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Daño en el genoma paterno y reparación embrionaria en peces: efectos en la descendencia

DNA damage in the paternal genome and embryo repair in fish: effects in the progeny

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Memoria presentada por

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Resumen

Durante los últimos años se ha demostrado que el estado de la cromatina espermática juega un papel fundamental desde el momento de la fecundación. Así, por ejemplo, determinados genes de origen paterno se expresan desde los primeros estadios del desarrollo embrionario, pudiendo afectar al éxito reproductor. Además, a lo largo de la espermatogénesis y hasta llegar a formar el espermatozoide maduro, se llevan a cabo una serie de procesos que van a dañar el ADN del espermatozoide, por lo que las células espermáticas presentan un nivel de daño genético basal, que puede incrementarse por diversos factores genotóxicos. Teniendo en cuenta que la capacidad de reparación del gameto masculino se va perdiendo a través de la espermatogénesis, dicho daño debe ser reparado por el zigoto inmediatamente después de la fecundación, para permitir que el desarrollo embrionario progrese adecuadamente.

La capacidad de fecundación de los espermatozoides depende de muchos factores tales como la integridad de su membrana, su morfología o su motilidad entre otros. En el caso de los mamíferos, se produce un proceso de selección a lo largo del tracto reproductor de las hembras, que impide que la inmensa mayoría de espermatozoides no aptos lleguen hasta el ovocito. Sin embargo, en especies con fecundación externa este proceso es menos restrictivo, aumentándose las probabilidades de que se produzca la fecundación con un espermatozoide dañado. Por ello, en el **primer capítulo** de la presente tesis doctoral, que está constituido por dos trabajos, se aborda el estudio de los efectos del daño genotóxico en muestras seminales de trucha (*Onchorynchus mykiss*) sobre el desarrollo de la progenie. Se lleva a cabo un análisis masivo de la expresión de genes en larvas obtenidas con semen portador de diferente nivel de daño en su cromatina debido al uso de protocolos de criopreservación no óptimos. Además, la mitad de las progenies fueron incubadas en

condiciones estándar, y la otra mitad fueron sometidas a la inhibición de la reparación durante la primera división zigótica. Los resultados revelaron un gran número de genes diferencialmente expresados (DEGs) en las larvas procedentes de embriones cuya reparación zigótica fue inhibida. Estos DEGs estaban fundamentalmente relacionados con las rutas de reparación del ADN, el control del ciclo celular y la apoptosis, revelando un patrón diferente de respuesta al daño en el ADN que las larvas control. Los transcritos implicados en actividad apoptótica mostraban una función antagonista, ya que genes pro-apoptóticos estaban reprimidos y los anti-apoptóticos estaban sobre- expresados a pesar de la inestabilidad genómica provocada en el embrión. Dichos resultados sugerían que las larvas supervivientes habían adquirido una respuesta de tolerancia al daño genotóxico, provocada a partir de la primera división del embrión. Además, se ha constatado que es esencial un control exhaustivo de la integridad del ADN espermático tras procesos de congelación/descongelación. Así, lesiones en la cromatina espermática causadas por la criopreservación, provocaron diferencias significativas en el perfil transcriptómico de las que pueden ser clave para su futuro crecimiento y desarrollo.

En el **segundo capítulo** quisimos determinar si los factores de manejo de reproductores que se aplican en piscifactorías, en concreto la manipulación del periodo natural de puesta, pueden afectar a la maquinaria de reparación del ADN de los ovocitos, y tener un impacto negativo en el desarrollo de la progenie, especialmente cuando el genoma paterno está dañado. Para ello, evaluamos el efecto del cambio de fotoperiodo, y por tanto del régimen térmico en el que ocurre la gametogénesis, en la calidad de los gametos de trucha arcoiris (*O. mykiss*). Mediante una técnica de PCR a tiempo real (qPCR) pudimos confirmar las lesiones promovidas en

el semen, así como la disminuida capacidad reparadora de los ovocitos obtenidos en regímenes térmicos inapropiados. Demostramos que, en estadios tempranos de desarrollo, los embriones obtenidos fuera del periodo natural de puesta poseen una intensa capacidad reparadora, pero una reducida capacidad para detener el desarrollo y reparar el daño en el ADN, perfil similar al de los ovocitos de los que proceden. La actividad apoptótica en estas mismas progenies se vio reducida durante la organogénesis, momento clave en el desarrollo normal de los órganos. La tolerancia al daño observada suprimió la inducción de la apoptosis, permitiendo progresar con el desarrollo en condiciones ambientales de estrés, que finalmente promovió una alta tasa de larvas malformadas que llegaron a la eclosión, pero no fueron capaces de sobrevivir a largo plazo.

La respuesta al daño en el ADN (DDR) es un mecanismo altamente conservado y coordinado de señalización que, tras la detección de la lesión en el ADN, activará alguna de las diferentes vías de reparación. El proceso se verá facilitado por la activación de un mecanismo de parada del ciclo celular para dar tiempo suficiente a estos mecanismos de reparación a eliminar el daño. De este modo se consigue evitar que las lesiones se transmitan a las células hijas. En el caso en el que el daño sea demasiado severo, los factores que regulan ésta respuesta al daño se encargarán de eliminar la célula dañada por un mecanismo de apoptosis o senescencia. Los peces comparten con los mamíferos muchos de los mecanismos implicados en la DDR y, aunque se ha observado la activación de diversas vías de reparación en embriones de pez cebra, poco se sabe sobre la respuesta al daño durante el desarrollo embrionario. Nuestros resultados previos mostraban una alta tolerancia de los embriones a la inestabilidad genética y por ello, en el **tercer capítulo,** quisimos abordar más en

profundidad qué ocurre en los primeros estadios de desarrollo embrionario. En este estudio usamos el pez cebra (*Danio rerio)* como especie modelo para analizar los efectos de la fecundación con semen portador de daños genotóxicos severos, causados por irradiación UV, en la activación de mecanismos de respuesta al daño, así como en el desarrollo de la progenie. Demostramos que espermatozoides con un alto nivel de fragmentación, superior al 10%, fueron capaces de fecundar el ovocito. Mediante técnicas de inmunodetección localizamos y cuantificamos la presencia de γH2AX y 53BP1, principales marcadores moleculares de daño y reparación, confirmando una alta actividad de reparación durante la activación transcripcional del zigoto, en aquellos embriones obtenidos con semen portador de daño genotóxico. Además, el análisis de la actividad transcripcional (medante qPCR) y postraduccional (mediante inmunodetección) de p53 reveló una sobre-expresión del gen y altos niveles de activación de la proteína a lo largo del desarrollo embrionario, demostrando una fuerte activación de los mecanismos de respuesta al daño. La activación de p53 promueve su función transcripcional, pudiendo activar vías de parada del ciclo celular, de reparación o de apoptosis. En este sentido, una vez que p53 está activado y estabilizado, se une a la región promotora de numerosos factores pro-apoptoticos en la ruta de muerte celular mitocondrial. Sin embargo, en nuestro estudio, en aquellas progenies obtenidas con semen dañado, los genes pro-apoptóticos mostraron menores niveles de expresión, mientras que los factores anti-apoptóticos revelaron una sobre-expresión, dando lugar a un escenario de represión apoptótica a pesar de la clara inestabilidad genómica presente en el embrión y sugiriendo una tolerancia al daño. Los resultados fueron confirmados tras observar una menor tasa de apoptosis revelada por el estudio con Anexina V. Esta tolerancia al daño en el genoma del

embrión promovió tal inestabilidad genómica, que disminuyo prácticamente a la mitad la supervivencia de las larvas obtenidas con semen dañado respecto a las progenies control. Además, la gran mayoría mostraron un cúmulo de malformaciones, que fueron incompatibles con la supervivencia a largo plazo. En este último trabajo, demostramos la activación de una fuerte respuesta al daño genotóxico, que implica a p53, pero que se resuelve con la tolerancia al daño. Se ha descrito recientemente en células de mamífero irradiadas con UV, un mecanismo en el que las lesiones del ADN evaden su detección y progresan con la replicación gracias a la cooperación de p53 con polimerasas específicas. La respuesta observada en pez cebra podría deberse a la activación de un mecanismo similar o representar una ruta específica de respuesta al daño en peces, ya que, al menos en pez cebra, se ha descrito la presencia de una isoforma alternativa (Δ113p53), que modifica la expresión de genes regulada por p53 y antagoniza la apoptosis. La tolerancia celular al daño en el ADN podría ser un mecanismo evolutivo propio de los animales con una estrategia reproductiva basada en la producción de un gran número de descendientes con una baja tasa de supervivencia a largo plazo. La tolerancia a la mutagénesis, favorecería la supervivencia de individuos que en otros grupos de vertebrados no superarían la fase embrionaria, y que podrían mostrar ventajas de adaptación a condiciones ambientales cambiantes.

Introducción

Las técnicas de reproducción asistida o fecundación artificial suponen un avance en la biotecnología reproductiva, no sólo en la clínica, sino también en la industria animal, donde se han empleado de forma satisfactoria. Las grandes ventajas que representa su aplicación han estimulado enormemente la investigación básica en el campo de la reproducción, contribuyendo a desentrañar los mecanismos moleculares que gobiernan el proceso reproductivo. Muchos de esos mecanismos de control son comunes a todos los vertebrados, pero otros muchos muestran diferencias aun no bien comprendidas entre aquellos que tienen fecundación externa e interna. Los primeros tienen una estrategia reproductiva basada en la producción de numerosos descendientes, conocida como estrategia de la "r" y característica de peces y anfibios. Sin embargo, aquellos vertebrados con fecundación interna presentan un intenso control y cuidado de la prole, propio de las estrategias de la "k", representativa de aves y mamíferos.

La reproducción de peces representa un amplio campo de investigación, con interés tanto para la ciencia básica cómo para la aplicación en producción animal o acuicultura, cuya producción se incrementa anualmente a nivel mundial. La competitividad en el campo de la producción acuícola reside en un mayor desarrollo biotecnológico que optimice la calidad de la producción, incrementando la tasa de crecimiento de las especies cultivadas y mejorando su manejo y conservación (Bostock et al., 2010). Uno de los factores clave para llevar a cabo este objetivo es la optimización de la reproducción y, con ella, la selección de individuos reproductores que proporcionen un alto número de peces con las cualidades deseadas (Migaud et al., 2013).

En la biotecnología de la reproducción, y especialmente en acuicultura, la calidad de la progenie siempre se ha relacionado con el factor materno, prestando menos atención de la que se merece a la importancia del esperma. En aquellas especies en las que se requiere fecundación artificial, la selección de machos reproductores se basa, con frecuencia, en la evaluación de distintos parámetros fenotípicos y fisiológicos, así como en la estimación de la capacidad fecundante del esperma a partir del análisis subjetivo de la motilidad (Bobe and Labbé, 2010; Lavara et al., 2005). La caracterización genética es también una importante herramienta usada para la selección de reproductores con el fin de controlar la variabilidad de la población, evitando tanto los riesgos de la endogamia como los de la deriva génica, que modificaría de forma incontrolada el perfil de la población (Frost et al., 2006; Ullah et al., 2015). Los análisis de paternidad revelan que, en aquellas especies en las que se permite la fecundación natural en los tanques de reproductores, y a pesar de haber realizado una selección previa de los individuos, la contribución de los machos al desarrollo de los alevines es muy desigual y la mayoría de ellos no proporcionan ningún descendiente (Borrell et al., 2011; Brown et al., 2005). Este hecho puede deberse a distintos factores, que abarcan desde la competición entre machos, hasta la presencia de individuos con una deficiente calidad seminal que reducirían sus posibilidades de fecundar la puesta (Ribolli and Zaniboni-Filho, 2009). También se ha establecido que, tras la fecundación, la supervivencia de aquellos juveniles procedentes de determinados machos es bastante inferior a la media (Bobe and Labbé, 2010), lo que sugiere que la herencia paterna tiene una gran importancia en el éxito reproductor a largo plazo.

La calidad espermática puede verse comprometida por muy diversos factores, ya sean naturales o relacionados con la manipulación inherente a la producción industrial, como el manejo de los animales, la modificación del periodo natural de puesta, el envejecimiento de los machos, la alimentación o el almacenamiento del semen en condiciones no óptimas. La influencia de todos estos factores sobre la motilidad espermática ya ha quedado demostrada en diferentes especies (Asturiano et al., 2001; Bobe and Labbé, 2010; Cabrita et al., 2008; Labbe et al., 1995; Migaud et al., 2013; Rurangwa et al., 2004). Del mismo modo se ha observado cómo dichos factores pueden afectar a la integridad de la cromatina, a pesar de que la capacidad fecundante del espermatozoide no se vea comprometida (Cabrita et al., 2011, 2005; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2009). El manejo de los animales y el almacenamiento del semen utilizando protocolos no optimizados, así como el cambio de fotoperiodo que se emplea para modificar los periodos de puesta (Bobe and Labbé, 2010; Rurangwa et al., 2004), son factores que pueden incrementar el estrés oxidativo, cuyos efectos en distintos componentes celulares son bien conocidos (Sabeti et al., 2016). En el caso del espermatozoide, el estrés oxidativo provoca la peroxidación de los lípidos de membrana comprometiendo su integridad, la señalización que se requiere para iniciar y mantener la movilidad y su capacidad de fusionarse con el ovocito (Aitken et al., 2013; Beirao et al., 2012). Pero además, como se verá más adelante, puede tener efectos directos en la estabilidad de la cromatina, ya que promueve la oxidación de bases y la fragmentación de la hebra de ADN (Aitken et al., 2013; Pérez-Cerezales et al., 2009).

En mamíferos, se ha demostrado una mayor implicación de la cromatina espermática en el control del desarrollo embrionario de la tradicionalmente

reconocida (Carrell and Hammoud, 2009; Hales et al., 2010; Ward, 2009). Además, desde que hace algunos años se demostrára la correlación entre la estabilidad genómica y el éxito reproductor, su integridad es considerada una de las características principales de calidad seminal (Evenson and Loma, 2000). Tradicionalmente se ha considerado al espermatozoide como un portador pasivo de su material genético, que sólo comenzaría a expresarse en el embrión a partir de la activación del genoma zigótico, fundamentalmente a partir del estadio de gástrula. Sin embargo los avances en el conocimiento de la organización de la cromatina de los espermatozoides, han permitido establecer que algunos genes paternos se expresan desde los primeros estadios, siendo cruciales durante el desarrollo embrionario temprano (Avendano et al., 2009; Wu et al., 2014, 2011). Así, cuando se fuerza la fecundación con espermatozoides que contienen daño en el genoma, se producen efectos en la descendencia que afectan a su desarrollo desde el momento de la fecundación, produciéndose la pérdida de embriones en fases previas a la implantación (Marchetti et al., 2007, 2004a) y otros efectos posteriores que comprometen la supervivencia, crecimiento, envejecimiento o susceptibilidad a enfermedades (Devaux et al., 2011; Gawecka et al., 2013; Hourcade et al., 2010; Marchetti et al., 2015; Moreira et al., 2008; Pérez-Cerezales et al., 2011). Este aspecto despierta cada vez más interés en el campo de la reproducción asistida, debido a las importantes implicaciones que puede tener. En peces se ha descrito la capacidad fecundante de espermatozoides con al menos un 10% de ADN fragmentado y se han observado alteraciones de la descendencia que podrían estar relacionadas con estos daños (Pérez-Cerezales et al., 2011). En ratón se ha estimado que los espermatozoides con un porcentaje similar de daño en el ADN son aptos para fecundar (Ahmadi and Ng,

1999) y, en el caso de humanos, este porcentaje aumenta hasta un 30% (Evenson and Loma, 2000).

1. **Conformación del núcleo del espermatozoide durante la espermatogénesis y contribución paterna al desarrollo**

1.1 Conformación del núcleo espermático

La cromatina espermática sufre enormes cambios a los largo del proceso de la espermatogénesis, que implica i) la sucesión de numerosos y cortos ciclos mitóticos, a través de los cuales se producen varias generaciones de espermatogonias, ii) la reducción y recombinación propia de la meiosis, que implica a las generaciones de espermatocitos y iii) el procesamiento y compactación progresiva a lo largo de la espermiogénesis, que permite a los espermatocitos transformarse en espermatozoides (Figura 1).
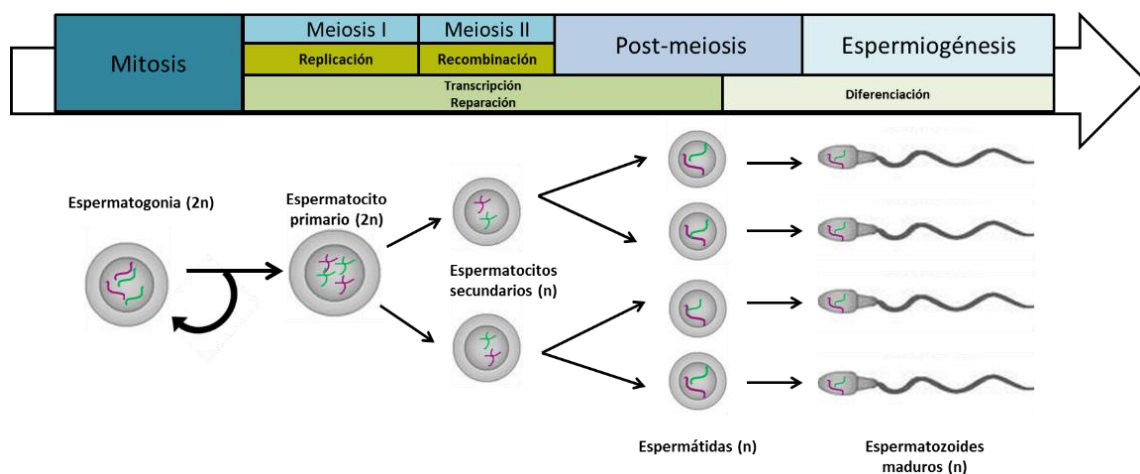

Figura 1. Esquema representativo del proceso de espermatogénesis.

El ADN espermático es procesado y empaquetado progresivamente a lo largo de la espermatogénesis, pero es en la espermiogénesis donde se produce una intensa remodelación de la cromatina. Al principio de este proceso la cromatina empieza a estar altamente compactada, permitiendo una importante reducción del volumen del núcleo celular. Esta compactación conlleva la sustitución de histonas por otras proteínas específicas conocidas por su acrónimo en inglés SNBPs (*sperm nuclear basic proteins* o proteínas básicas nucleares del esperma) (Ausio, 1999; Saperas et al., 1994), que se dividen en tres grupos: histonas, protaminas y proteínas tipo-protamina (*protamine-like proteins*). Las histonas corresponden a un grupo de proteínas cromosomales ricas en aminoácidos básicos (lisina y arginina). Las protaminas son ricas en arginina, a veces también en cisteínas, y desplazan y reemplazan casi totalmente a las histonas en la espermiogenesis de mamíferos. Por último, las *protamine-like proteins* son ricas en lisinas y argininas y pueden también reemplazar a las histonas aunque en momentos diferentes durante la espermiación (revisado por Herráez et al., 2015). Parece ser que la principal función de las SNBPs está ligada a la protección mecánica del ADN frente a factores dañinos para su estabilidad, principalmente durante su trayecto hasta el ovocito (Gonza, 2014; Olive and Banáth, 2006). Para ello, las proteínas se intercalan en la hebra de ADN con una gran eficacia, dando lugar a unas estructuras toroidales de 50 Kb altamente compactadas (Ward, 2009), que afectan a la actividad nuclear al reducir la accesibilidad a la hebra de ADN de la maquinaria de replicación, transcripción, reparación, etc. Sin embargo, parece ser que esta condensación no es homogénea y algunos elementos cromosómicos no sufren ese recambio de proteínas. Se ha determinado que, en mamíferos, una pequeña cantidad, alrededor de un 10%, de la cromatina espermática, permanece unida a histonas y que

otras regiones del ADN se unen a las proteínas de la matriz nuclear (Figura 2) (Singh y Agarwal, 2011; Ward, 2010).
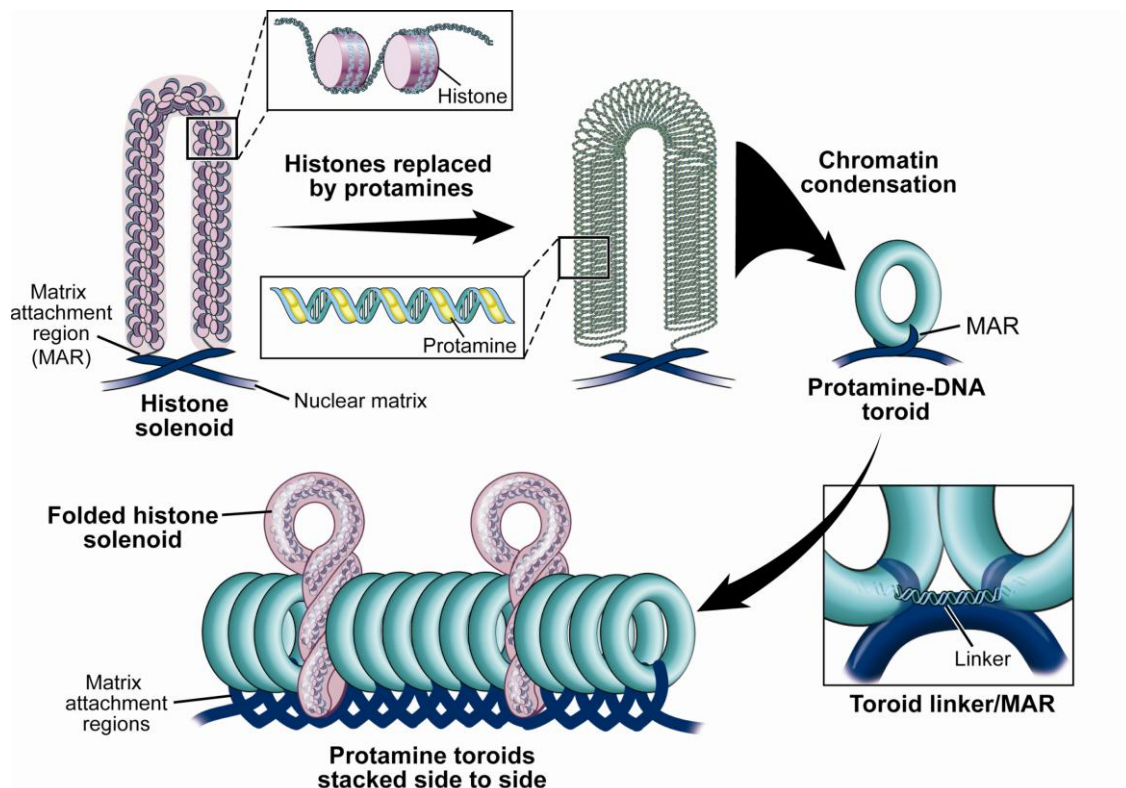
Figura 2. Esquema de la organización del ADN espermático en mamíferos. (Singh y Agarwal, 2011).

Al parecer estas regiones no protaminadas son necesarias para que el embrión acceda a algunas secuencias del genoma paterno, que serían esenciales para el control del programa del desarrollo embrionario tras la fecundación, y por lo tanto imprescindibles para que se produzca una embriogénesis correcta, contribuyendo a la totipotencia e *imprinting* paterno (Arpanahi et al., 2009; Brykczynska et al., 2010; Hammoud et al., 2009). Este hecho puede hacer que estos genes estén más expuestos y, por lo tanto, sean más susceptibles a sufrir daño (Gonza, 2014), despertando una cierta inquietud respecto a la futura calidad de los descendientes obtenidos y a su desarrollo embrionario. La configuración final del gameto masculino y la accesibilidad

de su cromatina a la maquinaria de transcripción, se debe también a ciertos cambios epigenéticos que tienen lugar a lo largo de la espermatogénesis. Así, estudios recientes demuestran que la retención de un patrón especifico de metilación del ADN y la correcta modificación de las histonas en el genoma del espermatozoide, son necesarias para lograr el éxito en la fecundación (Gannon et al., 2014).

En el espermatozoide de peces el patrón de condensación de la cromatina es muy diversificado: hay especies con la cromatina completamente protaminada, otras en las que no se produce el reemplazamiento de las histonas y algunas en las que se encuentran ambos tipos de proteínas (Herráez et al., 2015). En sus etapas iniciales de diferenciación, al inicio de la espermiogénesis, la cromatina presenta una estructura fibro-granular y contiene exclusivamente histonas. A medida que avanza la espermiogénesis y dependiendo del tipo de SNBP, se pueden distinguir cuatro grupos principales de compactación. En el primer grupo no se produce reemplazamiento, las SNBPs son de tipo histona (típica en *Sparus aurata*), pero las estructuras fibro-granulares observadas al principio de la espermatogénesis se unen y compactan a medida que la diferenciación avanza (Kurtz et al., 2009). En el segundo grupo, representado por *Mullus surmuletus*, las SNBP de tipo *protamine–like* desplazan a las histonas formando un complejo de nucleoproteínas altamente entrelazado (Saperas et al., 2006). El tercer grupo se caracteriza por SNBPs de tipo protaminas, las cuales reemplazan a las histonas formando un complejo de nucleoproteínas altamente condensado, como ocurre en *Dicentrarchus labrax* (Balhorn, 2007). El último grupo también está formado por SNBPs de tipo protaminas, ricas no solo en arginina, sino también en cisteína, que reemplazan a las histonas formando estructuras en lámina. Esta composición es típica de vertebrados de la clase Condrictios o peces cartilaginosos

como *Scyliorhinus canicula*. Una vez se ha producido la sustitución de las histonas, la siguiente transición de la cromatina, en todos estos grupos, es la convergencia de las estructuras de la cromatina en una organización nuclear altamente electrodensa, como resultado de procesos termodinámicos que suelen estar relacionados con modificaciones postraduccionales (acetilación y fosforilación) de las SNBPs implicadas (Kasinsky, 2011). Esta heterogeneidad en cuanto al tipo de SNBPs es propia de peces y anfibios, mientras que reptiles, aves y mamíferos contienen casi exclusivamente protaminas en el núcleo espermático (Ausió et al., 2007), pudiendo indicar que esta variabilidad, observada en especies mayoritariamente con fecundación externa, representa diferentes modelos de adaptación a dicha estrategia reproductiva.

1.2. <u>Transcritos presentes en el espermatozoide maduro</u>

El espermatozoide, además de su genoma nuclear y mitocondrial, contiene información clave en su citoplasma en forma de moléculas de ARN. Durante la espermatogénesis el ADN es transcripcionalmente activo, al menos hasta la formación de las espermátidas (Bukowska et al., 2013), silenciándose la transcripción durante el proceso de compactación de la cromatina (Johnson et al., 2010). Por ello, hasta hace relativamente poco tiempo, se creía que el espermatozoide era una célula altamente diferenciada y especializada que servía únicamente como medio de transporte del genoma paterno hasta el ovocito. Sin embargo, en los últimos años, un creciente número de estudios ha identificado la presencia de diferentes ARNs en espermatozoides de diferentes especies (Schuster et al., 2016). Desde que Miller y cols. (Miller et al., 1999) observaron en humanos que el espermatozoide contenía

diferentes moléculas de ARNm, hasta la fecha, se han descrito numerosos transcritos. Ostenmeier identificó hasta 7000 transcritos diferentes (Ostermeier et al., 2002) y dado el volumen de información al respecto, Schuster y colaboradores (Schuster et al., 2016) han desarrollado una base de datos dedicada a la caracterización de los ARNs presentes en espermatozoides de diversas especies: www.spermbase.org. Los ARN espermáticos están codificados por el genoma diploide y son los mismos en todos los espermatozoides de un mismo eyaculado, independientemente del haplotipo de cada uno de ellos (Hosken and Hodgson, 2014). Este hecho inicialmente se explicó por su síntesis en las fases premeióticas y una incompleta eliminación del ARN durante la espermiogenénesis, momento en el que se produce la reducción del citoplasma y el contenido en ARN hasta un nivel residual (Cooper et al., 2005). Sin embargo nuevos hallazgos sugieren una retención selectiva de determinados transcritos (Hamatani, 2011; Hosken and Hodgson, 2014; Johnson et al., 2010). La idea de que las células espermáticas puedan utilizar transcritos de ARN estables y presentes durante toda la espermatogénesis abre una nueva puerta de investigación en el campo de la biotecnología de la reproducción.

Los ARN espermáticos presentes en los espermatozoides pueden ser de diferente naturaleza, dividiéndose en transcritos codificantes, que incluyen ARNm, ARNt (transferentes) y no codificantes, como si-RNAs (*small interfering*), mi-ARN (micro), pi-ARN (*piwi-interacting*) y lnc-ARN (*long non-coding*) (Hosken and Hodgson, 2014; Jodar et al., 2013; Schagdarsurengin et al., 2012). Estudios de secuenciación (RNA-sec) y PCR a tiempo real (RT-PCR) han demostrado que la mayoría de los ARN codificantes están fragmentados o, al menos, presentan alteraciones en su estructura (Jodar et al., 2013). Aproximadamente un cuarto de todos los transcritos presentes en

el espermatozoide poseen sitios adicionales de poliadenilación (poli A), conservando la integridad de la región codificante a pesar de contener una corta región no codificante (UTR) en el extremo 3´ (Jodar et al., 2013). Este rasgo también se encuentra en los transcritos presentes en testículos (Liu et al., 2007), sugiriendo que dicha modificación puede ayudar a la estabilidad del trascrito así como a modular su localización y transporte (Jodar et al., 2013). Los análisis ontológicos parecen demostrar que la fracción de ARNs codificantes que no están fragmentados, está implicada en el control de la fertilidad masculina, fecundación y desarrollo temprano (Sendler et al., 2013), encontrándose patrones específicos que podrán servir de marcadores de fertilidad (Bieniek et al., 2016; García- Herrero et al., 2011; Guerra et al., 2013; Jodar et al., 2013). A pesar de que muchos de los transcritos están bien caracterizados, la función biológica que desempeñan es todavía, en muchos casos, una incógnita.

El grupo de Ostermeier (Ostermeier et al., 2004) y, posteriormente, el de Krawetz (Krawetz, 2005) sugirieron que algunos de estos ARNm eran liberados dentro del ovocito durante la fecundación. Para llegar a esta conclusión, fecundaron ovocitos de hámster con espermatozoides humanos y posteriormente identificaron que determinados transcritos estaban presentes tanto en espermatozoides como en zigotos, pero no en ovocitos sin fecundar (Krawetz, 2005). A día de hoy el papel de estos transcritos está siendo discutido, pero parece cada vez más clara su implicación en el control de la fecundación, la formación del zigoto, así como en los primeros estadios del desarrollo embrionario.

En cuanto a los transcritos no codificantes parece ser que, en el caso de mamíferos, el ARN con mayor presencia en el espermatozoide es el mi-ARN, el cual regula la expresión de genes (Hamatani, 2011), preferiblemente uniéndose a los ARNm

por su extremo 3´- UTR (Jia and Zeng, 2015). Se ha determinado que cientos de mi-ARNs están presentes en el espermatozoide humano (Krawetz et al., 2011; Ostermeier et al., 2002), estando algunos de ellos relacionados con el control de la proliferación celular, la apoptosis y el desarrollo embrionario (Wang y Xu 2015). Su implicación en este último puede deberse a su capacidad de reprimir la traducción de determinados transcritos en el zigoto, o a su capacidad de regular la trascripción interaccionando con promotores específicos (Jodar et al., 2013). Para llevar a cabo dicha función es necesario que estos transcritos de origen paterno, estén en la cantidad necesaria en el espermatozoide (Bourc'his y Voinnet 2010).

Una gran cantidad de estudios muestran que los mi-ARN regulan la expresión de genes ligados con la actividad reproductiva (Herráez et al., 2015; Jodar et al., 2013), así como con la protección genómica y desarrollo de las células de la línea germinal paterna (Bourc'his y Voinnet 2010; Donnell and Boeke, 2007; Krawetz et al., 2011; Siomi et al., 2011). Govindaraju y cols. (Govindaraju et al., 2012) también encontraron un gran número de mi-ARNs en espermatozoides bovinos y especularon que podrían ser un reflejo de su papel durante la espermatogénesis. También, en espermatozoides humanos y porcinos se han identificado gran cantidad de mi-ARNs, habiéndose correlacionado su perfil con la motilidad del espermatozoide, su integridad estructural o su metabolismo (Curry et al., 2008; Hamatani, 2011).

Uno de los miARN más abundantes en espermatozoide de mamífero es el miR-34c (Jodar et al., 2013; Krawetz et al., 2011), que podría ser crucial para controlar la primera división del zigoto del ratón. En el caso de teleósteos, en concreto en pez cebra (*D. rerio*), Jia y cols. (Jia et al., 2015) observaron una predominante expresión del transcrito miR-202-5p durante la diferenciación de las células germinales, así como en

los espermatozoides maduros. Sin embargo, en este caso, no está claro si éste u otros mi-ARNs son liberados al interior del ovocito durante la fecundación. Se sabe que los mecanismos de acción de los mi-ARNs son similares entre vertebrados, por lo que los avances en el conocimiento de su papel en el desarrollo de mamíferos podrían ser la base para su estudio en diferentes especies de peces. De hecho, la evaluación de patrones de mi-ARN en el espermatozoide podría ser utilizada como marcador de calidad espermática, tanto en medicina reproductiva, como en producción animal.

2. Alteraciones de la integridad del ADN espermático

La integridad del ADN se define como la ausencia de roturas y de bases modificadas en la hebra de ADN. Estas lesiones se pueden generar en cualquier etapa de la espermatogénesis, durante el transporte de las propias células espermáticas por el tracto genital masculino (Shamsi et al., 2011) o tras la eyaculación, pudiendo afectar tanto al ADN mitocondrial como al nuclear, si bien en el presente trabajo nos centraremos en el ADN nuclear. Los agentes potencialmente dañinos para la cromatina espermática son muy variados y pueden ser clasificados en intrínsecos, debidos al propio proceso fisiológico, o extrínsecos, causados por factores externos.

La etapa meiótica de la espermatogénesis genera roturas de doble cadena (DSB) que suelen ser reparadas tras la recombinación gracias a la activación en los espermatocitos de los mecanismos de respuesta al daño genético (Gunes et al., 2015). Sin embargo, si se producen errores en su reparación, estas roturas pueden pasar los sucesivos puntos de control del ciclo celular, pudiendo así llegar a estar presentes en el eyaculado (Shamsi et al., 2011). La posterior compactación del núcleo espermático incrementa el número de roturas en la hebra de ADN, debido a que requiere la

actividad de topoisomerasas que rompen la molécula de ADN para liberar estrés de torsión y favorecer el reemplazamiento de las histonas por otro tipo de SNBPs (Bach and Schlegel, 2016). Si los cortes realizados en este proceso no se reparan, bien por el exceso de actividad de las topoisomerasas o por la falta de inhibidores de las mismas, se produce una maduración incompleta (Ahmed et al., 2015; De Luliis et al., 2009; Hendrich and Bickmore, 2001; Li et al., 2014; Rathke et al., 2014; Wallach et al., 2010), que se ha podido relacionar con fragmentación del ADN en espermatozoides humanos (De Luliis et al., 2009). Hay que considerar que durante el proceso de la espermiogénesis no existe capacidad de reparación del ADN: tras la meiosis se frena el ciclo celular y los mecanismos de control de daño genotóxico inherentes a él, mientras que la compactación de la cromatina impide la actividad de las enzimas implicadas en la reparación que, por otra parte, se pierden durante la diferenciación del espermatozoide (Gunes et al., 2015). Un factor de riesgo añadido es la apoptosis que tiene lugar durante la espermatogénesis, cuya función está relacionada con la homeostasis de células de la línea germinal y la eliminación de células con un nivel de daño elevado (Shamsi et al., 2011). Las células que van a ser eliminadas expresan el marcador Fas/FasL. Sin embargo, parece que algunas células espermáticas destinadas a entrar en apoptosis escapan a este proceso debido a algún tipo de alteración en el mecanismo Fas, promoviendo la llamada apoptosis abortiva. Este proceso contribuye a que los espermatozoides maduros presenten daños en el ADN que han sido relacionados con infertilidad masculina (Gorczyca et al., 1993; Vicar et al., 2004). Por todos estos motivos el espermatozoide presenta, ya al ser liberado del epitelio testicular, niveles basales de fragmentación en su material genético relativamente altos incluso en condiciones fisiológicas normales (Bach and Schlegel, 2016).

Durante el transporte espermático a través de los túbulos seminíferos y el epidídimo se incrementa el daño genético. Así, se observan mayores niveles de daño en espermatozoides procedentes de eyaculado en comparación con los obtenidos de testículo (Cui et al., 2015) y se han encontrado asociaciones entre el nivel de daño del ADN espermático y el periodo de abstinencia sexual en la especie humana (Jonge et al., 2004). Estudios en modelos murinos muestran una disminución de las tasas de fertilización de espermatozoides obtenidos de la cola del epidídimo con respecto a aquellos obtenidos del testículo o de la cabeza del epidídimo (Suganuma et al., 2005). Se ha sugerido que el tránsito a lo largo de éstos conductos podría dañar el ADN de espermatozoides ya de por sí defectuosos (Bach and Schlegel, 2016; Gawecka et al., 2013), fundamentalmente debido al estrés oxidativo generado por desequilibrios entre la generación de ROS y los antioxidantes seminales. Los espermatozoides inmaduros son importantes productores de ROS por lo que, si se liberan al semen en elevadas cantidades, incrementan el estrés oxidativo (De Luliis et al., 2009). Además, se ha observado que las membranas de las células inmaduras son muy susceptibles a sufrir peroxidación lipídica, cuyos productos son genotóxicos y mutagénicos, pudiendo afectar también a los espermatozoides maduros (Aitken et al., 2014). La presencia de un exceso de ROS puede causar diferentes tipos de daños en el ADN: modificaciones de bases, creación de sitios abásicos, roturas en la hebra de ADN, cross-links entre hebras, etc. (Aitken et al., 2013; Shamsi et al., 2011; Thomson et al., 2009). La modificación las bases nitrogenadas se traduce con frecuencia en una posterior fragmentación del ADN (Aitken y De Luliis, 2007; Pérez-cerezales et al., 2011). La 8-hidroxilación de la guanina (8-OHdG) constituye uno de los marcadores más sensibles de daño en el ADN inducido por estrés oxidativo (Gonza, 2014) y su presencia se ha

relacionado con la fragmentación del ADN espermático (Luliis et al., 2009). Si su nivel se mantiene elevado, se considera mutagénico, pudiendo aumentar la mortalidad embrionaria y la tasa de malformaciones en la progenie (De Luliis et al., 2009).

Otros factores a considerar son aquellos factores exógenos que afectan al eyaculado: los tóxicos ambientales provenientes de la agricultura, industria, transporte etc. (Phillips et al., 2008; Tse et al., 2013; Wigle et al., 2008), el uso de irradiación UV, que es conocida por inducir fragmentación en el ADN y se utiliza para inactivar el genoma del gameto masculino durante procesos de ginogénesis en acuicultura (Fopp-Bayat et al., 2007; Piferrer et al., 2004), o la utilización de protocolos de criopreservación no óptimos (Ezz et al., 2017; Olaciregui et al., 2016; Pérez-Cerezales et al., 2011), que podrían ser responsables de dañar el ADN espermático, e incluso incrementar las lesiones persistentes producidas durante la espermatogénesis, son algunos ejemplos.

La manipulación de las muestras seminales en las piscifactorías es uno de los factores que podrían afectar la contribución paterna a la descendencia. El almacenamiento seminal a corto plazo con temperaturas por encima de 0ºC, es ampliamente utilizado debido a su bajo coste, pero como demostraron Pérez Cerezales y cols. (Pérez-Cerezales et al., 2009), el ADN de células espermáticas de trucha arcoíris almacenadas durante cinco días a 4ºC con aireación constante, en condiciones similares a las utilizadas en las plantas de producción, mostraba altos niveles de fragmentación y elevados niveles de guaninas oxidadas, indicativos de un importante estrés oxidativo. El incremento en la producción de ROS también fue descrito, después de dos días de almacenamiento, en el semen de esturión ruso (*Acipenser gueldenstaedti*) y siberiano (*Acipenser baerii),* que mostraba, en esta última especie,

un aumento de la fragmentación del ADN (Shaliutina-Kolešová et al., 2015). La criopreservación, como modo de almacenar el semen a largo plazo, es una de las prácticas más utilizadas en reproducción animal, tanto para fines productivos como de conservación de recursos genéticos. En acuicultura, la creación de bancos de germoplasma, es una herramienta muy utilizada en los programas de selección emprendidos por diferentes empresas dedicadas a la producción de especies con un alto valor comercial como la trucha (*O. mykiss*), la lubina (*D. labrax*), o la dorada (*S. aurata)* (Labbe and Maisse, 2001; Martínez-Páramo et al., 2016; Zilli et al., 2003). Así mismo, también son útiles para el mantenimiento de líneas transgénicas de especies modelo, como el pez cebra (*D. rerio*), o para la conservación de especies en peligro de extinción (Lahnsteiner, 2004; Martínez-Páramo et al., 2016; Routray et al., 2002). Sin embargo, también se ha documentado un cierto nivel de daño en el ADN del semen descongelado cuando los protocolos de criopreservación aplicados no están totalmente optimizados (Cabrita et al., 2005; Pérez-Cerezales et al., 2011, 2009). Este hecho es especialmente importante cuando el objetivo final es la creación de bancos para la conservación de perfiles genéticos particularmente valiosos. La congelación y posterior descongelación incrementa el estrés oxidativo en las células (Aitken et al., 2013; Watson, 2000), promoviendo daño genotóxico, tal y como se ha observado en diferentes especies de teleósteos como la trucha arcoíris (*O. mykiss*) (Labbe and Maisse, 2001), la lubina (*D. labrax)* (Zilli et al., 2003), la dorada (*S. aurata*) (Cabrita et al., 2005) o la locha (*Chromobotia macracanthus*) (Kopeika et al., 2004). Pero además, durante la criopreservación, el descenso de la temperatura hace que se formen cristales de hielo en el exterior celular y se altere el flujo de agua a través de la membrana plasmática, generando un estrés osmótico que deshidratara a la célula

(Watson, 2000). Posteriormente, si no se logra la deshidratación total, se forman cristales de hielo también en el interior celular, pudiendo éstos dañar los componentes celulares de los espermatozoides. Todos estos procesos generan un estrés mecánico que también puede afectar directamente a la integridad de la cromatina (Paoli et al., 2014). Algunos autores han observado que tras la criopreservación se generan roturas simples en el ADN telomérico (Honda et al., 2001). El incremento de estas roturas se relaciona en células somáticas con el acortamiento de los telómeros durante la replicación y con la aceleración de la entrada de las células en senescencia (Hiyama and Hiyama, 2007). Pérez-Cerezales y cols. demostraron la existencia de un acortamiento telomérico y fragmentación del ADN tras la criopreservación de espermatozoides de trucha arcoíris (*O. mykiss*) (Pérez-Cerezales et al., 2011), aunque Cartón-García y cols (Cartón-García et al., 2013) no corroboraron este efecto en semen de dorada (*S. aurata*).

El manejo de reproductores también puede afectar la estabilidad de la cromatina. La modificación de la estacionalidad de la reproducción es una práctica habitual en acuicultura y se logra mediante tratamientos hormonales o, más habitualmente, por cambios en determinados factores ambientales (Mañanos et al., 2008). Estos procedimientos implican que la espermatogénesis se produce durante un régimen térmico inadecuado que podría alterar los mecanismos de compactación del ADN, aumentando también el estrés oxidativo (Labbe and Maisse, 2001). Los desequilibrios entre un fotoperiodo artificial y los regímenes de temperatura en periodos más fríos o cálidos del año, afectan particularmente a la calidad de los ovocitos y disminuyen la supervivencia de los embriones y larvas fuera del periodo natural de puesta (Bobe and Labbe, 2010; Migaud et al., 2013). La cromatina

espermática también se ve afectada, como demuestra el aumento de la fragmentación del ADN que se ha descrito en espermatozoides de trucha arco iris (*O. mykiss*) al final del periodo reproductivo (Pérez-Cerezales et al., 2011, 2010, 2009) o en lenguado (*Solea senegalensis*) fuera de las temporadas naturales de desove (Beirão et al., 2011). Además, la cromatina de los espermatozoides obtenidos fuera de temporada muestra una mayor susceptibilidad a la criopreservación o a la irradiación UV, aumentando significativamente la tasa de fragmentación (Pérez-Cerezales et al., 2010, 2009) respecto a lo observado en el periodo natural, que se acompaña de un menor éxito reproductivo, y un aumento de las tasas de abortos y de larvas malformadas (Pérez-Cerezales et al., 2011).

Uno de los abordajes más frecuentes para evitar el efecto del estrés oxidativo en la calidad del gameto masculino, consiste en la adición de antioxidantes al plasma, aunque los resultados que ofrece la bibliografía son contradictorios. Así, por ejemplo, determinadas combinaciones de antioxidantes añadidos al medio de crioconservación mejoran la calidad del semen descongelado de lubina (*D. labrax*) (Liu et al., 2015; Martínez-Páramo et al., 2013, 2012) o la fertilidad de trucha arco iris (*O. mykiss*) (Kutluyer et al., 2014) y favorecen el almacenamiento a corto plazo en semen de dorada japónica (*Pagrus major*) (Liu et al., 2015). Sin embargo, hay evidencias de que en otras especies comerciales el efecto no es tan ventajoso o es, incluso, contraproducente. Así, por ejemplo, ensayos previos a los realizados por Kutluyer y cols. (Kutluyer et al., 2014) con trucha arcoíris (*O. mykiss*), no mostraron ninguna mejora con el uso de antioxidantes para la criopreservación de semen de esta especie (Lahnsteiner, 2004). Igualmente, ciertos antioxidantes no causan un aumento en parámetros como la motilidad o viabilidad espermática de lubina (*D. labrax),* y

aumentan la fragmentación de ADN en las muestras congeladas (Cabrita et al., 2011). La combinación adecuada de antioxidantes no sólo es específica de cada tipo celular, sino también de cada especie.

A la vista de la vulnerabilidad de la cromatina espermática, es necesario contar con herramientas que permitan hacer una valoración de la integridad de la hebra de ADN. Los primeros que desarrollaron una técnica para valorar la fragmentación de ADN en células fueron Ostling y cols. en 1984 (Ostling et al., 1984) los cuales propusieron el ensayo cometa o S*ingle Cell Gel Electrophoresis* (SCGE) en inglés. Esta técnica es una de las más utilizadas para la valoración del daño en el ADN, aunque otras muchas son también utilizadas en espermatología, siendo las más comunes el ensayo de la estructura de la cromatina espermática o S*perm Chromatine Structure Assay* (SCSA), el marcaje terminal de dUTP (TUNEL, *Terminal deoxynucleotidyl transferase dUTP nick end labelling*) y la prueba de dispersión de la cromatina espermática o prueba de halo (SCD, *Sperm Chromatin Dispersion*). En el campo de la acuicultura el ensayo de cometas es el más utilizado y se considera una herramienta fiable para el análisis de la integridad genómica del gameto masculino, así como para la evaluación de la sensibilidad de los espermatozoides a diferentes agentes (Cabrita et al., 2011; Martínez-Páramo et al., 2013).

3. <u>**Efecto de los daños en el genoma paterno tras la fecundación: impacto en la progenie**</u>

Para que se produzca la fusión entre los gametos masculino y femenino y por lo tanto la fecundación, es necesario que factores como la integridad de la membrana

plasmática, la motilidad y la adecuada morfología del espermatozoide, entre otros, se den en un mismo momento (Cosson et al., 1999). Sin embargo, para conseguir un correcto desarrollo del embrión y de la progenie el factor clave es contar con una buena integridad de la cromatina espermática (Delbès et al., 2009; Wagner et al., 2004; Ward, 2009).

La importancia de la integridad de la cromatina del espermatozoide se pone más de manifiesto con el uso de técnicas de reproducción asistida en reproducción animal y humana, ya que se elimina la influencia de muchos de los factores que hacen a un espermatozoide incompetente para la fecundación. En mamíferos es conocida la fuerte selección que existe en los espermatozoides durante su tránsito a través del tracto reproductor femenino (Holt and Van Look, 2004). Miles de millones de espermatozoides que son depositados en el tracto genital de la hembra deberán superar barreras anatómicas y fisiológicas en dirección a los ovocitos, sufriendo cambios bioquímicos que los harán aptos para la fecundación (Chow et al., 2010). El tracto genital establece las condiciones apropiadas para el transporte y preparación de ambos gametos, pero también para almacenar o eliminar los espermatozoides con daño genotóxico (Hourcade et al., 2010). Este proceso selectivo es muy importante en mamíferos y ha de ser muy riguroso, ya que tras la fecundación se invertirá una gran cantidad de energía en obtener un reducido número de embriones. Estudios realizados con semen portador de ADN dañado en ratón y humano, han demostrado la capacidad fecundante del espermatozoide, ya sea de manera natural o mediante inyección intracitoplasmática de espermatozoides (ICSI) (Fernández-González et al., 2008; García-Ferreyra et al., 2015; Simon et al., 2014, 2010). La fecundación mediante ICSI con semen portador de determinado nivel de daño genera anomalías en progenies de

ratón, encontrándose una eleva tasa de abortos, así como alteraciones del cariotipo, cambios de fenotipo y distintas patologías en los embriones obtenidos y en los recién nacidos (Barroso et al., 2009; Bowdin et al., 2007; Chen and Heilbronn, 2017; Fernández-González et al., 2008; Lim et al., 2009; Marchetti et al., 2015; Schulte et al., 2010). Los daños del material hereditario masculino se han relacionado con fallos durante el desarrollo embrionario y diversos problemas en la descendencia, que incluyen el aumento de las posibilidades de sufrir cáncer, tamaño corporal anormal o desarrollo de problemas de tipo cognitivo (Chen and Heilbronn, 2017; Henningsen et al., 2015; Schulte et al., 2010; Speyer et al., 2010; Sundh et al., 2014). La utilización de técnicas de reproducción asistida como el ICSI o la fecundación in vitro, que evitan la selección natural que ocurre de forma normal en el tracto reproductivo, y da opciones a espermatozoides que en un proceso de fecundación natural no hubieran llegado a fecundar, exige un control exhaustivo de la integridad de la cromatina del espermatozoide.

Entre los peces la estrategia reproductiva es diferente ya que, al tener fecundación externa, poseen unos mecanismos de selección espermática más débiles, aumentando el riesgo de fecundar con espermatozoides portadores de daño en la cromatina espermática (Pérez-Cerezales et al., 2010). En condiciones naturales los espermatozoides activan su movilidad en el momento de ser expulsados sobre los ovocitos y establecen contacto con el corion (la envuelta externa del ovocito) guiados por sustancias contenidas en el líquido folicular, con características de feromonas acuáticas (Cosson et al., 2015). Los espermatozoides permanecen móviles un máximo de 90 segundos (dependiendo de la especie), mientras el micropilo, o poro a través del cual a de traspasar el corion, permanece abierto durante aproximadamente 3 minutos.

Los espermatozoides que muestran un patrón alterado de motilidad tienen menos oportunidades de alcanzar el corion y atravesar el micropilo, lo que representa una barrera que impide la fecundación por espermatozoides fisiológicamente incompetentes (Cosson et al., 2015). No obstante, dependiendo de cuál sea el origen del daño en el genoma, muchos espermatozoides pueden mantener la capacidad fecundante a pesar de ser portadores de un genoma dañado. Así se ha documentado que la fecundación con espermatozoides portadores de daño genómico aumenta la mortalidad en embriones de carpa (*Cyprinus carpio*) (Kutluyer et al., 2014) y tenca (*Tinca tinca*) (Rodina et al., 2007). En cuanto al uso de semen congelado, se ha comprobado que los espermatozoides de trucha arcoíris portadores de diferente grado de daño en el ADN debido a la criopreservación, son capaces de fecundar, comprometiendo seriamente la supervivencia de la progenie y provocando defectos en la expresión de determinados genes relacionados con el crecimiento y desarrollo (Pérez-Cerezales et al., 2011). Kopieka y cols (Kopeika et al., 2004) también refirieron fallos en la supervivencia de las progenies de locha (*C. macracanthus*), Hayes y cols (Hayes et al., 2005) observaron mayores tamaños corporales y diferente respuesta al estrés en algunos grupos de larvas de trucha (*O. mykiss*) obtenidas con semen criopreservado y Horváth y cols (Horváth et al., 2007) detectaron efectos clastogénicos en larvas de carpa (*C. carpio*) obtenidas con semen congelado.

La exposición a determinados xenobioticos presentes en aguas continentales, también puede provocar efectos genotóxicos en el esperma de especies acuáticas mediante diferentes mecanismos, incluyendo el aumento de radicales libres de oxígeno. Devoux y cols. (Devoux et al., 2011) demostraron en peces una clara deficiencia de la capacidad reproductiva de los machos adultos tras su exposición

durante su periodo de freza a MMS (metil metao sulfonate), un tóxico representativo de los componentes alcalinos que hay en el medio acuático. Santos y cols. (Santos et al., 2013) también observaron un efecto similar en el pez espinoso (*Gasterosteus aculeatus*) tras ser expuesto al mismo tóxico (MMS) y, además, pudieron relacionar significativamente el daño sobre el genoma paterno con los defectos observados en las progenies. Algunos de los contaminantes emergentes como productos farmacéuticos, desinfectantes, agentes de uso industrial etc., son difíciles de detectar y eliminar. Muchos estudios evidencian que la exposición a estos químicos ambientales, durante la gestación en mamíferos o durante la vida posnatal, produce efectos que podrían tener consecuencias a largo plazo (Tse et al., 2013), e incluso heredarse de manera transgeneracional por vía paterna (Lombó et al., 2015).

La inestabilidad genómica que las lesiones del gameto masculino puede provocar durante el desarrollo del embrión, tiene como consecuencia el aumento en la tasa de mortalidad de los mismos (Schulte et al., 2010), pero como hemos resumido, puede persistir un nivel de daño sub-letal compatible con la supervivencia embrionaria, que podría comprometer la salud de la progenie a largo plazo.

4. Mecanismos moleculares de respuesta al daño genotóxico e implicaciones del factor materno

La reparación del ADN es una de las funciones celulares más importantes en el control de la homeostasis. Los mecanismos de reparación son los encargados de la protección del genoma ante la presencia de metabolitos endógenos o agentes exógenos que dañen el ADN (Davis and Chen, 2013). El organismo, ante estas situaciones, responde poniendo en marcha mecanismos de control y reparación del

daño en la hebra de ADN, o activando en su caso, mecanismos de parada del ciclo celular o de apoptosis. El conjunto de respuestas se conoce como DDR (*DNA damage response*) y puede activarse tanto en las células somáticas, como en las células de la línea germinal.

4.1. Respuesta celular al daño en el ADN: puntos de control del ciclo celular

La DDR se produce fundamentalmente cuando la cromatina es más accesible: en genes transcripcionalmente activos o durante la replicación. Es una respuesta altamente conservada y coordinada de señalización que, tras la detección de la lesión en el ADN, activará alguna de las diferentes vías de reparación. El proceso se verá facilitado por la activación de un mecanismo de parada del ciclo celular para dar tiempo suficiente a estos mecanismos de reparación a eliminar el daño. De este modo, se consigue evitar que las lesiones se transmitan a las células hijas (Jackson and Bartek, 2010; Stucki et al., 2005). En el caso en el que el daño sea demasiado severo, los factores que regulan esta respuesta al daño se encargarán de eliminar la célula dañada por un mecanismo de apoptosis o senescencia (Giglia-Mari, 2011; Jackson and Bartek, 2010) (Figura 3). Los peces comparten con los mamíferos muchos de los mecanismos implicados en la DDR y, aunque se ha observado la activación de diversas vías de reparación en embriones de pez cebra (*D. rerio*) (Fortier et al., 2009; He et al., 2015; Pei and Strauss, 2013), poco se sabe sobre la respuesta al daño durante el desarrollo embrionario.

La DDR comienza con la detección del daño por elementos sensores. Como hemos indicado, el espermatozoide muestra diversas lesiones, incluyendo numerosas

roturas en la hebra de ADN. Las roturas de simple hebra (SSBs, *Single strand breaks*) se transforman tras la replicación en roturas de doble hebra (DSBs, *Double strand breaks*), lesión que genera gran inestabilidad genómica y que representa el mayor riesgo tras la fecundación (Hilton et al., 2013; Lovato et al., 2012; Marchetti et al., 2015). Las lesiones en el ADN bloquean el avance de las polimerasas por las horquillas de replicación generando el bloqueo de las mismas y, por tanto, cadenas simples de ADN. La señalización de las DSBs es llevada a cabo por el complejo MRN, formado por tres proteínas MRE11, RAD50, NBS1, mientras que el ADN de simple cadena generado en las horquillas bloqueadas es identificado por la proteína A (RPA) (Jackson and Bartek, 2010; Stucki et al., 2005). La traducción de la señal está controlada por dos fosfatidil inositol quinasas (PIKK): ATM (Ataxia Telangiectasia Mutada) y ATR (ATM and Rad3-related), la primera es reclutada tras la generación de DSBs, y la segunda como resultado de la parada de la horquilla de replicación. (Cimprich and Cortez, 2008; Lavin, 2008; Smith et al., 2010). Tras el reclutamiento, ATM sufre un cambio conformacional y se autofosforila, disociándose en un monómero activo (Bakkenist and Kastan, 2003). Por su parte ATR, se activa mediante su interacción con la proteína TopBP1, que funciona como un inductor alostérico (Kumagai et al., 2006).

Una vez que ATM y ATR se activan, fosforilan diferentes proteínas esenciales para la respuesta y reparación de las lesiones. La histona H2AX (una variante de la histona canónica H2A) es fosforilada en la serina 139, formando γH2AX que, a su vez, recluta y activa a otras proteínas e inicia la remodelación de la cromatina. En concreto, γ-H2AX recluta la proteína adaptadora MDC1 (Stucki et al., 2005), que mediante la interacción con ATM y NBS1 (del complejo MRN) forma un bucle de retroalimentación positiva que amplifica la señal de γ-H2AX (Stucki et al., 2005). Otras proteínas

reclutadas a sitios de DSB incluyen, 53BP1 y BRCA1, los cuales son sustratos ATM y mediadores en la respuesta al daño del ADN. Estas proteínas formaran spots o *foci* que inician la remodelación de la cromatina necesaria para la reparación del daño y amplifican la señalización. 53BP1, una de las proteínas más estudiadas, tiene una ubicación pan-nuclear y, después de la exposición a agentes genotóxicos, se posiciona claramente en el lugar de los *foci* (Peuscher et al., 2011). La detección de los *foci*, mediante la localización de γ-H2AX o 53BP1, se emplea como marcador de daño genético.

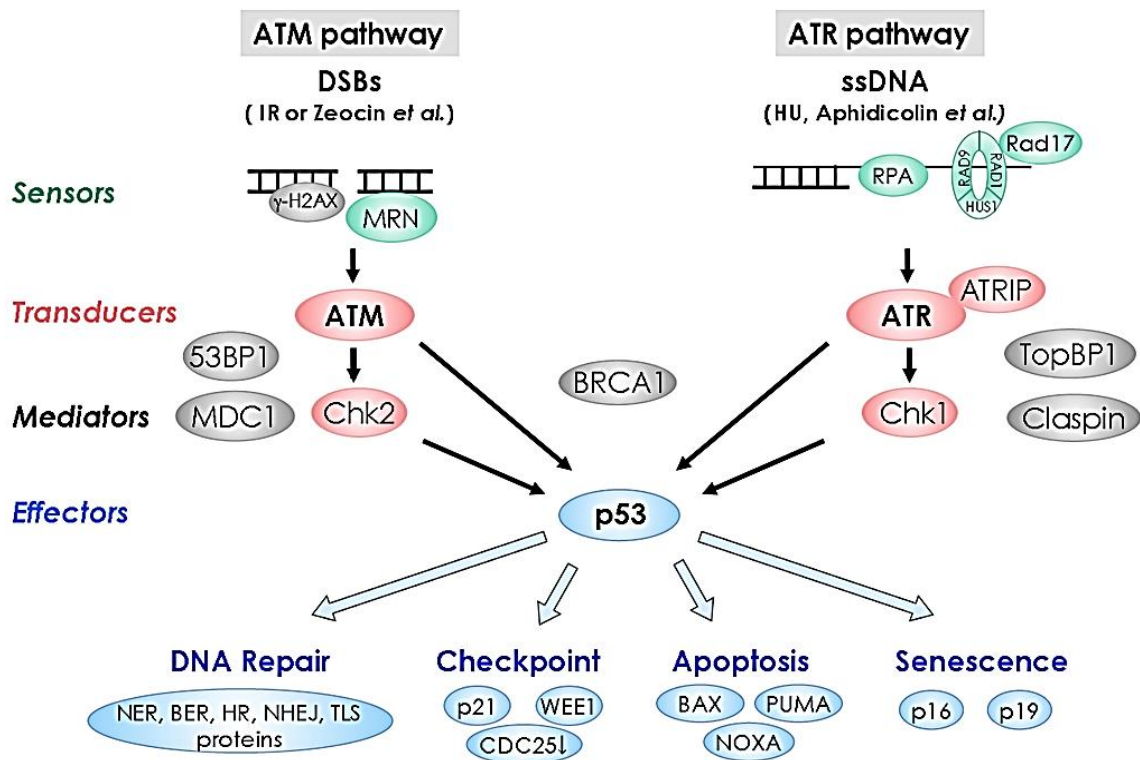**Figura 3.** Esquema representativo de la DDR en mamíferos. (Yosiyama et al., 2013).

Las roturas de doble hebra pueden también desencadenar un mecanismo más simple de señalización, mediado por otro miembro de la familia PIKK, el DNA-PK, que estimula actividades de reparación de forma local, sin provocar una cascada de señalización. Una vez detectado el daño en la doble hebra ADN-PK se activa en el sitio

de rotura, activando y promoviendo la ruta de reparación no homologa (NHEJ), que como veremos más adelante, es propensa a introducir errores de copia (Doherty and Jackson, 2001). La señalización la lleva a cabo el heterodímero Ku70-Ku80, que se une y estabiliza el extremo roto de la hebra de ADN. Esta unión recluta a la ADN-PK que se encarga de activar los efectores de la vía de reparación NHEJ. DNA-PK promueve una rápida respuesta de reparación, pero tiene un rol muy limitado en el control del daño, salvo, en ausencia de ATM. En este caso, puede estar más tiempo en el sitio de la lesión y contribuir a las funciones de control (Doherty and Jackson, 2001; Weterings and Van Gent, 2004).

Las vías de transducción iniciadas por ATM y ATR permiten la fosforilación de mediadores como Chk2 y Chk1 respectivamente, proteínas quinasas que van a ser responsables de la activación del principal efector en la DDR, la proteína p53, también llamada el guardián del genoma (Jackson and Bartek, 2010; Stucki et al., 2005). p53 es un factor de transcripción que regula la transcripción de numerosos genes, a su vez responsables de ejecutar la respuesta al daño detectado. Cuando p53 es fosforilado y, dependiendo de la presencia de diferentes proteínas que regulan su función, activará la transcripción de los genes necesarios para proceder con la detención del ciclo celular, la reparación del ADN, la activación de la apoptosis o de otros tipos de muerte celular. Las quinasas Chk1 y Chk2 fosforilan p53 en distintos residuos, activando este factor de transcripción (Jackson and Bartek, 2010; Stucki et al., 2005). Además, ATM inactiva mediante su fosforilación a Mdm2, una ubiquitin ligasa necesaria para la degradación de p53. De esta manera, ATM y ATR activan y acumulan p53 fosforilado.

Como hemos mencionado, una de las respuestas generadas tras la detección del daño en el ADN, es la parada del ciclo celular, que puede producirse en los

diferentes puntos de control en G1/S, intra - S y G2/M, activando mecanismos que frenan el avance a través del ciclo, hasta conseguir la correcta replicación y reparación del ADN (Jackson and Bartek, 2010; Stucki et al., 2005). La activación de p53 en el punto de control G1/S resulta en la activación de p21 (Bourdon et al., 2003), un inhibidor del complejo Cdk2/ciclina E, provocando el bloqueo del ciclo celular. Durante la fase S, la actividad de las quinasas Chk1 y Chk2, conduce a la parada de la síntesis de ADN, frenando la replicación y, por tanto, el ciclo. Por otro lado, durante el paso de G2 a la mitosis, la activación de las quinasas Chk2 y Chk1 promueve la fosforilación de Cdc25C, responsable de la progresión a través de G2. La fosforilación, conlleva en este caso, la inactivación de Cdc25C y tiene dos consecuencias: facilitar su unión al transportador p14-13-3, lo que resulta en su salida del núcleo y secuestro en el citoplasma o, bloquear la activación del complejo Cdc2/ciclina B y con ello, la entrada en mitosis (Lord and Ashworth, 2008). Todos estos mecanismos permiten a la célula activar las vías de reparación necesarias para eliminar los errores acumulados en el ADN.

4.2. Rutas de reparación del ADN

Los diferentes tipos de daño en la hebra de ADN requieren la activación de diferentes mecanismos de reparación, que implican un gran número de proteínas y proporcionan diferente grado de fidelidad (Jaroudi and SenGupta, 2007; Marchetti et al., 2007; Matsuda and Tobari, 1988). Las vías de reparación incluyen: la vía de reversión directa (DR, Direct Reversal), encargada de ciertos tipos de modificaciones de las bases nitrogenadas que no requieren su eliminación, como ocurre con las metilaciones de las guaninas, que pueden ser corregidas por las enzimas encargadas

de eliminar el aducto; la vía de reparación por malapareamiento (MMR, DNA Mismatch Repair), encargada de la reparación de deleciones o inserciones generadas erróneamente durante la duplicación del ADN; la vía de reparación por escisión de un nucleótido (NER, Nucleotide Excision Repair), encargada de la eliminación de grandes lesiones que distorsionan la hélice, como los dímeros de timina causados por exposición a radiación UV; la vía de reparación por escisión de bases (BER, Base Excision Repair), la cual se encarga de reparar las lesiones en un único nucleótido, como las oxidaciones, mediante la escisión de la hebra y la sustitución de la base afectada; y, por último, las vías de recombinación homóloga (HR, Homologous Recombination) y la de unión de extremos no homologos (NHEJ, Non-Homologous End Joining), encargadas de la reparación de dobles roturas (Hakem, 2008). La presencia de todos los mecanismos de reparación descritos ha sido observada en peces, sugiriendo que estas vías han sido bien conservadas durante la evolución, por lo que se ha llegado a proponer al pez cebra (*D. rerio*) como modelo para estudios de daño en el ADN y reparación (Pei and Strauss, 2013). En cuanto a su actividad durante la espermatogénesis, en mamíferos se ha descrito actividad de las vías BER, NER, MMR y NHEJ y los fallos en algunas de ellas han sido relacionados con problemas de fertilidad (Gunes et al., 2015). Sin embargo, la actividad reparadora se pierde en células meióticas y posmeióticas, de modo que los errores se acumulan sin reparar en el espermatozoide.

La vía BER de reparación es la principal vía de eliminación de SSBs y de oxidación de bases, lesiones frecuentemente provocadas en el esperma durante el manejo de reproductores y/o la conservación seminal, como detallamos anteriormente. Su actividad depende de la enzima PARP (poli (ADP – ribosa)

polimerasa), que ha sido descrita en peces (Fortier et al., 2009). El papel de esta enzima en la reparación del ADN se basa en su habilidad para modificar transitoriamente, mediante poli-ADP ribosilación (parylación), ciertas proteínas que están involucradas en la conformación de la cromatina (principalmente histonas), dando lugar a una alteración de la misma (Amours et al., 1999). De esta forma, la cromatina se convierte en una estructura más accesible para las proteínas implicadas directamente en la reparación. PARP reconoce las roturas simples y dobles de la hebra de ADN y los entrecruzamientos y, tras la unión con el ADN dañado, se activa su función catalítica. Su actividad se incrementa enormemente tras el estrés celular o el daño en el ADN (Beneke, 2012; Lovato et al., 2012). La inhibición de PARP utilizando agentes como el 3-AB (3- aminobenzamida) provoca el fallo de la vía BER e incrementa el número de DSBs generadas por las roturas simples durante la replicación. Dichas dobles roturas se podrán reparar por la vía de recombinación homóloga (HR), que copia la secuencia de una cromátida hermana no dañada a partir del cromosoma homólogo, o por la de unión de extremos no homólogos (NHEJ). Esta última actúa reparando los extremos alterados o inapropiados en las hebras de ADN, ligándolos de una forma rápida a expensas de generar posibles microdeleciones locales, por lo tiene más probabilidades de cometer errores y generar mutaciones (Hilton et al., 2013; Metzger et al., 2013).

La activación de la vía NHEJ depende de la detección de los extremos rotos del ADN por el heterodímero Ku70/80, que atrae a DNA-PKcs (Hefferin and Tomkinson, 2005). A pesar de su baja fidelidad, esta vía puede actuar de forma urgente y, a diferencia de HR, no depende de que exista una molécula de ADN hermana, cosa que solo ocurre después de la replicación y, por tanto, puede ser utilizada en cualquier fase

del ciclo celular. Liu y cols. han demostrado que, en el caso de pez zebra, esta vía, propensa al error, es la que predomina ante DSBs durante la embriogénesis (Liu et al., 2012).

La recombinación homóloga (HR) involucra más factores e implica más pasos que NHEJ. En primer lugar, para la búsqueda de homología en la cromátida hermana, las DSBs son reseccionadas para generar largos segmentos de ADN de una hebra (Hiom et al., 2000). Diferentes helicasas y exonucleasas son responsables de este proceso (Huertas and Jackson, 2009; Mimitou and Symington, 2008). A continuación, RPA con ayuda de otras proteínas, promueve la formación de un filamento nucleoprotéico de Rad51, que es capaz de invadir la hebra homóloga que servirá como molde para la correcta resíntesis del fragmento dañado. De esta forma se llega a una estructura que es resuelta para separar ambas cromátidas con ayuda de una endonucleasa, una ligasa y una resolvasa (West, 2003). Entre los factores implicados en HR, algunos son de gran importancia en la supresión tumoral en humanos como son BRCA1 y BRCA2, los cuales son necesarios para reclutar de forma eficiente a Rad51 (Yoshida and Miki, 2005).

Se ha descrito que el genoma del pez cebra (*D. rerio*) contiene los genes involucrados en todas las vías de reparación mencionadas anteriormente (Pei and Strauss, 2013). Sin embargo, y a pesar de que el material genético de *D. rerio* presenta la secuencia de la enzima que elimina las alquilaciones de la guanina (Mgmt) en la vía DR, no se han encontrado estudios que avalen la actividad reparadora de esta vía en particular. Además existe en peces un sistema adicional, la vía de reparación fotoencimática (PER), presente tanto en el reino animal como en el vegetal, pero ausente en animales placentarios (Sancar, 2000). Esta ruta revierte la formación de CPDs (dímeros de ciclopirimidina) por acción de la irradiación UV (B y C) (Sancar, 2000),

y su actividad depende de la dosis de irradiación que cause el daño (Mitchell et al., 2009). Se ha comprobado que PER actúa en larvas de pez cebra (*D. rerio*), pero no es capaz de eliminar el daño oxidativo causado por la irradiación UV, que sería resuelto usando la vía BER (Dong et al., 2007).

Las rutas de reparación del ADN son consideradas un mecanismo de defensa, a nivel celular, para evitar alteraciones que pongan en peligro la integridad del ADN. Sin embargo, algunas de estas vías (en particular NHEJ), podrían introducir cambios en las secuencias de nucleótidos del ADN, desencadenando efectos antagonistas.

4.3 Apoptosis

La apoptosis es un mecanismo de muerte que puede desencadenarse tras la detección del daño. La decisión de optar por la muerte celular y no por otras respuestas menos severas, viene determinada fundamentalmente por el número y gravedad de las lesiones, y por la sensibilidad de cada tipo celular al daño. Sin embargo, se desconoce cómo está regulada esta decisión. La apoptosis en respuesta a daño genotóxico es activada por p53 siguiendo dos caminos diferentes: inhibiendo la interacción de p53 con su regulador negativo Mdm2, o a través de varias modificaciones postraduccionales que promuevan su actividad transcripcional (Kruse and Gu, 2009; O' Reilly et al., 2009). Una vez que p53 está activado, promueve la transcripción de varios factores pro-apoptóticos de la superfamilia Bcl-2, como son PUMA, NOXA y BAX (Li et al., 2006; Nakano and Vousden, 2001; Oda et al., 2000). Estos genes cooperan en la despolarización de la membrana mitocondrial permitiendo la salida de varias proteínas como son el citocromo C, Smac/Diablo, AIF, Endo G al citosol

(Willis, 2011) y formando el apoptosoma. El apoptosoma activa directamente a la caspasa-9, y ésta a su vez a las caspasas efectoras, como la caspasa-3, desencadenando las últimas fases de la apoptosis.

El gen supresor de tumores p53 de pez zebra (*D. rerio*) es muy similar en estructura y función al de mamíferos y, como en éstos y en otros modelos animales, es el principal mediador de la apoptosis (Storer and Zon, 2010). Sin embrago, en el caso de peces existe bastante controversia, ya que mientras algunos autores han descrito un aumento en la expresión de p53 (Storer and Zon, 2010), otros reflejan una falta de inducción tras daños genotóxicos (Liu et al., 2011). Embriones inyectados con un morfolino de p53 en pez cebra parecen mostrar menos actividad apoptótica que embriones normales ante la presencia de determinados agentes genotóxicos (Langheinrich et al., 2002), señalando su papel en la inducción de muerte ante el daño. Sin embargo, en algunos casos parece que, tras una exposición a irradiación γ, a parte de no inducirse apoptosis, tampoco se produce un aumento de p21, ni se activan los puntos de control para parar la replicación del ADN (Berghmans et al., 2005). Estos datos contradictorios muestran la necesidad de un estudio más completo del papel de p53 en la DDR en peces. Por otra parte, la actividad de p53 puede ser modulada por Mdm2, como ya hemos mencionado, pero en el caso del pez cebra (*D. rerio*) se ha descrito un modulador adicional: Δ113p53, que es una isoforma alternativa de p53. Esta isoforma, actúa modificando la expresión de genes regulada por p53, y antagoniza la apoptosis mediante la activación del factor antiapoptótico bcl2. Este efecto ha sido observado en embriones de *D. rerio* tras su exposición a agentes promotores de DSBs (Chen et al., 2009). Esto indica que p53 podría activar diferentes respuestas durante el desarrollo de *Danio rerio*, pudiendo provocar tanto una activación como una inhibición

de la apoptosis, en función de la presencia de otros factores implicados en su regulación.

4.4. Mecanismos de tolerancia al daño genotoxico

La apoptosis mediada por p53 es uno de los mecanismos más importantes por el cual las células previenen una transformación maligna (Junttila and Evan, 2009; Schmitt et al., 2002). Sin embargo, la respuesta al daño también puede conducir a la activación de mecanismos de tolerancia. Así, por ejemplo, se ha descrito cómo los corales sometidos a condiciones térmicas inadecuadas que promueven un aumento del daño oxidativo, ven suprimida la actividad de las caspasas (Tchernov et al., 2011), probablemente como mecanismo de supervivencia en condiciones extremas. Los mecanismos de tolerancia pueden ser especialmente relevantes durante los primeros estadíos de desarrollo embrionario, donde los ciclos celulares son cortos y altamente sincrónicos, las fases G1 y G2 están ausentes y la duración de la síntesis de ADN puede ser extraordinariamente corta (Kermi et al., 2017). El avance por el ciclo, con una menor activación de los puntos de control, se vería favorecido por el aumento de la tolerancia al daño (Lerner et al., 2017).

Estos mecanismos de tolerancia podrían también, paradójicamente, ser facilitados por el "guardian del genoma". Parece que p53 en su dominio de unión al ADN codifica una exonuclesa 3´- 5´, la cual está implicada en replicación y reparación del ADN entre otras funciones (Hampp et al., 2016). Su contribución permite superar lesiones presentes en el ADN de la horquilla de replicación facilitando a la célula sobrevivir con el daño mediante un mecanismo llamado en inglés *trans lesión synthesis*

(Hampp et al., 2016). Esta vía de tolerancia al daño del ADN, junto con la recombinación homóloga, facilita el *bypass* de las lesiones que bloquean la replicación. Se ha demostrado que estos eventos son cruciales para la supervivencia y la rápida proliferación de células cancerígenas y de las células madre que se duplican activamente durante la renovación de tejidos (Hampp et al., 2016).

p53 estimula la reparación homologa de manera espontánea durante la fase S para superar el bloqueo de la horquilla de replicación y, así, evitar el colapso de la misma (Gatz and Wiesmuller, 2006; Ireno et al., 2014). Debido a su implicación en este mecanismo, se cree que p53 se encarga de proteger el ADN replicativo (Gatz and Wiesmuller, 2006; Ireno et al., 2014). En el momento en el que se encuentra un obstáculo/lesión en la horquilla de replicación, la maquinaria de replicación se para promoviendo la ubiquitinación de PCNA, que es una proteína involucrada en la síntesis de ADN, al mismo tiempo que se recluta p53 y una ADN polimerasa (Pol). Cuando el ADN se daña por efecto de radiación UV o agentes químicos, PCNA puede sufrir modificaciones que le permiten llevar a cabo la síntesis haciendo un *bypass* en el ADN dañado (Hampp et al., 2016). Diferentes ADN polimerasas, capaces de acomodar las hebras molde dañadas, están implicadas en este mecanismo de tolerancia (Sale et al., 2013). Sin embargo, las mismas adaptaciones estructurales que permiten que estas polimerasas eviten las lesiones, promueven una muy baja fidelidad al copiar la cadena molde dañada, aumentando el riesgo de mutagénesis (Sale et al., 2013). Las principales polimerasas de este mecanismo de tolerancia pertenecen a la familia Y e incluyen eta, iota, kappa y rev1 (Lerner et al., 2017). Se ha demostrado que ratones transgénicos que sobreexpresaban rev1 presentaban una alta tasa de mutaciones y una muy baja actividad apoptótica (Sasatani et al., 2017).

Los procariotas, deben su enorme capacidad de adaptación a los cambios ambientales a su gran tolerancia a la acumulación de mutaciones. En bacterias es conocida la respuesta SOS, una respuesta global al daño del ADN que se activa cuando los daños acumulados son tantos que impiden el avance de la maquinaria de replicación. Consiste en emplear polimerasas *bypass* para rellenar huecos con nucleótidos al azar. La respuesta SOS en bacterias es altamente mutagénica debido a la utilización de polimerasas propensas al error (Lerner et al., 2017). Las similitudes entre el sistema translesión en células de mamífero y la respuesta SOS en bacterias (Lerner et al., 2017), resaltan la importancia de la tolerancia al daño en diferentes especies y apuntan a las diferencias entre la activación de mecanismos de muerte o tolerancia como parte esencial de las estrategias evolutivas.

4.5. Capacidad de reparación del zigoto: factor materno

Los gametos masculino y femenino son propensos a sufrir diferentes daños genotóxicos, pero mientras que el espermatozoide va perdiendo su actividad reparadora a lo largo de la espermatogenesis, el ovocito contiene ADN, ARN polimerasas, distintos factores de transcripción y traducción, así como transcritos maternos y proteínas involucrados en mecanismos de reparación del ADN (Lubenzs et al., 2010; Sullivan et al., 2015). Por ello, una vez que se produce la fecundación, la reparación de los daños en el genoma paterno recae en la maquinaria reparadora del ovocito, hasta que se produce la activación transcripcional del zigoto. Se ha comprobado que la buena funcionalidad de las diferentes rutas de reparación del ovocito durante los primeros estadios del desarrollo embrionario, resulta ser una

condición fundamental para el desarrollo normal del embrión (Hakem, 2008; Marchetti et al., 2015; Pérez-Cerezales et al., 2010). De hecho, cuando la capacidad reparadora del zigoto no es suficiente para contrarrestar el daño genotóxico del espermatozoide, se produce la aparición de aberraciones cromosómicas en el embrión de ratón (Marchetti et al., 2015). La reparación zigótica se considera clave tras la fecundación, ya que aquellas lesiones que no sean reparadas en este momento, serán transmitidas a las células hijas tras la primera división, pudiendo perpetuar los errores en los primeros blastómeros (Marchetti et al., 2015, 2007, 2004b; Shimura et al., 2002). De hecho, los estudios citogenéticos realizados por Marchetti y su grupo de investigación, en embrión de ratón indican que el tipo de aberraciones cromosómicas que se producen en el zigoto tras la reparación del daño paterno, ya es predictivo del destino del embrión, indicando la enorme importancia que tiene en mamíferos la reparación que ocurre antes de que finalice la fase G1 del primer ciclo de desarrollo (Marchetti et al., 2004). Por tanto, las consecuencias de la fecundación con semen dañado no pueden entenderse sin considerar la capacidad de reparación del ovocito, que puede también verse afectada por distintos factores. Así, se ha determinado que los ovocitos de ratones viejos presentan un gran número de transcritos diferencialmente expresados (DEGs) relacionados con la integridad y estabilidad del ADN respecto de los ovocitos de hembras jóvenes, mostrando que la actividad de los mecanismos de reparación se reduce considerablemente durante el envejecimiento (Hamatani et al., 2004). También, mediante el estudio del transcriptoma, se ha observado una menor calidad reproductora de los ovocitos de mujeres mayores, que afecta a diferentes rutas de reparación del ADN (Grondahl et al., 2010).

La reparación defectuosa de SSBs por la vía BER da lugar a un aumento de DSBs después de la replicación (Hilton et al., 2013; Metzger et al., 2013), el tipo de lesión más perjudicial y que genera más inestabilidad genómica (González-Marín et al., 2012). Como hemos mencionado, las vías encargadas de eliminar este tipo de lesiones son la vía HR y NHEJ. La vía NHEJ es activa durante el ciclo celular zigótico después de la fertilización, el punto de control más activo para reparar el daño del ADN de origen paterno (Marchetti and Wyrobek, 2008; Shimura et al., 2002). La inhibición de la vía NEHJ en embriones de pez cebra, utilizando morfolinos, en los estadíos de 1 célula y 6 horas post fecundación (hpf), aumentó la actividad apoptótica y la tasa de malformaciones (Bladen et al., 2005), lo que sugiere la importancia de la reparación de dobles roturas en esos estadios tempranos. Así mismo, la inhibición de la vía BER en zigotos de trucha arcoíris (*O. mykiss*), también indujo un importante aumento de abortos (Pérez-Cerezales et al., 2010). Una proteína crucial en esta vía es Apex1 que se encarga de eliminar la base anómala. Embriones *knockdown* de pez cebra (*D. rerio*) para esta proteína sufrieron fallos embrionarios justo durante la activación del zigoto, mientras que *knockdown* parciales de apex1 presentaron defectos en los ojos, notocorda, cerebro, células sanguíneas y corazón (Wang and Höög, 2006), poniendo en relieve la importancia de una correcta maquinaria de reparación inmediatamente después de la fecundación.

Según la información proporcionada, la sinergia entre los factores paternos y maternos es esencial para poder determinar el resultado final del proceso reproductivo. Por ello, en la presente tesis doctoral, se pretende evaluar el efecto de diferentes agentes genotóxicos sobre el genoma paterno y la capacidad de los embriones, de dos especies de peces, para progresar con el desarrollo tanto en

condiciones normales, como cuando la capacidad de reparación ha sido comprometida. Se estudiará el fenotipo y el perfil transcriptómico de la descendencia, así como la actividad apoptótica y de reparación del ADN durante el desarrollo. Esta tesis doctoral aportará información acerca de cómo el daño genómico paterno afecta al desarrollo embrionario de peces y permitirá desarrollar marcadores moleculares de calidad de los gametos y embriones.

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Objetivos

Ha sido demostrado que los mecanismos de respuesta al daño en el genoma paterno se activan en el embrión de forma muy temprana. Sin embargo, no hay información acerca de los efectos que la reparación incompleta de ese daño puede tener en el desarrollo embrionario de peces. Entre los teleósteos parece demostrado que la selección de espermatozoides tras la eyaculación es poco restrictiva, incrementándose la probabilidad de fecundar con espermatozoides dañados. Por otra parte, los mecanismos de respuesta al daño durante el desarrollo embrionario de peces son muy poco conocidos, existiendo datos contradictorios en la bibliografía sobre la actividad apoptótica en caso de daño genotóxico. Dada la diferente estrategia reproductiva de peces -basada en la producción de numerosos descendientes que tienen pocas probabilidades de sobrevivir tras la eclosión, y mamíferos -basada en el control exhaustivo de un reducido número de embriones, podrían existir diferencias relevantes entre ambos grupos animales en el manejo de las lesiones en el ADN del espermatozoide.

La hipótesis de trabajo de la presente tesis doctoral es que el daño genotóxico en el esperma, si no es completamente reparado tras la fecundación, puede comprometer la salud y desarrollo de la futura progenie, conduciendo a fallos en la regulación de la expresión de genes clave en el desarrollo, particularmente en peces. Esta tesis doctoral se desarrollará en dos especies de peces: trucha arcoíris, dado su interés como especie comercial, y en pez cebra, por sus ventajas como modelo experimental.

Los objetivos concretos del trabajo de tesis son:

1. Evaluar el efecto de la inhibición de la reparación zigótica en el perfil transcriptómico de larvas de trucha arcoíris obtenidas con semen portador de diferente nivel de daño en su cromatina.

2. Comparar el perfil transcriptómico de larvas de trucha arcoiris obtenidas con semen dañado tras aplicar diferentes protocolos de criopreservación seminal con aquellas obtenidas con semen control.

3. Analizar si la alteración de la época natural de puesta mediante el cambio del fotoperiodo afecta a la estabilidad del genoma paterno y a la actividad reparadora del ovocito, evaluando el impacto de ambos factores sobre el desarrollo de la progenie.

4. Evaluar en pez cebra la calidad de la progenie, así como la activación de la respuesta (DDR) y de la tolerancia (DDT) al daño en el ADN, tras la fecundación con semen portador de un alto nivel de daño genotóxico.

Capítulo 1: Efectos de la inhibición de la vía de reparación BER en el zigoto y consecuencias de utilizar protocolos de criopreservación subóptimos en el perfil transcriptómico de la progenie.

Inhibition of zygotic DNA repair: transcriptome analysis of the offspring in trout (*Oncorhynchus mykiss*)

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Abstract

Zygotic repair of the paternal genome is a key event after fertilization. Spermatozoa accumulate DNA strand breaks during spermatogenesis and can suffer additional damage by different factors, including cryopreservation. Fertilization with DNA damaged spermatozoa (DDS) is considered to promote implantation failures and abortions, but also long-term effects on the progeny that could be related with a defective repair. Base excision repair (BER) pathway is considered the most active in zygotic DNA repair, but healthy oocytes contain enzymes for all repairing pathways. In this study, the effects of the inhibition of the BER pathway in the zygote were analyzed on the progeny obtained after fertilization with differentially DDS. Massive gene expression (GE; 61 657 unique probes) was analyzed after hatching using microarrays. Trout oocytes are easily fertilized with DDS and the high prolificacy allows live progeny to be obtained even with a high rate of abortions. Nevertheless, the zygotic inhibition of Poly (ADP-ribose) polymerase, upstream of BER pathway, resulted in 810 differentially expressed genes (DEGs) after hatching. DEGs are related with DNA repair, apoptosis, telomere maintenance, or growth and development, revealing a scenario of impaired DNA damage signalization and repair. Downregulation of the apoptotic cascade was noticed, suggesting a selection of embryos tolerant to residual DNA damage during embryo development. Our results reveal changes in the progeny from defective repairing zygotes including higher malformations rate, weight gain, longer telomeres, and lower caspase 3/7 activity, whose long-term consequences should be analyzed in depth.

Introduction

Recent studies have changed our perception of the contribution of sperm chromatin in early embryo development. The differential gene packaging in the spermatozoa, as well as the presence of epigenetic marks in specific genes at both histone and DNA levels, point to an essential role of the paternal chromatin in the genetic control of the first events occurring after fertilization (Carrell & Hammoud 2010, Speyer et al. 2010, Ward 2010). Fertilization with DNA damaged spermatozoa (DDS) is considered as an important factor leading to implantation failures and abortions both in mammals (Lin et al. 2008, Zini et al. 2008, Speyer et al. 2010) and fish (Ciereszko et al. 2005, Perez-Cerezales et al. 2010a). In addition, DDS has been related with later paternal effects such as abnormal weight at birth, higher susceptibility to certain diseases, or even premature aging (Evenson & Wixon 2006, Bowdin et al. 2007, Fernandez-Gonzalez et al. 2008, Barroso et al. 2009, Lim et al. 2009, Zini & Sigman 2009, Schulte et al. 2010, Zini 2011). Apart from intrinsic factors acting during spermatogenesis, many external agents, such as those produced by cold or frozen storage, could promote DNA injury in the ejaculate, being most of them related with the generation of reactive oxygen species (ROS; Evenson & Wixon 2006, Thomson et al. 2009, Perez-Cerezales et al. 2010a). Spermatozoa have a very limited capacity to repair DNA damage (Smith et al. 2013) and easily accumulate DNA lesions. DNA repair capacity declines during the latter part of spermatogenesis, and this function relies on the oocyte after fertilization. Therefore, repair of the paternal DNA takes place mainly in the zygote and during early developmental stages, being dependent on the mRNAs and proteins stored in the oocyte and on genes expressed very early in development (Derijck et al. 2008, Jaroudi et al. 2009). Mammalian oocytes express genes involved in

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all DNA repair pathways, as revealed in the transcriptome analyses performed at different stages of maturation providing the regulatory machinery required to avoid transmitting mutations into the next generation until the embryo genome is fully activated. Fertilization of oocytes from female mice defective in DNA repair machinery has demonstrated that this is a key factor in ensuring the genomic integrity of the conceptus (Marchetti et al. 2007, Derijck et al. 2008). Nevertheless, this ability to repair is limited. Yamauchi et al. (2007) observed the inability of the zygote to repair such damage after ICSI with DNA cleaved in 50 kb fragments. Moreover, DNA repair could, in turn, introduce new mutations depending on the kind of DNA damage and on the active repairing pathway. Therefore, as Gonzalez-Marin et al. (2012) stated, the effect of sperm DNA fragmentation on the offspring depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it.

Among the damages promoted in spermatozoa chromatin during cold or cryo-storage, oxidization of bases and the production of simple and double strand breaks (ssDNA and dsDNA respectively) are frequently reported. Shortening of spermatozoa telomeres has also been reported after cryopreservation of trout sperm (Perez-Cerezales et al. 2011). Oxidized bases and simple strand breaks are repaired by the base excision repair (BER) pathway (Mitra et al. 2001). Double strand breaks can, in turn, be repaired either using the homologous end joining (HR) or non-homologous end joining (NHEJ) pathways (Hilton et al. 2013), both of them operative during the zygotic cell cycle in mouse (Marchetti et al. 2007, Derijck et al. 2008) and zebrafish (Bladen et al. 2005). One of the signaling enzymes upstream the BER pathway is the poly(ADP-ribose) polymerase (PARP), catalyzing the so-called 'PARylation', the post-translational addition of ADP-ribose chains to a wide range of substrates, including the

same PARP. Its role in DNA signaling and repair is well known and its inhibition generates an increase in double strand breaks, leading to genetic instability (Beneke 2012, Lovato et al. 2012, Metzger et al. 2013). PARP activity has also been strongly suggested in dsDNA repair in the zygote (Matsuda & Tobari 1989). PARP inhibition is currently being used to promote cytotoxicity (Lovato et al. 2012, Hilton et al. 2013) being 3-aminobenzamide (3AB) one of the classical inhibitors. Teleost are external fertilizers and pose a weak spermatic selection process, providing DDS with higher chances of fertilizing than in mammals (Perez-Cerezales et al. 2010b). Artificial fertilization procedures are easy to perform and, given their high fecundity, large progenies are obtained from the same mating. In addition, the external embryo development gives access to the embryos at any developmental stage, all these factors make fish a good model to analyze the role of sperm chromatin and oocyte repair in the reproductive out- come. In a previous study by our team (Perez-Cerezales et al. 2010a), we analyzed in *Oncorhynchus mykiss* the reproductive outcome of sperm carrying different levels of DNA fragmentation in trout and the effect of the zygotic inhibition of PARP. Our results revealed that fertilization rates decreased in batches fertilized with DDS according to the sperm fragmentation ratio, and embryo loss rates increased more significantly. Surprisingly, PARP inhibition did not affect fertilization rates and led to increased embryo loss in batches fertilized with high DDS, but not in those fertilized with intermediate degree DDS, revealing that the increase in genetic instability promoted by the inhibitor was only critical for the most susceptible embryos. This previous work indicates that the embryo has a high capacity to repair paternal DNA damage, estimated in a 10% of fragmented chromatin, as well as a high capacity to do this regardless of PARP activity.

A more in depth analysis of larvae resulting from this fertilization procedure, able to progress within development and to successfully hatch, could help in the understanding of the extent of later effects of repairing activity on the offspring. In this study, we examined the possible consequences of PARP inhibition in the *O. mykiss* progeny by analyzing the transcriptome of 1 day old larvae obtained by spermatozoa carrying different degree of DNA damage, as well as the effects of defective repair on the larvae performance.

Materials and methods

Reagents

All media components were purchased from Sigma–Aldrich except when otherwise stated.

Experimental procedure

The experiments were carried out in accordance with European Union Council Guidelines (86/609/CEE), following Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals, and were approved by the Scientific Committee of the University of Leon.

Larvae were obtained from fertilization procedures performed previously as described in Perez-Cerezales et al. (2010a). Briefly, fresh sperm and eggs were obtained from four males and two female rainbow trout (*O. mykiss*) from Las Zayas fish-farm (Leon, Spain). Fish were maintained under natural photoperiod, and gametes were obtained during the natural reproductive season (January–February, with a 6–8 ºC water temperature). The sperm were collected by cannulation and their motility

was checked by visual inspection using light microscopy. All samples scored more than 90% motile spermatozoa. The oocytes were extracted by stripping from females anesthetized with MS222. The eggs were pooled and divided into 200 egg batches for fertilization trials.

Immediately after collection, sperm from each male was divided into three aliquots: one used as fresh control (F samples) and two frozen in 0.5 ml French straws using different additives in the extender. For freezing, sperm was diluted 1:3 (sperm: extender) in #6 from Erdahl and Graham (0.7 mM CaCl2!2H2O, 1.08 mM MgCl2!6H2O, 1.49 mM Na2HPO4, 34.30 mM KCl, 100 mM NaCl, 0.52 mM citric acid, 55.5 mM glucose, 4.52 mM KOH, 6.48 mM bicine, and 323 mOsm/kg, pH 7.4) containing 7% Me2SO (v/v) as a permeable cryoprotectant and either 10% (v/v) egg yolk (EY samples) or 12% (w/v) (LDL samples) as a membrane protector. LDL was obtained following the protocol described previously (Moussa et al. 2002). DNA fragmentation was evaluated in fresh and frozen/thawed sperm using the comet assay as described previously (Perez-Cerezales et al. 2011).

Egg batches were fertilized with sperm from each male using fresh sperm diluted 1:3 (sperm: extender) in #6 from Erdahl and Graham, or sperm cryopreserved with egg yolk, or LDL. Four batches of 200 eggs were fertilized for each experimental condition (2400 eggs/male). Ten minutes later, the eggs were washed with water and two batches of each treatment were incubated in the dark, for 6 h at 10 ºC in 15 mM 3AB to inhibit the PARP activity (two batches per treatment) or in water (two batches per treatment). Then, the eggs were washed with water and incubated in the dark, at 10 ºC and with a continuous water flow until hatching. The fertilization rate was calculated over a subsample of 30 embryos extracted from each batch at blastula stage

to evaluate embryo development. One day after hatching, ten live larvae from each batch were taken and frozen at -80 ºC for RNA extraction.

Total RNA extraction

Total RNA was obtained using a Trizol Reagent kit (Applied Biosystems) following the manufacturer's instructions. RNA integrity was checked with the Agilent Bioanalyzer (Agilent Technologies, Massy, France) and the yield was estimated using a Nanodrop ND-1000 spectrophotometer (Labtech, Palaiseau, France). Total RNA was stored at -80 ºC until its processing.

GE microarrays processing

RNA from ten larvae from each batch was pooled, therefore four samples (corresponding to the progeny from each male) were analyzed per treatment (sperm type and fertilization/ incubation procedure). Rainbow trout GE profi ing was performed using an Agilent 8!60 K high-density oligonucleo- tide microarray (GEO platform #GPL15840). The 8X60K rainbow trout microarray design was done using eArray (an Agilent freeweb-based application). Each area of the slide contains 62 976 features. Among those 62 976 features, 61657 are unique probes (with a hit on Swiss-Prot Database) and the 1319 remaining features are controls. Several probes can match for a same gene. Labeling and hybridization steps were performed following the 'One-Color Microarray-Based GE Analysis (Low Input Quick Amp labeling)' Agilent protocol. For each sample, 150 ng total RNA were amplified and labeled usinCy3-CTP. Yield (O0.825 ng cRNA) and specific activity (O6 pmol of Cy3/mg of cRNA) of Cy3-cRNA produced were checked with the Nanodrop: 600 ng of Cy3-cRNA was fragmented and hybridized on each array. Hybridization was carried out for 17 h at 65 ºC in a rotating

hybridization oven before washing and scanning with an Agilent Scanner (Agilent DNA Microarray Scanner, Agilent Technologies) using the standard parameters for a GE microarray (channel: green, resolution: 5 mm, TIFF: 20 bits). Data were then obtained using the Agilent Feature Extraction Software (10.7.3.1). Before analysis, saturated spots, non-uniform spots, and spots not significantly different from background (k=5) were flagged using the Agilent Gene-spring GX Software (10.5.0). The probes were considered valid when corresponding spots remained present in at least 80% of the replicates of each experimental condition after the flagging procedure. Data were subsequently scale-normalized using the median value of each array.

Gene ontology analysis

Gene ontology was performed using DAVID Bioinformatics Resources v6.7 to explore functional annotation (Huang da et al. 2009). Gene names of differentially expressed gene (DEG) and total microarray genes were both imported into the web-software as gene list and background list respectively. In order to show functional classification, clustering annotation was used and a threshold score of 5 was applied (Supplementary File 2, see section on supplementary data given at the end of this article).

qPCR validation

The differential expression of five genes from those with higher sequence similarity with *O. mykiss* annotations, implied in cell cycle control (*e2f4* and *sumo1*), mitochondrial activity (*hint2*), maintenance and regulation of telomere length (*myc* and *ptges3*), morphogenesis (*myc*), DNA repair pathways (*sumo1*), and apoptosis (*sumo1*), was validated by q-PCR. Total RNA (1 µg) was reverse transcribed using the

High Capacity cDNA Kit (Applied Biosystems) following the manufacturer's instructions. The conditions applied for reverse transcription were 25 ºC for 10 min, 37 ºC for 120 min, and final extension at 85 ºC for 5 min. Real-time PCR was performed using a Step-One Plus thermocycler (Applied Biosystems). Reverse transcription products were diluted 1: 3 and 2 ml were used for each q-PCR. The primers for q-PCR were designed using Primer Express (Software v2.0, Applied Biosystems) and Primer Select (Software v10.1 DNA Star, Lasergene Core Suit). The primer nucleotide sequences and annealing temperature from rainbow trout related transcripts can be found in additional material (Table 1). The q-PCR conditions were optimized for the different primers to achieve similar amplification efficiencies. Product specificity was tested by melting curves and product size was visualized by electrophoresis on agarose gel (data not shown).

Reaction mixtures (total volume 20 µl) contained 2 µl of cDNA, 10 µl of 1 X SYBR Green Master mix (Applied Biosystems), and 2 µl of 500 nM each forward and reverse primer. q-PCR was initiated with a pre-incubation phase of 10 min at 95 ºC followed by 40 cycles of 95 ºC denaturation for 10 s and the temperature for primer extension for 1 min. Three technical replicates were done per sample.

Expression level for each transcript was normalized to 18S gene using the Delta-Ct ($2-\Delta Ct$) method (FoldChange) to analyze relative changes in GE concerning the housekeeping expression.

Table 1: List of primers for gene expression. Secquences start from 5´to 3´. Anneling temperature, accession number and amplicon size are specified for each pair of primers.

Primer name	Primer set	Anneling Tm (ºC)	Accesion number	Amplicon size (bp)
ptges3	F- CCAGCGACTGCTAAGTGGTATG	55	BT073211.1	62
	R- TCCCCTACGCAGAACTCGAT	55		
myc	F- TTGCTGTCCACTCCTCCTC	60	EU086537.1	197
	R- AACCCGCTCCACATACAGTC	60		
sumo1	F- GGTCAGGACAACAGCGAAAT	61	NM001160594.1	175
	R- CCTCATCTTCCATTCCAAGC	61		
e2f4	F- CAGACACCTCCTGCACTGAA	60	NM001140310.1	241
	R- CGGAGGAGTGGAGAGAACAC	60		
hint2	F- GGTCACCTCCTAGTGGTTGC	64	NM001165053.1	158
	R- TAACCTGGAGGCCAGTTCAG	64		

Offspring evaluation

After the evaluation of GE, progenies from four males were obtained with and without inhibition of the BER pathway following the same procedure above previously. Next, parameters were analyzed in the offspring obtained from both types of fertilization: percentage of malformations one-day post hatching (1 dph), weight 1 and 30 dph, telomere length, apoptotic activity (activity of caspases 3/7), and ploidy.

Telomere length was measured in larvae 1 dph. Genomic DNA extraction was performed using the protocol described by our group (Carton-Garcia et al. 2013), including a previous step of larvae digestion with 6 mg/ml collagenase 2 h at 37 ºC. To stop the reaction, the cells were washed with two volumes of PBS and the pellet was resuspended in 700 µl of extraction buffer (10 mM Tris–HCl, pH 8.0; 100 mM EDTA, pH 8.0; 0.5% (v/v) SDS, supplemented with 0.5 µl proteinase K (1 mg/ml)). DNA quantity and yield were determined spectrophotometrically at 260 nm (Nanodrop ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples showed high purity (A260/A280 O1.8). Total genomic DNA was used for measuring telomere length

by real-time PCR assay, as described previously (Perez- Cerezales et al. 2011). PCRs were performed using telomeric primers and primers for 18S ribosomal DNA as housekeeping gene (Table 2). The telomere signal was normalized dividing by the signal of 18S DNA, the average of this ratio was reported as relative telomere length in comparison with control larvae. To analyze the apoptotic activity, ten larvae per batch were anesthetized with MS-222 (80 mg/l) and cut in small pieces. Larvae fragments were incubated 3 h under agitation in a dissociation solution containing 3.6% trypsine, 2.4 ml DNAse I (Applied Biosystems), and 10% FBS in Leibovitz's (L-15) medium. The larvae fragments were gently and repeatedly pipetted to facilitate the dissociation process. Then, cells were filtered by 140 mm nylon mesh and washed twice with a solution containing L-15 medium. The activity of caspases 3/7 was analyzed using the Caspase-Glo 3/7 Assay Systems Kit (Promega) following the instructions of the manufacturer.

Ploidy analysis was performed with somatic cells extracted from the gills of 30 dph larvae, following the protocol described by Zhang & Arai (1996). The cells were labeled with propidium iodide (PI) and DNA content was evaluated by flow cytometry using a FACScalibur (Becton Dickinson, San Diego, CA, USA) cytometer adjusted for blue excitation (488 nm) line for the detection of PI (670/30). A total of 10 000 events were acquired per sample and data were analyzed using the Weasel 3.1 free software. Nuclear DNA content was expressed as DNA index (DI), the ratio between median G0/G1 peak of control and treated larvae.

Data analysis

To identify DEGs among treatments, a two-way ANOVA was performed using Genespring GX Software. A Benjamini–Hochberg correction was applied (FDR cut off <0.005). Hierarchical clustering analysis was performed on genes and samples using Cluster and Tree View softwares (Eisen et al. 1998) using the following parameters: median-centered (genes), Pearson correlation, and average linkage. For annotation, blastX was performed on Swissprot database and the best blast hit was chosen (e-value<$5X10^{-2}$ with a minimum id of 85%).

Significant differences in GE, and all the parameters analyzed in the progeny, were validated by an unpaired t-test using the GraphPad Prism v5.0 Software, San Diego, CA, USA (P value <0.05).

Results

Spermatozoa DNA fragmentation, fertility rates, and hatching rates were shown as reported by Perez- Cerezales et al. (2010a) and some results are summarized in Fig. 1.

Corresponding data were deposited in GE Omnibus database (GEO Series accession number GSE52217). Out of the 61 657 probes, 810 features showed a significant differential expression (FDR cut off <0.005) (Supplementary Table 1, see section on supplementary data given at the end of this article).
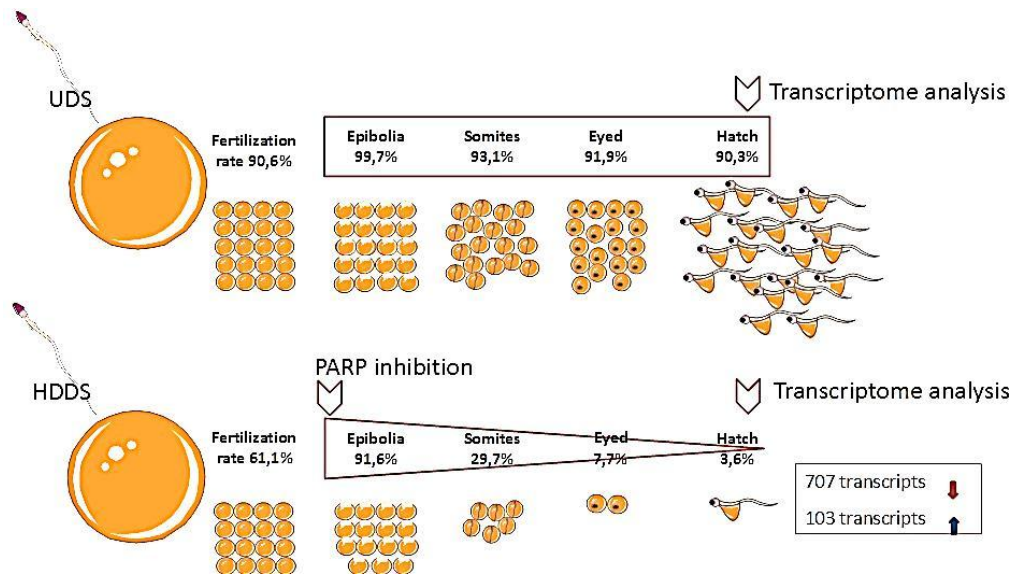
Figure 1. Schematic representation of the experimental procedure and the survival rates at different stages after fertilization in the most extreme treatments: fertilization with undamaged sperm (USD) and incubation under normal conditions, or fertilization with highly damaged sperm (HDDS) (frozen with egg yolk) followed by inhibition of the Base Excision Repair Pathway (PARP inhibition). Red and blue arrows represent down- or up-regulated transcripts in larvae obtained after PARP inhibition respectively.

The clustering analysis of these 810 probesets (Fig. 2) allowed two major clusters of genes to be identified. The first cluster included 103 probesets (given in Supplementary Table 2, see section on supplementary data given at the end of this article) that were upregulated in larvae obtained after fertilization and incubation with 3AB during the first cleavage. In contrast, the second cluster included 707 (Supplementary Table 3) downregulated probesets in the same batches. The expression analysis of the probesets by qPCR confirmed G1/S, S and points and cell the differential expression provided by the array showing a downregulation of *myc*, *e2f4*, *sumo*1, and *ptges3* and upregulation of *hint2* with the inhibition of DNA repair (Fig. 3).
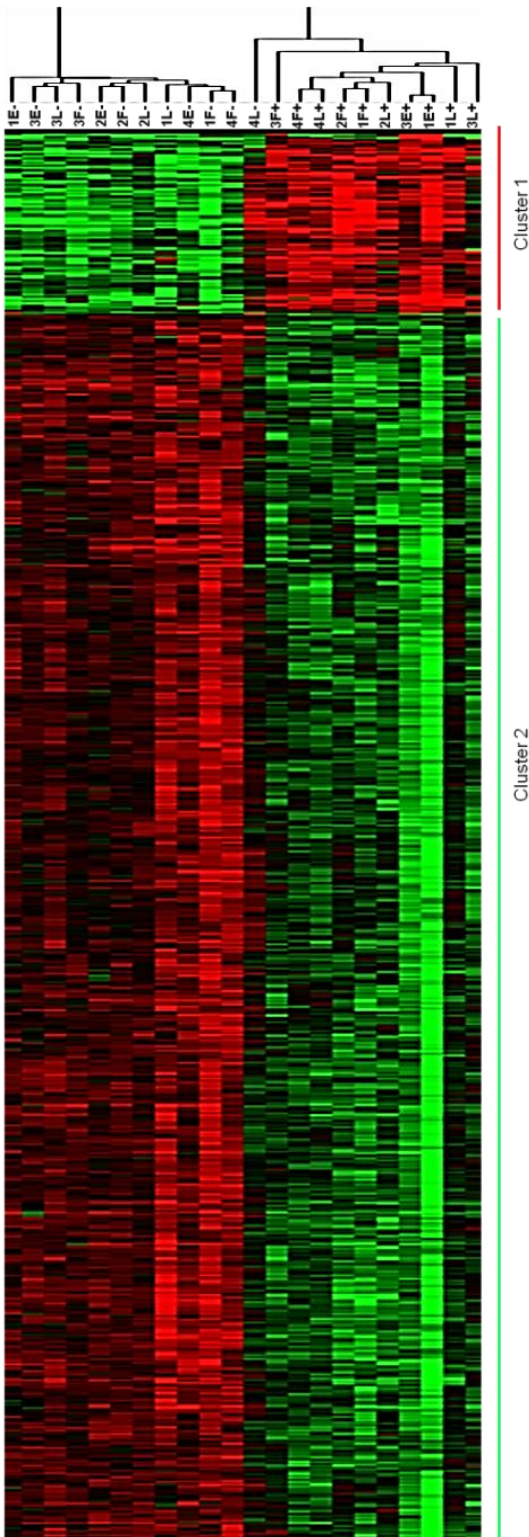
Figure 2. Image provided by cluster analysis showing the expression profiles of the transcriptome in the larvae progenies of rainbow trout (Oncorhynchus mykiss) 1 day after hatch. Cluster 1 shows 103 probesets with a higher expression in batches treated with 3AB, PARP inhibitor. Cluster 2 represents 717 probesets with a lower expression in the same batches. Letters and numbers identify the batches: numbers identify the male used for fertilizing; (C/K) batches incubated with/without (3AB); E: sperm cryopreserved with egg yolk; L: sperm cryopreserved with LDL and F: fresh sperm.

The cluster analysis showed different expression patterns according to type of fertilization: the larvae obtained from standard artificial fecundation showed a normal profile, and the larvae obtained after incubation of fertilized eggs with the PARP inhibitor showed an altered profile (Table 3).

Table 3: Expression patterns on rainbow trout larvae progenies from zygotes incubated or not with the inhibitor of PARP. ♂ Male used for fertilization. Fresh: fresh sperm, EY: cryopreserved sperm with egg yolk, LDL: cryopreserved sperm with LDL. N: normal pattern, A: altered pattern, NH: no hatch.

	Incubated without 3AB				Incubated with 3AB			
	♂1	♂2	♂3	♂4	♂1	♂2	♂3	♂4
Fresh	N	N	N	N	A	A	A	A
EY	N	N	N	N	A	NH	A	NH
LDL	N	N	N	A	A	A	A	A
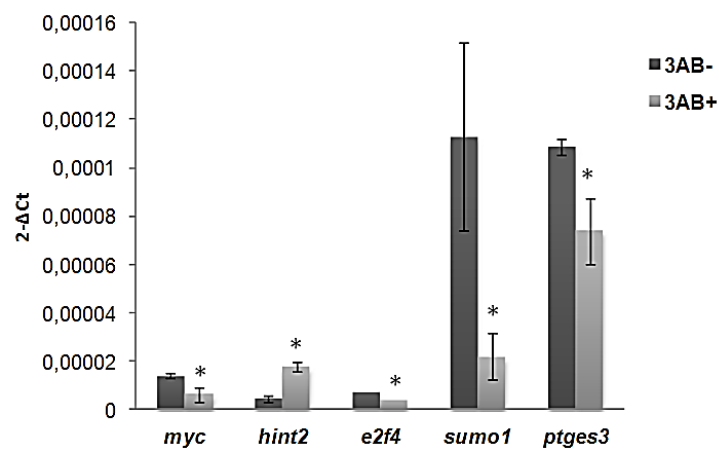
Figure 3. mRNA levels (fold change: 2KDCt) for *myc*, *hint2*, *e2f4*, *sumo1*, and *ptges*3 at 1 dph from trout larvae obtained after normal artificial fertilization or after zygotic inhibition of BER DNA repair pathway using 3AB. Data represent mean values S.D. (n=40). Asterisks show significant differences between treatments (P<0.05).

The progenies from one male carrying DDS and non-treated with the inhibitor (Fig. 2 column 4L-) better clustered with batches showing the 'altered' profile, but with a very low *P* value (*P*=0.05806), displaying a much more reduced number of genes up- or down-regulated with respect to the normal pattern than the inhibited ones. Among the 810 DEG, 604 have a gene name, 553 being unique gene names. Among these 553 unique genenames, 370.cor- respond to at least one gene ontology (GO) biological process term and are included in nine different clusters according to cellular components, biological functions, or molecular processes (Suplementary file 4). Cluster

6 contains at least 52 genes (Table 4) related with the cell cycle control (92.3% of them down- regulated; Fig. 4). All of these genes related with cell cycle checkpoints and arrest were downregulated in larvae hatched after the inhibition of the BER pathway.

Table 4: Differentially expressed genes with unique gene name involved in cell cycle.

G1/S, S and G2/M	Cell cycle checkpoints and cell cycle arrest	Mitosis	Others
upf1*	atm	cenpk4	fgfr4*
anapc11	triap1b	mms19	ccnl
e2f4	mad2	haus2	e2f4
khdrbs1	spc25	stmn3	mapksp1
ube2	spc22	titin	rad51
znf473	ppm1l	cenpa	triap1b
pole		exo1	kiaa0892
khdrbs1		dusp13	npm1
Ing4			Cetn2
			Cetn1
			Cetn3
			smarca4
			cenpj
			ube2e1
			psme3
			psmd6
			psma1
			psma2
			psma3
			psma7
			psma6
			myc
			cdc42
			vps4
			erh
			pa2g4
			pfdn1
			fnta
			chmp1b

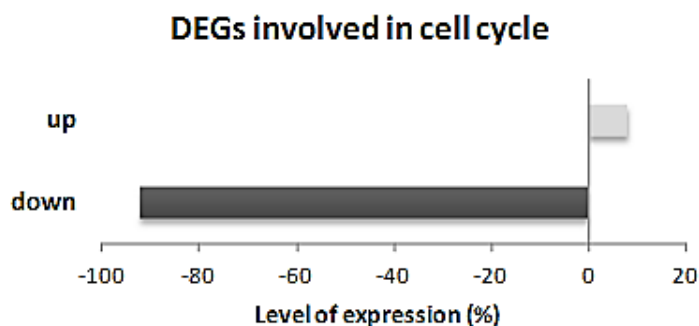## DEGs involved in cell cycle

Figure 4. Percentage of (DEGs) with unique gene name involved in cell cycle process according to the up- or down-regulation after PARP inhibition.

Cluster 4 included at least 37 genes related with programed cell death (Table 5) and all the proapototic genes were downregulated after the inhibition of PARP activity (Fig. 5).

Table 5: Differentially expressed genes with unique gene name involved in apoptotic activity.

Apoptotic process	Regulatio of cell death		Execution	Signaling pathways	Others
	Positive	Negative			
ywhaz	ing4	dnajb6	hmgb1	fis1	capn10*
prkce*	psenen	fas	madd*	psme3	tm2
actn2*	pdia3	angpt1		myc*	fxr1*
fnta	atm	psme3		madd*	rtn3
actn3*	btg1	igf1r*		ddx47	nckap1
traf3		madd*		pttg1	pdcd2
		dad1		triap1	trim69
		triap1		diablo	cln
					npm1
					rpb2

*Transcripts with a higher expression in larvae with the "altered" expression profile (FDR < 0.005).
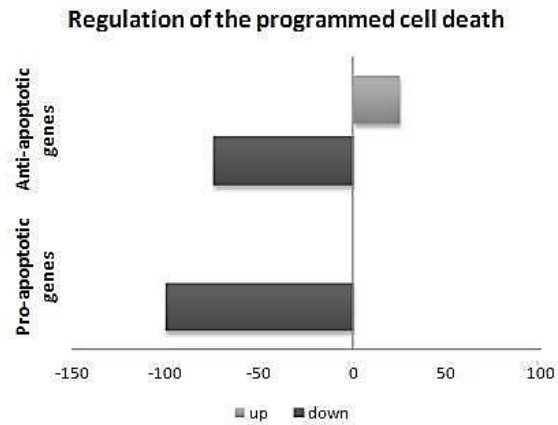
Figure 5. Percentage of DEGs with unique gene name involved in pro- or anti-apoptotic activity according to the up- or down-regulation after PARP inhibition.

With regard to the control of the transcription mechanism two of the functional clusters (three and eight) aggregate an important number of genes, related with different pathways involved in morphogenesis, DNA repair, cell cycle, etc.

Among the 370 genes included in the nine GO clusters, at least 23 are involved in DNA repair (Table 6), most of them being downregulated in larvae with the altered profile. A number of DEGs are related with the control of telomere length (Table 7), only one of them showing upregulation in larvae with an altered profile, *terf1*, and the rest being downregulated. At least 64 DEGs were related with growth and development (Table 8). We found genes involved in the early embryo development control (symmetry, axis, segmentation, gastrulation, etc.), pluripotency maintenance, organogenesis, tissues differentiation, and nervous system development. Focusing on the mitochondrial process (Table 9), at least, 22 DEGs were found and all of them were downregulated.

Table 6: Differentially expressed genes with unique gene name involved in DNA repair pathways: Base excision repair (BER) pathway, Nucleotid excision repair (NER) pathway, Homologous recombination (HR), Non-homologous end joining (NHEJ), Double strand breaks (DSB) pathway, checkpoint ATM/ATR and, others.

BER pathway	NER pathway	HR pathway	NHEJ pathway	DSB pathway	ATM/ATR signaling	Others
apex1	*rad23*	*xrcc3*	*nono*	*atm*	*atm*	*exo1*
triap1b	*triap1b*				*triap1b*	*rad23*
	ube2b				*pttg1*	*xrcc3*
						pole
						hmgb1
						upf1*
						rpa
						rfc2
						asf

*Transcripts with a higher expression in larvae with the "altered" expression profile (FDR < 0.005)

Table 7: Differentially expressed genes with unique gene name involved in maintenance and regulation of telomere length.

Maintenance	Regulation
terf1*	terf1*
atm	*myc*
hspb11	
myc	
ptges3	
rad23	
rfc2	
tcerg1	

*Transcripts with a higher expression in larvae with the "altered" expression profile (FDR < 0.005)

Table 8: Differentially expressed genes with unique gene name involved in growth and development.

Axis/Symmetry/ Segmentation	Embryo development	Gastrulation	Nervous system development	Morphogenesis/Tissues	Pluripotency/ Cell differentation	Eye	Growth factors signaling	Others
nr2f2*	wnt16	zbtb17	Dmd*	nr2f2*	fgfr4*	mab21l2	fgfr4*	ing4
	id2		myo1b*	Prkce*	smarca4*	actl6a	glg1*	triap1b
	ube2b		nptn*	angpt1	Vcan*	ephb2	igf1r*	
	kif3a		smarca4*	atm	vps37d	pknox1	Adipoq	
	angpt1		atm	myc	tspy2	mab21l2	Ptn*	
	dad1		hmgb1	tbca	btg1		sertad2	
	hlx		hlx	wnt16	hlx		fgffr4	
			actl6a	fhl1	fnta		e2f4	
			hes5	serp1	e2f4		fgfr4	
			ephb2	titin	ybx1		nptn	
			tnr	esrrb	esrrb			
			cdc42	vcan	angpt1			
			dspp	trappc2	pknox1			
			hlx	mab21l2	nus1			
			eif2b	hlx	btg1			
				ppp2r1b	itgv			
				rab26	rpl22			
				jpk1	ap3			
				mesdc2				

Table 9: Differentially expressed genes with unique gene name involved in mitochondrial processes.

ATP production	Ribosomal proteins	Others
atp5g1	mrpl32	hint2*
atp5g3	mrpl37	c1qbp
atp5s	mrpl38	diablo
cmc1	mrpl40	dut
sdhaf1	mrpl42	exog
	mrps6	fis1
		myg1
		sco1
		timm13
		tomm34
		tomm6

*Transcripts with a higher expression in larvae with the "altered" expression profile (FDR < 0.005).

The analysis of larvae showed that zygotic inhibition of DNA repair promoted a slight increase in the rate of larval malformations 1 dph, from 8.4 to 12.11% (Fig. 6A), mainly skeletal malformations and defective yolk sac. Larvae from inhibited batches showed an increased growth rate (66% weight gain from 1 to 30 dph in treated batches vs 59% in non-treated batches; Fig. 6B). Apoptosis was also affected by the treatment revealing a lower activity of caspases 3/7 in larvae from the treated animals (Fig. 6C), which also showed longer telomeres (Fig. 6D). All the treated larvae showed a DIZ1, indicating the same ploidy than control larvae (Fig. 7).
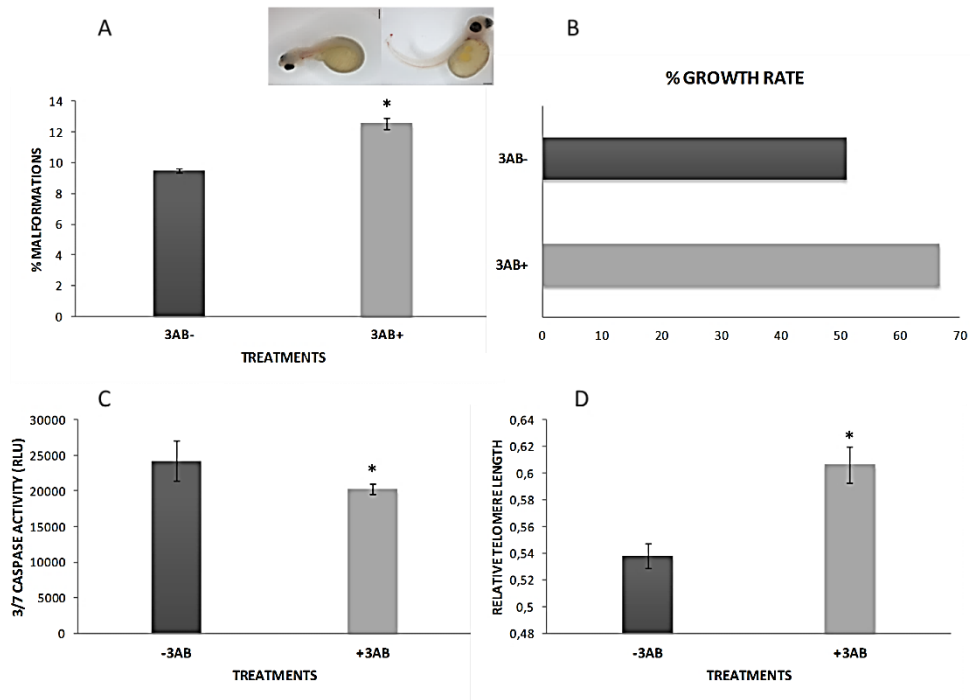
Figure 6. Malformation rates (A), weight gain from 1 to 30 dph (B), caspase 3/7 activity (C), and relative telomere length (D), in larvae obtained after normal artificial fertilization or after zygotic inhibition of BER DNA repair pathway using 3AB. Images show the most common malformations. Asterisks show significant differences between treatments ($P<0.05$).
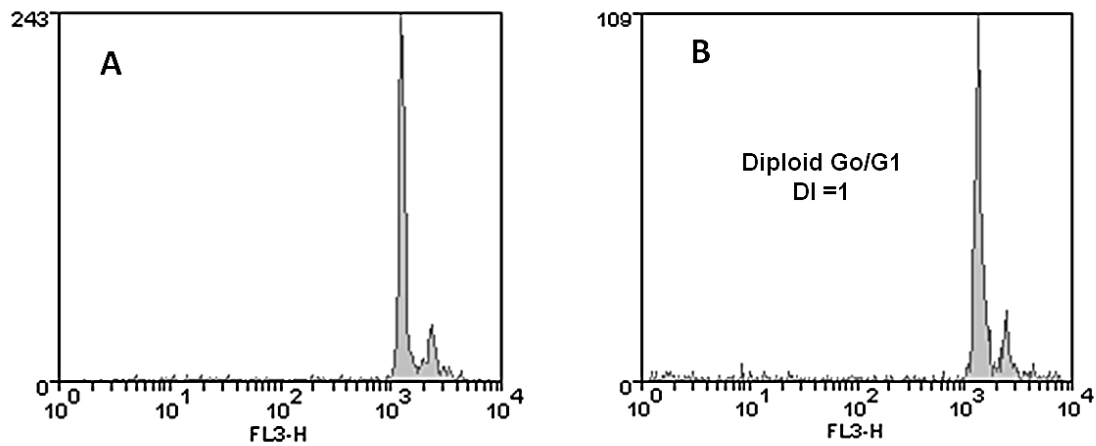

Figure 7. Nuclear DNA content from trout larvae. (A) Histogram from diploid trout progeny obtained after normal fertilization. (B) Histogram from diploid trout progeny obtained after zygotic inhibition of BER DNA repair pathway using 3AB.

Discussion

Zygotic DNA repair is a process required to repair injuries in the paternal DNA, produced by intrinsic as well as external factors, which spermatozoa is exposed to, and which could accumulate during the final steps of spermatogenesis and beyond (Derijck et al. 2008). DNA previously quoted, oocytes seem to have the required SenGupta 2007). The sperm used in this work for fertilization was analyzed by Perez-Cerezales et al. (2011), which contained different degrees of damage. Therefore, fertilization with the different sperm samples would require a different repairing effort in the zygote, and promote different degree of genetic instability in the obtained embryos. In fact, a higher loss rate of embryo was reported in frozen sperm batches, mainly with EY. Zygote seems to have the ability to effectively repair at least a 10% of fragmented paternal chromatin, because EY samples, carrying at least a 10% fragmentation in all the spermatozoa, generated normal progeny as reported by the same team (Perez-Cerezales et al. 2010a). Nevertheless, as revealed in the transcriptome analysis, once embryo development is completed, hatched larvae did not show great differences according to the kind of sperm used for fertilization and only one of the progenies obtained with damaged sperm showed an expression profile different from the one considered as 'normal' pattern in absence of DNA repair inhibition. This could reflect that most embryos carrying chromosomal or genetic aberrations from paternal origin are lost during development and does not progress to hatch, being survivors from the less damaged spermatozoa.

The maternal ability to repair paternal injuries thus seems to be very effective, but risk could arise when repairing pathways are impaired. It is well known that defective repair of single-strand breaks by the BER pathway results in an increase in double-strand breaks after replication (Hilton et al. 2013, Metzger et al. 2013). This is the basis for using PARP enzyme inhibitors such as 3AB in cancer therapy: PARP inhibition promotes failure in the BER pathway, increasing dsDNA, which are repaired using the HR or the NHEJ pathway. Tumor cells lacking functional HR system are forced to use the NHEJ pathway, accumulating genetic errors that promote cell death (Lovato et al. 2012, Hilton et al. 2013, Metzger et al. 2013). In our study, the use of 3AB immediately after fertilization was expected to generate an intense genetic stress, considering that first cell cycle lacking the G1-S checkpoint is able to arrest replication (Shimura et al. 2002) and then embryos will progress with cleavage. Moreover, transient double-strand breaks are also promoted during sperm chromatin remodeling after penetrating in the mouse oocyte (Bizzaro et al. 2000, Derijck et al. 2006). A high number of double-strand breaks should then be generated after PARP inhibition, particularly in the EY progeny. Nevertheless, PARP inhibition only increased the embryo loss rates in batches fertilized with the most highly damaged sperm (Perez-Cerezales et al. 2010a), demonstrating that only those batches were unable to repair the increased number of dsDNA. Apparently, the level of damage in F and LDL sperm was repaired by alternative pathways compatible with successful embryo development. The use of alternative pathways in addition to BER was confirmed in mice after ICSI with irradiated sperm carrying dsDNA. When oocytes from strains defective in the NHEJ or HR pathway for repairing double-strand breaks where fertilized by ICSI, the embryonic lethality significantly increased (Marchetti et al. 2007).

Both pathways are active in the mammalian zygote, as was also demonstrated by Derijck et al. (2008) in a similar experience fertilizing with irradiated sperm oocytes from defective mouse. The inhibition of the NEHJ pathway in zebrafish using morpholines at 1 cell and 6 hpf also increased the apopototic activity and malformations rate, confirming in fish the importance of this alternative pathway (Bladen et al. 2005). Our results also suggest the ability of the oocyte to repair paternal damage by alternative pathways without introducing lethal genetic errors.

In spite of the ability to provide apparently normal progeny, massive differences in the transcriptome were caused by treatment with the inhibitor of PARP after fertilization. Considering that hatching took place 26 days after fertilization, PARP inhibition did not promote a transient modification of the repairing activity, but a long-term change in the regulation of different processes. The treated embryos could have required the activation of compensatory mechanisms to bypass the reduction in PARP activity and these changes produced a number of DEGs. Results showed that a wide range of DEGs involved in a variety of functions. Some of them are not known substrates of PARP neither are down- stream PARP in known pathways, suggesting the activation or inhibition of alternative mechanisms. The expression level was modified (mostly downregulated) in transcripts involved in all DNA repairing pathways, suggesting changes in the regulation of the processes and reinforcing the idea that all the repairing pathways have suffered an alternative regulation. Alteration in these genes can have devastating consequences at both the cell and organism level as described by Tebbs et al. (1999) and Kucherlapati et al. (2002), who observed that targeted inactivation of enzymes acting downstream of the glycosylases in BER pathway resulted in embryonic or post-natal lethality in mice. Genes related with

growth and development (at least 25 DEGs) could be respon- sible for the increase in malformed larvae and the increased rate of weight gain during the first 30 days. Increase in skeletal malformations could be related with a number of DEGs including those related with muscle development, such as dmd, myo9b, tpm1, tpm3 (Supplementary Table 1), and the increased growth rate to the upregulation of growth factor receptors such as *igf1r* and *fgfr4* or to the downregulation of the inhibitory growth factor *ing4*.

A high number of DEGs were related with the control of cell cycle and apoptosis, suggesting a scenario of apoptotic inhibition: atm, implied in the checkpoints of DNA damage and proapototic genes, *diablo*, *ddx47*, and *pttg1* are down-regulated, whereas anti-apoptotic transcripts madd and *igf1r* are upregulated. This down- regulation of the apoptosis was confimated by the analysis of the caspases 3/7 activity and of the atm/atr signaling pathway, represents a source of potential mutations, and is also a mechanism allowing survival under stress conditions. The genotoxic stress promoted in the progenies was expected to generate an apoptotic response that could determine the increase in the abortion rates. Nevertheless, the analyzed larvae were able to progress with embryo development in spite of the increased genetic instability scenario. In the absence of a proper repair, residual DNA damage would lead to either apoptosis or tolerance (Menezo et al. 2007) and our results demonstrate that the hatched larvae actually showed a lower apoptotic activity, suggesting an increasing tolerance.

Tchernov et al. (2011) demonstrated that coral surviving to the thermal stress responsible for the process of bleaching and death in reef-forming corals are those

animals in which the apoptotic cascade is downregulated. Inhibition of the apoptotic process represents a selective process preventing death under stressful conditions and boosting survival of individuals with specific characteristics.

With regard to telomere maintenance and elongation, one DEG was upregulated in treated larvae and involved in telomere maintenance, terf1. Downregulated DEGs have both positive (*ptges3* and *smn1*) and negative (*tcerg1*) effects on elongation. The telomere length analysis showed that the actual regulation of telomerase activity in treated larvae conduced to a higher elonga- tion, which could be related with the impaired mechanism of DNA damage signaling of the cell cycle control and whose effects on long-term survival or aging should be analyzed.

The wide range of pathways and functions affected by the PARP inhibition could also be explained by the large number of PARP substrates, and their involvement in different regulatory activities apart from DNA repair. PARP activity has been correlated with DNA damage since it was discovered, but modification by PARylation modulates numerous cellular processes including transcription, chromatin remodeling, apoptosis, maintenance of telomere length, etc. (Beneke 2012).

The analysis of the ploidy revealed that none of these effects are due to an interference of the inhibition treatment applied after fertilization with the mechanism of polar body extrusion, which is relatively easy to prevent in fish providing triploid animals.

Our results reinforce the importance of egg metabolism in repairing sperm DNA damage, suggesting that oocytes defective in some transcripts or proteins involved in repair could also progress with development after fertilization by damaged sperm. A

reduction in DNA repair mechanisms has been described in oocytes from aged mouse (Hamatani et al. 2004) and other factors from genetic background to exogenous factors such as cryopreservation, in vitro culture, etc., could also affect the repairing activity of the zygote and compro- mise the long-term outcome of the progeny. In conclusion our results suggest that the oocyte has a high capacity to repair paternal DNA damage, activating more than one single repairing pathway, but long-term consequences for the progeny of any treatment affecting this repairing activity should be analyzed in depth.

Supplementary data

This is linked to the online version of the paper at http://dx.doi. org/10.1530/REP-14-0382.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Changes in transcriptomic profile of trout larvae obtained with frozen sperm

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Under review

Abstract

Sperm cryobanking is a common procedure in animal production, including aquaculture, and animal conservation. The accuracy of the method relies in the faithful preservation of the sperm quality in order to provide a healthy progeny. Cryopreservation is prone to promote DNA damage that could compromise this objective. As we demonstrated in a previous study, trout embryos obtained with sperm damaged with UV irradiation, can generate tolerant conditions to genotoxic damage that allow the progression of development. This tolerant scenario would increase the chances of affecting gene expression in the progeny. In order to check whether the DNA damage promoted by cryopreservation provides a similar progeny to those from fresh sperm, we have developed an *in silico* analysis of the transcriptomic profile of trout larvae, with apparently normal phenotype, obtained from fresh sperm or from sperm frozen using two procedures that rendered two levels of DNA damage. Data, deposited in the Gene Expression Omnibus database (GEO bank_Accession number GSE52217), were processed and the Panther online database was used for the functional and ontological study. We found differential expressed genes (DEGs) in those progenies from cryo-damaged sperm, which were mainly implied in metabolic processes and in other cellular processes such as differentiation, signalization, trafficking, etc. We have demonstrated that the effects of sperm DNA cryodamage go beyond from its fertilization ability and, particularly in fish, affect the transcriptomic profiling of the obtained larvae. Our results encourage to strictly evaluating the DNA integrity prior to the adoption of any cryopreservation sperm protocol, mainly in the aquaculture field

Keywords

Sperm cryopreservation; DNA damage; trout development; transcriptome

Highlights

Trout larvae from DNA cryodamaged sperm show an altered transcriptomic profile.

Modified transcripts affect metabolism, signalization and apoptotic pathways.

DNA integrity is critical to assure the accuracy of trout sperm cryobanking.

Introduction

Sperm cryopreservation is a very advantageous methodology for the management of reproduction in aquaculture, mainly in seasonal breeders (Cabrita et al., 2005; Martínez-Páramo et al., 2016). One of the risks associated to frozen/thawing methodologies is related to the sensitivity of the chromatin during the process. Sperm cryopreservation generates different levels of DNA damage, mostly fragmentation and base oxidation, as has been reported in different species including trout (Cabrita et al., 2005; Herráez et al., 2015; Pérez-Cerezales et al., 2010, 2009; Thomson et al., 2009). Fertilization with DNA cryo-damaged sperm could impair embryo development. In a previous work published by our group we showed that trout, as external fertilizer, has weak mechanisms for sperm selection allowing the spermatozoa with high levels of DNA fragmentation to proceed with fertilization. We also demonstrated the relationship between the sperm DNA damage and the abortion rates in batches fertilized with cryopreserved sperm (Pérez-Cerezales et al., 2010). Moreover, fertilizing

batches with fresh sperm or with samples frozen with two different membrane cryoprotectants (low density lipoprotein (LDL) and egg yolk (EY)), that promote three levels of DNA damage (low (F), medium (LDL) or high (EY)), we identified in larvae 1 day post hatch (dph) from EY sperm, the differential expression of 6 genes related to growth and development as well as differences in telomere length (Pérez-Cerezales et al., 2011).

Further studies developed with UV irradiated sperm demonstrated that trout embryo development largely depends on the egg repairing activity: both good and low quality oocytes allowed progression with development, but progenies from the sub-optimal oocytes, in which DNA repair was compromised, generated an environment tolerant to the genotoxic stress (Fernandez-Diez et al., 2016). The previous studies suggested that trout -and probably fish- embryo development is highly tolerant to sperm DNA damage. In such conditions our hypothesis is that trout embryos derived from sperm containing damaged DNA have a different gene expression profile than those derived from undamaged sperm. In that case, the use of cryopreserved sperm for farming practices or gene cryobanking, without an appropriate control of the DNA integrity, could provide progenies with modified genetic profiles that could affect gene expression even in the absence of gross malformations.

Aiming to deeply analyze such potential differences among the progeny that proceed with embryo development and successfully hatch, an *in silico* analysis of the transcriptome of larvae 1dph from sperm with three different rates of DNA damage (F, LDL and EY) was performed. The analyzed transcriptome corresponds to a previous experiment devoted to the analysis of the effect of DNA repair inhibition in the

progenies (Fernández-Díez et al., 2015). The results were re-analysed in order to evaluate the potential transcriptomic differences exclusively promoted by DNA damage in cryopreserved sperm.

Materials and Methods

Experimental procedure and transcriptomic datasheet

In a previous work, as indicated in Fernández-Díez et al., (2015) sperm from four tout males was separately divided in three aliquots: one was used as fresh (F), and two cryopreserved using egg yolk (EY) or LDL (LDL) in the extender. The comet assay analysis revealed different DNA fragmentation rates (Fresh 39% ± 1; LDL 49% ±1; EY 57% ± 1). Samples were used to fertilize 200 eggs batches in triplicate. One day after hatching ten alive larvae were taken and total RNA was extracted to perform a gene expression profiling study using an Agilent 8X60K high-density RNA oligonucleotide microarray (GEO platform #GPL15840). In the referred study half of the batches were submitted to the inhibition of zygotic DNA repair and then the transcriptomic changes were analyzed (Fernández-Díez et al., 2015). The data were deposited in the Gene Expression Omnibus database (GEO bank_Accession number GSE52217). This dataset has been now reanalyzed, in order to evaluate the potential differences of gene expression among larvae from fresh and frozen sperm. Only the data from batches whose repairing ability was not modified have been compared.

Data analysis

Data from 12 samples (3 experimental conditions / 4 replicates), summarized in Table 1, were compared. Values normalization and statistical analysis were performed

using *library* limma (v. 3.20.9) (Smyth, 2005) within the R environment (v. 3.1.1). The background correction of the fluorescence signal (Gentleman et al., 2004) was made applying the "mle" option of the "normexp" method (Silver et al., 2009). Data were scale-normalized using the "quantile" method and the probes whose fluorescence intensity value was not 10% higher than the 95% percentile of the negative controls, were removed from the analysis. Average values from probes with the same sequence in the 4 replicates were used for the analysis. The data from the three experimental conditions showed in Table 1 were compared in order to identify differentially expressed genes (DEGs). Contrast 1 compares the transcriptome of larvae from EY cryopreserved sperm and fresh sperm (EY-F), contrast 2 of larvae from LDL cryopreserved sperm and fresh sperm (LDL-F) and contrast 3 larvae from cryopreserved sperm with EY and LDL (EY-LDL). To identify DEGs in each contrast, an unpaired *t*-test was performed, applying a Benjamini–Hochberg correction ($p<0.01$).

The Panther online database (http://www.pantherdb.org/) was used for functional annotations (Mi et al., 2013) of the DEGs. In each contrast the duplicate probes were removed and unique probes were only considered. The molecular function (MF), biological processes (BP), cellular component (CC) and cellular pathways associated to the DEGs were represented.

Results

The gene expression analysis revealed different expression pattern according to the type of sperm used to fertilize. When samples from sperm frozen with EY and fresh sperm were compared (contrast 1), 255 probesets were differentially expressed, most of them downregulated, and 218 of them were unique probes (Supplementary file1).

Larvae from milt frozen with LDL shown 119 DEGs respect to the control from fresh milt (contrast 2), most of them downregulated and 100 of them corresponding to unique probes (Supplementary file 2). The third contrast among larvae from EY-LDL, revealed 92 DEGs, 77 were unique probes (Supplementary file 3). The shared DEGs between contrasts are shown in the figure 1 and revealed that contrasts 1 and 2, both of them comparing larvae from fresh and frozen sperm, shared 14 DEGs, 12 of them downregulated (Fig. 1).
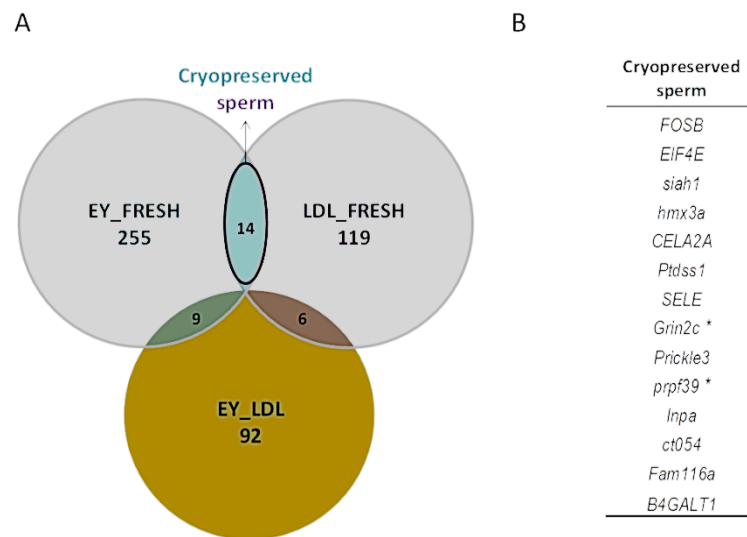

Figure 1: Schematic representation of the differentially expresed genes (DEGs) in each analyzed contrast and the shared ones between group of transcript sets. List of shared genes within interesting group. Asterisks represent the upregulated genes in larvae from frozen sperm.

The functional annotations revealed that a relevant number of genes in contrast 1 and 2 are implied in cellular process (89 and 38 respectively) and metabolic process (83 and 30 respectively) (Figs 2 and 3).
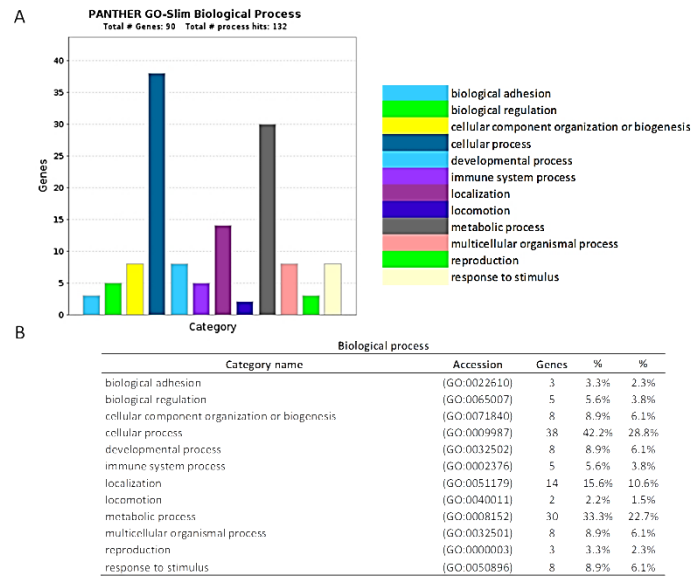
Figure 2: Biological process of DEGs from EY-F samples (contrast 1). A: Representation of gene ontology terms. B: Category name, accession, genes, percent of gene hit against total genes and percent of gene hit against total process hits.

Figure 3: Biological process of DEGs from LDL-F samples (contrast 2). A: Representation of gene ontology terms. B: Category name, accession, genes, percent of gene hit against total genes and percent of gene hit against total process hits.
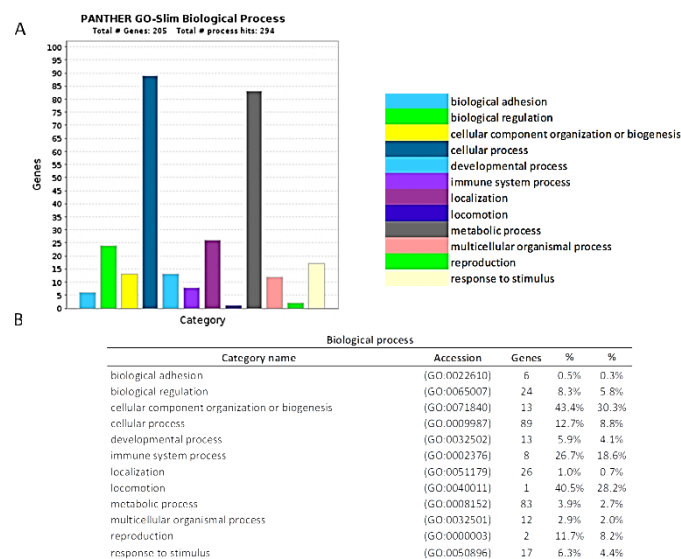
The Panther pathways study showed that in contrast 1 (Fig 4) the DEGs correspond to 91 genes involved in 53 pathways: 7 genes are related to the

gonadotropin-releasing hormone receptor pathway, 6 with the CCKR receptor map and the pentose phosphate pathway, 4 with inflammation signaling pathway and 12 with different neurotransmitter receptor pathways (glutamate or acetylcholine). The cellular processes which are affected by these pathways are related to transcription, translation, signalization, differentiation, endocrine regulation or apoptosis (5 genes related to the p53 pathways), among others. Contrast 2 (Fig. 5) identified 75 genes involved in 50 pathways in larvae frozen with LDL, most of them shared with contrast 1: gonadotropin-releasing hormone receptor pathway and Wnt pathway have 4 affected genes, 3 are involved in CCKR signaling map and inflammation signaling pathway, 13 genes were related to neurotransmitter receptor pathways, and the apoptotic pathways were represented by 5 genes.
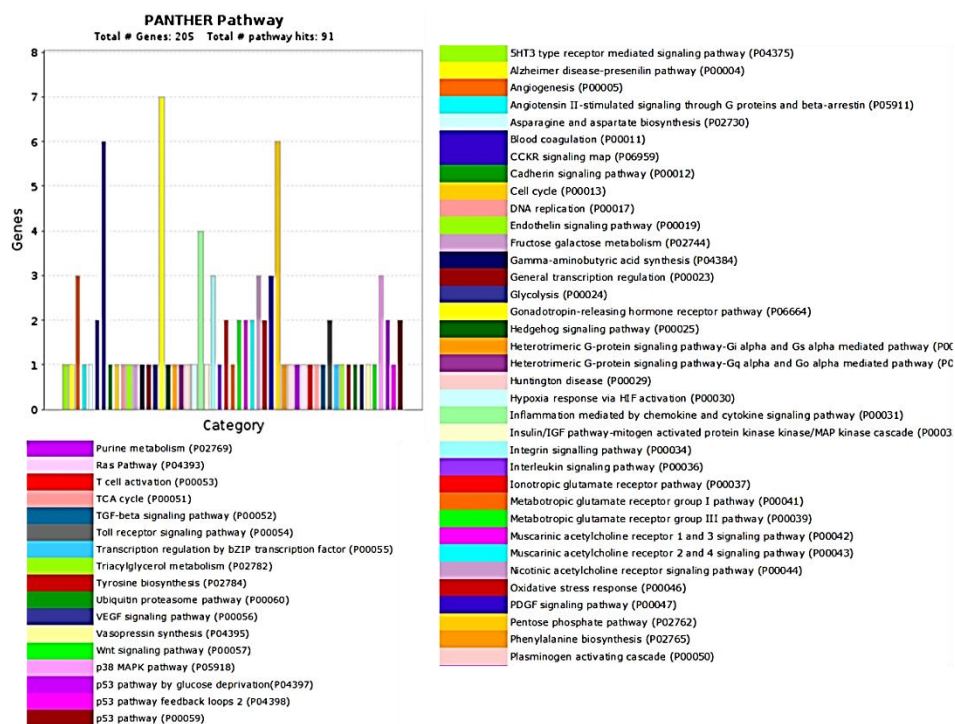

Figure 4: Representation of pathway associations of DEGs from EY-F samples (contrast 1).

PANTHER Pathway
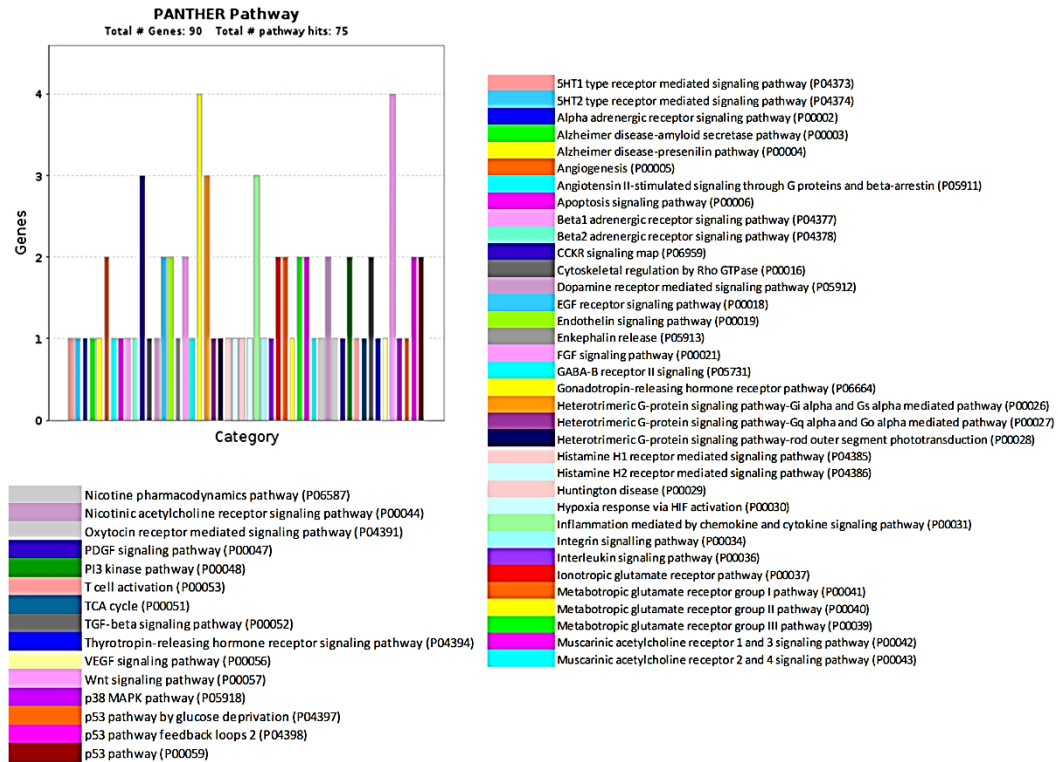Total # Genes: 90   Total # pathway hits: 75

Figure 5: Representation of pathway associations of DEGs from LDL-F samples (contrast 2).

The functional and ontological analysis of the 14 shared DEGs among frozen larvae showed that 4 are involved in cellular process (*prpf39, cela2s, ptdss1* and *fam116a*) and other 4 in metabolic process (*prpf39, sele ptdss1* and *fam116a)* (Fig 6). Eleven pathways were identified as affected by these 14 DEGs (Fig. 7), including 4 neurotransmiters receptor pathways, 2 apoptotic pathways as well as the gonadotropin-releasing hormone receptor, the Wnt and the CCKR signalling map pathways.

Figure 6: Biological process of shared DEGs from cryopreserved samples. A: Representation of gene ontology terms. B: Category name, accession, genes, percent of gene hit against total genes and percent of gene hit against total process hits.
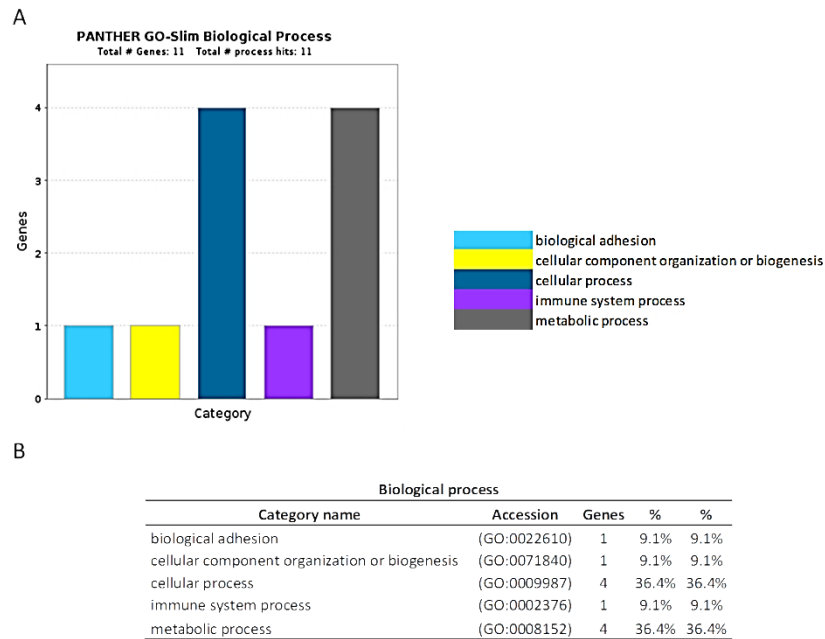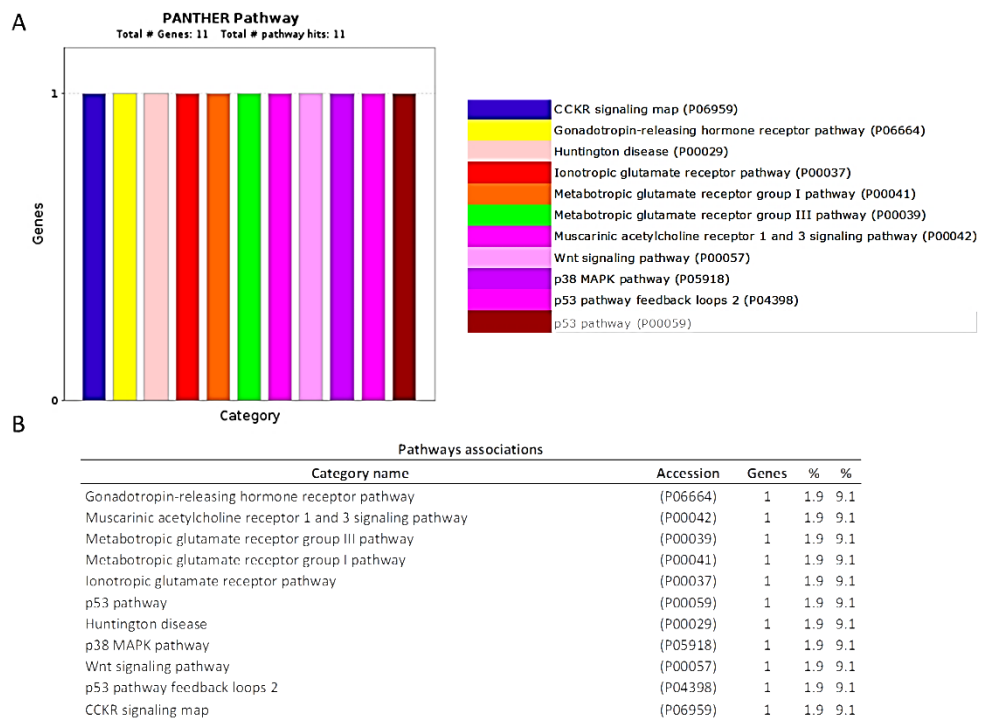

Figure 7: Pathway associations of shared DEGs from cryopreserved samples. A: Representation of gene ontology terms. B: Category name, accession, genes, percent of gene hit against total genes and percent of gene hit against total pathway hits.

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Discussion

Sperm cryopreservation, performed in proper conditions, is considered a safe reproductive technology. Brown trout milt cryopreserved with a method that did not increase DNA fragmentation, provided an offspring whose genotypic determination by microsatellite analysis revealed a similar profile than progenies from fresh sperm, demonstrating the suitability of sperm cryobanking for the preservation of specific brown trout strains (Martínez-Páramo et al., 2009). Nevertheless, the integrity of the spermatic chromatin is a crucial factor which must be strictly controlled during the process, a certain degree of DNA fragmentation and oxidization being frequently reported after cryopreservation in human, cattle and fish (Baumber et al., 2003; Pérez-Cerezales et al., 2010; Thomson et al., 2009). Sperm DNA damage has been related to implantation failures in mammals, increasing abortion rates, and different developmental failures (Fernandez-Diez et al., 2016; Gawecka et al., 2013; Speyer et al., 2010). Upon fertilization, the zygote has the ability to repair a certain degree of the spermatic damage (Fernandez-Diez et al., 2016; Fernández-Díez et al., 2015; Marchetti et al., 2015; Pérez-Cerezales et al., 2010, 2009), but additional lesions could overtake the repairing capacity of the embryo. It is rational to hypothesize that those embryos suffering an incomplete repair would collapse increasing the abortion rates or, alternatively, suffer from developmental failures.

In this study we have analyzed the transcriptome of larvae which had completed the embryo development, succeeded to hatch and presented an apparently normal phenotype, suggesting a proper repair of spermatic DNA. Nevertheless, the results showed that larvae from frozen sperm display at hatching a different gene

expression profile that those embryos from fresh milt indicating an alternative gene regulation in those batches. The analysis of the transcriptome has revealed that the more damage in the DNA, the more DEGs displayed the larvae 1dph (119 probes (DEGs) in LDL larvae and as much as 255 in EY larvae). Only 14 of those DEGs were common in batches from frozen sperm, revealing the RNAs whose presence was most affected by fertilization with frozen sperm. The high capacity of trout embryo to progress with development in spite of having unrepaired DNA has been suggested by our previous studies of embryos from UV irradiated sperm, particularly when batches from sub-optimal reproductive period were used for fertilization (Fernandez-Diez et al., 2016). Early trout embryos displayed an enhanced DNA repairing activity and a decreased apoptotic activity, generating tolerant conditions to DNA damage, which allows the progression with the development in absence of a proper repair (Fernandez-Diez et al., 2016). In contrast, induction of apoptosis has been noticed in mammalian early embryos that accumulates DNA damage (Heyer et al., 2000). This tolerant scenario to DNA damage has been also noticed in other external fertilizers such as corals (Tchernov et al., 2011) and could represent an evolutionary mechanism in stressful conditions. The variability generated by the defective repair could provide some advantages in changing environmental conditions, but could increase the risk of promoting undesirable effects in fish farming.

The ontological study of the 119 DEGs in LDL larvae and the 225 DEGs in EY larvae revealed that the miss regulated genes affect the expression of different kind of proteins (enzymes, transcription factors, receptors, structural proteins, etc), most of them downregulated. Those genes are mainly implied in metabolic processes -that could affect the further growth and development- and in other cellular processes such

as differentiation, signalization, trafficking, etc. Several pathways are affected in both types of larvae, being relevant the miss regulation of an important number of genes which are implied in particular pathways: CCKR signaling map, implied in digestion, appetite control and body weight regulation (Tripathi et al., 2015); gonadotropin-releasing hormone receptor pathway, essential to the control of the reproductive axis, that mediates the gonadotropin synthesis and secretion (Perrett and McArdle, 2013); multiple neurotransmitter receptors pathways, that could affect neural development at different levels and a range of neurological functions; the Wnt pathway, related to embryo development, cell proliferation or cell migration, and whose alteration is known to result in increased risk of several pathologies related to cell proliferation (cancer) and insulin sensitivity (diabetes) (Chen et al., 2009; García-Jiménez et al., 2013); and the apoptotic pathways, crucial to control the genotoxic damage. Moreover, the analysis of the common 14 DEGs indicates that among the affected genes are *eif4e* and *fosb1*, essentials in cell growth, cell proliferation and differentiation; *grinc2c*, related to neural development, specifically with learning, memory and synaptic development; and *siah1*, implied in apoptosis induction.

The modification of these specific transcripts points to increased chances of affecting the larval growth, their neural development, its reproductive performance and their susceptibility to suffer from pathologies at later times. All these risks could have an important impact on the progeny performance and long term survival. The downregulation of the apoptotic activity (*siah1*) would be essential to facilitate the survival of embryos up to hatching, as was already suggested in early trout embryos from sperm whose DNA was damaged with UV (Fernández-Díez et al., 2016).

We can conclude that fertilization with frozen/thawed sperm harboring fragmented DNA, promotes changes in the transcriptomic profile of the obtained larvae that could affect the progeny performance. The control of DNA damage during sperm cryopreservation is absolutely essential in order to obtain a healthy progeny, especially in fish, whose embryo development seems to be tolerant to a significant degree of genotoxic damage. Considering that sperm quality deals to the ability to generate a healthy offspring, the evaluation of fertilization rates is not a parameter that could inform about the accuracy of a cryopreservation procedure and evaluation of chromatin integrity should be always required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Capítulo 2: Efectos de la alteración de la época natural de puesta en el genoma espermático y la actividad de reparación del ovocito: impacto sobre el desarrollo de la progenie.

Impact of sperm DNA damage and oocyte repairing capacity on trout development

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Abstract

Zygotic repair of paternal DNA is essential during embryo development. In spite of the interest devoted to sperm DNA damage, the combined effect with defective repairing oocytes has not been analyzed. Modification of the breeding season is a common practice in aquaculture that reduces developmental success and could affect both factors: sperm DNA integrity and oocyte repair capacity. To evaluate the maternal role, we analyzed the progeny outcome after fertilizing in-season trout oocytes with untreated or with UV irradiated sperm, as well as the offspring obtained out- of season with untreated sperm. The analysis of the number of lesions in 4 sperm nuclear genes revealed an increase of 1.22 - 11.18 lesions/ 10 kb out-of-season, similar to that obtained after sperm UV irradiation (400 µW/cm2 5 min). Gene expression study showed in out of season oocytes the overexpression of repair genes (ogg1, ung, lig3, rad1) and downregulation of tp53, indicating an enhanced repairing activity and reduced capacity to arrest development upon damage. The analysis of the progeny revealed in out-of-season embryos a similar profile tolerant to DNA damage, leading to a much lower apoptotic activity at organogenesis, lower hatching rates and increased rate of malformations. The effects were milder in descendants from in-season irradiated sperm, showing a enhanced repairing activity at epibolia. Results point out the importance of the repairing machinery provided by the oocyte and show how susceptible is to environmental changes. Transcripts related to DNA damage signalization and repair could be used as markers of oocyte quality.

Introduction

DNA integrity of germinal cells is a crucial factor allowing the proper transmission of parental genetic information to the progeny (Santos et al. 2013, Marchetti et al. 2015). During spermatogenesis, particularly in meiotic steps, DNA is very prone to suffer lesions. Moreover, spermatic chromatin is very sensitive to environmental stress generated by factors like UV irradiation, thermal changes, presence of chemicals, etc. that could increase oxidative stress or affect the DNA compaction mechanisms (Shamsi et al. 2011). In addition, the DNA repair ability of male germ cells is high during mitotic and meiotic phases, but is loosed in postmeiotic spermatids with the reduction of the cytoplasmic content and the compaction of the nuclei, which limits the access of the DNA repair machinery (Baarends et al. 2001, Marchetti et al. 2015). Chromatin stability is better preserved in the oocytes, because even if during arrest at meiosis I, prophase and the final stages of follicular growth, may accumulate errors involved in the correct chromosome segregation (Carroll & Marangos 2013) and may be susceptible to damage by reactive oxygen species (ROS) (Menezo et al. 2010), they have a fully active machinery leading to repair genomic lesions promoted during oogenesis (Kurus et al. 2013).

It has been repeatedly shown both in mammals and fish that fertilization capacity can be preserved in spermatozoa showing standard quality in terms of motility and morphology but carrying a damaged genome (Upadhya et al. 2010, Avendano & Oehninger 2011, Perez-Cerezales et al. 2011, Fernandez-Diez et al. 2015). Fertilization with DNA damaged sperm increases the risk of promoting pregnancy loss in mammals (Speyer et al. 2010), arrest in embryo development or embryo death

(Perez-Cerezales et al. 2010b, Gawecka et al. 2013), birth defects, chromosomal abnormalities and other genetic diseases (Fernandez-Gonzalez et al. 2008, Barroso et al. 2009, Schulte et al. 2010, Marchetti et al. 2015). The DNA repairing activity relies on the oocyte machinery once the fertilization takes place (Aitken et al. 2014, Fernandez-Diez et al. 2015), being the zygote responsible for repairing the sperm damage. In previous works we estimated that trout oocytes are able to repair at least a 10% of sperm DNA fragmentation (Perez-Cerezales et al 2010) and demonstrated that the suppression of the repairing activity in trout zygotes, using an inhibitor of the Poli (ADP-ribose) polymerase (PARP) activity, involved in the base excision repair (BER) pathway, has severe consequences for the progeny: alternative repairing mechanisms were activated, embryo survival was reduced and surviving larvae displayed massive differences in the transcriptomic profile (Fernandez-Diez et al. 2015). The zygotic repairing ability is thus a key factor guarantying the progress of a proper development, mainly when paternal DNA integrity is compromised. Nevertheless, different intrinsic and extrinsic factors with effects on the oocyte, from genetic background, to ageing (Hamatani et al. 2004), cryopreservation, in vitro culture or environmental clues, could also affect the repairing activity of the zygote. Therefore, those factors potentially harmful for male and female gametes could have synergic effects with a serious impact on the conceptus development, compromising the long term outcome of the progeny.

Fish have been used as models in the study of the effects of sperm DNA damage on the reproductive success given the advantage provided by its high prolificacy (allowing the study of a high number of descendants from the same mating), easy process of artificial fertilization and weak mechanism of sperm selection (facilitating fertilization with damaged spermatozoa) and external development (giving

free access to embryos throughout development) (Kopeika et al. 2004, Pérez-Cerezales et al. 2009, Pérez-Cerezales et al. 2010b, Perez-Cerezales et al. 2011, Fernandez-Diez et al. 2015).

Most fish species from temperate areas are seasonal breeders, but reproduction out of season is easily induced in many commercial species by environmental or hormonal stimulation. Among salmonids -breeding in cold water- photoperiod manipulation is usually applied in order to obtain an all year round production (Billard 1978). Nevertheless, due to the mismatch between the artificial photoperiod and temperature regimes in warmer periods of the year, overall oocyte quality, embryonic survival and larval performance are usually lower out of the natural breeding period (Bobe & Labbe 2010, Migaud et al. 2013). An increase of DNA sperm damage (Perez-Cerezales et al. 2010a) and defective vitellogenesis in oocytes (Bobe & Labbe 2010) that could affect embryo development have been identified in out-of-season breeders, but the oocyte repairing activity and their combined effect with the sperm chromatin integrity have never been explored.

The hypothesis is that warmer temperature during gametogenesis in out-of-season breeders, apart from affecting sperm DNA integrity, could modify the oocyte repairing activity, compromising the offspring development at short and long term. DNA repair related transcripts and lesions in DNA will be analyzed in oocytes and sperm respectively, both during optimal breeding season (ORS) (from breeders maintained with natural photoperiod) and at a later period (LRS) (from breeders under artificial photoperiod and warmer temperature regimes). The offspring development will be evaluated at decisive stages during embryo development up to hatching. The

ability to progress with development when sperm genotoxic damage is promoted by UV irradiation will be also assessed at ORS.

Materials and methods

<u>**Reagents**</u>

All media components were purchased from Sigma-Aldrich except when otherwise stated.

<u>**Experimental procedures**</u>

The experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU, modified by 2010/62/EU), following Spanish regulations (RD 1201/2005, abrogated by RD 53/2013) for the use of laboratory animals, and were approved by the Committee on the Ethics of Animal Experiments of the University of León (Permit Number: 15-2011). Animals were sacrificed using the concussion method by qualified personal and all efforts were made to minimize suffering.

<u>**Sperm and eggs collection**</u>

Sperm was obtained from sex-reversed ripe rainbow trout (Oncorhynchus mykiss) females (neomales), which were maintained under artificial photoperiod in a fish-farm (Lugo, Spain). Neomales from two different batches were slaughtered, 4 from a batch breeding during ORS (December- January) and 5 from a batch breeding at LRS (February- March). The testicles were dissected and blood vessels were removed. Sperm was extracted directly from testicle doing several incisions with a scalpel. Sperm

was diluted 1:10 in a commercial maturation medium, Storfish® (IMV, France) and samples were kept 2 h at 4ºC with oxygenation before processing. Eggs were obtained by abdominal massage from 2 different females in each collection time. After checking their proper quality by visual inspection, the eggs were pooled and divided in batches for fertilization procedures.

Sperm UV irradiation

Sperm from each male from ORS was divided in 2 aliquots, one of them was used as control and the other one was subjected to UV-C irradiation in those samples obtained in-season. Prior to irradiation, sperm was diluted 1:3 with SFMM (Seminal Fluid Mimicking Medium) (110 mM NaCl, 28.18 mM KCl, 1.22 mM MgSO4•7H2O, 1.77 mM CaCl2•2H2O, 10.05 mM bicine, 9.9 mM HEPES, pH 7.4). Twenty mL of diluted sperm were transferred to a Petri dish 10 cm diameter, giving a thin layer of sperm. The Petri dish was located 15 cm under a UV lamp (Vilber Lourmat, Germany) (254nm) receiving 400 µW/cm2 irradiation during 5 min. Then, treated and control samples were kept in the dark at 4ºC until further analysis.

Fertilization and embryo incubation

Pooled eggs were divided in 26 batches with 400 eggs / batch and 2 batches per treatment that were fertilized with fresh sperm or irradiated sperm from each neomale separately, both of them diluted 1:3 with SFMM, as previously stated (2 replicates/treatment) (Figure 1). One mL of sperm was added over the eggs and stirred with a feather. After 10 min of incubation the sperm was activated with 10 mL of DIA solution (94.97 mM NaCl, 49.95 mM, Glicine, 19.98 mM Tris, pH 7.4). Ten min later, the

eggs were washed with water and incubated in the dark at 10 °C with a continuous water flow of 24 L/min until hatching.

Fifteen embryos from each replicate were collected at different developmental stages following the classification from (Vernier Ann. Embryol. Morpho. 1969) (Vernier 1969): stage 13 (epibolia), stage 19 (somita), stage 23 (organogenesis) and stage 30 (larvae 1day post hatch (1dph)). Embryos were cleared with a solution containing 50 mL formaldheyde, 40 mL acetic acid and 60 mL glycerine to check the proper development and identify the aborted embryos. Ten additional embryos at each stage per treatment were frozen at -80ºC for DNA and RNA extraction and ten more for the analysis of the apoptotic activity. During development all the aborted embryos were removed and, after hatching, the hatching rates and cumulative percentage of abortions were calculated.
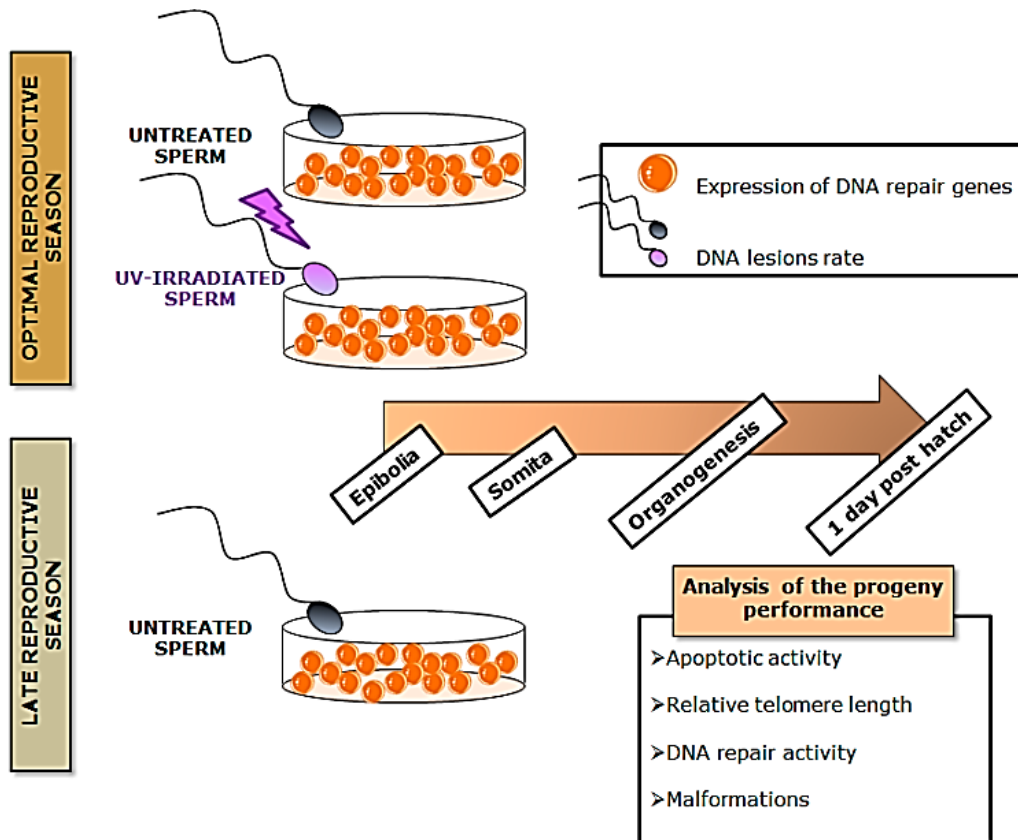
Figure 1: Scheme of the experimental design.

Genomic DNA extraction

Total genomic DNA was extracted from untreated and irradiated sperm following the protocol described by our group (Cartón-García et al. 2013). For late embryos (stage 13 and 23) and larvae (stage 30) a previous digestion step with 6 mg/mL collagenase in PBS 1X during 2 h at 37 ºC was performed. To stop the reaction, the cells were washed with two volumes of PBS 1X and the pellet was resuspended in 700 μL of extraction buffer (10 mM Tris–HCl, pH 8.0; 100 mM EDTA, pH 8.0; 0.5% (v/v) SDS, supplemented with 1 μL proteinase K (1 mg/mL)). DNA quantity and purity were determined spectrophotometrically at 260 nm (Nanodrop ND-1000

Spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples showed high yield (A260/A280>1.8), and were kept at -20 ºC until further processing.

RNA extraction and reverse transcription

Total RNA from embryos at stages 13 and 23, larvae 1dph and from 20 unfertilized eggs collected at two different moments of the reproductive season, was extracted using Trizol® Regeant kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. RNA integrity was confirmed by electrophoresis analysis of total RNA samples prior to reverse transcription (data not shown). Total RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). One µg of total RNA from embryos, larvae and eggs were reverse transcribed using the High Capacity cDNA Kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions. The conditions applied for reverse transcription were 25 ºC for 10 min, 37 ºC for 120 min, and final extension at 85 ºC for 5 min. RT-qPCR was performed using a Step-One Plus thermocycler (Applied Biosystems, Madrid, Spain). Complementary DNA products were kept at -20 ºC until further analysis.

DNA lesions rate in sperm

The RT-qPCR approach described by (Rothfuss et al. 2010) was applied to analyze the number of lesion in four nuclear genes previously studied in rainbow trout by (Gonzalez-Rojo et al. 2014), hoxc4a-2, eifib, sox2 and 18S rRNA. For each gene, two genomic DNA fragments of different length in the same region were amplified: long

fragments with up to 700 bp and short ones with less than 100 bp. Reaction mix for the long amplicons contained 4 µL Fast Start DNA Master plus SYBR Green I (Roche, Germany), 0.4 µL 50X ROX passive reference dye (BioRad, Foster City, CA, USA), 3 ng of genomic DNA and 0.5 µL of each 10 µM forward and reverse primer. RT-qPCR started with a pre-incubation of 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 sec, annealing temperature specific for each couple of primers for 10 sec and 72 ºC for 50 sec. Reaction mix to the short fragments contained 10 µL 2X SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain), 0.5 µL of each 10 µM forward and reverse primer and 3 ng of genomic DNA. RT-qPCR began with a pre-incubation of 95ºC for 10 min followed by 40 cycles of 95 ºC for 15 sec, annealing temperature for 1 min. Three technical replicates were performed per sample. The RT-qPCR conditions were optimized for the different primers to achieve similar amplification efficiencies. Product specificity was tested by melting curves and product size was visualized by electrophoresis on agarose gel (data not shown). The nucleotide sequences and GenBank accession numbers are shown in table 1.

The differences of Ct values between irradiated sperm samples and untreated sperm samples for each long and short fragments, was calculated according the formula described by (Rothfuss et al. 2010).

$$Lesion\ rate\ = 1 - 2\ \frac{\Delta Ct\ long -\ \Delta Ct\ short}{size\ of\ long\ fragment\ (bp)} X\ 10000\ (bp)$$

Table 1: Oligonucleotide sequences of the PCR primers used to assay the DNA lesions by RT-qPCR.

Primer name	Primer set (5`-3`)	Amplicon size (bp)	Annealing Tm (ºC)	Accesion number
hoxc4a-2 (long)	F: AACAGCTACATCCCCGACCACAG R: TCGCGCACATAGGCTACATAACAG	643	65	AY567804
hoxc4a-2 (short)	F:GTGCCTCTAACTCCCATCTCC R:CAAAAGCTTCTCCCCTATCGT	57	63	
eif1b (long)	F:CCCAGAGTATGGGGAAGTGA R: GTTGGTAGCCCAGCATCAAT	634	63	NM_001165193
eif1b (short)	F: GGCTGCATACGTCCATGTTA R:GGCTGCGATGATCAGAACTT	56	63	
sox2 (long)	F:AGTTGTCAAGGCTCTGGCGA R: GCCTCCCCCTACACCCACT	651	65	NM_001141718
sox2 (short)	F:ATGGGTTCGGTGGTCAAGTC R:GGAGTGAGACGACGACGTGA	66	63	
rRNA 18S (long)	F:CCGCAGCTAGGAATAATGGA R:CTCAATCTCGTGTGGCTGAA	632	65	FJ710873
rRNA 18S (short)	F:ATGGCCGTTCTTAGTTGGTG R: CCGGAGTCTCGTTCGTTATC	63	63	

For DNA lesion rate analysis untreated samples from ORS were used as reference. For each nuclear gene the difference in the Ct values (long fragment/ short fragment) was used as a measure of relative DNA lesion frequency with the 2-ΔΔCt method (Livak & Schmittgen 2001) in relation to the amplification size of the long fragment.

Evaluation of DNA repair related transcripts in the oocytes

Reverse transcribed products from RNA extracted from twenty unfertilized oocytes obtained in both breeding seasons (ORS and LRS) were used to perform a RT-qPCR assay. Five genes implied in at least one DNA repair pathway or DNA damage checkpoint were tested: ung (Uracil- DNA- Glicosylase) and ogg1 (8- oxoguanine DNA glycosilase) involved in base excision repair (BER) pathway, lig 3 (ligase 3) implied in both excision repair pathways (BER and NER), tp53 (tumor protein P53) regulating

different genes involved in DNA repair, cell cycle and apoptosis, and rad1 (DNA repair exonuclease) regulating the cell cycle progress in response to DNA damage.

cDNA products were diluted 1: 3 in nuclease free water and 2 μL were used for each RT-qPCR. The primers were designed using Primer Express (Software v2.0, Applied Biosystems, Madrid, Spain) and Primer Select (Software v10.1 DNA Star, Lasergene Core Suit). The primer nucleotide sequences and annealing temperature from rainbow trout repair genes are reported in Table 2. The RT-qPCR conditions were optimized for the different primers to achieve similar amplification efficiencies. Product specificity was checked by melting curves and product size was visualized by electrophoresis on agarose gel (data not shown).

Reaction mixtures (total volume 20 μL) contained 2 μL of cDNA, 10 μL of 1X SYBR Green Master mix (Applied Biosystems, Madrid, Spain), and 0.5 μL of 10 μM each forward and reverse primer. RT-qPCR was initiated with a pre-incubation phase of 10 min at 95 ºC followed by 40 cycles of 95 ºC denaturation for 10 sec and the temperature for primer annealing for 1 min. Three technical replicates were done per sample. Expression level for each repair gene was normalized to 18S RNA gene using the Delta-Ct (2ΔCt) method to analyze relative changes in gene expression concerning the housekeeping expression.

Analysis of the progeny performance

Next parameters were checked in all the obtained progenies at epibolia, somita, organogenesis and larvae 1dph stages.

Apoptotic activity

Quantitative apoptotic activity was analyzed using ten alive embryos/ larvae per treatment anaesthetized with MS-222 (80 mg/L), following the protocol described by Fernandez-Diez et al. 2015 including a previous step of dechorionization for embryos at stage 13 at 23. Embryos and larvae cut in small pieces and fragments were incubated 3 h under agitation in a dissociation solution containing 3.6% (w/v) trypsine, 2.4 ml DNAse I (Applied Biosystems, Foster City, CA, USA), and 10% (v/v) FBS in Leibovitz's (L-15) medium. Embryos at epibolia stage were kept in the dissociation solution for 1 h. The embryo and larvae fragments were gently and repeatedly pipetted to facilitate the dissociation process. Then, cells were filtered using a 140 mm nylon mesh and washed twice with a solution containing L-15 medium. The activity of caspases 3/7 was analyzed using the Caspase-Glo 3/7 Assay Systems Kit (Promega, MA, USA) following the instructions of the manufacturer.

Relative telomere length

Telomere length was measured by RT-qPCR assay using telomeric primers and genomic DNA. Reaction mix (total volume 20 µL) for each sample contained 0.03 µg of genomic DNA, 10 µL of Quantimix easy SYBR kit (Biotools, Madrid, Spain), 0.4 µL of 50X ROX passive reference dye (Biorad, Foster City, CA, USA), and 0.5 µL of 10 µM each forward and reverse primer. RT-qPCR began with a pre- incubation of 94ºC for 3 min and 40 cycles of 94 ºC of denaturation for 10 sec, 56 ºC of annealing for 15 sec and two extension steps of 72 ºC for 10 sec and 78 ºC for 15 sec. Three technical replicates were done per sample. Telomeric primer signal was normalized dividing by primers for

18S ribosomal DNA as reference gene (Table 3). The average of this ratio compared with control embryo/larvae or control sperm was reported as relative telomere length.

Offspring DNA repair activity

DNA repair activity was evaluated analyzing the presence of specific transcripts in embryos at 13 and 23 stage and larvae 1dph following the same procedure above described for unfertilized oocytes.

Malformations

One day after hatching, the malformations rate was analyzed in each progeny, classifying the malformed larvae in five types: axial axis malformed (AAM), half hatching (HH) and yolk- sac malformed (YSM), cyclopodia (C) and prognathism (P).

Statistical analysis

Data analysis was carried out using the computerized package generated by SPSS 21.0 software for Windows (IBM, EEUU). The results were expressed as mean ± SEM. One-way ANOVA test followed by the DMS or Games Howell post hoc test (P <0.05) was used to analyze parametric data corresponding with cumulative percentage of abortions, hatching and growth rates, total malformations and telomere length. A non- parametric Kruskal- Wallis was used to analyze the apoptosis and repair activity data on the progeny. Significant differences in gene expression on eggs (collected at ORS and LRS) and the number of sperm DNA lesions at ORS respect to the untreated sperm samples, were validated by an unpaired t-Test followed by the Welch´s correction using the GraphPad Prism v5.0 Software, San Diego, CA, USA. (*P* value <0.05).

Results

Genotoxic damage in sperm

Results shown that UV-C irradiated sperm and sperm collected at LRS have a higher number of lesions than untreated sperm samples obtained at ORS (Dec-Jan) (Figure 2 A-B). This increase has a similar range in both types of samples (average number of lesions 4.25 in UV sperm and 3.8 in LRS sperm). Irradiation promote a homogeneous damage in the analyzed genes, whereas higher differences among genes where observed in LRS sperm.
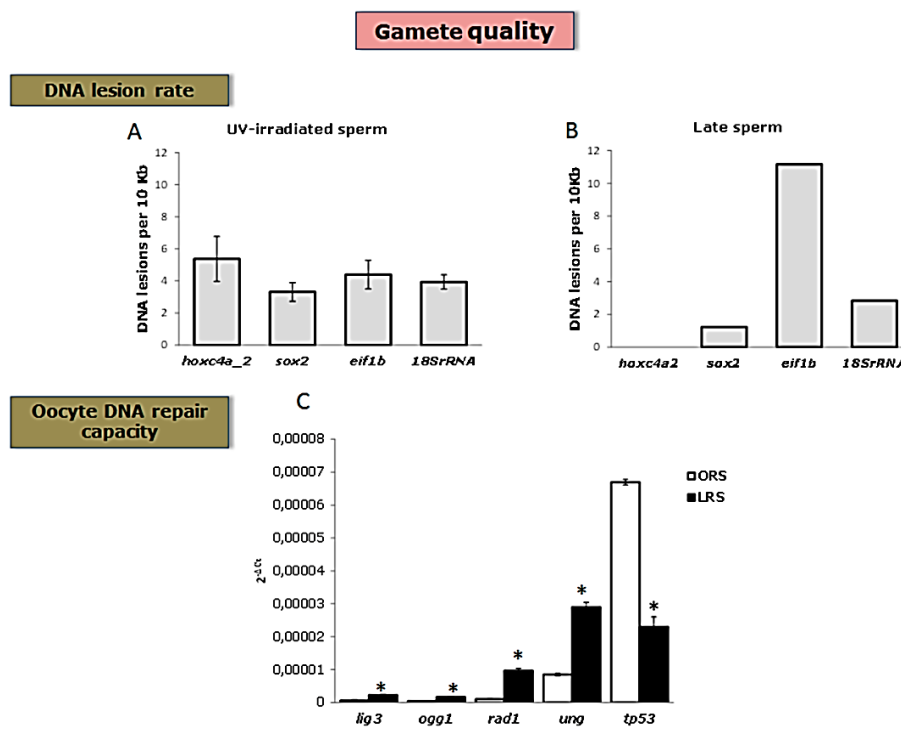

Figure 2: Rate of lesions in sperm DNA (A, B) and oocyte repairing capacity. A: DNA damage of sperm treated with UV-C irradiation (400 µW/cm2 during 5 min) respect to untreated sperm at ORS. Data show mean values ± SEM. (n= 4) B: Mean DNA damage of sperm obtained out of season (LRS) (n=5) respect to the sperm collected on–season. C: mRNA levels (Fold change 2ΛCt) for lig3, ogg1, rad1, ung and tp53 in oocytes from different reproductive periods. Data show mean values ± SEM (n= 20). Asterisks show significant differences between samples (*P*< 0.05). ORS: optimal reproductive season (December-January); LRS: late reproductive season (February- March).

Oocyte DNA repair ability

The expression of genes related to DNA repair (lig3, ogg1, rad1, ung and tp53) showed significant differences among the oocytes obtained at different reproductive periods. All the analyzed transcripts, except tp53, were significantly over-expressed in LRS oocytes. lig3, ogg1 and ung genes increased their expression about 3.5 times and rad1 as much as 9.15 times. In the case of tp53 the level of expression at ORS was higher (2.9 times) than in oocytes from females maturating at later time (Figure 2 C).

Performance of the progeny

As shown in figure 3 A, UV irradiation of the sperm did not increase the abortion rates during embryo development. Nevertheless, a significant increase of embryo loses was noticed in batches fertilized during LRS that resulted in a lower survival at hatching (53.83 ± 10.54 and 29.68 ± 3.46 for control batches at ORS and LRS respectively). The quantitative analysis of the caspases activity (Figure 3 B) revealed that apoptosis increases during embryo development reaching at organogenesis the highest level, and dropping at hatching. The same pattern is observed in batches fertilized with UV damaged sperm which show higher activity than control batches at epibolia and 1dph. Apoptotic activity displays a different pattern in LRS batches in which the level of caspase 3/7 activity did not increase at organogenesis.
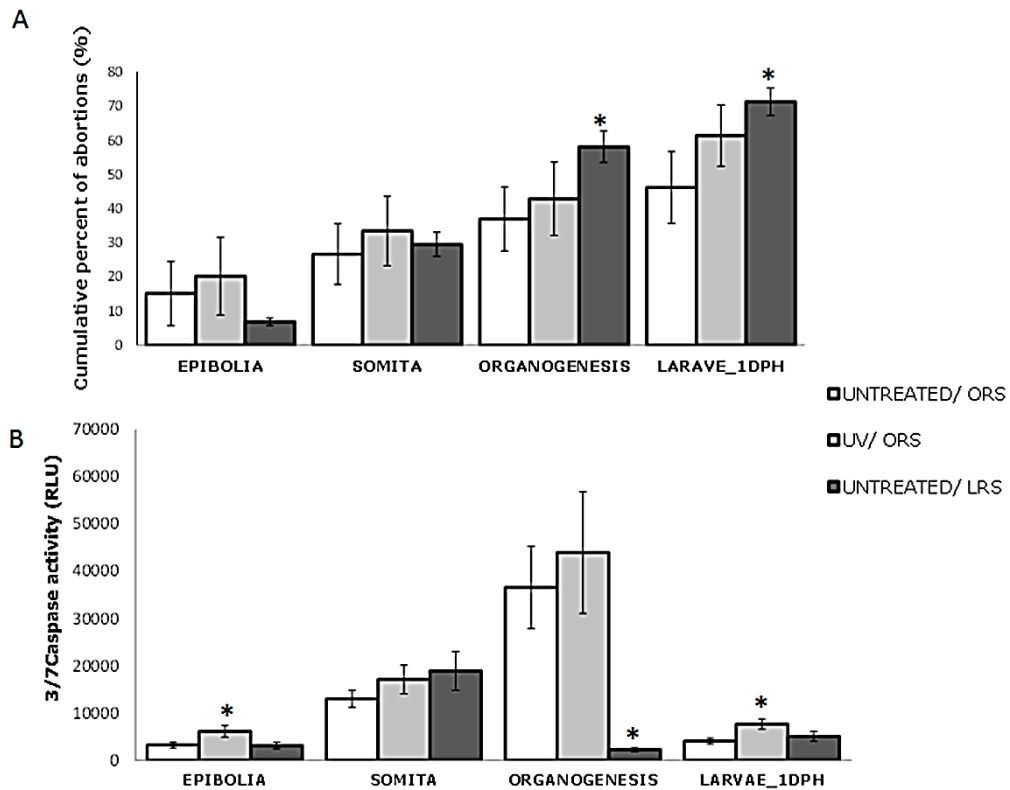
Figure 3: A: Cumulative percentage of abortions and B: caspase 3/7 activity in relative luminescence units (RLU) at epibolia, somita, organogenesis and larvae (1dph) stages from progenies obtained in and out of season (ORS and LRS respectively) with untreated sperm (UNTREATED) and UV-C irradiated sperm (UV); Data show mean values ± SEM. (2 batches per treatment, 400 eggs per batch for the abortions rate and 5 embryos per batch for caspase 3/7 activity). Asterisks indicate significant differences respect to the untreated samples at ORS ($P< 0.05$).

The percentage of malformed larvae 1dph was higher in batches obtained at LRS than at optimal period (4.31 ± 1.27 and 11.59 ± 0.53 from ORS and LRS respectively) (Figure 4 B). The obtained progenies developed different types of malformations as shown in Figure 4 A. The malformations related to the axial axis formation were the most common among larvae being the higher percentage in ORS batches from control sperm (Figure 4C). A high percentage of embryos showing defective hatching were recorded in batches fertilized at LRS (45%) (Figure 4E). Prognathism was only identified in batches from ORS (18% with UV sperm) (Figure 4C). Larvae from UV sperm displayed the higher percentage of yolk-sac defective larvae (25%) (Figure 4C), being cyclopodia only observed in these batches.
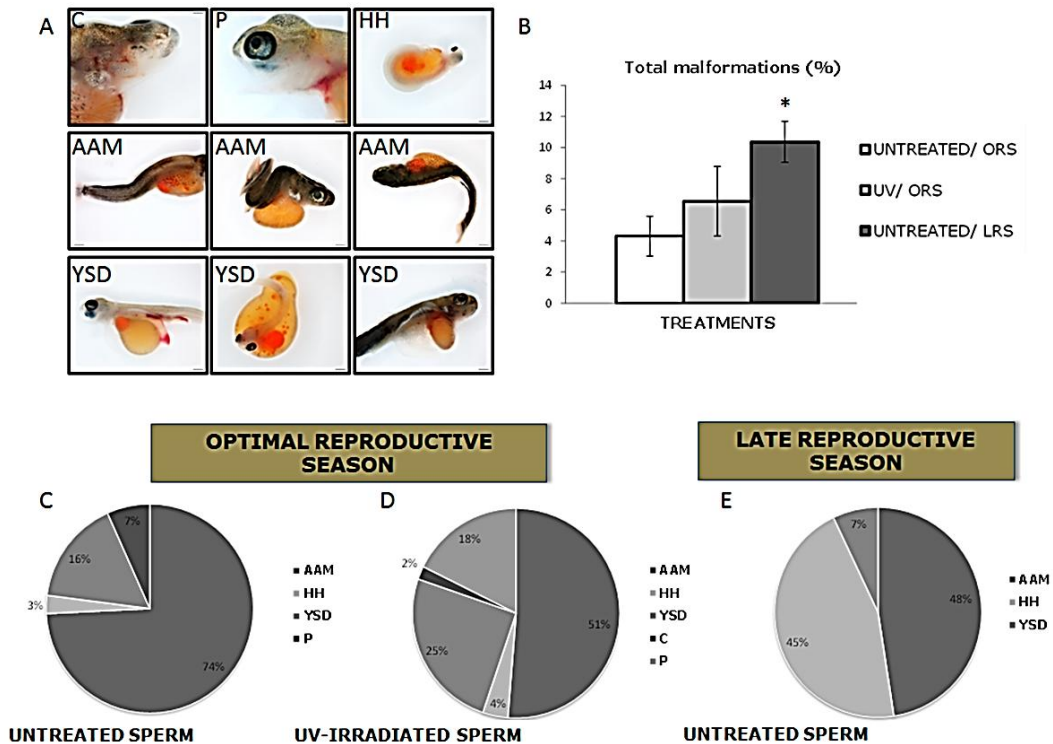
Figure 4: Phenotype analysis of larvae 1dph in batches obtained in and out of season (ORS and LRS respectively) with untreated sperm (UNTREATED) and UV-C irradiated sperm (UV). A: Types of malformations observed in larvae. C: cyclopodya; P: Prognathism; HH: Half hatching; AAM-F: axial axis malformed; YSD: Yolk –sac defects. B: Percentage of total observed malformations. C-E: Percentage of specific malformations. Data show mean values ± SEM. Asterisks indicate significant differences respect to the untreated samples at ORS (P< 0.05) (2 batches per treatment, 400 eggs per batch).

As shown in Figure 5 telomere length changed during embryo development. The shorter telomeres were found in sperm samples, which did not show differences between treatments, whereas progenies at epibolia stage showed the longer telomeres. Shorter telomeres respects to the control were observed in batches from UV sperm at epibolia and in LRS batches at epibolia and organogenesis. At hatching the telomere length was similar in all the analyzed progenies.
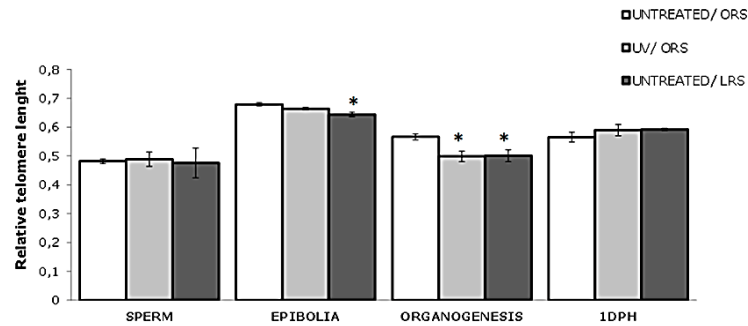
Figure 5: Relative telomere length in sperm and embryos at epibolia, organogenesis and larvae (1dph) stages from progenies obtained in and out of season (ORS and LRS respectively) with untreated sperm (UNTREATED) and UV-C irradiated sperm (UV). Asterisks indicate significant differences respect to the untreated samples at ORS (P< 0.05). (n=4 for samples obtained at ORS and n= 5 for samples obtained at LRS).

Gene expression analysis revealed a significantly modified profile in LRS batches at epibolia, with overexpression of genes related with DNA repair pathways BER and NER (ogg1, ung and lig3) (Figure 6 A-C) and rad1 expression (Figure 6 D) and a lower expression level of tp53 (Figure 6 E). At the same stage UV progenies showed overexpression of rad1 respect to the ORS control. One-day post hatch the expression of tp53 was significantly higher in UV batches and lower in late progenies than in those obtained at the optimal period.
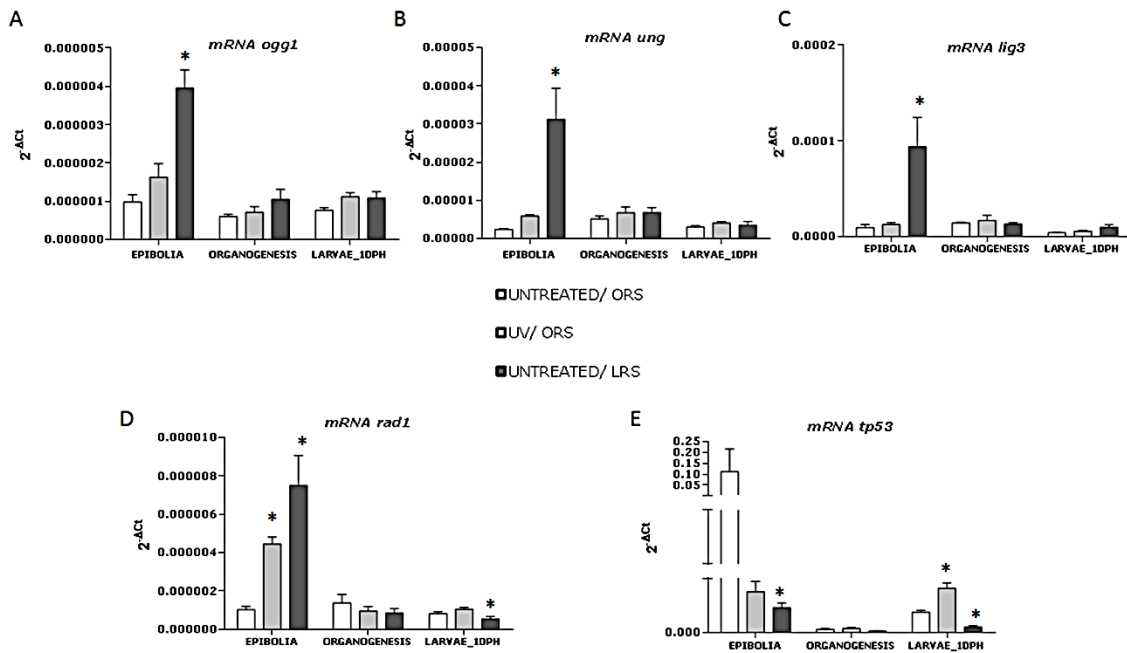
Figure 6: mRNA levels (2ΔCt) for rad1, ogg, ung, tp53 and lig3 at different stages during development from embryos obtained in and out of season (ORS and LRS respectively) from fresh sperm (UNTREATED) and UV-C irradiated sperm (UV). Asterisks indicate significant differences respect to the untreated samples at ORS (P< 0.05). (n=4 for embryos obtained at ORS and n= 5 for embryos obtained at LRS).

Discussion

The proper embryo development largely relies on the integrity of the genomic information provided to the zygote. Physiological conditions during gametogenesis are crucial to ensure that the lesions promoted during recombination at meiosis or during chromatin condensation at spermiogenesis, do not exceed the species' natural repairing ability. It is well known the detrimental effect of thermal stress on spermatogenesis, different studies showing that increased temperature entails an increase of DNA fragmentation in the sperm, both in mammals (Durairajanayagam et al. 2015) and fish (Lahnsteiner & Leitner 2013). The performed analysis of DNA lesion by RT-qPCR showed an increase of DNA damage (up to 11 DNA lesions/ 10 Kb) in

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rainbow trout sperm samples collected at LRS, breeding at higher temperatures than those from the optimal period. Similar results were observed when DNA fragmentation was analyzed using the Comet assay at the end of the natural reproductive period in rainbow trout (Perez-Cerezales et al. 2010a) or in sole Solea senegalensis breeding out of season (Beirao et al. 2011), revealing in fish that an inadequate environment promotes DNA chromatin susceptibility, as was also demonstrated in human (Takata et al. 2013). The loss of DNA integrity is commonly considered a result of failing sperm chromatin packaging during spermatogenesis, defective apoptosis and excessive ROS production (Shamsi et al. 2011, Durairajanayagam et al. 2015) and should generate an increased repairing effort upon fertilization. Nevertheless, environmental stress could also imply defective repairing mechanisms in the zygote, being the maternal contribution essential in this respect.

It is known that UV exposure increases the formation ROS through the endogenous photosensitizers (Zan-Bar et al. 2005, Kim et al. 2015) promoting genotoxic stress. UV-C irradiation at the tested doses (400 µW/ cm2) increased in the ORS sperm the number of DNA lesion in a similar range to those promoted by out of season breeding. Higher doses than the one used in our study are commonly used in aquaculture to inactivate the sperm genome for gynogenetic procedures (Lin & Dabrowski 1998, Ciereszko et al. 2005, Dietrich et al. 2005).

As demonstrated in a variety of vertebrates unrepaired DNA lesions from paternal origin promote a slower paternal DNA replication (Gawecka et al. 2013), de novo mutations propagated to the next generation like hereditary diseases (Carroll & Marangos 2013), chromosomal structural aberrations (Marchetti et al. 2015) and even

lead to arrest in embryo development (Gawecka et al. 2013). As has been demonstrated in human and mouse, zygotic DNA repair initially depends on the oocyte-born mRNAs and proteins and, additionally, on genes expressed very early in development (Derijck et al. 2008, Jaroudi et al. 2009), Therefore, the combined effect of sperm DNA damage and the capacity of the oocyte to repair it, will determine the final impact on the offspring development (Gonzalez-Marin et al. 2012).

In our study, the genotoxic treatment applied to the sperm, had transitional consequences for the offspring development. Ciereszcko and colleagues (Ciereszko et al. 2005) observed in trout a decrease in the survival rate from 2-cell stage to pre-hatch stage on the progeny obtained with UV-C irradiated sperm with higher doses (up to 1720 J/m2). Our results did not reveal an increase of the accumulative abortions neither of the hatching rates respect to the batches of the same reproductive period, revealing that ORS oocytes have the ability to properly repair the sperm lesions. Similar results were reported by Perez-Cerezales et al (2010), who estimated that trout oocytes are able to repair sperm with a 10% fragmented chromatin. Genomic instability promoted by UV had effects early in development: an increased apoptotic activity and overexpression of rad1 were observed at epibolia, revealing the activation of mechanisms protecting against genotoxic damage. Moreover, telomere length was reduced at organogenesis. The shortening of telomeres has been previously described in human cells submitted to UV irradiation as a result of the erroneous repair of the pyrimidinepyrimidone (6-4) photoproducts (6-4 PPs) (Stout & Blasco 2013) which are generated by UV irradiation in the DNA strand (reviewed by Batista et al. 2009). Sperm DNA damage seems to be identified and safely repaired early during development.

Different results were obtained when the fertilized oocytes display an altered repairing profile. We have observed that oocytes collected out of season showed a significant increase in all the analyzed repair transcripts (up to 9.15 times in rad1mRNA) but in tp53 mRNA which was downregulated. The analyzed transcripts are involved in DNA damage control and the repair of different kind of lesions that could be promoted during gametogenesis: ogg1 is involved in the removal of 8-oxoguanyne, mutagenic base produced after ROS (Gagne et al. 2013); DNA lig3 (lig3) plays a crucial role in the repairing of DNA single-strand breaks during embryo development that cannot be replaced by other DNA ligases (Puebla-Osorio et al. 2006); ung gene encodes for uracil-DNA glycosylase which is involved in excision of uracil residues from U:G and U:A mispairs in DNA molecule, participating in base excision repair pathway -recent reports in zebrafish have demonstrated its implication in a correct embryo development during post-fertilization (Wu et al. 2014)-; rad1 mRNA encode to Rad1, checkpoint protein which is involved in cell cycle arrest (Bozdarov et al. 2013), playing an important role during the correct embryo development (Han et al. 2010); and tp53 is one of the most important transcriptional factors with suppressive and proapoptotic functions upon genotoxic stress (Storer & Zon 2010). The expression of tp53 is usually enhanced under conditions of genomic instability, but different researchers have reported diverse response in fish cells, some of them noticing induction and some others failing to detect any increase after UV irradiation, treatment with hydroxyurea or with chemotherapeutic agents with effect on mammalian cells (Liu et al. 2011). The overexpression of repairing genes that we have observed in oocytes at LRS is compatible with a higher repairing effort, required to repair the genomic lesions promoted during oogenesis in sub-optimal conditions (Harrouk et al. 2000). The

increased expression of rad1, also involved in the telomere length maintenance (Khair et al. 2010), is a common surveillance mechanism activated in trout in response to genotoxic stress such as UV light or ionizing radiation, etc. (Bozdarov et al. 2013), whereas the downregulation of tp53 generates a more tolerant environment to genotoxic stress. In such conditions the zygote shows a modified repairing profile that should face the damaged male pronuclei.

The development of the batches fertilized in the two analyzed periods showed significant differences. Respect to the natural season, batches from LRS showed at epibolia the same repairing profile than oocytes: overexpression of all the analyzed transcripts and downregulation of tp53, revealing an intense repairing activity but a reduced capacity to arrest the development upon damage. Tolerance to damage, suppressing the induction of apoptosis, is a known mechanism allowing to progress with development in stressful environmental conditions, as was observed in corals (Tchernov et al. 2011) or in rainbow trout embryos with inhibited repairing machinery (Fernandez-Diez et al. 2015). tp53 is in fish very similar to mammalian P53, and is highly and ubiquitously expressed in zebrafish early embryos (Storer & Zon 2010). As reviewed by Liu et al. 2011 is functionally similar to mammalian P53, being regulated by MDM2 and regulating the transcription of the same genes that drive to apoptosis. Zebrafish knockdown for tp53 are developmentally normal in homeostatic conditions, but display a reduced induction of apoptosis after genotoxic damage (Duffy & Wickstrom 2007, Storer & Zon 2010). This effect was observed in a late stage, at organogenesis, being the apoptotic activity clearly reduced in LRS batches. Apoptosis is a key event during organogenesis allowing the bulk remodeling suffered by the embryo tissues that drive to a cascade of morphogenetic events required for the

proper organ development. All the embryos obtained at the optimal season undergo a very significant increase of apoptosis which is not observed in the late embryos. This is in agreement with the downregulation of tp53 observed in the late oocytes, and probably promotes developmental failures that entail the observed increase in embryo death later on, the abortions rate increasing very significantly. Moreover, shorter telomeres are observed at epibolia and organogenesis in the LRS embryos. The percentage of hatching is lower than in ORS, whereas the percentage of malformations increases, being the half hatching (HH) the most relevant. These malformed larvae died shortly after hatching, indicating an excess of genomic instability accumulated during embryo development which is not compatible with life. The surviving larvae still showed a lower level of tp53 at 1 dph, revealing potential risks of accumulations of genetic damage at later times. Different reports have already showed that artificial photoperiod in rainbow trout entails lower survival of embryos at eyeing stage and increased incidence of deformities (Bobe & Labbe 2010), but our results showed how the oocyte repairing capacity is involved in the defective development. All the results showed that out of season breeding affect the oocyte DNA signalization and repair profile, generating an increased repairing activity in the offspring and a lower ability to induce apoptosis. Therefore, the reproductive performance is reduced: developmental failures are accumulated, promoting embryo death and decreasing the hatching rates. In addition, the rate of malformed larvae increased.

Our results have shown the synergic effects of sperm DNA damage and oocyte repairing ability on embryo development, pointing out the importance of the repairing machinery provided to the zygote and showing how susceptible is to environmental stress. The differential expression of DNA damage surveillance and repair genes in the

oocyte deeply affect the embryo development, promoting defective apoptosis and impaired embryo development that entails lower hatching rates and higher rates of malformed larvae. The presence of specific transcripts representatives for the capacity of DNA damage signalization and repair could be used as markers of oocyte quality.

Declaration of interest

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Capítulo 3: Respuesta al daño genotóxico
paterno durante el desarrollo embrionario de pez cebra.

Tolerance to paternal genotoxic damage promotes survival during embryo development in zebrafish (*Danio rerio*)

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Under review

Abstract

Mammals have a strong sperm selection mechanism and DNA damage response (DDR) which is activated in the zygote upon fertilization in order to guarantee the genomic conformity of the reduced progeny. However, previous results from our group showed a downregulation of apoptotic activity in trout embryos with a defective DNA repairing ability, suggesting that tolerance mechanisms to damaged DNA were activated in order to maintain cell survival and progression in development. In this work zebrafish embryos were obtained from control or UV irradiated sperm, carrying more than 10% of fragmented DNA. The genotoxic damage in those sperm cells did not affect the fertilization ability. DNA repair ability (γH2AX and 53BP1*foci*), apoptotic activity, expression of genes related to DDR and malformation rates, were analyzed during development. Results showed, in the progeny from damaged sperm, an enhanced repairing activity at the mid blastula transition stage, that returned to the basal level at later stages, and a very high rate of multimalformed larvae. The study of transcriptional and post-translational activity of p53 revealed the activation of an intense DDR in those progenies. However, the downstream pro-apoptotic factor *noxa* showed a significant downregulation, and the anti-apoptotic gene *bcl2* was upregulated, triggering a repressive apoptotic scenario in spite of a clear genomic instability. Our results suggest that p53 switches DNA damage tolerance (DDT) pathways allowing the embryo survival regardless the paternal DNA damage. DDT could be an evolutionary mechanism in fish: tolerance to unrepaired sperm DNA could introduce new mutations, some of them potentially advantageous to face a changing environment.

Introduction

Sexual reproduction is a key evolutionary event allowing the combination of genetic information from two progenitors to perform a newborn. In order to assure the genomic conformity of the progeny a highly coordinated series of processes is required, both during gametogenesis and at fertilization. All the process entails specific genomic rearrangements in conditions not always compatible with the canonical pathways of DNA damage control and repair, increasing the chances of introducing mutations.

The male gamete is considered the main source of the *de novo* mutations produced during fertilization (Crow and Weinberg, 2000; Kong et al., 2012; Marchetti et al., 2015, 2007). The spermatogenesis promotes a number of DNA strand breaks during the chromosome recombination at meiosis and during the nuclear condensation. These injuries remain unrepaired in the mature spermatozoa because the post-meiotic spermatids have limited or absent repairing mechanisms and a highly compacted nucleus which hinders the access to DNA repairing machinery ((Baarends et al., 2001; Herráez et al., 2015; Olsen et al., 2005). In addition, many genotoxic agents are able to promote different types of DNA lesions post ejaculation, compromising the sperm genomic stability (reviewed by (Herráez et al., 2015). In contrast, DNA repairing activity is maintained during oogenesis, the mature oocyte containing the mRNAs and proteins in charge of handling a certain level of DNA paternal damage after fertilization ((Jaroudi et al., 2009; Jaroudi and Sengupta, 2007; Langley et al., 2014; Marchetti et al., 2007; Ménézo et al., 2010). The genetic conformity of the zygote is the master piece to obtain a healthy progeny. Different works have identified a relationship between

sperm DNA damage and the rate of embryo loss and defects at birth (González-marín et al., 2012; Hourcade et al., 2010; Jaroudi and Sengupta, 2007). Moreover, studies in mammals and fish demonstrated that efficient repairing machinery from oocytes is a mandatory condition to allow a correct embryo development. In all cases, the changes in gene expression related to DNA damage checkpoints and DNA repair, deeply affected the embryo development (Fernandez-Diez et al., 2016; Fernández-Díez et al., 2015; González-marín et al., 2012; Jaroudi and Sengupta, 2007; Marchetti et al., 2007). Fertilization with DNA damaged sperm (DDS), seems to activate in mammals some mechanisms of cell cycle arrest at zygotic G2/M and increases the activity of different repairing pathways immediately after fertilization (mainly BER, MMR and HR pathways) (Chen et al., 2012; González-marín et al., 2012; Jaroudi and Sengupta, 2007; Kumar et al., 2013; Wang et al., 2013). Nevertheless, the variable efficiency of those repairing pathways can generate DNA double strand breaks (DSBs) in the unrepaired spots upon DNA duplication. Marchetti and cols., (Marchetti et al., 2015, 2007) showed that paternal exposure to ionizing irradiation or mutagenic chemicals promoted chromosomal aberrations affecting to sister chromatids in 64.2% of the zygotes, attributed to defective repairing of interstrand crosslinks and which promoted death postimplantation in 45% of the embryos. Studies from this group also demonstrated in mouse that the genomic aberrations in zygotes promoted by the spermatic damage are highly predictive of abnormal embryonic development (Marchetti et al., 2015). The reproductive outcomes largely depend on the fidelity of the zygotic repair achieved: only those embryos properly repaired or carrying reciprocal translocations after zygotic repair can produce viable offspring, whereas all those zygotes in which failures

of DNA repair conduct to other genomic aberrations, resulting in preimplantation loss or in dead implants (Marchetti et al., 2004).

Later on, the mammalian preimplantation period is a critical stage in which the transition from maternal to zygotic transcripts takes place (Clift and Schuh, 2014; Li et al., 2013) but the control of DNA damage still relies on the maternal factors (Jaroudi et al., 2009; Jaroudi and Sengupta, 2007). DNA replication and cell proliferation are fast and cell cycle is short, increasing the risk of losing genomic integrity and reducing the chances to activate the cycle checkpoints in order to control the DNA stability (Jaroudi and Sengupta, 2007).

Throughout the development, the embryo acquires a greater ability to generate an integral DNA damage response (DDR), activating different genes which operate in DNA damage detection, the different pathways of DNA repair, the control of cell cycle arrest and the apoptosis activation (Jaroudi and Sengupta, 2007). In that way, embryos acquire the capacity to take the appropriate developmental decision upon damage: proceed with repairing allowing the development, and tolerate the damage in spite of their impact on cell metabolism a long term, or activating the apoptosis. During mammalian embryo development, all the data indicate that the repairing effort starts at the 1 cell stage, but cell death or apoptosis are only observed at late cleavage or blastocyst stages, revealing a tolerant period during cleavage, but a strict control at the pre-implantation stages (Jaroudi and Sengupta, 2007; Langley et al., 2014). Increased apoptotic activity is also observed at organogenesis upon genotoxic stress (Jaroudi and Sengupta, 2007) and a high rate of post implantation dead is observed in embryos with residual unrepaired damage (Marchetti et al., 2004).

Most teleost, as external fertilizers, display a very different reproductive strategy based on the increased quantity of embryos with low survival probabilities after birth: large egg batches are fertilized and the embryos which are exposed to changing environmental conditions, predation, etc., have reduced chances to survive. In these species, it has been observed a less restrictive mechanisms for sperm selection than in mammals allowing fertilization with DDS (Pérez-Cerezales et al., 2010). Moreover, the study of transcripts in trout embryos and larvae suggested a tolerance to genotoxic damage after the inhibition of DNA repair in the zygotes (Fernández-Díez et al., 2015) as well as in embryos from sub optimal quality oocytes, which showed altered expression of DDR related genes (Fernandez-Diez et al., 2016). Our hypothesis is that fish embryo development displays a high degree of tolerance to paternal DNA damage, allowing the development of embryos with a high degree of genomic damage up to hatch. In this study we use zebrafish as model species in order to analyze the effects of fertilization with DNA damaged sperm on the activation of DDR mechanisms and on the progeny performance. Zebrafish share with mammals the DNA repairing pathways, cell cycle control mechanisms and the main apoptotic pathways.

Materials and Methods

Reagents

All media components were purchased from Sigma-Aldrich (Spain) except when otherwise stated.

Ethics statement

The experiments were carry out in accordance with the Guidelines of the European Union Council (86/609/EU, modified by 2010/62/EU), following Spanish regulations (RD 1201/2005, abrogated by RD 53/2013) for the use of laboratory animals and were approved by the Ethics and Scientific Committee of the University of León and the Competent Organism of the Junta de Castilla y León (Project number ULE008-2016).

Collection of gametes and *in vitro* fertilization

Mature eggs and sperm were obtained by gentle squeezing of males and females following the procedures by (Hagedorn and Carter, 2011). The animals were immersed in a solution of tricaine methane sulfonate (MS-222) according to Westerfield (Westerfield, 2002) until gill movements have slowed down. Males were placed in a sponge with the ventral surface up. The anal fin area was dried and both sides, from pectoral to the anal fin, were pressed using forceps. The sperm was collected with a 10 µL pippete and then placed into an eppendorf tube containing 20 µL of PBS on ice. The same tube was used to pool the sperm from five males. Pooled sperm was divided in 3 aliquots, each one containing about 1×10^7 cell/ml. One aliquot was used as control, and the rest were subjected to different levels of UV-C irradiation.

To collect the eggs anesthetized females were dried and placed in a Petri dish. Pressure was applied with a slight movement of the fingers on the ventral side, back towards the pelvic fins. The eggs were immediately fertilized using 50 µL of diluted pooled sperm (from control or treated samples) and incubated 2 min with 750 µL of water to activate the gametes.

Sperm UV irradiation

Each cell suspension was transferred to 35 mm plastic Petri dish which was placed on ice at 15 cm under a UV lamp (Vilber Lourmat, Germany) (254nm) receiving 400 CW/cm2 irradiation during 30 or 40 sec depending on the treatment. Then, treated and control samples were kept in the dark at 4ºC.

Analysis of the sperm chromatin integrity

Comet assay:

DNA fragmentation was analysed using the Single Cell Gel Electrophoresis (SCGE) or Comet assay. Control and treated samples were diluted in PBS 1X to a final concentration of 1 X 106 cell/mL. Samples treated with 20 µM H2O2 during 15 min on ice were used as positive control. All samples were centrifuged at 8000 x g during 5 min at 4ºC, and the pellet was resuspended in 10 µL PBS 1X. Sperm cells were mixed with 180 µL of 0.5% low melting point agarose (Promega Biotech Iberica) and then, 75 µL of cell suspension were pippeted over a 3-Aminopropyl-triethoxysilane (A.T.E.) treated slide and covered with a glass cover slip. Slides with gels were kept at 4ºC during 30 min to solidify. Then the coverslips were removed and the slides incubated 1 h at 4ºC in lysis solution (100 mM EDTA-Na2, 2.5 M NaCl, 10 mM Tris-HCl and 1% Triton X-100, pH 10). The slides were placed into an electrophoresis buffer (1 mM EDTA-Na2, 0.3 M NaOH, pH 13) for 20 min to promote the DNA unwind followed by 20 min of electrophoresis (25 V, 280-350 mA). The slides were washed using a neutralization solution (0.4 M Tris-HCl, pH 7.5). This procedure was repeated three times to ensure the elimination of all alkali and detergents. The slides were fixed with

absolute methanol for 3 min and were then left to dry in the air protected from the light.

20 µL of 0.5 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) were pippeted over the slides for comet visualization and then were covered with a coverslip. Samples were observed with an epifluorescence microscope (Nikon Eclipse E800) fitted with a 510–560 nm excitation filter and a 590 nm barrier filter. Images were obtained with a Nikon DXM1200F digital camera, acquiring approximately 50 cells from each slide using Nikon ACT-1 software (v. 2.62, Nikon). All the images were analysed with the free CaspLab software (1.2.3beta2) and the percentage of tail DNA (% DNAt) was used to quantify the DNA damage.

Tunel assay:

Terminal deoxynucleotidyl transferase dUTP nick end labelling (Tunel) kit (Roche, Germany) was used to detect DNA fragmentation. Control and treated (30s and 40s UV-irradiation) samples were washed two times with PBS 1X and then fixed with 4% paraformaldehyde in PBS 1X, 20 min at room temperature (RT). Samples were centrifuged at 8000 x g at 4ºC, 5 min and the pellet was resuspended with 100 µL distilled water. 20 µL of the cell suspension were drooped over a slide. The slides were dried at 37ºC overnight (ON).

The samples were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, 5 min at RT and washed in PBS 1X. 30 µL of TUNEL reaction mixture were added per drop and the slides were kept in a wet chamber for 1 h at 37°C in the darkness. A negative staining was performed as control using 30 µL of label solution without enzyme solution. The positive control of fragmentation samples was treated 10 min at

25ºC with 30 µL of a solution containing 1 µL TURBO DNase (Applied Biosystems, Foster City, CA, USA), 1 µL TURBO DNase (Applied Biosystems, Foster City, CA, USA) buffer 10X and 26 µL of distillate water, prior to perform the TUNEL reaction.

All slides were washed twice in PBS 1X and were labelled with 50 µM DAPI during 5 min at RT. The slides were washed thrice and were mounted with ProLong Gold Antifade reagent (Thermo Fisher Scientific) and covered with a coverslip. The slides were analysed using a confocal microscope LSM 800 (Axio Observer Z1, Zeiss).

Analysis of the progeny performance

Fertilization rates and embryo development

Embryos were kept at 28ºC in darkness until hatching and then, fertility rates at 3 hpf, accumulative mortality rates through embryo development at 1k, 8 hpf, 24 hpf, 48 hpf and 72 hpf, and survival rates at hatching (72 hpf) were evaluated.

Malformations

At hatching the percentage of embryos displaying malformations was analysed. Those embryos unable to naturally hatch were manually dechorionized in order to characterize the developmental abnormalities. Malformations were classified in 8 types: axial torsion, distal torsion, defective yolk-sac, cardiac edema, no hatching, no caudal fin, no pectoral fin and defective pigmentation. To characterize the head skeletal malformations, the cartilage was stained in larvae 6 days post hatch (dph). 10 larvae from each batch were fixed using 4% paraformaldehyde solution at 4ºC ON. The next day the larvae were washed 3 times in tap water and then in PBT solution (0.1% Tween-20 in PBS 1X). Then, the embryos were cleared using 1.5% H_2O_2 in1% KOH

during 30 min and washed twice in PBT. The whole larvae were stained with 0.1% alcian blue in 70% EtOH). Then, they were washed with 70% EtOH in 5% HCl and 0.25% KOH in 20% glycerol during 3 h each time. Finally, they were kept in 0.25% KOH in 50% glycerol ON and the next day were stored at 4ºC in 0.1% KOH in 50% glycerol to acquire the images using a stereomicroscope.

DNA damage response (DDR)

DNA repairing activity: γH2AX and 53BP1 immunodetection

Ten alive embryos from bathes derived from control or treated sperm at 1 K, 8 hpf and 24 hpf were fixed with 4% paraformaldehyde in PBS 1X ON at 4ºC. The embryos were washed three times in water and then were dechorionized and permeabilized with pure methanol during 2 h at -20ºC. Then, they were incubated with 2 N HCl 1 h at RT to denaturize the DNA. The embryos were washed twice with PDT (1X PBST (0.1% Tween 20 in PBS 1X), 0.3% Triton and 1% DMSO) for 20 min at RT, incubated 1 h at RT in blocking solution (20% goat serum, 3% BSA in PBST) and then incubated two days at 4ºC with primary antibodies diluted in blocking solution: mouse monoclonal to γ-H2AX (phospho S139) (ab 26350, 1:100 at the two early stages and 1:50 at 24hpf) and rabbit polyclonal to 53BP1 (ab36823, 1:200 to two first stages and 1:100 at 24hpf). Embryos were washed with PDT solution and then incubated 1 day at 4ºC with secondary antibodies (A865 and A11008 respectively from Thermo Fisher Scientific) in blocking solution.

All samples were washed twice and labelled with DAPI during 10 min at RT. The slides were washed, mounted with ProLong Gold Antifade reagent and coverd with a coverslip. The samples were observed with a confocal microscope LSM 800 (Observee

Z1, Zeiss). Fluorescence emission in the nuclei of 300 randomly selected cells per embryo, and 5 embryos per treatment was quantified using Image J software.

tp53 activation analysis

The immunodetection of p53 phosphorylated (Phospho-p53 (Ser15) Antibody from Cell Signaling Technology) was performed as previously described for DNA repairing activity. The primary antibody (1:50) was incubated 2 days at 4ºC in blocking buffer in embryos at 1 K, 8 hpf and 24 hpf. The secondary antibody (A11008 from Thermo Fisher Scientific) was incubated 1 day at 4ºC in blocking buffer. The samples were analyzed per triplicate and were observed with a confocal microscope LSM 800 (Observee Z1, Zeiss). Fluorescence nuclei emission in 300 randomly selected cells per embryo and 5 embryos per treatment were quantified using Image J software.

Apoptotic activity

Apoptotic activity was analysed using cells pooled from ten alive embryos at different developmental stages (1k, 8 hpf, 24 hpf and 72 hpf). 8 hpf and 24 hpf embryos and larvae at 72hpf were dechorionized and cut in small pieces. The fragments were incubated 2 h under agitation in a dissociation solution containing 6 mg/ml collagenase I, 2.4 µl DNAse I (Applied Biosystems, Foster City, CA, USA), and 10% (v/v) FBS in Leibovitz's (L-15) medium. 1K embryos were also dechorionized and repeatedly pipetted in L-15 medium to promote the dissociation process. Then, cells were filtered using a 100 mm nylon mesh and washed twice with L-15 medium. The apoptotic activity was detected using a FITC annexin V Apoptosis Detection Kit (Molecular probes by Life Technologies) following the manufacturer's instruction. The samples were analysed with an ImageStream multispectral imaging flow cytometer

(Amnis Corporation, Seattle, WA) using a 488 nm laser and the data analysis was made using IDEAS software.

Expression of genes related to DDR

RNA extraction and reverse transcription:

Total RNA from embryos at 1 K, 8 hpf and 24 hpf and from larvae at 72 hpf was obtained using Trizol® Regeant kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions. RNA integrity was confirmed by electrophoretic analysis of total RNA samples prior to reverse transcription (data not shown). Total RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1 µg of total RNA from embryos and larvae were reverse transcribed using the High Capacity cDNA Kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions.

Gene expression

Reverse transcribed products from the extracted RNA were used to perform a qPCR assay to analyse the expression of genes involved in DNA damage response: noxa (pro-apoptotic gene), bcl2 (anti-apoptotic gene) and p53 (considered as "genome guardian"). 600 ng of cDNA products were used for each qPCR. The primers were designed using Primer Express (Software v2.0, Applied Biosystems, Madrid, Spain) and Primer Select (Software v10.1 DNA Star, Lasergene Core Suit) (Table 1). The primer sequences and accession number are summarized in table xx. Product specificity was checked by melting curves and product size was visualized by electrophoresis on agarose gel (data not shown). Reaction mixtures (total volume 20 µL) contained 600ng

of cDNA, 10 µL of 1X SYBR Green Master mix (Applied Biosystems, Madrid, Spain) and

0.5 µL of 10 µM each forward and reverse primer. qPCR was initiated with a pre-

incubation phase of 10 min at 95 ºC followed by 40 cycles of 95 ºC denaturation for 10

sec, annealing for 1 min at the optimal temperature for each pair of primers (tabla

xxx). Three technical replicates were done per sample. Expression level for each repair

gene was normalized to 18S RNA gene using the Delta-Ct (2ΔCt) method to analyse

relative changes in gene expression concerning the housekeeping expression.

Table 1: Oligonucleotide sequences of the PCR primers used to assay the repair activity by RT-qPCR.

Primer name	Primer set (5`-3`)	Amplicon size (bp)	Annealing Tm (ºC)	Accesion number
Bcl2	F: GTCACTCGTTCAGACCCTCA R: AAGAATGCAATGATCCGCCC	203	60	NM_001030253.2
noxa	F: ATGGCGAAGAAAGAGCAAAC R: CGCTTCCCCTCCATTTGTAT	134	62	NM_001045474.3
p53	F: CCCATATGAAGCACCACAGC R: CACACACGCACCTCAAAAGA	171	63	XM: 005165104.3

Statistical analysis

Data analysis was carried out using a computerized package generated by SPSS 24.0 software

for Windows (IBM, EEUU). The results were expressed as mean ± SEM. One-way ANOVA test

followed by DMS or Games Howell posthoc test (p<0.05) was used to analyze parametric data.

Results

Genotoxic damage in sperm

The analysis of the percentage of cells with different degree of fragmented DNA

using the comet assay showed that UV-irradiation significantly increased the DNA

fragmentation. The average of tail DNA, that indicates the percentage of fragmented

DNA in cells, increased from 3.84% ± 1.12 in control sperm to 42.09% ± 2.15 and 39.2% ± 0.68 in treated samples (30s and 40s of UV irradiation respectively) (Figure 1A). In addition, 94.17% ± 5.82 of the control cells contained less than 10% fragmented DNA, whereas in the treated samples there were no cells in that range, most of them harbouring more than 30% fragmented DNA (75.6% ± 5.9 and 78.2% ± 8.22 in samples irradiated 30 and 40 s respectively) (Figure 1A). The Tunel assay (Figure 1B) also revealed a significant increase of DNA fragmentation, with almost 12% more positive cells in UV irradiated samples (19% ± 0.38 and 18.89% ± 0.47 in 30s and 40s irradiated samples respectively) than in control samples (7.91% ± 0.46).
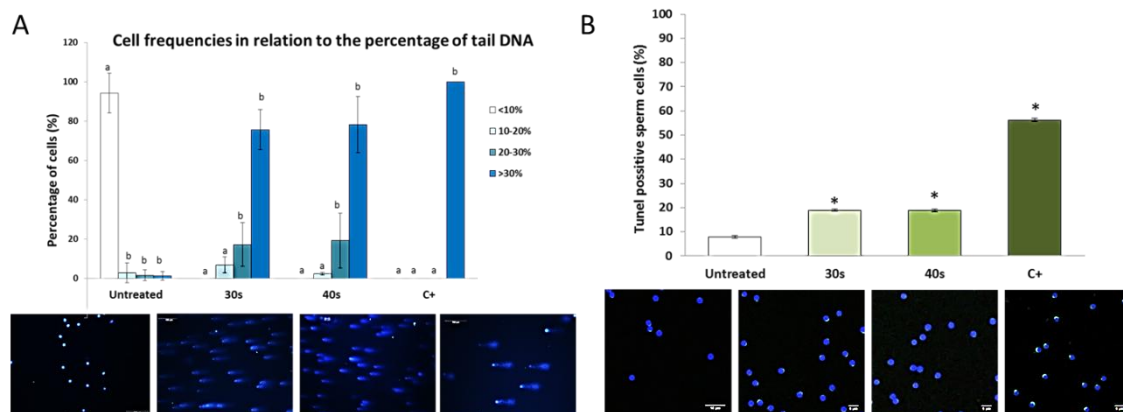

Figure 1: Sperm DNA integrity. A: Cell frequencies in relation to the percentage of tail DNA by comet assay. Four ratios of DNA fragmentation are shown for each type of spermatic cells (untreated sperm and treated with 30 s or 40 s of UV irradiation). Representative comet assay images (20x) from sperm cells are shown   B: Relative intensity of Tunel positive cells. Representative comet assay images (40x) from sperm cells are shown. Positive control is shown in both cases. Data indicate mean ± SEM (n=3). Different letters and asterisks indicate significant differences (*p*<0.05).

Progeny performance

As showed in figure 2, the sperm treatment did not affect the fertility rates (Figure 2A). The survival rate at 72 hpf was clearly lower in progenies from treated sperm than in those from control ones (Figure 2B), showing an increase in the mortality rate from 8 hpf to 24 hpf and 72 hpf (Figure 2C).
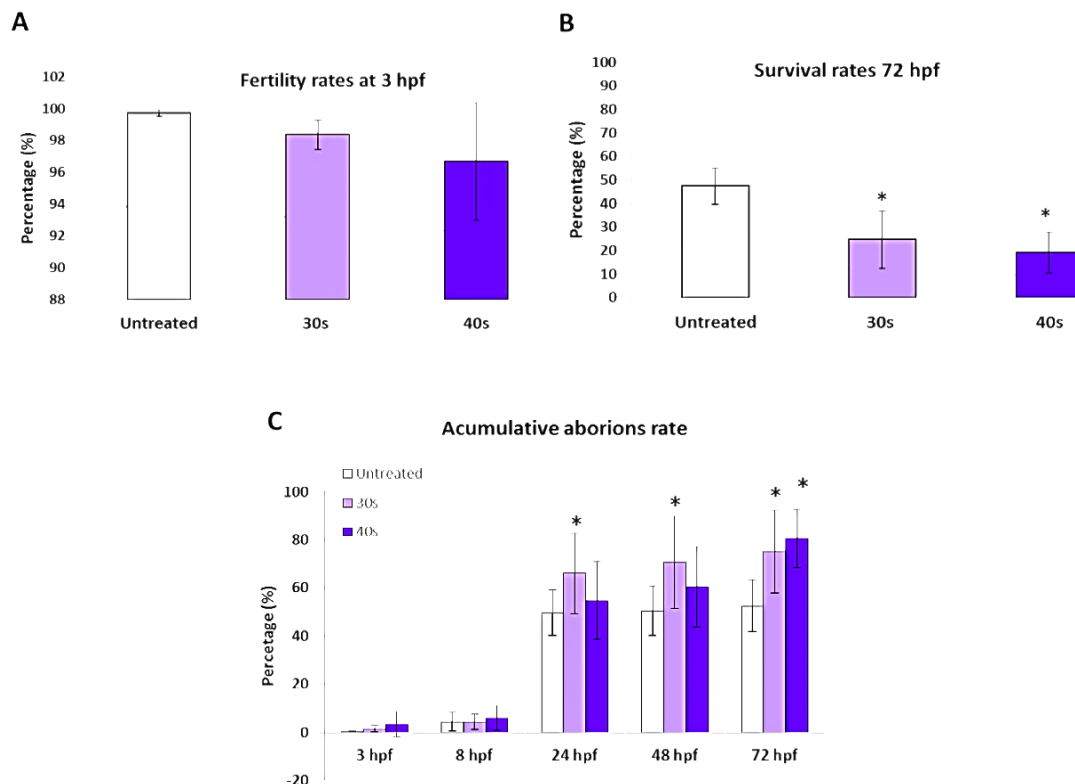

Figure 2: A: Capacity of sperm to fertlize measuring at 3 hours post fecundation (hpf) and B: Survival rates at 72 hpf in progenies obtained from untreated sperm and treated with 30 s and 40 s of UV irradiation; C: Acumulative abortions rate at 3 hpf, 8 hpf, 24 hpf, 48 hpf and 72 hpf. Data are shown as mean ± SEM (n=4). Asterisks indicate significant differences ($p<0.05$).

The percentage of malformed larvae 72 hpf was extremely high in those batches from treated sperm, reaching almost 100% malformation rate (96.87% ± 3.12 and 89.28 ± 10.71 in batches from sperm treated 30s and 40s respectively) (Figure 3A). Most of the embryos displayed several lesions simultaneously (Figure 3), having a

global phenotype modification (axial torsion, cardiac edema, defects in yolk conformation, inability to hatch and lower level of pigmentation). In addition, the chondrogenesis was deeply affected. As it is shown in the figure 3, the cartilage is not well defined in those progenies from treated sperm, deeply affecting the craneofacial skeleton formation.
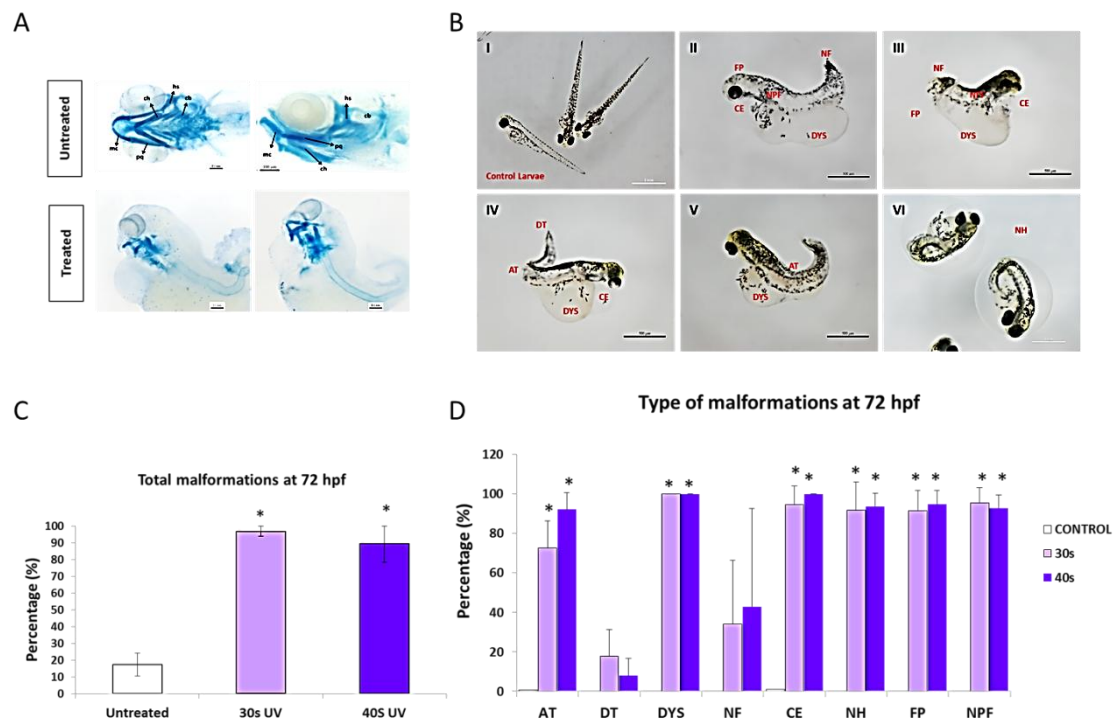

Figure 3: Phenotype of larvae from untreated sperm and treated with 30 s and 40 s of UV irradiationA: Representation of mandibular cartilage from zebrafish embryos 6 days post fecundation (dpf). Abbreviations used: mc, meckel´s cartilage., pq, palatoquadrate., bh, basihyal., ch, ceratohyal., hs, hyosymplectic., cb, ceratobranchial. B: Types of malformations observed at 72 hpf. AT: Axial torsion., DT: Distal torsion., DYS: Defective Yolk-sac., NF: No fin., CE: Cardiac edema., NH: No hatching., FP: Failed pigmentation., NPF: No pectoral fin. C: Percentage of total malformations., D: Percentage of specific malformations. Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences ($p<0.05$).

Immunodetection of the hallmarks of DNA repair (yH2AX and 53BP1) (Figure 4) revealed that at the earlier stage (1K) the embryos showed the more intense repairing activity, particularly in batches from treated sperm. The presence of both, γ-H2AX and 53BP1 was twice the detected in embryos from untreated sperm. A slight decrease in the repairing activity was observed 8 hpf in the same batches respect to the control ones and no differences respect to the control batches were observed at 24 hpf.
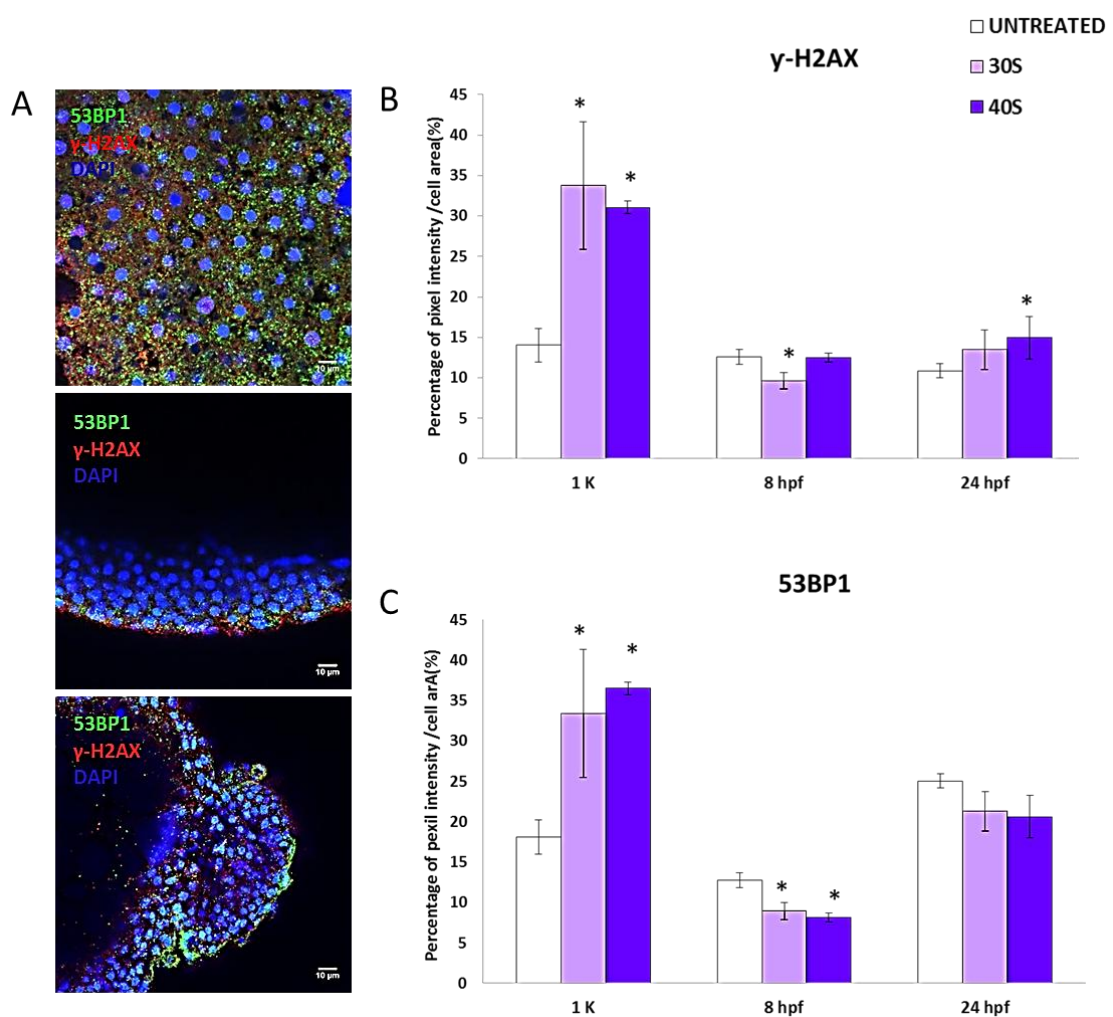

Figure 4: Repair ability during embryo development at 1 K, 8 hpf, 24 hpf and 72 hpf in progenies from untreated sperm and treated with 30 s and 40 s of UV irradiation. A: 53BP1 and γ-H2AX immunolocalization. Representative confocal images (40x) from whole embryos. Cell nuclei was contrasted with DAPI. Percentage of pixel intensity per celular area for B: γ-H2AX and C: 53BP1. Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences ($p<0.05$).

The immunodetection of p53-phosphorilated also indicated a very significant increase in the activation of p53 in the progenies from irradiated sperm (Figure 5A-B), particularly at 8hpf and in embryos from samples irradiated 40s. This pattern was consistent with the expression of p53 in the same developmental stages, showing an up-regulation in those batches from DNA damaged sperm (Figure 5C).
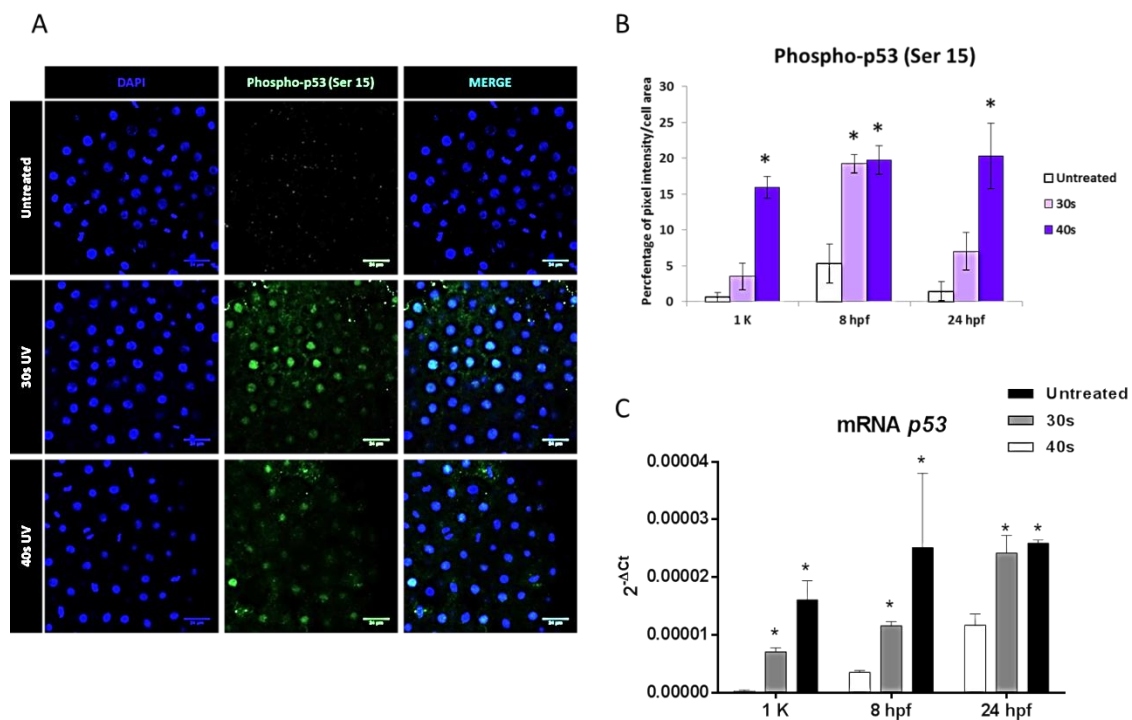

Figure 5: A: Phopho p53 (Ser 15) immunolocalization. Representative confocal images (40x) from whole embryos. Cell nuclei was contrasted with DAPI. B: Percentage of pixel intensity per celular area and C:: mRNA levels of *p53* at different stages during development (1 K, 8 hpf and 24 hpf in progenies obtained from untreated sperm and treated with 30 s and 40 s of UV irradiation. Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences (*p*<0.05).

The expression of pro-apoptotic gene *noxa* (Figure 6A) was significantly lower in embryos from irradiated sperm at 8 hpf and 24 hpf, but no changes were noticed during mid-blastula transition. *bcl2* showed an opposite expression pattern. A great increase in *bcl2* was observed from zygotic activation (1K) to organogenesis (24hpf) in

progenies from treated sperm (Figure 6B). The apoptotic activity evaluated by the detection of annexin V varied during development, being maximum at 75% epibolia (8hpf). Progenies from treated sperm displayed a slightly enhanced apoptotic activity than those from control sperm at that stage (Figure 6C).
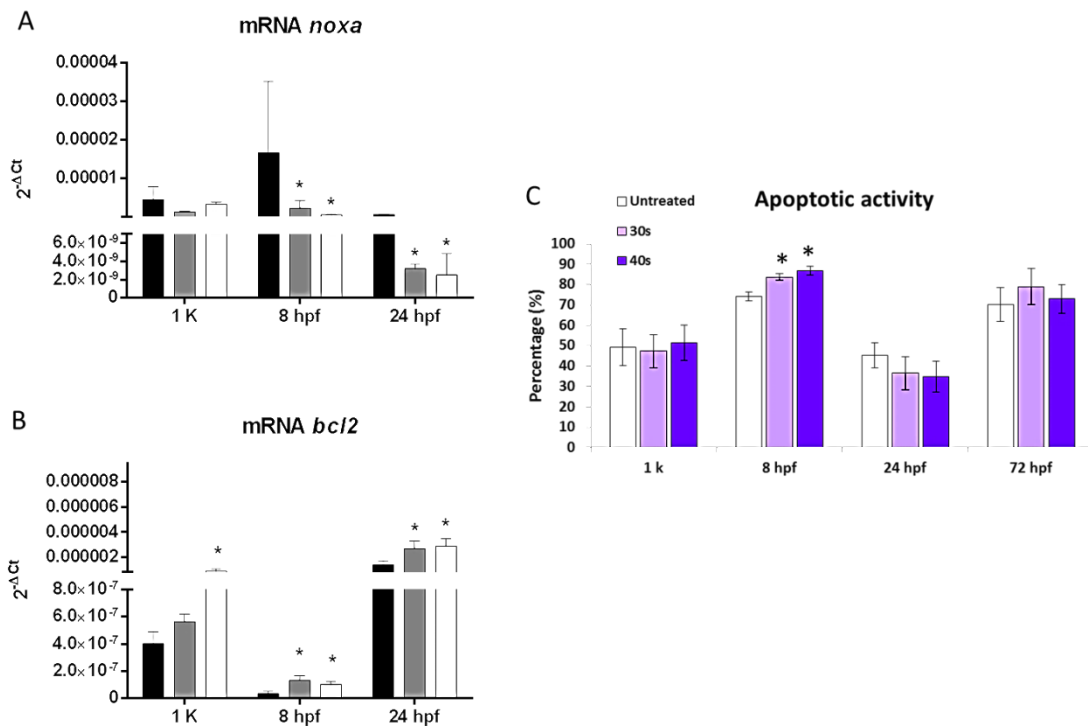

Figure 6: A-B: mRNA levels of genes involved in DNA damage response (DDR) during embryo development at 1 K, 8 hpf and 24 hpf in progenies from untreated sperm and treated with 30 s and 40 s of UV irradiation. A: Expression of *noxa*., B: Expression of *bcl2.* C: Apototic activity measured by flow citometry at different stages during development (1 K, 8 hpf, 24 hpf and 72 hpf). Data are shown as mean ± SEM (n=3). Asterisks indicate significant differences ($p<0.05$).

Discussion

The good quality of the germinal cells is a mandatory condition to generate a healthy progeny. Upon the fusion of male and female gametes a cascade of interactive events among oocyte and sperm components will determine the embryo performance

(Anifandis et al., 2014). It is known that, in mammals, DNA damaged spermatozoa (DDS) increase the embryo genomic instability and lead to pregnancy loss, increase of abortions, birth defects and genetic diseases in the offspring (González-marín et al., 2012; Marchetti et al., 2007, 2004; Robinson et al., 2012). The sperm used in this work was submitted to different levels of UV irradiation (30s and 40s) promoting severe lesions in all the treated cells in absence of oxidative stress (data no showed). The genotoxic damage did not affect the fertilization ability, but severely compromised the progeny performance. It is known that the oocyte contains the transcripts and proteins in charge of repairing the sperm DNA lesions before the zygotic genomic activation (Jaroudi et al., 2009; Menezo et al., 2007). In most cases the repairing ability compensates the spermatic damage and the development progresses properly, but the percentage of DNA lesions that can be repaired is limited (Gunes et al., 2015; Pérez-Cerezales et al., 2011). It is considered than trout oocytes are able to repair up to 10% of DNA damage (Pérez-Cerezales et al., 2011), Ahmadi and cols., (Ahmadi and Ng, 1999) estimated that less than 8% is repaired in mouse and, according to Evenson (Evenson and Loma, 2000) human oocyte could be able to repair up to 30% of DNA damaged sperm (DDS). In any case the repairing capacity will depend not only on the extent, and on the type of DNA damage, but also on the oocyte characteristics. Some studies revealing huge variations in the oocyte repairing ability (Fernandez-Diez et al., 2016; Generoso WM., 1980). The analyses performed in this work, revealed that approximately 80% of the sperm cells showed a range of fragmented DNA higher than 30% using comet assay, and around 20% of sperm cells were positive using Tunel assay, very likely overtaking the normal capacity of zygotic repair. When DDs overcome the repairing ability, two scenarios can be generated: the damage is non-compatible

with development conducting to embryo death or the development progresses in spite of having an unrepaired genome (González-marín et al., 2012; Marchetti et al., 2004).

Different studies have shown the importance of early developmental stages in this respect. It has been demonstrated that, in mammals, zygotic checkpoints are sensitive to DNA lesions activating the cell cycle arrest, the repair pathways or even the apoptosis, before the first mitosis to prevent chromosome fragmentation, embryo loss, and infertility (Chen et al., 2012; Ladsta, 2016; Marchetti et al., 2015; Wang et al., 2013). Some lesions can escape this surveillance mechanism and suffer defective repairing which leads to chromosomal aberrations in the daughter cells (Marchetti et al., 2015). Marchetti and cols., (Marchetti et al., 2004) performed a cytogenetic analysis of the progenies from male mouse treated with different mutagens, concluding that the chromosomal aberrations paternally transmitted to the progeny were formed before the end of the first S phase after fertilization.

Our data revealed a significant genetic stress which required an intense repairing activity at the mid blastula transition stage (MBT) (3 hpf) in those embryos obtained with treated sperm. This is the stage during which the zebrafish undergone the progressive transition from maternal to zygotic genome (Haberle et al., 2014; Lee et al., 2014). The increase in γH2AX and 53BP1, indicates the recruiting of repairing factors at double strand breaks (DSB) sites (Durik et al., 2015; Podhorecka et al., 2010), clearly suggesting an increased repairing effort in those embryos from DDS, more genetically unstable. Nevertheless, we did not show a delay in development nor a decrease in embryo viability, revealing that embryos go ahead with development independently of the presence of a significantly increased in the number of DNA

damaged spots. The dependence of the maternal genome (transcripts and proteins) at this stage could allow to the normal development in the presence of embryo genotoxic damage. It is also known during mammalian development, the death of embryos with structural aberrations does not seems to occur during the first cycles of development (Marchetti et al., 2004). This delay has been related to the control exercised by the maternal factors up to the full activation of the zygotic genome and by the inefficiency of the cell cycle check points at early development.

At 75% epibolia (8hpf) development progresses in all embryos, both from control and treated sperm, without showing signs of increased mortality rates. The DNA repair foci decrease even at lower levels than in control batches, suggesting the end of the massive repairing activity of the paternal damage. An increase of the apoptosis is observed at this stage, mainly in samples from DDS, which could reflect the activation of mechanisms avoiding the survival of cells with residual damage at this stage (Hakem et al., 2008), when the dependence of the embryonic transcripts is established (Haberle et al., 2014). This process represents a programing cell death to facilitate normal embryonic development or to prevent the spread of a localized lesion, allowing for the survival of the organism as a whole (Montero et al., 2016).

Embryo death clearly increases at organogenesis (24hpf), more intensively in embryos from DDS but the DNA repair effort and the apoptotic activity show the same levels than in control embryos. Approximately half of the embryos than in control batches reach the hatching stage (72hpf) in those batches from treated sperm, but almost all of them show severe malformations incompatible with long term survival. The malformations affect different processes: chondrogenesis, skeleton

morphogenesis, pigmentation, heart morphogenesis, angiogenesis and lymphatic vessels formation (or edemas), showing that organogenesis was severely impaired in embryos obtained with damaged sperm. The affected developmental processed are regulated by different pathways, indicating that development has progressed in spite of showing a miss regulation of a range of essential processes in development. This fact indicates that the big repairing effort developed during the earlier stages was insufficient to properly repair the inherited DNA lesions, which probably promoted different kind of chromosomal rearrangements in the embryo cells similar to those observed by Marchetti and cols., in mouse blastomeres (Marchetti et al., 2015). Moreover, it indicates that the surveillance mechanisms of genotoxicity, are not very astringent during embryo development and were not activated in spite of such an intense instability.

Nevertheless, the study of p53 expression and activation (amplification of *p53* transcripts by RT-PCR and immunodetection of p53P) showed a clear activation of the tumour suppressor factor p53 in the progenies from DDS at all the developmental stages, both at transcriptional and posttranslational levels. This activation is usually followed by the downstream activation of the surveillance mechanisms against genotoxic damage. These downstream events are mediated by the transcriptional activity of p53 which could affect a wide number of genes driving to cell cycle arrest, to DNA repair or to apoptosis (Vousden KH and Lu X, 2002). Our results showed that development progresses and there are no signs of an increased apoptotic activity. Moreover, in spite of the high expression and activation of p53, the downstream pro-apoptotic factor *noxa* showed a significant downregulation, whereas the anti-apoptotic gene *bcl2* was upregulated. The balance between pro and anti-apoptotic

genes suggest a repressive apoptotic scenario in batches from DDS that would repress the loss of cells with unrepaired damage.

The observed progression with development in the embryos from DDS, together with the activation of p53 in absence of an enhanced apoptosis or repairing activity, points to the potential implication of p53 in other pathways or to a downstream inhibition of p53. In fact, a role of p53 as enhancer of cell tolerance to DNA damage has been recently described in UV irradiated cells (Hamp et al., 2016; Lerner et al., 2017). DNA unrepaired lesions may evade detection and persist into S-phase. It is often more advantageous to circumvent replicative arrests and postpone repair of the offending damage to complete the cell cycle and maintain cell survival (Lerner et al., 2017). This process is referred as DNA damage tolerance DDT or lesion bypass. DNA lesions usually block the advance of polymerases at replicative forks, but from a cell survival perspective, bypassing of stalled forks can be accomplished by specific DNA polymerases (POL eta, POLi), dependent of the activation of p53 (Hamp et al., 2016; Lerner et al., 2017). In these particular cases, p53 plays a key role on the deceleration of nascent DNA elongation at replication barriers, facilitating the resolution of stalled forks (Hamp et al., 2016). The direct role of p53-in the translesion DNA synthesis has thus a protective effect on cell survival and a key role in DDT. Moreover, in zebrafish embryos submitted to genotoxic agents, an alternative isoform of p53 (Δ113p53) has been identified to antagonize the p53-depending apoptosis through the activation of the anti-apoptotic factor bcl2 (Chen et al., 2009).

Our results reveal a high level of tolerance to DNA damage up to hatch that could be mediated by a p53 dependent mechanism. A dowregulated apoptosis was

observed in embryos from other external fertilizers submitted to different kind of genotoxic stress: corals in acidic environment (Tchernov et al., 2011) trout embryos whose zygotic DNA repair activity was inhibited (Fernández-Díez et al., 2015) and trout embryos from oocytes with a compromised quality (Fernandez-Diez et al., 2016). The variable efficiency of repairing pathways during gametogenesis and some steps of embryo development tolerate a limited number of mutations, allowing that evolution takes place (Jaroudi and Sengupta, 2007), however the range of damage tolerated by fish embryos seems to overtake the mammalian degree of tolerance. Our study seems to confirm that embryo tolerance to DNA damage could be a survival mechanism in animals with a reproductive strategy based on the production of a high number of siblings with a low rate of long term survival. Tolerance to unrepaired DNA from sperm could introduce new mutations, some of them potentially advantageous to face a changing environment. The enhanced expression and activity of p53 and the repressed apoptotic activity, point to the activation of p53 dependent DDT pathways as the mechanism responsible for such genotoxic tolerance that deserves a deeper study.

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Conclusiones

Las conclusiones que derivan de los trabajos que constituyen la presente tesis doctoral son las siguientes:

1. La inhibición de poli (ADP – ribosa) polimerasa (PARP), esencial en la vía de reparación BER, durante la primera división zigótica de trucha arcoíris (*O. mykiss*), afecta al perfil transcriptómico de la progenie un día después de la eclosión, donde se muestra un amplio número de transcritos diferencialmente expresados implicados en diversas rutas y funciones celulares.

2. La inhibición de la ruta BER de reparación en zigotos de trucha arcoíris (*O. mykiss*) durante la primera división, muestra una regulación alternativa de genes implicados en el control del ciclo celular, la reparación del ADN y la apoptosis, que sugiere la supervivencia, hasta la eclosión, de aquellos embriones que han activado la reparación del genoma paterno usando rutas alternativas y generado un ambiente antiapoptótico tolerante al daño genético.

3. Las progenies de trucha arcoíris (*O. mykiss*) obtenidas con semen congelado/ descongelado y portador de ADN fragmentado, muestran cambios en el perfil transcriptómico un día tras la eclosión, que afectan fundamentalmente a la expresión de genes implicados en rutas metabólicas, lo que podría tener efectos sobre el desarrollo de la progenie a largo plazo; considerándose por tanto, el control del ADN dañado durante la criopreservación seminal absolutamente esencial para obtener una progenie saludable.

4. El retraso del periodo natural de puesta en neomachos y hembras de trucha arcoíris (*O. mykiss*) incrementa el daño genético en el esperma y afecta al perfil de transcritos implicados en la señalización y reparación del ADN del ovocito. Ambos factores, generan un aumento en la actividad reparadora en la descendencia

durante el desarrollo temprano y una baja habilidad para inducir apoptosis en fases posteriores del desarrollo, tolerando cierto nivel de daño genotóxico, que aumenta el porcentaje de descendientes malformados.

5. Las alteraciones de la capacidad de reparación del ovocito, como consecuencia del retraso del periodo natural de puesta, muestran su susceptibilidad a los cambios ambientales y afectan al desarrollo de la progenie de forma más intensa que las lesiones producidas en el ADN espermático. El perfil de transcritos de reparación del ovocito podría ser usado como marcador de calidad de las puestas.

6. La fecundación de ovocitos de pez cebra (*D. rerio*) con semen portador de daño genotóxico severo, provoca un gran aumento en la presencia de foci de reparación del ADN en los primeros estadios de desarrollo embrionario (1K). Las alteraciones fenotípicas producidas a la eclosión demostraron que el esfuerzo reparador fue insuficiente para corregir adecuadamente las lesiones heredadas del genoma paterno.

7. El desarrollo embrionario del pez cebra (*D. rerio*) obtenido con semen portador de daño genotóxico, progresa sin un aumento de actividad apoptótica ante una gran inestabilidad genómica. A pesar de mostrar una alta expresión y activación de p53, noxa (factor proapotótico) se mostró reprimido, mientras que bcl2 (factor antiapoptótico) se mostró sobre-expresado, indicando un escenario de represión de la apoptosis en aquellas progenies obtenidas con semen dañado. Los datos sugieren la implicación de p53 en la activación de rutas de tolerancia al daño genético o su represión por algún otro factor aguas abajo.

8. Nuestros resultados demuestran que, en peces, existe un alto grado de tolerancia al daño del ADN paterno, que podría ser un mecanismo evolutivo relacionado con

su propia estrategia reproductiva, basada en la producción de un alto número de descendientes con una baja tasa de supervivencia a largo plazo y una alta capacidad de adaptación a los cambios ambientales.