UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Implicación de la adición de N-acetilcisteína en los medios de cultivo de gametos y embriones criopreservados

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2017

AGRADECIMIENTOS:

Agradece a la llama su luz, pero no olvides el pie del candil que paciente la sostiene. (Rabindranath Tagore). Por ello, empezaré por mostrar mi gratitud a Nicolás Montero (hombre Cluster) y a José Mijares por vincular mi beca Santander al CCMIJU, que permitió comenzar la aventura piramidal. A Paco y D. Jesús por la oportunidad de formar parte de "la casa". A Dieguito Celdrán por hacer más fácil el comienzo.

A mi director Ignacio Santiago Álvarez "Santi" por confiar en mí y obsequiarme con la oportunidad de encaminar mi futuro hacia la investigación. A Eva Miguel, por agilizar, mover, preguntar…..por facilitar los trámites de este proyecto desinteresadamente.

A mi querida amiga Carolina, mi salvavidas durante 5 años, mi ying y mi yang, mi hermana del CCMI, mi tesoro Mohedano… Y a mi otra reproductora, Nuria, gracias por las risas, los llantos, por ser mi compañera de cruzada. A Bea Moreno, por compartir codo con codo (literal) el día a día, los cotilleos y los buenos y no tan buenos ratos (emoticono de cara con corazones en los ojos).

A mis psicólogos personales Jorge, Mario y Alberto, mis hombros para llorar y mis fuerzas para seguir adelante. Gracias por vuestros consejos, opiniones, por abrirme los ojos y no permitir que me derrumbe.

A Antequera, Joaquín, Juanmi, Rebe, Vero, Javi, todo el "Club del taper", Eva, las Mari Joses, Pepi y Seba, a Iván, Ferni y Juan Carlos, gracias por hacer más grato el tiempo, por sacarme la sonrisa y por esos pequeños momentos que hacen especial el día.

No me olvido de Guillen, porque no habría podido continuar sin el hombre de calidad. Gracias por los desahogos y los silencios necesarios, por ser copiloto, conductor y amigo. A Chache, por los consejos de jardinería y las risas entre curvas, y resoplos de Guillermo, gracias.

Aristóteles dijo que algunos creen que para ser amigos es suficiente con querer, como si para estar sano bastara con desear la salud, por eso, Sami, Julia, Virginia, Claudia, Esther, Nuria (ilustración), Vela, Teresa (Mangas claro!), Vicente, Natalia, Luisa, Óscar, Añover y anteriormente mencionados, gracias por las cañas, los viajes, las barbacoas, los cafés después de sesiones clínicas, los cumpleaños, las despedidas…la terapia de grupo… por el mejor regalo de todos, la amistad!

Gracias al equipo de Animalario por cuidar, alimentar, medicar a los animalitos de la tesis y por las tertulias de almacén de pienso y sala de curas.

Al grupo SINTREP y al departamento de Fisiología de la UEx, en especial a Fran, por permitir usar sus equipos y ayudarme a analizar datos de esta Tesis.

Gracias a todos aquellos, miembros o no del CCMIJU, que no nombro personalmente pero han influido y marcado esta etapa de mi vida.

A todo el equipo de Alter Real, en especial a Elisa, Pipo, Ana Rita y Sofia por acogerme y cuidarme, por enseñarme y ayudarme sin pedir nada a cambio y por supuesto, por darme la oportunidad de optar a la mención internacional.

A Mariví y Patri, lo mejor y más bonito que saqué de la carrera y a José Ramón, mi apoyo en la distancia, por las tan preciadas y necesarias risas.

A mis padres y mi hermana, por su amor y apoyo incondicional. A mi abuela que gracias a que he heredado su cabezonería, testarudez y fuerza (bruta) he logrado llegar a la meta. Gracias al resto de familia (de sangre y política) por quererme. A mis apoyos pacenses (Lola, Raquel, Mada, Mariola, Humberto, Julia, Elena, Roci…) que sin ser conscientes, habéis hecho que momentos de desconexión sean la mejor de las terapias.

A mi maravilloso marido, el hombre más bueno de este mundo. Por tu comprensión, paciencia, amor infinito, por tus valores, virtudes y sobre todo por hacerme mejor persona.

Sin ti, San Lauro no habría citometría y sin ella no habría tesis… Gracias Dr. González-Fernández por dedicarme tiempo, por corregir, opinar, valorar, por ayudarme a publicar, por ser y estar.

A Bea aunque cuando la gratitud es tan absoluta las palabras sobran, dejo para el final el pilar más importante de este trabajo. Por aceptarme como doctoranda, por tu constancia y paciencia, por tus consejos y enseñanzas, por valorar mis virtudes y animarme,¡por hacer realidad lo que parecía imposible! por ser una fuente inagotable de conocimientos, por ser mi mentora, maestra y amiga. ¡GRACIAS!

GRACIAS A TODOS

Quien obra puede equivocarse,

pero quien no hace nada ya está equivocado.

Sta. Teresa de Ávila.

ÍNDICE

ABREVIATURAS

- ACPs: agentes crioprotectores
- ADN: ácido desoxirribonucleico
- ADN-mt: ácido desoxirribonucleico mitocondrial
- ALH: amplitud del desplazamiento lateral de la cabeza del espermatozoide
- ARN: ácido ribonucleico
- ATP: adenosín trifosfato
- CV: Coeficiente de variación
- DMF: dimetilformamida
- DMSO: dimetil sulóxido
- EO: estrés oxidativo
- EROs: especies reactivas de oxígeno
- FIV: fecundación *in vitro*
- GSH: glutatión
- hMMP: alto potencial de membrana mitocondrial
- ICSI: inyección intracitoplasmática de espermatozoides
- LH: hormona luteinizante
- LIN: índice de linealidad del espermatozoide
- MCI: masa celular interna
- MII: metafase II
- MMI: membrana celular interna
- NAC: N-acetilcisteína
- NADH: Nicotinamida adenina dinucleótido (coenzima I)
- NADPH: Nicotinamida adenina dinucleótido fosfato (enzima lactaldehído reductasa)
- REDOX: reacción reducción-oxidación
- PNs: pronúcleos
- VAP: velocidad media del espermatozoide
- VCL: velocidad curvilínea del espermatozoide
- VG: vesícula germinal
- VSL: velocidad rectilínea del espermatozoide
- ZP: zona pelúcida

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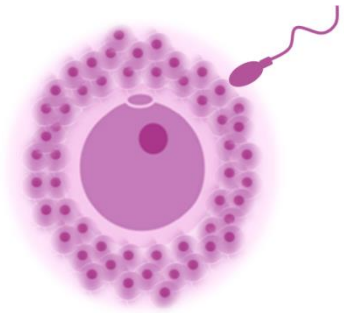
- ºC: grados centígrados
- min: minutos
- ml: mililitros
- (v/v): volumen/ volumen: porcentaje que representa el soluto dentro de la disolución
- mM: milimolar



INTRODUCCIÓN

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Los gametos masculinos y femeninos proceden de una estirpe celular específica denominada línea germinal que da lugar a las células germinales primordiales. En las etapas tempranas del desarrollo, las células germinales primordiales se diferencian en ovocitos (u oocitos) en el caso de las hembras y espermatozoides en el sexo masculino.

ESPERMATOZOIDE

Los espermatozoides son células altamente diferenciadas que se forman en los testículos en un proceso denominado espermatogénesis. Las células precursoras de los espermatozoides sufren una serie de divisiones y modificaciones en los túbulos seminíferos hasta alcanzar su pleno desarrollo morfológico. Aún después de esta maduración en el testículo, los espermatozoides deberán seguir sufriendo transformaciones en el epidídimo donde adquirirán capacidad móvil y fecundante.

A) ESTRUCTURA DEL ESPERMATOZOIDE

Desde un punto de vista morfológico, las estructuras principales del espermatozoide son la cabeza y la cola o flagelo y entre ambas se localiza una estructura denominada pieza conectora o intermedia [1]; rodeando a todas estas estructuras se encuentra la membrana plasmática.

1) Cabeza

La cabeza se encuentra recubierta por el acrosoma y contiene el núcleo y una pequeña cantidad de citoplasma. En la cabeza se pueden distinguir varias regiones, la región apical, la región ecuatorial y la región postacrosómica [2]. La región apical está situada en la parte anterior de la cabeza y contiene el acrosoma, en el extremo contrario se encuentra la región postacrosómica y entre estas dos regiones se encuentra la región ecuatorial. El acrosoma es una estructura vesicular derivada del aparato de Golgi que

se forma durante las últimas fases de la espermatogénesis. Está compuesto por una membrana acrosómica externa y una membrana acrosómica interna adyacente a la membrana plasmática que rodea a todo el espermatozoide. En su interior se encuentran diversas enzimas hidrolíticas que se liberarán al unirse el espermatozoide a la zona pelúcida del ovocito en la denominada reacción acrosómica. Esto permite la degradación de la zona pelúcida y la subsiguiente fusión del espermatozoide con el ovocito y, por tanto, la fecundación. El núcleo es la estructura que ocupa la mayor parte de la cabeza del espermatozoide y contiene el ADN en forma de cromatina muy compactada gracias a la presencia de protaminas. El alto grado de compactación facilita el transporte del ADN al ovocito evitando que el material genético se dañe durante el trayecto.

2) Flagelo

El flagelo es la estructura responsable del movimiento y está constituido a su vez por tres partes: la *pieza intermedia*, la *pieza principal* y la *pieza terminal* (Figura 1). La *pieza principal* ocupa la mayor parte del flagelo y está compuesta por una vaina fibrosa que rodea a una estructura llamada axonema y a las fibras densas. El "axonema", está constituido por 9 pares de microtúbulos de dineína dispuestos longitudinalmente y un par central, siendo la estructura responsable de la motilidad. Dado que el axonema está anclado a la base de la cabeza, la fuerza de deslizamiento global de esta estructura será la que genere el desplazamiento del espermatozoide. En la *pieza intermedia* se encuentra la vaina mitocondrial formada por mitocondrias dispuestas helicoidalmente que generarán la energía necesaria para el movimiento [3]. Por último, la *pieza terminal* constituye el extremo final de la cola y está compuesta únicamente por el axonema sin vaina fibrosa.

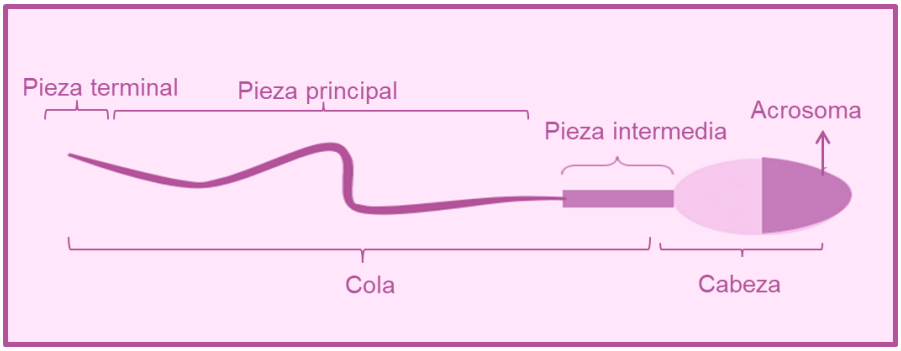


Figura 1: Estructura del espermatozoide.

B) ESPERMATOGÉNESIS

Durante la espermatogénesis tiene lugar la producción de espermatozoides y se divide en dos procesos básicos, la espermatocitogénesis y la espermiogénesis. La espermatocitogénesis comprende divisiones mitóticas y meióticas que finalmente originan los espermatozoides, mientras que la espermiogénesis se refiere a la fase de maduración de las espermátidas formadas en la fase anterior. En la mayoría de las especies de mamíferos la primera oleada de la *espermatocitogénesis* se inicia en el feto, cuando las células germinales llamadas gonocitos primordiales se diferencian para formar las espermatogonias (2n). El proceso se reinicia poco antes de llegar a la pubertad, cuando las espermatogonias se diferencian en espermatogonia tipo A (2n) que marca el inicio de la espermatogénesis y va a durar toda la vida del individuo sano. Las espermatogonias tipo A proliferan mediante divisiones mitóticas y algunas se diferencian para formar espermatogonias tipo B (2n) [4]. Cuando los individuos alcanzan la madurez sexual, las espermatogonias tipo B incrementan su tamaño para convertirse en espermatocitos primarios. Estos espermatocitos entran en una profase prolongada seguida de una finalización rápida de la meiosis I y la formación transitoria de espermatocitos secundarios que, al finalizar la segunda división meiótica, formarán espermátidas haploides. En la *espermiogénesis* se produce la transformación de la espermátida globular en un espermatozoide aún inmaduro desde un punto de vista funcional, pero prácticamente conformado desde un punto de vista estructural. La maduración de las espermátidas para dar lugar a los espermatozoides implica fundamentalmente la reducción del tamaño nuclear, la condensación del material genético y la sustitución de las histonas por protaminas. Además, tiene lugar la formación de la vesícula acrosómica a partir del aparato de Golgi, la formación de un flagelo a partir de la región centriolar, la ordenación de las mitocondrias en la pieza intermedia, la reducción del citoplasma y la formación del cuerpo residual [4].

C) MADURACIÓN EN EL EPIDÍDIMO

Los túbulos seminíferos vacían su contenido en la *rete testis* que transportan los espermatozoides y el fluido contenido en dichos túbulos hasta el epidídimo. En los

túbulos, las células principales crean una barrera inmunoprotectora que es necesaria para la correcta maduración de los espermatozoides [5]. El epidídimo es un conducto sinuoso que se divide anatómicamente en 3 segmentos: *cabeza, cuerpo y cola*.

El epidídimo también aporta un medio adecuado en el que los espermatozoides se concentran, maduran y adquieren capacidad fecundante y mótil. Para ello, los espermatozoides procedentes de la *rete testis* (red de testis) deben migrar a través de la cabeza y el cuerpo del epidídimo donde sufrirán modificaciones morfológicas y funcionales gracias a las secreciones procedentes de las células epiteliales que lo forman. En conjunto a estas modificaciones se las denomina "maduración epididimaria". En la mayoría de los mamíferos, el espermatozoide tarda de 2 a 5 días en atravesarlo. Una vez que los espermatozoides completan este tránsito se almacenan en la cola del epidídimo hasta la eyaculación. La duración del almacenamiento es variable entre especies, pero puede oscilar entre 3 y 13 días. Finalmente, los espermatozoides que desembocan en la cola del epidídimo junto con los procedentes del conducto deferente, constituyen un reservorio espermático conocido como "reserva extragonadal"[5-7].

OVOCITOS:

Los ovocitos son célula germinales femeninas que está en proceso de convertirse en un óvulo maduro. Su función es la formación de un zigoto al fusionar su núcleo con el del gameto masculino.

A) ESTRUCTURA DEL OVOCITO

El ovocito presenta una estructura celular en forma de esfera. En su etapa más temprana de crecimiento, la mayoría de los orgánulos subcelulares se encuentran agrupados alrededor del *núcleo* en el *citoplasma*, formando lo que se conoce como *corpúsculo de Balbiani* [8]. La *membrana plasmática* es una estructura que engloba a las células y define sus límites. Dicha membrana está formada principalmente por fosfolípidos, colesterol, glúcidos y proteínas. La principal característica de esta barrera

es su permeabilidad selectiva, lo que le permite seleccionar las moléculas que entran y salen de la célula. De esta forma se mantiene estable el medio intracelular, regulando el paso de agua, iones y metabolitos, a la vez que mantiene el potencial electroquímico.

Los ovocitos de mamíferos (Figura 2) se encuentran rodeados por varias capas de células de la granulosa que constituyen el cúmulo oóforo (*cumulus oophorus*) [9]. La parte más interna de la capa de células se denomina corona radiada y esta formada por dos o tres capas de células de la granulosa unidas a la capa protectora más externa del ovocito, la zona pelúcida (Figura 2). Las células de la corona radiada se comunican con el ovocito a través de uniones intercelulares tipo "gap" y permiten el intercambio de moléculas entre el ovocito, las células de la granulosa y la circulación sanguínea, con una finalidad nutritiva y reguladora [10]. La cubierta más externa del ovocito es la *zona pelúcida* (ZP) [11]; está compuesta por distintas glicoproteínas y su composición varía dependiendo de las especie [12] y se encuentran agrupadas en familias: ZP1, ZP2, ZP3 y ZP4 según sus propiedades inmunológicas y funcionales. La zona pelúcida se encarga de la protección del ovocito y del embrión. Durante la fecundación lo espermatozoides son los encargados de llevar a cabo la disgregación de las células del cúmulo gracias a la liberación de enzimas hidrolíticas localizadas en el acrosoma. La importancia de la ZP en mamíferos radica en que está implicada en la unión específica espermatozoide-ovocito, la inducción de la reacción acrosómica y el bloqueo de la polispermia. El espacio existente entre el citoplasma y la ZP se denomina *espacio perivitelino*. Adyacente a la membrana plasmática, hacia el lado citosólico, se encuentran los *gránulos corticales* de secreción, constituyendo el córtex del citoplasma del óvulo. Los gránulos de secreción son un tipo especial de lisosomas primarios formados a partir del complejo de Golgi y del retículo endoplasmático y compuestos por glicoproteínas y enzimas hidrolíticas. El ovocito en estado maduro (MII); es decir, cuando posee la capacidad de ser fecundado, presenta el llamado corpúsculo polar.

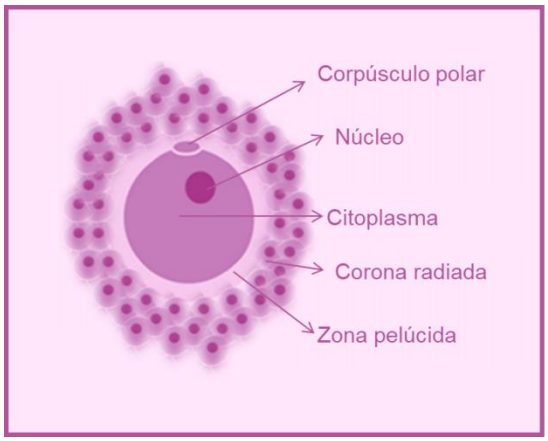


Figura 2: Estructura del ovocito.

B) OVOGÉNESIS

La ovogénesis es el desarrollo y diferenciación del gameto femenino (ovocito) mediante una división meiótica produciéndose, a partir de una célula diploide, una célula haploide funcional (óvulo). La formación de los ovocitos es un evento temprano en el desarrollo embrionario que se produce en el endodermo del saco vitelino a partir de células germinales primordiales que emigran hasta la cresta gonadal antes de diferenciarse. Durante el desarrollo fetal, las células germinales primordiales del ovario van a sufrir un proceso de diferenciación mediante sucesivas divisiones mitóticas transformándose en ovogonias, que darán lugar a los ovocitos primarios al iniciarse la primera división meiótica [13]. Toda la población de ovocitos entra en meiosis sincrónicamente en la vida fetal coincidiendo con el nacimiento o momentos posteriores cercanos, produciéndose la primera detención de la meiosis en profase I. En este momento, el ovocito comienza a ser rodeado por células pre-granulosas que forman una membrana basal generando el compartimento folicular [14].

MADURACIÓN

El proceso de maduración hace referencia a todos los cambios nucleares, citoplasmáticos y de membrana que sufre el ovocito con el fin de prepararse para la fecundación y el desarrollo embrionario [15]. La maduración es un proceso que

requiere de una red de señalización endocrina, paracrina y autocrina que implica la interacción directa entre el ovocito y las células de la granulosa que forman el cúmulo [16].

Los ovocitos en estado latente en la profase I presentan un núcleo prominente denominado vesícula germinal (VG) y están rodeados de una capa de células epiteliales (foliculares) no proliferativas conocidas como células pre-granulosas, que están implicadas en el crecimiento folicular y en el mantenimiento de la inhibición de la meiosis del ovocito. El conjunto del ovocito primordial y la capa de células de la granulosa que lo rodean forma una unidad funcional denominada folículo primordial, siendo estos la fuente de gametos femeninos en el organismo sexualmente maduro.

La maduración nuclear comprende toda una serie de procesos moleculares que permiten la reanudación de la meiosis (desde profase I) hasta la progresión hacia el estadio MII, con una nueva detención en este estadio a la espera de la fecundación. La maduración nuclear se inicia con la disolución de la membrana nuclear, un proceso conocido como "rotura de la vesícula germinal" progresando al estadio de metafase I en el que se produce la segregación de los cromosomas homólogos; la progresión de la meiosis dará lugar a la extrusión del primer corpúsculo polar y el paso de ovocito al estadio MII. La maduración nuclear finaliza cuando el ovocito alcanza el estadio de MII tras el pico de LH [17]. *La maduración citoplasmática* se refiere a los procesos o mecanismos moleculares no directamente relacionados con la progresión de la meiosis que acompañan a la maduración nuclear y preparan al ovocito para su activación, la formación de los pronúcleos, la fecundación y el posterior desarrollo embrionario [18]. Tras la rotura de la VG se produce una redistribución de los gránulos corticales y de las mitocondrias hacia una posición perinuclear que es necesaria para la progresión de la maduración ovocitaria [19, 20]. Además, los gránulos corticales aumentan de número al final del periodo de maduración y migran hacia la periferia del ovocito, situándose debajo de la membrana plasmática.

DESARROLLO EMBRIONARIO:

Tras la fecundación, es decir, la unión espermatozoide-ovocito, se formarán los pronúcleos (PNs), uno procedente del óvulo y otro del espermatozoide y se observará

la presencia de dos corpúsculos polares. A este estadio embrionario se le denomina zigoto (Figura 3). El tiempo de desarrollo embrionario, marcado por la división celular mediante divisiones mitóticas, va a depender de la especie. Tras la primera división celular por mitosis, el embrión presentará 2 células; cada una de esas células a su vez sufrirá otra división mitótica y dará lugar a un embrión de 4 células. Tras sucesivas mitosis, el embrión alcanza el estadio de mórula en el que presentan generalmente de más de 16 células o blastómeras. En ese estadio, comienzan a observarse uniones entre las membranas de las células pero éstas pueden distinguirse unas de otras y se denominan mórulas no compactadas. Estas uniones van en aumento hasta una compactación completa o mórula compacta. El siguiente estadio embrionario se denomina blastocisto y se caracteriza por que el embrión presenta al menos 64 células [21].

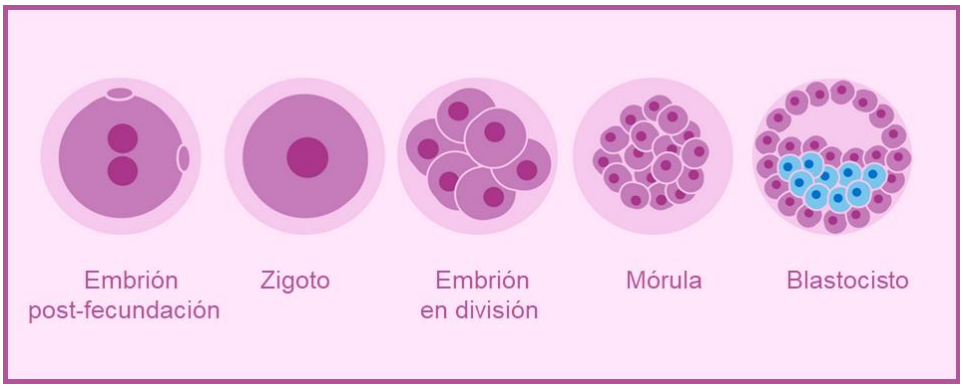


Figura 3. Desarrollo embrionario.

Los blastocistos presentan dos estructuras claves en su morfología: la masa celular interna (MCI), que va a originar las capas embrionarias que darán lugar a los órganos del feto y el trofoectodermo o masa celular externa, que originará la placenta. Entre ambas estructuras se encuentra el blastocele que es una cavidad central llena de líquido apenas visible en el blastocisto temprano.

Durante su posterior desarrollo, el blastocisto comienza a cavitar para formar blastocele y el trofoblasto y la MCI se distinguen claramente. Paralelamente, la zona pelúcida se vuelve más delgada permitiendo la expansión del blastocisto (blastocisto expandido) y su salida para la implantación en el endometrio (eclosión o hatching) [22] (Figura 4). La expansión del blastocisto es imprescindible para que pueda tener lugar la

implantación del embrión en el útero, por lo que el desarrollo embrionario *in vitro* hasta este estadio se considera como un buen marcador de calidad embrionaria [23].

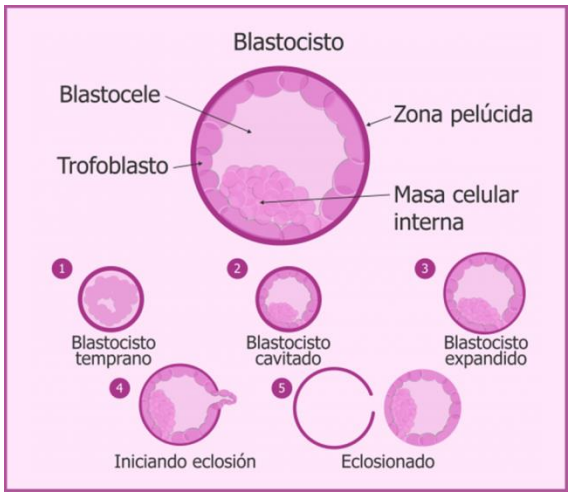


Figura 4. Descripción y evolución del blastocisto [24].

CRIOPRESERVACIÓN:

La capacidad de criopreservar gametos o embriones de mamíferos se ha convertido en una técnica fundamental en reproducción asistida para preservar especies en peligro de extinción con el fin de crear de criobancos y para la producción *in vitro* de embriones de especies ganaderas de interés [25, 26].

A pesar de las diferencias fisiológicas, el éxito de los procedimientos de criopreservación van a depender del tipo celular, de la distribución de los compuestos crioprotectores en los espacios intra y extracelular, de la velocidad de enfriamiento (enfriamiento lento, rápido o muy rápido) y del mecanismo de solidificación de la solución que contiene la muestra biológica (congelación o vitrificación).

AGENTES CRIOPROTECTORES

Los agentes crioprotectores (ACPs) son sustancias que evitan o minimizan la formación de cristales de hielo. Estas sustancias se añaden a los medios de congelación

y vitrificación y actúan reduciendo el estrés físico y químico que se genera durante el proceso. Los crioprotectores se clasifican en 2 grandes grupos: permeables y no permeables. Los *permeables* (glicerol, dimetilsulfóxido, etilenglicol, amidas, propilenglicol, etc.) producen una reorganización de los lípidos y las proteínas de la membrana dando lugar a un incremento en la fluidez, mayor deshidratación a temperaturas más bajas, menor formación de hielo intracelular y mejor supervivencia al proceso de criopreservación. Estos crioprotectores permeables tienen además la capacidad de disolver azúcares y sales en el medio de congelación. Por otro lado, los crioprotectores *no permeables* (trehalosa, aminoácidos, dextranos y azúcares) no atraviesan la membrana plasmática y solo actúan en el medio extracelular. Estos crioprotectores pueden alterar la membrana plasmática o actuar como solutos, disminuyendo la temperatura de congelación del medio y la formación de hielo extracelular [27].

La adición de crioprotectores provoca un cambio de volumen en las células en respuesta a los cambios osmóticos extracelulares perdiendo o captando agua. La capacidad de las células para cambiar su volumen viene determinada por la velocidad de transporte de agua a través de la membrana celular y la energía de activación para el transporte de agua [28]. A su vez, la permeabilidad de la membrana está influida por la composición de fosfolípidos, proteínas, canales iónicos, contenido de colesterol y elementos del citoesqueleto [29, 30]. Los agentes crioprotectores aumentan la permeabilidad de la membrana al agua y, por tanto, facilitan la deshidratación celular durante la congelación [30, 31]. Una vez compensada la fuerza osmótica, la célula vuelve a hidratarse.

TIPOS DE CRIOPRESERVACIÓN

Hablamos de congelación convencional a baja velocidad o congelación lenta cuando se reduce la temperatura de la muestra a una velocidad controlada durante la criopreservación. Se basa en la deshidratación celular por enfriamiento en una solución con bajas concentraciones de crioprotectores en la que se induce una disminución gradual de temperatura antes de almacenarse en nitrógeno líquido. La *congelación lenta* es un proceso largo de más de 110 minutos aunque su duración depende de la

especie y del tipo celular, utilizándose distintas rampas de enfriamiento. Este tipo de criopreservación comienza con una tasa de enfriamiento de 2ºC/min hasta los -7ºC para ovocitos y embriones y de 0,1º C /min para espermatozoides, temperatura a la que se realiza el *seeding*. El *seeding* consiste en la inducción de la formación del primer núcleo de hielo en la solución donde se encuentran las células. Ese proceso provoca el aumento de la concentración de solutos de la parte líquida lo que supone una deshidratación celular. La siguiente rampa abarca de 0,3ºC a -30ºC/min para embriones y de entre 10 a -60ºC/min hasta llegar a -80º C para los espermatozoides. Una vez alcanzada las temperatura anteriormente mencionadas, las muestras continúan con una rampa de enfriamiento de -50ºC/min hasta los -196ºC [32], temperatura a la que se almacena. El término vitrificación se aplica a procesos muy rápidos de inmersión directa en nitrógeno líquido. Aunque el tiempo del proceso depende de la especie y del tipo celular, éste no supera los 15 minutos de duración. Dependiendo del tipo de soporte (abierto o cerrado) y del volumen de la solución, la velocidad de enfriamiento oscilará entre 1.000ºC/min y 50.000ºC/min [33, 34]

Las principales diferencias entre ambos procesos radican en la adición y concentración de los agentes crioprotectores y la velocidad de enfriamiento [35] (Figura 5). La criopreservación de ovocitos y embriones en la mayoría de especies animales se lleva a cabo principalmente mediante vitrificación [36, 37] y en el caso de los espermatozoides, la congelación lenta es el método utilizado [38].

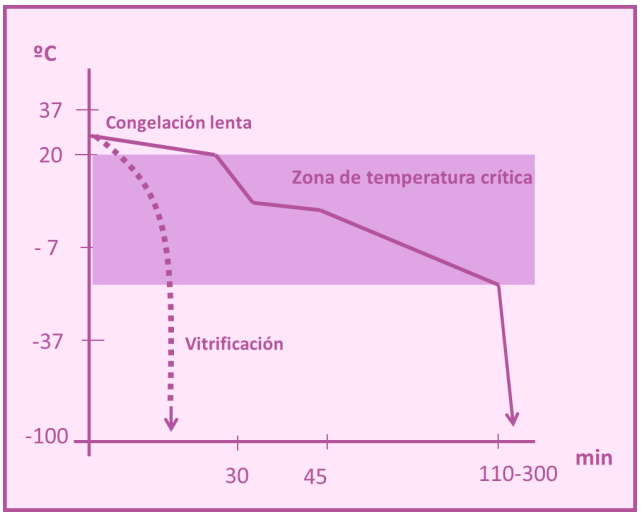


Figura 5. Diagrama representativo de las rampas de enfriamiento.

CONGELACIÓN LENTA: PARTICULARIDADES

La criopreservación de gametos facilita el transporte y el almacenamiento para su uso en técnicas de reproducción asistida a largo plazo. Sin embargo, existe un alto grado de variación en la supervivencia después de la descongelación entre especies, individuos y tipos celulares congelados. La congelación lenta es la más utilizada para la criopreservación de espermatozoides y, por haber sido el método utilizado en nuestros experimentos, a partir de ahora solo nos referiremos a su uso en gametos masculinos.

En general, el ACP más utilizados para la congelación de espermatozoides es el glicerol y se añade a concentraciones que oscilan entre un 5 y 7% (v/v) [39-42]. Recientemente, se ha combinado con el uso de amidas (metilformamida y dimetilformamida), que gracias a su bajo peso molecular, mejora las tasas post-descongelación [43], motivo por el cual, en la presente Tesis Doctoral hemos utilizado esta combinación de crioprotectores para la congelación de semen de la especie bovina.

Además, los medios de congelación lenta de espermatozoides suelen suplementarse con agentes modificadores y protectores de membrana como la leche desnatada o la yema de huevo [32, 44, 45]. La congelación convencional del semen envasado en pajuelas se realiza usando diferentes velocidades de enfriamiento que a su vez dependen del volumen de las pajuelas utilizados (0.25 o 0.5 ml). Debe resaltarse que la composición del medio de congelación debe ser adaptada para cada especie e incluso a cada individuo con el fin de optimizar el proceso y maximizar el número de espermatozoides que mantengan su capacidad fecundante [46].

VITRIFICACIÓN: PARTICULARIDADES

Cada año, cientos de miles de animales domésticos se producen a partir de embriones y ovocitos criopreservados [25, 47, 48]. Concretamente la vitrificación, es una técnica de criopreservación en la que no se produce cristalización ni formación de hielo [49], sino que la solidificación viene determinada por la extrema viscosidad del

medio, que se enfría a velocidades muy elevadas permitiendo que la muestra atraviese con rapidez los intervalos térmicos nocivos. Otra propiedad positiva de la vitrificación es la acusada disminución de las lesiones por enfriamiento; sin embargo, tiene el inconveniente de requerir una alta concentración de crioprotectores, que aumenta el efecto tóxico de los mismos [50]. El procedimiento básico de vitrificación consiste en someter a los embriones u ovocitos a solución de crioprotector o mezcla de ellos entre el 5 % y el 15% durante un tiempo variable, según la cantidad de agua, de entre 5-15 minutos; este paso se conoce comúnmente como equilibrado. Posteriormente, se exponen brevemente (< 1 minuto) a una solución de vitrificación que presenta una concentración de crioprotector más elevada que oscila entre el 15-40% (v/v). El porcentaje y tipo de crioprotector utilizados depende de la especie. Los agentes crioprotectores permeables más usados son el etilenglicol, el propilenglicol, el DMSO y el glicerol en combinación con un crioprotector no permeable (generalmente sacarosa o galactosa) [51-53]. La alta presión osmótica de los crioprotectores causa una rápida deshidratación de las células. En ese tiempo, los embriones u ovocitos deben colocarse en los soportes especiales diseñados para su almacenamiento en nitrógeno líquido.

SISTEMAS DE ALMACENAJE

Encontramos dos tipos distintos de dispositivos de almacenaje: los *sistemas abiertos*, los cuales ponen el material biológico en contacto directo con el nitrógeno líquido y los *dispositivos cerrados*, que aíslan las células del nitrógeno líquido. Normalmente, los eyaculados se almacenan en sistemas cerrados en pajuelas de plástico de 0.25 o 0.5 ml (dependiendo de la especie) proporcionando una superficie/volumen grande. Sin embargo, existe controversia sobre cuál es el soporte de almacenamiento más adecuado para ovocitos y embriones. Normalmente, se utilizan soportes que consisten en una fina tira a modo de lengüeta unida a un mango de plástico resistente al nitrógeno líquido. Estos soportes los podemos encontrar a su vez de las dos maneras anteriormente descritas: abiertos o cerrados. Su diseño permite realizar la carga del material biológico con un volumen mínimo de medio de vitrificación (0.1 µl aprox.) [54]. Al disminuir considerablemente el volumen del medio que contiene las células que se van a vitrificar, se incrementa la velocidad de

vitrificación, lo que a su vez permite reducir la concentración de crioprotectores, y con ello, los efectos tóxicos [55]. Algunos estudios sugieren que el uso de sistemas cerrados es más seguro puesto que eliminan la posibilidad de contaminación durante el proceso y la conservación [56]. Aunque en la bibliografía encontramos estudios contradictorios respecto a los sistemas descritos [57] son común e indistintamente utilizados para la criopreservación de ovocitos y embriones.

SENSIBILIDAD A LA CRIOPRESERVACIÓN

Existe una óptima tasa de enfriamiento para cada tipo de célula que depende del volumen y de la composición de la membrana. Los ovocitos y los primeros estadios de desarrollo embrionario presentan una tolerancia menor a la criopreservación que los embriones en estadios de desarrollo más avanzados [54]. Este fenómeno es debido a que la relación núcleo-citoplasma es menor cuanto mayor es el número de células del embrión [58] y a la menor permeabilidad de la zona pelúcida [59, 60].

La sensibilidad para la congelación también está asociada al contenido de lípidos de la membrana y del citoplasma, que varía dependiendo de las condiciones de producción y cultivo de los embriones (*in vivo* vs. *in vitro*); por ejemplo, los embriones producidos *in vivo* presentan tasas de supervivencia mayores tras la criopreservación que los generados *in vitro* [54].

DAÑOS INDUCIDOS POR LA CRIOPRESERVACIÓN

Los daños asociados a la criopreservación se pueden dividir en 2 tipos: daños físicos (producidos por la formación de cristales de hielo y los cambios de volumen) y daños químicos provocados por los cambios de las concentraciones de solutos [28]. Durante el proceso de criopreservación, el agua del medio se congela por lo que la concentración relativa de los solutos en el medio extracelular incrementa, aumentando la osmolaridad del mismo [61]. Además, el incremento relativo de la concentración de crioprotectores produce un efecto tóxico [62] y un choque hiperosmótico induciendo una disminución del volumen celular durante la congelación. Durante el proceso de descongelación encontramos el fenómeno inverso; es decir, a medida que

el agua del medio se descongela, la célula experimenta un choque hipotónico y un incremento de volumen. Estos cambios de volumen producen un efecto deletéreo *per se* en las células, afectando específicamente a la viabilidad en el caso de los espermatozoides [63]. En espermatozoides, ovocitos y embriones, se ha demostrado previamente que las alteraciones en las propiedades físicas y químicas producidas por la criopreservación, afectan la integridad de la membrana celular y del citoesqueleto, inducen la despolarización mitocondrial y disminuyen la concentración de ATP intracelular, desencadenando la apoptosis y aumentando la producción de especies reactivas de oxígeno (EROs) [64-67]. Está descrito que el estrés oxidativo conduce a una disminución en la tasa de supervivencia y de desarrollo de los embriones tras la descongelación, siendo las mitocondrias la principal fuente de EROs [68, 69]. Las EROs, a su vez, inducen peroxidación lipídica de la membrana que afecta a la división celular, al transporte de metabolitos, inducen la fragmentación del ADN nuclear y disfunciones mitocondriales. Por otro lado, la adecuada distribución de estos orgánulos y el correcto mantenimiento del potencial de membrana mitocondrial aseguran el desarrollo y la función celular adecuada en embriones, espermatozoides y ovocitos [70].

ESTRUCTURA Y FUNCIÓN DE LA MITOCONDRIA:

Las mitocondrias son orgánulos subcelulares altamente organizados. En los ovocitos maduros las mitocondrias se sitúan en una posición perinuclear, siendo de vital importancia para la fecundación. En los embriones, las mitocondrias se encuentran distribuidas por el citoplasma de las blastómeras y en los espermatozoides las encontramos dispuestas helicoidalmente en la pieza intermedia. Estos orgánulos poseen una membrana externa, una membrana interna, un espacio intermembranoso y un espacio interno delimitado por la membrana interna denominado matriz mitocondrial. La membrana mitocondrial externa es altamente permeable y contiene canales que permiten el intercambio de sustancias. En la membrana mitocondrial interna se localizan las denominadas crestas mitocondriales que forman un compartimento distinto del resto de la membrana interna. El número y forma de las crestas supone un reflejo de la actividad celular, siendo su función principal la

producción de energía en forma de adenosín trifosfato (ATP) en un proceso conocido como fosforilación oxidativa. La fosforilación oxidativa consta de dos tipos de reacciones: la cadena de transporte de electrones que libera energía y un proceso endergónico de síntesis de ATP, que utiliza esa energía para llevar a cabo sus reacciones. La cadena de transporte de electrones está formada por tres complejos de proteínas principales (complejo I, III, IV), y varios complejos "auxiliares", utilizando una variedad de donantes y aceptores de electrones. Los tres complejos se asocian en supercomplejos para canalizar las moléculas transportadoras de electrones, la coenzima Q y el citocromo c, haciendo más eficiente el proceso. El movimiento de protones crea un gradiente electroquímico a través de la membrana donde la energía se almacena mayormente como la diferencia de potenciales eléctricos en la mitocondria. La ATP-sintasa situada en la membrana mitocondrial interna (MMI), libera esta energía almacenada completando el circuito y permitiendo a los protones fluir a través del gradiente electroquímico. Tanto la cadena de transporte de electrones como la ATP-sintasa, están embebidos en la membrana, y la energía se transfiere desde la cadena de transporte de electrones a la ATP-sintasa por el movimiento de protones a través de la membrana, en un proceso llamado quimiósmosis.

Pesar de que la fosforilación oxidativa es una parte vital del metabolismo, produce una pequeña proporción de especies reactivas de oxígeno tales como aniones superóxido y peróxido de hidrógeno, lo que lleva a la propagación de radicales libres.

La mitocondria también participa en otros procesos fundamentales como son la modulación del balance osmótico, la regulación y homeostasis de Ca^{2+}, así como el metabolismo de los ácidos grasos mediante un proceso denominado β-oxidación. Además, interviene en la respuesta celular ante múltiples y variadas situaciones de estrés, así como en complejos procesos como la proliferación celular y la senescencia y la defensa antioxidante [71].

A) IMPORTANCIA DE LAS MITOCONDRIAS EN LOS ESPERMATOZOIDES

Durante la espermatogénesis las mitocondrias sufren modificaciones significativas tanto en el número, como en el tamaño de las crestas mitocondriales lo

que provoca un aumento de la actividad respiratoria [72-74]. La versatilidad funcional mitocondrial comienza en la espermatogénesis y termina en los acontecimientos de fecundación [75-77]. La función principal de las mitocondrias en los espermatozoides es la producción de energía química en forma de ATP que es necesaria para el mantenimiento de la motilidad espermática y depende de la integridad funcional de la mitocondria [78]. Para que las mitocondrias sean funcionales deben mantener intacta la impermeabilidad de la membrana interna. Durante el proceso de criopreservación, la membrana mitocondrial se daña, se liberan EROs al citosol del espermatozoide, disminuyendo su vida media y la supervivencia al proceso.

B) IMPORTANCIA DE LA MITOCONDRIA EN OVOCITOS Y EMBRIONES

La capacidad de generar ATP es crucial para la maduración del citoplasma y del núcleo y la preparación para la fecundación. En ovocitos inmaduros las mitocondrias se dispersan por todo el ooplasma. En el momento de la maduración, la mitocondria forma agregados con el retículo endoplasmático liso y se sitúa cerca del núcleo [70]. La calidad del ovocito es dependiente del número de mitocondrias debido a la alta demanda de energía que requiere la fecundación. Los ovocitos presentan alrededor de 6.000 mitocondrias en el desarrollo del estado primordial y tras la maduración a metafase II (MII), el número asciende de 300.000 a 400.000 mitocondrias [79]. El papel de la mitocondria en la producción de ATP y en la fosforilación oxidativa es crucial en los primeros estadios embrionarios [80], de esta forma, cuando la función mitocondrial se ve alterada, disminuyen las tasas de fecundación e incrementan las aneuploidías [81-84]. En las primeras etapas del embrión no se presentan replicaciones del ADNmt con lo que el número proporcional respecto al número de células disminuye drásticamente hasta estadio de blastocisto. Por otro lado, se ha demostrado que en estas primeras etapas, estas mitocondrias presentan escasas crestas mitocondriales debido a su inmadurez [85]. Debe tenerse en cuenta que los blastocistos producidos *in vitro* presentan un mayor número de mitocondrias inmaduras y mayor vacuolización de las mismas comparados con los embriones obtenidos *in vivo*[86].

ESTRÉS OXIDATIVO

La oxidación es un proceso bioquímico de pérdida de electrones. Esta oxidación participa en los procesos de obtención de la energía celular, sin embargo, cuando existe un exceso de oxidación aparece el fenómeno conocido como estrés oxidativo. Las especies reactivas de oxígeno (EROs) son necesarias para el correcto funcionamiento de las células pero en cantidades excesivas pueden modificar la función celular y poner en peligro la supervivencia de las mismas [87].

A) ESTRÉS OXIDATIVO EN ESPERMATOZOIDES

Los espermatozoides son particularmente sensibles al daño producido por el estrés oxidativo (EO), ya que poseen en su membrana plasmática una cantidad elevada de ácidos grasos poliinsaturados y su citoplasma contiene una cantidad baja de captadores de especies reactivas [87]. Además el EO también afecta a la integridad del ADN y puede tener graves consecuencias en la fertilidad [88, 89]. Para poder disminuir los efectos de las EROs, en el plasma seminal se encuentran diferentes agentes antioxidantes [90]. Las EROs participan en reacciones en cadena que producen nuevas especies reactivas, lo que las hace tan perjudiciales [91]. Se ha detectado la formación de EROs por parte de los espermatozoides [92] por dos vías: por el sistema NADPH oxidasa a nivel de la membrana plasmática y por la oxido-reductasa dependiente de NADH a nivel de la mitocondria. La criopreservación genera EROs que dañan las defensas antioxidantes del eyaculado [93] afectando negativamente a la membrana plasmática, el acrosoma y la integridad mitocondrial [94]. El plasma seminal es la fuente de defensas antioxidantes [95] con lo que la centrifugación previa a la adición del diluyente de congelación para retirar el plasma seminal o la extracción de espermatozoides de la cola epididimaria para su conservación, afecta negativamente el equilibrio oxidante/antioxidante.

B) ESTRÉS OXIDATIVO EN OVOCITOS Y EMBRIONES

En el caso de los embriones se han detectado multitud de efectos negativos debido al EO. El metabolismo del embrión genera especies reactivas de oxígeno siendo la producción de estos radicales particularmente importante durante las manipulaciones *in vitro* [96], aunque los niveles de EROs varían según la etapa del desarrollo embrionario [97]. Las EROs son capaces de difundir a través de las membranas biológicas y alterar diferentes tipos de moléculas, como lípidos, proteínas y ácidos nucleicos. Las consecuencias son múltiples e incluyen alteraciones mitocondriales, bloqueo del desarrollo del embrión, disminución de la concentración de ATP y la inducción de la apoptosis [98]. Las EROs dan lugar a fragmentación del ADN y peroxidación lipídica que afecta a la división celular, al transporte de metabolitos e inducen disfunciones mitocondriales [99]. Debido a que el ADN mt no tiene histonas éste presenta 4 veces más mutaciones que el ADN nuclear. Teniendo en cuenta que el ADN mt codifica enzimas esenciales para la fosforilación oxidativa [100], los defectos en dicho ADN producen un mal funcionamiento del metabolismo intermediario, ralentizando e incluso inhibiendo el desarrollo embrionario.

1.6.1 ANTIOXIDANTES COMO MECANISMOS DE DEFENSA CONTRA LAS EROS

Se define un antioxidante como la sustancia que, a bajas concentraciones comparadas con el sustrato oxidable, retrasa significativamente o inhibe la oxidación de dicho sustrato [101]. Los antioxidantes se clasifican en hidrofílicos que actúan a nivel de citoplasma celular o hidrofóbicos, que protegen las membranas de la célula contra la peroxidación de lípidos [102]. El propio organismo posee un sistema de defensa antioxidante con componentes producidos de manera endógena o adquirida por micronutrientes dietéticos [103].

Entre los antioxidantes más comunes encontramos: el ácido ascórbico (Vitamina C) que es un agente reductor que neutraliza especies reactivas del oxígeno y no puede ser sintetizado por el organismo. El glutatión (GSH) es sintetizado en las

células desde sus aminoácidos. Es uno de los antioxidantes celulares más importantes, ya que mantiene el estado redox de la célula. El α-tocoferol (vitamina E) es el más importante de los antioxidantes liposolubles y protege las membranas de la célula contra la oxidación. También encontramos enzimas tales como la catalasa, superóxido dismutasa y varias peroxidasas con capacidad antioxidante. Los niveles bajos de antioxidantes o la inhibición de las enzimas antioxidantes causan estrés oxidativo y pueden provocar daños por los excesos de radicales libres. En el ámbito de la reproducción asistida, estos efectos se traducen en disfunción mitocondrial, daños en el ADN, ARN y proteínas [104], además de fallos en la fusión de núcleos durante la fecundación in vitro o FIV [105]. La adición de antioxidantes al medio de cultivo puede tener efectos positivos en el desarrollo embrionario, mejorando la función mitocondrial, regulando los niveles de EROs [106, 107] y aumentando los niveles de ATP [108]. Se ha demostrado que la adición de antioxidantes a los medios de maduración [109], fecundación [110, 111], de desarrollo [112], congelación seminal [113, 114] y de vitrificación [115] mejora la tasas de éxito de dichos procesos.

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

La cisteína es un precursor de glutatión (GSH) en ovocitos [116], que juega un papel importante como antioxidante cuando se adiciona al medio de cultivo. La N-acetil-L-cisteína (NAC) es la forma estable de la L-cisteína, que después de la absorción, des-acetilación, y la conversión a glutatión, funciona tanto como un tampón REDOX como captador de EROs [117-119]. Puede ser considerado como complemento para disminuir los radicales libres formados durante el estrés oxidativo y facilitar la biosíntesis de GSH [120, 121]. La N-acetilcisteína es un inhibidor eficaz de muerte fisiológica de células [118, 122, 123] además de paliar los efectos citotóxicos de distintos tipos celulares como neuronas o células hepáticas entre otras. En el campo de la reproducción asistida Whitaker y Knight en 2010 [124] evaluaron el efecto de la N-acetilcisteína en la maduración *in vitro* de ovocitos porcinos mejorando las tasas de desarrollo a blastocisto. También se ha descrito que la adición de antioxidantes (incluido NAC) al medio de cultivo de ovocitos de ratonas de edad avanzada mejora la actividad mitocondrial, la expresión génica y el desarrollo embrionario [125]. Además, este análogo de la cisteína tiene un gran potencial de fertilización en los ovocitos de ratón debido a la reducción de enlaces disulfuro en la zona pelúcida (ZP) [126]. Michael *et al.* 2010 demostraron que la suplementación de diluyentes de semen con N-acetilcisteína durante la refrigeración es beneficioso para mantener la motilidad de los espermatozoides en perro [127]. También, reducen la fragmentación del ADN y la peroxidación lipídica en espermatozoides de verracos [128] y protegen los espermatozoides frente a las EROs sin comprometer la viabilidad, la integridad del ADN o el desarrollo de los embriones tras la ICSI en semen bovino [129]. Teniendo en cuenta todo lo anteriormente expuesto, en la presente Tesis Doctoral nos planteamos los siguientes objetivos:

OBJETIVO GENERAL

- Analizar el efecto de la adición de N-Acetilcisteína a ovocitos, embriones y espermatozoides criopreservados de diferentes especies con el fin de determinar la utilidad de dicho aditivo en los procesos de congelación lenta y vitrificación.

OBJETIVOS ESPECÍFICOS

1- Determinar si distintas combinaciones de crioprotectores (glicerol y dimetilformamida) en medios de congelación lenta mejora los parámetros de motilidad del semen epididimario de toro de lidia tras el almacenamiento prolongado previo a la criopreservación.

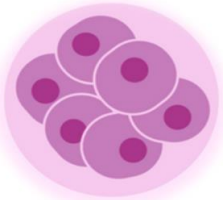
2- Comprobar el efecto de la adición de N-acetilcisteína en los medios de congelación de semen epididimario de toro de lidia sobre la viabilidad, producción de EROs, potencial de membrana mitocondrial, integridad del acrosoma y del ADN tras la descongelación.

3- Comprobar si la adición de N-acetilcisteína a los medios de cultivo de embriones de 2 células frescos y vitrificados mejora la calidad de los blastocitos generados.

4- Estudiar el efecto de la suplementación con N-acetilcisteína antes y después de la vitrificación en los ovocitos de ratón.

5- Comprobar si dicha suplementación mejora la capacidad de producción de ATP, agregación mitocondrial y producción de EROs de los ovocitos.

6- Establecer si existen diferencias significativas en la calidad de los blastocistos y la capacidad de desarrollo *in vitro* de los embriones derivados de los ovocitos vitrificados suplementados con NAC.



PUBLICACIONES

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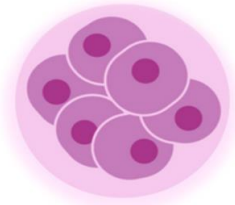
1.- **N-Acetylcysteine does not improve sperm motility of Lidia bull after prolonged epididymal storage.** 2017 Veterinary Journal of Andrology Vol. 2 (1).

2.- **Outlining adecuate protocols for Lidia bull epididymal storage and sperm cryopreservation: use of glycerol, dimethylformamide and N-Acetylcysteine** 2017 Spanish Journal of Agricultural Research, Volume 15, Issue 3, e0405. DOI: https://doi.org/10.5424/sjar/2017153-11463

3.- **Improvement of blastocyst quality by supplementation of the culture medium with N-Acetylcysteine of vitrified embryos at the two-cell stage.** En revisión.

4.- **N-Acetylcysteine improves oocyte mitochondrial polarization status and quality of embryos derived from vitrified murine oocytes.** En revisión.



ARTÍCULO 1

JOURNAL OF VETERINARY ANDROLOGY ISSN 2542-3045
VOL 2(1) (ENERO-JUNIO/JANUARY-JUNE 2017)

Research Article/Artículo de Investigación

N-ACETYLCYSTEINE DOES NOT IMPROVE SPERM MOTILITY OF LIDIA BULL AFTER PROLONGED EPIDIDYMAL STORAGE

LA N-ACETILCISTEINA NO MEJORA LA MOTILIDAD ESPERMATICA EN TOROS DE LIDIA LUEGO DEL ALMACENAMIENTO EPIDIDIMARIO PROLONGADO

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ABSTRACT

The Lidia bovine breed is considered a hallmark of Spanish cattle industry. Assisted reproductive techniques like cryopreservation of epididymal spermatozoa could be considered as an important tool to obtain more offspring and store its genetics. As these bulls are not selected by their reproductive performance or sperm freezability, the quality of their ejaculates is poor and addition of antioxidants prior cryopreservation could exert beneficial effects on the post-thaw sperm quality. The aim of this study was to evaluate the effect of the supplementing a tris-fructose-egg yolk based freezing extender with 1 mM and 2.5 mM of N-acetylcysteine to sperm recovered from epididymis stored at 4ºC for 24, 48, 72 or 96 hours prior cryopreservation. Motility values and sperm kinematic parameters were compared against control (epididymis stored for 24 hours and no antioxidant addition). Our results showed that N-acetylcysteine addition did not improve sperm motility parameters at any of the time points or dosages tested. In addition, storage of bullfight epididymis up to 96 hours did not significantly affect sperm kinematic parameters or total and progressive motility.

Keywords: Lidia breed; Epididymal sperm; Cryopreservation; N-acetylcysteine.
JOURNAL OF VETERINARY ANDROLOGY (2017) 2(1):23-29

RESUMEN

La raza de Lidia es considerada una insignia de la industria ganadera española. Las tecnologías de reproducción asistida como la criopreservación de espermatozoides epididimarios podría ser considerara como una herramienta importante para obtener más crías y conservar su genética. Ya que estos toros no son seleccionados por su desempeño reproductivo o congelabilidad espermática, la calidad de sus eyaculados es pobre y la adición de antioxidantes antes de la criopreservación podría tener efectos beneficiosos sobre la calidad del semen descongelado. El propósito de este estudio fue evaluar el efecto de la suplementación del medio a base de tris-fructosa y yema de huevo con 1 mM y 2,5 mM de N-acetilcisteína a espermatozoides recuperados de epidídimos almacenados a 4°C por 24, 48, 72 o 96 horas antes de la criopreservación. Los valores de movilidad y los parámetros de cinética espermática fueron comparados con el control (epidídimos almacenados por 24 horas y sin la adición de antioxidantes). Nuestros resultados muestran que la adición de N-acetilcisteína no mejora los parámetros de motilidad espermática en ninguno de los momentos o dosis evaluadas. Además, el almacenamiento de epidídimos refrigerados hasta 96 horas no afecta significativamente los parámetros de cinética espermática o la movilidad total o progresiva.

Palabras clave: Raza de Lidia; Espermatozoides epididimarios; Criopreservación; N-acetilcisteína.
JOURNAL OF VETERINARY ANDROLOGY (2017) 2(1):23-29

Received/Recibido: 11/11/2016; Accepted/Aceptado: 12/12/2106

INTRODUCTION

The Lidia bovine breed is an Iberian breed considered a hallmark of Spanish cattle industry. As a general rule, Lidia bulls are selected based upon temperament and aggressiveness disregarding their reproductive performance (Jiménez et al., 2007). The descendants are not allowed to sire, as they are not tested until they reach the fighting ring and this fact has lead to an increased in inbreeding (Canon et al., 2008). Assisted reproductive techniques (ARTs) could be considered as an important tool to obtain more offspring from certain maternal lineages or from a particular bull killed during the bullfight (Katska-Ksiazkiewicz et al., 2006).

Cryopreservation of epididymal spermatozoa allows for an efficient use of the genetic material as it can be successfully cryopreserved and used for in vitro embryo production or artificial insemination (Martins et al., 2007). Usually, assisted reproductive facilities are far from bullrings and thus, transport and/or storage of the epididymis is necessary prior sperm harvesting. It has to be noted that even when epididymal spermatozoa are known to be moderately protected by a variety of antioxidant enzymes (Chen et al., 2003), seminal plasma is recognized as their predominant source of antioxidant defenses (Vernet et al., 2004). Cryopreservation exerts deleterious effects on sperm cells impairing their fertility due to the thermic, osmotic and oxidative stresses triggered during the freezing and thawing cycles (Amidi et al., 2016). In fact, these processes have been demonstrated to negatively impact plasma membrane, acrosome and mitochondrial integrity (Januskauskas et al., 2003). Sperm cryopreservation has also been demonstrated to generate reactive oxygen species (ROS) which rapidly overwhelms the antioxidant defenses of the spermatozoa (Bilodeau et al., 2000). Accordingly, sperm removal from the epididymal tail followed by cryopreservation detrimentally affects the oxidant/antioxidant balance (Silva and Guerra, 2011).

The addition of antioxidants to the freezing media has been demonstrated to reduce the negative effects induced by ROS in spermatozoa (Aitken, 1995; Bilodeau et al., 2000; Yoshimoto et al., 2008; Taylor et al., 2009; Gadea et al., 2011) and thus, antioxidants may have beneficial effects on the function of epididymal spermatozoa submitted to cryopreservation. N-acetylcysteine (NAC) is a potent free radical scavenger that can be considered as a supplement to alleviate Glutathione (GSH) depletion and free radical formation during oxidative stress as previously reported (Wu et al., 2006). Accordingly, our study aimed to evaluate the effect of NAC (1 mM and 2.5 mM) addition to Tris-fructose-egg-yolk (TEY) freezing medium with 7% glycerol (v/v) on kinematics of epididymal Lidia bull sperm cooled at 4ºC for 24, 48, 72 or 96 hours prior cryopreservation.

MATERIALS AND METHODS

Reagents

All the reagents used were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise stated.

Media

The base medium was composed of Tris-fructose-citrate (TF) (Tris 250 mM, citric acid 86.9 mM, fructose 36 mM, 1 mg/ml penicillin and 0.5 mg/ml gentamicin in sterile Milli-Q water) as previously described (Van Wagtendonk-De Leeuw et al., 2000; Chaudhari, et al., 2015). Base freezing medium was composed of Tris-fructose added with 20% (v/v) of egg yolk (Van Wagtendonk-De Leeuw, et al., 2000), and 7% glycerol (TEY). Treatment groups were added with 1 mM NAC (TEY-1) and 2.5 mM NAC (TEY-2.5); non-NAC added groups were considered as controls and are referred as TEY-C.

Sperm harvesting and processing

Spermatozoa were collected from the epididymis of 18 Lidia Spanish bulls aged 3-4 years. Testes with attached epididymis were obtained post-mortem at the bullring. Immediately after removal, the testis were placed into plastic bags in an isothermal box at 4ºC and shipped to the laboratory overnight. Once arrived to the laboratory the epididymis were separated from the testis, as previously described (Yu and Leibo, 2002). Epididymis were stored in the fridge (4ºC) and processed at 4 different time points: a) 24 hours (n = 5), b) 48 hours (n = 5), c) 72 hours (n = 5) and d) 96 hours (n = 3). Connective tissue was carefully dissected, and the cauda epididymis was straightened to allow for flushing medium passage. A 20G needle attached to a 10 ml plastic syringe was used to flush the cauda epididymis of each bull using 5 ml of TF medium pre-warmed at 37ºC (Chaudhari et al., 2015). Each sample obtained was aspirated using a Pasteur plastic pipette and transferred to a 15 ml tube. Sperm was then centrifuged at 600 g for 10 minutes at room temperature, the supernatants were discarded and the pellets were diluted in 1.5-2 ml of TF at room temperature (22-25ºC) and centrifuged again. The supernatant was discarded once more and the remaining pellets were resuspended in 600-800 µl of TF. Sperm concentration was determined using a Neubauer chamber and freezing medium was slowly added to reach a final concentration of 100×10^6 spz/ml. The diluted semen was packed into 0.25 ml "french" straws at room temperature, and closed using an ultrasound sealer (Ultrasound Welding Machine, Vitrolife, Sweden). The straws were placed horizontally in a rack, and

placed in the fridge at 4°C for 2 hours; then, the straws were placed 4 cm above liquid nitrogen vapors for 20 minutes, seeded and subsequently plunged into liquid nitrogen as previously described (Chaveiro et al., 2006). The straws were stored for at least 1 month prior thawing and subsequent analysis. Thawing was achieved by immersing the straws for 1 minute in a water bath set at 37°C. After thawing 50 µl of each sample were resuspended with an equal volume of TF and subjected to a short spin (MiniSpin®, eppendorf) for 10 seconds. The supernatant was removed and the remaining pellet was resuspended in 90 µl of pre-warmed TF.

Motility Assay

All samples were examined using a CASA system (ISAS®, Proiser R+D, Paterna, Valencia, Spain). Two microliters of each sample were placed in a pre-warmed counting chamber (Leja®, Nieuw-Vennep, The Netherlands). Sperm motility was assessed with a microscope (Nikon Eclipse 50i) equipped with a 10x negative-phase contrast objective and a heated stage at 38°C. Analysis was based on the examination of 25 consecutive digitalized images and at least 200 spermatozoa per sample were analyzed. After acquiring at least 3 representative fields, the following sperm motility descriptors were recorded: total motility (TM) and progressive motility (PM), VCL (curvilinear velocity in µm/sec), VSL (straight-line velocity in µm/sec), VAP (average path velocity in µm/sec), LIN (linearity coefficient in %), STR (straightness coefficient in %), ALH (amplitude of lateral head displacement in µm) and BFC (beat cross frequency in Hz).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test; results are reported as mean ± standard error of the mean (SEM). Groups were compared using an ANOVA on ranks due to their non-Gaussian distribution. When statistically significant differences against the control (TEY-C at 24 hours) were found, a Dunn's post-hoc test was used. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered as statistically significant when $p < 0.05$.

RESULTS

Total motility values did not show statistically significant differences among groups (Table 1; $p > 0.05$). Conversely, for progressive motility, statistically significant differences were found between TEY-C at 24 h (14.22% ± 3.51; mean ± SEM), and TEY-C and TEY-1 at 96 hours (2.8% ± 0.95 and 2.23% ± 0.96; mean ± SEM; respectively). No statistically significant differences were found in the velocity parameters (VCL, VSL, VAP, LIN and ALH) among control (TEY-C) and treatment groups (TEY-1 and TEY-2.5) at any storage time studied (Tables 2 and 3; $p > 0.05$). Conversely, significant differences among control (TEY-C) and TEY-1 (68.70% ± 2.48 vs. 52.47% ± 2.60; mean ± SEM) and TEY-2.5 (68.70% ± 2.48 vs. 57.27% ± 1.93; mean ± SEM) at 96 hours were found for STR. BCF differed only among control (TEY-C) and TEY-1 after 96 hours ($p < 0.05$; Table 3).

Table 1. Total and progressive motility of frozen-thawed Lidia bull epidydimal sperm

Storage time(4ºC)	Freezing medium	n	TM (%)	PM (%)
24 hours	TEY-C	5	30.22 ± 5.96	14.22 ± 3.51
	TEY-1	5	26.54 ± 5.21	11.44 ± 2.28
	TEY-2.5	5	25.08 ± 6.19	11.36 ± 2.92
48 hours	TEY-C	5	21.72 ± 5.10	6.44 ± 2.51
	TEY-1	5	21.18 ± 5.4	5.98 ± 1.76
	TEY-2.5	5	19.66 ± 4.9	6.78 ± 2.18
72 hours	TEY-C	5	21.08 ± 2.87	7.36 ± 2.22
	TEY-1	5	23.52 ± 1.88	7.12 ± 0.89
	TEY-2.5	5	25.46 ± 2.67	9.16 ± 2.03
96 hours	TEY-C	3	12.16 ± 3.10	2.8 ± 0.95*
	TEY-1	3	15.1 ± 5.25	2.23 ± 0.96*
	TEY-2.5	3	16.26 ± 4.60	3.56 ± 0.35

Total and progressive motility of thawed Lidia bull sperm from epididymis stored at 4 ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24h using an ANOVA and are represented as mean ± SEM; values marked with * differ statistically $p < 0.05$.

Table 2. Sperm velocity parameters of frozen-thawed Lidia bull epidydimal sperm

Storage time (4ºC)	Freezing medium	n	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})
24 hours	TEY-C	5	82.45 ± 4.48	27.78 ± 2.07	40.29 ± 2.17
	TEY-1	5	86.47 ± 5.2	27.43 ± 2.01	40.17 ± 2.51
	TEY-2.5	5	74.79 ± 12.9	23.21 ± 4.35	34.19 ± 6.22
48 hours	TEY-C	5	81.22 ± 5.12	21.34 ± 2.71	34.21 ± 3.57
	TEY-1	5	82.16 ± 1.80	20.41 ± 1.31	33.81 ± 1.92
	TEY-2.5	5	82.31 ± 3.14	22.62 ± 2.23	35.32 ± 2.49
72 hours	TEY-C	5	80.53 ± 4.64	22.36 ± 2.89	34.70 ± 2.65
	TEY-1	5	80.47 ± 2.99	22.37 ± 2.90	35.76 ± 1.73
	TEY-2.5	5	86.55 ± 5.76	22.50 ± 1.82	41.63 ± 4.15
96 hours	TEY-C	3	69.35 ± 2.49	27.76 ± 3.59	31.54 ± 2.18
	TEY-1	3	64.59 ± 8.65	19.02 ± 1.07	30.83 ± 6.49
	TEY-2.5	3	64.43 ± 8.65	15.88 ± 2.59	28.12 ± 4.53

Sperm velocity parameters of frozen-thawed Lidia bull sperm recovered from epididymis stored at 4 ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24 h using an ANOVA and represent the mean ± SEM; no statistically significant differences were detected among groups $p > 0.05$.

Table 3. Sperm kinematic parameters of frozen-thawed Lidia bull epididymal sperm

Storage time(4ºC)	Freezing medium	n	LIN (%)	STR (%)	ALH (μm)	BCF (Hz)
24 hours	TEY-C	5	33.94 ± 2.78	68.70 ± 2.48	3.47 ± 0.20	10.52 ± 0.29
	TEY-1	5	31.94 ± 2.33	68.22 ± 2.30	3.74 ± 0.23	10.37 ± 0.55
	TEY-2.5	5	30.85 ± 1.49	67.36 ± 2.03	3.17 ± 0.52	9.33 ± 1.43
48 hours	TEY-C	5	26.03 ± 2.23	62.01 ± 2.19	3.57 ± 0.52	8.85 ± 1.23
	TEY-1	5	24.78 ± 1.22	60.39 ± 1.70	4.34 ± 0.33	8.63 ± 1.21
	TEY-2.5	5	27.37 ± 2.11	63.67 ± 2.73	3.72 ± 0.17	8.46 ± 0.82
72 hours	TEY-C	5	27.81 ± 2.99	63.98 ± 4.30	4.04 ± 0.32	9.54 ± 0.65
	TEY-1	5	27.82 ± 1.36	62.59 ± 2.24	3.92 ± 0.33	9.25 ± 0.78
	TEY-2.5	5	27.82 ± 1.56	65.96 ± 2.16	4.29 ± 0.30	10.60 ± 0.62
96 hours	TEY-C	3	31.64 ± 2.22	60.65 ± 3.32	3.42 ± 0.6	8.13 ± 1.59
	TEY-1	3	27.47 ± 1.47	52.47 ± 2.60*	2.76 ± 1.12	4.81 ± 1.35*
	TEY-2.5	3	24.40 ± 1.05	57.27 ± 1.93*	2.44 ± 0.28	6.70 ± 1.93

Sperm kinematics of frozen-thawed Lidia bull sperm recovered from epididymis stored at 4ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24 h using an ANOVA; values marked with * differ statistically $p < 0.05$.

DISCUSSION

This study evaluated the effects of different dosages of NAC (1 mM and 2.5 mM NAC) on thawed Lidia bull sperm motility parameters after epididymal storage at 4ºC (24 to 96 hours). Our results show that total and progressive motility did not vary in thawed epididymal Lidia bull sperm despite prolonged epididymis storage at 4ºC. Furthermore, NAC addition at none of the dosages used exerted any significant effect in total or progressive motility (Table 1). Coinciding with our results, a high variability between bulls has been reported after 24 hours of refrigerated storage prior sperm freezing in Lidia breed (Posado et al., 2008). This variability observed between bulls explains why despite the apparent vivid differences in the total and progressive motility after epididymal refrigeration for 24 and 96 hours at 4ºC (30.22-12.16%

and 2.23-14.22%; respectively) no significant differences were found. Furthermore, although the total motility values obtained may appear to be low, epididymal sperm have been demonstrated to yield lower total and progressive motility, and lower straightness and linearity than freshly ejaculated bovine sperm (Goovaerts et al., 2006). However, it has to be noted that reference motility parameters are not established yet in the Lidia breed for epididymal or ejaculated sperm and ours are among the first results published.

Regarding the advanced sperm kinematic parameters, no changes were detected in VCL, VSL, VAP, ALH and LIN despite NAC addition or prolonged epididymal storage (Tables 2 and 3) and only STR and BCF experienced statistically significant alterations after 96 hours in the NAC supplemented groups. Individual velocities have been shown to predict the fertilizing potential of frozen-thawed semen in many species (Byrd et al., 1990; Fetterolf and Rogers, 1990; Adoyo et al., 1995) including the bull (Nagy et al., 2015). In this sense, it has been suggested that VAP may be the most useful parameter with clinical relevance to predict fertility in bulls (Nagy et al., 2015). Hence, our results suggest that prolonged storage of Lidia bull epididymis up to 96 hours may not significantly impair the fertility of the retrieved sperm and thus, processing of Lidia bull sperm epididymis can be done after epididymal storage for up to 96 hours at 4ºC.

On the other hand, prolonged storage is critical due to the accumulation of reactive oxygen species (ROS), which leads to the so called 'oxidative stress' (Nichi et al., 2007). Epididymal samples are particularly susceptible to attack by ROS, as they are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognized as the prime source of anti-oxidant protection (Chen et al., 2003). Antioxidant addition to sperm freezing media is backed by many authors (Bilodeau et al., 2000; Gadea et al., 2005; Yoshimoto et al., 2008; Taylor et al., 2009; Gadea et al., 2011; Olfati Karaji et al., 2014; Sapanidou et al., 2014; Mata-Campuzano et al., 2015), although its use is controversial (Seifi-Jamadi et al., 2016), as high antioxidant concentrations can also exert deleterious effects such as apoptosis due to cell pro-oxidation (Aisen et al., 2005; Atessahin et al., 2008; Kang et al., 2016). A number of studies have demonstrated the positive effect of ROS scavenger addition (such as superoxide dismutase, catalase, cytochrome C or Vitamin E among others) to ejaculated semen of ruminants for preservation at either 15 or 5ºC (Maxwell and Stojanov, 1996; Upreti et al., 1997; Upreti et al., 1998). NAC scavenging properties have been previously studied on fresh human spermatozoa incubated at room temperature (Oeda et al., 1997), and it has also been demonstrated a beneficial effect of NAC on refrigerated and cryopreserved canine and bovine spermatozoa motility and viability (Bilodeau et al., 2001; Michael et al., 2010; Pérez et al., 2015). However, our results are in agreement with previous research in different species in which it was concluded that antioxidant addition does not improve sperm motility parameters after 96 hours of refrigerated storage (Ball et al., 2001; Fernández-Santos et al., 2009) (horse and deer respectively). Our data and the previously mentioned works suggest that thermal and osmotic shocks (or others insults rather than ROS) are the main damages impairing sperm post-thaw quality and that these insults cannot be prevented by antioxidants. NAC addition to TEY medium at either 1 or 2.5 mM prior freezing does not induce any beneficial or detrimental effect on Lidia bull sperm after epididymal cooled storage. Furthermore, it was observed a high variability between males to withstand the sperm freezing and thawing processes.

CONCLUSIONS

In conclusion, in our study the possible beneficial effect of NAC in the freezing semen extender of Lidia bulls could not be demonstrated. Additionally, storage of bullfight epididymis up to 96 hours after animal slaughter, does not significantly affect total motility and progressive motility or sperm kinematic parameters. Due to the scant data available in Lidia bull sperm, more effort has to be put in establishing accurate protocols that maintain good quality of frozen epididymal sperm after the bullfight and to ensure its fertility.

ACKNOWLEDGMENTS

Elvira Matilla holds a predoctoral grant funded by the Jesús Usón Minimally Invasive Surgery Centre. Beatriz Macías-García holds a postdoctoral grant Juan de la Cierva Incorporación (IJC-2014-19428) from the Spanish Ministry of Economy, Industry and Competitiveness. Lauro González-Fernández holds a postdoctoral grant of the Fundação para a Ciência e a Tecnologia (Portuguese Ministry for Science, Technology and Higher Education) co-funded by Programa Operacional Potencial Humano (POPH) financed by European Social Fund (ESF) and Portuguese national funds from Ministry for Science, Technology and Higher Education; Grant reference: SFRH/BPD/85532/2012. V. Calle-Guisado holds a FPU Predoctoral grant (FPU-014/03449) from the Spanish Ministry of Education, Culture and Sport. The testicles used in this study were kindly donated by the Luis Terrón Díaz cattle raising farm. We thank the SINTREP group of the University of Extremadura for the unlimited access to the sperm motility evaluation facilities.

REFERENCES

Adoyo PA., Moore A., Moore HDM. 1995. A strategy for identifying candidate sperm antigens for immunocontraception: isolation of human testis cDNA clones using polyclonal antisera directed against hamster acrosomal membrane preparation. **International Journal of Andrology** 18:32-38.

Aisen E., Quintana M., Medina V., Morello H., Venturino A. 2005. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. **Cryobiology** 50:239-249.

Aitken R. 1995. Free radicals, lipid peroxidation and sperm function. **Reproduction, Fertility and Development** 7:659-668.

Amidi F., Pazhohan A., Shabani Nashtaei M., Khodarahmian M., Nekoonam S. 2016. The role of antioxidants in sperm freezing: a review. **Cell and Tissue Banking** 17:745-756.

Atessahin A., Bucak MN., Tuncer PB., Kızıl M. 2008. Effects of anti-oxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process. **Small Ruminant Research** 77:38-44.

Ball BA., Medina V., Gravance CG., Baumbe J. 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. **Theriogenology** 56:577-589.

Bilodeau JF., Blanchette S., Gagnon C., Sirard MA. 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. **Theriogenology** 56:275-286.

Bilodeau JF., Chatterjee S., Sirard MA., Gagnon C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. **Molecular Reproduction and Development** 55:282-288.

Byrd W., Bradshaw K., Carr B., Edman C., Odom J., Ackerman G. 1990. A prospective randomized study of pregnancy rates following intrauterine and intracervical insemination using frozen donor sperm. **Fertility and Sterility** 53:521-527.

Canon J., Tupac-Yupanqui I., Garcia-Atance MA., Cortes O., Garcia D., Fernandez J., Dunner S. 2008. Genetic variation within the Lidia bovine breed. **Animal Genetics** 39:439-445.

Chaudhari DV., Dhami AJ., Hadiya KK., Patel JA. 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (-196 °C) of buffalo semen. **Veterinary World** 8:239-244.

Chaveiro A., Machado L., Frijters A., Engel B., Woelders H. 2006. Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. **Theriogenology** 65:1875-1890.

Chen H., Chow PH., Cheng SK., Cheung ALM., Cheng LYL., O W-S. 2003. Male genital tract antioxidant enzymes: their source, function in the female, and ability to preserve sperm dna integrity in the golden hamster. **Journal of Andrology** 24:704-711.

Fernández-Santos MR., Domínguez-Rebolledo AE, Esteso MC., Garde JJ., Martínez-Pastor F. 2009. Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin c supplementation. **Reproduction in Domestic Animals** 44:212-220.

Fetterolf PM., Rogers BJ. 1990. Prediction of human sperm penetrating ability using computerized motion parameters. **Molecular Reproduction and Development** 27:326-331.

Gadea J., Gumbao D., Matás C., Romar R. 2005. Supplementation of the thawing media with reduced glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after cryopreservation. **Journal of Andrology** 26:749-756.

Gadea J., Molla M., Selles E., Marco MA., Garcia-Vazquez FA., Gardon JC. 2011. Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. **Cryobiology** 62:40-46.

Goovaerts IGF., Hoflack GG., Van Soom A., Dewulf J., Nichi M., de Kruif A., Bols PEJ. 2006 Evaluation of epididymal semen quality using the Hamilton–Thorne analyser indicates variation between the two caudae epididymides of the same bull. **Theriogenology** 66:323-330.

Januskauskas A., Johannisson A., Rodriguez-Martinez H. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. **Theriogenology** 60:743-758.

Jiménez JM., Criado M., Molina A. 2007. Las razas bovinas de fomento andaluzas: Retinta y Lidia. In: **Las razas ganaderas de Andalucía**, p. 43.

Kang J-T., Moon JH., Choi J-Y., Park SJ., Kim SJ., Saadeldin IM., Lee BC. 2016. Effect of antioxidant flavonoids (quercetin and taxifolin) on in vitro maturation of porcine oocytes. **Asian Australasian Journal of Animal Science** 29:352-358.

Katska-Ksiazkiewicz L., Lechniak-Cieslak D., Korwin-Kossakowska A., Alm H., Rynska B., Warzych E., Sosnowski J., Sender G. 2006. Genetical and biotechnological methods of utilization of female reproductive potential in mammals. **Reproductive Biology** 6(Suppl 1):21-36.

Martins CF., Rumpf R., Pereira DC., Dode MN. 2007. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses in vitro embryo production. **Animal Reproduction Science** 101:326-331.

Mata-Campuzano M., Álvarez-Rodríguez M., Álvarez M., Tamayo-Canul J., Anel L., de Paz P., Martínez-Pastor F. 2015. Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. **Theriogenology** 83:520-528.

Maxwell WM., Stojanov T. 1996. Liquid storage of ram semen in the absence or presence of some antioxidants. **Reproduction, Fertility, and Development** 8:1013-1020.

Michael AJ., Alexopoulos C., Pontiki EA., Hadjipavlou-Litina DJ., Saratsis P., Ververidis HN., Boscos CM. 2010. Effect of N-acetyl-L-cysteine supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. **Reproduction in Domestic Animals** 45:201-207.

Nagy Á., Polichronopoulos T., Gáspárdy A., Solti L., Cseh S. 2015. Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. **Acta Veterinaria Hungarica** 63:370-381.

Nichi M., Goovaerts IGF., Cortada CNM., Barnabe VH., De Clercq JBP., Bols PEJ. 2007. Roles of lipid peroxidation and cytoplasmic droplets on in vitro fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34 °C. **Theriogenology** 67: 334-340.

Oeda T., Henkel R., Ohmori H., Schill WB. 1997. Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? **Andrologia** 29:125-131.

Olfati Karaji R., Daghigh Kia H., Ashrafi I. 2014. Effects of in combination antioxidant supplementation on microscopic and oxidative parameters of freeze–thaw bull sperm. **Cell and Tissue Banking** 15:461-470.

Pérez L., Arias ME., Sánchez R., Felmer R. 2015. N-acetyl-L-cysteine pre-treatment protects cryopreserved bovine spermatozoa from reactive oxygen species without compromising the in vitro developmental potential of intracytoplasmic sperm injection embryos. **Andrologia** 47:1196-1201.

Posado R., Hernández M., García J., Bartolomé D., Olmedo S., Rodríguez L., López-Fernández C., Gosálvez J. 2008. DNA fragmentation in frozen semen samples of fighting bulls. In: **24th Annual Meeting Association Europeenne de Transfert Embryonnair**, p. 220. Association Europeenne de Transfert Embryonnair PAU, France, 12th -13th September 2008.

Sapanidou VG., Margaritis I., Siahos N., Arsenopoulos K., Dragatidou E., Taitzoglou IA., Zervos IA., Theodoridis A., Tsantarliotou MP. 2014. Antioxidant effect of a polyphenol-rich grape pomace extract on motility, viability and lipid peroxidation of thawed bovine spermatozoa. **Journal of Biological Research-Thessaloniki** 21:1-6.

Seifi-Jamadi A., Kohram H., Zare Shahneh A., Ansari M., Macías-García B. 2016. Quercetin Ameliorate Motility in Frozen-Thawed Turkmen Stallions Sperm. **Journal of Equine Veterinary Science** 45:73-77.

Silva SV., Guerra MMP. 2011. Efeitos da criopreservação sobre as células espermáticas e alternativas para redução das crioinjúrias. **Revista Brasileira de Reprodução Animal** 35:370-384.

Taylor K., Roberts P., Sanders K., Burton P. 2009. Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. **Reproductive Biomedicine Online** 18:184-189.

Upreti G.C, Jensen K., Munday R., Duganzich DM., Vishwanath R., Smith JF. 1998. Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. **Animal Reproduction Science** 51:275-287.

Upreti GC., Jensen K., Oliver JE., Duganzich DM., Munday R., Smith JF. 1997. Motility of ram spermatozoa during storage in a chemically-defined diluent containing antioxidants. **Animal Reproduction Science** 48:269-278.

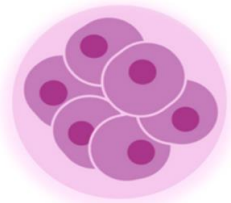
Van Wagtendonk-De Leeuw AM., Haring RM., Kaal-Lansbergen LMTE., den Daas JHG. 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. **Theriogenology** 54:57-67.

Vernet P., Aitken RJ., Drevet JR. 2004. Antioxidant strategies in the epididymis. **Molecular and Cellular Endocrinology** 216:31-39.

Wu W., Goldstein G., Adams C., Matthews RH., Ercal N. 2006. Separation and quantification of N-acetyl-l-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection. **Biomedical chromatography: BMC** 20:415-422.

Yoshimoto T., Nakamura S., Yamauchi S., Muto N., Nakada T., Ashizawa K., Tatemoto H. 2008. Improvement of the post-thaw qualities of Okinawan native pig spermatozoa frozen in an extender supplemented with ascorbic acid 2-O-α-glucoside. **Cryobiology** 57:30-36.

Yu I., Leibo SP. 2002. Recovery of motile, membrane-intact spermatozoa from canine epididymides stored for 8 days at 4 degress C. **Theriogenology** 57:1179-1190.



ARTÍCULO 2

RESEARCH ARTICLE

Spanish Journal of Agricultural Research
Volume 15, Issue 3, e0405.
DOI: https://doi.org/10.5424/sjar/2017153-11463
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

Outlining adequate protocols for Lidia bull epididymal storage and sperm cryopreservation: use of glycerol, dimethylformamide and N-acetylcysteine

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Received: 27 Mar 2017.

ABSTRACT

The Lidia bovine breed is an important hallmark of the Spanish cattle industry. Bulls are selected based upon aggressiveness and epididymal sperm cryopreservation is the way to obtain and store their genetics. There are not specifically designed protocols yet to perform Lidia bull sperm cryopreservation. The present study aimed to determine if a tris-fructose-citrate-egg yolk (20% v/v; TFY) extender supplemented with 7% glycerol (TFY1) or 3.5% glycerol plus 3.5% dimethylformamide (DMF; TFY2) are suitable media for cryopreservation of epididymal Lidia bull sperm. Moreover, the effect of N-acetylcysteine (NAC), a potent antioxidant, was evaluated. The epididymis were stored at 4°C for 24, 48, 72 or 96 h, and both freezing media were tested as such or supplemented with 1 or 2.5 mM of NAC. Our data demonstrated that post-thaw viability was well maintained (TFY1: 50.8% ± 1.9 at 24 h and 52.4% ± 0.8 at 96 h and TFY2: 52.6% ± 1.6 at 24 h and 56.1% ± 1.8 at 96 h; mean % ± SEM; $p>0.05$) as also were total and progressive sperm motility, high mitochondrial membrane potential, ROS production, DNA status and acrosomal intactness of Lidia bull sperm up to 96 h of epididymal storage, all extender variations being similar ($p>0.05$). In conclusion, the use of TFY medium supplemented either with 7% glycerol alone or the combination of 3.5% glycerol and 3.5% DMF were equally safe choices for epididymal Lidia bull sperm cryopreservation, and NAC addition did not significantly improve sperm post-thaw quality.

INTRODUCTION

The Lidia bovine breed or fighting bull is an autochthonous breed of the Iberian Peninsula. The cows and the sires are selected based upon temperament and aggressiveness, disregarding their reproductive performance. In addition, the descendants are not allowed to breed and are not tested until they reach the fighting ring, thus increasing the lineage inbreeding (Canon *et al.*, 2008). The use of assisted reproductive techniques (ARTs) is considered as an important tool to obtain offspring or preserve the genetics of a particular bull killed during the bullfight (Katska-Ksiazkiewicz *et al.*, 2006). Usually, bullrings are far from ART facilities and thus, transport and cooled storage of the epididymis become necessary to allow for sperm harvesting (Malcotti *et al.*, 2012). Fortunately, epididymal sperm can be successfully cryopreserved and used for artificial insemination or *in vitro* production of embryos (Martins *et al.*, 2007; Lopes *et al.*, 2015), although the maximum refrigerated epididymal storage time still needs to be determined in the Lidia bovine breed.

One of the most damaging events occurring during sperm refrigerated storage and freezing-thawing cycles is an increased production of reactive oxygen species (ROS), which overwhelms the sperm's antioxidant defenses (Nichi *et al.*, 2007). This oxidant/antioxidant imbalance results in DNA damage (Gürler *et al.*, 2016), plasma membrane lipid peroxidation and cytoskeletal alterations (Agarwal *et al.*, 2014), among other injuries. Two strategies have been followed to partially alleviate the detrimental effect of ROS: a) antioxidant addition to freezing extenders and b) the use of alternative cryoprotective agents (CPAs) of potentially lower toxicity.

Regarding antioxidants, a wide variety have been tested as supplements to freezing extenders (Fernández-Santos *et al.*, 2009a). Among them, N-Acetylcysteine (NAC) has demonstrated to alleviate glutathione (GSH) depletion and free radical formation during oxidative stress (Wu *et al.*, 2006), and has yielded satisfactory results in different species (Ciftci *et al.*, 2009; Mata *et al.*, 2012; Pérez *et al.*, 2015). However, until now, there is only one report regarding NAC addition to extenders used for freezing Lidia bull epididymal sperm, showing that sperm kinematics remain unchanged after prolonged epididymal cooled storage (Matilla *et al.*, 2017).

Glycerol is the most commonly added cryoagent to bovine sperm freezing media (Forero *et al.*, 2012; Almeida *et al.*, 2017) although it increases plasmalemmal permeability and induces the disruption of the actin cytoskeleton in equine sperm (Macias *et al.*, 2012). Recently, in the horse, alternative cryoagents such as dimethylformamide (DMF) have been tested with great success (Alvarenga *et al.*, 2005). The lower molecular weight of amides compared to glycerol improves sperm membrane permeation thus decreasing the pre-cooling interval (Squires *et al.*, 2004; Alvarenga *et al.*, 2005). In addition, DMF has been demonstrated to enhance equine sperm post-thaw quality when used as single CPA (Olaciregui *et al.*, 2014) or combined with glycerol (Morillo *et al.*, 2012). However, the use of DMF as single CPA at 3% (v/v) in bull ejaculates has been shown to detrimentally affect sperm cells (Forero *et al.*, 2012; Martins *et al.*, 2015). Accordingly, this study was designed to evaluate the effect of glycerol alone (7%; v/v) or a combination of 3.5% glycerol and 3.5% DMF (v/v/v) in presence or absence of N-acetylcysteine (1 mM or 2.5 mM) to the freezing extender of Lidia bull epididymal sperm stored at 4°C for 24, 48, 72 and 96 h.

MATERIAL AND METHODS

Reagents and media

All the reagents were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise stated. The base medium was composed of tris-fructose-citrate (TF) consisting of tris (250 mM), citric acid (86.9 mM), fructose (36 mM), 1 mg/mL penicillin and 0.5 mg/mL gentamicine in sterile Milli-Q water (Chaudhari *et al.*, 2015). Base freezing medium was composed of TF added with 20% egg yolk (v/v; TFY) (van Wagtendonk-de Leeuw *et al.*, 2000) and the following cryoprotectants: 7% glycerol (v/v;

referred as TFY1) or 3.5% glycerol and 3.5% DMF (v/v; referred as TFY2). Furthermore, N-acetylcysteine was added to each extender and TFY1 or TFY2 devoid of NAC were considered as controls (TFY1-C and TFY2-C respectively). Both extenders were supplemented with 1 or 2.5 mM of NAC, resulting in three groups per freezing medium.

Sperm harvesting and processing

Sperm were collected from the epididymis of 17 Lidia Spanish bulls aged 3-4 years from June to September of 2016. Testes with attached epididymis were obtained post-mortem from the bulls at the bullring. Immediately after removal, the testis were placed into plastic bags in an isothermal box at 4ºC and shipped overnight to the laboratory. Once at the laboratory, the epididymis were separated from the testis, as described by Yu & Leibo (2002) and stored in the fridge (4ºC) prior processing at four time points: 24 h, 48 h,72 h and 96 h. The connective tissue was carefully dissected, and the cauda epididymis was straightened to allow for flushing extender passage. A 20G needle-attached to a 10 mL plastic syringe was used to flush the cauda epididymis of each bull using 5 mL of pre-warmed (37ºC) buffered TF (Chaudhari *et al.*, 2015). Each sample obtained was aspirated using a Pasteur plastic pipette and transferred to a 15 mL tube. Sperm was then centrifuged at 600 × g for 10 min, the supernatant was discarded and the pellets were diluted in 1.5-2 mL of TF at room temperature (22-25ºC). Sperm concentration was determined using a Neubauer counting chamber. Each sample was divided into six groups, one per treatment, and then centrifuged at 600 × g for 10 min. The supernatant was discarded and the freezing medium (TFY1 or TFY2) was slowly added to reach a final concentration of 100×10^6 sperm/mL in the presence or absence of NAC (no addition, 1 or 2.5 mM). Once the extender was added, subjective total motility was assessed; sperm samples with total sperm motility below 40% were discarded. The diluted semen was packed into 0.25 mL French straws at room temperature and closed using an ultrasound sealer (Ultrasound Welding Machine, Vitrolife, Sweden). The straws were placed horizontally in a rack, and preserved in the fridge at 4°C for 2 h; then, the straws were located 4 cm above liquid nitrogen vapors for 20 min, seeded and subsequently plunged into liquid nitrogen (Forero *et al.*, 2012). The straws were stored for at least 1 month prior analysis. Thawing was achieved by immersing the straws for 1 min in a water bath set at 37°C. After thawing, 50 µL of each sample were resuspended with an equal volume of TF, subjected to a short spin (MiniSpin®, eppendorf) for 10 s, the supernatant was discarded and the pellet was resuspended in 90 µL of pre-warmed TF to achieve final concentration of 50×10^6 sperm/mL.

Motility assay

All samples were examined using a CASA system (ISAS®, ProiserR+D, Paterna, Valencia, Spain). Two microliters of sperm were placed in a pre-warmed motility chamber of 20 µm depth (Leja®, Nieuw-Vennep, The Netherlands). Sperm motility was assessed with a Nikon Eclipse 50i microscope equipped with a ×10 negative-phase contrast objective and a heated stage at 38°C. Analysis was based on the examination of 25 consecutive digitalized images obtained from 3 fields and at least 200 sperm per sample were analyzed; total and progressive motility were measured (Amann & Waberski, 2014).

Flow cytometry

All fluorescence signals of labeled sperm were analyzed by flow cytometry (ACEA NovoCyte™; ACEA Biosciences, Inc., San Diego, CA, USA) using the ACEA NovoExpress™ software. A minimum of 10.000 sperm were examined for each assay at a minimum flow rate of 300 cells/s. The sperm population was gated using forward and side scatter light signals to exclude debris and aggregates. The fluorophores were excited with a 200 mV argon ion laser operating at 488 nm.

Mitochondrial potential status

The metachromatic dye JC-1 (Thermo Fisher, Madrid, Spain) differentiates between mitochondria with

high and low mitochondrial membrane potential (MMP). When MMP is high (hMMP), the JC-1 molecules form aggregates that emit in the orange wavelength while JC-l monomers emitting green fluorescence are detected when the MMP is low (Garner & Thomas, 1999). From each sperm sample, 5 µL were diluted in 245 µL of isotonic buffered diluent (PBS) containing 0.9 µM of JC-1 and was incubated at 38°C for 30 min prior evaluation.

Acrosomal status

The sperm acrosomal status was assessed using peanut agglutinin conjugated with fluorescein thiocyanate PNA-FITC as a marker for acrosomal integrity and propidium iode for viability (PI, component B of sperm viability kit, Thermofisher, Madrid, Spain). Aliquots of 5 µL of each sperm sample (100 × 10^6 cells/mL) were incubated at 38ºC in the dark for 5 min with 1 µg/mL PNA-FITC and 6 µM PI in 45 µL of isotonic buffered diluent. Then, 200 µL of isotonic buffered diluent were added to each sample and mixed before flow cytometry analysis (Hurtado de Llera *et al.*, 2013).

Reactive oxygen species production

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate or H_2DCFDA (Thermo Fisher, Madrid, Spain) is a non-fluorescent probe that becomes de-esterified intracellularly and turns into highly fluorescent 2´,7´-dichorofluoresecein upon oxidation by ROS; cell viability was simultaneously assessed by detection of fluorescence of the non-permeant probe PI. The protocol used is a modification of the one described by Macias *et al.* (2015). In brief, aliquots of 5 µL of each semen sample (100 x 10^6 cells/mL) were diluted in 245 µL of isotonic buffered diluent and incubated for 30 min with 20 µM of H_2DCFDA. In the last 2 min, PI was added (final concentration, 6 µM). Results represent the percentage of living cells producing ROS.

Sperm chromatin status

The SCSA technique (Evenson *et al.*, 2002) is based on the metachromatic stain acridine orange; this probe exhibits green fluorescence when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). Sperm were diluted in TNE buffer (0.15 M NaCl, 0.01 M TrisHCl, 1 mM EDTA; pH 7.4) to reach a final concentration of 2-4 × 10^6 sperm/mL). Samples were flash frozen in LN_2 and stored at -80°C until analysis. For the analysis, the samples were thawed on crushed ice and 200 µL were transferred to a cytometry tube. Then, 400 µL of an acid detergent solution consisting of 80 mM HCl, 0.15 M NaCl and 0.1% Triton X-100 at a 1.2 pH were added. Exactly, 30 s after the acid-detergent solution addition, 1.2 mL of staining solution were added (6 µg/mL of acridine orange chromatographically purified (Polysciences, Warrington, PA, USA) in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH 6). Sample acquisition was carried out with the CellQuestv.3 software (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data (FCS files) were processed by using the R statistical environment with the Bioconductor flow Core package. The DNA fragmentation index (DFI) was calculated for each spermatozoon as the ratio of red fluorescence with respect to total fluorescence (red + green) and expressed as a percentage. The percentage of sperm with high fragmentation index (%DFI) was calculated as the percentage events with DFI>25%. The high DNA stainability index (HDS) was obtained as the percentage of sperm with green fluorescence intensity above channel 600 (0–1023 channels).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test and the obtained results are represented as mean ± standard error of the mean (SEM). Groups were compared using an ANOVA on ranks due to their non-Gaussian distribution. When statistically significant differences were found, a Dunn's post-hoc test was used to compare groups. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered as statistically significant when $p<0.05$.

RESULTS

Total and progressive motility analysis

An apparent decrease in total motility was observed after prolonged storage. Statistically significant differences were observed in progressive motility between TFY1-C at 24 h and TFY1-C at 96 h (16.2 ± 0.6 *vs*. 2.8 ± 0.4; mean % ± SEM; $p<0.05$). Although, values obtained for total motility did not vary significantly between TFY1-C at 24 h and TFY2-C at 96 h (33.1 ± 1.4 *vs*. 15.3 ± 2.2; mean % ± SEM). Additionally, NAC addition or the use of glycerol or the combination of glycerol and DMF did not significantly affect total or progressive motility. No statistically significant differences were found for the total motility parameter in any of the groups tested (Table 1; $p>0.05$).

Determination of the percentage of live cells producing ROS

Our data showed an apparent increase in the percentage of live sperm producing ROS when TFY2-C was used compared to TFY1-C at 24, 48, 72 and 96 h. In addition, a tendency for ROS production alleviation was observed when TFY2 was supplemented with NAC either at 1 mM or 2.5 mM at any time point studied (Table 1), although the values obtained did not vary significantly. Nevertheless, statistically significant differences in the percentage of live cells producing ROS were not found between groups disregarding the length of the epididymal storage, freezing medium used and/or NAC addition ($p>0.05$; Table1).

Determination of sperm with high mitochondrial membrane potential

Our data showed that storage time of the epididymis, freezing media or NAC addition did not significantly affect the percentage of sperm with high mitochondrial membrane potential ($p>0.05$; Table 1) which ranged between 33.5% ± 1.0 for TFY2-2.5 mM at 72 h (mean ± SEM; minimum value) to 52.2% ± 1.81 for TFY1-C at 48 h (mean ± SEM; maximum value).

Determination of the sperm viability and acrosomal integrity

Our data showed that sperm viability and acrosomal intactness was maintained despite prolonged epididymal cooled storage, varying freezing medium or NAC addition. No statistically significant differences were observed between groups (Table 2; $p>0.05$).

Determination of sperm chromatin status

The percentage of sperm showing DNA fragmentation (%DFI) or high DNA stainability (%HDS) did not significantly vary despite NAC addition, freezing extender used or prolonged refrigerated epididymal storage (Table 2; $p>0.05$); DFI was below 2.2% and HDS remained below 5.7% for all cases, indicating good chromatin status.

Discussion

Sperm recovery from the cauda epididymis for subsequent cryopreservation is the most practical method to obtain and store semen from Lidia bulls due to their risky field handling. However, glycerol concentrations currently added to bovine freezing extenders vary from 3% to 7% depending on the research laboratory (Chaveiro *et al.*, 2006; Fernández-Santos *et al.*, 2009a), and marked differences in sperm tolerance to glycerol has been demonstrated between breeds of the same species (*i.e.* goats and turkeys) (Kulaksiz *et al.*, 2013; Long *et al.*, 2014). Moreover, glycerol and DMF have also been shown to detrimentally affect sperm motility, viability and mitochondrial status of bull and stallion semen when used as single CPA (Alvarenga *et al.*, 2005; Forero *et al.*, 2012). Therefore, in our study the alternative cryoprotectant DMF combined with glycerol was tested, as this CPA mixture has yielded promising results in the equine species (Álvarez *et al.*, 2014). Our data demonstrate that total and progressive sperm motility, viability, mitochondrial membrane potential, ROS production, DNA status and acrosomal intactness of Lidia bull sperm post-thaw are well maintained in TFY medium added either with 7% glycerol or the combination of 3.5% glycerol and 3.5% DMF for up to 96 h post-mortem (Tables 1 and 2; $p>0.05$). Coinciding with previous observations in

boar, our work demonstrates that, in Lidia bull, the combination of DMF and glycerol is not better than glycerol alone for epididymal sperm cryopreservation (Malo *et al.*, 2012). However, the combination of DMF and glycerol seems to effectively decrease the toxic effect of the latter, as DMF alone at 3% or 5% (v/v) significantly impairs motility, mitochondrial function and acrosomal intactness of bull sperm (Forero *et al.*, 2012). However, it has to be mentioned that the percentage of live sperm producing ROS was higher and the hMMP was lower when the combination of 3.5% glycerol and 3.5% DMF was used compared to glycerol alone (Table 1). Thus, as the use of a single CPA agent facilitates the preparation of freezing extenders, glycerol seems to be the cryoagent of election for Lidia bull sperm.

Even when total motility decreased during prolonged storage and a similar pattern was followed by progressive motility (Table 1), no statistically significant differences were found between treatments except for PM of sperm frozen in TFY1-C at 24 h and 96 h (Table 1). This lack of significance can be explained due to the high individual variability existing between bulls and their sperm tolerance for the cooling and freezing-thawing cycles (Posado *et al.*, 2008). A recent report by Nichi *et al.* (2016) established that total motility of post-thaw epididymal sperm from Charolais, Limousin and Belgian Blue bovine breeds after 2-3 h of refrigerated storage was 16.2% ± 4.3, which is comparable to the results obtained in the present study after 96 h (Table 1), highlight the fact that Lidia bull tolerance for prolonged epididymal cooled storage is substantial as previously shown in other species of ruminants (Fernández-Santos *et al.*, 2009b).

In addition, the percentage of viable sperm was well maintained in our setting disregarding the freezing medium used or storage time (over 44.5 %, see Table 2). These results coincide with previous reports in which viability after freezing-thawing decreased approximately to 50% in bovine sperm (Gürler *et al.*, 2016; Nichi *et al.*, 2016) and validates our freezing media and cooling/freezing protocols as suitable for routine Lidia bull epididymal storage and sperm cryopreservation.

Interestingly, despite the fact that total and progressive motility were lower after 96 h, the percentage of sperm with high mitochondrial membrane potential remained unchanged for all treatments (Table 1). Numerous studies have shown that the number of viable sperm with hMMP are significantly related to field fertility in buffalo (Minervini *et al.*, 2013), human (Marchetti *et al.*, 2002) and ram (Windsor, 1997), and thus, the fertility of Lidia bull epididymal sperm may be well preserved along the 96 h cooling period. In human and equine sperm, high mitochondrial potential and membrane integrity have also been positively correlated with sperm motility (Agnihotri *et al.*, 2016; Swegen *et al.*, 2016). However, in view of our results and previously published reports in bovine (Rocha *et al.*, 2006), this correlation needs to be more deeply studied in Lidia bulls.

Very few reports have been published in the post-thaw quality of Lidia bull breed, although Guijarro *et al.* (2014) described that in epididymal Lidia bull sperm cooled for 24 h, the percentage of PNA-/PI- subpopulation after thawing with Bioxcell® or Biladyl® was 58.0% ± 1.0 and 67.0% ± 1.0 respectively. This apparent divergence in sperm viability compared to the results obtained in the present work can be explained in part because sperm viability post-thaw depends on a wide variety of factors such as the pre-freezing sperm quality, cryopreservation medium and also on the individual male sperm freezability (Takahashi *et al.*, 2012). As previously stated, our study is amongst the first flow cytometry analysis of Lidia bull sperm, and therefore, there are not substantial reference values to compare with.

The last experiments of our work tried to determine if increased epididymal storage and sperm cryopreservation rendered a higher oxidative burst that could result in enhanced ROS production and DNA damage (Chatterjee & Gagnon, 2001). Theoretically, oxidative damage is increased in epididymal sperm, since they are not exposed to the accessory sex glands secretions (seminal plasma) (Chen *et al.*, 2003). Supporting this theory, Martínez-

Pastor *et al.* (2006) demonstrated that epididymal sperm post-thaw quality can be substantially increased when seminal plasma is added to deer samples prior freezing. As Lidia bull seminal plasma cannot be easily obtained, antioxidant addition was tried to partially alleviate the oxidative burst. NAC antioxidant properties have been shown to exert a beneficial effect on fresh human sperm incubated at room temperature (Oeda *et al.*, 1997), and also on refrigerated and cryopreserved sperm of different species including cattle (Bilodeau *et al.*, 2001; Partyka *et al.*, 2013; Pérez *et al.*, 2015). Our results show that the percentage of live cells producing ROS were higher when the freezing extender included the combination of 3.5% glycerol and 3.5% DMF (Table 1), although the differences found were not significant. Also, we found a decrease in ROS production when NAC was added to TYF2. On the other hand, ROS imbalance and sperm cryopreservation have been shown to detrimentally affect the DNA intactness in bovine sperm (Gürler *et al.*, 2016). Our results did not show any significant increase in DNA damage as total DNA fragmentation remained under 2% and HDS was below 5.7 % for all treatments, coinciding with previous reports (Martínez-Pastor *et al.*, 2009). Interestingly, DFI below 10% have been related to high fertility status in humans and bulls (Evenson & Wixon, 2006; Waterhouse *et al.*, 2006), suggesting that epididymal Lidia bull sperm fertility is maintained despite prolonged storage.

In conclusion, our study demonstrates for the first time that Lidia bull epididymis can be refrigerated at 4ºC up to 96 h prior cryopreservation and that prolonged storage does not impair sperm quality. Furthermore, glycerol alone (7%) or the combination of glycerol and dimethylformamide (3.5% v/v each) added to TFY are equally safe choices as cryoprotective agents in Lidia bull sperm. N-acetylcysteine addition to sperm freezing extenders does not affect post-thaw sperm quality in Lidia bulls. More studies are needed to elucidate the *in vitro* and *in vivo* fertility of frozen Lidia bull epididymal sperm and to test if other antioxidants, CPAs or other combinations of dimethylformamide and glycerol help to increase its post-thaw quality.

Acknowledgments

The testicles used in this study were kindly donated by the Luis Terrón Díaz cattle raising farm. We thank the SINTREP group of the University of Extremadura for the unlimited access to the sperm evaluation facilities.

REFERENCES

Agarwal A, Virk G, Ong C, du Plessis SS, 2014. Effect of oxidative stress on male reproduction. World J Mens Health 32 (1): 1-17.

Agnihotri SK, Agrawal AK, Hakim BA, Vishwakarma AL, Narender T, Sachan R, Sachdev M, 2016. Mitochondrial membrane potential (MMP) regulates sperm motility. In Vitro Cell Dev Biol Anim 52 (9): 953-960.

Almeida FC, Silva SV, Souza HM, Gomes WA, Lima Filho JAC, Wicke AA, Batista AM, Guerra MMP, 2017. Effects of glycerol, equilibration time and antioxidants on post-

thaw functional integrity of bovine spermatozoa directly obtained from epididymis. Andrologia 49 (3): Epub 2016 Jun 2.

Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros A, 2005. Amides as cryoprotectants for freezing stallion semen: A review. Anim Reprod Sci 89 (1–4): 105-113

Álvarez C, Gil L, González N, Olaciregui M, Luño V, 2014. Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender. Cryobiology 69 (1): 144-148.

Amann RP, Waberski D, 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81 (1): 5-17.

Bilodeau J F, Blanchette S, Gagnon C, Sirard M A, 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 56 (2): 275-86.

Canon J, Tupac-Yupanqui I, Garcia-Atance M A, Corte, O, Garcia D, Fernandez J,

Dunner S, 2008. Genetic variation within the Lidia bovine breed. Anim Genet 39 (4):439-45.

Chatterjee S, Gagnon C, 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 59 (4): 451-458.

Chaudhari DV, Dhami AJ, Hadiya KK, Patel JA, 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (-196 degrees C) of buffalo semen. Vet World 8 (2): 239-44.

Chaveiro A, Machado L, Frijters A, Engel B, Woelders H, 2006. Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. Theriogenology 65 (9): 1875-1890.

Ciftci H, Verit A, Savas M, Yeni E, Erel O, 2009. Effects of N-acetylcysteine on semen parameters and oxidative/antioxidant status. Urology 74 (1): 73-76.

Chen, H, Chow, P H, Cheng, S K, Cheung, A L M, Cheng, LYL, O, WS, 2003. Male Genital Tract Antioxidant Enzymes: Their Source, Function in the Female, and Ability to Preserve Sperm DNA Integrity in the Golden Hamster. Journal of Andrology 24 (5): 704-711.

Evenson DP, Larson KL, Jost, LK, 2002. Sperm Chromatin Structure Assay: Its Clinical Use for Detecting Sperm DNA Fragmentation in Male Infertility and Comparisons With Other Techniques. Journal of Andrology 23 (1): 25-43.

Evenson DP, Wixon R, 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 65 (5): 979-991.

Fernández-Santos, M R, Domínguez-Rebolledo, AE, Esteso MC, Garde JJ, Martínez-Pastor F, 2009a. Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity. Int J Androl 32 (4): 353-359.

Fernández-Santos MR, Domínguez-Rebolledo AE, Esteso MC, Garde JJ and Martínez-Pastor F, 2009b. Refrigerated Storage of Red Deer Epididymal Spermatozoa in the Epididymis, Diluted and with Vitamin C Supplementation. Reprod Domest Anim 44 (2): 212-220.

Forero-Gonzalez RA, Celeghini ECC, Raphael CF, Andrade AFC, Bressan FF, Arruda RP, 2012. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia 44: 154-159.

Garner DL, Thomas CA, 1999. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. Mol Reprod Dev 53 (2): 222-229.

Guijarro RA, Katchicualula A, Alvarez J, Fernández L, de la Varga S, Carbajo MT, 2014. Effect of freezing extender on quality after thawing in lidia bulls epididymal sperm cell for conservation postmortem. Reprod Domest Anim 49: 2.

Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, Bollwein H, 2016. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology 86 (2): 562-571.

Hurtado de Llera A, Martin-Hidalgo D, Rodriguez-Gil JE, Gil MC, Garcia-Marin LJ, Bragado, MJ, 2013. AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa. Biochimica et Biophysica Acta (BBA) - Biomembranes 1828 (9): 2143-2151.

Katska-Ksiazkiewicz L, Lechniak-Cieslak D, Korwin-Kossakowska A, Alm H, Rynska B, Warzych E, Sosnowski J, Sender G, 2006. Genetical and biotechnological methods of utilization of female reproductive potential in mammals. Reprod Biol 6 Suppl 1: 21-36.

Kulaksiz R, Ari Ç, Daşkin A, Üner AG, 2013. The effect of different glycerol concentrations on freezability of semen from Angora, Kilis and Saanen goats. Slovak Journal Animal Science 46: 5.

Long JA, Purdy PH, Zuidberg K, Hiemstra SJ, Velleman SG, Woelders H, 2014 Cryopreservation of turkey semen: Effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching. Cryobiology 68 (3): 371-378.

Lopes G, Soares L, Ferreira P, Rocha A, 2015. Tris–Egg Yolk–Glycerol (TEY) Extender Developed for Freezing Dog Semen is a Good Option to Cryopreserve Bovine Epididymal Sperm Cells. Reprod Domest Anim 50 (1): 97-103.

Macias Garcia B, Ortega Ferrusola C, Aparicio IM, Miro-Moran A, Morillo Rodriguez A, Gallardo Bolanos J M, Gonzalez Fernandez L, Balao da Silva CM, Rodriguez Martinez H, Tapia JA, Pena FJ, 2012. Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology 77 (7): 1280-9.

Macias-Garcia B, Gonzalez-Fernandez L, Loux SC, Rocha AM, Guimaraes T, Pena FJ, Varner DD, Hinrichs K, 2015. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction 149 (1): 87-99.

Malcotti V, Pelufo V, Bergamo N, Aisen E, 2012. Recovery of epididymal spermatozoa from bull and red deer, stored at different times and temperatures before freezing–thawing. Anim Sci 52 (8): 741-745.

Malo C, Gil L, Cano R, Martínez F, García A, Jerez RA, 2012. Dimethylformamide is not better than glycerol for cryopreservation of boar semen. Andrologia 44: 605-610.

Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P, 2002. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 17 (5): 1257-1265.

Martínez-Pastor F, Anel L, Guerra C, Álvarez M, Soler AJ, Garde JJ, Chamorro C, de Paz P, 2006. Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. Theriogenology 66 (8): 1847-1856.

Martínez-Pastor F, Del Rocío Fernández-Santos M, Domínguez-Rebolledo A, Esteso MC, Garde JJ, 2009. DNA Status on Thawed Semen from Fighting Bull: A Comparison Between the SCD and the SCSA Tests. Reprod Domest Anim 44 (3): 424-431.

Martins CF, Rumpf R, Pereira DC, Dode MN, 2007. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses in vitro embryo production. Anim Reprod Sci 101 (3–4): 326-331.

Martins CF, Silva AEDF, Dode MN, Rumpf R, Cumpa HCB, Silva CG, Pivato I, 2015. Morphological characterization and conservation of bovine spermatogenic cells by refrigeration at 4 °C and freezing using different cryoprotective molecules. Cryobiology 71 (1): 47-53.

Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Anel L, de Paz P, Garde JJ, Martínez-Pastor F, 2012. Effect of Several Antioxidants on Thawed Ram Spermatozoa Submitted to 37°C up to Four Hours. Reprod Domes Anim 47 (6): 907-914.

Matilla E, González-Fernández L, Hernández N, Tobajas C, Calle-Guisado V, Mijares J, Sánchez-Margallo F, Álvarez I, Macías-García B, 2017, N-Acetylcysteine does not improve sperm motility of Lidia bull after prolonged epididymal storage. Journal of Veterinary Andrology 2 (1): 23-29.

Minervini F, Guastamacchia R, Pizzi F, Dell'Aquila ME, Barile VL, 2013. Assessment of Different Functional Parameters of Frozen–Thawed Buffalo Spermatozoa by Using Cytofluorimetric Determinations. Reprod Domest Anim 48 (2): 317-324.

Morillo Rodriguez A, Balao da Silva C, Macías-García B, Gallardo Bolaños JM, Tapia JA, Aparicio IM, Ortega-Ferrusola C, Peña FJ, 2012. Dimethylformamide Improves the In vitro Characteristics of Thawed Stallion Spermatozoa Reducing Sublethal Damage. Reprod Domest Anim 47 (6): 995-1002.

Nichi M, Goovaerts IGF, Cortada CNM, Barnabe VH, De Clercq JBP, Bols PEJ, 2007, Roles of lipid peroxidation and cytoplasmic droplets on in vitro fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34 °C. Theriogenology 67 (2): 334-340.

Nichi M, Rijsselaere T, Losano JDA, Angrimani DSR, Kawai GKV, Goovaerts IGF, Van Soom A, Barnabe VH, De Clercq JBP, Bols PEJ, 2016. Evaluation of epididymis storage temperature and cryopreservation conditions for improved mitochondrial membrane potential, membrane integrity, sperm motility and in vitro fertilization in bovine epididymal sperm. Reprod Domest Anim 52 (2): 257-263.

Oeda T, Henkel R, Ohmori H, Schill WB, 1997. Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? Andrologia 29 (3): 125-131.

Olaciregui M, Gil L, Montón A, Luño V, Jerez RA, Martí JI, 2014. Cryopreservation of epididymal stallion sperm. Cryobiology 68 (1): 91-95.

Partyka A, Niżański W, Bajzert J, Łukaszewicz E, Ochota M, 2013. The effect of cysteine and superoxide dismutase on the quality of post-thawed chicken sperm. Cryobiology 67 (2): 132-136.

Pérez L, Arias ME, Sánchez R, Felmer R, 2015. N-acetyl-L-cysteine pre-treatment protects cryopreserved bovine spermatozoa from reactive oxygen species without compromising the in vitro developmental potential of intracytoplasmic sperm injection embryos. Andrologia 47 (10): 1196-1201.

Posado R, Hernández M, García J, Bartolomé D, Olmedo S, Rodríguez L, López-Fernández C, Gosálvez J, 2008. DNA Fragmentation in frozen semen samples of fighting bulls. In '24th Annual Meeting Association European Embryo Transfer', PAU, France, 12th - 13th September, p. 220

Rocha A, Oliveira E, Vilhena MJ, Diaz J, Sousa M, 2006. A novel apical midpiece defect in the spermatozoa of a bull without an apparent decrease in motility and fertility. Theriogenology 66 (4): 913-922.

Squires EL, Keith SL, Graham JK, 2004. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology 62 (6): 1056-65.

Swegen A, Lambourne SR, Aitken RJ, Gibb Z, 2016. Rosiglitazone Improves Stallion Sperm Motility, ATP Content, and Mitochondrial Function. Biol. Reprod 95 (5): 107, 1-12

Takahashi T, Itoh R, Nishinomiya H, Katoh M, Manabe N, 2012. Effect of Linoleic Acid Albumin in a Dilution Solution and Long-term Equilibration for Freezing of Bovine Spermatozoa with Poor Freezability. Reprod Domest Anim 47 (1): 92-97.

Van Wagtendonk-de Leeuw AM, Haring RM, Kaal-Lansbergen LMTE, den Daas JHG, 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology 54 (1): 57-67.

Waterhouse KE, Haugan T, Kommisrud E, Tverdal A, Flatberg G, Farstad W, Evenson DP, De Angelis PM, 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod, Fertil Dev 18 (7): 781-788.

Windsor DP, 1997. Mitochondrial function and ram sperm fertility. Reprod Fertil Dev. 9 (3): 279-84.

Wu W, Goldstein G, Adams C, Matthews RH, Ercal N, 2006. Separation and quantification of N-acetyl-l-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection. Biomed Chromatogr 20 (5): 415-22.

Yu I, Leibo SP, 2002. Recovery of motile, membrane-intact spermatozoa from canine epididymides stored for 8 days at 4°C. Theriogenology 57 (3): 11.

Table 1. Total and progressive motility, percentage of living cells producing ROS and percentage of sperm depicting high mitochondrial membrane potential of thawed Lidia bull sperm from epididymis stored at 4°C at different time points prior cryopreservation.

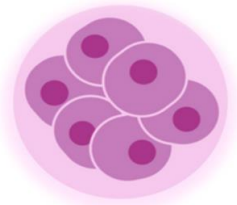
Storage time 4°C	Freezing medium[1]	n	TM (%)[2]	PM (%)[3]	% Living cells ROS+[4]	hMMP(%)[5]
24 hours	TFY1-C	4	33.1 ± 1.4	16.2 ± 0.6[a]	16.5 ± 0.9	40.6 ± 1.3
	TFY1-1mM	4	29.3 ± 3.8	12.6 ± 1.9	16.2 ± 1.4	37.0 ± 4.8
	TFY1-2.5 mM	4	29.1 ± 0.8	12.9 ± 0.9	16.0 ± 1.4	34.7 ± 2.0
	TFY2-C	4	26.1 ± 2.8	10.5 ± 1.8	28.4 ± 2.8	34.4 ± 2.3
	TFY2-1mM	4	34.6 ± 1.6	18.3 ± 2.6	19.9 ± 2.5	37.0 ± 3.9
	TFY2-2.5 mM	4	33.4 ± 2.1	17.0 ± 2.8	18.2 ± 1.8	34.4 ± 2.6
48 hours	TFY1-C	5	21.7 ±1.6	6.4 ± 1.0	11.4 ± 1.7	52.2 ± 1.8
	TFY1-1mM	5	21.1 ± 2.1	5.9 ± 0.6	11.9 ± 1.1	45.0 ± 2.3
	TFY1-2.5 mM	5	19.6 ± 0.9	6.7 ± 0.7	13.6 ± 1.3	44.4 ± 2.4
	TFY2-C	5	19.5 ± 1.1	5.8 ± 0.7	17.4 ± 1.2	43.0 ± 2.6
	TFY2-1mM	5	19.6 ± 1.5	5.1 ± 0.8	10.2 ± 3.3	38.6 ± 3.3
	TFY2-2.5 mM	5	19.8 ± 0.8	5.1 ± 0.9	14.0 ± 3.0	40.5 ± 1.5
72 hours	TFY1-C	5	21.0 ± 1.4	7.3 ± 1.0	21.9 ± 3.5	38.6 ± 0.8
	TFY1-1mM	5	23.5 ± 1.8	7.1 ± 0.3	16.2 ± 1.7	40.9 ± 1.8
	TFY1-2.5 mM	5	25.4 ± 1.5	9.1 ± 0.9	18.6 ± 1.4	40.4 ± 1.2
	TFY2-C	5	24.3 ± 1.5	7.3 ± 0.8	29.1 ± 2.5	34.5 ± 1.5
	TFY2-1mM	5	24.8 ± 2.2	7.1 ± 0.6	24.0 ± 2.1	34.7 ± 1.0
	TFY2-2.5 mM	5	24.3 ± 0.5	6.4 ± 0.6	18.7 ± 3.0	33.5 ± 1.0
96 hours	TFY1-C	3	12.1 ± 0.4	2.8 ± 0.4[b]	24.0 ± 1.3	42.8 ± 1.0
	TFY1-1mM	3	15.1 ± 1.8	2.2 ± 0.5	24.9 ± 4.5	40.9 ± 1.9
	TFY1-2.5 mM	3	16.2 ± 2.8	3.5 ± 0.5	22.8 ± 3.4	46.8 ± 1.8
	TFY2-C	3	15.3 ± 2.2	2.5 ± 0.5	34.9 ± 3.4	41.5 ± 0.7
	TFY2-1mM	3	17.2 ± 2.1	4.0 ± 0.7	30.6 ± 2.9	38.7 ± 0.6
	TFY2-2.5 mM	3	19.2 ± 1.1	4.1 ± 0.4	27.8 ± 5.1	40.0 ± 2.3

[1] Cryopreservation media were supplemented with varying doses of NAC (0, 1 and 2.5 mM). TFY-1: TFY with 7% glycerol, TFY-2: TFY with 3.5% glycerol and 3.5% dimethylformamide (v/v). [2] TM: total motility. [3] PM: progressively motility [4] % Living cells ROS+: percentage of live cells producing ROS. [5]hMMP: percentage of high mitochondrial membrane potential. [a,b] Values between TFY1-C at 24 h and TFY1-C at 96 h differ statistically (p <0.05). All groups were compared between them.

Table 2. Simultaneous determination of viability, live acrosome-intact sperm and DNA fragmentation of thawed Lidia bull sperm from epididymis stored at 4°C at different time points prior cryopreservation.

Storage time 4ºC	Freezing medium[1]	n	PNA+/PI-[2]	Viability[3]	TDFI (%)[4]	HDS (%)[5]
24 hours	TFY1-C	4	3.1 ± 4.0	50.8 ± 1.9	0.8 ± 0.1	4.5 ± 02
	TFY1-1mM	4	3.9 ± 5.0	44.5 ± 3.5	0.9 ± 0.1	5.1 ± 0.2
	TFY1-2.5 mM	4	3.5 ± 0.5	45.4 ± 3.0	1.0 ± 0.3	4.1 ± 0.4
	TFY2-C	4	6.1 ± 1.1	52.6 ± 1.6	0.8 ± 0.1	4.3 ± 0.2
	TFY2-1mM	4	4.3 ± 0.3	56.3 ± 1.7	1.2 ± 0.3	5.1 ± 0.2
	TFY2-2.5 mM	4	3.2 ± 0.5	53.3 ± 3.1	0.6 ± 0.1	5.1 ± 0.4
48 hours	TFY1-C	5	3.0 ± 0.1	52.5 ± 2.9	1.5 ± 0.2	4.9 ± 0.2
	TFY1-1mM	5	2.9 ± 0.2	47.9 ± 2.8	1.4 ± 0.1	5.6 ± 0.5
	TFY1-2.5 mM	5	2.9 ± 0.4	46.2 ± 1.9	1.6 ± 0.2	5.5 ± 0.2
	TFY2-C	5	3.4 ± 0.1	49.1 ± 2.6	1.0 ± 0.1	5.6 ± 0.2
	TFY2-1mM	5	3.9 ± 0.9	45.1 ± 1.5	1.4 ± 0.1	5.1 ± 0.4
	TFY2-2.5 mM	5	2.5 ± 0.5	46.0 ± 2.4	1.5 ± 0.1	5.6 ± 0.2
72 hours	TFY1-C	5	2.0 ± 0.2	51.6 ± 3.1	1.5 ± 0.1	3.4 ± 0.2
	TFY1-1mM	5	1.8 ± 0.3	49.3 ± 2.0	2.1 ± 0.3	3.3 ± 0.2
	TFY1-2.5 mM	5	1.4 ± 0.2	53.2 ± 2.0	1.7 ± 0.0	3.3 ± 0.2
	TFY2-C	5	2.3 ± 0.3	56.6 ± 1.1	1.6 ± 0.2	3.4 ± 0.1
	TFY2-1mM	5	2.6 ± 0.3	55.8 ± 0.7	1.5 ± 0.2	3.3 ± 0.2
	TFY2-2.5 mM	5	1.8 ± 0.2	58.9 ± 2.0	1.7 ± 0.1	3.6 ± 0.2
96 hours	TFY1-C	3	10.2 ± 1.5	52.4 ± 0.8	1.4 ± 0.1	3.0 ± 0.1
	TFY1-1mM	3	11.4 ± 1.2	53.6 ± 1.4	0.9 ± 0.1	2.9 ± 0.4
	TFY1-2.5 mM	3	8.5 ± 1.9	55.7 ± 1.4	1.4 ± 0.3	3.0 ± 0.4
	TFY2-C	3	13.9 ± 0.4	56.1 ± 1.8	1.1 ± 0.1	2.7 ± 0.2
	TFY2-1mM	3	12.1 ± 1.1	57.7 ± 1.9	1.1 ± 0.3	3.2 ± 0.5
	TFY2-2.5 mM	3	9.9 ± 2.5	59.7 ± 1.6	1.3 ± 0.2	3.5 ± 0.8

[1] Cryopreservation media were supplemented with varying doses of NAC (0, 1 and 2.5 mM). TFY-1: TFY with 7% glycerol, TFY-2: TFY with 3.5% glycerol and 3.5% dimethylformamide (v/v). [2] PI-/PNA$^+$: live sperm with intact acrosome
[3]Viability: sperm viability was extracted from the sum the PI-. [4]TDFI (%): percentage of total DNA fragmentation index.[5]HDS (%): percentage of immature sperm with intact DNA. All groups were compared between them. Values do not differ statistically (p >0.05).



ARTÍCULO 3

Improvement of blastocyst quality by supplementation of the culture medium with N-Acetylcysteine of vitrified embryos at the two-cell stage

Running head: Effect of NAC addition to vitrified murine 2 cell embryos

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Key words: N-acetylcysteine, vitrification, early embryos, mouse, blastocyst quality.

Contents

Vitrification is the best method for embryo cryopreservation although it increases endogenous reactive oxygen species (ROS) production. As N-acetylcysteine (NAC) is a free radical scavenger, the aim of this study was to investigate if its addition to fresh or vitrified and warmed 2-cell embryos at different time points or during the entire culture improves their developmental competence or the cell number at the blastocyst stage. Thus, 2-cell embryos were obtained *in vivo* or by *in vitro* fertilization (IVF), and were vitrified or cultured fresh in presence or absence of 1 mM of NAC during: a) the entire embryo culture, b) for 24 hours with NAC at days 1.5 (G1) or 2.5 (G2) and returned to basal embryo culture (KSOM) or c) cultured in the presence of NAC for 12 hours at day 3.5 (G3). Despite NAC addition to fresh or vitrified embryos produced *in vivo* or by IVF, blastocyst rates remained unchanged. In vitrified-warmed IVF-derived embryos, total cell number significantly increased only when NAC was added at day 1.5 (61.1 ± 1.6 vs. 49.8 ± 3.1 for G1 and control respectively; mean cell number ± SEM, $p < 0.05$) while in vitrified *in utero*-derived embryos a significant increase was observed for G1 and G2 *vs.* control (57.3 ± 2.0 and 58.8 ± 1.5 vs. 51.9 ± 1.1 respectively; mean cell number ± SEM, $p < 0.05$). Hence, supplementation of the embryo culture medium with 1 mM of NAC in the first days of development improves blastocyst quality of murine embryos.

INTRODUCTION

Embryo cryopreservation is an assisted reproductive technology that has been extensively used in conservation programs and livestock species [25, 26]. Vitrification is the safest and most rapid method for embryo cryopreservation as it avoids the formation of intracellular and extracellular ice crystals [130, 131]. This method consists on the solidification of the cell components at low temperatures reaching an amorphous-ice state by combining: a) extremely rapid cooling rates (over 1000ºC/min) and b) high concentrations of permeable cryoprotectants (typically ethylene glycol or EG and dimethyl sulfoxide or DMSO) and an non-permeable cryoprotectants agents (a carbohydrate) [132]. This method expedites the freezing process reducing the exposure to the toxic effect of the cryoprotectants [133] although embryo survival and the subsequent development is lower than in fresh embryos [36, 134]. It has been previously shown that cryopreservation

causes alterations in the embryos by affecting the integrity of the cell membrane and cytoskeleton, inducing mitochondrial depolarization and increasing the production of reactive oxygen species (ROS) [135, 136]. Among these insults, oxidative stress derived from mitochondrial damage is known to trigger the apoptotic cascade leading to a decrease in the survival rate and developmental competence of embryos after thawing [69, 137]. Additionally, *in vitro* culture of mammalian embryos, further enhances free radical production overwhelming the embryos' endogenous antioxidant capacity [138], being especially notable in vitrified embryos. For this reason antioxidant addition to the embryo culture medium has been tried and has shown to improve gamete quality and embryo development [108]. N-acetylcysteine (NAC) is a potent free radical scavenger that can be considered as a supplement to alleviate glutathione (GSH) depletion and free radical formation during oxidative stress in mice [108]. GSH is one of the major non-enzymatic antioxidants present in oocytes and embryos and is essential for formation, maintenance and protection against oxidative stress [139].

Thus, the aim of our study was to investigate the impact of N-acetylcysteine addition to murine embryos after vitrification. Two cell embryos were recovered *in vivo* or after *in vitro* fertilization (IVF), vitrified and incubated for 24 hours with 1 mM of NAC during different time points (day 1.5, day 2.5 or day 3.5) or during the entire culture to the blastocyst stage; total cell number and the percentage of embryos reaching the expanded blastocyst stage were assessed.

MATERIAL AND METHODS

Reagents

Unless otherwise stated, all the reagents were purchased from Sigma-Aldrich (Barcelona, Spain).

Animals and superovulation protocol

All the experimental procedures were reviewed and approved by the Ethical Committee of the Junta de Extremadura (Spain; Ref. SBC/mbr). B6D2F1/J mice were housed under a 12 h light/12 h dark cycles at a controlled temperature (19-23ºC) with free access to food and water. Females were intraperitoneally (IP) injected with 8 IU of equine chorionic gonadotropin (eCG, Veterin Corion, Divasa Farmavic) followed 47 h later by 8 IU of IP human chorionic gonadotropin (hCG, Foligon, MSD) to trigger ovulation.

In Vitro Fertilization

Male B6D2F1/J mice aged 8-12 weeks were euthanized by cervical dislocation and ventrally dissected to remove the cauda epididymis. Once located, the epididymis and attached *vas deferens* were sectioned and transferred to a Petri dish containing 500 µl of pre-equilibrated human tubal fluid (HTF; at 37°C in a 5% CO_2/ 95% air atmosphere at 100% humidity) covered with mineral oil. Sperm were obtained by gently pressing the cauda epididymis through the vas deferens and were allowed to capacitate for 45 minutes at 37°C in a 5% CO_2/ 95% air atmosphere at 100% humidity. At the end of the incubation, sperm concentration was measured using a Makler chamber (Sefi-Medical instruments LTD, CA, USA). Cumulus-oocyte complexes (COCs) were recovered from oviducts following female euthanasia and placed in 500 µl of pre-equilibrated HTF covered with mineral oil; COCs were inseminated using 2 x 10^6 sperm/ml and were co-incubated for 6 hours and then transferred to equilibrated potassium-supplemented simplex optimization medium (KSOM). The day at which IVF was performed was considered as day 0. The next morning, cleaved embryos were retrieved and allocated to an experimental group as described in the experimental design section.

In vivo embryo recovery

Female mice were hormonally stimulated to trigger ovulation as previously described; after hCG injection, females were paired with B6D2 males in a 1:1 ratio. After 24 hours, females were sacrificed by cervical dislocation and the embryos were collected from the oviducts; these 2-cell embryos

were allocated into an experimental group (see experimental design section).

Vitrification and warming

In vivo and *in vitro* produced two cell embryos were equilibrated in M2 medium added with 7.5% of DMSO (v/v), 7.5% ethylene glycol (v/v) and 20% (v/v) fetal bovine serum (FBS) for 3 min. Afterwards, the embryos were transferred to a vitrification solution consisting of M2 supplemented with 20% FBS added with 15% ethylene glycol (v/v), 15% DMSO (v/v) and 0.5 M sucrose for 1 minute. An average of 15 embryos were loaded in 0.25 ml French straws (IMV, L'Aigle, France) at room temperature and sealed by ultrasounds (Superultrasonic Co, Taiwan). After that, the straws were plunged into liquid nitrogen and stored for at least 7 days. Embryos were warmed at 37ºC for 3-4 minutes in M2 medium added with 0.5 M sucrose and 20% FBS (v/v) and washed in M2 medium drops for further 3 minutes.

Experimental design

Fresh or vitrified-warmed *in vivo* and *in vitro* produced two cell embryos (1.5 days of development) were separately allocated to one of the following experimental groups:
Control: embryos were cultured in KSOM to the blastocyst stage; G1: embryos were cultured for 24 hours in KSOM supplemented with 1 mM NAC (day 1.5 to 2.5), after this incubation embryos were transferred to KSOM until day 4; G2: embryos were cultured in KSOM, transferred for 24 hours to KSOM supplemented with 1 mM NAC (day 2.5 to 3.5), and returned to KSOM until day 4; G3: embryos were cultured in KSOM for 12 hours supplemented with 1 mM NAC (day 3.5 to 4); C-NAC: embryos were cultured in KSOM supplemented with 1 mM NAC to the blastocyst stage. The number of embryos reaching the blastocyst stage was recorded visually by a stereomicroscope. All the embryos were moved to a new droplet of their corresponding medium each day, including the embryos included in the control and the continuous NAC culture groups.

Total cell number

The number of cells in an embryo is the most critical indicator of embryo quality [140]. Therefore, in view of the previous data, expanded blastocysts were fixed in 4% formaldehyde in PBS added with 0.01% of polyvinyl alcohol (PVA; w/v) at 4ºC for 12 hours and stained with 2.5µg/ml of Hoechst 33258 (Eugene, OR, USA) in PBS added with PVA for 10 minutes at 37ºC. Then, the blastocysts were mounted on glass slides with glycerol, covered with coverslips and sealed using nail polish. The embryos were then visualized using a fluorescence microscope (Nikon Elipse TE2000-S) equipped with an ultraviolet lamp. Cell number was analyzed using the Fiji Image-J Software (1.45q, Wayne Rasband, NIH, USA).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test; the results are reported as mean ± standard error of the mean (SEM). Treatment groups were compared using ANOVA on ranks due to their non-Gaussian distribution. Between groups, all pair wise comparisons were made using a Holm-Sidak post-hoc test. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered as statistically significant when $p < 0.05$.

RESULTS

Embryo development

No significant differences were found between treatments in the development to the blastocyst stage ($p > 0.05$; Table 1). Additionally, homologous treatments between groups (control, G1, G2, G3 or C-NAC) were compared but statistically significant differences were not found ($p > 0.05$).

Total cell number determination

No significant differences were found between groups in fresh *in utero* retrieved embryos despite NAC addition ($p > 0.05$; Table 2). However, when *in utero*-derived embryos were vitrified and warmed, significant differences were found when embryo culture medium was supplemented with NAC in G1 (58.8 ± 1.5; mean cell number ± SEM) and G2 (57.3 ± 2.0; mean cell number ± SEM) compared to control (51.9 ± 1.1; mean cell number ± SEM, $p < 0.05$; Table 2) and also between G1 and G2 compared to C-NAC. Addition of NAC to fresh IVF derived embryos did not significantly affect total cell number ($p > 0.05$; Table 2). Conversely, when IVF derived embryos were vitrified and warmed, NAC addition at day 1.5 increased the blastomere number in G1 (61.6 ± 1.6; mean cell number ± SEM) compared to control and day 3.5 of embryo development (49.8 ± 3.1 and 50 ± 2.9; mean cell number ± SEM, control and G3 respectively; $p < 0.05$, Table 2 and Figure 1).

DISCUSSION

Embryo vitrification is an important hallmark of the assisted reproductive technology industry. The murine model has been extensively used to test and develop vitrification protocols due to its high capacity to withstand the process. However, depending upon the developmental stage, their tolerance to vitrification notably varies [141]. For example, it has been described that vitrified 2-cell embryos exhibit a similar survival rate after warming compared to 4-cell, 8-cell, morulae and blastocysts, although their development to the blastocyst stage is significantly lower compared to vitrified-warmed embryos at the 8-cell stage [141, 142]. This difference has been attributed to a lower cryoprotectant permeability of the zona pellucida at the earlier embryo stages and to increased ROS production after vitrification, being this stage the less suitable for cryopreservation [59, 143]. Thus, in our setting, vitrified 2-cell embryos produced *in vivo* and *in vitro* were used to study the effect of NAC supplementation during the entire culture or at different time points. In our setting no statistically significantly differences were observed in the developmental competence of the 2-cell embryos despite NAC addition, *in vitro* or *in vivo* embryo production and/or cryopreservation (Table 1; $p > 0.05$); additionally our results also demonstrate that 1 mM of NAC does not induce a toxic effect on embryos, even when NAC is present during their entire culture to the expanded blastocyst stage.

However, it has to be mentioned the high development to the expanded blastocyst stage obtained after embryo vitrification in our setting (82.9 ± 3 and 84.4 ± 2.1; blastocyst rate for vitrified-warmed *in vivo vs.* IVF produced embryos respectively; Table 1). Previous works have reported up to 69.4% blastocyst rates after *in utero* retrieved 2-cell embryo vitrification using the Cryotop method [141, 142] or a 97.3% blastocyst rate when the embryos were produced by IVF and vitrified using the Kitasato System [144]. Although the mouse strain used and the vitrification protocol (close in our setting *vs.* open in the mentioned reports) might influence the results obtained, our results suggest that *in vivo* and *in vitro* produced 2-cell embryos similarly withstand vitrification when a closed system is used.

As similar blastocyst rates were found among groups, we decided to compare the number of blastomeres between groups, as it has been shown to be a reliable indicator of embryonic quality [145, 146]. Coinciding with our findings it has been reported that the mean cell number in mouse blastocysts recovered from uterus is 74.5 ± 2.3 [147] and that total cell number significantly drops in IVF-derived embryos [148]. The lower cell number of *in vitro*-derived embryos has been linked to a higher cell death compared to *in utero*-derived embryos [149] and to an enhanced ROS production occurring during *in vitro* embryo production [150].

However, in our settings, even when NAC was added during the entire embryo culture, no statistically significant differences in the total cell number were found in fresh or

vitrified embryos disregarding their source (*in vitro* or *in utero*; Table 1). Similar findings have been described in vitrified *in vitro*-derived porcine embryos in which addition of L-ascorbic acid to the embryo culture medium ameliorated ROS production but did not result in enhanced total cell number [151]. The results by Castillo-Martín et al. (2014) and our own results suggest that, antioxidants added during the entire embryo culture can exert effective ROS scavenging that is not reflected by an enhanced total cell count in the resulting embryos. Interestingly, addition of 1 mM of NAC to the embryo culture at the different time points tested (day 1.5, 2.5, 3.5 or the entire embryo culture) to vitrified-warmed 2-cell embryos produced *in vitro* or *in vivo* exerted different effects. The total cell number of the *in utero*-derived embryos significantly increased when NAC was added at days 1.5 or 2.5 compared to the control or continuous NAC addition (Table 2 and Figure 1). Conversely, in vitrified-warmed *in vitro*-derived embryos total cell number significantly increased only at day 1.5 (G1) compared to control and NAC added at day 3.5 (G3). Our results suggest that NAC addition exerts its maximum beneficial effect right after embryo warming (*in vitro* and *in vivo* produced embryos), and during the progression from the 4-cell to the morula stage in the *in utero* derived embryos. It is known that physiological ROS production is required for correct embryo division and pre-implantation development [152]. Thus, our data suggest that after vitrification and warming the NAC scavenging properties might be ameliorating the increased ROS production triggered by the cryopreservation process. This effect was observed only when NAC was added during the first two thirds of the embryo culture (*in vivo* produced embryos), but in the last third of embryo development, the damages induced seem to be irreversible. The fact that continuous NAC addition did not result in an enhanced total cell number suggests that excessive ROS scavenging could be interfering with blastomere cytokinesis explaining why total cell number does not increase [153].

In conclusion, NAC addition enhances total cell number but not embryo development of vitrified murine 2-cell embryos obtained *in vivo* when added at days 1.5 and 2.5 of culture and *in vitro* at day 1.5. NAC supplementation during the entire culture to the blastocyst stage does not improve the quality of fresh or vitrified-warmed 2-cell embryos. The vitrification process detrimentally affects *in vivo*-derived 2-cell embryos more vividly than *in vitro* produced embryos, as the number of blastomeres is significantly lower after reaching the blastocyst stage. More studies are necessary to clarify the optimal concentrations of NAC necessary to improve quality of vitrified murine embryos at different developmental stages.

ACKNOWLEDGEMENTS

The authors acknowledge the help of Dr. Lauro González-Fernández for his unconditional backing during development of this work, critical reading and review of the manuscript. Elvira Matilla holds a predoctoral grant funded by the Jesús Usón Minimally Invasive Surgery Centre. Beatriz Macías-García holds a postdoctoral grant Juan de la Cierva Incorporación (IJCI-2014-19428) from the Spanish Ministry of Economy, Industry and Competitiveness. This work was performed at the Singular Scientific Technological Infrastructures (ICTS) Nanbiosis (Units 23 and 14).

REFERENCES

Ali A. A.; Bilodeau J. F.; Sirard M. A., 2002: Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. **Theriogenology**, *59* 939-949.

Azadbakht M.; Valojerdi M. R., 2008: Development of vitrified-warmed mouse embryos co-cultured with polarized or non-polarized

uterine epithelial cells using sequential culture media. **Journal of Assisted Reproduction and Genetics**, *25* 251-261.

Bedaiwy M. A.; Falcone T.; Mohamed M. S.; Aleem A. A. N.; Sharma R. K.; Worley S. E.; Thornton J.; Agarwal A., 2004: Differential growth of human embryos in vitro: Role of reactive oxygen species. **Fertility and Sterility**, *82* 593-600.

Castillo-Martín M.; Bonet S.; Morató R.; Yeste M., 2014: Comparative effects of adding β-mercaptoethanol or l-ascorbic acid to culture or vitrification–warming media on IVF porcine embryos. **Reproduction, Fertility and Development**, *26* 875-882.

Covarrubias L.; Hernández-García D.; Schnabel D.; Salas-Vidal E.; Castro-Obregón S., 2008: Function of reactive oxygen species during animal development: Passive or active? **Developmental Biology**, *320* 1-11.

ESHRE, 2011: The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting†. Human Reproduction, Vol. 26. pp. 1270-1283.

Gao C.; Han H.-B.; Tian X.-Z.; Tan D.-X.; Wang L.; Zhou G.-B.; Zhu S.-E.; Liu G.-S., 2012: Melatonin promotes embryonic development and reduces reactive oxygen species in vitrified mouse 2-cell embryos. **Journal of pineal research**, *52* 305-311.

Ghandy N.; Karimpur Malekshah A. A., 2017: Which Stage of Mouse Embryos Is More Appropriate for Vitrification? **International Journal of Fertility & Sterility**, *10* 357-362.

Guérin P.; El Mouatassim S.; Ménézo Y., 2001: Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. **Hum Reprod Update**, *7* 175-189.

Jurisicova A.; Rogers I.; Fasciani A.; Casper R. F.; Varmuza S., 1998: Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development. **Mol Hum Rep**, *2* 139-145.

Kong X.; Yang S.; Gong F.; Lu C.; Zhang S.; Lu G.; Lin G., 2016: The Relationship between Cell Number, Division Behavior and Developmental Potential of Cleavage Stage Human Embryos: A Time-Lapse Study. **PloS one**, *11* e0153697.

Kuleshova L. L.; Lopata A., 2002: Vitrification can be more favorable than slow cooling. **Fertility and Sterility**, *78* 449-454.

Liang Y.; Ning F. Y.; Du W. J.; Wang C. S.; Piao S. H.; An T. Z., 2012: The type and extent of injuries in vitrified mouse oocytes. **Cryobiology**, *64* 97-102.

Mallol A.; Santaló J.; Ibáñez E., 2013: Comparison of three differential mouse blastocyst staining methods. **Systems Biology in Reproductive Medicine**, *59* 117-122.

Mandawala A. A.; Harvey S. C.; Roy T. K.; Fowler K. E., 2016: Cryopreservation of animal oocytes and embryos: Current

progress and future prospects. **Theriogenology**, *86* 1637-1644.

Marí M.; Morales A.; Colell A.; García-Ruiz C.; Fernández-Checa J. C., 2009: Mitochondrial Glutathione, a Key Survival Antioxidant. **Antioxidants & Redox Signaling**, *11* 2685-2700.

Momozawa K.; Matsuzawa A.; Tokunaga Y.; Abe S.; Koyanagi Y.; Kurita M.; Nakano M.; Miyake T., 2017: Efficient vitrification of mouse embryos using the Kitasato Vitrification System as a novel vitrification device. **Reproductive Biology and Endocrinology**, *15* 29.

Pedro P. B.; Yokoyama E.; Zhu S. E.; Yoshida N.; Valdez Jr D. M.; Tanaka M.; Edashige K.; Kasai M., 2005: Permeability of Mouse Oocytes and Embryos at Various Developmental Stages to Five Cryoprotectants. **Journal of Reproduction and Development**, *51* 235-246.

Rall W.F.; G.M. F., 1985: Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. **Nature**, *313(6003)* 3.

Rall W. F., 1987: Factors affecting the survival of mouse embryos cryopreserved by vitrification. **Cryobiology**, *24* 387-402.

Sawicki W.; Mystkowska E. T., 1990: In vivo staining of mouse preimplantation embryos with hoechst 33342. **The Anatomical Record**, *227* 359-362.

Silva E.; Greene A. F.; Strauss K.; Herrick J. R.; Schoolcraft W. B.; Krisher R. L., 2015: Antioxidant supplementation during in vitro culture improves mitochondrial function and development of embryos from aged female mice. **Reproduction, Fertility and Development**, *27* 975-983.

Somfai T.; Ozawa M.; Noguchi J.; Kaneko H.; Kuriani Karja N. W.; Farhudin M.; Dinnyés A.; Nagai T.; Kikuchi K., 2007: Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. **Cryobiology**, *55* 115-126.

Somoskoi B.; Martino N. A.; Cardone R. A.; Lacalandra G. M.; Dell'Aquila M. E.; Cseh S., 2015: Different chromatin and energy/redox responses of mouse morulae and blastocysts to slow freezing and vitrification. **Reprod Biol Endocrinol**, *13* 22.

Tatone C.; Di Emidio G.; Vento M.; Ciriminna R.; Artini P. G., 2010: Cryopreservation and oxidative stress in reproductive cells. **Gynecological Endocrinology**, *26* 563-567.

Tsang W.H.; Chow L.K., 2009: Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula. **Biotechniques**, *46(7)* 3.

Vajta G., Vitrification of the oocytes and embryos of domestic animals. **Animal reproduction science**, *60* 357-364.

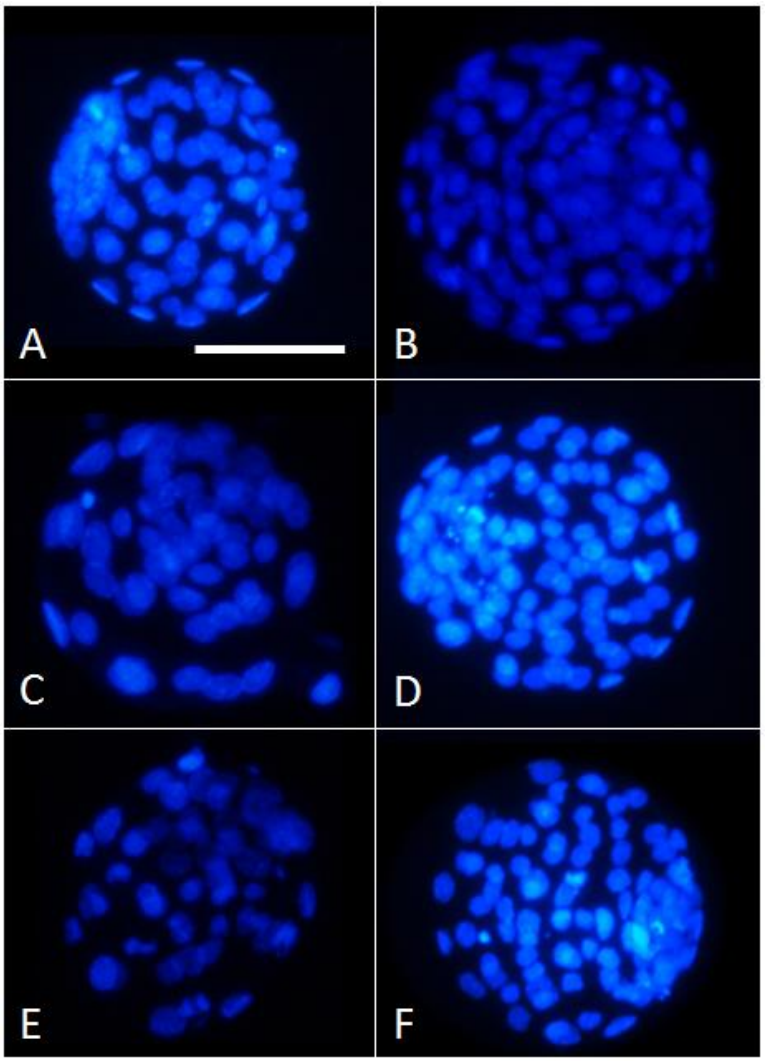
Van der Elst J; Amerijckx Y; A. V. S., 1998: Ultra-rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in-vitro cultured blastocysts. **Human Reproduction**, *13(6)* 5.

Yan C. L.; Fu X. W.; Zhou G. B.; Zhao X. M.; Suo L.; Zhu S. E., 2010: Mitochondrial behaviors in the

vitrified mouse oocyte and its parthenogenetic embryo: effect of Taxol pretreatment and relationship to competence. **Fertil Steril**, *93* 959-966.

Zhang J.; Cui J.; Ling X.; Li X.; Peng Y.; Guo X.; Heng B. C.; Tong G. Q., 2009: Vitrification of mouse embryos at 2-cell, 4-cell and 8-cell stages by cryotop method. **Journal of Assisted Reproduction and Genetics**, *26* 621-628.

Figure 1- Representative micrographs of murine expanded blastocysts



In vivo or IVF-derived murine 2-cell embryos were obtained, vitrified and cultured to the blastocyst stage in the presence or absence of 1 mM NAC. Representative micrographs are shown of blastocyst derived from 2-cell embryos that were obtained after **A)** IVF (fresh); **B)** In utero harvesting (fresh); **C)** 2-cell embryos produced in utero followed by vitrification; **D)** produced in utero followed by vitrification, cultured in presence of 1 mM NAC for 24 hours (day 1.5) and allowed to develop; **E)** IVF derived 2-cell embryo subjected to vitrification and warming; **F)** IVF derived 2-cell embryo subjected to vitrification, cultured in presence of 1 mM NAC for 24 hours (day 1.5) and allowed to develop. White bar represents 100 µm; the micrographs were taken using a 40x objective.

Table 1- Embryo development to the blastocyst stage

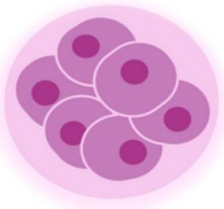
Embryo source	Treatment	n	Blastocyst rate
Fresh *In utero* (IU)	Control	36	89.0 ± 4.1
	NAC G1	41	95.2 ± 2.7
	NAC G2	38	95.0 ± 2.8
	NAC G3	35	94.0 ± 3.4
	C-NAC	37	89.7 ± 4.0
IU- vitrified embryos	Control	40	82.9 ± 3.0
	NAC G1	37	84.3 ± 5.9
	NAC G2	38	84.5 ± 3.5
	NAC G3	37	85.4 ± 6.5
	C-NAC	39	83.0 ± 5.5
Fresh IVF	Control	40	84.0 ± 2.4
	NAC G1	41	85.1 ± 1.9
	NAC G2	39	84.1 ± 3.9
	NAC G3	42	82.7 ± 6.0
	C-NAC	46	78.9 ± 3.1
IVF – Vitrified	Control	42	84.4 ± 2.1
	NAC G1	42	85.1 ± 1.7
	NAC G2	44	73.4 ± 4.8
	NAC G3	43	72.4 ± 4.3
	C-NAC	42	71.1 ± 5.5

Blastocyst rates of fresh and vitrified mouse embryos obtained by IVF or *in vivo* in presence or absence of NAC. The groups studied were: Control: embryos cultured in the absence of NAC; NAC G1: 1 mM NAC was added for 24 hours to the embryo culture medium at day 1.5 of embryo development; NAC G2: 1 mM NAC was added for 24 hours to the embryo culture medium at day 2.5 of embryo development; NAC G3: 1 mM NAC was added for 24 hours to the embryo culture medium at day 3.5 of embryo development; C-NAC: culture medium was supplemented with 1 mM of NAC during the entire embryo culture. Statistically significant differences were not found between treatments in the same group or between homologous treatments in the different groups studied. Values are expressed as the mean percentage ± SEM ($p > 0.05$).

Table 2- Cell number of murine blastocyst

Embryos	Treatment	n	Cell number
Fresh *in utero* (IU)	Control	20	70.6 ± 3.5
	NAC G1	20	78.5 ± 3.9
	NAC G2	20	67.7 ± 4.5
	NAC G3	20	68.2 ± 3.4
	C-NAC	20	67.9 ± 4.6
IU- vitrified embryos	Control	21	51.9 ± 1.1^{b,c}
	NAC G1	21	58.8 ± 1.5^{a}
	NAC G2	20	57.3 ± 2.0^{a}
	NAC G3	23	55.3 ± 1.3^{a,b,c}
	C-NAC	23	51.3 ± 1.0^{b}
Fresh IVF	Control	20	51.3 ± 2.1
	NAC G1	17	47.7 ± 3.1
	NAC G2	16	46.9 ± 2.8
	NAC G3	19	54.3 ± 3.6
	C-NAC	16	49.0 ± 2.9
IVF - Vitrified	Control	16	49.8 ± 3.1^{a}
	NAC G1	16	61.6 ± 1.6^{b}
	NAC G2	17	54.2 ± 2.9^{a,c}
	NAC G3	16	50.0 ± 2.9^{a}
	C-NAC	17	56.3 ± 1.7^{a,b}

Total cell number of vitrified mouse embryos obtained by IVF or *in vivo* in presence or absence of NAC. Control: embryos cultured in the absence of NAC; NAC G1: 1 mM NAC was added for 24 hours to the embryo culture medium at day 1.5 of embryo development; NAC G2: 1 mM NAC was added for 24 hours to the embryo culture medium at day 2.5 of embryo development; NAC G3: 1 mM NAC was added for 24 hours to the embryo culture medium at day 3.5 of embryo development; C-NAC: culture medium was supplemented with 1 mM of NAC during the entire embryo development. Values are expressed as the mean percentage ± SEM. Values bearing different letters in the same group differ statistically ($p < 0.05$).



ARTÍCULO 4

N-Acetylcysteine improves oocyte mitochondrial polarization status and quality of embryos derived from vitrified murine oocytes

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ABSTRACT

Vitrification is the safest method to cryopreserve oocytes, however this process alters mitochondrial function resulting in increased production of reactive oxygen species (ROS). The aim of the present study was to alleviate ROS stress in vitrified mice oocytes using N-acetylcysteine (NAC at 1 mM), a ROS scavenger, being the final goal to improve their developmental competence. In our setting four experimental groups were compared: fresh oocytes (F-C), vitrified oocytes (V-C), NAC addition prior oocyte vitrification (V-NAC-Pre) and NAC addition after vitrification (V-NAC-Post). V-NAC-Pre and V-NAC-Post exhibited higher mitochondrial polarization values than vitrified control (36.5 ± 3.1, 37.7 ± 1.3 and 27.2 ± 2.4 measured as the spatial coefficient of variation/oocyte respectively, mean ± SEM; $p < 0.05$). However, ROS production increased in vitrified oocytes added with NAC compared to the vitrified control (794.6 ± 164.9 (V-C) *vs.* 1124.7 ± 102.1 (V-NAC-Pre) and 1063.2 ± 82.1 (V-NAC-Post) arbitrary fluorescence units/oocyte (mean ± SEM; $p < 0.05$). ATP content significantly decreased when NAC was added prior vitrification compared to NAC addition after vitrification (18.5 ± 6.9 *vs.* 54.2 ± 4.6 fmol/oocyte respectively, mean ± SEM; $p < 0.05$), being the ATP content of the latter treatment similar to F-C and V-C. Blastocyst rates derived from F-C oocytes was higher than those of V-NAC-Pre (90.7 ± 1.8 *vs.* 79.1 ± 1.8, mean % ± SEM, respectively; $p < 0.05$) but similar to those of V-NAC-Post (90.7 ± 1.8, mean % ± SEM, $p > 0.05$). Total blastomere count of blastocysts derived from V-NAC-Post oocytes was higher than in embryos produced using vitrified oocytes (76.8 ± 4.1 *vs.* 58.9 ± 2.5 total cell number respectively, mean ± SEM; $p < 0.05$). Our results demonstrate a significant improvement of the *in vitro* quality of vitrified mature murine oocytes when NAC is added after vitrification.

Keywords: mouse; N-acetylcysteine; oocyte; vitrification.

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1. INTRODUCTION

Mitochondria are core organelles in the oocyte as they determine their meiotic and developmental competence [154]. These organelles are responsible for energy production in the form of ATP through the oxidative phosphorylation chain resulting in controlled Reactive Oxygen Species (ROS) production [155]. Specifically, mitochondrial membrane potential is an important parameter defining mitochondrial and cellular status [156]. In mature oocytes, mitochondrial size, function as well as their overall number, are critical factors for fertilization and subsequent embryo development [157]. In fact, during the progression of the primordial follicle to the mature oocyte stage (metaphase II or MII), the number of mitochondria vividly increases due to the high energy demands required for oocyte fertilization and subsequent cleavage [158]. The oocyte represents the female germline and its long term maintenance enables for the protection and more flexible use of selected female genetics [159]. In this sense, vitrification is the most rapid and safest method to cryopreserve oocytes, but it alters mitochondrial membrane potential resulting in increased production of ROS, thus eroding their developmental competence [136]. In addition, cryopreservation severely affects the oocyte's mitochondrial status and function which has been associated with implantation failure in IVF-derived embryos [160, 161]. However, such dysfunctions do not induce evident morphological abnormalities that could help to select the best embryos to transfer [162]. During cryopreservation, oxidative stress occurs due to gradual accumulation of free radicals causing a decrease in the survival rate and developmental competence of the oocytes [137]. In this regard, increased ROS production impairs multiple physiological processes such as oocyte maturation, fertilization and embryo development [163], and thus, exogenous antioxidant addition could help to alleviate ROS production and support mitochondrial function [160]. Supplementation of media with antioxidants could possibly help to improve gamete quality and fortify the developing embryo [109] as previously shown in vitrified-warmed bovine mature oocytes [164]. However, the appropriate antioxidants and concentrations required, as well the best moment to add the selected antioxidants for different assisted reproduction techniques still remain an ongoing area of research [165]. Some antioxidants such as Glutathione (GSH) have been reported to mitigate the toxic effects of oxidative stress when added to vitrified murine oocytes at different time points of the process [166]. For example, in the murine model, it has been demonstrated that supplementation with a glutathione donor prior vitrification improves the cryotolerance of MII oocytes [167]. Additionally, immature murine oocytes vitrified and warmed in presence of glutathione exhibit increased blastocyst rates compared to the non-supplemented controls [168]. In this sense, the use of N-acetylcysteine or NAC (a precursor of GSH) during MII oocyte cryopreservation, could be advantageous as it actively scavenges free radicals increasing oocyte quality in aged mice and in vitrified immature murine oocytes [169, 170]. Thus, the aim of the present study was to investigate the impact of NAC supplementation to mature murine oocytes before and after vitrification as well as its effects on ATP production, mitochondria polarization, ROS production and embryo development and quality after IVF.

2. MATERIAL AND METHODS

2.1. Reagents

All the reagents were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise stated.

2.2. Animals

All the experimental procedures were reviewed and approved by the Ethical Committee of the Junta de Extremadura Spain (Spain, Ref. SBC/mbr). B6D2F1/J mice were housed under 12 h light/12 h dark cycles at a controlled temperature (19-23ºC), with free access to food and water. Females were intraperitoneally (IP) injected

with 8 IU of equine chorionic gonadotrophin (eCG, Veterin Corion, Divasa Farmavic) followed 47 h later by 8 IU of IP human chorionic gonadotrophin (hCG, Foligon, MSD) to trigger ovulation. Female mice were euthanized 12 hours after hCG administration by cervical dislocation, and cumulus-oocyte complexes (COCs) were recovered from oviducts and placed in 500 µL of Human Tubal fluid (HTF) covered with mineral oil. One hour later, COCs were denuded using hyaluronidase (80 IU/mL) in M2 medium for no more than one min to remove granulosa cells and washed in M2 medium. After denudation, MII oocytes were separated in the different experimental groups (Fig. 1).

2.3. Vitrification and warming

MII denuded oocytes were equilibrated in M2 medium added with 7.5% of DMSO (v:v), 7.5% ethylene glycol (v:v) and 20% (v:v) fetal bovine serum (FBS) for 3 min. Afterwards, the oocytes were moved to a vitrification solution consisting of M2 medium supplemented with 20% FBS added with 15% ethylene glycol (v:v), 15% DMSO (v:v) and 0.5 M sucrose for 1 min. Groups of 10 to 15 oocytes were loaded in 0.25 mL French straws (IMV, L'Aigle, France) at room temperature and sealed by ultrasounds (Superultrasonic Co; Ltd. Taiwan). After that, the straws were plunged into liquid nitrogen and stored for at least 7 days. Oocytes were warmed at 37ºC for 3-4 min in M2 medium added with 0.5 M sucrose and 20% FBS and washed in M2 medium for further 3 min.

2.4. In Vitro Fertilization

Male B6D2F1/J mice aged 8-12 weeks were euthanized by cervical dislocation and ventrally dissected to remove the cauda epididymis. Once located, the epididymis and attached vas deferens were sectioned and transferred to a Petri dish containing 500 µL of pre-equilibrated HTF covered with mineral oil. Spermatozoa were obtained by gently pressing the cauda epididymis through the vas deferens and were allowed to capacitate for 45 min at 37°C in a 5% CO_2/ 95% air atmosphere at 100% humidity. At the end of the incubation, sperm concentration was measured using a Makler chamber (Irvine Scientific, CA, USA). Oocytes were inseminated with 2×10^6 sperm/mL; gametes were co-incubated for 3 hours, moved to clean KSOM medium and followed in culture until they reached the expanded blastocyst stage.

2.5. Experimental design

Oocytes were separately allocated to one of the following experimental groups (Fig. 1):

- Fresh control group (F-C): the denuded oocytes were cultured in KSOM medium for 2 hours prior IVF; presumptive zygotes were transferred to fresh KSOM medium and cultured to the blastocyst stage.

-Vitrified control group (V-C): the denuded oocytes were cultured in KSOM medium for two hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF afterwards; presumptive zygotes were transferred to fresh KSOM medium and cultured to the blastocyst stage.

-Vitrified pre-treatment group (V-NAC-Pre): denuded oocytes were cultured in KSOM medium supplemented with 1 mM NAC for two hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in regular KSOM medium before IVF. After gamete co-incubation presumptive zygotes were transferred to fresh KSOM medium and cultured to the blastocyst stage.

-Vitrified post-treatment group (V-NAC-Post): denuded oocytes were cultured in regular KSOM medium for two hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC. Then, the oocytes were subjected to IVF. After gamete co-incubation, presumptive zygotes were transferred to fresh KSOM medium and cultured to the blastocyst stage.

2.6. Mitochondrial polarization status

Denuded oocytes were photosensitized using the mitochondrial-specific fluorophore Rhodamine 123 (R123) at 5 µM at 37ºC added to 4-(2-hydroxyethyl)-1-

piperazineethanesulphonic acid (HEPES)-base medium containing (mM): 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 20 glucose, 10 HEPES, with the pH adjusted (NaOH) to 7.4 for 10 min as described previously [171]. After loading, the chamber containing the oocytes was perfused for 2 min with Na+-HEPES-based medium in order to remove excess of R123. The cells were then imaged with a confocal scanning laser microscopy (Eclipse Ti, Nikon) at × 40 magnification using a 488 nm argon laser and the emission was collected through a 525/550 nm filter following the method described by Toescu et al. (2000). This method is based on the determination of the heterogeneity of the signal from R123 as an indicator of the depolarization of the mitochondrial network. The heterogeneity is calculated by the spatial coefficient of variation (CV; standard deviation/average) of the whole body fluorescence, thus, the higher the CV, the more hyperpolarized is the mitochondrial network. In our setting, in the oocytes with polarized mitochondria, the image taken contained a number of pixels with very high intensity, corresponding with mitochondria accumulating lipophilic dyes and many pixels with low intensity which coincide with the cytosolic and nuclear regions; as a result, both the SD and CV will be large. In oocytes with depolarized mitochondria, the distribution of individual pixel intensities will be more homogeneous, as the mitochondrial signal will decrease and, at the same time, the signal from cytosol will increase, being their CV lower [171]. Images were acquired using NIS Elements software (Nikon, Japan) and analysed with Fiji (NIH [172]). In these experiments 12 oocytes/treatment collected in 3 different days from at least 9 different animals were analyzed; four different sessions were used to analyze the oocytes.

2.7. ROS assessment

To measure ROS levels, the oocytes were transferred to HEPES base medium added with 4 MM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Merck; Darmstadt, Germany) at 37°C for 15 min in the dark. Then, the solution was removed and the oocytes (12 oocytes/treatment collected in 3 different days from at least 9 different animals were analyzed in 4 different sessions) were washed for 1 min with HEPES-base medium. The cells were then imaged with a confocal microscope (confocal scanning laser (Nikon A1) coupled to an inverted microscope (Eclipse Ti, Nikon)) using a 480 nm argon laser; the emission was collected through a 525/550 nm filter and the intensity of the fluorescence was determined with Fiji (NIH [172]).

2.8. Oocyte ATP content determination

ATP concentrations were determined using the ATP Bioluminescent Somatic Cell Assay Kit, as described previously with minor modifications [173]. Ten to fifteen oocytes from each group were snap-frozen in a sterile microtube containing 100 µL of ultrapure water and stored at -80ºC. The oocytes (38 to 45 per treatment) were collected at 3 different days from at least 9 different animals and the analysis was performed in 4 different sessions. The day of the experiment, a volume of 50 µL of ATP assay mix Working Solution was added to individual wells in an opaque 96-well plate and kept at room temperature for 3 min to allow for endogenous ATP hydrolysis. Fifty microliters of the thawed samples were mixed with Somatic Cell ATP Releasing Reagent (1:100). This mixture was transferred to the wells and the amount of bioluminescence emitted was measured immediately using a Synergy 2 plate reader (Varioskan, ThermoFisher Scientific). The background luminescence was subtracted from all readings. ATP concentration was calculated by comparison against a standard curve.

2.9. Development to the blastocyst stage

The presumptive zygotes were followed for 4 days to assess their development to the expanded blastocyst stage; the blastocyst formation rate was recorded using a stereomicroscope for each group individually.

2.10. Blastocyst cell number determination

Blastocyst of each group were fixed in 4% parafomaldehyde and stained with Hoechst

33258 (2.5 µg/mL in PBS) added with 0.2% of polyvinyl alcohol (PVA) for 10 min at 37ºC in the dark. At the end of the incubation, the expanded blastocysts were mounted using glycerol on glass slides, covered with coverslips, sealed with nail polish and analyzed using a fluorescence microscope (Nikon Eclipse TE2000-S) equipped with an ultraviolet lamp. Pictures were taken and the number of blastomeres of each blastocyst was determined. Cell number was analyzed using the Fiji Image-J software (1.45q, Wayne Rasband, National Institutes of Health, USA).

2.11. Statistical analysis

Data were tested for normality using a Shapiro–Wilk test; results are reported as mean ± standard error of the mean (SEM). Groups were compared using ANOVA on ranks due to their non-Gaussian distribution. When statistically significant differences were found, a Holm-Sidak or Dunn´s post-hoc test was used to compare pairs of values. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Mitochondrial polarization analysis

When all the treatments were compared between them, frozen-thawed oocytes exhibited the lowest mitochondrial polarization compared to the rest of the treatments (Fig. 1; $p < 0.05$). Interestingly, NAC addition prior and after vitrification exhibited similar mitochondrial polarization compared to the fresh control group (36.5 ± 3.1, 37.7 ± 1.3 and 42.6 ± 1.7, CV/oocyte in % respectively) (Fig. 2 and 5; $p > 0.05$).

3.2. ROS production assessment

After vitrification overall ROS production increased when NAC was added prior or after vitrification compared to the vitrified control ($p < 0.05$); interestingly, although ROS production was higher when NAC was added, this increase was not statistically significant compared to fresh control group (695.3 ± 32.1 (control) *vs.* 1124.7 ± 102.1 (prior vitrification); 1063.2 ± 82.1 (after vitrification) arbitrary fluorescence units/oocyte (mean ± SEM) (Fig. 3 and 6; $p > 0.05$).

3.3. Determination of ATP content

Our results showed that fresh and frozen-thawed control groups did not differ in their ATP content (42.5 ± 3.0 *vs.* 38 ±8.7 fmol/oocyte respectively, mean ± SEM; $p > 0.05$). However, addition of NAC to vitrified-warmed oocytes significantly decreased ATP content when NAC was added prior vitrification compared to NAC addition after vitrification (18.5 ± 6.9 *vs.* 54.2 ± 4.6 fmol/oocyte respectively, mean ± SEM; $p < 0.05$). No significant differences were observed when all the rest of the treatments were compared among them (Fig. 4; $p > 0.05$).

3.4. Development to the blastocyst stage and total blastomere number

Our results showed significant differences between blastocyst rates derived from fresh oocytes and those obtained from vitrified-warmed oocytes added with 1 mM NAC prior vitrification (90.7 ± 1.8 *vs.* 79.1 ± 1.8, mean % ± SEM, respectively; Table 1, $p < 0.05$). Also, statistically significant differences were found in the blastocyst rates between vitrified-warmed oocytes added with NAC prior vitrification and oocytes added with NAC after vitrification (79.1 ± 1.8 *vs.* 90.1 ± 1.8, mean % ± SEM, respectively; Table 1, $p < 0.05$). Interestingly, total cell number of blastocyst derived from the vitrified NAC-Post group significantly increased compared to vitrified control (76.8 ± 4.1 *vs.* 58.9 ± 2.5 total cell number respectively, mean ± SEM ($p < 0.05$; Table 1 and Fig. 7).

4. DISCUSION

Vitrification and warming significantly affects oocytes' structures impairing their developmental competence. Among other insults, vitrification induces mitochondrial depolarization of the oocyte [136]

decreasing their ATP content and enhancing ROS production [174]. As mitochondria play a core role determining the oocyte's developmental competence, any deleterious impact on mitochondrial function will negatively affect the ability of the zygotes to reach the expanded blastocyst stage [80, 155]. In addition, it is known that the mitochondrial network is highly vulnerable to temperature fluctuations and vitrification is known to alter mitochondrial distribution of matured oocytes [175]. Regarding mitochondrial distribution, a previous report has determined that a fine homogeneous pattern of active mitochondria could be an indicator of poor developmental competence in ovine oocytes [176]. In our experiments a higher mitochondrial polarization was observed in fresh oocytes compared to their vitrified counterparts, and NAC addition prior or after vitrification enhanced mitochondrial polarization to the level observed in fresh oocytes (Figs. 2 and 5). Our results are in agreement with those of Lei et al. (2014) who found that vitrification induced an abnormal mitochondrial distribution and a decreased mitochondrial activity in mouse oocytes [175]. In addition, NAC addition prior and after vitrification significantly increased mitochondrial polarization coinciding with the results reported by Yue et al. (2016) for immature mouse oocytes [170]. These results suggest that the assessment of mitochondrial polarization status using the CV coincides with the analysis of mitochondrial distribution pattern, being therefore a reliable indicator of mitochondrial status and distribution.

Oocyte vitrification alters the normal mitochondrial function and provokes damage to the endogenous antioxidant systems which are responsible for the increase in the levels of ROS [137]. In porcine oocytes, it has been demonstrated that ROS production increases after oocyte vitrification [177]. To alleviate this oxidative burst, antioxidant addition to the medium prior vitrification [177] or during the recovery culture after warming [164] has been used to avoid the loss of the physiological balance between ROS production and antioxidant defenses. Interestingly, our results showed that ROS production in vitrified-warmed oocytes was similar to that of fresh oocytes, while oocyte vitrification in presence of NAC enhanced ROS production. The homogeneity in ROS production of fresh and vitrified oocytes is in contrast with those of Tatone et al. (2011) who showed that vitrification enhanced ROS production in murine oocytes. This divergence can be explained because of the different mice strains used (CD-1 in their work *vs.* B6D2 in ours) or could be due to the fact that, in our setting, oocytes are incubated for moderate times prior and after vitrification, inducing a slight oocyte aging. It has been demonstrated that *in vitro* oocyte ageing induces lower ROS production after vitrification (that is similar to aged fresh oocytes) compared to young oocytes [178]. Even when this could seem to be a matter of concern, the incubation times used in our experimental design (maximum of 5 hours when NAC was added after vitrification) have been demonstrated not to detrimentally affect the oocyte's developmental competence [179]. To further confirm this theory, when NAC was added prior vitrification, blastocyst rates remained unaffected compared to fresh controls (Table 1) even at the longest oocyte incubations, validating our experimental design.

Regarding ROS increase in vitrified NAC-added oocytes (Fig. 3), over those of vitrified control group, this result could be explained in view of the higher mitochondrial polarization observed in our experiments being the mitochondria more active and capable of producing ROS as demonstrated in other germ cells [180]. Furthermore, the increase observed is similar to that of the fresh group, suggesting that NAC addition to murine oocytes prior or after vitrification does not induce a deleterious ROS imbalance. However, it has been demonstrated that intracellular ROS scavenging using antioxidants does not necessarily correlate with enhanced cryotolerance or developmental competence in bovine embryos, not being ROS production a reliable indicator of cryopreservation or *in vitro* fertilization outcomes [181].

In view of these results, ATP production was measured in all the groups as it is known that mitochondrial damage affects the oocyte's developmental competence impairing ATP production and increasing embryo arrest [155]. Interestingly, ATP production remained unchanged in vitrified and fresh oocytes, and vitrified oocytes added with NAC after vitrification, contrasting with previous research that demonstrated an ATP decrease after oocyte cryopreservation [182, 183]. However, ATP production significantly decreased when NAC was added prior vitrification, suggesting that its addition before vitrification is not indicated. Furthermore, the fact that the development to the blastocyst stage in this group after IVF is lower than for fresh, vitrified and vitrified oocytes supplemented with NAC after vitrification groups, suggests that NAC addition prior vitrification exerts a toxic effect on mitochondrial function. It has to be noted that in our experiments the number of blastomeres significantly decreases after vitrification compared to the fresh counterparts, as also demonstrated for mice cryopreserved embryos [184]. Notably, after vitrification and NAC addition, the number of blastomeres significantly increased compared to the vitrified controls or vitrified oocytes added with NAC prior cryopreservation, demonstrating an improvement on embryo quality [145, 146]. Although total blastomere number was lower in vitrified embryos added with NAC after vitrification than fresh control, total cell number was almost doubled compared to vitrified embryos added with NAC before vitrification (Table 1 and Fig. 7). These results together with the lower developmental competence and diminished ATP production in the oocytes added with NAC before vitrification demonstrate that NAC should be added to murine oocytes after vitrification. In addition, ROS production or mitochondrial polarization do not seem to be good indicators of mitochondrial status, being ATP content more adequate in murine oocytes. Although previous reports showed that glutathione donors improve murine oocyte's cryotolerance and maintain ATP content [167], in this work ATP was measured right after oocyte thawing while in our work oocytes were allowed to re-equilibrate, possibly explaining the observed differences.

5. CONCLUSIONS

In conclusion, supplementation of mice oocytes with NAC after vitrification improves mitochondrial status and total cell number in expanded blastocysts. NAC addition prior cryopreservation is advised against, as it impairs the oocyte´s ATP content and erodes its developmental competence. More research is necessary to understand the mechanism by which NAC exerts its effects on murine oocytes and to develop and improve post-warming equilibration media by NAC supplementation. Our findings could be of clinical interest and more studies are required to test if our results can be extrapolated to other domestic species.

REFERENCES

[1] Chiaratti MR, Meirelles FV. Mitochondrial DNA Copy Number, a Marker of Viability for Oocytes1. Biology of Reproduction. 2010;83:1-2.

[2] Chappel S. The Role of Mitochondria from Mature Oocyte to Viable Blastocyst. Obstetrics and Gynecology International. 2013;2013:183024.

[3] Toescu E.C., A. V. Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining. Pflugers Arch. 2000;440(6):941-7.

[4] Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, et al. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Human Reproduction. 2001;16:909-17.

[5] Jansen RPS, de Boer K. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Molecular and cellular endocrinology. 1998;145:81-8.

[6] Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Human Reproduction Update. 2017;23:139-55.

[7] Liang Y, Ning FY, Du WJ, Wang CS, Piao SH, An TZ. The type and extent of injuries in vitrified mouse oocytes. Cryobiology. 2012;64:97-102.

[8] Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, et al. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Hum Reprod 2001;16(5):909-17.

[9] Lei T, Guo N, Liu J-Q, Tan M-H, Li Y-F. Vitrification of in vitro matured oocytes: effects on meiotic spindle configuration and mitochondrial function. International Journal of Clinical and Experimental Pathology. 2014;7:1159-65.

[10] Thouas GA, Trounson AO, Jones GM. Developmental Effects of Sublethal Mitochondrial Injury in Mouse Oocytes. Biol Reprod. 2006;74:969-77.

[11] Somfai T, Ozawa M, Noguchi J, Kaneko H, Kuriani Karja NW, Farhudin M, et al. Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. Cryobiology. 2007;55:115-26.

[12] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:28.

[13] Khalil WA, Marei WFA, Khalid M. Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. Theriogenology.80:161-8.

[14] Yashiro I, Tagiri M, Ogawa H, Tashima K, Takashima S, Hara H, et al. High revivability of vitrified–warmed bovine mature oocytes after recovery culture with α-tocopherol. Reproduction. 2015;149:347-55.

[15] Agarwal A, Durairajanayagam D, du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reproductive Biology and Endocrinology. 2014;12:112.

[16] Shan XQ, Aw TY, Jones DP. Glutathione-dependent protection against oxidative injury. Pharmacol Ther. 1993;47(1):61-7.

[17] Trapphoff T, Heiligentag M, Simon J, Staubach N, Seidel T, Otte K, et al. Improved cryotolerance and developmental potential of in vitro and in vivo matured mouse oocytes by supplementing with a glutathione donor prior to vitrification. MHR: Basic science of reproductive medicine. 2016;22:867-81.

[18] Moawad AR, Tan SL, Taketo T. Beneficial effects of glutathione supplementation during vitrification of mouse oocytes at the germinal vesicle stage on their preimplantation development following maturation and fertilization in vitro. Cryobiology. 2017;76:98-103.

[19] Liu J, Liu M, Ye X, Liu K, Huang J, Wang L, et al. Delay in oocyte aging in mice by the antioxidant N-acetyl-l-cysteine (NAC). Human Reproduction. 2012;27:1411-20.

[20] Yue S-L, Zhang Y-T, Wang S-W, Sun M, Xing Y-C, Wen J, et al. Effect of NAC on mouse GV oocyte survival and subsequent embryonic development following vitrification2016.

[21] Toescu EC, Verkhratsky A. Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining. Pflugers Arch. 2000;440:941-7.

[22] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji - an Open Source platform for biological image analysis. Nature methods. 2012;9:10.1038/nmeth.2019.

[23] Simsek-Duran F, Li F, Ford W, Swanson RJ, Jones HW, Jr., Castora FJ. Age-Associated Metabolic and Morphologic Changes in Mitochondria of Individual Mouse and Hamster Oocytes. PloS one. 2013;8:e64955.

[24] Szurek EA, Eroglu A. Comparison and Avoidance of Toxicity of Penetrating Cryoprotectants. PloS one. 2011;6:e27604.

[25] Thouas GA, Trounson AO, Jones GM. Effect of Female Age on Mouse Oocyte Developmental Competence Following Mitochondrial Injury. Biol Reprod. 2005;73:366-73.

[26] Lei T, Guo N, Tan M-h, Li Y-f. Effect of mouse oocyte vitrification on mitochondrial membrane potential and distribution. J Huazhong Univ Sci Technolog Med Sci. 2014;34:99-102.

[27] Leoni GG, Palmerini MG, Satta V, Succu S, Pasciu V, Zinellu A, et al. Differences in the Kinetic of the First Meiotic Division and in Active Mitochondrial Distribution between Prepubertal and Adult Oocytes Mirror Differences in their Developmental Competence in a Sheep Model. PLoS ONE. 2015;10:e0124911.

[28] Giaretta E, Spinaci M, Bucci D, Tamanini C, Galeati G. Effects of Resveratrol on Vitrified Porcine Oocytes. Oxidative Medicine and Cellular Longevity. 2013;2013:920257.

[29] Tatone C, Di Emidio G, Barbaro R, Vento M, Ciriminna R, Artini PG. Effects of reproductive aging and postovulatory aging on the maintenance of biological competence after oocyte vitrification: insights from the mouse model. Theriogenology. 2011;76:864-73.

[30] Karami A, Bakhtiari M, Azadbakht M, Ghorbani R, Khazaei M, Rezaei M. Impact of prolonged oocyte incubation time before vitrification on oocyte survival, embryo formation, and embryo quality in mice. In Vitro Cellular & Developmental Biology - Animal. 2017;53:525-31.

[31] Macías-García B, González-Fernández L, Gallardo-Bolaños JM, Peña FJ, Johannisson A, Morrell JM. Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS. Animal Reproduction Science. 2012;132:74-82.

[32] Rocha-Frigoni NAS, Leão BCS, Nogueira É, Accorsi MF, Mingoti GZ. Reduced levels of intracellular reactive oxygen species and apoptotic status are not correlated with increases in cryotolerance of bovine embryos produced in vitro in the presence of antioxidants. Reproduction, Fertility and Development. 2014;26:797-805.

[33] Manipalviratn S, Tong Z-B, Stegmann B, Widra E, Carter J, DeCherney A. Effect of vitrification and thawing on human oocyte ATP concentration. Fertility and Sterility. 2011;95:1839-41.

[34] Salvetti P, Buff S, Afanassieff M, Daniel N, Guérin P, Joly T. Structural, metabolic and developmental evaluation of ovulated rabbit oocytes before and after cryopreservation by vitrification and slow freezing. Theriogenology. 2010;74:847-55.

[35] Zander-Fox D, Cashman KS, Lane M. The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development. Journal of Assisted Reproduction and Genetics. 2013;30:107-16.

[36] Kong X, Yang S, Gong F, Lu C, Zhang S, Lu G, et al. The Relationship between Cell Number, Division Behavior and Developmental Potential of Cleavage Stage Human Embryos: A Time-Lapse Study. PloS one. 2016;11:e0153697.

[37] Mallol A, Santaló J, Ibáñez E. Comparison of three differential mouse blastocyst staining methods. Systems Biology in Reproductive Medicine. 2013;59:117-22.

Figure 1.

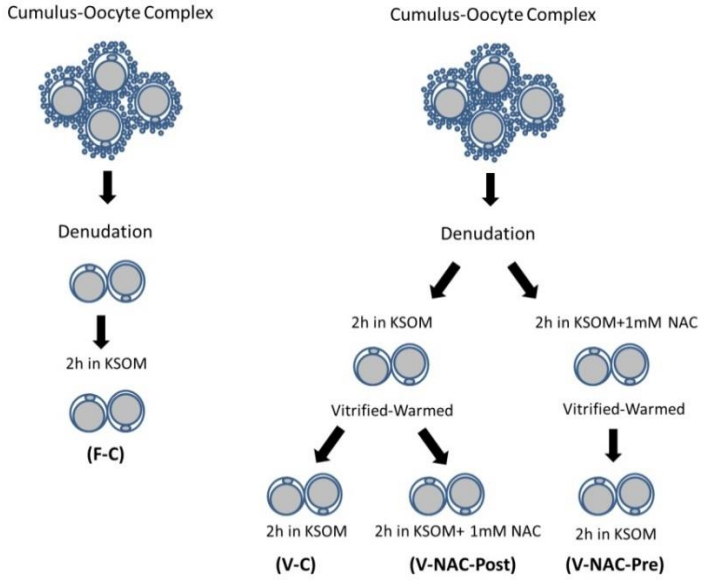
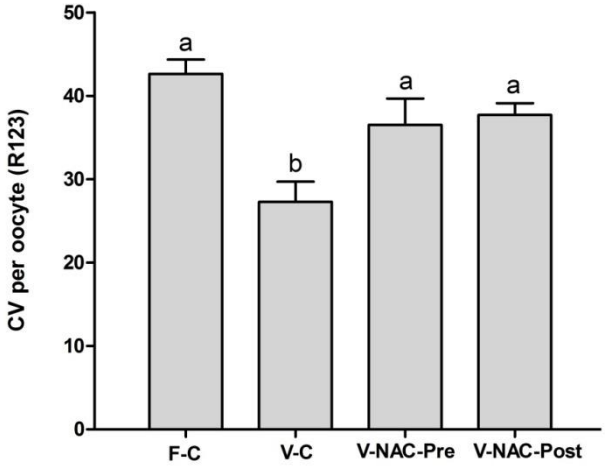


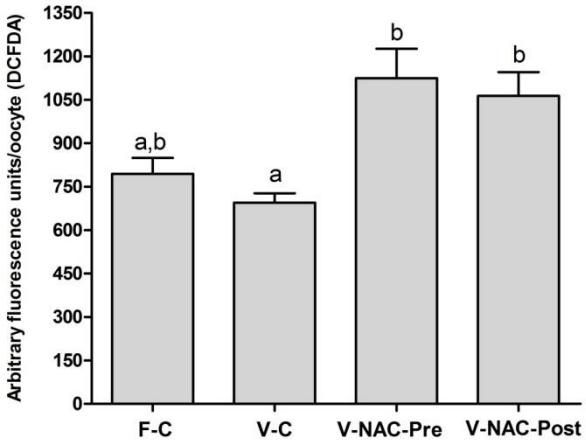
Diagram explaining the experimental design used in our study. F-C: MII denuded oocytes were cultured in KSOM medium for 2 hours prior IVF; V-C: MII denuded oocytes were cultured in KSOM medium for 2 hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF; V-NAC-Pre: MII denuded oocytes were cultured in KSOM medium supplemented with 1 mM NAC for two hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium before IVF; V-NAC-Post: MII denuded oocytes were cultured in KSOM medium for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC before IVF.

Figure 2.



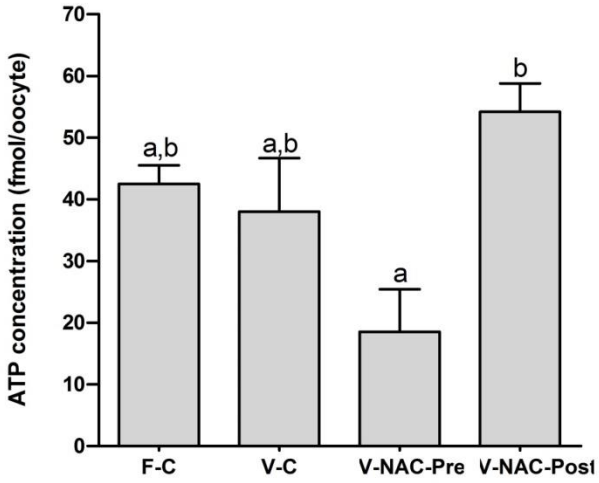
Mitochondrial polarization status of fresh and vitrified murine oocytes in presence or absence of NAC. The groups studied were: F-C: oocytes were cultured in KSOM medium for 2 hours prior IVF; V-C: oocytes were cultured in KSOM medium for 2 hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF; V-NAC-Pre: oocytes were cultured in KSOM medium supplemented with 1 mM NAC for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium before IVF; V-NAC-Post: oocytes were cultured in KSOM medium for two hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC before IVF. Bars bearing different letters differ statistically ($p < 0.05$); values are expressed as the mean ± SEM.

Figure 3.



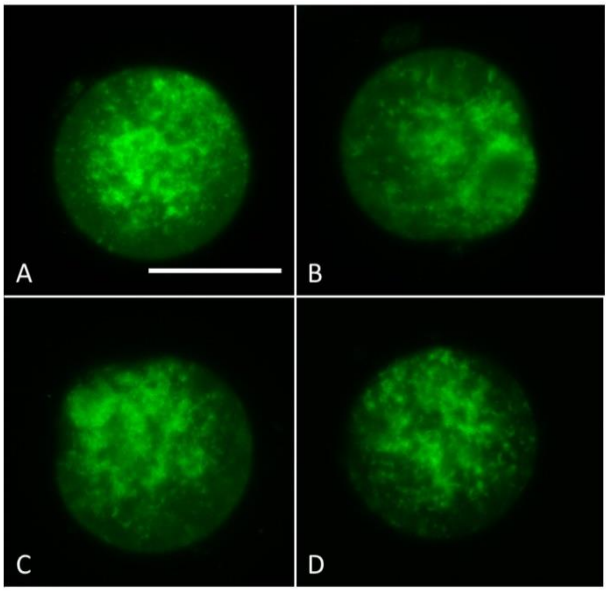
Assessment of ROS production in fresh and vitrified murine oocytes in presence or absence of NAC. The groups studied were: F-C: oocytes were cultured in KSOM medium for 2 hours prior IVF; V-C: oocytes were cultured in KSOM medium for 2 hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF afterwards; V-NAC-Pre: oocytes were cultured in KSOM medium supplemented with 1 mM NAC for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM before IVF; V-NAC-Post: oocytes were cultured in KSOM medium for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC before IVF. Bars with different letters differ statistically ($p < 0.05$); values are expressed as mean ± SEM.

Figure 4.



ATP content of fresh and vitrified oocytes in presence or absence of NAC. The groups studied were: F-C: oocytes were cultured in KSOM medium for 2 hours prior IVF; V-C: oocytes were cultured in KSOM medium for 2 hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF; V-NAC-Pre: oocytes were cultured in KSOM medium supplemented with 1 mM NAC for two hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium before IVF; V-NAC-Post: oocytes were cultured in KSOM medium for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC before IVF. Bars with different letters differ statistically ($p < 0.05$); values are expressed as mean ± SEM.

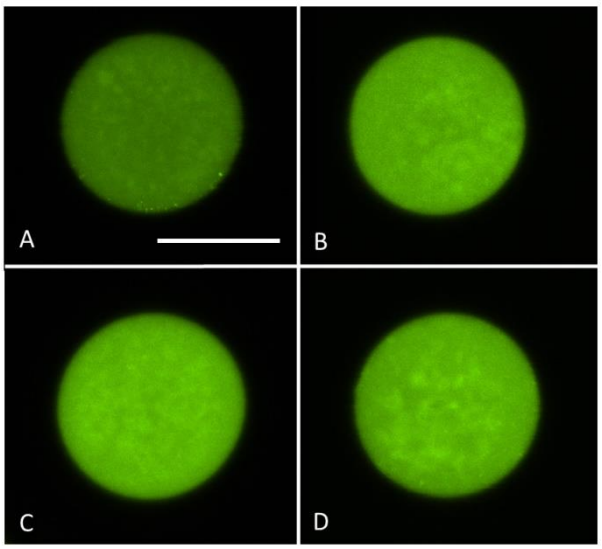
Figure 5.



Representative images of the mitochondrial polarization status of fresh and vitrified murine oocytes. The letters in each panel represent: **A)** Fresh oocytes cultured in KSOM medium for 2 hours; **B)** Vitrified oocytes cultured in KSOM medium for 2 hours after warming; **C)** Oocytes cultured in KSOM medium supplemented with 1mM of NAC for 2 hours prior vitrification and cultured in KSOM medium for 2 hours after warming; **D)** Vitrified oocytes cultured in

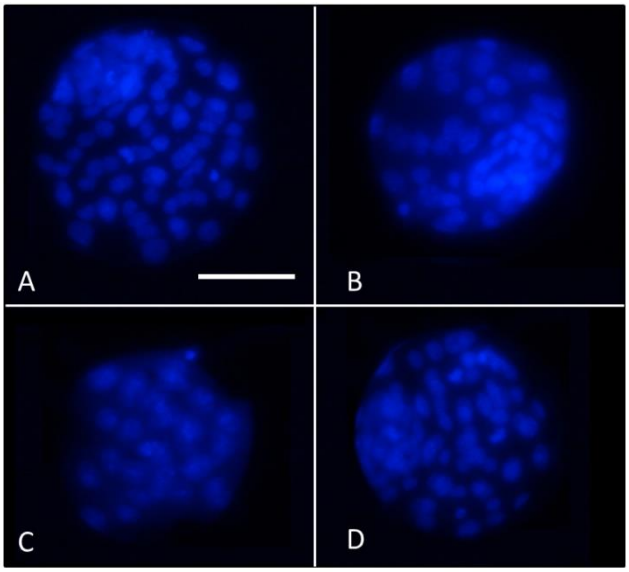
KSOM medium supplemented with 1mM of NAC for 2 hours after warming. The scale represents 40 µm and the micrographs were taken using a × 40 objective.

Figure 6.



Representative images for mouse oocytes producing ROS. The letters in each panel represent: **A)** Fresh oocytes cultured in KSOM medium for 2 hours; **B)** Vitrified oocytes cultured in KSOM medium for 2 hours after warming; **C)** Oocytes cultured in KSOM medium supplemented with 1mM of NAC for 2 hours prior vitrification and cultured in KSOM for 2 hours after warming; **D)** Vitrified oocytes cultured in KSOM medium supplemented with 1mM of NAC for 2 hours after warming. The scale represents 40 µm; the micrographs were taken using a × 40 objective.

Figure 7.



Representative micrographs of expanded blastocysts stained with Hoechst 33342 derived from fresh and vitrified murine oocytes after IVF. The letters in each panel represent: **A)** Fresh oocytes cultured in KSOM medium for 2 hours**; B)** Vitrified oocytes cultured in KSOM medium for 2 hours after warming; **C)** oocytes cultured in KSOM medium supplemented with 1mM of NAC for 2 hours prior vitrification and cultured in KSOM medium for 2 hours after warming; **D)** Vitrified oocytes cultured in KSOM medium supplemented with 1mM of NAC for 2 hours after warming. The scale represents 100 µm; the micrographs were taken using a × 40 objective.

Table 1.

Oocyte treatment	n	Blastocyst rate (%)	n	Cell count
Fresh control	67	90.7 ± 1.8^{a}	12	86.8 ± 2.5^{a}
Vitrified Control	92	$85.9 \pm 1.5^{a,b}$	15	58.9 ± 2.5^{b}
Vitrified NAC-Pre	81	79.1 ± 1.8^{b}	12	36.7 ± 2.3^{c}
Vitrified NAC-Post	80	$90.1 \pm 1.8^{a,c}$	12	76.8 ± 4.1^{d}

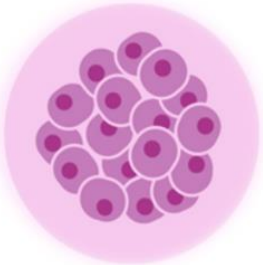
Table 1. Development to the blastocyst stage and total blastomere number.
Development to the blastocyst stage and total cell number determination of embryos obtained by IVF using fresh or vitrified murine oocytes in presence or absence of NAC. The groups studied were: **F-C**: oocytes were cultured in KSOM medium for 2 hours prior IVF**; V-C**: oocytes were cultured in KSOM medium for 2 hours prior vitrification. After warming, oocytes were allowed to recover in KSOM medium for 2 hours and subjected to IVF afterwards; **V-NAC-Pre:** oocytes were cultured in KSOM medium supplemented with 1 mM NAC for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium before IVF; **V-NAC-Post:** oocytes were cultured in KSOM medium for 2 hours prior vitrification. Warmed oocytes were allowed to recover for 2 hours in KSOM medium added with 1 mM NAC before IVF. Different letters in the same column represent statistically significant differences ($p < 0.05$); values are expressed as the mean ± SEM.

OTRAS PUBLICACIONES DERIVADAS DE ESTA TESIS DOCTORAL

- Comunicación 1: **Adición de N-acetilcisteína al medio de congelación seminal de Sementales Pura Sangre Lusitanos.** 2017; ITEA vol. 113 (2). Issn: 2386-3765. DOI: http://dx.doi.org/10.12706/itea.

- Comunicación 2: **Post-thaw sperm quality of Merino rams ejaculates with low sperm freezability: effect of N-acetylcysteine addition.** 2017 Aceptado en Reproduction in domestic animals. Issn: 1439-0521.

- Comunicación 3: **Incubation of murine oocytes with N-acetylcysteine after thawing prevents mitochondrial depolarization.** 2017 Reproduction in Domestic Animals vol. 52 (S3) p110. Issn: 1439-0521. DOI: 10.111/rda.13026.



RESUMEN/ SUMMARY

RESUMEN

La capacidad de criopreservar gametos o embriones de mamíferos se ha convertido en una técnica fundamental en las clínicas reproductivas, ya que facilita el transporte, flexibiliza su uso y permite el almacenamiento a largo plazo. Se ha demostrado previamente que la criopreservación y los crioprotectores alteran las propiedades físicas y químicas de la membrana celular y del citoesqueleto, inducen la despolarización mitocondrial, aumentan la producción de especies reactivas de oxigeno (EROs) y desencadenan la apoptosis. Todos estos factores menoscaban la capacidad fecundante de los gametos y la capacidad de desarrollo de los embriones. Además, la tolerancia al proceso varía dependiendo del material genético que se pretenda criopreservar. Concretamente, la congelación de semen epididimario es más dificultosa que la del semen eyaculado debido a que éste no ha tenido contacto con el plasma seminal (fuente principal de enzimas antioxidantes y colesterol en el eyaculado). Por otro lado, la vitrificación de los ovocitos y los primeros estadios de desarrollo embrionario (de zigotos a 8 células) toleran peor el proceso debido a la baja permeabilidad de la zona pelúcida a los medios; además, los embriones producidos *in vitro* tienen una calidad inferior que los producidos *in vivo* siendo más susceptibles de sufrir daños irreversibles durante la vitrificación. Por lo tanto, en los experimentos realizados en la presente Tesis Doctoral nos centramos en la mejora de los procesos de criopreservación de gametos y embriones en los estadíos que presentan peor tolerancia al proceso. Para ello analizamos el efecto de la adición de N-Acetilcisteína a ovocitos, embriones de ratón (estadio de 2 células) y espermatozoides epididimarios criopreservados de toro de Lidia con el fin de determinar si el uso de este antioxidante mejora la calidad de los embriones y gametos tras la criopreservación. Además, se intentó determinar si tras la refrigeración prolongada a 4ºC (24-96 horas) el uso de crioprotectores alternativos como la dimetilformamida (DMF) mejoraba la calidad del semen de toro de Lidia post-descongelación.

Los resultados obtenidos mostraron que el almacenamiento refrigerado del epidídimo de toro de Lidia hasta 96 horas no afecta significativamente los parámetros avanzados de de motilidad VCL, VSL, VAP, ALH y LIN cuando los medios criopreservación se suplementaron con glicerol al 7% (v/v) o glicerol y DMF (al 3.5% cada uno, v/v). La adición de NAC (1 o 2.5 mM) no mejoró los parámetros de motilidad espermática total y

progresiva, la viabilidad, el potencial de membrana mitocondrial, la producción de EROs, la fragmentación de ADN, ni el estado del acrosoma de los espermatozoides de raza bovina de Lidia después de la descongelación.

Para los embriones murinos vitrificados en estadio de dos células, nuestros experimentos demostraron que la adición de 1 mM de NAC no indujo un efecto tóxico incluso cuando se mantuvo la suplementación durante todo el cultivo hasta fase de blastocisto. Además, la calidad de los embriones vitrificados producidos *in utero* aumentó significativamente cuando se añadió NAC a los días 1.5 o 2.5 tras la descongelación y los producidos *in vitro* cuando se suplementó el día 1.5 de desarrollo.

En ovocitos, observamos una mayor polarización mitocondrial tras la adición de 1 mM de NAC antes y después de la vitrificación, cuando se compararon con ovocitos vitrificados en ausencia del antioxidante. La producción de EROs y ATP en ovocitos desvitrificados fue similar a la producida por ovocitos frescos. Sin embargo, el contenido de ATP disminuyó cuando la adición de NAC fue anterior a la vitrificación, comparado cuando la suplementación fue posterior. Mientras que la producción de EROs se incrementó en presencia de NAC comparado con ovocitos vitrificados. Por otro lado se observó un aumento significativo en el número de blastómeras cuando la adición de NAC se efectuó después de la vitrificación en comparación con la suplementación previa a la vitrificación.

En conclusión, la adición del antioxidante N-acetilcisteína no parece tener un efecto beneficioso en los medios de congelación de semen epididimario en el toro de Lidia; sin embargo, su adición en embriones desvitrificados de 2 células y en ovocitos murinos después de la vitrificación parece mejorar la calidad de los blastocistos generados dependiendo del momento de su adición.

SUMMARY

Gamete and embryo cryopreservation is a core assisted reproductive tool that facilitates transport, use and long-term storage of valuable genetics. It has been demonstrated that the cryoprotectants used in the cryopreservation extenders as well as the process itself alter the chemical and physical proprieties of the plasma membrane of gametes/embryos. Specifically cryopreservation induces structural changes in the plasma membrane and cytoskeleton, induces mitochondrial depolarization, increases ROS production and in turn triggers apoptosis. Furthermore, tolerance for cryopreservation notably varies depending upon the type of genetic material to be cryopreserved. Specifically, epididymal sperm cryopreservation is less successful than ejaculated sperm because it has had no contact with seminal plasma (the main source of spermatozoal antioxidant enzymes, lipids and cholesterol). On the other hand, oocytes and early embryonic stages (zygotes to 8 cells) exhibit lower tolerance for vitrification due to the low permeability of their zona pellucida; in addition, the quality of the embryos produced *in vitro* is lower than the quality of those produced *in vivo* being more susceptible to irreversible damage during vitrification. Therefore, in the experiments carried out in the present Doctoral Thesis we focused our efforts towards the improvement of gamete and embryo cryopreservation at the more susceptible stages to freezing-induced damage. To achieve this goal we analyzed the effect of N-acetylcysteine addition to vitrified murine oocytes and embryos (2-cell stage) at different time points as well as to cryopreserved Lidia bull epididymal spermatozoa in order to determine the usefulness of this antioxidant. In addition, an attempt was made to determine whether the use of alternative cryoprotectants such as dimethylformamide (DMF) improved post-thaw semen quality of Lidia bull epididymal sperm after prolonged storage at 4° C (24-96 hours).

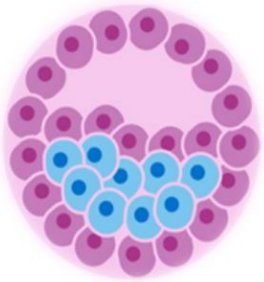
Our results showed that prolonged storage of Lidia bull epididymis up to 96 hours did not seem to negatively affect sperm fertility, as significant differences in their main advanced motility parameters VCL, VSL, VAP, ALH and LIN were not found when sperm were cryopreserved using an extender added with glycerol (7%, v/v) or glycerol and DMF (at 3.5% v/v each). When 1 or 2.5 mM of NAC were added to any of the sperm freezing extenders total and progressive motility, viability, mitochondrial membrane

potential, ROS production, and DNA fragmentation or acrosomal intactness remained unaffected despite prolonged cooled storage.

On the other hand, when murine 2 cells embryos produced *in vivo* or *in vitro* were vitrified and warmed, vitrification or addition of 1 mM of NAC did not negatively affect their development to the expanded blastocyst stage compared to the fresh controls. Interestingly, the quality of the expanded blastocysts derived from vitrified 2-cell embryos produced *in vivo* significantly increased when the antioxidant was added at days 1.5 and 2.5 after freezing and at day 1.5 in IVF-derived embryos, as the total blastomere count at day 4 was significantly enhanced compared to the vitrified control devoid of NAC.

In our last batch of experiments, murine oocytes were vitrified in presence or absence of NAC (1 mM) which was added prior to or after vitrification. In oocytes added with NAC prior or after vitrification higher mitochondrial polarization was observed compared to the vitrified controls. Interestingly, ROS production ATP content was similar in fresh and vitrified oocytes. However, ATP content significantly decreased when NAC was added prior to vitrification, compared to NAC supplementation after warming. On the other hand, a significant increase in the number of blastomeres was observed when the addition of NAC was carried out after vitrification compared to its addition prior to vitrification.

In conclusion, the addition of N-acetylcysteine does not seem to exert any beneficial effect on the post-thaw quality of Lidia bull epididymal sperm; however, its addition to murine oocytes and early embryos improves the quality of the derived expanded blastocysts depending upon the moment of addition.



DISCUSIÓN

DISCUSIÓN

La necesidad de utilizar protocolos de criopreservación seguros y efectivos que garanticen la conservación óptima de gametos y embriones ha llevado a la realización de la presente Tesis Doctoral. En el momento actual, según un informe publicado en 2013 del Ministerio de Agricultura, Alimentación y Medio Ambiente, solamente el 13,2% de nuestras razas o variedades autóctonas (incluyendo bovino, ovino, caprino, aviar porcino y equino) disponen de un banco de germoplasma que permitiría la reintroducción completa de las razas o variedades (banco completo) [185]; dichos bancos contemplan el almacenamiento de dosis seminales, embriones, células somáticas y ovocitos.

Aunque el estudio de mejora de medios de criopreservación ha sido desarrollado ampliamente en la literatura, la conveniencia de la adición de antioxidantes (dosis y tipos) a los medios de preservación aún no está esclarecida. Por un lado, está descrito que la manipulación *in vitro* de gametos y embriones da lugar a la producción excesiva de EROs afectando al balance oxidante-antioxidante endógeno. Además, como se ha expuesto previamente, determinadas técnicas utilizadas en reproducción asistida tales como la criopreservación inducen estrés oxidativo celular aumentando las citadas EROs. Por ello, durante el desarrollo de la presente Tesis Doctoral los estudios realizados se centraron en el uso de crioprotectores alternativos en gametos masculinos y en la adición de NAC en espermatozoides, oocitos y embriones de dos células, ya que constituyen los estadíos más sensibles al proceso.

En la primera parte de la presente Tesis Doctoral se eligió como modelo el semen de la raza bovina de Lidia, clasificada como raza autóctona de fomento en el Catálogo Oficial de razas de ganado de España. Actualmente existen alrededor de 10.000 dosis seminales conservadas procedentes solamente de 64 sementales [186]. La dificultad de extracción de eyaculados de esta raza unida a su conservación basada en la importancia genética y no en base a su capacidad de congelación, obstaculizan la preservación de dicha raza. Puesto que no existen protocolos de congelación específicos para el semen del ganado de lidia, ni tampoco se ha estipulado el tiempo máximo de almacenamiento del epidídimo antes del inicio del proceso de criopreservación, en el Artículo 1 de la presente Tesis Doctoral se evaluaron los parámetros de motilidad de espermatozoides descongelados después del almacenamiento epidídimo a 4ºC (de 24 a 96 horas). Para ello

se utilizó como medio base un medio basado en Tris-fructosa citrato adicionado con yema de huevo y glicerol previamente validado para la criopreservación de espermatozoides bovinos epididimarios [187]. En la criopreservacion del semen bovino uno de los crioprotectores más utilizado es el glicerol por su capacidad de estabilizar la membrana plasmática [188]. Este agente crioprotector (ACP) se añade en concentraciones que oscilan del 3% al 7% (v/v) dependiendo del laboratorio de investigación [189, 190], aunque hay que tener en cuenta que existen diferencias en la tolerancia de los espermatozoides al glicerol entre especies e incluso entre razas [191, 192]. Por otra parte, existen estudios que demuestran que el glicerol cuando se usa como único ACP en el medio de congelación, afecta negativamente la motilidad del espermatozoide, la viabilidad y al potencial de membrana mitocondrial [41]. Por ello, en nuestro estudio, combinamos el uso del glicerol con dimetilformamida (DMF) debido a sus buenos resultados en la especie equina [40]. Esta Tesis describe el primer análisis de parámetros avanzados de motilidad de espermatozoides de toro de Lidia, por lo que hay pocos valores de referencia previos para comparar.

En estudios previos se ha demostrado que los parámetros avanzados de velocidad individuales predicen el potencial fertilizante del semen descongelado en muchas especies [193-195] incluyendo la bovina [196]. En este sentido, se ha sugerido que el promedio de VAP puede ser el parámetro más útil con relevancia clínica para predecir la fertilidad en los toros [196]. En nuestro estudio, en los resultados obtenidos en los medios adicionados con glicerol al 7% o glicerol al 3.5% y 3.5% de DMF hasta 96 horas post-mortem no encontramos diferencias para MT, MP, VCL, VSL, VAP, ALH y LIN (Artículo 1). Por lo tanto, el almacenamiento refrigerado del epidídimo de toro de Lidia hasta 96 horas no afecta a los parámetros de motilidad de esta raza. Nuestro estudio demuestra que el procesamiento del semen de toros de Lidia puede realizarse tras un almacenamiento epididimario de hasta 96 horas a 4ºC sugiriendo que la fertilidad podría no verse afectada. Ya que la adición de glicerol o la combinación de DMF y glicerol no mostraron diferencias y debido a la mayor sencillez en la preparación de medios con un solo crioprotector, no parece necesario el uso de DMF en la congelación de semen de esta raza.

Siguiendo el diseño experimental del Artículo 1, en nuestro segundo trabajo se almacenaron los epidídimos a 4ºC de 24 a 96 horas antes de su criopreservación. Una vez descongelados se evaluó la motilidad total y progresiva, la viabilidad, el potencial de membrana mitocondrial, la producción de EROs, la fragmentación de ADN y el estado

del acrosoma de los espermatozoides de raza bovina de lidia. Los resultados obtenidos no mostraron diferencias entre los medios de congelación adicionado con glicerol al 7% o glicerol al 3.5% y 3.5% de DMF hasta 96 horas post-mortem (Artículo 2). Sin embargo, hay que mencionar que el porcentaje de espermatozoides vivos produciendo EROs aumentó y el porcentaje de espermatozoides con alto potencial de membrana mitocondrial (hMMP) disminuyó ligeramente cuando se utilizó la combinación de 3.5% de glicerol y 3.5% de DMF en comparación con el uso de glicerol solamente (Artículo 2), aunque estas diferencias no fueron significativas. Por lo tanto, no parece que existan ventajas obvias en el uso de DMF en el semen de toro de Lidia (al menos a las dosis utilizadas en esta Tesis Doctoral). Estudios previos han demostrado que el porcentaje de espermatozoides viables con hMMP está significativamente relacionado con la fertilidad en distintas especies como búfalos [197] y en carneros [198]. Por tanto, y coincidiendo con los datos obtenidos en el Artículo 1, estos datos refuerzan la idea de que la fertilidad de los espermatozoides del toro de Lidia conservados en los epidídimos durante 96 horas se mantiene intacta.

Como se ha indicado anteriormente, el plasma seminal es la fuente más importante de factores antioxidantes con el que cuenta el espermatozoide entre los que se encuentran la superóxido dismutasa, la catalasa y el GSH, entre otros [188]. Teniendo en cuenta que el semen epididimario carece de secreciones de las glándulas sexuales accesorias (plasma seminal) éste queda desprovisto de antioxidantes y el daño oxidativo debido a la criopreservación es mayor que en los eyaculados completos [199]. Debido a que en nuestros estudios utilizamos semen epididimario de Toro de Lidia, con el fin de paliar este daño oxidativo y mitigar los efectos adversos de las EROs durante la criopreservación, se probó la suplementación del medio de congelación seminal con NAC en combinación con crioprotectores alternativos. Este antioxidante se eligió en base a los estudios previos que demuestran los efectos beneficiosos de la N-acetilcisteína en los espermatozoides eyaculados bovinos [129, 200]. Por ello, durante la fase experimental de la presente Tesis se evaluaron los efectos de diferentes dosis de NAC (1 mM y 2,5 mM NAC) añadidos al medio de congelación seminal. Nuestros resultados coinciden con investigaciones previas en diferentes especies (caballo y ciervo) en las que se concluyó que la adición de antioxidantes no mejora los parámetros de motilidad espermática después de 96 horas de almacenamiento refrigerado [190, 201].

En la presente Tesis también se ha comprobado el efecto de la adición de 1 y 2.5 mM de NAC en el medio de congelación seminal de eyaculados de las especies ovina y equina (datos no mostrados). Coincidiendo con los resultados obtenidos en Toro de Lidia, la adición de NAC a los medios de congelación seminal no mostró mejoras significativas respecto a los controles (datos publicados pero no incluidos en la presente Tesis). Estos resultados pueden ser debidos a que el choque térmico y osmótico son los principales daños que afectan la calidad de los espermatozoides después de la descongelación y no pueden prevenirse mediante el uso de NAC a las dosis utilizadas en este estudio.

En el momento actual, la vitrificación de los ovocitos y embriones es una técnica importante de la industria de la tecnología de reproducción asistida. Además, el número de embriones criopreservados en el Banco nacional de Germoplasma es notoriamente menor que el de pajuelas de semen, existiendo menos de 200 embriones almacenados de razas autóctonas Españolas. Esta diferencia se debe a que la obtención de ovocitos y embriones es más costosa y su tolerancia a la criopreservación notablemente inferior a la del semen. Por lo tanto, en la segunda parte experimental nuestros esfuerzos se centraron en el estudio de los efectos negativos del proceso de vitrificación en los ovocitos y embriones murinos de 2 células, así como el efecto protector de la adición de NAC a los mismos. El modelo murino ha sido ampliamente utilizado para probar y desarrollar protocolos de vitrificación debido a su alta capacidad para soportar el proceso. Por ese motivo y por la fácil disponibilidad de un gran número de ovocitos y embriones, se ha utilizado esta especie en nuestros experimentos. Además, dependiendo de la etapa de desarrollo, la tolerancia a la vitrificación de los embriones varía notablemente siendo mejor en estadio de mórula y blastocistos expandidos que en fases anteriores [141]. Debido a la mayor dificultad para su vitrificación, esta Tesis Doctoral se centra en los estadios más delicados de vitrificación, el ovocito y el embrión de dos células.

La tolerancia a la vitrificación de los ovocitos se ha atribuido a una menor permeabilidad de la zona pelúcida a los crioprotectores y a una mayor producción de EROs después de la vitrificación en ovocitos y embriones en las primeras etapas [59]. Además, la sensibilidad para la congelación varía dependiendo de las condiciones de producción y cultivo de los embriones (*in vivo vs. in vitro*). Con el fin de disminuir las EROs producidas por la vitrificación se ha evaluado el efecto de la suplementación de 1 mM de NAC a distintos tiempos de ovocitos y embriones murinos (Artículos 3 y 4).

Para los embriones de 1.5 días post-fecundación (dos células) se ha valorado la adición de NAC en el momento de la desvitrificación, a las 24 h (día 2.5 de desarrollo), a las 48 horas (día 3.5 de desarrollo), o durante el cultivo completo de embriones. Nuestros experimentos demostraron que la adición de NAC no altera la capacidad de desarrollo de los embriones de 2 células obtenidos *in vivo* o *in vitro*, incluso cuando el NAC se añade durante todo el cultivo hasta fase de blastocisto (día 4). Sin embargo, considerando que el recuento del número de blastómeras es un indicador fiable de la calidad embrionaria [146] observamos un menor número de blastómeras en los embriones producidos *in vitro* en comparación con los embriones obtenidos del útero (Artículo 3), como se ha descrito previamente [202]. Este resultado puede explicarse debido a que, durante la producción *in vitro* de embriones se produce una mayor liberación de EROs en comparación con la producción *in vivo* induciendo la muerte de una parte de las células embrionarias [203].

Debe mencionarse que el número de células totales de los embriones derivados *in utero* aumentó significativamente cuando se añadió NAC a los días 1.5 o 2.5 en comparación con el control o la adición continua de NAC. Ya que en los embriones vitrificados obtenidos *in vitro* el número total de células solo aumentó significativamente en día 1.5, nuestros datos sugieren que después del proceso de vitrificación-desvitrificación, la adición de NAC podría estar amortiguando la producción excesiva de EROs desencadenada por el proceso de criopreservación, mejorando de esta forma la calidad embrionaria (Artículo 3). Nuestros resultados sugieren que la adición de NAC ejerce su máximo efecto tras la descongelación y durante el paso del estadío de 4 células a mórula, aunque más allá de este estadío embrionario, los daños ejercidos por las ROS no parecen poder prevenirse mediante la adición de NAC. Por otro lado, está descrito que la producción fisiológica de EROs es necesaria para la correcta división de los embriones y el desarrollo previo a la implantación [152]. Sin embargo, la eliminación excesiva de EROs podría interferir con la citocinesis blastómera explicando por qué aunque el desarrollo embrionario se mantiene, el número total de células no aumenta cuando la adición de NAC se realiza durante la duración total del desarrollo embrionario [204]. El hecho de que el desarrollo hasta el estadío de blastocisto fuera similar entre todos los grupos estudiados (frescos *vs* vitrificados) en presencia o ausencia de NAC pero hubiera diferencias significativas en el número de blastómeras dependiendo del grupo en estudio demuestran que el recuento de blastómeras es un indicador más fiable de calidad embrionaria que el porcentaje de desarrollo. Por otro lado, la adición de NAC incrementa

el número de blastómeras significativamente dependiendo del día de adición (día 1.5 en embriones vitrificados producidos mediante FIV vs. Días 1.5 y 2.5 en embriones producidos *in vivo*) sugiriendo la utilidad de su adición en cultivos secuenciales.

Debido a que la vitrificación induce la despolarización mitocondrial, disminuye el contenido ATP e incrementa la producción de EROs [174, 205], en el trabajo desarrollado en ovocitos maduros de ratón, hemos determinado el efecto de la adición de 1 mM de NAC antes y después de la criopreservación (Artículo 4). Cabe destacar que hemos validado por primera vez la polarización mitocondrial de los oocitos basándonos en el coeficiente de variación de la intensidad de fluorescencia de la rodamina 123 (Comunicación 3), una técnica utilizada previamente en neuronas [171]. En nuestro último trabajo (Artículo 4) observamos una mayor polarización mitocondrial tras la adición de NAC antes y después de la vitrificación comparado con los ovocitos vitrificados en ausencia del antioxidante sin observar diferencias respecto a la polarización de ovocitos frescos. En ovocitos porcinos, se ha demostrado que la producción de EROs aumenta después de la vitrificación, lo que justifica la adición de antioxidantes al medio de vitrificación [177] o en el medio de recuperación tras la desvitrificación [206]. Nuestros resultados mostraron que la producción de EROs en ovocitos murinos desvitrificados es similar a la de ovocitos frescos mientras que su producción aumentó en presencia del NAC. Este incremento en la producción de EROs no parece ser perjudicial, ya que las tasas de desarrollo embrionarias obtenidas fueron similares en los ovocitos frescos y en los vitrificados suplementados con NAC.

Ya que la polarización de la membrana mitocondrial y la producción de EROs son medidas indirectas de la funcionalidad de la mitocondria, en nuestros experimentos nos propusimos estudiar la producción de ATP. Debido a que la fosforilación oxidativa tiene lugar en la mitocondria, la producción de ATP se ve alterada cuando existe daño mitocondrial siendo una medida más directa de la funcionalidad mitocondrial [155]. En el presente trabajo demostramos que los niveles de ATP no varían entre ovocitos frescos y desvitrificados; sin embargo, en presencia de NAC, el contenido de ATP disminuye cuando la adición de NAC es anterior a la vitrificación y aumenta significativamente cuando la suplementación es posterior a la vitrificación. Este hecho unido a la menor tasa de desarrollo hasta blastocisto cuando la adición de NAC es anterior a la vitrificación, sugiere que, en ese momento, la adición de NAC ejerce un efecto tóxico sobre la cadena de fosforilación oxidativa mitocondrial.

Por otro lado, observamos que el desarrollo a blastocisto no muestra diferencias estadísticamente significativas si los embriones son frescos o vitrificados en ausencia de NAC, pero la adición del antioxidante previa o posterior a la criopreservación influye en en el desarrollo embrionario y el número de blastómeras. Cabe destacar que la adición de NAC antes de la vitrificación disminuyó significativamente el recuento de blastómeras y el desarrollo de los embriones en comparación con los derivados de ovocitos frescos o vitrificados adicionados con NAC tras la criopreservación. En vista de estos resultados, la adición de NAC previa a la vitrificación de los ovocitos parece ser perjudicial mientras que la suplementación de NAC después de la vitrificación mejora el estado de polarización mitocondrial, aumenta los nieles de ATP y el número de blastómeras. Estos resultados deben tenerse en cuenta con el fin de aplicarlos a especies de interés pecuarios, con el fin de mejorar las tasas de producción de embriones derivados de oocitos vitrificados. Al igual que en estudios anteriores [184], hemos comprobado que el recuento de blastómeras después de la vitrificación disminuye notablemente con respecto a los ovocitos frescos.

CONCLUSIONES

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- Los epidídimos de toro de Lidia pueden refrigerarse a 4ºC durante 96 horas antes de la criopreservación sin que las calidad espermática varíen tras la descongelación.

- La combinación de DMF y glicerol (3.5% v/v cada ACP) no presenta ventajas evidentes con respecto al uso de glicerol al 7% (v/v) en los diluyentes de congelación epididimarios de Toro de Lidia.

- La adición de N-acetilcisteína a los medios de congelación de espermatozoides epididimario de Toros de Lidia no afecta significativamente a la calidad post-descongelación de los espermatozoides.

- La adición de N-acetilcisteína aumenta el número de células de embriones desvitrificados en día 1.5 y 2.5 de cultivo producidos *in vivo* y de embriones *in vitro* en día 1.5 de cultivo.

- La adición de NAC previa a la vitrificación de los ovocitos disminuye los niveles de ATP, el desarrollo y el número total de blastómeras de los blastocitos murinos generados por FIV.

- La suplementación de NAC después de la vitrificación mejora el estado de polarización mitocondrial, aumenta los nieles de ATP y el número de blastómeras de los blastocistos generados mostrando resultados comparables a los embriones frescos.

CONCLUSIONS

- Lidia bull epididymis can be refrigerated at 4ºC up to 96 hours prior cryopreservation and prolonged storage does not impair their sperm quality post-thaw.

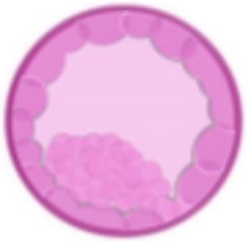
- The combination of DMF and glycerol at 3.5 % (v/v, each) is not better than the use of glycerol at 7% (v/v) in epididymal Lidia bull freezing extenders.

- N-Acetylcysteine addition to sperm freezing extenders does not affect post-thaw sperm quality in Lidia bulls.

- NAC addition enhances total cell number of vitrified murine 2-cell embryos obtained *in vivo* when added at days 1.5 and 2.5 of culture and *in vitro* at day 1.5.

- NAC supplementation prior oocyte vitrification decreases ATP production, developmental competence and total blastomere count in murine embryos produced by IVF.

- NAC supplementation after murine oocyte cryopreservation improves mitochondrial polarization status, ATP levels and total expanded blastocyst blastomere count over vitrified embryos devoid of NAC and the results are comparable to those of fresh oocytes.



Bibliografía

BIBLIOGRAFÍA

1. Neill's, K.a., *Physiology of Reproduction*. 2006: Academic Press. 2684.

2. Boerke, A., et al., *Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: Functional relationship with zona binding and the zona-induced acrosome reaction.* Theriogenology, 2008. **70**(8): p. 1188-1196.

3. Lindemann, C.B. and K.A. Lesich, *Functional anatomy of the mammalian sperm flagellum.* Cytoskeleton, 2016. **73**(11): p. 652-669.

4. Neils, K., *Physiology of Reproduction*, ed. Ed. 2006.

5. Cornwall, G.A., *New insights into epididymal biology and function.* Human Reproduction Update, 2009. **15**(2): p. 213-227.

6. Toshimori, K., *Maturation of mammalian spermatozoa: modifications of the acrosome and plasma membrane leading to fertilization.* Cell and Tissue Research, 1998. **293**(2): p. 177-187.

7. França, L.R., G.F. Avelar, and F.F.L. Almeida, *Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs.* Theriogenology, 2005. **63**(2): p. 300-318.

8. Kloc, M., et al., *Mouse early oocytes are transiently polar: Three-dimensional and ultrastructural analysis.* Experimental Cell Research, 2008. **314**(17): p. 3245-3254.

9. Diaz, F.J., K. Wigglesworth, and J.J. Eppig, *Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice.* Developmental Biology, 2007. **305**(1): p. 300-311.

10. Gilula, N.B., M.L. Epstein, and W.H. Beers, *Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex.* The Journal of Cell Biology, 1978. **78**(1): p. 58-75.

11. Litscher E.S. and W. P.M., *Egg extracellular coat proteins: From fish to mammals.* Histol Histopathol, 2007. **22**: p. 337-347

12. Wassarman P.M. and L. E.S., *Mammalian fertilization:the egg's multifunctional zona pellucida.* Int. J. Dev. Biol. , 2008. **52**: p. 665 - 676.

13. Mehlmann, L.M., et al., *The G-Linked Receptor GPR3 Maintains Meiotic Arrest in Mammalian Oocytes.* Science, 2004. **306**(5703): p. 1947-1950.

14. Mehlmann, L.M., *Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation.* Reproduction, 2005. **130**(6): p. 791-799.

15. Picton, H.M., et al., *The in vitro growth and maturation of follicles.* Reproduction, 2008. **136**(6): p. 703-715.

16. Sun, Q.-Y., et al., *Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: Effects of gonadotropins, cumulus cells, follicular size, and protein synthesis inhibition*.* Mol Reprod Dev, 2001. **59**(2): p. 192-198.

17. Peters, J.-M., *The Anaphase-Promoting Complex.* Molecular Cell, 2002. **9**(5): p. 931-943.

18. Abeydeera, L.R., *In vitro production of embryos in swine.* Theriogenology, 2002. **57**(1): p. 257-273.

19. Thibault C., Szöllösi D., and G. M., *Mammalian oocyte maturation.* Reprod Nutr Dev., 1987. **27(5)**: p. 865-96.

20. Moor R and D. Y., *Maturation of pig oocytes in vivo and in vitro.* Reprod Suppl. , 2001. **58:**: p. 91-104.

21. White, M.D., et al., *Chapter Seven - Mouse Embryo Compaction*, in *Current Topics in Developmental Biology*, L.D. Melvin, Editor. 2016, Academic Press. p. 235-258.

22. Bloxham, P.A., *From ovulation to blastocyst attachment. A review of early embryonic life in the mouse.* Folia Vet Lat., 1976. **6(4)**: p. 319-34.

.

23. Kirkegaard, K., et al., *Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study.* Human Reproduction, 2013. **28**(10): p. 2643-2651.

24. https://www.reproduccionasistida.org/clasificacion-embriones-calidad/.

25. Mandawala, A.A., et al., *Cryopreservation of animal oocytes and embryos: Current progress and future prospects.* Theriogenology, 2016. **86**(7): p. 1637-1644.

26. Somoskoi, B., et al., *Different chromatin and energy/redox responses of mouse morulae and blastocysts to slow freezing and vitrification.* Reprod Biol Endocrinol, 2015. **13**: p. 22.

27. Whittingham, D.G., S.P. Leibo, and P. Mazur, *Survival of Mouse Embryos Frozen to -196° and -269°C.* Science, 1972. **178**(4059): p. 411-414.

28. Mazur, P., *Kinetics of Water Loss from Cells at Subzero Temperatures and the Likelihood of Intracellular Freezing.* The Journal of General Physiology, 1963. **47**(2): p. 347-369.

29. Akhoondi, M., et al., *Membrane hydraulic permeability changes during cooling of mammalian cells.* Biochim Biophys Acta, 2011. **1808**(3): p. 642-8.

30. Oldenhof, H., et al., *Membrane permeability parameters for freezing of stallion sperm as determined by Fourier transform infrared spectroscopy.* Cryobiology, 2010. **61**(1): p. 115-22.

31. Devireddy, R.V., et al., *Cryopreservation of Equine Sperm: Optimal Cooling Rates in the Presence and Absence of Cryoprotective Agents Determined Using Differential Scanning Calorimetry.* Biol Reprod, 2002. **66**(1): p. 222-231.

32. Sieme, H., H. Oldenhof, and W.F. Wolkers, *Sperm Membrane Behaviour during Cooling and Cryopreservation.* Reprod Domest Anim, 2015. **50 Suppl 3**: p. 20-6.

33. Arav, A. and J. Saragusty, *Directional freezing of spermatozoa and embryos.* Reproduction, Fertility and Development, 2013. **26**(1): p. 83-90.

34. Hochi, S., et al., *Effects of Cooling and Warming Rates during Vitrification on Fertilization of in Vitro-Matured Bovine Oocytes.* Cryobiology, 2001. **42**(1): p. 69-73.

35. Kasai M. and M. T., *Cryopreservation of animal and human embryos by vitrification.* Reproductive BioMedicine Online, 2004. **9 (2)**: p. 6.

36. Kuleshova, L.L. and A. Lopata, *Vitrification can be more favorable than slow cooling.* Fertility and Sterility, 2002. **78**(3): p. 449-454.

37. Vajta, G., *Vitrification of the oocytes and embryos of domestic animals.* Anim Reprod Sci, 2002. **60**: p. 357-364.

38. Sieme, H. and H. Oldenhof, *Cryopreservation of Semen from Domestic Livestock*, in *Cryopreservation and Freeze-Drying Protocols*, W.F. Wolkers and H. Oldenhof, Editors. 2015, Springer New York: New York, NY. p. 277-287.

39. Squires, E.L., S.L. Keith, and J.K. Graham, *Evaluation of alternative cryoprotectants for preserving stallion spermatozoa.* Theriogenology, 2004. **62**(6): p. 1056-65.

40. Álvarez, C., et al., *Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender.* Cryobiology, 2014. **69**(1): p. 144-148.

41. Alvarenga, M.A., et al., *Amides as cryoprotectants for freezing stallion semen: A review.* Anim Reprod Sci, 2005. **89**(1–4): p. 105-113.

42. Dziekońska, A., et al., *Effects of storage in different semen extenders on the pre-freezing and post-thawing quality of boar spermatozoa*, in *Polish Journal of Veterinary Sciences*2015. p. 733.

43. Morillo Rodriguez, A., et al., *Dimethylformamide Improves the In vitro Characteristics of Thawed Stallion Spermatozoa Reducing Sublethal Damage.* Reproduction in Domestic Animals, 2012. **47**(6): p. 995-1002.

44. Layek, S.S., et al., *Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders.* Anim Reprod Sci, 2016. **172**: p. 1-9.

45. Sieme, H., H. Oldenhof, and W.F. Wolkers, *Mode of action of cryoprotectants for sperm preservation.* Anim Reprod Sci, 2016. **169**: p. 2-5.

46. Barbas, J.P. and R.D. Mascarenhas, *Cryopreservation of domestic animal sperm cells.* Cell and Tissue Banking, 2009. **10**(1): p. 49-62.

47. Bielanski, A., *A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices.* Theriogenology. **77**(3): p. 467-482.

48. Mullen, S.F. and G.M. Fahy, *A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep.* Theriogenology. **78**(8): p. 1709-1719.

49. Liebermann, J., et al., *Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now?* Reprod Biomed Online, 2003. **7**(6): p. 623-633.

50. Fuller B. and P. S., *Fundamentals of cryobiology in reproductive medicine.* Reprod Biomed Online, 2004. **9 (6)**: p. 12.

51. Vajta G. and N. Z.P., *Are programmable freezers still needed in the embryo laboratory? Review on vitrification.* Reprod Biomed Online. **12(6)**: p. 17.

52. Vajta, G. and M. Kuwayama, *Improving cryopreservation systems.* Theriogenology, 2006. **65**(1): p. 236-244.

53. Leibo, S.P. and N. Songsasen, *Cryopreservation of gametes and embryos of non-domestic species.* Theriogenology, 2002. **57**(1): p. 303-326.

54. Massip, A., *Cryopreservation of Embryos of Farm Animals.* Reproduction in Domestic Animals, 2001. **36**(2): p. 49-55.

55. Saragusty, J. and A. Arav, *Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification.* Reproduction, 2011. **141**(1): p. 1-19.

56. Parmegiani, L., et al., *Efficiency of aseptic open vitrification and hermetical cryostorage of human oocytes.* Reproductive BioMedicine Online, 2011. **23**(4): p. 505-512.

57. Kuwayama M, et al., *Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination.* Reprod Biomed Online, 2005. **11(5)**: p. 608-614.

58. Leibo, S.P. and N.M. Loskutoff, *Cryobiology of in vitro-derived bovine embryos.* Theriogenology, 1993. **39**(1): p. 81-94.

59. Pedro, P.B., et al., *Permeability of Mouse Oocytes and Embryos at Various Developmental Stages to Five Cryoprotectants.* Journal of Reproduction and Development, 2005. **51**(2): p. 235-246.

60. Gao, C., et al., *Melatonin promotes embryonic development and reduces reactive oxygen species in vitrified mouse 2-cell embryos.* Journal of Pineal Research, 2012. **52**(3): p. 305-311.

61. P.F., W., *Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function.* Reprod Fertil Dev, 1995. **7 (4)**: p. 11.

62. Woods, E.J., et al., *Fundamental cryobiology of reproductive cells and tissues.* Cryobiology, 2004. **48**(2): p. 146-56.

63. Pommer, A.C., J. Rutllant, and S.A. Meyers, *The role of osmotic resistance on equine spermatozoal function.* Theriogenology, 2002. **58**(7): p. 1373-1384.

64. Jones, A., et al., *Cryopreservation of metaphase II human oocytes effects mitochondrial membrane potential: implications for developmental competence.* Hum Reprod, 2004. **19**(8): p. 1861-6.

65. Zhao, X.M., et al., *Effect of vitrification on mitochondrial distribution and membrane potential in mouse two pronuclear (2-PN) embryos.* Mol Reprod Dev, 2009. **76**(11): p. 1056-63.

66. Zhao, X.M., et al., *Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture.* Mol Reprod Dev, 2011. **78**(12): p. 942-50.

67. Demant, M., et al., *Vitrification at the pre-antral stage transiently alters inner mitochondrial membrane potential but proteome of in vitro grown and matured mouse oocytes appears unaffected.* Hum Reprod, 2012. **27**(4): p. 1096-111.

68. Ozawa, M., et al., *Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage.* Mol Reprod Dev, 2006. **73**(8): p. 998-1007.

69. Tatone, C., et al., *Cryopreservation and oxidative stress in reproductive cells.* Gynecological Endocrinology, 2010. **26**(8): p. 563-567.

70. Van Blerkom, J., *Mitochondria as regulatory forces in oocytes, preimplantation embryos and stem cells.* Reprod Biomed Online, 2008. **16**(4): p. 553-69.

71. Dumollard, R., et al., *Mitochondrial function and redox state in mammalian embryos.* Seminars in Cell & Developmental Biology, 2009. **20**(3): p. 346-353.

72. De Martino, C., et al., *Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats.* Cell and Tissue Research, 1979. **196**(1): p. 1-22.

73. Petit, J.M., et al., *Mouse testis cell sorting according to DNA and mitochondrial changes during spermatogenesis.* Cytometry, 1995. **19**(4): p. 304-312.

74. Ruiz-Pesini, E., et al., *The Role of the Mitochondrion in Sperm Function: Is There a Place for Oxidative Phosphorylation or Is This a Purely Glycolytic Process?*, in *Current Topics in Developmental Biology*. 2007, Academic Press. p. 3-19.

75. Ramalho-Santos, J., et al., *Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells.* Hum Reprod Update, 2009. **15**(5): p. 553-572.

76. Rajender, S., P. Rahul, and A.A. Mahdi, *Mitochondria, spermatogenesis and male infertility.* Mitochondrion, 2010. **10**(5): p. 419-428.

77. Luo, S.-M., H. Schatten, and Q.-Y. Sun, *Sperm Mitochondria in Reproduction: Good or Bad and Where Do They Go?* Journal of Genetics and Genomics, 2013. **40**(11): p. 549-556.

78. Paoli, D., et al., *Mitochondrial membrane potential profile and its correlation with increasing sperm motility.* Fertility and Sterility, 2011. **95**(7): p. 2315-2319.

79. Jansen, R.P. and K. de Boer, *The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate.* Mol Cell Endocrinol, 1998. **145**(1-2): p. 81-8.

80. Thouas, G.A., A.O. Trounson, and G.M. Jones, *Effect of Female Age on Mouse Oocyte Developmental Competence Following Mitochondrial Injury.* Biol Reprod, 2005. **73**(2): p. 366-373.

81. Spikings, E.C., J. Alderson, and J.C.S. John, *Regulated Mitochondrial DNA Replication During Oocyte Maturation Is Essential for Successful Porcine Embryonic Development.* Biol Reprod, 2007. **76**(2): p. 327-335.

82. Liu, L., J.R. Trimarchi, and D.L. Keefe, *Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes.* Biol Reprod, 2000. **62(6)**: p. 9.

83. Thouas, G.A., et al., *Mitochondrial Dysfunction in Mouse Oocytes Results in Preimplantation Embryo Arrest in Vitro1.* Biol Reprod, 2004. **71**(6): p. 1936-1942.

84. Chiaratti, M.R., et al., *Ooplast-mediated developmental rescue of bovine oocytes exposed to ethidium bromide.* Reproductive BioMedicine Online. **22**(2): p. 172-183.

85. Senger, P.L. and R.G. Saacke, *UNUSUAL MITOCHONDRIA OF THE BOVINE OOCYTE.* The Journal of Cell Biology, 1970. **46**(2): p. 405-408.

86. Shamsuddin, M. and H. Rodriguez-Martinez, *Fine Structure of Bovine Blastocysts Developed Either in Serum-Free Medium or in Conventional Co-Culture With Oviduct Epithelial Cells.* Journal of Veterinary Medicine Series A, 1994. **41**(1-10): p. 307-316.

87. Zini, A., E. de Lamirande, and C. Gagnon, *Low Levels of Nitric Oxide Promote Human Sperm Capacitation In Vitro.* Journal of Andrology, 1995. **16**(5): p. 424-431.

88. Lopes, S.M.S., et al., *Sperm Deoxyribonucleic Acid Fragmentation is Increased in Poor-Quality Semen Samples and Correlates with Failed Fertilization in Intracytoplasmic Sperm Injection ¹.* Fertil Steril. **69**(3): p. 528-532.

89. Aitken, R.J. and K.M. West, *Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients.* Int J Androl, 1990. **13**(6): p. 433-451.

90. Donnelly, E.T., N. McClure, and S.E.M. Lewis, *Antioxidant supplementation in vitro does not improve human sperm motility.* Fertil Steril. **72**(3): p. 484-495.

91. Sies, H., *Strategies of antioxidant defense.* European Journal of Biochemistry, 1993. **215**(2): p. 213-219.

92. Gil-Guzman, E., et al., *Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation.* Hum Reprod, 2001. **16**(9): p. 1922-1930.

93. Bilodeau, J.-F., et al., *Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase.* Theriogenology, 2002. **57**(3): p. 1105-1122.

94. Januskauskas, A., A. Johannisson, and H. Rodriguez-Martinez, *Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility.* Theriogenology, 2003. **60**(4): p. 743-758.

95. Vernet, P., R.J. Aitken, and J.R. Drevet, *Antioxidant strategies in the epididymis.* Mol Cell Endocrinol, 2004. **216**(1–2): p. 31-39.

96. Goto, Y., et al., *Increased generation of reactive oxygen species in embryos cultured in vitro.* Free Radical Biology and Medicine, 1993. **15**(1): p. 69-75.

97. Nasr-Esfahani, M.M. and M.H. Johnson, *The origin of reactive oxygen species in mouse embryos cultured in vitro.* Development, 1991. **113**(2): p. 551-560.

98. Lee, H.C. and Y.H. Wei, *Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging.* Exp Biol Med (Maywood). 2007. **232(5).** p. 592-606.

99. Munne, S. and A. Estop, *The effect of in-vitro ageing on mouse sperm chromosomes.* Hum Reprod, 1991. **6**(5): p. 703-708.

100. Taanman, J.-W., *The mitochondrial genome: structure, transcription, translation and replication.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1999. **1410**(2): p. 103-123.

101. Gutteridge, J.M. and B. Halliwell, *Iron toxicity and oxygen radicals.* Baillieres Clin Haematol., 1989. **2(2)**: p. 195-256.

102. Sies, H., *Oxidative stress: oxidants and antioxidants.* Experimental Physiology, 1997. **82**(2): p. 291-295.

103. Vertuani S., Angusti A., and Manfredini S., *The Antioxidants and Pro-Antioxidants Network: An Overview.* Current Pharmaceutical Design, 2004. **10**(14): p. 1677-1694.

104. Comporti, M., *Three models of free radical-induced cell injury.* Chemico-Biological Interactions, 1989. **72**(1): p. 1-56.

105. Aitken, R., *Free radicals, lipid peroxidation and sperm function.* Reproduction, Fertility and Development, 1995. **7**(4): p. 659-668.

106. Olson, S.E. and G.E.J. Seidel, *Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients.* Biol Reprod. , 2000. **62(2):**: p. 248-52.

107. Yuan, Y., M.B. Wheeler, and R.L. Krisher, *Disrupted Redox Homeostasis and Aberrant Redox Gene Expression in Porcine Oocytes Contribute to Decreased Developmental Competence1.* Biol Reprod, 2012. **87**(4): p. 78, 1-10-78, 1-10.

108. Silva, E., et al., *Antioxidant supplementation during in vitro culture improves mitochondrial function and development of embryos from aged female mice.* Reproduction, Fertility and Development, 2015. **27**(6): p. 975-983.

109. Khalil, W.A., W.F.A. Marei, and M. Khalid, *Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development.* Theriogenology. **80**(2): p. 161-168.

110. Ali, A.A., J.F. Bilodeau, and M.A. Sirard, *Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development.* Theriogenology, 2003. **59**(3-4): p. 939-49.

111. Li, X.X., et al., *Glutathione and cysteine enhance porcine preimplantation embryo development in vitro after intracytoplasmic sperm injection.* Theriogenology, 2014. **81**(2): p. 309-14.

112. Abeydeera, L.R., et al., *Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization.* Theriogenology, 1998. **50**(5): p. 747-56.

113. Michael, A., et al., *Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa.* Theriogenology. **68**(2): p. 204-212.

114. Olfati Karaji, R., H. Daghigh Kia, and I. Ashrafi, *Effects of in combination antioxidant supplementation on microscopic and oxidative parameters of freeze–thaw bull sperm.* Cell and Tissue Banking, 2014. **15**(3): p. 461-470.

115. Castillo-Martín, M., et al., *Addition of L-ascorbic acid to culture and vitrification media of IVF porcine blastocysts improves survival and reduces HSPA1A levels of vitrified embryos.* Reprod Fertil Dev. , 2015 **27(7):**: p. 1115-23.

116. de Matos, D.G. and C.C. Furnus, *The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: Effect of β-mercaptoethanol, cysteine and cystine.* Theriogenology. **53**(3): p. 761-771.

117. Roederer, M., et al., *N-Acetylcysteine: A New Approach to Anti-HIV Therapy.* AIDS Research and Human Retroviruses, 1992. **8**(2): p. 209-217.

118. Mayer, M. and M. Noble, *N-acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(16): p. 7496-7500.

119. Burgunder, J.M. and B.H. Lauterburg, *Decreased production of glutathione in patients with cirrhosis.* European Journal of Clinical Investigation, 1987. **17**(5): p. 408-414.

120. Wu, W., et al., *Separation and quantification of N-acetyl-l-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection.* Biomed Chromatogr, 2006. **20**(5): p. 415-22.

121. Whitaker, B.D. and J.W. Knight, *Mechanisms of oxidative stress in porcine oocytes and the role of anti-oxidants.* Reprod Fertil Dev, 2008. **20**(6): p. 694-702.

122. Cossarizza, A., et al., *Protective effect of N-acetylcysteine in tumor necrosis factor-alpha-induced apoptosis in U937 cells: the role of mitochondria.* Exp Cell Res, 1995. **220**(1): p. 232-40.

123. Delneste, Y., et al., *Thiols prevent Fas (CD95)-mediated T cell apoptosis by down-regulating membrane Fas expression.* European Journal of Immunology, 1996. **26**(12): p. 2981-2988.

124. Whitaker, B.D. and J.W. Knight, *Effects of N-acetyl-cysteine and N-acetyl-cysteine-amide supplementation on in vitro matured porcine oocytes.* Reprod Domest Anim, 2010. **45**(5): p. 755-9.

125. Cheraghi, E., et al., *N-Acetylcysteine improves oocyte and embryo quality in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection: an alternative to metformin.* Reprod Fertil Dev, 2014.

126. Takeo, T., et al., *Cysteine analogs with a free thiol group promote fertilization by reducing disulfide bonds in the zona pellucida of mice.* Biol Reprod, 2015. **92**(4): p. 90.

127. Michael, A.J., et al., *Effect of N-acetyl-L-cysteine supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa.* Reprod Domest Anim, 2010. **45**(2): p. 201-7.

128. Whitaker, B.D., S.J. Casey, and R. Taupier, *N-acetyl-l-cysteine supplementation improves boar spermatozoa characteristics and subsequent fertilization and embryonic development.* Reprod Domest Anim, 2012. **47**(2): p. 263-8.

129. Perez, L., et al., *N-acetyl-L-cysteine pre-treatment protects cryopreserved bovine spermatozoa from reactive oxygen species without compromising the in vitro developmental potential of intracytoplasmic sperm injection embryos.* Andrologia, 2015. **47**(10): p. 1196-201.

130. Rall W.F. and F. G.M., *Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification.* Nature, 1985. **313(6003)**: p. 3.

131. Vajta, G., *Vitrification of the oocytes and embryos of domestic animals.* Anim Reprod Sci. **60**: p. 357-364.

132. Rall, W.F., *Factors affecting the survival of mouse embryos cryopreserved by vitrification.* Cryobiology, 1987. **24**(5): p. 387-402.

133. Tsang W.H. and Chow L.K., *Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula.* Biotechniques, 2009. **46(7)**: p. 3.

134. Azadbakht, M. and M.R. Valojerdi, *Development of vitrified-warmed mouse embryos co-cultured with polarized or non-polarized uterine epithelial cells using sequential culture media.* Journal of Assisted Reproduction and Genetics, 2008. **25**(6): p. 251-261.

135. Yan, C.L., et al., *Mitochondrial behaviors in the vitrified mouse oocyte and its parthenogenetic embryo: effect of Taxol pretreatment and relationship to competence.* Fertil Steril, 2010. **93**(3): p. 959-66.

136. Liang, Y., et al., *The type and extent of injuries in vitrified mouse oocytes.* Cryobiology, 2012. **64**(2): p. 97-102.

137. Somfai, T., et al., *Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage.* Cryobiology, 2007. **55**(2): p. 115-126.

138. Ali, A.A., J.F. Bilodeau, and M.A. Sirard, *Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development.* Theriogenology, 2002. **59**(3): p. 939-949.

139. Marí, M., et al., *Mitochondrial Glutathione, a Key Survival Antioxidant.* Antioxidants & Redox Signaling, 2009. **11**(11): p. 2685-2700.

140. ESHRE. *The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting†*. in *Human Reproduction*. 2011.

141. Ghandy, N. and A.A. Karimpur Malekshah, *Which Stage of Mouse Embryos Is More Appropriate for Vitrification?* International Journal of Fertility & Sterility, 2017. **10**(4): p. 357-362.

142. Zhang, J., et al., *Vitrification of mouse embryos at 2-cell, 4-cell and 8-cell stages by cryotop method.* Journal of Assisted Reproduction and Genetics, 2009. **26**(11-12): p. 621-628.

143. Gao, C., et al., *Melatonin promotes embryonic development and reduces reactive oxygen species in vitrified mouse 2-cell embryos.* J Pineal Res, 2012. **52**(3): p. 305-311.

144. Momozawa, K., et al., *Efficient vitrification of mouse embryos using the Kitasato Vitrification System as a novel vitrification device.* Reproductive Biology and Endocrinology, 2017. **15**(1): p. 29.

145. Kong, X., et al., *The Relationship between Cell Number, Division Behavior and Developmental Potential of Cleavage Stage Human Embryos: A Time-Lapse Study.* PLoS One, 2016. **11**(4): p. e0153697.

146. Mallol, A., J. Santaló, and E. Ibáñez, *Comparison of three differential mouse blastocyst staining methods.* Systems Biology in Reproductive Medicine, 2013. **59**(2): p. 117-122.

147. Sawicki, W. and E.T. Mystkowska, *In vivo staining of mouse preimplantation embryos with hoechst 33342.* The Anatomical Record, 1990. **227**(3): p. 359-362.

148. Van der Elst J, Amerijckx Y, and V.S. A., *Ultra-rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in-vitro cultured blastocysts.* Human Reproduction, 1998. **13(6)**: p. 5.

149. Jurisicova, A., et al., *Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development.* Mol Hum Rep, 1998. **2**: p. 139-145.

150. Guérin, P., S. El Mouatassim, and Y. Ménézo, *Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings.* Hum Reprod Update, 2001. **7**(2): p. 175-189.

151. Castillo-Martín, M., et al., *Comparative effects of adding β-mercaptoethanol or l-ascorbic acid to culture or vitrification–warming media on IVF porcine embryos.* Reproduction, Fertility and Development, 2014. **26**(6): p. 875-882.

152. Covarrubias, L., et al., *Function of reactive oxygen species during animal development: Passive or active?* Developmental Biology, 2008. **320**(1): p. 1-11.

153. Bedaiwy, M.A., et al., *Differential growth of human embryos in vitro: Role of reactive oxygen species.* Fertility and Sterility, 2004. **82**(3): p. 593-600.

154. Chiaratti, M.R. and F.V. Meirelles, *Mitochondrial DNA Copy Number, a Marker of Viability for Oocytes1.* Biology of Reproduction, 2010. **83**(1): p. 1-2.

155. Chappel, S., *The Role of Mitochondria from Mature Oocyte to Viable Blastocyst.* Obstetrics and Gynecology International, 2013. **2013**: p. 183024.

156. Toescu E.C. and V. A., *Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining.* Pflugers Arch., 2000. **440(6)**: p. 941-7.

157. Wilding, M., et al., *Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos.* Human Reproduction, 2001. **16**(5): p. 909-917.

158. Jansen, R.P.S. and K. de Boer, *The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate.* Mol Cell Endocrinol, 1998. **145**(1–2): p. 81-88.

159. Rienzi, L., et al., *Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance.* Human Reproduction Update, 2017. **23**(2): p. 139-155.

160. Wilding, M., et al., *Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos.* Hum Reprod. , 2001. **16(5)**: p. 909-17.

161. Lei, T., et al., *Vitrification of in vitro matured oocytes: effects on meiotic spindle configuration and mitochondrial function.* International Journal of Clinical and Experimental Pathology, 2014. **7**(3): p. 1159-1165.

162. Thouas, G.A., A.O. Trounson, and G.M. Jones, *Developmental Effects of Sublethal Mitochondrial Injury in Mouse Oocytes.* Biol Reprod, 2006. **74**(5): p. 969-977.

163. Agarwal, A., S. Gupta, and R.K. Sharma, *Role of oxidative stress in female reproduction.* Reprod Biol Endocrinol, 2005. **3**: p. 28.

164. Yashiro, I., et al., *High revivability of vitrified–warmed bovine mature oocytes after recovery culture with α-tocopherol.* Reproduction, 2015. **149**(4): p. 347-355.

165. Agarwal, A., D. Durairajanayagam, and S.S. du Plessis, *Utility of antioxidants during assisted reproductive techniques: an evidence based review.* Reproductive Biology and Endocrinology, 2014. **12**(1): p. 112.

166. Shan, X.Q., T.Y. Aw, and D.P. Jones, *Glutathione-dependent protection against oxidative injury.* Pharmacol Ther, 1993. **47(1)**: p. 61-67.

167. Trapphoff, T., et al., *Improved cryotolerance and developmental potential of in vitro and in vivo matured mouse oocytes by supplementing with a glutathione donor prior to vitrification.* MHR: Basic science of reproductive medicine, 2016. **22**(12): p. 867-881.

168. Moawad, A.R., S.L. Tan, and T. Taketo, *Beneficial effects of glutathione supplementation during vitrification of mouse oocytes at the germinal vesicle stage on their preimplantation development following maturation and fertilization in vitro.* Cryobiology, 2017. **76**: p. 98-103.

169. Liu, J., et al., *Delay in oocyte aging in mice by the antioxidant N-acetyl-l-cysteine (NAC).* Human Reproduction, 2012. **27**(5): p. 1411-1420.

170. Yue, S.-L., et al., *Effect of NAC on mouse GV oocyte survival and subsequent embryonic development following vitrification*. Vol. 37. 2016. 295-302.

171. Toescu, E.C. and A. Verkhratsky, *Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining.* Pflugers Arch, 2000. **440**(6): p. 941-7.

172. Schindelin, J., et al., *Fiji - an Open Source platform for biological image analysis.* Nature methods, 2012. **9**(7): p. 10.1038/nmeth.2019.

173. Simsek-Duran, F., et al., *Age-Associated Metabolic and Morphologic Changes in Mitochondria of Individual Mouse and Hamster Oocytes.* PLoS One, 2013. **8**(5): p. e64955.

174. Szurek, E.A. and A. Eroglu, *Comparison and Avoidance of Toxicity of Penetrating Cryoprotectants.* PLoS One, 2011. **6**(11): p. e27604.

175. Lei, T., et al., *Effect of mouse oocyte vitrification on mitochondrial membrane potential and distribution.* Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban, 2014. **34**(1): p. 99-102.

176. Leoni, G.G., et al., *Differences in the Kinetic of the First Meiotic Division and in Active Mitochondrial Distribution between Prepubertal and Adult Oocytes Mirror Differences in their Developmental Competence in a Sheep Model.* PLoS ONE, 2015. **10**(4): p. e0124911.

177. Giaretta, E., et al., *Effects of Resveratrol on Vitrified Porcine Oocytes.* Oxidative Medicine and Cellular Longevity, 2013. **2013**: p. 920257.

178. Tatone, C., et al., *Effects of reproductive aging and postovulatory aging on the maintenance of biological competence after oocyte vitrification: insights from the mouse model.* Theriogenology, 2011. **76**(5): p. 864-873.

179. Karami, A., et al., *Impact of prolonged oocyte incubation time before vitrification on oocyte survival, embryo formation, and embryo quality in mice.* In Vitro Cellular & Developmental Biology - Animal, 2017. **53**(6): p. 525-531.

180. Macías-García, B., et al., *Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS.* Animal Reproduction Science, 2012. **132**(1): p. 74-82.

181. Rocha-Frigoni, N.A.S., et al., *Reduced levels of intracellular reactive oxygen species and apoptotic status are not correlated with increases in cryotolerance of bovine embryos produced in vitro in the presence of antioxidants.* Reproduction, Fertility and Development, 2014. **26**(6): p. 797-805.

182. Manipalviratn, S., et al., *Effect of vitrification and thawing on human oocyte ATP concentration.* Fertility and Sterility, 2011. **95**(5): p. 1839-1841.

183. Salvetti, P., et al., *Structural, metabolic and developmental evaluation of ovulated rabbit oocytes before and after cryopreservation by vitrification and slow freezing.* Theriogenology, 2010. **74**(5): p. 847-855.

184. Zander-Fox, D., K.S. Cashman, and M. Lane, *The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development.* Journal of Assisted Reproduction and Genetics, 2013. **30**(1): p. 107-116.

185. Ministerio de Agricultura, A.y.M.A.S.D.L.B.D.G.D.L.R.A.E.A.f.h.w.m.g.e.e.g.

186. http://www.mapama.gob.es/es/ganaderia/temas/zootecnia/razas-ganaderas/razas/catalogo/autoctona-fomento/bovino/lidia/default.aspx.

187. Lopes, G., et al., *Tris–Egg Yolk–Glycerol (TEY) Extender Developed for Freezing Dog Semen is a Good Option to Cryopreserve Bovine Epididymal Sperm Cells.* Reproduction in Domestic Animals, 2015. **50**(1): p. 97-103.

188. Almeida, F.C., et al., *Effects of glycerol, equilibration time and antioxidants on post-thaw functional integrity of bovine spermatozoa directly obtained from epididymis.* Andrologia, 2016: p. n/a-n/a.

189. Chaveiro, A., et al., *Significant variability among bulls in the sperm membrane permeability for water and glycerol: possible implications for semen freezing protocols for individual males.* Cryobiology, 2006. **53**(3): p. 349-59.

190. Fernández-Santos, M.R., et al., *Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity.* Int J Androl, 2009. **32**(4): p. 353-359.

191. Long, J.A., et al., *Cryopreservation of turkey semen: Effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching.* Cryobiology, 2014. **68**(3): p. 371-378.

192. Kulaksiz, R., et al., *The effect of different glycerol concentrations on freezability of semen from Angora, Kilis and Saanen goats.* Slovak Jpurnal Animal Science, 2013. **46**: p. 5.

193. Adoyo, P.A., A. Moore, and H.D.M. Moore, *A strategy for identifying candidate sperm antigens for immunocontraception: isolation of human testis cDNA clones using polyclonal antisera directed against hamster acrosomal membrane preparation.* Int J Androl, 1995. **18**: p. 32-38.

194. Byrd, W., et al., *A prospective randomized study of pregnancy rates following intrauterine and intracervical insemination using frozen donor sperm*.* Fertil Steril, 1990. **53**(3): p. 521-527.

195. Fetterolf, P.M. and B.J. Rogers, *Prediction of human sperm penetrating ability using computerized motion parameters.* Mol Reprod Dev, 1990. **27**(4): p. 326-331.

196. Nagy, Á., et al., *Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis.* Acta Veterinaria Hungarica, 2015. **63**(3): p. 370-381.

197. Minervini, F., et al., *Assessment of Different Functional Parameters of Frozen–Thawed Buffalo Spermatozoa by Using Cytofluorimetric Determinations.* Reproduction in Domestic Animals, 2013. **48**(2): p. 317-324.

198. Windsor, D.P., *Mitochondrial function and ram sperm fertility.* Reproduction, Fertility and Development, 1997. **9**(3): p. 279-284.

199. Chen, H., et al., *Male Genital Tract Antioxidant Enzymes: Their Source, Function in the Female, and Ability to Preserve Sperm DNA Integrity in the Golden Hamster.* Journal of Andrology, 2003. **24**(5): p. 704-711.

200. Bilodeau, J.F., et al., *Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen.* Theriogenology, 2001. **56**(2): p. 275-86.

201. Ball, B.A., et al., *Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5dEC.* Theriogenology, 2001. **56(4)**: p. 12.

202. Jurisicova, A., et al., *Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development.* MHR: Basic science of reproductive medicine, 1998. **4**(2): p. 139-145.

203. Guérin, P., S. El Mouatassim, and Y. Ménézo, *Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings.* Human Reproduction Update, 2001. **7**(2): p. 175-189.

204. Ballester, L., et al., *Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency.* Fertil Steril, 2014. **102**(6): p. 1762-1768.

205. Somfai, T.s., et al., *Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage.* Cryobiology, 2007. **55**(2): p. 115-126.

206. Yashiro, I., et al., *High revivability of vitrified-warmed bovine mature oocytes after recovery culture with Î±-tocopherol.* Reproduction, 2015. **149**(4): p. 347-355.