DNA.
- Determinar las alteraciones en las marcas postraduccionales de la proteína XPA, por ser la principal proteína de andamio.
- Evaluar la interacción del complejo proteico XPA-RPA, que participa en el reclutamiento de la maquinaria de reparación de la vía NER.
- Determinar el efecto sobre la actividad de la vía de NER.

## 6. Material y métodos

### 6.1 Muestreo y recolección de $PM_{10}$

Las partículas suspendidas $PM_{10}$ utilizadas en este trabajo se recolectaron en la Zona Urbana Residencial (ZUR) de la Ciudad de México (ubicada en Ciudad Universitaria, UNAM, Instituto de Ciencias de la Atmósfera) a través del uso de un colector de partículas de grandes volúmenes (GMW modelo 1200 VFC HVPM10 Sierra Andersen), que cuenta con un flujo constante de 1,13 $m^3$/min y filtros de nitrocelulosa con un tamaño de poro de 3,0 µm (Sartorius AG, Goettingen). Los filtros de nitrocelulosa se mantuvieron en un desecador a 4°C en obscuridad y posteriormente las $PM_{10}$ se recuperaron por medio del raspado de las membranas con ayuda de una hoja de bisturí y se almacenaron en viales de vidrio libres de contaminantes y endotoxinas, los cuales se guardaron a 4°C y en obscuridad hasta su uso. La caracterización (HAP, metales y endotoxinas) de las $PM_{10}$ utilizadas en este estudio se describe en trabajos previos (Chirino et al., 2017).

### 6.2 Cultivo celular y exposición a $PM_{10}$

Para el desarrollo de este trabajo se utilizaron células A549 (derivadas de un adenocarcinoma de pulmón humano) que fueron adquiridas en la ATCC (American Type Culture Collection Ref: CCL-185). Se cultivaron en medio Ham´s F12K (Kaighn´s) (Gibco, 21127-022), suplementado con 10% de Suero Fetal Bovino (SFB) (Gibco, 16000044) de acuerdo con las recomendaciones de la ATCC (temperatura de 37°C y ambiente de 5% de $CO_2$). Los experimentos se realizaron mediante la siembra de las células A549 en placas de 10 $cm^2$ y laminillas de cultivo de 0.8 $cm^2$. A las 24 h de sembrado (confluencia celular del 70%) las células se expusieron a 10 µg/$cm^2$ de $PM_{10}$ en un curso temporal de 6, 12, 24 y 48 h. A la par, se mantuvieron células sin exponer como control (CT) y células expuestas a Benzo(a)pireno (BaP) a una concentración de 1µM, utilizadas como control positivo de la formación de aductos en el DNA y de activación de la vía de NER (Abbas et al. 2011; Reed et al. 2020).

### 6.3 Determinación de aductos de Benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) en el DNA

Transcurridas 24 y 48 h de exposición a $PM_{10}$ o BaP, se retiró el medio de cultivo de la placa y se realizó un lavado con 1 ml con Buffer de fosfatos (PBS), posteriormente se colocó 1 ml de Tripsina-EDTA (Gibco,25300-054) con el fin de obtener el botón de células a partir del cual se realizó la purificación de DNA mediante el método estándar de extracción con Fenol-

cloroformo-alcohol isoamílico (Sambrook 1989), utilizando RNAsa A (Thermo Scientific, EN0531) y Proteinasa K (Invitrogen, 25530-015). El DNA se disolvió en 60 µl de agua libre de nucleasas y se precipitó toda la noche a 4°C. Al día siguiente se llevó a cabo la cuantificación del DNA y se determinó la pureza mediante la relación A260/280 utilizando el espectrofotómetro ND-1000 (NanoDrop Technologies); además, se evaluó la integridad del DNA mediante electroforesis en geles de agarosa al 1.2%.

La concentración de aductos de BPDE (metabolito derivado del BaP) en el DNA se determinó mediante un ensayo de ELISA (Oxi-Select Cell biolabs, Inc, Ref STA-357) siguiendo las especificaciones del fabricante, que se describe en breve. Se utilizó 1 µg de DNA y una curva estándar de BPDE-DNA (1.56-100 ng/ml) incluida en el kit. Se adicionaron 50 µl de cada muestra de DNA a la placa contenida en el kit y posteriormente se agregaron 50 µl de solución de unión a DNA, se realizó la mezcla con la punta de la pipeta y se incubó toda la noche a temperatura ambiente en un agitador orbital. Al día siguiente se removió la solución y se realizaron dos lavados con PBS, posteriormente se adicionaron 200 µl de la solución diluyente a cada pozo y se realizó el bloqueo por 1 h a temperatura ambiente. Se removió el sobrenadante y se adicionaron 100 µl de anticuerpo anti-BPDE, el cual se incubó por 1 h a temperatura ambiente y en agitación orbital. Se realizaron 5 lavados con buffer de lavado, al término de los lavados la placa se dejó totalmente seca y se colocaron 100 µl de anticuerpo secundario anti-HRP por 1 h a temperatura ambiente y en agitación orbital.

Nuevamente, se realizaron 5 lavados como se especificó anteriormente. Al finalizar los lavados, se agregaron 100 µl por pozo de solución de sustrato y se incubó en un agitador orbital por 30 minutos monitoreando el cambio de color de la reacción. Al momento de visualizar el cambio de color se agregaron 100 µl de solución de paro y se midió la absorbancia a una longitud de onda de 450 nm en el espectrofotómetro Infinite 200 Pro (modelo 6305/UV, Jenway). Las mediciones de los niveles de aductos se realizaron en duplicado interno y triplicado biológico. Los resultados se expresaron como niveles relativos de aductos de BPDE basado en la cuantificación de nanogramos de aductos de BPDE por microgramo de DNA calculados con la curva estándar de BPDE incluida en el kit y normalizando respecto al Control en el que no se encontró presencia de aductos.

### 6.4 Determinación de los niveles de proteínas

Los niveles de proteínas involucradas en la vía de NER se evaluaron mediante Western blot (WB) a partir de lisados de proteínas tanto totales como nucleares posterior a las 6, 12, 24 y 48 h de exposición a $PM_{10}$ o BaP, siguiendo los protocolos que se describen a continuación.

#### 6.4.1 Extracción de proteínas totales

Después de que las células fueron expuestas a $PM_{10}$ y BaP, se retiró el medio de cultivo de la placa y se realizó un lavado con 1 ml de PBS por pozo, posteriormente para la extracción de proteínas totales se agregaron 120 µl de amortiguador de lisis (20mM Tris, 1% NP-40 y 150mM NaCl pH8) complementado con inhibidor de proteasas y fosfatasas (Thermo Fisher, 78440) manteniendo la placa en agitación durante 10 minutos a 4°C. Posterior a la agitación, cada pozo fue raspado con un raspador de células (Corning, 3008) y las muestras se colocaron en microtubos de 1.5 ml, se centrifugaron a 13 000 rpm por 5 minutos a 4°C y se recuperó el sobrenadante. Se almacenaron a -20°C hasta su uso.

#### 6.4.2 Extracción de proteínas nucleares

Para la obtención de proteínas nucleares, las células se removieron de la placa de cultivo realizando un lavado con PBS y colocando 1 ml de tripsina por 5 minutos. Se obtuvo el botón celular el cual se mantuvo en hielo hasta su procesamiento. La extracción se llevó a cabo con el kit de extracción nuclear Chemicon´s (Millipore, 2900), añadiendo 500 µl de buffer de lisis citoplásmico complementado con inhibidor de proteasas y fosfatasas (Thermo Fisher, 78440), las células se resuspendieron mediante inversión del microtubo y se incubaron en hielo por 15 minutos. Posteriormente, el lisado se centrifugó a 2000 rpm por 5 minutos a 4°C, se descartó el sobrenadante y el botón celular se resuspendió en 50 µl de buffer de lisis citoplásmico. La suspensión celular se traspasó a una jeringa con aguja de 1 ml y 0.40mm respectivamente (BD-insulina, 326710) por la cual se expulsó el lisado, este proceso se repitió 10-15 veces y se centrifugó a 11 000 rpm por 30 minutos a 4°C, se recuperó el sobrenadante como fracción citoplásmica almacenándolas a -80°C. El botón celular se resuspendió en 30 µl buffer de extracción nuclear y la suspensión celular se traspasó a una jeringa con de 1 ml y 0.40 mm respectivamente (BD-insulina, 326710), por la cual se expulsó el contenido como se indica anteriormente, se recuperó el sobrenadante y se almacenó a -80°C toda la noche. Al día siguiente la suspensión se agitó a baja velocidad por 30 minutos a 4°C y se centrifugó a 13 000 rpm por 5 minutos a 4°C. Se recuperó el sobrenadante como fracción nuclear y se almacenó a -80°C.

### 6.4.3 Cuantificación de proteínas

La concentración de proteínas (totales y nucleares) se determinó mediante el método de ácido bicinconínico, utilizando como parámetro la curva de albúmina sérica bovina de 1.25-10 µg/µl (Thermo Fisher, 23209) y leyendo las muestras en el espectrofotómetro Infinite 200 Pro (modelo 6305/UV) a una longitud de onda de 570 nm.

### 6.4.4 Electroforesis de proteínas

La determinación de los niveles de proteínas se realizó mediante Western blot bajo las siguientes condiciones: en el caso de la determinación de proteínas totales se cargaron 30 µg de muestra en geles de poliacrilamida-SDS al 10% y para la determinación de proteínas nucleares se cargaron 15 µg de muestra en geles de poliacrilamida-SDS al 15%. Las muestras se prepararon con Tris 10 mM pH 7.4 más buffer Laemmli al 95% (Bio-Rad, 161-0737) y 2-Mercaptoetanol (BME) al 5% (Sigma, M6250) para obtener una relación 1:1 entre el volumen proteína/Tris y Laemmli/BME. La electroforesis se realizó por 1 h y 35 minutos a un voltaje de 150 V en buffer Tris/glicina/SDS (Bio-Rad, 161-0772). Se realizó la transferencia de proteínas a membranas de PVDF con tamaño de poro de 0.45 µm en buffer Tris/glicina/metanol utilizando el sistema de transferencia semiseco (Trans-Blot-Turb, Bio-Rad) a un voltaje de 25 V y 1 ampere durante 30 minutos.

Para evitar unión inespecífica de los anticuerpos, las membranas se bloquearon con leche baja en grasa al 5% en TBS-Tween 0.1%, manteniéndolas en agitación por 1 h. Al finalizar el bloqueo se realizó la incubación con el anticuerpo primario de cada una de las proteínas a evaluar: Anti-RAD23 (Cell signaling, 24555), anti-XPD (Cell signaling, 11963), anti-XPA (Santa Cruz, sc-56497) y anti-WIP1 (Cell signaling, 11901) a una dilución 1:1000, Anti-pXPAs196 (Thermo Fisher, 64730) a una dilución 1:500 y anti-H4K20me2 (Abcam, ab9052) a una dilución 1:2000, durante toda la noche a 4°C en agitación constante. Para el control de carga de proteínas totales se utilizó Anti-Beta-Actina (β-Actina) en una dilución 1:3000 (anticuerpo monoclonal donado por el Dr. Manuel Hernández, Cinvestav-IPN) (Morales-Barcenas et al. 2015; Sanchez-Perez et al. 2009) y para proteínas nucleares se utilizó el anticuerpo anti-Histona H3 (Abcam, ab1791) en una dilución 1:5000, ambos anticuerpos fueron incubados 1 h a temperatura ambiente en agitación.

Posteriormente se realizaron 3 lavados de 10 minutos con TBS-Tween 0.1% en agitación y se incubó por 1 h anticuerpo secundario anti-conejo (Amersham, NA934V) 1:3000 o anti-ratón (Amersham, NA931) 1:3000 según corresponda a cada anticuerpo primario. Se realizaron 3 lavados de 10 minutos con TBS-Tween 0.1% en agitación y se llevó a cabo la inmunodetección utilizando el sustrato de quimioluminiscencia peroxidasa HRP (Millipore, WBKLS0100). Para la visualización y captura de imágenes se utilizó el fotodocumentador ChemiDoc-It Imager UVP. Las densitometrías de cada membrana se llevaron a cabo con el programa Image J Software.

### 6.5 Ensayo de Co-inmunoprecipitación

Posterior a la exposición de las células a $PM_{10}$ y BaP se evalúo la formación de complejos de proteínas mediante la técnica de co-inmunoprecipitación utilizando el kit Dynabeads Co-Inmunoprecipitation (Invitrogen, Thermo Fisher Scientific, 14321D) de la siguiente manera:

Para la formación del complejo perlas anticuerpo, se pesó 1 mg de perlas magnéticas, se colocaron en un tubo de 1.5 ml y se incubaron toda la noche con 5 µg de anticuerpo anti XPA (Santa Cruz, sc-56497) o 5 µg de Normal goat IgG (R&D systems, AB-108-C) en buffer C1-C2 manteniéndolas en agitación constante a 37°C. Al día siguiente se colocaron los tubos en el magneto permitiendo que las perlas se adhirieran a la pared del tubo, se removió el sobrenadante y se realizaron lavados de 1 minuto con los buffers HB, LB y SB contenidos en el kit (composición no especificada). Posteriormente, se realizó un lavado de 15 minutos incubando las perlas con buffer SB en agitación constante, el tubo se colocó nuevamente en el magneto y se retiró el sobrenadante. Las perlas se resuspendieron en 100 µl de buffer SB y se almacenaron a 4°C hasta su uso.

Para la extracción de proteínas, las células se removieron de la placa realizando un lavado con 1 ml de PBS y colocando 1 ml de tripsina por 5 minutos, a través de centrifugación se obtuvo el botón celular el cual se resuspendió en buffer de extracción IP (0.5% de tritón) con inhibidores de proteasas y fosfatasas (Thermo Fisher, 78440, Rockford, Illinois, United States) en un radio de 1:9 respecto al peso de las células, se llevó a cabo la incubación en hielo por 15 minutos y se centrifugó a 13 000 rpm por 5 minutos a 4°C, el sobrenadante se colocó en un tubo nuevo y el lisado se utilizó inmediatamente.

El complejo de perlas-AB, previamente formado, se lavó con buffer de extracción durante 1 minuto y se removió el sobrenadante. Dicho complejo se mezcló con el lisado celular y se incubó a 4°C en agitación constante por 25 minutos, posteriormente los tubos se colocaron en el magneto y se removió el sobrenadante. El inmunocomplejo se lavó con buffer de extracción y buffer LWB mediante resuspensión con punta de pipeta. Al finalizar cada lavado los tubos se colocaron en el magneto por 1 y 5 minutos respectivamente y se retiró el sobrenadante. Se transfirió la suspensión a un tubo limpio, se colocó en el magneto y se removió el sobrenadante. Para finalizar, el inmunocomplejo se resuspendió en 60 µl de buffer de elución, se incubó 5 minutos, se colocó en el magneto y el sobrenadante (contiene la proteína de interés purificada con él complejo) se colocó en un tubo nuevo y se almacenó a 4°C.

Para visualizar la interacción de las proteínas, se realizó una electroforesis del complejo inmune en un gel de poliacrilamida-SDS concentración 12% siguiendo los pasos descritos en la sección electroforésis de proteínas. En este caso se utilizó el anticuerpo Anti-RPA (Cell signaling, 2267) en una dilución 1:1000 y anticuerpo secundario anti-conejo (Amersham, NA934V) 1:3000.

### 6.6 Actividad de la vía de NER mediante el ensayo de síntesis de DNA no programada.

La actividad de reparación del DNA por la vía de NER se evaluó en células sembradas en laminillas de 0.8 $cm^2$ las cuales fueron expuestas a $PM_{10}$ y BaP por 24 y 48 h, mediante el ensayo de síntesis de DNA no programada (UDS) utilizando el kit Click-iT EdU Imaging (Invitrogen, C10337) en el que se utiliza 5-Ethynyl-2´-deoxyuridine (EdU) como análogo de timidina, el cual se incorpora al DNA durante la síntesis de la cadena del DNA posterior a la eliminación de los aductos. Previo a la exposición a $PM_{10}$ y BaP las células se trataron con 5 mM de hidroxiurea (Sigma, H8627), para bloquear la replicación del DNA, y evitar la incorporación inespecífica del EdU el cual se adicionó a una concentración final de 10 µM al medio de cultivo (Kelly and Latimer 2005; Wienholz et al. 2017).

Al concluir las horas de exposición a $PM_{10}$ y BaP, las células fueron lavadas con PBS y fijadas con paraformaldehído al 3.7% por 20 minutos, posteriormente se realizó un lavado con buffer de lavado compuesto por albúmina al 3% en PBS (BSA-PBS) y se incubaron con buffer de permeabilización (0.5% Tritón-PBS) por 25 minutos, se retiró el buffer y se realizaron dos lavados (BSA-PBS). Posteriormente las células se incubaron por 30 minutos

protegidas de la luz con 300 µl de cocktail Click-iT, compuesto por Click-iT EdU reaction buffer, $CuSO_4$, Alexa Fluor azida y Click-iT EdU buffer additive cuyos volúmenes se adicionaron de acuerdo a las instrucciones del fabricante. Concluido el tiempo de incubación, se retiró el reactivo y se realizó un lavado con PBS. Al finalizar el procedimiento las laminillas se secaron completamente, se adicionó medio de montaje DAPI (Invitrogen, 8961S) y se colocó un cubreobjetos. Se almacenaron a 4°C toda la noche y al día siguiente se realizó el sellado de la laminilla con barniz transparente. Las laminillas se observaron en el microscopio de fluorescencia AxioKop2 Mot Plus.D2 (Carl Zeiss, Oberkochen, Germany) y se tomaron microfotografías de los diferentes tratamientos.

Se analizaron 1500 células de las cuales se contaron el número de células EdU-positivas (en las que se observan focos de reparación en el núcleo). Las células que presentaron una tinción completa del núcleo se excluyeron del conteo de reparación, debido a que en este caso el EdU se incorporó durante la fase de síntesis, según lo reportado por Kelly y colaboradores (Kelly and Latimer 2005). Los niveles de actividad de la vía de NER se calcularon como el porcentaje de células EdU positivas en cada tratamiento divididas entre el porcentaje de células EdU positivas presentes en las células CT.

### 6.7 Análisis estadístico

Los datos presentados son resultados de al menos tres experimentos independientes. Se presentan como media ± desviación estándar. Las diferencias estadísticas entre los aductos de BPDE y la actividad de la vía de NER se probaron mediante el análisis de varianza con prueba post hoc de Bonferroni. Los niveles de proteína se analizaron aplicando la prueba t de Student. Todos los análisis se realizaron con el software GraphPad, versión 6. Se consideró estadísticamente significativo un valor de $p \leq 0,05$.

## 7. Resultados

### 7.1 Las $PM_{10}$ indujeron la formación de aductos de BPDE en el DNA

Las $PM_{10}$ generaron un incremento significativo de los aductos de BPDE-DNA a las 24 h de exposición (2.01 vs 0.0, $p < 0.05$) respecto al control (CT). A las 48 h también se observó un incremento en los niveles de los aductos de BPDE-DNA (2.54 vs 0.0, $p < 0.05$) en las células A549 comparado con el CT. En el caso de las células expuestas a BaP se encontró un aumento significativo en los aductos de BPDE-DNA a las 24 h (2.85 vs 0.0, $p < 0.05$) y a las 48 h (1.30 vs 0.0, $p < 0.05$) comparado con el CT; sin embargo, se observó una disminución significativa en la concentración de aductos de BPDE-DNA en las células expuestas a BaP por 48 h comparado con las células expuestas a BaP por 24 h (1.30 vs 2.85, $p < 0.05$) (Figura 5).

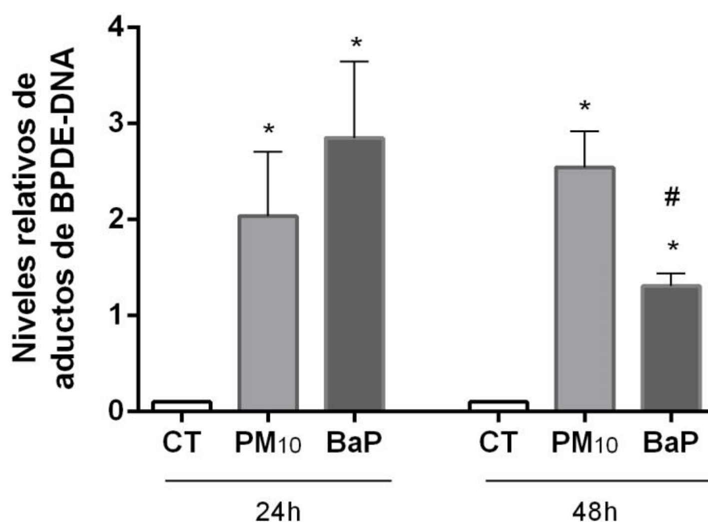


***Figura 5. Formación de aductos de BPDE-DNA en células A549 expuestas a 10 μg/cm² de $PM_{10}$ y 1 μM de BaP por 24 y 48 h.*** Los aductos de Benzo[a]pireno diol epoxide en DNA (BPDE-DNA) se evaluaron en células de pulmón (A549) expuestas a $PM_{10}$ (10 μg/$cm^2$) y BaP (1μM) por 24 y 48h. La detección de los aductos BPDE-DNA se expresó como niveles relativos y los valores representan los resultados de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística contra el grupo control (CT), $p < 0.05$. (#) indica diferencia estadística entre tiempos de exposición $p < 0.05$.

### 7.2 Las $PM_{10}$ desregularon los niveles de proteínas de diferentes puntos de la vía de NER.

Se determinó que RAD23, una de las proteínas de la fase de reconocimiento, presentó incremento a las 6 h de exposición a $PM_{10}$ (1.16 vs. 1.00; $p < 0.05$) respecto al control (CT). La exposición a BaP también generó incremento en RAD23 a las 6 h de exposición (1.20 vs. 1.00; $p < 0.05$) respecto al CT (Figura 6). No se observaron diferencias en el nivel de RAD23 después de las 12 h de exposición a $PM_{10}$ y BaP (1.04 and 1.02 vs. 1.00, respectivamente), 24 h (1.01 and 0.94 vs. 1.00, respectivamente) y 48 h (0.95 and 0.90 vs. 1.00, respectivamente), comparado con su grupo CT.

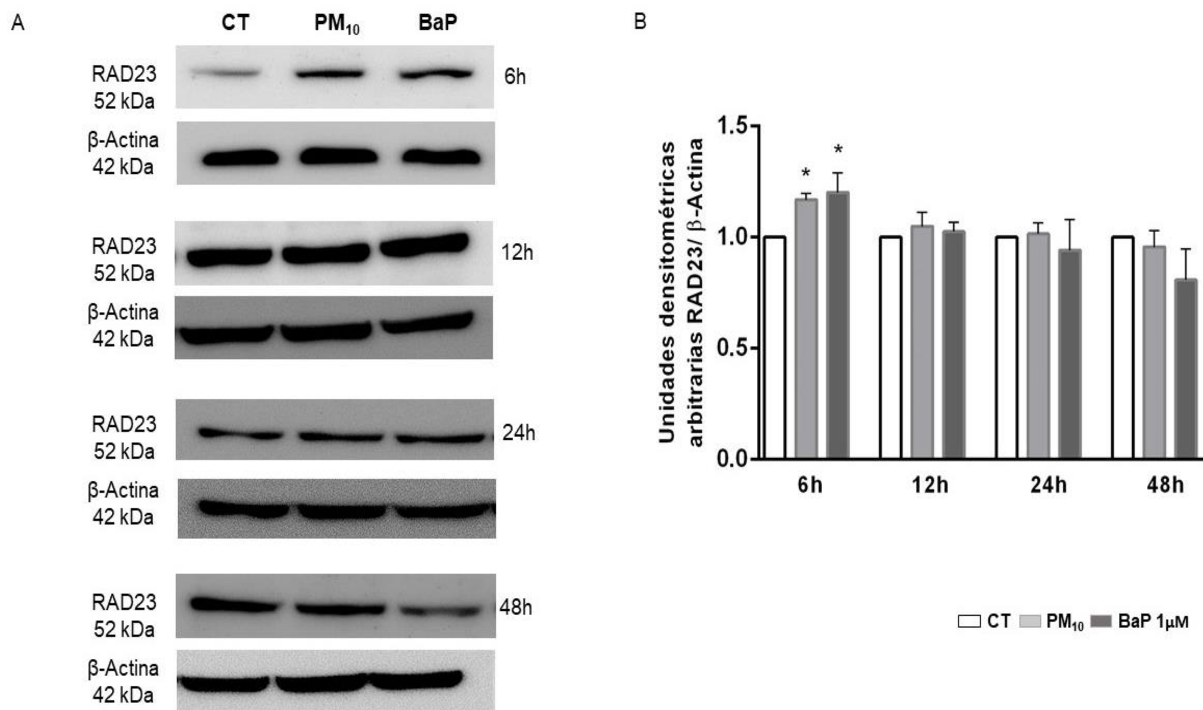


***Figura 6. Nivel de la proteína RAD23 evaluados en lisados totales de células A549 expuestas a 10 μg/cm² de $PM_{10}$ y 1 μM de BaP por 6, 12, 24 y 48 h.*** A) Imágenes representativas de los niveles de la proteína RAD23. B) Análisis densitométricos utilizando β-Actina como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

El análisis del nivel de la proteína XPD, una helicasa de la vía de NER, mostró un aumento estadísticamente significativo (1.20 y 1.00; $p < 0.05$) en las células expuestas a $PM_{10}$ por 24 h comparado con el grupo CT y en ese mismo tiempo se encontró incremento (1.15 y 1.00; $p < 0.05$) de XPD inducido con BaP. Sin embargo, en otros tiempos de exposición no se observaron diferencias significativas en células expuestas a $PM_{10}$ o BaP después de las 6 h (1.01 y 1.09 vs. 1.00, respectivamente), 12 h (1.05 y 1.03 vs. 1.00, respectivamente) y 48 h (0.97 y 0.98 vs. 1.00, respectivamente), comparado con sus grupos CT (Figura 7).

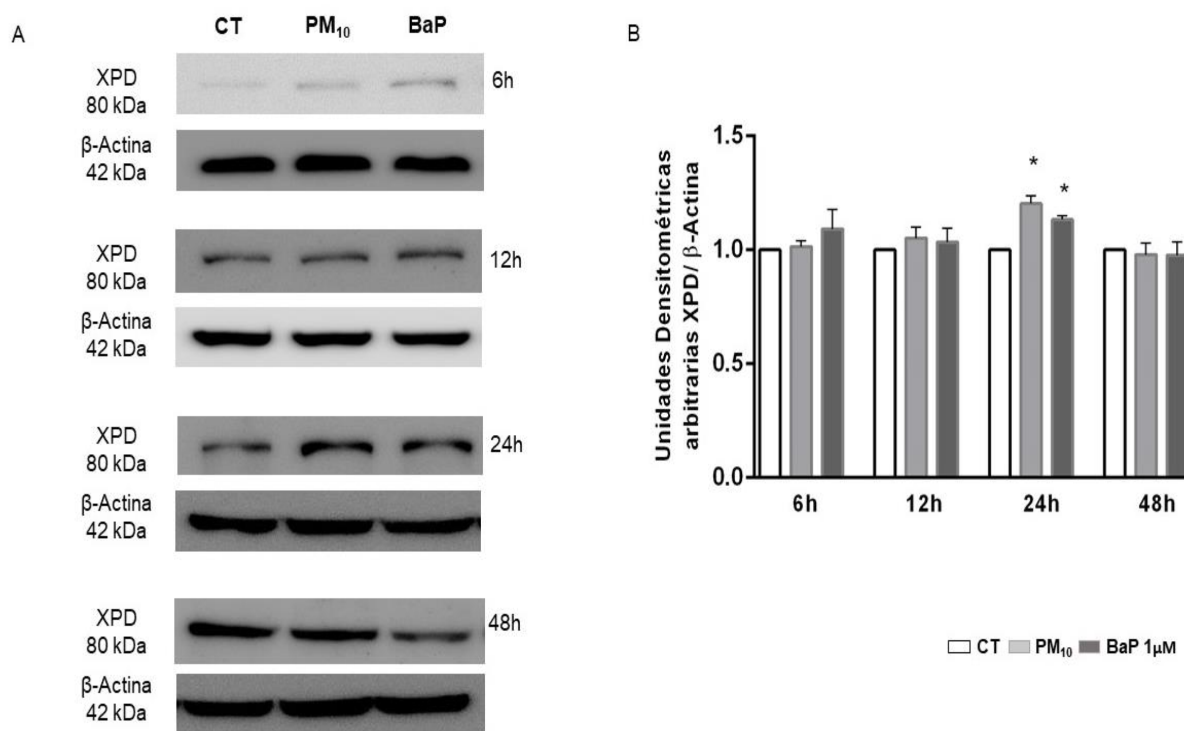


***Figura 7. Niveles de la proteína XPD evaluados en lisados totales de células A549 expuestas a 10 μg/cm² de $PM_{10}$ y 1 μM de BaP por 6, 12, 24 y 48* h.** A) Imágenes representativas de los niveles de la proteína XPD. B) Análisis densitométricos utilizando β-Actina como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

Respecto a XPA, se determinó que las $PM_{10}$ disminuyeron significativamente el nivel de la proteína XPA en las células expuestas por 24 h comparado con el CT (0.85 vs. 1.00; $p < 0.05$). Por el contrario, a las 24 h, el BaP incrementó los niveles de la proteína XPA comparado con el CT (1.17 vs. 1.00; $p < 0.05$). No se encontraron cambios en los niveles de XPA, proteína encargada de formar complejos efectores, en las células expuestas a $PM_{10}$ o BaP después de 6 h de exposición (1.00 y 0.99 vs. 1.00, respectivamente), 12 h (1.03 y 1.07 vs. 1.00, respectivamente) y 48 h (0.98 and 0.96 vs. 1.00, respectivamente) comparado con sus grupos CT (Figura 8).

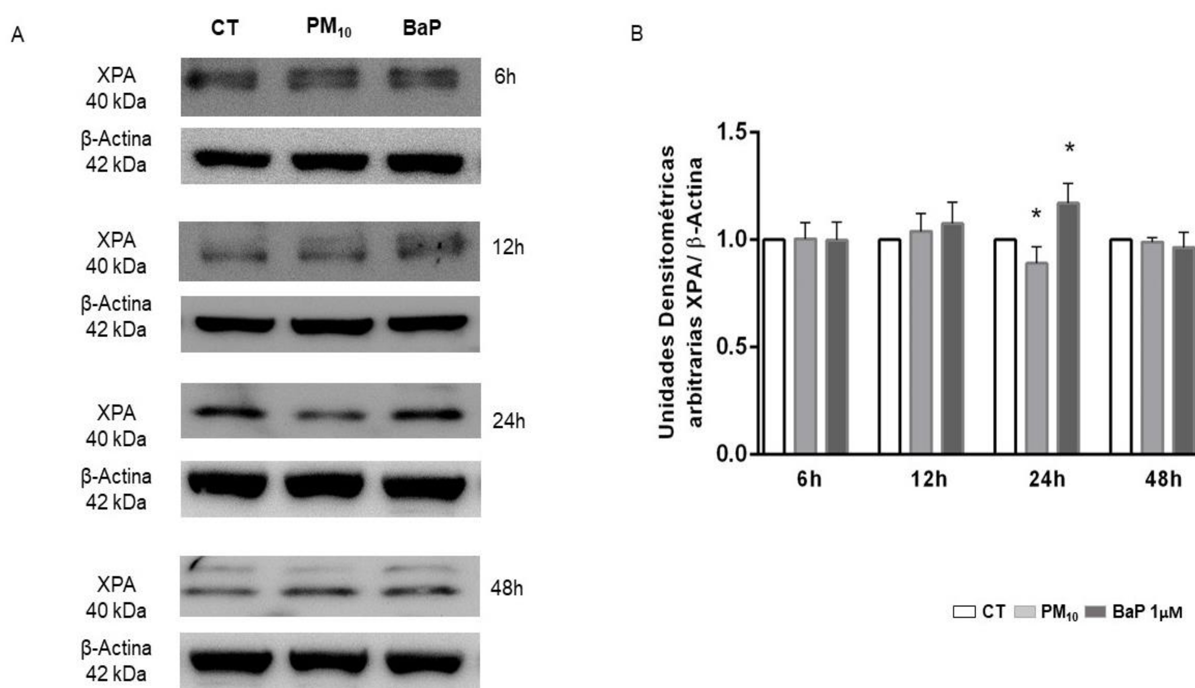


***Figura 8. Niveles de la proteína XPA evaluados en lisados totales de células A549 expuestas a 10 μg/cm² de $PM_{10}$ y 1 μM de BaP por 6, 12, 24 y 48 h.*** A) Imágenes representativas de los niveles de la proteína XPA. B) Análisis densitométricos utilizando β-Actina como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

### 7.3 Las $PM_{10}$ indujeron la dimetilación de la lisina 20 en la histona 4 en el núcleo.

Se analizó la dimetilación de la lisina 20 en la histona 4 (H4K20me2) como señal de reclutamiento de XPA. Se observó que las $PM_{10}$ generaron un incremento estadísticamente significativo en el nivel de H4K20me2 comparado con el grupo CT (2.09 vs. 1.00; $p < 0.05$). En el caso de las células expuesta a BaP no se observó un cambio en el nivel de H4K20me2 respecto al CT (1.10 vs. 1.00) (Figura 9).

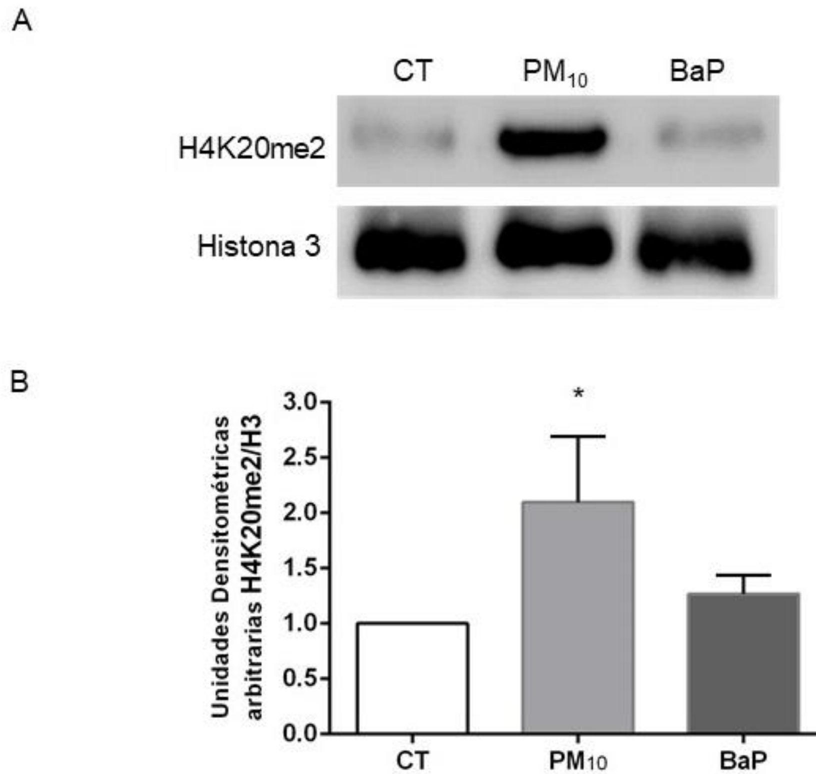


***Figura 9. Niveles de la histona 4 dimetilada en el residuo de lisina 20 (H4K20me2) evaluados en proteínas nucleares de células A549 expuestas a 10 μg/cm² de $PM_{10}$ y 1 μM de BaP por 24 h.*** A) Imágenes representativas de los niveles de la proteína H4K20me2. B) Análisis densitométricos utilizando Histona 3 como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

### 7.4 Las $PM_{10}$ disminuyeron la fosforilación de XPA e incrementaron los niveles de la fosfatasa WIP1.

Se encontró que en las células expuestas a $PM_{10}$ por 24 h, el niveles de XPA fosforilada en su residuo de serina 196 (principal modificación postraduccional) disminuyó significativamente comparado con el grupo CT (0.74 vs. 1.00; $p < 0.05$). El BaP indujo una tendencia al incremento en la fosforilación de XPA respecto al grupo CT (1.13 vs. 1.00) sin lograr una significancia estadística (Figura 10).

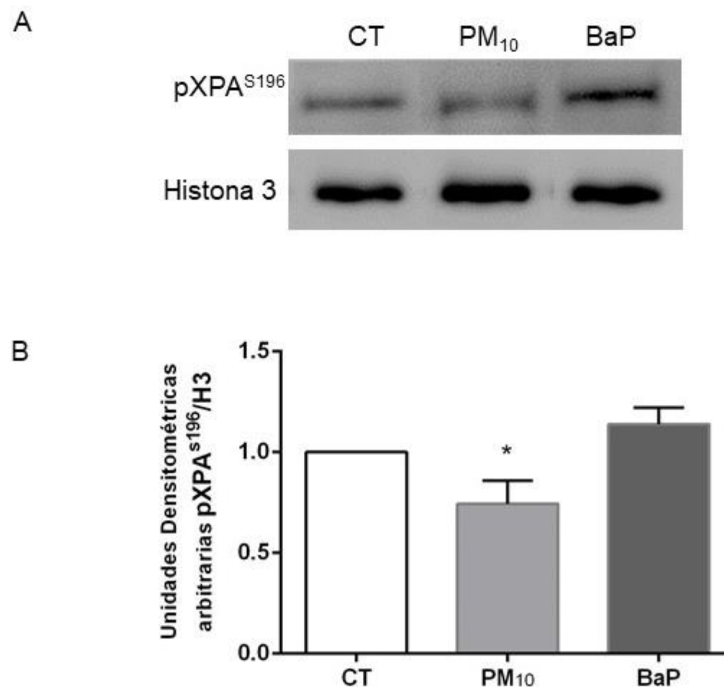


***Figura 10. Niveles de fosforilación de la proteína XPA en el residuo serina 196 ($XPA^{S196}$) evaluados en proteínas nucleares de células A549 expuestas a 10 $\mu g/cm^2$ de $PM_{10}$ y 1 µM de BaP por 24 h.*** A) Imágenes representativas de los niveles de la proteína $pXPA^{S196}$. B) Análisis densitométricos a nivel nuclear respecto a los niveles de XPA en núcleo. Se utilizó Histona 3 como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

Adicionalmente, se determinó que el nivel de la fosfatasa WIP1, encargada de desfosforilar a XPA, incrementó en las células expuestas a $PM_{10}$ por 24 h comparado con el grupo CT (1.29 vs. 1.00; $p < 0.05$). En las células expuestas a BaP no se encontró diferencias en el nivele de WIP1 después de las 24 h de exposición respecto al CT (1.02 vs. 1.00) (Figura 11).

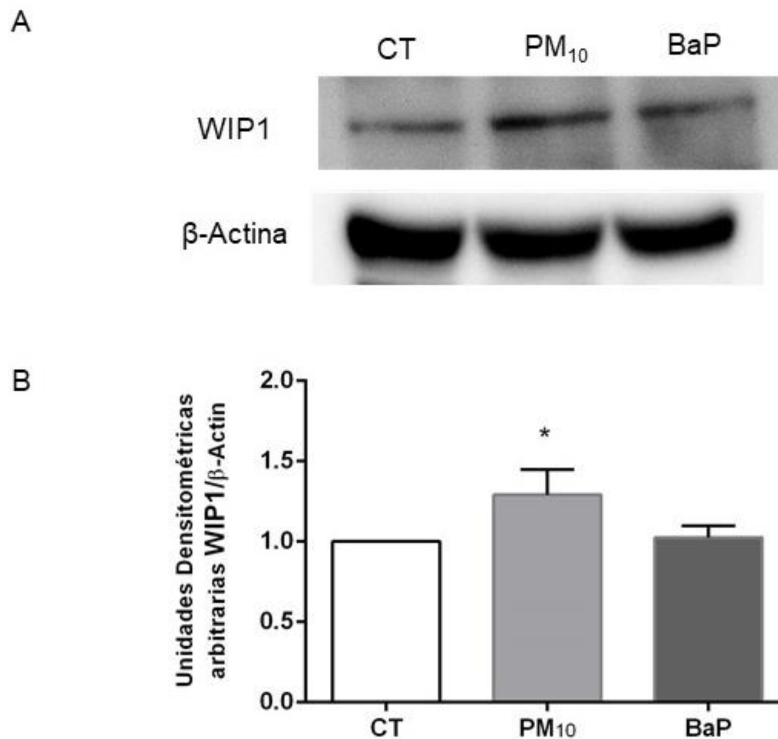


***Figura 11. Niveles de la proteína WIP1 evaluados en lisados totales de células A549 expuestas a 10 µg/cm² de $PM_{10}$ y 1 µM de BaP por 24 h.*** A) Imágenes representativas de los niveles de la proteína WIP1. B) Análisis densitométricos utilizando β-Actina como control endógeno. Los valores representan las veces de cambio respecto al control (CT), los resultados provienen de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$.

### 7.5 Las $PM_{10}$ inhibieron la interacción entre XPA y RPA

Se observó que en las células expuestas a $PM_{10}$ por 24 h no se formó el complejo entre XPA y RPA (Figura 12), el cual induce la remoción de aductos y la reparación del DNA. Por otra parte, en las células expuestas a BaP se encontró una interacción entre XPA y RPA.

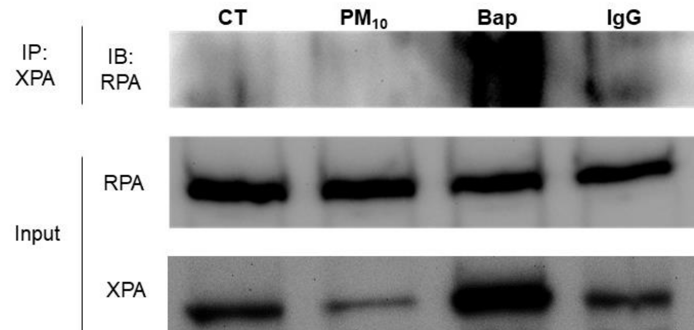


***Figura 12. Formación del complejo entre XPA y RPA en células A549 expuestas a 10 µg/cm² de $PM_{10}$ y 1 µM de BaP por 24 h.*** Western Blot representativo de tres experimentos independientes en los que se detectó la interacción entre XPA y RPA mediante un ensayo de inmunoprecipitación realizado en lisados totales. IP: Inmunoprecipitación. IB: Inmunoblot.

### 7.6 La actividad de la vía de NER se inhibió en las células expuestas a $PM_{10}$

Las células expuestas a $PM_{10}$ por 24 y 48 h no mostraron diferencias en la actividad de la vía de NER respecto a las células del grupo control (CT) (0.99 vs. 1.00 y 0.99 vs. 1.00, respectivamente) a pesar de los aductos detectados previamente (Figura 13 y Figura 14).

En las células que fueron expuestas a BaP se observó un incremento estadísticamente significativo en la actividad de la vía de NER a las 24 h comparado con el CT (1.50 vs. 1.00; $p < 0.05$), mientras que las células expuestas a BaP por 48 h no mostraron diferencias en la actividad de la vía de NER respecto al CT(1.09 vs. 1.00) (Figura 13 y Figura 15).

La comparación entre tiempos de exposición mostró una disminución significativa en la actividad de la vía de NER entre las células expuestas a BaP por 48 h comparado con las células expuestas a BaP por 24 h (1.09 vs. 1.50; $p < 0.05$) (Figura 13).

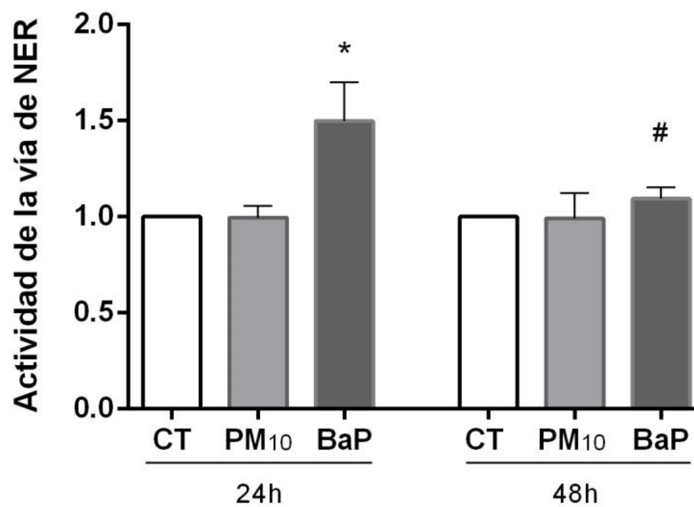


***Figura 13. Actividad de la vía de reparación por escisión de nucleótidos (NER) en células A549.*** La actividad de la vía NER se evaluó mediante el ensayo de síntesis de DNA no programada UDS en células de pulmón A549 expuestas a 10 μg/cm$^2$ de $PM_{10}$ y 1μM de BaP por 24 y 48 h. La gráfica muestra los resultados cuantitativos expresados como actividad de NER posterior al conteo de 1500 células por condición comparado con el control (CT). Valores representan los resultados de 3 experimentos independiente con desviación estándar por tratamiento. (*) Indica diferencia estadística vs. CT, $p \leq 0.05$. (#) indica diferencia estadística entre tiempos de exposición $p < 0.05$.

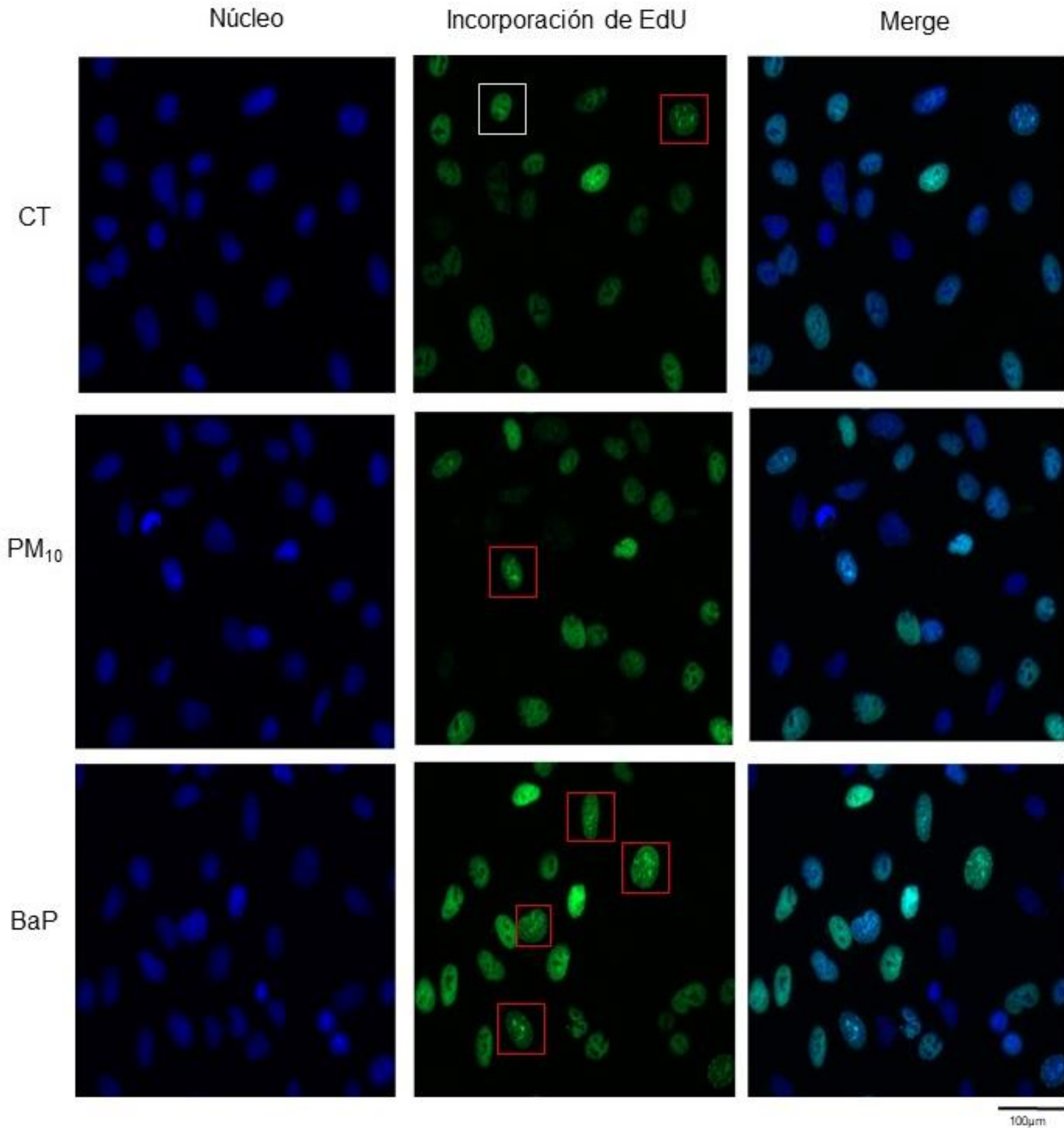


***Figura 14. Imágenes representativas de la actividad de la vía NER en células expuestas a $PM_{10}$ y BaP por 24 h.*** Microfotografías de la detección de la incorporación del análogo de timidina (EdU) durante la reparación del DNA por vía de NER en cada grupo. La actividad de NER es reconocida por la formación de focos de reparación, cuyas células se encuentran marcadas en recuadros rojos. La incorporación inespecífica durante la replicación del DNA se identifica como una tinción homogénea del núcleo (el ejemplo de una célula con marcaje inespecífico se presenta en un recuadro blanco), estas células no se consideraron en el conteo para el análisis de la actividad de reparación.

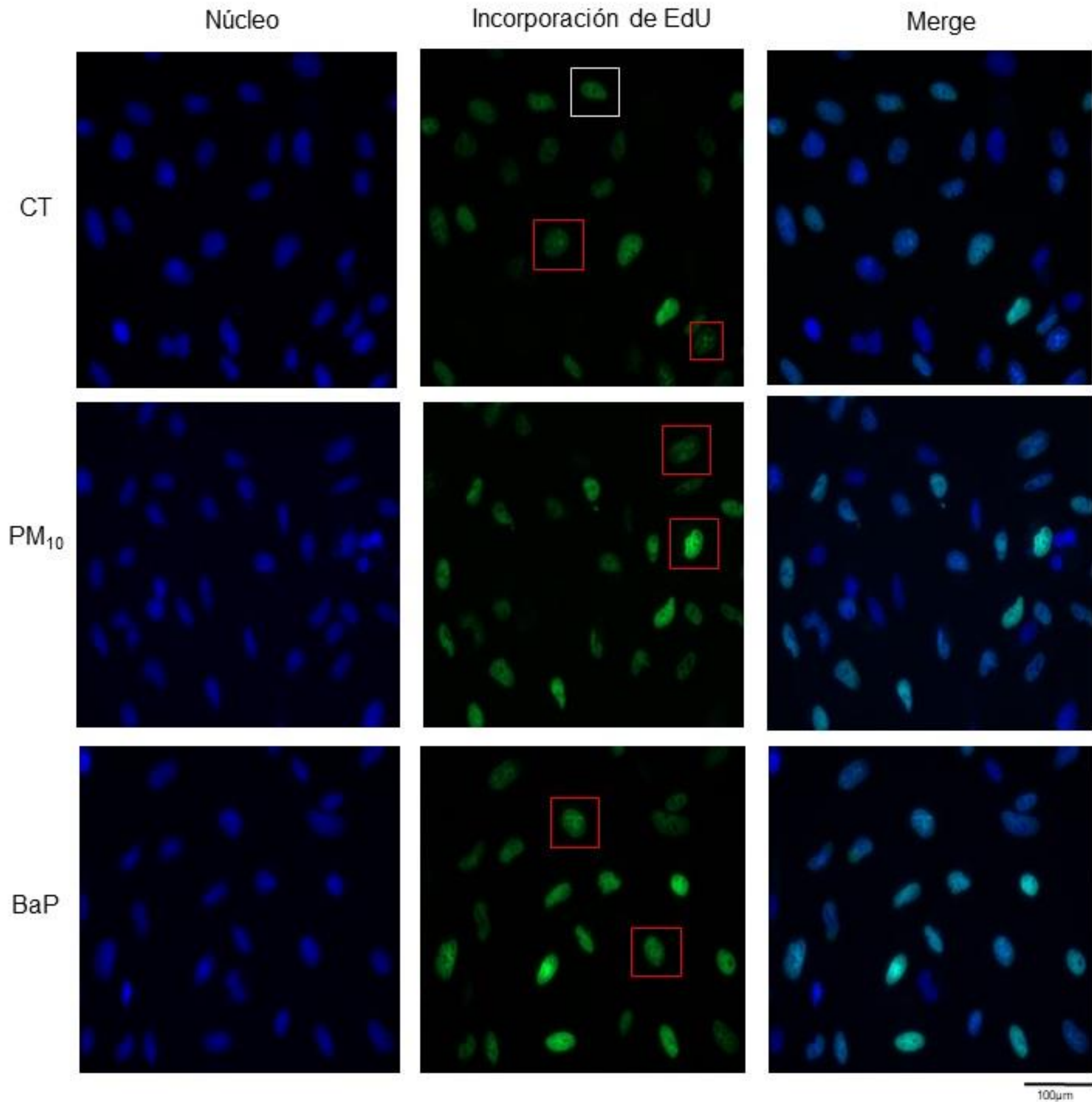


***Figura 15. Imágenes representativas de la actividad de la vía NER en células expuestas a PM10 y BaP por 48 h.*** Microfotografías de la detección de la incorporación del análogo de timidina (EdU) durante la reparación del DNA por vía de NER en cada grupo. La actividad de NER es reconocida por la formación de focos de reparación, cuyas células se encuentran marcadas en recuadros rojos. La incorporación inespecífica durante la replicación del DNA se identifica como una tinción homogénea del núcleo (el ejemplo de una célula con marcaje inespecífico se presenta en un recuadro blanco), estas células no se consideraron en el conteo para el análisis de la actividad de reparación.

## 8. Discusión:

Las $PM_{10}$ son un factor de riesgo para el desarrollo de cáncer, principalmente de pulmón (Chen et al. 2016a; Yu et al. 2021; Zhou et al. 2017a). Entre los diferentes componentes contenidos en las $PM_{10}$ se encuentran diferentes metales como los de transición y diversos HAP, de los cuales algunos han sido clasificados como carcinógenos y se conoce los mecanismos asociados a su carcinogenicidad. Si bien, actualmente se han descrito diferentes efectos a nivel celular y molecular inducidos por las $PM_{10}$, aún se busca dilucidar los mecanismos que favorecen el desarrollo de cáncer, especialmente porque hasta el momento no se han detectado mutaciones asociadas a la exposición a $PM_{10}$ (Guo et al. 2018). Por lo anterior, el estudio de la genotoxicidad y de los efectos inducidos por las PM sobre las vías de reparación de daño al DNA podría ayudar a entender el papel de las $PM_{10}$ en la carcinogénesis pulmonar; debido a que además de las mutaciones, la evasión de la reparación del DNA se considera un factor para la generación de tumores, en particular si se presentan deficiencias en la vía de NER encargada de eliminar los aductos voluminosos (Barnes et al. 2018; Cheng et al. 2000; Hoeijmakers 1993; Topinka et al. 2011).

En este estudio, se expusieron células de epitelio pulmonar (A549) las cuales son un modelo ampliamente utilizado en toxicología (Andre et al. 2011; Santibanez-Andrade et al. 2019; Yang et al. 2016), a una concentración sub-letal de 10 $\mu g/cm^2$ de $PM_{10}$ que simula 5 días de exposición en los humanos (Ferecatu et al. 2010; Li et al. 2003) y se analizaron los efectos tanto en las proteínas como en el funcionamiento de la vía de NER. En primer lugar, encontramos que las $PM_{10}$ inducen la formación de aductos de BPDE en el DNA, como se ha demostrado en trabajos previos (Abbas et al. 2011; Salcido-Neyoy et al. 2015b). La literatura indica que más del 60% de los aductos de tipo voluminoso inducidos por luz UV y BaP son removidos a las 48 h desde su generación (Grosskopf et al. 2010; Piberger et al. 2018; Schwerdtle et al. 2010a). Sin embargo, en este trabajo determinamos que en las células expuestas a $PM_{10}$ estas lesiones persisten hasta las 48 h. Contrariamente, la misma línea celular expuesta al carcinógeno BaP mostró una disminución de los aductos de BPDE-DNA a las 48 h, sugiriendo que más de la mitad de estas lesiones se han removido a este tiempo. Estos resultados sugieren que el daño generado en el DNA por las $PM_{10}$ podría persistir y acumularse. El daño genotóxico persistente genrado por las $PM_{10}$ podría inducir inestabilidad genómica, a diferencia del daño en el DNA generado por un solo carcinógeno como lo es el BaP.

El segundo hallazgo de este estudio es la identificación de los cambios en las proteínas que participan en la vía de NER. Al evaluar cada paso de la vía de reparación mediante la determinación de los niveles de proteínas clave, encontramos que en las células expuestas a $PM_{10}$, los niveles de RAD23, H4K20me2 y XPD incrementaron, por el contrario, los niveles de XPA disminuyeron. Los estudios de actividad de la vía de NER han demostrado que en las células A549 expuestas a HAP, el comienzo de la reparación del DNA ocurre entre las 4 a 6 horas después de la generación del daño en el DNA (Shi et al. 2017). Por lo tanto, el aumento de la proteína RAD23 que observamos en las células expuestas a $PM_{10}$ en las primeras horas posteriores de exposición, sugiere que las células reconocen el daño en el DNA, debido a que RAD23 se une a la proteína XPC reconociendo el aducto, lo cual permite el reclutamiento de las helicasas que se encargarán de la apertura de la cadena de DNA (Ng et al. 2003). Además, en este trabajo no se observaron cambios en RAD23 en las siguientes horas de exposición ni en $PM_{10}$ ni en BaP, lo que indica que esta proteína solo es necesaria durante el paso inicial de reparación, tal y como se ha demostrado en diferentes estudios (Riedl et al. 2003; Sugasawa et al. 1998).

Adicionalmente, las $PM_{10}$ incrementaron los niveles de H4K20me2, lo que confirma que en estas células, el paso de reconocimiento del DNA dañado funciona correctamente, por lo que esperaríamos que se genere el reclutamiento de otras proteínas en el sitio de daño, como XPA (Chitale and Richly 2018a). Después del reconocimiento del daño, el DNA probablemente se desenrolla en las células expuestas a $PM_{10}$ debido a que observamos aumento de la proteína XPD, que funciona como helicasa (Kuper et al. 2014). El incremento de estas dos proteínas podría funcionar como señal para continuar con la reparación del DNA. En células expuestas a BaP, se encontró un aumento en los niveles de XPD; sin embargo, no se observaron cambios en los niveles de H4K20me2, por lo que es probable que este evento haya ocurrido horas antes de que se midiera la proteína, la cual solo se analizó a las 24 h. Aunque XPA no tiene actividad enzimática, las alteraciones de esta proteína pueden generar una inhibición en la progresión de la actividad de la vía de NER, porque participa como proteína de ensamblaje del complejo de incisión en el sitio de daño al DNA y este paso permite el corte de la cadena dañada (Krasikova et al. 2010; Matsuda et al. 1995). Importantemente, los niveles de XPA disminuyen en las células expuestas a $PM_{10}$, a pesar de que la señal de reclutamiento H4k20me2 si está activa, como sugiere el incremento de dicha marca. Por el contrario, XPA incrementa en las células expuestas a BaP, lo que sugiere que en las células expuestas a $PM_{10}$ el paso de verificación se encuentra alterado.

Con base en estos resultados, proponemos que la disminución de XPA en células expuestas a $PM_{10}$ podría estar relacionada con algunos metales de transición contenidos en $PM_{10}$, debido a que XPA está estructurado por dedos de zinc (Hu et al. 2016) y se ha reportado que el níquel, zinc, vanadio y cobre pueden oxidar a los grupos tiol presentes en los dominios de dedos de zinc de distintas proteínas que comparten esta conformación (Hartwig et al. 2002; Kopera et al. 2004; Schwerdtle et al. 2010b). Además, las especies reactivas de oxígeno producidas por la exposición a $PM_{10}$ (Jan et al. 2020) podrían alterar la actividad y estabilidad de esta proteína. Por otra parte, se ha descrito que las modificaciones postraduccionales, principalmente la desacetilación y la fosforilación, son necesarias para el adecuado funcionamiento de XPA (Fan and Luo 2010; Sugitani et al. 2016; Wu et al. 2006). Es importante destacar que las $PM_{10}$ disminuyeron la fosforilación de XPA, lo que sugiere que las $PM_{10}$ alteran el funcionamiento de la proteína XPA, debido a que XPA desfosforilada pierde su capacidad de interactuar con otras proteínas (Shell et al. 2009b; Wu et al. 2006). De hecho, la alteración en la fosforilación puede conducir a una disminución en el nivel de XPA, porque el XPA desfosforilado es un sustrato para la ubiquitinación de HERC2 y su degradación por vía proteasoma (Lee et al. 2012; Shell et al. 2009b).

La disminución de fosforilación de XPA en serina 196 observada en este estudio podría estar asociada con el incremento de la proteína WIP1 inducida por la exposición a $PM_{10}$, debido a que WIP1 es una fosfatasa de residuos serina/treonina de distintas proteínas de reparación incluyendo a XPA (Nguyen et al. 2010a). Además, en las células expuestas a BaP no se encontraron alteraciones ni en los niveles de fosforilación de XPA ni en los niveles de WIP1. Debido a que WIP1 incrementó solo en presencia de las $PM_{10}$, sugerimos que los metales presentes en las $PM_{10}$ (Hadei et al. 2020) podrían ser inductores de esta proteína dado que la actividad de las fosfatasas está regulada por magnesio y manganeso (Shi 2009; Tanoue et al. 2013) y las $PM_{10}$ contienen estos metales (Chirino et al. 2017; Dergham et al. 2012; Hadei et al. 2020). Es relevante señalar que en otros modelos, las células con altos niveles de WIP1 presentan una reducción en la cinética de reparación mediante NER (Fiscella et al. 1997; Nguyen et al. 2010a) y la sobreexpresión de WIP1 ha sido reportada en tumores de pulmón (Bai et al. 2018; Zhao et al. 2016). Adicionalmente. proponemos que la desfosforilación de XPA inducida por las $PM_{10}$ modifica su capacidad para interactuar con la proteína RPA, porque este complejo no se detectó en las células expuestas a $PM_{10}$. Dado que el complejo XPA-RPA promueve el reclutamiento y la unión de proteínas que participan en la última etapa de la reparación como ERCC1-XPF

(Krasikova et al. 2018; Topolska-Wos et al. 2020; Yang et al. 2002), hipotetizamos que en las células expuestas a $PM_{10}$ los aductos de DNA no son removidos adecuadamente, dado que durante el paso de verificación de la vía de NER no se forma el complejo XPA-RPA, que está encargado de reclutar proteínas del último paso de la reparación del DNA.

Considerando la persistencia de aductos de DNA en células expuestas a $PM_{10}$ y las alteraciones en XPA, una de las principales proteínas de la vía NER, decidimos medir la actividad de la vía NER. A través de este análisis, confirmamos que, a pesar del reconocimiento del daño, el funcionamiento de la vía NER se inhibió después de la exposición a $PM_{10}$. Por el contrario, las células expuestas a BaP mostraron un aumento en la actividad de la vía NER, lo que es consistente con la disminución en la concentración de aductos de BPDE encontrada a las 48 h. Por lo tanto, el uso de BaP como control positivo de daño genotóxico nos permitió determinar que la vía NER está activa en las células A549. La inadecuada capacidad para eliminar el daño del DNA en las células expuestas a $PM_{10}$ indica que las alteraciones en los niveles de XPA y en la fosforilación reducen la respuesta de la vía NER, lo que destaca la probable participación de WIP1 en la inhibición de la reparación del DNA, hallazgos que deben confirmarse más adelante. BaP, un componente cancerígeno de $PM_{10}$, no tuvo ningún efecto sobre la actividad de la vía NER, lo que destaca que los efectos sobre la actividad de reparación del DNA podrían ser el resultado de los efectos sinérgicos de todos los componentes de $PM_{10}$ (Lepers et al. 2014b) que generan una inhibición del funcionamiento de la vía de NER y puede predisponer a las células a la acumulación de daño el DNA (Figura 16).

Por otro lado, es probable que la vía de NER no sea la única vía de reparación del DNA inhibida por la exposición a $PM_{10}$. Existe clara evidencia de los diferentes tipos de daño en el DNA inducido por $PM_{10}$, incluida la detección de procesos oxidantes (8-hidroxi-2-desoxiguanosina), rompimientos de las cadenas de DNA y la formación de focos γH2AX (Calderon-Garciduenas et al. 2020; Chirino et al. 2010; Sanchez-Perez et al. 2009); además, se ha descrito la formación de micronúcleos (Santibanez-Andrade et al. 2021), que es considerada como evidencia indirecta del daño en DNA y de su probable acumulación. Hasta el momento se han evaluado algunas alteraciones en las proteínas involucradas en las vías de reparación del DNA indicando la desregulación de proteínas de la vía BER, la recombinación homóloga (HR) y las vías de unión de extremos no homóloga (NHEJ) sugieren un posible deterioro en la reparación global del DNA después de la exposición a $PM_{10}$ (Quezada-Maldonado et al. 2021). Es relevante señalar que si bien todas las vías de

reparación del DNA protegen al genoma, la vía de NER es una de las vías con mayor implicación en la carcinogénesis, mientras que otras vías como BER tienen un impacto muy bajo en el desarrollo de cáncer (Yoshioka et al. 2021), lo que destaca la importancia de conocer los efectos de las $PM_{10}$ sobre la vía de NER.

Aunque se necesitan estudios adicionales para evaluar el impacto final de las evidencias obtenidas en este trabajo, los resultados sugieren que las $PM_{10}$ comprometen la estabilidad del genoma de las células de pulmón al causar alteraciones en la vía de NER, comprometiendo la reparación del DNA y así predisponer a las células a la generación de procesos carcinogénicos atribuido a la acumulación de lesiones voluminosas. No descartamos que la acumulación de daño en el DNA, específicamente de aductos de HAP, inducida por la exposición a $PM_{10}$ pueda dar lugar a mutaciones que aún no han sido detectadas en los diferentes estudios con PM.

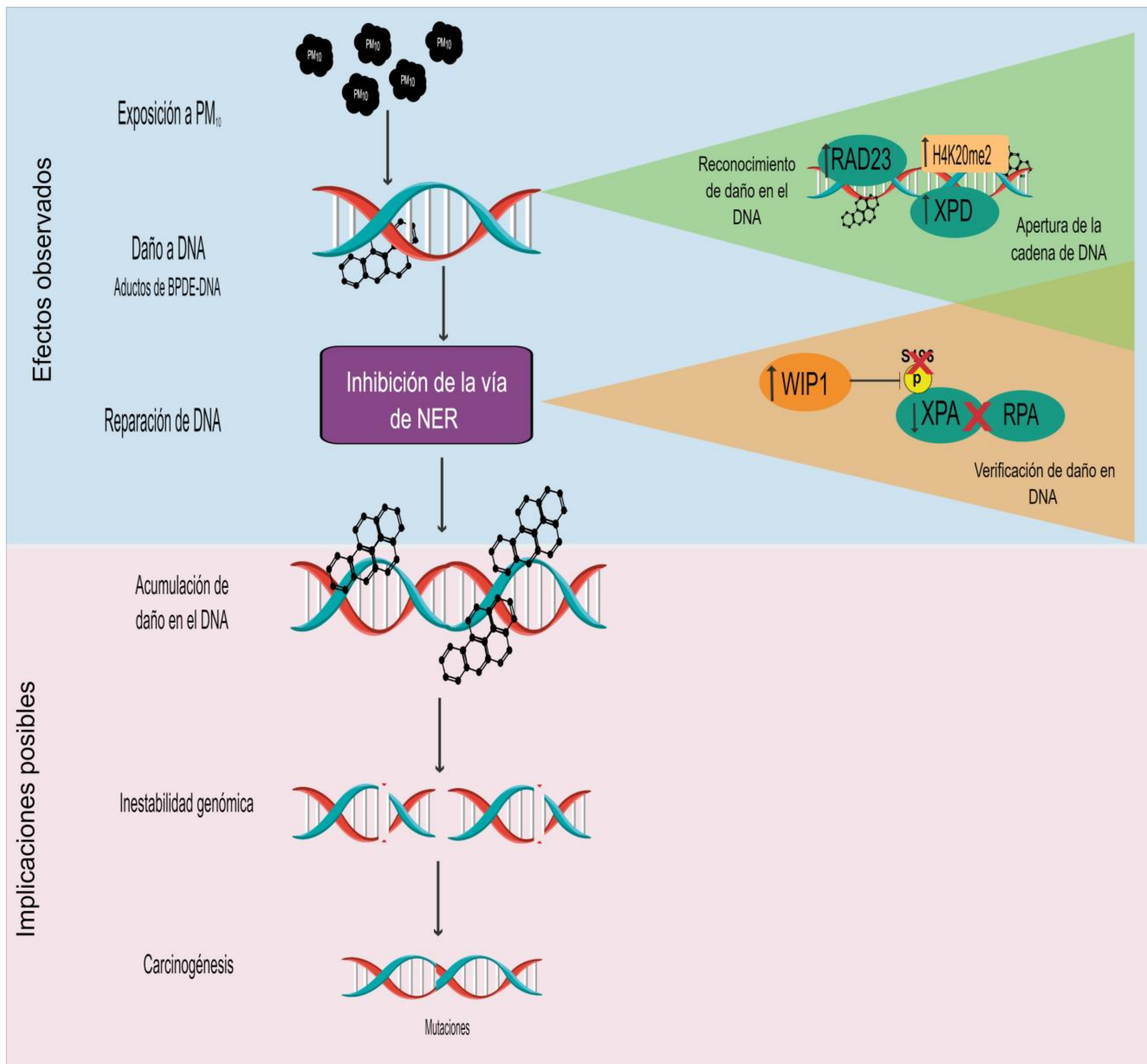


***Figura 16. Representación esquemática de los posibles efectos de la exposición a $PM_{10}$ asociados a la inhibición de la actividad de la reparación del DNA por la vía de NER.*** En este trabajo se observó que las $PM_{10}$ inducen daño en el DNA a través de la formación de aductos de HAP, este daño es reconocido por RAD23, lo cual induce la actividad de las proteínas XPD y H4K2me2. Sin embargo, la eliminación de estos aductos se inhibe como resultado de la disminución del nivel de XPA, así como su desfosforilación en el residuo de serina 196, lo cual se asocia al incremento de WIP1. La alteración de XPA conlleva a la inhibición de la formación del complejo entre XPA y RPA lo que podría modificar el paso final de la reparación del daño. Por lo tanto, sugerimos que la alteración en el funcionamiento de la vía de NER predispone a las células a la acumulación de daño en el DNA y contribuye a la generación de inestabilidad genómica, lo cual a su vez podría llevar a la aparición de mutación y a un proceso carcinogénico mediado por las $PM_{10}$.

## 9. Conclusión:

La exposición a $PM_{10}$ induce la formación de aductos de BPDE en el DNA, los cuales son reconocidos por RAD23, sin embargo, las $PM_{10}$ desregulan el paso de verificación del daño a través de la disminución y desfosforilación de XPA en serina 196, lo que inhibe la formación del complejo proteico con RPA, dando como resultado la no inducción de la actividad de la vía de NER en las células A549. Estos hallazgos proporcionan evidencia del deterioro de la actividad de la vía NER y la probable acumulación de DNA dañado, mecanismos que podrían explicar el potencial carcinogénico de las aeropartículas, contribuyendo al entendimiento de las $PM_{10}$ como un factor de riesgo para el desarrollo de cáncer de pulmón.

## 10. Perspectivas:

-Determinar la participación de los metales presentes en las $PM_{10}$ como los principales inhibidores de la actividad de la vía de NER a través del uso de quelantes de metales previos a la exposición.

-Comprobar el efecto de la fosfatasa WIP1 sobre la pérdida de fosforilación de XPA y analizar si el bloqueo de WIP1 incrementa la actividad de la vía de NER aún en presencia de $PM_{10}$.

-Realizar experimentos en los que se compruebe la inhibición del funcionamiento de la vía de NER en las células expuestas a $PM_{10}$ a través del tratamiento con compuestos genotóxicos que induzcan aductos del DNA (BaP, luz UV) posterior a las $PM_{10}$.

-Profundizar en el estudio de los tipos de daño en el DNA producidos por las PM, así como sus efectos sobre las distintas vías de reparación del DNA que podría dilucidar diferentes mecanismos a través de los cuales las PM ejercen su efecto carcinogénico.

-Es importante entender el impacto del material particulado sobre las distintas vías de reparación del DNA para poder dilucidar otros de los mecanismos a través de los cuales el material particulado ejerce su efecto carcinogénico.

-Son necesarios los estudios que permitan entender los efectos del material particulado en los diferentes mecanismos celulares asociados con el desarrollo de cáncer, para contribuir con evidencia toxicológica que pueda ser elemento de difusión en los foros de políticas en salud ambiental.

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## 12. Anexo Artículos publicados

- Quezada-Maldonado EM, Sanchez-Perez Y, Chirino YI, Garcia-Cuellar CM. 2021. Airborne particulate matter induces oxidative damage, DNA adduct formation and alterations in DNA repair pathways. Environ Pollut 287:117313. Review.

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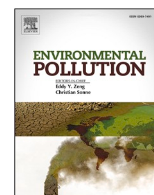

Review

# Airborne particulate matter induces oxidative damage, DNA adduct formation and alterations in DNA repair pathways☆

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ABSTRACT

Air pollution, which includes particulate matter (PM), is classified in group 1 as a carcinogen to humans by the International Agency for Research in Cancer. Specifically, PM exposure has been associated with lung cancer in patients living in highly polluted cities. The precise mechanism by which PM is linked to cancer has not been completely described, and the genotoxicity induced by PM exposure plays a relevant role in cell damage. In this review, we aimed to analyze the types of DNA damage and alterations in DNA repair pathways induced by PM exposure, from both epidemiological and toxicological studies, to comprehend the contribution of PM exposure to carcinogenesis. Scientific evidence supports that PM exposure mainly causes oxidative stress by reactive oxygen species (ROS) and the formation of DNA adducts, specifically by polycyclic aromatic hydrocarbons (PAH). PM exposure also induces double-strand breaks (DSBs) and deregulates the expression of some proteins in DNA repair pathways, precisely, base and nucleotide excision repairs and homologous repair. Furthermore, specific polymorphisms of DNA repair genes could lead to an adverse response in subjects exposed to PM. Nevertheless, information about the effects of PM on DNA repair pathways is still limited, and it has not been possible to conclude which pathways are the most affected by exposure to PM or if DNA damage is repaired properly. Therefore, deepening the study of genotoxic damage and alterations of DNA repair pathways is needed for a more precise understanding of the carcinogenic mechanism of PM.

## 1. Introduction

World Health Organization (WHO) estimates that air pollution causes approximately 4.2 million premature deaths worldwide annually, and Particulate Matter (PM) is considered the main pollutant associated with the impact on population health (WHO, 2018). PM is defined as a complex mixture of organic, inorganic, and biological compounds. Endotoxins, bacteria, and pollen are some of the biological components of the PM, while inorganic components include nitrates, sulfates and transition metals such as iron, copper, zinc and vanadium. Elemental carbon, dioxins and polycyclic aromatic hydrocarbons (PAH), such as benzo(a)pyrene (BaP), benzo(b)fluoranthene and benzo(g)perylene, are included in the organic components of PM (Sugita et al., 2004; Chirino et al., 2015; Dominici et al., 2015; Morakinyo et al., 2016). PM is classified according to its aerodynamic diameter into $PM_{10}$, $PM_{2.5}$ and $PM_{0.1}$ ($\leq$10 μm, $\leq$2.5 μm and $\leq$0.1 μm respectively). These PM are considered inhalable fractions because their ability to enter the respiratory system and to reach deep regions, such as bronchioles and alveoli, causing adverse health effects (Gilmour et al., 2004; Huttunen et al., 2012; Turner et al., 2020).

Long-term exposure to PM is linked to an increase in mortality and the incidence of lung cancer (Pope et al., 2002; Badyda et al., 2016; Chen et al., 2016a; Consonni et al., 2018; Turner et al., 2020), and based on scientific evidence, International Agency for Research on Cancer (IARC)

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declared air pollution, including PM, as a carcinogen to humans (Loomis et al., 2014). To date, it is known that PM specifically induces biological effects, including inflammatory processes, oxidative stress and genotoxicity (Sanchez-Perez et al., 2009; Li et al., 2018; Shao et al., 2018), which might commence with direct or indirect damage to the DNA, and this process is considered an initial event in the course of carcinogenesis leading to changes in gene expression but also mutations if the DNA is not repaired correctly (Hoeijmakers, 2001). Although a mutational footprint associated with PM exposure has not currently been described, some toxicological studies based on Ames tests, which determine possible mutagenesis in bacterial models, have shown that PM can induce mutations; additionally, in vitro models have demonstrated that PM generates genomic instability (Saint-Georges et al., 2009; Feretti et al., 2019; Santibanez-Andrade et al., 2019; Velali et al., 2019), suggesting that DNA damage is not repaired correctly. Importantly, deficiencies in the DNA repair pathways predispose individuals to genomic instability, which can increase or accelerate the acquisition of mutations, mostly if DNA damage persists and accumulates (Hanahan and Weinberg, 2011).

In this review, we aimed to identify the types of DNA damage induced by airborne particulate matter exposure as well as the effects of $PM_{10\text{-}0.1}$ exposure on DNA repair pathways. In addition, we identified polymorphisms in genes of DNA repair pathways that could influence the elimination of genotoxic damage generated by PM.

### *1.1. Types of DNA damage*

DNA damage refers to different chemical or structural alterations of the DNA strands that modify the functionality of the encoded genes but also to the altered tridimensional structure. **Oxidative DNA damage** is generated by the imbalance between pro-oxidant molecules and the antioxidant defenses of cells. Interactions between reactive oxygen species (ROS) and DNA can lead to the formation of oxidized DNA bases, such as 8-oxoguanine (8-OxoG) adducts and 8-hydroxy-2-deoxyguanosine (8-oxodG) lesions, which are the main effects of oxidative damage. However, 8-oxodG is the best characterized oxidative DNA damage because it is highly mutagenic since it can induce G-T transversions, which are among the most frequent mutations in human cancers (Klungland et al., 1999; Pilger and Rudiger, 2006; Hoeijmakers, 2009). Metals are considered the highest mediators of oxidative stress by Fenton reactions (Valko et al., 2016); however, it has been described that compounds derived from the metabolism of PAH also generate oxidative damage (Bolton and Dunlap, 2017).

**DNA adducts** are formed by the covalent attachment of a chemical compound to a DNA base (Rajalakshmi et al., 2015). The most common bulky adducts are those produced by PAH, specifically by BaP, which is activated through the enzymatic system of cells (CYP450), and benzo [a] pyrene diol epoxide (BPDE) metabolites are generated, forming adducts in the N2 position of guanine; however, other PAH, nitroaromatic compounds, aromatic amines, and chemotherapeutic agents can induce DNA adducts.

**Double-strand DNA breaks (DSBs)** are complex lesions considered the most serious type of DNA damage that can be generated because of direct exposure to chemical or physical agents or that can also occur because of the unusual secondary structures of DNA caused by oxidative or bulky lesions, abasic sites, polymerase blockages, or crosslinks that were not repaired. DSBs can induce genomic instability by translocation and loss of genetic material or inactivate essential genes (Mehta and Haber, 2014). If it is necessary to delve into some of these topics, we recommend consulting the following reviews (Khanna and Jackson, 2001; Poirier, 2004; Sancar et al., 2004; van Attikum and Gasser, 2005; David et al., 2007; Marteijn et al., 2014).

### *1.2. DNA repair pathways*

Cells have a set of proteins involved in the maintenance of DNA that ensure the integrity of the transcribed genome. In general, each type of damage is repaired through a specialized DNA repair pathway, which consists of several proteins that form a cascade of three stages: damage recognition, activating of the repair machinery, and injury removal (Chatterjee and Walker, 2017).

**Excision repair pathways** that repair single-strand DNA damage include the base excision repair (**BER**) and nucleotide excision repair (**NER**) systems that operate through "cut and patch" mechanisms that excise and remove nucleotides that contain the alteration and subsequently incorporate the corresponding bases through nonreplicative DNA polymerases. BER eliminates nucleotide lesions up to a maximum of twelve bases and generally removes oxidative damage, alkylated nucleobase lesions and apurinic/apirimidine (AP) sites. Moreover, 8-Oxoguanin DNA glycosylase-1 (OGG1) and apurinic/apirimidine endonuclease (APE1) are the main proteins responsible for the functioning of the BER pathway (Krokan and Bjoras, 2013). It is important to note that inactivation of the *OGG1* gene has been documented in sporadic tumors associated with the prevalence and increase of oxidative damage (Nascimento et al., 2017). On the other hand, NER is a critical multistep process that involves more than 30 different proteins, including XPC, XPA, XPD, ERRC1 and RPA, for the recognition, verification and elimination of lesions that distort the helix as well as bulky adducts. In this way, approximately 25 nucleotides are cut around the lesion, which are then resynthesized by DNA polymerase (Volker et al., 2001; Hakem, 2008). Importantly, mutations in NER genes affect DNA damage repair functionality and increase susceptibility to cancer development (Hakem, 2008).

**Recombination repair pathways** include homologous recombination (**HR**) and nonhomologous end-joining (**NHEJ**) pathways. Failures in HR and/or NHEJ promote chromosomal aberrations and sister chromatid exchange (Chatterjee and Walker, 2017), and then pathways maintain genome stability (Cahill et al., 2006; Hakem, 2008). More than 90% of DSBs are repaired by NHEJ, which acts in the G1 phase of the cell cycle, and a template of DNA is not required. Proteins that participate in NHEJ include the Ku subunits, DNA–PKCs, XRCC4 and DNA ligase IV. Moreover, HR requires several proteins, such as ATM, XRCC3, RAD50 and RAD51, and operates at S or G2 phase due to the requirement for the sister chromatid as a template.

## 2. Method

A search for all existing published studies in English from 2000 to 2021 using PubMed, Scopus, Scielo and Google Scholar databases was used for this review using the search items "particulate matter", "PM", "$PM_{10}$", "$PM_{2.5}$", "$PM_{0.1}$"; "DNA damage", "DNA damage repair", "DNA repair", "DNA damage repair pathways", "repair mechanisms", "DNA adducts", "oxidative damage", "single strand breaks", and "double strand breaks"; and "BER", "NER", "HR", and "NHEJ".

## 3. Particulate matter leads to different types of DNA damage

### *3.1. Oxidative DNA damage*

#### *3.1.1. Reactive oxygen species (ROS)*

ROS induction has been extensively studied in relation to PM exposure and oxidative stress is considered a central contributor to PM-associated carcinogenesis (Pope and Dockery, 2006). PM exposure induces ROS generation by metals such as iron, copper, nickel and vanadium through Fenton reactions (Valavanidis et al., 2005) and through quinones derived from PAH metabolization by phase I enzymes (CYP450) (Bolton and Dunlap, 2017). $PM_{10\text{-}0.1}$ exposure is associated with high levels of ROS-mediated oxidization in several experimental models. The 8-oxoG adducts and 8-oxodG lesions are the best characterized oxidative DNA damage, which is possibly due to their mutagenic potential. Importantly, in human blood cells, a positive correlation has been found between 8-oxodG levels in lymphocyte DNA and an increase

of 10 μg/$m^3$ in $PM_{2.5}$ concentration levels in people living in central Copenhagen (Sorensen et al., 2003) as well as high levels of 8-oxodG in the DNA of lymphocytes from taxi–moto drivers continually exposed to ambient air pollution (Ayi Fanou et al., 2006). The presence of 8-oxodG in urine has been accepted as a biomarker of exposure to air pollution; in this sense, workers exposed to $PM_{2.5}$ containing high concentrations of metals showed high levels of 8-oxodG in the urine (Kim et al., 2004), and bus drivers of a downtown city characterized by high levels of air pollution showed a greater excretion of 8-oxodG in the urine compared to drivers of rural areas (Loft et al., 1999; Rossner et al., 2007). Additionally, an increase in 8-oxodG urinary excretion has been reported in taxi drivers in a highly polluted Taiwan city (Chuang et al., 2003). It was described that $PM_{0.1}$ causes oxidative stress at the systemic level, which was determined by the increase in oxidized purines measured as formamidopyrimidine DNA glycosylase (FPG) in mononuclear blood cells during controlled exposure to urban air particles (Brauner et al., 2007), as well as in inhabitants of cities with high air pollution in which the concentration of FPG was correlated with the abundance of $PM_{0.1}$ (Avogbe et al., 2005).

Inhalation exposure to $PM_{2.5}$ in mice leads to increased levels of 8-oxodG detected in the urine, which correlates with multiple pathological damage in different organs (Li et al., 2019). The instillation of $PM_{2.5}$ in rats, increases the levels of 8-oxodG in the lungs and causes hyperemia, inflammation and bronchial epithelial hyperplasia (Li et al., 2017). In addition, exposure to $PM_{2.5}$ generates oxidative stress (8-oxodG) in the lungs and kidneys of mice (de Oliveira et al., 2018), and in the hearts of rats (Zhao et al., 2019). In the same context, $PM_{2.5}$ induces 8-oxoG in mouse skin tissue (Piao et al., 2018).

These results demonstrated that exposure to high levels of $PM_{10\text{-}0.1}$ has systemic effects despite being a pollutant whose main route of exposure is inhalation; however, no comparison has been made between DNA damage in the respiratory tract and that of different tissues that may be altered.

$PM_{10}$ and $PM_{2.5}$ induce the formation of 8-oxoG adducts in A549 cells and in human HaCaT keratinocytes, respectively (Chirino et al., 2010; Zhen et al., 2019a, 2019b) and $PM_{2.5}$ induces the formation of 8-oxodG in different cell types, including BEAS-2B and 16HBE lung cells (Dergham et al., 2012; Niu et al., 2020). It has also been demonstrated that $PM_{0.1}$ exposure increases 8-oxodG levels in peripheral blood lymphocyte cultures (Bhargava et al., 2018) and induces oxidative stress in a murine macrophage cell line (Li et al., 2003).

However, in addition to its role as an oxidative stress generator, $PM_{10\text{-}0.1}$ exposure also promotes an imbalance in antioxidant enzymatic defense, which plays an important role in the elimination of ROS. For A549, Chirino et al. reported that $PM_{10}$ induces a significant decrease in the activity of superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione-S-transferase (GST) (Chirino et al., 2010), and the activity levels of these enzymes are depleted in peripheral blood lymphocytes exposed to $PM_{0.1}$ (Bhargava et al., 2018). A decrease in reduced glutathione (GSH) levels was observed in human bronchial epithelial cells (16 H BE) exposed to $PM_{2.5}$ and in RAW264.7 cells exposed to $PM_{0.1}$ (Li et al., 2003; Niu et al., 2020), which is an important nonenzymatic defense against ROS, and a cofactor of antioxidant enzymes such as GST.

$PM_{2.5}$ decreases the activity of GR and SOD directly in the organs of exposed mice, mainly in the lungs and liver and in the heart of exposed rats (de Oliveira et al., 2018; Zhao et al., 2019). Demonstration of impairment in antioxidant enzymes in human samples has not yet been performed, but it would be possible that the increase in oxidative markers seen in human samples is accompanied by a decrease in the enzymatic antioxidant response in targeted tissues.

The exposure of fibroblasts, calf thymus DNA and lung epithelial cells to soluble extracts derived from urban $PM_{10\text{-}2.5}$ (metal-rich extraction) induced a concentration-dependent formation of 8-oxodG (Karlsson et al., 2004; Shi et al., 2006; Andre et al., 2011). Trolox treatment, which is an antioxidant, prior to $PM_{10}$ exposure prevented the damage associated with ROS production in lung epithelial cells (Chirino et al., 2010). In addition, the use of deferoxamine, an iron chelator, before treatment with ambient particulate matter (unspecific size) prevented the formation of 8-oxodG in a rat lung epithelial cell line, which was associated with the prevention of hydroxyl radical generation (Knaapen et al., 2000). Organic extractable matter of PM (unspecific size) causes 8-oxodG lesions, a decrease in SOD activity, an increase in the DNA binding activity of nuclear erythroid factor 2 (NRF2), and deregulation of the expression of *HMOX* and *NQO1* (Abbas et al., 2011).

#### 3.1.2. Reactive nitrogen species (RNS)

Reactive nitrogen species (RNS) might also contribute to oxidative DNA damage. These species are derived from nitric oxide, which is produced by nitric oxide synthase (NOS). This enzyme catalyzes the production of nitric oxide using L-arginine as a substrate and has 3 isoforms, neuronal, inducible, and endothelial NOS. Neural nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutive enzymes, but the inducible isoform (iNOS) is responsible for large amounts of nitric oxide, which together with superoxide anion results in peroxynitrite formation. This species is highly oxidant and is produced by immune cells during the inflammatory response.

In this regard, exposure to $PM_{2.5}$ induces iNOS overexpression in macrophages accompanied by proinflammatory markers (Jayawardena et al., 2020; Kim et al., 2020; Rahmani et al., 2020). The overexpression of iNOS suggests increased nitric oxide production, which could lead to RNS being able also to cause DNA damage. Indeed, inhalation of air pollution induces pulmonary inflammation and increased peroxynitrite levels (Rao et al., 2018), and recently, the detection of proteins modified by peroxynitrite was detected in experimental models (Ziegler et al., 2020). An increase in RNS has also been detected in epithelial and lung cells exposed to diesel exhaust particles, which are also components of air pollutants (Manzo et al., 2012).

Peroxynitrite-mediated DNA damage has not been completely elucidated in particulate matter exposure experimental models, but activation of immune cells derived from inflammation induced by PM exposure causes peroxynitrite-induced DNA damage (Ahmed et al., 2020). In addition, peroxynitrite has also been detected in lung diseases such as obstructive lung diseases, suggesting DNA damage (Di Stefano et al., 2020) and suggesting possible DNA damage associated with RNS. However, the generation of peroxynitrite depends on superoxide anion formation, which could be produced by an increase in enzymatic sources such as phagocyte NADPH oxidase (Caceres et al., 2020). In contrast, nonphagocytic NADPH oxidase is a ubiquitous enzyme, and air pollutant exposure induces an impairment in its activity, decreasing physiological superoxide anion production (Du et al., 2013). The mitochondrial dysfunction induced by PM exposure has been well demonstrated and indeed can directly interact with mitochondrial components (Sharma et al., 2021), leading to an increase in superoxide anion (Caceres et al., 2020; Sotty et al., 2020) contributing not only to peroxynitrite formation but also to DNA damage.

The oxidative DNA damage induced by $PM_{10\text{-}0.1}$ and some of its components is mediated not only by the induction of ROS but also by the impairment of antioxidant defense, which includes antioxidant enzymes, including SOD, catalase, and GST, and nonenzymatic molecules, such as GSH. There is clear evidence that compounds such as metals can deregulate or inactivate some genes or proteins of the DNA repair pathways, which is, due to the inhibition of expression or oxidation of the active or catalytic sites specifically OGG1, XPA, Ape1, HSP70 and N-methylpurine-DNA glycosylase (Asmuss et al., 2000; McNeill et al., 2004; Bravard et al., 2006; Wang et al., 2006; Al Bakheet et al., 2013; Hsu et al., 2013); these findings suggest that ROS induced by exposure to $PM_{10\text{-}0.1}$ could also inactivate proteins involved in DNA repair, which might explain why $PM_{10\text{-}0.1}$ exposure has a large impact on DNA damage.

### 3.2. DNA adducts

Analysis of leukocytes from residents of polluted cities and from workers exposed to long-term traffic showed high levels of DNA adducts (Palli et al., 2001, 2008; Li et al., 2014). Gestational exposure to $PM_{10}$, $PM_{2.5}$ and PAH was significantly correlated with the presence of DNA bulky lesions in both the mother and newborn. Adducts have been found in the umbilical cord and in the blood of the mother and child, furthermore, in fetuses the presence of polymorphisms in *CYP1B1*, a gene that participates in PAH metabolism, is associated with higher levels of adducts (Jedrychowski et al., 2013; Maciel-Ruiz et al., 2019), which could confer greater susceptibility to the development of a carcinogenic process in later stages of life.

The genotoxicity of $PM_{10}$ can vary spatially and temporally, which was demonstrated in nonsmoker residents of Mexico City, and that presented significantly higher levels of DNA adducts in the dry season compared to the rainy season, because $PM_{10}$ concentrations decreased during the wet station (Garcia-Suastegui et al., 2011). Similar results have been obtained in cell models since $PM_{10}$ collected in winter induce higher levels of PAH-DNA adducts in comparison to samples collected in summer in hepatocellular carcinoma cells (HepG2) (Binkova et al., 2007b; Sevastyanova et al., 2007, 2008). On the other hand, $PM_{10}$ from an industrial zone induced twice the formation of DNA adducts compared to $PM_{10}$ obtained from a residential area of the same city (Topinka et al., 2000, 2011). These results show that measurement of DNA adduct levels can reflect the average exposure to PM and suggest that DNA damage is closely related to the intensity of air pollution and likely with PAH concentrations (Chen et al., 2016b).

Levels of PAH tend to be higher in PM of smaller aerodynamic diameter ($PM_{2.5}$), however, studies show that PM with a larger diameter ($PM_{10}$) also induce the formation of adducts. Lung epithelial A549 and BEAS-2B cells showed DNA PAH-Adducts after exposure to $PM_{10}$ and $PM_{2.5}$ associated with PAH bioactivation, which is reflected in increased expression of the CYP1A1 and CYP1B1 gene and protein activity after 24 h of exposure to $PM_{10}$ and 24, 48 and 72 h of exposure to $PM_{2.5}$ (Lepers et al., 2014a; Salcido-Neyoy et al., 2015). The formation of DNA adducts is partially attributable to PAH; however, other compounds such as inorganic elements also contribute to bulky adduct formation through the increase of CYP1A1 and CYP1B1 activity (Lepers et al., 2014a).

Cocultures of alveolar macrophages and epithelial cells (L132) exposed to organic compounds extracted from $PM_{2.5}$ showed formation of adducts in alveolar macrophages but not in epithelial cells, suggesting that alveolar macrophages can participate as a defense mechanism, phagocytizing $PM_{2.5}$, detoxifying of organic compounds, and preventing the formation of adducts in epithelial cells (Abbas et al., 2011). Nevertheless, low exposure to $PM_{2.5}$ is also associated with DNA adduct formation, as demonstrated by Rossner et al. in Prague inhabitants, who presented high levels of DNA blood adducts (Rossner et al., 2013b), despite living in a city with low levels of pollution, suggesting there is no threshold in relation to PM exposure and DNA damage.

### 3.3. Double-strand DNA breaks

$PM_{10}$ and $PM_{2.5}$ exposure induces double-strand breaks in the DNA indicated by increased phosphorylation of H2AX in serine 139 (γH2Ax) a biomarker of DSBs formation (Kuo and Yang, 2008). High levels of γH2Ax were found in the prefrontal white matter of young adults, who were exposed to elevated levels of $PM_{10}$-$PM_{2.5}$ (Calderon-Garciduenas et al., 2020). In addition, γH2Ax incremented in the lungs of mice exposed to $PM_{2.5}$ for a prolonged period and this effect was greater in mice with defects in the DNA repair protein XPC (de Oliveira Alves et al., 2020).

An increase in γH2Ax has been detected in A549 cells exposed to $PM_{10}$ and in human embryonic lung fibroblasts and BEAS-2B exposed to $PM_{2.5}$ as well as in peripheral blood lymphocytes treated with $PM_{0.1}$ (Sanchez-Perez et al., 2009; Rossner et al., 2014; Bhargava et al., 2018; Billet et al., 2018). Cells pretreated with Trolox before exposure to $PM_{10}$, prevented the appearance of breaks in the DNA strands. In addition, exposure to $PM_{2.5}$ in the presence of deferoxamine decreased the DNA damage, which was analyzed by comet assay, as well as, the use of a hydroxyl radical scavenger (5,5-dimethyl-1-pyrroline) that prevented DNA strand breaks induced by $PM_{2.5}$ (Knaapen et al., 2002; Healey et al., 2005; Shi et al., 2006; Sanchez-Perez et al., 2009).

Different studies support the hypothesis of the effect of organic components on genotoxicity, among which the study of Rossner et al. described that exposure of human embryonic lung fibroblasts to BaP and organic compounds extracted from $PM_{2.5}$ induced stable chromosomal aberrations as a result of DSBs (Rossner et al., 2014). Subsequent studies conducted with BEAS-2B cells showed that $PM_{2.5}$ increases DSBs (γH2Ax) according to the abundance of PAH, because $PM_{2.5}$ collected in autumn-winter induced more DSBs than $PM_{2.5}$ collected in spring-summer, and the concentration of organic compounds is higher in $PM_{2.5}$ of autumn-winter (Lepers et al., 2014b). PAH-derived quinones induce DSBs in lung epithelial A549 cells and overexpression of *CYP1A1*, *CYP1B1* and ROS (Shang et al., 2013). Organic PM (unspecific size) extracts generated *CYP1A1* overexpression, ROS production and DSBs generation in breast cancer MCF7 cells (Chen et al., 2013).

Because $PM_{10\text{-}2.5}$ induces multiple types of DNA damage, these alterations could occur simultaneously and at any site in the genome, predisposing patients to processes such as mutagenesis if the damage is not properly repaired, which would have a greater impact if tumor suppressor genes or oncogenes are affected. Together, with the demonstration of cell death evasion (Reyes-Zarate et al., 2016) and a "senescence-like state" without cell cycle arrest (Sanchez-Perez et al., 2014; Ryu et al., 2019), induction by $PM_{10}$ exposure in lung cells suggests that PM exposure might induce carcinogenesis by a permanent induction of DNA damage accompanied by an inappropriate scenario to be repaired.

Based on the above information, the DNA damage induced by $PM_{10\text{-}0.01}$ exposure might start with oxidative stress, and oxidative DNA damage could be the first type of DNA lesion formed and metals through Fenton reactions are the main contributors to this damage, while organic components are responsible for the formation of DNA adducts. However, massive DNA damage induced by oxidative DNA damage or by DNA adducts might occur together or independently cause DSBs.

## 4. Particulate matter exposure modifies DNA repair pathways

The elimination of different types of DNA damage follows a certain order based on the complexity of the injury, in general apurinic sites, single-stranded DNA breaks and small base distortions are repaired faster than some photoproducts, bulky adducts and DSBs (Kiwerska and Szyfter, 2019). However, in the case of $PM_{10\text{-}0.1}$ exposure, it would be difficult to determine which pathway is activated first or which type of damage is eliminated as a priority when the cells detect multiple DNA lesions produced at a similar time. Alterations in the functionality of DNA repair pathways can lead to the accumulation of genotoxic damage, alter cellular functions and predispose people to mutagenic and carcinogenic processes (Lengauer et al., 1997). Until now, it has been clearly demonstrated that PM exposure activates all DNA repair pathways, but failure in the systems or deregulation in their proteins can also be detected.

### 4.1. Alterations in excision repair pathways

Inhabitants of Mexico City, a city with high levels of air pollution, had in the olfactory bulb and in the frontal cortex a tendency to have decreased expression of *OGG1* and an increased expression of *XPA,* which belong to the BER and NER repair pathways, respectively. These deregulations correlated with the accumulation of nickel, chromium and arsenic in the frontal cortex (Calderon-Garciduenas et al., 2013). The decrease in *OGG1* expression could lead to failures in the BER repair

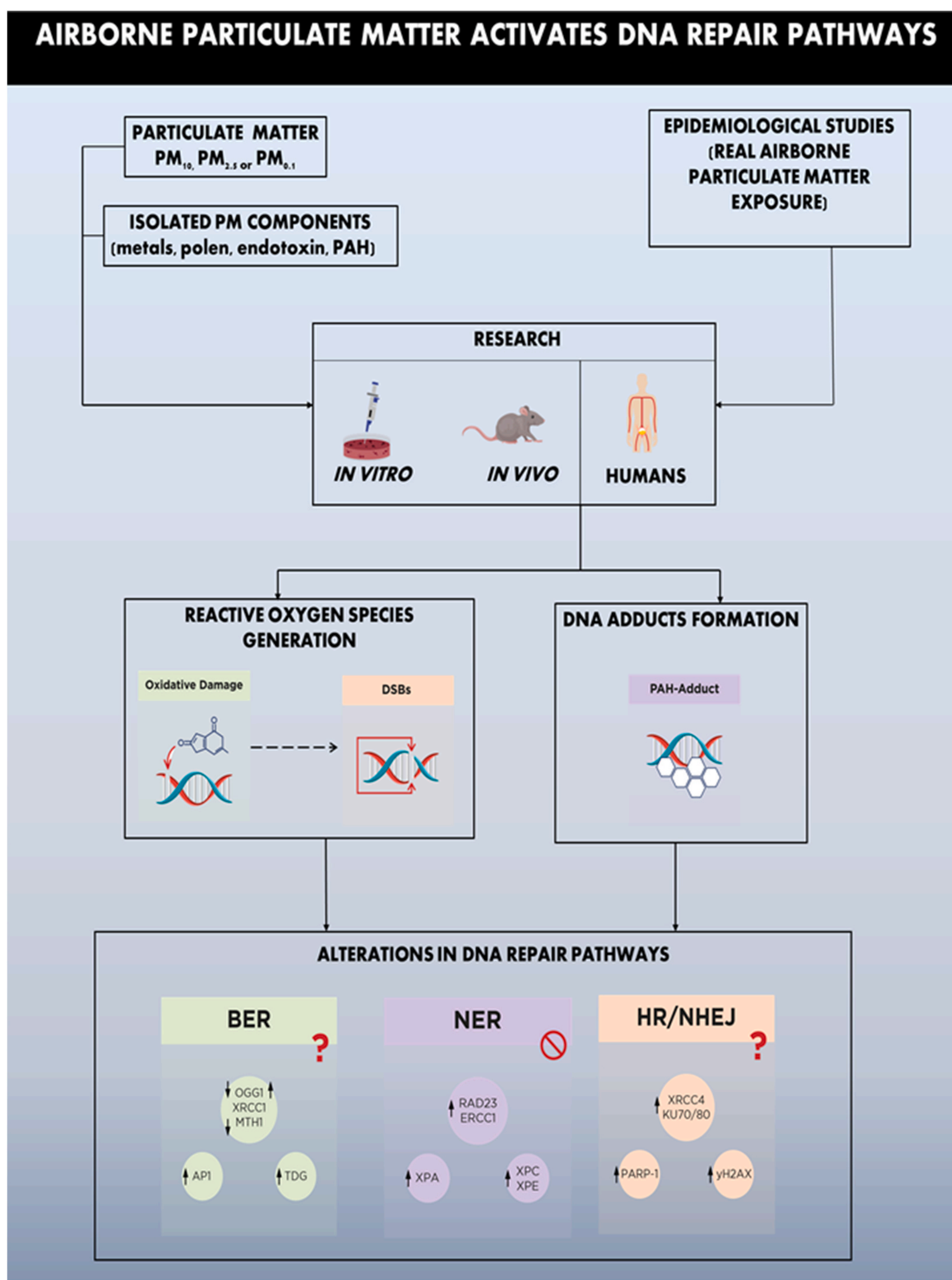


**Fig. 1. Airborne particulate matter activates DNA repair pathways.** PM sized ≤10 μm ($PM_{10}$), ≤2.5 μm ($PM_{2.5}$) and ≤0.1 μm ($PM_{0.1}$) are considered the inhalable fraction of air pollution. Research has been conducted not only using $PM_{10\text{-}0.1}$ but also the main components, including metals (iron, copper, zinc and vanadium), pollen, endotoxin (found in the membrane of Gram-negative bacteria) and polycyclic aromatic hydrocarbons (PAH) in experimental models (in vivo and in vitro). In addition, epidemiological studies have been conducted in humans living in highly polluted cities. Both, human and experimental studies have provided strong evidence that reactive oxygen species generation causes DNA damage, mainly by the oxidation of DNA. The formation to 8-hydroxy-2-deoxyguanosine is one of the most common DNA damages, and precedes double-strand breaks (DSBs) of DNA. In addition, PAH is also a common bulky adduct of DNA detected. There is a strong association between DNA damage and alterations in DNA repair pathways, and those are related to deregulation in the protein expression of components of base excision repair (BER), nucleotide excision repair (NER) and homologous recombination (HR) and nonhomologous end-joining (NHEJ) pathways. Together, there is robust evidence of the DNA damage induced by PM exposure and the alterations in DNA repair pathways in human and experimental models; however, the efficiency of DNA repairment and some biological effects are still under research. APE1: Human apurinic/apyrimidinic endonuclease 1; OGG1: 8-Oxoguanin DNA glycosylase-1; XRCC1: X-ray repair cross complementing group; MTH1: 7,8-dihydro-8-oxoguanine triphosphatase; TDG: Thymine DNA Glycosylase; XPA: Xeroderma pigmentosum complementation group A; RAD23: Homolog A, Nucleotide Excision Repair Protein; ERCC1: DNA excision repair protein ERCC-1; XPC: Xeroderma pigmentosum complementation group C; XPE: Xeroderma pigmentosum complementation group E; PARP-1: Poly (ADP-ribose) polymerase 1; XRCC4: DNA repair protein XRCC4; KU70/80: X-ray repair cross-complementing protein 6; H2AX: H2A histone family member X.

system, which in turn would be conducive to oxidative lesions generated by the high concentration of metals present in the PM. On the other hand, the increase in *XPA* expression could be indicative of the activation of the NER system in response to the presence of bulky lesions generated by PAH present in the $PM_{2.5}$.

$PM_{10}$ induced mRNA deregulation in 87 genes, including DNA repair genes, specifically *TDG* of the BER pathway and *RAD23A* and *ERCC1* of the NER pathway, in exposed human U937 macrophages (Bastonini et al., 2011). In both 16HBE cells and rat lungs, $PM_{2.5}$ increased oxidative damage, induced an increase in OGG1, and suppressed the expression of XRCC1 and MTH1 mRNA and protein levels (Li et al., 2017; Niu et al., 2020), which possibly generated alterations in the DNA repair process. Exposure to $PM_{2.5}$ inhibited the repair of damage generated by UV light and by BaP in lung epithelial A549 cells, which is carried out by the NER pathway. This repair failure was associated with the increased frequency of both spontaneous and UV-induced mutations (Mehta et al., 2008). Subsequent studies in human embryonic lung fibroblasts showed that organic compounds extracted from $PM_{2.5}$ induced the overexpression of *APE1* in the BER pathway and increased the expression of *XPC* and *XPE*, which are responsible for the recognition phase of the NER pathway; however, NER functionality was reduced (Rossner et al., 2013a; Traversi et al., 2015). These results suggest that $PM_{10\text{-}2.5}$ alters DNA repair capacity and therefore leads to the accumulation of damage both by oxidation and by the presence of DNA adducts, which could represent a problem in lung cells as well as in the microenvironmental cells of this organ. Therefore, it is important to delve into the effects that $PM_{10\text{-}0.1}$ can induce in the different repair pathways and to determine if PM exposure negatively influences DNA damage repair.

### 4.2. Alterations in double strand break repair

Demonstration of DBSs repair induced by $PM_{10}$ exposure was based on increased phosphorylation of H2AX (γH2Ax) and overexpression of 53BP1 protein, an important element for the repair of DSBs required for the accumulation of p53 at the site of damage, in addition to an increase in total and phosphorylated protein p53 (p53 in serine 15), indicating the activation of repair signals (Sanchez-Perez et al., 2009). In breast cancer cells (MCF-7), the exposure to organic solvent extracts from PM (total suspend particles) induced the activation of PARP-1, an enzyme that mediates the cellular response to DNA damage and contributes to the repair of DSBs (Chen et al., 2013). In BEAS-2B cells, exposure to $PM_{2.5}$ decreased RAD51 mRNA and protein levels (Liu et al., 2020), and an increase in XRCC4 and Ku70/80 protein levels was found in lung fibroblast cells after exposure to PM organic extracts and to BaP (Rossner et al., 2014). These findings suggest that cells exposed to $PM_{2.5}$ detect and respond to DNA breaks; however, it is important to note that in the study of Rossner et al., NHEJ repair activity was low (Rossner et al., 2014). In this sense, inadequate repair of DSBs can lead to effects such as aneuploidy or genomic instability and it is known that exposure to PM generates chromosomal instability (Santibanez-Andrade et al., 2019), which could then be associated with probable failures in the DNA repair pathways in cells exposed to PM. Until now, there has been clear evidence for different types of DNA damage followed by activation of DNA repair pathways. However, the whole PM or some components are also related to impairment in DNA damage repair by decreasing activity or by deregulation of gene expression (Fig. 1).

### 4.3. Exposure to individual components of PM affects DNA repair pathways

We compiled studies that evaluated the effect of at least one of the components present in PM, such as metals or PAH on DNA repair pathways. The results might be different from those studies using the entire PM; however, they have been useful to understand the contribution of each PM component.

Exposure to cadmium (II) and zinc (II) inactivated the DNA glycosylase activity of OGG1 and decreased the ability of cells to repair oxidative DNA damage in a murine model, while an in vitro analysis concluded that cadmium (II), lead (II) and iron (II) inhibited APE1 nuclease activity (Zharkov and Rosenquist, 2002; McNeill et al., 2004). In addition, nickel exposure prevented the removal of PAH-DNA adducts and increased the frequency of BPDE-induced mutations, specifically through base substitution in lung fibroblast cells (Hu et al., 2004). Similarly, cadmium decreased the repair capacity of HeLa cells to eliminate bulky DNA lesions caused by BaP (Mukherjee et al., 2004). These results suggest that transition metals have an impact on the proteins involved in NER repair and therefore affect the functionality of the pathway.

Several studies indicate that cadmium, copper, nickel, and cobalt, induce inactivation of XPA and PARP, which are essential proteins for excision repair, and zinc specifically alters the DNA binding capacity of XPA through disruption of the zinc finger domains, inducing structural loss and subsequent XPA inactivation. The structural change of XPA affects the interaction with other proteins of the NER system and decreases the ability of this protein to bind to DNA, which consequently reduces the repair of DNA damage (Asmuss et al., 2000; Kopera et al., 2004; Hu et al., 2016). According to these results, transition metals, in addition to inducing oxidative damage, have the potential to inhibit repair pathways, specifically NER, because zinc-finger proteins seem to display higher susceptibility to metal-induced damage; however, the exact mechanism is not known with certainty (Cooper et al., 2016; Huestis et al., 2016; Ding et al., 2017).

In the case of DSBs effects induced by individual PM components, it has been reported that heavy metals significantly inhibit the function of DSBs repair proteins. Occupational exposure to nickel was shown to reduce the expression of 29 repair genes (*XRCC5, CLK2, ATM, PRKDC, RAD50, PARP1, GTF2H3, RECQL, MRE11A, PRPF19, POLH, GTF2H1, MSH2, DDB1, UNG, PCNA, RPA2, PALB2, HLTF, DCLRE1A, TP53, APEX2, NHEJ1, FANCF, GTF2H4, RPA1, OGG1, MUS81 and DDB2*); therefore, it is suggested that metals could induce epigenetic changes leading to the simultaneous silencing of different repair genes (Arita et al., 2013).

Prolonged exposure to chromium (VI) negatively regulated the RAD51 protein and inhibited its nuclear import, preventing its function even in the presence of DNA damage. The inhibition of RAD51 induced by chromium, correlated with an increase in chromosomal aberrations, specifically with the presence of chromosomes and with the exchange of sister chromatids. The inhibition of RAD51 induced by chromium, correlated with an increase in chromosomal aberrations, specifically with the presence of dicentric chromosomes and the exchange of sister chromatids (Qin et al., 2014; Browning et al., 2016; Browning and Wise, 2017). In addition, in human endothelial cells (HMEC-1), the presence of cadmium favored the persistence of γH2AX foci and decreased the repair capacity of DSBs associated with the overactivation of the MRE11-dependent repair pathway (Viau et al., 2008).

Interestingly, in addition to the fact that it has been observed that some metals such as arsenic, can inhibit the HR repair pathway, it has also been shown that the concentration of metals can influence the way that cells repair damage, specifically DSBs. Low concentrations of nickel chloride promoted the resolution of DSBs through HC; however, higher concentrations of $NiCl_2$ inhibited HR but stimulated NHEJ pathway function (Morales et al., 2016). This finding clearly shows that cells have a limit to cope with DNA damage and over this limit, inhibition of DNA repair pathways might occur.

PAH also induces changes in DNA repair pathways, specifically, BaP exposure significantly reduces XPD helicase protein and expression levels in H1299 lung cancer cells. The decrease in XPD correlates with NER inhibition (Lin et al., 2016). Furthermore, BaP decreases the expression of ATM and Xrcc6 (Ku80) in the liver and lung of mice, as well as the expression of Xrcc5 (Ku70) and DNA-PKcs only in the lungs. Despite this decrease, it was determined that NHER increases; however, activation of this pathway associated with a decrease in repair genes

could result in a higher incidence of aberrant DNA repair (Tung et al., 2014).

Tetrachlorobenzo(p)dioxin (TCDD), a component of PM (Kouimtzis et al., 2002), negatively regulates the expression of some genes of different DNA repair pathways, including EXO1, FEN1, PCNA, POLE2, RAD51AP1, and RAD54L, which could lead to the inhibition of repair in a human prostatic carcinoma cell line (Hruba et al., 2011). In addition, TCDD, repress the expression of BRCA-1 in MCF-7 breast cancer cells. BRCA-1 belongs to the repair pathway of DSBs (Papoutsis et al., 2010).

Additionally, lipopolysaccharide (LPS), a bacterial endotoxin present in the PM, decreases BRCA levels in macrophages (Morrone et al., 2019), deregulates the levels of APE1 in the liver of mice (Kovalchuk et al., 2013) and decreases the pulmonary NER capacity of mice associated with the downregulation of XPC, XPA and XPF genes as well as of the XPA protein (Gungor et al., 2010).

The usage of individual components of PM has some limitations because exposure alone is not sufficient to explain the entire effects of PM; indeed, isolated effects of components might be the opposite of those observed in studies of PM exposure; however, these studies explain the contribution of some components of PM.

### *4.4. Gene polymorphisms present in repairing enzymes influence the response to toxic agents, including PM*

Polymorphisms in DNA damage repair genes influence repair, and it was described that bus drivers with variants of the *OGG1* (Cys/Cys) or *XPD23* (Gln/Gln) genes exposed to high levels of PAH have greater oxidative damage in lymphocyte DNA compared to subjects with the wild-type genotype (WT) of these genes. Furthermore, the presence of polymorphisms increased the susceptibility to presenting DSBs related to exposure to air pollution, probably because allelic variants reduce the functioning of the proteins they encode and modify the ability to eliminate DNA damage, which leads to the accumulation of oxidative stress that can eventually induce DNA breaks and mutations (Bagryantseva et al., 2010).

Polymorphisms in the genes responsible for metabolizing PAH, mainly *CYP1B1* and *GTSM1*, increased susceptibility to DNA damage in humans and more adducts in subjects exposed to $PM_{10}$ are detected compared to subjects with the wild-type genotype (Garcia-Suastegui et al., 2011). Polymorphs in *ERCC2/XPD,* one of the main effectors of the NER pathway, increased the levels of bulky adducts after exposure to $PM_{2.5}$ (Binkova et al., 2007a). In addition, the *ERCC2* variant (Lys751Gln) induced a greater presence of sister chromatid exchanges in subjects exposed to air pollution (Zijno et al., 2006), and the variation in exon 23 of the *XPD* gene increased the frequency of micronuclei in workers exposed to PM (unspecific size) because DNA repair enzyme activity was altered (Sellappa et al., 2010). In the same way, a positive association was described between exposure to PAH derived for traffic emission and the incidence of breast cancer in women with DNA repair gene polymorphisms, specifically in *ERCC2* (Lys751), *XRCC1* (Arg194Trp) and *OGG1* (Ser326Cys). The variations described in these genes affect the repair activity and, consequently, the elimination of lesions derived from exposure to PAH (Mordukhovich et al., 2016). The study by Etamadi et al. found an association between ERCC5 polymorphisms and DNA adduct levels in which NER capacity was affected by ERCC1 gene polymorphisms in women exposed to PAH (Etemadi et al., 2013).

## 5. Discussion

Although $PM_{10\text{-}0.1}$ exposure causes oxidative DNA damage, DNA adduct formation and DSBs, in some cases, PM deregulates the gene expression of DNA repair pathways; however, it has not been demonstrated that exposure to PM leads to the appearance of mutations, probably because the experimental strategies used have not allowed the detection of mutations since they would require longer exposure systems. Activation of DNA damage repair pathways prevents the appearance of mutations by preventing the accumulation of damage in the genome; however, in cells exposed to PM, the balance between DNA damage and repair capacity has not yet been completely described.

The scientific evidence of DNA damage induced by $PM_{10\text{-}0.1}$ exposure performed in cell cultures and animal models is associated with the DNA damage found mainly in urine and blood of citizens living in highly PM polluted cities, which highlights the usefulness of experimental models as a predictive model of PM exposure effects in humans, specifically since the respiratory tract is the main target of PM inhalation, and studies using samples derived from this system would be an invasive method with ethical complications. However, one of the strongest limitations of experimental models in which no mutations are detected, is the lack of a combination of risk factors to which citizens from highly polluted cities are exposed. For instance, smoking, alcohol consumption, polymorphisms, and even subjacent diseases together might explain the association between PM exposure and risk for lung cancer.

In addition, 8-oxo-G lesions and PAH-DNA adducts are the most frequent type of damage detected, perhaps because laboratory methods are well established. However, there are other types of oxidative and bulky DNA lesions, such as thymine glycol, abasic sites and exocyclic DNA adducts formed from a predominant product of lipid peroxidation, which are probably generated by $PM_{10\text{-}0.01}$ but have not been analyzed. Although there are different techniques, such as liquid chromatography-tandem mass spectrometry, monoclonal antibodies, biotin-containing aldehyde-reactive probes (ARPs) and nanoflow LC–nanospray ionization tandem mass spectrometry, to measure these types of DNA damage (Atamna et al., 2000; Bailey et al., 2006; Chen and Lin, 2011), they have probably not been applied for research on the effects of PM.

Evaluation of DNA repair pathways has been performed in experimental models and humans, but epidemiological studies are still missing. In addition, most of the studies identify DNA damage markers and proteins from DNA repair pathways, but whether DNA damage is correctly and completely repaired has not been defined. There is also no information yet on which DNA repair pathway is the most susceptible to PM exposure. Until now, we know that excision repair pathways are the first line of defense against oxidative damage and adduct formation, while HR and NEHJ respond to DSBs; however, there is not enough evidence to conclude which pathway is most altered by exposure to PM or if DNA damage is being efficiently repaired. Thus, there is still a gap in the knowledge of DNA damage, DNA repair and carcinogenesis linked to PM exposure; therefore, studies should be designed to define this phenomenon, which would expand the information on the role of PM in DNA damage generation and repair related to carcinogenic processes.

In this context, methodologies for DNA damage and DNA repair detection have limitations that need to be solved to move ahead with other mechanisms that could be involved in the carcinogenesis associated with PM exposure. Among those mechanisms, epigenetic, post-transcriptional and posttranslational regulation is related to DNA repair, and genetic polymorphisms in DNA repair genes need deeper analysis since they might provide evidence of PM exposure and carcinogenesis. Something less explored is a detailed comparison among PM exposure effects on cell linages. The rate of cell division may have an impact on the damage induced by PM exposure. For instance, unrepaired DNA damage induced by PM exposure might have less risk in cells with a low rate of cell division, such as neurons, than in epithelial cells.

Furthermore, some studies have revealed that specific PM components induce DNA damage, while others impair DNA damage repair, suggesting that the entire PM particle may synergize with these effects. For this reason, studies performed with complete PM particles and isolated components are complementary. However, further comparisons among single components and the entire PM using proper concentrations and doses and avoiding unrealistic models of exposure are necessary to elucidate how DNA damage and failure in DNA repair pathways are linked to carcinogenesis.

Based on the above information, a detailed detection of DNA damage

**Table 1**
DNA damage induced by particulate matter in different organs and its association with diseases.

| Airborne particulate matter (PM) with an aerodynamic diameter ≤10 or ≤2.5 or ≤0.1 μm ($PM_{10}$ or $PM_{2.5}$ or $PM_{0.1}$ [a]) | Organ | Direct evidence of DNA damage | Indirect evidence of DNA damage | Associated disease | Reference |
|---|---|---|---|---|---|
| $PM_{10}$ | Human lung | Non demonstrated | Micronuclei formation | Chronic obstructive pulmonary disease | Wunnapuk et al. (2019) |
| | Liver, kidney and heart in rat model | Increased index of DNA tail length | No data | Cancer and metabolic disorders | Busso et al. (2016) |
| | Brain in rat model | Non demonstrated | No data | Neurodegeneration | Guo et al. (2012) |
| $PM_{2.5}$ | Lung | Phosphorylated form of H2AX (γH2AX) | No data | Cancer | de Oliveira Alves et al., 2020 |
| | Lung, brain, heart, testis, and intestine in mouse model | Increased index of DNA tail length 8-hydroxy-2-deoxy-guanosine in urine | Micronuclei formation in periphery blood cells | Inflammatory disease pathologies Cardiovascular Neurodegeneration | Li et al. (2019) |
| | Lung and kidney in mouse model | 8-hydroxy-2-deoxyguanosine | No data | Cancer | de Oliveira et al. (2018) |
| | Lung in rat model | Increased index of DNA tail length 8-hydroxy-2-deoxyguanosine | DNA repair proteins deregulation (MTH1, XRCC1) | Cancer | Li et al. (2017) |
| | Liver in mouse model | Non demonstrated | Increase of malondialdehyde levels | Hepatic disease | Ge et al. (2020) |
| | Heart in rat model | 8-hydroxy-2-deoxyguanosine and DNA strand breaks | No data | Cardiovascular | Zhao et al. (2019) |
| | Human brain and brain in mouse model | Phosphorylated form of H2AX (γH2AX) | No data | Alzheimer | Calderón-Garcidueñas et al., 2020 |
| | Human brain | Non demonstrated | DNA repair genes deregulation | Neurodegeneration | Calderón-Garcidueñas et al., 2013 |
| | Brain in mouse model | Non demonstrated | Micronuclei formation | Neurodegeneration | Di Domenico et al. (2020) |
| | Brain in rat model | Non demonstrated | Increase of malondialdehyde levels | Neurodegeneration | Fagundes et al. (2015) |

[a] No evidence was found of DNA damage induce in a specific organ by $PM_{0.1}$; H2AX, H2A histone family member X; MTH1, Human MutT Homolog 1; X-ray repair cross-complementing protein 1, XRCC1.

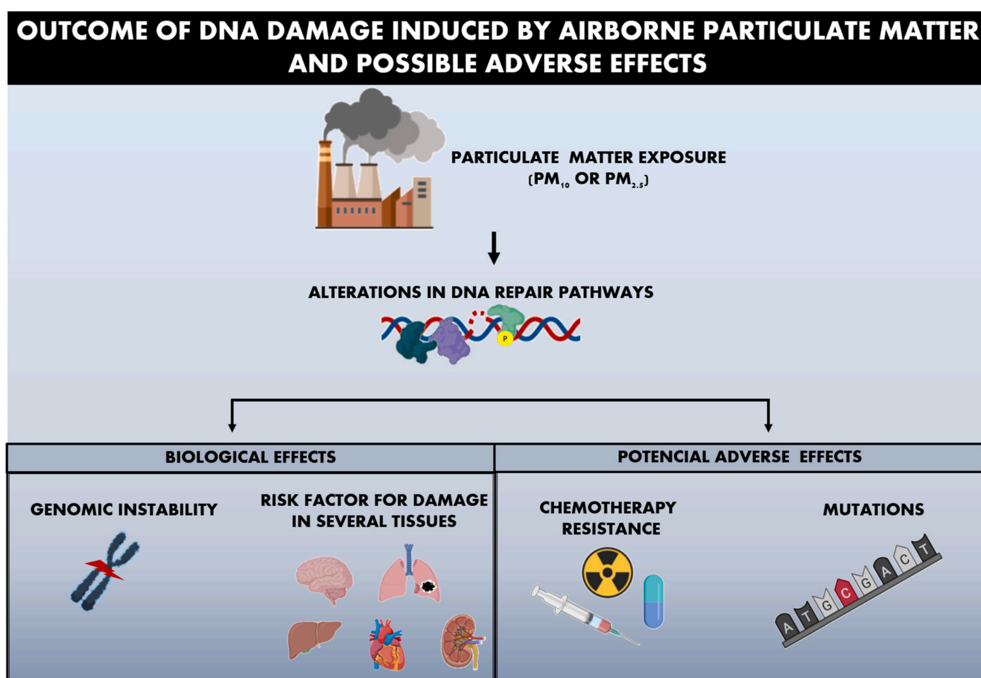


**Fig. 2. Outcome of DNA damage induced by airborne particulate matter and possible adverse effects.** The source of airborne particulate matter in highly polluted cities is mainly the anthropogenic activity, which includes the burning of fossil fuels, road traffic, and industry, but also, might contain biological particles such as pollen and endotoxin. PM sized ≤10 μm ($PM_{10}$), ≤2.5 μm ($PM_{2.5}$) and ≤0.1 μm ($PM_{0.1}$) have been the subject of intense studies and currently, air pollution is a carcinogen to humans according to International Agency for Research on Cancer. The exact mechanism by which air pollution is associated with lung cancer is unclear. The $PM_{10\text{-}2.5}$ exposure induce alterations in DNA repair pathways, and genomic instability is a well-demonstrated effect besides to be considered as a risk factor for damage in several tissues including the lung, brain, liver, heart and kidney; however, there are other potential effects. Since DNA repair pathways are probably deregulated in highly exposed subjects in polluted cities, the failure of antineoplastic treatments might be an additional adverse effect for cancer patients. Mutations might be another adverse effect that has not been reported until now, but precise alterations in the DNA sequence induced by $PM_{10\text{-}2.5}$ exposure are still to be unveiled.

and the alterations in DNA repair pathways induced by PM exposure, together with more complex and longer experimental models and studies in humans, will help to fill the knowledge gap regarding whether PM inhalation is classified as a carcinogen to humans.

## 6. Conclusions

Regardless of the in vitro, animal model or epidemiological study, city of PM collection and aerodynamic size of PM, the induction of multiple types of DNA damage is conclusive, and oxidative stress, DNA adducts and DSBs are footprints of PM exposure. Among the types of DNA damage, 8-oxodG has been the most reported in PM exposure, first detected in experimental models, then in vivo and more recently, used as a marker of oxidative stress in human samples. Furthermore, 8-oxodG might not be the only main type of damage generated by PM but also, one of the easiest in terms of methodological tools available to quantify this damage, compared to the measurement of other DNA lesions, such as adducts and DSBs. In this regard, the measurement of DSBs could be described more precisely using techniques such as DNA double-strand break sequencing, DNA break immunocapture or breaks labeling, enrichment on streptavidin and next-generation sequencing. The evidence suggests an accumulation of damage in different organs (Table 1); however, it has not been possible to conclude which DNA repair pathway is most affected by exposure to PM, because studies are still limited; hence, DNA repair deficiency would generate multiple effects at the cellular and systemic levels (Fig. 2). DNA damage, the unrepaired DNA, polymorphisms in some genes related to detoxification, together with other mechanisms such as apoptosis evasion might contribuite to the generation of genomic alterations, which in the long term could lead to the development of various diseases including cancer.

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## Author contributions

Conceptualization, E.M.Q.-M., Y.S.-P; writing-original draft preparation, E.M.Q-M., Y.S.-P; writing—review and editing, E.M.Q.-M., Y.S.-P., Y.I.C. and C.M.G.-C. All authors have read and agreed to the published version of the manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Nucleotide Excision Repair Pathway Activity Is Inhibited by Airborne Particulate Matter ($PM_{10}$) through XPA Deregulation in Lung Epithelial Cells

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**Abstract:** Airborne particulate matter with a diameter size of ≤10 μm ($PM_{10}$) is a carcinogen that contains polycyclic aromatic hydrocarbons (PAH), which form PAH–DNA adducts. However, the way in which these adducts are managed by DNA repair pathways in cells exposed to $PM_{10}$ has been partially described. We evaluated the effect of $PM_{10}$ on nucleotide excision repair (NER) activity and on the levels of different proteins of this pathway that eliminate bulky DNA adducts. Our results showed that human lung epithelial cells (A549) exposed to 10 μg/cm$^2$ of $PM_{10}$ exhibited PAH–DNA adducts as well as an increase in RAD23 and XPD protein levels (first responders in NER). In addition, $PM_{10}$ increased the levels of H4K20me2, a recruitment signal for XPA. However, we observed a decrease in total and phosphorylated XPA (Ser196) and an increase in phosphatase WIP1, aside from the absence of XPA–RPA complex, which participates in DNA-damage removal. Additionally, an NER activity assay demonstrated inhibition of the NER functionality in cells exposed to $PM_{10}$, indicating that XPA alterations led to deficiencies in DNA repair. These results demonstrate that $PM_{10}$ exposure induces an accumulation of DNA damage that is associated with NER inhibition, highlighting the role of $PM_{10}$ as an important contributor to lung cancer.

**Keywords:** DNA adducts; DNA repair inhibition; nucleotide excision repair pathway; particulate matter; lung cancer

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## 1. Introduction

Outdoor air pollution, specifically particulate matter (PM), has an impact on the incidence and mortality of lung cancer worldwide [1,2]. Epidemiological evidence and research in animal and in vitro models supported the classification of PM as a class 1 carcinogen by the International Agency for Research on Cancer (IARC) [3,4]. PM is divided according to its aerodynamic diameters into $PM_{10}$ (≤10 μm), $PM_{2.5}$ (≤2.5 μm), and ultrafine particles (UFPs) (≤0.1 μm). $PM_{10}$ contains all of the smaller fractions, and all of them are deposited in the respiratory tract during breathing [5–9]. $PM_{10}$ is made up of a variety of metals, such as zinc, copper, and vanadium; biological agents, such as pollen, bacteria, and endotoxins; and polycyclic aromatic hydrocarbons (PAH), including benzo(g)perylene (BghiP), dibenzo(a)anthracene (DBahA), and benzo(a)pyrene (BaP). The composition of

$PM_{10}$ plays an important role in its genotoxic and carcinogenic potential because some of these components modify the DNA [10,11].

There are several studies in which the role of $PM_{10}$ components as mediators in the DNA damage has been demonstrated. For instance, metals can mediate an increase in reactive oxygen species (ROS), which, in turn, leads to the oxidation of DNA [12,13]. In this regard, base excision repair (BER) is the pathway responsible for repairing the oxidative DNA lesions that cause small distortions in the helical structure of DNA, but enzymatic and non-enzymatic antioxidant defense also protects cells from the ROS generation induced by $PM_{10}$ exposure [14,15]. However, some other components of $PM_{10}$, such as PAH, mediate genotoxic effects through the induction of bulky DNA lesions, mainly BaP, which is biotransformed by cytochrome P450 enzymes in combination with microsomal epoxide hydrolase, leading to the generation of the reactive species benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), which can form adducts with DNA (BPDE–DNA) and cause significant distortions in the helical structure of this biomolecule [16–21]. The antioxidant defenses and the BER pathway are insufficient for coping with such distortions. The nucleotide excision repair (NER) pathway, which is composed of more than 30 proteins, acts in the removal of bulky lesions formed in the DNA, including the PAH–DNA adducts, through one of two sub-pathways: the global-genome (GG-NER) pathway or the transcription-coupled (TC-NER) pathway [22,23].

Damage recognition is the first step of the pathway and is carried out by the XPC-RAD23 proteins in GG-NER. Unwinding of the DNA strand is the next step and is performed by the helicases XPD and XPB [22,24]. A DNA damage verification step is carried out by the XPA protein, which is a key scaffold protein required for the verification of lesions and for the recruitment of other NER pathway proteins. Proper binding of XPA to the site of DNA damage depends on histone 4 (H4), which is dimethylated in lysine 20 (H4K20me2) in the presence of bulky DNA lesions [25–27]. Together with RPA, XPA forms the pre-incision complex in damaged DNA, which ensures that lesions are properly excised and thus represents an important rate-limiting step. In the third step, the XPF-ERCC1 complex cleaves the damage at the 5′-DNA strand, whereas the 3′ incision is mediated by the XPG endonucleases; at the end, δ or ε DNA polymerases synthesize the new DNA strand using the undamaged strand as a template [28].

Post-translational modifications regulate DNA repair by modulating some factors of the NER pathway, including XPA [29]. The phosphorylation of XPA at serine residue 196 ($pXPA^{S196}$) by kinase ATR enhances the stability of XPA and is required for the formation of the XPA-RPA protein complex. WIP1 phosphatase catalyzes the dephosphorylation of XPA at S196, thus inactivating this protein and reducing the NER pathway's functionality, which might increase the carcinogenic potential of different compounds that cause DNA damage [30–35]. DNA adducts can modify DNA conformation and deregulate replication and transcription. Therefore, the accumulation of this type of DNA lesion can induce genomic instability, lead to the appearance of mutations, and promote carcinogenic processes [36–38]. In relation to this, alterations in the NER repair system have been described in different types of cancer, including lung neoplasms [39,40]. However, the effect of $PM_{10}$ exposure in the NER pathway remains unclear. The aim of this study was to investigate the deregulation of the RAD23, XPD, XPA, $pXPA^{s196}$, H4k20me2, and WIP1 proteins, which are the main components of NER pathway responsible for the removal of bulky DNA damage, establishing an association with the NER pathway activity in A549 lung epithelial cells exposed to $PM_{10}$ for 24 and 48 h.

## 2. Results

### 2.1. *$PM_{10}$ Induced the Formation of BPDE–DNA Adducts*

$PM_{10}$ exposure for 24 h increased the levels of BPDE–DNA adducts in A549 cells compared with the control group (FC = 2.01 vs. 0.0; $p < 0.05$) (Figure 1). $PM_{10}$ exposure for 48 h showed a further increase in BPDE–DNA adducts (FC = 2.54 vs. 0.0; $p < 0.05$). BaP exposure for 24 h increased the levels of BPDE–DNA adducts in A549 cells compared with

the control group (FC = 2.85 vs. 0.0; $p < 0.05$). BaP exposure for 48 h also showed an increase in BPDE–DNA adducts (FC = 1.30 vs. 0.0; $p < 0.05$). The comparison of the times exhibited a significant decrease in the levels of BPDE–DNA adducts among cells treated with BaP for 48 h compared with that in those treated for 24 h (FC = 1.30 vs. 2.85; $p < 0.05$).

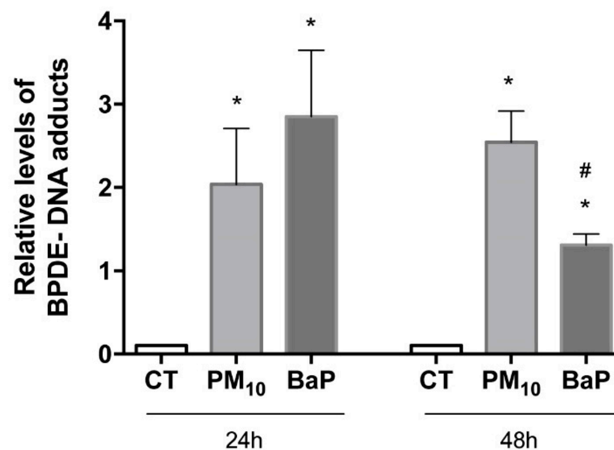


**Figure 1.** $PM_{10}$ exposure induces the formation of BPDE–DNA adducts in A549 cells. Benzo(a)pyrene diol epoxide-DNA (BPDE–DNA) adducts were evaluated in A549 cells exposed to 10 $\mu g/cm^2$ of $PM_{10}$ and 1 $\mu$M of BaP for 24 and 48 h. The concentration of BPDE–DNA adducts was measured using the OxiSelect BPDE DNA Adduct ELISA Kit and expressed according to the relative levels, and the values represent results from three experiments with the mean ratio $\pm$ SD per treatment. BaP was used as a positive control for DNA adduct generation. The images are representative of the data obtained. (*) indicates statistical differences versus the control group; $p < 0.05$. (#) indicates statistical differences between the amounts of time; $p < 0.05$.

### *2.2. $PM_{10}$ Deregulated the RAD23, XPD, and XPA Proteins Used in the Recognition and Verification Step of the NER Pathway*

$PM_{10}$ exposure for 6 h increased the RAD23 protein levels in A549 cells compared with the control group (1.16 vs. 1.00; $p < 0.05$) (Figure 2A). BaP exposure for 6 h also increased the RAD23 protein levels compared with the control group (1.20 vs. 1.00; $p < 0.05$). No differences were observed in the RAD23 protein levels after $PM_{10}$ or BaP exposure for 12 h (1.04 and 1.02 vs. 1.00, respectively), 24 h (1.01 and 0.94 vs. 1.00, respectively), or 48 h (0.95 and 0.90 vs. 1.00, respectively) compared with their control groups.

No differences were observed in the XPD protein level after $PM_{10}$ or BaP exposure for 6 h (1.01 and 1.09 vs. 1.00, respectively), 12 h (1.05 and 1.03 vs. 1.00, respectively), or 48 h (0.97 and 0.98 vs. 1.00, respectively) compared with the control groups (Figure 2B). However, $PM_{10}$ exposure for 24 h increased the XPD protein level in A549 cells compared with the control group (1.20 vs. 1.00; $p < 0.05$). BaP exposure for 24 h also increased the XPD protein level compared with the control group (1.15 vs. 1.00; $p < 0.05$).

No differences were observed in the XPA protein level after $PM_{10}$ or BaP exposure for 6 h (1.00 and 0.99 vs. 1.00, respectively), 12 h (1.03 and 1.07 vs. 1.00, respectively), or 48 h (0.98 and 0.96 vs. 1.00, respectively) compared with the control groups (Figure 2C). However, $PM_{10}$ exposure for 24 h decreased the XPA protein level in A549 cells compared with the control group (0.85 vs. 1.00; $p < 0.05$). On the other hand, BaP exposure for 24 h increased the XPA protein level compared with the control group (1.17 vs. 1.00; $p < 0.05$).

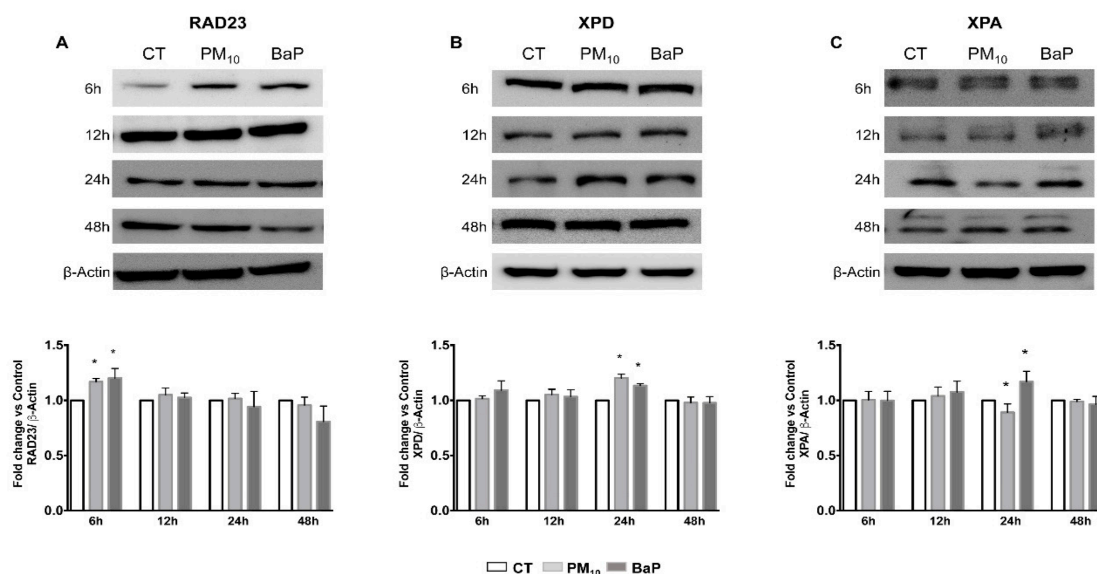


**Figure 2.** $PM_{10}$ deregulated proteins used in the NER pathway during different stages of exposure in A549 cells. The protein levels of (**A**) RAD23, (**B**) XPD, and (**C**) XPA were evaluated with a Western blot in the total protein lysates of A549 lung epithelial cells exposed to 10 μg/cm$^2$ of $PM_{10}$ and 1 μM of BaP for 6, 12, 24, and 48 h. Representative images of protein levels in protein lysates (upper panels) and an analysis of densitometry levels (lower panels) using β-Actin as a housekeeping control are shown. β-Actin blot housekeeping control is representative of all time point experiments (see Supplementary Figure S1). The values represent results from three independent experiments with the mean ± SD per treatment. BaP was used as a positive control for NER pathway activation. The images are representative of the data obtained. (*) indicates statistical differences versus the control group; $p < 0.05$.

### *2.3. $PM_{10}$ Induced Nuclear Recruitment (H4K20me2) and Dephosphorylation of XPA Associated with WIP1 Increase*

Cells treated with $PM_{10}$ for 24 h exhibited an increase in nuclear H4K20me2 protein levels compared with the control group (2.09 vs. 1.00; $p < 0.05$), whereas cells treated with BaP for 24 h showed no statistically significant differences (1.10 vs. 1.00) (Figure 3A). In addition, cells treated with $PM_{10}$ for 24 h exhibited a decrease in nuclear $pXPA^{S196}$ protein levels compared with the control group (0.74 vs. 1.00; $p < 0.05$), whereas cells treated with BaP for 24 h showed no differences (1.13 vs. 1.00) (Figure 3B). Interestingly, cells treated with $PM_{10}$ for 24 h exhibited an increase in phosphatase WIP1 protein levels compared with the control group (1.29 vs. 1.00; $p < 0.05$), whereas cells treated with BaP for 24 h showed no differences (1.02 vs. 1.00) (Figure 3C).

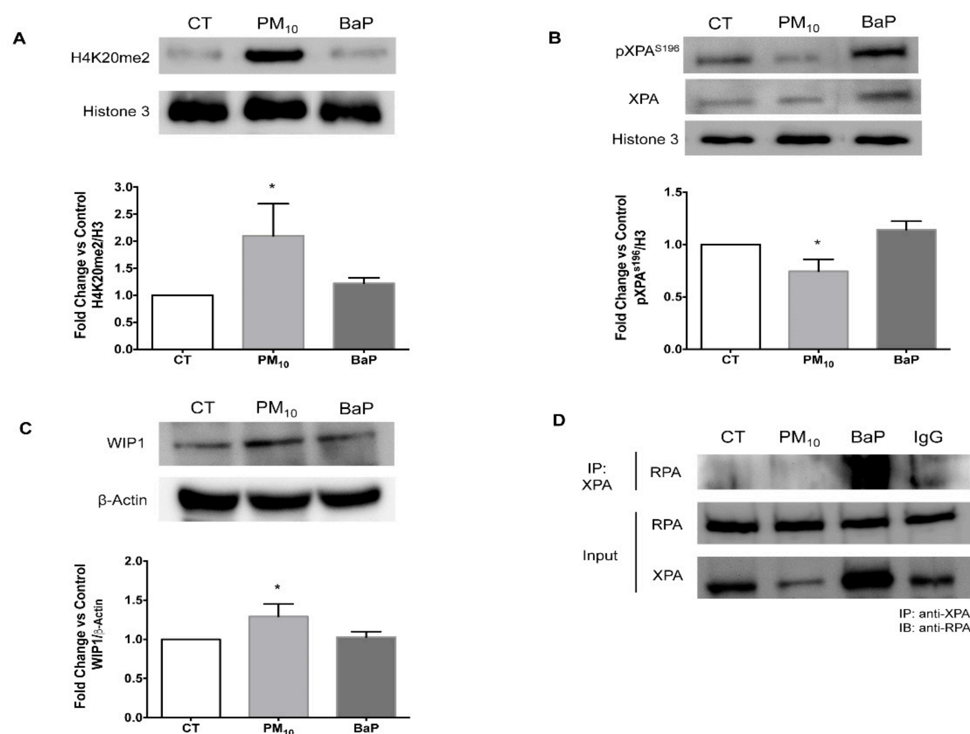


**Figure 3.** $PM_{10}$ exposure induced signals for the recruitment of XPA into the nucleus but decreased its phosphorylation, thus inhibiting the formation of the XPA-RPA complex in A549 cells. Protein levels were evaluated with a Western blot in nuclear protein lysates of A549 lung epithelial cells after exposure to 10 μg/cm$^2$ of $PM_{10}$ and 1 μM of BaP for 24 h. BaP was used as a positive control for NER pathway activation. (**A**) Representative Western blot of H4K20me2 in nuclear protein lysates (upper panel) and levels of a densitometry analysis using H3 as an endogenous control (bottom panel). (**B**) Representative Western blot of pXPA$^{S196}$ in cytoplasm and nuclear protein lysates, total XPA is displayed (upper panel) and levels of a densitometry analysis using histone 3 as an endogenous control (bottom panel). (**C**) Representative Western blot of WIP1 levels in total protein lysates (upper panels) and levels of a densitometry analysis using β-Actin as an endogenous control (bottom panels). The images and values represent results from three independent experiments with the mean ± SD per treatment. The images are representative of the data obtained. (*) indicates statistical differences versus the control group; $p < 0.05$. (**D**) Representative Western blot of the interaction between XPA and RPA detected by an immunoprecipitation assay after 24 h. IP: immunoprecipitation, IB: immunoblot. Representative image of three independent experiments.

### 2.4. *$PM_{10}$ Impaired the Formation of the XPA-RPA Complex*

Cells treated with $PM_{10}$ for 24 h did not display the XPA-RPA interaction, indicating that the complex between these proteins is not formed, whereas cells treated with BaP for 24 h showed an effective interaction between XPA and RPA, exhibiting a successful formation of this complex (Figure 3D).

### 2.5. *The NER Pathway Was Inactive in Cells Exposed to $PM_{10}$*

Cells treated with $PM_{10}$ for 24 and 48 h showed no differences in NER pathway activity compared with the control groups (0.99 vs. 1.00, and 0.99 vs. 1.00, respectively) (Figure 4). Cells treated with BaP for 24 h showed an increase in NER pathway activity compared with the control group (1.50 vs. 1.00; $p < 0.05$), whereas cells treated with BaP for 48 h showed no differences in NER pathway activity compared with the control group (1.09 vs. 1.00). The comparison between the amounts of time exhibited a significant decrease in NER pathway activity among cells treated with BaP for 48 h compared with those treated for 24 h (1.09 vs. 1.50; $p < 0.05$).

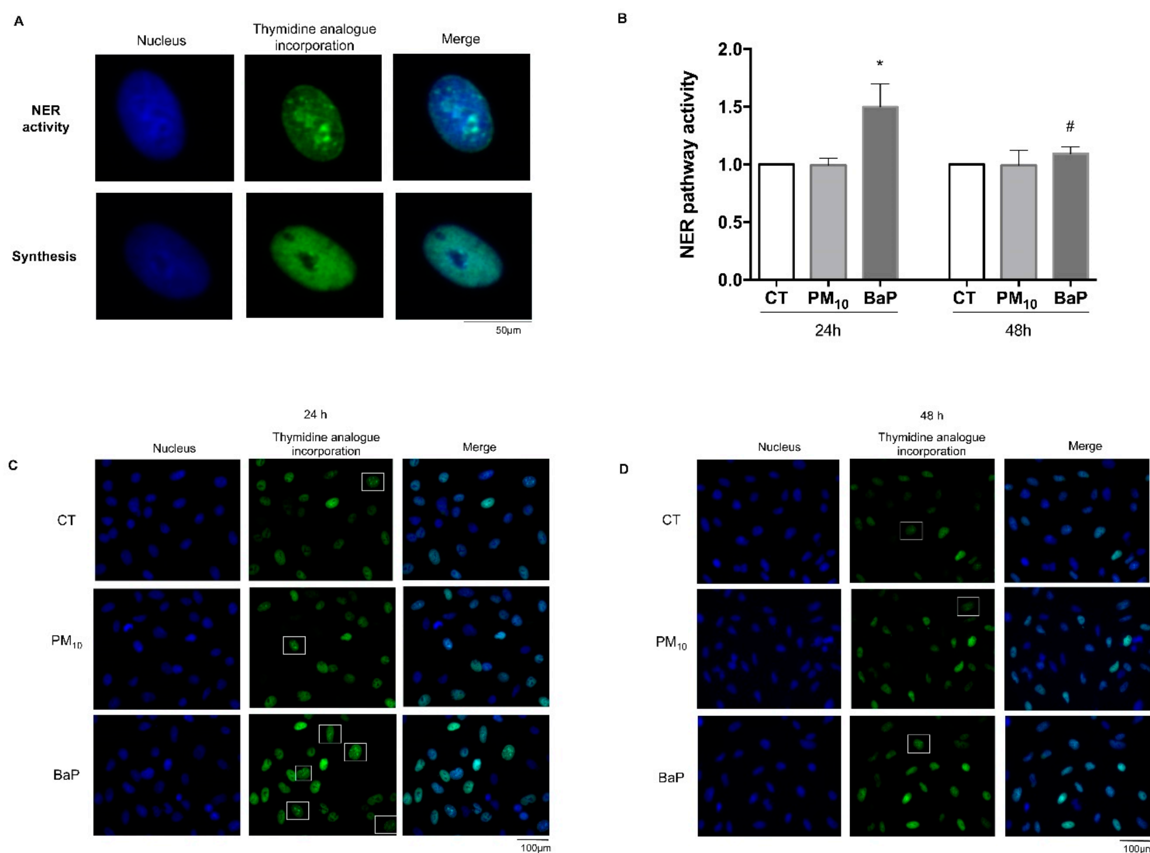


**Figure 4.** The NER pathway is inactive in A549 cells that are exposed to $PM_{10}$. The NER pathway's activity was evaluated through unscheduled DNA synthesis (UDS) in A549 lung epithelial cells exposed to 10 µg/cm$^2$ of $PM_{10}$ and 1 µM of BaP for 24 and 48 h. (**A**) The panel shows representative fluorescence micrographs of the detection of the incorporation of thymidine analog (EdU), and NER pathway activity is recognized according to the formation of foci (upper panel). Non-specific incorporation during DNA synthesis was identified by green homogeneous nucleus staining (lower panel), and these cells were not considered in the cell count for the repair analysis. (**B**) The quantitative results of the NER pathway activity were expressed after counting 1500 cells per condition. The values represent results from three experiments with the mean ratio ± SD per treatment. BaP was used as a positive control for NER pathway activation. (*) indicates statistical differences versus the control group; $p < 0.05$. (#) indicates statistical differences between the amounts of time compared; $p < 0.05$. (**C**,**D**) Representative images of NER pathway activity (positive cells are marked in white squares) at 24 and 48 h, respectively. The magnification of the NER pathway activity panels can be observed in Supplementary Figure S2 (24 h) and Supplementary Figure S3 (48 h).

## 3. Discussion

$PM_{10}$ is a well-known risk factor for the development of lung cancer [5,7]. Some of the components of $PM_{10}$ contained in this complex mixture are highly toxic or have been classified as carcinogens, including metals and PAH [17]; however, until now, a mutational fingerprint associated with PM exposure has not been detected [41]. Therefore, the study of the genotoxicity and effects of PM on DNA damage repair pathways could help in understanding the mechanism of $PM_{10}$ in lung carcinogenesis because the evasion of DNA repair induces the accumulation of damaged DNA. It is mainly the altered activity of NER, which is responsible for repairing bulky lesions, that leads to cancer [19,24,42,43]. In this study, A549 cells were exposed to a sub-lethal concentration of 10 µg/cm$^2$, which simulated human $PM_{10}$ exposure for five days [44,45], and the effects on proteins and the functionality of NER were analyzed.

We showed that $PM_{10}$ induced the formation of BPDE-DNA, and the literature indicated that 60% of bulky lesions are removed within 48 h of their generation [46–48]; however, in cells exposed to $PM_{10,}$ these lesions persisted for over 48 h. By contrast, when the same cell line was exposed to BaP, it showed a decrease in BPDE–DNA adducts at 48 h, suggesting that more than half of the BPDE adducts were eliminated. This highlights that DNA damage induced by $PM_{10}$ could be accumulated more in comparison with the DNA damage induced by BaP, despite being a well-characterized carcinogen. The second main finding of our study is related to a detailed identification of the proteins in the NER pathway. When each step of the repair was evaluated by determining the levels of key NER pathway proteins, we found that in cells exposed to $PM_{10}$, the RAD23, H4K20me2, and XPD proteins increased, whereas there was a decrease in XPA protein.

NER activity studies have shown that in A549 cells exposed to PAH, the beginning of DNA repair occurs 4 to 6 h after the generation of DNA damage [49]. Therefore, the increase in the RAD23 protein that we observed in cells exposed to $PM_{10}$ in the first hours after exposure suggested that the cells could recognize DNA damage. In addition, in this work, no changes were observed in RAD23 in the following hours of exposure in either $PM_{10}$ or BaP, which indicates that this protein is only necessary during the initial repair step, as has been demonstrated in other studies [26,28,50]. It was also found that after damage recognition, the DNA probably unwinds in cells exposed to $PM_{10}$ because of the increase in the XPD protein, which functions as helicase [27]. Furthermore, $PM_{10}$ increased the levels of H4K20me2, confirming that in cells exposed to $PM_{10}$, the damaged DNA recognition step works correctly, so this could induce the recruitment of other proteins, such as XPA [25]. In cells exposed to BaP, an increase in XPD levels was found; however, no changes were detected in H4K20me2 levels, so it is likely that this event occurred hours before the protein was measured; further experiments need to be conducted to determine these changes.

Alterations in the XPA protein generate a disruption in the progression of the NER pathway because although XPA does not possess enzymatic activity, it plays a critical role in the assembly of the pre-incision complex in damaged DNA [51]. Importantly, the levels of XPA decrease in cells exposed to $PM_{10}$ even though the recruitment signal for XPA is active, as suggested by the increase in H4K20me2 levels. However, in cells exposed to BaP, an increase in XPA levels was observed, suggesting that in cells exposed to $PM_{10}$, the verification step is altered. We proposed that the decrease in XPA in cells exposed to $PM_{10}$ could be a consequence of the metals contained in $PM_{10}$ because XPA is structured by zinc fingers [52], and nickel, zinc, cadmium, and copper can oxidize the thiol groups of these domains, thus inducing conformational and structural changes in this protein [48,52–54]. In addition, the reactive oxygen species produced by $PM_{10}$ exposure could also be responsible for alterations in enzymatic activity [55]. We must emphasize that $PM_{10}$ exposure decreased the rate of phosphorylation of XPA, suggesting that $PM_{10}$ alters the function of the XPA protein because dephosphorylated XPA loses its ability to interact with other proteins [33,56]. In addition, altered phosphorylation can lead to a decrease in XPA level, because dephosphorylated XPA is a substrate for HERC2 ubiquitination and proteasome degradation [30,33].

The loss of phosphorylation of XPA in serine 196 observed in this study could be the result of the increase in $PM_{10}$-induced WIP1 phosphatase levels; Nguyen et al. reported that WIP1 dephosphorylates the serine/threonine residues of different repair proteins, including XPA [31]. In addition, the cells exposed to BaP did not show alterations in either the levels of $pXPA^{S196}$ or WIP1. Cells expressing higher levels of WIP1 have shown reduced repair kinetics for the NER pathway [31,57], and WIP1 overexpression has been reported in some tumors, including lung adenocarcinoma [58,59]. We suggest that $PM_{10}$ exposure could stimulate the phosphatase activity of WIP1 because the functionality of phosphatases depends on low concentrations of metals, such as magnesium or manganese [60,61], and $PM_{10}$ has been shown to contain these metals [18,20]. We propose that the dephosphorylation of XPA induced by exposure to $PM_{10}$ has an impact on the interaction between XPA and RPA because this complex was absent in cells exposed to $PM_{10}$. Since this complex

performs the verification of damage and recruitment of the excision proteins, such as ERCC1 [51,62,63], we suspect that in cells exposed to $PM_{10}$, the DNA adducts could not be adequately removed during this step of the NER pathway, in contrast to cells exposed to BaP, which showed a higher rate of DNA adduct removal associated with the presence of the XPA-RPA complex.

Because we observed the persistence of DNA adducts in cells exposed to $PM_{10}$ and we also found alterations in XPA, one of the main proteins of the NER pathway, we decided to measure the activity of the NER pathway. Through this analysis, we confirmed that despite the recognition of damage, the functioning of the NER pathway was inhibited after exposure to $PM_{10}$. On the contrary, cells exposed to BaP showed a clear increase in the activity of the NER pathway, which is consistent with the decrease in the concentration of BPDE adducts found at 48 h. Therefore, the use of BaP as a positive damage control allowed us to determine that the NER pathway works correctly in A549 cells. The inadequate ability to remove damage indicates that alterations in XPA levels and in the phosphorylation reduce the response of the NER pathway in A549 cells exposed to $PM_{10}$, highlighting the likely role for WIP1 in inhibition of DNA repair; these are findings that need to be confirmed later. BaP, a carcinogenic component of $PM_{10}$, had no effect on NER pathway activity, which highlights that the effects on DNA repair activity might be the result of synergistic effects of all $PM_{10}$ components [20].

On the other hand, it is highly likely that NER is not the only DNA repair pathway disrupted by $PM_{10}$ exposure. There is clear evidence of $PM_{10}$-induced DNA damage, including the detection of 8-hydroxy-2-deoxyguanosine, DNA strand breaks, and formation of γH2AX foci [12,64,65], which is also supported by indirect evidence of DNA damage, such as micronucleus formation [66]; however, the accuracy of the alterations in proteins involved in the DNA repair pathways is still being assessed. For now, some hints of protein dysregulation of the BER pathway, homologous recombination (HR), and nonhomologous end-joining (NHEJ) pathways suggest potential impairment in the global DNA repair after $PM_{10}$ exposure [67]. Together, all DNA repair pathways protect the genome and its fidelity, but according to the literature, deficiencies in the NER and mismatch repair pathways have greater implications for carcinogenesis, whereas alterations in the BER pathway have a very low impact on cancer development [68,69].

Therefore, although additional studies are needed to assess the significance of the results of our study, the data indicate that lung cells exposed to $PM_{10}$ can accumulate DNA damage, which could predispose cells to genomic instability, and, in turn, this could lead to carcinogenesis [70,71]. $PM_{10}$ exposure induces the formation of DNA adducts, and their removal by the NER pathway is impaired in lung epithelial cells so that the risk of lung cancer development attributed to $PM_{10}$ inhalation can be explained by DNA accumulation more than by mutations in early stages of exposure. However, we do not discard that DNA accumulation induced by $PM_{10}$ exposure could lead to mutations that are still not detected (Figure 5).

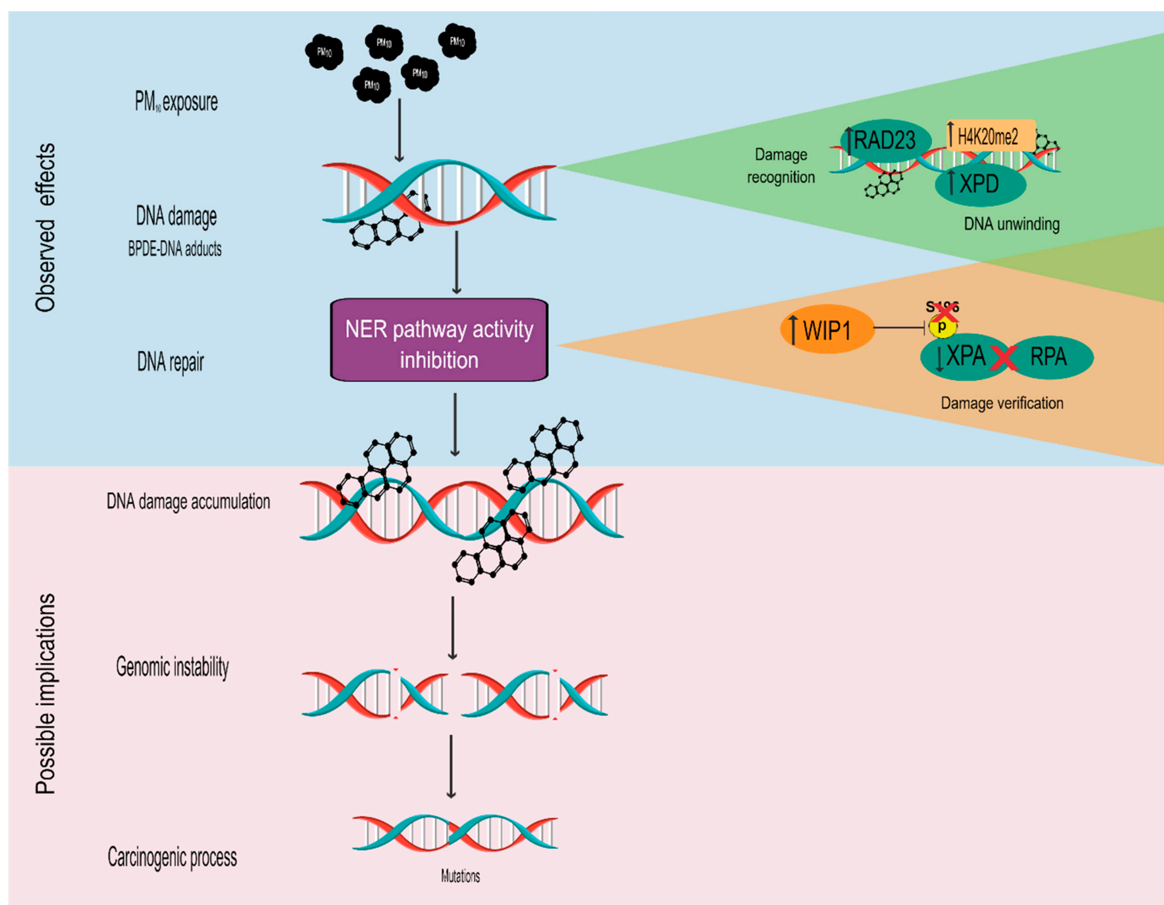


**Figure 5.** Schematic representation of the possible effects of $PM_{10}$ exposure associated with NER pathway inactivation. Exposure to $PM_{10}$ induces DNA damage through the formation of DNA adducts and this damage is recognized by the overexpression of RAD23, which induces the activity of the XPD and H4K2me2 proteins. Nevertheless, the removal of these adducts is inhibited as a result of the decrease in XPA levels and their dephosphorylation at serine residue 196 mediated by the upregulation of WIP1, which, in turn, disrupts the formation of the complex between XPA and RPA. Therefore, we suggest that the alteration in the functioning of the NER pathway predisposes cells to the accumulation of DNA damage and contributes to genomic instability, which could lead to the generation of mutations and, ultimately, to carcinogenic processes mediated by $PM_{10}$, which could be aggravated if some other DNA repair pathways are also inactivated.

## 4. Materials and Methods

### *4.1. $PM_{10}$ Collection*

$PM_{10}$ was collected from a residential urban area of Mexico City, one of the main sources of air pollution in the city, using a high-volume air collector (GMW model 1200 VFC HVPM10 Sierra Andersen, Smyrna, GA, USA) with a constant flow of 1.13 $m^3$/min. To recover $PM_{10}$, we used nitrocellulose filters with a pore size of 3.0 µm (Sartorius AG, Goettingen, Germany), which were then scraped with a surgical blade to collect the PM. $PM_{10}$ was stored in endotoxin-free glass vials at 4 °C in the dark until use. The $PM_{10}$ utilized in this study was characterized in previous studies through the analysis of PAH, metals, and endotoxins [72].

### *4.2. Cell Culture and $PM_{10}$ Exposure*

The A549 human lung epithelial cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in F-12 Kaighn's medium (Gibco BRL, 21127022, Grand Island, NY, USA) supplemented with 10% heat-inactivated

fetal bovine serum (FBS; GIBCO, 16000044, Life Technologies) at 37 °C using a 5% $CO_2$ atmosphere. One milligram of the stock suspension of $PM_{10}$ was resuspended in one milliliter of F-12 Kaighn's medium to obtain a $PM_{10}$ suspension of 1 mg/mL, as previously described [73]. After reaching 70% confluence, cells were exposed to 10 µg/cm$^2$ of $PM_{10}$ in F-12K medium supplemented with 10% FBS. Cells with only F-12K medium supplemented with 10% FBS were used as a control (CT), and cells treated with BaP (1 µM) (Sigma, B1760, USA) were used as a positive control for DNA adduct generation and the activation of the NER pathway [19,74].

### 4.3. Measurement of the Benzo(a)pyrene-7,8-diol-9,10-epoxide-DNA Adducts (BPDE-DNA Adduct)

After the cells were exposed to $PM_{10}$ and BaP for 24 or 48 h, DNA was isolated using the phenol–chloroform–isoamyl alcohol extraction protocol of Sambrook et al., 1989 [75]. DNA was dissolved in nuclease-free water and quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). The DNA integrity was evaluated using agarose gel electrophoresis. The BPDE-DNA concentration was measured using the OxiSelect BPDE DNA Adduct ELISA Kit (Cell Bio-labs, Inc., STA-357, San Diego, CA, USA) using the BPDE-DNA standard curve, according to the manufacturer's protocol. The absorbance was read in fluorescence plate reader (Tecan, GENios Plus, Männedorf, Switzerland) at 450 nm. The results were expressed as relative levels represented as fold changes (FCs) based on the calculation of nanograms of BPDE–DNA adducts per microgram of DNA.

### 4.4. Evaluation of the Total Protein Levels of the NER Pathway

The protein levels of RAD23, XPD, XPA, and WIP1 were evaluated at 6, 12, 24, and 48 h in cells exposed to $PM_{10}$ and BaP. Cells were washed with PBS, and protein extraction was performed using RIPA lysis buffer (20 mM Tris pH 8.0, 1% NP-40, and 150 mM NaCl at pH 8.0) with protease and phosphatase inhibitors (Thermo Fisher, 78440, Rockford, IL, USA). Protein quantification was performed by using the bicinchoninic acid method with a bovine serum albumin curve as a standard (Thermo Fisher, 23209, California, UK). Thirty micrograms of protein were used for electrophoresis on 12% SDS polyacrylamide gels, and the proteins were transferred to 0.45 µm polyvinylidene difluoride (PVDF) membranes using a semidry blotting system (Trans-Blot-Turbo, transfer system; Bio-Rad, California, UK). Membranes were blocked with 5% low-fat milk in TBS-Tween 0.1% under agitation for 1 h. Primary antibodies were incubated in a dilution of 1:1000 (anti-RAD23 cell signaling, 24555, anti-XPD cell signaling, 11963, anti-XPA Santa Cruz, sc-56497, and anti-WIP1 cell signaling, 11901) overnight at 4 °C under constant agitation. Anti-beta-actin (β-Actin) was used as a housekeeping protein in a dilution of 1:3000 (monoclonal antibody donated by Dr. Manuel Hernández, Cinvestav-IPN) [64,76]. After incubation, membranes were washed with TBS–Tween 0.1% and incubated with HRP-secondary anti-rabbit antibody (Amersham, NA934V) 1:2000 or HRP-secondary anti-mouse antibody (Amersham, NA931) 1:3000 for 1 h. Immunodetection was performed with chemiluminescence peroxidase substrate (Millipore, WBKLS0100, UK) and with the ChemiDoc-It Imager UVP. A densitometry analysis was performed by using the Image J software.

### 4.5. Measurements of Nuclear Protein Levels of the NER Pathway

The nuclear protein levels of H4K20me2 and XPA Ser196 were evaluated after the A549 cells were exposed to $PM_{10}$ and BaP for 24 h. Protein extraction was performed by separating the nuclear protein fraction and cytoplasmic protein fraction using Chemicon's nuclear extraction kit (Millipore, 2900, Billerica, MA, USA) according to the manufacturer's instructions. Protein quantification was performed using the bicinchoninic acid assay, as previously mentioned. Fifteen micrograms of nuclear protein fraction was loaded into a 15% SDS-polyacrylamide gel, and the levels of proteins were determined as previously described. Anti-H4K20me2 antibody (Abcam, ab9052) at 1:2000 and anti-phospho-XPA (Ser196) antibody (Thermo Fisher, 64730) at 1:500 were incubated overnight at 4 °C under

constant agitation. Histone 3 (H3) (Abcam, ab1791) was used as a housekeeping protein at 1:5000 for 1 h at room temperature, followed by the incubation of HRP-secondary anti-mouse antibody (Amersham, NA931).

### 4.6. Detection of the XPA-RPA Protein Complex

The immunoprecipitation assay was performed using the Dynabeads Co-Immunoprecipitation kit (Invitrogen, Thermo Fisher Scientific, 14321D) according to the manufacturer's protocol for detection of the XPA-RPA complex. One milligram of magnetic beads and 5 μg of XPA antibody (Santa Cruz, sc-56497) or 5 μg of normal goat IgG antibody (R&D systems, AB-108-C) were used for the formation of complex ab-magnetic beads. One milligram of protein cell lysates was incubated with the complex ab-magnetic beads. The elution of the immune complex was electrophoresed on 12% SDS-polyacrylamide gel, according to the steps described in the protein determination subsection. Anti-RPA (cell signaling, 2267) antibody at 1:1000 and HRP-secondary anti-rabbit antibody (Amersham, NA934V) at 1:3000 were used.

### 4.7. Measurement of NER Activity

The activity of the NER pathway was evaluated with an unscheduled DNA synthesis (UDS) assay. This technique provides a direct measurement of the excision and repair of damage after in vitro exposure to the compounds of interest by incorporating the thymidine analogue (5-ethinyl-2'-deoxyuridine (EdU) into DNA during the synthesis of the new strand in the final step of NER [77,78]. DNA replication was blocked (for prevention of nonspecific incorporation of EdU) by incubating the cells with 5 mM hydroxyurea for 2 h before the treatments, and it was left in the culture medium until the end of the corresponding exposure [79]. Subsequently, the cells were exposed to $PM_{10}$ or BaP, and 10 μM EdU was added to the culture medium. After 24 and 48 h of exposure, cells were washed with PBS and fixed using 3.7% paraformaldehyde in PBS for 20 min at room temperature. The incorporation of EdU was visualized with fluorescence microscopy using the Click-iT EdU Imaging Kit (Invitrogen, C10337, Carlsbad, CA, USA) according to the manufacturer's recommendations. At the end of the procedure, the slides were dried, and the nuclei were stained with Prolong Gold antifade DAPI (Invitrogen, 8961S, CA, USA). The UDS slides were observed under an AxioKop2 Mot Plus.D2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Micrographs of the different treatments were taken, and the number of EdU-positive cells (foci present in the nucleus) was counted among 1500 cells. Cells that presented a complete staining of their nuclei with EdU (incorporation during the replication of DNA) were excluded from the analysis [78,80] (Figure 4). The activity of the NER pathway was calculated as the percentage of EdU-positive cells in each treatment divided by the percentage of EdU-positive cells present in the control cells.

### 4.8. Statistical Analysis

The results of at least three independent experiments are presented as means ± standard deviation (SD). Statistical differences between DNA adducts and NER activity were tested by using one way analysis of variance and Bonferroni's post hoc test. Protein levels were tested by applying the two-tailed Student's t-test. All analyses were performed using the GraphPad Software, version 6 and a value of $p \leq 0.05$ was considered statistically significant.

## 5. Conclusions

Exposure to $PM_{10}$ induces the formation of BPDE–DNA adducts, which are recognizable by RAD23; however, $PM_{10}$ deregulates the damage verification step through the dephosphorylation of XPA at serine 196, thus preventing the formation of the protein complex with RPA, which results in the inhibition of the NER pathway's activity in A549 cells. These findings provide evidence that the impairment of the NER pathway's activity and the damaged DNA might be involved in the carcinogenic potential of airborne particulate

matter, thus helping explain why $PM_{10}$ is considered a risk factor for the development of lung cancer.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23042224/s1.

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