universidad de león

FACTORES QUE AFECTAN A LA CALIDAD ESPERMÁTICA Y HERRAMIENTAS EMERGENTES EN SU ANÁLISIS

Departamento de Biología Molecular

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A todos los que creyeron y creen en mí,

Eppur si muove

Galileo Galilei

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RESUMEN

En la presente tesis doctoral se utilizan técnicas de biología molecular y celular para analizar el impacto de distintos factores antropogénicos que afectan a la calidad del espermatozoide y se exploran vías para la mejora de la misma y para la selección de subpoblaciones espermáticas óptimas. Los factores de estudio están vinculados principalmente a dos áreas de conocimiento: la biotecnología de la reproducción humana y la producción animal. Para abarcar estos focos de interés biotecnológico, se utilizan tres especies de vertebrados distintas. Cada una de ella presenta características concretas que hacen posible abordar la investigación planteada: (I) el trabajo con el ser humano (*Homo sapiens*), permite profundizar en el estudio de técnicas aplicables en clínica y avanzar en el desarrollo de soluciones a los problemas reproductivos a los que nos enfrentamos, cuyo incremento ha sido significativo durante los últimos años; II) el lenguado senegalés (*Solea senegalensis*) presenta un interesante problema reproductivo asociado al factor masculino, vinculado a la estabulación de los ejemplares en el campo industrial de la acuicultura; y III). el pez cebra (*Danio rerio*) proporciona todas las ventajas propias de un animal modelo extensamente utilizado, permitiendo realizar investigación básica y facilitando el estudio del efecto transgeneracional de las alteraciones en el espermatozoide

En el **primer capítulo** de esta Tesis Doctoral se analiza el efecto sobre genes y transcritos concretos de un protocolo de criopreservación, utilizado de forma rutinaria en clínicas de reproducción asistida. La criopreservación del semen es un elemento relevante en cualquier protocolo de reproducción asistida, tanto en el ser humano como en otras especies animales. Durante años se han evaluado los efectos negativos del protocolo de congelación y descongelación sobre los espermatozoides de formas muy variadas. En este primer capítulo utilizamos una técnica novedosa de análisis y cuantificación de daño en genes concretos en muestras seminales humanas provenientes de donantes normozoospérmicos. La técnica está basada en un protocolo de PCR cuantitativa (qPCR) que permite estimar el número de lesiones sufridas en un gen concreto. Se estudiaron genes relacionados con éxito de la fecundación, con desarrollo embrionario temprano y dos relacionados con síndromes con mayor incidencia en los nacidos tras el uso de estas técnicas de reproducción asistida. Nuestro estudio corroboró la presencia de lesiones en los genes estudiados tras la criopreservación, siendo aquellos relacionados con las citadas enfermedades unos de los más afectados.

La segunda parte de este capítulo centra su atención en la posible alteración que un protocolo de criopreservación puede causar en los ARNm presentes en los espermatozoides. Hoy en día la importancia de los ARN espermáticos en la fecundación y desarrollo embrionario temprano es un hecho totalmente aceptado. Nuestros resultados han constatado la disminución de un transcrito descrito como potencial marcador de éxito

del embarazo y tres transcritos destacados como potenciales marcadores de calidad espermática. Tras la realización de este estudio hipotetizamos que la criopreservación puede alterar de forma estocástica determinados transcritos espermáticos. Es importante destacar, que en ambos casos, la inducción de lesiones en genes concretos y la disminución significativa de determinados transcritos, no tiene por qué traducirse en alteraciones directas en el desarrollo embrionario, pues también han de ser tenidos en cuenta los potentes mecanismos de reparación presentes en el ovocito. No obstante, estos estudios permiten desarrollar técnicas más precisas y eficaces en la detección de daños concretos potencialmente transmisibles al embrión, que pueden revertir en un mejor análisis de las muestras seminales y en una mejor selección de los protocolos de criopreservación utilizados. Estos estudios constituyen los dos primeros artículos derivados de esta Tesis Doctoral, publicados en *Andrology* y *Cryobiology*, y un capítulo de libro (incluido éste último en el apartado de ANEXOS).

El **segundo capítulo** se centra en el efecto adverso que la estabulación puede ejercer sobre la calidad espermática de una especie con elevado interés comercial en la industria de la acuicultura, el lenguado senegalés. El cultivo de esta especie se ve frenado por la disfunción reproductiva presente en los machos nacidos en cautividad (F1). Este hecho hace de este animal un interesante modelo para el estudio del efecto de la estabulación en la calidad seminal. Por un lado, se realizó una valoración mediante citometría de flujo del porcentaje de espermatozoides apoptóticos presentes en muestras seminales de reproductores nacidos en cautividad, utilizando diferentes protocolos de análisis (YO-PRO 1 y caspasas). El estudio reveló un incremento en el porcentaje de células apoptóticas en animales nacidos en cautividad en comparación con muestras seminales de animales nacidos en libertad. Se corroboró que el método de análisis mediante caspasas es más específico a la hora de cuantificar apoptosis. En este primer trabajo se implementó por primera vez en peces teleósteos la tecnología MACS (*magnetic activated cell sorting*). Esta tecnología podría, por tanto, ser utilizada antes de la criopreservación de muestras seminales permitiendo mantener en los bancos de germoplasma de las empresas o los centros de investigación material biológico de calidad para su uso posterior en fecundación artificial hasta que se consiga solucionar la fecundación natural en las generaciones criadas en cautividad.

Además, realizamos una descripción comparativa entre los machos capturados en el medio natural y y los ejemplares de la primera generación (F1, nacidos en cautividad) desde otros dos nuevos enfoques: analizando viabilidad y los niveles de especies reactivas del oxígeno (ROS) por citometría de flujo. Nuestros resultados proporcionaron porcentajes semejantes de viabilidad y especies reactivas del oxígeno en los dos grupos de estudio. Se

constató que la presencia de ROS en los espermatozoides de esta especie es, por lo general, elevada, pero no siempre indicativa de disfunción espermática. De hecho, los machos F1 con espermatozoides con motilidad alta presentaron índices de ROS elevados, de forma semejante a lo observado en las muestras provenientes de animales salvajes Los estudios por microscopía confocal sugieren que la distribución intracelular de ROS puede ser clave para la interpretación de los resultados, ya que mientras que una elevada presencia de ROS a nivel mitocondrial puede responder a una característica propia de la especie, altos niveles en el núcleo podrían ser claramente negativos.

Por otro lado, en este capítulo también se estudió el efecto de la criopreservación en los niveles de ROS en ambos grupos experimentales (nacidos en el medio natural *versus* nacidos en cautividad). La criopreservación afectó significativamente la viabilidad en los machos de la primera generación y las especies reactivas del oxígeno mostraron una tendencia a la disminución.

Estos resultados han dado lugar a los dos artículos científicos que constituyen este segundo capítulo (publicados en *Theriogenology* y *Reproduction*). Además, los estudios realizados dentro de esta tesis doctoral, en aras de una mejor estabulación y un mejor cultivo de los ejemplares de lenguado senegalés, han permitido la realización de un prototipo que ha resultado ganador en el Concurso Transfronterizo de Prototipos orientados al Mercado. La memoria de dicho prototipo ha sido incluida en el apartado de ANEXOS.

El **tercer capítulo** aborda el efecto potencial que los disruptores endocrinos de naturaleza antropogénica pueden tener sobre los machos de las poblaciones salvajes de peces y su potencial efecto en sus progenies. Se estudió la exposición al disruptor endocrino 17-α-etinilestradiol (principio activo de las píldoras anticonceptivas encontrado en aguas superficiales) capaz de mimetizarse con el ligando natural estradiol uniéndose a los receptores de estrógenos. Para ello, se expusieron machos de pez cebra a dosis ambientalmente relevantes durante las primeras etapas de la espermatogénesis. Los resultados indicaron una modificación en la expresión testicular de receptores de estrógenos que también fue observada en los espermatozoides. Además, la exposición reveló patrones de expresión de marcadores moleculares semejantes a los reportados previamente en malos reproductores indicando un efecto negativo sobre la capacidad reproductiva de los machos. Las progenies obtenidas tras cruzar los animales expuestos con hembras no expuestas revelaron una mayor tasa de malformaciones en aquellas provenientes de machos tratados frente a las progenies control. Las anomalías más

abundantes fueron relacionadas con la formación de edemas linfáticos principalmente en la parte anterior de los embriones.

A través de estudios de expresión génica de genes relacionados con linfoangiogénesis y con migración de las células endoteliales se comprobó la existencia de una desregulación genética en esta zona del embrión. Estos resultados se correlacionaron con variaciones en la expresión de los receptores de estrógenos en los mismos tejidos. Además de estas observaciones moleculares, se comprobó una modificación en el desarrollo del otolito en las larvas y una variación en su patrón de comportamiento, indicando que el efecto del tóxico es heredable vía paterna causando efectos perdurables. Estos resultados se encuentran enviados para publicación en la actualidad.

En el **cuarto** y último **capítulo** de esta Tesis Doctoral se explora una posible vía de mejora de la calidad seminal mediante la administración de cepas de microorganismos probióticos como suplemento alimenticio. En el primero de los dos artículos que constituyen este último capítulo (*Journal of Applied Ichthyolog*y) analizamos el efecto de la administración de una cepa comercial de probióticos sobre una batería de transcritos cuya expresión había sido previamente descrita como marcador de buenos y malos reproductores en el pez cebra. Esta novedosa utilización de los probióticos como posibles agentes moduladores de la calidad seminal, proporcionó interesantes resultados, mostrando variaciones significativas en la expresión de tres de los marcadores estudiados, sugiriendo efectos beneficiosos a nivel molecular

En la segunda parte de este capítulo, exploramos la opción de la administración de dos cepas de probióticos con capacidad antioxidante y antiinflamatoria suministrada por la empresa Biópolis S.A. como elemento de mejora de la calidad espermática en el ser humano. La ingesta mejoró significativamente la motilidad en muestras provenientes de donantes astenozoospérmicos. Considerando que este aumento podría estar correlacionado con las capacidades antioxidantes de las cepas, se realizaron estudios de fragmentación del ADN mediante la técnica SCSA (*sperm chromatin structure assay*) y se analizó la presencia de especies reactivas del oxígeno mediante citometría de flujo. Nuestros resultados demostraron descensos significativos en los niveles de fragmentación y de especies reactivas del oxígeno, avalando la hipótesis planteada. Estos novedosos resultados han sido patentados (patente incluida en el apartado de ANEXOS) y publicados en la revista *Beneficial Microbe*s.

INTRODUCCIÓN

La reproducción en los animales vertebrados requiere de multitud de procesos moleculares y celulares perfectamente orquestados. La correcta sucesión de estos procesos bajo el complejo conjunto de señalizaciones celulares que los regulan, aseguran la producción de una descendencia viable.

La calidad de los gametos incide de forma crucial en el éxito reproductivo. Históricamente, se ha atribuido el mayor peso en el éxito o el fracaso reproductor al factor femenino, posiblemente en relación con la influencia materna durante el proceso de gestación en los mamíferos. Este hecho motivó que durante décadas no se le otorgara suficiente importancia al factor masculino. Sin embargo hoy en día, existen estudios recientes que señalan que el aporte masculino y femenino son equivalentemente importantes en la contribución a un embrión totipotente que pueda diferenciarse con éxito en un individuo sano. La presente tesis doctoral focaliza su campo de estudio en el factor masculino.

Existen parámetros que definen la calidad seminal y permiten estimar su fertilidad potencial. Dichos parámetros son claves para estudiar el efecto de factores externos en la calidad del espermatozoide. De este modo, los métodos de evaluación de calidad espermática se convierten en unas herramientas de gran valor y utilidad en los campos de estudio de la biología de la reproducción y la biotecnología en diferentes especies de vertebrados. Se permite, con ellos, abordar diseños experimentales que tengan como objetivo evaluar el efecto, directo o indirecto, de diferentes factores extrínsecos sobre las células germinales masculinas. Las conclusiones derivadas de estos estudios pueden proporcionar: 1) un mayor conocimiento teórico en el campo de la biología reproductiva, mediante la obtención de conclusiones globales para varias especies, a partir de estudios en especies modelo o 2) conclusiones enfocadas a la búsqueda de soluciones a problemas prácticos concretos en especies con elevado valor económico en el mercado. Estos hallazgos pueden tener gran interés desde el punto de vista económico si son relevantes para la biotecnología reproductiva humana o de otras especies de alto interés comercial. En esta tesis se abordan ambos enfoques, pretendiéndose profundizar en conocimientos teóricos mediante el empleo de especies modelo como el pez cebra, sin abandonar la búsqueda de la aplicación de dichos hallazgos en la solución de problemas concretos, bien sea para la especie humana, o para especies de interés comercial dentro de la industria de la acuicultura como el lenguado senegalés.

Biología del espermatozoide

Los espermatozoides son células haploides altamente diferenciadas (Alberts et al. 2008). Los gametos masculinos podrían ser considerados, de forma simplificada, como vectores móviles que tendrían como fin último el transporte de la dotación genética paterna al embrión. Durante los últimos años se ha comprobado que además de la información genética, el patrón epigenético masculino parece tener una importancia clave (Puri et al. 2010). Además, el ADN no es la única molécula masculina crucial para el desarrollo embrionario temprano que es transferida en la fecundación. Existen diversos ARN y proteínas (Jodar et al. 2016; Schuster et al. 2016) que han demostrado ser claves en las primeras etapas de desarrollo embrionario.

Los espermatozoides son generados tras un complicado proceso biológico denominado espermatogénesis. En los vertebrados, este fenómeno tiene lugar en los testículos de forma continuada durante toda la vida sexual del macho. El proceso se caracteriza por una secuencia de cambios morfológicos y fisiológicos de las células germinales en el que un pequeño número de células troncales diploides produce un gran número de células haploides con genoma recombinado (Costa et al. 2005). Comprende tres etapas sucesivas: la espermatocitogénesis, la división meiótica y la espermiogénesis (Schulz and Miura 2002).

La espermatocitogénesis está caracterizada fundamentalmente por la división mitótica y la diferenciación celular de las espermatogonias (células derivadas de las células germinales primordiales). En el individuo adulto, según las características nucleares, estas células diploides se pueden subdividir en: tipo A (con un núcleo de cromatina laxa y un nucléolo por lo general muy próximo a la membrana nuclear) y tipo B (con núcleos de cromatina densa y nucléolos en posición central). Un grupo de espermatogonias de tipo A, por sucesivas mitosis, da origen a nuevas generaciones de espermatogonias de tipo A, actuando como células de reserva o células madre, conocidas como SSC (del inglés: *spermatogonial stem cells*) (de Rooij 2001). Otro grupo, independientemente, se diferencia para dar lugar a las de tipo B, que se diferenciarán, a su vez, hacia espermatocitos. (**Fig. 1A)**

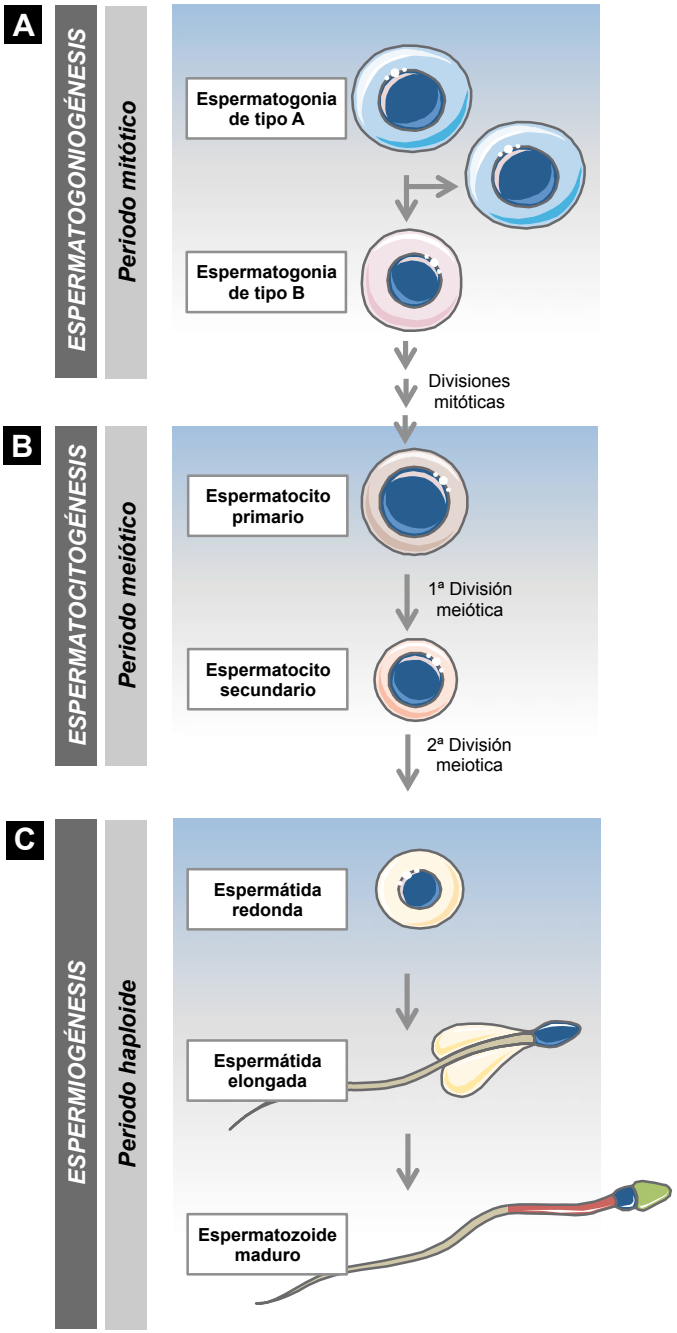

Figura 1.- Proceso de espermatogénesis. Tipos celulares y etapas. A: Espermatogénesis. B: espermatocitogénesis. C: Espermiogénesis.

La etapa de división meiótica conduce a la reducción del número de cromosomas y permite la recombinación cromosómica. Los espermatocitos primarios (diploides) por la primera división meiótica dan lugar a los espermatocitos secundarios (haploides). Esta primera división meiótica es muy prolongada (principalmente la profase) y dependiendo de la especie puede llegar a durar varias semanas. Los espermatocitos secundarios

generados, por la segunda división meiótica, dan lugar a las espermátidas, completándose así las divisiones celulares de la espermatogénesis **(Fig. 1B).**

La espermiogénesis comprende una serie de transformaciones celulares de las espermátidas. Tras el fin de la meiosis, estas células compactan densamente su cromatina con las proteínas condensadoras, manteniendo la expresión de los genes necesarios para el embalaje de la cromatina y la morfogénesis celular (Braun 1998). A pesar de estos importantes cambios nucleares, la mayoría de las modificaciones que sufren las espermátidas son citoplásmicas. Como resultado de esta fase, se diferencian los espermatozoides maduros. **(Fig. 1C)**

Los espermatozoides maduros de vertebrados constan de tres partes fundamentales fácilmente distinguibles por microscopía: cabeza, la pieza media y la cola (Müller 1997), limitada su estructura, como toda célula, por su propio plasmalema. La cabeza se corresponde con el núcleo celular. En la pieza media se encuentran el centriolo y las mitocondrias necesarias para proporcionar la energía suficiente para el movimiento celular. Por último, la cola (o colas dependiendo de la especie), está compuesta por el flagelo cuyas vainas fibrosas junto con el sistema de microtúbulos, permiten la mecánica del movimiento espermático.

A pesar de los rasgos generales comunes, existen diferencias en el proceso de espermatogénesis entre grupos de vertebrados (Schulz et al. 2010).

En vertebrados amniotas (reptiles, aves, mamíferos) como en la especie humana, los testículos contienen un número fijo de células de Sertoli “inmortales" que apoyan las ondas sucesivas de la espermatogénesis. Durante estas ondas, una célula de Sertoli proporciona soporte al mismo tiempo a células germinales en diferentes etapas de desarrollo (Schulz et al. 2005). Por ejemplo, una célula de Sertoli contacta basalmente con las espermatogonias, mientras que con las partes laterales lo hace con espermatocitos y espermátidas primarias, y las partes adluminal y apical con espermátidas tardías (**Fig. 2A**).

Por otro lado, en los vertebrados anamniotas (peces y anfibios) nos encontramos con espermatogénesis de tipo quísitica (Engel and Callard 2007). Existen dos diferencias principales en comparación con los testículos de vertebrados superiores. En primer lugar, dentro de los túbulos de espermatogénesis, las células de Sertoli generan extensiones citoplasmáticas que forman quistes que envuelven un solo grupo de células germinales derivadas de una única espermatogonia que se desarrollan de forma sincrónica. En segundo lugar, las células de Sertoli que conforman los quistes conservan su capacidad de proliferación también en peces adultos (Schulz et al. 2005). Por lo tanto, la unidad funcional básica del tejido de la espermatogénesis en los peces es un quiste formado por un grupo

dinámico de las células de Sertoli que rodea y soporta de forma sincrónica el desarrollo de un clon de células germinales (Schulz et al. 2005). **(fig. 2B y 2C)**.

En los peces teleósteos se puede realizar una subdivisión de la espermatogénesis (Schulz et al. 2010): en el primer tipo (espermatogonias con distribución restringida), las regiones distales del compartimiento germinal, cerca de la túnica albugínea, están ocupadas por las células de Sertoli que rodean las espermatogonias primarias indiferenciadas. A medida que las células se dividen y entran en la meiosis, los quistes migran hacia la región de los conductos espermáticos, situados en el centro del testículo, donde se produce la liberación de los espermatozoides. Este tipo de disposición se encuentra los órdenes: Atheriniformes, Cyprinodontiformes y Beloniformes (Parenti and Grier 2004). En el segundo tipo (sin restricciones en la distribución de espermatogonias), las espermatogonias se extienden a lo largo del compartimiento germinal en todo el testículo. Los quistes no migran ni se desplazan durante su desarrollo (Grier 1981). La distribución sin restricciones de espermatogonias se considera un patrón más primitivo que se encuentra en grupos taxonómicos menos evolucionados, tales como el orden Characiformes, como las pirañas; el orden Salmoniformes como las truchas o los salmones; o el orden Cypriniformes en el que se incluye al pez cebra (Parenti and Grier 2004). Sin embargo, también existe una forma de distribución intermedia o semicística tal y como la descrita en Perciformes, como la tilapia (Vilela et al. 2003); Pleuronectiformes como el lenguado senegalés (García-López et al. 2005) o Gadiformes, como el bacalao común (Almeida et al. 2008). En estas especies, las espermatogonias indiferenciadas muestran una ubicación preferida, pero no exclusiva, cerca de la túnica albugínea. Esto conlleva a una zonificación de los testículos: las primeras etapas de desarrollo de las células germinales residen en la periferia (cortex) y las fases avanzadas se encuentran cerca del conducto colector (médula) (**Fig. 2C**).

De forma general, el control endocrino del proceso reproductivo es semejante en todos los vertebrados (Chauvigné et al. 2016). Está regulado principalmente por las gonadotropinas generadas en la adenohipófisis: la hormona estimulante del folículo (FSH) y hormona luteinizante (LH), que son hormonas glicoproteicas heterodiméricas formadas por una subunidad específica β (Fshβ o Lhβ) y una subunidad común α, que activan sus receptores afines, el receptor de FSH (FSHR) y el receptor de LH y de la coriogonadotropina (LHCGR), en las gónadas (Holdcraft and Braun 2004; Pierce and Parsons 1981). En el testículo, la FSH se une el FSHR en las células de Sertoli para estimular la secreción de varios factores de crecimiento, que inician y mantienen la espermatogénesis, mientras que en las células de Leydig la unión de FSH/FSHR desencadena la síntesis de hormonas esteroideas tales como los andrógeneos: testosterona (T) y 11-cetotestosterona (11-KT) (Chauvigné et al. 2012; Schulz et al. 2010).

Como ejemplo de la complejidad celular y molecular del proceso de espermatogénesis, en el ser humano más de 2300 genes son temporal y espacialmente regulados para lograr generar una célula espermática funcional completamente diferenciada (Carrell et al. 2016). Es evidente, que cualquier fallo que acontezca durante todo el proceso puede afectar a la calidad final del gameto.

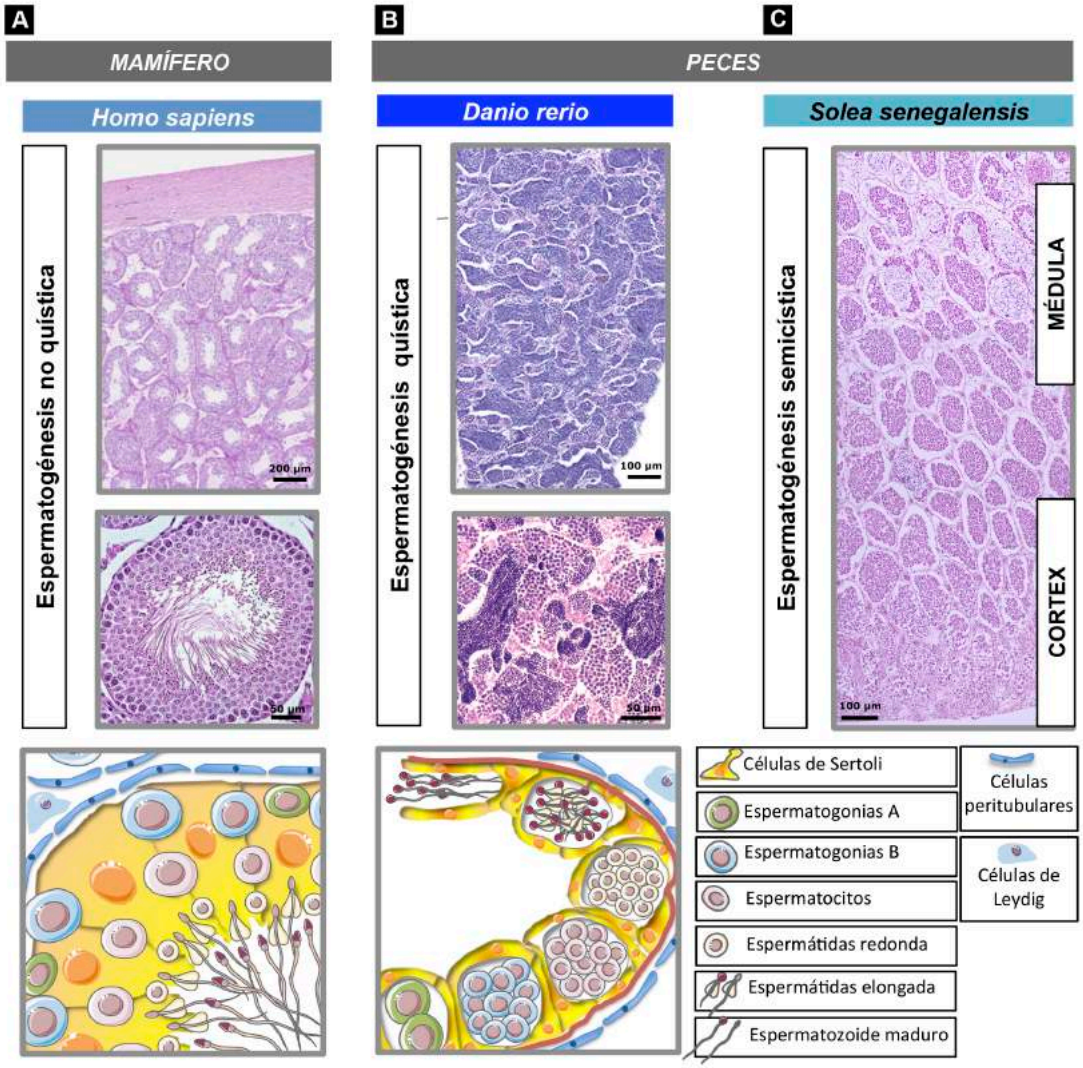

Figura 2.- Tipos de espermatogénesis en mamíferos y peces. Fotografías de microscopía óptica (tinción de hematoxilina-eosina). A: Mamíferos con espermatogénesis no quística (*Homo sapiens*). Micrografías obtenidas de www.histology.leeds.ac.uk B: Peces con espermatocitogénesis quística (*Danio rerio*) C: Peces con espermatogénesis semicística (*Solea senegalensis*).

Especies de estudio

La utilización de diferentes especies de vertebrados alejados evolutivamente entre sí permite la realización de diversos estudios enmarcados en diferentes campos de conocimiento. Mientras que por un lado el trabajo directo con la especie humana permite avanzar experimentalmente en las técnicas existentes ante los retos reproductivos que afrontamos como especie, el uso de animales modelo, permite realizar investigaciones previas para poder trasladar los resultados de unos organismos a otros. El trabajo con especies de interés en producción animal, a su vez, permite introducir los resultados de la investigación básica en el sector comercial en un ejercicio de aplicación directa y transferencia de conocimiento desde el laboratorio a la industria.

Bajo este prisma multiespecífico, en la presente tesis se trabaja con tres especies de vertebrados diferentes: la especie humana (*Homo sapiens* (Linnaeus, 1758)), el pez cebra (*Danio rerio* (Hamilton-Buchanan, 1822)) y el lenguado senegalés (*Solea senegalensis* (Kaup,1858)) **(Fig. 3)**. Los resultados esperan aportar avances en el campo de investigación básica, la biomedicina (en el área de la biotecnología de la reproducción), la ecotoxicología y en el sector industrial.

Existen evidentes diferencias entre mamíferos y peces extensibles a sus mecanismos reproductivos y sus gametos.

Los espermatozoides de mamíferos, como nuestra especie, una vez en el ambiente del tracto reproductivo femenino, sufren un fenómeno denominado capacitación espermática que consiste en una serie de cambios fisiológicos y bioquímicos dependientes del tiempo (Shur et al. 2004). Los cambios bioquímicos asociados con el proceso de capacitación incluyen: 1) un flujo de salida de colesterol de la membrana plasmática que conduce a un aumento de la fluidez de la membrana y la permeabilidad a bicarbonato e iones de calcio; 2) la hiperpolarización de la membrana plasmática (Hernández-González et al. 2006); 3) cambios en fosforilación de proteínas y en la actividad de la proteína quinasa (Arcelay et al. 2008; Baldi et al. 2000; Visconti 2009) y 4) el aumento de la concentración de bicarbonato y el pH intracelular, Ca^{2+} y los niveles de adenosín monofosfato cíclico (AMPc) (Ickowicz et al. 2012).

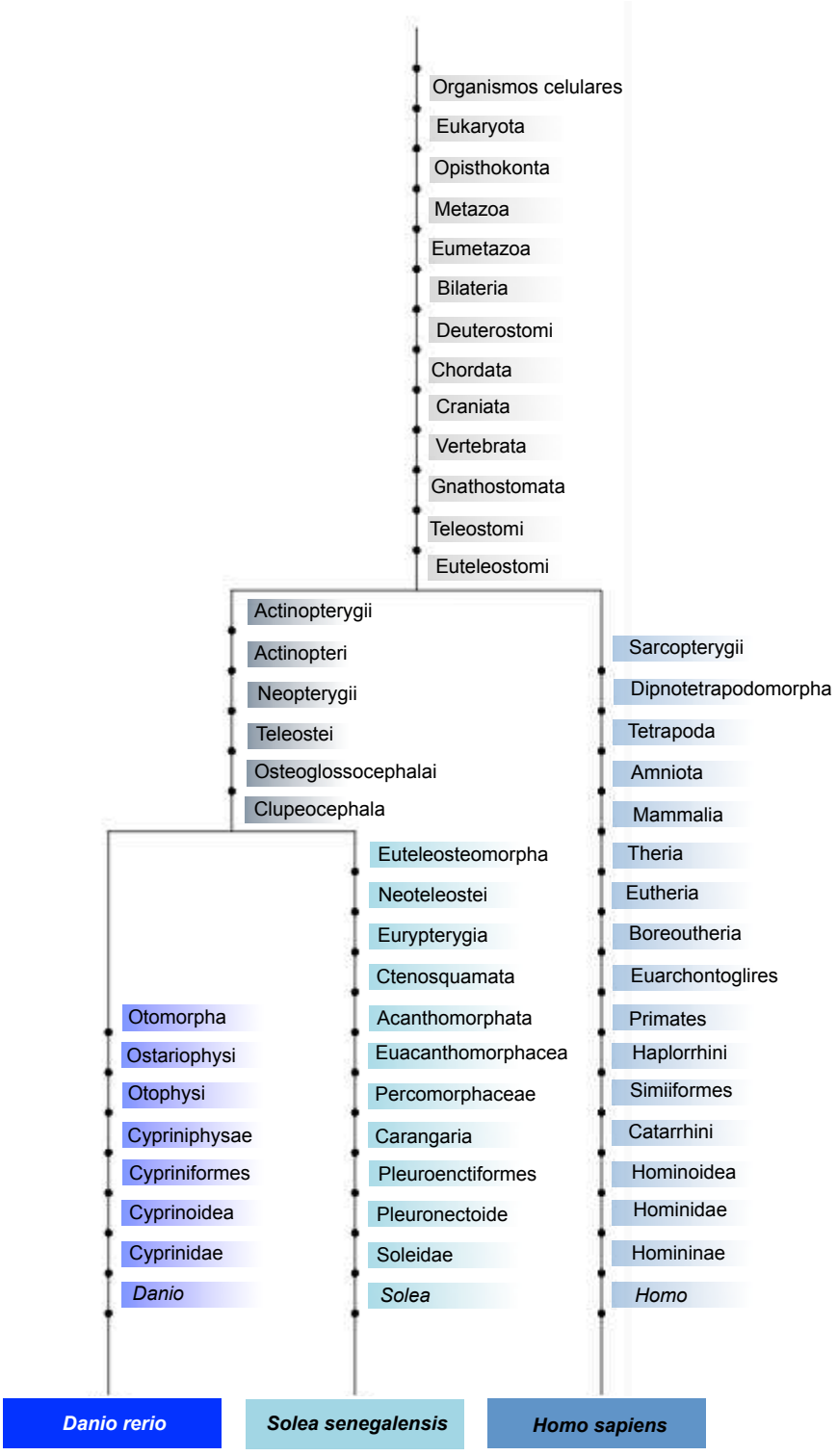

Figura 3.- Árbol filogenético con las tres especies de vertebrados a estudio: *Homo sapiens*, *Danio rerio* y *Solea senegalensis*. El diagrama ha sido generado con el *software online* PhyloT basado en los datos taxonómicos del NCBI y posteriormente se ha editado.

El espermatozoide capacitado penetra en el *cumulus oophorus* del óvulo y, a continuación, se une a la zona pelúcida con su membrana plasmática intacta. Esta unión ocurre a través de unos receptores para las glicoproteínas específicas de la zona pelúcida denominadas ZP, cuyos mecanismos de actuación siguen siendo investigados (Avella et al. 2013). Posteriormente, el espermatozoide se somete a un proceso de exocitosis denominado reacción acrosómica (Breitbart 2003; Florman et al. 2008; Roldan and Shi 2007; Shur et al. 2004). Este evento es necesario para la fecundación ya que permite el paso del espermatozoide a través de la zona pelúcida y su posterior fusión con el oolema del ovocito. Las enzimas y moléculas necesarias para que ocurra la reacción acrosómica son almacenadas en el acrosoma (Abou-Haila and Tulsiani 2000), una vesícula membranosa derivada del aparato de Golgi generada en la espermatogénesis que se encuentra rodeando la parte superior del núcleo en los espermatozoides maduros **(Fig. 4A)**.

Los peces son animales acuáticos y su fecundación es, por lo general, externa salvo excepciones en algunas especies (Huang et al. 2004). Esto implica que el esperma sea liberado en el agua y que los espermatozoides deban alcanzar y fecundar los ovocitos en este medio especialmente hostil (Cosson et al. 2008). Este hecho afecta directamente a las características fisiológicas de los espermatozoides **(Fig. 4B y 4C)**. Las diferencias principales con los espermatozoides de mamíferos están relacionadas directamente con el mecanismo de fecundación de este grupo de animales. Los ovocitos de peces teleósteos condicionan las características espermáticas. La mayoría de los peces presentan unos ovocitos de gran tamaño rodeados por un corion. Esta envuelta membranosa rígida está perforada por uno o más canales denominados micrópilos (Jalabert 2005). El gameto masculino debe alcanzar este poro, atravesarlo, llegar a la membrana plasmática y fecundar los ovocitos. El contacto entre membranas tiene lugar de forma directa entre el ovocito y el espermatozoide; esto provoca que, salvo excepciones como el esturión (Psenicka et al. 2007), no se requieran enzimas específicas para lisar las envueltas del ovocito, tales como las contenidas en el acrosoma de las especies de mamífero. Esta característica es resultado de la simplificación secundaria durante la evolución debido al desarrollo de las envueltas del ovocito (Cabrita et al. 2009).

La especie humana (*Homo sapiens*)

Interés científico

Durante las últimas décadas, la comunidad científica y médica ha registrado tendencias negativas en la salud reproductiva masculina en nuestra especie en los países con mayor desarrollo económico (Cissen et al. 2016). Hoy en día existe un incremento de la incidencia de diversos problemas reproductivos incluyendo el cáncer testicular, trastornos

del desarrollo sexual, criptorquidia, hipospadias, niveles bajos de testosterona y mala calidad del semen. Dichos trastornos conllevan al aumento de la demanda de técnicas de reproducción asistida (Skakkebaek et al. 2016). La comunidad científica y médica necesitan perfeccionar las potentes tecnologías existentes así como explorar nuevas estrategias para conseguir mejorar la calidad espermática y por lo tanto reducir los problemas de infertilidad.

Descripción del espermatozoide

El espermatozoide humano presenta una cabeza ovoide compuesta por el núcleo celular y el acrosoma localizado en la parte superior (**Fig. 4A**). El ADN nuclear está compactado mayoritariamente mediante protaminas (Kotaja 2014). El corto cuello espermático humano donde se localizan los centriolos (Palermo et al. 1997) es seguido por una pieza media que contiene un elevado número de mitocondrias que se encargan de generar la energía para el movimiento de la cola (Visco et al. 2010). El patrón del axonema es el habitual "9x2 +2" microtúbulos (Linck et al. 2016). Esto proporciona la fuerza propulsora permitiendo la locomoción de la célula espermática hacia el ovocito para la penetración. Una célula espermática humana tiene una longitud aproximada entre 45-50 μm (Mossman et al. 2013).

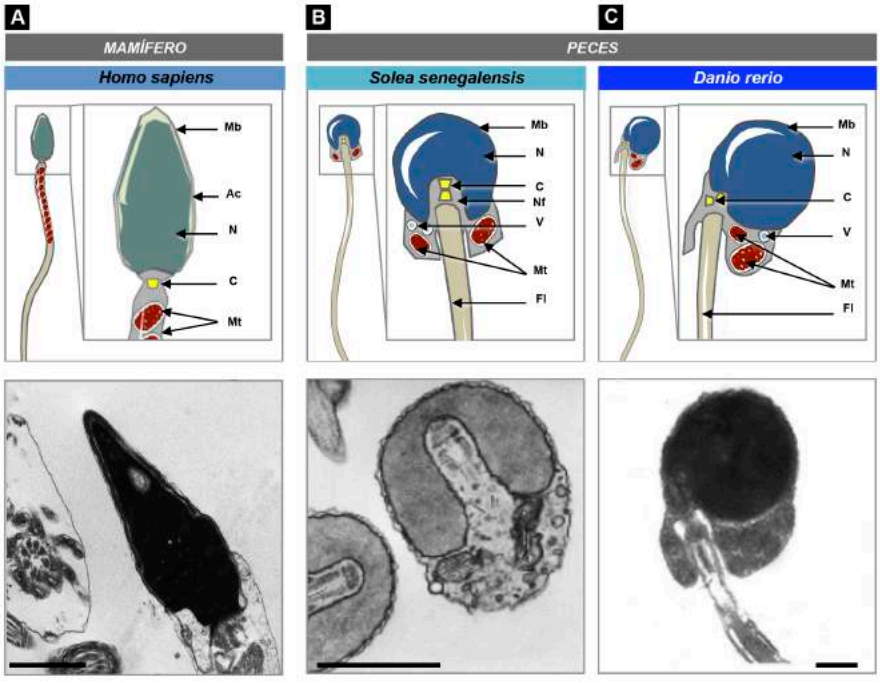

Figura 4.- Diagramas de los espermatozoides de las tres especies de estudio y fotografías tomadas por microscopía electrónica. A: *Homo sapiens*; micrografía (Moretti et al. 2016) B: *Solea senegalensis*; micrografía (Medina et al. 2000). C: *Danio rerio*; micrografía (Zhang et al. 2014) Barras de escala: 1 μm. Mb: membrana; Ac: acrosoma; N: núcleo; C: Centriolos; Mt: mitocondrias; Nf: fosa nuclear; Fl: flagelo; V: vesículas.

El pez cebra (*Danio rerio*)

Interés científico

A pesar de ser un vertebrado primitivo, el pez cebra ha sido utilizado como un excelente organismo modelo durante los últimos años gracias a las numerosas ventajas que proporciona. En primer lugar existe un elevado número de técnicas disponibles para esta especie que facilitan la investigación en este modelo animal, incluyendo: transgénesis, ensayos de expresión génica transitorios *in vivo*, experimentos de sobreexpresión mediante la inyección de ARNm, de *knockdown* por inyección de morfolino, de *knockout* y de edición génica por el sistema CRISPR/Cas9 y mutagénesis (Lin et al. 2016). Además, este animal es un excelente modelo para biología del desarrollo por la transparencia de sus embriones, la cual permite también visualizar la expresión dinámica de un gen específico durante el desarrollo (Lee et al. 2015). Además, algunas líneas de peces transgénicos y mutantes disponibles muestran alteraciones fenotípicas similares a las de las enfermedades humanas (Lieschke and Currie 2007). Existen numerosos trabajos que han abordado el desarrollo de corazón (Hami et al. 2011; Liebling et al.), de músculo (Lee et al. 2009; Wang et al. 2008), de tejido ocular (Hu et al. 2006) o tejido nervioso (Tu et al. 2012) en el pez cebra. Es un animal altamente utilizado como biosensor ante tóxicos medioambientales (Amanuma et al. 2000, 2002). Así mismo, la especie es utilizada en investigaciones vinculadas con estudios de comportamiento (Kalueff et al. 2014b, 2014a), regeneración (Fuller-Carter et al. 2015), hematopoiseis (Hsia and Zon 2005), enfermedades cardiovasculares (Shin and Fishman 2002), melanoma (Wang et al. 2006), metástasis (Zhang et al. 2015), leucemia (Yeh et al. 2009) o hepatoma (Zheng et al. 2014) entre otros. El aumento exponencial de los estudios con este organismo también ha hecho posible que en la actualidad existan numerosas secuencias anotadas que facilitan su estudio. Además, es el pez para el cual hay más disponibilidad de información sobre la metilación global del ADN. En el campo de la biología reproductiva también es una valiosa especie modelo y existen numerosas líneas de investigación vinculadas al estudio de las células primordiales germinales (Beer and Draper 2013; Kawasaki et al. 2016; Raz 2004; Riesco et al. 2014), espermatología (Fechner et al. 2015; Hagedorn et al. 2012; Jia et al. 2015), endocrinología (Morais et al. 2013; Nóbrega et al. 2015), disruptores endocrinos (Huang et al. 2015; Lombó et al. 2015) y criopreservación del esperma (Wang et al. 2015; Yang et al. 2016) entre otras.

Descripción del espermatozoide

La estructura del espermatozoide del pez cebra es semejante a otros peces con fecundación externa (Mattei 1991) pero presenta una clara asimetría debida a la localización medio-lateral de la fosa nuclear (Zhang et al. 2014), promoviendo un desequilibrio con el

flagelo insertado excéntricamente en la cabeza (**Fig. 4B)**. El ADN del núcleo se encuentra compactado con histonas (Ausió et al. 2014). Los centriolos se posicionan el uno del otro a 125 º. La pieza media está muy poco desarrollada, siendo esto una característica habitual en los peces con fecundación externa (Cabrita et al. 2009) y presenta unas pocas mitocondrias redondeadas. El flagelo presenta el patrón común de axonema "9x2 +2" (Zhang et al. 2014) también presente en otros ciprínidos (Fürböck et al. 2009). La longitud de los espermatozoides de pez cebra es aproximadamente de 30-35 μm (Zhang et al. 2014).

El lenguado senegalés (*Solea senegalensis*)

Interés en acuicultura

Los peces planos (Pleuronectiformes) componen un orden relativamente amplio de teleósteos, la mayor parte marinos, que representan una fuente nutricional importante de alto valor comercial a lo largo del mundo (Chauvigné et al. 2016). En el sur de Europa, el lenguado senegalés (*Solea senegalensis*) se ha convertido en una de las especies de mayor interés en el campo de la acuicultura debido a sus altas tasas de crecimiento. Este hecho se ha visto reflejado en el incremento de la producción de esta especie de pez plano durante los últimos años (Morais et al. 2016). Sin embargo, el cultivo sostenible del lenguado senegalés está obstaculizado por la falta de métodos de control de la reproducción en cautividad (Chauvigné et al. 2016), y particularmente, por la disfunción reproductiva que presenta la primera generación (F1) de los machos nacidos en cautividad (Morais et al. 2016). A pesar de las múltiples líneas de investigación existentes que abordan este problema reproductivo (Chauvigné et al. 2016; Fatsini et al. 2016; Tapia-Paniagua et al. 2014), es necesario avanzar en el conocimiento de esta especie para desbloquear el impulso de la misma a nivel comercial, sobre todo en la Península Ibérica, donde esta especie es firme candidata para la diversificación de la acuicultura y el desarrollo de esta industria (APROMAR 2016).

Descripción del espermatozoide

El espermatozoide de *Solea senegalensis* consta de una cabeza esférica ligeramente ovoide con un núcleo bilobular (**Fig. 4C**). La corta pieza media contiene mitocondrias irregulares embebidas en una masa citoplasmática conformando un anillo y una larga cola con dos aletas laterales (Medina et al. 2000). El axonema presenta el patrón convencional de "9x2 + 2" microtúbulos. Los centriolos están localizados en una profunda fosa nuclear y están orientados coaxialmente en la dirección longitudinal del

espermatozoide. La longitud del espermatozoide es aproximadamente 40-45 μm (Medina et al. 2000).

Parámetros de calidad espermática

Un espermatozoide de calidad ha de presentar: 1) habilidad para alcanzar el ovocito (supervivencia en el tracto femenino o en el medio externo); 2) capacidad de cruzar y atravesar las envueltas del ovocito y, en el caso de los peces, entrar a través del micrópilo; 3) capacidad de interacción y fusión de los gametos ya sea en mamíferos (reacción acrosómica) o en peces (reconocimiento del oolema y fusión de membranas); 4) capacidad de activación de las vías metabólicas en el ovocito y 5) integridad de su genoma y otros ácidos nucleicos que permita la correcta contribución al futuro embrión.

La definición de parámetros de calidad espermática es una tarea difícil debido a la complejidad y la naturaleza multifactorial de la fertilidad masculina. Para otorgar a una muestra un valor de calidad general es necesario analizar múltiples aspectos **(Fig. 5).** El avance en el descubrimiento de predictores eficaces de la calidad espermática supone un claro beneficio tanto en la especie humana como en aquellos animales de interés en producción animal. Además, permite una mejor comprensión y análisis de los efectos que diversos factores extrínsecos a la biología del espermatozoide pueden suponer sobre su integridad y funcionalidad.

De forma rutinaria y convencional, en un laboratorio de reproducción, la determinación de la calidad está basada en una combinación de evaluación macroscópica y microscópica. Se evalúan parámetros tradicionales como: volumen, pH, número total de espermatozoides, concentración espermática, porcentaje de espermatozoides móviles y porcentaje de espermatozoides con morfología normal. Algunos de estos parámetros son correlacionados con la fecundación aunque numerosos estudios indican que no siempre un único parámetro puede vincularse con calidad y fertilidad de forma certera (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010).

Resulta evidente que los resultados de las pruebas funcionales (tales como los test de penetración (Binor et al. 1980; Martínez-Rodríguez et al. 2012), los test de unión a la zona pelúcida (Burkman et al. 1988; Liu et al. 1988), los test de respuesta hipoosmótica (Jeyendran et al. 1984) o los estudios directos de evaluación de fecundación (Cabrita et al. 2009), presentan mejor correlación con el éxito de la fertilización si se compara con las

evaluaciones seminales convencionales (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010). Sin embargo, estos métodos presentan un limitado valor pronóstico para el éxito reproductivo del macho donante (Lefievre et al. 2007; Ramalho-Santos et al.) y una difícil estandarización, ya que los resultados otorgados son también dependientes de la calidad del ovocito o de la ratio ovocito:esperma, así como de las condiciones de incubación.

Utilizando los parámetros clásicos de calidad, a menudo no se diagnostican anormalidades discretas pero que pueden menoscabar el éxito reproductivo de los espermatozoides y su interacción con el ovocito.

En las últimas décadas, los intentos de superar las limitaciones de las técnicas tradicionales, han conducido a la introducción en los laboratorios de algunos análisis sofisticados. Campos como la proteómica, bioquímica, los enfoques inmunocitoquímicos o el estudio de ácidos nucleicos están empezando a destacar permitiendo determinar el éxito espermático de una forma más precisa.

La combinación de las evaluaciones de parámetros más convencionales junto con aquellas metodologías de análisis más sofisticadas y novedosas permite una determinación de la calidad seminal de forma más certera y garantizar ausencia de alteraciones en la progenie.

Evaluación de características generales de la muestra seminal:

- *Volumen*

Este parámetro es una de las primeras evaluaciones macroscópicas analizadas en un laboratorio de reproducción. El volumen de eyaculado es muy variable dependiendo de las especies: desde unos pocos microlitros como en el caso pez cebra (Chen et al. 2017) o el lenguado senegalés (Cabrita et al. 2011) a más de un mililitro en humanos (World Health Organization 2010). Un bajo volumen de eyaculado fuera de los límites habituales puede reflejar anormalidades en las glándulas sexuales accesorias o en su secreción de fluidos (Roberts and Jarvi 2009). En las instalaciones de producción animal este parámetro permite hacer una selección preliminar de la calidad de la muestra seminal, no siempre correlacionada con la calidad de los espermatozoides.

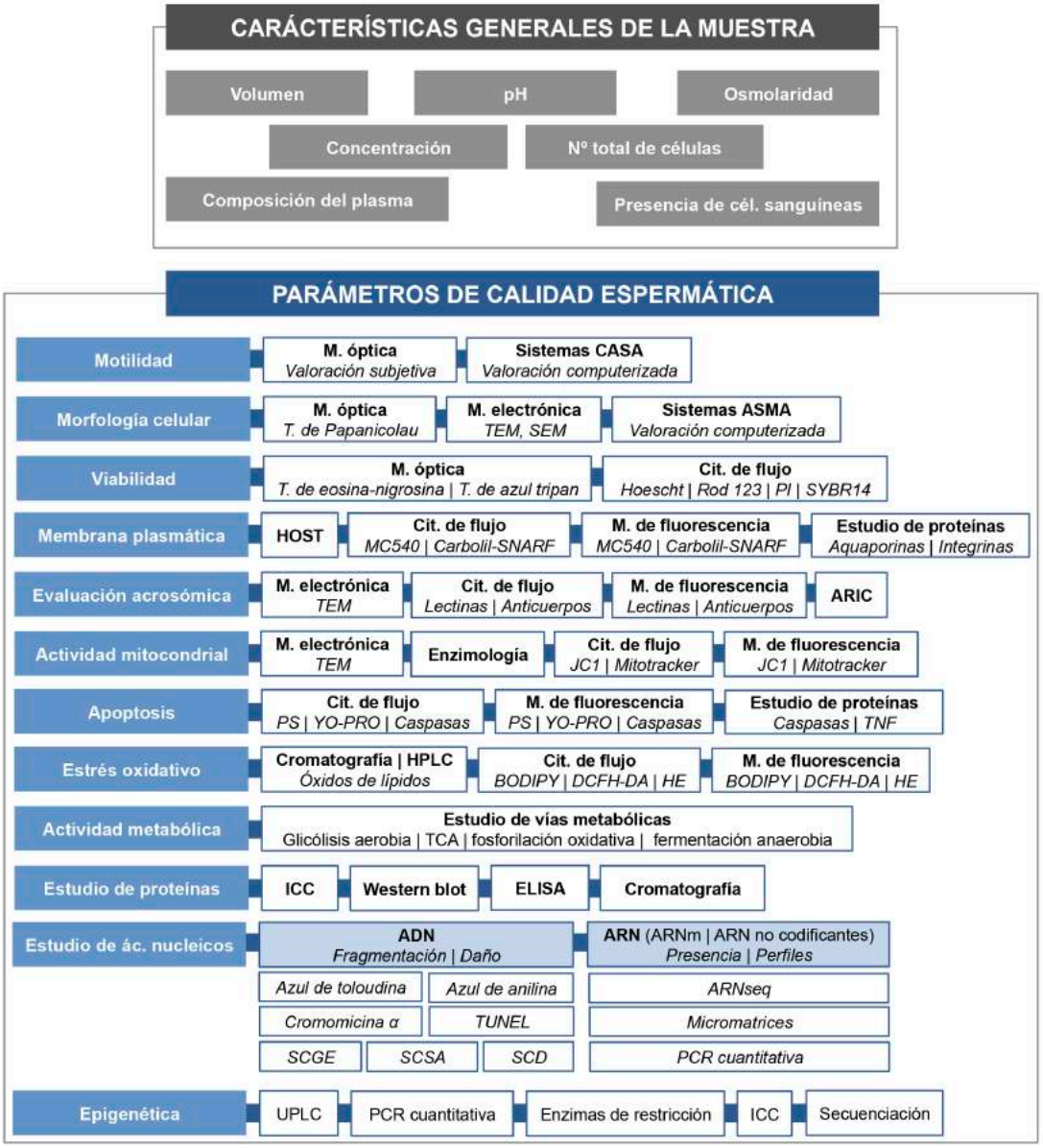

Figura 5.- Resumen de parámetros de calidad espermática

- *pH*

El pH seminal se determina por las secreciones ácidas de las glándulas accesorias en el sistema reproductivo. Variaciones importantes fuera de los rangos de distribución normales de cada especie pueden indicar infección derivada en una disminución de las secreciones tales como el ácido cítrico (Qian et al. 2016). Un valor de pH anormal también puede ser registrado en los casos de eyaculación incompleta. Registros excesivamente ácidos son encontrados en casos de agenesia de las vesículas seminales (Mehta G. and Woodward 2013).

- *Osmolaridad*

Este parámetro es atendido principalmente en las investigaciones vinculadas con acuicultura. Bajas osmolaridades son correlacionadas con contaminaciones con orina, suponiendo el descarte de la muestra para una técnica de fecundación artificial en escala industrial (Beirão et al. 2015). La importancia de este parámetro radica en que la motilidad espermática es altamente dependiente de la osmolaridad del medio externo. Los cambios en la concentración iónica intracelular causada por la hinchazón o contracción promovida por la osmolaridad externa parecen regular la motilidad espermática en la mayoría de peces teleósteos (Cabrita et al. 2009).

- *Número total de células y concentración*

La concentración espermática suele ser determinada de rutina utilizando algún método basado en hemocitómetros (Brillard and McDaniel 1985) utilizando un microscopio de contraste de fases y realizando varias réplicas técnicas. Estos métodos tradicionales de contaje pueden ser sustituidos por un dispositivo electrónico como un contador Coulter (Paulenz et al. 1995). En la especie humana, la OMS establece el límite umbral para diagnosticar oligozoospermia en 15 millones de células/mL (World Health Organization 2010). El número total de espermatozoides por eyaculado otorga una idea sobre la producción por espermatogénesis en los testículos así como las posteriores modificaciones de maduración. Este valor, así como la concentración espermática han sido relacionados con las tasas de embarazo (Slama et al. 2002; Zinaman et al.) y han sido clasificados como predictores de la concepción tanto en humanos (Larsen et al. 2000) como en peces (Aas et al. 1991).

- *Composición del plasma*

El plasma seminal contiene sustancias capaces de mantener el metabolismo espermático y moléculas que pueden reflejar las funciones del sistema reproductivo. Se han realizado estudios de plasma tanto en peces teleósteos (Bozkurt et al. 2009; Lahnsteiner et al. 1998) como en humanos (Schmid et al. 2013) correlacionando este parámetro con calidad espermática. Habitualmente se realizan estudios con kits o analizadores automáticos la cantidad de iones (Na^+, K^+, Ca^{2+}, Mg^{2+} o Cl^- entre otros) y metabolitos (glucosa, proteínas, colesterol, triglicéridos, urea…).

- *Presencia de células sanguíneas, bacterias o células en estadios previos al espermatozoide maduro*

Por lo general, el eyaculado contiene células que no son espermatozoides (World Health Organization 2010) Estas pueden ser células poligonales del tracto genital, bacterias, glóbulos blancos o incluso eritrocitos (World Health Organization 2010). La presencia de un

número excesivo de células germinales en estadios previos de diferenciación sugiere patologías a nivel del epitelio seminífero con una espermatogénesis inadecuada y/o liberación prematura de células en estadios previos a espermatozoide maduro. A través de microscopía óptica sumada a tinciones específicas (World Health Organization 2010) es posible diferenciar los diferentes tipos celulares.

Evaluación de parámetros de calidad espermática

- *Motilidad*

La motilidad es el parámetro tradicional de calidad por excelencia dada su frecuente correlación con fertilidad. El análisis convencional de la motilidad, sin recurrir a un equipo complejo, está basado en la clasificación a través de observación subjetiva bajo microscopía óptica de las células móviles en una extensión celular sin teñir (en el caso de peces tras la activación espermática). A pesar de que en muchos centros de reproducción la motilidad sigue siendo asignada de forma subjetiva por personal entrenado, la aparición en la década de los 80 de los sistemas computerizados de análisis de espermatozoides (CASA; del inglés: *computer assisted sperm analysis*) supuso una revolución en el campo (Lu et al. 2014). Estos equipos permiten valorar un mayor número de parámetros cinéticos del movimiento de esperma de forma objetiva, sensible y precisa (World Health Organization 2010). La técnica fue inicialmente utilizada en mamíferos pero en hoy en día existen múltiples descripciones de motilidad específicas de especie y líneas de estudio en diversos animales: peces (Dietrich et al. 2007; Gallego et al. 2017), invertebrados marinos (Gallego et al. 2014), aves (Kleven et al. 2009), mamíferos marinos (Montano et al. 2012), reptiles (Al-Dokhi et al. 2015) o incluso insectos (Al-Lawati et al. 2009).

De forma sencilla, esta tecnología captura campos microscópicos que son escaneados de forma sistemática permitiendo trazar la trayectoria de cada célula en el campo y extraer mediante un *software* múltiples parámetros cinéticos de cada espermatozoide de forma individualiza. De forma generalista, el equipo permite asignar valores de: motilidad progresiva rápida, motilidad progresiva lenta, motilidad no progresiva e inmovilidad además de otros parámetros cinéticos.

Cabe destacar que los equipos CASA son actualizados de forma continua y hoy en día existen módulos de análisis que permiten estudiar morfología, concentración y fragmentación del ADN.

- *Morfología celular*

Las células espermáticas representan una población única en la que existen elevados porcentajes de malformaciones morfológicas. En el caso de una muestra de especie humana la presencia de un 4% de células con la forma canónica es considerado suficiente para ser etiquetada como normal (World Health Organization 2010). Las variaciones pueden presentarse como malformaciones de la cabeza, defectos de tamaño, problemas en el cuello, en la pieza media o a nivel flagelar.

La forma más tradicional para la evaluación de la morfología celular es la observación en microscopio óptico de una extensión celular tras tinción de Papanicolau (Cabrita et al. 2009; Rurangwa et al. 2001). La utilización de microscopía electrónica de transmisión (TEM; del inglés: *transmission electron microscopy*) y microscopía electrónica de barrido (SEM; del inglés: *scanning electron microscopy*) proporcionan mayor información pero es un procedimiento complejo y alejado de la rutina habitual.

La utilidad de la evaluación de la morfología del esperma como un predictor del potencial de fecundación de un macho a menudo ha sido cuestionada debido a los diferentes sistemas de clasificación, la diversidad de la preparación de las muestras y los problemas con la reproducibilidad debido a subjetividad de observación (Ombelet et al. 1995). Mientras que en mamíferos se ha correlacionado con fallos en el éxito de la gestación (Lundin et al. 1997) las investigaciones en peces teleósteos se centran en estudios taxonómicos y evolutivos (Wei et al. 2007).

Al igual que en motilidad, existen sistemas computerizados para la evaluación de la morfología espermática, los denominados sistemas ASMA (del inglés: *assisted sperm morphology analysis*) que son una herramienta mucho más precisa para la valoración de este parámetro de calidad seminal (Gravance and Davis 1995).

- *Viabilidad*
    - *Tinciones tradicionales*

Las tinciones vitales de espermatozoides permiten la cuantificación de la fracción de células vivas, independientemente de su motilidad. La evaluación tradicional se realiza con eosina-nigrosina o *trypan blue* que permite diferenciar entre los dos tipos de células fácilmente. Este tipo de rutina permite evaluar si existe una reducción de espermatozoides viables, reflejando anomalías estructurales de los espermatozoides.

    - *Otros métodos*

Hoy en día los laboratorios de reproducción tienen a su disposición una batería de pruebas fluorescentes altamente específicas y combinables entre sí que permiten una

evaluación específica de los espermatozoides con equipos de citometría de flujo. Muchas de las pruebas están vinculadas con el estatus de la membrana ya que habitualmente son moléculas de unión a ADN impermeables en células sanas con membrana íntegra. Dentro de este grupo encontramos: yoduro de propidio (Lopez-Urueña et al. 2016) o rodamina 123 (Eskandari and Momeni 2016). Inversamente, SYBR14 es permeable en células sanas (Smith et al. 2016).

- *Membrana plasmática*

La integridad, fluidez y composición de la membrana espermática son esenciales para la supervivencia de los espermatozoides y para la fertilización (Holt 2000) ya que viabilidad y estatus de membrana están directamente relacionados.

Independientemente del grupo de animales al que pertenezcan, los espermatozoides son expuestos a variaciones osmóticas drásticas. La membrana plasmática juega un papel crucial siendo irreversible el daño causado si esta se ve comprometida (Meyers 2005). La funcionalidad de la membrana puede analizarse mediante la prueba HOST (del inglés: *hypo-osmotic swelling test*) (Ramu and Jeyendran 2013) o mediante combinaciones de colorantes de membrana fluorescentes por citometría de flujo, microscopía de fluorescencia o confocal (Malama et al. 2017)

- *Evaluación acrosómica*

En mamíferos y algunas excepciones de peces que presentan acrosoma como es el caso del esturión (Psenicka et al. 2007), una opción para la evaluación acrosómica es la microscopía electrónica. No obstante, es un método caro y laborioso imposibilitando el uso rutinario. Con el mismo fin, se utilizan de forma habitual ensayos de microscopía óptica, de microscopía de fluorescencia, microscopía confocal o citometría de flujo utilizando lectinas o anticuerpos específicos (Ashworth et al. 1995). Otra opción de análisis es el ensayo ARIC (del inglés: *acrosome reaction following ionophore challenge*) que permite separar los espermatozoides que se someten a reacción acrosómica espontánea de aquellos que han sido inducidos, ya que el resultado de la reacción del acrosoma inducida es más significativo (Zeginiadou et al. 2000).

- *Actividad y funcionalidad mitocondrial*

La actividad mitocondrial de los peces es diferente, de forma general, a la de los mamíferos debido al reducido número de mitocondrias presentes en estos vertebrados y la consecuente menor duración de la motilidad (Cabrita et al. 2009). La evaluación mitocondrial se puede realizar por morfología de los orgánulos mediante microscopía electrónica, integridad de la membrana mitocondrial (Ogier de Baulny et al. 1999; Taddei et al. 2001), o cambios en el potencial de membrana. El fluorocromo catiónico lipofílico

permeable a la membrana mitocondrial JC-1 es uno de los más utilizados para la evaluación de la funcionalidad mitocondrial (Agnihotri et al. 2016). Otros fluorocromos utilizados para la visualización de mitocondrias son MitroTracker Deep Red o Green (Nagy et al. 2016). Para el análisis de la actividad y funcionalidad mitocondrial también se han estudiado actividades enzimáticas mitocondriales (Ford 2006).

- *Evaluación de la apoptosis*

Uno de los cambios más importantes asociados a la apoptosis es la externalización de la fosfatidilserina (PS), una molécula generalmente confinada en la cara interna de la membrana plasmática (Tavalaee et al. 2014). Para realizar su evaluación, se utiliza con frecuencia la anexina V, una proteína dependiente de calcio que se une a la fosfatidilserina cuando esta es translocada a la cara externa de la membrana plasmática en el esperma dañado. La Anexina V se puede conjugar con fluorocromos tales como FITC (isotiocianato de fluoresceína) en análisis de citometría de flujo. La externalización de la PS y su unión a la anexina ha permitido desarrollar técnicas para la eliminación de subpoblaciones espermáticas apoptóticas, como es el MACS (del inglés *Magnetic Activated Cell Sorting*) en el que los espermatozoides apoptóticos quedan retenidos en un campo magnético al unirse a microbolas magnéticas recubiertas de anexina V. Un evento celular vinculado de forma intrínseca a la apoptosis es la activación del sistema de caspasas (Paasch et al. 2004). Se pueden utilizar anticuerpos contra las caspasas 7 y 9 (iniciadoras de la apoptosis) o contra la caspasa 3 activa (ejecutora en el proceso apoptótico). Vinculados a la apoptosis también se han descrito fenómenos tales como la fragmentación del ADN, la perdida de la integridad mitocondrial y el aumento de la permeabilidad de la membrana de la célula (Agarwal et al. 2009). En algunas especies de mamífero como el cerdo, se han realizado también estudios proteómicos enfocados a la detección de la proteína TNF (receptor de muerte que inicia la vía extrínseca de la apoptosis) realizando correlaciones con calidad espermática (Payan-Carreira et al. 2012).

- *Estrés oxidativo*

El metabolismo de los espermatozoides en condiciones aeróbicas origina moléculas oxidativas: las especies reactivas del oxígeno (ROS; del inglés: *reactive oxygen species*) que son productos químicos intermedios de corta duración, altamente reactivos que oxidan lípidos, proteínas y glúcidos. Las células contribuyen al mantenimiento de la homeostasis oxidativa mediante el control de la cantidad de ROS, convirtiéndolos en moléculas menos perjudiciales (Agarwal et al. 2009; Garrido et al. 2004). El exceso de producción de ROS daña la membrana de los espermatozoides, reduce la motilidad (por la disminución de potencial de membrana), induce daño irreparable del ADN y está estrechamente asociada con la apoptosis (Kim et al. 2010b; Natali and Turek 2011). El estrés oxidativo que sufren los

espermatozoides puede ser evaluado por múltiples vías. Una de las más habituales es a través de la cuantificación de malonaldehido (uno de los productos resultante de la oxidación de lípidos) por métodos indirectos usando espectrofotometría (Agarwal et al. 2016). También es habitual el uso de la sonda fluorescente BODIPY$^{581/591}$-C11 en citometría de flujo para evaluar estrés oxidativo (Gallardo Bolaños et al. 2012; Sanocka and Kurpisz 2004). Existen otras opciones para el análisis del estrés oxidativo, como la cuantificación de la reacción de la glutation peroxidasa, la medición de la intensidad de fluorescencia de los compuestos oxidados por ROS como DCFH-DA (diclorofluoresceina diacetato) (Mahfouz et al. 2009) o DHE (di-hidroetidio) (Mahfouz et al. 2009) o la identificación de óxidos de lípidos por cromatografía y HPLC (Agarwal et al. 2008b; Sanocka and Kurpisz 2004).

- *Actividad metabólica*

El estudio del metabolismo espermático está altamente relacionado con el análisis de la composición del plasma seminal. Los niveles de ATP han sido utilizados habitualmente como un parámetro predictivo de la motilidad espermática (Ferramosca et al. 2014). La razón radica en que los mecanismos moleculares por los que la motilidad es iniciada están vinculados con el ATP como proveedor de energía (Mansour et al. 2003). Existen diferentes rutas que proporcionan energía a los espermatozoides: glicólisis aerobia, ciclo del ácido tricarboxílico, fosforilación oxidativa, fermentación anaerobia, catabolismo lipídico, beta oxidación e incluso existen referencias a fuentes externas de energía (Frolikova et al. 2016; Gwo 1995; Lahnsteiner et al. 1999).

- *Estudio de proteinas*

En la actualidad existen líneas de investigación que utilizan técnicas como: inmunocitoquímica, *Western blot*, cromatografía o ELISA (del inglés: *enzyme-linked immunosorbent assay*) y estudios de proteómica. Algunas de ellas están focalizadas en el estudio de grupos proteicos específicos: integrinas, adhesinas (Töpfer-Petersen et al. 1998), proteínas ancladas a la membrana (Tomes 2015), aquaporinas espermáticas (Boj et al. 2015) o las proteínas compactadoras de ADN (Kasinsky et al. 2011).

- *Ácidos nucleicos*

ADN

Se ha sugerido que la integridad del ADN espermático puede ser un importante predictor de la fertilidad masculina. Hay pruebas de que el esperma de hombres infértiles contiene más daño en el ADN que los hombres fértiles y de que este daño tiene un efecto negativo sobre la fertilidad potencial de estos pacientes (Evenson et al. 1999; Guzick et al. 2001; Kodama et al. 1997; Zini et al. 2001a). Altos niveles de daño en el ADN espermático a menudo se correlacionan con parámetros seminales pobres, tales como recuentos celulares

bajos, baja motilidad o morfología anormal (Irvine et al.; Lopes et al. 1998; Muratori et al.). No obstante, un 8% de los hombres con parámetros seminales normales también presentan daños en el ADN de sus espermatozoides (Zini et al. 2001a, 2001b). Al igual que en la especie humana, en peces también se ha correlacionado fertilidad con daño en ADN (Cabrita et al. 2014; Pérez-Cerezales et al. 2010)

Existen numerosas pruebas que evalúan diferentes tipos de daño en el ADN espermático. Desafortunadamente, la mayoría de las técnicas disponibles proporcionan una información limitada sobre la naturaleza de las lesiones evidenciadas, y no permiten poner de relieve la alteración exacta del ADN espermático.

Los métodos menos costosos para evaluar la estructura de la cromatina espermática utilizan sondas o colorantes, tales como el naranja de acridina (Evenson 2013), el azul de anilina (Schill and Henkel 1999), cromomicina α (Singh et al. 2016a) o el azul de toluidina (Asmarinah et al. 2016).

Hoy en día, las pruebas más utilizadas para el análisis de la fragmentación del ADN espermático son: el ensayo de electroforesis en gel de una sola célula, también conocido como ensayo de cometas o SCGE (del inglés: *single cell gel electrophoresis*) (Albert et al. 2016), la técnica del marcado de final de corte de dUTP por la desoxi-transferasa terminal (TUNEL; del inglés: *Terminal deoxynucleotidyl transferase dUTP nick end labeling*) (Palermo et al. 2014), el análisis de la estructura de la cromatina espermática (SCSA; del inglés: *sperm chromatin structure assay*) (Evenson et al. 2007) y la prueba de dispersión de la cromatina de los espermatozoides (SCD; del inglés: *sperm chromatin dispersion*) (Evenson 2016). Los tres primeros ensayos se centran en la detección de la fragmentación del ADN, mientras que el último es un ensayo de matriz nuclear espermática que permite detectar reparación del ADN deficiente o desorganización de la cromatina (Evenson 2016).

ARN

Históricamente, se creía que los espermatozoides eran sólo un vehículo que proporcionaba el genoma masculino al ovocito. Sin embargo, en los últimos años la investigación ha demostrado que los espermatozoides entregan al ovocito algo más que solo el genoma haploide paterno. La célula espermática madura conserva una población de ARN compleja, incluyendo ARNs no codificantes que pueden funcionar como reguladores celulares que silencian o suprimen la expresión de genes, como los micro ARNs (miRNAs) (Kotaja 2014; Schuster et al. 2016). La utilidad de ARNs espermáticos como marcadores para la infertilidad ha sido explorada (Miller 2000; Ostermeier et al. 2002; Steger 2001) encontrando diferencias en los niveles de transcritos en el esperma con diferentes motilidades (Lambard et al. 2004) o entre muestras normales y anormales (Jodar et al.

2013, 2012; Montjean et al. 2012; Platts et al. 2007; Steger et al. 2003). Todos estos estudios intentaron correlacionar los niveles de ARNm con la calidad del esperma e infertilidad.

Debido a estos descubrimientos, la comunidad científica está atendiendo cada vez más al perfil transcriptómico del espermatozoide. Existen estudios en seres humanos, ratones y otros animales domésticos centrados en el análisis de ARNs espermáticos (Card et al. 2013; Das et al. 2013; Gilbert et al. 2007; Goodrich et al. 2007; Kawano et al. 2012; Singh et al. 2016b; Yang et al. 2009). Los resultados indican la existencia en los espermatozoides de una combinación de ARNs codificantes y no codificantes (lncRNAs, miRNAs, piRNAs) (Kotaja 2014; Meikar et al. 2014; Yadav and Kotaja 2014). La importancia funcional de los ARN espermáticos sigue sin estar completamente clarificada. Sin embargo, al menos algunos de los transcritos específicos son transmitidos al ovocito en la fecundación y parecen poseer una función potencial después de la fecundación en el desarrollo temprano de los embriones (Ostermeier et al. 2004) o en la herencia epigenética (Luteijn and Ketting 2013). Los miRNAs espermáticos han sido sugeridos como elementos importantes durante la embriogénesis temprana para el control transcripcional de la expresión génica embrionaria temprana (Jodar et al. 2013; Liu et al. 2012). A pesar de los recientes y prometedores avances en el estudio de los ARNs espermáticos, en el campo de la acuicultura, su estudio se encuentra en estadios más iniciales y de momento las investigaciones son escasas aunque prometedoras (Guerra et al. 2013).

El estudio de estas moléculas se basa principalmente en análisis por qPCR de presencia de transcritos, estudios de transcriptómica por microarrays o RNAseq.

- *Epigenética*

La epigenética estudia las modificaciones estructurales genómicas que afectan a la expresión de genes sin alterar la secuencia de nucleótidos subyacente (Molaro et al. 2014; Verma et al. 2014). Los mecanismos epigenéticos implicados en la regulación de la expresión génica incluyen la regulación por ARN no codificante, remodelación de la cromatina, la metilación del ADN y modificaciones de las histonas (Schagdarsurengin and Steger 2016; Verma et al. 2014). De estos mecanismos, la metilación del ADN se ha correlacionado con numerosas funciones biológicas, tales como el desarrollo de los espermatozoides y los embriones tempranos, y la represión de retrotransposones endógenos, mientras que también tiene una amplia gama de efectos en la expresión génica (Albertini 2014; Cheng et al. 2014). La desregulación de la metilación del ADN se ha asociado con diversos trastornos humanos, y se ha demostrado que aumenta el riesgo de fracaso de la fecundación, la disfunción en la embriogénesis, la mortalidad perinatal,

anomalías congénitas, parto prematuro y bajo peso al nacer (Chen et al. 2015; Katib et al. 2014). Las alteraciones de la metilación del ADN han sido altamente relacionadas con infertilidad masculina (Gunes et al. 2016; Xu et al. 2016) y su estudio está comenzando a extenderse como elemento crucial a tener en cuenta en el campo de la espermatología.

Factores que pueden afectar la calidad espermática

La calidad de los espermatozoides puede verse afectada por múltiples factores. La manipulación de los gametos es, evidentemente, uno de los factores que más puede comprometer la calidad espermática ya que afecta de forma directa a la célula. Pero también se ha comprobado que la exposición a radiación (Agarwal et al. 2008a), a metales pesados (Benoff et al.) o a disruptores endocrinos (Rozati et al. 2002; Spanò et al. 2005) tiene un efecto menoscabador en la calidad espermática. En el ser humano elementos vinculados al estilo de vida tales como el consumo de alcohol (Li et al. 2011), de cafeína (Jensen et al. 2010), de nicotina (Calogero et al. 2009; Mitra et al. 2012) o drogas ilegales (Battista et al. 2008; George et al. 1996) también han sido descritos como elementos con efecto negativo sobre la calidad espermática. Por el contrario la actividad física (Vaamonde et al. 2009) y los hábitos nutricionales saludables (Mendiola et al. 2010; Wong et al. 2003) han sido correlacionados con mejoras de la calidad espermática.

La presente tesis doctoral focaliza la investigación en cuatro de estos factores. Por un lado, se estudia el efecto de la criopreservación espermática como manipulación directa previa a una técnica de reproducción asistida; por otro, se analiza el efecto de la estabulación sobre la calidad espermática de una especie de elevado interés en acuicultura, el lenguado senegalés y también se evalúa el efecto de la exposición a un contaminante emergente, el 17-α-etinilestradiol en pez cebra como animal modelo. Finalmente, se estudia el efecto de la ingesta de suplementos alimenticios como posible modulador de la calidad seminal en el pez cebra y en humano.

Tecnologías de la reproducción

Las tecnologías de reproducción asistida, ARTs (del inglés *assisted reproductive technologies*) son una herramienta de gran relevancia tanto en biología reproductiva humana como en producción animal de especies de animales domésticos.

Estas tecnologías conllevan la manipulación *in vitro* de ovocitos, de espermatozoides o de embriones, con el objetivo de conseguir un embarazo exitoso (Sullivan et al. 2013). A nivel mundial, se estima que más de 5 millones de niños han nacido a partir de tecnologías de reproducción asistida (Pinborg et al. 2016). Estos valores son consecuencia directa del importante incremento de la infertilidad, definida como la incapacidad de lograr un embarazo clínico después de más de doce meses de relaciones sexuales regulares sin existencia de métodos anticonceptivos (Boivin et al. 2007; Sullivan et al. 2013). Las ARTs surgen a finales de los años 70 de la mano de Patrick Steptoe y del Nobel en Medicina de 2010 Robert Edwards (Steptoe and Edwards 1978) como una herramienta contra un importante reto reproductivo al que se enfrenta la especie humana.

Sin embargo, a pesar del éxito de estas ARTs durante las pasadas décadas, las tecnologías aún no están libres de problemas. Los bebés concebidos mediante estas técnicas en general, siguen presentando un ligero aumento del riesgo de parto prematuro y de ser pequeños para la edad gestacional. Los trastornos en el *imprinting* también se presentan una mayor tasa de incidencia en los nacidos por estas técnicas, teniendo consecuencias para la salud en la edad adulta (Grace and Sinclair 2009).

Estas deficiencias pueden ser parcialmente explicadas por las características reproductivas de los padres (Pinborg et al. 2013), sin embargo, los procesos intrínsecos de la técnica tales como la estimulación ovárica hormonal, a la que son expuestas las mujeres que acuden a una clínica de reproducción o las técnicas de manejo de gametos o embriones, también pueden afectar directamente a la descendencia.

Existen principalmente tres tipos de ARTs: la fertilización *in vitro* convencional (FIV) (Steptoe and Edwards 1978), la inyección intracitoplasmática de espermatozoides (ICSI, del inglés *intracytoplasmic sperm injection*) (Palermo et al. 1992) y la inseminación intrauterina (IIU) (Barwin 1974). Los tratamientos de fertilidad son procedimientos complejos y cada ciclo de reproducción asistida conlleva varios pasos (Brezina et al. 2015) que han de ser ejecutados de forma correcta para el éxito de la concepción.

Cada una de las tecnologías disponibles es seleccionada en función del tipo de problema reproductivo que presente la pareja al acudir a la clínica de reproducción asistida. Se ha estimado que el factor masculino es directamente responsable de aproximadamente el 50% de los problemas de concepción (Bisht et al. 2017) y afecta al 10-15% de los hombres (Esteves 2016). La sub-fertilidad o infertilidad masculina es una condición común entre las parejas con problemas para la concepción natural. A lo largo de los años, la Organización Mundial de la Salud ha ido definiendo de formas diferentes la subfertilidad masculina. Una muestra con calidad de semen normal fue inicialmente descrita como

aquella que presentara una concentración de espermatozoides mayor o igual que 20 millones/mL, con motilidad total por encima 50% de células, morfología celular normal en 50% de los espermatozoides y libre de anticuerpos (World Health Organization 1987). En 1992, la institución modificó sus criterios para la morfología de los espermatozoides normales reduciendo del 50% al 30% el valor umbral (World Health Organization 1992). Cuando se utilizaron criterios estrictos de morfología, sólo con un 14% la muestra fue considerada normal (Grow and Oehninger 2009). Desde la última revisión de la OMS en 2010, los valores básicos de referencia de calidad de semen fueron establecidos en: volumen de muestra mayor o igual a 1,5 mL, una concentración de espermatozoides de igual o mayor que 15 millones/mL, la motilidad total por igual o mayor al 40% y morfología normal en igual o mayor al 4% (Cooper et al. 2010; World Health Organization 2010). La tendencia negativa actual en la salud reproductiva masculina es evidente. Desde el momento de la aparición de la criopreservación como opción viable para la conservación de esperma en el ser humano en la década de los 50 (Bunge and Sherman 1953) el uso de la técnica se convirtió en el método dominante de preservación de la fertilidad para los hombres que se enfrentan a problemas reproductivos o a tratamientos médicos agresivos que pueden afectar directamente a sus espermatozoides tales como la quimioterapia en enfermos de cáncer (Osterberg et al. 2014).

Los hombres que presentan espermiogramas subóptimos pueden elegir criopreservar una muestra de esperma antes de continuar con los tratamientos de fertilidad como una "póliza de seguro" en el caso de que sus parámetros de calidad espermática continúen disminuyendo en el futuro. Además, el uso de donantes anónimos de esperma es común en el tratamiento de fertilidad de las mujeres que desean lograr el embarazo en los casos muy graves de infertilidad masculina en su pareja o en aquellos casos en los que no existe pareja masculina (Klock 2014).

La criopreservación de espermatozoides es relativamente sencilla. La muestra se obtiene clásicamente mediante masturbación tras un periodo de abstinencia sexual. En algunos casos en los que no es posible obtener el esperma a través de la eyaculación, existen técnicas de recuperación de esperma mediante técnicas quirúrgicas (Wang et al. 2013). Tras un periodo de tiempo a temperatura ambiente para favorecer que la muestra se licúe, se analizan los parámetros de calidad de la muestra (ver capítulo anterior). En la actualidad muchas clínicas de reproducción asistida realizan procesos de selección como el *swim-up* o la centrifugación para la selección de la población de células más viable en la muestra. Tras este proceso el esperma es criopresevado y almacenado en bancos de germoplasma durante el tiempo necesario de manera estable hasta el momento de su utilización en un protocolo de ARTs.

Las muestras seminales criopreservadas se consideran estables y pueden utilizarse incluso décadas más tarde obteniendo embarazos exitosos (Feldschuh et al. 2005). Existe una aceptación generalizada de la seguridad del uso de estas muestras tras décadas de embarazos saludables.

Sin embargo, existen evidencias científicas que demuestran efectos nocivos de los protocolos de congelación/descongelación de esperma. De forma general, se provoca una reducción en la viabilidad del esperma debido a alteraciones en la estructura y funciones de los espermatozoides. Los efectos secundarios incluyen motilidad alterada, cambios en la membrana plasmática y la integridad acrosomal y aumento de la fragmentación del ADN. Todas estas alteraciones inducen una reducción de la capacidad de esperma para sobrevivir en el tracto reproductivo femenino y por lo tanto, para interactuar con el ovocito en la fecundación (Kim et al. 2010a; Rodriguez-Martinez 2012). En un intento de compensar estos efectos secundarios, las dosis seminales se preparan habitualmente con un número excesivo de espermatozoides con el fin de mejorar la tasa de éxito de la fecundación (Payan-Carreira et al. 2011; Rodriguez-Martinez 2012). Las técnicas de criopreservación disponibles tienen una serie de problemas potencialmente perjudiciales, tales como lesiones físicas y químicas que los espermatozoides propensos a la muerte celular y la disfunción (**Fig. 6**). Estos incluyen:

• *Cambios semejantes a la capacitación derivados de la congelación/ descongelación.*

Los espermatozoides se comportan como capacitados, lo que disminuye su capacidad de sobrevivir en el tracto genital de la mujer y de fusionarse con los ovocitos (Naresh and Atreja 2015).

• *Deterioro de la motilidad*

Se observa una disminución de la motilidad en los espermatozoides-post descongelado, que tienden a exhibir un grado variable de debilitamiento de la motilidad, con obstaculización subsiguiente de la progresión de los espermatozoides hasta los oviductos y una disminución en el potencial de fertilidad (Yeste 2016).

• *Daños oxidativos*

Este tipo de daños pueden desencadenar la apoptosis y daño en el ADN cuando se sobrepasa un valor umbral. El daño oxidativo compromete la función mitocondrial, la motilidad y predispone a la fragmentación de ADN (Di Santo et al. 2012).

• *Variaciones en la composición de la membrana y la integridad del acrosoma*

La pérdida de la integridad de la membrana afecta al transporte iónico en la célula, en particular, el equilibrio de calcio y el agua, con la consiguiente pérdida de la capacidad del esperma para la regulación de volumen y su osmo-regulación. Asimismo, hay modificación en la exposición de proteínas en la superficie de la célula, lo que afecta negativamente a la supervivencia de los espermatozoides (Sieme and Oldenhof 2015) y la interacción entre gametos masculinos y femeninos. Además, un acrosoma con la integridad comprometida (Pukazhenthi et al. 2007) puede afectar directamente a la capacidad del esperma para penetrar las capas del ovocito durante la fertilización.

• *Cambios moleculares en ácidos nucleicos*

El daño genético se correlaciona frecuentemente con errores en el desarrollo embrionario posterior. La presencia de daño en el ADN en la línea germinal masculina se ha relacionado con una variedad de resultados adversos, tales como tasas bajas de fertilización, disminución en la implantación embrionaria, aborto espontáneo, cáncer y otras enfermedades en la descendencia (Aitken et al. 2009; Fernández-Gonzalez et al. 2008; Lewis and Aitken 2005). Además, el daño en el ADN del esperma se ha relacionado con un mayor riesgo de pérdida del embarazo en humanos después de los procedimientos de reproducción asistida (Zini et al. 2008). Más allá del estudio del ADN, confirmado el aporte por parte del espermatozoide en la fecundación de ARNs en los últimos años (Ostermeier et al. 2004; Schuster et al. 2016), hoy en día se deben responder aún a muchas preguntas sobre el papel de los transcritos espermáticos en la fecundación y el desarrollo embrionario y cómo la estabilidad de estas moléculas puede ser crucial o no para el desarrollo embrionario.

La presente tesis doctoral se va a centrar en este punto de análisis molecular de daños en genes y transcritos concretos por la importancia que puede tener para garantizar la ausencia de alteraciones en la progenie.

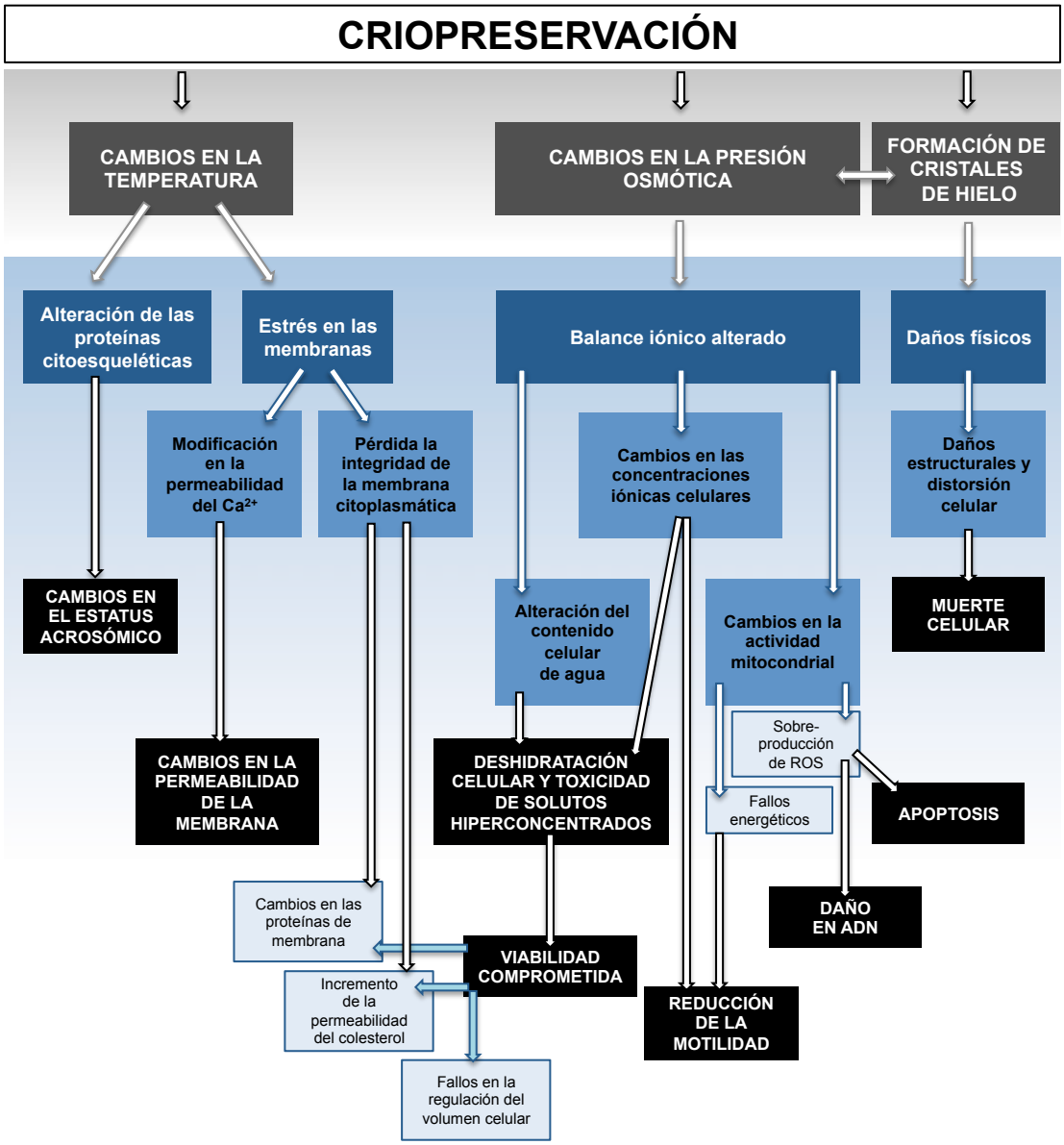

Figura 6.- Resumen de los efectos derivados de la criopreservación a nivel celular y correlación con procesos celulares.

La estabulación en los centros de producción animal a nivel industrial

El mayor reto al que se enfrenta la humanidad en las próximas décadas, más allá de la obtención de fuentes de energía, es sustentar a los 9700 millones de personas que se estima poblarán la Tierra en el año 2050 (UN 2015). Bajo el marco del cambio climático y un mundo con grandes desigualdades económicas, apremia la necesidad de incrementar el rendimiento de la producción agropecuaria de manera sostenible. Más allá de la posible

solución que la acuicultura pueda suponer a nivel global, es un sector económicamente importante en nuestro país que aún no ha desarrollado todo su potencial. La acuicultura no es sólo un complemento de la pesca, sino que, dentro del campo de la producción animal industrial, es el sector con mayor proyección de futuro (APROMAR 2016). Tiene a su favor que: el 70% de la superficie del planeta es agua, que no requiere del consumo de agua dulce en el caso de las especies de cultivo marino, que los animales acuáticos convierten mejor su alimento que los vertebrados terrestres y que sus tasas de reproducción son varios órdenes de magnitud superiores. Sin embargo, el sector presenta a su vez varios retos: se han de mejorar los conocimientos sobre la salud de los animales criados, conseguir la optimización de los piensos y de sus materias primas, mejorar la gestión de las instalaciones, así como la domesticación de nuevas especies y la mejora genética de las actuales (APROMAR 2016).

En la actualidad, uno de los retos de la acuicultura es la diversificación de especies cultivadas. En los países del sur de Europa, sobre todo en España y Portugal existe gran interés en el cultivo del lenguado senegalés (*Solea senegalensis*). Este foco de atención es debido a su valor comercial (Fatsini et al. 2016) y a las dificultades de rentabilidad que las empresas de cultivos marinos sufren frente al aumento de la oferta por parte de otros países en los mercados de dorada y lubina (Dinis et al. 1999). En consecuencia, en las últimas tres décadas se ha realizado un considerable esfuerzo de investigación para optimizar las metodologías de cría a nivel industrial (Morais et al. 2016). Sin embargo, la reproducción de *Solea senegalensis* en cautividad depende de reproductores de origen salvaje (**Fig. 7**), ya que los machos criados en las instalaciones no fertilizan los ovocitos (Agulleiro et al. 2007; García-López et al. 2006) a pesar de tener la capacidad de producir gametos viables adecuados para la fecundación *in vitro* (Agulleiro et al. 2007; Carazo et al. 2011; Rasines et al. 2012). Las terapias hormonales no tuvieron éxito para corregir esta disfunción y los estudios de comportamiento han demostrado que la falta de conducta apropiada de cortejo en los machos cultivados podría estar detrás de este problema (Guzmán et al. 2011).

El mantenimiento de animales estabulados en cautividad es necesario no sólo en instalaciones de producción animal sino también en centros de conservación de especies o en institutos de investigación experimental. Existe una amplia variación en la respuesta que tienen los animales a la cautividad, y en algunos casos el estrés crónico derivado de esta situación parece incidir en múltiples aspectos sobre el animal (Mason 2010). Los animales salvajes, una vez mantenidos en cautividad suelen recibir elevadas dosis de comida, alta atención veterinaria, y protección contra la depredación y las situaciones de conflicto. En consecuencia, los ejemplares en cautividad son a menudo más saludables y viven más tiempo (Mason and Veasey 2010; Müller et al. 2010; Patton et al. 2007).

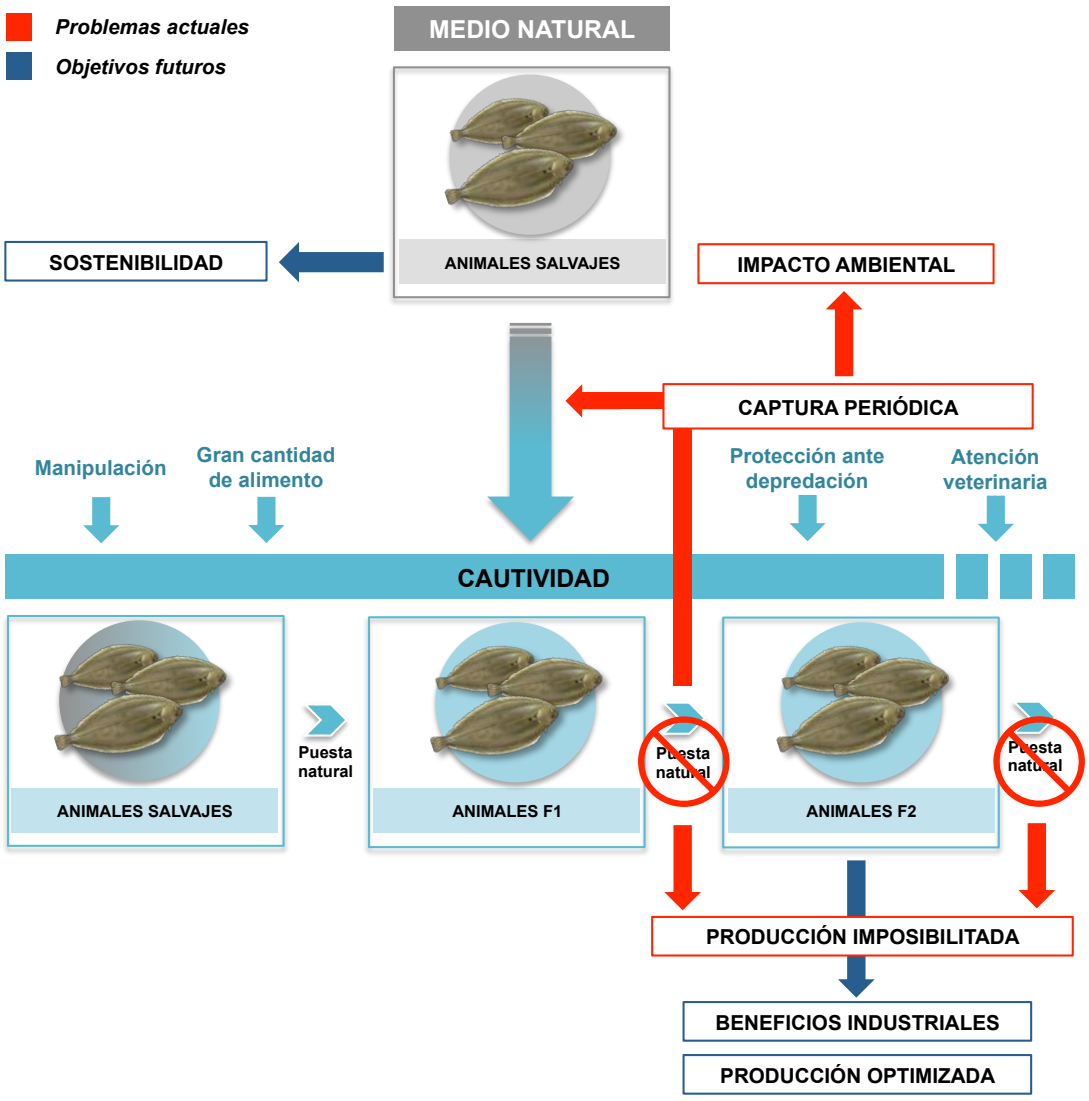

Figura 7.- Diagrama descriptivo de los problemas actuales y objetivos futuros en el cultivo del lenguado senegalés (*Solea senegalensis*)

La reproducción exitosa de los animales estabulados ha sido difícil de conseguir en muchos casos (Mason 2010). Esta dificultad puede derivarse de los efectos del estrés inducido por el cautiverio (Owen et al. 2004; Terio et al. 2004), ya que normalmente se acepta que el estrés desregula directamente la fisiología reproductiva y el comportamiento (Wingfield and Sapolsky 2003). Por otra parte, los animales en cautiverio tienden a presentar perfiles fisiológicos y conductuales que no coinciden con los de sus homólogos salvajes (Calisi and Bentley 2009; Zerani et al. 1991). Este tipo de alteraciones pueden afectar la capacidad de reproducción de un animal. Existen estudios que muestran la evidencia de que perfiles taxonómicos semejantes pueden diferenciarse enormemente en

su adaptación a la cautividad (Mason 2010). Mientras que algunas especies tienen una adaptación y comportamientos aceptables o incluso buenos, grupos próximos pueden mostrar niveles de estrés altos en condiciones similares. Un ejemplo en acuicultura es el caso de los peces planos, mientras que el rodaballo (*Scopthalmus maximus*) es hoy en día fácilmente cultivable (Chereguini et al. 2003; Labatut and Olivares 2004), el lenguado senegalés presenta numerosos problemas reproductivos, previamente mencionados.

El efecto de la cautividad y el estrés puede reflejarse a nivel celular y afectar directamente a la calidad de los gametos, especialmente en lo relacionado con estrés oxidativo y apoptosis. Esta tesis se centra en este tipo de estudios.

Contaminantes emergentes

Durante las últimas décadas, la comunidad científica ha centrado su atención en los potenciales efectos adversos que pueden provocar los denominados disruptores endocrinos, EDCs (del inglés: *endocrine dirupting compounds*). Este interés ha sido generado principalmente por dos elementos: 1) las observaciones de desórdenes reproductivos y/o de desarrollo en las poblaciones salvajes expuestas a productos químicos sintéticos liberados por la actividad antropogénica 2) la disminución, cada vez más preocupante, de la capacidad reproductiva humana en las parejas de los países industrializados y 3) la posibilidad de que los efectos perjudiciales de estos tóxicos puedan ser heredables transgeneracionalmente.

Los EDCs son un grupo heterogéneo de moléculas que abarcan agentes farmacéuticos, pesticidas, materiales de construcción o componentes de plásticos (Hampl et al. 2016; Tavares et al. 2016). Estas moléculas se encuentran en muchos de los productos diarios utilizados por el ser humano y son subproductos en procesos industriales o domésticos (Adeel et al. 2017). Las plantas de tratamiento de aguas residuales no consiguen la eliminación de estas moléculas en la actualidad (Esteban et al. 2014; Qiang et al. 2013) por lo que son liberadas al medio natural en concentraciones ambientalmente relevantes. Centrándonos en las poblaciones de animales salvajes acuáticas, existe evidencia acumulada sobre el efecto de estos compuestos en: perturbaciones en el desarrollo (Hotchkiss et al. 2008), feminización de machos y reducción del tamaño testicular (Arnold et al. 2014; Tetreault et al. 2011), reducción de la capacidad reproductiva (Rose et al. 2013), inducción de la producción de vitelogenina (Kidd et al. 2007), reducción considerable en la biomasa de peces, interrupción de la cadena alimentaria acuática (Hallgren et al. 2014) o aparición de disfunciones cardiacas (Salla et al. 2016) entre otras muchas.

Un grupo de EDCs de elevado interés son los estrógenos esteroideos. Este subgrupo se ha convertido en una preocupación emergente debido al rápido aumento de sus concentraciones en suelos y aguas de todo el mundo amenazando a las poblaciones salvajes, a los recursos hídricos y potencialmente al ser humano (Adeel et al. 2017). La presente tesis doctoral se centra en el estrógeno sintético 17-α-etinilestradiol (EE2). El EE2 es el principal estrógeno en la mayoría de las píldoras anticonceptivas orales y en los tratamientos de reemplazo hormonal (Nazari and Suja 2016) y su efecto ambiental es elevado debido a que su vida media en el medio ambiente es más persistente que la de los estrógeneos naturales (Adeel et al. 2017). Las mujeres que toman anticonceptivos orales excretan aproximadamente 10 μg de EE2 al día de media (Johnson et al. 2000; Johnson and Williams 2004). En aguas superficiales europeas las concentraciones de EE2 están por lo general por debajo de 10ng/L, pero estos valores están muy por encima de los estándares de calidad ambiental (EQS) propuestos para este producto químico sintético (Tiedeken et al. 2017). En peces, la exposición directa a EE2 se ha correlacionado con un desarrollo sexual alterado (Länge et al. 2001; Orn et al. 2003), alteraciones de las características sexuales secundarias (Länge et al. 2001; Robinson et al. 2003), reducción de la fecundidad (Fenske et al. 2005; Länge et al. 2001; Nash et al. 2004) e intersexualidad (Balch et al. 2004; Länge et al. 2001). En lo referente al espermatozoide, la exposición directa a EE2 ha sido correlacionada con una peor calidad (Santos et al. 2007), reducción en el recuento de espermatozoides (Oropesa et al. 2015) o alteración de parámetros cinéticos (Montgomery et al. 2014; Oropesa et al. 2015).

Más allá de los efectos provocados por la exposición directa, recientemente se ha publicado que la exposición paterna a otro disruptor endocrino, el bisfenol A (BPA), modifica el contenido de los transcritos espermáticos de los receptores de insulina (*insrs*), correlacionándose estos cambios con malformaciones cardiacas hasta la generación F2 (Lombó et al. 2015). En concordancia con estos resultados, hay cada vez más evidencias que confirman que los efectos a ciertos factores ambientales pueden ser transmitidos vía paterna a la descendencia en ausencia de cambios en el genoma del esperma (Klosin et al. 2017; Öst et al. 2014). La información paterna que aporta el espermatozoide de peces no sólo reside en su genoma, sino también en su patrón específico de marcas epigenéticas, su contenido de mRNA y sus ARNs no codificantes (Herráez et al. 2017; Robles et al. 2017). Así los factores ambientales pueden alterar la información epigenética contenida en las células germinales y escapar de los procesos de reprogramación que ocurren durante la gametogénesis y el desarrollo embrionario temprano (Klosin et al. 2017). Además, se ha descrito cómo los cambios en el comportamiento reproductivo y no reproductivo (ansiedad y

shoaling (formación de cardúmenes)) promovidos por la exposición embrionaria a EE2 son transmitidos a la generación F1 no expuesta (Volkova et al. 2015).

Recogiendo toda esta información, en el presente trabajo se explora el efecto de la exposición paterna a dosis ambientalmente relevantes de EE2 durante la espermatogénesis temprana. Se evalúan los cambios testiculares a nivel molecular que pueden alterar la población de transcritos en los espermatozoides teniendo como consecuencia efectos negativos en las progenies. De este modo, se estudia la transmisión de los efectos ambientales (en este caso utilizando un disruptor endocrino) a la progenie por vía paterna.

Factores nutricionales

Se ha demostrado que la nutrición del macho puede correlacionarse positiva o negativamente con la calidad espermática. Una elevada ingesta de cafeína (Jensen et al. 2010), carne o productos lácteos (Mendiola et al. 2009), grasas saturadas (Attaman et al. 2012) o isoflavonas de soja (Chavarro et al. 2008) se han asociado con una disminución de la calidad del esperma. Sin embargo, la dieta también puede tener una contribución positiva sobre la calidad del semen, tal y como se ha observado tras la ingesta de antioxidantes (Mendiola et al. 2010). Es imprescindible no pasar por alto que en materia de salud reproductiva, las células diana primarias que pueden ser afectadas por modificaciones nutricionales son las células germinales testiculares cuyas alteraciones derivan en la calidad espermática final. Las dos estrategias generales en este área de investigación son la suplementación de la dieta con moléculas beneficiosas y la reducción de elementos negativos en la alimentación.

La suplementación con aminoácidos es una de las opciones utilizadas para modular la calidad seminal, ya que el rendimiento reproductivo masculino está inevitablemente relacionado con su utilización y metabolismo (Dai et al. 2015). Las cantidades adecuadas de aminoácidos en la circulación, especialmente arginina, son esenciales para la generación, diferenciación y maduración de los espermatozoides, lo que afecta a su cantidad y calidad (Eskiocak et al. 2006; Wu 2009). Estudios preliminares en este campo mostraron una deficiencia de arginina en la dieta en los hombres con bajos recuentos seminales y malos valores de motilidad (Holt and Albanesi 1944). Por el contrario, la suplementación dietética con arginina aumentó notablemente el número de espermatozoides y la motilidad, conduciendo a embarazos exitosos (Tanimura 1967). Las dietas suplementadas con argininia no sólo mejoran la calidad del esperma, también producen un aumento en la concentración de poliaminas, ornitina, arginina y prolina en el fluido seminal, que es crucial para la fecundación en mamíferos (Wu et al. 2009).

Las moléculas con propiedades antioxidantes como suplemento dietético han sido otra opción estudiada. Los espermatozoides son particularmente susceptibles al daño inducido por estrés oxidativo por ROS, porque sus membranas plasmáticas contienen grandes cantidades de ácidos grasos poliinsaturados (PUFA) (Alvarez and Storey 1995) y su citoplasma contiene bajas concentraciones de secuestradores (*scavengers*) de estas moléculas (Jones et al. 1979). Los ataques derivados del estrés oxidativo no sólo afectan a la membrana plasmática del espermatozoide, sino también a la integridad del ADN nuclear (Aitken 1999). Se ha demostrado que, en el plasma seminal de hombres subfértiles, los niveles de ROS son mayores y los niveles de antioxidantes menores que en los controles fértiles normales (Abd-Elmoaty et al. 2010; Kao et al. 2008). Varios estudios han informado de la existencia de una mejora de la calidad del esperma humano a través de la suplementación dietética con folato (Mendiola et al. 2010; Schmid et al. 2012), vitamina C (Mendiola et al. 2010; Minguez-Alarcon et al. 2012; Schmid et al. 2012), vitamina D (Blomberg Jensen et al. 2011; Yang et al. 2012), vitamina E (Schmid et al. 2012), β-caroteno (Minguez-Alarcon et al. 2012), licopeno ((Mendiola et al. 2010; Minguez-Alarcon et al. 2012) y zinc (Schmid et al. 2012).

Esta tesis explora la incorporación de los probióticos como suplemento alimenticio para el estudio de su efecto en la calidad de los gametos bajo el prisma nutricional, a pesar de que su uso ha estado en práctica en otros campos desde hace más de un siglo (Hill et al. 2014). Los probióticos son, según la Asociación científica internacional de probióticos y prebióticos (ISAPP; del inglés: *International Scientific Association for Probiotics and Prebiotics*), "microorganismos vivos que, cuando son administrados en cantidades adecuadas, confieren un beneficio de salud en el hospedador" (Hill et al. 2014). Dentro de los beneficios para la salud promovidos por los probióticos se incluyen entre otros: actividades antimicrobianas contra patógenos intestinales, modulación del sistema inmunológico, disminución de los niveles de colesterol en la sangre, reducción de colitis e inflamación, prevención de cáncer de colon o la regulación del metabolismo energético del organismo de acogida (Lievin-Le Moal and Servin 2014).

Las bacterias del ácido láctico (LAB; del inglés: *lactic acid bacteria*) son un grupo heterogéneo de bacterias Gram-positivas, que no contienen lipopolisacáridos (LPS) unidos a la membrana celular y se están erigiendo como importantes microoganismos probióticos (Cano-Garrido et al. 2015). La ausencia de endotoxinas evita la generación de shock anafiláctico cuando son administradas en seres humanos (Rueda et al. 2014) y por lo tanto son apreciadas como una opción atractiva para la producción industrial (Konings et al. 2000; Ross et al. 2002). La seguridad de estas bacterias en la especie humana está certificada tras su uso durante siglos en la producción y preservación de alimentos (Cano-Garrido et al.

2015; Konings et al. 2000; Ross et al. 2002). Las bacterias del ácido láctico han sido clasificadas como microorganismos de grado alimentario (GRAS) por la FDA (del inglés: *Food and Drug Administration*) estadounidense y cumplen con los criterios QPS (del inglés: *Quality Presumption of safety*) de presunción de seguridad europeos de la EFSA (del inglés: *European Food Safety Authority*) (Cano-Garrido et al. 2015). El grupo LAB incluye microorganismos de diferentes géneros, tales como *Bifidobacterium*, *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *Streptococcus* y *Pedicoccus*. La administración de probióticos no ha sido relacionada con mejoras sobre la calidad seminal y dentro del campo de la reproducción su utilización esta muy limitada a tratamientos de problemas en el tracto reproductor femenino (Mastromarino et al. 2013).

En peces, existen algunos estudios que demuestran la importancia de cepas LAB en la mejora de su capacidad reproductora aunque no existen evidencias previas a esta tesis doctoral sobre su efecto sobre calidad seminal. La mayoría de los estudios se han realizado en las especies modelo como el pez cebra (*Danio rerio*), demostrando que la administración oral de probióticos estimula la reproducción mediante el aumento de la expresión de *gnrh3* (Gioacchini et al. 2010), una diferenciación sexual acelerada, e influencia en la proporción de sexos, probablemente a través de la modulación de *sox9*, un gen autosómico que participa en el control de la diferenciación cromosómica testicular (Avella et al. 2013; Carnevali et al. 2013).

La presente tesis estudia el efecto de determinadas cepas de probióticos (algunas con características antiinflamatorias o antioxidantes) sobre la calidad seminal tanto a nivel molecular (en el caso del pez cebra) como a nivel de otros parámetros de calidad seminal (en el caso de humano).

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OBJETIVOS

La presente tesis doctoral pretende proporcionar conocimiento de relevancia en el campo de la espermatología que pueda ser utilizado en biotecnología de la reproducción, tanto en el campo de la reproducción humana como en el de la producción animal. Bajo este prisma multidisciplinar, esta trabajo busca profundizar en el conocimiento de los efectos concretos, a nivel celular y molecular, de determinados factores externos, tales como la manipulación/criopreservación espermática, la estabulación de los ejemplares reproductores o la presencia de contaminentes en el medio, y explora el efecto de la ingesta de probióticos como suplemento nutricional potencialmente útil para revertir bajas calidades espermáticas. Los objetivos concretos de esta tesis son:

1.- Evaluar el efecto de un protocolo de criopreservación rutinario en las clínicas de reproducción asistida sobre los ácidos nucleicos espermáticos: 1a) Analizar el efecto sobre genes concretos relacionados con síndromes con mayor incidencia en los nacidos tras el uso de tecnologías de reproducción y sobre genes relacionados con el éxito en fecundación y en desarrollo embrionario temprano. 1b) Estudiar el efecto sobre una batería de ARNm espermáticos descritos como marcadores potenciales de calidad espermática y de éxito de fecundación.

2.- Estudiar, desde un punto de vista molecular y celular, el efecto de la estabulación sobre los gametos de machos reproductores de lenguado senegalés y desarrollar herramientas para la selección de subpoblaciones espermáticas óptimas: 2a) Analizar las poblaciones apoptóticas en las muestras de ejemplares nacidos en el medio natural y reproductores nacidos en cautividad con disfunción reproductiva. 2b) Implementar en peces teleósteos la técnica MACS (*Magnetic activated cell sorting*) para la selección de poblaciones libres de células apoptóticas como paso previo para la fecundación artificial. 2c) Evaluar el efecto de la estabulación y de la criopreservación sobre los niveles de especies reactivas del oxígeno en los espermatozoides.

3.- Analizar la transmisión por vía paterna de los efectos derivados de la exposición medioambiental a contaminantes emergentes utilizando 17-α-etinilestradiol como molécula de estudio. Correlacionar la exposición al tóxico en la espermatogénesis temprana con la capacidad de este compuesto para: 3a) desregular la expresión génica testicular y las poblaciones de ARNm espermáticas y 3b) afectar a las progenies no expuestas, prestando atención a los tipos de malformaciones registradas y las vías celulares implicadas.

4.- Explorar el efecto de la administración de cepas bacterianas probióticas sobre la calidad de las células germinales masculinas como posible herramienta útil para paliar problemas reproductivos en el macho: 4a) Evaluar en pez cebra, como modelo, el efecto de la ingesta de microorganismos probióticos sobre transcritos testiculares descritos como

predictores de éxito de fecundación; 4b) Evaluar el efecto de la ingesta de probióticos sobre los espermatozoides de hombres con baja calidad seminal y analizar el impacto del tratamiento a través de diferentes parámetros de calidad espermática.

CAPÍTULO I

Efecto de las tecnologías de la reproducción
sobre la calidad espermática:
efecto de la criopreservación en espermatozoides humanos

I.A

Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development

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Abstract

Sperm cryopreservation is widely used in clinic for insemination, *in vitro* fertilization and other procedures such as intracytoplasmic sperm injection. The assessment after freezing/thawing of spermatozoa viability, motility and sometimes DNA integrity (mainly using fragmentation assays) has been considered enough to guarantee the safety and effectiveness of the technique. However, it is known that, even when fragmentation is absent, a significant DNA damage could be detected in some genome regions. This is particularly important considering that, during the last years, several studies have pointed out the importance of key paternal genes in early embryo development. In this study, using normozoospermic donors, we present a candidate gene approach in which we quantify the number of lesions produced by freezing/thawing over key genes (*PRM1*, *BIK*, *FSHB*, *PEG1/MEST*, *ADD1*, *ARNT*, *UBE3A*, *SNORD116/PWSAS*) using quantitative PCR. Our results demonstrated that the cryopreservation protocol used, which is routinely employed in clinic, produced DNA lesions. The genes studied are differentially affected by the process, and genome regions related to Prader-Willi and Angelman syndromes were among the most damaged: *SNORD116/PWSAS* (4.56 ± 1.84 lesions/10 kb) and *UBE3A* (2.22 ± 1.3 lesions/10 kb). To check if vitrification protocols could reduce these lesions, another experiment was carried out studying some of those genes with higher differences in the first study (*FSHB*, *ADD1*, *ARNT* and *SNORD116/PWSAS*). The number of lesions was not significantly reduced compared to cryopreservation. These results could be relevant for the selection of the most adequate available cryopreservation protocol in terms of the number of lesions that produced over key genes, when no differences with other traditional techniques for DNA assessment could be detected.

Introduction

During the last years, cryopreservation has become an integral part of assisted reproductive technologies (ARTs) (Di Santo et al., 2012). This is a widely employed procedure in fertility clinics for the conservation of spermatozoa that will be later used for insemination, *in vitro* fertilization (IVF) and intracytoplasmatic sperm injection (ICSI). It is well-known that cryopreservation has detrimental effects on spermatozoa. Besides this procedure causes structural and functional alterations (O'Connell et al., 2002; Ozkavukcu et al., 2008; Di Santo et al., 2012), many studies have also pointed out the damaging effects that freezing/thawing processes have on DNA. The increase in reactive oxygen species (ROS) during cryopreservation seems to be the main cause of DNA alterations (Mazzilli et al., 1995; Wang et al., 1997; Aitken & De Iuliis, 2007, 2010) rather than other processes as apoptosis (Thomson et al., 2009). ROS affect DNA integrity causing abasic sites, cross-linking, modification in nitrogen bases or even DNA strand breakages (Box et al., 2001; Baumber et al., 2003). The presence of DNA damage in the male germ line has been linked with a variety of adverse outcomes such as low fertilization rates, decrease in embryo implantation, miscarriage, cancer and other diseases in the offspring (Lewis & Aitken, 2005; Fatehi et al., 2006; Fernandez-Gonzalez et al., 2008; Aitken et al., 2009). Moreover, sperm DNA damage has been related with an increased risk of pregnancy loss after ARTs, such as IVF or ICSI (Zini et al., 2008). Most of the studies to test DNA integrity in human spermatozoa are limited to fragmentation analysis; however, it is known that even when fragmentation is absent, a significant DNA damage could be detected in some genome regions (Riesco & Robles, 2012). It is also known that some paternal genes are crucial for early embryo development. In fact, most genes in spermatozoa are hypermethylated, and those regions hipomethylated are usually related to development regulators, biosynthetic or metabolism loci (Wu et al., 2011). Both, in human and mice, genes important for embryonic development lack methylations (Hammoud et al., 2009; Brykczynska et al., 2010). According to Wu et al. (2011), this fact would make easier early gene activation in the embryo. Taking this into account, we can assume that DNA lesions could be particularly detrimental when affects to those genes. To study the number of lesions promoted by cryopreservation in those relevant genes is particularly interesting considering that, as we have previously said, there are several DNA lesions, different to fragmentation, which cannot be detected with the methods traditionally employed in clinic (Halosperm, Halomax; Halotech, Madrid, Spain). We have employed a quantitative PCR (qPCR) approach previously described by Rothfuss et al. (2010) to quantify the number of lesions in six genes with roles in fertilization and embryo development: BCL2-interacting killer (*BIK*), FSHb polypeptide (*FSHB*), protamine 1 (*PRM1*), mesoderm specific transcript homolog (mouse) (*PEG1/MEST*), adducin 1 alpha (*ADD1*) and

aryl-hydrocarbon receptor nuclear translocator (*ARNT*). Moreover, as ARTs have been recently associated with a higher incidence of some epigenetic syndromes (Maher et al., 2003; Amor & Halliday, 2008; Carrell & Hammoud, 2010; van Montfoort et al., 2012), two genome regions related with Prader-Willi and Angelman syndrome were also analysed (*UBE3A* and *SNORD116/PWSAS*). Moreover, to check if the freezing rate is a key factor affecting lesions ratio, we have quantified the number of lesions in *FSHB*, *ADD1*, *ARNT* and *SNORD116/PWSAS* after vitrification following the protocol previously published by Isachenko et al. (2012).

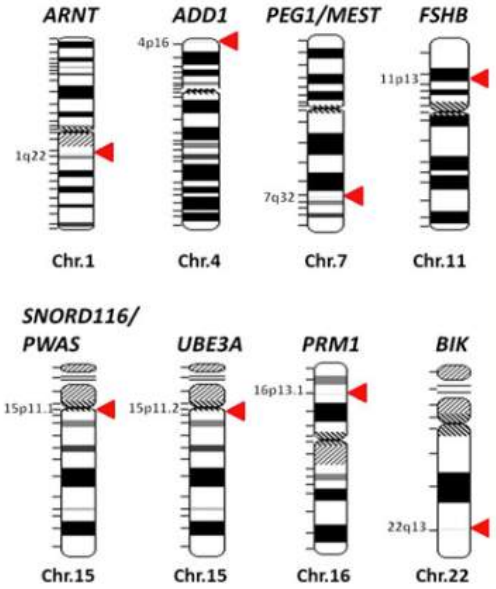

Figure 1 Localization of all the studied genes in their chromosomes (from NCBI).

Materials and methods

Collection and analysis of human samples

Human semen samples were donated by young men (24– 28 years old), in accordance with the institutional standards approved by the Research Ethical Committee of our University. All donors gave written informed consent to take part in this study. All the ejaculates were allowed to liquefy for 20 min at 37 °C. Then, a preliminary analysis of the samples was performed analysing: volume of semen, sperm concentration, motility and morphology at light microscopy, as well as leucocyte concentration. Only the samples reaching the standards in sperm volume, total spermatozoa in the ejaculate, spermatozoa per mL, motility and percentage of morphologically normal cells proposed by the World Health Organization

(WHO, 2010) were selected. A total of six samples were studied for the cryopreservation assay and four for the vitrification experiment.

Cryopreservation procedure and thawing

Sperm samples were cryopreserved strictly following the clinic procedure. First, semen samples were diluted 1:1 in a commercial cryoprotective medium (from 2 to 6 × 10^7 sptz/mL), Sperm Freezing Medium (Irvine Sciencific, Barcelona, Spain). The mixture was equilibrated during 10 min at RT and loaded in 0.5 mL French straws. Then, the straws were exposed horizontally to liquid nitrogen vapours (2 cm over the surface) for 30 min. The straws were then plunged into liquid nitrogen and stored until used. Thawing was carried out at RT for 5 min. Cell viability and motility after cryopreservation was always confirmed under light microscopy and DNA isolation was immediately performed.

Vitrification procedure and thawing

The vitrification protocol was performed using four new normozoospermic donors following the protocol described by Isachenko et al. (2012). The seminal plasma was discarded after centrifugation (340 g for 5 min). After that, 15 × 10^6 cells were diluted in 15 μL of human tubal fluid (HTF) (EMD Millipore, Madrid, Spain) supplemented with 1% human serum albumin (HSA; Invitrogen, Madrid, Spain). Then, this spermatozoa suspension was diluted (1:1) with a vitrification solution (HTF + 1% HSA supplemented with 0.5 M sterilized sucrose) and stored in cut standard straws (CSS) (Isachenko et al., 2007). These straws were generated using standard 0.25 mL straws cut at an angle approximately 45°. Three 10 μL aliquot of spermatozoa suspension was located on the end of the inner part of the CSS. Later on, CSS were placed inside sterile 0.5-mL straws, which were hermetically sealed with a hand-held sealer. Equilibration time (5 min) was conducted, at 37 °C in 5% CO_2. Immediately after this, direct plunging into liquid nitrogen (vitrification) was performed. Thawing was performed as following: CSS from the liquid nitrogen were collected, removed from the packaged straw and quickly immersed in pre-warmed (37 °C) 1.8 mL HTF+1% HSA in a 2 mL tube.

H_2O_2 treatment of spermatozoa

Hydrogen peroxide (H_2O_2) treatment was used as DNA damage positive control. H_2O_2 (30%) was diluted in PBS to a final concentration of 4 mM. An aliquot containing 10^7 cells was obtained from each cryopreserved sample and centrifuged at 4500 g for 4 min. The obtained pellet was re-suspended in 0.2 mL of 12% H_2O_2 (freshly prepared to avoid H_2O_2 degradation) and incubated for 1 h at RT in the dark with gentle agitation. Each sample was centrifuged at

13200 g for 4 min to eliminate H_2O_2 solution and DNA extraction was performed. All the chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

DNA isolation

DNA extraction was conducted starting from 10^7 cells of each fresh, cryopreserved and H_2O_2-treated samples. The pellet obtained by centrifugation was resuspended in STE buffer (10 mM Tris-Cl pH 8; 50 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8 and 1% sodium dodecyl sulphate (v/v). Proteinase k (final concentration of 2 mg/mL) and dithiothreitol (1 mM) were added to the lysis buffer (final incubation volume of 0.7 mL). Cells were allowed to lyse at 55 °C with gentle agitation for 2 h. Sperm lysates were subsequently extracted adding one volume (0.7 mL) of phenol/chloroform/isoamyl (25:24:1). After 4 min of vigorously agitation, the aqueous phase was obtained by centrifugation (13200 g for 5 min) and washed twice with 0.7 mL chloroform. DNA was ethanol precipitated overnight at -20 °C. The precipitated DNA was washed with 70% ethanol cooled to -20 °C and resuspended in TE buffer (10 mM Tris-Cl pH 8; 1 mM EDTA). All samples were extracted in a time period of 48 h and stored at -80 °C until use. After thawing, samples were diluted and quantified before qPCR analysis. Samples obtained for each treatment were subjected to the same processes and storage time to reduce variations that can disturb final results. All the chemicals were obtained from Sigma-Aldrich except chloroform (AppliChem, Barcelona, Spain) and ethanol (VWR, Barcelona, Spain). DNA quantity and purity were determined in a Nanodrop spectrometer (Nanodrop 1000; Thermo Scientific, Madrid, Spain). Only high purity DNA (A260/A280 > 1.8) was used for the qPCR assays.

Gene selection and primers design

In this study, we have analysed six genes with roles in fertilization and embryo development: *PRM1*, *BIK*, *FSHB*, *PEG1/MEST*, *ADD1* and *ARNT*. Moreover, two regions with importance in Prader-Willi and Angelman syndrome diseases are ubiquitin protein ligase E3A (*UBE3A*) and small nuclear RNA, C/D box 116 cluster (Prader-Willi/Angelman syndrome region) (*SNORD116/PWAS*) (Fig. 1). For these genes, primers were designed employing Primer Express v 3.0 software (Applied Biosystems). To analyse DNA lesions, two set of primers for long and short fragment amplification were designed within the same region of each gene. All the gene sequences used for primers design were obtained from the NCBI database. Their GenBank references, primers sequences, PCR product length and annealing temperature are shown in Table 1 (ANEXOS).

Quantitative PCR

Quantitative PCR assays were performed on a StepOnePlus Real-Time PCR system

(Applied Biosystems, Foster City, CA, USA). All the assays were performed in 96-well plates (Applied Biosystems, Madrid, Spain). To compare DNA lesions, two fragments of different lengths located in the same gene were amplified as was described previously by Rothfuss et al. (2010). Quantitative PCR assays for long and short fragments were simultaneously performed in the same plate. For long fragments, the reaction mixture (20 μL) contained 4 μL of 5X Fast Start DNA Master plus SYBR Green I (Roche, Germany, Madrid, Spain), 1 μL of each 10 μM forward and reverse primer, 0.4 μL of 50X ROX passive reference dye (BioRad, Madrid, Spain), template DNA (3 ng) and sterile bidistilled water up to 20 μL. For short fragments, the reaction mixture consisted of 10 μL of 2X SYBR Green PCR (Applied Biosystems), 1 μL each of 10 μM forward and reverse primer, 3 ng of template DNA and bidistilled water up to 20 μL. Technical replicates were prepared per each sample (triplicates for long fragments and duplicate for short fragments) and a non-template control was included for each set of primers. The cycling conditions included a pre-incubation phase of 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C, 10 sec at the annealing temperature shown in Table 1 and 50 sec at 72 °C. Product specificity was verified running a 2% agarose gel electrophoresis (data not shown). Threshold cycles (Cts) were measured by StepOnePlus version 2 software (Applied Biosystems). To determine primers efficiencies and validate qPCR results, 1 μg/mL of sperm DNA was diluted (1:10) to 1:100000, corresponding to ~1000–0.01 ng of DNA, and amplified with the primers for long and short fragments employing the cycling conditions above described.

DNA lesions analysis

Number of DNA lesions per 10 kb was calculated according to the formula described by Rothfuss et al. (2010). The difference in the Ct value between the treated samples respect the untreated (fresh samples) for each long and short fragment was calculated and expressed as lesions per 10 kb. Lesions produced by cryopreservation, vitrification and H_2O_2 treatment were calculated for each gene in all normozoospermic samples. The lesions rate value is displayed as an average of the lesions rate calculated for each sample and gene,

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software (IBM, Madrid, Spain). Comparison between the two treatments in each gene was performed by one-way ANOVA. Differences among genes were analysed using one-way ANOVA with S-N-K post hoc test. Significance level of 0.05 was used throughout.

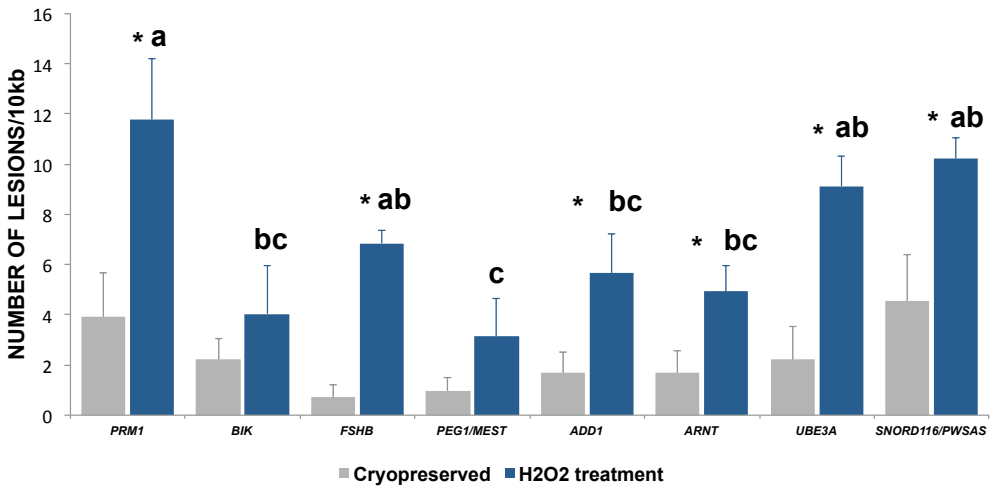

Figure 2 Number of DNA lesions per 10 kb in eight different genes (associated with fertilization, embryo development and epigenetic syndromes) after sperm cryopreservation or treatment with H_2O_2 (4 mM for 1 h in the dark). DNA damage was calculated using the $2^{-}(\Delta\Delta Ct)$ and transformed into DNA lesions rate. Data are expressed as mean values ± SEM (n = 6). Asterisks show differences among the same gene and letters show differences among genes for the same treatment ($p < 0.05$).

Results

DNA lesions rate after cryopreservation procedure

As expected, DNA lesion rates in H_2O_2 treated samples (positive control for DNA damage) were higher than lesion rates in cryopreserved samples in all the studied genes. Significant differences were detected for *PRM1*, *FSHB*, *ADD1*, *ARNT*, *UBE3A* and *SNORD116/PWSAS* (Fig. 2). The maximum number of lesions per 10 kb after H_2O_2 treatment was detected in *PRM1*, *SNORD116/PWSAS*, *UBE3A* and *FSHB*, corresponding to 11.78 ± 2.43, 10.23 ± 0.8, 9.13 ± 1.2 and 6.85 ± 0.53 respectively. The number of lesions was significantly lower in *PEG1/MEST* gene (3.14 ± 1.52), which corresponds to the minimum observed value (Fig. 2). For *ADD1*, *ARNT* and *BIK* genes, the lesions rate calculated after H_2O_2 was 5.67 ± 1.55, 4.94 ± 1.04 and 4.04 ± 1.94 showing significant differences with *PRM1* (Fig. 2). The number of lesions calculated for the cryopreserved samples was lower than for the H_2O_2-treated samples in all cases and no significant variations were found among genes. However, some differences can be observed (Fig. 2). The maximum number of lesions after freezing/thawing was detected in *SNORD116/PWSAS* (4.56 ± 1.84) and *PRM1* (3.91 ± 1.75). These values are around 2 lesions higher than the numbers detected for *BIK* and *UBE3A* (2.21 ± 0.85 and 2.22 ± 1.3) and around 2.5 lesions respecting *ADD1* and *ARNT* (1.69 ± 0.84 and 1.68 ±

0.89). The minimum lesions rates after cryopreservation were 0.72 ± 0.46 and 0.97 ± 0.52 calculated for *FSHB* and *PEG1/MEST* genes (Fig. 2). In these cases, the differences respecting *PRM1* and *SNORD116/PWSAS* values were around 3.5 lesions.

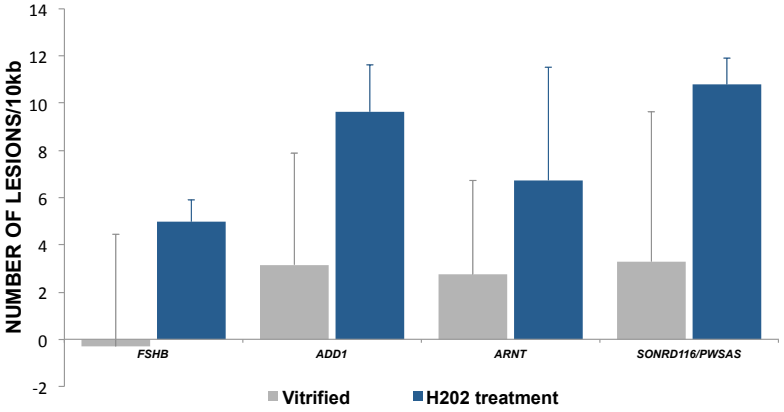

Figure 3 Number of DNA lesions per 10 kb in four different genes (associated with fertilization, embryo development and epigenetic syndromes) after sperm vitrification or treatment with H_2O_2 (4 mM for 1 h in the dark). DNA damage was calculated using the $2^{-}(\Delta\Delta Ct)$ and transformed into DNA lesions rate. Data are expressed as mean values ± SEM (n=4). No significant differences between treatments and genes were found.

DNA lesions rate after vitrification procedure

As expected, DNA lesion rates after H_2O_2 treatment were higher than lesion rates in vitrified samples in all the studied genes. No significant differences were detected for the studied genes: *FSHB*, *ADD1*, *ARNT* and *SNORD116/PWSAS* (Fig. 3).

Q-PCR validation

The PCR efficiencies ranged from 89.4 to 112.6% for long fragments, and from 97.3 to 112.9% for short fragments. The relationship between the Ct and the DNA dilution value was linear with coefficient of regression r^2 higher than 0.9 in all cases. Similar efficiencies were observed for all the primers over the same range of template concentration, and therefore, validating the results obtained.

Discussion

A major concern within the field of assisted reproduction is to ensure the safety of the techniques used. Therefore, it is necessary to understand in depth the molecular causes of a failed embryonic development or the increase in the incidence of certain syndromes associated with these technologies which have been already described (Maher et al., 2003;

Amor & Halliday, 2008; Carrell & Hammoud, 2010; van Montfoort et al., 2012). The technique of sperm cryopreservation is widely used in gynaecological centres performing insemination, IVF and ICSI. In fact, the effects of freezing/thawing on sperm chromatin integrity and their possible origins have been widely studied (Aitken & De Iuliis, 2007, 2010). There are many different techniques commonly employed for DNA damage evaluation such as COMET assay, sperm chromatin structure assay, TdT-mediated dUTP nick end labelling and sperm chromatin dispersion (SCD) test (Chohan et al., 2006; Aitken et al., 2009). Moreover, SCD technology, initially described by Fernandez et al. (2003) has become the basis of some commercial kits in the recent years (Halosperm, Halomax), extensively used in fertility clinics for their quick set (Fernandez et al., 2005). Essentially, all these techniques provide information about DNA fragmentation degree and the general status of chromatin integrity.

However, an absence of detectable DNA fragmentation after cryopreservation is not always correlated with an absence of DNA damage (Riesco & Robles, 2012). The limitations of the global DNA integrity analysis can be overcome employing qPCR methods. These methods are based on the ability of certain DNA lesions (as oxidized bases, abasic sites or thymidine dimers, generated during cryopreservation) to retard and block the polymerase progression along DNA template. Those lesions are finally translated to less amplification and consequently a Ct delay (Sikorsky et al., 2004; Rothfuss et al., 2010). Thus, DNA damage which would be undetectable with traditional techniques can be analysed and quantified in specific genes or chromatin regions employing qPCR approaches. These approaches are particularly important in those genes relevant in early development. In our study, the lesions detected after H_2O_2 treatment were higher than those produced by cryopreservation in all cases (Fig. 2). These expected results demonstrated that ROS-generated lesions are detectable by qPCR. The highest number of lesions/10 kb, generated after H_2O_2 treatment, were detected in *PRM1* (11.78), *UBE3A* (9.13) and *SNORD116/PWSAS* (10.23), whereas the lowest in *PEG1/MEST* (3.14). As a consequence of the freezing/thawing processes, the maximum number of lesions were detected in *SNORD116/PWSAS* (4.56), *PRM1* (3.91), *UBE3A* (2.22) and *BIK* (2.21) whereas the minimum in *PEG1/MEST* (0.97) and *FSHB* (0.72), which correlates with the lesions quantified after H_2O_2 treatment in most genes. These results suggest different vulnerability of genome regions to undergo damage as we have observed in our laboratory in previous studies (Riesco & Robles, 2012). The structure of sperm chromatin could explain this different vulnerability to damage. As Ward (2010) reviewed, human sperm chromatin is divided into three structural domains: the vast majority of DNA is associated with protamines which confer protection during fertilization; a small part remained associated with histones (10–15%) (Gineitis et al., 2000; Zalenskaya et al., 2000; Wykes & Krawetz, 2003) and therefore with a lower degree of compaction; and another part

of DNA would be attached to the sperm nuclear matrix at matrix attachment regions associated also with histones and situated near the periphery of the nucleus. The human protamine domain (*PRM1-PRM2-TNP2*) on chromosome 16p13.13 would be one of these regions associated with histones (Fig. 4) and bounded by two spermatozoa-specific attachment regions as demonstrated by Wykes & Krawetz (2003).

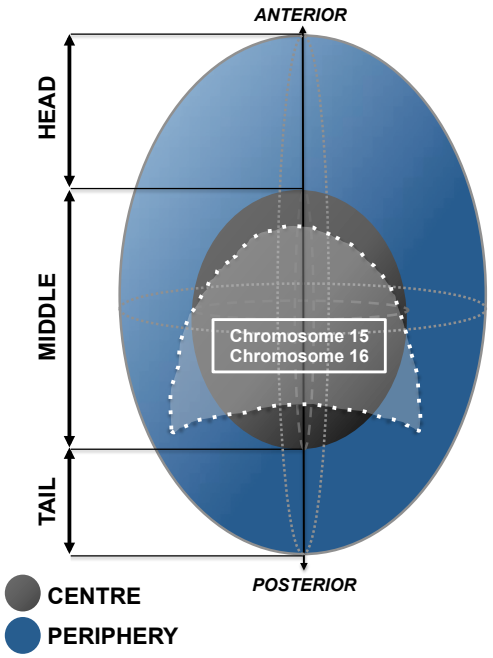

Figure 4 Representation of human sperm nucleus following Manvelyan et al. (2008) and Li et al. (2008) scheme. Dark grey represents the middle part of the nucleus and light grey the periphery. Anterior (head) and posterior (tail) parts are also represented. White shadow shows the approximate localization of the core histones in decondensed and permeabilized sperm nuclei. Following the ISCN human chromosomes sub groups (Shaffer & Tommerup, 2005) and Manvelyan et al. positional studies, authors hipothesized that chromosomes 15 and 16 (where the higher degree of damage was observed in the genes *PRM1*, *SNORD116* and *UBE3A*) are localized in the centre, co-localizing with the core histones represented.

These observations are in concordance with our results for *PRM1* gene, in which we observed higher number of lesions per 10 kb (3.91) after cryopreservation and the highest after H_2O_2 treatment (11.78). Moreover, according to Li et al. (2008), the histone-compacted chromatin is also localized in the post-acrosomal region, extending to the sperm nuclear annulus, being coincident with the localization of chromosomes 15 and 16 (Shaffer & Tommerup, 2005; Manvelyan et al., 2008) where the major number of lesions have been detected in the genes *PRM1*, *SNORD* and *UBE3A* after cryopreservation and peroxide treatment (Fig. 2). These results pointed out that those genes located in regions with low degree of compaction are also more susceptible to undergo damage after cryopreservation.

These assumptions become more important as these histone enriched regions correspond to genes with important roles in embryo development. These regions correspond to developmental promoters (Hammoud et al., 2009; Wu et al., 2011), *HOX* gene cluster and transcription and signalling factors (Wykes & Krawetz, 2003; Hammoud et al., 2009; Miller et al., 2010; Ward, 2010; Johnson et al., 2011). It is known that some genetic or epigenetic disruptions affecting imprinted genes can lead to abnormalities related to growth and neurodevelopmental disorders. Prader-Willi syndrome (PWS), characterized by infantile hypotonia, hypogonadism, obesity and hyperphagia, developmental delay, intellectual disability and other phenotype features, is caused by deficiency in one or more paternally expressed imprinted transcripts within chromosome 15q11-q13 (Cassidy et al., 2012). Many studies have targeted to the paternally expressed SNORD116 snoRNAs (small nucleolar RNA, C/D box 116 cluster) located within the *SNURF/SNRPN* locus to have an important role in the PWS aetiology (Ding et al., 2008; Sahoo et al., 2008; de Smith et al., 2009). Moreover, Angelman syndrome (AS), characterized for severe cognitive and neurological disability, results from an underexpression of the maternally imprinted gene *UBE3A* (encoding for the ubiquitin protein ligase E3A) located also within chromosome 15q11-q13 (Cassidy et al., 2000; Ishida & Moore, 2012). Although the main causes for both syndromes have been attributed to chromosomal deletions, duplications or uniparental disomy, epimutations and DNA methylation defects are present in a frequency of 1–3% (PWS) and 2–4% (AS) (Ishida & Moore, 2012). In this study, we have also analysed both *SNORD116/PWSAS* region and *UBE3A*, obtaining interesting results. The lesions quantified for *SNORD116/PWSAS* were the highest observed after cryopreservation (4.56 ± 1.84) following a similar pattern after H_2O_2 treatment (10.23 ± 0.8). The lesions detected for this region were similar to those detected for *PRM1* gene, showing significant differences with some of the other genes (Fig. 2). These results suggest a higher susceptibility of *SNORD116/PWSAS* region to cryodamage. For *UBE3A*, the lesions observed after cryopreservation were also one of the highest values observed (2.22 ± 1.3), as well as those observed after H_2O_2 treatment (9.13 ± 1.2), showing a similar pattern than *PRM1* and *SNORD116/PWSAS*. In spite of AS being originated for an underexpression of the maternally *UBE3A* gene, the study of this gene in spermatozoa could provide more information about the susceptibility of this *PWAS* region, as both *SNORD116* and *UBE3A* are located within chromosome 15q11-q13. On the other hand, imprinted *PEG1/MEST* gene (paternally expressed) was found to show a low number of lesions; 0.97 after cryopreservation and 3.14 after H_2O_2 treatment (significantly different respecting *PRM1*, *UBE3A*, *SNORD116/PWSAS* and *FSHB*). These observations pointed out a higher resistance to cryodamage and damage generated by ROS. The chromosome region 7q32, where this gene is located, has been delineated as a candidate area for Silver–Russell

syndrome, another epigenetic disease (Amor & Halliday, 2008; Ishida & Moore, 2012). The association of ARTs with a higher incidence of some epigenetic syndromes that some authors have suggested during the last years (Maher et al., 2003; Amor & Halliday, 2008; Carrell & Hammoud, 2010; van Montfoort et al., 2012) was essentially attributed to the effect that embryo culture conditions could have on methylation patterns. However, our observations suggest that sperm cryopreservation could have also a detrimental effect in some important imprinted regions.

DNA damage has been associated with many detrimental effects in the offspring such as apoptosis after the first cleavages (Fatehi et al., 2006), decrease in embryo implantation, miscarriage and diseases in the newborn (Aitken et al., 2009). Altered transcription rates for different genes involved in growth and differentiation have been documented in the offspring obtained with cryopreserved spermatozoa in other species (Perez-Cerezales et al., 2011). Moreover, sperm DNA damage has been related with an increased risk of pregnancy loss after IVF or ICSI (Zini et al., 2008). However, some studies did not find correlation between DNA damage in spermatozoa and its fertilization ability (Aitken et al., 1998; Ahmadi & Ng, 1999). Therefore, DNA damaged spermatozoa are able to reach and fertilize the oocyte, both *in vivo* and especially *in vitro*, where the natural selection barriers are bypassed (Perez-Cerezales et al., 2010). The oocyte has multiple mechanisms and a great capacity of DNA repair (Menezo et al., 2010), the problem arises in establishing the level of DNA damage that exceeds this capacity, as DNA having specific lesions may cause misreading errors during replication generating de novo mutations, or impairing DNA methylations.

Cryopreserved normozoospermic samples are commonly used in clinic for insemination procedures and therefore, to know the 'molecular healthy' of sperm samples after the process would be crucial. The technique presented in this work could be useful for the selection of the most adequate cryopreservation protocol when other traditional techniques to evaluate DNA status fail in reporting differences among them. The comparison established in this study between cryopreservation and vitrification protocols demonstrated that results are highly variable among males after vitrification and therefore, the number of lesions was not significantly reduced compared with cryopreservation.

This qPCR-based technique for DNA evaluation in specific genes could help in selecting and improving cryopreservation protocols used in clinic. Any possible improvement in this respect could enhance, even more, the safety and success of assisted reproduction techniques, a major biotechnological advance in recent decades.

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Conflict of interest

There is no competing interest.

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Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development

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Abstract

During recent years, several studies have pointed out the importance of key paternal transcripts in early embryo development. Sperm cryopreservation is commonly applied in assisted reproductive technologies (ARTs) and it is important to know if it produces any relevant effect at this level. In this study, using normozoospermic donors, we present a candidate transcript approach in which we quantify the presence of sperm mRNAs considered as markers for male fertility and pregnancy success. Analyses were done using quantitative PCR. Our results demonstrated that the used cryopreservation protocol, which is routinely employed in clinical practice, alter transcripts considered as spermatozoa quality markers and markers for pregnancy success. Most of the studied transcripts considered as male quality markers (*PRM1*, *PRM2*, and *PEG1/MEST*) and one of studied mRNAs reported as markers of pregnancy success (*ADD1*) were reduced after cryopreservation. In order to check if vitrification protocols could reduce this alteration, another assay was carried out analysing those transcripts with higher differences in the first study (*PRM1* and *PRM2*). The results showed the same tendency. Although maternal mRNAs can compensate these deficiencies, these results could make advisable the optimization of freezing/thawing procedures.

Introduction

In vitro fertilization (IVF) and intracitoplasmatic sperm injection (ICSI) have revolutionized the field of human reproduction. Recently, a higher incidence of diseases such as Prader–Willi [24] has been associated with the use of these technologies. Since 2004, when Ostermeier and colleges [28] showed the contribution of spermatic RNA to the oocyte during fertilization, many studies have been carried out to disentangle the role of these molecules in early stages of embryonic development [14,30]. Previous to the mentioned publication, no function was described for spermatic RNA, due to: the low abundance of transcripts present in these cells [21], the confirmed absence of spermatic ribosomes [27] and consequently, the fact that RNA stored in sperm cells is residual after spermatogenesis [1]. Therefore, many questions must been answered regarding the role of the spermatic transcripts on fertilization and embryo development, opening the door to possible future targets to improve assisted reproductive technologies (ARTs). Some of these questions have been answered in publications which assigned different roles to these RNAs such as compaction and structural functions [15,26], epigenetic modulation functions in the early stages of embryonic development [30] and specific biological functions [32]. Transcriptomics has been used in some recent studies as a tool to compare transcripts pat- terns presented in diverse spermatozoa samples [5,37,38] and differences between fertile and infertile individuals have been found [29]. Thus, spermatic mRNAs could be possible clinical markers associated with semen quality and pregnancy success [10,12]. This fact makes necessary to study in depth the potential modifications that these reproductive assisted technologies can produce on bio- logical-key RNA population. Moreover, cryopreservation is a technique widely used for the conservation of spermatozoa that will be later used for ARTs and it is known that this technique can produce changes in transcripts [11], and DNA damage [7] and/or epigenetic modifications [3]. We consider that this evidence makes necessary to carry out an in-depth study of potential modifications that the freezing/thawing processes can produce in biological-key RNA populations with roles in early embryonic development. Interestingly, the transcripts found in mature spermatozoa correlated with hypomethylated DNA [34] and therefore with those genes potentially relevant for early embryo development. In this study we have analysed the effect of cryopreservation on human spermatozoa transcripts. Using this candidate transcript approach, we studied two different groups of transcripts: one proposed as male fertility markers: BCL2-interacting killer (*BIK*), FSHb polypeptide (*FSHB*) [11], protamine 1 (*PRM1*), protamine 2 (*PRM2*) [6] and mesoderm specific transcript homolog (mouse) (*PEG1/MEST*) [20]; and a the second one proposed as pregnancy success markers: activin A receptor type II like 1 (*ACVRL1*), adducin 1 alpha (*ADD1*), androgen receptor (*AR*), aryl-hydrocarbon receptor nuclear translocator (*ARNT*) and

endothelial PAS domain protein (*EPAS1*) [11]. Moreover, in order to check if the freezing rate is a key factor affecting transcript stability, we have compared the results obtained with the protamine transcripts after cryopreservation and vitrification [17].

Materials and methods

Ethics

The study protocol was performed in accordance with the institutional standards approved by the Research Ethical Committee of the University of León. All donors gave written informed consent to take part in this study in accordance with the Declaration of Helsinki.

Collection and analysis of human samples

Human semen samples were donated by young men (24–28years old). All the ejaculates were allowed to liquefy for 20 min at 37 °C. Then, a detailed morphological characterization and counting of the spermatozoa was carried out before any assay. Phase contrast microscopy was used on fresh ejaculate samples. The amount of detected leukocytes or immature spermatids represented less than 0.1% of the total number of cells. Only the samples reaching the standards proposed by the World Health Organization (WHO, 2010) were selected. A total of twelve samples classified as normozoospermic were individually studied. Nine of them were used for the cryopreservation assay (n=9) and the three remaining for the comparative among cryopreservation and vitrification protocols study (n=3).

Cryopreservation procedure and thawing

Each of the sperm samples, used in this assay (n=9), was cryopreserved strictly following a protocol used in clinic. First, they were diluted 1:1 in a commercial cryoprotective medium (from 2 to 6 $\times$ 10^7 sptz/mL), Sperm Freezing Medium (Irvin Sciencific, Barcelona, Spain). The mixture was equilibrated during 10 min at RT and loaded in 0.5 mL French straws. Then, the straws were exposed horizontally to liquid nitrogen vapours (2 cm over the surface) for 30 min. The straws were then plunged into liquid nitrogen and stored until used. Thawing was carried out at RT for 5 min. Cell viability after cryopreservation was always confirmed under micros- copy and immediately after, RNA isolation was performed.

Vitrification procedure and thawing

The vitrification protocol was performed in parallel to cryopreservation in three new normozoospermic donors following the protocol described by Isachenko et al. [17]. After removing the seminal plasma (340 g for 5 min), 30 $\times$ 10^6 spermatozoa were diluted in 15 μL

of human tubal fluid (HTF) (EMD Millipore, Madrid, Spain) supplemented with 1% human serum albumin (HSA; Invitrogen, Madrid, Spain). This cell suspension was then diluted (1:1) with a vitrification solution (HTF + 1% HSA supplemented with 0.5 M sterilized sucrose) and loaded in cut standard straws (CSS) [18]. These straws were created using standard 0.25 mL straws cut at an angle approximately 45°. Three 10 μL aliquot of cell suspension was placed on the end of the inner part of the CSS. After that, CSS were placed inside sterile 0.5 mL straws, which were sealed hermetically with a hand-held sealer. The equilibration was con- ducted, at 37 °C in 5% CO_2 for 5 min., immediately after, vitrification (direct plunging into liquid nitrogen) was performed. For thawing, CSS were recovered from the liquid nitrogen, removed from the packaged straw and quickly immersed in 1.8 ml HTF + 1% HSA which had been pre-warmed to 37 °C in a 2 mL tube.

RNA isolation

RNA was extracted from each fresh, cryopreserved and vitrified sample. The samples were centrifuged at 9000 rpm, for 4 min, at RT. After that, seminal plasma or preservation procedure residues (supernatant), was discarded. Trizol Reagent (Invitrogen, Madrid, Spain) protocol was carried out following commercial advices.

RNA quantity and purity were determined in a Nanodrop spectrometer, (Nanodrop 1000, Thermo Scientific). Only high purity RNA (A_{260}/A_{280} >2.00) was used for cDNA synthesis.

cDNA synthesis

For each fresh, cryopreserved and vitrified sample, complementary DNA was synthesized from 1 μg of RNA using a commercial kit, the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Madrid, Spain). The final reaction was incubated in a thermocycler using a single cycle program which included a first step of 55 min at 50°C followed by 5 min at 85°C. After that, it was kept at 4°C until its storage at −20 °C.

Gene selection and primer design

For transcript analysis, we focused on two sets of genes: those ones reported as possible male fertility markers and those associated with pregnancy success. Within the first group the studied genes were: BCL2-interacting killer (*BIK*), FSHb polypeptide (*FSHB*), protamine 1 (*PRM1*), protamine 2 (*PRM2*) and mesoderm specific transcript homolog (mouse) (*PEG1/MEST*). In the second one, the set of genes included were: activin A receptor type II like 1 (*ACVRL1*), adducin 1 alpha (*ADD1*), androgen receptor (*AR*), aryl- hydrocarbon receptor nuclear translocator (*ARNT*) and endothelial PAS domain protein (*EPAS1*). Primers pairs for the amplification of each mentioned gene were designed using Primer Blast

software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) leaving an intron within the amplicon in order to avoid amplification of residual genomic DNA.

Moreover, primers pairs described by Cavalcanti and colleges [2] for four genes proposed as reference genes (beta-actine (*B-ACT*), ATP synthase subunit beta (*ATP5B*), glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) and heat shock protein 1, beta (*HSPCB*) were studied.

All gene sequences used for primers design were obtained from the NCBI database. Their GenBank references, primers sequences, PCR product length and annealing temperature are shown in Table 1 (ANEXOS).

Quantitative PCR

Quantitative PCR assays were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). All the assays were performed in 96-well plates (Applied Biosystems, Madrid, Spain).

Quantitative PCR assays for fresh and cryopreserved samples for each male (n=9) were simultaneously performed in the same plate. Each reaction mixture (20 ll) contained 10 μL of 2× SYBR Green PCR (Applied Biosystems, Madrid, Spain), 1μL of each 10 μM forward and reverse primer (See Table 1), 0.5 μL of complementary DNA (fresh-cryopreserved) and 7.5 μL of bidistillated water up to 20 μL. The same protocol was exactly performed for the comparative assay for fresh, cryopreserved and vitrified samples for each male (n=3) using *PRM1* and *PRM2* primers (See Table 1).

The program used for the amplification consisted of 10 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 60 s at the annealing temperature shown in Table 1 for each gene. A melting curve analysis was included after the amplification program. Three technical replicates were done for each sample and a non-template control was included for each set of primers. Product size and specificity were verified running a 2% agarose gel electrophoresis (data not shown).

Primers efficiency tests were carried out as well. Starting from 2 μL of cDNA, five serial dilutions were done (1:2) corresponding to 100 ng to 3.125 ng of cDNA. These dilutions were amplified with the same cycling conditions previously described.

Two step RT-PCR analysis

After complementary DNA synthesis, conventional PCR was carried out using specific primers pairs for the four reference genes proposed by Cavalcanti [2] as well as for *BIK* and *ADD1* (See Table 1). For each reaction mixture, 0.5 μL of cDNA (from each fresh and cryopreserved sample) was amplified employing commercial the GoTaq Flex DNA commercial kit (Promega, Madrid, Spain). Cycling conditions included a pre-incubation

phase of 5 min at 94 °C, followed by 30 cycles of 30s at 94°C, 30s at 62°C and 20s at 72 °C; and a final extension of 7 min at 72 °C. In order to detect the PCR final products, the total volume of each sample (20 μL) were examined by electrophoresis in a 2% agarose gel stained with 0.5 μg/mL Gel Red (Biotium, Madrid, Spain). The results were visualized under a UV transilluminator (GenoSmart, VWR).

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 soft- ware (IBM, USA). Two variables were generated with the mean values of each of the nine male for each gene (fresh, Ct_gene_f, and cryopreserved, Ct_gene_c) in order to compare the transcripts population before and after freezing. Fresh and cryopreserved mean values for each gene were compared by one-way ANOVA test with a cut-off value $p < 0.05$. The same statistical analysis was per- formed with the data obtained from the comparative assay. In this case three variables, with three mean values each, were generated (fresh, Ct_gene_f, cryopreserved, Ct_gene_c and vitrified Ct_gene_v) for *PRM1* and *PRM2* transcripts.

Results

All the studied transcripts from fresh semen samples showed earlier Ct (''threshold cycle'') than those from cryopreserved samples (Fig. 1), indicating a clear decrease in the presence of transcripts after the cryopreservation process. Interestingly, reference genes also showed this delay in Ct values after cryopreservation in some of the studied males (Fig. 2).

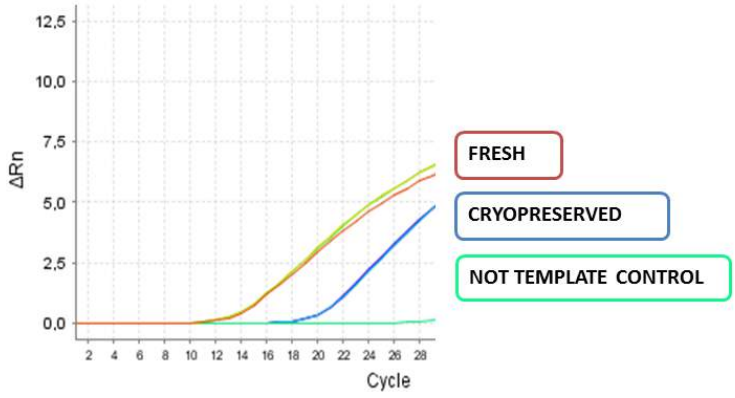

Fig.1.- General amplification pattern of the qPCR results for the studied transcripts.

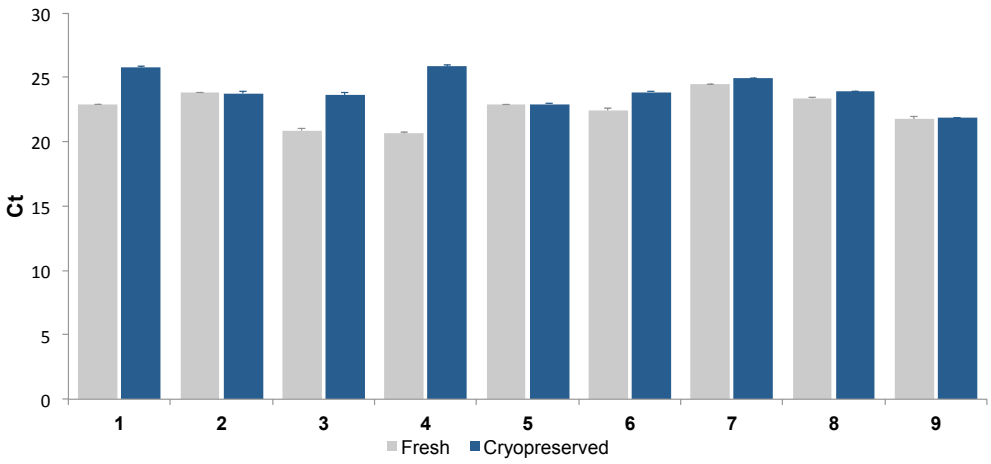

Fig.2. Threshold cycle (Ct) variation among males for the reference gene actin-beta.

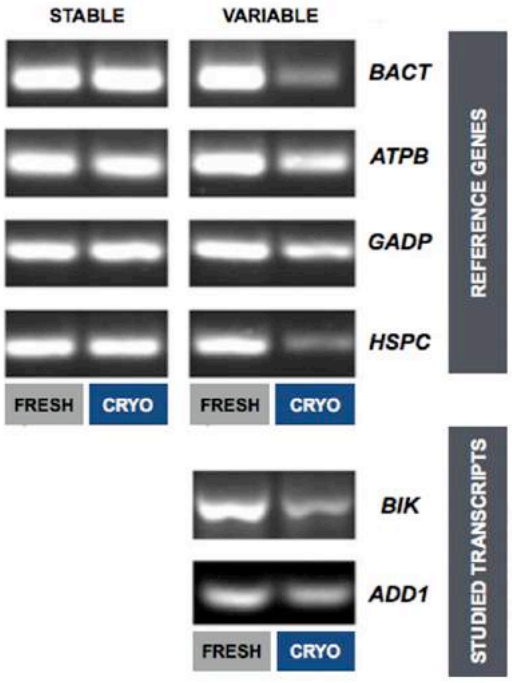

Fig. 3. Final products of the RT-PCR assay for described reference genes (*B-ACT*, *ATPB5*, *GAPD* and *HSPCB*) and for two studied transcripts (*BIK* and *ADD1*) before (FRESH) and after cryopreservation (CRYO). In reference genes, all transcripts could be either remained stable or variable after cryopreservation, making the use of a stable reference gene unviable.

The 2 step RT-PCR assay corroborated the results obtained by the qPCR experiment. After cryopreservation, the amount of final product for reference genes (*B-ACT*, *ATP5B*, *GAPD* and *HSPCB*) could be either stable or variable depending on the male, whereas transcripts of study showed band variation in all cases (Fig. 3). Despite individual differences, the protamine 1 (*PRM1*) and 2 (*PRM2*) had a very early Ct, also showing the earliest amplification in cryopreserved samples. The remaining transcripts (*BIK*, *PEG1/MEST*, *FSHB*, *ACVRL1*, *ARNT*, *AR, ADD1* and *EPAS1*) showed later Ct values, especially in cryopreserved samples (Fig. 4).

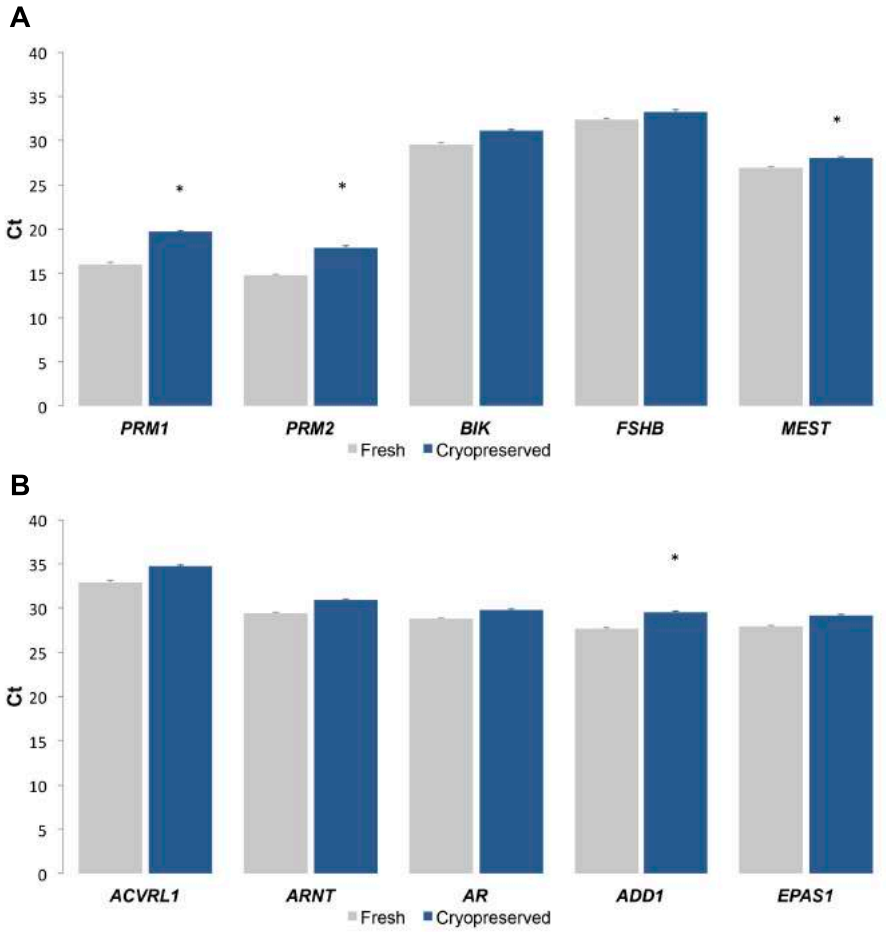

Fig. 4. Comparison of threshold cycle (Ct) values of the transcripts before and after cryopreservation. Data are expressed as mean values ± SEM (n=9). Asterisks show significant differences among fresh and treated samples ($p < 0.05$). (A) Transcripts published as potential markers for sperm quality. (B) Transcripts published as potential markers for pregnancy success.

Taking into account the variability of reference genes, the quantification of transcripts and the analysis of the results were performed using Ct values. Mean values of ‘‘Ct_gene_f’’ and

"Ct_gene_c" variables were compared by one-way ANOVA between pairs of fresh-cryopreserved variables. Significant differences were obtained for *PRM1*, *PRM2*, and *PEG1/MEST* within the group of transcripts associated with sperm quality, and *ADD1* within the group of pregnancy success (Fig. 4).

The comparative assay between freezing rates (cryopreservation and vitrification protocol) studying *PRM1* and *PRM2* (those transcripts with higher differences among fresh and cryopreserved samples in the first assay) showed the same tendency independently on the protocol used. A decrease in the amount of transcripts (later Ct) in both treatments (cryopreservation and vitrification) was observed. Significant differences were obtained for *PRM1* and *PRM2* between fresh and vitrified samples after per- forming a one-way ANOVA. No significant differences were found between the two freezing methods (cryopreservation and vitrification) (Fig. 5).

Q-PCR validation

All primers employed for the qPCR analysis showed PCR efficiencies from 90% to 110%. The relationship between the Ct and the DNA dilution value was linear in all cases. The coefficient of regression r^2 was always higher than 0.9. Similar efficiencies were observed for all the primers over the same range of template con- centration, and therefore, validating the results obtained.

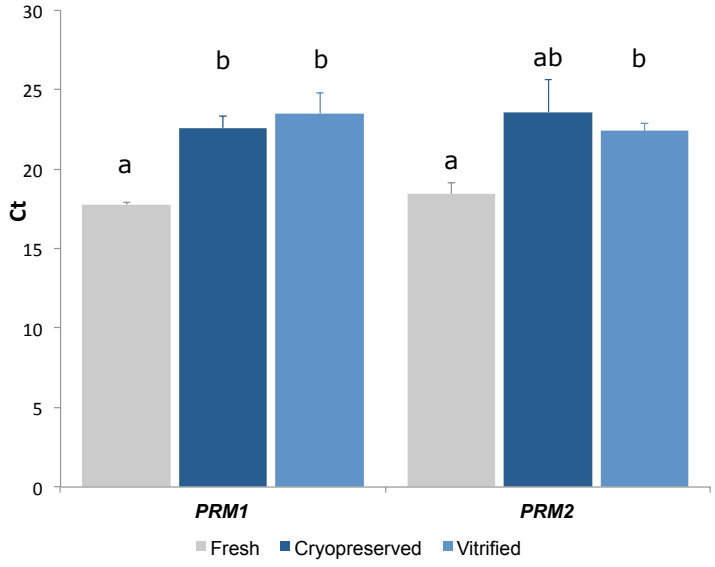

Fig. 5. Comparison of threshold cycle (Ct) values of the protamine transcripts in fresh, cryopreserved and vitrified samples. Data are expressed as mean values ± SEM (n=3). Letters show differences among treatments ($p < 0.05$).

Discussion

Paternal contribution is not limited to DNA. Spermatozoa RNAs are also transferred to the oocyte and seem to have a relevant role in early embryo development. Recent publications have demonstrated, using microarray technology, that several transcripts in human spermatozoa disappear after sperm cryopreservation [9]. It is possible that this deficiency in the paternal contribution could be overcome by maternal mRNAs, however it makes necessary to evaluate the real effect of cryopreservation over key transcripts. Transcriptional activity has never been demonstrated in spermatozoa [27]. Thus, it can be assumed that mRNA molecules present in spermatozoa represent a remaining of the transcripts from spermatogenesis [31]. Taking this into consideration, it is easy to understand that, in most cases, the transcripts are present at very low concentrations, although their role may be relevant to the embryo in many ways, as it has been reported by many authors [14,15,26,30]. This small population of mRNAs explains the late Ct obtained in most of the transcripts studied when these results were compared with other data from transcriptionally active cells. Only *PRM1* and *PRM2* transcripts showed early Ct values (Fig. 4). This fact can be explained because these transcripts are stored in spermatozoa as residual RNAs in very high quantities from the final stages of spermatogenesis [25,31] where *PRM1* and *PRM2* proteins play a very important and necessary role in achieving the correct hyper-compaction of the paternal genetic material [10]. However, attending to the results and the pattern of the amplification curves for each studied gene, qPCR can be considered as an accurate method for transcripts quantification even in this cell type [2].

The relative quantification of the transcripts by the usual method $2^{-}(\Delta\Delta Ct)$ for gene expression requires a constant reference gene with a Ct value lower than the rest of the transcripts analyzed [13]. Despite the existence of these references in spermatozoa, the fact that this specific study focuses on the possible elimination of transcripts after cryopreservation makes changes in reference genes or target genes equally likely. We corroborated this fact by observing the Ct obtained in reference and target genes (Fig. 2). The evidence that commonly used housekeeping genes are not totally stable throughout the chilling and freezing conditions has been re- ported in other species like zebrafish [23]. In our study, this variation was also corroborated with the 2-step RT-PCR assay which showed variance among males (Fig. 3). Since finding an appropriate reference it is highly improbable, the results were analysed in terms of Ct delay. Therefore, statistical analysis was carried out with raw Ct values obtained for each gene. Ct delay is significant in the genes: *PRM1*, *PRM2* and *PEG1/MEST*, within the group of potential sperm quality markers, and *ADD1* in the group of potential successful pregnancy markers. These transcripts are significantly reduced after cryopreservation. Based on the genes analysed, cryopreservation

significantly affects four of the five studied transcripts proposed as fertility markers and two to five mRNAs proposed as successful pregnancy markers. Our hypothesis is that cryopreservation is affecting transcripts stability making some of them prone to degradation. It is known that some proteins can interact with specific maternal mRNAs and repress their expression, supporting the stability of these transcripts [16,19,36]. We hypothesize that the interaction of proteins-spermatic RNAs could be also responsible for the stabilization of paternal transcripts until oocyte fertilization or until first stages in early development. In this case, cryopreservation could be affecting mRNA–protein interaction making mRNA molecules more susceptible to degradation. In fact it is known that cryopreservation could affect nucleoprotein structure in the sperm DNA [9]. It is feasible that, in the same way, freezing-thawing could affect the interaction of proteins with mRNAs, which would remain unstable and finally degraded.

Based on these results, the transcripts *PEG1/MEST*, and *ADD1*, presented by Garcia-Herrero [11] as potential fertility and pregnancy markers, might be affected by cryopreservation. Moreover, our study suggests that cryopreservation may also reduce the presence of protamines transcripts which have been included within the transcripts associated to male fertility [6]. These results imply that cryopreservation protocols can affect molecular elements with an important role in fertilization success and correct early embryonic development. It would either be a random or stochastic effect, not only among individuals but also in different freezing processes of the same ejaculate. Thus, even when maternal contribution could totally or partially overcome these deficiencies, it would be advisable to perform an assessment of relevant transcripts prior to fertilization. However, it is obvious that this advice could be seen as unrealistic, at least nowadays, due to the cost of the technique and the complexity of the assays.

After this first study, we decided to check if ultrafast freezing rates (vitrification) could affect in a lower level at the molecular status of the spermatozoa transcripts. This procedure, which has been used for embryo and oocyte preservation [33,35], has been success- fully applied in assisted reproductive technologies during recent years [4,22]. Vitrification protocols have risen up as an important part of human reproductive medicine [8]. Moreover, sperm vitrification experiments have been performed leading to birth of healthy babies [17]. Following the CSS protocol described by Isachenko et al. [18] we studied the population of *PRM1* and *PRM2* transcripts (those with a higher Ct decrease after cryopreservation) before and after vitrification, in order to compare this protocol against cryopreservation (using the same ejaculates in both protocols). Both transcripts presented Ct delay after the vitrification protocol (Fig. 5), having the same tendency as the one described in the first assay (Fig. 4). No significant differences were found among treatments but different tendencies were noticed. Vitrified samples showed earlier Ct values than the cryopreserved ones in *PRM2*

contrary to *PRM1* in which the results were the opposite. Anyway, our results indicate that despite the freezing rate used, the molecular status of the spermatozoa after them is poorer than the fresh ones.

It should be mentioned that the male donors used in this study were young and normozoospermic. Cryopreserved normozoospermic samples are commonly used in clinical practice for insemination procedures and therefore, knowing the ‘‘molecular health’’ of sperm samples after the process would be crucial. Future work, in collaboration with a Gynecological Center, will address the effect of cryopreservation on males presenting different sperm pathologies, which are usually the patients in ARTs clinics. The detection of these alterations could help in the development of optimized cryopreservation and vitrification protocols, and consequently enhance the safety and success of assisted reproduction techniques, a major biotechnological advance in recent decades.

Conflict of interest

There is no competing interest

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CAPÍTULO II

Efecto de la estabulación en la calidad espermática en especies de interés comercial: el lenguado senegalés

II.A

Selection of nonapoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*)

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Abstract

Senegalese sole (*Solea senegalensis*) is a promising species in aquaculture. However, owing to decreased sperm quality in F1 generations and the absence of courtship in those individuals born in captivity, artificial fertilization is being used to generate new progenies. The objective of this study was to implement a sperm selection method for nonapoptotic sperm subpopulation recovery before sperm cryopreservation. In particular, magnetic-activated cell sorting is used to eliminate apoptotic spermatozoa. This study represents the proof-of-concept for magnetic-activated cell sorting applicability in teleost species relevant in aquaculture. Apoptotic cell population was studied by flow cytometry using YO-PRO-1 and a caspase detection kit. Also, reactive oxygen species were measured in sperm samples. Our data demonstrated that caspase detection is more specific than YO-PRO-1 in the identification of apoptotic cells in *S senegalensis* seminal samples. The results showed that the percentage of apoptotic cells (caspase positive) was significantly higher (P=0.04) in seminal samples from F1 than that from wild individuals. Magnetic-activated cell sorting removed a significant number of apoptotic cells from the samples (54% and 75% in wild and F1 individuals, respectively), decreasing the level of cells positive for reactive oxygen species (P=0.17). In conclusion, this technique reduces the percentage of nonfunctional spermatozoa in a seminal sample before cryopreservation. This novel technique can be applied directly in the aquaculture industry.

Introduction

Senegalese sole (*Solea senegalensis*) is a relevant species in European aquaculture but shows important limitations in commercial culture [1]. The lack of courtship and natural spawns from F1 animals bred in captivity is the major drawback [2]. Moreover, Senegalese sole present poor and variable semen quality [3–6], thus reducing the opportunities of successful fertilization. In F1 breeders, sperm quality parameters are even worse [5]. This problem has a direct effect on industrial production because aquaculturists can only rely on wild-captured animals for breeding programs. Experiments performed by Mañanós and Carazo [7] corroborated that reproductive failure was focused on males. The absence of courtship in F1 individuals [8] means that artificial fertilization methods have to be used [9], and these require sperm cryopreservation to conserve seminal samples until fertility trials are carried out. It is well-known that good quality sperm samples must be used in cryopreservation protocols to guarantee good fertility results after thawing [10]. When initial sperm quality is poor, as in this particular case, the possibility of selecting sperm subpopulations within the same seminal sample could be particularly important. This is the main objective of the present study. We evaluated apoptotic status in wild-captured individuals and in F1 males. We also provided the proof-of-concept in this species of a simple, fast, inexpensive, and highly specific method for use in routine protocols in the fish farm industry, thus enabling nonapoptotic spermatozoa to be cryopreserved. For the first time in fish seminal samples, we propose magnetic-activated cell sorting (MACS) as a selection method for nonapoptotic sperm subpopulation recovery (Fig. 1). To confirm the efficacy of the technique, an apoptotic cell population was studied by flow cytometry using YO-PRO-1 and a caspase detection kit. The analysis of reactive oxygen species (ROS) levels before and after the MACS protocol was also assessed.

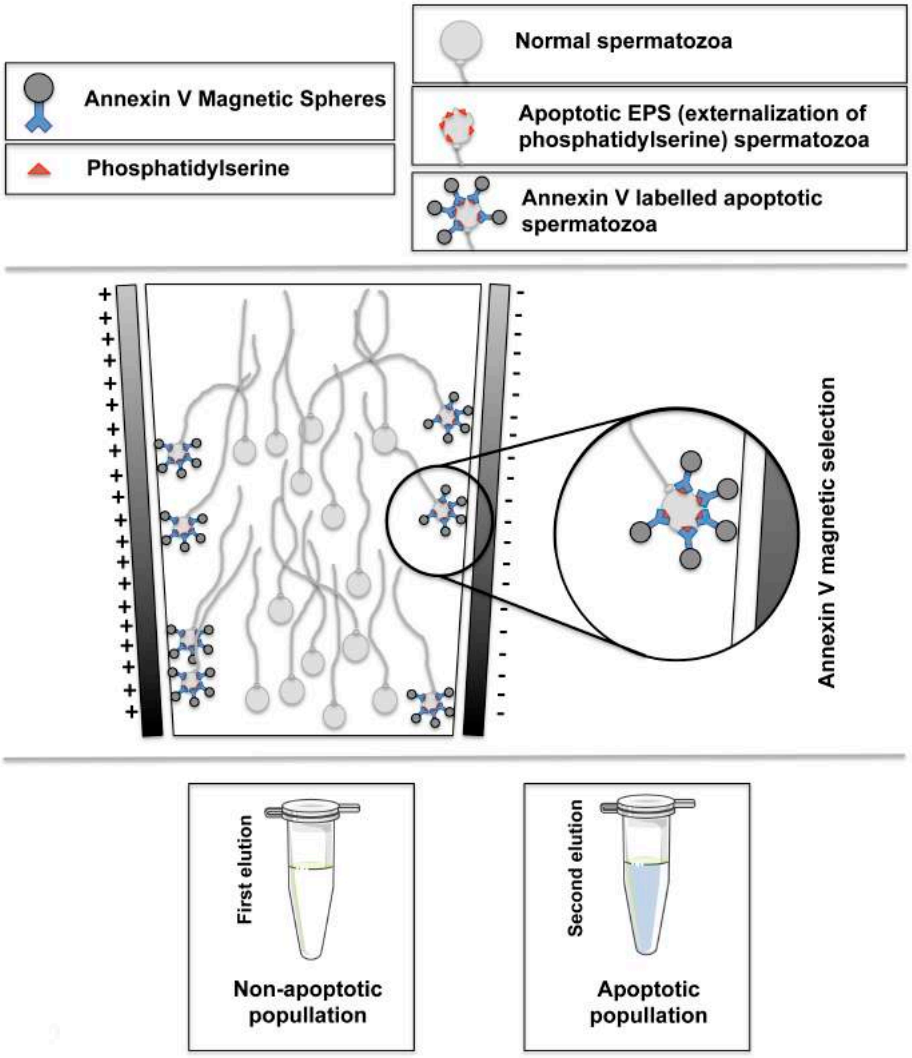

Figure 1.- Magnetic cell sorting (MACS) selection basis. After incubation with MACS microbeads (annexin-V beads), the cell suspension is loaded onto the separation column. The unlabelled cells run through while the magnetically labelled cells (externalized phosphatidylserine/annexin-V) are retained in the column. The retained material is washed with buffer to remove unlabelled material.

Materials and methods

Ethics

Fish handling (routine management and research) was performed according to the national and institutional guidelines and the European Union Directive (2010/63/EU) for the protection of animals used for experimental and other scientific purposes.

Animal maintenance

The wild-captured and F1 broodstocks (stock density around 5 kg/m^3) of the Spanish Oceanographic Institute in Santander (Marine Culture Plant El Bocal) used in the experiment were kept indoors in tanks (1:1, female:male) 4 to 14 m^2 in area and 1 m in depth. An open flow circuit (33% tank/h of water renewal) and constant aeration maintained optimal conditions in each tank. The continual artificial photoperiod was 16 hours of light and 8 hours of darkness. Mesh shading was placed over the tanks to reduce light intensity. Animals were fed (0.5% of the total biomass) 6 days a week with a commercially extruded feed (Vitalis Cal and Vitalis Repro, Skretting). Each animal was monitored with a passive integrated transponder tag (Pit-tags, AVID) on the dorsal musculature. The fish were kept under environmental temperatures (around 13 °C) until the end of January. The temperature was then artificially increased 0.5 °C every week. Once the water reached 16 °C, the thermostat was programmed to perform ± 2 °C periodic peaks to induce gonad maturation. Sperm sampling was performed during this period [9].

Sperm sampling, analysis and pooling

Animals were anesthetized with 40-ppm clove oil during 2 minutes before sperm collection. The urogenital pore was cleaned to eliminate water, feces, and mucus. Sperm was collected with a syringe without a needle by gently pressing the testes on the fish-pigmented side. Ejaculates were kept in microcentrifuge tubes on ice until further analysis. Samples contaminated with urine or seawater were rejected. Sperm was collected identically in both types of males (wild type and F1 males).

A total of 27 fluent males were evaluated: nine from the wild-captured broodstock and nine from the F1 broodstock. Sperm volume was analyzed, and cell concentration determined for each ejaculate. Sperm was prediluted 1:10 in 200 mOsm/kg Ringer solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES; pH 7.7). Sperm motility was measured after activating 1 mL of prediluted sperm with 500 mL artificial seawater (450 mM NaCl, 10 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2$·6H2O, and 16 mM $MgSO_4$·$7H_2O$; pH=7.7) kept at 4 °C. Motility was determined under light contrast microscopy (magnification: 200 ×). Billard et al. [11] percentage scores were used for motility evaluation. Each sperm sample was analyzed in duplicate.

For the apoptosis and membrane status assays, ejaculates were pooled (three pools for wild-captured males and three for F1 males; 3 males/pool) attending to similar motility parameters to prevent sperm quality from being affected, to get volume enough to perform the study and following the routine in aquaculture companies before artificial fertilization. Each pooled sample was split into two aliquots. For ROS evaluation assay, three pools (3

males/pool) were created from nine ejaculate samples from wild-captured males. These pools were directly processed by MACS.

Cryopreservation protocol

One of the aliquots was directly cryopreserved following the published protocol for this species [9]. Seminal plasma was removed by centrifugation (1 minute, 1000 g). Cells were resuspended in Mounib without cryoprotectants. Sperm was diluted (1:2 ratio) in Mounib extender with cryoprotectants (10% BSA and 10% DMSO), loaded into 0.5-mL French straws (IMV Technologies, France), exposed to liquid nitrogen vapor during 7 minutes and rinsed in liquid nitrogen until used. This aliquot was left as the control in the experiment. The cryopreservation protocol was performed in exactly the same way with the other aliquots after MACS separation.

Magnetic-activated cell sorting

A MiniMACS separation unit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for MACS following commercial recommendations. Briefly, sperm cells were incubated with Annexin V-conjugated microbeads at 4 °C for 15 minutes. For a total cell number of 10^6 separated cells, 10 mL of microbeads were used. The spermatozoa-microbeads mixture was loaded into an MS separation column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) containing a coated matrix with iron balls, fitted in a magnet device. The magnetic field enabled two fractions to be separated: one enriched on viable cells (with no interaction with the matrix, Annexin-V negative) and one enriched on apoptotic cells (retained in the column by the microbeads-matrix interaction, Annexin-V positive). The first fraction was eluted and held with buffer for cryopreservation. The second one, obtained by flushing, was discarded.

Thawing protocol

Samples were thawed in a 40 °C bath for 7 seconds. Cryoprotectants were removed by centrifugation, and the cells were resuspended in Ringer solution.

Flow cytometry analysis

Thawed samples (both control and MACS elution) were stained with a CaspGLOW Fluorescein Active Caspase Staining Kit (eBioscience, San Diego, USA) following the manufacturer's instructions. This kit contains the inhibitor specific for all caspases, Z-VAD-FMK, which is directly conjugated to fluorescein isothiocyanate for the detection system of the active caspases. It is a nontoxic cell permeable reagent and binds directly to the active

enzymes. The samples were costained with 2 mg/mL propidium iodide (PI; Invitrogen, Leiden, The Netherlands) for live cell detection.

Membrane viability analysis was performed by double staining PI/YO-PRO-1. YO-PRO-1 (Invitrogen) was added to the sperm (150 nM) and incubated 10 minutes in the dark at 4 °C. Then, PI (Invitrogen) was added (2 mg/mL) and left to incubate for 5 minutes in the dark at 4 C.

After the incubation time (PI/YO-PRO-1 and PI/CaspGLOW), samples were analyzed twice in a FACSort Plus Analyzer (Becton–Dickinson, USA) acquiring 10,000 events per replicate. The red fluorescence emitted by PI was detected using a 610 nm filter, and the green fluorescence emitted by YO-PRO-1 or CaspGLOW (fluorescein isothiocyanate) was detected with a 516 nm filter.

Reactive oxygen species evaluation

Reactive oxygen species determination was performed using dichlorofluorescein diacetate (DCFH-DA; Sigma, Madrid, Spain), which can specifically reveal intracellular H_2O_2 emitting green fluorescence. Fresh cells from each pool (before and after MACS) were incubated in 25 mM DCFH-DA (7 °C, 40 minutes) and costained with 2 mg/mL PI (Invitrogen; 7 °C, 10 minutes). Nuclei were stained with 40 6-diamidino-2-phenylindole (DAPI), and the slides were evaluated under a fluorescence microscope. Acquired images were processed using FIJI software. 200 cells were randomly analyzed for each slide. Positive cells for DCFH-DA (green fluorescence) and negative for PI were counted as live ROSþ cells.

Statistical analysis

The results were expressed as means ± standard error of the mean. The Shapiro–Wilk normality test was carried out for all variables. Significant differences between resulting flow cytometry variables were analyzed using a Student's t test ($P < 0.05$). All statistical analyses were conducted with SPSS software (version 20.0).

Results

Flow cytometry analysis revealed a significantly different apoptotic status ($P<0.05$) between wild-captured males and F1 males. Following the PI/CaspGLOW staining protocol, wild-captured males presented a low percentage of apoptotic cells, whereas F1 males reported a higher percentage of this subpopulation of cells, around 2.6 times more (Fig. 2A).

PI/YO-PRO-1 staining revealed no significant differences between the two experimental groups with regard to sperm membrane status. The percentage of YO-PRO^{+}/PI^{-} (live and apoptotic) cells for the wild-captured group was similar to that of the F1 group (Fig. 2B).

The effectiveness of MACS for this species was corroborated by the two analyzed parameters (% of caspases^{+} cells and % of membrane-damaged cells). CaspGLOW/PI staining showed a reduction in the percentage of apoptotic cells before and after the MACS protocol in both experimental groups ($P<0.05$). The percentage of apoptotic cells in the wild-captured group comparing to the aliquot treated with MACS decreased by approximately 54%. (Fig. 3A). In the F1 group, approximately 75% of the sample was cleaned after MACS protocol. (Fig. 3C).

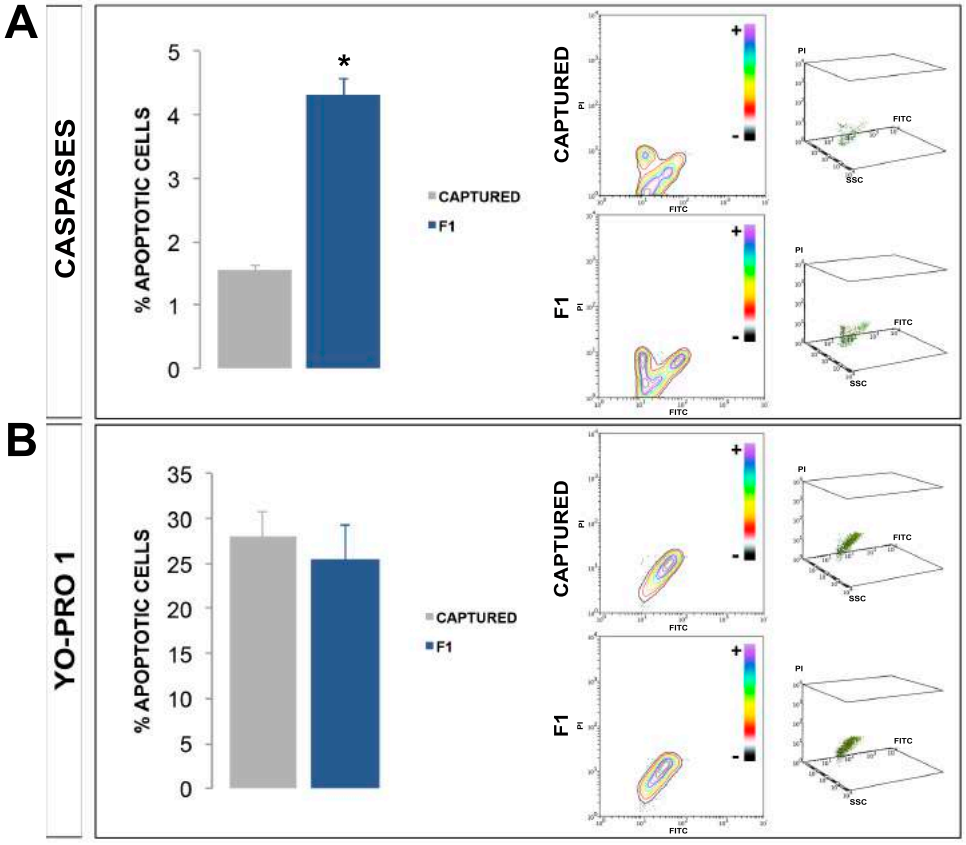

Fig. 2. Apoptotic cell percentage (mean ± standard error of the mean) in wild-captured males (CAPTURED; n=9) and F1 males (F1; n=9) bred in captivity. Two independent staining protocols were performed before flow cytometry: (1) CaspGLOW Fluorescein Active staining Kit combined with PI staining (CASPASES) (A) and (2) YO-PRO-1 staining combined with PI (YO-PRO-1) (B). Those cells positive for green fluorescence and negative for PI were considered as apoptotic cells. (For selected apoptotic population, see contour and 3-Dimensional dot plot figures on the right). Asterisk shows statistical differences (Student's t test; $P < 0.05$). FITC, fluorescein isothiocyanate; PI, propidium iodide; SSC, side scatter.

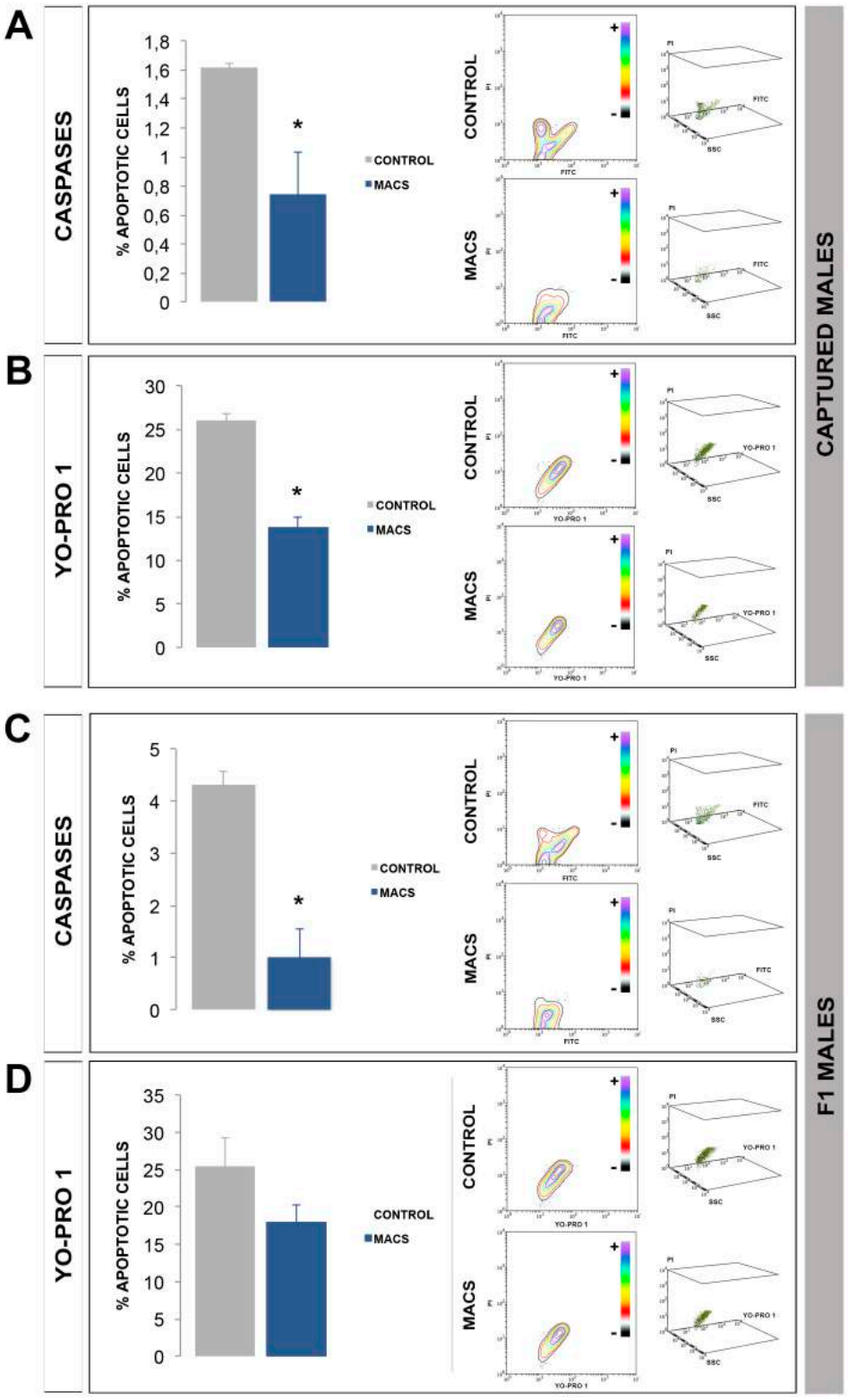
A
CASPASES
% APOPTOTIC CELLS
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Figure 3 (Previous page)- Apoptotic cell percentage in wild-captured males (CAPTURED) (A and B) and F1 males (F1) (C and D) bred in captivity before (CONTROL) and after MACS protocol (MACS). Before flow cytometry, two independent staining protocols were performed: 1) CaspGLOW Fluerescein Active staining Kit combined with PI staining (CASPASES) (A and C) and 2) YO-PRO-1 staining combined with PI (YO-PRO-1) (B and D). Those cells positive for green fluorescence and negative for PI were considered as apoptotic cells. (For selected apoptotic population, see contour and 3D dot plot figures on the right). Asterisks show statistical differences (t-Student; p<0.05).

The YO-PRO-1/PI staining protocol showed similar results. The wild-captured group reported a reduction ($P < 0.05$) of damaged cells of around 50% (Fig. 3B). No statistically significant reduction in the percentage of damaged cells was observed in the F1 group. However, the samples presented the same reduction tendency (Fig. 3D).

Concomitantly, to the evident reduction in apoptotic cells and membrane-damaged cells, the levels of ROS^+ cells ($PI^-/DCFH^+$; Fig. 4A) were determined by comparison before and after the MACS protocol (Fig. 4B).

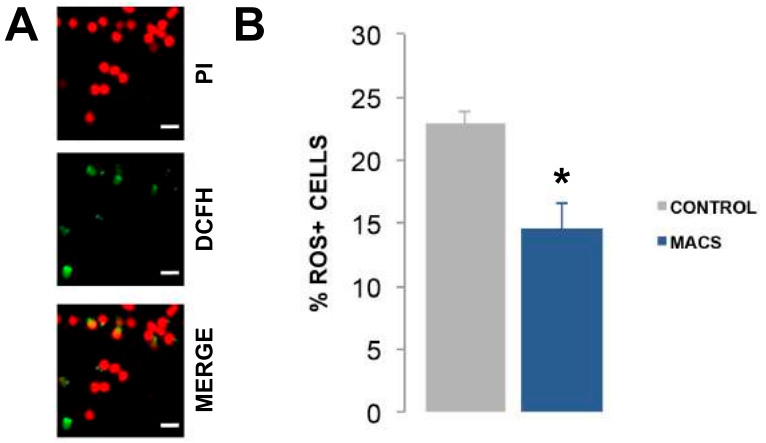

Fig. 4. Oxidation levels before (CONTROL) and after the MACS protocol (MACS) in wild-captured males (n=9). (A) Confocal image of dichlorofluorescein diacetate (DCFH-DA) labeling. (1) propidium iodide (PI); (2) 2',7'-Dichlorofluorescein (DCFH); and (3) merge. (B) Apoptotic cell percentage (mean ± standard error of the mean) before (CONTROL) and after the MACS protocol (MACS) in wild-captured males. Asterisk shows statistical differences (Student's t test; P < 0.05). MACS, magnetic-activated cell sorting; ROS, reactive oxygen species.

Discussion

Soleidae flatfish are considered a family of potential species that can diversify Southern Mediterranean Aquaculture, mainly in Spain and Portugal. Market saturation with European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Spaurus aurata*) has reduced fish prices in recent years, directly affecting the industry. Common sole (*Solea solea*) and

Senegalese sole (*S. senegalensis*) are important candidate species of the Soleidae family. Scientist have developed on-going methods for both of these high commercial value species [12–14] reporting that Senegalese sole could have better growth ratios in captivity than common sole. For this reason, research lines have focused on the improvement of *S. senegalensis* using reliable technologies and methods for high-scale production. Significant progress has already been made. Natural spawns from wild Senegalese sole adapted to captivity [15] and optimized weaning methods for larvae [16–21] have been reported. Nevertheless, considerable drawbacks still hinder their culture. Senegalese sole broodstock (F1 generation) show inadequate spawning performance compared to their wild-captured counterparts [1,2]. Males in the F1 generation are responsible for this reproductive failure, not females [8]. Lack of courtship, together with low-quality semen samples, hinders the expansion of sole aquaculture. Many different strategies have been used to find solutions for low-quality sperm in F1 Senegalese sole, such as enriched diets and hormonal treatments [22], but sperm quality has not improved significantly or permanently, making it necessary to implement new technologies to optimize fertilization results. F1 sperm quality is poorer than that of wild-captured males in different parameters [5], and high numbers of apoptotic cells have been reported in seminal samples [3]. The removal of apoptotic spermatozoa is particularly relevant to prevent failure in fertilization [23–25]. Selection of nonapoptotic sperm subpopulations is particularly important in low-quality semen samples, and even more so if those samples are to be cryopreserved, as in this particular case, until used in artificial fertilization trials. It is well-known that the spermatozoa and/or egg ratio is relevant in fish fertility trials. The presence of low-quality spermatozoa that do not contribute in fertilization and could hinder fertility and modify the real ratio of spermatozoa participating in fertilization should be avoided. If the sample is to be cryopreserved, it is essential to start with good-quality sperm sample because the undesirable effects produced by the process could differentially damage high- and low-quality spermatozoa.

The aims of this study were two: (1) to analyze the spermatozoa apoptotic status of wild-captured males and F1 generation males and (2) to evaluate, for the first time in a fish species, the effectiveness of a protocol (MACS) for the removal of apoptotic sperm cells in *S. senegalensis* (wild-captured and F1 males).

In this work, we assessed differences between wild-captured and F1 from a cellular point of view by evaluating apoptosis in sperm from both groups. Apoptosis is a common process during spermatogenesis. However, apoptotic cells should be phagocytized while in the testis, in agreement with publications conducted in mammals [26]. Nevertheless, in mammals, the presence of apoptotic cells in ejaculated spermatozoa is widely documented [27–30]. Beirão et al. [3] described the presence of an apoptotic population in Senegalese sole sperm samples in a range of 6% to 20% (varying in males) after performing flow cytometry analysis.

The results were similar to their previous studies [30]. For the identification of this population, the authors used Annexin V, a calcium-dependent binding protein with a high affinity for phosphatidylserine (externalized in the cell membrane in early apoptosis). In our study, we checked apoptosis status before and after the process using two different methods: (1) evaluation of the active caspases present in the spermatozoa (a highly specific method) using a specific kit for active caspase staining and (2) evaluation of damage to the sperm membrane using the YO-PRO-1 probe (correlated to early stages of apoptosis). Our results after flow cytometry analysis showed that using the first specific method (staining active caspases) the percentage of apoptotic cells in wild-captured males was lower than that in F1 males (Fig. 2A). However, the analysis performed using YO-PRO-1 (membrane integrity) did not report any differences between the two types of samples: wild-captured and F1 males (Fig. 2A). The percentages obtained in our assays were lower in the case of active caspases and higher in the case of YO-PRO in comparison with the range reported by Beirão [3]. Different staining protocols (with a higher or lower specificity) can show different percentages. The evaluation of active caspases seems to be the best option for the detection of all types of apoptotic cells because the protocol is directed straight at the main proteins regulating the apoptotic pathway, rather than evaluating cellular processes derived from programmed cell death.

Once the higher percentage of apoptotic cells had been confirmed in the F1 seminal samples, we evaluated for the first time in a fish species, a MACS protocol as a method for selecting an apoptotic-free sperm sample. In the apoptosis process, one of the early markers is the loss of membrane integrity, which leads to phospholipid phosphatidylserine externalization [31] (a molecule with a high affinity for Annexin V) [32]. Consequently, Annexin V can be used for cell sorting when it is conjugated with magnetic beads, which are then exposed to a magnetic field in an affinity column. This way, apoptotic spermatozoa are retained in the affinity column and isolated from nonapoptotic sperm. In mammals, there is evidence that probe MACS efficiency decreasing sperm DNA fragmentation levels [33,34], and removing nonapoptotic spermatozoa from ejaculates [32,35,36] MACS selection has been correlated with an improvement in sperm quality and functionality [23,37]. The technology impacts positively on sperm motility [38,39] and improves the sperm deformity index [23,35,37,40]. Some authors reported better fertilization rates after MACS [41] and described better [38] embryo quality because the best sperm cells were selected compared with standard selection methods before artificial reproductive technologies. Magnetic-activated cell sorting has not yet been used for sperm selection in fish species. However, it has been used with carp germ cells for the isolation and enrichment of spermatogonial stem cells from *Labeo rohita* testis [39] using Thy1 (CD90) antibody conjugated with the magnetic microspheres as marker for positive selection. Our results demonstrated that *S.*

senegalensis sperm can be cleaned of apoptotic cells using Annexin V-beads (Fig. 1). The effectiveness of this protocol was corroborated by flow cytometry using two staining techniques in wild-captured males: YO-PRO-1 (Fig. 3B), which detects membrane damage in cells (a decrease of 50%), and the CASPGlow kit (Fig. 3A) which detects active caspases in cells (a decrease of 54%). These results were found in F1 males (around 75% reduction using CASPGlow; Fig. 3C). The MACS protocol also reduced the percentage of positive cells for ROS by approximately 35% (Fig. 4B). Reactive oxygen species affect DNA integrity resulting in abasic sites, cross-linking, modification in nitrogen bases, or even DNA strand breakages [42–44]. DNA damage in the male germ line has been linked to a variety of adverse outcomes [45]. Magnetic-activated cell sorting can be used as an easy protocol in Senegalese sole fish farms as a method for subpopulation selection before cryobanking, ensuring that only the best cells are stored for artificial fertilization.

Conclusions

In conclusion, we showed for the first time in Senegalese sole that sperm apoptotic status in wild-captured males is significantly different to that of F1 males. Our results also point to caspase positive cell quantification as the most specific method for apoptotic spermatozoa evaluation in this species. We also corroborated that MACS is an effective technique for the selection of sperm nonapoptotic subpopulations in *S. senegalensis* (nonapoptotic spermatozoa recovery), providing a useful protocol for reproductive and productive programs in the aquaculture industry.

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II.B

Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa

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Reproduction

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Abstract

Reactive oxygen species have a great impact on spermatozoa function. Gametes from sole males born in captivity (F1) display lower quality than those from wild individuals. In this paper, the percentage of cells positive for dichlorofluorescein (DCF^+) was determined by flow cytometry in wild and F1 animals, the effect of cryopreservation on DCF^+ cells was evaluated in both groups and the distribution of H_2O_2 within the cell was studied by confocal microscopy. Our results indicated that there are no differences in either viability or DCF^+ cells between wild and F1 animals when fresh samples were evaluated. However, when data were analyzed considering two different sperm populations in terms of motility, a significant decrease in viability and DCF^+ cells was reported in low-motile F1 spermatozoa. Cryopreservation did not alter the viability or the presence of DCF^+ cells in sperm samples from wild animals, but significantly decreased the viability in F1 samples. Distribution patterns of H_2O_2 have been established by confocal microscopy in *Solea senegalensis* spermatozoa: co-localization of H_2O_2 with active mitochondria ($MitoTracker^+$) and co-localization with nuclear DNA (DAPI). Compared with H_2O_2 distribution in other marine species such as *Scophthalmus maximus*, *Solea senegalensis* spermatozoa showed widespread presence of H_2O_2 particularly in the nuclei, which could potentially compromise DNA integrity.

Introduction

Marine aquaculture producers from Southern Europe show an increasing interest in a promising species: Senegalese sole (*Solea senegalensis* Kaup, 1858). Sole has both a good marketable assessment and very good consumer acceptance. Despite the fact that sole is an appealing candidate for marine aquaculture, this species shows important limitations in industrial culture (Howell *et al.* 2006, 2009). The absence of courtship in F1 males (Porta *et al.* 2006) together with poor and variable semen quality, particularly in F1 males, are major obstacles in large-scale production (Cabrita *et al.* 2006, Martínez-Pastor *et al*. 2008, Beirão *et al.* 2009, 2011). This absence of natural reproduction in animals born in captivity makes the use of artificial fertilization methods necessary (Rasines *et al.* 2013). These methods involve fish manipulation for gamete collection, sperm classification by motility, and cryopreservation for storing the best sperm samples (Sieme *et al*. 2015) until artificial fertilization trials are performed. Only optimal samples should be used for cryopreservation so as to guarantee good *in vitro* fertilization results.

Reactive oxygen species (ROS) formation has been recognized as a problem for sperm survival and fertility (Guthrie & Welch 2012). In mammals, oxidative stress damage competence of spermatozoa by peroxidation of lipids, induction of oxidative DNA damage and formation of protein adducts are well known (Aitken *et al.* 2012). It has also been established that ROS production could initiate an intrinsic apoptotic cascade causing spermatozoa to lose their motility (Aitken *et al.* 2012). In Senegalese sole, a significantly higher percentage of apoptotic cells in F1 males was reported than those in wild-captured animals (Valcarce *et al.* 2016). The aim of this work was to investigate the relationship between ROS levels and sperm quality in *Solea senegalensis,* which presents two particular problems: (1) low sperm quality in F1 samples and (2) the need for sperm to be cryopreserved before artificial fertilization. For this purpose, the intracellular levels of ROS species and viability of sperm samples from wild-captured and F1 males were analyzed by flow cytometry in fresh and cryopreserved samples. Moreover, the distribution of H_2O_2 in the spermatozoa was studied by confocal microscopy and compared with other marine flat fish species. This analysis gave a molecular insight into sole spermatozoa providing new keys for understanding reproduction failure in *Solea senegalensis*.

Materials and methods

Ethics

Animal manipulation was carried out following the national and institutional bioethical guidelines and European Union Directive 2010/63/EU for the protection of animals used for

experimental and other scientific purposes. The experiments performed in this study are part of project AGL2015 68330C2-1R approved by the IEO Ethics Committee (1/2016).

Animal maintenance and experimental groups

Wild-captured (WT) and F1 broodstocks (F1) were used in this experiment. Fish labeled as wild-captured males (WT) refers to those born in their natural environment and able to reproduce naturally, whereas those labeled F1 are born in captivity and are unable to reproduce. WT males were captured two years before starting this experiment and, according to their size and weight, were approximately 4 years old. F1 males were 4 years old. Both groups of animals were held indoors at a 1:1 female:male ratio in tanks (4–14m^2 in area and 1m deep) with a stock density around 5 kg/m^3 in the Spanish Institute of Oceanography in Santander (Marine Culture Plant *El Bocal*). Each tank maintained constant aeration and an open flow circuit (33% tank/h of water renewal). They were exposed to a 16:8 h (light:darkness) continual artificial photoperiod. Light intensity was reduced with mesh shading placed over the tanks. The fish were fed (0.5% of the total biomass) 6 days a week with commercially extruded feed (Vitalis Cal and Vitalis Repro, Skretting). Each animal in the culture plant was monitored with passive integrated transponder tags (PIT tags, AVID) inserted in the dorsal musculature. Three WT (n=3) and six F1 (n=6) fish were used for this experiment.

Sperm sample collection

Before collection, the males were anesthetized with 40 ppm clove oil for 2 min. The urogenital pore was always toweled to remove feces, water and mucus before sperm sampling. Contaminated sperm was rejected. Ejaculates were collected with a syringe without a needle by gently pressing the testes on the pigmented side of the fish. The samples were individually maintained at 4 °C in microcentrifuge tubes until processing.

Sperm analysis

For each ejaculate (WT: n=3 and F1: n=6), cell concentration and volume were analyzed. Percentage of motile spermatozoa was assessed using the scores by Billard and coworkers (1995). For this purpose, 1µL of prediluted sperm (1:10) in 200mOsm/kg Ringer solution (116 mM NaCl; 2.9 mM KCl; 1.8 mM $CaCl_2$; 5 mM HEPES, pH 7.7) was activated with 500 µL artificial seawater (24.6 g/L NaCl, 0.67 g/L KCl, 1.36 g/L $CaCl_2 \cdot 2H_2O$, 6.29 g/L $MgSO_4 \cdot 7H_2O$, 4.66 g/L $MgCl_2 \cdot 6H_2O$, 0.18 g/L $NaHCO_3$; 1100 mOsm/kg; pH 8.0) kept at 4°C. Motility was determined under light contrast microscopy (magnification: 200×). This procedure was performed twice for each sperm sample following the method described by Billard (Billard *et al.* 1995).

Cryopreservation protocol

Each sample was split into two: (1) a fresh aliquot (FRESH) and (2) a cryopreserved aliquot (CRYO). For cryopreservation, cells were diluted (1:2 ratio) in Mounib extender with cryoprotectants (10% BSA and 10% DMSO) following the protocol published by Rasines (Rasines *et al.* 2013). The samples were loaded into 0.5 mL straws and exposed to liquid nitrogen vapor for 7 min. After this time, the straws were immersed in liquid nitrogen.

Thawing protocol

The samples were thawed in a 40 °C bath for 7 s. The cryoprotectants were discarded after centrifugation (1 min; 1000 g) and the cells were resuspended in Ringer solution.

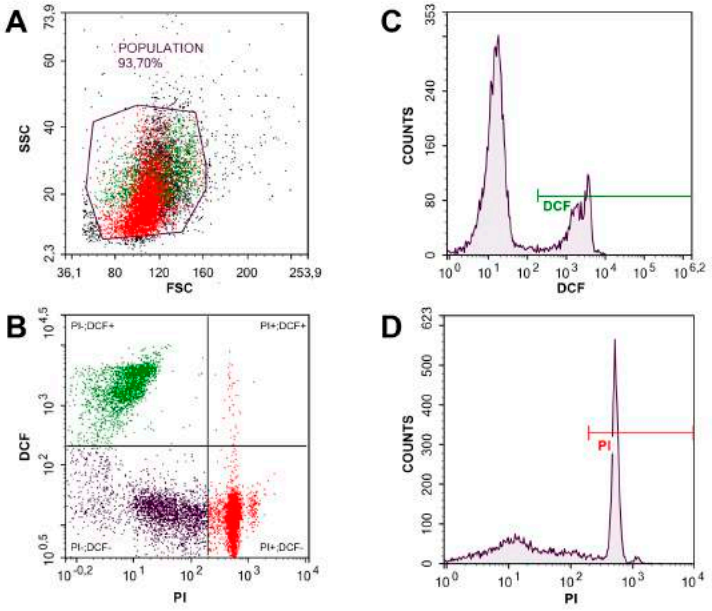

Figure 1 Flow cytometry examples. (A) Dot plot cytograms showing population gating (SSC vs FSC), (B) simultaneous measurements of intracellular reactive oxygen species (DCF) and dead cells (PI). Single count histograms for (C) DCF and (D) PI. DCF, dichlorofluorescein; FSC, forward scatter; PI, propidium iodide; SSC, size scatter.

Flow cytometry analysis

Analyses were performed using a NovoCyte Flow Cytometer (Acea Biosciences, San Diego, CA, USA). Viability was measured using propidium iodide (PI) (Sigma), and intracellular sperm ROS levels were studied using dichlorofluorescein diacetate (DCFH-DA) (Sigma), which can precisely reveal intracellular ROS, emitting green fluorescence when oxidized (Fig. 1). Both aliquots (FRESH and CRYO) for each individual (WT and F1) were incubated in 25 µM DCFH-DA (7 °C, 40 min) and co-stained with 2 µg/mL PI (Invitrogen) (7 °C, 10min).

Overall, 10,000 events were acquired per sample, and the acquisition was performed twice for each sample. Data analysis was performed using NovoExpress software (Acea Biosciences). As control of the technique for the detection of ROS with DCFH-DA in *Solea senegalensis* sperm, three samples were split into three: (i) a non-treated aliquot, (ii) an aliquot treated with 100 mM sodium pyruvate (Sigma-Aldrich), a scavenger of reactive oxygen species and (iii) an aliquot treated with 10% H_2O_2 (Sigma-Aldrich) for 15 min as a positive control. Controls were washed in 1× PBS after treatment and subsequently processed in the usual way.

Measurement of reduced glutathione

To analyze the antioxidant activity in WT and F1 spermatozoa, we studied the intracellular reduced glutathione content for both wild-captured (n=3) and F1 spermatozoa cryopreserved samples (n=3). For this purpose, we used a Glutathione Colorimetric Assay Kit (Biovision). In brief, 1×10^6 cells from wild-captured and F1 sperm respectively were used as samples. After lysis and centrifugation, the supernatants were used for the reduced glutathione measurement in a microplate reader as per the manufacturer's protocol.

Confocal microscopy analysis

To localize the presence of intracellular ROS in Senegalese sole sperm, $1–2\times10^6$ cells/mL of WT, F1 and controls (15 min; 10 mM sodium pyruvate and 15 min 10% hydrogen peroxide) were incubated with 25 µM DCFH-DA (Sigma) (7 °C, 40 min) and 100 nM MitoTracker Deep Red (Invitrogen) (7 °C, 10 min). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich; 1:1000). A 5 µL cell suspension drop was placed on a slide and immediately analyzed under a LSM 800 confocal microscope (Zeiss). To compare the localization of intracellular H_2O_2 in other flatfish species, confocal microscopy analysis was used following the same protocol described previously with turbot (*Scophthalmus maximus*) sperm cells.

Statistical methods

Data were analyzed using SPSS, version 20 for Macintosh (SPSS). Data are presented as mean ± s.e.m. in all cases. Mean values of each variable were compared by a one-way ANOVA ($P<0.05$). Duncan post hoc test was performed to analyze homogenous subgroups in each parameter.

Results

Sperm viability

Wild-captured vs F1 males

No statistical differences were found between wild-captured (WT-FRESH) and F1 (F1-FRESH) males in fresh samples (Fig. 2 – darker bars). Good viability (over 60% in both cases) was reported in fresh samples.

Fresh vs cryopreserved samples

Two different patterns were shown: cryopreservation did not significantly decrease viability in wild-captured males (WT-CRYO) (only a reduction of less than 5% of viable cells was recorded) (Fig. 2). On the other hand, males born in captivity (F1-CRYO) were strongly affected by the protocol reporting a statistically significant reduction of around 35%.

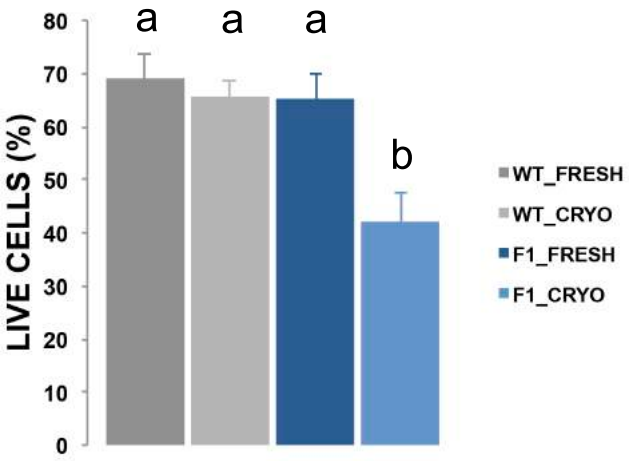

Figure 2 Viability analysis by flow cytometry: WT (n=3) vs F1 (n=6). CRYO, cryopreserved/thawed samples; F1, F1 males; FRESH, control samples before cryopreservation; WT, wild-captured males. Superscript letters show differences among groups (*P* < 0.05).

F1 generation: high-motility samples vs low-motility samples

To check if the best F1 samples in terms of motility showed a similar tendency to wild male samples, data were reanalyzed in F1 attending to motility. Wild-captured male samples were kept as reference. The F1 group was split into two groups according to their motility (Supplementary Material 1, *ANEXOS*). Three of them were labeled 'low-motility samples' (F1-L MOT; *n=3)* with a percentage of motile cells ≤ 30%, and another three were

considered high-motility samples (F1-H MOT; n=3) with motility values ≥ 45%). In F1 fresh high-motility samples (Supplementary Material 1A; F1-FRESH-H MOT), viability was similar to sperm samples from wild animals (WT-FRESH), but in low-motility samples (Supplementary Material 1B; F1-FRESH-L MOT), a significant reduction in viability was observed in F1 fresh spermatozoa (54.66 ± 5.41%). After cryopreservation, sperm viability in F1 males decreased in both high- (F1-CRYO-H MOT) and low-motility samples (F1-CRYO-L MOT) (high motility: 56.71 ± 3.94% and low motility: 27.39 ± 2.44%) (Supplementary Material 1A and B – light blue bars).

Sperm ROS level by flow cytometry

The evaluation of intracellular ROS species in *Solea senegalensis* spermatozoa with DCF by flow cytometry was corroborated by an independent experiment (Supplementary Material 2). Samples treated with sodium pyruvate (scavenger of ROS) presented significantly fewer DCF^+ cells, whereas those treated with H_2O_2 reported higher values than the non-treated sample.

Wild-captured vs F1 males

WT fresh samples (WT-FRESH) presented high levels of DCF^+ cells over 50%. There were no statistical differences between this group and F1 fresh samples (F1-FRESH) although a clear tendency of reduction can be seen in the results (Fig. 3 – darker bars).

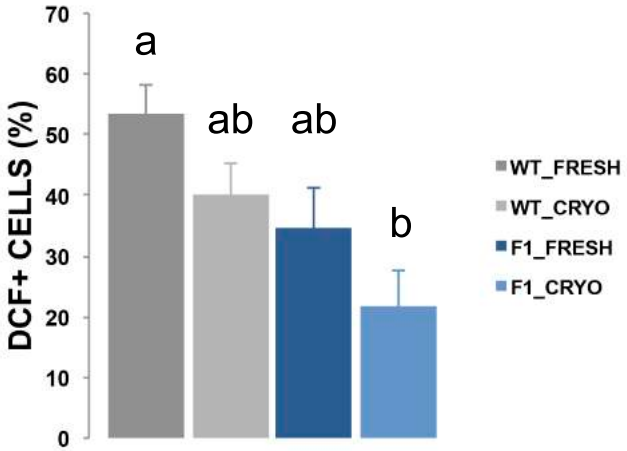

Figure 3 Intracellular reactive oxygen species analysis (DCF^+ cells) by flow cytometry. WT (*n*=3) vs F1 (*n*=6). CRYO, cryopreserved/thawed samples; DCF, dichlorofluorescein; F1, F1 males; FRESH, control samples before cryopreservation; WT, wild-captured males. Superscript letters show differences among groups (*P* < 0.05).

Fresh vs cryopreserved samples

No significant differences were found between fresh (WT-FRESH) and cryopreserved samples (WT-CRYO) in wild-captured males, although lower levels of DCF^+ cells were found in cryopreserved samples (WT-FRESH: 53.32 ± 4.89% and WT-CRYO: 40.10 ± 5.40) (Fig. 3 – gray bars). The F1 males were not significantly affected by the freezing–thawing process. F1 fresh samples (F1-FRESH) reported 34.76 ± 6.61% DCF^+ cells (mean value ± s.e.m.) and cryopreserved spermatozoa showed 21.72 ± 5.81% (mean values ± s.e.m.) (Fig. 3 – dark blue bar).

F1 generation: high-motility samples vs low-motility samples

The previously described split was carried out in this analysis for the F1 generation: high-motility samples (F1-H MOT) vs low-motility samples (F1-L MOT). High-motility samples reported the same tendency as the global analysis (Supplementary Material 1C and D). Statistical differences were only found between WT-FRESH and F1-CRYO-H MOT samples (Supplementary Material 1C). F1 low-motility samples (F1-L MOT) presented a different profile (Supplementary Material 1D) compared with high-motility ones. These samples reported a reduction of 66% ROS^+ cells compared with WT fresh samples. Following the same tendency, F1 low-motility cryopreserved samples (F1-CRYO-L MOT) showed a reduction of around 84% compared with the WT-CRYO group. No statistical differences were found between fresh and cryopreserved in F1 low-motility samples.

Reduced glutathione levels by colorimetric assay

The colorimetric assay reported a statistically significant higher level of reduced glutathione in F1 samples compared with that in wild-captured ones (Fig. 4).

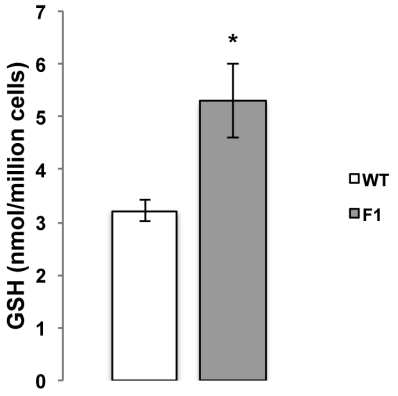

Figure 4 Reduced glutathione levels by colorimetric assay. WT (n=3) vs F1 (n=3). F1, F1 males; WT, wild-captured males. Asterisk shows statistically significant differences ($P < 0.05$).

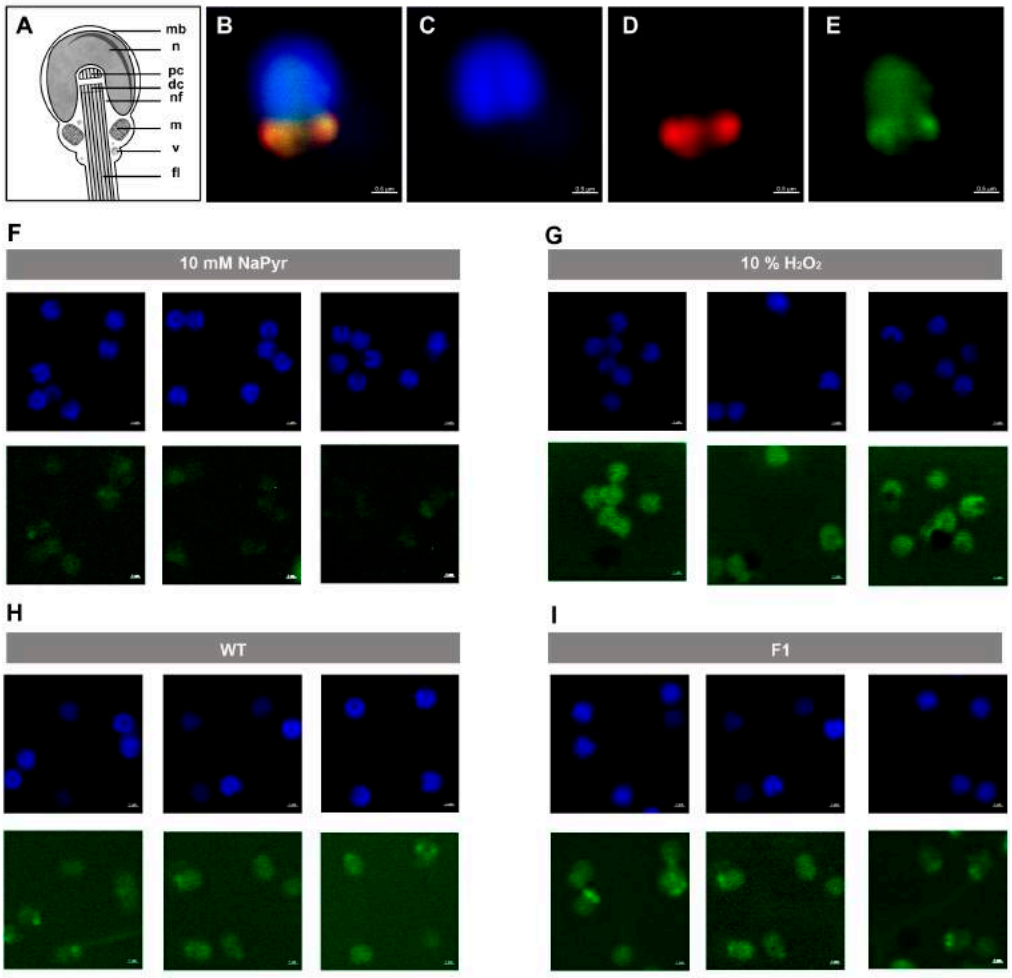

Figure 5 Intracellular reactive oxygen species localization by confocal microscopy. (A) *Solea senegalensis* sperm diagram. mb, membrane; n, nucleus; pc, proximal centriole; dc, distal centriole; nf, nuclear fossa; m, mitochondria; v, vesicles; fl, flagellum. (B) merge (C) DNA labeling (DAPI). (D) Mitochondria labeling (MitoTracker Deep Red). (E) Intracellular ROS labeling (DCF). Examples of fields (upper fields: DAPI; lower fields: DCF) for (F) 10 mM NaPyr (ROS scavenger); (G) 10% H_2O_2 (positive control) (H) WT and (I) F1. DAPI, 4′,6-diamidino-2-phenylindole; DCF, dichlorofluorescein; F1, F1 males; NaPyr, Sodium pyruvate; WT, wild-captured males.

Cell localization of intracellular H_2O_2

Confocal microscopy images showed a co-localization of ROS (green fluorescence, DCF labeling) with mitochondria (red fluorescence, MitoTraker labeling) (Fig. 5). The mitochondrial ring is located at the base of the heads of *Solea senegalensis* spermatozoa, which is observed as intense fluorescent areas in the confocal captures (Fig. 5D). H_2O_2 is also detected in nuclei (blue, DAPI labeling) showing that oxidative stress is not only present in mitochondria but also could be damaging DNA. This localization pattern was reported in

WT and F1 samples (Fig. 5H and I). The intensity of the DCF labeling in the nuclei of *Solea senegalensis* spermatozoa pointed to a larger presence of ROS species in this compartment of the spermatozoa from *Solea senegalensis* than in other flat fish such as *Scophthalmus maximus* (Supplementary Materials 3 and 4).

Discussion

Reactive oxygen species (ROS) have usually been associated to defective sperm function causing cellular damage at different levels. The major source of ROS in spermatozoa is mitochondria. It is well known that oxidative stress produces peroxidation of lipids (Cabrita *et al.* 2014). It is described that mammalian spermatozoa are particularly vulnerable to this type of damage due to the high amount of polyunsaturated fatty acids (PUFA), which are very vulnerable to free radical attack (Aitken *et al.* 2012). Lipid peroxidation is very harmful, having a clear negative effect on sperm motility and fertilization (Aitken & Curry 2011). ROS can even trigger an intrinsic apoptotic cascade. It is thought that the only product generated during this cascade capable of producing DNA damage is the H_2O_2 released from the mitochondria because it is a small chargeless molecule, able to penetrate the nucleus (Aitken *et al.* 2012). The mechanism by which DNA breakage is caused is by the oxidation of vulnerable bases, particularly guanines, which generates 8-hydroxy,2-deoxyguanosine (8OHdG). All these events lead to a decrease in motility, viability and DNA integrity and therefore significantly reduce sperm quality (Aitken *et al.* 2012).

Our study aims to determine the effect the processes such as cryopreservation has on the levels of intracellular H_2O_2 in *Solea senegalensis* spermatozoa. Moreover we try to elucidate if the sperm sample's origin (from wild animals vs animals born in captivity) has an effect on the presence of ROS in these cells. Finally, we studied the distribution of this molecule in the spermatozoa.

To date, studies carried out on oxidative stress of fish could be divided into different types: (1) some reports study the ROS detoxification mechanism (Chauvigné *et al.* 2015), (2) others study the effect of UV irradiation or other treatments inducing oxidative stress on spermatozoa (Dietrich *et al.* 2005, Gazo *et al.* 2015), (3) other approaches evaluate the effect of the addition of antioxidant compounds or the effect of enriched diets (Beirão *et al.* 2015), (4) others study lipid peroxidation and its relation to decrease in sperm quality (Martínez-Páramo *et al.* 2012), and finally (5) others evaluate the antioxidant status of seminal plasma and spermatozoa (Shaliutina *et al.* 2013, Słowińska *et al.* 2013). Interestingly, in mammals, it has been suggested that seminal plasma could be considered as one of the most powerful antioxidant fluids (Aitken *et al.* 2012). The common feature of all these types of studies in fish is that ROS are considered deleterious for spermatozoa. From

this perspective, our results would indicate that *Solea senegalensis* spermatozoa have high levels of oxidative stress (DCF^+ cells, on average: 53.32±4.89% in WT and 34.76 ± 6.60% in F1) (Fig. 3), and this could make them prone to suffering different types of damage. Surprisingly, the number of DCF^+ cells is significantly lower in cryopreserved F1 samples. It is well known that cryopreservation could induce oxidative stress, accelerating ROS production (Thomson *et al.* 2009, Kim *et al.* 2010, Aitken *et al.* 2012). So how can this fact be explained? It is known that the induction of ROS generation by sperm mitochondria is a very early stage within the whole oxidative stress process (Koppers *et al.* 2008, 2011), which, as expected, happens before DNA damage is produced and cell viability decreases. One possibility is that the oxidative stress process in F1 cryopreserved spermatozoa is in a later stage, so there is much more damage, but levels of ROS are not as high. In fact, when we look at viability results, we observe a significantly lower percentage of viable cells in this group (42.05 ± 5.61%, Fig. 2). In accordance with this hypothesis, when we divided the sperm samples in terms of motility for data analysis, we observed that low-motility samples have a lower percentage of DCF^+ cells not only after cryopreservation but also when fresh (Supplementary Material 1). When we look at the viability results, once again a significant decrease in cell viability in F1 fresh low-motility samples is observed. As expected, reduced glutathione levels are also lower in WT cells (with high oxidative stress) than in F1 spermatozoa. In conclusion, our results could be indicating that (1) spermatozoa in this species have high levels of oxidative stress regardless of whether the males were wild or born in captivity, and these high levels of ROS could be an early indicator of susceptibility to damage but (2) in very low-quality samples, high levels of ROS are not necessarily expected because the oxidative stress process could be more advanced and specific spermatozoa damage could already be happening, producing, as an example, a direct decrease in cell viability.

However, there is a point in our study that could not be easily explained considering ROS as a factor that causes defective sperm function. When we plotted the samples, DCF^+ cells vs live cells considering motility ranges (Fig. 6), we observed that fast spermatozoa samples consistently appeared with high ROS levels, whereas slow spermatozoa showed low ROS levels. Although most studies in fish address the ROS issue from a negative perspective, it is intriguing how such vulnerable cells can generate these numbers of cells that are potentially dangerous for them. In mammals, it has been established that ROS play an important physiological role in spermatozoa. It is known that low levels of ROS generation are crucial for normal sperm function and are involved in important signal transduction pathways (Aitken *et al.* 2012). In fact, in mouse, it has been shown that sperm lipid peroxidation using a combination of ferrous ions and ascorbate increased the fertilizing potential of mouse spermatozoa by 50% (Kodama *et al.* 1996). Moreover, it is also known that ROS are

required for compacting sperm chromatin and, paradoxically, providing protection against oxidative DNA damage (Pfeifer *et al.* 2001, Aitken *et al.* 2012). Therefore, although the underlying mechanisms of these positive effects are yet to be completely elucidated, particularly in fish, it seems clear that ROS could also be important for the regulation of sperm function.

ROS localization is also another factor that matters. In our study, we observed that ROS colocalized with mitochondria, as expected, but it is also found in the nucleus, being a potential inductor of DNA damage (Fig. 5). When compared with ROS distribution in other marine flat fish (*Scophthalmus maximus*) spermatozoa, we observed higher levels of ROS in *Solea senegalensis* nuclei (Supplementary Materials 3 and 4). Whether this species has particularly high levels of oxidative stress in their spermatozoa or they are necessary for normal sperm function, we could conclude that the beneficial or detrimental effects of ROS will always be in a delicate balance and should not always be considered alarming if they are not accompanied by other negative consequences on spermatozoa.

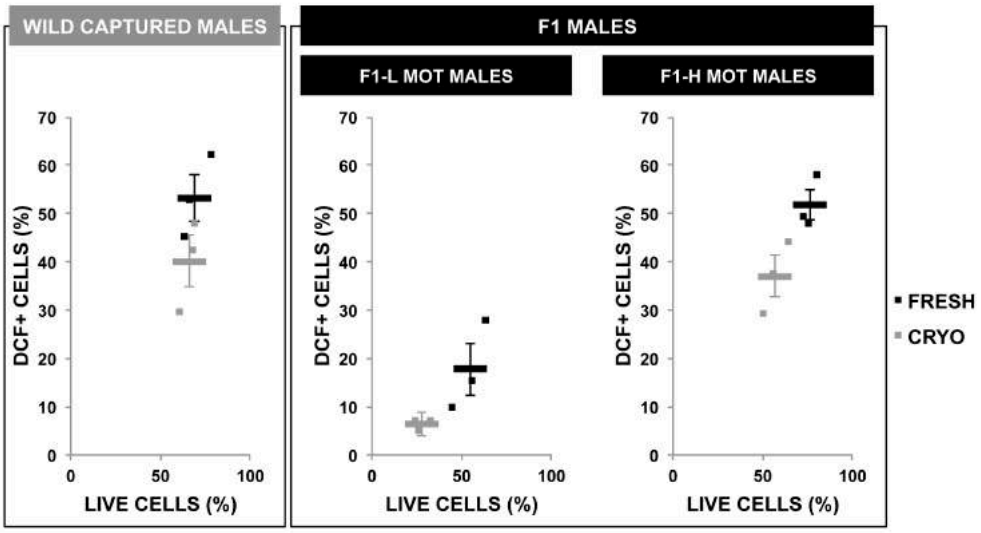

Figure 6 Viability, motility and intracellular ROS species correlation diagram. CRYO, cryopreserved/thawed samples; DCF, dichlorofluorescein; F1, F1 males; FRESH, control samples before cryopreservation; H MOT, high-motility samples; L MOT, low-motility samples; WT, wild-captured males.

Supplementary data

This is linked to the online version of the paper at http://dx.doi. org/10.1530/REP-16-0270.

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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CAPÍTULO III

Efecto de los contaminantes emergentes sobre la calidad seminal y consecuencias en su progenie:
efecto del 17-α-etinilestradiol

III.A

Paternal exposure to environmental 17-α-ethinylestradiol concentrations modifies the sperm transcripts affecting the offspring performance in zebrafish

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Abstract

Background

The synthetic estrogen 17-α-ethinylestradiol (EE2), major constituent in contraceptive pills, is an endocrine disrupting chemical (EDC) present in the aquatic environment at concentrations of ng/L. Developmental exposure to these low concentrations in fish can induce several disorders. Zebrafish (*Danio rerio*) is a perfect organism for monitoring the effects of environmental contaminants. Our hypothesis is that changes promoted by EE2 in the germ line of male adults could be transmitted to the unexposed progeny.

Methods:

We exposed male zebrafish to 2.5, 5 and 10 ng/L of EE2 during spermatogenesis and mated them with untreated females. Detailed progeny development was studied attending to survival, hatching and malformation rates. Due to the high incidence of lymphedemas within larvae, we performed qPCR analysis of genes involved in lymphatic development (*vegfc* and *vegfr3*) and endothelial cell migration guidance (*cxcr4a* and *cxcl12b*). Estrogen receptors (ERs) transcripts presence was also evaluated in sperm, testis and embryos.

Results:

Progenies showed a range of disorders: skeleton distortions, uninflated swimbladder, lymphedema formation, cartilage deformities and otolith tethering. Swimming evaluation revealed alterations in behaviour. All these processes are related to pathways involving ERs (*esr1*, *esr2a* and *esr2b*). mRNAs analysis revealed that environmental EE2 causes the up-regulation of *esr1* an *esr2b* in testis and the increase of *esr2b* transcripts in sperm pointing to a link between lymphedema in embryos and ERs expression impairment .

Conclusion:

We demonstrate that the effects induced by environmental toxics can be paternally inherited and point to the changes on the sperm transcriptome as the responsible mechanism.

Introduction

Environmental release of endocrine-disrupting chemicals (EDCs) has become a major worldwide focus of attention due to their ability to disturb the endocrine system in wildlife populations (Adeel et al. 2017). 17-α-ethinylestradiol (EE2) is a potent endocrine modulator used in contraceptive pills globally found in the aquatic environment at biologically active concentrations (Nazari and Suja 2016). The presence of EE2 in sewage-treatment works (STW) effluents (Esteban et al. 2014; Qiang et al. 2013) is due to the absence of efficient or convenient removal technologies (Duong et al. 2010; Nazari and Suja 2016).

EE2 water concentrations are typically below 10ng/L in Europe, but these values are largely over the proposed environmental quality standards (EQS) values for this synthetic chemical (Tiedeken et al. 2017). In fish, direct exposure to EE2 has been correlated to disturbed sexual development (Länge et al. 2001; Orn et al. 2003), secondary sexual characteristics alterations (Länge et al. 2001; Nash et al. 2004; Robinson et al. 2003), reduction of fecundity (Fenske et al. 2005; Länge et al. 2001; Nash et al. 2004) and a leading to intersex (Balch et al. 2004; Länge et al. 2001).

On the other hand, there is increasing evidence confirming that the effects of certain environmental factors can be paternally transmitted to the offspring in the absence of changes in the sperm genome (Klosin et al. 2017; Lombó et al. 2015; Öst et al. 2014). Paternal information does not only reside in its genome, but also in its specific pattern of epigenetic marks, its mRNA content and its non-coding RNAs (Herráez et al. 2017; Robles et al. 2017). There exists data suggesting that variety environmental factors can alter epigenetic information contained in germ cells and escape the reprogramming processes that occur during gametogenesis and early embryo development (Klosin et al. 2017). It has also been observed in zebrafish that the paternal exposure to the endocrine disrupting chemical bisphenol A (BPA) modifies the content of spermatic transcripts of the insulin receptors (*insrs)*, being these changes linked with of cardiac malformations up to F2 (Lombó et al. 2015). Moreover, it has been described that the effects on reproductive and non-reproductive behaviour (anxiety and shoaling) promoted by zebrafish developmental exposure to EE2 were similarly observed in the non-exposed F1 generation (Volkova et al. 2015).

The presence of transcripts in sperm has been related to seminal quality in fish (reviewed by Robles et al. 2017). Sperm mRNAs are remnants of spermatogenesis because of the transcriptionally quiescent nature of these cells (Johnson et al. 2011) and, as proved in mammals by Swann and Lai (Swann and Lai 2016), could be translated by the embryo in the early stages of development, before midblastula transition (MBT). Furthermore, previous studies identified certain transcripts showing different expression in good or bad breeding zebrafish males, that can be used as markers of seminal quality (Guerra et al. 2013).

Our hypothesis is that the changes promoted in gene expression during phases of spermatogenesis, prior to transcriptional and translational silencing, is one of the mechanisms that could explain the transmission of environmental effects to the progeny by paternal pathway: those environmental factors that modify the spermatic transcripts population, can cause alterations in the development of the progeny.

In order to validate this hypothesis, in this work we use zebrafish (*Danio rerio)* as model for screening EE2 toxic effects. This vertebrate is an optimal model in ecotoxicology because of its embryo transparency, easy reproduction, low cost maintenance, genetic manipulation tools availability, conservation of cell signalling pathways, and similarities with mammalian developmental phenotypes (Lee et al. 2015; Sipes et al. 2011). EE2 endocrine disrupting capacity in zebrafish is well known, as it interferes with estrogen receptors (ERs) affecting expression of large numbers of genes brain (Porseryd et al. 2017), liver (Rose et al. 2015) or gonads (Luzio et al. 2016a). Estrogen receptors (*esrα* and *esrβ* in mammals and their homologs *esr1*, *esr2a* and *esr2b* in zebrafish (Heldring et al. 2007)) are expressed and their cognate ligands produced in all vertebrates, indicative of important and conserved functions (Bondesson et al. 2015). Several studies have shown how the presence of the ligands and natural or synthetic agonists (such as EE2), regulates the expression of these receptors (Baker and Hardiman 2014). Chandrasekar and collaborators (Chandrasekar et al. 2010) demonstrated that testis was one of the target organs, reporting an overexpression of two of the receptors after natural ligand exposure.

In order to verify whether the parental exposure to EE2 affects mRNA content in the sperm and has subsequent effects for the development of progeny, we treated adult male zebrafish with different environmental concentrations of the toxic during the first phases of spermatogenesis. We have analysed the population of spermatic ERs transcripts in the exposed males as well as the expression of specific genes in the F1 progeny based on their phenotypic effects.

Materials and methods

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise stated.

Ethics statement

All experimental protocols and procedures were approved by the Competent Organism at the Junta de Castilla y León (project number ULE009-2016). All the animals were standard handled (Westerfield 1995) 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.

Experimental fish

Adult zebrafish used in the present study were selected from the wildtype AB strain colony of the University of León (Spain). The animals were raised in a standard recirculating system (Aquatic Habitats) at 28 ± 1°C with a 14 h light/10 h dark photoperiod. 24 spawning males were used for the experimental groups and 48 spawning females were used for mating. Male breeders were randomly selected and distributed in groups of three individuals in glass tanks (1.5 L) for exposure. The experiment was performed twice (6 males per experimental condition).

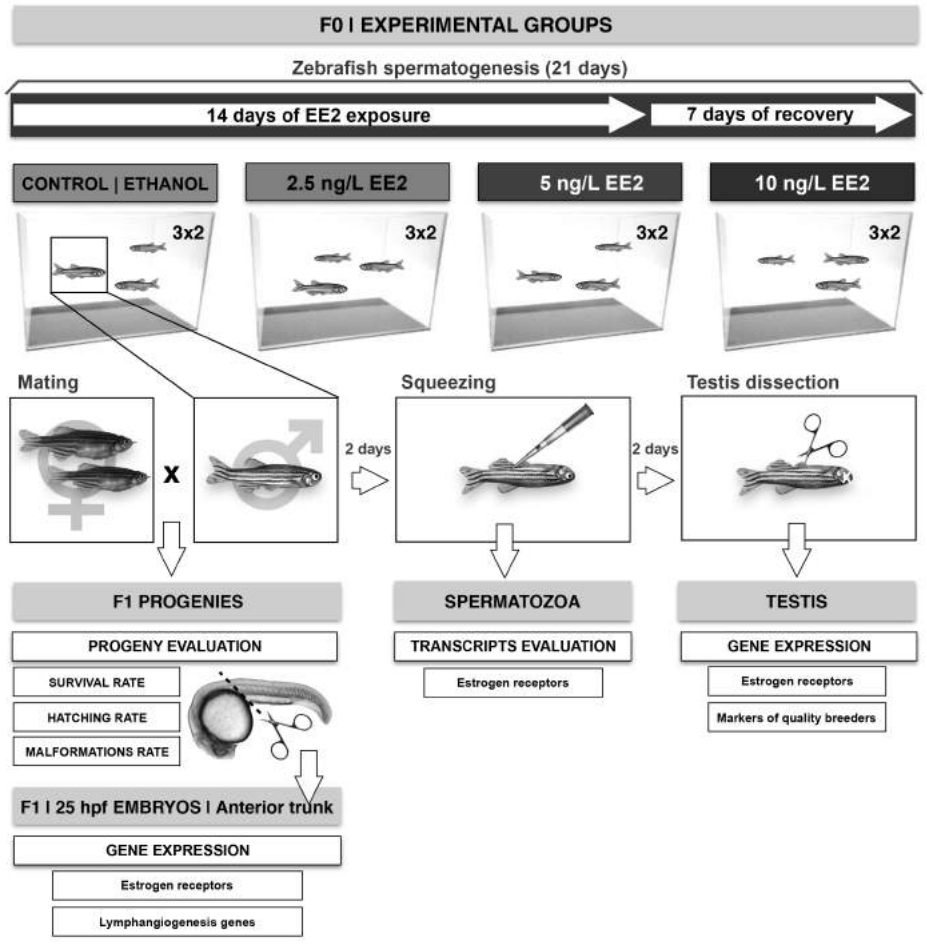

Figure 1.- Experimental design scheme.

17-α-ethinylestradiol exposure

The exposure concentrations were selected regarding the environmental relevance previously reported (Belfroid et al. 1999; Cargouët et al. 2004; Luzio et al. 2016b; Nash et al. 2004; Rocha et al. 2013). Males were exposed to 0 (control), 2.5, 5 and 10 ng/L of EE2 and a final concentration of 1ppm ethanol (vehicle) during 14 days (Fig. 1). After this exposure time, animals were kept for 7 days under standard conditions. Mature spermatozoa at the end of the 21 days period, derive from cells that were exposed to the toxic at meiotic and premeiotic stages, the transcriptionally active period.

Mating

After the recovery week, males were mated with untreated females (*sex ratio* 1:2, male:female). Spawning was induced in the morning as the light cycle was turned on. Fertilized embryos were rinsed and then randomly distributed in Petri dishes (50-100 embryos/dish). The progeny resulting from each exposed male was considered as a biological replicate (n=6). F1 animals were incubated at 28 ± 1 °C from 0 hours post fertilization (hpf) until 8 days post fertilization (dpf).

Progeny evaluation

Survival, hatching and malformation rates

Dead embryos or larvae were removed from the dishes at 4 hpf, 12 hpf and 1 to 7 dpf. We established the hatching rate: percentage of hatched larvae per live animals within the batch, at 72 hpf. Malformations rate (percentage of malformed larvae per live animals within the batch) at 5 dpf (when major organs morpohogenesis has finished (Chu and Sadler 2009)) was scored under stereomicroscope (Leica MZ16F). The survival rate (percentage of live animals per initial batch) was evaluated at 7 dpf.

Otolith development

Otolith development was assessed at 5 dpf. For this purpose, 14 randomly selected larvae from control and 5 ng/L treatments were anaesthetised in 0.02% tricaine methanesulfonate, placed under a Leica stereomicroscope and lateral images were captured. Saccule, utricle and the sum of both areas were measured using Adobe Photoshop CC 2017 for Macintosh. Three blind measurements were performed for each larva.

Swim bladder development

With the objective to analyse swim bladder inflation and yolk lipid consumption, whole mount Oil-Red-O staining was performed on 5 dpf F1 larvae (control and 5 ng/L EE2) as

previously described (Bhandari et al. 2016). Briefly, larvae were fixed in 4% phosphate buffered PFA overnight, washed 3 times in 1× PBS, bleached in 1.5% H_2O_2, rinsed 3 times in 1× PBS and pre-incubated 30 min in 60% isopropanol. Larvae were later incubated 3 h in a freshly prepared 0.3% solution of Oil-Red-O in 60% isopropanol and washed several times with 1× PBS. Larvae were laterally photographed.

Cartilage development

F1 cartilage development analysis was performed by whole mount Alcian blue staining. For this purpose, 7 dpf larvae from control and 5 ng/L batches were fixed in 4% phosphate buffered PFA overnight. After two washes in 1× PBS for 5 minutes, larvae were incubated twice in phosphate buffered saline with 0.1% Tween 20 (PBT) for 5 min and bleached with 1.5% H_2O_2 in 1% KOH for 30 min. Following two washes in 1× PBS 5 min, embryos were incubated in 0.1% Alcian blue solution overnight at 25 °C on a shaker. After three washes (1h) in acidic ethanol (70% ethanol, 5% HCl) and two washes in 0.25% KOH in 20% glycerol (1h), larvae were incubated at 25 °C in 0.25% KOH in 50% glycerol overnight. Samples were stored in 0.1% KOH in 50% glycerol until photographed.

Anterior trunk dissection at 25 hpf

In order to evaluate gene expression in the anterior part of the embryos at 25 hpf, 20-25 embryos were randomly selected from control and 5 ng/L EE2 progenies (3 pools per treatment), sacrificed using a lethal dose of tricaine methane sulfonate (MS-222) solution (300 mg/L) following the standard protocol (Westerfield 1995), and manually dechorionized using fine watchmaker's forceps under a stereomicroscope. Anterior trunks were dissected cutting the larvae between the caudal border of the yolk sac junction and the cranial part of the genital ridges. The caudal fraction of the embryos, were discarded (shown in Fig. 1). The samples were kept in cold 1× PBS until RNA extraction.

Sperm collection

Squeezing procedure was performed following standard methods (Westerfield 1995) 2 days after mating. Briefly, males were anesthetized in tricaine methane sulfonate (MS-222) solution (168 mg/L) until gill movements clearly reduced (around 30 s). Males were placed in a sponge with the dorsal part down. Using a dissecting microscope, the surface close to the anal fin was carefully cleaned and gentle abdominal pressure was applied to get the sample. Sperm was collected with a 10 µL capillary and placed into an Eppendorf tube containing 20 µL of 1× PBS on ice until subsequent RNA extraction. After squeezing, males were kept

in a rescue tank the time required for their recovery and then returned to the maintenance tanks.

Testis dissection

2 days after squeezing, animals were sacrificed using a lethal dose of tricaine methane sulfonate (MS-222) solution (300 mg/L) following the standard protocol (Westerfield 1995). Testis were extracted, cleaned and washed as previously described (Gupta and Mullins 2010) under a dissecting microscope. The organs were kept in cold 1× PBS until posterior RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from sperm, testis and 25 hpf anterior trunk samples using Trizol reagent (Invitrogen, Spain) according to the manufacturer's protocol. RNA concentration and purity were established using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples showed high purity (A260/A280 > 1.8) and RNA integrity was checked by Gel Red staining of 28S and 18S rRNA fragments on 1% agarose gel in testis and trunk samples.

Complementary DNA (cDNA) was transcribed from 1 µg of the total RNA using a High Capacity cDNA Kit (Applied Biosystems) following the manufacturer's instructions. The RT-PCR cycle was: 25 ºC for 10 min, 37 ºC for 120 min and a final extension at 85 ºC for 5 min. cDNA samples were stored at −20°C until quantitative PCR (qPCR) analysis.

Gene selection and primer design

To evaluate the effect of EE2 on spermatic ERs transcripts population (*esr1*, *esr2a* and *esr2b*) we selected primers pairs previously described for zebrafish by Chandrasekar and collaborators (Chandrasekar et al. 2010). In testis, we studied the expression of the three ERs with the same primers pairs and a set of transcripts previously described as markers of good and bad breeders (Guerra et al. 2013): *bdnf* (brain-derived neurotrophic factor), *lepa* (leptin a), *dmrt1* (double-sex and mab-3 related transcription factor 1), *fshb* (FSH beta polypeptide) *bik* (BCL2-interacting killer (apoptosis-inducing) and *hsd17b4* (17 beta-hydrosysteroid dehydrogenase 4). To assess the potential effect of EE2 on the molecular regulation of lymphatic angiogenesis and guidance of endothelial cell migration in the anterior part of 25 hpf embryos, we focused on four genes with important roles in these processes (Schuermann et al. 2014): *vegfc* (vascular endothelial growth factor c), its receptor vegfr3 (vascular endothelial growth factor receptor 3), *cxcr4a (chemokine (C-X-C motif) receptor 4a)* and its receptor *cxcl12b (chemokine (C-X-C motif) ligand 12b)*. Primers pairs for these four genes were designed using NCBI Primerblast tool

(www.ncbi.nlm.nih.gov/tools/primer-blast/). GenBank references, primers sequences, amplicon length and references are shown in Table 1.

Gene	Primer set (5'-3')	Accesion number	Amplicon size (bp)	References
esr1	F: CAGGACCAGCCCGATTCC R: TTAGGGTACATGGGTGAGAGTTTG	NM_152959.1	87	Chandrasekar et al., 2010
esr2a	F: CTCACAGCACGGACCCTAAAC R: GGTTGTCCATCCTCCCGAAAC	NM_180966.2	88	Chandrasekar et al., 2010
esr2b	F: CGCTCGGCATGGACAAC R: CCCATGCGGTGGAGAGTAAT	NM_174862.3	80	Chandrasekar et al., 2010
cxcr4a	F: TGGCTTATTACGGACACATCGT R: CACATCACACGGGACCTCAA	NM_131882.3	107	Self-designed
cxcl12b	F: ACTGAGACGCCACACTGAGC R: ACTGATGGGCTTTGCGTCA	NM_001320414.1	150	Self-designed
vegfc	F: TCAAGCAGATGCCATGCAGGA R: GAAGTAAGCGCGACCCCATC	NM_205734.1	149	Self-designed
vegfr3	F: GCCAGTGTGCCAGCTATGTA R: CGAATCCTTCAGGGATAGTGGT	NM_130945.2	100	Self-designed
bdnf	F: GCCGGACAACCCAGTCTTAC R: ATAAACCGCCAGCCGATCTT	NM_001308648.1	70	Guerra et al., 2013
lepa	F: AGCATGACCGGAAAAATGTC R: CAGCGGGAATCTCTGGATAA	NM_001128576.1	122	Guerra et al., 2013
dmrt1	F: ACGGGTCGCTGTCCATCA R: GTGACACGAAGCCGTGGTTT	NM_205628.2	75	Guerra et al., 2013
fshb	F: TGTGGAGAGCGAAGAATGTG R: AGACCTTCTGGGTGTGCTGT	NM_205624.1	119	Guerra et al., 2013
bik	F: TGGCTGTCAGGAGGCTAGAAA R: CAGTCAGAAACATGCAAGTTGGA	NM_001045038.2	60	Guerra et al., 2013
hsd17b4	F: GATGTGGACTGGGACCTGAT R: TCTGCTGCTTCATGTGGTTC	NM_200136.1	88	Guerra et al., 2013
b-act 2	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	NM_181601.4	86	Riesco et al., 2014

Table 1.- Primer sets

Real-time qPCR

Transcript abundance in sperm and relative gene expression in testis and embryo trunks were measured using SYBR green mix (Promega, USA) on a StepOnePlus system™ (Applied Biosystems). Reactions were performed in a 20 µL volume mix containing: 10 µL SYBR Green PCR Master Mix (Applied Biosystems), 0.5 µL (50 ng) of cDNA template and 1 µL (500 nM) of each forward and reverse primer and bidistillated water up to 20 µL. The cycling conditions were as follows: 10 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 60 s at 62°C (annealing temperature). To check the specificity of qPCR reactions,

melting curve analysis was also included: 1 cycle of 95 °C for 15 s, 60 °C for 1 min, followed by slow ramping of the temperature to 95 °C and finally 95 °C for 15 s. Three technical replicates were used in the qPCR analysis. The levels of these genes expression were normalized to *actb2* (actin, beta 2) levels using the formula $2^{-(\triangle\triangle Ct)}$ (Livak and Schmittgen 2001).

Motor function assessment

The assessment of swimming activity in newly hatched embryos was performed following a previously described test for zebrafish (Levin and Cerutti 2009). At 8 dpf, 64 live larvae (16 animals from 4 different progenies) were randomly selected and evaluated in control and 5 ng/L EE2 experimental groups. Each larva was individually placed in a cylindrical evaluation arena (area: 9.5 cm^2; swimming volume: 3 mL). Larvae were let to acclimate to the new environment for 2 min and right after animal behaviour was filmed (1920×1080 px) for 2 min. Individual larva swimming activity was monitored using the free digital video tracking software Tracker (physlets.org/tracker/). The actual position of the animal was manually located every 50 frames to avoid possible inaccuracies of the automatic option of the software. Onwards, each resulting track was evaluated using a virtual grid pattern (with 8 outer and 8 inner areas) in order to allow quantification and comparison between experimental groups.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 for Macintosh (International Business Machines Corp., USA). Experimental results were expressed as mean ± standard error (SEM). Normality was evaluated before statistic analysis. Non parametric variables were analysed using Mann-Whitney U test, Kruskal-Wallis or Wilconxon test. Normally distributed gene expression variables were analysed using a t-Student test. Values with $p<0.05$ (indicated by asterisks) were considered to be statistically significant.

Results

F1 progeny evaluation

Significant differences were not revealed after the analysis of survival rate at 7 dpf between progenies derived from control males and those from exposed males (Fig. 2A). In all cases, survival rate remained over 50%, indicating that the tested concentrations did not drastically affect the survival of the progeny in the studied temporal frame. A reduction trend in batches from 2.5 and 5 ng/L replicates was observed with mean values of 63.78 ± 12.86 % and 57.4

± 9.85 %, respectively versus the 74.04 ± 3.78 % in the control progenies. Offspring survival ratio in batches from 10 ng/L was higher comparing to the other experimental progenies (79.2 ± 2.36 %), suggesting a U shape doses/response curve.

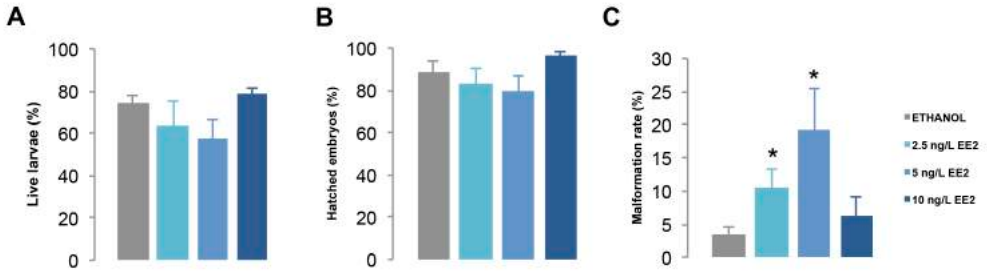

Figure 2.- Phenotype analysis of F1 larvae from control males and males exposed to 2.5 ng/L, 5 ng/L and 10 ng/L of EE2. A: Survival rate at 7 dpf. B: Hatching rate at 72 hpf. C: Percentage of total observed malformations. Asterisk indicates significant differences ($p<0.05$) when compared to control group (n=6 replicate batches 50-100 embryos each).

A similar pattern was obtained in the hatching rate at 72 hpf with no significant statistical differences (Fig. 2B). The minimum registered hatching rate value was found in progenies from 5 ng /L treated males with a mean value of 79.83 ± 6.83 %.

The phenotypic evaluation at 5 dpf was performed considering the total malformations rate (Fig. 2C) with special focus on the malformation types found within the replicates (Fig. 3A). We grouped the malformations in three types: (1) presence of uninflated swimming bladder (Fig. 3B), (2) axial skeletal distortions (Fig. 3C) and (3) formation of lymphoedema (Fig. 3D). The results obtained in total malformations showed significant differences respect to the control in progenies from 2.5 and 5 ng/L treated males with p=0.018 and p=0.010 respectively. The highest number of malformations recorded was in larvae from 5 ng/L treatment with a percentage around 20 % versus 3 % in the control samples. Offspring from 2.5 ng/L reported a value around 10 %, whereas the progeny from the higher concentration exhibited lower values, close to the control ones (6.23 ± 2.9 %), showing again a U shape like doses/response curve.

The study of the malformation types did not show significant differences in malformations related to swim bladder impaired development nor in axial skeleton distortions (Fig. 3A).

Lymphedema formation ratio showed a similar tendency to that found in total malformations, indicating the relevance of this type of lesion within the global data. Offspring from 5 ng/L treated males rose up to 13.29 ± 4.09 %, significantly higher compared to the control progeny (1.02 ± 0.65 %). Batches from 2.5 and 10 ng/L treatments did not report significant differences.

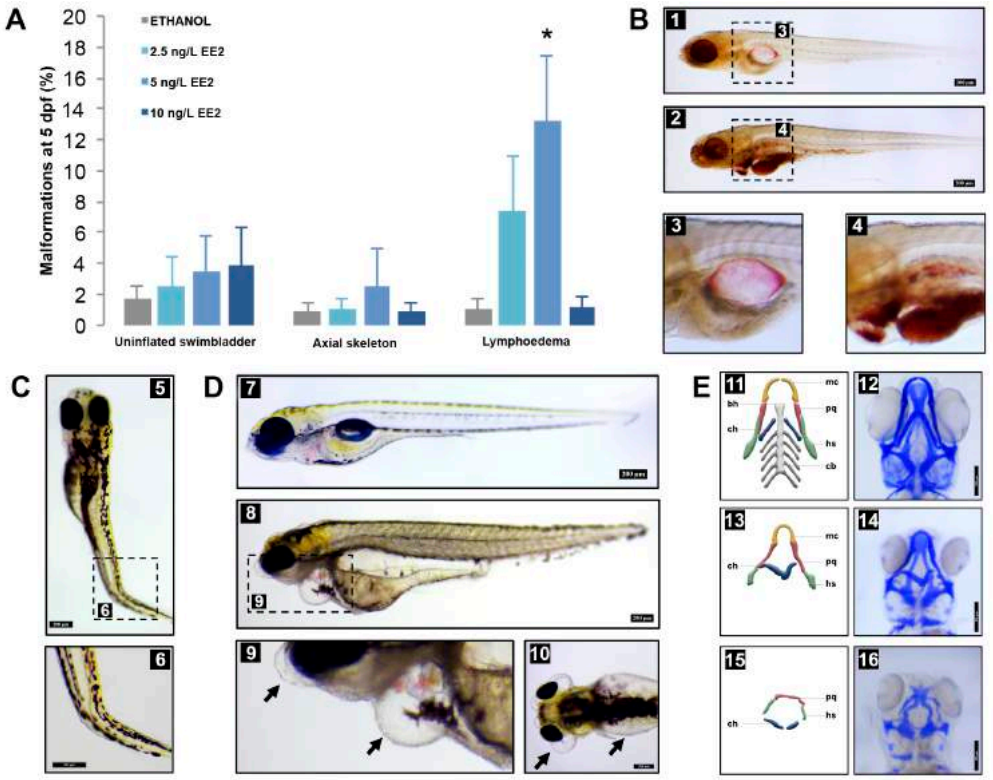

Figure 3.- Types of malformation in F1 larvae from exposed males to 5 ng/L of EE2. A: Percentages of the three types of malformations registered: uninflated swimbladder, axial skeleton deformities and lymphoedema formation. Asterisk indicates significant differences ($p<0.05$) when compared to control group (n=6 replicate batches of initial 50-100 embryos each). B: Examples of oil red staining in control larvae (1,3) and larvae derived from 5 ng/L EE2 paternal exposure (2,4) at 5 dpf. C: Examples of axial skeleton deformities found in exposure-derived larvae (5,6) at 5 dpf. D: Lateral views of control (7) and 5 EE2 ng/L derived larvae (8,9) showing lympoedemas in the anterior part. Top view of an EE2-derived larvae at 7 dpf (10). Black arrows point to lymphoedemas. E: Examples cranial cartilage development of control (11,12) and 5 ng/L EE2-derived larvae (13-16) at 7 dpf. mc: Meckel's cartilage. bh:basihyal. pq: palatoquadrate. ch: ceratohyal. hs: hyosymplectic. cb: ceratobranchial.

Considering the noticed malformation rates, progenies from 5 ng/L treated males were selected for molecular analysis in tissues (sperm, testis and embryo trunks) as well as for the study of cranial cartilage morphogenesis, otoliths development, and swimming behaviour.

Whole mount alcian blue staining revealed that the cartilage has a different pattern in 5 ng/L EE2 malformed larvae (oedema, uninflated swimbladder) with respect to the control ones (Fig. 3E). Different severity degrees were found in the progeny. In general, the ceratohyal cartilages are not obliquely placed, but are almost perpendicular to the basibranchial. Also, bent palatoquadrate cartilages were noticed, giving rise together with the Meckel's cartilages

to a non-canonical and shorter snout. Furthermore, shorter ceratobranchial cartilages were found.

Otholith area comparison revealed significant differences between control and larvae from treated males within the three evaluated areas (Fig. 4A): saccule, utricle and the sum of total areas (Fig. 4B). Globally our data indicate a disruption within otolith development, leading to an area increment. In some of larvae from experimental batches otolith tethering was observed (Fig. 4C)

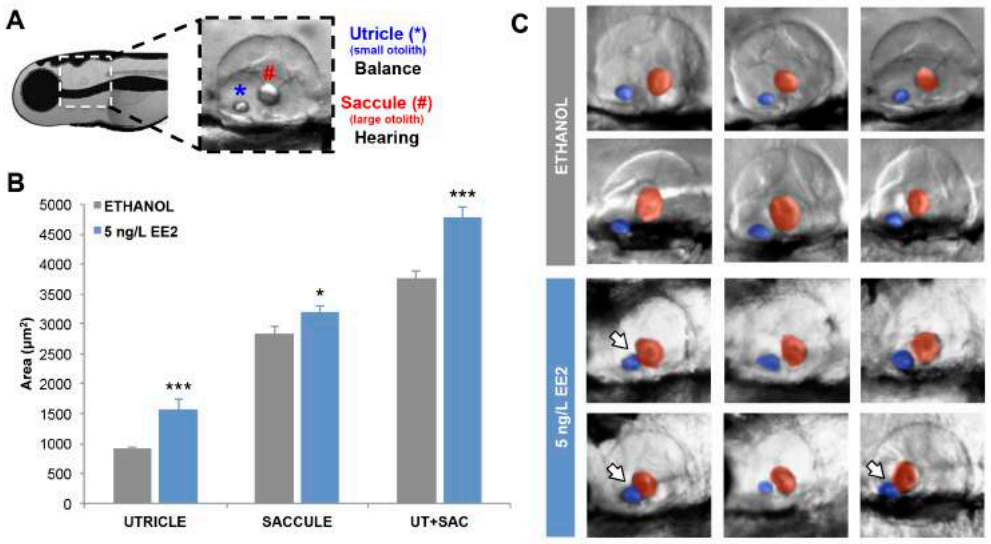

Figure 4.- Otolith development analysis in 4.5 dpf larvae from control and exposed males to 5 ng/L of EE2. A: Scheme of the zebrafish otolith. B: Area of the utricle, saccule and total area (utricle and saccule). C: Examples of otoliths in control and experimental larvae. Asterisks indicate significant differences (*p<0.05; *<0.001) when compared to control group (n=14).**

Gene expression analysis in F0 exposed testis

Testes from exposed males to 5 ng/L EE2 reported significant differences compared to control organs (Fig. 5A and 5B). In the case of the markers of bad breeders, a general down-regulation tendency was found within genes whose down regulation (*bdnf*, *lepa*, *dmrt1*, *fshb*) or up regulation (*bik* and *17hsd4b*) have been linked in testes to bad zebrafish breeders. Significant differences were found in the *bdnf* and *dmrt1* gene expression which expression was 1.42 and 1.64 lower than the control respectively (Fig. 5A). The analysis of ERs in exposed males testis revealed an overexpression in *esr1* (p=0.029) and *esr2b* (p =0.043) with 2.36 ± 0.44 and 1.74 ± 0.27 fold changes respectively (Fig. 5B). The over-expression tendency was also recorded in *esr2a*, without reporting significant differences.

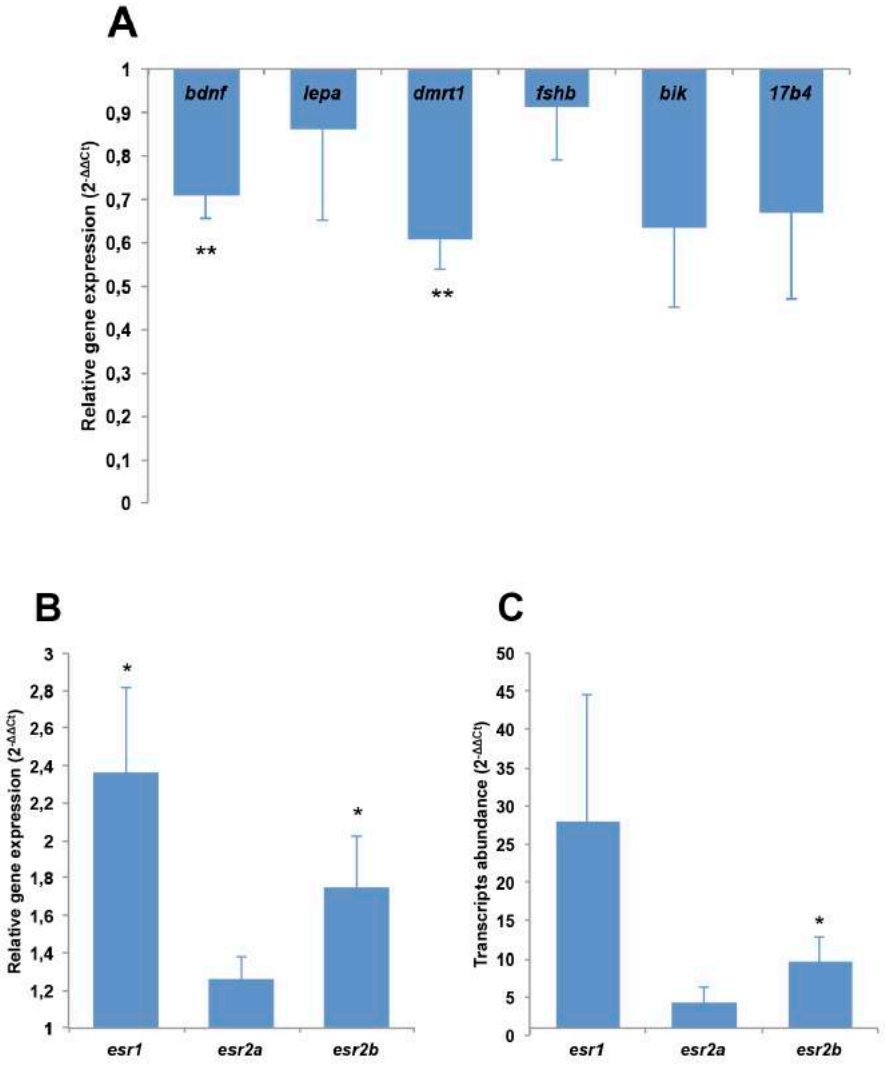

Figure 5.- Relative expression of zebrafish genes in 5 ng/L EE2 exposed males. A: Testicular markers of bad (*bdnf*, *lepa*, *dmrt1* and *fshb*) and good (*bik* and *17b4*) breeders. B: Zebrafish estrogen receptors *esr1*, *esr2a* and *esr2b* expression in testis. C: Relative quantity of remnant esr1, esr2a and esr2b mRNAs in spermatozoa. Expression levels relative to actin beta were calculated using $2^{-}(\Delta\Delta Ct)$ method. Asterisks indicate significant differences (*p<0.05; **p<0.010) when compared to control group (n=6).

mRNA population analysis in semen

The study of the effect of male exposure to 5 ng/L showed a trend to increase the number of estrogen receptors mRNAs (Fig. 5C). However, only significant differences were reported in the transcript *esr2b* with a fold change of 7.81 ± 2.78 comparing to the control samples.

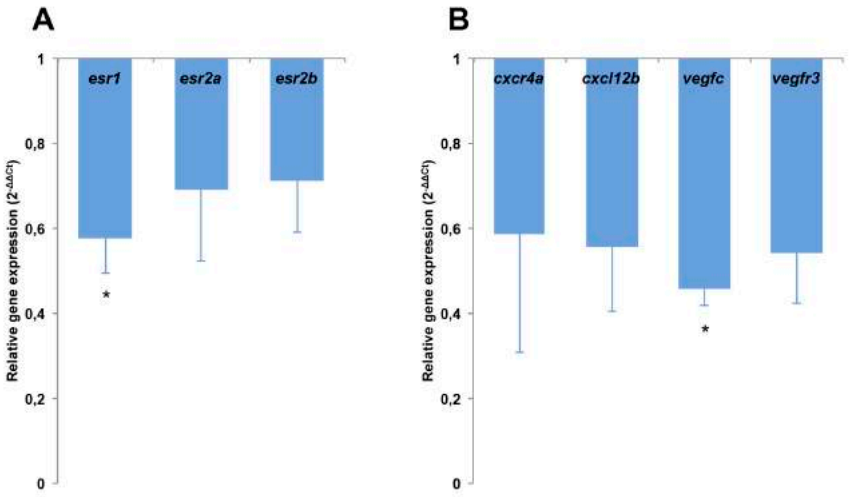

Figure 6.- Relative expression of zebrafish genes in anterior trunks of 25 hpf embryos from 5 ng/L EE2 exposed males. A: Zebrafish estrogen receptors *esr1*, *esr2a* and *esr2b.* B: Zebrafish genes with key roles in endothelial cell migration guidance *cxcr4a* and *cxcl12b* and lymphangiogenesis *vegfc* and *vegfr3*. Expression levels relative to actin beta were calculated using $2^{-}(\Delta\Delta Ct)$ method. Asterisks indicate significant differences (*p<0.05) when compared to control group (n=3; 20-25 embryos per replicate).

Gene expression in the anterior embryonic trunks of F1 larvae at 25 hpf

The paternal exposure to 5 ng/L of EE2 caused a global down-regulation in all the genes studied at this developmental stage (Fig. 6). Among the three ERs only *esr1* showed significant differences (p=0.014), being around 1,7 less expressed in larvae from EE2 treated males (Fig. 6A). The study of genes linked to the migration of endothelial cells showed a non-statistical tendency to downregulation in *cxcr4a* receptor *cxcl12b*. Genes associated with lymphatic angiogenesis in the anterior part of the embryo revealed significant down-regulation in the one of the studied genes: *vegfc* (p=0.027) (Fig. 6B).

Motor function analysis

The behaviour evaluation revealed an effect of EE2 male exposure on the F1 generation. The larvae from 5 ng/L males placed in the arena of study showed a more restricted use of the available space (Fig. 7A). Control larvae crossed more times the virtual grid in which the arena was divided for the analysis (around 11 crossings) comparing to progenies from EE2 (around 7 crossings) (Fig. 7B and 7C). A subdivided analysis, paying attention to the inner or outer areas scored by the animals, showed statistical significant differences in the outer areas, indicating that larvae from EE2 males do not screen the arena as much as control ones do (Fig. 7D and 7E). This conclusion was corroborated when we analysed the larval ability to score all the arena subareas by quartiles. We found a clear difference between control larvae and the experimental ones (Fig. 8E). The percentage of animals that score

more than the half of the total subareas of the area was over 60 % whereas the larvae from EE2 males the percentage was reduced below 50%.

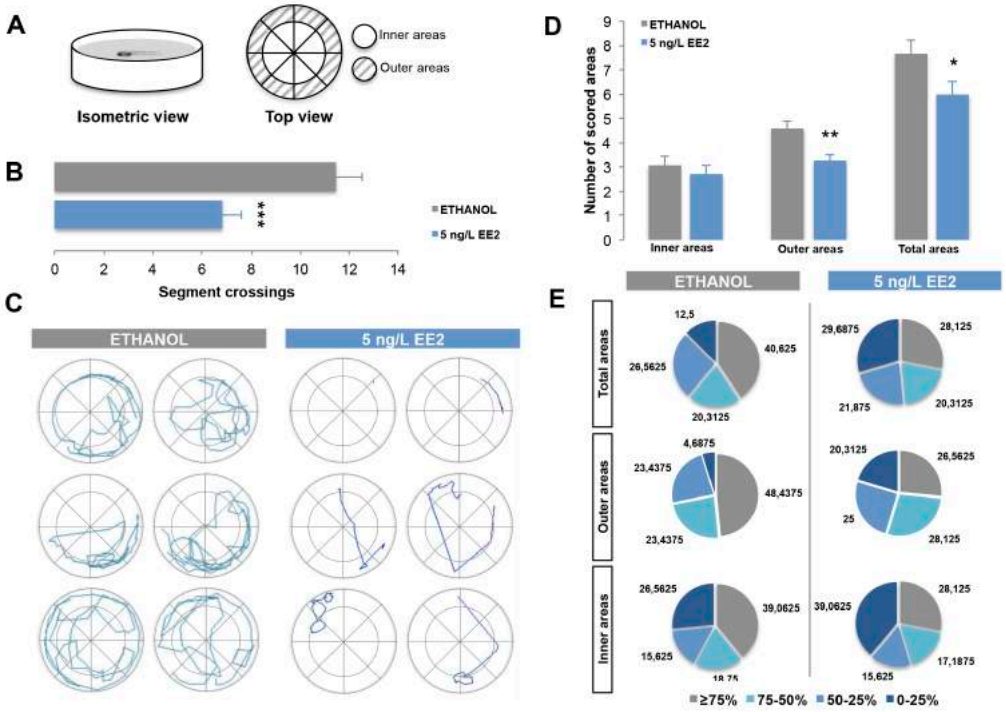

Figure 7.- Motor assessment in control larvae and larvae derived from males exposed to 5 ng/L of EE2. A: Schemes (isometric and top views) of the arena and the virtual grid used for the analysis. B: Number of segment crossings of the swimming tracks of the larvae. C: Examples of tracks. D: Number of scored areas (inner, outer and total areas) in larvae tracks. E: Percentage of areas explored by the larvae divides into quartiles (0-25%; 25-50%; 50-75% and ≥75%). Asterisks indicate significant differences (*p<0.05; **p<0.010; *<0.001) when compared to control group (n=64; 4 replicates of 16 larvae per replicate)**

Discussion

The presence of environmental contaminants, including pharmaceutically-active compounds, in aquatic ecosystems is an ubiquitous phenomenon nowadays (Tiedeken et al. 2017). One of these emerging concern chemicals is the estrogenic component used in hormone replacement therapies and in oral contraceptive pills 17-alpha-ethynylestradiol, EE2 (a synthetic estrogenic hormone), which, as other EDCs, interferes with estrogen receptor (ER) signalling pathways (Clouzot et al. 2008; Wise et al. 2011). Zebrafish estrogen receptors, ESR1 (homologous to mammalian ESRα), and ESR2a and ESR2b (subtypes homologous to mammalian ESRβ) (Bondesson et al. 2015), bind to the natural ligand 17-β-estradiol with similar affinities as to the corresponding mammalian receptors

(Menuet et al. 2002; Shelby et al. 1996). It has been repeatedly reported in mammals that natural and synthetic estradiol agonists positively regulate the expression of estrogen receptors (Mattison et al. 2014). Considering that EE2 is a potent estrogen agonist, it was expected that the exposure to this molecule would modify the testicular expression of estrogen receptors in zebrafish. Our results confirmed that exposure to the environmental relevant concentration of 5 ng/L EE2 during early stages of spermatogenesis, effectively affected gene expression of the receptors in adult male testis, showing an overexpression of testicular estrogen receptors isoforms *esr1* and *esr2b*. Consistent with these results, *esr1* overexpression was also registered in zebrafish testis after adult exposure to the natural ligand 17-β-estradiol (Chandrasekar et al. 2010).

The ER genes code to a steroid/nuclear receptor superfamily of enhancer proteins, which are important ligand-activated transcription factors modulating estrogen-target gene transcription (Heldring et al. 2007). Nowadays, it is accepted that estrogen receptors present different mechanistically molecular pathways in their regulatory actions (Heldring et al. 2007). Within the classical (direct) pathway, after specific interaction, ligand-receptor complexes recognize the estrogen response elements (EREs) and bind DNA. Estrogen receptors are powerful signal molecules with a relevant role in the control of the expression of a number of genes with a broad spectrum of performance within the cell and with well-known important roles within vertebrate development (Bondesson et al. 2015; Hao et al. 2013). As consequence, changes in the ligand concentrations or on their receptors could have severe developmental consequences. Changes in the expression of other genes, which could be a direct consequence of the estrogen receptor pathways disruption, were effectivelynoticed in the treated males: we found significant downregulation of two of the transcripts whose low level of expression was described as marker of bad zebrafish male breeders (Guerra et al. 2013): *bdnf* and *dmrt1*. Interestingly, these two markers were positively modulated in zebrafish testis after probiotic administration (Valcarce et al. 2015). The data point to the expression of these genes as more robust as marker of breeder quality than the rest of the proposed set of transcripts when exogenous factors regulate testicular gene expression.

Spermatogenesis takes 21 days in zebrafish testicle (Schulz et al. 2010). Considering the experimental design, differentiated spermatozoa at mating come from germ cells that were exposed to EE2 at pre-meiotic and meiotic stages, during differentiation from spermatogonia to spermatids. The transcriptional inactive nature of the spermatozoa is globally accepted (Johnson et al. 2011). However, the mature sperm still contains a variety of RNA species (Schuster et al. 2016), which provide a record of past events during spermatogenesis, before and after the last burst of transcription (Estill and Krawetz 2016; Sendler et al. 2013). Estrogen receptors in testicles are present in somatic and

germinal cells, including spermatogonia and spermatocytes (Carreau et al. 2011; Chauvigné et al. 2017), therefore, it is rational to expect that the observed effects on testicular transcription, would affect the estrogen receptors expression in germinal cells and, as consequence, could have effects on the number of transcripts transmitted to the sperm. The results effectively showed a higher amount of *esr2b* within sperm after EE2 exposure. Thus, paternal exposure to 17-α-ethinylestradiol during early spermatogenesis causes an overexpression of certain genes leading to an unbalanced transport of transcripts to the zygote upon fertilization. It has been suggested that some spermatic mRNAs have a correlation to human reproduction success (Bieniek et al. 2016; García-Herrero et al. 2011; Jodar et al. 2013) and a set of murine spermatozoal transcripts has been described to be key elements during early embryo development (Fang et al. 2014). Recently, due to the high amount of reports regarding spermatozoal transcriptomic profiles, Schuster and collaborators (Schuster et al. 2016) have developed a database dedicated to sperm-borne RNA profiling of multiple species: the spermbase (www.spermbase.org) in which it is indicated the presence of both *esr1* and *esr2* transcripts on human, mouse and rabbit sperm. We hypothesize that the contribution of a supra or infra-physiological number of paternal mRNAs to the zygote from the spermatozoon causes an unnatural cellular landscape in the zygote leading to an important gene missregulation (Lombó et al. 2015). As it happens with the maternal mRNAs present in the oocyte (Rauwerda et al. 2016; Sheets et al. 2017) sperm-derived transcripts could be translated by maternal ribosomes before mid-blastula transition (MBT). The translation of an increased number of paternal ERs mRNAs would entail an overpopulation of estrogen receptors within the early stages of development. The translated receptors may bind to the estrogens present within the embryo, as it has been suggested (Celeghin et al. 2011), potentially exerting profound effects in development by different ER pathways (Fig. 8).

Previous investigations have revealed ERs expression in head and trunk of zebrafish embryos at 1 dpf (Kinch et al. 2016; Tingaud-Sequeira et al. 2004). Moreover, using a transgenic mutant containing EREs driving the expression of GFP, Hao and collegues (Hao et al. 2013) demonstrated that EE2 treatment enhanced GFP expression at 1 dpf, mainly in the head region. Our qPCR results of the expression in F1 embryos 25 hpf, showed that instead an increase of transcripts, a significant downregulation of *esr1* and a similar trend in *esr2a* and *esr2b*, was produced in the un-exposed progeny. The increased expression of receptors during early development from paternally inherited mRNAs, could promote a negative feedback triggering the estrogen receptors down-regulation at post-MBT embryonic stages.

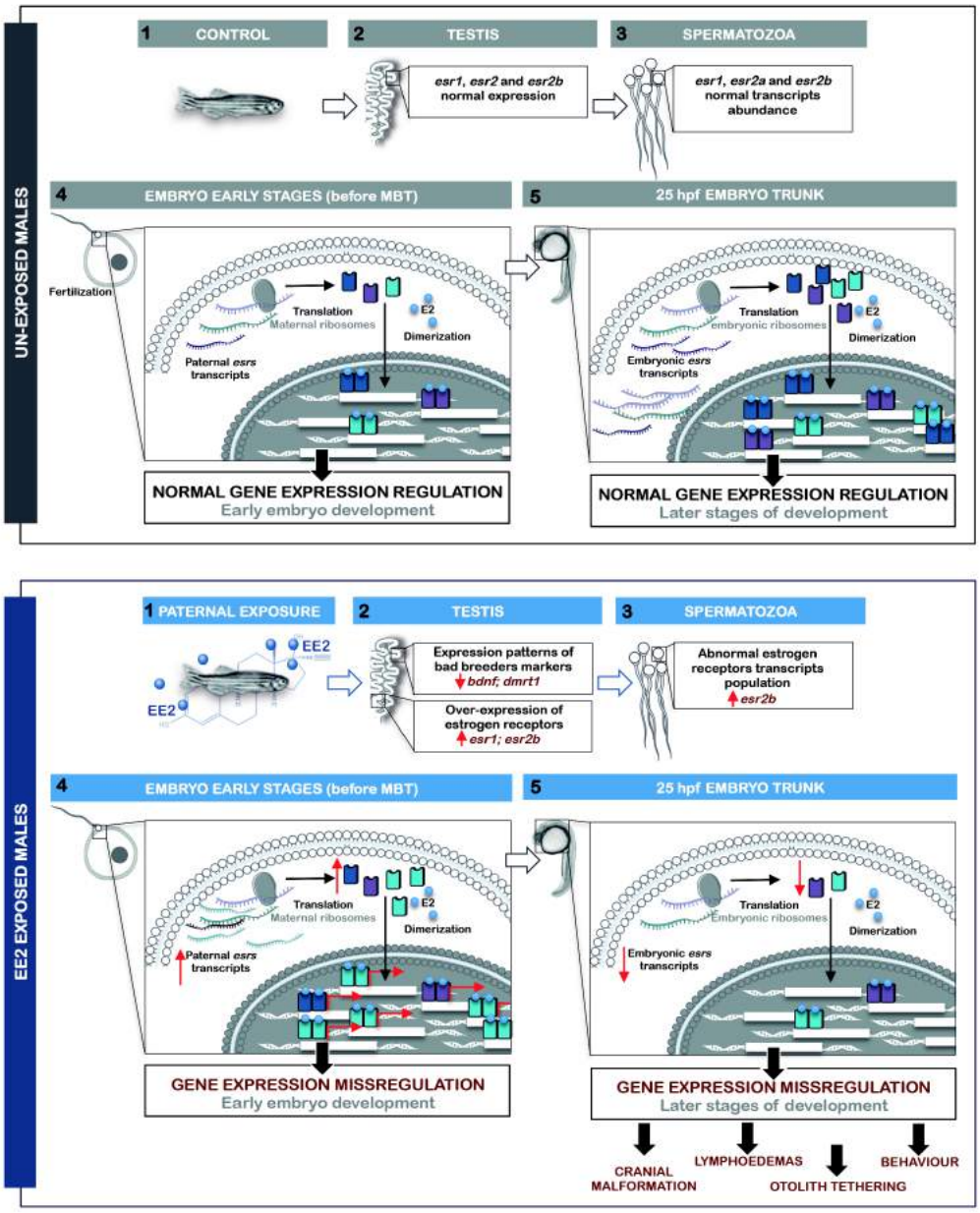

Figure 8.- Mechanism explaining the effects of paternal EE2 exposure and the transmission of gene regulation impairment in the progeny derived from these males. Over expression of testicular estrogen receptors promoted by EE2 entails the spermtatic over population of estrogen receptors mRNAs. Paternal transcripts delivery to the oocyte affects gene regulation before mid-blastula transition (MBT) leading to severe effects in the progeny.

Estrogen receptor pathways are related to a wide range of growth and morphogenetic events during development. The molecular missregulation observed on the non-exposed

progeny could thus be responsible from the observed phenotypic effects paternally inherited. Survival rates did not report statistical changes and mortality did not exceed 50% in any case, the effects being milder than those promoted by similar paternal exposure to bisphenol A (BPA) (Lombó et al. 2015). Likewise, the rate the total number of malformations recorded in our experiments (20 % aprox.) was comparatively lower than those recorded in the BPA assay, where mean values reached around 70% (Lombó et al. 2015). These data indicate a greater disruption capacity of paternal exposure to BPA in comparison with EE2. However, a sharp severity of malformations was found in 5 ng/L EE2 progenies. Some of the experimental larvae showed axial skeleton abnormalities although this type of malformation was not statistically significant. Nevertheless cranial skeleton was severely affected. Failures in skeletal development have been previously reported after direct exposure to other EDC (Lam et al. 2011), heavy metals (Salvaggio et al. 2016) and also after paternal exposure experiments to BPA (Lombó et al. 2015). Chondrogenesis impairment has been described in *esr2a* knockdown larvae (Celeghin et al. 2011) and similar phenotypes to those observed in our study were reported in Cohen and collaborators experiments (Cohen et al. 2014) after the inhibition of aromatase activity which naturally promotes the estrogens synthesis. More specifically, a number of transcripts related to skeletogenesis, jaw elongation or extracellular matrix (ECM) formation, were identified in zebrafish developing heads as targets of estrogen pathways (Ahi et al. 2016). In that study, both the expression of estrogen receptors and their target genes, were affected by E2 treatment, promoting effects on craniofacial skeletogenesis. Hence, the down-regulation of ERs expression in the progeny of the treated males could be responsible for the observed craniofacial malformations in our study.

An unexpected finding derived from our observations was the increased otolith area in progenies from treated males. Direct exposure to different estrogenic compounds mostly leads to smaller saccule and utricle as well as otholith tethering by interference with estrogenic pathways (Gibert et al. 2011; Tohmé et al. 2014). In those studies the direct treatment promoted estrogenic over-signalling in the treated animals, but in our study the effects were observed in the untreated progeny, which seems to suffer from a defective estrogenic signalling, compatible with the opposed phenotypic effects.

The most common malformation within EE2-derived larvae was lymphedema formation, mainly in the cranial part of the embryo. Lymphoangiogenesis is a dynamic process that involves the sprouting of lymphatic endothelial cells (LECs) from veins to form lymphatic vessels. Vascular endothelial growth factor (VEGFC) activates its receptor VEGFR3 (also known as Fms Related Tyrosine Kinase 4, FLT4) to induce lymphatic development (Shin et al. 2016). Mutations interfering with VEGFR3 signal transduction avoid normal lymphatic vascular function and cause lymphoedema (Ferrell et al. 2000): *vegfr3* null

zebrafish larvae (Shin et al. 2016) or embryonic injection of *vegfc*-specific morpholinos (Yaniv et al. 2006), promote severe lymphedemas similar to those observed in our study in F1 5ng/L EE2 progeny. The analysis of the expression of *vegfc* showed a significant downregulation in F1 larvae. Moreover *cxcr4a* and *cxcl12b* play also key roles during angiogenesis and lymphatic vessels formation, guiding endothelial cell migration in the cranial part of zebrafish embryos (Schuermann et al. 2014), however the downregulation was not significant in these two genes. The expression study, therefore, confirms a scenario compatible with a defective lymph vessels formation and lymphedema development. Different studies reveal close interactions between both signallization pathways (VEGFC/VEGFR3 and CXCR4A/CXCL12B) and estrogenic pathways. On the one hand, natural estradiol induces CXCR4/CXCL12B pathway in mammals via the activation of ERβ/E2 pathway (Rodriguez-Lara et al. 2017). On the other hand, it has been published that *vegf* gene has in mammals an upstream estrogen response element (ERE) (Mueller et al. 2000), the absence of mammalian ERα leading to down-regulation of the gene. In addition, *esr2a* knockdown zebrafish larvae developed edemas (Celeghin et al. 2011). All the data indicate that the reported ERs down-regulation in the anterior trunk of larvae from treated males, derived from the modified profile in paternal mRNAs, may induce the down-regulation of the morphogenetic pathways responsible for the phenotypic observations.

According to the behavioural disorders, links have been reported between direct EE2 exposure with reproductive behaviour alterations in adult zebrafish (Colman et al. 2009; Dzieweczynski and Kane 2017), short-term exposure triggering shift behaviour in males. In a different approach, other groups have analysed the persistent effects of developmental EE2 exposure on in adult zebrafish brain transcriptome (Porseryd et al. 2017). Moreover, direct developmental zebrafish exposure to environmental relevant low concentrations of EE2 during development led to changes in adult non-reproductive behaviour (anxiety and shoaling parameters) that were not remediated in chemical-free water (Volkova et al. 2015). Additionally, this group found that the behaviour changes due to EE2 were transferred to the untreated progenies, suggesting that the effects are transgenerational. Similar transgenerational observations have also been found in other vertebrates as rodents after endocrine disruptors exposure at embryo development (Skinner et al. 2008; Wolstenholme et al. 2012). Our results showed an effect on the swimming patterns of progenies from EE2 males. Larvae resulting from exposed males registered shorter and more erratic tracks and the animals did not recognize the arena as much as their control counterparts. This behavioural change may be explained as estrogen receptors are involved in many aspects of the development of the

neuroendocrine system, influencing brain development (Bondesson et al. 2015; Coumailleau et al. 2015; Pellegrini et al. 2005). The deficient development of organs implied in swimming activity, such as ototliths and swim bladder as well as the derangement of the head cartilages could also contribute to a behavioural alteration.

Overall, our results provide evidence that EE2 exposure at environmentally relevant concentrations during early stages of spermatogenesis is able to affect testicular gene expression, leading to modifications in spermatic transcripts population and disturbing the normal development of the resulting untreated progenies, by mechanisms compatible with an upstream event of estrogen receptors missregulation. These findings provide evidence that the toxic effects of the endocrine disruptive chemical 17-α-ethinylestradiol can be transmited through paternal inheritance.

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Conflicts of interest

The authors declare no competing financial interests.

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CAPÍTULO IV

Efecto de los suplementos nutricionales sobre la calidad seminal: efecto de los probióticos

IV.A

Effect of diet supplementation with a commercial probiotic containing *Pediococcus acidilactici* (Lindner, 1887) on the expression of five quality markers in zebrafish (*Danio rerio* (Hamilton, 1822)) testis

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Abstract

The effects of a commercial probiotic diet supplement (Bactocell®), containing lactic acid bacteria *Pediococcus acidilactici* were evaluated by identifying five zebrafish (*Danio rerio*) sperm quality markers. *P. acidilactici* (10^6 colony-forming units per gram) was supplied to young zebrafish males (age 8 months) as a feed probiotic additive during a 10 days test period. Five transcripts, previously described as male zebrafish quality markers, were studied: brain-derived neurotrophic factor (*bdnf*), BCL2-interacting killer (*bik*), double-sex and mab-3 related transcription factor 1 (*dmrt1*), follicle-stimulating hormone beta subunit (*fshb*) and leptin a (*lepa*). After exposition, males fed on with the supplemented diet with a commercial probiotic containing *P. acidilactici* presented an over-expression on three fertility markers comparing to the control group: *lepa*, *dmrt1* and *bdnf*. These results suggest that *P. acidilactici* has a potential use as probiotic supplement in zebrafish diet for the improvement of molecular parameters in testicular cells, indicating that probiotic supplementation could affect male reproductive performance.

Introduction

Probiotics contain live microorganisms that are believed to confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2002). During recent years, probiotics have increasingly been used as diet supplementation not only in feeds per se (John et al., 2006) but also as profilactic treatments against many diseases after colonizing the gastrointestinal tract. Many mechanisms of action have been proposed to clarify the stabilizing effects of probiotics on the gut tract including immunomodulation or stimulation (Sharifuzzaman and Austin, 2009), pathogen exclusion and production of inhibitory substances such as organic acids and bacteriocins (Cladera-Olivera et al., 2004; Corr et al., 2009).

Bactocell® (*Pediococcus acidilactici* CNCM MA18/5 M), is a lactic acid producing bacteria strain which is authorized for use in aquaculture in the European Union. Previous studies have focused on the effect of this probiotic on rainbow trout (*Oncorhynchus mykiss*) growth rate (Aubin et al., 2005; Merrifield et al., 2010; Ramos et al., 2013), immunity assays in tilapia (*Oreochromis niloticus*) (Ferguson et al., 2010; Standen et al., 2013) or antioxidant effect on blue shrimp (*Litopenaeus stylirostris*) under infection challenge (Castex et al., 2010).

In zebrafish, it has been proposed that oocyte maturation could be increased by probiotic administration (Gioacchini et al., 2010). Many studies attended to the reproductive benefits produced by *Lactobacillus rhamnosus* when administered at 10^6 colony-forming units per gram (CFU g^{-1}) (Gioacchini et al., 2010, 2012, 2013; Giorgini et al., 2010). However, to our knowledge, there is no report that correlates probiotic supplement with male reproductive performance.

The mRNA profile could be used as an indicator for male gamete quality and predictor of fertilization ability in different species (Guerra et al., 2013; Valcarce et al., 2013). Recently, potential sperm quality markers were differentially expressed in testicular cells in good and bad zebrafish breeders (Guerra et al., 2013).

In the present study, we evaluated the effects of a probiotic containing diet (containing 10^6 CFU g^{-1} of *P. acidilactici)* at 10 days of administration on the expression of five quality markers in zebrafish testicular cells: brain-derived neurotrophic factor (*bdnf*), BCL2-interacting killer (*bik*), double-sex and mab-3 related transcription factor 1 (*dmrt1*), follicle-stimulating hormone beta subunit (*fshb*) and leptin a (*lepa*).

Materials and methods

Wild type zebrafish (*Danio rerio*) were kept in an aquarium under standard protocols: 12 h light: 12 h darkness photoperiod cycle and 28 ± 1°C (Nusslein-Volhard and Dahm, 2002) in the AZTI-Tecnalia zebrafish facilities EU-BI-10 (Derio, Spain). Fish were fed a pelleted formulated diet (Gemma Micro 300; Skretting, Stavanger, Norway) twice a day to achieve a total daily feed input of 5% of body weight.

The control group (CTRL in Fig. 1) was fed only with the commercial diet whereas the experimental group was fed with the same diet but supplemented with Bactocell® PA 10 (Lallemand Animal Nutrition S.A., Blagnac, France) formulated with live *P. acidilactici* MA 18/5 M (Institut Pasteur, Paris, France) for ten 10 days (PROBIO in Fig. 1). The experiment was done in triplicate. Six adult males (8 months; weight: 0.5 ± 0.1 g) belonged to each replicate. For the treated group (PROBIO in Fig. 1), Bactocell® PA 10 (*P. acidilactici* in powder form) was coated on the pellets using vegetable oil as a carrier and giving a final concentration of 10^6 CFU of *P. acidilactici* per gram of diet, concentration previously used in probiotic assays related to reproduction in female zebrafish (Gioacchini et al., 2010). The probiotic concentration in the feed was systematically checked after processing by counting *P. acidilactici* strains on MRS plates using serial dilution. The control diet (CNTRL in Fig. 1) was also coated with vegetable oil.

After the time of exposure, testes were carefully removed from adult zebrafish under a dissecting microscope (Morris et al., 2003) and treated with 0.5% bleach in phosphate-buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.02 M PO_4 (Na_2HPO_4/KH_2PO_4), pH 7.3) for 2 min (Sakai, 2002). Subsequently, RNA extraction was performed (testicles from six males were pooled per replicate): clean testes were dissociated in TRIzol Reagent (Invitrogen Dynal AS, Oslo, Norway) to obtain RNA following commercial advices. One microgram of RNA was used as substrate for cDNA synthesis. This protocol was performed following commercial Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Madrid, Spain) guidelines.

Target genes selected for quantitative PCR (qPCR) analysis (*bdnf*, *lepa*, *bik*, *dmrt1* and *fshb*) were selected attending to our previous results that indicate that these genes are differentially expressed in testicular cells from bad and good zebrafish breeders (Guerra et al., 2013). QPCR was carried out in a StepOnePlus System (Applied Biosystems, Foster City, CA) according to guidelines provided and using reaction volumes, cycle conditions and primers described before (Guerra et al., 2013).

For the expression analysis, relative quantification of the transcripts (ΔC_t) was calculated using beta-actin 1 (*actb1*) as reference gene in order to avoid variation in mRNA and cDNA quantity. Modification of gene expression after treatment is represented with respect to the control. Data are presented as mean ± SD for n=3. Resulting variables were tested for normality using Shapiro-Wilk test. One-way ANOVA was carried out to compare mean values with a cut-off value of $P < 0.05$. SPSS version 20.0 software (IBM, NY, USA) was used for statistical analysis.

Results

After 10 days of probiotic administration of *P. acidilactici*, all molecular markers analyzed (*bdnf*, *bik*, *dmrt1*, *fshb* and *lepa*) showed the same overexpression tendency in testes after probiotic fed except *bik* (Fig. 1). However, only three markers (*bdnf*, *dmrt1* and *lepa*) reported significant statistical differences.

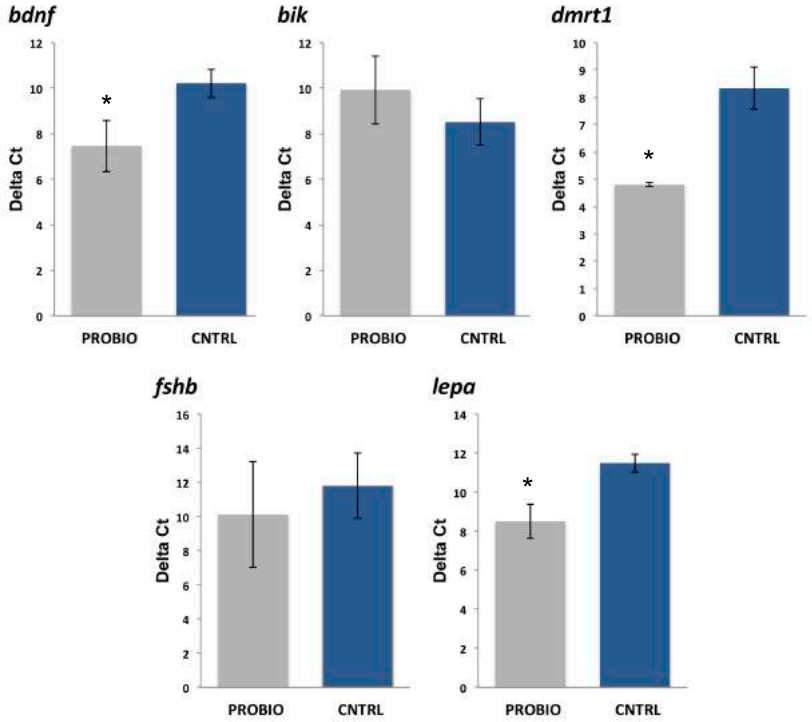

Fig. 1. *bdnf*, *bik*, *dmrt1*, *fshb*, *lepa* relative mRNA levels in testicular cells normalized against b-actin gene (ΔC_t), in zebrafish males fed *Pediococcus acidilactici* (PROBIO) and in control males (CNTRL). Data are given as mean ± SD (n=3). Asterisks denote significant differences from the control samples (P<0.05) analyzed using one-way ANOVA. Higher values of ΔC_t indicate lower levels of mRNA

Discussion

A key issue for the aquaculture industry is the improvement of reproductive performance of breeders and the identification of predictive markers of successful breeders would have major uses in research, fish farm and biotechnological companies. For example, these markers can be used to test the positive effect of probiotic diets on reproductive parameters, which is very promising in aquaculture. So far, some approaches have been carried out directly on commercial aquaculture species of interest, as for example in gilthead seabream (*Sparus aurata*) (Guerra et al., 2013), but the use of zebrafish as a model take advantage of the variety of molecular tools to apply making this model the ideal candidate for this type of studies. In present study, five transcripts (*bdnf*, *bik*, *dmrt1*, *fshb* and *lepa*), previously described as male zebrafish quality markers (Guerra et al., 2013) were studied after 10 days of probiotic administration. *bdnf*, *dmrt1* and *lepa* reported relative transcripts abundance showed statistical differences comparing control samples. Interestingly, all of them were overexpressed on testicular cells from good zebrafish breeders (Guerra et al., 2013). As a matter of fact, our results indicated that probiotic treatment with *P. acidilactici* had an effect on zebrafish testicular cells gene expression. The overexpression of markers related to males with good reproductive performance indicates that probiotic supplementation has a direct beneficial effect on treated animals. Moreover, *bik* transcript, with an opposite tendency, was previously defined as a marker of bad zebrafish breeders (Guerra et al., 2013) and after probiotic supplementation, the relative amount of this transcript is lower, although not significant.

Studying the effect at testicular level and not at spermatozoa is based on the difficulty of obtaining sperm sample enough for cDNA desirable quantities but also because it is a fact that, in this species, somatic cells play key roles during spermatogenesis. Moreover, some of the transcripts from the analyzed set, proposed by our group as quality markers in testicular cells, were also differentially presented in high and low motility gilthead seabream sperm samples (Guerra et al., 2013).

In previous studies on female zebrafish endocrine control, probiotic administration with other lactic bacteria, *Lactobacillus rhamnosus*, induced a significant increase in the gene expression of leptin, a key hormone in energy homeostasis and neuroendocrine functions (Gioacchini et al., 2010). In accordance with this study, our results also showed over expression of this gene in the testicular cells. The other two transcripts significally overexpressed after probiotic exposure are *dmrt1* and *bdnf*. Doublesex/mab-3 related transcription factor 1 (*dmrt1*) is a transcription factor that act upstream in the signaling cascades that regulate sexual differentiation in fish playing a regulator role during early testicular differentiation (Jørgensen et al., 2008; Siegfried and Nusslein-Volhard, 2008). A decrease of brain-derived neurotrophic factor (*bdnf*) transcript and protein has been

associated to some sperm pathogenesis in humans (Zheng et al., 2011). Interestingly, this transcript was overexpressed in good zebrafish breeders comparing to bad breeders and is downregulated in low motile gilthead seabream spermatozoa as well (Guerra et al., 2013). As a matter of fact, our results suggest potential beneficial effect of *P. acidilactici* in zebrafish sperm quality.

In addition, the expression profile of another marker of good zebrafish breeders, follicle-stimulating hormone beta subunit (*fshb*) which plays critical roles in reproduction, showed a remarkably similar tendency with the other transcripts of this group in our study, but results are not statistically significant. Collating with other teleosts, zebrafish FSHB cysteines exhibit unique distribution. Interestingly, in addition to the pituitary, *fshb* is also expressed in some extrapituitary tissues, particularly the gonads and brain (So et al., 2005).

This evidence together with our results makes the reported overexpression as well as the reported tendencies truly interesting, revealing that probiotic consumption can improve the expression of some genes with key roles in reproduction in zebrafish.

Conclusions

This study represents the first step forward the possibility of selecting probiotic diets according to their effect on specific teleost quality markers of reproductive performance. We have revealed that after 10 days of administration, zebrafish males fed on a supplemented diet with the probiotic *P. acidilactici* presented an over-expression on three fertility markers: doublesex/mab-3 related transcription factor 1 (*dmrt1*), brain-derived neurotrophic factor (*bdnf*), and leptin a (*lepa*). *P. acidilactici* has a potential use as probiotic supplementation in zebrafish diet for the improvement of molecular parameters in testicular cells.

Acknowledgements

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IV.B

Probiotic administration improves sperm quality in asthenozoospermic human donors

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Beneficial Microbes

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Abstract

The objective of this study was to analyse the effect of the ingestion of two selected antioxidant probiotics strains (*Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347) on sperm quality parameters in asthenozoospermic males after three and six weeks of administration. Nine asthenozoospermic men without any medical treatment under similar diet conditions participated in the study. The quality of individual sperm samples was evaluated before (previous to ingestion), during (after 3 and 6 weeks of ingestion) and after probiotic administration (3 and 6 weeks after finishing the treatment). Sperm motility was evaluated by computer-assisted sperm analysis system (CASA), DNA fragmentation by sperm chromatin structure assay (SCSA), cell viability by flow cytometry and measurement of intracellular H_20_2 (reactive oxygen species) by flow cytometry using dichloro-dihydro-fluorescein diacetate (DCFH-DA). Sperm motility was drastically improved after the treatment (approximately 6 fold change), DNA fragmentation was statistically reduced after the administration of probiotics from (approximately 1.2 fold change) and intracellular H_20_2 level was decreased (approximately 3.5 fold change). Cell viability was not affected by the treatment. The significant improvement on sperm motility and the decrease in DNA fragmentation reported in this study provides preliminary evidence that probiotics could be administrated to improve motility and decrease DNA fragmentation and reactive oxygen species levels in asthenozoospermic human males.

Introduction

Nowadays, 15% of couples are affected by infertility (Hull MG et al. 1985). It has been suggested that 40% of these events are caused by the male factor (Fleming S et al. 1995). World Health Organization (WHO) establishes different subtypes of sperm abnormalities: asthenozoospermia, oligozoospermia, teratozoospermia or their combinations (WHO, 2010). Asthenozoospermia pathology is described by reduced motility or absent sperm motility in the fresh ejaculate. It is known that 1 of 5000 men is affected by absolute asthenozoospermia (Eliasson et al. 1977) and this condition involves a poor fertility prognosis. Male gamete motility is critical for spermatozoa migration in the female reproductive tract, for penetration of the oocyte, and for processes involved in fertilization (Ortega *et al.* 2011). Consequently, the possibility of natural reproduction and sperm motility is strongly correlated (Beauchamp et al. 1984).

Since the introduction of the intracytoplasmatic sperm injection (ICSI) the low fertilization prognosis of these patients has improved drastically with successful gestations and live births after injection of immotile cells (Ortega *et al.* 2011). However, new fields should be explored to try an improvement of sperm motility and facilitate assisted reproductive technologies in clinical centres or natural pregnancies.

Probiotics have been defined as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (FAO, 2002). The intestinal microbiome is a complex ecosystem, which provides numerous crucial functions to the host, embracing: protection against pathogen invasion, carbohydrate metabolism and modulation of the immune response. The host is closely involved in the preservation of a healthy gut microbial community. This mutualism between the host and its microbiome is fundamental for the maintenance of the homeostasis of a healthy individual (Leser and Mølbak 2009). Both harmful and beneficial bacteria must be, respectively, avoided and adapted in the host in order to succeed in this ecosystem (Gibson et al. 2014). Probiotics colonize the intestinal mucus layer where they can affect the gut immune system, displace enteric pathogens, supply anti-mutagens and antioxidants, and many other possible effects by cell signalling process (Kanmani et al. 2013).

Most commonly used probiotics are lactic acid bacteria (LAB), an ecologically varied group of microorganisms united by the formation of lactic acid as the primary metabolite of sugar metabolism, and bifidobacteria. Recent publications demonstrated that these microorganisms could be effectively used in the treatment of diarrhea (Chouraqui et al. 2008; Gaón et al. 2003), food allergies (Pohjavuori et al. 2004), inflammatory bowel disease (Azcárate-Peril et al. 2011; del Carmen S et al. 2011) and colorectal cancer (Hirayama and Rafter 2000; Rafter 2002).

LAB and bifidobacteria strains have also been reported for production of antioxidants (Amaretti et al. 2013). These metabolic antioxidant activities may be assigned to reactive oxygen species (ROS) scavenging, enzyme inhibition, and reduction activity or inhibition of ascorbate autoxidation in the intestine by neutralizing free radicals (Amaretti et al. 2013).

The aim of this study was to evaluate the effect of the administration of a combination of two probiotic strains (*Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347) with tested antioxidant activity on sperm quality (assessing sperm concentration, volume, motility, DNA fragmentation status, cell viability and intracellular H_20_2 levels) from males suffering asthenozoospermia. On human reproduction field the use of these bacteria has not been tested in males. Only studies in the female factor have been carried out using probiotic as a therapy for bacterial vaginosis reporting positive results in some clinical trials (Borges et al. 2013; Mastromarino et al. 2013). This the first report evaluating the effect of probiotic ingestion on human sperm quality parameters.

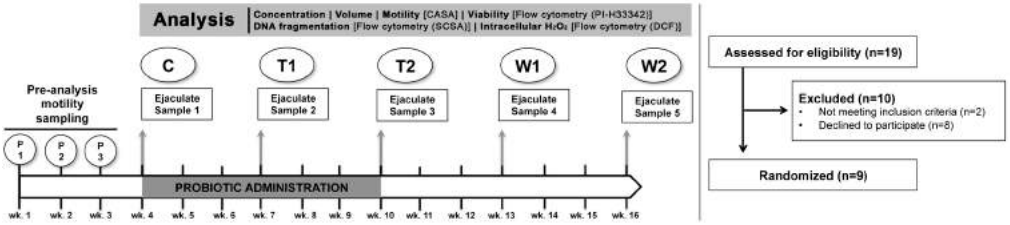

Figure 1.Experimental timeline. Capital letters represent key points along the timeline. P1, P2 and P3 represent ejaculate samples for pre-analysis motility assessment. C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1 (three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration).

Material and Methods

Ethics

This project was approved by the Research Ethical Committee of the University of León (#15 2013). All donors gave written informed consent to take part in this study in conformity with the Declaration of Helsinki and Spanish legislation of confidentiality.

Donors recruitment

This study included a set of nine donors under similar healthy diet conditions and with no medication.

Study design

In order to assess motility, three weekly pre-analysis were performed (P1, P2, P3) prior to the beginning of the study. Seven days after pre-analysis, control sampling (C) was carried out before probiotic administration started. Second sampling (T1) was collected after three weeks after daily probiotic ingestion. Third sampling (T2) was picked after six weeks of daily ingestion. After the treatment, two washout samplings (W1 and W2) were performed, three and six weeks respectively after the end of the probiotic ingest. It is important to clarify that this study did not present a placebo control due to the difficulty to enrol participants enough to have two groups and this could be found as a caveat.

A schematic representation of the study design is presented in Figure 1.

Oxidative stress assays in *C. elegans*

C. elegans wild type strain N2 (*Caenorhabditis* Genetics Center at the University of Minnesota, USA) was used to study the *in vivo* antioxidant activity of the probiotic strains *L. rhamnosus* CECT8361 and *B. longum* CECT7347. Protocol was carried out as previously described (Grompone et al., 2012; Martorell et al, 2016). Age-synchronized worms were grown in NG agar medium (Nematode Growth medium: agar 17.5 g/L, NaCl 3.0 g/L, peptone 2.5 g/L, cholesterol 0.005 g/L) with a lawn of *Escherichia coli* OP50 as standard food (control media). To test the antioxidant activity of the probiotic strains, *L. rhamnosus* CECT8361 and *B. longum* CECT7347 were grown at 37 °C for 18 h on MRS-Cys (0.05%) medium, in an anaerobic atmosphere generated by means of an AnaeroGen system (Oxoid). Concentrated cultures (50 µL, OD = 30) were then added to the NGM surface, previously seeded with *E. coli* OP50 to ensure standard nutrition conditions. NG supplemented with Vitamin C (10 µg/mL) was used as positive control medium. Young adult worms were incubated at 20 ºC for 5 days, transferring them to new plates every two days, to separate them from progeny. Afterwards worms were transferred to a S-Basal medium (5.85 g/L NaCl, 1 g/L K_2HPO_4, 6 g/L KH_2PO_4, 1 ml/L cholesterol (5 mg/mL in ethanol)), supplemented with 2 mM of H_2O_2 to provide an acute oxidative stress. After 5 h of incubation, worm survival was scored in each fed condition. Worms were considered dead when they no longer responded to prodding.

Probiotic administration

Lactobacillus rhamnosus CECT8361 and *Bifidobacterium longum* CECT7347 strains were provided to volunteers in a capsule containing a combination of both microorganisms at 50%. Probiotics were supplied by the company Biopolis S.L. (Valencia, Spain). Each volunteer took a daily capsule corresponding to the administration of 10^9 cfu/day. The carrier

for the lyophilized probiotics was maltodextrin. The mixture was encapsulated in hypromellose capsules.

Semen analysis

Semen samples were collected in a sterile recipient and according to the procedure recommended by the World Health Organization (WHO, 2010).

Concentration assessment

Concentration was evaluated following World Health Organization (WHO, 2010) routing counting procedure using a Neubauer chamber (VWR, Madrid, Spain).

pH evaluation

Ejaculate pH was measured with Hydrion® Brilliant pH dip stiks pH-range 6.5-13 (Sigma, Madrid, Spain)

Sperm motility evaluation

Sperm were diluted in PBS 1X to 10 to 20X 10^6 spermatozoa/mL and loaded into a Makler counting chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) at 37 °C. The computer assisted sperm analysis (CASA) system consisted of a triocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) using a 10X negative phase-contrast objective, equipped with a warming stage at 37 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured and analysed using a computer-assisted motility analyzer (ISAS; Proiser, Valencia, Spain) with specific settings to human spermatozoa. The software rendered the following global parameters: (i) percentage of motile spermatozoa, (ii) percentage of progressive spermatozoa, and (iii) percentage of static spermatozoa.

Cell viability

The analysis was performed with a double staining with Hoechst 33342 (H342-Sigma, Madrid, Spain) and propidium iodide (PI-Sigma, Madrid, Spain). Each ejaculate was diluted in 500 µL of PBS 1X (1–2 million spermatozoa/mL) with Hoechst 33342 (5 µM) and PI (1.5 µM). After 10 min at room temperature and darkness, stained samples were evaluated by flow cytometry. Forward scatter and side scatter (FSC/SSC) were used to distinguish sperm population from other events. Once spermatozoa were isolated from other events for analysis, they were classified in two cell populations: non-viable (sperm positive for IP with red fluorescence in the nucleus) and viable sperm, negative for IP. Sample acquisition was carried out using a CYAN flow cytometer (CyAn ADP, Beckman Coulter. Fullerton, CA, USA)

adjusted for both UV (351 nm) and blue excitation (488 nm) lines for the detection of Hoechst 33342 (450/65) and PI (670/30) fluorescence, respectively. All analyses were performed applying Weasel 3.1 free software. A total of 5,000 events were counted for each sample.

DNA fragmentation analysis

Sperm chromatin structure assay (SCSA) technique (Evenson 2013) was performed to assess DNA fragmentation using metachromatic staining Acridine Orange (AO; Polysciences INc., Waringtron, PA). AO fluoresces in the red band when combined with denatured DNA and in the green band when combined with the undamaged double DNA helix. Ejaculates were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA; pH 7.4) at a final cell concentration of approximately 1-2X10^6 cells/mL. Samples were kept immediately in liquid nitrogen until processed. Samples were thawed in a 37 °C bath and mixed with 200 µL of an acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.15 Triton X 100; pH 1.4). After 30 seconds of acid detergent exposition, AO staining was performed adding 1.2 mL of stain solution containing 6 µg of AO per mL buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA (di-sodium), 0.15 M NaCl, pH 6.0). Just 3 min after staining, samples were analysed on a FACS calibur flow cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon laser tuned at 488 µm. Flow rates were around 200 cells/s and 5,000 events were counted for each sample. Data corresponding to the red (FL3 photodetector; 670 long pass filter) and green fluorescence (FL1 photodetector; 530/30 band pass filter) of acquired particles were recorded and analysed with Weasel 3.1 free software. The main parameter, DNA Fragmentation Index (DFI), corresponds to a ratio between red to total (red and green) fluorescence.

Cryopreservation and thawing

Sperm samples were cryopreserved following clinic protocols. 6 × 10^6 cells/mL were 1:1 diluted in a commercial cryoprotective medium (Sperm Freezing Medium (Irvin Scientific, Barcelona, Spain)). After 10 min of equilibration time at RT, the mixture was loaded in 0.5 mL French straws. The straws were then exposed to liquid nitrogen vapors horizontally (2 cm over the surface of liquid nitrogen) for 30 min. After that, they were plunged into liquid nitrogen and stored until used for the Intracellular H_2O_2 analysis. Thawing was carried out at RT for 5 min.

Intracellular H_2O_2 analysis

Flow cytometry

2×10^6 cells/mL from each thawed sample were incubated with 25 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Sigma, Madrid, Spain) 40 min at RT. DAPI was used as counter stain dye. Each aliquot was analysed using a CyAn flow cytometer (ADP; Beckman Coulter, Inc). Green fluorescence (DCF) was evaluated between 500 and 530 nm. Data analysis was perforemed applying WEASEL 3.1 free software. 10000 events were counted for each sample.

Confocal microscopy

In order to localize the presence of intracellular ROS H_2O_2 in human asthenozoospermic samples, 1-2X10^6 cells/mL were incubated with 25 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Sigma, Madrid, Spain) 40 min at RT and 10 min at RT with 100 nM MitoTracker Deep Red (Invitrogen, Madrid, Spain). Two different aliquots were processed: one used as negative control and one used as oxidized control, exposed to 5 % H_20_2 (Sigma, Madrid, Spain). A 5 μL cell suspension drop was place on a slide and immediately analysed under a LSM 800 confocal microscopy (Zeiss, Jena, Germany).

Statistical methods

Data were analysed using SPSS version 20 for Macintosh (SPSS Inc., Chicago, IL). Data are presented as mean SE in all cases. Mean values of each variable were compared by t-Student test for correlated variables ($p<0.05$).

Results

Antioxidant activity of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347

The percentage of *C. elegans* survival worms after the incubation with *L. rhamnosus* CECT8361 and *B. longum* CECT7347 reported higher values (58.5% and 66% respectively) than 10 μg/mL Vit. C positive control replicates confirming the antioxidant activity of both strains (Fig. 2).

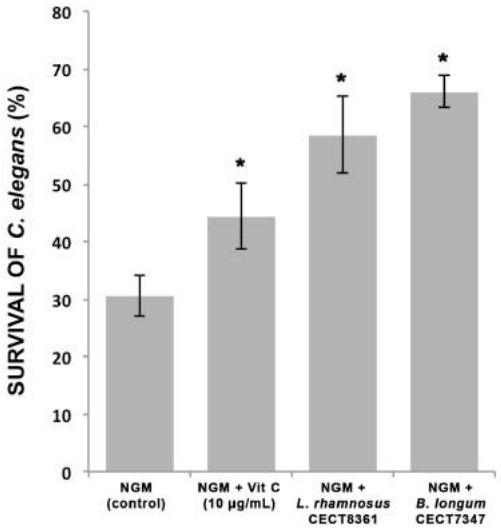

Figure 2.Survival of *C. elegans* N2 (wild-type strain) treated with 2 mM of H_2O_2 on NGM (control), vitamin C (positive control), and the probiotic strains *L. rhamnosus* CECT8361 and *B. longum* CECT7347. Asterisks show significant differences (p<0.05).

Concentration, pH and volume

Probiotic ingestion did not significantly modify these three parameters among the key points evaluated in the experimental design (C, T1, T2, W1 and W2).

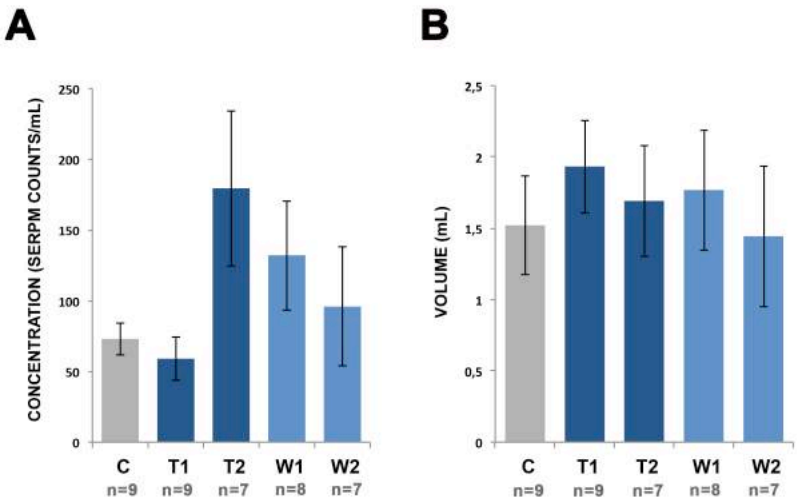

Figure 3.A) Spermatozoa concentration (sperm counts/mL) variation along the experimental design. B) Ejaculate volume (mL) variation along the experimental design. Capital letters represent key points along the timeline (C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1 (three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration)). No significant differences were found after statistical analysis.

Mean concentration values (mean ± SE) were lower in control and treatment 1 sampling (73.04 ± 11.28 and 59.07 ± 15.56 million cells/mL) comparing with treatment 2, which registered the highest mean value (179.56 ± 54.91 million cells/mL). A decrease after stopping probiotic administration was observed in W1 and W2 (132.145 ± 38.68 and 96.02 ± 41.93 million cells/mL) but just as a no statistical significant tendency (Fig. 3A). Concentration variance among males was high (Table 1).

Reported pH values were the same (pH=7) in all cases for all donors along the experimental design.

Volume data were variable among males (Table 2) with no statistical significant differences along the experimental procedure. The lowest registered mean volume (mean ± SE) was in the second washout, W2 (1.44 ± 0.05 mL) similar to control mean value (1.52 ± 0.035 mL). The highest volume was reported after three weeks of probiotic administration, T1 (1.93 ± 0.33 mL). However no significant differences were found after statistical analysis was performed (Fig. 3B).

Motility

All donors included in this study were classified as asthenozoospermic following WHO standards (WHO, 2010) after three pre-analysis (P1, P2 and P3) motility evaluations by the computer-assisted sperm analysis (CASA) (Fig. 4A). Asthenozoospermic conditions were also confirmed in the control sampling (C), prior to the beginning of the probiotic administration. Registered motile cells percentages in P1, P2 and P3 were: 3.5 ± 1.89; 11.29 ± 3.20 and 6.22 ± 2.59 % respectively (mean value ± SE).

Control sampling reported a motility percentage of 6.43 ± 2.63 % (mean value ± SE) in accordance with the pre-analysis results. After three weeks of probiotic administration (T1), motile spermatozoa percentage increased around six times comparing to the previous samplings, reaching 31.88 ± 6.01% (mean value ± SE). This increment was maintained after six weeks of probiotics exposure (T2), as well as both washouts performed after the end of the ingestion of the bacteria (W1 and W2). Reported data were respectively: 28.57 ± 7.58; 33.25 ± 6.38 and 39.57 ± 8.52 % (Fig. 4B).

Individual data (static, motile and progressive cell percentages) of each donor are collected in Table 3.

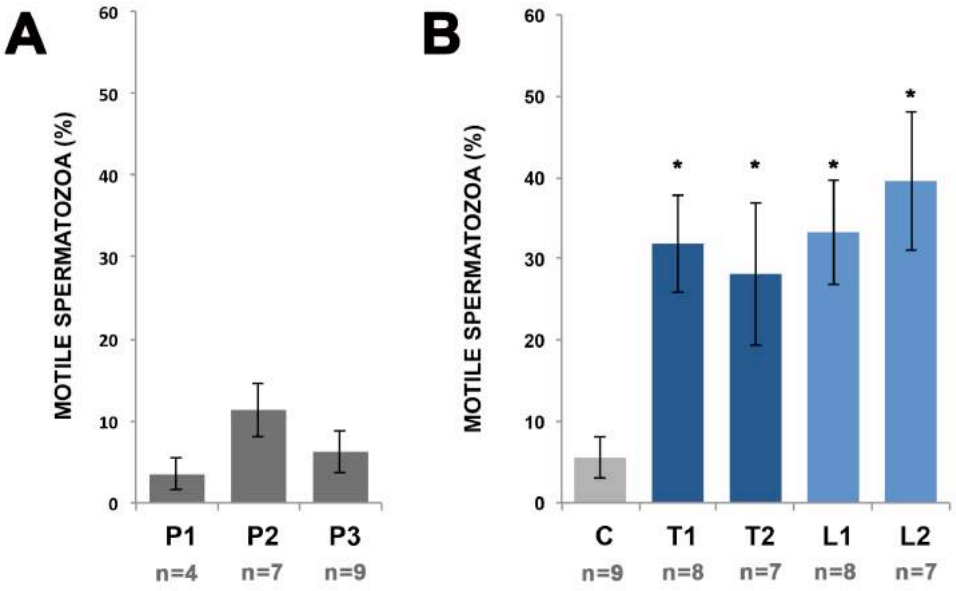

Figure 4.A) Motile spermatozoa (%) variation along the pre-analysis motility assessment B) Motile spermatozoa (%) variation along the experimental design. Values with superscript asterisks are significantly different to the control after t-Student test for correlated variables (p<0,05). Capital letters represent key points along the timeline (P1, P2 and P3 represent ejaculate samples for pre-analysis motility assessment; C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1 (three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration)).

Sperm chromatin structure analysis

Obtained DNA Fragmentation Index (DFI) for each volunteer can be found in Table 4. Fig. 5 shows the mean percentage values obtained in each key point along the experimental design. Control (C) reported the highest DFI with 25.74 ± 0.59 % ratio. During the six weeks of probiotic administration this fraction was reduced in both samplings 21.11 ± 1.00 and 21.58 ± 0.93 % (T1 and T2 respectively). After the washout, the recorded improvement seen during the administration started to change. Washotut 1 and 2 (W2) acquired a higher DFI comparing to T1 and T2 with a DFI ratio of 21.64 ± 1.73 % and 23.09 ± 1.27 % respectively, changing the trend.

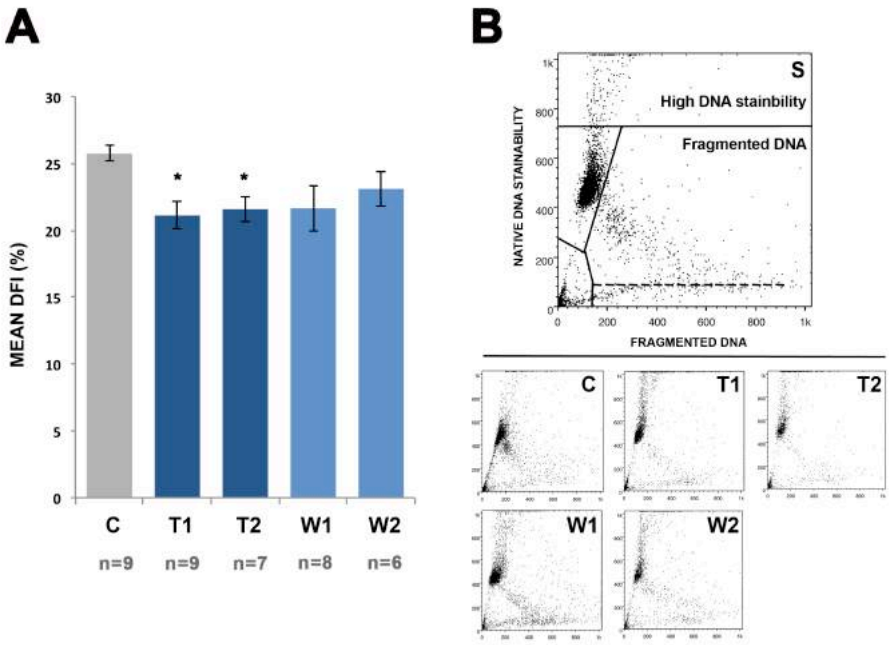

Figure 5.A). Mean DNA Fragmentation Index (%) variation along the experimental design. Values with superscript asterisks are significantly different to the control after t-Student test for correlated variables (p<0,05). B). Sperm chromatin structure assay (SCSA)-derived cytograms. They show green (normal double stranded DNA) versus red (fragmented DNA, denatured single-stranded DNA) fluorescence. Capital letters represent: S: cytogram example and key points along the timeline (C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1 (three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration).

Viability

The percentage of live cells obtained after flow cytometry in each sampling was homogeneous during the experimental procedure (Table 5). Any statistical significant difference was established. Living cells mean percentages were over 55% in all samplings (Fig. 6). No correlation between viability and the ingestion of probiotics can be done.

Intracellular H_2O_2 levels

The percentage of dichloro-dihydro-fluorescein (DCF) positive cells obtained after flow cytometry reported a statistical significant difference between control replicates prior to probiotic ingestion (16.57 ± 3.34 %) and T1 (5.02 ± 0.93 %), T2 (6.2 ± 1.77 %), W1 (7.27 ± 2.37 %) and W2 (7.9 ± 1.36 %) (Fig. 7).

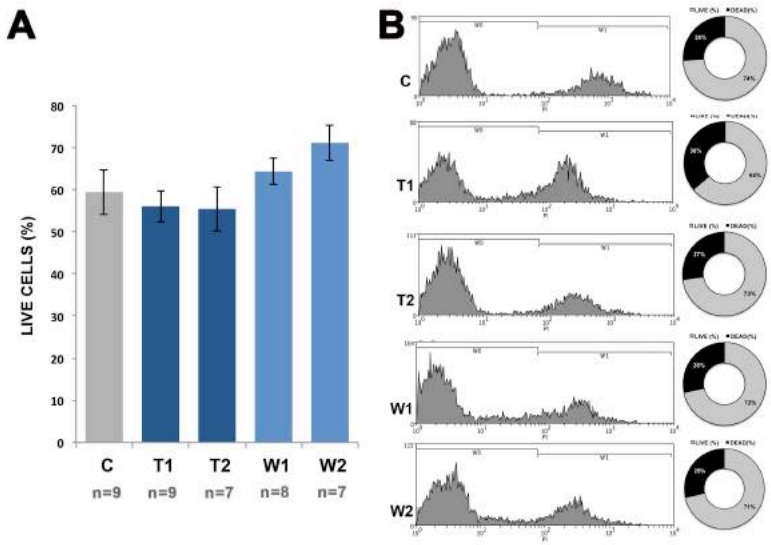

Figure 6.A) Live cells (%) variation along the experimental timeline. No significant differences were found after statistical analysis. B). Representative histograms obtained after Propidium Iodide-Hoeschst 33342 obtained in one case of study. Rings show percentages of cells in each histograms. Capital letters represent key points along the timeline (C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1 (three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration)).

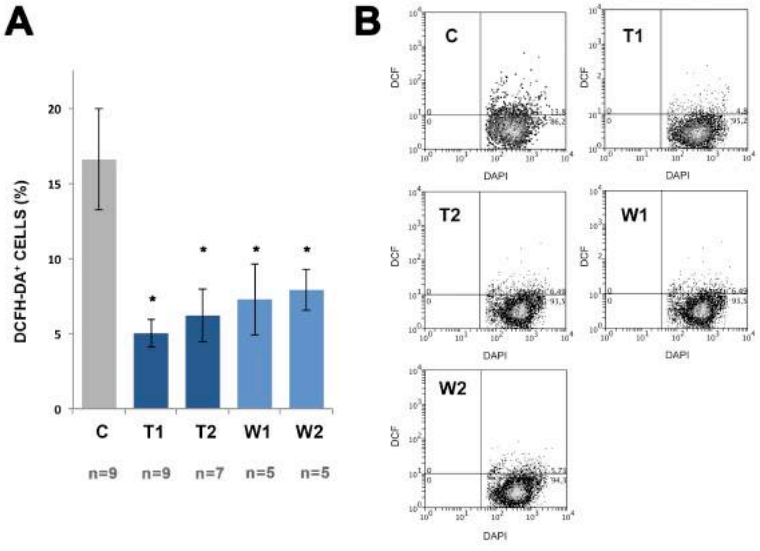

Figure 7.Intracellular H_20_2 (% DCFH-DA$^+$ cells) along the experimental timeline. Control value is significantly different (asterisk) to the rest of values after one-way ANOVA test (p<0,05). Capital letters represent key points along the timeline (C: Control (before probiotics administration); T1: treatment 1 (after three weeks of probiotics administration); T2: treatment 2 (after six weeks of probiotics administration); W1: washout 1(three weeks after the end of probiotics administration) and W2: washout 2 (six weeks after the end of probiotics administration)).

Intracellular H_2O_2 localization

Confocal microscopy showed the co-localization in the sperm cell (Fig. 8A) of intracellular H_2O_2 with mitochondria in the middle piece in both negative and oxidized controls (Fig. 8B and C). Moreover, cells exposed to 5% H_2O_2 showed a high amount of ROS also in the nucleus (Fig. 8C).

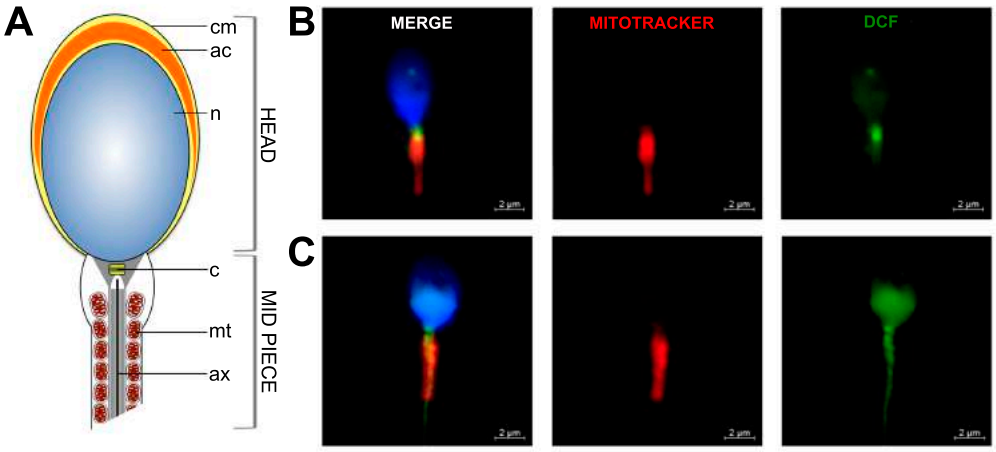

Figure 8.Intracellular H_20_2 localization in spermatozoa. A. Diagram of the head and mid piece of a human spermatozoa. cm: cell membrane; ac: acrosome; n: nucleus; c: centriole; mt: mitochondrial sheath; ax: axoneme. Confocal microscopy images. DNA stained with DAPI, mitochondria stained with Mitotracker Deep Red, intracellular H_20_2 stained with DCFH-DA. B. Control cell. C. 5% H2O2 exposed cell.

Discussion

The most usual origin of infertility cases presented at assisted reproductive technologies (ARTs) clinics is spermatic dysfunction. Asthenozoospermia is one of the male subfertility pathologies defined by the World Health Organization (WHO, 2010) as a condition in which the percentage of progressively motile sperm is abnormally low.

Intracytoplasmic sperm injection (ICSI) is nowadays widely used in those cases with male-factor infertility and it is clear that this technique has risen as a key revolutionary tool in ARTs (de Mendoza et al. 2000). However, with immotile spermatozoa, the positive pregnancy rates are still low (Liu et al. 1995, 1997) mainly due to the struggle of discriminating non-viable from viable immotile spermatozoa. The identification of new ways to increase sperm quality could be really useful to improve reproductive performance of individuals and in reproductive clinics prior to insemination, *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). In the present study the effect of oral administration of a combination of two strains of probiotic bacteria with tested antioxidant effect on sperm quality from asthenozoospermic donors is analysed.

Probiotics consumption is increasing worldwide as a therapy for many different diseases and disorders. Scientific community is focusing on isolating bacterial strains able to improve human health and describing the biological molecular basis of probiotic function. *Lactobacillus* and *Bifidobacterium* were selected in this study since they are common genus of the endogenous intestinal tracts of mammals (Guarner and Malagelada 2003), they have been widely used as probiotics and they are able to stabilize the intestinal microbiome, inducing host immunomodulation and reducing the symptoms of a wide range of gastrointestinal disorders (Manley et al. 2007; Pant et al. 2007). *C. elegans* is a soil nematode that feeds on bacteria. This nematode is an extremely powerful and well-studied biological system, which has been used as a model to study aging and oxidative stress. In addition, this nematode has become an excellent model to evaluate ingredients and probiotics exerting antioxidant and antiinflamatory properties (Grompone et al. 2012; Martorell et al. 2011, 2016). The two selected strains used in this experiment have a clear antioxidant activity as it has been demonstrated in *C. elegans* (Fig. 2). The use of antioxidants in semen quality studies is not new. Over the last decades clinical studies have been carried out to establish the possible beneficial effects of treatment (Ross et al. 2010) with antioxidants on improvement of sperm parameters in men as well as fertilization or pregnancy rates in their partners. The most commonly studied antioxidants were vitamin C, vitamin E, selenium, glutathione, zinc, N-acetyl-cysteine and L-carnitine (Ross et al. 2010). Some of these studies evaluate the effect of *in vitro* antioxidants (added as supplements in the solution with cryoprotectants) and they concluded a beneficial effect of these additives in protecting spermatozoa from exogenous oxidative molecules and freezing-thawing protocols (Gharagozloo and Aitken 2011). But, could be oral administration of antioxidant compounds still effective in improving sperm quality? Bejarano and coworkers (Bejarano et al. 2014) observed that couples whose males were treated with melatonin increase seminal total antioxidant capacity and improve human sperm quality. Other studies evaluated the effect of oral antioxidant administration on sperm parameters. In 2010, Ross (Ross et al. 2010) reviewed beneficial effects of the ingestion of antioxidants. In this work, 75% of the analyzed trials showed an improvement in sperm in at least one sperm parameter: 63% improved motility, 33% improvement of in sperm concentration, 17% an improvement in sperm morphology. On the female factor, several studies have reported beneficial effects of the use of probiotic strains in reproductive biology field, focusing only on its properties as therapy against bacterial vaginosis (Borges et al. 2013; Mastromarino et al. 2013). Until now, our results are the first report describing the effect of probiotic ingestion on human male germ cells but taking into account the significant effect of ingested antioxidants on human sperm quality, it is reasonable to think that the antioxidant properties of the probiotic strains used in the present study could be potentially beneficial for sperm quality.

Participants in the present study were defined as asthenozoospermic men (Table 4) following WHO criteria. Six-week probiotics consumption of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 did not affect parameters like concentration, volume or pH (Fig. 3). Following the same tendency, viability was not modified along the experimental design and was considered normal following WHO criteria (Fig. 6). However, a significant improvement was registered in sperm motility comparing control (C) and treatment samplings (T1 and T2) after 3 and 6 weeks respectively, revealing a clear effect of the probiotics on this parameter (Fig. 4). Unpredictably, the increase was maintained after the end of the exposure in both washouts (W1 and W2). Thus, probiotic administration significantly improved sperm quality attending to motility parameters.

Concomitantly, an analysis of sperm DNA fragmentation was performed. It is well known that there is a correlation between poor motility and high levels of DNA damage (Aitken *et al.* 2014). The test used was Sperm Chromatin Structure Assay (SCSA) (Evenson 2013). This protocol measures the percentage of sperm with a high vulnerability to low pH-induced DNA denaturation and is expressed as the DNA fragmentation index (DFI%). This percentage is a highly accurate, repeatable measure of DNA quality that is proportional to the level of DNA strand breaks (Sailer *et al.* 1995; Aravindan *et al.*, 1997). In our study, control sampling showed the highest DNA Fragmentation Index along the whole experimental design (25.74 ± 0.59%). Treatment samplings values were lower in both cases, correlating to the improvement of the motile cells percentages (Fig. 5). Following the same tendency as motility, registered fragmentation reduction was maintained after washout although only statistical differences were found during probiotic administration samplings. The DFI percentage threshold establishing male subfertility is variable depending on different publications: ≥20% (Boe-Hansen et al. 2006), ≥27% (Larson-Cook et al. 2003) and ≥30% (Payne et al. 2005; Zini et al. 2005). However, it is clear that a higher fragmentation level is correlated with poor motility and worse fertility success although high levels of DNA fragmentation are compatible with on going pregnancy using ART (Boe-Hansen et al. 2006).

Reactive oxygen species (ROS) are considered an important cause of DNA fragmentation in human spermatozoa (Lopes et al. 1998) and intracellular H_2O_2 is one of the most common forms of ROS. H_2O_2 were measured levels in order to confirm if the observed effect of probiotic ingestion on preventing DNA fragmentation was produced by a decrease in ROS due to their antioxidant properties. DCFH-DA was used as probe due to its confirmed ability to measure H_2O_2 (Guthrie 2006; Mahfouz et al. 2009). Reported intracellular H_2O_2 levels in the control group (16.57 ± 3.34 %) are similar to previous Mahfouz results, around 20% in mature sperm (Mahfouz et al. 2009). The probiotic ingestion showed the same effect in this parameter, following the previously described trend: a positive reduction after the beginning of the administration which is maintained until the washout samplings. Therefore, the

decrease in intracellular H_2O_2 levels supports our hypothesis, suggesting that antioxidant capacity of probiotic strains could decrease intracellular reactive oxygen species preventing DNA from fragmentation. The reduction in DNA fragmentation could be also explaining the improvement of sperm motility.

The antioxidant effect of the LAB bacteria and bifidobacteria has been widely studied (Guo et al. 2013; Lin and Chang 2000). This effect could be causing that DNA would be less affected by ROS and this would explain the registered improvement in DNA Fragmentation Index.

Sperm cells are rich in mitochondria because they need a constant resource of energy for their motility. The formation of superoxide in the electron transfer chain in these organelles and, in a supplementary way, NADPH oxidase are the central sources of ROS in sperm (Koppers et al. 2010; Kothari et al. 2010). Specific probes for mitochondria-produced ROS (mROS) shows that unnecessary production results in membrane peroxidation and loss of motility (Aitken et al. 2012; Koppers et al. 2008). Furthermore, a higher content of unsaturated fatty acid on sperm is also associated to an increase in mROS again leading to motility loss and DNA damage (Koppers et al. 2010). Our results could be explained considering that the administration of antioxidant probiotic bacteria to asthenozoospermic males act as a defence for the sperm mitochondria against ROS and, as consequence, motility is improved.

In conclusion, the results of our study demonstrated the stimulatory role of *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347 administration as a feed antioxidant additive improving sperm motility, reducing DNA fragmentation and reducing intracellular H_2O_2 sperm levels. The specific molecular mechanisms through which probiotics stimulate sperm motility are still unknown and further studies are needed in order to clarify this aspect. This work opens a new window for male infertility treatment therapy, using probiotic strains prior to ARTs or natural reproduction.

Declaration of interest

Authors from Biopolis S. L. provided the probiotic strains and the evidence of their antioxidant properties in *C. elegans* but they had no other role in study design, data collection and analysis, decision to publish or preparation of the manuscript**.**

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DISCUSIÓN

La contribución paterna al embrión, aunque imprescindible, se ha visto habitualmente relegada a un segundo plano, otorgándosele al espermatozoide una función "todo o nada" en su papel en la fecundación, y olvidándose, en la mayoría de los casos, su potencial contribución en el desarrollo embrionario. La presente tesis doctoral, sin pretender abarcar los innumerables factores que pueden afectar a la calidad espermática, ha seleccionado alguno de dichos factores para estudiar en detalle su efecto sobre parámetros espermáticos concretos, buscando paralelamente desarrollar nuevos métodos de análisis de calidad seminal que complementen a los ya existentes. La especie utilizada no ha supuesto una barrera en esta búsqueda, utilizando en cada uno de los casos de estudio aquella especie que siendo accesible para la investigación, se ha considerado más adecuada para los objetivos perseguidos. Ante los numerosos factores que pueden afectar de forma negativa a la calidad seminal, existen algunos que han de resultar beneficiosos para la misma, y esta tesis no podía concluir sin estudiar uno de ellos, la ingesta de probióticos, nunca antes considerado como elemento beneficioso a este nivel en ninguna especie animal.

Efecto de las tecnologías de la reproducción sobre la calidad espermática: efecto de la criopreservación en el espermatozoide humano

El primer objetivo de esta Tesis Doctoral, abordó el efecto de la criopreservación sobre el ADN genómico y sobre los transcritos espermáticos en el ser humano. La criopreservación es una herramienta muy útil para la conservación del material genético en diversos campos biotecnológicos, desde la producción animal a la conservación de especies en peligro de extinción o a la biotecnología de la reproducción humana. Este último campo es el marco de trabajo de nuestros estudios. Las tecnologías de reproducción asistida, de forma casi general, requieren de la criopreservación de muestras seminales. Existen numerosos estudios previos sobre la criopreservación de muestras espermáticas en diferentes especies de animales. La ausencia de daño en el ADN de los gametos masculinos es un factor crucial a considerar a la hora de elegir un protocolo de criopreservación (Kopeika et al. 2015). Es por esta razón que muchos grupos de investigación centran su interés en las posibles consecuencias negativas que el proceso de congelación y descongelación puede tener sobre el ADN del espermatozoide (Kalthur et al. 2008; Thomson et al. 2009; Zribi et al. 2010). La mayoría de los estudios proporcionan análisis de daño de forma global, centrándose en la evaluación de la fragmentación del ADN y en la integridad de la cromatina. Para ello, utilizan diferentes técnicas de análisis tales como el COMET *assay* (Albert et al. 2016), SCSA (*sperm chromatin structure assay*)

(Evenson et al. 2007), TUNEL (*TdT-mediated dUTP Nick end labelling*) (Palermo et al. 2014) o SCD (*spermchromatin dispersión test*) (Evenson 2016). Para profundizar en el análisis del daño potencial que la criopreservación puede ejercer sobre el ADN espemático humano, en la primera parte de este capítulo se ha empleado una técnica novedosa basada en un protocolo de PCR cuantitativa (qPCR). El análisis molecular que hemos aplicado tras la criopreservación logra cuantificar lesiones producidas en regiones de genes concretos. La técnica fue descrita por Rothfuss y colaboradores (Rothfuss et al. 2010) para cuantificar daño genético derivado de la exposición de peróxido de hidrógeno. Nuestro grupo había utilizado este protocolo para evaluar daño en otros tipos celulares en teleósteos (Cartón-García et al. 2013; Riesco and Robles 2013), pero el uso de este protocolo para estudiar daño en genes concretos producidos por la criopreservación en espermatozoides humanos ha sido totalmente novedoso en la presente Tesis Doctoral. La técnica empleada se basa en analizar el bloqueo de la progresión de la ADN polimerasa debido a la presencia de algunas lesiones en la cadena de ADN. Este bloqueo produce un retraso en la amplificación de la hebra molde de ADN y consecuentemente provoca un retraso en el Ct (*threshold cycle*) de la región estudiada. La utilización de esta técnica como complemento de los protocolos de valoración global de la integridad del ADN presenta claras ventajas, permitiendo detectar de forma precisa las lesiones moleculares en genes concretos y desenmascarar daños no detectables con el uso exclusivo de los métodos globales.

En base a los objetivos generales de esta Tesis Doctoral, se analizó un protocolo de criopreservación rutinario en los centros de reproducción asistida con el fin de proporcionar más información sobre esta herramienta biotecnológica de uso clínico. De este modo, se utilizó el medio de criopreservación comercial (*Sperm Freezing Medium*), el envasado (pajuelas de 0,5 mL), el tiempo de equilibrado (10 min) y la rampa de congelación (2 cm por encima de vapores de nitrógeno durante 30 min) y descongelación (5 min a temperatura ambiente) utilizados de forma rutinaria en las clínicas. Los seis genes seleccionados para este estudio han sido descritos como relevantes en fecundación y en desarrollo embrionario temprano (Depa-Martynów et al. 2007; García-Herrero et al. 2011; Kagami et al. 2007): *BCL2-interacting killer* (*BIK*), *FSHb polypeptide* (*FSHB*), *protamine 1* (*PRM1*), *mesoderm specific transcript homolog (mouse)* (*PEG1/MEST*), *adducin 1 alpha* (*ADD1*) y *aryl-hydrocarbon receptor nuclear translocator* (*ARNT*). A esta batería de genes, sumamos dos regiones genómicas (*UBE3A* y *SNORD116/PWSAS)* relacionadas respectivamente con dos síndromes epigenéticos: el síndrome de Prader-Willi y el síndrome de Angelman. El motivo de la incorporación de estas dos regiones se debió a la evidencia documentada de un incremento en la incidencia de estas enfermedades en los nacidos a partir de tecnologías de reproducción asistida (Amor and Halliday 2008; Carrell and Hammoud 2010; Maher et al.

2003; van Montfoort et al. 2012). Con el objetivo de tener un control positivo de daño genético se realizó un tratamiento con peróxido de hidrógeno en alícuotas de las muestras.

Los resultados obtenidos, demostraron que el protocolo de criopreservación testado provoca lesiones en las regiones genómicas estudiadas. Estas lesiones, que podrían ser de variable naturaleza (bases oxidadas, posiciones abásicas, *crosslinking* o dímeros de timidina), fueron encontradas en mayor número en las regiones correspondientes a *PRM1*, *UBE3A* y *SNORD116*/PWSAS. En nuestro estudio, como era de esperar, todos los genes analizados presentaron mayor número de lesiones en los controles positivos expuestos a peróxido de hidrógeno. Estos resultados corroboraron la capacidad de la técnica utilizada para detectar lesiones producidas por especies reactivas del oxígeno. Existen evidencias de que estas moléculas son generadas en los protocolos de criopreservación así como de su capacidad para causar la aparición de lesiones en el ADN (Baumber et al.; Box et al. 2001). Resultó interesante constatar que dentro de las muestras expuestas a peróxido de hidrógeno, las mismas regiones (*PRM1*, *UBE3A* y *SNORD116*/PWSAS) fueron las más dañadas. Estos resultados nos sugirieron la existencia de zonas de diferente vulnerabilidad al daño genético dentro del genoma espermático. Con el objetivo de explicar estas variaciones, centramos nuestra atención sobre las diferentes zonas de compactación de la cromatina espermática. Existen tres dominios estructurales diferentes (Ward 2010): por un lado, la gran mayoría del ADN se encuentra asociado a protaminas; por otro lado, un porcentaje pequeño (10-15%) de ADN está asociado a histonas; y por último, una tercera parte se encuentra asociado a la matriz nuclear espermática en regiones asociadas a histonas en la zona periférica. Realizamos un estudio de la arquitectura cromosómica, en base a datos previamente publicados (Li et al. 2008; Manvelyan et al. 2008), encontrando una asociación entre localización y vulnerabilidad. Los cromosomas 15 y 16, en los que se encuentran las regiones genéticas estudiadas que resultaron más dañadas tanto por criopreservación como por el peróxido de hidrógeno (*PRM1* (16p13.13), *UBE3A* (15q11-q13) y *SNORD116*/*PWSAS* (15q11-q13)), se sitúan en la zona post-acrosómica central del núcleo espermático (Manvelyan et al. 2008), colocalizando con la cromatina asociada a histonas (Li *et al.*, 2008). El ADN unido a histonas presenta un menor grado de compactación, lo que podría derivar en una mayor vulnerabilidad ante el daño genético, explicando nuestros resultados. De este modo pudimos concluir que existe un mayor grado de vulnerabilidad de la región *PWAS* en el cromosoma 15 vinculada a los síndromes de Prader-Willi y Angelman al daño producido por la criopreservación.

En la segunda parte de este primer capítulo analizamos el efecto que el mismo protocolo de criopreservación puede tener sobre las poblaciones de transcritos presentes en las células espermáticas. Hoy en día, conocemos que la contribución paterna en la fecundación no está limitada al ADN. Los transcritos espermáticos son también transferidos

al ovocito (Ostermeier et al. 2004) y existen evidencias de su importancia a nivel molecular en el desarrollo embrionario temprano (Amdani et al. 2016). A través de estudios de transcriptómica se han revelado diferencias en las poblaciones de ARNm entre los espermatozoides de pacientes sanos y con patologías (García-Herrero et al. 2011). Cabe destacar que una reducción de estos transcritos, al igual que las lesiones en el ADN, puede ser corregida por la potente maquinaria celular del ovocito o del embrión (Ménézo et al. 2010). Sin embargo, es necesario evaluar el efecto real sobre dichos transcritos para garantizar que los protocolos utilizados en las clínicas de reproducción humana estén completamente optimizados para garantizar la ausencia de alteraciones durante el desarrollo embrionario. Para ello, basándonos en resultados de transcriptómica previamente publicados, seleccionamos los transcritos candidatos para el estudio realizado. Dichos transcritos pertenecieron a dos grupos: I) potenciales marcadores de fertilidad masculina (Depa-Martynów et al. 2007; García-Herrero et al. 2011; Kagami et al. 2007): *BCL2-interacting killer* (*BIK*), *FSHb polypeptide* (*FSHB*), *protamine 1* (*PRM1*), *protamine 2* (*PRM2*) y *mesoderm specific transcript homolog (mouse) (PEG1/MEST)* y II) potenciales marcadores de éxito de embarazo (García-Herrero et al. 2011): *activin A receptor type II like 1* (*ACVRL1*), *adducin 1 alpha* (*ADD1*), *androgen receptor* (*AR*), *aryl-hydrocarbon receptor nuclear translocator* (*ARNT*) y *endothelial PAS domain protein* (*EPAS1*). Dada la naturaleza transcripcionalmente inactiva de la célula espermática (Ostermeier et al. 2002), la cantidad de ARNm presentes en el espermatozoide es muy baja (Swann and Lai 2016). Los transcritos existentes son remanentes de la espermatogénesis. A pesar de esta realidad, nuestros experimentos permitieron su detección por qPCR, validando esta técnica para este propósito. Los resultados mostraron que la presencia de tres de los transcritos, pertenecientes al grupo de marcadores de fertilidad masculina (*PRM1, PRM2 y PEG1/MEST*) y un marcador de éxito del embarazo (*ADD1*), se redujo significativamente tras el protocolo de criopreservación. Además la tendencia a la reducción de las poblaciones fue percibida en todos los transcritos estudiados, traduciéndose en un retraso en los Ct (*threshold cycle*) en las muestras criopreservadas. Este dato nos condujo a hipotetizar que la criopreservación afecta a la estabilidad de los transcritos espermáticos haciéndoles más susceptibles a la degradación. Existen publicaciones de definen la existencia de interacciones ARNm-proteína que estabilizan los transcritos maternos protegiéndolos ante una potencial degradación (Igreja and Izaurralde 2011; Jeske et al. 2011; Zaessinger et al. 2006). En el gameto masculino podría existir un mecanismo semejante que pudiera verse afectado por el protocolo de criopreservación. De hecho, estudios recientes muestran la importancia del *chromatoid body* (CB) en el almacenamiento y metabolismo del ARN en células germinales masculinas (Nagamori and Sassone-Corsi 2008). Esta estructura está formada por diversas proteínas así como distintos tipos de

ARNs y es sabido que transcritos específicos de espermatocitos y espermátidas están presentes en el CB y son protegidos de la degradación gracias a este almacenamiento. Estudios con ratones *knockout* que carecen de componentes cruciales para el CB y son estériles han demostrado la importancia del CB en la fertilidad masculina (Peruquetti 2015). Es posible que en el espermatozoide, aquellos transcritos relevantes para la fecundación e incluso para el desarrollo embrionario temprano puedan estar estabilizados, y por tanto protegidos de la degradación gracias a la interacción con proteínas, de forma semejante a lo que sucede en células germinales masculinas menos diferenciadas. De ser así, determinados protocolos de criopreservación, al igual que pueden modificar la estructura del nucleosoma podrían modificar esta interacción ARN-proteína y hacer a los transcritos más susceptibles a la degradación.

Como conclusión, en este primer capítulo se ha realizado un estudio preciso del efecto a nivel molecular de la criopreservación, proporcionando las herramientas necesarias para mejorar la evaluación de los protocolos utilizados en clínica con objeto de permitir una mejor selección de los mismos, y garantizar así la calidad de los gametos y el éxito de las técnicas de reproducción asistida.

Efecto de la estabulación sobre la calidad espermática en especies de interés comercial: el lenguado senegalés.

En el segundo capítulo de esta Tesis Doctoral, se analizaron las alteraciones moleculares producidas en los espermatozoides por la estabulación de reproductores de lenguado senegalés. Es importante destacar la importancia que esta especie tiene en el sector de la acuicultura nacional para contextualizar la necesidad de conseguir superar el problema reproductivo que sufre. *Solea senegalensis* se presenta como un candidato excelente para diversificar la producción industrial de la acuicultura en los países del sur de Europa. Se ajusta a este fin tanto por sus características propias como por su valor en el mercado (APROMAR 2016). El problema reproductivo que presenta este pez plano radica en la falta de reproducción natural en los machos nacidos en cautividad (F1) a diferencia de los animales nacidos en el medio natural (WT) (Agulleiro et al. 2006; Carazo et al. 2011; Rasines et al. 2012). Esta disfunción reproductiva hace necesaria la utilización de técnicas de fecundación artificial y además conduce, a la captura e incorporación de animales del medio natural de forma periódica produciendo una importante presión de pesca sobre las poblaciones salvajes. Para los ciclos de producción, las muestras seminales son extraídas y criopreservadas en bancos de germoplasma hasta su uso (Rasines et al. 2012). En la presente Tesis Doctoral se realiza un estudio detallado de la calidad seminal en los reproductores F1. En el primer trabajo del segundo capítulo recogemos los resultados de un estudio pormenorizado del grado de apoptosis presente en las células espermáticas de los

machos F1 mediante dos vías de análisis por citometría de flujo. El fundamento de esta investigación radica en el efecto negativo que las células apoptóticas pueden tener sobre la fecundación, (Grunewald et al. 2009; Said et al.; Weng et al. 2002), agravado por el hecho de que su presencia en la muestra fresca, puede incrementar los daños que se producen durante la congelación/descongelación. En este estudio, se analizaron las caspasas como elementos centrales en la vía apoptótica (Paasch et al. 2004) y se evaluó la integridad de la membrana como efecto derivado de la apoptosis con YO-PRO 1 (Beirao et al. 2010).

Atendiendo a la evaluación de las caspasas activas, el estudio nos permitió concluir que existe un porcentaje de células apoptóticas mayor en los machos estabulados desde su nacimiento (F1). No ocurrió lo mismo con el estudio con YO-PRO 1 donde los porcentajes fueron semejantes en ambos grupos. Esta variación puede ser debida a la especificidad de la técnica utilizada. El estudio de las caspasas es mucho más específico, ya que directamente evalúa un elemento central de la apotosis mientras que YO-PRO 1 estudia un efecto derivado de la misma o de otros procesos deletéreos. Los porcentajes de daño en membrana fueron en todos los casos elevados con valores semejantes a otros estudios previos publicados (Beirão et al. 2008).

Corroborada la existencia de un gran porcentaje de células apoptóticas y/o dañadas, nuestro objetivo se centró en la implementación de una técnica que nos permitiera la selección celular de aquellos espermatozoides óptimos previamente a la criopreservación seminal o a los ensayos de fecundación artificial. La tecnología seleccionada fue MACS (*magnetic activated cell sorting*). Esta técnica ampliamente utilizada en mamíferos incluso en clínica en humano (Rawe et al. 2009; Vermes et al. 1995), nunca había sido explorada con fines reproductivos en peces y los estudios en teleósteos han sido escasos en otras áreas de conocimiento (Panda et al. 2011). El fundamento molecular de este protocolo radica en la selección por la conjugación de un marcador de membrana celular con un anticuerpo específico o molécula contra él que está asociado a una pequeña esfera metálica. Tras la incubación, la suspensión celular es expuesta a un campo magnético donde se retienen las células que se quieren seleccionar para su uso o su descarte. En nuestro experimento la molécula conjugada a las micro-esferas fue la anexina V que presenta una gran especificidad contra la fosfatidilserina (Dirican et al. 2008). Esta molécula es externalizada en membranas de las células apoptóticas, como uno de los primeros marcadores de apoptosis (Said and Land 2011). De este modo, se permite la retención de las células espermáticas apoptóticas. Nuestros resultados mostraron la eficacia de este método tanto en los animales capturados en el medio salvaje como en los nacidos en cautividad. La reducción de células apoptóticas fue demostrada analizando tanto las caspasas como la integridad de la membrana con YO-PRO 1.

En consecuencia, nuestros resultados nos permitieron concluir que los niveles de apoptosis son mayores en los machos F1, que el análisis de la apoptosis en espermatozoides de lenguado senegalés mediante el estudio de las caspasas activas es mucho más específico que YO-PRO 1 y que la tecnología MACS puede ser utilizada con éxito en esta especie.

En el segundo trabajo de este capítulo se evaluaron las especies reactivas del oxígeno (ROS) en los espermatozoides de los machos nacidos en cautividad y se correlacionaron con valores de viabilidad. A pesar de que existen referencias sobre el estudio de niveles de peroxidación lipídica en los espermatozoides de esta especie (Beirão et al. 2015), nunca antes se había realizado la evaluación directa de especies reactivas del oxígeno y su posible relación con la calidad seminal. Las especies reactivas del oxígeno tienen un impacto muy importante sobre el espermatozoide. La presencia de ROS en los espermatozoides ha sido correlacionada en mamíferos con (Aitken et al. 2012): peroxidación lipídica, apoptosis, inducción de daño en el ADN y daño en proteínas. Los efectos negativos de las especies reactivas del oxígeno sobre espermatozoides de peces han sido explorados también desde diferentes perspectivas (Beirão et al. 2015; Chauvigné et al. 2015; Dietrich et al. 2005; Gazo et al. 2015; Martínez-Páramo et al. 2012; Shaliutina et al. 2013). Con nuestro estudio abordamos el efecto de la estabulación además de analizar el efecto de la criopreservación sobre la generación ROS, dada la importancia de esta técnica en la producción actual de esta especie, tal como se ha mencionado con anterioridad. Para realizar esta evaluación utilizamos citometría de flujo incubando las muestras con yoduro de propidio (viabilidad) y diclorofluoresceína (DCF) para revelar la presencia de ROS.

Nuestros resultados con las muestras frescas mostraron la existencia de un alto estrés oxidativo tanto en los espermatozoides de los machos capturados en el medio natural como en los estabulados. En base a las publicaciones existentes, esperaríamos que estos valores tuvieran consecuencias muy negativas sobre los espermatozoides. La viabilidad sin embargo no difirió en ambos grupos registrando porcentajes medios en torno al 70%. De forma sorprendente, el número de células que resultaron positivas para DCF fue mayor en las muestras de los machos nacidos en el medio natural. Estos resultados podrían indicar que en esta especie en concreto, la presencia de ROS no tiene que ser necesariamente negativa, y pudiera responder a patrones fisiológicos normales, tal y como se ha descrito en mamíferos (Aitken et al. 2012; Kodama et al.; Pfeifer et al. 2001). Para profundizar en este hecho, realizamos una subdivisión de los machos F1 en base a su motilidad. y los resultados mostraron que los machos F1 con buena motilidad presentaron valores semejantes a los observados en machos nacidos en el medio natural.

Discusión

Nuestra hipótesis considera que las especies reactivas del oxígeno presentes en los espermatozoides de esta especie no presentan necesariamente efectos negativos sobre su función, y que es la localización de las mismas (en mitocondrias o en núcleo) la que pudiera determinar en realidad un efecto positivo o negativo sobre el espermatozoide. El estudio por microscopía confocal demostró una colocalización de ROS con las mitocondrias de la pieza media (Medina et al. 2000) pero también pudimos revelar presencia de ROS en el núcleo. Con el objetivo de explorar si estos resultados se restringían a la especie de estudio, se analizó la presencia de de ROS por microscopía confocal en espermatozoides de rodaballo (*Scophthalmus maximus*). En este caso la localización nuclear de ROS fue mucho más aislada.

Llegados a este punto, un aspecto interesante a considerar era el efecto que la criopreservación, técnica ampliamente utilizada en esta especie como paso previo a las fecundaciones artificiales, tendría sobre ambos tipos de estas muestras seminales (WT y F1) valorando no sólo la viabilidad, si no la cantidad de ROS tras el proceso. El análisis del protocolo de criopreservación utilizado mostró resultados de viabilidad diferentes entre las muestras de machos nacidos en medio natural y los F1. Mientras que en el primer grupo la viabilidad se mantuvo en los mismos niveles tras la criopreservación, los machos F1 sufrieron una reducción significativa tras el protocolo. Cuando se analizaron las muestras seminales de los individuos nacidos en cautividad (F1) en función de la motilidad, también se observó que la viabilidad era mejor preservada tras la congelación en aquellas muestras de mayor motilidad. Por tanto, en esta especie, la calidad seminal no puede ser asociada de forma directa a la presencia de ROS.

De los resultados obtenidos a partir de estos dos trabajos concluimos que la estabulación de los machos de *Solea senegalensis* tiene un impacto a nivel celular en los espermatozoides afectando a los niveles de apoptosis y de especies reactivas del oxígeno sin afectar la viabilidad en muestras frescas. Estos estudios permitieron implementar una tecnología para la selección de células no apoptóticas que podría usarse previamente a la criopreservación y a la fecundación artificial.

Efecto de los contaminantes emergentes sobre la calidad seminal y consecuencias en su progenie: Efecto del 17-α-etinilestradiol

En este apartado de la tesis se aborda el efecto que los disruptores endocrinos pueden tener sobre las especies acuáticas salvajes o cultivadas en masas de agua que reciben efluentes con presencia de contaminentes. La gran mayoría de estudios previos realizan investigaciones a través de la exposición directa al tóxico, lo cual ha permitido destacar multitud de consecuencias negativas sobre los animales (Arnold et al. 2014; Hotchkiss et al. 2008; Salla et al. 2016; Tetreault et al. 2011). En este caso, el enfoque del

trabajo se circunscribió al potencial efecto que estas moléculas pueden jugar sobre la espermatogénesis temprana afectando como consecuencia por vía paterna a sus progenies no expuestas. Para desarrollar este objetivo se utilizó el pez cebra, cuyo uso como modelo en experimentos de ecotoxicología está altamente extendido (Lee et al. 2015; Sipes et al. 2011). Como disruptor endocrino se eligió el 17-α-etinilestradiol, principio activo en fármacos anticonceptivos (Nazari and Suja 2016) y cuya presencia en aguas superficales ha sido registrada a nivel mundial (Adeel et al. 2017). Como población experimental se utilizaron machos de cepas salvajes en edad reproductiva que fueron expuestos a tres dosis del tóxico relevantes en el medio natural. La duración de la exposición al tóxico fue de 14 días con el objetivo de abarcar las etapas iniciales de la espermatogénesis (estadios meióticos y premeióticos) en pez cebra (Schulz et al. 2010). Tras una semana de descanso los animales fueron cruzados con hembras no tratadas para estudiar de este modo el efecto paterno en las progenies obtenidas.

En un primer apartado se analizó la expresión génica a nivel testicular y se estudiaron las poblaciones de transcritos en muestras seminales de los machos expuestos. Se focalizó la atención en los receptores de estrógenos (ERs) debido a la capacidad manifiesta que presenta el tóxico para unirse a ellos (Mattison et al. 2014) pudiendo desencadenar alteraciones en las numerosas vías en las que juegan papeles cruciales (Bondesson et al. 2015). El estudio nos permitió concluir que el EE2 es capaz de alterar la expresión de dos de los tres receptores de estrógenos de pez cebra (Heldring et al. 2007) en los testículos (*esr1* y *esr2b*) de forma semejante a lo observado en experimentos previos realizados con el ligando natural 17-β-estradiol (Chandrasekar et al. 2010). Teniendo en cuenta que los receptores de estrógeno en los testículos están presentes en las células somáticas y germinales, incluyendo espermatogonias y espermatocitos (Carreau et al. 2011; Chauvigné et al. 2017), es racional esperar que los efectos observados en la expresión testicular sean encontrados de forma semejante en los espermatozoides. Así, los resultados mostraron, efectivamente, una mayor cantidad de *esr2b* en los espermatozoides tras la exposición a EE2. De este modo, se concluyó que la exposición paterna al 17-α-etinilestradiol durante la espermatogénesis temprana es capaz de provocar una sobreexpresión de ciertos genes que conducen al transporte de un número mayor de determinados transcritos al zigoto. Con el objetivo de evaluar si la exposición alteraba la capacidad reproductiva de los machos, se estudió también la expresión en el testículo de una batería de transcritos cuyos patrones de expresión habían sido descritos previamente como marcadores de buenos y malos resproductores en pez cebra (Guerra et al. 2013). Los resultados revelaron que los patrones de dos de ellos eran semejantes a los encontrados en

malos reproductores sugiriendo así que la exposición al tóxico disminuye la capacidad reproductiva de los animales.

Tal y como se ha comentado con anterioridad en esta tesis, en la actualidad existen numerosas evidencias que permiten afirmar que los perfiles transcriptómicos de los espermatozoides juegan papeles importantes en el éxito de la reproducción (García-Herrero et al. 2011) y el desarrollo embrionario temprano (Fang et al. 2014). En esta línea, en el grupo de investigación donde se ha realizado la presente tesis doctoral se abordaron experimentos en los que se correlacionaron bajas poblaciones de transcritos de receptores de insulina en espermatozoides con malformaciones cardiacas en las progenies no expuestas, que fueron heredadas hasta la generación F2 (Lombó et al. 2015). De este modo se pudo corroborar que los ARNm presentes en el espermatozoide tienen un impacto importante sobre el desarrollo embrionario. En concordancia, los resultados obtenidos en la evaluación de la expresión en la parte anterior de embriones a 25 hpf en las progenies experimentales revelaron un paisaje celular alterado. La expresión del receptor de estrógeno *esr1* se veía *down*-regulado en este estadio donde existe de forma natural expresión de ERs (Kinch et al. 2016; Tingaud-Sequeira et al. 2004). De este modo, la cantidad suprafisiológica de transcritos paternos presente en las primeras etapas embrionarias, produciría una grave desregulación en diferentes vías de señalización celular, afectando el desarrollo canónico del embrión. Así se comprobó en las progenies resultantes de los cruces, que presentaron malformaciones principalmente vinculadas a la formación de edemas linfáticos. Con el objetivo de profundizar y obtener mayores conclusiones se exploraron genes vinculados con la linfoangiogéneis (*vegfc* y *vegfr3*) y el control de la migración de las células endoteliales (*cxcr4a* y *cxcl12b*) (Schuermann et al. 2014) en las muestras de la parte anterior de embriones a 25 hpf, zona y estadio en la que existe expresión de genes regulados por elementos de respuesta a estrógenos (EREs) (Hao et al. 2013). Los resultados corroboraron una menor expresión de *vegfc* indicando una alteración en las vías vinculadas con la aparición de linfoedemas, que han sido relacionadas con los receptores de estrógenos (Mueller et al. 2000; Rodriguez-Lara et al. 2017). Así se pudo concluir que la alteración testicular derivada de la exposición a EE2 provoca un efecto indirecto sobre las progenies a través de la alteración de los transcritos espermáticos entregados en la fecundación.

La aparición de linfoedemas no fue la única consecuencia negativa percibida en las progenies experimentales. Se registró un incremento en el área de los otolitos de las larvas, diferente tendencia a la descrita en ensayos de exposisicón directa a tóxicos (Gibert et al. 2011; Tohmé et al. 2014). Sin embargo, algunas de las larvas estudiadas mostraban sáculos y utrículos que tendían a la fusión de forma semejante a lo descrito en los mismos estudios, indicando que la desregulación derivada por el factor paterno puede tener un

efecto diferente aunque igualmente perjudicial. Dada la presencia de malformaciones que podrían afectar al comportamiento y dada la desregulación del receptor de estrógeno 1 en la zona cranial, se realizó una evaluación de la capacidad de natación de las larvas ya que los receptores de estrógenos juegan un papel esencial en el desarrollo del cerebro (Bondesson et al. 2015). Los resultados reflejaron un patrón diferente entre las larvas derivadas de machos expuestos y las control, encontrando una reducción en las trayectorias recorridas así como una menor capacidad de reconocimiento y exploración del área de análisis.

Como conclusión, nuestros resultados proporcionan evidencia de que, en peces óseos, la exposición a EE2 en concentraciones ambientalmente relevantes durante las primeras etapas de la espermatogénesis es capaz de afectar la expresión génica testicular, conduciendo a modificaciones en la población de transcritos espermáticos y perturbando el desarrollo normal de las progenies no tratadas resultantes a través de una desregulación de los receptores de estrógeno.

Efecto de los suplementos nutricionales sobre la calidad seminal: efecto de los probióticos

A diferencia de los estudios previos, en este capítulo se explora la posibilidad de incrementar la calidad seminal mediante la ingesta de probióticos. Los probióticos son microorganismos vivos que pueden proporcionar beneficios en la salud de un hospedador cuando son administrados en dosis adecuadas (Hill et al. 2014). Sus potenciales aplicaciones se han enumerado en varios campos (Lievin-Le Moal and Servin 2014) y su uso se está expandiendo exponencialmente en los últimos años. En el campo de la reproducción se habían realizado experiencias con animales modelo en el factor femenino (Carnevali et al. 2013; Gioacchini et al. 2010) y también han sido empleados como un posible tratamiento clínico en mujeres (Mastromarino et al. 2013), pero nunca se había sugerido su empleo en la mejora de la calidad seminal. En nuestros estudios trabajamos con cepas de bacterias del ácido láctico (LAB) y bifidobacterias.

En la primera parte de este objetivo, utilizamos el pez cebra como especie modelo para valorar el efecto de la ingesta de probióticos a nivel molecular en las células testiculares. Para ello, utilizamos una serie de transcritos descritos con anterioridad como marcadores de buenos reproductores y malos reproductores por nuestro grupo (Guerra et al. 2013). El probiótico comercializado bajo el nombre Bactocell® (que contiene la bacteria láctica *Pediococcus acidilactici)* fue administrado a través de la dieta durante diez días a los machos en una dosis controlada. Los transcritos cuya expresión fue estudiada y comparada con un grupo control fueron: *bdnf*, *bik*, *dmrt1*, *fshb* y *lepa*. Nuestros resultados mostraron sobre expresión de los marcadores asociados a buenos reproductores (*bdnf*, *dmrt1*, *fshb* y *lepa*) mientras que el transcrito correlacionado con malos reproductores mostró la

tendencia contraria. Los análisis estadísticos permitieron encontrar diferencias significativas en los transcritos *bdnf*, *dmrt1* y *lepa* mostrando un claro efecto de la ingesta del probiótico a nivel molecular.

En base a los prometedores resultados obtenidos con la especie modelo, el segundo experimento de este capítulo se centró en evaluar el efecto de los probióticos sobre muestras espermáticas con baja calidad en el ser humano. Un grupo de 9 donantes voluntarios astenozoospérmicos con dieta homogénea fue seleccionado para el estudio. Se realizaron evaluaciones previas de motilidad mediante CASA para establecer la condición de astenozoospermia de los donantes participantes en el estudio (World Health Organization 2010). La administración de las dos cepas de probióticos utilizadas (*Lactobacillus rhamnosus* CECT8361 y *Bifidobacterium longum* CECT7347) duró 6 semanas y el estudio se realizó de forma quincenal y continuó durante otras 6 semanas denominadas "de lavado" tras la finalización de la ingesta del probiótico. Estos microorganismos fueron proporcionados por la empresa Biópolis S.A. donde se certificó la capacidad antioxidante y antiinflamatoria de esta combinación de microorganismos. Nuestros resultados no mostraron variaciones significativas en los parámetros generales estudiados (volumen, concentración y pH). Tampoco la viabilidad espermática se modificó durante el tratamiento. Por el contrario, la motilidad registrada tras la primera quincena de ingesta mejoró de forma significativa en comparación con la evaluación control antes de la ingesta. Esta mejora en la motilidad se mantuvo durante los demás muestreos, e incluso tras los lavados. La evaluación de la fragmentación del ADN mediante SCSA permitió explicar los resultados de motilidad ya que se registró una reducción significativa en el índice de fragmentanción en el ADN durante la administración de las cepas. Para analizar si el efecto de la ingesta de la combinación de cepas probióticas era debido a una disminución del estrés oxidativo que se traducía en el menor índice de fragmentación de DNA observado, y consecuentemente en mayores motilidades, analizamos por citometría de flujo la cantidad de especies reactivas del oxígeno presentes en las muestras. Los resultados mostraron una reducción significativa en la cantidad de células DCF^{+}. En base a estos resultados pudimos concluir que la capacidad antioxidante de los probióticos proporcionó protección frente al estrés oxidativo a los espermatozoides. La novedad del presente estudio permitió que pudiera ser patentado (ANEXO) además de ser publicado. A pesar de que pudiera resultar sorprendente que la ingesta de un suplemento alimenticio consiga tener los efectos descritos existen otros trabajos que también han observado mejoras en la calidad espermática tras la ingesta de otros compuestos (Mendiola et al. 2010; Minguez-Alarcon et al. 2012; Schmid et al. 2012; Yang et al. 2012). El punto en común con nuestra estudio radica en la capacidad antioxidante de las moléculas utilizadas. El uso de probióticos permite una administración segura y fácilmente aceptable por los potenciales

pacientes. En conclusión nuestros resultados demostraron el efecto positivo de la ingesta de las cepas probióticas *Lactobacillus rhamnosus* CECT8361 y *Bifidobacterium longum* CECT7347 sobre la calidad seminal diminuyendo la fragmentación del ADN, reduciendo los niveles intracelulares de ROS y mejorando significativamente la motilidad espermática.

Este trabajo abre una nueva ventana en el desarrollo de terapias contra infertilidad masculina mediante la utilización de nuevos tratamientos.

Consideraciones Finales

El desarrollo de la presente Tesis Doctoral se ha enmarcado en el campo de la biotecnología de la reproducción y ha buscado aportar conocimiento en el área clínica, la ecotoxicología y a nivel industrial.

En primer lugar, se han implementado nuevas herramientas moleculares para la valoración de los protocolos de criopreservación espermática en el ser humano, describiendo el efecto de la congelación sobre genes y transcritos con papeles claves en fecundación y éxito de embarazo. En segundo lugar, se ha estudiado el efecto de la estabulación animal en la calidad espermática, utilizando una especie de elevado valor en el mercado como es el lenguado senegalés y se ha implementado una técnica para la selección de subpoblaciones óptimas mediante *MACS*.

En tercer lugar, los hallazgos derivados de este trabajo proporcionan evidencias acerca de los efectos negativos heredables que el disruptor endocrino 17-α-etinilestradiol puede tener sobre las poblaciones acúaticas, al haber registrado la capacidad para la transmisión via paterna de efectos deletéreos.

Por último, se ha descubierto el efecto positivo sobre las células germinales masculinas de determinadas cepas probióticas tanto en peces como en humanos, llegado a formular una combinación de cepas patentada que mejora significativamente la motilidad de donantes astenozoospérmicos y reduce la fragmentación del ADN espermático.

El trabajo con tres especies distintas de vertebrados (humano, pez cebra y lenguado senegalés) ha permitido realizar una combinación entre investigación básica -que ha tenido como producción capítulos de libro, revisiones y artículos- y aplicada (prototipo y patente), proporcionando conclusiones relevantes en biología reproductiva.

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CONCLUSIONES

Las conclusiones derivadas de los trabajos que constituyen esta Tesis Doctoral son las siguientes:

Capítulo 1:

1- El protocolo de criopreservación de rutina estudiado, utilizado en las clínicas de reproducción asistida, provoca lesiones en regiones genómicas y genes clave vinculados con calidad espermática, éxito de embarazo y síndromes epigenéticos.
2- La utilización de qPCR permite la cuantificación del número de lesiones producidas por la criopreservación en regiones genómicas y genes concretos en el espermatozoide humano. Las regiones del ADN espermático asociadas a histonas, y por tanto con una compactación menor, presentan mayor vulnerabilidad al daño producido por la criopresevación.
3- El protocolo de criopreservación de rutina estudiado, utilizado en las clínicas de reproducción asistida, provoca una reducción en las poblaciones de transcritos espermáticos clave vinculados con la calidad espermática y éxito de embarazo. Este hecho puede explicarse por un efecto inductor de la degradación del ARNm debido a la desestabilización sobre interacciones ARN-proteína.

Capítulo 2:

4- Los reproductores de lenguado senegalés nacidos y criados en cautividad presentan un mayor porcentaje de células espermáticas apoptóticas en comparación con los individuos nacidos en libertad.
5- La tecnología MACS (*Magnetic Activated Cell Sorting*) puede ser utilizada como herramienta para la selección de poblaciones no apoptóticas de espermatozoides en el lenguado senegalés.
6- Los espermatozoides de lenguado senegalés están expuestos a un alto nivel de estrés oxidativo. Los espermatozoides de machos nacidos en libertad presentan mayores niveles de especies reactivas del oxígeno (ROS) que los machos nacidos y criados en cautividad.
7- Los niveles de especies reactivas del oxígeno no se correlacionan directamente con parámetros de mala calidad espermática, siendo su localización (mitocondrial o nuclear) determinante. Muestras con buena motilidad (independientemente de su origen) presentan mayores porcentajes de ROS.

Capítulo 3:

8- La exposición a dosis medioambientalmente relevantes del disruptor endocrino 17-α-etinilestradiol (principio activo de las píldoras anticonceptivas) durante las etapas

tempranas de la espermatogénesis afecta a la expresión génica de los testículos alterando específicamente a los receptores de estrógenos y algunos genes relacionados con bajo éxito reproductivo.

9- Los niveles de transcritos presentes en espermatozoides son igualmente afectados tras la exposición, encontrándose un mayor número de ARNm de los receptores de estrógenos que podrían jugar papeles cruciales en las primeras etapas de desarrollo embrionario y ser responsables de los efectos observados en la progenie.

10- Las progenies resultantes de los cruces de los machos expuestos con hembras no tratadas presentan un mayor grado de malformaciones, principalmente vinculadas a la formación de edemas linfáticos. Estudios de expresión génica corroboraron la desregulación de genes de receptores de estrógenos y genes relacionados con la linfoangiogénesis.

11- Las larvas derivadas de los machos tratados presentan cambios en el patrón de comportamiento.

Capítulo 4:

12- La suplementación alimentaria con el probiótico *Pediococcus acidilactici* en machos reproductores de pez cebra induce la sobreexpresión de los genes *bdnf*, *dmrt1* y *lepa* vinculados con éxito de fecundación.

13- La suplementación alimentaria con una combinación de cepas probióticas *Lactobacillus rhamnosus* CECT8361 y *Bifidobacterium longum* CECT7347 mejora la calidad espermática en hombres astenozoospérmicos incrementando la motilidad y reduciendo la presencia de especies reactivas del oxígeno y la fragmentación del ADN.

ANEXOS

ANEXO I

Material suplementario

M.S. CAPÍTULO I

I.A.-

Table 1

Table 1

Target genes	GenBank reference	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)
PRM1	AB281136.1	GGTGGTGCCCTGCTCGA	GCAGGCTCCTGATTTTTATTGG	403	62
		ACAGACCGCGATGTAGAAGACA	CAGGCAGGAGTTTGGTGGAT	62	62
BIK	AY245248.1	CCCCTCTCTTAGCGGTTTACTCT	TCTTGGTCCCCGCGCTAAC	678	62
		GCATGGGCCGGTCTGA	CCTGCCATGCCTCCAAAGT	65	62
PEG1/MEST	AB045582.1	CTGGACCCCAGCGTCATC	CCGCGAGACGAACCACTT	651	66
		TCTAACCCCCAGCATCGCCCT	TGAATCGCACCAGAGGGAAGCC	69	66
FSHB	NG_008144.1	GCAAGGCAGCCGACCACAGG	TGGTCTGCTCATCCTCCTCAATCAC	782	66
		GCAAGGCAGCCGACCACAGG	CACCACTTGAAAACGGTAGATGCCA	52	66
ADD1	NG_012037.1	TAACCCCAGGTTTTCCTTCTTCA	CATCACATATGCGATGACACACA	700	66
		GATAAGCTCATGGATTTGGGACTT	CTTTGCCAACTTCAATCAGTGAA	65	62
ARNT	NG_028248.1	AGCCATATAAATGCGGATACAGGTT	GTGGGTGCCTGTTGTTTATGAA	700	66
		GAGGCCAAAGGAATGTCCATT	TCCACTACCTGGCACCAGACT	69	62
UBE3A	NG_009268.1	GCAGCCCAAGGAAAACTGATC	CTGCTGTCCTTGAACTAAGCTGTAGA	600	66
		TGATGCCATTGTTGCTGCTT	CCCTCCCACTACATTTGCATAGT	64	62
SNORD116/PWAS	NG_002690.1	GGAGAGTCACGGAGGGATTG	TTTCAAAGCAAGAAAAAGGATCAAG	750	66
		GGTCTTGGGCTCTCATTTGG	CCGACCTGTCATCCATCAGA	65	62

I.B.-

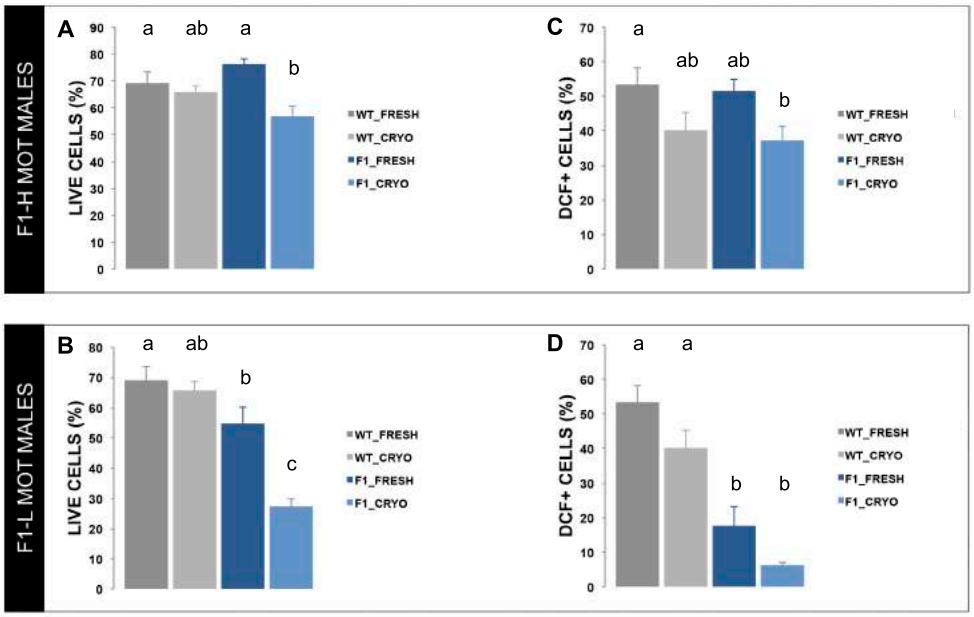
Table 1

Forward and reverse primers designed for each target RNA sequence. Gene Bank reference as well as amplicon size and annealing temperature are specified.

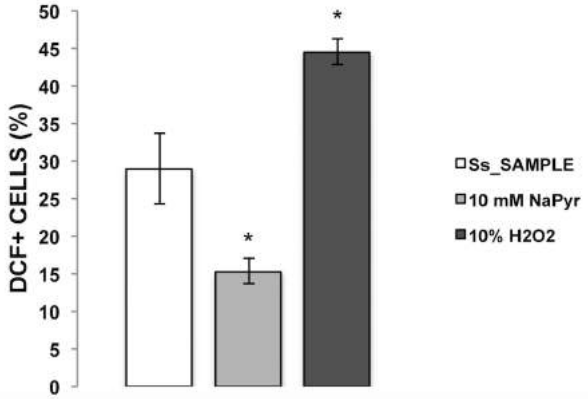
Target genes	Gen Bank reference	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
Olinonucleotides for transcripts analysis					
PRM1	AB281136.1	TCGCAGACGAAGGAGGCGGA	GGTCTGTACCTGGGGCGGCA	72	62
PRM2	AB281137.1	ATCCACAGGCGGCAGCATCG	TTCCAGCTGGGGGTGAGGGG	144	63
BIK	AY245248.1	CCCGGGTGGCTTACAGACGC	TCCATGGTCGGGGGTTCCAGG	131	62
PEG1/MEST	AB045582.1	GGCTTTGGCTTCAGTGACAAACCG	ACGATGCTGGCCTGCTCAAAT	65	64
FSHB	NG_008144.1	CACCACTTGGTGTGCTGGCT	TTGGGCCTGGCTGGGTCCTT	66	62
ACVRL1	NM_000020.2	TGGGCTCCCCCAGGAAAGGC	GCCCCGAGCGGCTTCACAG	80	63
ADD1	NM_176801.1	ACTCCGAGCCAGGAACACTTCCTCA	ACTCCCAGATTAGTGCTTCCACGA	127	62
AR	NM_000044.2	CCAGCTCACCAAGCTCCTGGAC	GTGAACTGATGCAGCTCTCTCGCA	60	62
ARNT	NM_001197325.1	TGGTACCAGGAAGAGATGTACTGGC	GGGGCTTGCTGTGTTCTGGTCC	90	62
EPAS1	NM_001430.4	CGGTCACCAACAGAGGCCGT	TGGCCCGTGCAGTGCAAGAC	70	64
Oligonucleotides for reference genes [2]					
BACT	NM_001101	TTCCTTCCTGGGCATGGAGT	TACAGGTCTTGCGGATGTC	90	62
ATPB5	NM_001686	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT	80	62
GAPD	NM_002046	TCTCCTCTGACTTCAACAGCGAC	CCCTGTTGCTGTAGCCAAATTC	126	62
HSPCB	NM_007355	AAGAGAGCAAGGCAAAGTTTGAG	TGGTCACAATGCAGCAAGGT	120	62

M.S. CAPÍTULO II

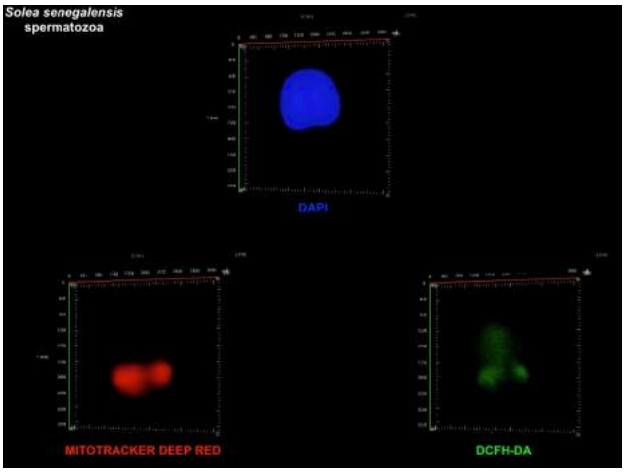
II.B.-



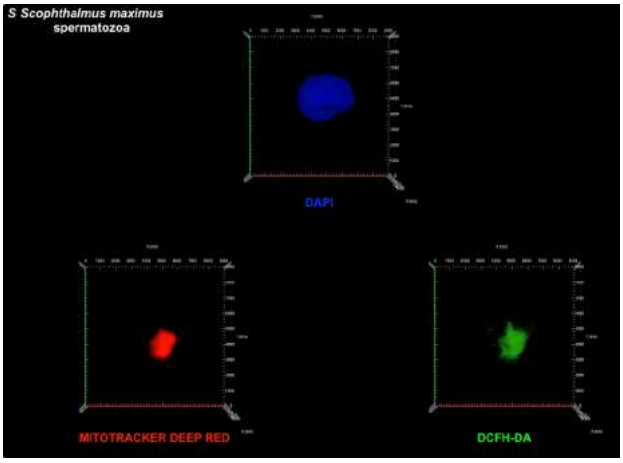
Supplemental material 1.- Analysis by flow cytometry after motility split in F1 samples: A and C: High motility samples (H MOT). B and D: Low motility samples (L MOT). WT: wild-captured males; F1: F1 males; FRESH: control samples prior to cryopreservation; CRYO: cryopreserved/thawed samples. Superscript letters show differences among groups (p<0.05).



Supplemental material 2.- Flow cytometry technique validation for reactive oxygen species detection in *Solea senegalensis* sperm. Ss sample: non treated cells. 10 mM NaPyr: sodium pyruvate (reactive oxygen species scavenger). H_2O_2: positive control. Asterisks show statistical significant differences (p<0.05).



Supplemental material 3.- *Solea senegalensis* sperm cell volume rendering. Merge composition, individual channels and stereo anaglyph.



Supplemental material 4.- *Scophthalmus maximus* sperm cell volume rendering. Merge composition, individual channels and stereo anaglyph.

M.S. CAPÍTULO IV

IV.B.-

Table 1.- Concentration data for each individual in each key point of the experimental design.

	CONCENTRATION (SPERM COUNTS/mL)				
	CONTROL	TREATMENT 1	TREATMENT 2	WASHOUT 1	WASHOUT 2
Individual 1	51.00	144.83		159.67	326.67
Individual 2	98.17	22.85	9.22	126.33	44.67
Individual 3	52.43	21.50	91.98		
Individual 4	58.90	30.88	69.64	87.20	
Individual 5	121.00	25.38	285.70	105.75	0.39
Individual 6	99.83	49.00		74.87	58.67
Individual 7	106.80	38.75	391.40	90.00	85.50
Individual 8	47.20	128.33	305.75	385.71	136.00
Individual 9	22.00	70.13	103.27	27.63	20.25

Table 2.- Volume data for each individual in each key point of the experimental design.

	VOLUME (mL)				
	CONTROL	TREATMENT 1	TREATMENT 2	WASHOUT 1	WASHOUT 2
Individual 1	0.20	0.90		1.20	0.50
Individual 2	2.50	1.30	0.50	1.70	2.00
Individual 3	0.90	1.30	1.30		
Individual 4	1.00	1.80	0.75	1.50	
Individual 5	1.00	1.50	2.50	2.00	0.60
Individual 6	1.40	2.00		0.80	0.50
Individual 7	3.00	4.00	3.50	4.00	4.00
Individual 8	3.00	3.00	1.50	2.80	1.90
Individual 9	0.70	1.60	1.80	0.15	0.60

Table 3.- Motility parameters for each individual in the three pre-analysis and in each of the key points of the experimental design.

	MOTILITY (%)								
	P1	P2	P3	CONTROL			TREATMENT 1		
	MOTILE	MOTILE	MOTILE	STATIC	MOTILE	PROGRESSIVE	STATIC	MOTILE	PROGRESSIVE
Individual 1			15.00	86.00	14.00	9.00	68.00	32.00	27.00
Individual 2			7.00	93.00	7.00	4.00	69.00	33.00	25.00
Individual 3	3.00	12.00	23.00	82.00	18.00	16.00	56.00	44.00	41.00
Individual 4		5.00	2.00	99.00	1.00	-	99.00	1.00	-
Individual 5		12.00	4.00	97.00	3.00	2.00			
Individual 6	1.00	1.00	2.00	99.00	1.00	-	42.00	58.00	53.00
Individual 7		28.00	1.00	99.00	1.00	-	65.00	35.00	30.00
Individual 8	9.00	12.00	2.00	98.00	2.00	-	83.00	17.00	14.00
Individual 9	1.00	9.00	-	100.00	-	-	65.00	35.00	30.00

	TREATMENT 2			WASHOUT 1			WASHOUT 2		
	STATIC	MOTILE	PROGRESSIVE	STATIC	MOTILE	PROGRESSIVE	STATIC	MOTILE	PROGRESSIVE
Individual 1				52.00	48.00	43.00	61.00	39.00	33.00
Individual 2	84.00	16.00	8.00	64.00	36.00	32.00	80.00	20.00	17.00
Individual 3	48.00	52.00	50.00						
Individual 4	94.00	6.00	3.00	95.00	5.00	2.00			
Individual 5	78.00	22.00	19.00	57.00	43.00	38.00	97.00	3.00	2.00
Individual 6				43.00	57.00	52.00	46.00	54.00	50.00
Individual 7	45.00	55.00	48.00	61.00	39.00	34.00	35.00	65.00	61.00
Individual 8	61.00	39.00	30.00	72.00	28.00	23.00	65.00	35.00	26.00
Individual 9	90.00	10.00	5.00	90.00	10.00	10.00	39.00	61.00	55.00

Table 4.- DNA Fragmentation Index (DFI) for each individual in each of the key points of the experimental design.

	DNA FRAGMENTATION (%)				
	CONTROL	TREATMENT 1	TREATMENT 2	WASHOUT 1	WASHOUT 2
Individual 1	27.92	22.57		21.82	23.20
Individual 2	26.32	22.03	23.13	24.88	28.93
Individual 3	24.89	18.40	18.29		
Individual 4	28.28	27.34	25.67	31.79	
Individual 5	24.54	17.11	21.85	15.87	
Individual 6	23.04	18.73		20.54	20.35
Individual 7	27.26	21.29	22.51	17.97	22.33
Individual 8	24.77	22.20	20.00	19.11	23.29
Individual 9	24.71	20.39	19.54	21.15	20.49

Table 5.- Viable and dead cell population percentage for each individual in each of the key points of the experimental design.

	VIABILITY (%)									
	CONTROL		TREATMENT 1		TREATMENT 2		WASHOUT 1		WASHOUT 2	
	LIVE	DEAD	LIVE	DEAD	LIVE	DEAD	LIVE	DEAD	LIVE	DEAD
Individual 1	84.12	15.88	62	38			76.96	23.04	67.96	32.04
Individual 2	54.64	45.36	66.08	33.92	53.26	46.74	58.29	41.71	49.85	50.15
Individual 3	81.13	18.87	66.68	33.32	59.86	40.14				
Individual 4	37.27	62.73	38.16	61.84	36.95	63.05	47.34	52.66		
Individual 5	43.46	56.54	56.94	43.06	57	43	60.69	39.31	75.54	24.46
Individual 6	45.8	54.2	56.96	43.04			67.9	32.1	76.67	23.33
Individual 7	73.97	26.03	63.89	36.11	72.68	27.32	71.56	28.44	71.43	28.57
Individual 8	45.81	54.19	55	45	69.96	30.04	72.9	27.1	75.8	24.2
Individual 9	69.07	30.93	39.22	60.78	38.46	61.54	59.03	40.97	81.03	18.97

ANEXO II

Otra producción científica derivada del capítulo I

A.II. Capítulo de libro

Chapter 19

Cryopreservation effect on genetic function:

Neonatal outcomes

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in ***Cryopreservation of Mammalian Gametes and Embryos***
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Chapter 19

Cryopreservation Effect on Genetic Function: Neonatal Outcomes

Vanesa Robles, Marta F. Riesco, and David G. Valcarce

Abstract

Cryopreservation is a well-established technique commonly used in clinical practice. It is used widely for the conservation of gametes and embryos that will be used later for insemination or in vitro fertilization. However, several studies have shown that this technique can produce changes in messenger RNA levels, in the epigenome and induce DNA damage. Although the embryo has potent mechanisms for DNA repair, and molecular changes in spermatozoa are not necessarily reflected in the embryo, it is important to explore new molecular tests and diagnostic tools to design optimal cryopreservation protocols and avoid undesirable molecular alterations. This chapter describes a protocol to quantify the lesions produced by cryopreservation using a protocol previously published by Rothfuss.

Key words Cryopreservation, DNA damage, Quantitative polymerase chain reaction, DNA lesions, DNA extraction

1 Introduction

Assisted reproductive technologies (ARTs) have been associated with a higher than normal risk of epigenetic syndromes [1] such as Beckwith-Wiedemann syndrome, Angelman syndrome (AS) and Prader-Willi syndrome (PWS) [2–4]. Moreover, higher than normal rates of low birth weight (LBW) (not attributable to maternal age or multiple births) have also been observed with the use of ARTs [5]. Cryopreservation is a technique used widely in ARTs but it has never been considered as a potential factor in these alterations. However, in humans, it is known that cryopreservation alters the presence of crucial transcripts in the spermatozoa [6], and in model animals such as zebrafish, it modifies the methylation pattern of certain promoters [7]. Additionally, cryopreservation affects DNA in the germline, producing not only fragmentation but also mutations in different genes that cannot be detected with traditional methods for DNA evaluation [8]. Spermatozoan DNA damage has been associated with a significant increase in embryonic loss

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after in vitro fertilization and intracytoplasmic sperm injection (ICSI) [9]. Most studies evaluating DNA status in human spermatozoa have been limited to fragmentation analysis. Our group used a quantitative polymerase chain reaction (q-PCR)–based technique published by Rothfuss et al. [10] to quantify lesions in specific genome regions after cryopreservation. First in animal models [7, 8, 11], and then in human sperm samples [12], our results demonstrated that, even when fragmentation is absent, significant DNA damage could be detected in some of the studied genes. This is particularly relevant for those genes considered key players in early embryo development or those genome regions particularly related to certain syndromes. Interestingly, among all the genes and genome regions studied, an elevated number of lesions were found in *SNORD116/PWSAS* (a key region in PWS) and *UBE3A* (key gene in AS) [12].

Thus, the qPCR technique initially described by Rothfuss et al. [10] can be applied successfully for the detection of DNA damage produced by cryopreservation (Fig. 1), and could be crucial in the selection of optimized cryopreservation protocols for human sperm that contribute greatly to the safety and success of ARTs. This chapter provides a detailed description of this technique and its application to the detection of lesions produced by cryopreservation.

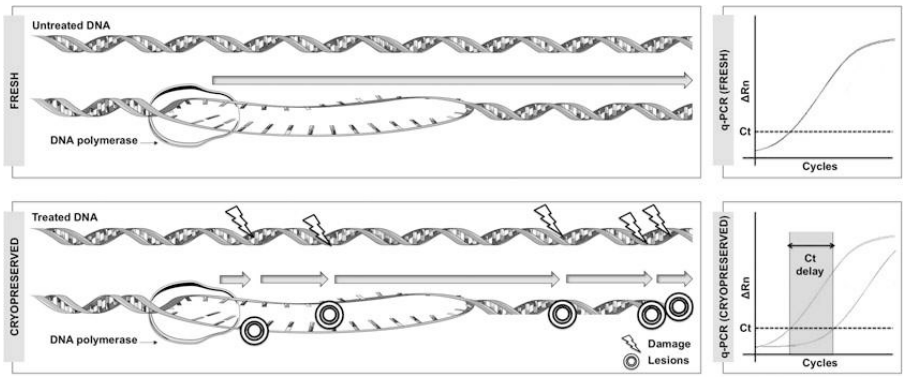

Fig. 1 Molecular basis of q-PCR-based technique for DNA lesion analysis described by Rothfuss et al. [10]. DNA lesions derived from damage caused after cryopreservation affects DNA polymerase amplification. This disruption in the polymerase complex progression is translated in a threshold cycle (Ct) delay when the analysis is monitored in a real-time PCR system. The graph illustrates non-treated DNA (fresh DNA) and treated DNA (cryopreserved DNA) differences schematically

2 Materials

Informed consent should be obtained from donors, and specific national legislation should be followed in order to work with human samples. The approval of the bioethics committee of the working institution is also required.

Perform all protocols at room temperature unless otherwise specified.

2.1 Sampling

1. Sterilized sample container.
2. Pasteur pipettes.
3. Calibrated 15 mL tubes.
4. Haemocytometer.
5. Optical microscope.
6. PBS 10×: to a flask, add 2 g KCl (27 mM), 14.2 g Na_2HPO_4 (100 mM), 2.4 g KH_2PO_4 (17 mM), and 80.8 g NaCl (1.37 M). Add bidistilled water up to 1 L. Adjust pH to 7.4. Prepare a 1:10 dilution to obtain PBS 1×, check the pH, and autoclave.

2.2 Cryopreservation and Thawing Protocol

Lesions produced by any cryopreservation protocol can be measured using this technique. In this chapter, one such protocol used for human spermatozoa in fertility clinics is described.

1. Sperm freezing medium (*Origio Medicult Media*).
2. 0.5 mL French straws.
3. Cooling device, consisting of a hermetic box with a lid (made of polystyrene or similar material) filled to half of its volume with liquid nitrogen. A rigid plastic rack is then positioned inside it at the appropriate distance from the surface of the liquid nitrogen where the samples will be placed.

2.3 DNA Extraction

1. Lysis buffer. Prepare a solution of 10 mM Tris-Cl, 50 mM NaCl, 1 mM ethylenediamine tetra-acetic acid (EDTA) and 1 % v/v sodium dodecyl sulphate; adjust pH to 8 prior to addition of the sodium dodecyl sulphate. Immediately prior to experimentation, aliquot the required amount of this solution and add 2 mg/mL proteinase K and dithiothreitol to 1 mM.
2. Heater.
3. Phenol/chloroform/isoamyl (25:24:1).
4. Chloroform.
5. Flow hood.
6. Centrifuge.
7. Absolute ethanol.

8. 70 % pre-cooled ethanol (−20 °C).
9. TE buffer. Add 1 mL of 1 M Tris pH 7.5 and 0.2 mL 0.5 M ethylenediamine tetra-acetic acid (EDTA), pH 8, to a flask. Add bidistilled water up to 100 mL, and adjust the pH to 7.4.

2.4 Primer Validation and Establishment of Optimal Melting Temperature

1. Gradient thermocycler.
2. PCR commercial kit (DNA polymerase, dNTPs, $MgCl_2$, specific DNA polymerase buffer).
3. Agarose.
4. Electrophoresis system.

2.5 q-PCR Validation and q-PCR Assays:

1. Real-time PCR system.
2. Fluorescent dye for short fragments.
3. Fluorescent dye for long fragments.
4. Adapted 96-well optical plates for the real-time PCR system.

3 Methods

3.1 Sampling

1. Collect the ejaculates in sterilized sample containers previously provided to the patients or donors (*see* **Note 1**). If possible, following WHO guidelines [13], collect sample after 3 days of sexual abstinence.
2. Let the ejaculates liquefy for 20 min (*see* **Note 2**).
3. Evaluate the volume of the sample with a disposable Pasteur pipette (*see* **Note 3**).
4. Analyse sperm concentration using a hemocytometer (*see* **Note 4**). Flick the sample to dilute a homogeneous sample 1:10 in PBS 1× and charge the chambers. Evaluate the agglutination level of the sample, abnormal morphology, and leukocyte population, and take into account any unusual values for interpretation of results (*see* **Note 5**).

3.2 Cryopreservation Protocol and Thawing

Perform the protocol under evaluation at this point. The following is an example of a common cryopreservation protocol.

1. Divide the sample in two aliquots: one will be subjected to the cryopreservation protocol, and the other kept in fresh conditions for comparison. Note that this step ensures cell number count equivalence between cryopreserved and fresh samples.
2. Drop by drop, dilute the semen sample 1:1 in a commercial Sperm Freezing Medium (*Origio Medicult Media*) to a final concentration: 4×10^7 sptz/mL.
3. Loading of the straws should be performed after exactly 10 min, since this is the equilibration time needed in the cryoprotectant

solution. For loading of 0.5 mL French straws, a P-1000 micropipette can be used (*see* **Note 6**).

4. After loading, expose straws horizontally to liquid nitrogen vapour (2 cm over the surface) in the cooling device (*see* **Note 7**). Close the box to reduce nitrogen evaporation and keep the straws in this atmosphere for 30 min.
5. Immediately following the vapour freezing period, plunge the straws into liquid nitrogen. Then, transfer the straws to the storage liquid nitrogen tank, and keep them until needed (*see* **Note 8**).
6. For thawing, carefully remove the straws from the liquid nitrogen and allow them to thaw at room temperature for 5 min. Thereafter, rub them between your hands for 2 s to ensure they are completely thawed. Hold the straw vertically in an Eppendorf tube with a cotton plug outside. Cut the cotton plug with scissors. Blow gently from above with a micropipette to allow the mixture to collect in the tube.

3.3 DNA Extraction

This part of the protocol must be repeated identically with the non-cryopreserved aliquot.

Work in a gas extraction flowhood for **steps 3** and **4**.

1. Take an aliquot containing 10^7 cells from the thawed cryopreservation sample and centrifuge it at 400 *g* for 10 min.
2. Resuspend the pellet in 0.7 mL lysis buffer. With gently agitation, lyse the samples at 55 °C for 2 h.
3. Add one volume (0.7 mL) phenol/chloroform/isoamyl (25:24:1) and, by hand, agitate the tubes vigorously for 4 min.
4. Centrifuge the tubes at 13,200 × *g* for 5 min. Collect the aqueous phase (upper phase) with a P-1000 micropipette, and recover it to a new tube (*see* **Note 9**). Wash it with a volume (0.7 mL) chloroform by performing exactly the same procedure but this time with 2 min of vigorous agitation and 5 min of centrifugation at 13,200 × *g*. Discard the other phases (*see* **Note 10**).
5. Repeat the previous step with the upper phase after the first wash.
6. Split the volume into two new tubes (0.35 mL). Add 0.875 mL (2.5 volume) absolute ethanol to precipitate the DNA. Maintain it overnight at −20 °C. In many cases, after shaking, DNA is visible.
7. Centrifuge the sample at 13,200 × *g* for 10 min. Carefully discard the ethanol, keeping the precipitated DNA in the tube, then wash the DNA with 0.5 mL of 70 % ethanol cooled to −20 °C.

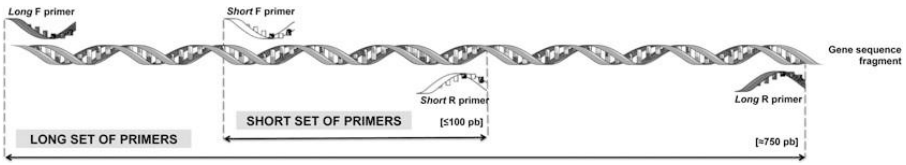

Fig. 2 Schematic representation of primer design. Two sets of primers are needed for the protocol: (1) *Long primers*: Generated product is ~750 bp; and (2) *Short primers*: generated product is <100 bp. The small amplicon must be contained within the big amplicon

8. Centrifuge the sample for 5 min at 13,200 × *g*. Discard the ethanol with a micropipette and, if needed, use a pulse centrifuge to collect as much ethanol residue as possible, then remove it with a micropipette.
9. Maintain the tubes open for 10–15 min to let the ethanol evaporate.
10. Carefully, resuspend DNA in 30 μL of TE buffer.
11. Evaluate DNA quantity and purity using a Nanodrop spectrometer. Select only high purity ($A_{260}/A_{280} > 1.8$) samples for q-PCR analysis.
12. Dilute DNA samples to the desired concentration for q-PCR, usually 20–25 ng/μL. Keep the DNA samples stored at −80 °C if they are not immediately processed (*see* **Note 11**).

3.4 Primer Design

1. Select sequences of the studied genes from a database.
2. Using primer design software, create two pairs of primers for each studied gene. The first set of primers should generate a longer amplicon of around 700–800 bp, and the second should create a shorter one (≤100 bp); this short fragment must be designed to be within the larger amplicon (Fig. 2). Primers should have the following characteristics: 19–25 bases long, G+C content not higher than 70 %, and melting temperature (Tm) between 58 °C and 62 °C (*see* **Note 12**).

3.5 Primer Validation and Establishment of Optimal Melting Temperature

1. Check optimal primer melting temperatures (Tm) by conventional PCR in a gradient thermocycler in order to confirm the product size and optimized Tm. Try varying the temperature around the Tm suggested by the primer design software (±1 or 2 °C). Use a commercial kit for this purpose, with a final concentration of around 20 ng/μL in the final reaction mix.
2. Carry out a standard PCR cycle adapted for the expected product length (*see* **Note 13**).
3. Separate the products using 2 % agarose gel electrophoresis to verify their size, and select the optimal Tm (the one yielding the cleanest specific band) for q-PCR assays.

3.6 q-PCR Validation

1. Evaluate the efficiency of the designed primers sets for validating q-PCR results. Prepare a dilution series starting from 1 μg/mL DNA sample from 1:10 to 1:100,000, corresponding to approximately 1000–0.01 ng DNA.
2. Amplify each dilution for each gene and set of primers (long and short) using the cycling conditions described below (Subheading 3.7, **steps 1** and **2**).
3. Acceptable efficiencies should range from 80% to 120 %. The relation between Ct and the DNA dilution value must be linear, ideally with regression, $R^2 = 1$.

3.7 q-PCR

Perform the q-PCR analysis for long and short fragments for the same gene simultaneously in the same 96 plate in the real-time system (*see* **Note 14**).

1. Prepare the reaction mixture for long fragments (20 μL): 4 μL 5× Fast Start DNA Master plus SyBr Green I (Roche), 1 μL each 10 μM forward and reverse primer, 0.4 μL 50× ROS passive reference dye (Bio-Rad), template DNA (3 ng) and bidistilled water up to 20 μL (*see* **Note 15**).
2. Prepare the reaction mixture for short fragments (20 μL): 10 μL 2× SYBER Green PRC (Applied Biosystems), 1 μL each 10 μM forward and reverse primers, template DNA (3 ng) (*see* **Note 16**).
3. Design the plate conditions for the q-PCR system.
4. Run a q-PCR cycle in a quantitative thermocycler consisting of a pre-incubation phase of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 10 s at the melting temperature of each primer set, and 50 s at 72 °C, with a final extension period of 7 min at 72 °C.
5. Always check product specificity by running a 2 % agarose gel electrophoresis.

3.8 DNA Lesion Analysis

Rothfuss and colleagues initially described the following analysis in 2010 (Rothfuss et al., 2010):

1. Calculate the difference in Ct value determined by q-PCR analysis between the cryopreserved (treated) and the fresh samples. Repeat this calculation for each long and short fragment for each gene. In this way, a collection of "Δlong" and "Δshort" variables will be generated.
2. Replace the reported values in this formula described by Rothfuss:

$$\text{Lesion rate}\,[\text{Lesion per 10kb DNA}] = \left(1 - 2^{-(\Delta\text{long}-\Delta\text{short})}\right) \times \frac{1000[\text{bp}]}{\text{size of long fragment}\,[\text{bp}]}$$

4 Notes

1. Note that any biological sample that has not been tested for infectious diseases is potentially hazardous, thus compliance with all health and safety measures is paramount in this respect.
2. If evaluation of motility is needed, keep the samples at 37 °C until this has been performed.
3. If motility is going to be analysed, avoid using plastic pipettes as they can negatively influence this parameter.
4. The most common haemocytometer types regularly used for sperm concentration evaluation are: Neubauer chamber and Makler chamber. Computer Assisted Sperm Analysis (CASA) software can also be used for motility and concentration evaluation.
5. If cell type selection is needed for a particular experiment (for example, a study may focus only on motile cells), perform a density gradient adapted to the requirements.
6. Loading French straws require practice. Training may be required before performing the protocol in an experimental setting. Using a P-1000 micropipette is the most common method of loading the straws; however, a system for loading French straws can be created easily with a syringe, a piece of thin plastic tube, and a P-200 tip. Attach the tip to the extreme end of the tube by its narrower end, and then connect this item to the syringe. Place the cotton plug part of the French straw into the tip, and the other end inside the mixture of the cryoprotectants and ejaculate. Aspire 0.5 mL of the mixture until the French straw is loaded (Fig. 3). When working with a lot of different samples, the use of coloured straws facilitates tracing afterwards, and avoids mistakes.
7. To create a cooling device, cut two identical square pieces of polystyrene with a width corresponding to the distance of exposure in your cryopreservation protocol. Then, remove the internal part of the squares to create two perimeters. Cut a plastic rigid net, or similar, to the same dimensions of the polystyrene perimeters. Glue the three parts together (Fig. 4).
8. Working with liquid nitrogen can be dangerous. Extreme caution and specific health and safety guidelines must always be followed.
9. Note that aqueous phases present very dense mucus textures that might hamper their recovery with a micropipette. It is recommended to attempt this step very slowly and carefully, trying to collect as much volume as possible without compromising the sample. It is preferable to work with a smaller volume than to risk contamination with the interphase.If it is not possible to recover the full starting volume, top up with TE to reach the required volume.

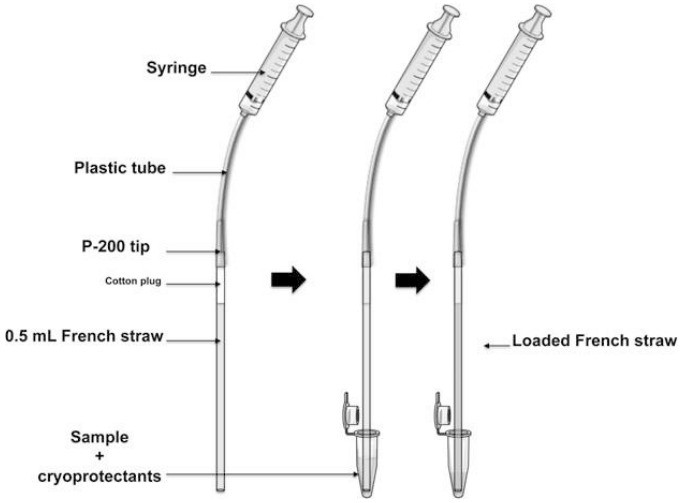

Fig. 3 Preparing a system for loading French straws. A syringe, a P-200 tip and a plastic tube create a helpful device for loading samples into French straws

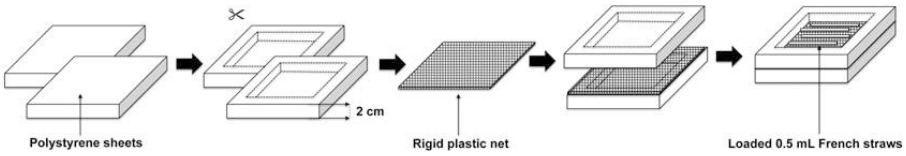

Fig. 4 Preparing a cooling device. Using polystyrene sheets and a rigid plastic net, a cooling device for French straw cryopreservation can be generated by hand in the laboratory. Adapt sheet width according to the protocol

10. All discarded solutions must be properly processed.
11. If samples are not processed immediately, DNA samples should be stored at −80 °C since, due to the high accuracy of the technique, −20 °C storage could induce detectable alterations in the DNA. For this reason, it is better to process both control and experimental samples instantly processed, or, if this is not possible, they should be kept for the same period of time at −80 °C.
12. It is possible to use the forward or reverse primer from the long amplicon as the forward or reverse primer for the short amplicon. In this way, you can reduce the number of primers you need to design, reducing both the time required and the cost.
13. A conventional PCR cycle usually consists of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C and 30 s at Tm, and

72 °C at the required amplification time (reference: 60 s for 1000 bp), with a final extension of 7 min at 72 °C.

14. In q-PCR experiments, pipetting precision is crucial. Always use calibrated micropipettes and the same set of materials for these experiments. Try to perform all procedures in a reproducible manner, avoiding variation among technical and biological replicates. Use one tip for each pipetting in order to avoid introducing changes in volumes.
15. For q-PCR assays, three technical replicates are needed. In order to avoid possible pipetting mistakes, prepare 1.5 times the volume of the reaction mixture required. q-PCR dyes must not be exposed to direct light (work in partial darkness).

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ANEXO III

Otra producción científica derivada del capítulo II

A.III. Prototipo

Sistema automático para la clasificación del lenguado senegalés (*Solea senegalensis*)

Vanesa Robles [a,b] , Javier Alfonso Cendón[c], David G. Valcarce [b,d]

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CONVENIO ESPECÍFICO DE COLABORACIÓN PARA EL DESARROLLO DE PROTOTIPOS ORIENTADOS AL MERCADO – PROTOTRANSFER, DENTRO DEL PROYECTO INESPO II

En León, a 1 de abril de 2014

REUNIDOS

De una parte, D. Humildad Rodríguez Otero, Directora de la Fundación General de la Universidad de León y de la Empresa, con domicilio en C/ Jardín de San Francisco s/n, 2ª planta, 24004- León, CIF: G-24356644, en adelante la FUNDACIÓN.

Y de otra parte, Dª Vanesa Robles Rodríguez con DNI 09804051W, D. Javier Alfonso Cendón con DNI 71434576A, D. David García Valcarce con DNI 71421079F, y domicilio en León, con vinculación con la Universidad de León, en adelante los PROMOTORES.

Actuando el primero en la representación indicada y el segundo en su propio nombre e interés, y reconociéndose capacidad legal para obligarse.

DECLARAN

PRIMERO: Que se reconocen recíprocamente capacidad y legitimación para la negociación y firma del presente documento.

SEGUNDO: Que el objeto del presente Convenio es formalizar la colaboración entre las partes firmantes para desarrollar un prototipo orientado hacia el mercado, que tenga la posibilidad de ser comercializado.

TERCERO: Que el contenido de esta colaboración será el desarrollo de actividades de transferencia de conocimiento mediante la materialización y desarrollo de un prototipo para conseguir un producto o proceso con posibilidades de ser comercializado en el mercado. Esta colaboración no tendrá naturaleza de carácter laboral, ya que se encuadra dentro del Proyecto INESPO II – RED DE TRANSFERENCIA DE CONOCIMIENTO UNIVERSIDAD-EMPRESA. REGIÓN CENTRO DE PORTUGAL – CASTILLA Y LEÓN. Este proyecto está cofinanciado con fondos FEDER, dentro de la 3ª convocatoria del Programa Operativo de Cooperación Transfronteriza 2007-2013 POCTEP y, en él participan los siguientes socios: Universidade da Beira Interior, Universidade de Aveiro, Universidade de Coimbra, Conselho Empresarial do Centro, Universidad Pontificia de Salamanca, Fundación General de la Universidad de León y de la

Empresa, Fundación General de la Universidad de Valladolid y Fundación General de la Universidad de Salamanca.

ACUERDAN

Suscribir el presente Convenio de Colaboración para desarrollo de prototipos orientados al mercado, con arreglo a las siguientes:

CLÁUSULAS

Primera: Localización.-

El proyecto de prototipo se desarrollará, si el promotor lo solicita, en un espacio físico que facilitará la Universidad de León, siempre que sea técnicamente posible y en función de su disponibilidad.

Segunda: Duración.-

El periodo de desarrollo del proyecto de prototipo estará comprendido entre el **1 de abril de 2014 y el 31 de enero de 2015**.

Tercera: Contenido del proyecto.-

El proyecto de prototipo que desarrollarán los promotores en el marco del presente convenio y que lleva por título ***"Sistema Automático para la Clasificación del Lenguado Senegalés (Solea Senegalensis)"*** deberá respetar los objetivos y fases planteados en la memoria inicial redactada por él y que se adjuntó en la candidatura de solicitud presentada al presente concurso.

Sin perjuicio de lo anterior, este proyecto de prototipo se adaptará lo máximo posible a los intereses de los promotores, de la Universidad de León y a los objetivos del propio proyecto INESPO II.

Ni este convenio ni las actuaciones que en él se contemplan suponen relación contractual alguna, de clase mercantil, civil o laboral, al no concurrir los requisitos que exigen las leyes. Las partes son totalmente libres de cualquier obligación mutua, pudiendo, en cualquier momento, dejar sin efecto la colaboración, con el acuerdo previo de las dos partes y por causa suficientemente motivada.

Cuarta: Obligaciones de la Fundación.-

- La Universidad de León siempre que sea técnicamente posible y en función de su disponibilidad, dotará a los promotores de un espacio físico para realizar el proyecto, si éste lo solicita.

- La Fundación se compromete a llevar a cabo el seguimiento del desarrollo del proyecto de prototipo, estando en continuo contacto con los promotores y con el tutor académico, en caso de que exista.

- Facilitar a los promotores el acceso a los contenidos digitales sobre protección industrial e intelectual alojados en www.innotransfer.eu.

- Programar en la Universidad de León el Curso sobre Competencias en la Creación de Empresas de Base Tecnológica – CEBT Ibérico, edición 2014, que incluirá:

    - Tutorización para la elaboración de un plan de negocio, al objeto de analizar la viabilidad de la posible creación de una empresa asociada al proyecto.

    - Realización de un plan de comercialización del prototipo resultante.

- Colaborar con los promotores en la preparación de una memoria de protección de resultados.

- La Fundación, conjuntamente con el resto de Universidades socias del proyecto INESPO II, organizarán presentaciones públicas de los resultados de los prototipos resultantes, previsiblemente en los primeros meses de 2015.

- Realizará el pago por un **importe bruto total de 1.500 €** (mil quinientos euros) al finalizar el proyecto del prototipo ***"Sistema Automático para la Clasificación del Lenguado Senegalés (Solea Senegalensis)"***. Este pago se realizará a nombre de la persona, miembro del equipo promotor y que haya sido designada por este, quedando condicionado al cumplimiento de sus obligaciones descritas en la cláusula quinta del presente documento.

  Las cantidades que se abonan al promotor en ningún caso tendrán carácter de salario o retribución, sino de ayuda económica.

Quinta: Obligaciones de los Promotores.-

- Se comprometen a aplicarse para llevar a cabo con la máxima diligencia el plan de trabajo fijado junto a la Fundación y al tutor académico, en caso de que exista, para el desarrollo del

proyecto de prototipo ***"Sistema Automático para la Clasificación del Lenguado Senegalés (Solea Senegalensis)"*** manteniendo contacto permanente con la Fundación en la forma que se le indique.

- A la finalización del proyecto, siempre antes del día 31 de enero de 2015, los promotores deberán presentar ante la Fundación, los siguientes materiales:

  - Memoria final que contenga (extensión máxima 30 páginas), al menos, los siguientes apartados:
    - Introducción.
    - Objetivos.
    - Motivación.
    - Especificaciones técnicas.
    - Análisis básico sobre las posibilidades de explotación del prototipo (mercado, creación de una empresa,...).
    - Manual de usuario.

  - CD-Rom, con los siguientes archivos:
    - Power Point de presentación del prototipo.
    - Video demostrativo.
    - Código de aplicación (*).
    - Ejecutable (*).

  (*): Archivos requeridos únicamente en proyectos relacionados con TIC's

  - Plan de negocio.
  - Plan de comercialización.
  - Memoria de protección de resultados.

  La entrega de la documentación final requerida condicionará el pago final de la ayuda económica concedida.

- Si la Fundación lo estima oportuno, los promotores deberán realizar una presentación de su proyecto ante la comisión transfronteriza de selección, previa a la liquidación de la bolsa de ayuda económica.

- Asistir a las sesiones formativas del Curso sobre Competencias en la Creación de Empresas de Base Tecnológica – CEBT Ibérico, edición 2014, que la Fundación programará en la Universidad de León, así como a colaborar activamente en el correspondiente plan de negocio y plan de comercialización del prototipo resultante, con el apoyo tutelar que se le facilitará al efecto. En el caso de que exista una causa justificada para la no asistencia a alguna de las sesiones, los promotores deberán presentar el correspondiente justificante ante la Fundación.

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- Presentar los resultados del prototipo en las jornadas públicas que organizarán conjuntamente la Fundación con el resto de Universidades socias del proyecto INESPO II, previsiblemente en los primeros meses de 2015.

- Los promotores se comprometen a guardar absoluta confidencialidad sobre los datos, resultados y cualquier información del proyecto desarrollado en el marco de este convenio, quedando prohibida su difusión sin previa autorización de la Universidad de León y/o su Fundación General.

- Los promotores aceptan expresamente la cláusula sexta del presente convenio.

Sexta: Producto final.-

Si el producto final (o productos intermedios) son susceptibles de ser protegido mediante cualquier modalidad de propiedad intelectual o industrial, la Universidad de León se reserva el derecho de hacerlo a su nombre, respetando el derecho de autoría de los inventores y el derecho a la percepción de los beneficios de explotación que les correspondan en virtud de la normativa interna aplicable al caso.

Séptima

Las partes colaborarán en todo momento de acuerdo a los principios de buena fe y eficacia, para asegurar la correcta ejecución de lo convenido.

Y en prueba de conformidad de cuanto antecede, firman las partes este documento, por duplicado y a un sólo efecto en la fecha al principio indicada.

La Fundación

Fdo.: Dª Humildad Rodríguez Otero
Directora

Los Promotores

Fdo.: Dª Vanesa Robles Rodríguez

INESPO II

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Los Promotores:

Fdo: D. Javier Alfonso Cendón

Los Promotores:

Fdo: D. David García Valcarce

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LA FUNDACIÓN GENERAL DE LA UNIVERSIDAD DE LEÓN Y DE LA EMPRESA (FGULEM)

CERTIFICA QUE,

D. Javier Alfonso Cendón
D. David García Valcarce
Dª Vanesa Robles Rodríguez

Han sido ganadores del **"I Concurso Transfronterizo de Prototipos Orientados al Mercado"** por su participación con el proyecto **"Sistema automático para la clasificación del Lenguado Senegalés (Solea Senegalensis)"**

Y para que así conste, lo firma en León, el 6 de marzo de 2015

fgulem
Fundación General de la Universidad de León y de la Empresa

Fdo. Humildad Rodríguez Otero

Esta actividad se incluye dentro del proyecto INESPO II (Innovation Network Spain- Portugal), que se encuentra enmarcado en el Programa Operativo de Cooperación Transfronteriza España- Portugal (POCTEP 2007-2013)

COOPERACIÓN TRANSFRONTERIZA ESPAÑA - PORTUGAL COOPERAÇÃO TRANSFRONTEIRIÇA

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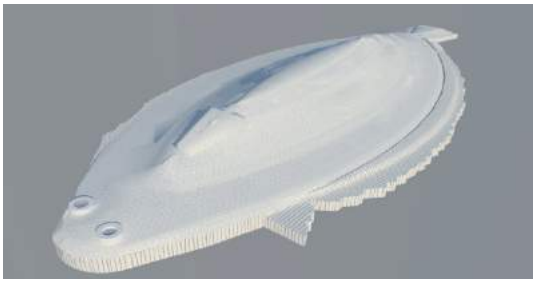
ESPECIFICACIONES TÉCNICAS II

Etapa 1. Muestreo de lenguados (*Solea Senegalensis*). Se realizó un muestreo de lenguados (*Solea Senegalensis*) en el Instituto Español de Oceanografía (IEO) ubicado en Santander, con el objetivo de definir los parámetros más característicos de los mismos, así como realizar un exhaustivo muestreo de tallas y pesos con el objetivo de obtener patrones para su posterior integración en el prototipo software.

ESPECIFICACIONES TÉCNICAS III

Etapa 2. Construcción de un modelo de lenguado (*Solea Senegalensis*) en 3D. Con los datos obtenidos en el muestreo realizado en la Etapa 1 y con el objetivo de reducir costes a la hora de realizar las pruebas de laboratorio, se decidió realizar un modelo que permitiera imprimir lenguados en una impresora de 3D, no siendo necesario por lo tanto adquirir y mantener lenguados en las etapas de desarrollo del prototipo. En la Figura 2 se puede observar el modelo 3D y en la Figura 3 se puede ver un lenguado impreso en 3D.

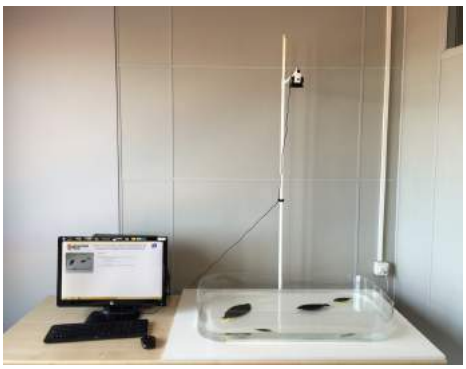


ESPECIFICACIONES TÉCNICAS IV

Etapa 3. Desarrollo del prototipo software. El prototipo software fue desarrollado utilizando MatLab (abreviatura de *MATrix LABoratory*, "laboratorio de matrices") es una herramienta de software matemático que ofrece un entorno de desarrollo integrado (IDE) con un lenguaje de programación propio (lenguaje M).

ESPECIFICACIONES TÉCNICAS V

Etapa 4. Construcción de una maqueta real a escala. Para poder realizar pruebas en el laboratorio se desarrolló una maqueta real a escala de un tanque de engorde de lenguados (*Solea Senegalensis*) y una estructura para soportar la cámara que sirve de entrada de información al prototipo software. En la Figura 4 se muestra una imagen de la maqueta construida.



ANEXO IV

Otra producción científica derivada del capítulo IV

A.IV.1 Patente nacional

(19) OFICINA ESPAÑOLA DE PATENTES Y MARCAS

ESPAÑA

(11) Número de publicación: **2 575 828**

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A61K 35/747 (2015.01)
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(12) SOLICITUD DE PATENTE A1

(22) Fecha de presentación:

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(43) Fecha de publicación de la solicitud:

01.07.2016

(71) Solicitantes:

UNIVERSIDAD DE LEÓN (50.0%)
Avda. de la Facultad, 25
24071 León ES y
BIOPOLIS, S.L. (50.0%)

(72) Inventor/es:

ROBLES RODRÍGUEZ, Vanesa ;
GARCÍA VALCARCE, David;
RAMÓN VIDAL, Daniel;
GENOVÉS MARTÍNEZ, Salvador;
MARTORELL GUEROLA, Patricia y
CHENOLL CUADROS, Mª Empar

(74) Agente/Representante:

PONS ARIÑO, Ángel

(54) Título: **Empleo de probióticos en el incremento de la fertilidad masculina**

(57) Resumen:
Empleo de probióticos en el incremento de la fertilidad masculina.
La presente invención se refiere al uso de Lactobacillus rhamnosus en combinación con Bifidobacterium longum en la elaboración de una composición para incrementar la fertilidad masculina en un sujeto. En particular, dichas cepas son las cepas de Lactobacillus rhamnosus CECT 8361 y Bifidobacterium longum CECT 7347. Asimismo, también se describe la composición que comprende dichas cepas Lactobacillus rhamnosus CECT 8361 y Bifidobacterium longum CECT 7347.

ES 2 575 828 A1

A.IV.2 Patente internacional (Solicitud PCT)

(12) SOLICITUD INTERNACIONAL PUBLICADA EN VIRTUD DEL TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES (PCT)

(19) Organización Mundial de la Propiedad Intelectual
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7 de julio de 2016 (07.07.2016)

WIPO | PCT

(10) Número de Publicación Internacional
WO 2016/107948 A1

(51) **Clasificación Internacional de Patentes:**
C12N 1/20 (2006.01) *A23L 29/00* (2016.01)
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(21) **Número de la solicitud internacional:**
PCT/ES2015/070948

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(30) **Datos relativos a la prioridad:**
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31 de diciembre de 2014 (31.12.2014) ES

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(74) **Mandatario: PONS ARIÑO, Ángel**; Glorieta Rubén Darío, 4. 28010 Madrid (ES).

(81) **Estados designados** *(a menos que se indique otra cosa, para toda clase de protección nacional admisible):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Estados designados** *(a menos que se indique otra cosa, para toda clase de protección regional admisible):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), eurasiática (AM, AZ, BY, KG, KZ, RU, TJ, TM), europea (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Publicada:

— *con informe de búsqueda internacional (Art. 21(3));*

— *con la parte de lista de secuencias de la descripción (Regla 5.2(a));*

WO 2016/107948 A1

(54) **Title:** USE OF PROBIOTICS TO INCREASE MALE FERTILITY

(54) **Título :** EMPLEO DE PROBIÓTICOS EN EL INCREMENTO DE LA FERTILIDAD MASCULINA

(57) **Abstract:** The present invention relates to the use of *Lactobacillus rhamnosus* in combination with *Bifidobacterium longum* to manufacture a formulation to increase male fertility in a subject. In particular, said strains are the strains *Lactobacillus rhamnosus* **CECT** 8361 and *Bifidobacterium longum* **CECT** 7347. Additionally, the formulation comprising the said strains *Lactobacillus rhamnosus* CECT 8361 and *Bifidobacterium longum* CECT 7347 is also described.

(57) **Resumen:** La presente invención se refiere al uso de *Lactobacillus rhamnosus* en combinación con *Bifidobacterium longum* en la elaboración de una composición para incrementar la fertilidad masculina en un sujeto. En particular, dichas cepas son las cepas de *Lactobacillus rhamnosus* CECT 8361 y *Bifidobacterium longum* CECT 7347. Asimismo, también se describe la composición que comprende dichas cepas *Lactobacillus rhamnosus* CECT 8361 y *Bifidobacterium longum* CECT 7347.

ANEXO V

Revisiones científicas

A.V.1. Revisión científica

Factors enhancing fish sperm quality and emerging tools for sperm analysis

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Factors enhancing fish sperm quality and emerging tools for sperm analysis

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ABSTRACT

With this review we try to give a comprehensive overview of the current methods used in research to assess sperm quality. In addition, we identify some of the most important factors for enhancing sperm production and quality, including, broodstock nutrition, epigenetics and sperm management (cryopreservation). Sperm quality can be assessed by analyzing different parameters from simple methods to very sophisticated approaches involving molecular tools. Parameters related with sperm composition or function (e.g. spermatozoa plasma membrane lipids, seminal plasma composition, motility activation) have successfully characterized a sperm sample but could not respond to the causes behind sperm defects. Reactive oxygen species (ROS) are one of the causes of the impairment of sperm traits. High contents of ROS are capable of producing cell apoptosis, DNA strand breakages, mitochondria function impairment, and changes in membrane composition due to sugars, lipids, and amino acid oxidation, affecting at later times sperm fertilization ability. Recently, the importance of spermatozoa RNAs in the fertilization and early embryo development has been clearly demonstrated in different species, including fish. Spermatozoa delivers more than the paternal genome into the oocyte, carrying also remnant mRNA from spermatogenesis. These RNAs have been found in sperm from human, rodent, bovine, and recently in several fish species, demonstrating the important predictive value of spermatozoa transcripts present only in those samples with high motility or from males with higher reproductive performance. The content of those transcripts can be changed during gametogenesis process influencing their content in spermatozoa.
We will focus this review on sperm quality markers, in new trends on sperm analysis, and in the use of these tools for the identification of factors enhancing gamete quality. Basic research in this field is helping to develop appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications.

1. Introduction

Control of sperm quality is a major issue for the aquaculture industry, both for the production of well-established commercial species and for the introduction of new ones with high commercial interest. The identification of predictive estimators or markers of sperm quality would also have major applications in other research fields and in the development of biotechnological companies. However it has been extremely difficult to accurately estimate the quality of sperm and to correlate these quality estimators with the capacity of these cells, not only to reach the oocyte and fertilize the egg, but as it will be demonstrated, to contribute to a successful early embryonic development. This quality evaluation may be relevant in order to better understand the mechanisms by which sperm is affected and to control some of the factors influencing overall gamete quality. Most of these factors are related with male breeder performance, life history, social context, or to husbandry conditions such as broodstock nutrition, environmental manipulation and spawning induction protocols or procedures for sperm handling and management.

This review gives an overview of the new trends on sperm analysis and emerging tools for sperm quality determination, with a focus on the most relevant factors affecting sperm production and quality, including, epigenetics, broodstock nutrition, and sperm management (cryopreservation). The range of optimal indicators should be defined according to the different species, sperm fate or reproductive strategy such as artificial fertilization, cryopreservation, gene banking, or mass

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0044-8486/

production. Basic research in this field is helping develop appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications for early prediction of gamete quality.

2. Factors enhancing sperm quality

There are several extensive reviews on sperm quality highlighting the potential factors that may contribute to an improvement of sperm quality or that produce detrimental effects. Most reviews deal with aspects related with broodstock improvement (Cabrita et al., 2011b) while others directly identify factors capable of affecting sperm quality traits in several fish species (Bobe and Labbé, 2010; Cabrita et al., 2009; Fauvel et al., 2010; Rurangwa et al., 2004) or relate broodstock management procedures with gamete features (Migaud et al., 2013). In this sense, this review will particularly be focused on the principal aspects for enhancing sperm quality trying to give updated information on the latest highlights in research.

2.1. The role of breeders' nutrition, epigenetics and cryopreservation on sperm quality enhancement

Although it may seem strange these three subjects are related when we speak about factors enhancing sperm quality. Nutrition enhancement has proved to ameliorate the quality of sperm by improving several spermatozoa traits. In fact not only seminal or plasma membrane composition may be positively modulated through the incorporation of certain compounds in the feeds, although most of the reports deal with an increase of DHA and ARA in plasma membrane of fish feed on polyunsaturated fatty acids (PUFA) enriched diets in species such as European seabass, *Dicentrarchus labrax* (Asturiano et al., 2001), and carp, *Catla catla* (Nandi et al., 2007). Plasma membrane fluidity is determined by the phospholipids (PL) and cholesterol (CHO) composition, and CHO regulates the lipid chain order and molecular organization of the membranes (Muller et al., 2008; Wassall and Stillwell, 2009). Therefore their modulation has a direct impact on sperm physiology and functionality. Several authors have investigated the PL, CHO and fatty acids contents in sperm and their interaction to understand how its composition affects sperm quality and influence its ability to successfully fertilize the eggs (Beirão et al., 2012; Henrotte et al., 2010; Lahnsteiner et al., 2009; Muller et al., 2008). These compounds are also inducers of sex steroid and eicosanoid production, particularly prostaglandins, and thereby their involvement in gonad development may also potentiate sperm quality (Alavi et al., 2009; Izquierdo et al., 2001). In Senegalese sole (*Solea senegalensis*), the supplementation of PUFA on diet increased the percentage of progressive spermatozoa and sperm velocity as a consequence of DHA, ARA and EPA increase in sperm membranes, particularly if this supplementation was complemented by an addition of antioxidants (Beirão, 2011). However, modulation of fish nutritional requirements may affect other parts of the fish spermatozoa. Although there are no reports on this subject in fish species, several recent reviews, exploring the promising field of nutrigenomics, discussed on the important role that epigenetic mechanisms play at the nexus between nutrition and the genome (Farhud et al., 2010). Nutrient–gene interactions enable various nutrients to transiently influence the expression of specific subsets of genes. It is becoming increasingly evident that by interacting with epigenetic mechanisms, that regulate chromatin conformation, transient nutritional stimuli at critical ontogenic stages can yield lasting influences on the expression of genes or in the methylation process of certain genes during gametogenesis. If such epigenetic changes occur in the gametes, and they are transmittable to the next generation, the quality of sperm not only in our breeders but also on the breeders of future generations in captivity can be affected. Therefore, it seems clear the positive concomitant effects of nutrition and epigenetics on sperm quality enhancement.

Breeder's nutrition can play an important role as an enhancement factor of sperm quality. There are several reports on the incorporation of antioxidant substances into feed proving to have a favorable effect on sperm quality, especially when it needs to be reinforced to sustain manipulations such as cryopreservation. It has been widely assumed that cryopreserved sperm quality is influenced by the quality of initial fresh samples (Cabrita et al., 2009), and therefore once more the cascade events of enhanced nutritional factors may also interfere in this process. It is assumed that high quality samples enhanced by nutritional factors are more prone to resist to cryodamage and therefore will better sustain the cryopreservation process.

The incorporation of probiotics in fish feeds has been one of the new areas of research for the improvement of gamete quality. There are some studies that demonstrate the importance of *Lactobacillus rhamnosus* in improving fish reproductive performance. Most of the studies have been performed in model species like the zebrafish (*Danio rerio*) where it was shown that oral administration of probiotics (10^6 CFU) stimulated reproduction by increasing *gnrh3* expression (Gioacchini et al., 2010), anticipated sex differentiation, and influenced sex ratio, probably through modulation of *sox9*, an autosomic gene also involved in chromosomal control of testis differentiation (Avella et al., 2012; Carnevali et al., 2013). Regarding the improvement of sperm quality, another study in zebrafish demonstrated the relevance of *L. rhamnosus* in target genes such as *fhsb* and *lepa* expressed in testicular cells (Riesco et al., 2013) that were considered in other species as markers of sperm quality and fertility.

Recent studies have demonstrated the effect of probiotics in European eel spermatogenesis using different concentrations of *L. rhamnosus* IMC 501® (10^3, 10^5, 10^6 CFU/ml) (Sinbyotec, Italy) added daily to the rearing water from the 6th week of hCG treatment for inducing maturation (Santangeli et al., 2013). These authors recorded significant higher sperm volume, and an improvement in the percentage of motile cells and straight swimming velocity compared to controls and to the lowest dose. These changes were also associated with the increasing levels of *Activin*, *arα*, *arβ*, *pr1* and *fshr* expression during the first 2 weeks of treatment, proving some evidences of the involvement of these molecular markers in physiological spermatozoa processes.

Although with some controversies, the replacement of fish meal by plant proteins in some cases seems to improve the quality of sperm. In a recent study, Nyina-Wamwiza et al. (2012) demonstrated that total replacement of fish meal by agriculture plants meal produced an increase in sperm volume, spermatocrit, spermatozoa integrity and motility parameters of sperm in African catfish (*Clarias gariepinus*). Although the positive effects could be associated with a different source of plant protein, some effects could be also associated with the modified proportion of fatty acids in each tested meal, since an increase in the quantity of PUFAs due to the replacement of fish meal by a high amount of plant ingredients (including oils) had a positive impact on sperm motility. Previously, we have debated that PUFAs are also implicated in physiological functions linked to sperm activity.

Sperm cryopreservation is a safe method to store and preserve the male genetic material. Its use should benefit the fish farming industry at different levels, from management of reproduction and genetic selection of males with high reproductive value to the use of high quality selected samples. Protocols for fish sperm cryopreservation have been successfully developed for several teleost species (Cabrita et al., 2009). Despite the identification of some cell damage, there are several ways to enhance the quality of samples. One approach was previously mentioned by nutritional incorporation of certain compounds that will benefit sperm traits. Another approach is by the incorporation of certain substances in the extender media of samples. This effect increases post-thaw sperm survival rates. Martínez-Páramo et al. (2012a,b) found higher motility rates and a decrease in DNA fragmentation in European seabass sperm cryopreserved with the addition of antioxidants such as vitamins (α-tocoferol and ascorbic acid) or sulfur-containing amino acids like taurine and hypotaurine. These amino

acids were also shown to promote a decrease in DNA fragmentation in gilthead seabream, *Sparus aurata* cryopreserved sperm (Cabrita et al., 2011a).

Cryopreservation may also ensure sperm quality by the selection of disease-free material being a safety method for seed supply.

3. Emerging tools for sperm analysis

Although most of the time the requests for sperm quality analysis are coming from the industry, the new demands on research nowadays imply new techniques for sperm analysis, that probably, in a not so far future, will be used by fish farming companies. In this sense there are several factors that in our opinion may have contributed to this new generation of tools that in various ways will improve the knowledge on sperm quality assessment. Some of these advancements can be related with the development of hundreds of protocols for the cryopreservation of sperm from different species or to the use of sperm in toxicological assays.

In the last decade the number of publications in sperm cryopreservation doubled, corresponding to the introduction of new cultivated species, and therefore to an increased need to apply this tool in new reproductive management strategies. There has been also a huge concern in the conservation of threatened species due to an increase in their number, being cryopreservation an excellent way to store this valuable material. Although cryopreservation can be a useful tool it can also induce certain types of species-specific damage, as mentioned before. Consequently, an exhaustive quality analysis of samples is important to guarantee the benefits of the cryopreservation technique. In this regard, research has associated oxidative stress to certain types of damage such as peroxidation of lipids, induction of oxidative DNA damage, formation of protein adducts, ROS production involving electron leakage from the sperm mitochondria, and electron transport chain impairment. The net result of mitochondrial ROS generation is the damage of these organelles and the initiation of an intrinsic apoptotic cascade, and as consequence spermatozoa lose motility, DNA integrity and vitality (Aitken et al., 2012). The mammalian background research on oxidative stress performed since the early 40s and the awareness of the importance of oxidative stress in the etiology of male infertility (last 25 years, Alvarez et al., 1987; Aitken and Clarkson, 1987) helped to understand these concepts and their importance in sperm quality. As in mammalian research, the analysis of fish sperm quality does not come close to evaluating the full range of properties that spermatozoa need to express in order to search for predictable markers of sperm quality. The latter will be only possible when we have a complete understanding of the molecular mechanisms regulating sperm function and appreciate how this process can be influencing sperm functionality. The discovery of new-born mRNA in mammalian species, and more recently in fish, may open a hole in this window, conducting to new advancements and to new trends in sperm quality research (see more details in next section).

Another point that raised the interest of sperm analysis has been the application of sperm as a biomarker in toxicity studies. Current strategies in monitoring programs for marine–coastal areas usually require the integration of chemical analyses and biological testing in order to better evaluate the bioavailable fraction of toxicant interacting with living organisms (Coulaud et al., 2011; Macova et al., 2010). Therefore test batteries need to include organisms representing different phyla and different trophic levels (Macken et al., 2009). In recent years ecotoxicological tests have been standardized for gametes and embryos from a range of aquatic species. These tests have gained more relevance due to EU regulations on the use of animals in experimentation, because in some species this material can be easily collected without sacrificing the animal, can be stored until the tests are carried out and sperm from commercial species can be used for this purpose (Fabbrocini et al., 2012). Therefore, sperm quality tests proposed as a bioassay are a promising starting point for the development of toxicity tests that are increasingly tailored to the needs of ecotoxicology and environmental quality evaluation strategies (Fabbrocini et al., 2012).

3.1. Oxidative stress markers

In living organisms, the production of reactive oxygen species (ROS) occurs as consequence of the normal metabolisms of the cells. Thus, free radicals generation can be from the mitochondrial respiratory chain or from intracellular enzyme systems as xanthine- and NADH-oxidases (Baker and Aitken, 2004). Within the most common ROS known are the hydroxyl radical ($-OH$), the hydrogen peroxide (H_2O_2), the superoxide radical ($O_2^{\cdot-}$) and some derivates such as the hydroperoxyl radical ($OOH\cdot$). These reactive species play an important role in many cellular pathways regulated by redox events. However, ROS are highly reactive molecules that can interact with proteins, lipids, DNA and RNA, promoting cell injury at several levels. Because of this injuring potential, the level of ROS is strictly controlled by cell-specific antioxidant systems (electron-scavengers and redox-sensitive enzymes) to avoid an "oxidative stress situation" as a result of an imbalance in the production and detoxification of ROS. In aquatic species, environmental factors such as changes in temperature, oxygen levels and salinity, besides exposure to transition metals (i.e. iron, copper, chromium, mercury) or pesticides (i.e. insecticides, herbicides, fungicides and oil and related pollutants) can induce oxidative stress (reviewed by Lushchak, 2011). The exposure to all of these factors in their natural environment makes aquatic organisms good models for the study of oxidative stress damage.

The fish spermatozoa characteristics make this type of cells very prone to suffer oxidative stress-related damage, mainly due to the high content of PUFA that makes the membrane a good target for ROS, but DNA (inducing single- and double-strand DNA breaks) and proteins could be also affected. Therefore in recent years, several oxidative markers have been developed in fish sperm by analyzing different structures in the spermatozoa (Fig. 2).

In fish sperm, seminal plasma provides the major defense against ROS, due to the low content of cytoplasm in spermatozoa (Shiva et al., 2011). Several authors have analyzed the antioxidants and oxidant defensive enzymes present in spermatozoa and seminal plasma of different fish species. Ascorbic acid and uric acid are considered important antioxidants in teleost fish, being the metabolites occurring in highest concentration in sperm of several species such as burbot (*Lota lota*), perch (*Perca fluviatilis*), bleak (*Alburnus alburnus*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) (Ciereszko and Dabrowski, 1995; Lahnsteiner and Mansour, 2010; Lahnsteiner et al., 2010). Besides metabolites scavengers of ROS, there are several enzymatic systems that can be used as markers of oxidative stress. The main enzymes responsible for the detoxification of reactive oxygen species in fish sperm are: catalase, superoxide dismutase (SOD), glutathione reductase (GSR) and peroxidase (GPX) (Lahnsteiner and Mansour, 2010; Mansour et al., 2006).

Sperm can be exposed to oxidative stress from spermatogenesis, because of the high rates of mitochondrial oxygen consumption inherent to the extremely active replicative process (Aitken and Roman, 2008). Although during gametogenesis ROS can play an important role in oxidative damage, compared to other germ cells, such as spermatogonia type A, B or even spermatocytes, the resistance of spermatozoa to this attack is lower, being more easily attacked by oxygen species than other germ cells (Fig. 1). This fact is associated with the high protection given by scavengers to spermatogonia and to the fact that the cytoplasm where these enzymes act is reduced in spermatozoa (Miura and Miura, 2011).

Mature spermatozoa can suffer the attack of free radicals when is preserved to be used in artificial fertilization practices (Shaliutina et al., 2013a). For instance, during cryopreservation, the antioxidant barrier provided by seminal plasma is extremely weakened, mainly due to sperm dilution in the extender that reduces the concentration of seminal plasma compounds (Cabrita et al., 2011a).

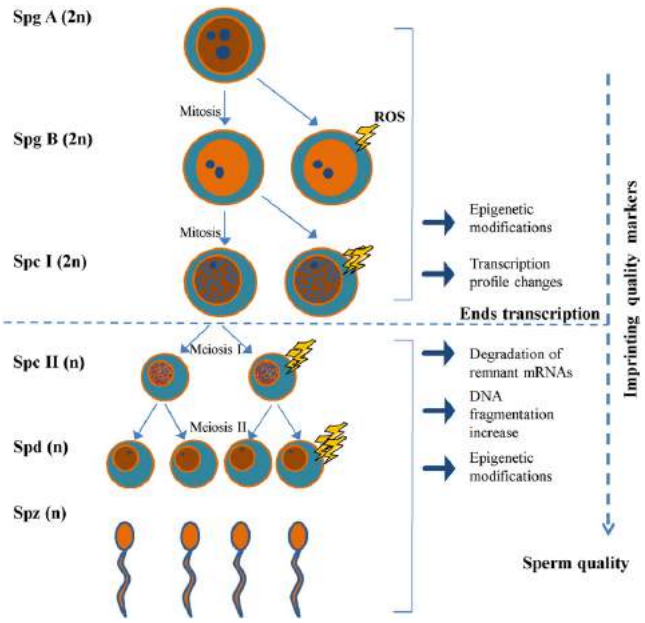

Fig. 1. Steps during fish spermatogenesis targeting points of damage that could be transmitted to spermatozoa, imprinting a quality marker in each cell.

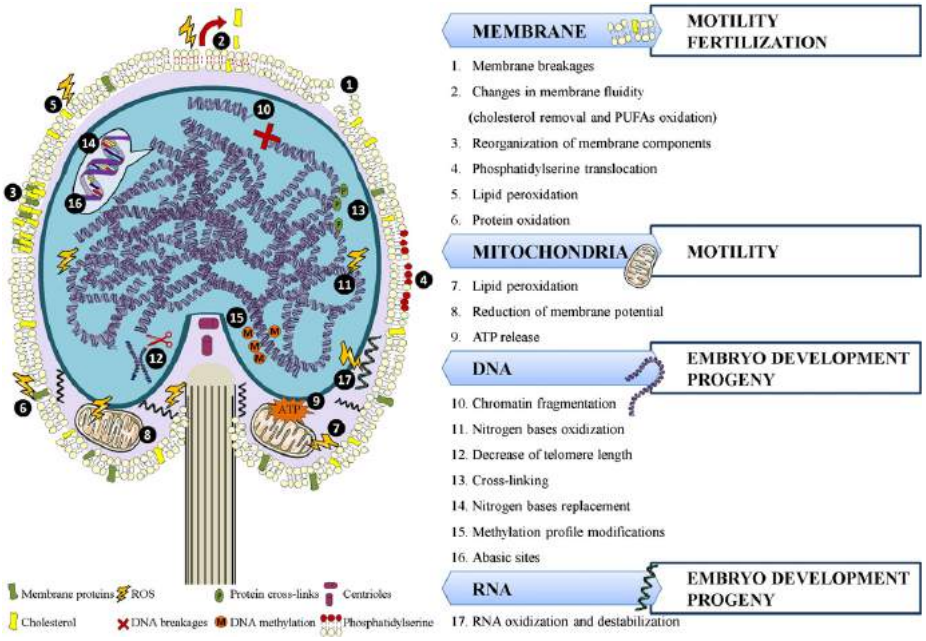

Fig. 2. Fish spermatozoa illustration showing the principal types of damage occurring in plasma membrane, mitochondria, DNA and RNA that could be used as sperm quality markers. These markers can identify changes in sperm motility, fertilization ability, embryonic development and progeny fitness.

In European seabass post-thawed sperm, it was observed that there is a significant increase in the activity of the enzymatic antioxidant system, probably to counteract the destabilization of the antioxidant barrier after dilution in the extender (Martínez-Páramo et al., 2012b, 2013a). This reaction seemed to be enough to protect lipids and proteins from oxidative damage, though DNA remained exposed to ROS attack showing levels of chromatin fragmentation around 55%, which were reduced by addition of antioxidants into the freezing media (Martínez-Páramo et al., 2013a). DNA damage induced by oxidative stress, is not limited to direct effect on chromatin fragmentation, but may alter gene expression or induce epigenetic deregulation by way of posttranslational histone modifications after hypo- or hypermethylation (Chervona and Costa, 2012; Ziech et al., 2011). In recent studies, genes related with apoptosis (pro-apoptotic-*bax and bad*; anti-apoptotic-*bcl-2*, *bcl-xl*) induced by oxidative stress revealed to be good biomarkers not only in fish germ cells exposed to xenobiotics or contaminants, but also in the assay of sperm cryopreservation (Jeong et al., 2009; Nadzialek et al., 2010). These markers revealed to be good indicators of the effect of antioxidants (Zn, Se, Vit. E and C) supplementation in fish diets on germ cell quality (Cabrita et al., 2013).

Several techniques have been used to quantify oxidative damage in fish sperm. However, the evaluation of oxidative stress markers is a key question in the investigation of oxidative stress because of the impossibility to monitor the level of ROS *in vivo* in the biological processes (Lushchak, 2011). Therefore, the most common approach to evaluate oxidative stress, is quantifying the products of cellular constituents modified by ROS. We summarize some of the markers and techniques used for the evaluation of oxidative stress in fish (Table 1).

3.1.1. Quantification of ROS

The use of fluorescent probes is a very useful technique to quantify the level of ROS in the cell. There are several fluorophores that after being incorporated into the cell can be modified by oxidative reaction, emitting fluorescence. The intensity of the fluorescence emitted is supposed to be proportional to the levels of ROS, and can be quantified by several methods such as fluorescence microscopy, flow cytometry or spectrophotometry. The dihydroethidium (DHE) is a reagent used to quantify superoxide anion (Hagedorn et al., 2012). This molecule exhibits blue-fluorescence in the cytoplasm until oxidized, where it intercalates within the DNA, labeling the nucleus with red fluorescence. Similarly, the acetate ether of dichlorofluorescein penetrates inside the cell where it is cleaved by esterases to dichlorofluorescein (DCF) that is easily oxidized to the green fluorescent form (Pérez-Cerezales et al., 2010a,b). These probes were used in zebrafish (Hagedorn et al., 2012) and rainbow trout (Pérez-Cerezales et al., 2010a,b) to compare the levels of ROS in fresh and cryopreserved sperm, showing a significant increment of these reactive species in post-thaw samples.

3.1.2. Total antioxidant status

The analyses of the antioxidant status in the sperm samples must include the analyses of low and high molecular mass antioxidants. Among the low molecular mass antioxidants are metabolites such as tocopherol, ascorbic acid, uric acid, reduced glutathione, selenium, and zinc, among others. The analyses of the high molecular mass antioxidants include the enzymatic activity of GPX, GSR, SOD and catalase. There are commercial kits that provide an overall indication of antioxidant status, including metabolites and antioxidant enzymes but they can also be measured by routine enzymatic assays (Lahnsteiner and Mansour, 2010; Li et al., 2010a,b; Mansour et al., 2006; Shaliutina-Kolesova et al., 2013). This methodology has been used to characterize the level of total antioxidants in seminal and blood plasma of European seabass and gilthead seabream (Martínez-Páramo et al., 2013b) and in Atlantic cod (*Gadus morhua*), where it was demonstrated that the antioxidant capacity was positively related to motility and velocity of frozen-thawed sperm (Butts et al., 2011). Markers of the sperm antioxidant system were also determined in burbot, perch, bleak, brown trout (Lahnsteiner and Mansour, 2010), common carp (*Cyprinus carpio*) (Li et al., 2010a,b), Arctic char (*Salvelinus alpinus*) (Mansour et al., 2006), brook trout (*Salvelinus fontinalis*), Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon

Table 1
Techniques used for the analysis of sperm quality in fish species.

Assay	Structure	Reference
Cell viability		
Probes: Hoechst, PI/SYBR-14, acridine orange, DAPI, eosine, trypan blue, YO-PROl	Plasma membrane	Cabrita et al. (2009)
Lytic enzymes: acid phosphatase, alkaline phosphatase (LD-glucuronidase	Sperm	Lahnsteiner et al. (1998)
Metabolic enzymes: malate dehydrogenase, lactate dehydrogenase, aspartate aminotransferase, adenosine triphosphatase	Seminal plasma	Lahnsteiner et al. (1998)
HOS-test		
Hyper or hyperosmotic test	Plasma membrane	Cabrita et al. (2009)
Spermatozoa motility		
CASA, analysis of images captured with stroboscopic light, subjective scoring	Sperm	Rurangwa et al. (2004); Cosson et al. (2008)
ATP metabolism		
ATP and ADP levels, creatine phosphate, ATPase, adenylate kinase	Sperm	Lahnsteiner et al. (1998)
Mitochondria functionality		
Probes: JC1, Rhodamine 123; Mitotracker	Mitochondria	Cabrita et al. (2005a,b); Liu et al. (2007)
MTT assay	Mitochondria	Aziz (2006)
Spermatozoa morphology		
Electron microscopy, ASMA	Spermatozoa	Marco-Jiménez et al. (2006)
Oxidative stress		
Anti-oxidative enzymes: catalase, glutathione peroxidase, superoxide dismutase	Seminal plasma	Martínez-Páramo et al. (2012a,b)
Anti-oxidative potential	Seminal plasma	Martínez-Páramo et al. (2013a,b)
Lipid peroxidation		
Free radicals: dihydroethidium-DHE assay, dichlorofluorescein-DCF assay)	Seminal plasma	Pérez-Cerezales et al. (2010a,b); Hagedorn et al. (2012)
MDA determination (TBARS assay), 8-isoprostane level	Spermatozoa	Martínez-Páramo et al. (2012a,b); Khosrowbeygi and Zarghami (2008)
Fluorescent dyes: Bodipy-Cll	Spermatozoa	Hagedorn et al. (2012)
Protein oxidation		
Carbonyl group (DNPH-2,4-dinitrophenilhidrazine reaction)	Spermatozoa	Martínez-Páramo et al. (2012a,b)
DNA fragmentation and oxidation		
Comet assay (with or without FISH or endonucleases)	Chromatine	Cabrita et al.(2009); Pérez-Cerezales et al. (2009)
TUNEL assay	Chromatine	Cabrita et al. (2011a,b)
SCSA	Chromatine	Evenson et al. (1999)
OxiDNA assay (8-oxoguanine assay)	Chromatine	Cambi et al. (2013)
Fertility assay	Sperm	Cabrita et al. (2009)

(*Acipenser baerii*) and sterlet (*Acipenser ruthenus*) (Shaliutina-Kolesova et al., 2013).

Besides these techniques, new techniques characterizing the antioxidant system are being developed in mammals. In humans, it has been demonstrated that *gpx1* and *gpx4* are differentially expressed in different donors, suggesting a role of these enzymes in male infertility (Garrido et al., 2004). Moreover, low expression of the *gpx* family in sperm has been related with asymmetric divisions in embryos (Meseguer et al., 2006). Paralogs of mammalian *gpx1* and *gpx4* were found to be present in teleosts such as zebrafish (Thisse et al., 2003), common carp (Hermesz and Ferencz, 2009), Southern bluefin tuna (*Thunnus maccoyii*) (Thompson et al., 2010), goldfish (*Carassius auratus*) (Choi et al., 2007), amberjack (*Seriola lalandi*) (Bain and Schuller, 2012) and olive flounder (*Paralichthys olivaceus*) (Choi et al., 2008). These markers of oxidative stress were used in brown trout (Hansen et al., 2006) and Atlantic salmon (Olsvik et al., 2005) exposed to different environmental conditions or to pollutants in the water. In fish, studies related to the expression of antioxidant enzyme genes have been focused in the detection of these enzymes in different tissues (i.e. gill, intestine and liver) and not specifically in sperm. However, considering the previous results obtained in mammals, the correlation of the mRNA expression of the antioxidant enzymes in sperm could be promising to understand the molecular causes of fertilization failure related to oxidative stress damage.

3.1.3. Lipid peroxidation

Lipid peroxidation is a marker currently used to evaluate oxidative stress in fish sperm because of the high amount of polyunsaturated fatty acids that constitute the spermatozoa membrane (Li et al., 2010a, b; Mansour et al., 2006; Shaliutina et al., 2013a). Lipid peroxidation is usually measured by the quantification of a final product of the oxidation of lipids, the malondialdehyde (MDA). The TBARS assay is the technique usually employed to evaluate lipid peroxidation, by quantification of the malondialdehyde present in the sample that reacts with the thiobarbituric acid used as reagent and with the final product being measured using a spectrophotometer. Mansour et al. (2006) used this technique to quantify lipid peroxidation in Arctic char sperm demonstrating a reduction of this parameter in those fish fed an antioxidant supplemented diet. Other authors, using the same technique in gilthead seabream, determined that lipid peroxidation was associated with the impairment of the fertilizing capability of sperm after acute exposure to surfactants (Rosety et al., 2007). Other works demonstrated that sperm preservation processes (cryopreservation or short-term storage) induce higher levels of lipid peroxidation in common carp (Li et al., 2010a,b), Russian and Siberian sturgeons (Shaliutina et al., 2013a). This technique was also used to determine lipid peroxidation in brook trout and sterlet sperm (Shaliutina-Kolesova et al., 2013).

The TBARS reaction is relatively nonspecific because thiobarbituric acid, besides MDA, also reacts with many types of compounds, such as aldehydes, amino acids, and carbohydrates. Thus, new commercial kits have been designed to minimize interference from other lipid peroxidation products. This kind of kits (Oxis BIOXYTECH MDA-586) has been used in European seabass, showing that sperm lipid peroxidation is correlated with differences in sperm quality during reproductive season and could be used as a biomarker of peroxidation in this species (Martínez-Páramo et al., 2012a).

The lipophilic probe BODIPY® can be also used to quantify lipid peroxidation. This fluorescent probe is incorporated into the cell membranes exhibiting a change in the spectral emission, shifting from red to green after interaction with peroxyl radicals. This technique usually used in mammals (Aitken et al., 2007; Ortega-Ferrusola et al., 2009) has been recently applied in zebrafish sperm by Hagedorn et al. (2012).

3.1.4. Protein oxidation

Similarly to the analyses of lipid peroxidation, oxidization of proteins is measured by quantification of carbonyl groups, that can be spectrophotometrically quantified through DNPH (2,4-dinitrophenilhidrazine) reaction at 360 nm (Levine et al., 1990).

This method has been used to quantify protein oxidization in several fish species, such as European seabass (Martínez-Páramo et al., 2012b), Russian and Siberian sturgeons (Shaliutina et al., 2013a), sterlet, common carp and brook trout (Shaliutina-Kolesova et al., 2013). In Russian and Siberian sturgeons and common carp, similarly to the result obtained from the lipid peroxidation analyses, the level of protein oxidation increased in the preserved samples (Li et al., 2010a,b; Shaliutina et al., 2013a).

3.1.5. DNA damage

According to Aitken et al. (2012), oxidative stress is one of the main mechanisms responsible for DNA strand fragmentation. In the DNA, the nitrogen bases, particularly guanine, are the main targets of ROS attack, generating 8-hydroxy, 2′-deoxyguanosine (8OHdG). This reaction weakens the bond between the guanine and the adjacent ribose unit, leading to loss of the oxidized base, destabilizing the DNA structure, and resulting in localized strand breakages. Several methods have been used to quantify the effect of ROS on DNA integrity. For instance, the presence of 8 OHdG can be quantified by flow cytometry using assays incorporating labeled avidin, which binds to 8OHdG with great affinity. However, the most widely method used to quantify the percentage of fragmented chromatin in fish sperm is the comet assay (Cabrita et al., 2005b; Martínez-Páramo et al., 2009; Shaliutina et al., 2013a; Zilli et al., 2003). This technique will be explained in detail in the next section.

3.1.6. Mitochondria dysfunction

In the majority of eukaryotic cell types, mitochondrial energy metabolism is the most important source of ROS (Kowaltowski et al., 2009). The complex redox mechanisms occurring in mitochondrial microenvironment are able to control ROS production at the levels required for the normal functionality of this organelle. However, oxidative stress situations leading to damage in the mitochondrial membrane can impair the mitochondrial respiratory efficiency promoting the release of ROS, and creating a vicious circle in which mitochondria might be the generator and the victim of the oxidative damage (Ferramosca et al., 2013). In human sperm, it was reported that oxidative stress negatively affects sperm mitochondrial respiration by an uncoupling between electron transport and ATP synthesis, and as a result it was suggested that this reduced mitochondrial respiratory efficiency decreased the progressive motility of spermatozoa (Ferramosca et al., 2013).

There are several dyes that could be used to evaluate the mitochondria membrane potential and functionality. Through the use of rhodamine 123 in red seabream (*Pagrus major*) and in rainbow trout and catfish it was showed that the percentage of spermatozoa with functional mitochondria was significantly reduced after cryopreservation (Liu et al., 2007; Ogier de Baulny et al., 1997, 1999). In striped bass (*Morone saxatilis*) and gilthead seabream, the JC-1dye was successfully used to evaluate the mitochondrial transmembrane potential of sperm incubated at different osmolarities (Guthrie et al., 2008) or exposed to different dilution ratios in the extender media during cryopreservation (Cabrita et al., 2005a).

3.2. Genome analysis as marker of sperm quality

Traditionally the chromatin damage has been poorly considered in the assessment of sperm quality. Chromatin integrity has been clearly related with the success of fertilization in mammals. Evenson et al. (1999) established the correlation between chromatin fragmentation index in a sperm sample and the ability to fertilize oocytes. Most of the factors potentially damaging for chromatin are also aggressive for other cell structures related to fertilization, resulting in a positive selection of spermatozoa carrying undamaged chromatin by the exclusion of damaged cells, unable to reach or penetrate the egg (Hourcade et al.,

2010). Nevertheless chromatin modifications could happen in the absence of measurable effects on other sperm characteristics, allowing sperm with damaged DNA to fertilize the oocytes. In rainbow trout, Pérez-Cerezales et al. (2010b) demonstrated that spermatozoa carrying at least 10% of fragmented DNA fertilize the egg and produced larvae. It is now admitted that sperm chromatin damage has higher effects on early embryo development than on the process of fertilization (Speyer et al., 2010), given its role in the control of gene expression from the first steps of embryo development (Carrell and Hammoud, 2010; Delbés et al., 2010; Ward, 2010). Different authors described the consequences of the use of bad quality sperm with low chromatin integrity on the offspring outcome both at embryonic and at later larval stages (Speyer et al., 2010; Zini, 2011). In rainbow trout the increase of abortion rates at different developmental stages promoted by fertilization with DNA-damaged sperm was reported (Pérez-Cerezales et al., 2010b) and a differential expression of 8 genes related to growth and development in the obtained larvae was later identified (Pérez-Cerezales et al., 2011). The importance of sperm chromatin integrity goes beyond the fertilization ability, contributing to the overall progeny development with a wider role than the simple transmission of their genome.

Many different factors could affect fish sperm chromatin stability, from exposure to toxicants or radiations (Dietrich et al., 2005, 2007, 2010), to aging, cold storage (Pérez-Cerezales et al., 2009), cryopreservation (Cabrita et al., 2005b; Labbé et al., 2001; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2009, 2010a; Zilli et al., 2003), or changes in thermal regime during spermiogenesis, etc. The reported results showed a different susceptibility to damage between species that could be related to differences in the chromatin structure. Chromatin compactation follows different patterns during fish spermiogenesis, being the sperm nuclear basic protein (SNBP) composition very heterogeneous. In some species like the gilthead seabream, histones are not substituted by protamines and the DNA remains associated to histone-like proteins in mature spermatozoa (Kurtz et al., 2009), whereas other species, such as chum salmon, have fully replaced somatic histones by protamines (Frehlick et al., 2006). As an example seabream DNA seems to be less cryosensitive than that from brown trout and this one more cryoresistant than that from rainbow trout (Cabrita et al., 2005b; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2009).

3.2.1. Methods to assess chromatin integrity

Different methods can be applied to the evaluation of chromatin damage, most of them related to the detection of fragments or packaging failures, whose main characteristics are summarized in Table 2. The comet assay or SCGE (single cell gel electrophoresis) is the technique most commonly used to analyze chromatin fragmentation in fish (Cabrita et al., 2005a,b; Devaux et al., 2011; Dietrich et al., 2007, 2010; Labbé et al., 2001; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2010a,b, 2011; Zilli et al., 2003) and is based on the different electrophoretic migration patterns of DNA fragments according to the size of the strand. Cells immobilized over an agar covered slide are lysed and submitted to electrophoresis, promoting the migration of the small pieces of fragmented DNA. After staining DNA can be observed forming a comet-like tail structure in DNA fragmented cells, preceding the non-fragmented DNA (head of the comet). Specific software allows the analysis of this "comets" for each single cell and thus results can be expressed as mean values for all the analyzed cells in a given sample, or as percentage of cells per range of DNA damage (Beirão et al., 2008; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2010b). One of the advantages of this method relies on its versatility since modification of the pH lysis solution allows the differential identification of single or double strand breaks. Detection of lesions other than fragmentation is also possible by combining this technique with a previous digestion with specific endonucleases that will cut the strand in modified bases. In trout sperm digestion with ENDIII or FPG has been applied for the quantification of oxidized cytosines and guanosines, respectively, in non-fragmented positions of the DNA strand (Pérez-Cerezales et al., 2009). Another technique based on the differential migration of chromatin fragments is the sperm chromatin dispersion test (SCD). This method requires species-specific protocols for chromatin decondensation that should be set up for fish, considering the specific differences in the chromatin packaging pattern. This method was used in the evaluation of tench (*Tinta tinca*) sperm reporting a good correlation with the results obtained using the comet assay (López-Fernández et al., 2009).

Fragmentation can also be assessed more specifically using the TUNEL assay (terminal deoxynucleotidyl transferase mediated dUTP-biotin end-labeling), based on the addition of a fluorescent labeled nucleotide to the 3′OH end of the strand. Fragmentation increases the presence of free 3′OH ends, thus the more fragmented the DNA, the higher the fluorescence emitted by the nucleus. Analysis of the results can be made by fluorescence microscopy, but flow cytometry is also compatible with this method, increasing the accuracy with the evaluation of a high number of cells per sample. TUNEL assay is commonly used for the detection of apoptotic cells, but it will effectively reveal DNA fragmentation, being caused by apoptosis or by any other process. In fish sperm TUNEL was used to identify differences in several freezing protocols in gilthead seabream and European seabass sperm (Cabrita et al., 2011a,b). Among the methods compatible with flow cytometry, the sperm chromatin structure assay (SCSA®) developed by Evenson et al. (1980), is one of the most commonly used in the evaluation of human sperm chromatin status, with different commercial kits available for an easy and fast application. It is a simple method based on the metachromatic shift of acridine orange (AO) fluorescence from green, when intercalated into native double-stranded DNA, to red when stacked on single-stranded DNA. This method measures the susceptibility of sperm DNA to acid-induced *in situ* denaturation. Cells preserving DNA integrity emitted in green after treatment, whereas cells with a compromised chromatin emitted red fluorescence. An alternative methacromatic probe is chromomicine A3 (CMA3). Their advantages are the simplicity and the possibility to analyze results by flow cytometry, but there are no reports on their use for sperm fish evaluation, probably because setting up this method to fish also requires facing the different patterns of DNA/proteins packaging between species.

Immunofluorescence methods allow the detection of specific lesions in the DNA strand. Antibodies have been developed against cyclobutane-pyrimidine dimers (CPDs) or 6–4 photoproducts (6-4PPs), typically induced by UV irradiation (Rastogi et al., 2010) used in aquaculture to inactivate the sperm genome in gynogenetic processes. Commercial kits are also available to detect 8-hydroxy,2-deoxyguanosine (8-OHdG), the hallmark of oxidative DNA damage, and are starting to be used for the evaluation of oxidative damage in sperm (Cambi et al., 2013). These methods, still not assayed in fish, allow the evaluation by microscopy or flow cytometry and provide more specific information about the kind of damage present in the DNA.

3.2.2. Analysis of damage in specific regions or genes

All methods previously discussed allow the assessment of the global status of the nuclear genome being good markers of chromatin status. However, some chromosome regions have a differential susceptibility to damage, considering their position on the chromosome, linkage to the nuclear proteins, or other more specific factors. Moreover, some specific paternal genes could have a relevant role in the control of early embryo development (Carrell and Hammoud, 2010; Ward, 2010; Wu et al., 2011). In the sperm of mammals these particular genes seem to be located in chromatin regions more loosely packaged than the rest of the chromatin whereas in zebrafish display a lower DNA methylation degree, as well as specific methylation pattern in their associated histones (Wu et al., 2011). The different epigenetic pattern could render them more susceptible to be injured than other chromatin regions. Moreover, damage in these particular genes could have special relevance, considering that proper embryogenesis relies on their correct expression and that any damage should be immediately repaired after fertilization. Therefore the consideration of these group-

Table 2
Advantages and disadvantages of different methods used for sperm DNA analysis.

Technique	Analysis	Information provided	Technical requirement	Equipment	Reports in fish	Advantages	Disadvantages
Comet assay	Cell by cell	Overall fragmentation (single and double strand breaks), indirect measurements of specific lesions	Medium	Basic but specific software recommended	Abundant	Cheap and versatile	Time consuming
SCD	Cell by cell	Overall fragmentation	Low	Basic	Scarce	Easy to perform	Species-adaptation required
TUNEL	Cell by cell/average	Fragmentation	High	High	Scarce	Accuracy	Expensive
SCSA CMA3	Average	Chromatin packaging/integrity	Medium	High	None	Simplicity	Species-adaptation required
Immunofluorescence	Cell by cell	Specific lesions in the genome (i.e. presence of 8OHdG)	High	High	None	Accurate identification of lesions	Still not assayed in fish Expensive
qPCR	Average	Total number of lesions in specific regions/genes	High	High	Scarce	Accuracy and analysis of key genes	Expensive, long genomic annotations required

set of genes as markers of sperm quality and early embryogenesis development success could represent a step forward in the assessment of sperm quality.

Telomeres, highly conserved guanine-rich tandem DNA repeats of the chromosomal end, provide chromosomal stability (Agarwal et al., 2010; Meyne et al., 1989; Ocalewicz et al., 2004) and could be regions more prone to suffer damage. Thus, telomere length can be used as a parameter of sperm quality. This tool has been recently applied in fish, showing a decrease in telomere length of rainbow trout spermatozoa after cryopreservation (Pérez-Cerezales et al., 2011). This study revealed that DNA fragmentation promoted by sperm cryopreservation disturbs the telomere region, as it was also reported by some studies on human sperm (Fernandez-Gonzalez et al., 2004). In addition, there were evident changes in telomere length in the larvae obtained with these sperm samples. Telomere length analysis was used to analyze cryodamage in gilthead seabream sperm by Cartón-García et al. (2013) who demonstrated the stability of telomeres from this species under the tested conditions.

The idea of studying DNA damage not in large regions, but in concrete genes or sequences has arrived to the aquaculture field from previous studies performed in other species, mainly in mammals. The application of fluorescence *in situ* hybridization (FISH), using probes for target genes or regions in combination with Comet assay has been used for this purpose in somatic cells. Hybridization with a specific DNA probe of the cells spread over a slide show the position of the target sequence in the tail (fragmented) or the head (unfragmented) of the comet. To our knowledge this technique was never used in fish sperm, although its application in the study of DNA repair mechanisms and genotoxicity in other species has been very useful (Jha, 2008).

More accurate procedures based on quantitative polymerase chain reaction (qPCR) have arisen in the last decade. These methods can be applied to the evaluation of multiple genes or sequences giving a global assessment of sperm DNA damage (Ayala-Torres et al., 2000; San Gabriel et al., 2006; Santos et al., 2006). The analyses are based on the capacity of certain DNA lesions (abasic sites, cross-linking, double lesions, modification of nitrogen bases, strand breakages, DNA fragmentation), to reduce and block polymerase progression in template DNA, which finally results in a reduction of the template amplification and threshold cycle (Ct) delay (Rothfuss et al., 2010; Sikorsky et al., 2004). Nuclear and mitochondrial evaluation of specific DNA damage has been reported independently with success (Santos et al., 2006). Rothfuss et al. (2010) developed a rapid and quantitative method to evaluate the relative levels of damage in mitochondrial DNA by using a semi-long qPCR amplification of mitochondrial DNA fragments of different lengths. The formula proposed by this group is based the correlation of the Ct obtained from two different amplicons (a small and a large one). This analysis provides specific damage as number of lesions per 10 kb in a concrete gene/region. These novel approaches have been applied to aquaculture species by our group (Cartón-García et al., 2013) for the first time. We studied the number of lesions produced by different cryopreservation protocols in specific genes of gilthead seabream spermatozoa. Two nuclear genes with important roles in embryo development (*Igf1* and *Gh*) and two mitochondrial genes (*Cytb* and *CoI*) were studied and DNA fragmentation was analyzed by the comet assay. The number of lesions/10 kb registered in samples that showed very low fragmentation rate with the comet assay was always higher on the mitochondrial genes than on those nuclear ones related to embryo development. More recent studies with trout sperm clearly revealed a differential sensitivity to damage of nuclear genes which is dependent on the source of damage. Comparison between 7 early transcribed genes related to embryo development and 2 late transcribed ones non-required for early embryo development, revealed important differences in the susceptibility to ROS between both groups, being the late transcribed genes more resistant to oxidative damage. Nevertheless, susceptibility to UV irradiation was similar between them (González-Rojo et al., 2013). Studying gene-specific damage can be used as an excellent complement approach for traditional techniques and could help to identify particular genes whose integrity could be used as a biomarker of sperm quality. Nevertheless, this accurate and sensitive qPCR method is expensive and time consuming requiring highly-skilled technologies. Application to most of the commercial species could be hindered by the lack of gene sequence annotations available.

3.3. Transcripts as predictors of sperm quality

It is well known that spermatozoa are transcriptionally inactive cells (Lalancette et al., 2008) and during decades it was assumed that spermatozoa RNA population was non-functional because these molecules are remnant from spermatogenesis. However, nowadays it is totally accepted that spermatozoa provide more than the paternal genome into the oocyte and the residual mRNAs from spermatogenesis can have key roles in early embryonic development and success of fertilization, in humans and other mammalian species (Garcia-Herrero et al., 2011; Johnson et al., 2011; Lalancette et al., 2008; Ostermeier et al., 2002). In studies made in bull (*Bos taurus*), spermatozoa transcriptome profiling has been presented as a potential tool to evaluate semen quality (Bissonnette et al., 2009), and in humans a differential transcriptomic profile has been found in spermatozoa capable of producing pregnancy (Garcia-Herrero et al., 2011). To date, the use of microarrays in aquaculture has not focused on the evaluation of the RNA profile for predicting gamete quality. Most of the transcriptomic studies performed in fish in the field of reproduction have been descriptive and fall within one of these three categories: (1) spermatogenesis and testicular development and (2) evaluation of hormones, drugs or contaminants on these processes, and more recently (3) the study of sperm quality markers.

Within the first group, we can mention some studies carried in rainbow trout where more than 3000 differentially expressed genes were grouped according to their expression profiles in developing testes and isolated germ cells (Schulz et al., 2010). Taking into account that the transcripts in the spermatozoa are relevant for fertilization and early development and are remnants from spermatogenesis, the

establishment of these gene expression clusters of male gonad development in the rainbow trout could be useful as a tool to potentially predict sperm quality. With this in mind, and assuming spermatozoa quality markers not only as end-points in assays, it is important to consider that molecular markers could reflect the mechanisms through which "sperm quality" is imprinted during spermatogenesis (Fig. 1). In this direction Gardner et al. (2012) described the expression profiles of certain genes in mature gonadal tissues of Atlantic blue fin tuna (*Thunnus thynnus*). Expressed sequence tags (ESTs) potentially related to components significant to spermatogenesis were included such as meiosis, sperm motility and lipid metabolism. Microarray technology has also allowed the discovery of novel markers. Yano et al. (2009) identified *notch1* as a molecular marker for type A spermatogonia by microarray analysis in transgenic rainbow trout. In other studies in the same species, next-generation sequencing was combined with microarray for transcriptome analysis, confirming the expression of known spermatogonial markers to be higher in spermatogonia A than in testicular somatic cells (Hayashi et al., 2012). In this way, these results were extremely useful to understand gametogenesis in freshwater species, opening way for further more detailed characterization of specific transcripts present at key developmental stages of spermatogenesis.

Within the second group, different studies explore the effects of hormones like progestin, androgens and estrogens on testicular gene expression by microarray analysis. It has been found that Sertoli cells change the expression profile of growth factors in the presence of steroids, and that these changes in expression subsequently modulate germ cell proliferation and differentiation (Schulz et al., 2010). In zebrafish, the exposure to 17α-ethinyloestradiol compromised the reproductive health of breeding individuals and, by transcriptomic analysis of gonads, it was confirmed that the expression of 114 genes was altered after the exposure to this molecule. The most affected genes were those related to the regulation of cell cycle progression, the ubiquitin system and glutathione peroxidase were affected by the treatment and associated with the decrease in gamete quality in both genders (Santos et al., 2007). The ubiquitin system has been implicated in the degradation of paternal mitochondria at fertilization, and in degradation of poor quality sperm (Sutovsky, 2003).

However, all these transcriptomic studies are not focused on the use of microarrays as a direct tool to predict sperm quality in fish. Recently, our group has performed a different approach to investigate on the role of mRNAs as quality markers in fish spermatozoa. Using a qPCR approach, we have recently defined a set of transcripts which have a different profile in testicular cells from good and bad zebrafish breeders (Guerra et al., 2013). The transcripts identified as predictors of good breeding performance were *bdnf*, *lhcgr*, *lepa*, *bik*, *dmrt1*, *fshb* and *hsd17b4*, whereas *bik* and *hsd17b4* were more abundant in bad zebrafish breeders. Although these results were obtained with a model species, we were interested on exploring the possibility of transferring these findings to relevant teleost species from a commercial point of view. Spermatozoa transcripts from *S. aurata* were analyzed in males with either good or bad sperm motility, which has been described as one of the most reliable parameters for predicting fertility in some species (Cosson et al., 2008). Males with low sperm motility registered low levels of *bdnf*, *bik* and *kita* comparing to the control group males with high sperm motility. However, changes in sperm transcripts and/or transcriptome profile cannot be only understood as a way to predict fertilization ability of males, it can also be considered as an emerging tool to evaluate sperm analysis after a process such as cryopreservation. As it was mentioned before, cryopreservation can produce several types of damage to the cells, affecting sperm functionality at later stages. Some studies have reported the decrease or even the elimination of some transcripts after cryopreservation (Garcia-Herrero et al., 2011). Our group reported the absence of certain transcripts after human sperm cryopreservation (Riesco and Robles, 2013; Valcarce et al., 2013a,b) and we speculate that this fact could be due to a change caused by cryopreservation in the interaction protein–mRNA that can make the mRNA more susceptible to degradation, although until now difficult to prove in fish sperm. Contrary to these observations, we have reported a different tendency after *S. aurata* sperm cryopreservation. Although cryopreservation has been reported to decrease transcript levels in spermatozoa from other species, the protocol employed for gilthead seabream sperm cryopreservation did not affect mRNA levels. This is a relevant observation since it is known that changes in spermatozoa transcripts could have a serious effect on fertilization or even on the offspring. Therefore, transcripts level analysis in the spermatozoa could also be understood as a tool to select the most efficient and safe cryopreservation protocol. Moreover, it is known that fertilization with cryopreserved spermatozoa with DNA-damage alters gene expression in the surviving embryo and larva (Pérez-Cerezales et al., 2011), indicating that transcriptome analysis in the progeny could be reflecting sperm quality.

Reproductive aquaculture will undoubtedly obtain higher benefits from transcriptomics in the future, although the lack of genetic tools and annotated sequences in many fish species makes that in many cases microarrays and specific probes can only be designed using limited information from expressed sequence tags (ESTs) or heterologous-species (Yano et al., 2009). Nevertheless the great potential of these technologies in other species, together with the preliminary results that have been reported in fish, let us envision a great applicability of these techniques in the field of aquaculture in the near future.

3.4. Proteome markers

Proteomic analysis of sperm cells in several species including fish has provided valuable information about the proteins involved in sperm physiology and function, in the components of the sperm head, tail and nucleus and in seminal plasma composition (Ciereszko et al., 2012). Specific proteins with key roles on a variety of processes linked to reproductive performance and gamete quality have been reported. Most of the studies made with fish have been designed on species with commercial interest due to de need of improving broodstock management procedures, sperm handling techniques such as cryopreservation or breeding strategies. Proteomic analysis revealed to be a good indicator of cryopreservation success and an indicator of specific cryodamage. In common carp, Li et al. (2010b) found 14 spots corresponding to proteins that were significantly altered after the cryopreservation process. Some of those proteins were identified as being involved in cell metabolism, oxidoreductase activity and signal transduction, membrane trafficking, organization and cell movement. Other authors working in marine species showed, using two dimensional electrophoresis coupled with MALDI-TOF (matrix-associated laser desorption/ionization time-of-flight) analysis, that the use of freezing–thawing procedures caused degradation of 21 sperm proteins in European seabass and few others in gilthead seabream (Zilli et al., 2005, 2008). In gilthead seabream the authors reported a strong effect on the phosphorylation state of proteins responsible for motility activation. Therefore it seems clear that according to the species, some proteins could be more affected than others and that choosing some specific proteins as markers could be interesting not only to study the mechanisms involved in cryodamage but to identify protocols with better success. Defects in sperm proteins may compromise sperm motility, fertilization ability, and the early events after fertilization (Zilli et al., 2005).

Other use of proteomics as a tool for the study of potential markers came from studies on broodstock quality, stripping frequency and changes in quality during the reproductive season. The proteomic profiling of *S. senegalensis* males during spermatogenesis allowed identifying some markers relevant for the different reproductive changes found between F1 and wild males (Forne et al., 2009). In this particular study, the authors identified 49 proteins differentially expressed during the progression of spermatogenesis, sperm maturation and motility, or cytoskeletal remodeling, pointing towards possible causes of differences

in sperm quality, found previously by other authors between the two types of broodstocks (Cabrita et al., 2006).

Proteome markers were also found in seminal plasma of perch and sterlet related with sperm collection during the reproductive season and consecutive strippings (Shaliutina et al., 2012, 2013b). These authors found specific proteins that were changed in seminal plasma during the course of the reproductive season that could be associated with changes in other sperm traits. In sterlet, some of these proteins were associated with intracellular mechanisms responsible for regulating spermatozoa motility.

Proteomic studies would greatly increase the chance of identifying new biomarkers of male fertility. Such knowledge can contribute significantly to the enhancement of control in reproduction under aquaculture conditions, especially the efficacy of fertilization procedures and semen storage, including cryopreservation (Ciereszko et al., 2012).

4. Future perspective applications to industry: how far are we?

It seems now clear that new biomarkers of sperm quality are needed for a correct evaluation of sperm traits opening new perspectives in research. In addition to this, the commercial availability of those markers seems to be the additional step to follow with potential benefits to the biotechnological companies offering services to the aquaculture industry. Fish sperm selection programs based on certain molecular characteristics could be realistic in a not so far future. Research on biomarkers can allow the design of specific probes for the genes responsible for sperm quality, fertilization success and early indicators of embryonic development. Therefore, once we are able to determine the ideal molecular profile of sperm, selection techniques that enable the enrichment of a sperm sample with spermatozoa with the molecular characteristics considered adequate could increase the reproductive success and give more guarantees of a high quality progeny.

Recently, magnetic-activated cell sorting (MACS) emerged as an alternative technique for sperm selection based on the use of microscopic microbeads that are conjugated to proteins or antibodies to tag cells of interest. Once tagged, these cells are retained in columns and passed through a high gradient magnetic field. Its application as a method for selecting optimal spermatozoa has been extensively described in the literature (Said et al., 2008), particularly in the selection of apoptotic sperm cells via externalization of phosphatidylserine (PS). A similar technique using only PS labeling was already successfully used in characterizing fish sperm (Beirão et al., 2008).

In the case of fish sperm, not only the selection of certain spermatozoa within a sample is important, but most of all, the selection of samples of a certain quality may be more interesting for industry. This may be achieved by using specific mRNA probes labeled with a fluorochrome by fluorescent *in situ* hybridization combined with flow cytometry will allow researchers to easily select samples within a threshold, specific for the species. The same can happen with the selection of certain proteins as biomarkers and the use of flow cytometer immunoassay. All these tests may be available with the integrated use of proteomics, microarray techniques and mass sequencing of sperm samples allowing the design of specific kits for sperm quality analysis.

Nowadays there are several services provided by biotechnological companies to assure the availability of disease-free sperm from several species, such as Atlantic and Pacific salmon, trout, cod and halibut. These companies need to certify the quality of samples not only in terms of specific pathologies, but also by using a set of markers that can rapidly be tested and that can certify sperm quality for progeny control. This will be especially relevant for samples exposed to cryopreservation and commercialized for production purposes.

In summary, several of the techniques mentioned raises the possibility of customized sperm selection in cases where male potential is interesting or where the evaluation of sperm exposed to management procedures such as cryopreservation is needed, or even their use in toxicological tests. This is a step towards a tailored quality assessment depending on the needs, but also ensuring reliable tools that can be employed by the industry to improve the efficiency of reproductive cells and produce high quality progeny.

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A.V.2. Revisión científica

Molecular basis of spermatogenesis and sperm quality

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ABSTRACT

Spermatozoan quality can be evaluated in different ways, here we focus on the analysis of DNA, RNA and epigenetic status of germ cells. These characterizations also can be the bases for explaining sperm quality at other levels, so we will see how some of these molecules could affect other sperm quality markers. Moreover, we consider the possibility of using some of these molecules as predictors of sperm quality in terms of the ability to produce healthy offspring. The relevant effect of different types of RNA molecules in germ line specification and spermatogenesis and the importance of germ cell DNA integrity and a proper epigenetic pattern will be also discussed. Although most studies at this level have been performed in mammals, some information is available for fish; these recent discoveries in fish models are included. We provide a general overview on how these molecules could have a deep influence in the final sperm quality.

1. Functions of non-coding RNAs in the control of germline development: miRNAs, piRNAs and lncRNAs

RNAs are crucial from very early stages in germline development. Primordial germ cells (PGCs) (the precursors of gametes) acquire a specific gene expression program in which non-coding RNAs and ribonucleoproteins play an important role. Some ribonucleoproteins (RBPs) such as DND1 are from very early stages mainly expressed in germ cells having an essential role in germline development. They are such important proteins that if in zebrafish embryos their translation is blocked by using morpholinos, PGCs do not differentiate and adults which are derived from these embryos never develop functional gonads (Riesco et al., 2014). Micro RNA (miRNAs) are non-coding RNA molecules with the capacity to regulate other gene expression by different processes and it is known that differential susceptibility to micro RNAs contributes to tissue-specific gene expression. As an example, miR-430 targets the 3́UTRs of mRNAs from germline genes (Tani et al., 2010). In order to prevent degradation of mRNAs that are crucial for germline development, DND1 binds uridine rich regions in the 3′UTR, either sequestering mRNAs or physically displacing miRNA-Induced Silencing Complex (miRISC) to alleviate micro RNA mediated suppression (van Kouwenhove et al., 2011) and therefore, preventing mRNA from degradation.

If PGC specification and migration to the genital ridge is successful, and a functional gonad develops, spermatogenesis will take place. RNAs are also crucial in spermatogenesis. It is well known that male germ-cell differentiation is tightly controlled at transcriptional and post-transcriptional level. Transcriptional and

Abbreviations: BPA, bisphenol A; CB, deoxyribonucleic acid; Ct, threshold cycle; DCP1a, decapping mRNA 1A; *Dmr*, doublesex and mab-3 related transcription factor; DNA, Deoxyribonucleic acid; DND, dead end protein; ICSI, intracytoplasmatic sperm injection; Insrβ, insulin receptor beta; KIF17b, kinesin family member 17 b; lncRNA, long non-coding RNA; miR-430, micro RNA 430; miRISC, miRNA-mediated silencing complex; miRNA, micro RNA; mRNA, messenger RNA; MVH, mouse vasa homolog; PGC, primordial germ cell; piRNA, piwi-interacting RNA; piwi, P-element induced wimpy testis; qPCR, quantitative polymerase chain reaction; RBP, ribonucleoproteins; RNA, ribonucleic acid; siRNA, small interfering RNA; Spga-lncRNA 2, spermatogonia-specificlncRNA 2; Spga-lncRNA1, spermatogonia-specificlncRNA 1; Tsx, testis specific X-linked gene; UTR, untranslated region; zili, zebrafish piwi like RNA-mediated gene silencing 2 homolog; ziwi, zebrafish piwi homolog.

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post-transcriptional regulation are crucial processes that allow the cells to have a rigorous control in time and space over the genetic information displayed. Post-transcriptional regulation is particularly important during the late steps of spermatogenesis when the compacting sperm nucleus becomes transcriptionally inhibited (Yadav and Kotaja, 2014). In post-transcriptional processes, target mRNAs are controlled by RNA binding proteins and by non-coding RNAs (Yadav and Kotaja, 2014).

Male germ cells express several classes of small RNAs including Dicer-dependent micro-RNAs (miRNAs) and endogenous small interfering RNAs (siRNAs) as well as Dicer independent piwi interacting RNAs (piRNAs) (Meikar et al., 2011). Both miRNAs and siRNAs control mRNA translation and degradation either by triggering endonuclease cleavage, promoting translation repression, or accelerating mRNA decapping (Valencia-Sanchez et al., 2006; Yadav and Kotaja, 2014). The differences between them are in their biogenesis. First type is produced from stem loop transcripts and second type is produced from long double stranded RNA precursors (Yadav and Kotaja, 2014). Both types are Dicer dependent for the cytoplasm-endonuclease-processing, but miRNAs are also early processed in the nucleus by Drosha. In mammals it is well known that different miRNAs are crucial for different steps of spermatogenesis. Some of them are relevant for the maintenance of undifferentiated state of spermatogonia, others in the induction of differentiation, and some others will have an important role in early embryo development (Fig. 1) (Kotaja, 2014; Luo et al., 2015; Wang and Xu, 2015). Although miRNAs in fish have not been as studied as those in mammals, deep sequencing profiling in some fish species (*Takifugu rubripes*) have demonstrated that most miRNA sequences are conserved, which indicates that the basic functions of vertebrate miRNAs share a common evolution. Moreover, some miRNAs families are abundant in the gonads, but are expressed only at low levels in somatic tissue; this finding suggests miRNA has a specific function in germ cells (Wongwarangkana et al., 2015). Several miRNAs were reported to be particularly abundant in rainbow trout testis (Farlora et al., 2015) and a recent study revealed that dre-miR-202-5p is common in zebrafish spermatozoa and testis, suggesting that this miRNA might be related to spermatogenesis and spermatozoa functioning (Jia et al., 2015).

Germ cells also possess piRNAs. PiRNAs are single stranded RNAs which are present in high numbers in the male germ line. Although PiRNAs sequences are not conserved between species PiRNA clusters seems to be conserved. The PIWI pathway has an important effect on testis differentiation and development and it is also important in genome defense against transposable elements which contribute to genome integrity maintenance (Bao and Yan, 2012; Siomi et al., 2011). Moreover, piRNAs are also considered as potential mediators of epigenetic transgenerational inheritance. The zebrafish genome encodes two Piwi homologs, Ziwi and Zili (Houwing et al., 2007). As has been demonstrated in mouse Piwi mutants, *Ziwi* mutants display a progressive decline in the germ cells due to apoptosis (Houwing et al., 2007). Moreover, as a result of PGC loss, Ziwi mutants are phenotypically males, since PGCs are required during embryogenesis for female development (Dranow et al., 2013).

Not only are small non-coding RNAs crucial for germline development, Long non-coding RNAs (lncRNAs) are also relevant. Interestingly, this group of RNAs, that are transcribed by RNA polymerase II, or formed by processing other transcripts, do not have a unique association with a specific group of proteins for their processing and function, contrary to what was observed for small noncoding RNAs which depends on argonaut (Yadav and Kotaja, 2014) and PIWI proteins (Chuma and Nakano, 2013). These RNAs could act at different levels having a huge variety of regulatory roles: they could inhibit miRNA function, avoid translation, influence chromatin remodeling, promote DNA methylation or even act as a precursors of short RNA (Kung et al., 2013). It is known in mammals, that some lncRNAs have specific roles in male germ cell development. As an example, Testis-specific X-linked (*Tsx*) is relevant for progression of meiosis, *Dmrt1*-related gene (*Dmr*) is probably involved in the switching between mitosis and meiosis of the germ cell development and *Spga-lncRNA1* and *2*, may be important in maintaining spermatogonia stemness. LncRNAs expressed in different germ cells have been identified in mice: 50 expressed in type A spermatogonia, 35 in pachytene spermatocyte, 24 in round spermatids (Lee et al., 2012).

But do the germ cells have a specific RNA processing centre in which these regulator pathways could converge? A centre for RNA storage and metabolism? This is the Chromatoid Body (CB),

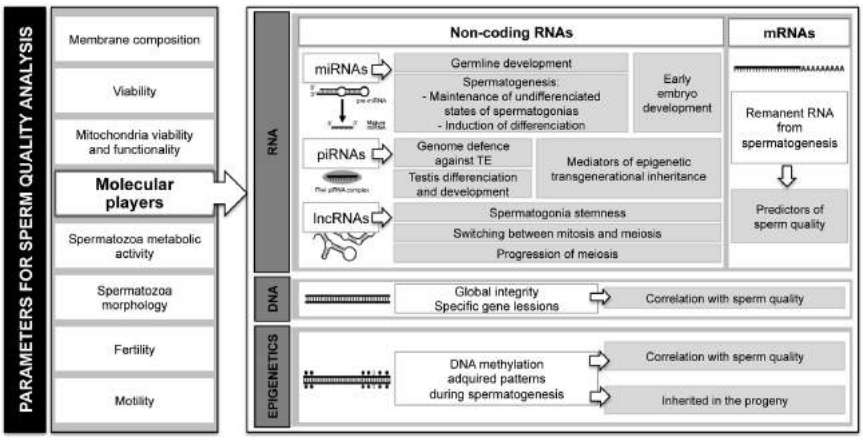

Fig. 1. Molecular players used as parameters for sperm quality analysis and their roles on male reproductive key process. RNA: non-coding RNAs (miRNAs, piRNAs and lncRNAs) and mRNAs; DNA and epigenetic status.

a lobulated perinuclear granule located in the cytoplasm of male germ cells.

2. The chromatoid body

The chromatoid body (CB) was described more than a hundred years ago (Benda, 1891; Kotaja, 2006). It is known that CB is involved in RNA storage and metabolism (Nagamori and Sassone-Corsi, 2008) being crucial for successful spermatogenesis.

The CB contains male-germ-cell-specific components, such as the mouse VASA homolog (MVH) and MIWI, as well as components of both the microRNA (miRNA) pathway (such as Argonaute proteins and the endonuclease Dicer) and the RNA-decay pathway (such as the decapping enzyme DCP1a and GW182). It is interesting that specific transcripts from spermatocytes or round spermatids are enriched in the CB. Although the molecular mechanism by which mRNAs are targeted to the CB are not well known, it has been suggested that the association of these mRNA in the CB is not due to CB targeting signals but probably to the absence of CB exclusion signals (Meikar et al., 2014). The CB importance in male reproduction is highlighted by the infertile phenotype of many knockout mice lacking CB components (Kotaja and Sassone-Corsi, 2007). An interesting aspect of CB is the intracytoplasmic movement. CB moves around the nucleus and makes frequent contact with the nuclear pore, collecting material from the nucleus (Kotaja, 2006; Söderström and Parvinen, 1976); miRNAs and mRNAs that are synthesized in the nucleus are transported to the cytoplasm and loaded to the CB through nuclear pores. The shuttling kinesin protein KIF17b, a component of the CB, could be involved in transporting RNA between the nucleus and the CB (Nagamori and Sassone-Corsi, 2008). Although most of the research on CB hast been focused in mammals, CB has been also described in spermatocytes of tilapia (Peruquetti et al., 2010) and medaka spermatids (Yuan et al., 2014).

3. mRNAs

From the first part of this review, it seems clear that the role of small and long non- coding RNAs in post-transcriptional regulation is completely necessary for successful spermatogenesis. However, mRNA are also crucial molecules that could be directly related to sperm quality. We have already pointed to the possible role of CB as a centre, not only for processing but also for the storage of mRNAs. This storage prevents mRNA from degradation; it is really interesting because recently it has been reported that certain mRNAs remnant from spermatogenesis in the adult spermatozoa are important in fertility and early embryo development in humans (García-Herrero et al., 2011; Gilbert et al., 2007). In humans differential transcriptomic profile in spermatozoa achieving pregnancy via intracytoplasmic sperm injection (ICSI) has been described (García-Herrero et al., 2011). But, what about fish? To evaluate the potential use of mRNA profile as a predictor of fertilization ability in fish, Guerra and colleges (Guerra et al., 2013) studied the presence of several transcripts in testicular cells from good and bad zebrafish breeders and in seabream spermatozoa. They reported that some of the transcripts were present in higher quantity in good breeders (Table 1). These findings, although preliminary, could have great impact in aquaculture, and further studies should be done in this direction.

Considering the huge importance of such transcripts, it might be interesting to explore whether common technologies like cryopreservation are able to modify these pools of transcripts, possibly, even including these types of studies as a method for validating particular protocols. In the case of cryopreservation, it has been found that freezing-thawing process (rather than cryoprotectant exposure) produced a decrease in certain transcripts in human spermatozoa and in zebrafish PGCs (Riesco and Robles, 2013; Valcarce et al., 2013a). Taking into account that these cells are transcriptionally inactive, making impossible the explanation of this effect by repressive transcriptional mechanisms, the authors hypothesized that cryopreservation affects mRNA stability making some of these molecules more susceptible to degradation. They stated that since cryopreservation can alter the chromatin structure in sperm, this process might also affect mRNA stability by altering their association with certain proteins (Riesco and Robles, 2013). However, this effect is not always observed in every species nor with all cryopreservation protocols. In gilthead seabream (*Sparus aurata*), a decrease of transcripts was not observed after the cryopreservation process (Guerra et al., 2013).

Table 1
Potential mRNA markers relevant for sperm quality in fish.

mRNA	Cell type	Transcript population modification	Species	Reference
bdnf lhcgr lepa fshb dmrt1	Testicular cells	Higher transcript population in good breeders	*Danio rerio*	Guerra et al., 2013
bik hsd17b4		Higher transcript population in bad breeders		
insrα insrβ	Sperm	Lower transcript population after bisphenol A exposure		Lombó et al., 2015
bdnf dmrt1 lepa	Testicular cells	Higher transcript population after probiotic supplementation		Valcarce et al., 2015
bdnf bik kita	Sperm	Lower transcript population in low motility sperm samples	*Sparus aurata*	Guerra et al., 2013
activin a bmp-15 ar-α ar-β pr1 fshr	Testicular cells	Higher transcript population after probiotic supplementation	*Anguilla anguilla*	Vílchez et al., 2015

Other external agents also can modify the sperm transcriptome. Lombó and partners (Lombó et al., 2015) demonstrated that an exposure to BPA decreased specific spermatic transcripts in adult zebrafish. The effect of the endocrine disruptor on the translation of particular genes during spermatogenesis could contribute to the reduction in certain transcripts. Moreover the decrease in the levels of transcripts for insulin receptor insrβ in sperm, was linked to heart malformations that were observed in the progeny from those BPA exposed males, as well as to the transgenerational inheritance of these malformations up to the F2 (Lombó et al., 2015).

But, what about the possibility of somehow increasing those molecules considered by some authors as markers for good breeders? In a study that we performed in zebrafish, three of these molecular markers were significantly upregulated in testicular cells after probiotic supplementation (Valcarce et al., 2015). Another recent study in European eel, reported changes in some transcripts that correlated with changes in sperm production and sperm motility; these were significantly increased after 2 weeks of probiotic treatment (Vílchez et al., 2015).

4. DNA

In addition to RNAs, DNA is also a crucial molecule in sperm quality. A correlation between DNA integrity and motility, and even fertility has been reported (Sheikh et al., 2009). Comet assay and Sperm Chromatin Dispersion tests have been widely used in

order to determine DNA integrity (Bungum, 2012). However, DNA damage could produce not only poor fertilization rates but also impaired embryo development. In addition to DNA fragmentation, there are several types of DNA damage that could not be detected with the previously mentioned methods. Novel methods allow the quantification of the lesions in the DNA in particular genome regions or particular genes using QPCR (Cartón-García et al., 2013; Valcarce et al., 2013b). This method is based on the decrease in polymerase amplification produced by the DNA lesions, delaying the Ct (threshold cycle). These data can be translated to a certain number of lesions using the formula published by Rothfuss (Rothfuss et al., 2010). Using this method, the effect of cryopreservation on single genes in *Sparus aurata* sperm has been evaluated (Cartón-García et al., 2013), and specific genes which are more sensitive to damage have been identified in rainbow trout (*Oncorhynchus mykiss*) sperm (González-Rojo et al., 2014). Authors claim that this method could be a good complement for comet assay studies, which provide a general picture of DNA integrity.

5. Epigenetic status

Epigenetic modifications or reprogramming is essential for development of sperm cells (Güneş and Kulaç, 2013; Schagdarsurengin et al., 2012). The best characterized epigenetic change is PGC reprogramming, deeply affecting DNA methylation which is almost globally erased in these cells (Messerschmidt et al., 2014). Interestingly, a recent study in zebrafish confirmed that cryopreservation of these cells could induce hypermethylation in the promoters of important genes such as *vasa* (Riesco and Robles, 2013), suggesting further consequences for gametogenesis.

Re-methylation occurs during gametogenesis and, contrary to PGCs, mature spermatozoa show a very high methylation degree and a condensed nuclei. Failure to establish the proper DNA methylation status during spermatogenesis has been linked to different infertility disorders in humans (Montjean and Ravel, 2015). Environmental or nutritional conditions could alter these processes and modify the epigenetic landmarks, affecting sperm quality or even producing paternal transgenerational effects. Given the epigenetic effects reported for several environmental contaminants (Fraga et al., 2005; Miao et al., 2014), it is plausible that such toxicants in the environment could affect the epigenetic remodeling in germinal cells during embryonic development or, at later phases, during adult life (Huang et al., 2015). The modification of the epigenetic pattern in the gametes could in that case promote developmental failures in the progeny such as those observed in the progeny of males that were exposed to BPA (Lombó et al., 2015). The changes perpetuated in the germ line may be inherited in subsequent generations (Manikkam et al., 2013). Lombó and colleges (Lombó et al., 2015) did not detect changes in global DNA methylation of spermatozoa from males exposed to BPA, but the malformations observed in the progeny (F1 and F2) could suggest undetected methylation changes in specific promoters.

6. Conclusions

Understanding the subjacent molecular regulation that control sperm quality would provide very interesting information on reproductive biology. Recent studies pointed to relatively new discovered molecules as principal actors in this respect. Although most of the knowledge is only available in mammals, recent studies in fish suggests a similar scenario. However, we are still far from understanding this complex network of molecular pathway interactions. Comparative studies analyzing all these coding and non-coding molecules in good and bad breeders using deep-sequencing technologies for transcriptome profiling together with candidate gene approach strategies, will help in developing deeper knowledge in this emerging and promising field. Moreover, studying how conserved are these markers among the different teleost species and their modification due to endogenous (changes in spermatogenesis) or exogenous (changes in environmental conditions or employment of biotechnologies) factors, would also provide important information about their potential importance and their function.

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ANEXO VI

Curriculum vitae

DAVID GARCÍA VALCARCE

Fecha de Nacimiento: 12-05-1988
D. N. I.: 71421079F
Dirección: Avd/ José María Fernández, 49, 7L
Teléfono de contacto: 695989720
Correo electrónico: dgvalcarce@gmail.com
Researcher ID: L-9791-2015
ORCID: orcid.org/0000-0001-9921-5949

EMPLEOS ANTERIORES (1)

Personal Docente e Investigador laboral a tiempo completo
Contrato dentro del Programa de la JCyL y el FSE: Ayudas para financiar la contratación predoctoral de personal investigador
Universidad de León Departamento de Biología Molecular. Área de Biología Celular. Periodo: Mayo 2013 – Mayo 2017

FORMACIÓN ACADÉMICA (3)

Doctorado en Biología Molecular y Biotecnología.
Universidad y Centro: Departamento de Biología Molecular (Área de Biología Celular) de la Universidad de León.
Fecha de terminación de estudios: En curso (lectura: 8 de septiembre de 2017).
Tesis: Factores que afectan a la calidad espermática y herramientas emergentes en su análisis.

Máster Universitario en Metodología de Investigación en Biología Fundamental y Biomedicina.
Universidad y Centro: Facultad de Ciencias Biológicas y Ambientales de la Universidad de León.
Fecha de terminación de estudios: Julio de 2012.
Trabajo Fin de Máster: Análisis genéticos en células de la línea germinal: Estudio del efecto de la criopreservación en los ARN mensajeros de espermatozoides humanos.
Calificación media obtenida (media en base 4): 2,26

Licenciatura en Biotecnología.
Universidad y Centro: Facultad de Ciencias Biológicas y Ambientales de la Universidad de León.
Fecha de terminación de estudios: Junio de 2011.
Calificación media obtenida (media en base 4): 2,04

FORMACIÓN ACADÉMICA COMPLEMENTARIA (16)

Denominación: 20th Annual ESDAR Conference
Organismos/Universidad/Centro: European Society for Domestic and Animal Reproduction
Duración/Número de créditos: 3 días Fechas inicio-fin: 27-29 Octubre de 2016.

Denominación: 2nd Training School of the Aquagamete COST Action FA1205 | *Molecular basis of fish gamete quality* Organismos/Universidad/Centro: Institut National de la Recherche Agronomique (INRA) Université de Rennes
Duración/Número de créditos: 4 días Fechas inicio-fin: 23-27 Junio 2014

Denominación: 1st Training School of the Aquagamete COST Action FA1205 | *Techniques for fish germline cryobanking* Organismos/Universidad/Centro: Instituto de Ciencias Marinas de Andalucía (Cádiz).
Duración/Número de créditos: 4 días. Fechas inicio-fin: 13-17 Mayo 2013

Denominación: VII Jornadas de Docencia de la Sociedad Española de Biología Celular
Organismos/Universidad/Centro: Universidad de León
Duración/Número de créditos: 2 días. Fechas inicio-fin: 23-24 de octubre 2014

Denominación: World Congress of Reproductive Biology 2014
Organismos/Universidad/Centro: Society for Reproduction and Fertility
Duración/Número de créditos: 2 días Fechas inicio-fin: 2-4 septiembre 2014

Denominación: Curso de Emprendimiento de base tecnológica (CEBT)
Organismos/Universidad/Centro: Fundación General de la Universidad de León y la Empresa (Fgulem)
Duración/Número de créditos: 50 horas Fechas inicio-fin: 3 abril-10 julio 2014

Denominación: 4th International Workshop on the Biology of Fish Gametes
Organismos/Universidad/Centro: Aquagamete COST Action FA1205
Duración/Número de créditos: 3 días Fechas inicio-fin: 17-10 septiembre 2013

Denominación: 8th European Zebrafish Meeting
Organismos/Universidad/Centro: European Zebrafish Network
Duración/Número de créditos: 3 días Fechas inicio-fin: 10-13 julio 2013

Denominación: IX Meeting of the Spanish Society for Developmental Biology
Organismos/Universidad/Centro: Sociedad Española de Biología del Desarrollo, Sociedad Portuguesa de Biología del Desarrollo y Sociedad America de Biología del Desarrollo.
Duración/Número de créditos: 2 días Fechas inicio-fin: 12-14 noviembre 2012

Denominación: "Foro Universidades: Patentes en los sectores químico-farmacéutico y biotecnológico".
Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 4 horas. Fechas inicio-fin: 8 octubre de 2010.

Denominación: I Congreso Nacional de la Biotecnología del vino. Jornadas "Vinomio: Biotec(E)nología". Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 10 horas. Fechas inicio-fin: 26-28 octubre de 2010.

Denominación: I Simposio "Bioderecho y Biotecnología".
Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 30 horas. Fechas inicio-fin: 12-13 noviembre de 2009.

Denominación: Proyecto DEBE (Divulgación de la Evolución y la Biología Evolutiva).
Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 10 horas. Fechas inicio-fin: 16 y 18 de noviembre de 2009.

Denominación: IV Congreso Interuniversitario de Biotecnología.
Organismos/Universidad/Centro: Universidad Politécnica de Valencia.
Duración/Número de créditos: 22 horas. Fechas inicio-fin: 8 - 11 de julio de 2009.

Denominación: III Congreso Interuniversitario de Biotecnología.
Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 10 horas. Fechas inicio-fin: 9 - 12 de julio de 2008.

Denominación: III Curso de Extensión Universitaria "Actualidad Científica y Cultural".
Organismos/Universidad/Centro: Universidad de León.
Duración/Número de créditos: 20 horas. Fechas inicio-fin: 17 de oct – 13 de dic 2007.

BECAS OBTENIDAS TRAS CONCURSO COMPETITIVO (9)

Organismo que concedió la beca: Aquagamete COST ACTION FA 1205
Ámbito de la beca (Local/ Nacional/ Internacional): Internacional
Finalidad de la beca (trabajo de investigación, etc.): Investigación | Estancia de doctorado.
Fechas de inicio y fin: 1-30 Junio 2016
Centro donde disfrutó de la beca: Centro de Ciéncias do Mar (CCMAR) Universidade do Algarve

Organismo que concedió la beca: Aquagamete COST ACTION FA1205
Ámbito de la beca (Local/ Nacional/ Internacional): Internacional
Finalidad de la beca (trabajo de investigación, etc.): Curso de especialización.
Fechas de inicio y fin: 23-27 Junio 2014
Centro donde disfrutó de la beca: Institut National de la Recherche Agronomique (INRA) Université de Rennes

Organismo que concedió la beca: Aquagamete COST ACTION FA1205
Ámbito de la beca (Local/ Nacional/ Internacional): Internacional
Finalidad de la beca (trabajo de investigación, etc.): Curso de especialización.
Fechas de inicio y fin: 13-17 Mayo 2013
Centro donde disfrutó de la beca: Instituto de Ciencias Marinas de Cádiz (ICMAN)

Organismo que concedió la beca: Junta de Castilla y León
Ámbito de la beca (Local/ Nacional/ Internacional): Regional
Finalidad de la beca (trabajo de investigación, etc.): Doctorado
Fechas de inicio y fin: Mayo 2013-Mayo 2017
Centro donde disfrutó de la beca: Departamento de Biología Molecular. Universidad de León

Organismo que concedió la beca: Banco Santander.
Ámbito de la beca (Local/ Nacional/ Internacional): Nacional.
Finalidad de la beca (trabajo de investigación, etc.): Prácticas Profesionales Santander CRUE – CEPYME.
Fechas de inicio y fin: 2 Julio – 28 de Septiembre de 2012.
Centro donde disfrutó de la beca: Instalaciones de MICROS VETERINARIA S.A. León.

Organismo que concedió la beca: Universidad de León.
Ámbito de la beca (Local/ Nacional/ Internacional): Nacional.
Finalidad de la beca (trabajo de investigación, etc.): Beca Erasmus Prácticas.
Fechas de inicio y fin: 1 de Julio – 30 de Septiembre de 2011.
Centro donde disfrutó de la beca: School of Life Sciences (Wellesbourne). University of Warwick (Reino Unido).

Organismo que concedió la beca: Universidad de León.
Ámbito de la beca (Local/ Nacional/ Internacional): Local.
Finalidad de la beca (trabajo de investigación, etc.): Prácticas de Cooperación Profesional.
Fechas de inicio y fin: 21 de Marzo – 28 de Junio de 2011.
Centro donde disfrutó de la beca: Instituto de Desarrollo Ganadero y Sanidad Animal de la Universidad de León (INDEGSAL).

Organismo que concedió la beca: Universidad de León.
Ámbito de la beca (Local/ Nacional/ Internacional): Local.
Finalidad de la beca (trabajo de investigación, etc.): Prácticas de Cooperación Profesional.
Fechas de inicio y fin: 2 de Agosto - 3 de Septiembre de 2010.
Centro donde disfrutó de la beca: Laboratorio Municipal de Aguas de la Ciudad de León.

Organismo que concedió la beca: Universidad de León.
Ámbito de la beca (Local/ Nacional/ Internacional): Local.
Finalidad de la beca (trabajo de investigación, etc.): Residencia de Verano en Grupos de investigación de la ULE.
Fechas de inicio y fin: 1 de Julio – 30 de Septiembre de 2009.
Centro donde disfrutó de la beca: Área de Genética de la Universidad de León.

IDIOMAS (1)

R=regular, B= bien, C= correctamente

Idioma	Habla	Lee	Escribe
INGLÉS	C	C	C
Certificado de Aptitud del Ciclo Superior de la Escuela Oficial de Idiomas de León (2008)			
Residente en Inglaterra durante 5 meses y medio.			

PUBLICACIONES (12)

CLAVE: Capítulo de libro
Autores (p. o. de firma): Robles V., Riesco M.F., **Valcarce D.G**
Título del capítulo: **Cryopreservation effect on genetic function: Neonatal outcomes.**
Título del libro: Cryopreservation of Mammalian Gametes and Embryos
Editors: Agarwal A., Nagy Z.P. and Varghese A.C.
Editorial: Springer Nature Año: 2017

CLAVE: Artículo
Autores (p. o. de firma): **Valcarce D.G**., Genovés S., Riesco M.F., Martorell P., Herráez M.P., Ramón D., Robles V.
Título: **Probiotic administration improves sperm quality in asthenozoospermic human donors**
Revista: Beneficial Microbes Año: 2017

CLAVE: Artículo
Autores (p. o. de firma): **Valcarce D.G.**, Robles V.
Título: **Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa.**
Revista: Reproduction Año: 2016

CLAVE: Artículo
Autores (p. o. de firma): **Valcarce D.G.**, Herráez M.P., Chereguini O., Rodríguez C., Robles V.
Título: **Selection of non-apoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*)**
Revista: Theriogenology Año: 2016

CLAVE: Revisión
Autores (p. o. de firma): Robles V., Herráez M.P., Labbé C., Cabrita E., Psenicka M., **Valcarce D.G.**, Riesco M.F.
Título: **Molecula basis of spermatogenesis and sperm quality**
Revista: General and Comparative Endocrinology Año: 2016

CLAVE: Artículo
Autores (p. o. de firma): Robles V., Riesco M.F., Psenicka M., Saito T., **Valcarce D.G.**, Cabrita E., Herráez M.P.
Título: **Biology of teleost primordial germ cells (PGCs) and spermatogonia: Biotechnological applications**
Revista: Aquaculture Año: 2016

CLAVE: Artículo
Autores (p. o. de firma): **Valcarce D.G.**, Pardo M.A., Riesco M.F., Cruz Z., Robles V.
Título: **Effect of diet supplementation with a commercial probiotic containing *Pediococcus acidilactici* (Lindner, 1887) on the expression of five quality markers in zebrafish (*Danio rerio* (Hamilton, 1822)) testis**
Revista: Journal of Applied Ichthyology Año: 2015

CLAVE: Artículo
Autores (p. o. de firma): Riesco M.F., **Valcarce D.G.**, Alfonso J., Herráez M.P. and Robles V.
Título:.***In vitro* generation of zebrafish PGC-like cells**
Revista: Biology of Reproduction Año: 2014

CLAVE: Revisión
Autores (p. o. de firma): Cabrita E., Martínez-Páramo S., Gavaia P.J., Riesco M.F., **Valcarce D.G.**, Sarasquete C., Herráez M.P., Robles V.
Título: **Factors enhancing fish sperm quality and emerging tools for sperm analysis**
Revista: Aquaculture Año: 2014

CLAVE: Artículo
Autores (p. o. de firma): **Valcarce D.G.**, Cartón-García F., Herráez M.P., Robles V.
Título: **Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development.**
Revista: Cryobiology Año: 2013

CLAVE: Artículo Autores (p. o. de firma):.**Valcarce D.G**., Cartón-García F., Riesco M.F., Herráez M.P., Robles V.
Título: **Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development**
Revista: Andrology Año: 2013

CLAVE: Artículo Autores (p. o. de firma): Guerra S.M., **Valcarce D.G.**, Cabrita E., Robles V.
Título: **Analysis of transcripts in gilthead seabream sperm and zebrafish testicular cells: mRNA profile as a predictor of gamete quality.**
Revista: Aquaculture Año: 2013

CONTRIBUCIONES EN CONGRESOS Y EVENTOS DE DIFUSIÓN CIENTÍFICA (13)

CONGRESOS INTERNACIONALES

Autores (p.o. de firma): **Valcarce D.G.**, Chereguini O., de la Hera M., Martín I., Herráez M.P., Rasines I., Rodríguez C., Robles V.
Título: ***Solea senegalensis* spermatozoa quality: are apoptotic cells and reactive oxygen species playing a role in F1 reproductive failure?**
Tipo de contribución (comunicación, póster, etc.): Póster.
Congreso /Evento: 20th Annual ESDAR Conference
Entidad/grupo organizador: European Society for Domestic and Animal Reproduction
Lugar de celebración:Lisboa (Portugal) Fecha: Octubre 2016

Autores (p.o. de firma): Robles V., Herráez M.P., Labbé C., Psenicka M., **Valcarce D.G.**, Riesco M.F.
Título: **Molecular basis of sperm quality**.
Tipo de contribución (comunicación, póster, etc.): Comunicación oral.
Congreso /Evento: 5th International Workshop on the Biology of Fish Gametes.
Entidad/grupo organizador: Aquagamete COST Action/Universita Politecnica Delle Marche.
Lugar de celebración:Ancona (Italia) Fecha: Septiembre 2015

Autores (p.o. de firma): **Valcarce D.G.**, Chereguini O., Herráez M.P., Rodríguez C., Robles V. Título: **Optimal sperm subpopulation selection in *Solea senegalensis***
Tipo de contribución (comunicación, póster, etc.): Póster.
Congreso /Evento: 5th International Workshop on the Biology of Fish Gametes.
Entidad/grupo organizador: Aquagamete COST Action/Universita Politecnica Delle Marche.
Lugar de celebración:Ancona (Italia) Fecha: Septiembre 2015

Autores (p.o. de firma): Riesco M.F., **Valcarce D.G.**, Lindo-Yugueros L., Sanz-Gómez N., Herráez M.P., Robles V. Título: ***vasa* promoter demethylation could be crucial on zebrafish Primordial Germ Cells in vitro generation**
Tipo de contribución (comunicación, póster, etc.): Póster. Congreso /Evento: 2014 Annual Conference of the Society for Reproduction and Fertility
Entidad/grupo organizador: Society for Reproduction and Fertility
Lugar de celebración:Edimburgo (Reino Unido) Fecha: Septiembre 2014

Autores (p.o. de firma): **Valcarce D.G**., Riesco M.F., Herráez M.P., Luengos-Martínez J.A., Suárez- Álvarez E.; Garrido-González F., Robles V.
Título: **Analysis of key sperm transcripts in human normozoospermic and asthenozoospermic samples after cryopreservation and vitrification.**
Tipo de contribución (comunicación, póster, etc.): Póster. Congreso /Evento: 2014 Annual Conference of the Society for Reproduction and Fertility
Entidad/grupo organizador: Society for Reproduction and Fertility
Lugar de celebración:Edimburgo (Reino Unido) Fecha: Septiembre 2014

Autores (p.o. de firma): Riesco M.F., **Valcarce D.G.**; Chereguini O., Herráez M.P., Cabrita E.
Título: **Cryopreservation of gilthead seabream (*Sparus aurata*) primordial germ cells**
Tipo de contribución (comunicación, póster, etc.): Póster.
Congreso /Evento: 4th International Workshop on the Biology of Fish Gametes
Entidad/grupo organizador: Centro de Ciéncias do Mar/Universidade do Algarve
Lugar de celebración: Albufeira (Portugal) Fecha: Septiembre 2013

Autores (p.o. de firma): Cabrita E., Martínez-Páramo S., Gavaia P.J., Pacchiarini T., **Valcarce D.G.**, Sarasquete C., Robles V.
Título: **Factors affecting sperm quality and emerging tools for sperm analysis**

Tipo de contribución (comunicación, póster, etc.): Comunicación oral.
Congreso /Evento: 6th Fish and Shellfish Larviculture symposium
Entidad/grupo organizador: Universitiet Gent, Norwegian University of Science and Technology and Larvanet
Lugar de celebración: Ghent (Bélgica) Fecha: Septiembre 2013

Autores (p.o. de firma): Riesco M.F., **Valcarce D.G.**, Gutiérrez L., Navarro-Lahuerta C., Sevilla-Movilla S., Robles V
Título: **Zebrafish blastomere differentiation to PGC-like cells**.
Tipo de contribución (comunicación, póster, etc.): Póster
Congreso /Evento: 8th European Zebrafish Meeting
Entidad/grupo organizador: European Zebrafish Network
Lugar de celebración:Barcelona (España) Fecha: Julio 2013

Autores (p.o. de firma): **Valcarce D.G.**, Cartón-García F, Herráez, M.P., Robles, V.
Título: **Study of the effect of cryopreservation on human mRNAs considered as potential fertility and pregnancy markers**.
Tipo de contribución (comunicación, póster, etc.): Póster.
Congreso /Evento: IX Meeting of the Spanish society for Developmental Biology.
Entidad/grupo organizador: Sociedad Española de Biología del Desarrollo.
Lugar de celebración:Granada (España). Fecha: Noviembre de 2012

Autores (p.o. de firma): Riesco, M.F.; **Valcarce, D.G.**; Robles, V.
Título: **An alternative cell source for genebanking in zebrafish: effect of cryopreservation procedures at genetic and epigenetic level**.
Tipo de contribución (comunicación, póster, etc.): Póster.
Congreso /Evento: IX Meeting of the Spanish society for Developmental Biology.
Entidad/grupo organizador: Sociedad Española de Biología del Desarrollo.
Lugar de celebración:Granada (España). Fecha: Noviembre de 2012

CONGRESOS NACIONALES

Autores (p.o. de firma): Herráez M.P., González-Rojo S., Lombó M., **Valcarce D.G.**
Título: **El aprendizaje mediante la comprensión, integración y divulgación de conocimientos: experiencia en estudiantes de primer curso de biotecnología**
Tipo de contribución (comunicación, póster, etc.): Comunicación Oral.
Congreso /Evento: VIII Jornadas de Docencia de la Sociedad Española de Biología Celular
Entidad/grupo organizador: Sociedad Española de Biología Celular
Lugar de celebración: Gerona (España) Fecha: Octubre 2016

Autores (p.o. de firma): Iturria I., Nacher-Vazquez M., **Valcarce D.G.**, Robles V., Rainieri S., López P., Pardo M.A.
Título**: El modelo *in vivo* pez cebra para evaluar las propiedades probioticas de bacterias en acuicultura**.
Tipo de contribución (comunicación, póster, etc.): Comunicación escrita.
Congreso /Evento: VI Workshop Probiotics, prebiotics and Health: Scientific Evidence.
Entidad/grupo organizador: Sociedad Española de Probióticos y Prebióticos.
Lugar de celebración:Oviedo (España) Fecha: Febrero 2015

Autores (p.o. de firma): González, J.; Polledo, L.; Martínez Fernández, B.; **Valcarce, D.G.**; García Iglesias, M.J.; Pérez Martínez, C.; García Marín, J.F.
Título: **Diagnóstico diferencial mediante técnicas anatomopatológicas de abortos ovinos causados por *Chlamydia abortus* o *Coxiella burnetti***.
Tipo de contribución (comunicación, póster, etc.): Comunicación oral.

Congreso /Evento: XVII Simposio Anual de la Asociación de veterinarios especialistas en diagnóstico de laboratorio (AVEDILA).
Entidad/grupo organizador: Asociación de veterinarios especialistas en diagnóstico de laboratorio (A VEDILA).
Lugar de celebración:Badajoz (España). Fecha: 18-19 de noviembre de 2012.

PROPIEDAD INDUSTRIAL E INTELECTUAL (1)

Título propiedad industrial registrada: **Empleo de probióticos en el incremento de la fertilidad masculina**
Inventores/autores/obtentores: Robles V., **Valcarce D.G.**, Ramón D., Genovés S., Martorell P., Chenoll M.E.
Código de ref: ES 2 575 828 A1 País de inscripción: España
Fecha de registro: 31/12/2014
Código de ref de Solicitud de PCT: WO 2016/107948 A1

ESTANCIAS EN CENTROS DE I+D+i RELACIONADOS CON SU FORMACIÓN UNIVERSITARIA (6)

Universidad/Centro: Instituto Español de Oceanografía (IEO) Planta de Cultivos El Bocal.
Motivo: Estancia de doctorado.
Lugar: Santander (España) Fecha inicio: 7 Noviembre 2016
Duración (semanas): 5

Universidad/Centro: Centro de Ciéncias do Mar (CCMAR) Universidade do Algarve
Motivo: Estancia de doctorado.
Lugar: Faro (Portugal) Fecha inicio: 1 Junio 2016
Duración (semanas): 4

Universidad/Centro: Instituto Español de Oceanografía (IEO) Planta de Cultivos El Bocal.
Motivo: Estancia de doctorado
Lugar: Santander (España) Fecha inicio: 26 Octubre 2015
Duración (semanas): 4

Universidad/Centro: Instituto Español de Oceanografía (IEO) Planta de Cultivos El Bocal.
Motivo: Estancia de doctorado
Lugar: Santande (España) Fecha inicio: 23 Febrero 2015
Duración (semanas): 8

Universidad/Centro: School of Life Sciences (Wellesbourne Campus). University of Warwick.
Motivo: Técnico de laboratorio colaborador en el grupo de investigación del Dr. J. Gutiérrez-Marcos.
Lugar: Coventry País: Reino Unido Fecha inicio: 1 Octubre 2011
Duración (semanas): 9

Universidad/Centro: School of Life Sciences (Wellesbourne Campus). University of Warwick.
Motivo: Beca Erasmus Prácticas.
Lugar: Coventry País: Reino Unido Fecha inicio: 1 Julio de 2011
Duración (semanas): 13

ACTIVIDAD DOCENTE

SUPERVISIÓN DE TRABAJOS DE FIN DE GRADO
Título del proyecto: Herencia paterna de los efectos de la exposición al disruptor endocrino EE2.
Alumna: Elena Vuelta Ramos
Tipo de docencia: Co-supervisión de proyecto de investigación
Titulación universitaria: Grado en Biotecnología Curso: 2016/2017
Créditos ECTS: 30

GRADO EN BIOLOGÍA
Nombre de la asignatura/curso: Organografía
Tipo de docencia: Prácticas de Laboratorio Curso que se imparte: 3
Frecuencia de la actividad: 1
Créditos Créditos ECTS: 4

GRADO EN BIOTECNOLOGÍA
Nombre de la asignatura/curso: Citología e Histología
Tipo de docencia: Seminarios Curso que se imparte: 2
Frecuencia de la actividad: 1
Créditos ECTS: 6

Nombre de la asignatura/curso: Biología Celular
Tipo de docencia: Prácticas de laboratorio. Curso que se imparte: 2
Frecuencia de la actividad: 1
Créditos ECTS: 8

GRADO EN CIENCIAS AMBIENTALES
Nombre de la asignatura/curso: Citología e Histología Animal y Vegetal
Tipo de docencia: Prácticas de Laboratorio Curso que se imparte: 2
Frecuencia de la actividad: 2
Créditos ECTS: 10

Nombre de la asignatura/curso: Biotecnología de la Reproducción
Tipo de docencia: Prácticas de Laboratorio Curso que se imparte: 4
Frecuencia de la actividad: 3
Créditos ECTS: 2

Nombre de la asignatura/curso: Biología Fundamental
Tipo de docencia: Prácticas de Laboratorio Curso que se imparte: 1
Frecuencia de la actividad: 2
Créditos ECTS: 8

CURSOS DE EXTENSIÓN UNIVERSITARIA
Nombre de la asignatura/curso: Técnicas experimentales de biología celular aplicadas a la biología de la reproducción
Tipo de docencia: Teoría y Prácticas de Laboratorio
Titulación universitaria: Curso de extensión universitaria.
Frecuencia de la actividad: 1
Créditos ECTS: 2 Entidad de realización: Universidad de León

PROYECTOS CON PARTICIPACIÓN

Como miembro del equipo investigador

Título: Mecanismos moleculares subyacentes al fallo reproductivo en teleósteos: desarrollo de nuevas estrategias y tratamientos para solventar la disfunción reprodutiva de la F1 de lenguado senegalés (Subproyecto 1)
IP: Vanesa Robles Rodríguez
Referencia del proyecto: AGL2015-68330-C2-1-R

Título: Efecto de contaminantes emergentes en peces: vías de actuación en la respuesta inmunitaria y en la reproducción (Subproyecto 3)
IP: M. Paz Herráez Ortega
Referencia del proyecto: AGL2014-53167-C3-3-R

Título: Aprendizaje basado en proyectos en entornos multidisciplinares para el fortalecimiento de las competencias transversales en titulaciones de grado de la Universidad de León
IP: Javier Alfonso Cendón

Como colaborador del equipo investigador

Título: Aplicación de análisis genéticos en la creación de bancos de germoplasma en teleósteos
IP: Vanesa Robles Rodríguez

Título: Caracterización, cultivo y criopreservación de PGCs y SSCs de peces
IP: Vanesa Robles Rodríguez
Referencia del proyecto: AGL2009-06994

PREMIOS

Descripción: Ganador del I Concurso Transfronterizo de Prototipos Orientados al Mercado (PROTOTRANSFER)
Entidad concesionaria: FUNDACION GENERAL DE LA UNIVERSIDAD DE LEON Y DE LA EMPRESA *Ciudad entidad concesionaria:* León (España)
Fecha de concesión: 01/04/2014

OTROS

Informática
Bioinformática Cálculo Estadística Análisis de imagen Análisis de vídeo
Herramientas del NCBI. Nociones básicas de Matlab. SPSS ImageJ | Photoshop | Adobe Illustrator | Tracker

Pertenencia a sociedades científicas
Miembro de la Sociedad Española de Biología Celular.
Miembro de la Sociedad Española de Acuicultura.

Participación en la organización de reuniones científicas o docentes
Miembro del Comité Organizador de las VII Jornadas de Docencia de la Sociedad Española de Biología Celular.

AGRADECIMIENTOS

Agradecimientos

Antes de comenzar: siento la extensión y la falta de seriedad académica propia de un documento como este en el presente apartado, pero oigan, para una vez que se puede explayar uno y le dejan unas líneas, tenía que aprovechar

***

Llegó el día, el último *deadline* y, la verdad, es que sólo puedo sonreír. Hace unos años jamás habría pensado estar escribiendo los agradecimientos de mi tesis doctoral y me hace mucha ilusión encontrarme ahora haciéndolo. El trabajo ha sido a veces desesperante, muy interesante pero sobre todo excitante: cambios de lugar, de especie y de compañeros. Ahora mismo no puedo más que encontrar todo como una grandísima oportunidad que se me ha brindado.

Tengo que dar las gracias a muchas personas: a unas académica/ profesionalmente, a otras muchas por haberme mantenido en pie durante este tiempo y a otras pocas por ambas cosas.

Me acuerdo perfectamente de cómo me fascinó aprender de pequeñín la existencia de las células y los orgánulos y de alucinar, literalmente, pensando en ellas como pequeñas ciudades. Del mismo modo, guardo en el cajón de recuerdos buenos cómo el primer día en la universidad entró por la puerta del aula una mujer pelirroja muy sonriente diciendo: "*Bienvenidos a Biotecnología, o mejor dicho, a corte y confección de genes".* Que esa mujer acabara siendo mi codirectora de tesis ha sido una suerte y un placer. **Paz**, gracias por ser tan cercana en todo momento (lejos de esos prototipos de catedráticos serios y de mirada imperturbable), por esforzarte en integrarme siempre, por ser un oráculo del conocimiento y por transmitirnos tu alegría constante e incansablemente. De menos joven, definitivamente, me gustaría llegar a parecerme un poco a ti. Eres sin duda, referente en múltiples aspectos.

Y *de tal palo tal astilla* (empiezo con refrán porque sé que te encantan), así llego a **Vanesa**, a mi directora de tesis, a la persona a la que más tengo que agradecer en este periodo. La verdad es que me pongo a pensar todo lo que me has enseñado en estos años y no puedo dejar de encontrar cosas. UN MONTÓN de cosas diferentes: a pensar de forma crítica, a desarrollar ideas, a buscar soluciones, a diseñar experimentos, a escribir, a corregir, a presentar, a entender cómo funciona este complicado y a veces extraño microcosmos científico, a priorizar en el trabajo, a aprovechar las oportunidades, a apostar cuando se cree en algo, a dirigir, a trabajar en equipo, a economizar, a sacarse ases de la manga, a encontrar sendas donde todo el mundo ve "imposibles", a ser previsor, a trabajar en plazos (tempranos si puede ser y *lo dejamos hecho :)*, a ser más políticamente correcto en los emails, a demostrar más mis emociones en el entorno laboral (tras aquellas nefastas reacciones robóticas iniciales) y un larguísimo etcétera. Además, has incluido un puñado de refranes babianos muy buenos en mi base de datos (aunque ese del arroz todavía me cuesta…). La verdad es que puedo presumir de supervisora y, ahora que acaba el vínculo académico, de amiga. Gracias por todo (menos por *la tabla de PGCs*, eso no te lo puedo agradecer ;) y por confiar en mí desde el principio. Espero que estés orgullosa de esta tesis-criatura.

A **Marta**, mi compañera en múltiples batallas, tengo mucho que agradecerle. Me enseñaste, sin dudarlo, todo lo que sabías de forma perfecta y lo mejor de todo, siempre envueltos en risas y un ambiente de amistad que hacen que tenga recuerdos increíbles de la primera etapa de tesis junto con Fer y Su. De **Fer** sólo puedo decir que es un verdadero orgullo tenerle como amigo, eres un crack de científico y mejor persona (estoy seguro de que la vida te traerá cosas buenas y si no es así tendremos que disfrutarla igual ;) De **Su** recordaré siempre como el respeto enorme que sentía por ti al llegar al INDEGSAL se

transformó en respeto+admiración+amistad. Gracias a los tres por todos aquellos momentos de felicidad en grupo.

Del resto del equipo inicial guardo millones de momentos geniales pero destaco: el veneno de **Reichel** (inolvidable, divertidísima y enérgica compañera), la risa de **Cintia,** la amistad de **Silvia S.,** la sinceridad y buen rollo de **Claudia,** la profesionalidad de **Natalia** y la simpatía de **Carmen**. Quiero recordar a **Cristina,** quien nos dejó demasiado pronto.

A mi equipo actual **Cris, Silvia y Marta Alevín** gracias por hacerme un huequecito y adoptarme. Os recordaré siempre como grandes científicas y trabajadoras incansables. Estoy seguro de que tendréis larguísimas carreras y podré presumir de conoceros. He aprendido mucho con vosotras. Me quedo con los buenos ratos que hemos pasado, con las risas en los cafés, los viajes, los miércoles *groumet*, los *zubrowkas* secretos y todos los momentos alegres.

Al Área de **Biología Celular** tengo que darle las gracias por haber sido un ecosistema INOLVIDABLE donde trabajar. Mis más sinceros agradecimientos a todos los habéis formado parte del área porque de todos he aprendido mucho de una forma u otra. Becarios: **Irene L., Neila, Diego, Berta, María, Quique, Irene F. y Paloma**; profesores **Felipe, Arsenio, Fito, Alberto, Carmen, Margot, Blanca y Paulino**; y técnicos de laboratorio **Pedro, Antonio, Indira, Cris** y **María R.** (gracias especialmente a ella por haberme enseñado histología, por hacerme los primeros minutos del día siempre tan alegres y entretenidos y por ser mi dietista particular :) Gracias también a **Elena V.** con la que he pasado muy buenos ratos trabajando codo con codo durante los últimos meses de tesis, y que además ha sido una *padawan* genial.

A **Eva** y a **Jenny**, mis compañeras de viaje académico desde primero de carrera hasta este año en el que acabamos la tesis los tres, gracias por haber sido más que colegas de facultad. Me hace mucha ilusión que los tres hayamos ido tan de la mano todos estos años. Sois unas científicas excelentes y tengo mucha suerte de teneros entre mis amigos.

También tengo que agradecer a las personas excepcionales que he conocido en mis estancias. La buena gente del IEO: **Chongui**, **Inma**, **Inés** pero sobre todo a **Nacho**, **Joserra** y a **Cris** que me trataron como uno más en mis estancias, y a los geniales **Elsa** y **Nacho** del CCMAR en Faro. También me gustaría recordar a toda la gente que he conocido en el entorno del **AQUAGAMETE y a la acción COST en sí, que me ha dado momentos internacionales irrepetibles**.

Fuera del mundo universitario tengo que dar las gracias a la gente de ***El Candil*** por hacerme ver todos los días la vida real fuera del laboratorio. Especialmente a **Estrella,** compañera y amiga desde aquellas reuniones en la facultad de Derecho a la hora de comer. Trabajar a vuestro lado intentando cambiar el mundo es un placer.

Evo (mi coordi y mi equipo). Apoyo aquí, allá o en el medio (café, cerveza, paseo, nada, comida de por medio…da igual). Cada uno de los recuerdos que compartimos (carrera, másteres, filosofadas, jueves de pizza en el 4 Kenilworth St…), cada conversación sanadora, cada risa "sin sentido" y más las "con sentido"...Todo me ha ayudado a conseguirlo. Porque sé que sabes leer en infinitivo, por eso: *agradecer.*

Vinculados a Evo en mi cabeza están **Javier Vences** y a **Paqui Vaquero** a los que quiero dar gracias por haberme dado mi primera oportunidad en un laboratorio y a **Lili Costa** y **José Gutiérrez Marcos** por inyectarme entre aquellas *Arabidopsis* en el HRI de Warwick esa pasión por la ciencia que derrocháis en todo lo que hacéis.

A mis amigos de TODA LA VIDA DESDE LA INFANCIA tengo una cosa que deciros: ¡chavales, que lo hice!¡Que lo conseguí *hojaldres*! **Pinchy, Kar, Lucy, Edu y Laury**: sois los mejores amigos del mundo (y los más *íntegros* como bien sabéis). Me habéis apoyado un montón todo este tiempo y siempre he sentido vuestro ánimo impulsándome incluso cuando os *daba la chapa* explicando cosas de *gafudo*, u os *sangraba la oreja* con mis peces. Sé que os hace mucha ilusión que lo haya conseguido *coro de chihuahuas.* A mí, ya sabéis que me *viene encantado* poder llegar al final de esta etapa de mi vida, entre otras cosas, porque la comparto con vosotros después de todos estos años, después de tantas etapas (y tapas), de historias, días *de estalle* y noches *de tormento*, asadurillas, yeres, tardes de café, blogs del Ejido, metrallas, viajes, terraceos, Plazas-del-Grano-en-todas-sus-versiones y todo lo demás. Al final no acabé estudiando *las uñas de las focas en Noruega* como profetizamos en 4º de la ESO pero bueno (*¿decimos mucho bueno?*) creo que estudiar *el semen de hombres y de peces* tiene su punto gracioso para explicar por el barrio (mención honorífica y pin de oro por el capítulo *Muestras de Villahierro*). Ojalá el Mali siguiera siendo nuestro Mali para celebrarlo allí, pero ya sabéis…*ya nada es lo que era, todo cambia menos nosotros* ;) Siento haber sido un poco pesado con el "hoy no puedo" y similares estos años pero creo que al final no quedé demasiado *psicopático*. Así que *un-cerebro-muchos-estómagos* a celebrarlo como si no hubiera mañana.

A **Ele**, mi musa azul, gracias por todo el apoyo que me has dado. Gracias por cogerme de la mano aquel invierno, por no haberla soltado y por haberla convertido en un andamio inamovible durante todo este tiempo. Has sido fundamental para que haya llegado hasta aquí. Gracias por animarme cuando estaba desanimado, por ilusionarme cada día, por compartir mis éxitos de forma tan sincera y real, por recordarme que sí que valgo en mis derrotas, por aconsejarme, ahuyentar mis fantasmas y por tus *misivas*. Gracias por mirarme con esos ojos y quemarme el azul del iris con alegría todos los días.

Ra, a ti tengo que agradecerte muchas cosas pero principalmente el hecho de ser siempre mi contrapunto en casi todo. Gracias por darme, como hermana mayor, lecciones de cruda realidad siempre que las he necesitado. Aunque creas que no es así, siempre he valorado mucho tus opiniones y recomendaciones. Sé que estás muy orgullosa de que lo haya logrado y me hace feliz sentirte así.

Y, por su puesto, gracias a **mi madre**. Quiero, aprovechando la oportunidad, agradecerte además de todo el apoyo, consejos y reflexiones que me has dado durante estos años de tesis, el haber sido mi referente vital. No conozco ninguna persona más fuerte, más buena, ni más amable y dudo que conozca a alguien como tú. Gracias por habernos sacado adelante a Ra y a mí como una leona, por saltar los obstáculos como nadie y por enseñarme a disfrutar de la vida. Menos mal que me *obligaste* cada mañana (en contra de mi voluntad) en primaria a ir al cole y que fuiste *tan* exigente conmigo *como dicen*. Sin eso, no estaría aquí. Siempre has dicho que lo más importante en esta vida es ser feliz y te prometo que lo estoy intentando lo máximo que puedo. Hoy lo estoy, entre otras cosas, porque sé que tú también lo estás.

Gracias también al resto de **mi familia** por haber mostrado siempre interés en mi trabajo, por trasladarme ánimos y sentirse orgullosos de mí.

Mentiría si dijera que no me pone nervioso empezar una nueva etapa, pero estoy ansioso por ver qué me depara el futuro. Cierro con estas líneas unos años de mi vida que recordaré con mucha alegría por encima de todo. Estoy muy feliz de haber conseguido terminar el doctorado y de que podáis estar leyendo esto. **Gracias, de verdad, a todos y a todas.**