UNIVERSIDAD NACIONAL

AUTÓNOMA DE MÉXICO

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

CAPACIDAD DE LA REPARACIÓN POR ESCISIÓN DE NUCLEÓTIDOS (NER) EN LA REMOCIÓN DE LAS LESIONES OXIDATIVAS EN UN NEUROBLASTOMA HUMANO

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS

PRESENTA

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MÉXICO , D.F. **2012**

El presente trabajo de investigación se llevó a cabo bajo la dirección de la Dra. Mahara Valverde Ramírez, en el departamento de Medicina Genómica y Toxicología Ambiental del Instituto de Investigaciones Biomédicas de la Universidad Nacional Autónoma de México (UNAM).

Este trabajo se realizó con el apoyo del Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT, proyecto IN212007), así como por la beca de doctorado otorgada por el Consejo de Ciencia y Tecnología (CONACYT, número de becario 205989).

A la Dra. Alexandra Rodríguez Sastre por su colaboración en el trabajo experimental.

Contenido

Resumen

El DNA es un blanco frecuente del estrés oxidante, la remoción del daño generado sobre esta macromolécula es un proceso crucial para prevenir el desarrollo de enfermedades degenerativas. La reparación del DNA es un sistema esencial que asegura la integridad de la secuencia genómica. Todas las células cuentan con distintos mecanismos de reparación y su actividad dependerá del tipo de lesión, la etapa en el ciclo celular en el que se encuentren y del estado de diferenciación. Entre los principales mecanismos de reparación de lesiones oxidativas se encuentran la reparación por escisión de bases (BER) y la de nucleótidos (NER). La BER es el empleado para remover lesiones oxidativas; mientras que la NER se encarga de reparar una variedad de lesiones que comprometen la tridimensionalidad del DNA, como aquellas que son consecuencia de la exposición a radiación UVC. Estudios recientes han propuesto la participación de la NER en la remoción de lesiones oxidativas. Se conoce poco acerca del papel de las proteínas que funcionan como sensores de daño y los factores participantes de la reparación en células terminalmente diferenciadas como las neuronas, las cuales son vulnerables al constante ataque de las especies reactivas de oxígeno.

En este trabajo se utilizó el modelo de neuroblastoma humano para determinar la capacidad de las neuronas terminalmente diferenciadas para reparar lesiones que distorsionan la doble hélice del DNA, tales como los anillos de ciclobutano, uno de los principales daños ocasionados por la UV, así como lesiones oxidantes como la guanina oxidada 7,8-dihidro-8-oxoguanina (8oxoG). Los resultados muestran que la reparación del DNA depende de la lesión producida y del estado de diferenciación. Las neuronas terminalmente diferenciadas, comparadas con las indiferenciadas muestran mayor sensibilidad a UV y una disminución en el daño al DNA. Por otro lado las células indiferenciadas exhibieron un incremento en el daño al DNA debido al reto oxidante y muestran una tendencia a acumular el daño en forma de guanina oxidada (8 oxoG). Las proteínas de la reparación global del genoma, de la reparación acoplada a la transcripción y la reparación por escisión de bases se encontraron relacionadas con la remoción de las lesiones evaluadas en este trabajo.

Abstract

DNA is a frequent target of oxidative damage, and DNA damage removal is therefore a crucial process in prevention of degenerative diseases. DNA repair is an essential system for maintaining the inherited nucleotide sequence of genomic DNA. Cells engage in efficient DNA repair mechanisms, the activity of which can vary depending on the type of lesion and the developmental stage. Base Excision Repair (BER) and Nucleotide Excision Repair (NER) are the major repair pathways addressed in this study. BER is the principal mechanism for repair of DNA oxidative lesions, while NER is the mechanism for repair of a variety of helix-distorting lesions such as those caused by UVC radiation. Recent studies suggest that NER plays a cooperative role in the removal of oxidative lesions. Little is known about the roles of DNA damage sensors and repair factors in terminally differentiated, non-proliferating cells such as neurons, which are vulnerable to oxidative damage from reactive oxygen species generated by endogenous or exogenous agents.

We used the human neuroblastoma MSN cell model to investigate whether terminally differentiated neuronal cells respond to lesions caused in the DNA helix, such as UVC-induced cyclopirimidine dimers (CPD) and the major DNA oxidative lesion7,8-dihidro-8-oxoguanine (8oxoG), and thereby clarify NER repair capacity. We observed differences in DNA damage removal depending on the challenge used and the differentiation state. Differentiated MSN cells, compared with undifferentiated cells, showed greater sensitivity to UVC and decreased DNA damage over time. In contrast, undifferentiated cells displayed genotoxicity induced by oxidative insult and tended to accumulate DNA damage and 8oxoG lesions over time. Our findings suggest the participation of GGR, TCR and BER proteins in the removal of 8 oxoG and CPDs indicating a dynamic role in overall response to damage.

Organización de la Tesis

Esta tesis está organizada en: introducción, planteamiento del problema, hipótesis, objetivo general, objetivos particulares, diseño experimental, resultados, discusión y conclusiones. En la sección del diseño experimental se incluye únicamente un diagrama de flujo con la metodología empleada, ya que esta se explica a detalle en los artículos que se encuentran en la sección de resultados.

En la sección de resultados se incluyen dos artículos:

Ramos-Espinosa P., Rojas E., Valverde M. 2007. **DNA damage and repair in neural cells**, publicado como parte del libro: The neurochemistry of Neuronal Death, editado por Massieu L., Arias C., y Morán J. Ed. Research Singpost, Kerala, India pp. 121-137. El contenido de este trabajo comprende una revisión de los mecanismos de reparación del DNA y la relación de estos con las enfermedades neurodegenerativas. En la parte final se muestran resultados experimentales en los que se determina la expresión génica de participantes clave de las vías de la reparación por escisión de nucleótidos en un modelo de diferenciación neuronal retado con estimulos oxidantes inducidos por la exposición a peróxido de hidrógeno 10 μM.

Ramos-Espinosa P., Rojas E., Valverde M. 2012. **Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model**. *J Neurotox*. En este trabajo se incluyen los hallazgos experimentales que conforman la parte medular del proyecto, la capacidad reparativa del mecanismo de escisión de nucleótidos que entabla un neuroblastoma humano en diferentes estados de diferenciación ante retos oxidantes inducidos por peróxido de hidrógeno y lesiones que comprometen la tridimensionalidad del DNA, inducidos por UVC.

También se incluyen como parte de los resultados, los experimentos realizados en el cultivo celular murino 3T3, dado que se empleó como modelo de referencia en el cual la capacidad de reparación por escisión de nucleótidos está parcialmente atenuada. Estos resultados se presentan en formato de artículo de investigación.

Finalmente en la sección de discusión se abordan de forma integral todos los resultados aquí presentados y su relevancia.

Índice de Tablas y Figuras

Introducción

Respuesta al Daño en el DNA

La exposición celular a agentes endógenos, propios del metabolismo y ambientales, genera una amplia variedad de lesiones sobre el material genético: el ácido desoxiribonucleico ó DNA por sus siglas en inglés, las cuales son removidas por una red de reparación que involucra alrededor de 130 genes (**Decordier et al. 2010**). El reconocimiento y señalización inducidos por el establecimiento de la respuesta al daño en el DNA es un requisito indispensable para el mantenimiento de la integridad génica. La integridad génica está coordinada por un conjunto de procesos como son: el ciclo celular, la activación de puntos de revisión ("checkpoints") del DNA dañado, la inducción de programas transcripcionales, la activación de vías de reparación, modificaciones y remodelamiento de cromatina **(Hoeijmakers 2001**); la activación de estos procesos aseguran la eliminación de errores en el material genético para evitar que se transmitan a la progenie (**Houtgraaf et al. 2006**). Sin embargo, cuando la reparación del DNA no es suficiente para remover los daños se inicia la muerte celular a través de diversas rutas. Debido a lo anterior, es de vital importancia el funcionamiento adecuado de los procesos de reparación, ya que la acumulación de daño podría acarrear repercusiones sobre la integridad génica, que sea detonante en la génesis de enfermedades.

Mecanismos de Reparación

Existe una gran variedad de daños que se generan en el DNA por causa de agentes ambientales o por productos del metabolismo celular. Se ha reportado que se generan 50,000 lesiones por célula al día en mamíferos, de las cuales, al menos 100 son modificaciones de tipo oxidativas (**Hoeijmakers 2001, Friedberg, 1995**). Para contrarrestar las alteraciones generadas en el DNA, la célula cuenta con mecanismos de reparación encargados de reconocer y remover las lesiones. Se han identificado diversos mecanismos de reparación, cada uno se enfoca en la remoción de lesiones muy particulares y se distinguen entre ellos por la manera en que el daño es reconocido. Los mecanismos de reparación tienen como finalidad restaurar al mal apareamiento de bases (MMR, por sus

siglas en inglés), la reparación por recombinación (HR, por sus siglas en inglés), la reparación de rompimientos de cadena doble (DSBR por sus siglas en inglés), la reparación por escisión de bases (BER, por sus siglas en inglés) y la reparación por escisión de nucleótidos (NER, por sus siglas en inglés), entre otros (Figura 1). Este trabajo se enfocó a los mecanismos de escisión, BER y NER, dado que son estos los involucrados en la remoción de lesiones oxidativas y aquellas que comprometen la tridimensionalidad del DNA, respectivamente.

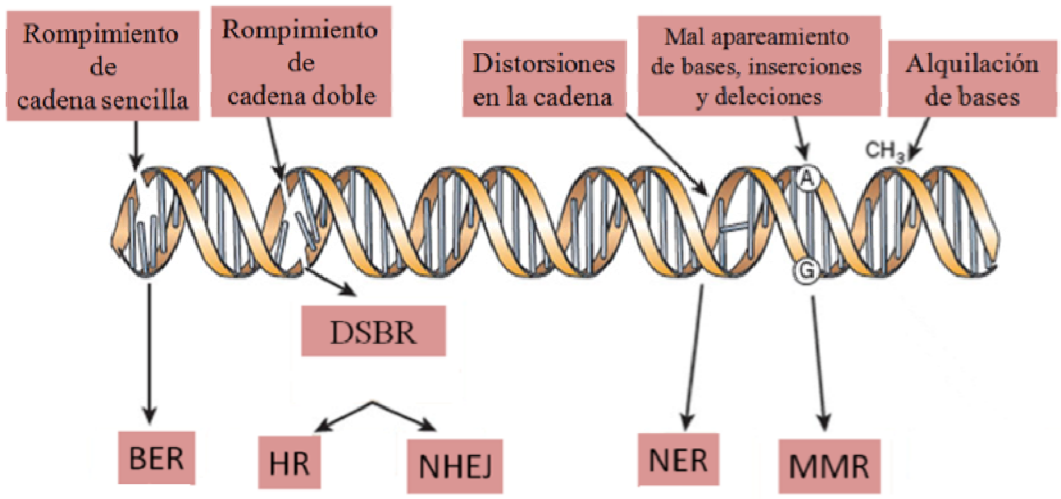

Figura 1. Resumen de los mecanismos de reparación. En el DNA se generan una gran variedad de lesiones las cuales son reparadas básicamente por 4 mecanismos de reparación: BER, DSBR, NER y MMR. Este trabajo se centra en los 2 mecanismos de escisión: NER y BER. Las proteínas que participan en estas vías son distintas y sus respectivas subvías son particulares.

Reparación por Escisión de Bases (Base Excision Repair, BER)

La reparación por escisión de bases es el principal mecanismo involucrado en la remoción de lesiones inducidas por especies reactivas, metilaciones, deaminaciones e hidroxilaciones. Éstas reacciones son generadas por el propio metabolismo celular, agentes xenobióticos o errores en la replicación del DNA. El primer paso de la BER es la remoción de las bases dañadas o modificadas mediante las DNA glicosilasas. En mamíferos se han reportado al menos 11 glicosilasas activas que reconocen un daño específico e

hidrolizan el enlace glicosídico, liberando la base dañada. Las glicosilasas están divididas en tipo I y II; las de tipo I remueven la base y producen un sitio abásico; mientras que las del tipo II no solo remueven la base si no que también cortan mediante la acción endonucleasa de sitios apurínicos (APE1, por sus siglas en inglés) en el extremo 3' de la lesión, dejando al DNA en cadena sencilla (**Christmann et al 2003**). El resultado de estas reacciones es la ausencia de una base con una desoxirribosa fosfato (dRP) en el extremo 5' y un radical hidroxilo (OH) en la región 3'. Una vez que la base dañada es removida y dependiendo de la extensión del sitio abásico se inicia la señalización hacía alguna de las vías de BER.

En la vía corta de BER, la DNA polimerasa β (DNA pol β) actúa como un polimerizador de los nucleótidos faltantes, removiendo el residuo de azúcar que carece de base a través de su actividad de liasa. Finalmente el fragmento recién polimerizado es sellado mediante el complejo XRCC1–ligasa 3. Mientras que la reparación por la vía larga involucra la participación de la DNA pol β, pol δ, pol ε y el antígeno nuclear de proliferación celular (PCNA) para la síntesis del fragmento reparado. Finalmente, la endonucleasa FEN1 remueve el DNA sobrante y la DNA ligasa 1 une los extremos reparados a la hebra de DNA (**Krokan 2000, Hoeijmakers 2001**). Para ilustrar la BER ver la Figura 2 de **Ramos-Espinosa et al 2007**, que se encuentra en la página 44.

BER y la lesión 8OHdG

La presencia de especies reactivas de oxígeno en el metabolismo celular es la razón por la que el daño oxidativo al DNA ocurre con alta frecuencia, evento que amenaza la integridad del genoma constantemente. Se han descrito más de 20 tipos de bases oxidadas en el DNA, dentro de estas se encuentra la 8 oxoG (**Grollman y Moriya 1993, Pravitel et al 2006**), (Figura 2). Esta base modificada es la más estudiada ya que es altamente mutagénica. En la Figura 2 se muestra su estructura y cómo se genera por la acción del radical hidroxilo, por el superóxido o por el peroxinitrito (Figura 2A) (**Barnes y Lindahl 2004, Cadet 2010**). La guanina oxidada puede encontrarse como 8-hidroxiG [Figura 2C) u como 8oxoG (Figura 2D). Se ha reportado que se generan 1,000 8oxoG por célula al día y 10,0000 en una célula cancerosa (**Bregeon 2011**).

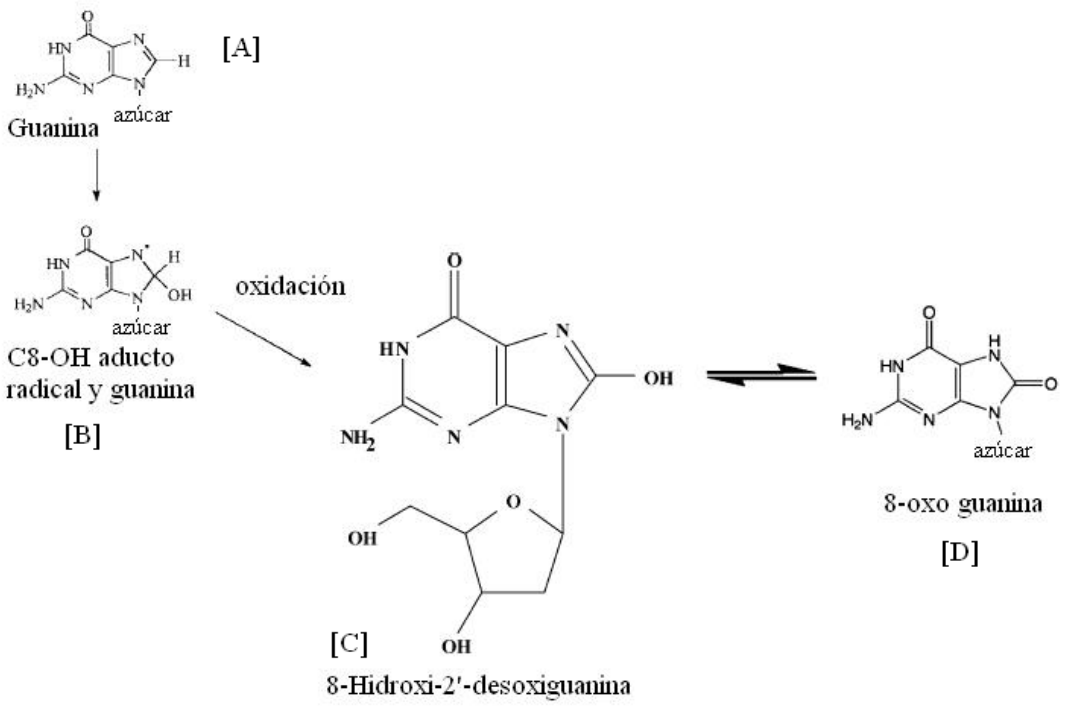

Fig. 2 Estructura de la guanina y de la 8oxoG. La guanina [**A**] es atacada en el carbono 8 por un radical hidroxilo (˙OH) [**B**] después de una reacción de oxidación produce la lesión 8-hidroxiguanina [**C**] ú 8-oxo guanina [**D**]. Modificado de **Alam** (1997), **Cooke** (2005) y **Wells** (2008).

La importancia de esta lesión, radica en que al no bloquear la actividad de la RNA polimerasa II (RNAPII), no hay reconocimiento por parte de los mecanismos de reparación y se favorece su permanencia en el DNA. Esto provoca que durante la replicación, las polimerasas incorporen una adenina por una 8 oxoG, ocasionando transversiones (G:C a T:A) (**Kino 2000**). Por otro lado, también impide la metilación de la citosina adyacente, afectando el señalamiento epigenético ya que altera la unión de factores de transcripción (**Zawia et al 2009**). Los eventos descritos pueden contribuir al desarrollo de enfermedades degenerativas y al envejecimiento (**Ames 1989, Cooke et al 2005**).

Se ha descrito a BER como el mecanismo predominante que remueve a la 8oxoG. Esta actividad recae sobre la 8-oxoguanina glicosilasa I (OGG1) (**Barnes y Lindahl 2004**), sin embargo, no se debe descartar la posibilidad de que otras enzimas de BER como la NTH1 endonucleasa III (NTH1) (**Duo et al 2003**) o la glicosilasa MYH involucradas en otros

mecanismos de reparación puedan intervenir en la eliminación de esta lesión (**Russo et al 2004 y 2006, Hazra et al 2003**).

Reparación por Escisión de Nucleótidos (Nucleotide Excision Repair, NER)

La NER es un mecanismo de reparación capaz de remover una amplia variedad de lesiones en el DNA. El reconocimiento está basado únicamente en la distorsión provocada en la doble hélice de DNA (**Lans et al 2010, Niedernhofer 2008**), característica que hace a este mecanismo sumamente versátil ya que no requiere de enzimas específicas para cada lesión (**Nouspikel 2009**). NER es un mecanismo altamente conservado que consiste en una serie de pasos en los que están involucradas más de 25 proteínas (**Lans et al 2010**).

La reparación se inicia por dos vías de reconocimiento diferentes: reparación global del genoma (GGR) y acoplada a la transcripción (TCR), sin embargo la manera en que se elimina el daño se hace por una vía común. Ambas vías consisten en el reconocimiento del daño, incisión y escisión del oligonucleótido que contiene la lesión, polimerización de un fragmento nuevo utilizando como templado la hebra complementaria y la ligación de éste a la hebra preexistente (**Araujo y Woods 1999**).

Reparación Global del Genoma (Global Genome Repair, GGR)

La GGR repara lesiones a lo largo del DNA pero su reparación varía dependiendo del estado de la cromatina (**Fousteri 2006**). El complejo que se encarga de reconocer censar la distorsión consiste de tres subunidades: la proteína del grupo C de complementación de *Xeroderma pigmentoso* (XPC), la proteína de reparación por escisión Rad23b (HR23 y la proteína centrina 2. Este complejo es indispensable en el reconocimiento y se recluta debido a que advierte el cambio estructural de la doble hélice, provocando que XPC y HR23B cambien su distribución en el DNA, migrando al sitio donde se encuentra la lesión (**Costa 2003**). XPC, HR23b y centrina 2 son proteínas de unión a DNA con una fuerte preferencia por el DNA dañado, la cual es esencial para la iniciación de GGR y para que se recluten otros factores de NER. Cuando el DNA no está dañado, las tres proteínas se

encuentran en el citoplasma (**van der Spek et al 1996, Araki et al 2001, Hoogstraten et al 2008**)

La mayoría de los genomas de mamíferos contienen dos ortólogos de las proteínas Rad23, HR23A y HR23B, ambos interaccionan con XPC e incrementa la actividad de NER. La mayor parte de la proteína XPC se encuentra en el complejo con HR23B, mientras que otra porción co-purifica con la proteína redundante HR23A, ambas proteínas son capaces de estabilizar y activar la función de XPC (**Okuda et al 2004**). Aunque HR23B no es esencial para NER *in vitro*, *in vivo* las células que no cuentan con HR23B tienen una reparación deficiente, lo que indica que HR23B es esencial para el correcto funcionamiento de NER (**Lans 2010**). Aunada al complejo de XPC-HR23B se encuentra la proteína centrina-2, la cual se ha sugerido que estabiliza al complejo, sin embargo su presencia parece no ser estrictamente necesaria para NER (**Shuck et al 2008**).

Reparación Acoplada a la Transcripción (Transcription-coupled Repair, TCR)

La reparación en los genes transcripcionalmente activos se lleva a cabo por la vía acoplada a la transcripción de la NER. Este tipo de reparación se activa por el impedimento físico que ocasiona la lesión al paso de la RNA polimerasa II (RNAPII) a través del DNA. Este bloqueo es señal suficiente para que se recluten factores específicos como las proteínas remodeladoras de la cromatina: las proteína del grupo A y B del síndrome de Cockayne, CSA y CSB respectivamente (**Lans et al 2010, Fousteri 2008**). Debido a los rearreglos que sufre la cromatina por estas enzimas, se puede inferir que la vía TCR es bastante rápida, ya que permite que el DNA se encuentre accesible a la maquinaria de reparación. CSB es una ATPasa dependiente de DNA que comparte homología con la familia de SWI/SNF (**Pazin y Kadonaga 1997, Citterio et al., 2000; Beerens et al., 2005, Newman et al 2006, Yuan 2007**). En lo que se refiere a su papel en la transcripción, CSB, ha mostrado interaccionar con el complejo DNA, RNAPII y RNA y se localiza en los sitios de transcripción, es parte del complejo proteico que contiene a Pol I y al factor de transcripción II H (TFIIH, por sus siglas en inglés) y es un prerrequisito para ensamblar a las proteínas de NER (**Bradsher et al 2002, Yuan 2007**).

Una vez que la RNAPII es bloqueada por la distorsión que ocasiona la lesión, la proteína CSB la desplaza del sitio de daño para permitir el acceso a otras proteínas relacionadas con la reparación. Posteriormente la RNAPII retrocede unos nucleótidos localizados antes del daño y favorece que la proteína CSA se mueva a la matriz nuclear (**Balajee y Bohr 2000**). Durante este evento, las características de la lesión cobran gran relevancia, debido a que el bloqueo de la RNAPII dependerá de la distorsión que provoque el daño, si no es suficiente puede permanecer en el DNA, amenazando su integridad. El destino de la RNAPII una vez desplazada se encuentra en debate, ya que se ha propuesto que ésta se ubiquitina para ser degradada, lo cual explicaría la disminución en la actividad de la RNAPII después de la inducción del daño. Otra hipótesis sostiene la participación de fosfatasas (FCP1) para el reciclaje de la polimerasa (**Laine y Egly 2006**); sin embargo, recientemente se ha sugerido que el destino de esta última es regresar al sitio donde se originó la lesión, ya que si se tuviera que prescindir de una RNAPII por cada daño, llevaría más tiempo transcribir un gen (**Hanawalt y Spivak 2008**).

La vía común

Después de la detección de la lesión ya sea vía GGR o TCR, el siguiente paso es ensamblar el TFIIH el cual es esencial en la NER. Dos principales componentes de TFIIH: las proteínas de complementación del síndrome de *Xeroderma pigmentoso* B (XPB) y D (XPD), ejercen su actividad de helicasa de forma dependiente de ATP, abriendo la doble hélice en sentidos opuestos, permitiendo el acceso a otros factores de reparación. En este paso se libera a la RNAPII que se detuvo (**Mellon et al 2005**) y de forma paralela tres subunidades de TFIIH: la proteína reguladora del ciclo celular H (ciclina H), cinasa dependiente de ciclina 7 (cdk7) y factor de ensamblaje activador de CDK (MAT1) forman el complejo CAK que fosforila la subunidad larga de la RNAPII permitiendo que cambie a la forma de elongación (**Santagatti et al 2001**). XPB y XPD junto con otras 5 subunidades, p62, p52, p44, p34 y TTD-A forman una estructura de anillo que se ancla a CAK, la función de estas subunidades es dar estabilidad al complejo (**Costa et al 2003**). Las proteínas XPA en conjunto con RPA son reclutadas para estabilizar el complejo y verificar la presencia el daño (**De Laat et al 1999**). XPA, es una proteína de unión al DNA, cuyo papel no está

claramente definido, aunque se le ha relacionado en el reconocimiento de la lesión junto con XPC. Datos recientes indican que la función de XPA es desplazar a XPC e identificar la hebra que lleva la lesión (**Hey et al 2002**). Este último paso es de suma importancia para asegurar que la hebra correcta sea cortada; es posible que esta tarea también sea llevada a cabo por TFIIH. Por otro lado, RPA, es una proteína que se sitúa en la apertura generada por las helicasas para evitar nuevamente el enrollamiento.

Una vez que ha quedado estable el sitio, la endonucleasa XPF en complejo con las proteínas de reparación de DNA por escisión (ERCC1) y proteínas de complementación del síndrome de *Xeroderma pigmentoso* G (XPG) escinden la hebra dañada en los extremos 3' y 5' respectivamente **(Mellon et al 2005).** XPG estabiliza al TFIIH y su ausencia lleva a la disociación del complejo CAK de la subunidad XPD. Mientras que el papel de ERCC1 es promover la estabilización recíproca entre ella y XPF. Por otro lado, ERCC1 contiene un dominio de unión a DNA lo que la vuelve responsable de llevar a XPF a su posición en el umbral de la burbuja de desnaturalización. Después de que el oligonucleótido que contiene la lesión ha sido removido, la proteína PCNA se recluta en el DNA en compañía de RFC. El resultado final es un espacio de 30 nucleótidos de cadena sencilla que es llenado por las DNA polimerasas delta y épsilon asociadas a PCNA con base en la secuencia de la hebra no dañada y ligado por la DNA ligasa I (**Lans et al 2010**). Algunos estudios han sugerido la participación de otras ligasas, tal es el caso de la DNA ligasa k y XRCC1-ligasa III (**Fousteri 2008, Nouspikel 2009**). Para ilustrar el mecanismo de NER, ver figura 3 de **Ramos-Espinosa et al 2007** en la página 46.

Las lesiones: 6-4 fotoproductos y los anillos de ciclobutano y la NER

La luz ultravioleta (UV) representa el 45% total del espectro solar, éste se encuentra dividido en 3segmentos de acuerdo a sus longitudes de onda: UVA (320-400nm), UVB (295-320nm) y UVC (100-295nm) (**Batista 2009**). A pesar de que la radiación UVC no tiene importancia ambiental, el hecho de que los picos máximos de absorción del DNA se encuentren en 260 nm, lo vuelve una molécula susceptible a este tipo de radiación (**Cadet et al 1997**). El primer paso en la generación de daño por la UV es la absorción de la energía de los fotones por el DNA, que actúa como un cromóforo celular (**Pattison et al**

2006). La absorción de los fotones de UV cambia la distribución de los electrones de la molécula fotosensible, lo que lleva a reacciones fotoinductoras entre dos purinas adyacentes, como se muestra en la Figura 3A, lo que genera lesiones conocidas como fotoproductos. Los más comunes son los anillos de ciclo butano (CPD por sus siglas en inglés) (Figura 3B) y los 6-4 fotoproductos (6-4PP, por sus siglas en inglés) (Figura 3C); un tercer fotoproducto poco conocido es el isómero de Dewar (DewPPs) que se origina por la fotoisomerización de los 6-4PPs (**Douki et al 2003, Perdiz et al 2000**).

El principal daño inducido en el DNA por la luz UVC son los CPDs que resultan de la unión covalente entre pirimidinas adyacentes a través del C5 y C6 (**Friedberg et al 2006, Hanawalt y Spivak 2008**) y el C4 y X4 de otra pirimidina, para dar origen a un anillo estable de cuatro miembros, cuando X es un oxígeno o nitrógeno e inestable cuando se trata de azufre (**Taylor JS et al 2006**): mientras que los 6-4PPs son producto de la apertura del anillo de un fotoproducto. Diversos estudios han mostrado que la formación de CPD y 6-4PP se genera en una relación de 3:1 (**Mitchell 1988, Prioetti et al 2001**) esto obedece a la secuencia de DNA y no es una consecuencia aleatoria. La distribución de timina y timina (T-T) y de timina-citosina (T-C) han mostrado ser más foto reactivas a la luz UV que la distribución citosina-timina (C-T) y citosina-citosina (C-C) por esta razón es que los anillos de ciclobutano se forman en una relación mayor que los 6-4PP (**Douki et al 2001**).

También es importante mencionar que un efecto secundario de la exposición a UV es la transferencia de energía a la molécula de oxígeno, lo que produce especies reactivas de oxígeno (EROs) que actúan sobre lípidos, proteínas y DNA (**Ravanat et al 2001**).

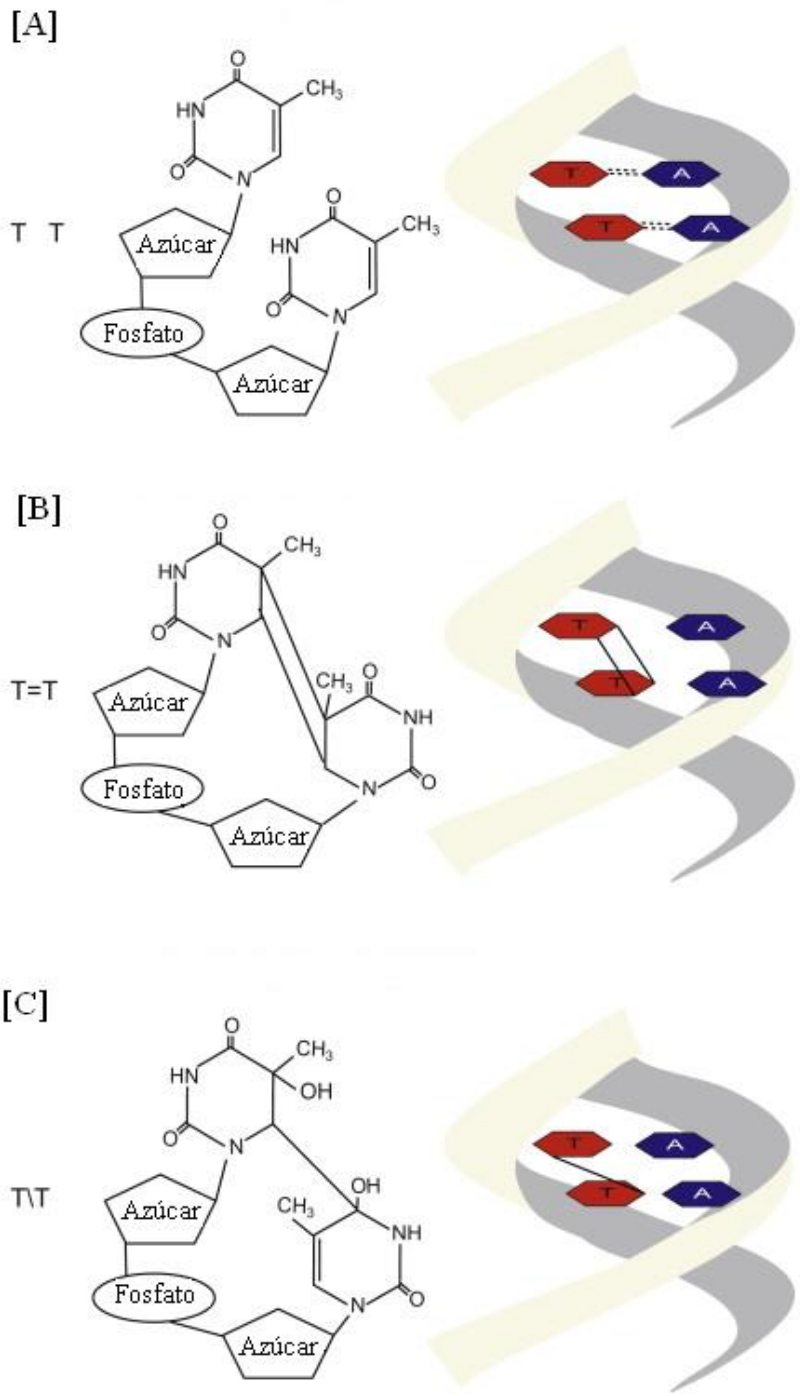

Figura 3. Estructura de las lesiones producidas por la radiación UVC. Los fotones de la radiación UVC estimulan los electrones de dos purinas adyacentes [**A**], produciendo dos lesiones sobre el DNA: los CPD [**B**] y los 6,4-fotoproductos [**C**]. Modificado de **Maverakis** (2010).

La NER caracterizó por remover fotolesiones 6-4PPs y los CPD **(Vrouwe 2010, Christmann M., et al 2003, Fousteri 2008)**. Los 6-4 fotoproductos causan una fuerte distorsión en el

DNA y por eso constituyen un buen sustrato para NER, contrario a lo que sucede con los CPDs que aunque son fuertes bloqueadores de la RNAPII, solo generan una distorsión modesta (**Bregeon 2011**). El reconocimiento de la NER como resultado del efecto de los fotoproductos se inicia por el complejo proteico de unión a DNA dañado (DDB), el cual es un heterodímero constituido por la proteína de unión a DNA dañado 1 (DDB1, por sus siglas en inglés) y el complejo formado entre la proteína de unión a DNA dañado 2 y la proteína del grupo de complementación E del síndrome *Xeroderma pigmentoso* (DDB2/XPE, por sus siglas en inglés). El heterodímero DDB alta afinidad por los 6-4 fotoproductos y los CPDs (**Wittschieben B et al 2005**). Se ha propuesto que la unión a la lesión por DDB induce una distorsión en el DNA que genera el reclutamiento de XPC y por ende a toda la maquinaria de NER (**van Hoffen et al 2003**). Este mecanismo no es tan relevante para el caso de los 6-4 fotoproductos que son reconocidos por el complejo XPC por sí solo, pero es crítico para la reparación eficiente de los CPDs.

Una peculiaridad de DDB2 es que puede interaccionar con la ubiquitin ligasa E3, que ubiquitina a la proteína XPC y la propia DDB2. La ubiquitinación en XPC incrementa la afinidad de esta proteína por el DNA dañado, mientras que DDB2 se degrada, permitiendo que XPC tenga el control sobre el reconocimiento del DNA dañado (**Nouspikel 2009**).

La relación entre NER y BER

Existe evidencia de que en las células de mamífero la 8 oxoG, sustrato de la OGG1 que participa en BER, también puede ser removida por la proteínas de NER. En ratones se ha observado que al mutar la glicosilasa OGG1, hay un aumento en la presencia de 8 oxoG. **Osterod y cols** (2002), compararon la capacidad de remover 8oxoG en ratones *ogg1*-/-, *csb* -/- y *csb-/-ogg1*-/-; encontrando que existía acumulación de la guanina oxidada tanto en ratones con el genotipo ogg1-/- como en los ratones csb-/- y de forma aditiva en los doble knockout (*csb-/-ogg1*-/-) indicando que la proteína CSB estaba relacionada con la remoción de 8oxoG, como un mecanismo alterno. De la misma forma **Trapp y cols. (2007**) han reportado que la proteína CSB truncada en conjunto con la ausencia de OGG1 ocasiona la acumulación de 8 oxoG en hepatocitos de ratones Big Blue. Otro ejemplo de la

estrecha relación que hay entre CSB y la remoción de 8oxoG lo reportó el grupo de **Davinov** (2002), el cual no solo evidenció que en los extractos nucleares de pacientes con síndrome de Cockayne (CS, por sus siglas en inglés) había una reducción en la incisión de 8oxoG, si no que la transcripción de la OGG1 disminuía a consecuanecia de la mutación en CSB. Tomando este antecedente, **Tuo y col** (2002) mostró en fibroblastos de pacientes con síndrome de Cockayne (CS) la colocalización nuclear entre CSB y OGG1. Mientas que el grupo de **Stevsner** (2008) y el de **Pastoriza** (**2003**), demostraron que en extractos mitocondriales CSB estimula la transcripción de OGG1. Por otro lado se ha sugerido que CSA tiene un papel similar al de CSB en la reparación de daños debido a estrés oxidante, sin embargo también hay resultados que indican lo contrario (**de Waard et al 2008**). Los queratinocitos y fibroblastos de pacientes con CSA mutada, expuestos a bromato de potasio ($KBrO_3$), un agente oxidante, mostraron ser altamente sensibles a este compuesto lo que sugiere que CSA está implicada en la reparación de daños oxidantes (**D'Errico et al 2007**). Otros autores han propuesto que las proteína XPC (**D'Errico et al 2006, Langie et al 2007**), XPG (**Langie et al 2007, Klungland et al 1999**) y XPA (**Dusinka et al 2006**) de la GGR también están relacionadas en la remoción de 8oxoG, sugiriendo que ambas vías de NER pueden participar en la reparación de bases oxidadas. Otras evidencias que hay que tomar en cuenta son las que sugieren que diversos genes que codifican para proteínas de NER tienen secuencias de unión en sus regiones promotoras reconocidas por factores de transcripción sensibles a óxido-reducción. Tal es el caso de la proteína ERCC1 que funciona como endonucleasa en el extremo 5' en la escisión de nucleótidos y su expresión puede ser mediada por el factor de transcripción AP-1 (**Langie et al 2007, Li et al., 1998, 1999**).

La Reparación de DNA en células en estado post-mitótico

La reparación juega un papel importante en las células que no se dividen, ya que debido a su baja tasa de replicación los puntos de revisión no se encuentran tan activos como ocurre en una célula en división durante el ciclo celular. Esto ocasiona una acumulación de mutaciones que podrían provocar procesos apoptóticos lo que resultaría en la descompensación del organismo entero.

Existen diferentes tipos de células terminalmente diferenciadas, ejemplos de ellos son los miotubos, los adipocitos, macrófagos, queratinocitos, hepatocitos y las neuronas (**Nouspikel y Hanawalt 2002**). La mayor parte de las células diferenciadas cuentan con el respaldo de sus precursoras que pueden reemplazarlas en caso de que sufran un daño irreparable. En el caso de las neuronas el respaldo es mínimo, ya que al momento de nacer contamos con un número determinado de éstas que deberán permanecer a lo largo de la vida y que a pesar de que existe neurogénesis en ciertas regiones del cerebro adulto, éste proceso no parece ser relevante en la respuesta al daño al DNA (**Nouspikel y Hanawalt 2002**, **Ming y Song 2005**).

En lo que se refiere a la reparación de DNA en neuronas algunos grupos han sugerido que el proceso de diferenciación neuronal y envejecimiento se encuentra relacionado con la disminución de la reparación. Desde 1972 el grupo de **Sanes y Okun** demostraron que las neuronas primarias de pollo tenían niveles bajos de reparación replicativa después de exposición a luz UV o a metil mentano sulfonato (MMS) en relación a los fibroblastos (**Tabla 1**). De la misma manera **Karran** y colaboradores **(1977**) en un modelo de neuronas de retina de pollo expuestas a acetoxi acetil aminofluoreno (AAAF) o a metil-metano sulfonato (MMS), evidenciaron una disminución de la eliminación de la lesión que producían estos agentes. Otros grupos han utilizado modelos murinos para explorar la capacidad de reparación del DNA en neuronas a través de la diferenciación, por ejemplo exponiendo neuronas primarias de rata a luz UV, rayos X, N-metilpurinas ó menadiona y han obtenido resultados similares a lo reportado en el modelo de pollo. Las neuronas terminalmente diferenciadas mostraron ser más sensibles a estos agentes en relación a los cultivos primarios de fibroblastos de pollo y rata, así como a los astrocitos de rata (**Subrahmanyam y Rao 1991, Wang y Wheeler 1978, Gobbel et al 1998, Hollensworth et al 2000, LeDoux et al 1998, Yamamoto et al 2007**). En lo que se refiere a cultivos celulares, los neuroblastomas han sido el modelo ideal *in vitro* debido a su capacidad para diferenciarse a neuronas a través de la incorporación de factores tróficos en los cultivos (**Abemayor y Sidell 1985, Reynolds, 1986**). Diversos grupos han utilizado modelos murinos de cultivos cancerosos (neuroblastoma, teratocarcinoma, embriocarcinoma y

neuroteratoma) para evaluar la reparación ante estimulos producidos como rayos X, luz UV y agentes alquilantes, y han observado una reducción en la remoción de las lesiones producidas por estos agentes, sugiriendo que los mecanismos de reparación se vuelven menos activos a lo largo del proceso de diferenciación neuronal.

Por todo lo anterior las neuronas, constituyen un sistema interesante en el cual estudiar estos mecanismos de reparación del DNA ya que la muerte neuronal y la respuesta al daño se pueden encontrar asociados al desarrollo de enfermedades degenerativas y al envejecimiento (**Fischel 2007**).

Tabla 1. Respuesta de la Reparación del DNA en Modelos Neuronales.

Modelo y Tipo Celular	Agente	Daños Generados	Reparación Evaluada	Respuesta	Referencia
Pollo, cultivos primarios embrionarios de neuronas. Ganglios de la raíz dorsal	UV y MMS	Agentes alquilantes del DNA. Agente no ionizante, distorsiona al DNA.	NER	Disminución de la NER en relación a los fibroblastos de pollo	Sanes y Okun 1972
Pollo, retina neural de embrión	AAAF o MMS	Agente radiomimético y alquilante.	NER	Disminución de NER a través de la diferenciación	Karran et al 1977
Rata, cultivos primarios de neuronas. Corteza cerebral	UV	Agente no ionizante. Distorsiona la estructura del DNA	NER	Disminución de NER a lo largo de la vida	Subrahmanyam y Rao 1991
Rata, cultivos primarios de neuronas cerebelares primarias	Rayos X	Agente ionizante. Rompe la doble cadena de DNA	DSBR	Neuronas tienen menor reparación que células tumorales o astrocitos	Wang y Wheeler 1978, Gobbel et al 1998
Rata, cultivos primarios de células gliales: astrocitos, oligodendrocitos y microglia.	Menadiona	Agente oxidante	BER	Los oligodendrocitos fueron más susceptibles a la menadiona que los astrocitos y microglía.	Hollensworth et al 2000
Rata, cultivos primarios de células gliales:	MNU	Agente alquilante	BER	Oligodendrocitos remueven menos daño que el resto de los tipos	LeDoux et al 1998

oligodendrocitos, astrocitos y microglia.				celulares	
Rata, cultivos primarios embrionarios de neuronas y astrocitos.	UV	Agente no ionizante	NER	Dismunución en la remoción de 6-4PPs en neuronas y astrocitos en relación a fibroblastos	Yamamoto et al 2007
Ratón, neuroblastoma	UV	UV	NER	Disminución de la reparación en el modelo diferenciado en relación al indiferenciado	McCombe et al 1976
Ratón, neuroblastoma	Rayos X	Agente Ionizante	DSBR	Aumento en la sensibilidad a los rayos X	Byfield et al 1975
Ratón, teratocarcinoma	UV	Agente no ionizante, distorsiona la estructura del DNA	NER	Disminución en la reparación en la diferenciación	Czibula et al 1997
Ratón, teratocarcinoma		Agente alquilante O^6-metilguanina	Reparación directa	Disminución en la actividad de la O^6 alquiltransfersa	Czibula et al 1997
Ratón, embrio-carcinoma	UV	Agente no Ionizante	NER	Disminución en la reparación de las lesiones generadas por UV	Rasko et al 1993
Humano, neuroteratoma	UV	Agente no Ionizante	NER	Disminución en la reparación de la lesiones producidas por UV	Nouspikel y Hanawalt 2000
Humano, células troncales vs cuerpos embrionarios	UV, H_2O_2, psoraleno +	UV: agente no ionizante. H_2O_2: agente oxidante.	BER y NER	Disminución en la reparación a lo largo del proceso de diferenciación	Maynard et al 2008

	UVA, radiación γ	Radiación γ: agente ionizante.			

MMS: metil metano sulfonato, AAAF: acetoxi acetilaminofluoreno, MNU: metilnitrosourea, NER: reparación por escisión de nucleótidos, BER: reparación por escisión de bases y DSBR: reparación de rompimientos de cadena doble.

Defectos en la NER y su posible relación en la etiología de la neurodegeneración

Algunas de las características neuropatológicas y bioquímicas de la pérdida neuronal en varias enfermedades neurodegenerativas, se pueden explicar de tres maneras en relación al DNA: 1) la acumulación anormal de DNA dañado, 2) la reducción en el flujo de información genética requerida para las funciones fisiológicas celulares y 3) el incremento en el número de errores transcripcionales que pueden causar muerte prematura de una población de neuronas. La propuesta de la acumulación de DNA no reparado debida a la disfunción de los mecanismos de reparación de DNA, fue detallada originalmente por **Robins** (1974) y está basada en observaciones que indican que algunas enfermedades como la esclerosis lateral amiotrófica, el síndrome de *Xeroderma pigmentoso*, el síndrome de Cockayne, la ataxia telangectasia, la ataxia espinocereblar con neuropatia axonal, entre otras, están relacionadas con la deficiencia en la reparación de DNA **(Robbins et al. 1974, 1983, 1985; Moshell et al. 1980; Scudiero et al. 1981; Bradley y Krasin 1982a,b; Li and Kaminskas 1985, Mazzarello et al 1992).** Hay al menos tres hipótesis que vinculan al daño persistente en el DNA con la neurodegeneración, la primera es que la disminución de reparación del DNA y el daño persistente alteran la transcripción de genes neuronales fundamentales; la segunda es que el daño al DNA que no es reparado puede llevar a la muerte neuronal masiva. La tercera hipótesis es que el daño persistente resulta en defectos en la neurogénesis, esta se ha reportado en ratones deficientes de BER, observándose menos proliferación en cerebelo durante el desarrollo (**Laposa et al 2001**).

Hay al menos 16 enfermedades hereditarias relacionadas con la reparación de DNA, en las cuales las anormalidades neurológicas son el principal o incluso el único síntoma clínico (**Brooks et al 2008**). Sin embargo, llama la atención los síndromes donde se encuentran mutadas algunas enzimas de reparación que incluyen síntomas neurológicos tales como *Xeroderma pigmentoso* (XP), el síndrome de *Cockayne* (CS) y la *Tricotiodistrofia* (TTD) (**Rolig et al 2000**).

El síndrome de *Xerderma pigmentoso* (XP) es un desorden hereditario, autosómico recesivo caracterizado por hipersensibilidad de la piel a luz UV, resultando en un

incremento de 1000 veces en la incidencia de cáncer de piel, por exposición solar o radiación UV y en la frecuencia de neoplasias en otros tejidos (**Rolig et al 2000, Mazzarello et al 1992, Fishel et al 2006**). En pacientes diagnosticados con este síndrome se ha observado degeneración neurológica, además de anormalidades genéticas que incluyen microcefalea, sordera y retraso mental (**Kraemer et al 2007**). El XP fue el primer desorden asociado a defectos en la reparación del DNA. En general la severidad de la enfermedad se correlaciona con la alteración en NER. Para explicar la neurodegeneración de XP, se ha propuesto que los genes neuronales que son transcritos son dañados continuamente por metabolitos producidos por la célula que mimetizan el efecto de la luz UV. Debido a los defectos en NER estas lesiones no son reparadas, por lo que el daño se acumula, repercutiendo en la transcripción y síntesis de proteínas necesarias para la funcionalidad y sobrevivencia de las neuronas, lo que resulta en la degeneración y muerte prematura de estas células (**Mazzarello et al 1992, Fishel et al 2006**).

Por otro lado, el síndrome de *Cockayne* (CS) se caracteriza por defectos en TCR debido a la mutación en las proteínas CSA y CSB. Los individuos con CS exhiben sensibilidad moderada a la luz UV y defectos en el crecimiento, con degeneración neurológica progresiva en todos los casos. La neuropatologías observadas en este síndrome son las alteraciones oculares (desprendimiento de la retina, ojos hundidos y atrofia óptica), la desmielinización primaria y periférica, sordera, enanismos, microcefalia, degeneración en la espasticidad, hipertensión, osteoporosis, demencia y calcificaciones intracranealies; mientras que no se observan anormalidades en la piel (**Pastoriza et al 2003, Mazzarello et al 1992**).

Los defectos en la transcripción se encuentran asociados también a *Tricotiodistrofia* (TTD), esta es una enfermedad caracterizada por cabello quebradizo deficiente en sulfuro, y fenotípicamente similar a XP y CS. La sensibilidad a luz UV se observa en 50% de los individuos. Las anormalidades neurológicas encontradas son microcefalea, retardo mental, sordera y ataxia. A diferencia de XP, la neuropatología TTD y CS se asocia también con desmielinización (**Rolig et al 2000**).

En individuos sanos el daño al DNA endógeno se produce constantemente pero se repara de la misma manera, lo que resulta en niveles constantes de daño, que son compatibles con la función celular (**Brooks et al 2008**). Sin embargo en personas con ausencia o deficiencia de alguna de las proteínas involucradas en la reparación del DNA, se puede producir la acumulación de lesiones a lo largo del genoma. En células post-mitóticas del cerebro que no son capaces de replicarse o que lo hacen raramente, se acumulan lesiones con el tiempo, incluyendo en los genes que se transcriben constantemente.

En la ausencia de TCR, como ocurre en CS, se incrementa la posibilidad de que la RNA pol II se detenga al detectar lesiones, generando alteraciones en la transcripción o traducción y llevando finalmente a la muerte neuronal. Sin embargo, también puede ocurrir que las lesiones no sean reconocidas por la RNA pol II, lo que puede generar alteración del RNA mensajero producido. Este mensajero generará proteínas mutadas o no funcionales, las cuales contribuirán a la disfunción o muerte celular (**Pastoriza et al 2003, Brooks et al 2008**).

De esta manera, los defectos en la vía de NER pueden conducir a la disfunción neuronal y a la neurodegeneración debido a que las alteraciones que pueden presentar las enzimas de esta reparación contribuyen a la acumulación de errores sobre el DNA que lleve a la muerte celular y con ello a una disfunción neuronal (**Fishel et al 2006**).

Planteamiento del Problema

Debido a que la reparación del DNA es un proceso fundamental para la estabilidad genómica, la pérdida, insuficiencia o ineficiencia de los principales mecanismos de reparación se encuentran relacionadas con el desarrollo de diversas patologías. Este trabajo, está enfocado en la reparación de lesiones oxidativas en el DNA de neuronas terminalmente diferenciadas, dado que este tipo celular es altamente vulnerable por el ambiente oxidativo del órgano en que se aloja. Se decidió estudiar la capacidad de las neuronas terminalmente diferenciadas en la remoción de lesiones oxidativas en el DNA tanto por la vía de reparación por escisión de bases, la cual es la que canónicamente remueve estas lesiones, y por la vía de escisión de nucleótidos, dado que lesiones oxidativas en el DNA pueden comprometer la tridimensionalidad de la molécula. Asimismo, es de interés caracterizar las lesiones oxidativas como guaninas oxidadas, ya que su acumulación en el genoma es considerada una lesión premutagéncia que conduce a alteraciones tales como la muerte celular, proceso que compromete el funcionamiento óptimo del sistema nervioso, manifestándose en el desarrollo de patologías.

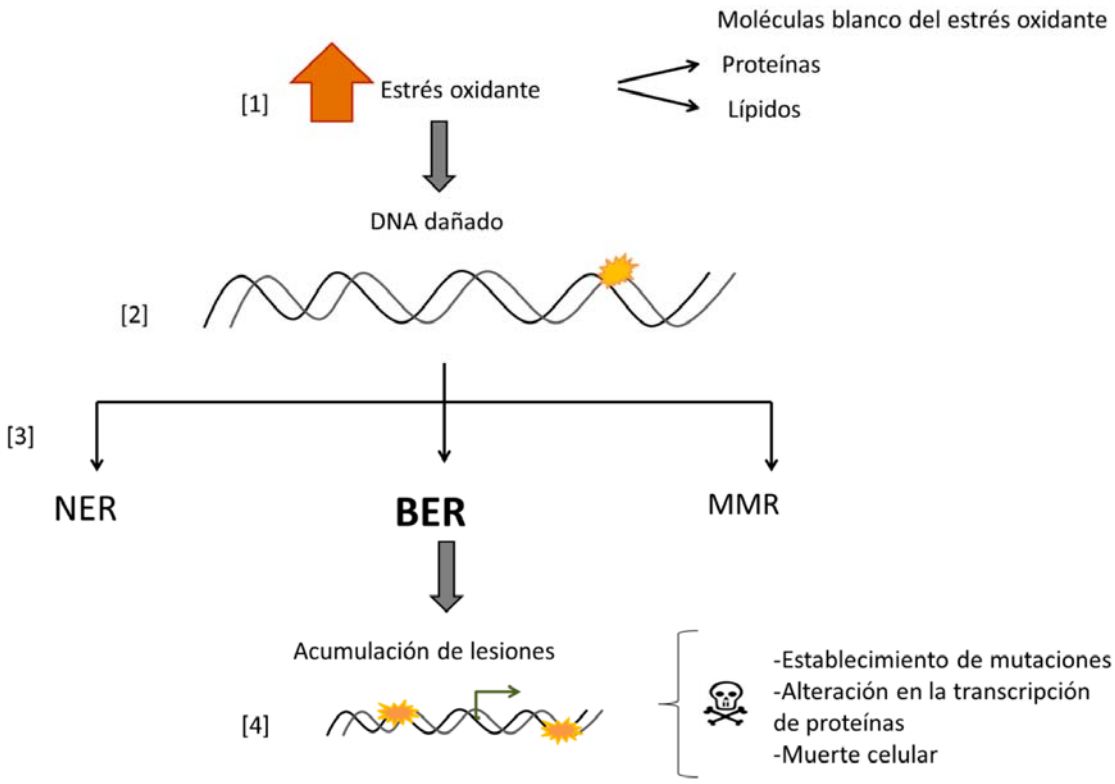

Fig. 4 Planteamiento del problema. Bajo un ambiente oxidante [1], el DNA es susceptible a ser oxidado [2]. La guanina es el blanco principal el cual puede ser removido por los mecanismos de reparación [3]. Cuando esto no ocurre por la deficiencia o alteración de los mecanismos de reparación, las lesiones se acumulan [4], lo que lleva al establecimiento de mutaciones, alteraciones en la transcripción de proteína y por ende en sus funciones, lo cual compromete la función neuronal. NER: Reparación por escisión de nucleótidos, BER: Reparación por escisión de bases y MMR: Reparación de bases mal apareadas.

Hipótesis

Las neuronas terminalmente diferenciadas acumulan más guaninas oxidadas en el DNA en comparación con neuronas indiferenciadas, lo cual se manifestará como una menor capacidad reparativa de dichas lesiones.

Objetivo

Evaluar la capacidad reparativa de los mecanismos de escisión en células indiferenciadas y neuronas diferenciadas ante los retos con peróxido de hidrogeno y UVC.

Objetivos Particulares

- Evaluar la capacidad reparativa por escisión de bases en las células de neuroblastoma humano MSN, en estado indiferenciado y diferenciado ante un inductor de este mecanismo de reparación (H_2O_2).
- Evaluar la capacidad reparativa por escisión de nucleótidos en las células del neuroblastoma humano MSN en estado indiferenciado y diferenciado por la exposición a un inductor de este mecanismo de reparación (luz UVC)
- Determinar las lesiones que se producen ante la exposición a H_2O_2 o UVC en el estado indiferenciado y diferenciado.
- Determinar la participación de las enzimas de reconocimiento de BER y NER ante ambos estímulos (H_2O_2 y UVC) en el estado indiferenciado y diferenciado.

Diseño Experimental

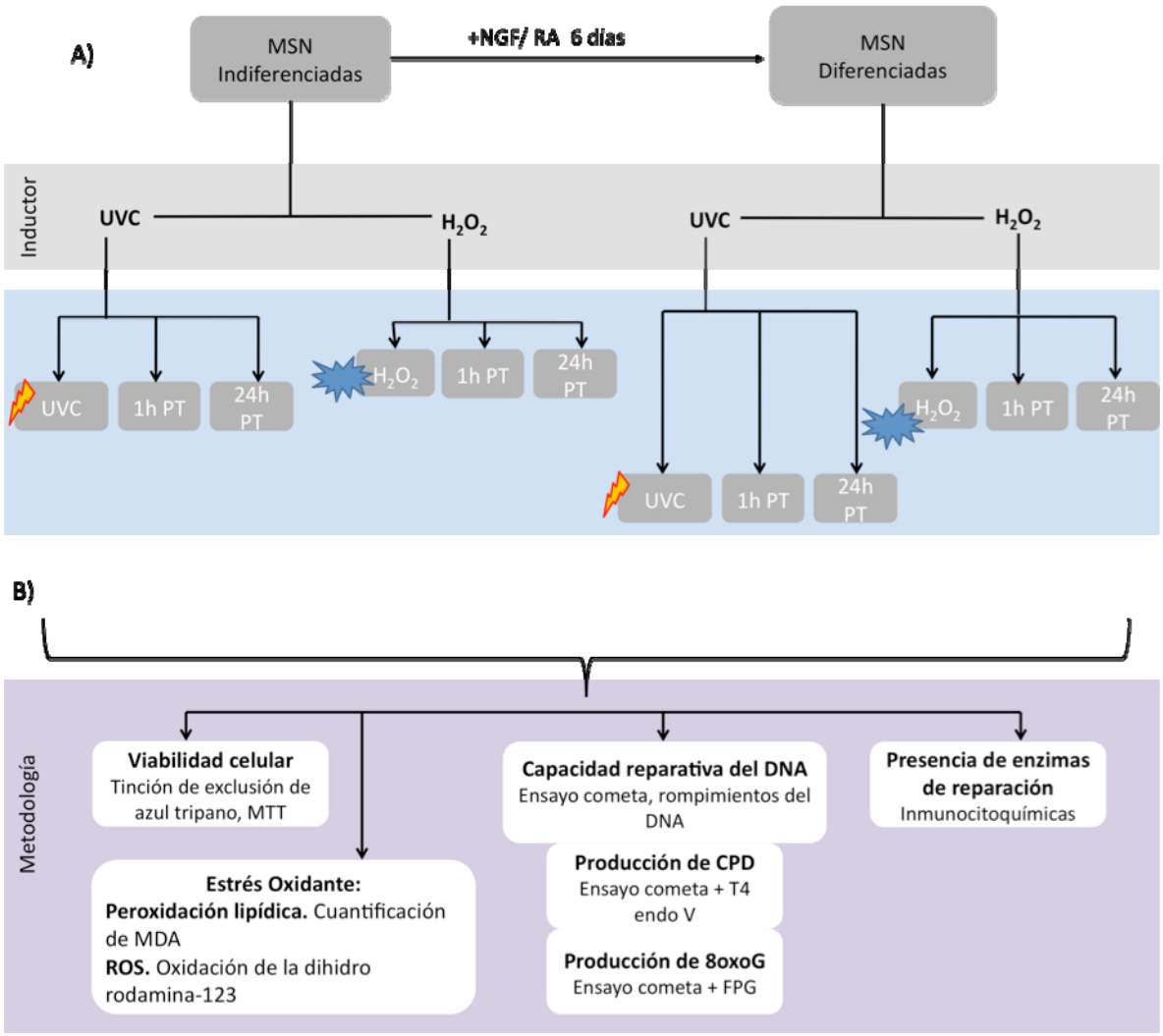

Figura 5. Diagrama del diseño experimental. **A)** Diseño experimental del modelo de neuroblastoma MSN el cual consiste en la comparación del estado indiferenciado en relación al diferenciado. El cultivo celular se expuso al factor de crecimiento neuronal (NGF, por sus siglas inglés) a una concentración de 10µM y ácido retinóico (RA, por sus siglas en inglés) a 50ng/ml por 6 días para diferenciarlos. Ambos se expusieron a dos distintos estímulos de manera independiente: luz UVC, como inductor de NER y peróxido de hidrógeno (H_2O_2), como inductor de BER. Los tratamientos

con H_2O_2 tuvieron una duración de 24 h continuas con el agente oxidante, al cabo del cual se eliminó el estímulo del medio de cultivo, mediante lavados. Posterior a este proceso se evaluaron las células 1h y 24h después, post-tratamiento (PT). La concentración de H_2O_2 utilizada en el primer trabajo fue de 10μM, mientras que en el segundo fue de 50μM. La dosis de exposición a UVC empleadas fue de 3.6 J/cm^2. Todos los tratamientos contaron con un cultivo indiferenciado y diferenciado que no fue expuesto a los estímulos (H_2O_2 y UVC) .**B)** Metodologías empleadas para determinar, viabilidad, estrés oxidante y capacidad reparativa del DNA, en el modelo de neuroblastoma humano en ambos estados de diferenciación.

Resultados

Diferenciación Neuronal y el Modelo del Neuroblastoma Humano: MSN

Contar con el modelo ideal para el estudio de neuronas que nos permita estudiar el desarrollo de enfermedades así como las estrategias terapéuticas para combatirlas, ha sido una limitación. La primera fuente de neuronas son los cultivos primarios de células humanas; esta opción representa dificultades técnicas entre las que están la obtención, manipulación, y mantenimiento; además las cuestiones éticas la vuelven difícil de obtener. Una alternativason las células de neuroblastoma que como ya se mencionó antes son comúnmente utilizadas en estudios de diferenciación neuronal (**Rubestein et al 1985, Prasad et al 1973, Perez-Polo et al 1979, Sidell et al 1983, Reynolds et al 1981, Sonnefeld y Ishii1983**). Estos cultivos neuronales provenientes de tumores proveen grandes ventajas como son una cantidad ilimitada de células de origen humano con características bioquímicas y morfológicas semejantes a las neuronas humanas (**Machin et al 1982**). Otra característica es la versatilidad con la que éstas se diferencian ante una gran variedad de estímulos como el uso de esteres de forbol (TPA), ácido retinóico, neurotrofinas y estaurosporina (**Xie et al 2010**). Sin embargo es importante considerar que el cultivo se encuentra en proliferación en su estado indiferenciado y que al iniciar el proceso de diferenciación el cultivo no se encuentra sincronizado.

En este trabajo se echó mano del modelo de diferenciación del neuroblastoma humano (MSN), el cual fue descrito por el grupo de **Reynolds** en 1986, estas células se originan de neuroblastos de la cresta neural. Se ha reportado que las células MSN son capaces de adquirir una morfología semejante a una neurona mediante la adición de NGF y AR (**Quiroz-Baez et al 2009**). En el laboratorio se determinó el tiempo de diferenciación con base en dos marcadores proteicos, el antígeno neuronal nuclear (NeuN por sus siglas en inglés) y la proteína asociada a microtúbulos 2 (MAP2, por sus siglas en inglés) que indicarían cuando las células se encontraban terminalmente diferenciadas a un tipo neuronal. Como primer acercamiento se siguió el crecimiento en cultivo del

neuroblastoma en estado indiferenciado por diez días (Figura 6 línea negra) y se comparó con el cultivo en diferenciación (Figura 6 línea roja). Como se puede observar las células indiferenciadas se continúan replicando a lo largo del tiempo; sin embargo, en el cultivo diferenciado, las células muestran una reducción en la proliferación celular, indicando que los factores empleados para la diferenciación impiden a las células seguir entrando al ciclo celular tal como ocurriría en las neuronas terminalmente diferenciadas.

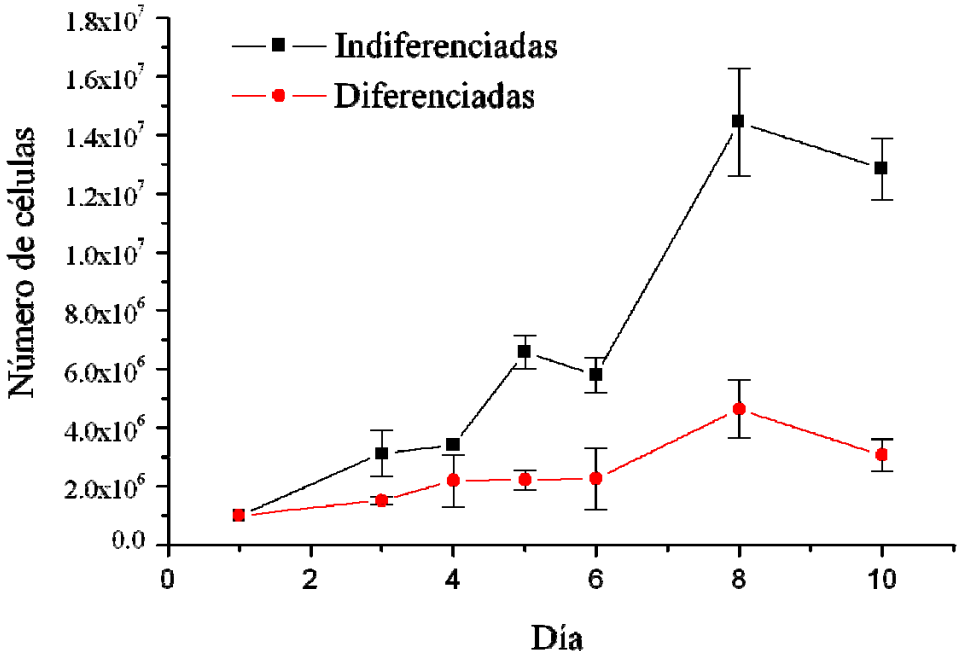

Figura 6. Curva de crecimiento del cultivo celular del neuroblastoma humano, MSN. Las células fueron contadas en un hemocitómetro mediante la tinción por exclusión con azul tripano con el fin de evaluar el número total de células indiferenciadas (línea negra) y en proceso de diferenciación (línea roja) en cultivos de diferente número de días. Los resultados son el promedio de tres experimentos independientes ± la desviación estándar.

En la figura 7 se muestra por inmunocitoquímica, la presencia de los marcadores proteicos de neurona madura: la proteína asociada a microtúbulos (MAP2, por sus siglas en inglés) en ambos estados de diferenciación (Figura 7A), se puede apreciar que la morfología del cultivo diferenciado muestra crecimiento de neuritas similar al de las neuronas humanas,

y que si bien, la presencia de los marcadores se presenta desde el estado indiferenciado, ésta se acentúa más después de la diferenciación.

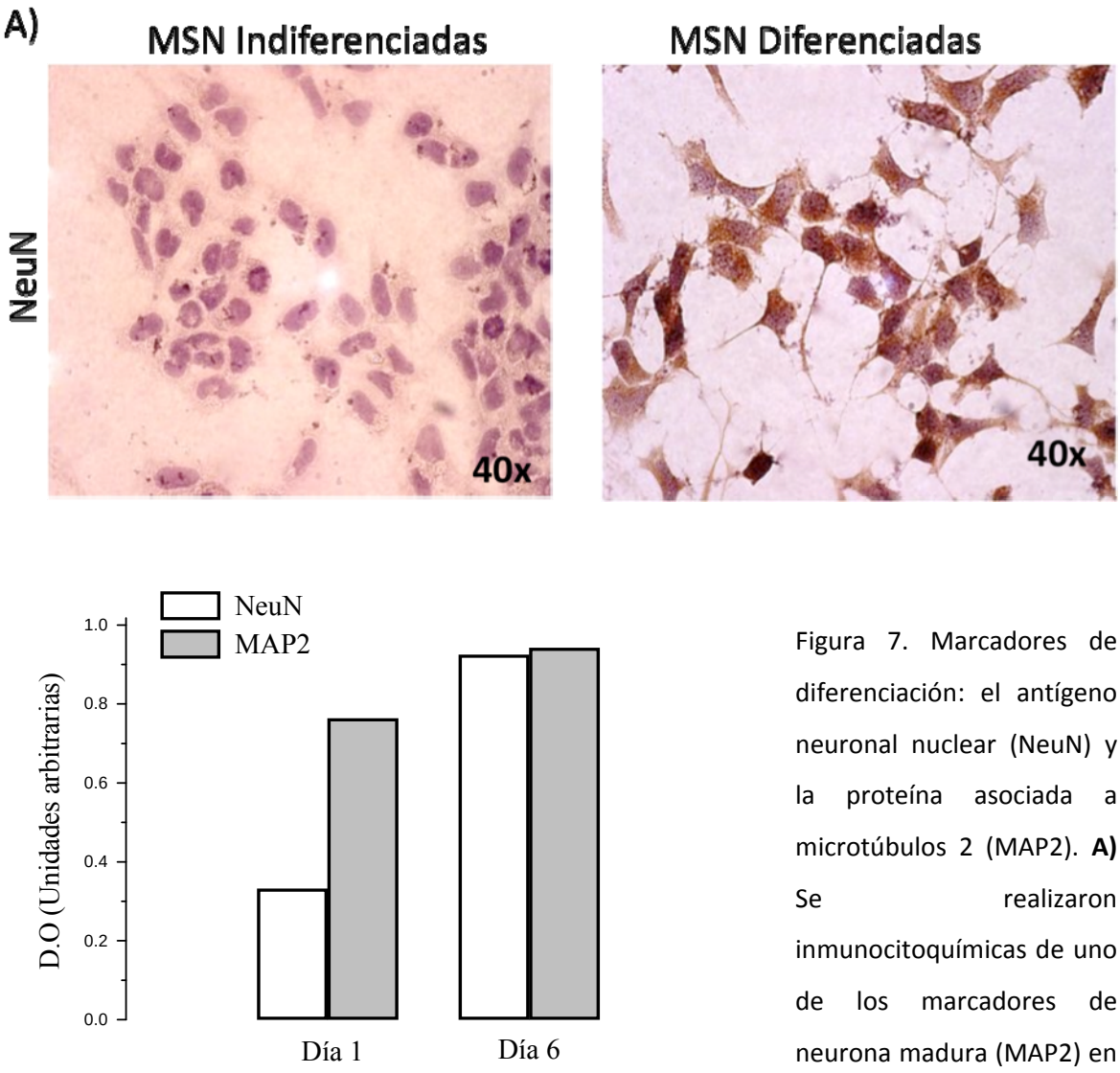

Figura 7. Marcadores de diferenciación: el antígeno neuronal nuclear (NeuN) y la proteína asociada a microtúbulos 2 (MAP2). **A)** Se realizaron inmunocitoquímicas de uno de los marcadores de neurona madura (MAP2) en ambos estados de diferenciación, utilizando una contra tinción con hematoxilina y diaminobencidina para evidenciar la marca positiva en la célula. **B)** La gráfica muestra la cuantificación proteica de NEUN y MAP2 en el día de inicio de la diferenciación y seis días después.

Con base en estos resultados se concluyó que el cultivo en diferenciación era estable y mostraba características similares a las de un tipo neuronal (morfología y marcadores).

Debido a que el cultivo mostraba características de neurona madura al día seis, se decidió utilizar estas condiciones para el trabajo de esta tesis.

Artículo 1:

"DNA damage and repair in neural cells"

The Neurochemestry of Neuronal Death

Research Signpost

En el siguiente trabajo se hace una revisión de los mecanismos de reparación del DNA y su relación con el desarrollo de enfermedades neurodegenerativas. También se incluyen los datos obtenidos del modelo del neuroblastoma humano (MSN) en estado indiferenciado y diferenciado expuesto a concentraciones de peróxido de hidrógeno equivalentes a las producidas por la agregación de la proteína β-amiloide (**Huang et al 1999**) con el fin de evaluar la capacidad reparativa en respuesta a un daño oxidante. A continuación el resumen de este trabajo:

El cerebro se encuentra en riesgo por el daño oxidativo inherente a su fisiología. Para preservar la integridad del DNA, la célula utiliza varios mecanismos de reparación, dentro de estos se encuentra la NER, el cual remueve diferentes lesiones distorsionantes del DNA. La NER está constituida por dos sub vías: GGR y TCR. La primera remueve las lesiones a lo largo del genoma, mientras que la segunda actúa únicamente en sitios en transcripción. Las células post mitóticas como las neuronas, por principio, podrían llevar a cabo la reparación de solo la porción del genoma encargado de sus funciones especializadas en lugar de detectar todo el DNA, lo cual favorecería la acumulación de daño en el DNA a lo largo de varios años en los genes no transcritos o durmientes durante su estado post mitótico. En este contexto las neuronas constituyen un sistema interesante para el estudio de la reparación del DNA, especialmente en relación a la transcripción. El objetivo de este estudio fue determinar el papel de la diferenciación neuronal en las vías de NER: TCR y GGR, utilizando al neuroblastoma humano MSN, como modelo de diferenciación ante un reto con peróxido de hidrógeno en las células indiferenciadas y diferenciadas MSN.

La estrategia con la que se abordó la parte experimental de este trabajo se resume en el siguiente diagrama:

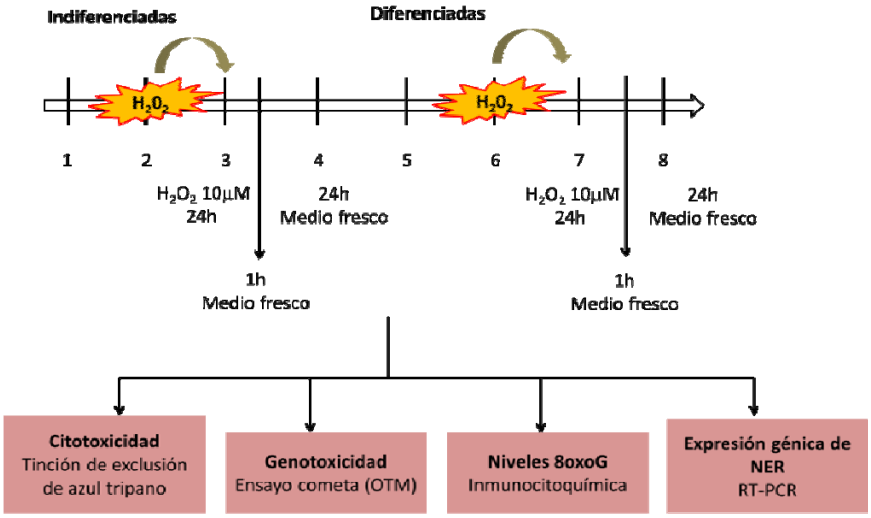

Figura 8. Estrategia experimental. El cultivo celular MSN fue expuesto a una concentración de peróxido de hidrógeno (H_2O_2) 10µM en el estado indiferenciado, una vez transcurridas 24 horas se evaluó la citotoxicidad, genotoxicidad, los niveles de 8oxoG y la expresión génica de las enzimas de reparación de NER. Posterior a este punto de muestreo, se cambió el medio con H_2O_2 por medio fresco y se realizaron las mismas determinaciones 1 y 24 horas posteriores al primer estímulo oxidante. Se realizó la misma estrategia para las células diferenciadas, se tomó como día de inicio 6 días después de haber sido inducida la diferenciación neuronal con la adición NGF y RA, tal y como se describió anteriormente.

La metodología empleada se describe brevemente a continuación:

Citotoxicidad: Para evaluar la toxicidad por los tratamientos se realizó un conteo de células vivas contra el número de células muertas. La determinación realizada fue por la tinción de exclusión del colorante azul tripano, donde las células vivas que no tienen comprometida la integridad de la membrana, no permiten que el colorante las penetre, observándose incoloras; mientras que las muertas se teñirán de color azul.

Genotoxicidad: Esta prueba permite evidenciar el DNA fragmentado mediante una electroforesis de células individuales en condiciones alcalinas, donde los fragmentos migran hacia el ánodo evidenciándose bajo el microscopio de fluorescencia, al ser teñidos con un cromóforo como el bromuro de etidio, lo que resulta en imágenes semejantes a un cometa cuya cola es proporcional al grado de daño. Se evaluaron 100 células por condición por experimento independiente midiendo el tamaño de la cola. Se estimaron las

frecuencias de los valores para cada muestra para tener una gráfica de histograma que representara el comportamiento por población en cada punto de muestreo.

Niveles de 8oxoG: Para determinar los niveles de 8oxoG, se realizó una inmunocitoquímica, donde se empleó un anticuerpo que reconoce de manera específica esta lesión y posteriormente se realizó una cuantificaciónde las células positivas a esta lesión. **Expresión génica:** Para determinar la expresión génica de las enzimas Rad23 ó HR23b, CSA, CSB y XPD, se recurrió a la transcripción reversa de la cadena de la polimerasa (RT-PCR). Las secuencias utilizadas para cada gen son los siguientes:

RAD23b-F AGAGCCAGTTTCAACAACCC

RAD23b-R GAAGCAAGGAAGGATTCTGC

CSA-F TGATGGACTTCACCTCCTCA

CSA-R CTCTCATCACTGCTGCTCCA

CSB-F CAGAAGAAGAGACCCTGGAG

CSB-R GTACATAATCTGGGCTGGCT

XPD-F AACTTCTCTGTGCAGCATTCC

XPD-R TCGGAAGACACAAGACTGTG

Los resultados obtenidos en este trabajo fueron los siguientes:

- El reto oxidante no fue citotóxico para las células indiferenciadas, no así para el estado diferenciado.

- En cuanto a lo que al daño genotóxico se refiere, los resultados muestran que el daño basal oxidante es mayor en las células diferenciadas, sin embargo en ambos estados de diferenciación se restaura el daño sobre el DNA.

- Para confirmar el daño oxidativo se evaluó la presencia de 8oxoG, cuyos niveles no se modificaron con el estado de diferenciación, pero si cambiaron en respuesta al estimulo oxidante y la desaparición de los rompimientos sobre el DNA.

- Finalmente, la expresión de los genes de NER en las células indiferenciadas sugieren la actividad de ambas vías en respuesta al estímulo oxidante, por otro lado las células diferenciadas carecieron de la expresión de CSA, sobre expresando únicamente los genes Rad23b y XPC, lo que podría sugerir una reparación dependiente de TCR.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

The Neurochemistry of Neuronal Death, 2007: 121-137 ISBN: 81-308-0086-1
Editors: Lourdes Massieu, Clorinda Arias and Julio Morán

6 DNA damage and repair in neural cells

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Abstract

Brain is at risk for oxidative damage due to its physiology. To preserve DNA integrity, cells utilize many repair mechanisms; one of these is the Nucleotide Excision Repair (NER), which removes different DNA-distorting lesions. Two main pathways are involved in NER: Global Genome Repair (GGR) and Transcription Coupled Repair (TCR). GGR removes lesions along the genome in non-transcribed sites, while TCR acts in the transcribed strand and requires the transcription machinery. Neurons and other post-mitotic cells could thus, in principle, afford to repair only the portion of their genome that is really needed for their specialized functions, as transcribed genes, promoting the DNA-damage accumulation in their

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dormant genes during their post-mitotic stage over many years. In this context, neurons constitute a particularly interesting system for the study of DNA-repair, especially in conjunction with transcription. The aim of our investigation is to determine the role of both NER pathways in neuronal differentiation, using the neuroblastoma cell line, MSN, as a differentiation model. We evaluated the basal oxidative DNA-damage and the efficiency of their repair mechanisms after H_2O_2 challenge, in the undifferentiated and differentiated MSN cells. In addition, to confirm the oxidative damage we evaluated the presence of 8-oxoguanine (8oxoG). Our results showed a higher cytotoxicity and basal DNA damage in differentiated than undifferentiated MSN cells. The NER's gene expression: Rad23 (GGR), CSA (TCR), and XPD (GGR and TCR), in undifferentiated MSN cells suggests the activity of both GGR and TCR mechanisms in response to the oxidative insult. However, in differentiated MSN cells, the lack of expression of CSA, cursing with the expression of Rad23 and XPD could suggest a TCR-independent DNA repair.

Introduction

DNA is constantly under the attack of endogenous and environmental agents. Endogenously formed reactive oxygen species (ROS) are reported as responsible for the elevated oxidative DNA damage detected in brains of subjects with stroke or chronic neurodegenerative disease [1]. In addition, exposure to environmental agents can potentially damage brain tissue DNA. Preservation of genomic stability is an essential biological function. Cells engage very efficiently mechanisms involving DNA surveillance/repair proteins that act to maintain inherited nucleotide sequence of genomic DNA over time. After DNA damage, which can arise both physiologically and pathologically during duplication, or after genotoxic stimuli, cells activate intracellular pathways, which are able to recognize damage, arrest cell cycle, recruit DNA repair factors, and repair damage or induce apoptosis. This definitely relevant process is finalized to prevent the generation and the persistence of impaired cells; which may ultimately be detrimental to the organisms. Very little is known about the role of DNA damage sensors and repair factors in terminally differentiated, not proliferating cells, such as neurons. It is well recognized that mutation of genes related to DNA damage repair is associated with specific cancer-prone syndromes. Many human pathological conditions with genetic defects in DNA damage response are also characterized by neurological deficits. These neurological deficits can manifest themselves during many stages of development, suggesting an important role for DNA repair system during the development maintenance of the brain. Relatively few studies have examined the expression of DNA repair proteins in the different brain cell types, including astrocytes, oligodendrocytes and microglia [2].

Neuronal oxidative damage

The aerobic organisms are susceptible to oxidative stress simply because semireduced oxygen species, superoxide and hydrogen peroxide, are produced by mitochondria during respiration [3]. Brain is considered abnormally sensitive to oxidative damage and in fact early studies demonstrating the propensity to peroxidation of brain membranes supported this notion [4,5]. The intrinsic brain physiology imply the generation of oxidative stress because: a) brain requires very high amounts of oxygen per unit weight (about 20% of the total amount used in humans); b) brain has a high content of Fe in certain regions and in general has high levels of ascorbate, both powerful lipid peroxidation generators; it has also a high content of easily peroxidizable unsaturated fatty acids; c) brain is not highly enriched in antioxidant protective defenses and this then adds to its otherwise readily poised potential for oxidative damage [6,7].

In detail, brain sensitivity to oxidative damage is due to nine principal features. One reason is its high demand for oxygen due to the high ATP consumption of neurons, for the maintenance of membrane potentials and the release and storage of neurotransmitters. This in turn means that neurons rely heavily on efficient mitochondrial function. In many neurodegenerative diseases both mitochondrial defects and oxidative damage co-exist [8]. A second potential problem for the brain is its extensive use of glutamate as a neurotransmitter. Impaired energy metabolism in the brain can lead to excess extracellular glutamate levels, whose receptor binding leads to a rising of intracellular Ca^{2+} to pathological levels. This overactivates phospholipase A_2, calpain and neuronal (n) NOS activities, leading to elevated $NO^{\cdot}$ production, release of free fatty acids and excessive proteolysis. Thirdly, many neurotransmitters are autoxidable molecules. Dopamine, its precursor levodopa and noradrenaline react with O_2 to generate $O_2^{\cdot-}$, H_2O_2 and reactivate quinones/semiquinones that can deplete GSH and bind to protein –SH groups [9]. Fourthly, iron is found throughout the brain. Important iron-containing proteins in brain include cytochromes, ferritin, aconitases, mitochondrial non-haem iron proteins, cytochromes P450 and the tyrosine and tryptophan hydroxylase enzymes, which catalyze the first step in the synthesis of dopamine and serotonin, respectively. Several brain areas (e.g. subtantia nigra, caudate nucleus, putamen, globus pallidus) have a high iron content. Most of the total iron in healthy brain is apparently stored as ferritin, which should limit its capacity to catalyse oxidative damage. However, damage to brain tissue readily releases iron (and copper) ions in forms that are capable of catalyzing such free radical reactions as $OH^{\cdot}$ formation from H_2O_2, lipid peroxidation, and autoxidation of neurotransmitters. Fifthly, neuronal membrane lipids contain highly polyunsaturated fatty-acid side-chains, especially docosahexaenoic ($C_{22:6}$) acid residues. These highly polyunsaturated

fatty acids are extremely susceptible to lipid peroxidation, at least *in vitro* [9]. For example, in some brain samples from patients with Alzheimer´s disease, elevated oxidation of docosahexanoic, but not of arachidonic, residues was detected [10]. Hydroxynonenal (HNE), a cytotoxic end product of the decomposition of lipid peroxides, seems especially cytotoxic to neuronal cells [11], and elevated levels of HNE in brain tissues have been detected in many neurodegenerative diseases [12]. Sixthly, brain metabolism generates H_2O_2. A major source of H_2O_2 is the oxidation of dopamine by monoamine oxidases (MAOs), flavoprotein enzymes located in the outer mitochondrial membrane. Seventhly, antioxidant defences in the brain are modest. In particular, levels of catalase are low in most brain regions. The catalase in brain appears to be located in small peroxisomes and thus could probably not deal efficiently with H_2O_2 generated in other subcellular compartments (e.g. MnSOD or MAOs, both located in mitochondria). In general, brain catalase in mammals is found at higher levels in hypothalamus and substantia nigra than in cortex or cerebellum [8]. Eightly, some of the glial cells found in the brain are microglia, which are resident macrophage-type cells. Like other macrophages, microglia can produce $O_2^{\cdot}$ and H_2O_2 upon activation and are capable of secreting cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α. Finally, cytochromes P450 (CYPs) are present in certain brain regions. Reactive species can 'leak away' from the catalytic intermediates in the P459 cycle, generating O_2^{+} and H_2O_2. Several isoforms of CYP have been detected in brain, for example, in rats CYP2E1 is present in hippocampus, in substantia nigra and in the blood-brain barrier [13]. Since CYP2E1 is more leaky than other CYPs, it is capable of producing more reactive species; it is another potential source of oxidative stress [8].

In addition, the fact that brain respiration almost exclusively utilizes glucose as energy source, probably explains why it consumes very high levels of oxygen per unit weight. Careful studies on isolated mitochondria from brain and other organs show that 2-5% of the total oxygen consumed in mitochondrial respiration yields reactive oxygen species (ROS), mostly as H_2O_2 [14,15]. Oxidative damage occurs as a result of processes involving ROS and trace metals causing damage to biomolecules as proteins, lipids, nucleic acids and metabolites. Unique oxidation products are formed, for instance, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) by the reaction of ROS with DNA [7]. At the time of oxidative damage formation, other processes occur that are involved in the removal or repair of these damage. Therefore, the total amount of oxidative damage products present is due to the balance between the rates of these processes. Oxidative damage to DNA produces strand breaks as well the oxidation of many bases. Typically, strand breaks are repaired and oxidized bases are removed by specific enzymes.

DNA damage and neurodegeneration

One of the most historically well known connections between abnormalities of the DNA damage response and neurodegeneration has been the human syndrome of ataxia telangiectasia (AT). However, others as xeroderma pigmentosum (XP), Nijmegan breakage syndrome (NBS), trichothiodystrophy (TTD) and Cockayne syndrome, have been associated with a defective DNA damage response and neurological disorders as a primary feature of their phenotypes [16]. There are many repair-genes, some of them are responsible

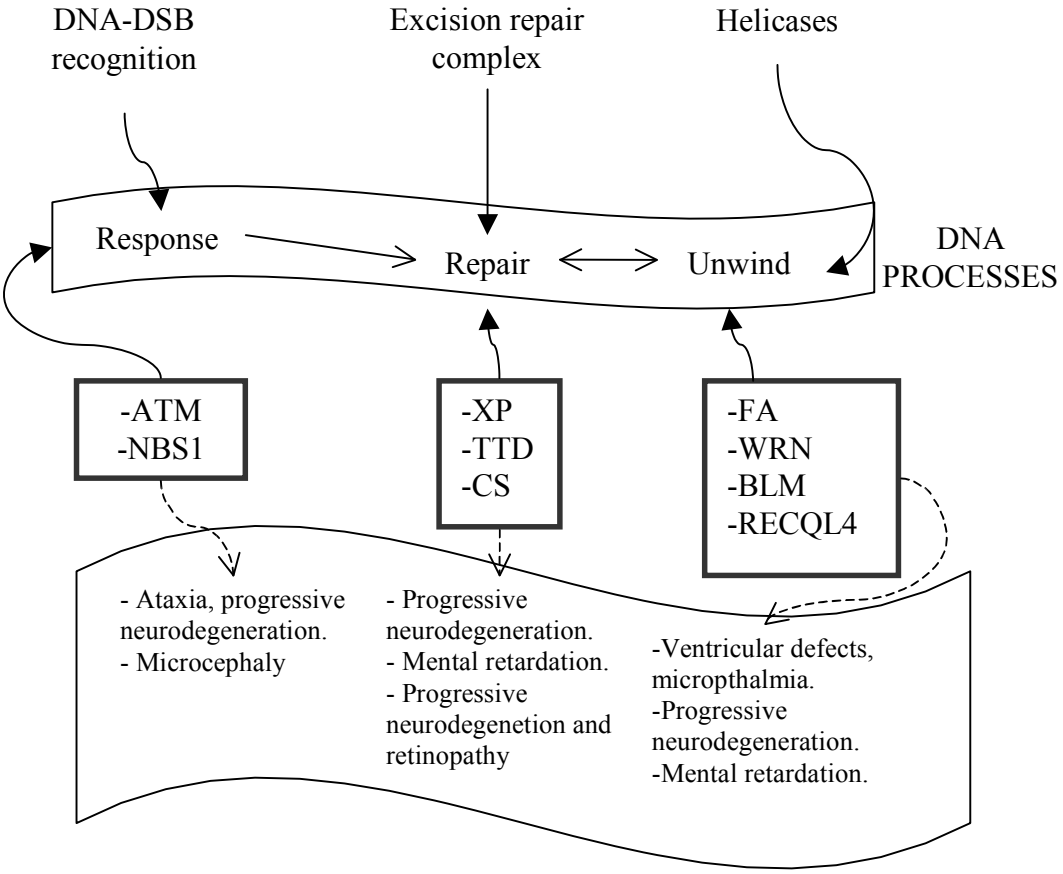

Figure 1. Dysfunction of DNA damage response and repair genes leads to neurodegeneration and some specific neuropathologies. Recognition to DNA double strand breaks sensed by response proteins as ATM and NBS1 is affected in pathologies as Ataxia Telangiectasia (ATM) and Nijmegan Breakage Syndrome (NBS). Defects on individual members of the excision repair complex as XP, TTP and CS trigger principally a progressive neurodegeneration and mental retardation, however in some subgroups of Xeroderma Pigmentosum (XP), neuropathology has not been found. Other pathologies as Fanconi Anemia (FA), Werner Syndrome, Bloom Syndrome and Rothmund-Thomson Syndrome have dysfunctional genes (FA, WRN, BLM and RECQL4) and proteins with helicase activity; however, all of these are more characterized by cancer predisposition than neurodegeneration, although they show neurological alterations.

for the diseases, other encode components of DNA-repair complexes however, repair deficiencies *per se* might not explain the pathology as we show in fig. 1. Together, these human syndromes provide an important insight into the fundamental processes that affect nervous system function and which probably involve DNA damage.

These kind of syndromes have defective DNA-repair or response machinery and varying degrees of neurological dysfunction. This suggests that defects in the repair of, or response to, DNA damage impact significantly on nervous system function. Although substantial in vitro data have accumulated regarding the molecular deficits in these repair or response deficiencies, few data are available that describe specific functions in the nervous system. Potentially endogenous reactive oxygen species, produced as by-products of cellular metabolism could act as genotoxic agents in the nervous system of repair-compromised individuals [16]. Analysis of age-dependent mutations in brain and liver showed a significantly lower level of genome rearrangements in brain compared to liver [17]. The postmitotic status of the brain might require efficient systems for avoiding mutations; this would have implications for the syndromes considered previously, particularly those with progressive neurodegeneration. DNA repair assays using extracts isolated from different brain regions are providing insights into this possibility. Another possibility to account for neurodegeneration, derived from cancer studies, is the concept of caretakers [18]. Many of the inherited mutations responsible for the syndromes discussed above lead to genomic instability and are therefore mutations of caretaker genes. This instability in the nervous system could promote mutations that ultimately lead to cell death rather than proliferation. The cell type and particular caretaker mutations might determine tissue and cellular selectivity associated with the different neurological alterations. It is also possible that in the nervous system many of the DNA repair- or response components might have other non-repair-associated roles, and defects in these functions could lead to neuropathology.

Oxidative DNA damage repair mechanisms

There have been many suggestions that ROS play an important role in, and may even be causative of neurodegenerative diseases, in particular tardive dyskinesia, Parkinson´s disease, Huntington´s disease, Alzheimer´s disease, Friederich´s ataxia and Down´s syndrome [8]. ROS generate mainly oxidative DNA damage as a principal target; challenging the repair mechanisms that safeguard the genomic integrity. DNA repair is one of the most essential systems for saving the inherited nucleotide sequence of genomic DNA over time, for such, the cells engage very efficient mechanisms as Base Excision Repair (BER) and Nucleotide Excision Repair (NER).

Base-excision repair (BER)

The most general DNA repair mode observed in nature is one in which damaged or inappropriate bases are excised from the genome and replaced by the normal nucleotide sequence and chemistry, also known as base excision repair [19]. Under normal circumstances DNA undergoes depurination at a measurable rate such that some 10,000 abasic sites/mammalian cell/day are generated [20-22]. In addition, a variety of lesions in DNA arise by spontaneous deamination of cytosine, from errors occurring during replication including insertion of damaged bases or of uracil, from reactions with endogenous ROS [22-24] or from exogenous exposure to toxic species in food and/or the environment. The lesions include among others uracil, 3 methyladenine, 8 oxoguanine, and thymine glycol.

Nonbulky lesions are generally recognized by the DNA glycosylases that remove the modified base and leave an abasic site (fig. 2). Higher eukaryotic cells have two classes of DNA glycosylases, those that remove the base only and those that remove the base and nick the DNA on the 3' side of the phosphodeoxyribose (dRP) residue.

Enzymes in the first category include uracil DNA glycosylase (UDG), G/T glycosylase, thymine glycol-DNA glycosylase, thymine DNA glycosylase, and 3 methyl adenine-DNA glycosylase [25]. UDG is by far the most abundant and active of these, because the presence of uracil in DNA occurs at high rates [26]. The second set of glycosylases includes 8-oxoguanine DNA glycosylase, hNth1, N-methylpurine/DNA glycosylase, and adenine-specific DNA glycosylases [22, 27, and 28].

The core BER reaction is initiated by strand incision at the abasic site by the APE1 endonuclease (fig. 2 reaction I). Poly(ADP-ribose) polymerase (PARP), which binds to and is activated by DNA strand breaks, and the recently identified polynucleotide kinase (PNK) [29], may be important when BER is initiated from a single strand break (SSB) to protect and trim the ends for repair synthesis (reaction II). In mammals, the so-called short-patch repair is the dominant mode for the remainder of the reaction. DNA pol β performs a one-nucleotide gap-filling reaction (reaction III) and removes the 5'-terminal baseless sugar residue via its lyase activity; this is then followed by sealing of the remaining nick by the XRCC1–ligase3 complex (reaction IV). The XRCC1 scaffold protein interacts with most of the above BER core components and may therefore be instrumental in protein exchange. The long-patch repair mode involves DNA pol β, pol δ and proliferating cell nuclear antigen (PCNA) for repair synthesis (2–10 bases) as well as the FEN1 endonuclease to remove the displaced DNA flap and DNA ligase 1 for sealing (reaction V-VII). The above BER reaction operates across the genome. However, some BER lesions block transcription, in this case TCR pathway take place as described below, including TFIIH, XPG (which also stimulates some of the glycosylases) and probably the remainder core of NER apparatus [30].

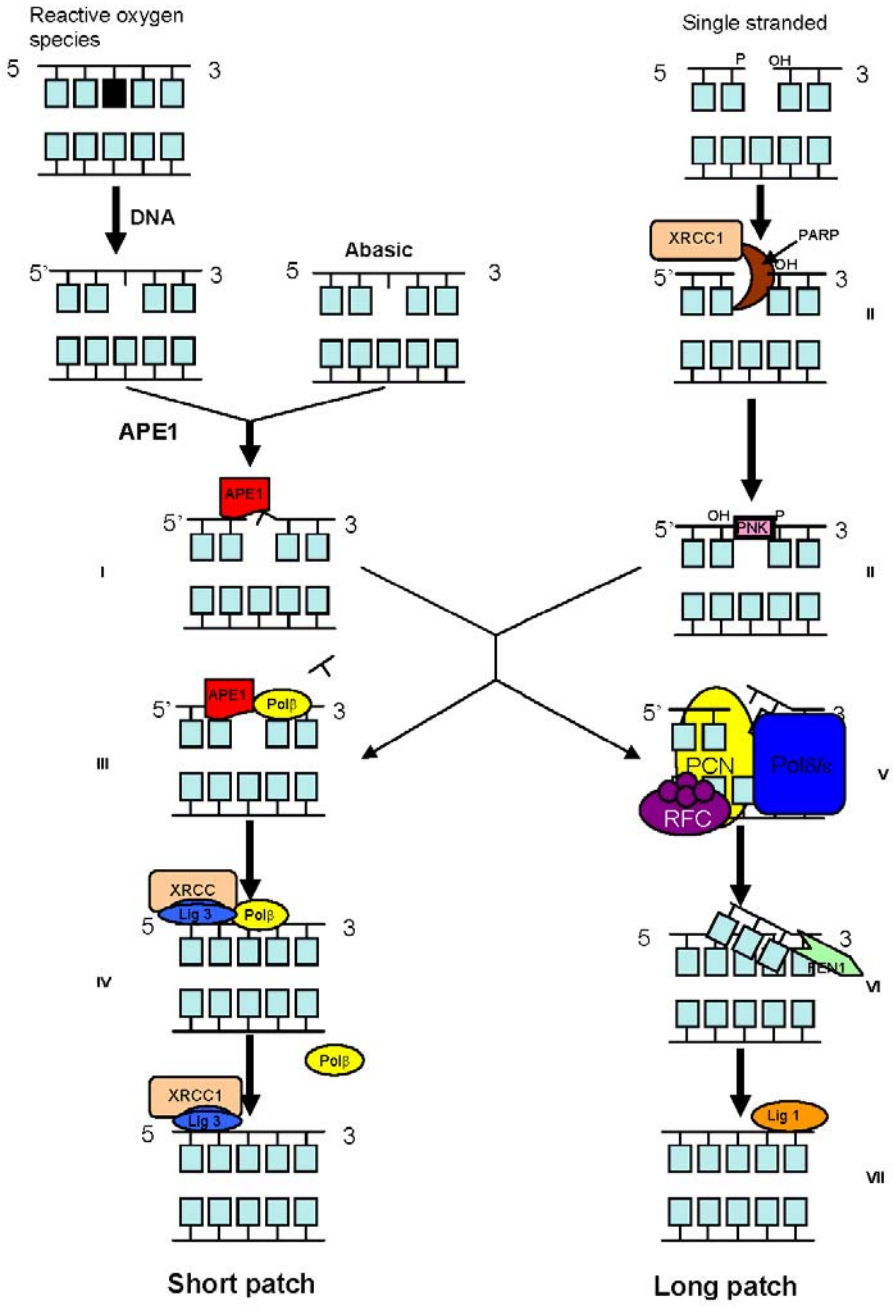

Figure 2. Base Excision Repair. The core BER reaction is initiated by strand incision at the abasic site by APE1 endonuclease (reaction I). In the other hand Poly(ADP-ribose) polymerase (PARP) is activated by DNA strand breaks and in junction with polynucleotide kinase (PNK) protects and trim the ends for repair synthesis (reaction II). In the Short patch pathway, DNA pol β performs a one nucleotide gap-filling reaction and removes the 5' terminal baseless sugar residue via its lyase activity (reaction III). The sealing of the remaining nick is performed by the XRCC1-ligase3 complex (reaction IV). The long patch repair mode involves DNA pol δ and proliferating cell nuclear antigen (PCNA) for repair synthesis, as well FEN1 endonuclease to remove the displaced DNA flap and DNA ligase for sealing (reaction V-VII).

Nucleotide-excision repair (NER)

NER is a particularly intriguing repair pathway because of its extraordinarily wide substrate specificity; it has the ability to recognize and repair a large number of structurally unrelated lesions, such as DNA damage formed upon exposure to the UV radiation from sunlight, and numerous bulky DNA adducts induced by mutagenic chemicals from the environment or by cytotoxic drugs used in chemotherapy. NER operates through a "cut-and-patch" mechanism by excising and removing a short stretch of DNA (24- to 32-nucleotides long) containing the damaged base; the original genetic sequence is then restored using the non damaged strand of the DNA double helix as a template for repair synthesis. Two distinct sub-pathways have been discerned (Fig. 3): global genome NER (GGR), which surveys the entire genome for distorting injury, and transcription coupled NER (TCR) that focuses on damage that blocks elongating RNA polymerases [31, 32].

The basic steps of nucleotide excision repair are (*a*) damage recognition, (*b*) dual incisions bracketing the lesion to form a 12–13-nt oligomer in prokaryotes or a 24–32-nt oligomer in eukaryotes, (*c*) release of the excised oligomer, (*d*) repair synthesis to fill in the resulting gap, and (*e*) ligation.

The GGR-specific complex XPC-hHR23B screens first on the basis of disrupted base pairing [33], instead of lesions per se. This explains why mildly distorting injury such as cyclobutane pyrimidine dimers are poorly repaired [34]. In TCR, the ability of a lesion (whether of the NER- or BER-type) to block RNA polymerase seems critical (Fig. 3 reaction I). The stalled polymerase must be displaced to make the injury accessible for repair [35], and this requires at least two TCR-specific factors: CSB and CSA. The subsequent stages of GG-NER and TCR may be identical. The XPB and XPD helicases of the multi-subunit transcription factor TFIIH open ~30 base pairs of DNA around the damage (reaction II). XPA probably confirms the presence of damage by probing for abnormal backbone structure [36], and when absent aborts NER [33]. The single-stranded-binding protein RPA (replication protein A) stabilizes the open intermediate by binding to the undamaged strand (reaction III). The use of subsequent factors, each with limited capacity for lesion detection *in toto*, still allows very high damage specificity [37]. The endonuclease duo of the NER team, XPG and ERCC1/XPF, respectively cleave 3' and 5' of the borders of the opened stretch only in the damaged strand, generating a 24–32-base oligonucleotides containing the injury (reaction IV). The regular DNA replication machinery then completes the repair by filling the gap (reaction V). In total, 25 or more proteins participate in NER. *In vivo* studies indicate that the NER machinery is assembled in a step-wise fashion from individual components at the site of a lesion. After a single repair event (which takes several minutes) the entire complex is disassembled again [38].

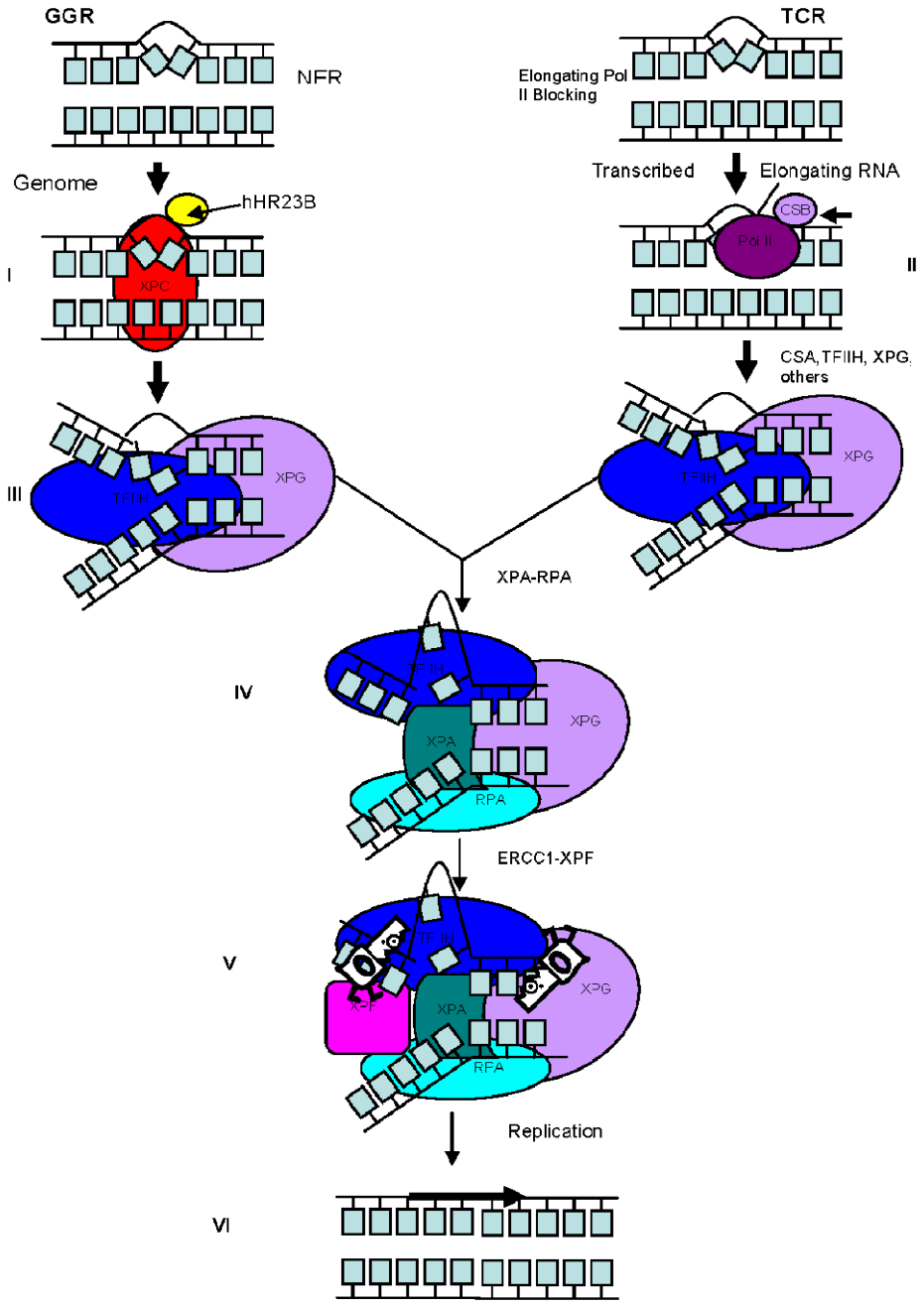

Figure 3. Model for mechanism of NER pathways; global genome nucleotide-excision repair and transcription-coupled repair. The GG-NER-specific complex XPC-hHR23B screens first on the basis of disrupted base pairing (reaction I). In TCR the ability of a lesion to block RNA polymerase seems critical, CSA and CSB helps to displace stalled RNA polymerase (reaction II). The helicases of the transcription factor TFIIH open ~30 base pairs of DNA around the damage (reaction III). The single-stranded binding protein RPA stabilizes the open intermediate by binding to the undamaged strand (reaction IV). The endonuclease duo XPG and ERCC1/XPF, respectively cleaves 3' and 5' of the damaged strand (reaction V) and finally the regular DNA replication machinery completes the repair by filling the gap (reaction VI).

Differentiation Associated Repair (DAR)

If a cell were not to ever replicate its DNA, it could conceivably accumulate numerous lesions in its genome, as long as it could maintain the integrity of those genes needed for viable cell function. The specialized repair pathway TCR can target several repair systems to transcribed genes [39, 40], thus potentially relieving post-mitotic cells from the chore and energy expense of repairing their entire genome.

Of course, this scenario assumes that these cells will not change their phenotype in the future, as this would imply transcribing genes that are currently silent and are potentially accumulating damage. Cells that do not divide and do not change phenotype are known as terminally differentiated cells and are very common in multicellular eukaryotes. It would therefore be of interest to examine the DNA repair capacity of such terminally differentiated cells [41].

Neurons are a perfect example of these kinds of cells; we are born with a given stock of them that is supposed to be sufficient for the rest of our lives. The status of the DNA repair machinery had been the focus of different groups reporting that neurons has lost any ability to repair cyclobutane pyrimidine dimmers, and markedly attenuated repair of benzo[a]pyrene diol-epoxide adducts at the global genome level [42,43]. Thus, turning off repair at the global genome level to concentrate on the repair of transcribed genes may be the best strategy to deal with the continuous induction of damage in postmitotic cells. Then, seems that the repair of the transcribed genes could be done by TCR mechanism; however, strangely enough, strand-specific analysis did not reveal the typical bias in favor of the transcribed strand, which is the hallmark of TCR [44]. This phenomenon, which was tentatively termed by Nouspikel and Hanawalt, [45] as differentiation-associated repair (DAR), seems to be confined to transcribed genes. Such a situation was previously encountered in two rodent model systems, rat myoblasts differentiating to myocytes [42] and rat PC12 pheochromocytoma cells that display a neuron-like phenotype upon differentiation [44].

The molecular mechanisms for DAR are currently not known, but several hypotheses have been proposed such as over-expressing incision enzymes, which may result in making better use of whatever trace of DNA repair activity is left in terminally differentiated cells; the possible role of genes involved in cell cycle checkpoints such ATM [46] and some others has been able to exclude an effect of chromatin structure, a decrease in expression of a key repair enzyme and an effect of p53 [41].

Indeed DAR might ensure that the non-transcribed strand will be equally well repaired in terminally differentiated cells. It has often been suggested that neuron aging and some forms of dementia could be due to the accumulation of unrepaired DNA lesions, which would eventually interfere with neuronal

function [20, 47]. DAR could be a way for normal neurons to delay, if not preclude, such events.

Then, there is sufficient evidence that DNA repair could play an important role in the establishment of some neurodegenerative diseases. In this respect in our laboratory we are studying the role of these DNA repair mechanisms in neuronal differentiation. In order to demonstrate whether neurons terminally differentiated which have accumulated oxidative damage throughout their life, cannot efficiently repair such injuries by TCR, because the GGR is inactive. This could be the events triggering an imbalance in neurons inducing an aberrant process, and leading it to the neuronal death. In other words, this event could drive to neuronal loss and consequently to the lost of neuronal functions such as cognitive functions implicated in some neurodegenerative diseases such as Alzheimer dementia.

We worked on the MSN cell model, which are cells derived from a human neuroblastoma, that are feasible to differentiate to neurons by the addition of soluble factors like retinoic acid and neuronal growth factor (NGF) [48]. This model allow us to explore if neuronal differentiation sensitize the cells to respond to an oxidative challenge similar to β-amyloid involved in neurodegeneration [49]. In addition, we evaluated NER gene expression changes before and after differentiation occurred (see fig. 4).

Our results indicate that the H_2O_2 challenge was not cytotoxic for undifferentiated MSN cells, meanwhile 24 hours after oxidative treatment differentiated MSN cells showed 45% of cytotoxicity as we show in figure 5. We could interpret this positive effect as neuronal death in a subpopulation of

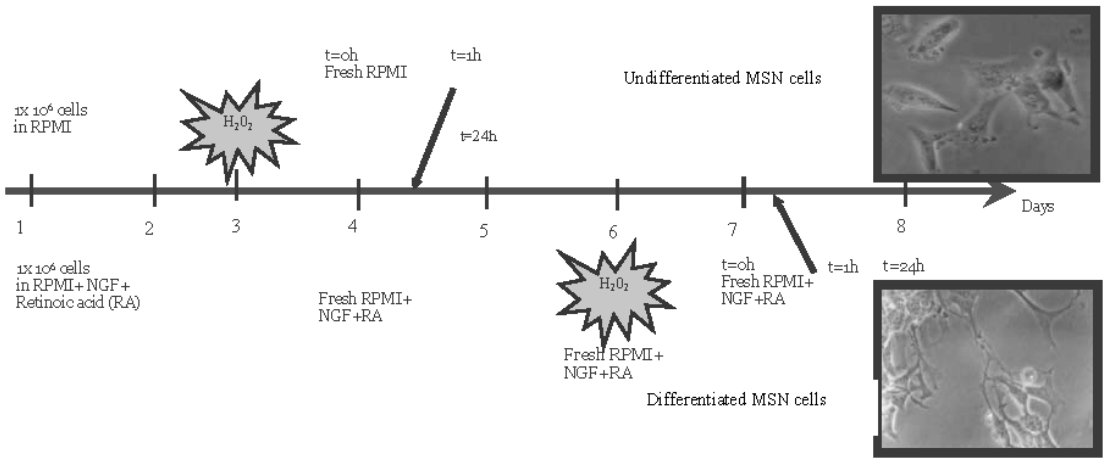

Figure 4. Experimental design to induce MSN cell differentiation and determine the presence of oxidative DNA damage, repair capacity and expression of repair genes. The upper panel represent the undifferentiated model meanwhile differentiated is show in the bottom panel. t=0 represent the H_2O_2 10μM treatment, t=1h represent the first recovery period as well as t=24h represent the largest recovery period.

the culture. The absence of toxic effect allows us to determine weather DNA damage turns on the DNA-repair machinery without any interference with the cell death process. It is worth noting that MSN differentiation increased the basal oxidative DNA-damage assessed by the comet assay. After oxidative challenge we observed a weak increase in DNA damage, which can be removed almost totally after 24 hours (fig. 6).

To confirm that we induced an oxidative damage by the treatment of 10 μM H_2O_2, we evaluated by immunocytochemistry the presence of 8-oxoguanine (figure 7). This particular oxidative damage is enough to induce the repair mechanisms. Our results indicate that basal levels of 8-oxoguanine are not affected by the differentiation process. However, it is evident that 8-oxoguanine lesions are differentially produced by both conditions: undifferentiated MSN cells accumulated a high amount of 8-oxoguanine by the treatment with 10 μM H_2O_2. In contrast, differentiated MSN cells showed a delay in the response because the 8-oxoguanine lesions appear 1 hour after the 10 μM H_2O_2 challenge finished.

Analyzing the correlation between the evaluation of DNA-damage (fig. 6) and 8-oxoguanine data (fig. 8) generated by 10 μM H_2O_2, we can suggest that the oxidative DNA damage assessed by the comet assay in undifferentiated MSN cells, involves the generation of 8-oxoguanine lesions; however this is not the case for differentiated neurons. With this approach, we determined a higher oxidative DNA-damage that present undifferentiated cells, which does not correlate with the 8-oxoguanine formation, suggesting that the comet assay damage detected involves oxidation processes different to 8-oxoguanine generation, such as abasic sites or DNA single strand breaks. Another point considered is that DNA-repair mechanisms different to NER, such as BER could be challenged. In this respect we were able to observe that APE1, a gene associated with BER, decreases its expression after oxidative damage (data not shown).

In order to explore the DNA repair mechanisms underlying this response we evaluated changes in the expression of different genes involved in DNA repair pathways. Considering Nouspikel and Hanawalt [45] proposal, about turning off the global genome repair in terminally differentiated cells, we began exploring Rad23B gene expression (GGR gene) before and after MSN differentiation at a time where we evaluated TCR genes such as CSA, CSB and XPD. XPD are involved in both subpathways of NER, GGR and TCR. The expression rates with respect to the controls are shown in fig. 8. They indicate that undifferentiated cells have mRNA expression of genes involved in both NER subpathways (GGR and TCR). On the other hand, differentiated neurons only showed mRNA expression for GGR, Rad23B gene. In addition it is worth noting that CSA gene (TCR) is not expressed.

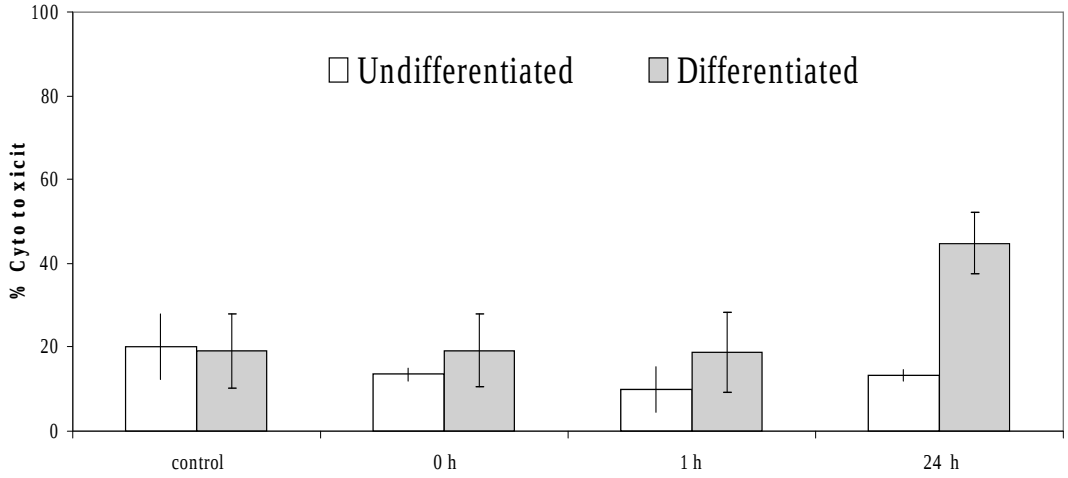

Figure 5. Cytotoxicity of MSN cells before and after differentiation. Basal cytotoxicity is show in control bars. 10µM H_2O_2 treatment and 1hour after recovery period did not show cytotoxic effect (t= 0h, and t=1h respectively), however at 24 hours after recovery we detected a cytotoxic effect only in differentiated cells.

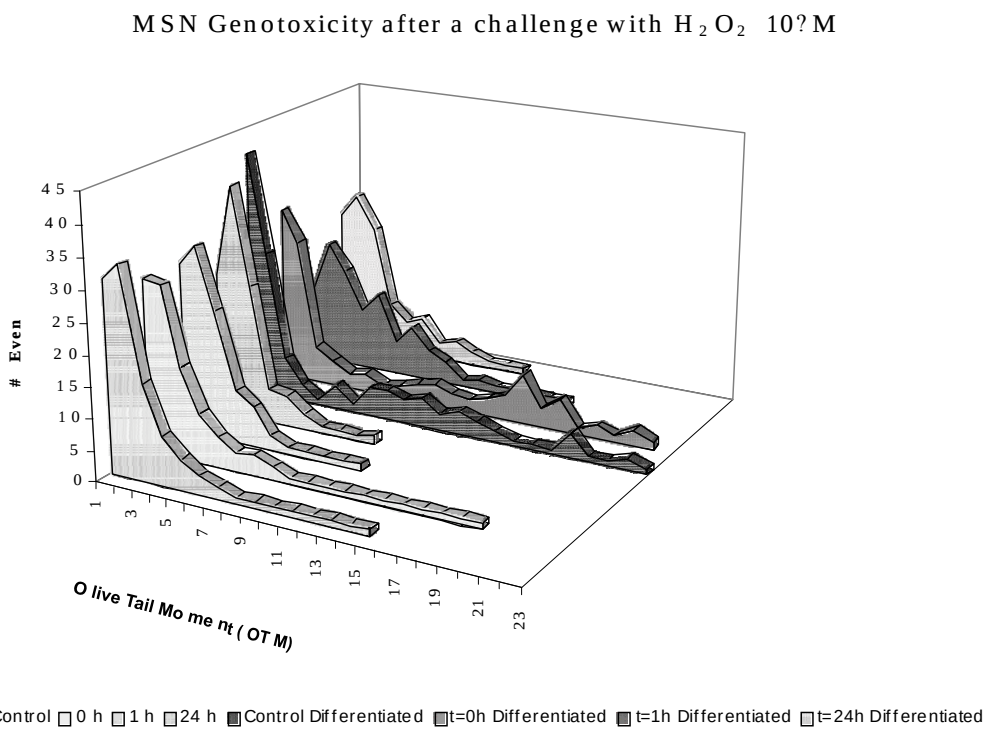

Figure 6. DNA damage of MSN cells before and after differentiation evaluated by comet assay, which represent the frequency of single-strand breaks. The first four histograms represents basal damage, damage induced by 10µM H_2O_2 (0h), damage 1 hour after recovery (1h), and damage 24 hours after recovery (24h) in undifferentiated cell model respectively. The last four histograms represent the same conditions for differentiated cells.

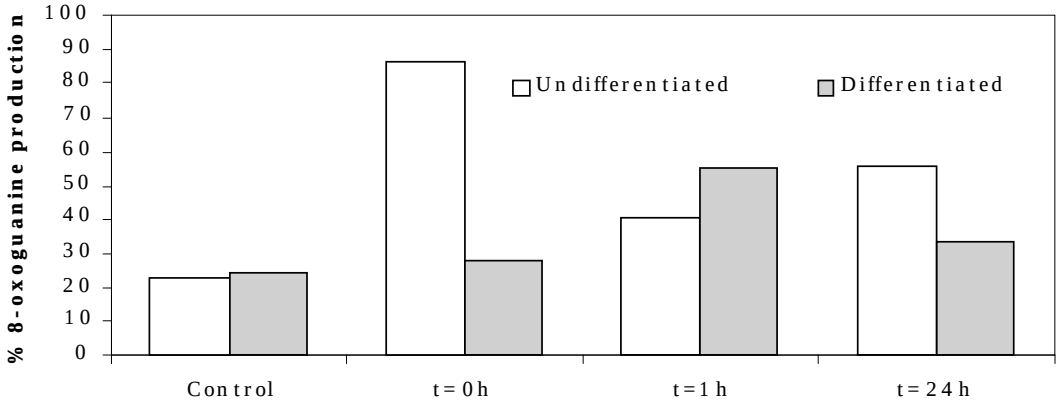

Figure 7. 8-Oxoguanine levels in MSN cell model before and after differentiation.

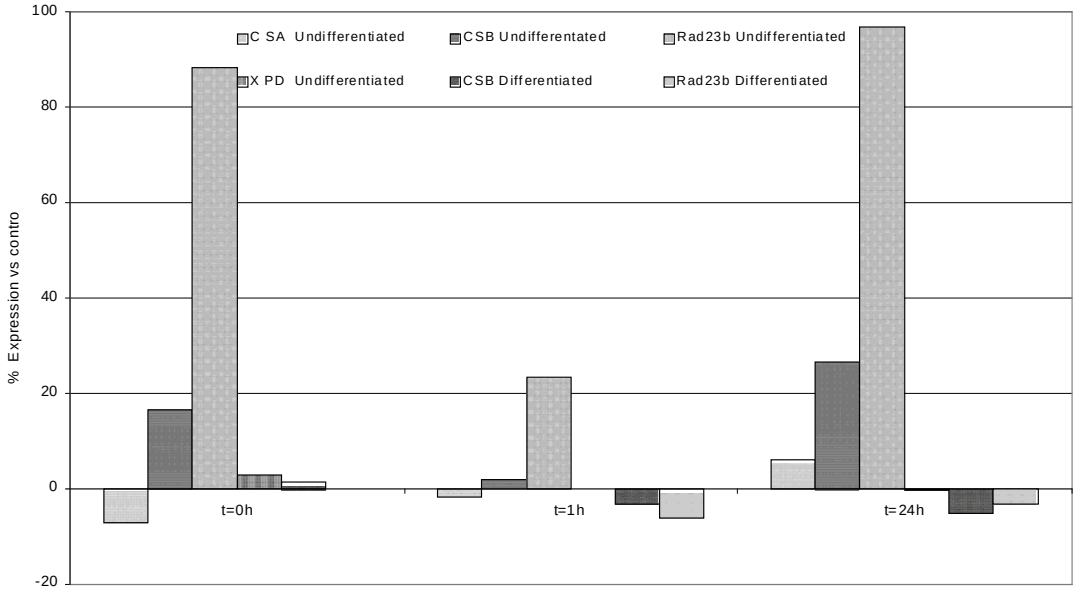

Figure 8. mRNA expression of NER repair genes in MSN undifferentiated and differentiated cells, during 10μM H_2O_2 challenge (0h), after 1 and 24 hours recovery periods (1h and 24h, respectively). CSA was not express in differentiated cells, and XPD expression of differentiated MSN was not determined.

Altogether, these results suggest that the removal of the oxidative damage in differentiated cells is independent of the TCR pathway, meanwhile both NER mechanisms in undifferentiated cells apparently function correctly. However, these studies are focused only in the mRNA expression. We are now working on the protein expression levels of these genes to establish a full picture of the process. If protein expression levels are in agreement with mRNA data, then our data will be in disagreement with the proposal of Nouspikel and Hanawalt [45] suggesting that the DNA repair mechanism that works after differentiation its nor related to TCR.

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Artículo 2

"Differential DNA damage to UV and hydrogen peroxide depending of differentiatin stage in neuroblastoma model"

Journal of NeuroToxicology

En este artículo se presentan los resultados obtenidos en el modelo del neuroblastoma humano en el que se evaluó la capacidad reparativa de lesiones oxidativas como 8oxoG y a lesiones provocadas por la exposición a luz UVC en dos estados de diferenciación. Se determinó la inducción de daño al DNA por dos agentes, la UVC como inductor canónico del mecanismo NER y el peróxido de hidrógeno como inductor de BER y NER, no canónico. Asimismo, se evaluó la capacidad reparativa de lesiones tridimensionales del DNA como los anillos de ciclobutano (CPDs) y la oxidación de guaninas, (8OHdG), comparando las respuestas entre neuronas indiferenciadas y diferenciadas.

Resumen de resultados:

-Se observaron diferencias en la remoción de daño del DNA de manera dependiente del reto inducido y del estado de diferenciación.

-Las células diferenciadas, comparadas con las indiferenciadas, mostraron una gran sensibilidad a la UVC, sin embargo el daño a lo largo del tiempo fue menor.

-Por el contrario, las células indiferenciadas mostraron una clara inducción de genotoxicidad en respuesta al daño oxidante, sin embargo la tendencia mostró una acumulación de 8OHdG a lo largo del tiempo.

-Este trabajo sugiere la participación de las dos sub vías de NER así como las de BER en la remoción de CPDs y de 8OHdG, indicando que la respuesta al DNA es dinámica y no solo depende un mecanismo de reparación.

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NEUTOX-1423; No. of Pages 10
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NeuroToxicology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model

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ARTICLE INFO

Article history:
Received 13 March 2012
Accepted 30 May 2012
Available online xxx

Keywords:
DNA damage
Neuroblastoma
UVC and hydrogen peroxide

ABSTRACT

DNA is a frequent target of oxidative damage, and DNA damage removal is therefore a crucial process in prevention of or recovery from degenerative diseases. DNA repair is an essential system for maintaining the inherited nucleotide sequence of genomic DNA over time. Cells engage in efficient DNA repair mechanisms, the activity of which can vary depending on the type of lesion and the developmental stage. Base excision repair (BER) and nucleotide excision repair (NER) are the major repair pathways addressed in this study. BER is the principal mechanism for repair of DNA oxidative lesions, while NER is the mechanism for repair of a variety of helix-distorting lesions such as those caused by UV radiation. Recent studies suggest that NER plays a cooperative role in removal of oxidative lesions. Little is known about the roles of DNA damage sensors and repair factors in terminally differentiated, non-proliferating cells such as neurons, which are vulnerable to oxidative damage from reactive oxygen species generated by endogenous or exogenous agents. We used the human neuroblastoma MSN cell model to investigate whether terminally differentiated neuronal cells respond to lesions cause in the DNA helix, such as UV-induced CPD and the major DNA oxidative lesion 8OHdG, and thereby clarify the role of NER capacity. We observed differences in DNA damage removal depending on the challenge insult and the differentiation state. Differentiated MSN cells, compared with undifferentiated cells, showed greater sensitivity to UVC and decreased DNA damage over time. In contrast, undifferentiated cells displayed genotoxicity induced by oxidative insult and tended to accumulate DNA damage and 8OHdG lesions over time. Our findings suggest the participation of GG-NER, TC-NER and BER proteins in the removal of 8-OHG and CPDs indicating a dynamic role in overall response to damage.

1. Introduction

The cell is a target for many types of endogenous and exogenous damage, ranging from the metabolism of the cell itself, to lesions caused by exposure to environmental agents. Such injuries target different types of macromolecules, including proteins, lipids and DNA. Damage to DNA has an impact leading to genomic instability; therefore, DNA damage repair is a crucial process in the prevention of disease development. Different types of cells respond to oxidative damage depending on their distinctive properties: antioxidant capacity, age, cell cycle state, transcription, differentiation status and DNA repair mechanisms. More importantly, several DNA repair systems can vary in their response to cellular differentiation (Nouspikel, 2006). Inefficient injury repair has been associated with the development of cancer and degenerative diseases. In this context, DNA repair is an essential system for maintaining the inherited nucleotide sequence of genomic DNA over time. For this purpose, cells engage in efficient DNA repair processes such as base excision repair (BER) and nucleotide excision repair (NER) (Christmann et al., 2003; Hoeijmakers, 2001). BER is the principal pathway for repair of DNA oxidative lesions (Seeberg et al., 1995), such as, fragmented pyrimidines, *N*-alkylated purines (7-methylguanine, 3-methyladenine, 3-methylguanine), thymine glycol and 8-oxo-7,8-dihydroguanine (8-OHdG). Oxidized bases are recognized by specific DNA glycosylases and are then cleaved, giving rise to an apurinic/apyrimidic site (AP). Abasic sites are split by an endonuclease, APE1, which initiates strand incision. BER operates *via* two pathways, termed short- and long-patch repair. In short-patch repair, DNA polβ fills the gap and the XRCC1–ligase3 complex seals the remaining nick. Long-patch repair involves the DNA polβ, polδ, and proliferating cell nuclear antigen (PCNA) for repair synthesis (2–10 bases), as well as the FEN1 endonuclease to remove the displaced DNA flap and DNA ligase 1 for sealing (Cooke et al., 2005). In contrast to BER, NER removes a wide variety of DNA lesions, such as UV-induced lesions (6–4 photoproducts and cyclobutane pyrimidine dimers (CPDs)) and chemical adducts that are mutagenic and may result in cell death. NER requires a variety of proteins that function in the

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0161-813X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neuro.2012.05.017

Please cite this article in press as: Ramos-Espinosa P, et al. Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model. Neurotoxicology (2012), http://dx.doi.org/10.1016/j.neuro.2012.05.017

recognition of damage (XPC-HR23B, DDB complex, RNA POL II, CSA and CSB), local opening of DNA helix (TFIIH, XPA and RPA), dual incision on both sides of the lesion (XPG, XPF-ERCC1), repair of the oligonucleotide containing the damage, gap filling by DNA synthesis (RFC, PCNA and DNA POL δ/ε/κ) and finally ligation of the new fragment to the previous DNA strand (DNA LIG I/III) (Kjelhede et al., 2011; Nouspikel, 2009). These mechanisms are divided into two subpathways: global genome repair (GG-NER) and transcription coupled repair (TC-NER). The subpathways differ in terms of their recognition proteins GG-NER: XPC and HR23B; TC-NER: RNA pol II, CSA and CSB (facilitator proteins) and their genome excision activity; GG-NER occurs over the entire genome and in non-transcribing strands of active genes, while TC-NER occurs in transcriptionally active genes (Costa et al., 2003; Shuck et al., 2008; Van Hoffen et al., 2003).

The 8OHdG lesion is the most common and important DNA-base lesion for two reasons: it is highly mutagenic, and it has been considered as a cause of many diseases including cancer and neurodegeneration (Cardozo-Pelaez et al., 2000; Davydov et al., 2003). This lesion can be removed through either BER or NER. In BER, the glycosylase considered to have the primary responsibility for 8-oxoG removal is the 8-OH guanine glycosylase (OGG1). There is increasing evidence for a cooperative role of NER in the removal of DNA oxidative lesions (Dianov et al., 1999; D'Errico et al., 2007). For example, Trapp et al. (2007) showed that mice without the CSB enzyme (TC-NER) accumulated 8OHdG in the genome and had elevated spontaneous mutation rates. De Waard (2004) demonstrated the accumulation of oxidative lesions in mouse embryonic fibroblasts deficient in CSB or CSA enzymes. D'Errico et al. (2006) observed a low rate of 8OHdG repair in XPC primary keratinocytes and fibroblasts.

The process of NER was shown to be reduced by differentiation in several cell types, including neurons (Nouspikel and Hanawalt, 2000). Neuroblastomas are therefore a useful model because they can be induced to differentiate into neurons *in vitro* and they allow the study of two cell lines with an identical genetic background, differing only in the fact that one has attained terminal differentiation (Nouspikel and Hanawalt, 2002). It is important to emphasize that the neuroblastoma cells represent an early stage in neuronal development where cells are pluripotent, and retain the capabilities for expressing multiple neural crest-derived phenotypes; turns the neuroblastoma model into a new vista in the analysis of tumor growth and differentiation (Abemayor and Sidelit, 1989).

We studied human neuroblastoma MSN cells to determine whether DNA damage repair in neuronal cells varies as a function of differentiation state rather than the type of lesion. We treated undifferentiated and differentiated neurons with UVC rays that cause DNA distortion such as CPDs or with prolonged oxidative challenge with hydrogen peroxide (H_2O_2). We observed differences in DNA damage repair processes that depended on both the challenge insult and the differentiation state. The differentiated cells, in comparison with the undifferentiated cells, showed a low survival to UVC and as well as decreased rates of repair of DNA damage over time. The undifferentiated cells showed the effects of genotoxicity induced by the oxidative insult and tended to accumulate DNA damage and 8OHdG over time. Our findings suggest that NER proteins participate in the removal of 8-OHG in conjunction with BER as a backup system to repair CPDs.

2. Materials and methods

2.1. Cell culture

Adherent human neuroblastoma MSN cells (Reynolds et al., 1986; Quiroz-Baez et al., 2009) were cultured in enriched RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 1% antibiotics, 1% non-essential amino acids, 1% QSN solution (serine, asparagine and glutamine) and 10% fetal bovine serum (Gibco, Life Technologies Corporation, Grand Island, NY, USA) in tissue culture dishes in an atmosphere of 5% CO_2 and 95% O_2, at 37 °C. Cells were plated at a density of 1×10^6 cells per dish, and harvested by gently pipetting. For differentiated neurons, the cells were seeded at a density of 1×10^6 per dish and differentiated by adding 10 μM retinoic acid (RA) and 50 ng/ml NGF (Invitrogen, Life Technologies Corporation, USA) for 6 days. The differentiated neurons were immunocytochemically stained for MAP2 and NeuN (a neuron specific marker).

2.2. Treatments

UVC light and hydrogen peroxide (H_2O_2) treatment and cell viability assay. To establish an appropriate UVC dose, undifferentiated MSN cells were washed with PBS buffer, placed in open dishes with PBS and irradiated with various doses of UVC (0, 1.8, 2.2, 3.6 and 5.1 J/cm^2, wavelength 254 nm at room temperature) from a germicidal lamp (Biological Safety Cabinet TUV 15 GITS). The cultures were monitored for 24 h after exposure. The cells were then harvested in physiological saline solution, and 10 μl of 0.4% trypan blue dye solution was added to 10 μl of cells for each experimental condition. The cells that excluded trypan blue were considered viable and were counted using a hemocytometer. To establish an appropriate H_2O_2 concentration, cells were washed with PBS buffer and then supplemented during 24 h with fresh medium containing H_2O_2 to achieve final concentrations of 0, 10, 25, 50 and 100 μM H_2O_2. The cultures were monitored for 24 h to simulate an oxidative environment. The medium was then removed, cells were harvested, and cell viability was assayed as described above.

2.3. Reactive oxygen species (ROS) measurement

Reactive oxygen species (ROS) were determined by a modified fluorometric assay (Lee et al., 2003) using dihydrorhodamine 123 (DHR) (Sigma) as the probe. DHR is oxidized by H_2O_2 and produces a fluorescent compound, rhodamine 123, which can be measured spectrophotometrically at a wavelength of 505 nm (Ferrera et al., 2008). After various treatment periods, cells were scraped off and centrifuged at 1200 rpm for 5 min. A total of 180 μl of a buffer containing 10 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM HEPES and 20 μl of 1 μM DHR were added to the pellet and incubated at 37 °C for 2 min. The fluorescent product was then measured spectrophotometrically, and the ROS level was estimated using a standard curve for rhodamine 123.

2.4. Lipid peroxidation

The thiobarbituric acid method was employed to measure malondialdehyde (MDA) levels. An aliquot of 100,000 cells was added to 100 μl trichloroacetic acid (10%, w/v) and centrifuged at 3000 × *g* for 10 min. The supernatant was added to 1 ml thiobarbituric acid reagent (0.375%) and was heated at 92 °C for 45 min. The absorbance of thiobarbituric acid–MDA complex was then measured at 532 nm using an ELISA spectrophotometer. The MDA level was estimated using a standard concentration curve of 1,1,3,3-tetraethoxypropane ranging from 1 to 10 nm.

2.5. Comet assay

For each experimental condition, at least 10,000 cells were mixed with 75 μl of 0.5% low melting point (LMP) agarose. The cells were loaded onto microscope slides pre-layered with 200 μl

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of 0.5% normal melting point agarose. The comet assay was performed as described by Vega et al. (2009). Briefly, after lysis of cells at 4 °C for at least 1 h in a medium consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, supplemented with 10% DMSO and 1% Triton X-100, slides, including a positive control (γ-irradiated lymphocytes), were placed in a horizontal electrophoresis chamber with running buffer solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13). The slides remained in the electrophoresis buffer for 10 min to allow the DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 25 V, ~0.8 V/cm. All steps were performed in the dark to avoid direct light. After electrophoresis, the slides were gently removed and rinsed with neutralization buffer (0.4 M Tris, pH 7.5) at room temperature for 15 min, dehydrated with absolute ethanol (15 min) and air-dried. Ethidium bromide (20 μl of 20 μg/ml solution) was added to each slide and a coverslip was placed on the gel. Individual cells were visualized at 20× magnification under an Olympus BX-60 microscope with fluorescence attachments (515–560 nm excitation filter, 590 nm barrier filter), and the DNA damage was determined using Komet 5.0 software (Kinetic Imaging). To evaluate DNA migration, 100 cells were scored for each experimental condition. The data were categorized into five categories according to the Olive tail moment score as described by Collins (2008). The total number of cells in each category was counted and multiplied by an assigned value 0–4 according to the damage class. The sum of all the categories was calculated and considered as the damage index. The overall score was expected to vary between 0 and 400 arbitrary units.

2.6. T4 endo V and FPG endonuclease Comet assays

Based on the results of the comet assay, we evaluated the UVC formation of cyclopyrimidine dimer (CPD) through T4 endo V-sensitive sites and of 8-hydroxy-2-deoxyguanosine (8-OHdG) through FPG-sensitive sites in control cells and in cells treated with UVC and H_2O_2. After treatment, the cells were layered on microscope slides and immersed in lysis buffer for at least 1 h at 4 °C. The slides were then rinsed with buffer solution (50 mM Tris-base, 10 mM EDTA, pH 7.6) for 5 min. For CPD detection, the slides were overlaid with 20 μl T4 endo V (New England BioLabs, MA, USA), which cuts DNA at CPD sites (Jiang et al., 2009). For 8-hydroxy-2-deoxyguanosine detection, the same procedure was performed, using FPG (Trevigen, CA, USA) enzyme instead of T4 endo V (Jiang et al., 2009). Coverslips were placed on the slides, and the slides were incubated for 30 min at 37 °C in a humidified atmosphere. A set of slides with irradiated cells in buffer (without enzyme) was included to confirm that the DNA strand breaks were enzyme-specific. Following enzyme incubation, the slides were rinsed with solution buffer (50 mM Tris-base, 200 mM EDTA, pH 7.6) and subjected to electrophoresis (~0.8 V/cm) for 10 min without unwinding incubation. Comet visualization was performed at 20× magnification using an Olympus BX-60 microscope with fluorescence attachments (515–560 nm excitation filter, 590 nm barrier filter). The images were digitized and analyzed using Komet 5.0 software. To evaluate DNA migration, 100 cells were scored for each experimental condition. The damage index was determined as described in the preceding section.

2.7. Immunocytochemistry

MSN cells were plated on coverslips coated with 0.1% gelatin and incubated overnight to allow the cells to adhere. After treatment with H_2O_2 or UV light, the medium was removed and the cells were fixed with 95% ethanol, 5% acetic acid and H_2O for 30 min at room temperature, followed by hydration of the sample in this order: 95% ethanol for 5 min, 50% ethanol for 5 min and H_2O for 5 min. The coverslips were then embedded in PBS–1% H_2O_2. The primary antibodies employed were rabbit polyclonal anti-CSA (1:50; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-CSB (1:50; Santa Cruz), rabbit polyclonal anti-XPC (1:50; Santa Cruz), goat anti-HR23B (1:100, Abcam, CA, USA), rabbit polyclonal anti-OGG1 (1:50; Santa Cruz), rabbit polyclonal APE1 (1:50; Santa Cruz), mouse ascites anti-8-oxoguanine (1:50; Becton, NJ, USA), mouse anti CPD (1:100; MBL, MA, USA) and anti-lamin A/C (1:100; Santa cruz), dissolved in PBS–1% H_2O_2 and incubated at 37 °C for 40 min. For each experiment, the coverslip was washed three times with PBS–1% H_2O_2 for 5 min. The secondary antibodies, goat anti-mouse HRP conjugate (1:250; Zymed, CA, USA) and goat anti-rabbit HRP conjugate (1:250; Zymed) were added for 40 min at 37 °C, and coverslips were washed three times for 5 min. The cells were stained with 3,3-diaminobenzidine (DAB) using a DAB kit (Vektor, CA, USA). Dehydration was performed in the following order: 50% ethanol for 5 min, 75% ethanol for 5 min, absolute ethanol, xylene. The coverslips were embedded in resin, set on slides and examined using an Olympus BX-60 microscope at 40× magnification. Images were digitized and analyzed using NIH Image J software. Values in Fig. 4 are expressed as optical density (arbitrary units) average per condition. Meanwhile data in Table 3 are expressed as percentage compared to unexposed cells.

2.8. Statistical analysis

Data for cell viability, ROS production, lipid peroxidation, and levels of repair recognition proteins were calculated as the mean ± S.D. from 3 independent experiments. The differences between experimental and control values were assessed by one-way Analysis of Variance (ANOVA; Sigma Stat v 3.5), with $p < 0.05$ considered to be significant. DNA fragmentation as determined by Comet assays was represented as the mean ± S.E.M. of damage indices from 3 independent experiments. The differences between values for various differentiation states were assessed by Student's "t" test (Sigma Stat v 3.5).

3. Results

3.1. Cell viability and oxidative status

To determine the doses of damaging environmental agents that pose a challenge to cell viability, undifferentiated MSN cell cultures were exposed various UVC light doses (0, 1.8, 2.2, 3.6, 5.1 J/cm^2) and H_2O_2 concentrations during 24 h. Cells were monitored for a subsequent 24 h because cell viability is not affected immediately by UVC exposure; as Batista (2009) suggested, biological effects such as cell death should be monitored for at least 8 h following experimental treatment (Batista et al., 2009). The results shown in Table 1 indicate a dose-dependent reduction in cell viability in cultures that excluded trypan blue after 1 h post-irradiation treatment. UVC induced a significant decrease in cell survival at lower doses (e.g., 1.8 J/cm^2). However, the major effect on cell viability, reflected as a 50% reduction in survival, was observed at a 3.6 J/cm^2 dose in undifferentiated cells. MSN cells were also treated with increasing H_2O_2 concentrations (0, 10, 25, 50, 100 μM) over 24 h to simulate a permanent oxidative environment. Viability of undifferentiated cells was evaluated by the trypan blue exclusion method. Because H_2O_2 does not persist for long periods of time in culture, but triggers an ROS cascade, H_2O_2 treatment was maintained for 24 h. The data on concentration-dependent reduction in cell viability are shown in Table 1. An H_2O_2 concentration of 50 μM during 24 h caused a 50% reduction in cell viability, similar to the effect of a UVC dose of 3.6 J/cm^2.

After establishing the UVC dose (3.6 J/cm^2) and H_2O_2 concentration (50 μM/24 h) that induced the desired effect in undiffer-

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Table 1
Cell viability assay for various UVC and H_2O_2 doses.

UVC exposure (J/cm^2)	% Cell viability	H_2O_2 (μM)	% Cell viability
0	100 ± 5.2	0	98.5 ± 2.1
1.8	73.1 ± 3.3***	10	79.5 ± 13.1
2.2	70.6 ± 9.8***	25	69.9 ± 21.7
3.8	45.9 ± 0.8***	50	51.6 ± 17.8**
5.1	44.6 ± 7.7***	100	61.3 ± 14.3*

Cell viability was evaluated by trypan blue exclusion assay in undifferentiated MSN cells 24 h post treatment with UVC and after 24 h of H_2O_2 treatment. All data are expressed as percentage and represent the mean ± S.D. of 3 independent experiments. Analysis of variance was performed by one-way ANOVA (Sigma Stat v 3.5).
* Significance of different from control value: $p < 0.05$.
** Significance of different from control value: $p < 0.005$.
*** Significance of different from control value: $p \leq 0.001$.

entiated cells, we determined the oxidative status of cells based on ROS levels and lipid peroxidation as percentages of values for control cells (Table 2). These determinations were performed for both differentiation stages immediately after treatment. We measured ROS production after both the UVC and H_2O_2 insults. Although ROS are produced mainly by longer UV wavelengths, there is evidence that UVC irradiation leads to production of $^1O_2$ as an indirect event (Zhang et al., 1997). UVC treatment did not increase intracellular ROS levels or lipid peroxidation in either undifferentiated or differentiated cells (Table 2). Undifferentiated cells treated with H_2O_2 (50 μM/24 h) showed ROS levels increased three times compared with control cells but did not show changes in lipid peroxidation. Also it is known that dividing cells (undifferentiated) accumulate α-tocopherol during replication suggesting the decreased peroxidizability as shown in Table 2. In contrast, differentiated cells treated with H_2O_2 showed a significant decrease in ROS levels and a three-fold increase in lipid peroxidation. This response could be attributed to trophic factors employed through differentiation (RA and NGF). NGF decreases α-tocoferol levels facilitating oxidative environment (Halliwell and Gutteridge, 1999). Parallel it has been reported that RA increases TBARS levels explaining lipid peroxidation increment. On the other hand RA increases antioxidant activity therefore decreasing ROS production (Conte da Frota et al., 2006).

After evaluating the effect of the intracellular oxidative environment, we focused on the induction of DNA damage and its repair over time. Utilising the comet assay, one of the most sensitive genotoxicity tests for strand break detection and oxidation-induced DNA damage (Speit et al., 2004; Tice et al., 2000), we determined DNA breaks in undifferentiated and differentiated MSN cells immediately UVC and H_2O_2 treatments (Fig. 1A). The UVC challenge induced a slight increase in DNA damage in undifferentiated cells, and induced increase (293%) in DNA strand breaks in differentiated cells compared with untreated differentiated cells. Other approach to determine the damage level induced by UVC between undifferentiated and differentiated cells was by CPD immune staining immediately after radiation (Fig. 1B). We found differences between differentiation stages however the change was not so evidently marked as determined by comet assay. We also compared the damage index of undifferentiated and differentiated cells following UVC treatment. Regarding the oxidative challenge with H_2O_2, we observed a statistically significant increase in the damage index only in undifferentiated cells; however, we did find differences between undifferentiated and differentiated cells. Notably, there was a differential response depending on the inducer and on the differentiation state. Because the most common cause of damage to terminally differentiated neurons is exposure to oxidation (Fischel et al., 2007), it is more important to consider the DNA damage induced by H_2O_2 than that induced by UVC. However, low-level DNA damage in differentiated cells could also result from continuous stimulation by differentiation factors (RA and NGF) that promote expression of survival genes (Ahlemeyer et al., 2001; Sofroniew et al., 2001; Conte da Frota et al., 2006).

A useful approach for assessing repair capacity is to evaluate the ability of cells to remove experimentally induced DNA damage, using the simple comet assay (Decodier et al., 2010). We performed a comet assay at various points during the 24 h following the treatment of cells, to evaluate the removal or persistence of DNA damage. Undifferentiated cells exposed to UVC showed a slight induction of DNA damage immediately after treatment and also 1 h and 24 h after treatment (Fig. 2A). However, an analysis of damage distribution (inset A) showed an increase in the percentage of cells in categories 2 and 3 over time. Differentiated cells showed a dramatic induction of DNA damage and an apparent recovery of DNA strand breaks 24 h after irradiation, but not enough to reach control levels (Fig. 2C). Undifferentiated cells showed significant induction of genotoxicity (160%) compared with untreated cells following H_2O_2 treatment and showed elimination of DNA damage over time in terms of the damage index (Fig. 2B). The damage distribution analysis (inset B) showed more cells in the low-damage categories 1 h after treatment but more cells in the high-damage categories 24 h after treatment, suggesting the accumulation of DNA damage. Differentiated cells did not show a significant difference in the damage index after H_2O_2 treatment (Fig. 2D) but did show a 39% increase compared with control cells. In contrast to the results for undifferentiated cells, the damage distribution analysis for differentiated cells (inset D) did not show an increased number of cells in the high-damage categories. Overall, the results suggest that differentiation status plays an important role in induction of DNA damage depending on the genotoxic challenge; undifferentiated MSN cells were more susceptible to H_2O_2 treatment whereas differentiated cells were more susceptible to UVC treatment. Regardless of the treatment, undifferentiated cells tended to accumulate DNA damage (insets A and B) while differentiated cells tended to show partial removal of DNA damage (inset C) or maintain the DNA-damage induced across time (inset D).

To further investigate the DNA lesions produced by UVC treatment in undifferentiated and differentiated cells immediately after exposure and 1 h and 24 h after treatment, we employed lesion-specific glycosylases in the comet assay. T4 endo V was used to recognize CPDs, and DNA formamidopyrimidine-DNA glycosylase

Table 2
Comparative oxidative status after UVC or H_2O_2 treatment: reactive oxygen species (ROS) production and lipid peroxidation in undifferentiated and differentiated MSN cells.

	ROS (% of control)		Lipid peroxidation (% of control)	
	Undifferentiated MSN	Differentiated MSN	Undifferentiated MSN	Differentiated MSN
Control	100 ± 18.8	100 ± 18.3	100 ± 12.8	100 ± 43
UV	101.6 ± 20.9	113.3 ± 18.8	104.9 ± 17.5	85 ± 70
H_2O_2	365.3 ± 68.9***	22.0 ± 12.3**	98.8 ± 12.9	396.4 ± 67.44***

Data are presented as the percentage of control value (mean ± S.D.) from 3 independent experiments. UVC: 3.6 J/cm^2, H_2O_2: 50 μM/24 h.
** Significance of difference from control by one-way ANOVA: $p < 0.005$.
*** Significance of difference from control by one-way ANOVA: $p \leq 0.001$.

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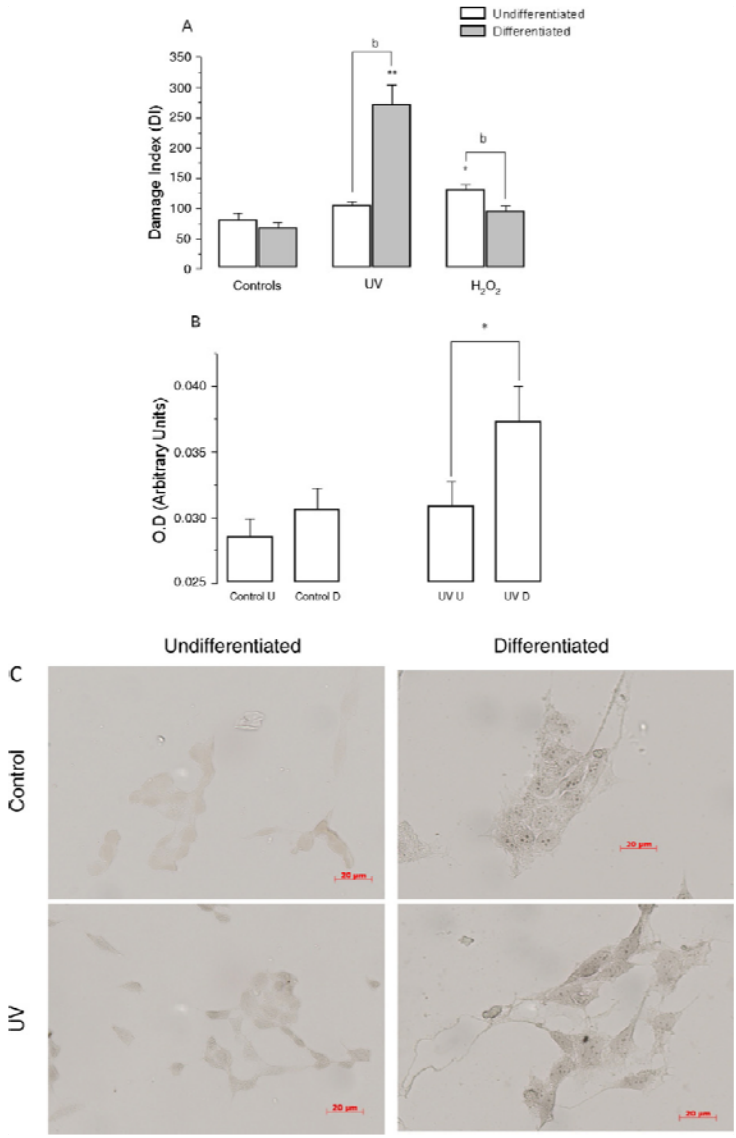

Fig. 1. Undifferentiated and differentiated MSN cells damage index exposed to UVC (3.6 J/cm²) or H_2O_2 (50 μM/24 h). (A) Cells were processed for Comet assay as described in Section 2. Results are mean ± S.E.M. of 3 independent experiments. Variance analysis was performed by One way ANOVA, significantly different to control: *$p < 0.05$, **$p < 0.005$. Differences between differentiation state were determined by Student *t*-test; [b]$p < 0.05$. UVC: 3.6 J/cm², H_2O_2: 50 μM/24 h. (B) Cells were processed by immune staining using anti-CPD antibody (MBL), representative images are present in different panels comparing undifferentiated and differentiated cells. Images were digitized and analyzed using NIH Image J software. Densitometry analysis values are expressed as optic density (arbitrary units) of 3 independent experiments (mean ± S.D.). *t*-Student was performed, significantly different to control *$p < 0.05$. UVC: 3.6 J/cm².

(FPG) was used to recognize 8OHdG. Recognition of DNA lesions as CPD or 8OHdG by these enzymes leads to the generation of a single strand break that can be revealed by the comet assay. An increase in DNA strand breaks after enzyme digestion in comparison with control cells directly reveals the presence of lesions. In order to guarantee T4 endo V linearity by comet assay, undifferentiated cells were irradiated with different UVC doses; these data are summarize in Fig. 3, were we show the linearity between UVC doses and two comet assay parameters: categorized DNA damage, damage index (D.I.) and Olive tail moment (OTM). In both we observed similar tendency, no enzyme saturation was shown at the employed dose (3.6 J/cm²), arguing that T4 endo V activity reflected in our data is proportional to the amount of CPD removal rather to saturation. Additionally we considered important to plot both parameters because showed same behavior, which justifies damage index.

UVC light exposure causes two principal types of lesions, CPD and 6–4 photoproducts, which can cause severe DNA distortion (Vreeswijk et al., 2008). We focused on CPD because it represents

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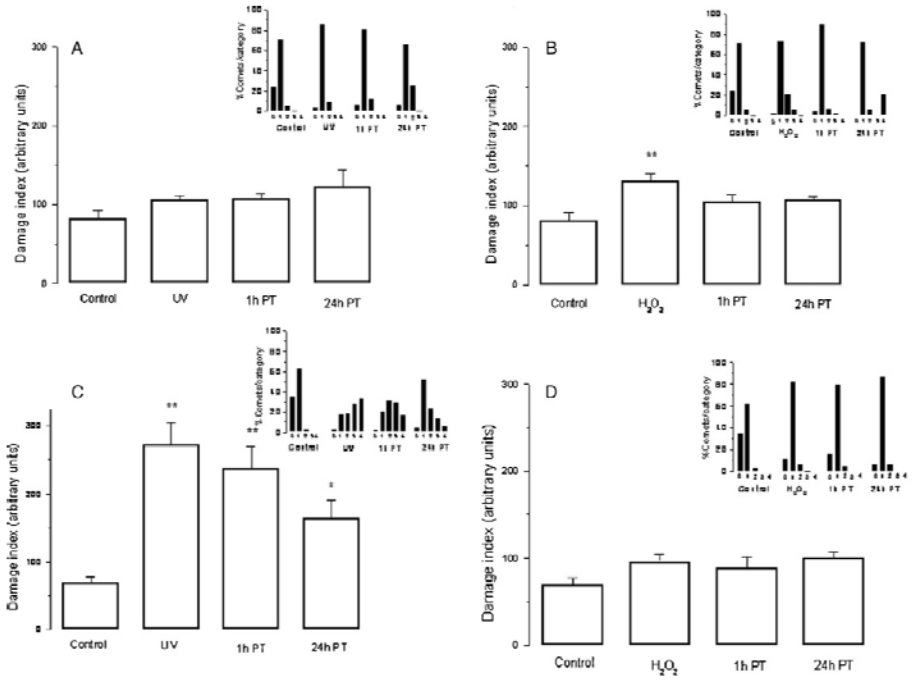

Fig. 2. Kinetic of DNA repair. This comet assay approach was conducted over 24 h post-treatment to follow DNA-damage removal or persistence measured as DNA damage index (DI) of undifferentiated MSN cells (A and B) and differentiated MSN cells (C and D) after treatments (H_2O_2 or UVC), an hour and 24 h after exposition. The values are expressed as the mean of DI ± S.E.M. of 3 independent experiments. Inserts represents DNA damage distribution by category as described in Section 2, where category 0 corresponds to undamaged nucleoids, category 1 to low damage, category 2 to medium damage, category 3 to high damage and category 4 to very high damage. One-way ANOVA *significantly different $p < 0.05$; **$p < 0.005$; ***$p \leq 0.001$. UVC: 3.6 J/cm^2, H_2O_2: 50 μM/24 h, PT: post treatment.

65–80% of both types of lesions (Proietti et al., 2002). Comparative CPD detection through T4 endo V digestion is illustrated in Fig. 4A. Both undifferentiated and differentiated cells showed CPD generation immediately after UVC (3.6 J/cm^2) exposure. However, the lesion persisted 24 h after treatment in undifferentiated cells, whereas it was removed 1 h after treatment in differentiated cells. These results suggest that only undifferentiated cells lack the DNA repair capacity to remove CPDs when are detected by T4 endo V. The detection of 8OHdG as FPG-sensitive sites generated after

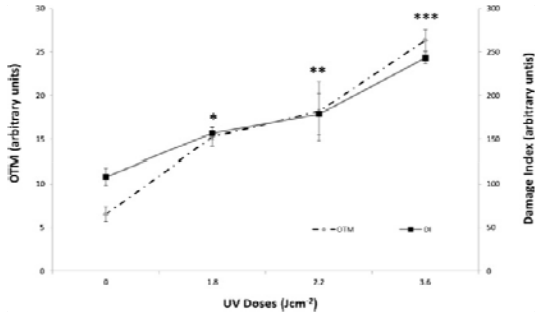

Fig. 3. Dose–response standard curve of T4 endo V sensitive sites. Undifferentiated cells were irradiated with various doses of UVC, nucleoids digested with T4 endo V and evaluated by comet assay. Continued line represents Olive Tail Moment (OTM) data, meanwhile dotted line shows damage index (DI) results. One-way ANOVA *significantly different with respect to control $p < 0.05$; **$p < 0.005$; ***$p \leq 0.001$.

H_2O_2 treatment differed depending on the differentiation stage (Fig. 4B). Undifferentiated cells displayed this type of DNA lesion at a high level only 24 h after treatment, suggesting a deficiency of DNA repair capacity as a consequence to the accumulation of other oxidative lesions besides 8OHdG, such as AP-site, 5,6-dihydrothymine, and 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine that has been reported to be detected also by *E. coli*-FPG enzyme (Jiang et al., 2009).

In contrast, differentiated cells presented FPG-sensitive sites immediately and 1 h after H_2O_2 treatment but not 24 h after treatment, indicating that these cells have the capacity to remove oxidative lesions.

3.2. NER recognition protein levels

The results of the comet assay in both UVC- and H_2O_2-treated undifferentiated cells revealed the accumulation of DNA damage and persistence of DNA lesions, which could be due to inefficient DNA repair systems. To elucidate the participation of repair recognition proteins, we compared GG-NER, TC-NER and BER basal protein levels in undifferentiated and differentiated cells by immunocytochemistry (Fig. 5). We found that protein levels decreased as follows when differentiation was induced: XPC 28% (GG-NER), CSA 47% (TC-NER), OGG1 43% and APE1 41% (BER). This result suggests an attenuation of repair systems as differentiation proceeds, as was also suggested by previous reports (Nouspikel and Hanawalt, 2000).

Protein levels normalized with respect to control conditions were compared between differentiation states and between 1 h

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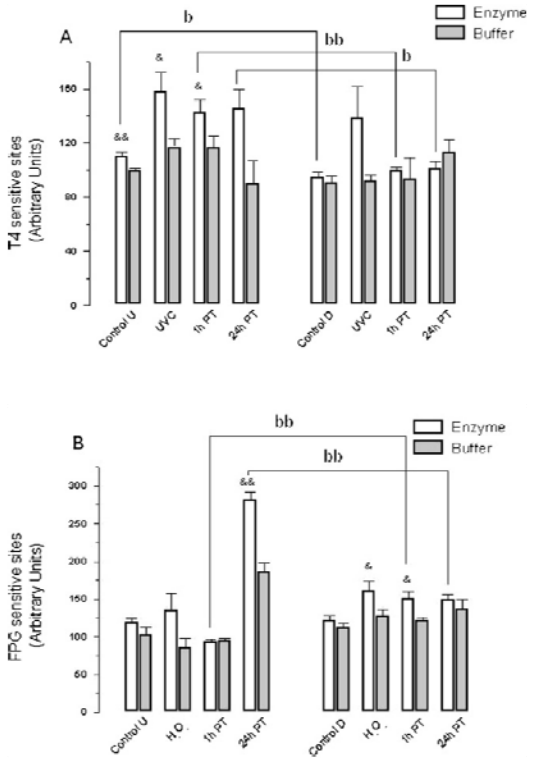

Fig. 4. T4 endo V and FPG sensitive sites induced by UVC or H_2O_2 in MSN cells. Nucleoids digested with enzyme (T4 endo V or FPG), or incubated only with buffer were assessed by comet assay to reflect CPD's or 8OHdG levels. (A) Comparison of MSN undifferentiated and differentiated CPD production by UVC exposition. (B) Comparison of 8OHdG production by H_2O_2 treatments. Difference between white and gray bars represents lesion (CPD or 8OHdG). Data represents the mean of DI ± S.E.M. of 3 or 5 independent experiments. t-Student significantly difference between enzyme and buffer: $^{b}p < 0.05$, $^{bb}p < 0.005$; $^{bbb}p \leq 0.001$; significantly difference between differentiation state: $^{\&}p < 0.05$, $^{\&\&}p < 0.005$; $^{\&\&\&}p \leq 0.001$. UVC: 3.6 J/cm^2, H_2O_2: 50 μM/24 h, PT: post treatment.

and 24 h after treatments with UVC and H_2O_2 (Table 3). Undifferentiated cells exposed to UVC showed an increase in HR23B and OGG1 proteins immediately after irradiation; however, increases in XPC, HR23B, CSB, CSA, OGG1 and APE1 proteins were observed 24 h after treatment, when we were still detecting CPDs (Fig. 3A). Differentiated cells showed an elevation of XPC, OGG1 and APE1 proteins immediately after UVC irradiation, but also showed increases of XPC, CSB, OGG1 and APE1 24 h after treatment, when we did not detect CPDs as T4 endo V sensitive sites (Fig. 3A). These findings suggest the participation of BER proteins in responses to UVC treatment, probably because of the presence of other lesions generated by UVC radiation, e.g., guanine oxidation, cytosine photohydration and adduct formation (Cadet et al., 2005). H_2O_2 treatment of undifferentiated cells induced decreased levels of XPC and increased levels of CSB and APE1 both 1 h and 24 h after treatment, when we still found DNA damage and FPG-sensitive sites. In contrast, H_2O_2 treatment of differentiated cells caused increased XPC levels at all times tested, but increased CSA and APE1 levels only immediately after treatment. APE1 level remained high even 24 h after treatment, although FPG-sensitive sites were not detected.

4. Discussion

DNA repair systems can vary in their responses, depending on the state of cellular differentiation (Nouspikel, 2006; Nouspikel and Hanawalt, 2000). Inefficient injury repair has been associated with the development of cancer and degenerative diseases. The aim of the present study was to determine, in a human neuroblastoma (MSN) model, whether DNA damage repair in neuronal cells varies as a function of the differentiation state rather than the type of lesion. For this purpose, we exposed undifferentiated and differentiated MSN cells to UVC irradiation (which causes DNA distortion) and to prolonged oxidative challenge with H_2O_2, to provide evidence regarding the effects of NER capacity. We observed differences in DNA damage repair, depending on the challenge insult and on the differentiation state. Differentiated cells, compared with undifferentiated cells, showed greater sensitivity to UVC and decreased DNA damage and DNA distortion repair over time. Studies of human NT2 cells and prenatal human neurons gave similar findings regarding UV sensitivity (Nouspikel and Hanawalt, 2000). These authors reported a striking decrease in nucleotide excision repair in differentiated cells by mRNA down-regulation. However, we observed loss of CPDs in differentiated cells even when we found low NER protein levels.

UVC irradiation induces the formation of DNA photoproducts, primarily CPD and 6–4 PPs (25). NER is the principal pathway capable of removing these lesions in mammals (Vrouwe et al., 2010). For this reason, UVC challenge has been applied frequently to assess this excision repair mechanism (Van der Wees et al., 2007). GG-NER is the preferred and most efficient DNA repair pathway for removing 6–4 PPs, which are totally eliminated from the overall genome 4 h after irradiation. In contrast, removal of CPD lesions by this pathway is more difficult; less than 50% are removed in 4 h (Riou et al., 1999; Costa et al., 2003). It is therefore easy to ascribe the CPD accumulation observed in undifferentiated cells to low GG-NER activity. CPD removal by TC-NER has been reported to be more efficient in transcribed strands (Nouspikel and Hanawalt, 2000), which may explain our finding of decreased levels of CPD lesions in differentiated cells exposed to UVC radiation. Even though TCR is difficult to measure by comet assay, our results for MSN neuronal cells suggest low NER capacity through differentiation at the global genome level; i.e., terminally differentiated cells do not replicate their genomic DNA, and can therefore dispense with the need to remove damage in non transcribed DNA from their genome (Nouspikel and Hanawalt, 2003).

There is increasing evidence for a cooperative role of NER in the removal of DNA oxidative lesions (Dianov et al., 1999; D'Errico et al., 2007). In the present study, we challenged undifferentiated and differentiated neuronal cells with UVC radiation and with prolonged H_2O_2 treatment to induce 8OHdG lesions, to compare the NER capacity for removal of various types of DNA damage. The H_2O_2 treatment simulated an endogenous oxidative environment like those found in biological systems (cytokines, growth factors, immune system, vascular rearrangement, etc.) (Choi et al., 2005; Veal et al., 2007) and in pathological disorders such as neurodegenerative diseases and cancer. The results from the oxidative insults indicated that undifferentiated cells partially removed the induced DNA damage but still had 8OHdG lesions 24 h after treatment. Differentiated cells required a long time to remove the DNA damage but did not present 8OHdG lesions 24 h after treatment. At this point it is important emphasize the fact that even to employ the MSN neuroblastoma, were genetic instability should be related with DNA-oxidative lesions as 8OHdG, we did not detect differences between basal 8OHdG levels due to differentiation stage (Fig. 4), allow us consider the inductions as consequences of H_2O_2 treatments rather to tumoral source.

Please cite this article in press as: Ramos-Espinosa P, et al. Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model. Neurotoxicology (2012), http://dx.doi.org/10.1016/j.neuro.2012.05.017

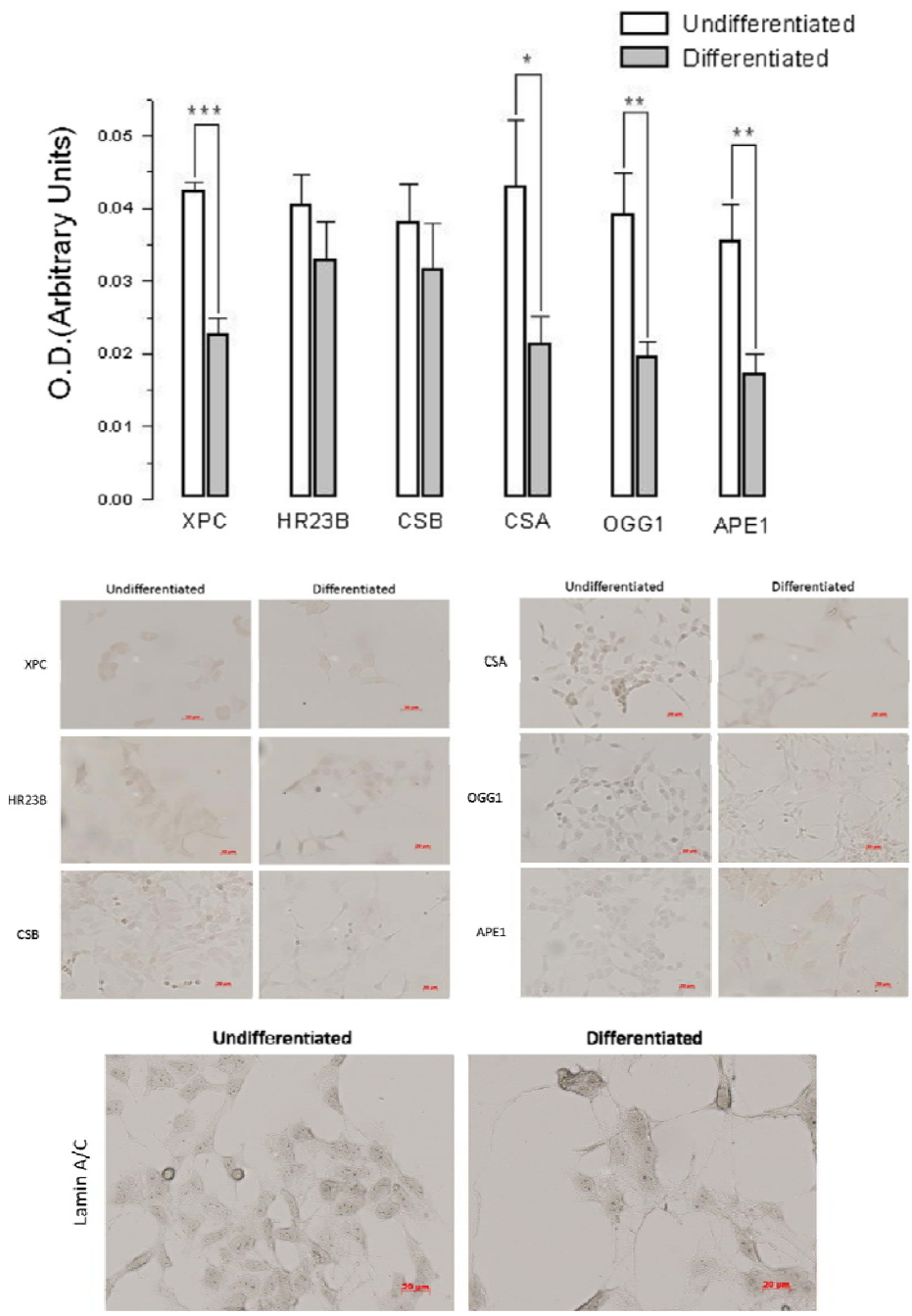

Fig. 5. Basal levels of recognition nucleotide excision repair proteins in undifferentiated and differentiated MSN cells. GG-NER: HR23B, XPC; TC-NER: CSA, CSB; BER: OGG1, APE1 and Lamine A–C proteins. Images were digitized and analyzed using NIH Image J software. Densitometry analysis values are expressed as optic density (arbitrary units) of 3 independent experiments (mean ± S.D.). t-Student was performed, significantly different to control $^{*}p < 0.05$; $^{**}p < 005$; $^{***}p \leq 0.001$. Representative images are present in different panels comparing undifferentiated and differentiated cells.

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Table 3
Comparison between repair recognition proteins levels in undifferentiated and differentiated MSN cells after exposure to UVC or H_2O_2. Data were normalized to its own differentiated status control and are represented as percentage value ± S.D. Results are the mean of 3 independent experiments.

	Protein	Control	UV	1 h PT	24 h PT	H_2O_2	1 h PT	24 h PT
Undifferentiated MSN cells								
GGR	XPC	100 ± 2.9	107.2 ± 28.7	89.0 ± 5.9	136.7 ± 17.3	69.0 ± 5.1*	65.7 ± 12.8*	79.5 ± 8.8*
	HR23B	100 ± 10.5	172 ± 25***	117.9 ± 9.8	175 ± 14***	102.3 ± 14.7	109.1 ± 25	83.9 ± 8.2
TCR	CSB	100 ± 13.2	114.5 ± 13	118.7 ± 8.1	142.2 ± 6.7**	158.5 ± 25**	121.3 ± 13	120.6 ± 5.9
	CSA	100 ± 12.5	87 ± 12	100.2 ± 13	133.8 ± 18.9*	100 ± 11.5	94.8 ± 10.8	89.8 ± 7.9
BER	OGG1	100 ± 12	124.7 ± 9.5	103.6 ± 12.5	191.2 ± 18*	104.7 ± 12.1	108.4 ± 17.1	107 ± 5
	APE1	100 ± 8.1	90.8 ± 5	126.7 ± 18	216.1 ± 49***	145.7 ± 9***	165.3 ± 15***	119.2 ± 6.4
Differentiated MSN cells								
GGR	XPC	100 ± 9.4	171.7 ± 10.2*	95.5 ± 11	176.6 ± 24***	181.0 ± 35**	134.8 ± 19.9	137.0 ± 12
	HR23B	100 ± 15.7	131.4 ± 14	63 ± 11.8	129.5 ± 26.9	102.9 ± 10.6	128.6 ± 15*	130 ± 4.7
TCR	CSB	100 ± 15.9	122.3 ± 18.9	77.8 ± 17.85	150 ± 15.8*	73.9 ± 23.4	55.7 ± 15	72.8 ± 8.4
	CSA	100 ± 16.7	121.6 ± 5.8	84.3 ± 17	116.7 ± 11	129.1 ± 4.5*	121.7 ± 6.5	83.9 ± 11.4
BER	OGG1	100 ± 8.9	161.1 ± 52.9	87.3 ± 18.7	193.9 ± 37.6	128.7 ± 15.3	110.6 ± 24.7	95.5 ± 12.9
	APE1	100 ± 14.8	144.4 ± 15*	105.1 ± 18.3	147.4 ± 10.5**	160.1 ± 15*	104 ± 5.3	155.7 ± 33*

* Significance of difference by one-way ANOVA: $p < 0.05$.
** Significance of difference by one-way ANOVA: $p < 0.005$.
*** Significance of difference by one-way ANOVA: $p \leq 0.001$.

Thus, the MSN cells were more efficient in recognising oxidative lesions than UVC helix-distorting damage, probably because oxidative lesions are the more common type of DNA damage. 8OHdG lesions, the main type of oxidative lesion, were removed without DNA strand preference (Thorslund et al., 2002), suggesting that the accumulation observed in the undifferentiated cells resulted from the deficient repair activity. The first defence against the most common base oxidative modification is the BER pathway (Christmann et al., 2003; Seeberg et al., 1995). 8OHdG lesions have been reported to be eliminated not only by the BER pathway but also by the cooperation of other repair mechanisms such as NER (Dianov et al., 1999). The oxidative damage removal observed in MSN cells may not result exclusively from BER, but also indicates indirectly a GG-NER activity (Table 3). 8OHdG is the most frequent mutagenic lesion caused by oxidative stress. Although cells use a specific DNA glycosylase, OGG1, to excise 8OHdG from DNA, recent studies suggest the existence of alternative pathways for removal of 8OHdG, including CSB-dependent transcription coupled repair (TCR) (Larsen et al., 2004). CSB may play a role in the removal of 8-oxoG from the overall genome independently from OGG1-mediated base excision repair and regular transcription. The repair of endogenous oxidative DNA damage in the absence of CSB and OGG1 was found to accumulate with age, leading to several-fold higher levels of oxidative purine (Osterod et al., 2002; Trapp et al., 2007). The acceleration of the rate of repair of CSB and CSA is due to the regulation of chromatin structure by ubiquitination and/or acetylation of histones, which increases accessibility to the DNA repair machinery (Citterio et al., 2000; Newman et al., 2006). Dianov et al. (1999) and Tuo et al. (2002) proposed that the CSB protein may affect the transcriptional regulation of several genes, including some involved in DNA repair, e.g., the OGG1 gene (Dianov et al., 1999; Tuo et al., 2002). The absence of XPC has been shown to reduce the repair rate of 8OHdG, suggesting that the XPC–HR23B complex acts as a cofactor in the base excision repair of this lesion and stimulates the activity of its specific DNA glycosylase OGG1 (D'Errico et al., 2006).

These findings, taken together, are relevant to reports of the attenuation of repair mechanisms as differentiation proceeds (Yamamoto et al., 2007). The present study showed a clear reduction in levels of almost all repair enzymes (XPC, CSA, OGG1, APE1) in differentiated cells compared with undifferentiated cells, indicating a high vulnerability to environmental insults.

Recognition enzymes, which are indispensable for initiation of repair, retained their activity following UVC or H_2O_2 treatment. Protein quantification was performed to determine whether the observed changes in levels of repair proteins during differentiation might be explained in terms of DNA damage removal. Table 3 summarizes the results of the analyses for proteins associated with the NER and BER mechanisms, normalized in relation to their controls depending on differentiation status. Interestingly, the GG-NER and BER proteins were induced by UV exposure regardless of cell cycle status (proliferation or differentiation), suggesting that even if the differentiation process reprograms a selected subset of genes (and non transcribed strands of active genes), the global genome repair system remains alert. Parallel oxidative treatments caused induction of NER and BER, indicating a dynamic role in overall response to damage, perhaps explainable in terms of XPC, CSB and CSA participation in 8OHdG removal.

5. Conclusions

In conclusion, we observed differential responses to diverse genotoxic insults depending on the differentiation stage. Differentiated MSN cells, compared with undifferentiated, showed greater sensitivity to UVC and decreased DNA damage over time. In contrast undifferentiated cells displayed slight genotoxicity induced by oxidative insult and tended to accumulate DNA damage and 8OHdG lesions over time. We suggest the participation of GG-NER, TC-NER and BER proteins in the removal of 8OHdG and CPDs indicating a dynamic role in overall response to damage. These findings are pointing out the impact DNA damage removal response carried out by terminally differentiated neurons to contribute to the knowledge in the biology of some neuro pathologies.

Funding

This work was supported by PAPIIT [IN202007].

Conflict of interest statement

None declared.

Acknowledgments

R-E. P. A. was supported by a fellowship from CONACyT.

Please cite this article in press as: Ramos-Espinosa P, et al. Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model. Neurotoxicology (2012), http://dx.doi.org/10.1016/j.neuro.2012.05.017

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Please cite this article in press as: Ramos-Espinosa P, et al. Differential DNA damage response to UV and hydrogen peroxide depending of differentiation stage in a neuroblastoma model. Neurotoxicology (2012), http://dx.doi.org/10.1016/j.neuro.2012.05.017

Artículo 3

” UVC response of fibroblast 3T3 versus undifferentiated and differentiated neurons. Participation of excision repair proteins”

Este artículo surge como un ejercicio comparativo de la actividad de NER y BER ante un reto con UVC entre un modelo murino (fibroblastos 3T3), donde se encuentra atenuada la reparación por escisión de nucleótidos (NER) (**Hanawalt 2001**), y el modelo de neuroblastoma humano MSN, en estado indiferenciado y diferenciado.

Los hallazgos encontrados en este trabajo se resumen a continuación:

- Los fibroblastos murinos son más sensibles a la luz UVC que las células indiferenciadas y diferenciadas del modelo del neuroblastoma humano.
- Los niveles proteicos de las enzimas de reparación de BER y NER varían a lo largo de la cinética de reparación, sin embargo, a este nivel no se detectaron sub-expresiones en los fibroblastos murinos, como para considerar una atenuación en la respuesta de las proteínas involucradas.
- Los tres tipos celulares responden al estímulo de la radiación con UVC, sin embargo, tanto los fibroblastos como las neuronas terminalmente diferenciadas presentan más rompimientos en el DNA que las células indiferenciadas.
- Contrario a lo que se esperaba, la principal lesión ocasionada por la radiación con UVC, anillos de ciclobutano (CPD), es reparada eficientemente por los dos tipos celulares terminalmente diferenciados, que además fueron los más sensibles.

UVC response of fibroblasts 3T3 versus undifferentiated and differentiated neurons. Participation of excision repair proteins.

1. INTRODUCTION

The NER mechanism is a multistep process able to recognize and remove helix-distorting lesions caused by UV irradiation and chemical mutagens (**Shuck et al 2008**). The two major classes of mutagenic DNA lesions induced by UV irradiation, and repaired primarily by NER, are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4PPs). This mechanism is a complex process consisting of the following steps: (i) recognition of a DNA lesion; (ii) single strand incision at both sides of the lesion; (iii) excision of the lesion-containing single stranded DNA fragment; (iv) DNA repair synthesis to replace the excised nucleotides; (v) ligation of the remaining single stranded nick (**van Hoffen 2003**). NER operates via two sub-pathways that differ in the mechanism of lesion recognition: transcription-coupled (TCR), which specifically targets lesions that block transcription of active genes, and global-genome (GGR), which repairs lesions throughout the genome (**Jackson and Bartek 2009**, **Palomera-Sánchez 2011**).

Inherited defects in NER cause two human syndromes with striking differences in symptoms and survival: Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) both characterized by extreme UV sensitivity and neurological alterations (**Niedernhofer 2008**). In this matter little is known about the relationship between NER protein defects favoring neuronal pathologies development. Since NER is the principal mechanism capable of removing UV-induced DNA lesions in mammals (**Nguyen 2010**), it becomes interesting to evaluate NER role in affected cells of neurological pathologies, such as neurons. Even though UV radiation cannot penetrate the skull and thus reach the brain, UV damage induction challenges neurons to repair through NER, and in this way elucidate DNA repair capacity. Unfortunately, growth characteristics make neurons a very challenging

experimental system, which is why many studies have been performed with tumor cell lines that can be differentiated into neuron-like cells.

On the other hand, most mammalian cells carry out excision repair; however some reports had shown that cells from different species responded to the same insult with vastly different rates and extents of repair replication (**Painter and Cleaver, 1969; Trosko et al 1965, Hanawalt 2001, Lohman et al 1976**). Different rodent cells such as fibroblast have shown poor UV lesions repair (**Ben-Ishai and Peleg 1975, Ganesan et al 1973, van Zeeland et al 1981 Vijg et al 1984**). An interesting issue is 3T3 mouse cell line which through cultivation showed almost no detectable excision repair (**Pelag et al 1976**) this characteristic turn's murine fibroblast a good NER attenuated model.

In this study, we were interested in evaluating DNA repair capacity in order to compare damage removal between murine fibroblast cells and undifferentiated and differentiated neurons. Taking advantage of 3T3 as attenuated excision repair model, our group was interested in evaluating in a comparative way, DNA repair in the terminally differentiated cells to find differences in lesion removal and damage accumulation favoring cell death and thus neural disease development.

2. MATERIALS AND METHODS

2.1 Cell culture.

3T3 fibroblast cells were grown at 37°C and 5% CO2 in culture dishes in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 1%antibiotics and 1% non-essential amino acids. Human neuroblastoma MSN cells (**Reynolds et al. 1986, Ramos-Espinosa et al 2012**) were cultured in enriched RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 1% antibiotics, 1% non-essential amino acids, 1% QSN solution (serine, asparagine and glutamine) and 10% fetal bovine serum (Gibco, Life Technologies Corporation, Grand Island, NY, USA) in

tissue culture dishes in an atmosphere of 5% CO_2 and 95% O_2, at 37°C, and were plated at a density of 1 X 10^6 cells per dish. For differentiated neurons, the cells were seeded at a density of 1 x 10^6 per dish and differentiated by adding 10mM retinoic acid and 50 ng/ml NGF (Invitrogen, Life Technologies Corporation, USA) for 6 days.

2.2 UVC irradiation dose-response.

To establish an appropriate UVC dose, undifferentiated MSN cells were washed with PBS buffer, placed in open dishes with PBS and irradiated with various doses of UVC (0, 1.8, 2.2, 3.6 and 5.1 J/cm^2, wavelength 254 nm at room temperature) from a germicidal lamp (Biological Safety Cabinet TUV 15 GIT5). The cultures were monitored for 24 h after exposure. The cells were then harvested in physiological saline solution, and 10 μl of 0.4% trypan blue dye solution was added to 10 μl of cells for each experimental condition. The cells that excluded trypan blue were considered viable and were counted using a hemocytometer.

2.3 Immunocytochemistry.

MSN cells were plated on coverslips coated with 0.1% gelatin and incubated overnight to allow the cells to adhere. After UVC irradiation, the medium was removed and the cells were fixed with 95% ethanol, 5% acetic acid and H_2O for 30 min at room temperature, followed by hydration of the sample in this order: 95% ethanol for 5 min, 50% ethanol for 5 min and H_2O for 5 min. The coverslips were then embedded in PBS-1% H_2O_2. The primary antibodies employed were rabbit polyclonal anti-CSA (1:50; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-CSB (1:50; Santa Cruz), rabbit polyclonal anti-XPC (1:50; Santa Cruz), goat anti-HR23B (1:100, Abcam, CA, USA), rabbit polyclonal anti-OGG1 (1:50; Santa Cruz), rabbit polyclonal APE1 (1:50; Santa Cruz), dissolved in PBS-1% H_2O_2 and incubated at 37°C for 40 min. For each experiment, the coverslip was washed three times with PBS-1% H_2O_2 for 5 min. The secondary antibodies, goat anti-mouse HRP conjugate (1:250; Zymed, CA, USA) and goat anti-rabbit HRP conjugate (1:250; Zymed)

were added for 40 min at 37°C, and coverslips were washed three times for 5 min. The cells were stained with 3,3-diaminobenzidine (DAB) using a DAB kit (Vektor, CA, USA). Dehydration was performed in the following order: 50% ethanol for 5 min, 75% ethanol for 5 min, absolute ethanol, xylene. The coverslips were embedded in resin, set on slides and examined using an Olympus BX-60 microscope at 40x magnification. Images were digitized and analyzed using NIH Image J software, values were expressed as optical density (arbitrary units) average per condition.

2.4 **Single strand breaks determination (Comet assay)**

For each experimental condition, at least 10,000 cells were mixed with 75 ml of 0.5% low melting point (LMP) agarose. The cells were loaded onto microscope slides pre-layered with 200 µl of 0.5% normal melting point agarose. The comet assay was performed as described by **Ramos-Espinosa** (**2012**). Briefly, after lysis of cells at 4°C for at least 1 h in a medium consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, supplemented with 10% DMSO and 1% Triton X-100, slides, including a positive control (g-irradiated lymphocytes), were placed in a horizontal electrophoresis chamber with running buffer solution (300 mM NaOH, 1 mM Na_2EDTA, pH >13). The slides remained in the electrophoresis buffer for 10 min to allow the DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 25 V, ~0.8 V/cm. All steps were performed in the dark; after electrophoresis, the slides were gently removed and rinsed with neutralisation buffer (0.4 M Tris, pH 7.5) at room temperature for 15 min, dehydrated with absolute ethanol (15 min) and air-dried. Ethidium bromide (20 ml of 20 mg/ml solution) was added to each slide and a coverslip was placed on the gel. Individual cells were visualized at 20x magnification under an Olympus BX-60 microscope with fluorescence attachments (515–560 nm excitation filter, 590 nm barrier filter), and the DNA damage was determined using Komet 5.0 software (Kinetic Imaging). To evaluate DNA migration, 100 cells were scored for each experimental condition. The data were categorized into five categories according to the Olive tail moment score as described by **Ramos-Espinosa et al** (**2012**). The total

number of cells in each category was counted and multiplied by an assigned value 0-4 according to the extent of damage. The sum of all the categories was calculated and considered as the damage index. The overall score was expected to vary between 0-400 arbitrary units.

2.5 T4 endo V sensitive sites (Enzymatic-Comet assay), cyclopyrimidine dimer determination.

Based on the results of the comet assay, we evaluated the UVC-induced formation of cyclopyrimidine dimer (CPD) through T4 endo V-sensitive sites in control and UVC irradiated cells. After treatment, the cells were layered on microscope slides and immersed in lysis buffer for at least 1 h at 4°C. The slides were then rinsed with buffer solution (50 mM Tris-base, 10 mM EDTA, pH 7.6) for 5 min. For CPD detection, the slides were overlaid with 20 mL T4 endo V (New England BioLabs, MA, USA), which specifically cuts DNA at CPD sites. Coverslips were placed on the slides, and the slides were incubated for 30 min at 37°C in a humidified atmosphere. A set of slides with irradiated cells in buffer (without enzyme) was included to confirm that the DNA strand breaks were enzyme-specific. Following enzyme incubation, the slides were rinsed with solution buffer (50 mM Tris-base, 200mM EDTA, pH 7.6) and subjected to electrophoresis (~ 0.8 V/cm) for 10 min without unwinding incubation. Comet visualisation was performed at 20x magnification using an Olympus BX-60 microscope with fluorescence attachments (515–560 nm excitation filter, 590 nm barrier filter). The images were digitized and analyzed using Komet 5.0 software. To evaluate DNA migration, 100 cells were scored for each experimental condition. The damage index was determined as described in the preceding section.

2.6 Statistical analysis.

Data for cell viability and levels of signal repair recognition proteins were calculated as the mean ± SD from 3 independent experiments. The differences between experimental and

control values were assessed by one-way Analysis of Variance (ANOVA; Sigma Stat v 3.5), with $p< 0.05$ considered to be significant. DNA fragmentation as determined by Comet assays was represented as the mean ± SEM of damage indices from 3 independent experiments. The differences between values for fibroblast 3T3 and neurons under various differentiation states were assessed by Student's "t" test (Sigma Stat v 3.5).

3 RESULTS

3.1 UVC dose-response determination.

To determine the doses of damaging environmental agents that pose a challenge to cell viability, 3T3 and MSN cell cultures were exposed various UVC light doses (0, 1.8, 2.2, 3.6, 5.1 J/cm^2). Cells were monitored for a subsequent 24 h because cell viability is not affected immediately by UVC exposure; as **Batista** (**2009**) suggested, biological effects such as cell death should be monitored for at least 8 h following experimental treatment. The results shown in Table 1 indicate a dose-dependent reduction in cell viability in cultures that excluded trypan blue after irradiation treatment. UVC induced a significant decrease in cell survival at lower doses (e.g., 0.7J/cm^2 for fibroblast 3T3, and 1.8 J/cm^2 for undifferentiated neurons). However, the major effect on cell viability, reflected as a 50% reduction in survival, was observed at a 2.2 J/cm^2 in 3T3 fibroblast, meanwhile 3.6 J/cm^2 dose in undifferentiated neurons.

Table 1. Cell viability measurement comparing 3T3 fibroblast and undifferentiated neurons exposed to different UVC dose.

3T3, Murine Fibroblast		MSN, undifferentiated neurons	
UVC exposure (J/cm^2)	% Cell viability	UVC exposure (J/cm^2)	% Cell viability
0	100 ± 2.8	0	100 ± 5.2
0.7	85 ± 3.4**	1.8	73.1 ± 3.3***
1.4	69.6 ± 5.1***	2.2	70.6 ± 9.8***
1.8	72.9 ± 2.8***	3.6	45.9 ± 0.8***
2.2	50.6 ± 6.4***	5.1	44.6 ± 7.7***
2.8	59.4 ± 10.4***		

Cell viability was evaluated by trypan blue exclusion assay in murine 3T3 fibroblast and MSN undifferentiated neurons 24 h post treatment with different doses of UVC irradiation. All data are expressed as percentage and represent the mean ± S.D. of 3 independent experiments. Analysis of variance was performed by one-way ANOVA (Sigma Stat v 3.5). Different from control value: *p<0.05; **p<0.005; ***p≤0.001.

3.2 **NER and BER protein expression**

As a first approach we tested DNA repair proteins prior to UV exposition in order to determine changes in protein levels among cell lines. We performed immunocytochemistry of recognition enzymes involved in NER and BER pathways comparing murine 3T3 fibroblasts, that have been reported as NER attenuated cells (**Pelag et al 1976**), with undifferentiated, and terminally differentiated neurons, MSN (Figure 1). Most differences occurred between 3T3 fibroblast and terminally differentiated neurons, were NER proteins as XPC, CSA, CSB, and both BER, OGG1 and APE-1 were sub-expressed in differentiated neurons, suggesting MSN differentiation model as another NER attenuated cell model.

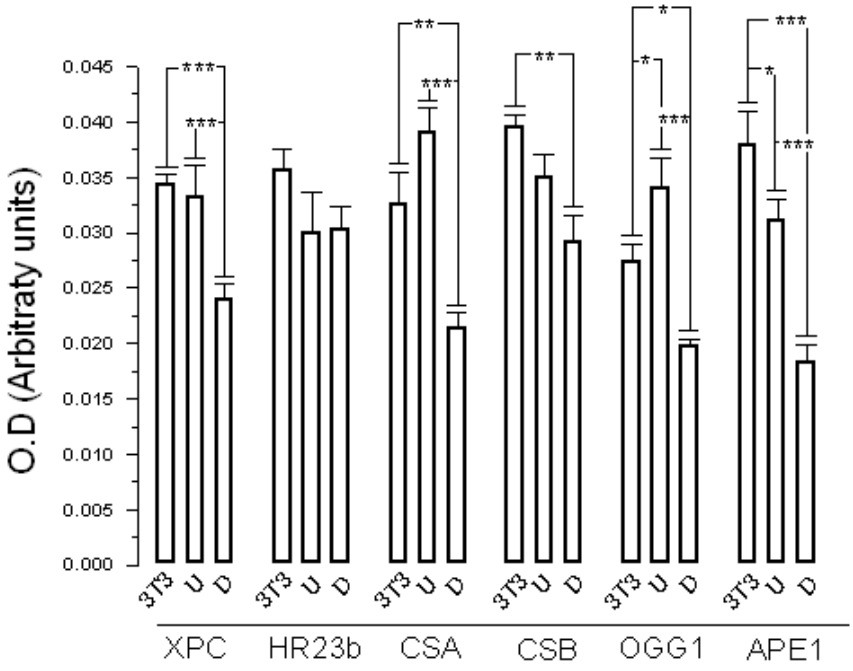

Figure 1. NER and BER recognition enzymes protein expression in (3T3) 3T3 murine fibroblast, (U) undifferentiated neurons and (D) differentiated neurons. GGR: XPC and HR23b; TCR: CSA and CSB; BER: OGG1 and APE1. Data are expressed as Mean ± S.E. One Way ANOVA was performed (Sigma Stat v 3.5). Significance of different from control value: *p<0.05; **p<0.005; ***p≤0.001.

3.3 **Response of NER and BER proteins against UVC irradiation challenge.**

We evaluated repair protein levels of NER and BER mechanisms in 3T3 fibroblast and undifferentiated and terminally differentiated neurons, before and after UV exposition in order to determine repair response to the genotoxic challenge. Cells were challenge with UVC dose that reflected 50% cell viability reduction (2.2 J/cm^2, 3T3 and 3.6 J/cm^2, neurons MSN) and maintained under physiological conditions during 1h and 24h as post treatments or recovery time. These results were summarized in Table 2. Immunocytochemistry was performed and quantitatively evaluated to determine protein in nucleus, in order to compare the response between fibroblasts and undifferentiated and differentiated neurons. The kinetic response of XPC was as follow, basal XPC protein level was decreased almost 30% in terminally differentiated neurons compared to undifferentiated neurons and fibroblast. After UVC irradiation, neurons had a dramatic increase (60%); meanwhile fibroblast and undifferentiated MSN had a slight induction (20-36%). In all cell lines XPC levels decreased an hour after irradiation. With respect to HR23b, this protein was induced only in MSN neurons and decreased 1h post treatment. Surprisingly on the fibroblast and undifferentiated neurons, HR23b protein increments were observed 1h post treatment. On the other hand, TCR protein, CSA, showed low basal levels in differentiated neurons comparing with fibroblast and undifferentiated neurons. After UV irradiation neurons showed CSA nuclear induction, while fibroblasts did not show changes in levels of this protein. CSB protein showed same basal behavior as its partner, CSA. Neurons showed increment of repair proteins after UVC challenge (46%), undifferentiated had almost half of neurons elevation and fibroblast had no significant changes compared to control. Even though, UVC stimuli, recruit in a particular way NER,

BER proteins suggest an oxidative DNA damage as a consequence to irradiation environment. BER protein expression in nucleus showed OGG1 and APE1 basal decrement in differentiated neurons compared to undifferentiated neurons. After UVC irradiation, undifferentiated and differentiated cells were able to recruit OGG1, but not APE1. Interestingly the presumed attenuated model, 3T3 had less OGG1 levels in nucleus than the NER proteins.

Table 2. Comparative levels of DNA repair enzymes after UVC challenge and 1h post treatment in 3T3 fibroblast, and undifferentiated and differentiated MSN neurons.

			3T3 Murine fibroblast	MSN Undifferentiated neurons	MSN Differentiated neurons
Global genome repair	XPC	Control	0.0345 ± 0.0009	0.0334 ± 0.0026^{ccc}	0.0242 ± 0.0016^{bbb}
		UV	0.0414 ± 0.0019**	0.0456 ± 0.0046*	0.0389 ± 0.0010***
		1h PT	0.0316 ± 0.0005^{aa}	0.0379 ± 0.0009^{ccc}	0.0219 ± 0.0011^{bbb}
	HR23B	Control	0.0357 ± 0.00189	0.0300 ± 0.0036	0.0304 ± 0.0019
		UV	0.0283 ± 0.0013*^{a}	0.0195 ± 0.0013*^{ccc}	0.0413 ± 0.0029**^{bbb}
		1h PT	0.0334 ± 0.0018^{aaa}	0.0461 ± 0.0021**^{ccc}	0.0209 ± 0.0017*^{bb}
Transcription coupled repair	CSA	Control	0.0326 ± 0.0029	0.0392 ± 0.0021^{ccc}	0.0215 ± 0.0013^{b}
		UV	0.0356 ± 0.0011	0.0375 ± 0.0019^{ccc}	0.0262 ± 0.0006*^{bbb}
		1h PT	0.0527 ± 0.0022***^{a}	0.0410 ± 0.0030^{ccc}	0.0181 ± 0.0016^{bbb}
	CSB	Control	0.0396 ± 0.0010	0.0351 ± 0.0019	0.0293 ± 0.0022^{b}
		UV	0.0389 ± 0.0009	0.0439 ± 0.0022*	0.0429 ± 0.0047*
		1h PT	0.0357 ± 0.0013^{aa}	0.0455 ± 0.0013**^{ccc}	0.0247 ± 0.0023^{bbb}
Base excision repair	OGG1	Control	0.0276 ± 0.0014^{a}	0.0342 ± 0.0025^{ccc}	0.0199 ± 0.0006^{b}
		UV	0.0277 ± 0.0008^{aaa}	0.0472 ± 0.0023**^{cc}	0.0320 ± 0.0047**
		1h PT	0.0313 ± 0.0029^{a}	0.0433 ± 0.0031*^{ccc}	0.0193 ± 0.0024^{b}
	APE1	Control	0.0380 ± 0.0029^{a}	0.0313 ± 0.0017^{ccc}	0.0185 ± 0.0014^{bbb}
		UV	0.0539 ± 0.0012*^{aaa}	0.0324 ± 0.0006^{c}	0.0270 ± 0.0021^{bbb}
		1h PT	0.0479 ± 0.0033*	0.0414 ± 0.0032**^{ccc}	0.0183 ± 0.0043^{bbb}

3T3 fibroblasts were exposed to 2.2 J/cm^2 UVC. Undifferentiated and differentiated cells were exposed to 3.6 J/cm^2 UVC. Images were digitized and analyzed using NIH Image J software. Densitometry analysis values represent 3 independent experiments (Mean ±S.E). One way ANOVA was performed, statistical difference between control and exposed cells is represented by *p<0.05; **p<0.005; ***p≤0.001; meanwhile difference between 3T3 and undifferentiated MSN cells are represented by ^{a}p<0.05; ^{aa}p<0.005; ^{aaa}p≤0.001; difference between 3T3 and terminally differentiated neurons are expressed as ^{bb}p<0.005; ^{bbb}p≤0.001, and statistical difference between MSN differentiation states are represented as ^{c}p<0.05; ^{cc}p<0.005; ^{ccc}p≤0.001.

3.3 **DNA strand breaks induction and kinetic of DNA repair after UVC challenge.**

Most NER proteins were recruited to the nucleus after UVC irradiation in some cases protein remained an hour later, suggesting that repair was not concluded. To support this idea, we evaluated DNA fragmentation (comet assay), as a strand breaks that remain as consequence of UVC challenge. Figure 2 show DNA strand breaks quantified as damage index (Arbitrary units) of basal condition (C), UV induced and 1h post treatment; comparing fibroblast with undifferentiated and differentiated MSN neurons. UVC irradiation induced DNA strand breaks in all cell lines studied and DNA damage was accumulated; probably as a consequence of poor DNA repair capacity. In addition, data were analyzed as percentage of DNA damage induction and percentage of DNA repair with respect to their own control (table 2). Fibroblast and undifferentiated cells showed 30% of DNA damage induction by UV challenge; meanwhile terminally differentiated neurons were extremely damaged (350%) by the same challenge. An hour post irradiation, differentiated neurons and fibroblast cell partially remove DNA damage and undifferentiated neurons did not repair.

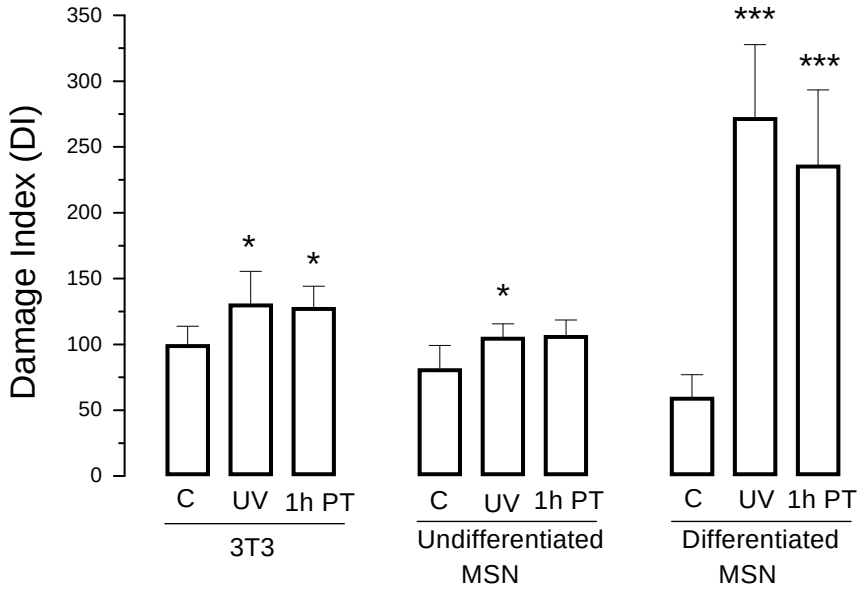

Fig. 2. Repair kinetics comparison between cell lines. Cells were irradiated corresponding 50% lethal UV dose and quantified by comet assay DNA as damage index (DI). DNA damage was measured in the basal condition (C), after UV irradiation (UV) and 1h post irradiation (1h PT) for repair kinetics. Analysis of variance was performed by one-way ANOVA (Sigma Stat v 3.5). Significance of different from control value: *p<0.05; **p<0.005; ***p≤0.001.

Table 2. Percentage of DNA Damage induction and repair after UVC challenge.

Cell type	%Damage induction after UV	%Damage induction 1 h after UV	%Damage repair 1h after UV
3T3 fibroblast	30.9	28.2	8.6
Undifferentiated MSN neurons	29.3	30.9	0
Differentiated MSN neurons	353.8	293.8	17

%Damage induction data are compared to control. %Damage induction an hour after UV-irradiation was calculated: (basal damage- 1h PT damage)/ induction damage x 100%. %Damage repair was calculated: 100%- 1h PT damage induction%.

3.4 **T4 endo V sensitive sites to determine CPD lesions after UVC challenge.**

The principal UV damage is known as CPD and is widely known to be a NER inducer, for this reason we decided to measure CPD loose as repair function, comparing fibroblast with undifferentiated and differentiated neurons. Enzymatic-Comet assay was performed with T4 endo V, reporting sensitive sites, because this enzyme detects with high specificity CPD, and cuts DNA in the lesion, which is turned into a strand break. Figure 3 shows T4 endo V sensitive sites detected in fibroblast, undifferentiated and differentiated neurons trough kinetic repair. All DNA cell lines had CPD production after UVC irradiation, however differentiated cells (fibroblast and neurons) were the only capable to remove this lesion. Table 3 summarizes the same data, analyzing percentage of CPD production and percentage of lesion remaining. CPD induction was evidently since UV irradiation; strand

breaks incremented compared to control in all cell lines. Interestingly only undifferentiated neurons had CPD remaining, indicating incapacity of DNA repair (94.9%).

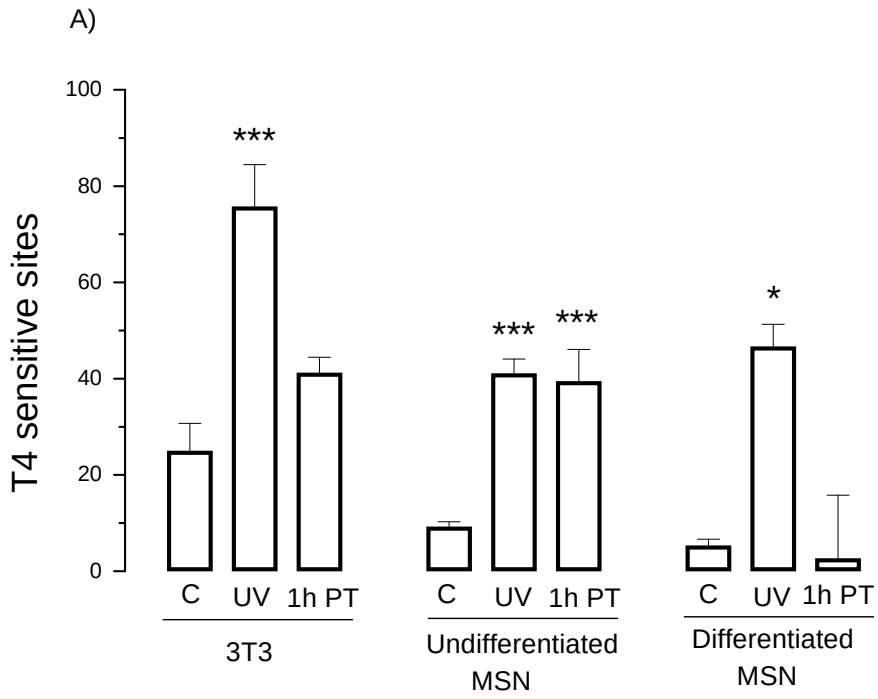

Fig. 3 Comparison of T4 endo V sensitive sites generation. Cells were irradiated with corresponding 50% lethal UV dose. Modified comet assay was performed in unexposed and exposed cells. CPD production was also measured an hour post irradiation for repair kinetics. Analysis of variance was performed by one-way ANOVA (Sigma Stat v 3.5). Significance of different from control value: *p<0.05; **p<0.005; ***p≤0.001.

Table 3. Percentage of CPD production after UV irradiation and percentage of lesion remain.

Cell type	%Damage induction after UV	%Damage induction 1 h after UV	%Damage repair 1h after UV
3T3 fibroblast	209.2	68.2	32.6
Undifferentiated MSN neurons	344	326	94.9

Differentiated MSN neurons	775	0	5.7

% CPD production with respect to control. % CPD production an hour after UV-irradiation was calculated: (basal damage- 1h PT damage)/ induction damage x 100%. % CPD remaining lesion was calculated: 100%- 1h PT damage induction%.

4.**Discussion**

Cells that proliferate to generate proliferating daughter cells are substantially different from cells that proliferate toward the formation of post-mitotic, differentiated progenies (**Simonatto 2007**). DNA repair systems can vary in their responses, depending on the state of cellular differentiation (**Nouspikel 2006, Nouspikel and Hanawalt 2000**). In this sense little is known about the role of DNA repair capacity of differentiated cells as neurons and its relation to degenerative diseases, aging and cancer. Taking advantage of 3T3 attenuated excision repair model, our group was interested to evaluate this capacity in a comparative way, DNA repair in the terminally differentiated cells in order to find differences in lesion removal and damage accumulation that could favor cell death and thus neural disease development. For this purpose, we exposed undifferentiated and differentiated MSN neurons and 3T3 fibroblast cells to UVC irradiation (which causes DNA distortion) to provide evidence regarding the effects of NER capacity. We observed differences in cell viability after increasing UV doses, 3T3 fibroblast compared to undifferentiated MSN neurons showed greater sensitivity to UVC. For this reason, we decided to work with different UV doses for each cell culture, which produce the same biological effect (50% of death). Prior to UV exposition, basal repair proteins were detected showing that almost all proteins were significantly different from all cell cultures. Interestingly 3T3 repair proteins were not drastically attenuated as we thought; in contrast, neuroblastoma cells had lower repair protein levels. Based on this data, neuroblastoma cells were expected to be more sensible to UVC damage, for this reason, cells were exposed to UVC and DNA fragmentation was measured. All cells had DNA damage induction after UVC exposition and remaining DNA-strand breaks. Similar sensibility was reported by **Maynard** (2008) where undifferentiated cells had less DNA

damage than differentiated cells after challenge. However in the same study, fibroblast were able to restore damage through time, this was not observed in our cells, probably because evaluation was performed only 1h post treatment. On the other hand, CPD production was measured in all cell cultures, CPD production was expected to correlate to DNA strand break as a consequence of UVC damage. Interestingly fibroblast and neurons removed this lesion an hour after irradiation, indicating that genotoxicity observed previously is not attributed to this lesion. In this matter, the assessment of repair of CPD has been reported by others groups, however tendency was contrary to our results showed. **Ganesan** (1973) found that mouse fibroblast had poorly CPD repair (10 to 30%) in contrast to human fibroblast (50 to 80%), indicating that lack of repair was attributed to a change on repair activity in mouse fibroblast.

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Discusión

El DNA se encuentra bajo constante amenaza de agentes exógenos, endógenos y químicos que producen varios tipos de lesiones a lo largo del genoma (**Lindahl 1993**). Para mantener la integridad del DNA, la célula cuenta con varios mecanismos de reparación, los cuatro más importantes son: la BER que repara modificaciones a la bases oxidadas (8oxoG), así como rompimientos de cadena sencilla del DNA; el cual a su vez tiene dos sub vías, la corta y la larga. La NER que repara lesiones distorsionantes y que tiene las sub vías GGR y TCR. La DSBR que remueve rompimientos de cadena doble del DNA con las sub vías de recombinación homóloga y no homóloga; y la MMR la cual remueve bases mal apareadas, inserciones y deleciones (**Brosh y Bohr 2007**).

Los agentes endógenos como las ROS y las especies reactivas de nitrógeno, constituyen el primer frente de batalla para los mecanismos de reparación, puesto que es el propio metabolismo celular quien los genera, y de forma importante en órganos altamente oxigenados como el cerebro (**Floyd y Hensley 2002**). Las repercusiones genotóxicas de estas especies reactivas son de gran relevancia en el desarrollo de enfermedades que van desde el cáncer hasta las neurodegeneraciones (**Salnikow y Zhitkovich 2008; Atamna H et al 2000; Goode et al 2002; Martin LJ, et al 2002**). Debido a esto es que, la diversas lesiones sobre el genoma debe ser reparados para prevenir la pérdida o transmisión incorrecta de la información genética, lo cual dependerá principalmente de la eficiencia de los mecanismos de reparación (**Branzei y Foiani 2008**). Si bien todas las células que constituyen un organismo cuentan con todos los mecanismos de reparación, se ha propuesto que la actividad de estos dependerán del tipo celular, del estado de desarrollo e incluso adoptar distintas respuestas en función del agente genotóxico (**Simonatto et al 2007**). Otro punto a considerar en la respuesta al daño en el DNA es el destino celular; las células que proliferan y que dan lugar a otras estirpes

celulares son sustancialmente diferentes a las células de su progenie. Las células en estado post-mitótico, es decir, progenies diferenciadas interpretaran el daño al DNA de manera diferente puesto que no dependerán de los puntos de revisión del ciclo celular (**Polesskaya and Rudnicki 2002**).

Por esta razón en el presente trabajo se decidió evaluar la capacidad reparativas de los mecanismos de reparación por escisión: NER y BER ante un estímulo oxidante y uno que genera distorsión en la tridimensionalidad del DNA en células post mitóticas como las neuronas; para determinar la acumulación o falta de remoción de lesiones como la antesala para el desarrollo de patologías.

El primer trabajo (**Ramos-Espinosa et al 2006**) tuvo como objetivo evaluar la NER ante un daño oxidante equivalente a 10 □M de H_2O_2 ocasionado por la acumulación del fragmento □-amiloide, determinado en personas con demencia tipo Alzheimer (**Huang et al 1999**). En este trabajo se mostró una alta sensibilidad al H_2O_2 (10□M) en las neuronas diferenciadas en relación a su contraparte indiferenciada, así mismo se observó que la expresión génica de las sub vías de NER (GGR y TCR) mostraba una dependencia al estado de diferenciación. Estos hallazgos encuentran semejanza con lo reportado por el grupo de **Nouspikel y Hanawalt** (2000), donde en un modelo de neuroblastoma humano (NT2) expuesto a luz UV (inductor de NER) mostraba mayor presencia de daño en el estado diferenciado que en el indiferenciado. Al evaluar la presencia de lesiones en el DNA en un periodo posterior a la exposición inicial encontraban que solo en el estado diferenciado permanecían las lesiones, sugiriendo que la remoción no era exitosa. Por otro lado, la determinación génica de las enzimas de NER también encontraba dependencia del estado de diferenciación. Dicho trabajo y el nuestro, aunados a otras evidencias hace suponer que el estado de diferenciación ofrece mayor sensibilidad a ciertos agentes, esto debido a un cambio en la actividad de los mecanismos de reparación encargados de eliminar los daños sobre el DNA. Un ejemplo más del fenómeno aquí observado es el envejecimiento mismo; donde se ha observado que las células diferenciadas de un organismo poseen menor actividad de enzimas de reparación, esto tal vez por la acumulación de lesiones a lo largo de los años. Estas evidencias nos llevan a confirmar la hipótesis planteada por

Nouspikel y Hanawalt tres años después de la publicación de su trabajo con el neuroblastoma (**Nouspikel y Hanawalt 2003**). Dicha hipótesis propone que las células diferenciadas tienen distinta capacidad de reparación que sus precursoras. La NER en este sentido tiene mayor relevancia sobre los otros mecanismos de reparación, dado que una de sus subvías de señalización, la acoplada a la transcripción, utiliza la propia maquinaria transcripcional para el reconocimiento de lesiones sobre el DNA a través de la RNAPII. La propuesta de este trabajo es que las neuronas al ser células terminalmente diferenciadas tendrán la sub vía, TCR, con mayor actividad, puesto que a la célula le confiere menor gasto energético detectar el daño solo de los genes en transcripción que hacerlo sobre todo el genoma a través de GGR. De ahí el que la sub vía de la TCR sea de gran importancia para las células terminalmente diferenciadas, como las neuronas, ya que la ineficiencia o insuficiencia de esta sub vía repercutiría en la acumulación de lesiones del DNA a lo largo de la vida, hasta representar una amenaza para la supervivencia neuronal. Dado que las neuronas tienen una discreta tasa de proliferación, la pérdida de ellas representaría un riesgo para el funcionamiento del cerebro.

Por otro lado debido a que el cerebro se encuentra bajo un ambiente oxidante y este tiene como blanco al DNA, fue de interés en el segundo trabajo, no solamente evaluar la capacidad reparativa de NER y sus sub vías sino determinar la presencia o eliminación de una lesión oxidativa en particular: la 8oxoG. Esta base oxidada adquiere relevancia puesto que está presente en diversas enfermedades degenerativas y es un biomarcador de estrés oxidante (**Mecocci et al 1998, Lovell et al 2001**). Para determinar si NER podía reparar este daño se decidió evaluar los niveles de las proteínas de este mecanismo ante este estímulo oxidante, sin embargo dado que la respuesta al daño es una red dinámica donde se interrelacionan varios mecanismos de manera simultánea, se decidió medir a la NER con un inductor específico para esta vía: la luz UVC.

En este trabajo fue interesante encontrar que había una respuesta diferencial en varios aspectos, el primero en la respuesta de los mecanismos de reparación: NER y el que tradicionalmente repara lesiones oxidativas, BER; en la reparación según el tipo de lesión que se indujo; y finalmente al estado de diferenciación. En este trabajo se observaron

diferencias en la remoción de daño al DNA de manera dependiente al reto inducido y al estado de diferenciación. Las células diferenciadas, comparadas con las indiferenciadas, mostraron una gran sensibilidad a la UVC, sin embargo el daño a lo largo del tiempo fue menor. Por el contrario, las células indiferenciadas mostraron una clara inducción de genotoxicidad en respuesta al daño oxidante y la tendencia mostró una acumulación de 8OHdG a lo largo del tiempo. Este trabajo sugiere también la participación de las dos sub vías de NER así como las de BER en la remoción de CPDs y de 8OHdG, indicando que la respuesta al DNA es dinámica y no solo depende un mecanismo de reparación.

Como ejercicio adicional de este trabajo fue comparar al modelo de diferenciación neuronal con otro cultivo neuronal, con el objeto de determinar si la deficiencia en la reparación se debía a una propiedad inherente al modelo de diferenciación o si había una tendencia de las células diferenciadas, en general, a comportarse de manera similar. Con base en lo que algunos grupos había observado en las células de murinos, decidimos incorporar una línea celular como control de la reparación de NER. Los fibroblastos murinos 3T3 representan un modelo de estudio interesante, puesto que son células diferenciadas y de acuerdo con algunos reportes, los murinos muestran una atenuación de la GGR en la mayoría de sus fibroblastos (**Hanawalt 2001**). Esta atenuación si bien no ha sido del todo documentada, sí se sabe que se encuentra en el reconocimiento de lesiones distorsionantes como las que ocasionan los fotoproductos producidos por la luz UVC. En el último trabajo de esta tesis se decidió incluir los resultados obtenidos en el modelo 3T3 comparando con el modelo de diferenciación neuronal, todos expuestos a luz UVC. Como primer resultado interesante es la alta sensibilidad que tienen los fibroblastos a la luz UVC, esto evidenciado por la citotoxicidad que produce su exposición. Debido a que la respuesta a luz UVC fue distinta para los tres cultivos celulares, se decidió utilizar diferentes dosis de UVC para los fibroblastos y el modelo de neuroblastoma, este hecho por si mismo, ya evidenciaba sensibilidad de los fibroblastos en relación al neuroblastoma. Para determinar si había alguna atenuación proteica de las enzimas que se estaban determinando, se realizó la evaluación de las enzimas de NER y BER en el estado basal de los fibroblastos y del neuroblastoma, observándose que los niveles basales de proteínas

en los fibroblastos no son menores al estado diferenciado del modelo de diferenciación neuronal, lo que hace suponer que si bien los fibroblastos podrían tener una GGR atenuada, las neuronas podrían presentar más susceptibilidad al daño al DNA por la disminución en sus condiciones basales. La actividad reparativa muestra que las células diferenciadas son eficientes para reparar lesiones distorsionante, contrario a lo que se pensaba. Se encontró al igual que en el segundo trabajo, una respuesta de las proteínas de reparación dinámica dependiente de la inducción de daño sobre el DNA.

Todos estos resultados en conjunto demuestran que la sensibilidad al daño al DNA esta íntimamente ligada a la respuesta eficiente, deficiente o suficiente de los mecanismos de reparación y que estos estarán determinados por el estado celular. Si el daño persiste sobre el DNA como consecuencia de la reducción de la actividad de los mecanismos de reparación, podría acarrear consecuencias importantes que van desde la alteración de la transcripción, mutaciones en el genoma o la muerte celular.

La acumulación de las lesiones a lo largo de la vida, puede ser el parteaguas para el desencadenamiento de la muerte neuronal con repercusiones sobre el organismo entero. Por esta razón el estudio de la capacidad reparativa puede dar una pista para el desarrollo de enfermedades como el cáncer y las de tipo neurodegenerativo.

Conclusiones

- La remoción de daño al DNA fue dependiente al reto inducido y al estado de diferenciación.
- Las células diferenciadas (neuronas y fibroblastos), comparadas con las indiferenciadas, mostraron una gran sensibilidad a los agentes utilizados (H_2O_2, agente oxidante; ó la luz UVC).
- La capacidad reparativa de lesiones tridimensionales del DNA como los anillos de ciclobutano (CPDs) y la oxidación de guaninas, (8OHdG), mostró ser dependiente del estado de diferenciación.
- Hay participación de las dos sub vías de NER así como las de BER en la remoción de CPDs y de 8OHdG, indicando que la respuesta al DNA es dinámica y no es exclusivo a un mecanismo de reparación.

Perspectivas

Con base a los resultados anteriores, surgen preguntas interesantes a responder que ampliarian el conocimiento de la respuesta al daño en neuronas, estas son:

- Determinar por otras estrategias experimentales la actividad de las enzimas de reparación de NER sobre las 8oxoG. Por ejemplo utilizando el ensayo del Faro Molecular o Molecular Beacon, este ensayo estaría dirigido a detectar la reparación de una lesión específica como la 8oxoG.
- Evaluar la NER ante estímulos crónicos oxidativos. Debido a que en este trabajo se empleó una concentración de peróxido elevada para retar al cultivo celular, resulta interesante evaluar si esta misma respuesta al daño se observa cuando el estímulo oxidativo es constante, en una concentración que no comprometa su viabilidad y por tiempos prolongados, ya que el cerebro se encuentra expuesto a un estímulo oxidativo constante a lo largo de la vida.
- Determinar la actividad de la TCR en las células neuronales por daños oxidativos. Aunque los daños oxidativos en muchas ocasiones no son suficientemente grandes para ser detectados por la TCR, es de vital importancia conocer si estos daños son los iniciadores de alteraciones en la transcripción. Para ello sería interesante evaluar la reparación ligada a la transcripción de genes, es decir, tomar un gen que se transcribe de forma única en una célula neuronal y evaluar la capacidad de reparación de este gen en particular. Se esperaría que la reparación se llevara a cabo, sin embargo sería interesante evaluara si esta reparación sería igual de eficiente con un daño oxidativo como las 8oxoG, donde la mayoría de las veces la lesión es tan pequeña que la TCR no es capaz de detectarla. Esto provocaría la acumulación de daños e incluso mutaciones que en la neurona llevaría a la muerte.
- Trabajo en cultivos primarios de neuronas humanas. Definitivamente el siguiente paso es tener un model apegado a la realidad. Debido a la dificultad para obtener neuronas humanas, en este trabajo se utilizó las células MSN, las cuales asemejan un cultivo neuronal, sin embargo siempre se debe de ser precavido al interpretar

estos resultados debido a que provienen de una linea tumoral, por lo que siempre será interesante determinar la capacidad reparativa en un modelo humano.

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