EFECTOS DEL GLUTATIÓN REDUCIDO Y LA PROCAÍNA EN LA RESISTENCIA A LA CRIOPRESERVACIÓN DE SEMEN PORCINO. ACCIONES A NIVEL DE LA ESTABILIDAD NUCLEAR Y SU EFECTO EN LA FERTILIDAD "IN VIVO"

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TESIS DOCTORAL

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Certifican:

Que la tesis titulada "Efectos del glutatión reducido y la procaína en la resistencia a la criopreservación de semen porcino. Acciones a nivel de la estabilidad nuclear y su efecto en la fertilidad *in vivo*". Presentada por **Efrén Estrada Paqui** para optar al grado de Doctor en Veterinaria se ha realizado bajo nuestra direccion y, considerandola acabada, autorizamos su presentación para que sea juzgada por la comision correspondiente.

Bellaterra (Cerdanyola del Vallés). 26 de febrero de 2014.

Dr. Joan Enric Rodriguez Gil Dr. Marc Yeste Oliveras

Efrén Estrada Paqui

(Doctorando)

CONTENIDO

RESUMEN

El objetivo de este trabajo fue el de evaluar los efectos de la suplementación con glutatión reducido (GSH) y clorhidrato de procaína (ProHCL) del medio de criopreservación de semen porcino sobre la estabilidad nuclear y la fertilidad “in vivo”.

En el primer estudio se comprobó el efecto del GSH y el ProHCL en concentraciones de 1 y 2 mM sobre la estabilización de los puentes disulfuro de las nucleoproteínas, así como la integridad de la cromatina. La fragmentación del DNA se observo a los 240 minutos posteriores a la descongelación. Estas mismas concentraciones de GSH pero no de ProHCL mejoraron parcialmente la integridad del acrosoma y la permeabilidad de membrana, la motilidad total y progresiva, así como los niveles de peróxidos y superóxidos productores de radicales libres de oxigeno (ROS).

En el segundo estudio se evaluó la magnitud de las alteraciones en la funcionalidad espermática entre eyaculados buenos congeladores (EBC) y malos congeladores (EMC) durante el proceso de criopreservación y el efecto de mejora del GSH en la criotolerancia de acuerdo con la congelabilidad del eyaculado. Se observaron diferencias entre ambos grupos a la descongelación, pero no durante el proceso de congelación, en la motilidad, integridad de la membrana celular y acrosoma, la estructura de nucleoproteínas y los niveles de fragmentación del ADN en eyaculados.

En el tercer estudio se comprobó que la adición de GSH mejoró significativamente la criotolerancia en las muestras EBC, sin diferencias entre 2 mM y 5mM. Por el contrario, la congelabilidad de los EMC se incrementó significativamente sólo cuando se suplementaron con 5 mM de GSH.

En el cuarto estudio se comprobó la resistencia a la criopreservación del espermatozoide durante el tiempo de mantenimiento (HT) a 17ºC, evaluando 2 diferentes tiempos (3 y 24 horas de mantenimiento), sobre los parámetros de sobrevivencia celular. A la vez se compararon los niveles de fosforilación de residuos de serina (pSer) en 30 proteínas implicadas en la regulación general de la función espermática. Las 24 horas de HT produjo un aumento significativo en los niveles de pSer de la proteína HSP70, que fue paralelo a un mejoramiento en la viabilidad, motilidad, integridad del acrosoma y estabilización de

puentes disulfuro de nucleoproteínas tras la congelación/descongelación. Estos resultados sugieren la existencia de una relación entre los niveles de pSer de proteínas como la HSP70 y la criotolerancia del semen porcino.

Por último, en el quinto artículo se determinó la capacidad fecundante del espermatozoide porcino "in vivo", con la adición de 2mM del GSH al medio de criopreservación conjuntamente con inseminación post-cervical en cerdas multíparas. Se observo un aumento importante y significativo en la tasa de no retorno a estro a 21 días, tasa de gestación a 30 días y tasa de parto, así como en el tamaño de camada determinado por lechones totales y vivos. Además, estos parámetros reproductivos se correlacionaron significativamente con los niveles de residuos de cisteína libre de la cabeza espermática y, en menor medida, con otros estimadores de la calidad seminal, como la fragmentación del ADN, la integridad de acrosoma, la viabilidad, motilidad progresiva y la producción de ROS. Por lo tanto, los resultados indican que la adición de GSH mejora de forma significativa los resultados obtenidos con la inseminación con semen congelado en porcino.

Como conclusión general, estos estudios muestran que la adición de GSH y ProHCL participan en grado similar en el mantenimiento de la integridad de la estructura nucleoproteica para la estabilización de la cromatina, punto crucial para el funcionamiento y fertilidad "in vivo" del espermatozoide porcino. También se demostró que la magnitud de daño en parámetros de función espermática durante el proceso de criopreservación es mayor en eyaculados EMC. Por lo tanto los EMC necesitan una mayor concentración de GSH que los EBC para mejorar su criotolerancia. Por otra parte, el espermatozoide porcino modula su funcionamiento durante el TH de 24 horas incrementando su criotolerancia mediante cambios en los niveles de pSer de proteínas ligadas a la resistencia ambiental. Finalmente, la adición de GSH en el medio de congelación parece ser una técnica de utilidad para la mejora de los resultados de inseminación con semen congelado en porcino.

SUMMARY

The aim of this study was to evaluate the effects of supplementation with reduced glutathione (GSH) and procaine hydrochloride (ProHCL) medium boar semen cryopreservation on nuclear stability and “in vivo” fertility.

In the first study, the effect of GSH and the ProHCL at concentrations of 1 and 2 mM of stabilizing disulfide bonds the nucleoproteins and chromatin integrity. DNA fragmentation was observed at 240 minutes after thawing. The same concentrations of GSH but partially improved ProHCL acrosome integrity and membrane permeability, total and progressive motility, as well as the levels of peroxides and superoxides producing oxygen free radicals (ROS).

In the second study the magnitude of the changes in functionality between good freezability ejaculates (GFE) or poor freezability ejaculates (PFE) evaluated during the cryopreservation process and the effect of GSH in improving cold tolerance according to ejaculate freezability. Differences between groups were observed thawing, but not during the freezing process, motility, integrity of the cell membrane and acrosome, the nucleoprotein structure and levels of DNA fragmentation in ejaculated.

In the third study found that the addition of GSH significantly improved cold tolerance in GFE samples, with no difference between 2 mM and 5 mM. In contrast, the PFE freezability increased significantly only when supplemented with 5 mM GSH.

In the fourth study resistance of sperm cryopreservation was found during the holding time (HT) at 17 °C, evaluating 2 different times (3 and 24 hours of maintenance), on parameters of cell survival. While the levels of phosphorylation of serine residues (pSer) in 30 proteins involved in the overall regulation of sperm function were compared. At 24 hours of HT produced a significant increase in the levels of pSer the HSP70 protein, which was parallel to an improvement in viability, motility, acrosome integrity and disulfide bonds stabilization the nucleoprotein after freezing / thawing. These results suggest the existence of a relationship between levels of pSer protein as HSP70 and cold tolerance of porcine semen.

Finally, in the fifth article the fertilizing capacity of boar spermatozoa “in vivo”, with the addition of 2 mM GSH to the cryopreservation medium together with post -cervical insemination in sows was determined. An important and significant increase in the rate of non-return to estrus 21 days, pregnancy rate at 30 days and farrowing rate and the litter size determined by total and live piglets was observed. Furthermore, these reproductive parameters were significantly correlated with the levels of free cysteine residues of the sperm head and to a lesser extent, other estimators of semen quality, such as DNA fragmentation, acrosome integrity, viability, progressive motility and production of ROS. Therefore, the results indicate that the addition of GSH significantly improves the results obtained with the pig insemination with frozen semen.

As a general conclusion, these studies show that the addition of GSH and similar degree ProHCL involved in maintaining the integrity of the nucleoprotein structure of chromatin stabilization, crucial for the functioning and improvement of fertility “in vivo” porcine sperm. It was also shown that the magnitude of damage in sperm function parameters during cryopreservation is higher in ejaculates PFE. Therefore the need PFE, GSH concentration greater than GFE to improve cold tolerance. Moreover, the boar spermatozoa modulate TH during 24 hours by increasing its cryotolerance changes of pSer levels of protein linked to the environmental resistance. Finally, addition of GSH in the freezing medium appears to be a useful technique for improving the results of pig insemination with frozen semen.

INTRODUCCIÓN

1. ESCENARIO ACTUAL DE LA PRODUCCIÓN PORCINA

La industria porcina es una actividad dinámica a nivel mundial. Así, en los últimos años se ha incrementado el inventario, la producción de carne y el consumo "per cápita". El pronóstico de crecimiento anual mundial durante la presente década es de un 2,8 %, alcanzándose un total de 86 millones de reproductoras y una producción de 110,46 millones de toneladas de carne al término de la misma (Pig Inter FAO: Food Outlook, 2012). Sin embargo, la actividad pecuaria tiene ciclos económicos poco predecibles, y ello determina la rentabilidad de las granjas dedicadas a la producción porcina (Orr y Shen, 2006).

En los últimos años la producción porcina ha experimentado cambios relevantes, que no han afectado sólo a la producción de carne y a los inventarios, sino que también han influido en las características de la carne de cerdo relacionadas principalmente con el aporte calórico y de colesterol. En efecto, las modificaciones que se han llevado a cabo durante los últimos 40 años han supuesto una reducción importante tanto del aporte calórico como del porcentaje de colesterol en la carne de cerdo (Trujillo et al., 2002). Estos cambios son debidos principalmente a la selección genética, lográndose que las cantidades de colesterol en la carne de cerdo sean incluso más bajos que los de otras especies (Hartog, 2005). Asimismo, otros aspectos importantes a considerar en el escenario de rápido desarrollo de la actividad porcina mundial son la selección de hembras reproductoras hiperprolíficas, la optimización de raciones alimenticias, la gestión en manejo reproductivo, el diagnóstico y control de las enfermedades, las medidas de bioseguridad y el bienestar animal (Trujillo et al., 2002). Finalmente, la gran expansión actual de la industria intensiva porcina se debe a factores como el ciclo corto de producción, tanto en la fase reproductiva como en la fase de engorde, el buen índice de conversión de alimento a carne y la consiguiente excelente ganancia de peso diario. Otros aspectos positivos son, además, la buena adaptación de la crianza en diferentes condiciones climáticas, así como la creación de puestos de trabajo directos e indirectos, ya que la cadena de comercialización porcina es muy diversificada comparada con la de otras especies productivas (Fig. 1).

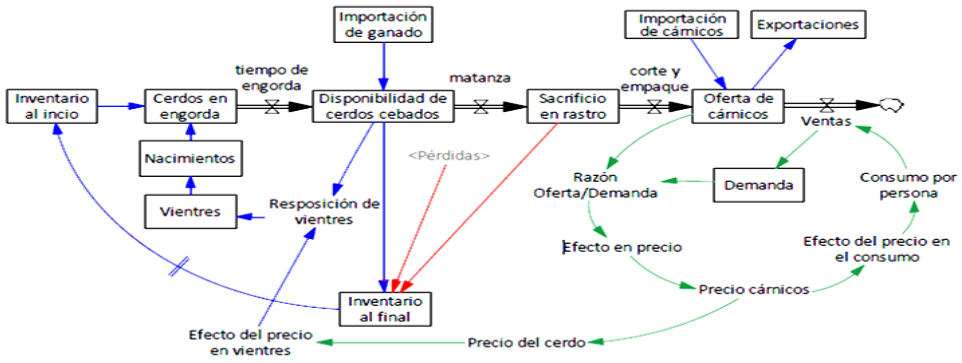


Figura 1. Cadena comercial de la producción porcina (Cabello y Torres, 2010)

Por otro lado, los sistemas de producción de grandes poblaciones de animales, como es el caso de la producción porcina, requieren de indicadores productivos y otros parámetros que deben valorarse durante este proceso. En efecto, la gestión de un gran número de animales hace necesario conocer y evaluar las explotaciones tanto de forma global, esto es, por áreas productivas y lotes de animales, como de forma individual, siendo esta última especialmente importante en el caso de los animales de pie de cría (verracos y hembras reproductoras). Además, es importante destacar que las evaluaciones pueden realizarse por lapsos de tiempo determinados, es decir por semana, mes, trimestre, y/o año (Trujillo et al., 2002). Entre los parámetros básicos a evaluar en las hembras reproductoras se encuentran los números de lechones nacidos totales y nacidos vivos por cerda y año, así como el de lechones destetados, el número de servicios por concepción, el porcentaje de parición, el número de días desde el destete a la concepción, los promedios de nacidos totales, nacidos vivos y destetados por parto, las tasas de reposición y desecho, el promedio de partos al desecho y la distribución de la camada por número de parto. En el caso de los verracos, la evaluación debe ser aún más rigurosa, puesto que el 70 % de la producción depende de éstos. Finalmente, hay que mencionar que los parámetros productivos más importantes a evaluar son las tasas de fertilidad y de prolificidad de la hembras, el peso de su progenie al nacimiento, al destete y al matadero y los estimadores de cantidad y calidad de la carne (Martínez, 2011).

2. GENERALIDADES DE LA REPRODUCCION DEL VERRACO

2.1. Anatomia y fisiologia del sistema reproductor

El aparato genital del verraco (Fig. 2) consta de manera sucinta de testículos, epidídimos, conductos deferentes, uretra, pene y glándulas sexuales accesorias.

Los **testículos** del verraco son de forma ovalada, con un contorno de tipo elíptico y tienen en un animal adulto un peso de 300-350 gramos. Estos órganos se ubican en la región perineal debajo de la abertura anal y están colocados de forma tal que el eje mayor está dirigido hacia arriba y atrás (Hafez, 2006; Bonet et al., 2012). El testículo está recubierto por la túnica albugínea que se continúa en el interior del parénquima testicular formando los septos testiculares. A continuación se encuentran los lóbulos o túbulos seminíferos, que tienen en su interior tejido intersticial, y los canalículos contorneados o conductos aferentes (Galina y Valencia, 2011). Los testículos se encuentran protegidos por el escroto que es una evaginación de la pared abdominal y que está situado a muy corta distancia del ano. En el verraco, el escroto no se encuentra tan marcadamente definido de las porciones circundantes como en los otros animales domésticos (Hafez, 2006).

Posteriormente y adyacente a cada testículo se encuentra el **epidídimo** que consta de tres partes: la cabeza, el cuerpo y la cola (Bonet et al., 2012). El epidídimo tiene una doble función, pues de un lado se produce la maduración de los espermatozoides en la cabeza, y de otro se conservan en la cola hasta su expulsión, que se produce gracias a las contracciones de las células musculares de esta parte del epidídimo. La continuación del epidídimo la constituye el **conducto deferente** que desemboca en la uretra. La pared de este conducto deferente tiene tres estratos musculares o membranas: externa o fibrosa, media o carnosa, e interna o mucosa. Este conducto es flexuoso en su porción testicular y está íntimamente unido a la túnica vaginal (Bonet et al., 2012).

La **uretra** es un conducto cutáneo-musculoso que consta de tres partes o porciones: la porción pelviana, la porción vulvar (raíz del pene) y la porción peneana. La porción pelviana es muy larga y está cubierta por el músculo uretral grueso, excepto dorsalmente donde existe una capa fibrosa densa.

El **pene** del verraco mide de 45-50 cm, con un diámetro que no excede de 1,5-2 cm. La parte terminal o glande tiene forma de tirabuzón y termina en punta y el tejido eréctil es escaso, por lo que este pene está clasificado como fibroelástico (Galina y Valencia, 2011). El prepucio es la parte más externa del pene y consta de cuatro sacos prepuciales. Estos sacos contienen normalmente restos de orina y descamaciones epiteliales como consecuencia de productos excretados como las feromonas, que producen un olor fuerte y desagradable que es característico del verraco. Al conjunto de secreciones prepuciales se le denomina esmegma (Cupps, 1991).

Dentro de las **glándulas sexuales accesorias** se encuentran la próstata, las vesículas seminales y las glándulas bulbouretrales o de Cowper. La **próstata** consta de dos partes: el cuerpo y la porción diseminada. Su secreción contiene ácido cítrico, ácido ascórbico, proteínas, lípidos y azúcares. Las **vesículas seminales** sirven básicamente de depósito de secreciones que contribuyen fundamentalmente a dar volumen y material energético al eyaculado total. Las **glándulas bulbouretrales** producen secreciones que llevan cantidades importantes de NaCl, K^+ y Ca^{2+}, con un efecto de naturaleza espermiocinética (Galina y Valencia, 2011)

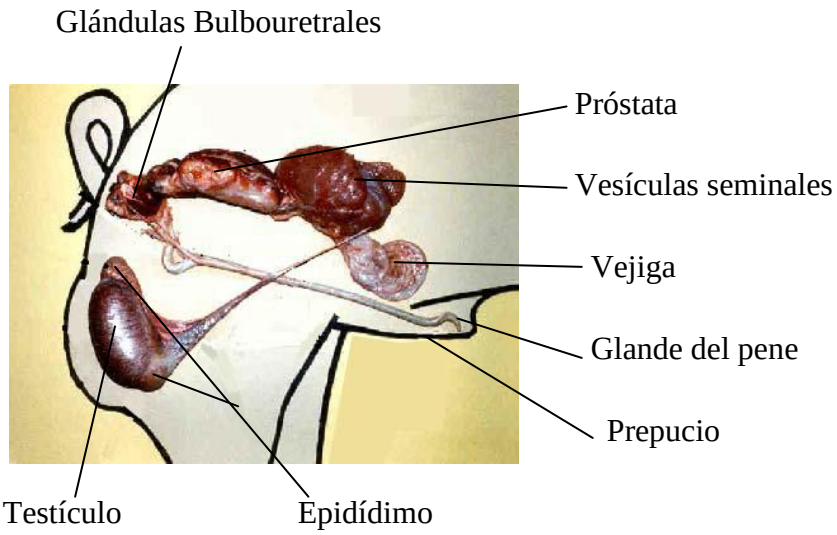


Figura 2. Órganos genitales del verraco (Barone, 1995)

2.2. **El espermatozoide**

2.2.1. Estructura y producción espermática

El espermatozoide de verraco es una célula altamente especializada de alrededor de 44 µm de longitud dividido en tres secciones principales: cabeza (7µm), cuello (0.7µm) y cola (37µm; ver Fig. 3). Estas secciones están rodeadas por una estructura lipoproteínica en forma de bicapa continua llamada plasmalema o membrana plasmática (Bonet et al., 2000; Holt et al., 2010).

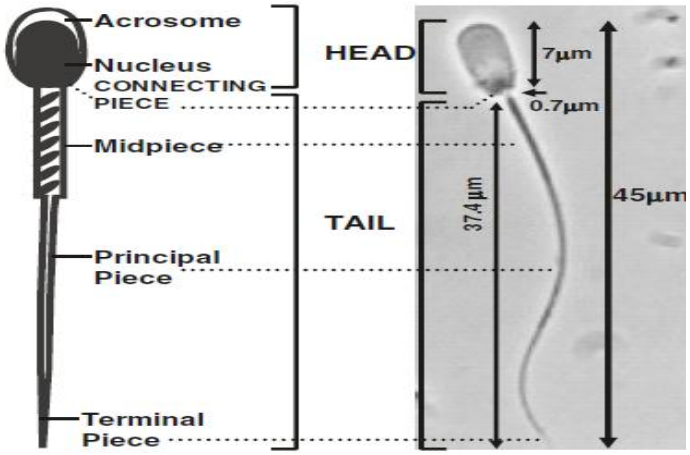


Figura 3. División y medidas del espermatozoide de verraco (Bonet et al., 2000)

La cabeza es plana bilateralmente y de forma ovalada conteniendo en su ápice una vesícula o acrosoma. En la cresta apical de una de las caras de la cabeza hay una protuberancia en forma de herradura de alrededor 0.4µm de ancho que causa la dilatación del acrosoma (Briz y Fábrega, 2013). El núcleo es una estructura rígida formada por la teca perinuclear y contiene los filamentos de DNA que se estructuran en complejos nucleoproteínicos al ligarse a protaminas y a histonas. Esta estructura forma la cromatina condensada (Hermo et al., 2010; Flores et al., 2011).

El acrosoma cubre la mitad del núcleo como un capuchón, extendiéndose desde la parte superior de la cabeza hasta la región ecuatorial. A esta zona también se le conoce con el nombre de región acrosomal. El acrosoma es similar a un lisosoma ya que tiene un pH muy ácido, aunque se origina a partir del aparato de Golgi (Ramalho-Santos et al., 2002). El

acrosoma contiene múltiples glucoproteínas que se condensan para formar la vesícula acrosómica (Hermo et al., 2010), así como distintas proteasas: (proacrosina/acrosina, hialurinidasas, glicohidrolasas y esterasas, etc., ver Senger, 2003). Aunque la superficie del acrosoma es una vesícula continua, está formado por dos membranas superpuestas: la membrana acrosomal externa y la interna (Hermo et al., 2010). La región acrosomal abarca el 80 % y está dividida en tres segmentos claramente diferenciados apical, principal y ecuatorial (Fig. 4). En este último segmento, la matriz acrosomal electrodensa es mayor que en los otros dos segmentos. El otro 20 % de la longitud nuclear corresponde a la región post-acrosomal, que consiste en una lámina densa y fibrosa. También se distingue el espacio sub-acrosomal o perinuclear que separa al núcleo de la membrana acrosomal interna (Briz y Fábrega, 2013).

A) Vista seccional : las líneas solidas continuas representan bicapas de membrana
1. Membrana plasmática o plasmalema
2 .Membrana acrosomal externa
3. Matrix acrosomal enzimática
4. Membrana acrosomal interna
5. Envoltura nuclear
6. Núcleo
7. Pieza conectora (cuello)
8. Pieza media
9. Axonema y mitocondrias
10. Anillo de Jensen´s
11. Vaina fibrosa
12. Axonema y fibras densas externas
B)Vista de la superficie de la cabeza y pieza media con subdominios
13. Anillo apical
14. Región acrosomal
15. Región ecuatorial
16. Región post-acrosomal

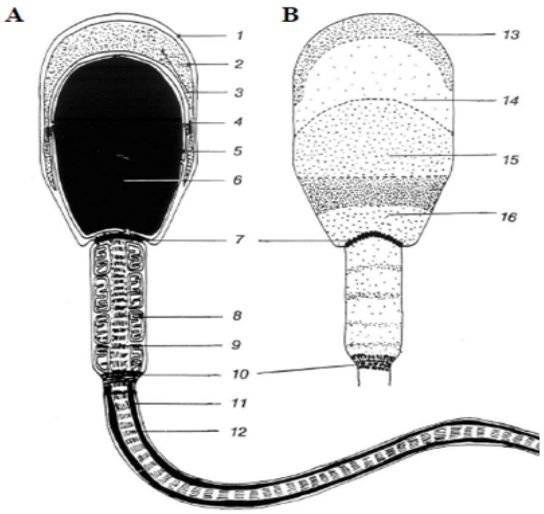


Figura 4. Estructura del espermatozoide de verraco (Gadella et al., 2008)

Otros elementos presentes son los dominios de membrana. Estos dominios tienen un composición glucosídica distinta, pues juegan distintas funciones durante el proceso de fecundación del ovocito por el espermatozoide (Brewis y Gadella, 2010).

La **pieza de conexión** o cuello une la cabeza con la pieza intermedia y tiene forma de trapecio (Bonet et al., 2000). Esta porción contiene las estructuras necesarias para transmitir el movimiento del flagelo a la cabeza. Además, el cuello contiene el centriolo a partir del

cual se llevará a cabo la primera división mitótica del embrión tras la fecundación del ovocito (Bonet et al., 2000).

El **flagelo** o cola tiene una forma filamentosa y cilíndrica (Fig. 3) compuesta de tres piezas: la pieza intermedia o mitocondrial, la pieza principal y la pieza terminal. La pieza intermedia o mitocondrial tiene 9 µm en longitud y 0,7 µm de diámetro, y presenta una estructura axonemática cubierta por la vaina mitocondrial, estructura formada por diversas mitocondrias helicoidalmente dispuestas alrededor del axonema (Phelps et al., 1990; Gadella et al., 1995). Esta pieza intermedia está conectada con la pieza principal a través del anillo de Jensen (Fig.4), un paquete de subunidades filamentosas que adhieren estas dos estructuras, evitando así el desplazamiento de la vaina mitocondrial (Guan et al., 2009).

La pieza principal mide 26,2 µm en longitud y 0,4 µm de diámetro y presenta una estructura axonemática cubierta con la vaina fibrosa. Finalmente, la pieza terminal tiene 2,2 µm en longitud y 0,2 µm en diámetro, así como un axonema desorganizado (Briz, 1994).

El axonema pasa a través de todos los segmentos de la cola y se compone de 9 fibras exteriores en forma de estrías cruzadas, 9 en el interior y 2 fibras centrales formado microtubulos dobles (subfibras A y B) en una disposición 9x2 permitiendo el movimiento lateral y hacia adelante del flagelo (Guraya ,1987; Hermo et al., 2010; ver Fig. 5).

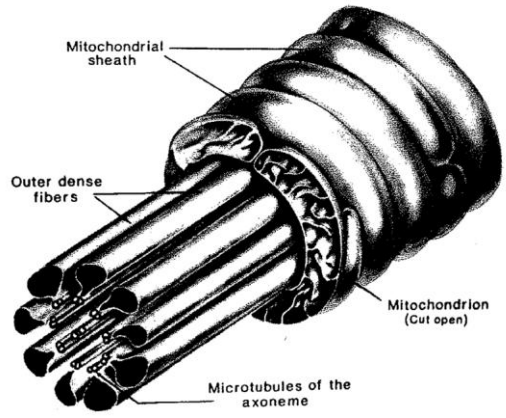


Figura 5 estructura completa del axonema (Guraya, 1987)

Espermatogénesis y control endocrino

La espermatogénesis se inicia con la espermatocitogénesis, en la cual hay proliferación por mitosis de un número fijo de células germinales dando lugar a células diploides denominadas espermatogonias A1 (Fig. 6; ver Senger, 2003). En el compartimento basal, las células germinales se unen a las células de Sertoli, que les proporcionan nutrición, protección con la barrera hematotesticular y aislamiento del sistema inmune (Jones y Dechereny, 2005). Las espermatogonias A1 llevan a cabo alrededor de 6 divisiones mitóticas para formar los tipos celulares subsiguientes (espermatogonias A1-A4, I, B y espermatocitos primarios). En suma, una espermatogonia inicial A1 produce 64 espermatocitos primarios (Senger, 2003). Los espermatocitos primarios llevan a cabo una última división por mitosis. De aquí en adelante comienza el proceso de reducción de material genético o fase de meiosis que se divide a la vez en meiosis I y meiosis II para dar lugar posteriormente a 256 espermatocitos secundarios haploides por cada espermatogonia A1 (Jones y Dechereny, 2005).

La espermiogenesis es la etapa de diferenciación morfológico-funcional de los espermatocitos una vez finalizada la meiosis. Comienza con la migración, controlada por las células de Sertoli, de las espermátidas hasta cerca de la luz del túbulo seminífero. Durante este movimiento se lleva a cabo el desarrollo del acrosoma y la formación de los centríolos en el flagelo, así como la condensación de la cromatina nuclear (Guraya, 1987). Las espermátidas llegan a introducirse dentro del citoplasma de las células de Sertoli, donde adquieren glucógeno como sustrato de reserva energético a utilizar durante los diferentes cambios estructurales que requieren (Hafez, 2006). Al mismo tiempo, el aparato de Golgi de la espermátida se transforma en el acrosoma y uno de sus centríolos organiza el axonema de la cola (Briz y Fábrega, 2013). También es en esta fase en la que las histonas nucleares son remplazadas en su mayor parte por protaminas, cambiando así la estructura nuclear. Asimismo, las mitocondrias migran hacia el flagelo para formar la vaina mitocondrial, y el contenido citoplasmático se reduce dejando como resto final la gota citoplasmática (Stone, 1981; Abou-Haila y Tulsiani, 2000). El ciclo espermático dura aproximadamente 34 días y el proceso se produce en varias secciones en una secuencia lineal de etapas a través del túbulo seminífero (Briz y Fábrega, 2013).

La producción espermática sigue un esquema de ondas que recorren todas las secciones de un túbulo seminífero en una secuencia consecutiva para completar el ciclo y proceder al siguiente nivel de desarrollo (Hafez 2006). La producción diaria se estima en unos $16x10^9$ espermatozoides y permite al verraco mantener la cantidad adecuada de reserva espermática durante su vida reproductiva (Senger, 2003).

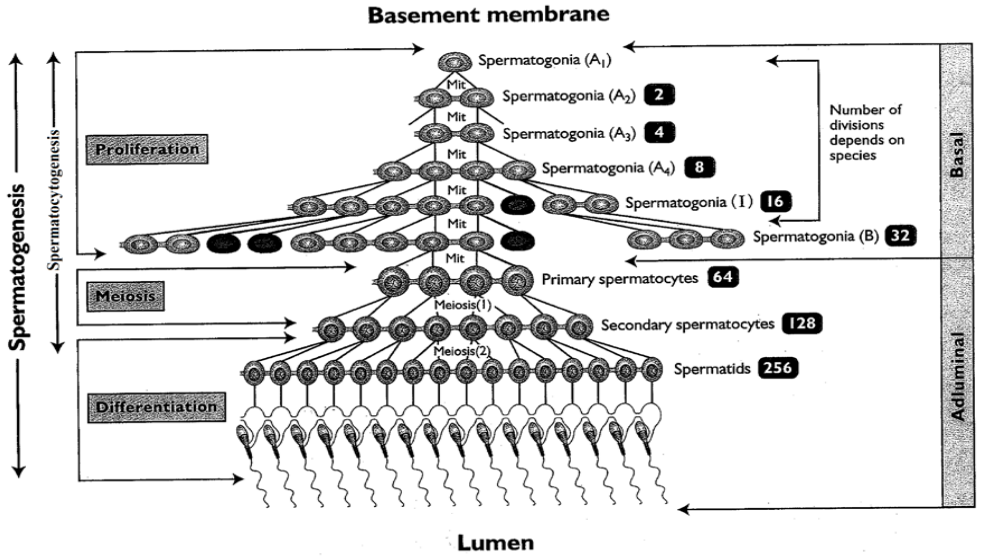


Figura 6. Secuencia del proceso de espermatogénesis en el verraco (Senger, 2003).

Al término de la espermatogénesis, los espermatozoides se liberan de las células de Sertoli en el proceso conocido como espermiación (Senger, 2003). Los espermatozoides se mueven hacia fuera de los túbulos seminíferos a través de la red testicular hacia el epidídimo para su maduración, en donde alcanzan su potencial completo de fertilidad después de unos 12-15 días de tránsito (Frandson et al., 2003; Martínez-Pastor et al., 2011). Todos estos eventos están controlados por mecanismos hormonales y paracrinos. La regulación endocrina se inicia a los 125 días de edad del macho como un proceso continuo controlado por la respuesta cerebral a diferentes estímulos externos como el fotoperiodo, la temperatura ambiental, el peso corporal y el medio ambiente social (Fig.7). Además, existen mecanismos hormonales de retroalimentación internos. Así, el incremento en la sensibilidad del hipotálamo a niveles basales de testosterona (Hafez, 2006) provoca pulsos de liberación de mayor duración de la hormona liberadora de gonadotropinas (GnRH) provenientes del hipotálamo. Este aumento provoca a su vez un estímulo sobre la liberación

de la hormona folículo estimulante (FSH), sintetizada en el lóbulo anterior de la glándula pituitaria (Senger, 2003). La FSH estimula a su vez a las células testiculares para regular su división celular (Germann y Stanfiel, 2005). También la FSH está involucrada en la síntesis de la proteína transportadora de andrógenos (ABP). La unión de los andrógenos a la ABP intratesticular permite mantener las cantidades necesarias de andrógenos dentro del túbulo seminífero, siendo a su vez responsable de la síntesis del enzima aromatosa que convierte la testosterona en estradiol (E2), en las células de Sertoli (Jones y Dechereny, 2005). El E2 testicular viaja a su vez por vía sanguínea hasta el hipotálamo, en donde tiene un efecto de retroalimentación negativa sobre la liberación de GnRH (Galina y Valencia, 2011).

Otra hormona producida en las células de Sertoli y regulada por la FSH es la inhibina, que a su vez controla la producción de FSH por retroalimentación negativa sobre la glándula pituitaria (Senger, 2003). La acción de la GnRH también estimula la producción de la hormona luteinizante (LH), la cual induce la producción de testosterona dentro de las células de Leydig (Jones y Dechereny, 2005). La producción de testosterona es necesaria para el correcto funcionamiento de las células de Sertoli y del último estadio de la espermatogénesis, así como para la regulación del comportamiento sexual del verraco (Germann y Stanfield, 2005). Se ha sugerido que las células de Sertoli tienen un papel en la regulación paracrina de las células de Leydig a través de la producción de estrógenos y, a su vez, las células de Leydig tienen un papel de regulación en la síntesis de FSH (Jones y Dechereny, 2005)

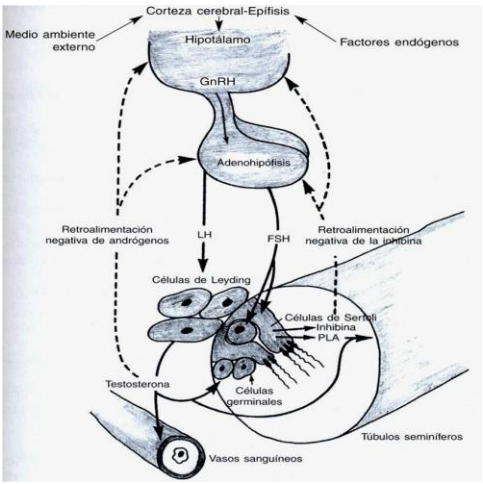


Figura 7. Control neuro-endocrino de la producción de espermatozoides del verraco (Hafez, 2006)

En la especie porcina la maduración espermática se lleva a cabo a través del tránsito epididimario, de duración aproximada entre 12 y 15 días (Martínez-Pastor et al., 2011). Se han distinguido entre 6 y 10 regiones en el epidídimo dependiendo de la composición del fluido epididimario y de las modificaciones que el espermatozoide experimenta en cada región (Fábrega et al., 2011). El proceso de maduración comprende la adquisición de la motilidad progresiva y el inicio de los procesos que conducen a los espermatozoides a desarrollar su capacidad fecundante plena. Estos procesos incluyen cambios como la estabilización de la cromatina nuclear a través del establecimiento de puentes disulfuro entre protaminas (Bonet et al., 1995; 2012). Otro cambio es la migración de la gota citoplasmática de la posición proximal a distal y su posterior eliminación (Couper, 2011). También aparecen cambios en la naturaleza y distribución de glucoproteínas en la membrana, lo que lleva a modificaciones de los dominios de membrana, así como en la capacidad de adhesión de espermoadhesinas liberadas por las glándulas accesorias. Estas espermoadhesinas se adhieren a la superficie espermática y mediante la interacción con las células del epitelio del oviducto forman el reservorio espermático oviductal (Topfer-Peterson et al., 2008). También durante la maduración espermática otro cambio importante es la modificación del metabolismo aeróbico y anaeróbico, optimizando el fenotipo energético de los espermatozoides para su vida post-eyaculación (Jones y Murdach, 1996).

El proceso de eyaculación del verraco dura entre 6 y 20 minutos. El volumen del eyaculado está entre 200 y 300 mL, alcanzando en casos excepcionales los 500 mL (Pinart et al., 1999). El volumen está sujeto a considerables variaciones como resultado de características individuales del verraco, raza, edad, condiciones fisiológicas y el medio ambiente (Setchell, 1991). En el verraco, tres fracciones pueden ser obtenidas de un eyaculado completo. Estas fracciones son las siguientes (Sancho, 2004; Peña et al., 2006).

1. La fracción pre-espermática: formada por secreciones producidas de próstata, vesícula seminal y glándulas bulbouretrales. Suele tener un volumen de 10-15 mL, no contiene espermatozoides y normalmente presenta una apariencia clara o trasparente.

2. La fracción espermática: el volumen de esta fracción es de 70 a 100 mL y tiene una apariencia lechosa, conteniendo los espermatozoides.

3. La fracción post-espermática: el volumen de esta fracción es alrededor de 150-200 mL de una apariencia blanca pálida y no contiene espermatozoides. Esta fracción proviene de la próstata y las glándulas bulbouretrales.

El semen constituye la parte funcional de un eyaculado y puede considerarse como un tejido líquido formado por la mezcla de las fracciones espermática y post-espermática del eyaculado. El semen está formado por una parte por la fracción celular, es decir, los espermatozoides, y por la fracción líquida que envuelve los espermatozoides, conocida como plasma seminal. El volumen constituido por los espermatozoides del eyaculado porcino representa el 10–30 % del semen, mientras que el plasma seminal, representa un 70-90 %. El plasma seminal está formado por la contribución de la secreción de testículo y epidídimo (2-5 % del volumen), vesícula seminal (15-20%), glándulas bulbouretrales (10-15%) y próstata (45-60%; ver Foote, 2002; Casas et al., 2009). El plasma seminal contiene componentes orgánicos e inorgánicos, principalmente carbohidratos, lípidos, aminoácidos y proteínas. La proporción entre los diversos componentes varía dependiendo de factores como la raza, el intervalo entre eyaculaciones y la salud del verraco (Caballero et al., 2012). Dentro los carbohidratos están la glucosa y, en mucha menor medida, la fructosa (Medrano et al., 2006; Foxcroft et al 2008). Por otra parte, las proteínas del plasma seminal influyen en la función y capacidad de fecundación del espermatozoide al interactuar con el tracto genital de la cerda durante el transporte espermático al sitio de fecundación (Moura et al., 2006). Otros componentes importantes que se encuentran en plasma seminal de verraco son los iones Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} y Cl^{-}, fosfato inorgánico, inositol, acido láctico, acido cítrico, ácido glutamico, glicerofosfocolina, glicerofosfatidilinositol, arginina, creatinina, ergotioneina, prostaglandinas y hormonas como el E2 (Martin- Rillo et al., 1996).

2.2. 2. Analítica seminal

El potencial fecundante del semen está inherentemente ligado a la calidad de sus espermatozoides. En este sentido, el examen del eyaculado es requerido principalmente cuando se utiliza para elaborar dosis de inseminación (Sancho y Vilagran, 2013). Los métodos convencionales del análisis de calidad espermática tales como el examen visual bajo microscopio óptico solo dan una idea general y vaga del potencial de fertilidad de un eyaculado. Por lo tanto, otros métodos más sofisticados deberían utilizarse para determinar

diferencias en la calidad de semen entre verracos seleccionados, especialmente si analizamos muestras sin patologías graves ni evidentes (Waberski et al., 2011). Además, el eyaculado debe ser evaluado teniendo en cuenta el conocimiento de fisiología de la célula espermática como parte esencial para medir su capacidad fecundante (Foxcroft et al., 2008). El análisis de la calidad espermática incluye parámetros celulares-funcionales y del estado metabólico del espermatozoide (Knobil y Neil, 1994). Los estimadores celulares-funcionales incluyen la concentración, la motilidad, la morfología, la integridad de membrana plasmática y la tolerancia osmótica (Gadea, 2005; Foxcroft et al., 2008). Los análisis fisiológicos y metabólicos son ensayos complementarios necesarios para predecir la capacidad fecundante de la célula espermática, valorando la fluidez de membrana, integridad nuclear, estado de la vaina mitocondrial, niveles de compuestos oxigenados reactivos (ROS), integridad del acrosoma, así como los niveles y actividad de diferentes metabolitos y enzimas (Bonet et al., 2012).

2.2.2.1. Evaluación física y visual del eyaculado

La primera evaluación de semen inmediatamente después de la extracción corresponde a un control macroscópico de las características del semen. La temperatura del eyaculado recién obtenido es de 36±2 ºC, mientras que su pH está usualmente entre 6,85 y 7,9. Por otra parte, la osmolaridad es variable, aunque siempre alrededor de los 300 mOsm/Kg. De hecho, las variaciones en la osmolaridad dependen de la composición seminal y a su vez están relacionadas con la secreción de testosterona como resultado de la actividad testicular (Martin-Rillo, 1982). El color de la fracción espermática es blanco-lechoso, aunque pueden existir variaciones que frecuentemente indican alguna alteración. Por ejemplo, el semen teñido de rojo significa presencia de sangre por lesión en uretra o replicación viral en el epidídimo (Sancho, 2002).

2.2.2.2. Concentración espermática

El número de espermatozoides por eyaculado varía entre razas y verracos, así como debido a la acción de factores como temperatura ambiental, nutrición, enfermedades, frecuencia de eyaculación y fotoperiodo (Sancho et al., 2004). Generalmente el eyaculado de verraco contiene una concentración espermática de 300 a 600 millones/mL (Casas et al., 2010).

Este valor es aceptado en los centros de procesamiento de semen para verracos fértiles (Martin- Rillo et al., 1996). Para la evaluación de la concentración se usan diferentes cámaras de contaje (Neubauer, Thoma, Burker) que permiten determinar el número de espermatozoides inmovilizados en un volumen conocido (Christensen et al., 2005). Variaciones entre 4 y 20% pueden ser observadas por el inadecuado uso de las cámaras y de la dilución espermática (Knox, 2004). Otras cámaras como la de Mackler se usan para valorar la concentración al mismo tiempo que la motilidad, destacando su simplicidad y su relativamente bajo coste (Knuth et al 1989). La concentración también puede ser medida con un sistema informático ("Computer Assisted Sperm Analysis", CASA; ver; Roca et al., 2011) así como por espectrofotómetros y colorímetros en centros de IA. Estos últimos miden el porcentaje de transmisión de luz a través de una alícuota de semen diluido con una curva estándar de calibración (Sancho y Vilagran, 2013). Finalmente, la concentración espermática también puede ser valorada por el citómetro de flujo. En este caso, el análisis de la concentración se asocia a una gran variedad de tinciones por fluorocromos para la determinación de una gran variedad de parámetros. De esta forma, el citómetro es utilizado como un procedimiento de referencia debido a su velocidad, sensibilidad y objetividad de las mediciones, aunque el uso está limitado a grandes laboratorios de investigación (Lu et al., 2007).

2.2.2.3. Motilidad espermática

La motilidad es conocida por ser una importante característica en la predicción del potencial de fecundación del espermatozoide en algunas especies (Johnson et al., 2000), si bien esta capacidad de predicción es a menudo poco clara, debido al menos en parte a que el espermatozoide depositado durante la inseminación es desplazado al sitio de fecundación principalmente por las contracciones uterinas y no por el movimiento intrínseco del espermatozoide (Langendijk et al., 2002). El método más utilizado para determinar la motilidad espermática es la observación directa de la muestra mediante un microscopio óptico ya que este método es práctico, económico y rápido. Sin embargo es muy subjetivo y de gran variabilidad en los resultados dependiendo del técnico que lo realiza. Además, las características especificas de motilidad de los espermatozoides de verraco hacen que este parámetro sea difícil de interpretar (Quintero-Moreno et al., 2004). De esta manera, el

contaje de la motilidad por sistema automatizado CASA es la herramienta de laboratorio más adecuada. Este sistema está basado en la captura de múltiples imágenes digitales con el movimiento individual del espermatozoide, lo que permite calcular la trayectoria basándose en diferentes parámetros de motilidad calculados por un software incorporado (Vyt et al., 2008). Sin embargo, la información obtenida a través del sistema CASA está todavía sujeta a factores externos tales como la preparación de la muestra o el tipo de cámara usada para su análisis, por lo que la estandarización del procedimiento utilizado es necesaria para un adecuado uso del sistema CASA (Tejerina et al., 2008). La evaluación por sistema CASA se lleva a cabo a 100x aumentos y el número aproximado de espermatozoides por muestra es de 100 a 200 (Martínez-Pastor et al., 2011).

Existen muchos factores que pueden afectar los resultados del análisis de motilidad. En este sentido, se ha reportado la pérdida del movimiento del espermatozoide con ligeras variaciones de temperatura del medio ambiente de 36 ºC a 38 ºC entre 15 y 20 minutos (Vyt et al., 2008). Asimismo, la contaminación bacteriana reduce la motilidad progresiva después del día 1 de almacenamiento a 15 ºC (Bussalleu et al., 2011). A pesar de todo ello, como ya se ha dicho, el análisis CASA es la mejor técnica existente hoy en día para la evaluación de la motilidad. Los parámetros de motilidad más utilizados obtenidos a partir del análisis CASA son los siguientes, aunque existen un número mucho mayor de índices y valores utilizables (Holt et al., 1996; Quintero-Moreno et al., 2004; ver Fig. 8):

1. Motilidad total: porcentaje de espermatozoides con una velocidad lineal superior a 10 µm/sg.

2. Velocidad curvilínea (VCL): distancia recorrida por el espermatozoide a lo largo de su trayectoria real en función al tiempo. Se expresa en µm/sg.

3. Velocidad rectilínea (VSL): distancia recorrida por el espermatozoide entre el primer punto y el último de su trayectoria en función del tiempo. Se expresa en µm/sg.

4. Velocidad lineal (VAP): distancia recorrida por el espermatozoide a lo largo de la trayectoria media en función al tiempo. Se expresa en µm/sg.

5. Índice de linealidad (LIN): relación porcentual entre la velocidad rectilínea y la velocidad curvilínea. Se expresa en %.

6. Índice de rectitud (STR): relación porcentual entre la velocidad rectilínea y la velocidad lineal. Se expresa en %.

7. Índice de oscilación (WOB): relación porcentual entre la velocidad lineal y la curvilínea. Se expresa en %.

8. Amplitud media de desplazamiento lateral (ALH): desplazamiento que efectúan las cabezas en su trayectoria curvilínea de un lado a otro de la trayectoria media o lineal. Se expresa en µm.

9. Frecuencia de batido de la cabeza (BCF): frecuencia con la que la trayectoria curvilínea a traviesa la trayectoria media en función al tiempo. Se expresa en Hz

10. Motilidad progresiva: espermatozoides que muestran un porcentaje de STR superior al 80%

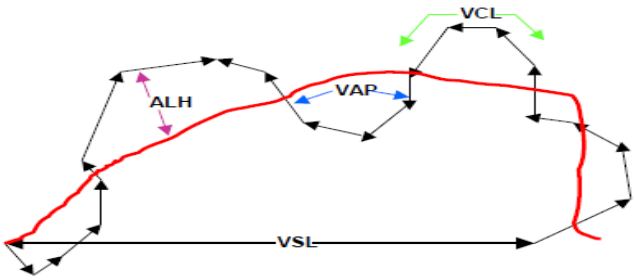


Figura 8. Presentación de parámetros de cinética espermática por el sistema CASA (Quintero-Moreno et al., 2004)

A pesar de todas sus limitaciones, el análisis por motilidad mediante CASA tiene cierta utilidad para el establecimiento de las cualidades fecundantes de un eyaculado. Así, cuando el espermatozoide es sometido a cambios ligados a capacitación muestra valores altos de VAP y bajo LIN (García et al., 2005). Holt et al, (1997) establecieron una asociación entre motilidad total y de motilidad progresiva con la fertilidad del eyaculado. Vyt et al. (2008) observaron que el incremento de motilidad en el semen diluido se relaciona con el aumento de 0,14 lechones nacidos por camada. También se ha observado una correlación

positiva débil entre motilidad total y habilidad del espermatozoide durante la fecundación medida como tasa de no retorno a estro dentro de 60 días (Yeste et al., 2010). En este sentido, la tasa de motilidad total de más del 60 % es aceptada como requerimiento mínimo para un eyaculado fértil (Donadeu, 2004; Broekhuijse et al., 2012).

Por otro lado, el análisis CASA permite obtener una información extremadamente detallada sobre los patrones de motilidad y velocidad de espermatozoides. Esta información puede ser usada para identificar diferencias entre subpoblaciones de espermatozoides dentro de un eyaculado (Peña et al., 2005). El análisis de subpoblaciones espermáticas podrían explicar la variabilidad en las poblaciones espermáticas con diferente comportamiento de motilidad dentro del eyaculado (Quintero-Moreno et al., 2004). En este sentido, los diferentes porcentajes de las diferentes subpoblaciones en el eyaculado de verraco pone en manifiesto la existencia de 3 a 4 subpoblaciones de espermatozoides con diferentes características de motilidad (Martínez-Pastor et al., 2011). Aunque la motilidad total no parece ser de gran importancia en la fertilidad en vivo, sí se ha observado que algunas subpoblaciones especificas pueden estar estrechamente relacionadas con la capacidad fecundante de un eyaculado (Quintero-Moreno et al., 2004). Sin embargo el valor del análisis aún no es completamente objetivo debido a que no existe método estadístico estándar para valorar las diferentes subpoblaciones (Martínez-Pastor et al., 2011).

2.2.2.4. Morfología espermática

La valoración de la morfología espermática es una herramienta efectiva para estimar la función del epitelio seminífero y de la maduración epididimaria. Además, también sirve como una medida adicional de la calidad del semen y potencial de fertilidad (Gadea, 2005). Usualmente el examen morfológico del eyaculado porcino compromete una clasificación cuantitativa y cualitativa de morfología espermática normal y anormal. La clasificación puede ser valorada por microscopio óptico con técnicas de tinción simples tales como la eosina-nigrosina, el azul tripán, el Giemsa, el Papanicolaou o el Diff-Quick© en microscopio de contraste de fases o también por el sistema CASA (Foxcroft et al., 2008; Bonet et al., 2012). Se considera un semen con morfología normal el que presenta un porcentaje mínimo de un 75-85 % de espermatozoides morfológicamente normales (Martin-Rillo et al., 1996; Rozeboom et al., 2000). El origen y tipología de alteraciones

espermáticas pueden ser primarias, secundarias y terciarias (Bonet, 2000). Las anormalidades primarias son producidas en el testículo durante la espermatogénesis, la espermiogenesis o la espermiacion (Donadeu, 2004)**.** Las causas fundamentales de estas alteraciones son a menudo congénitas y su heredabilidad es baja. Las malformaciones de cabeza en cuanto a tamaño y forma afectan el acrosoma y el núcleo así como la anormalidad de la vaina mitocondrial daña la función del flagelo (Pruneda et al., 2007). Las malformaciones de la cabeza pueden presentarse como aberraciones en número (2 o más cabezas) forma (triangular, ovoide, forma de pera) o tamaño (micro o macro cefálico). Malformaciones de cola pueden presentarse como aberraciones en número (2 o más colas) y longitud o trayectoria (colas enrolladas o plegadas; ver Sancho, 2002). Los porcentajes aberrantes en más del 50 % presentes en un eyaculado son conocidos como teratozoospermia y son causa de infertilidad (Who, 2000). Por otro lado, Pinart et al. (1999) estudiaron la calidad espermática con criptorquídia unilateral y observaron un porcentaje de células espermáticas con cola corta 6 veces más alto que verracos sanos. También el fotoperíodo puede afectar la morfología espermática principalmente el régimen de obscuridad de 14 horas (Sancho et al., 2004).

Las anormalidades secundarias ocurren en el epidídimo durante el proceso de maduración **(**Briz et al., 1996). Las gotas citoplasmática proximal y distal son las más habituales de entre estas anormalidades (Pruneda et al., 2007). Cuando el porcentaje de espermatozoides con anormalidades secundarias excede del 30%, este hecho es normalmente debido a una disfunción epididimaria que afecta el transito del espermatozoide. Dentro de alteraciones del tránsito epididimario las más comunes son las relacionadas con un aumento de frecuencia en el ritmo de colección de semen o con las primeras eyaculaciones, principalmente de verracos jóvenes (Pruneda et al., 2005).

Las anormalidades terciarias son adquiridas por el inapropiado manejo del semen, generalmente durante por cambios en la temperatura de almacenamiento, transporte, dilución y preparación de dosis para inseminación (Sancho et al., 2004). Dentro de estas anormalidades las más frecuentes son alteraciones morfológicas graves de las colas (Bonet et al., 2000).

Por otro lado, el semen de verracos con pobre morfología espermática pueden resultar en baja tasa de gestación y reducido tamaño de camada cuando se usa para inseminación (Alm et al., 2006). Por lo tanto, una alta morfología anormal puede ser un marcador importante para identificar a verracos subfértiles (Bonet et al., 2012), si bien las anormalidades secundarias y terciarias pueden ser compensadas mediante el incremento en el número de espermatozoides por dosis de inseminación (Martin-Rillo et al., 1996).

2.2.2.5. Aglutinación espermática

En ocasiones, los espermatozoides se unen entre ellos cabeza a cabeza o cabeza a cola. En condiciones normales este hecho, conocido como aglutinación, no debería ocurrir y puede ser causa de infertilidad (Sancho y Vilagran, 2013). Factores como los aniones bivalentes y trivalentes en el plasma seminal, un período de almacenamiento largo, factores inmunológicos y la contaminación bacteriana son causas de aglutinación (Yeste et al., 2008; Bussalleu et al., 2011**).** Por lo tanto, la presencia de aglutinaciones suele indicar un problema de manejo de la muestra post-eyaculación.

2.2.2.6. Resistencia osmótica del espermatozoide

El espermatozoide de verraco es muy sensible a cambios en el medio ambiente y su habilidad de responder al estrés osmótico está relacionada con la funcionalidad celular (Foxcroft et al., 2008; Yeste et al., 2010) y la habilidad de regular el flujo de electrolitos y agua por diferentes mecanismos de adaptación y resistencia a los repentinos cambios en la osmolaridad (Foxcroft et al., 2008). Estos mecanismos involucran complejas vías metabólicas, por ejemplo los canales iónicos relacionados a ATPasa-dependientes de iones Na^+/K^+ (Medrano et al., 2006) llevando al choque osmótico que afecta la difusión de fosfolípidos en la bicapa de membrana espermática (Chistova et al., 2002).

Dentro de los ensayos para evaluar la resistencia osmótica del espermatozoide, el más importante en la especie porcina es el test de resistencia osmótica (ORT). Este test consiste en valorar la capacidad del espermatozoide a resistir la incubación en un medio hipo-osmótico. En esta prueba se utilizan soluciones iso-osmóticas (305±7 mOsm) e hipo-osmóticas (102±5 mOsm), llevando a cabo la medición de la tolerancia espermática a los cambios de osmolaridad determinado por el porcentaje de alteración o irregularidad del

acrosoma. La valoración se lleva a cabo o bien directamente bajo observación en contraste de fase o bien con tinción de eosina-nigrosina (Rodríguez-Gil et al., 1994). Este test ha sido correlacionado con resultados de fertilidad en campo (Schilling et al., 1996; Pérez-Llano et al., 2001).

Existen otros tests osmóticos. Así, el test hipo-osmótico de hinchamiento (HOST) se utiliza en especies como la bovina o la canina. En este test, el espermatozoide es sometido a estrés hipo-osmótico y se evalúa la capacidad de hinchazón de la célula cuando trata de equilibrar el contenido iónico con el medio ambiente externo. Este fenómeno es observado en forma de cambios en la morfología normal de la cola del espermatozoide, aunque estos cambios pueden variar dependiendo de diferentes factores tales como tolerancia individual o composición del medio (Rodríguez-Gil et al., 1994; Vázquez et al., 1997; Bonet et al., 2006). También se han asociado los cambios de resistencia osmótica del espermatozoide porcino evaluados mediante HOST con la viabilidad, motilidad, morfología, acrosoma intacto y la tasa de no retorno a estro a 60 días y tamaño de camada (Yeste et al., 2010).

2.2.2.7. Viabilidad espermática

La integridad de la membrana espermática es un indicador de la viabilidad espermática, puesto que dicha integridad es necesaria para mantener la función celular (Sancho y Vilagran, 2013). El eyaculado porcino debe contener como mínimo un 85 % de espermatozoides con membrana intacta para ser considerado de buena calidad (Pinart et al., 1999; Sancho, 2002). Procedimientos de manejo tales como la dilución o almacenamiento a bajas temperaturas de semen diluido o destinado para criopreservación pueden dañar la membrana espermática, por lo que es importante su evaluación para determinar la fertilidad del espermatozoide (Waberski et al., 2011). La viabilidad puede ser evaluada por diferentes métodos. Así, en microscopía con luz convencional se utilizan tinciones vitales como la eosina-azul de anilina o la eosina-nigrosina (Kvist y Bjorndahl., 2002). La Tinción de eosina-nigrosina es la más usada para el análisis de viabilidad por su facilidad y coste. Esta tinción, además, permite el examen simultáneo de la integridad de membrana y de la morfología espermática. Este método se basa en que la membrana plasmática de los espermatozoides viables no es permeable a la tinción mientras los espermatozoides no viables se vuelven permeables, tiñéndose en consecuencia (Sancho, 2002). Sin embargo,

las tinciones para luz convencional pueden ser inadecuadas con ciertos medios, como los diluyentes para congelación, puesto que estos medios interfieren a menudo con la tinción, produciéndose así resultados erróneos (Casas et al., 2010). A pesar de ello, la bondad de este tipo de tinciones es indudable, existiendo incluso reportes de resultados de viabilidad espermática valorados mediante eosina-nigrosina que se correlacionan con la tasa de gestación de la cerda (Tsakmakidis et al., 2010).

Por otro lado, la medición de la viabilidad también puede evaluarse mediante fluorocromos monitoreados con microscopio óptico de fluorescencia o por citómetro de flujo. La citometría es, de hecho, el mejor método por su repetibilidad, la precisión de resultados confiables y por el elevado número de espermatozoides que son contados en corto tiempo. Además, la citometría permite combinar diferentes pruebas de funcionalidad espermática como la fluidez de membrana y acrosoma entre otros (De Andrade et al., 2007; Yeste et al., 2009). Tanto en microscopía de fluorescencia como en citometría, los fluorocromos se unen directamente a los orgánulos del espermatozoide ya sea por uniones covalentes u otras moléculas y son eficientes para ligarse a estructuras como núcleo, acrosoma y membrana plasmática (Gravance et al., 2000). La técnica de fluorocromos basada en la doble tinción con SYBR-14 y yoduro de propidio (PI) es la más comúnmente utilizada para el análisis de viabilidad. En esta técnica, los espermatozoides marcados son excitados por láser de argón emitiendo fluorescencia verde para SYBR-14 y Rojo con PI (Yeste et al., 2009).

2.2.2.8. Integridad del acrosoma

El acrosoma intacto es necesario para la penetración del ovocito y, por lo tanto, su integridad es considerada vital para su optima capacidad fecundante del espermatozoide (Jiménez et al., 2002; Bonet et al 2012). La técnica actualmente más usada para el análisis de la integridad acrosomal es el marcaje con lectinas. Las lectinas son glucoproteínas relacionadas con los sistemas de defensa de plantas y de animales invertebrados y por lo general sin actividad enzimática, si bien existen excepciones. Tienen como principal propiedad la de unirse a fracciones glucídicas específicas según el tipo de lectina utilizado. De esta manera, si se conjuga un tipo específico de lectina con un determinado fluorocromo, se consigue determinar de manera exacta la localización de la fracción glucídica detectada por la lectinas en la superficie de la membrana celular analizada.

Numerosos trabajos han establecido que lectinas como la obtenida de *Pisum Sativum* (PSA), la de *Arachis hypogaea* o aglutinina de maní (PNA) y la concanavalina A (Vázquez et al., 2005; Hernández et al., 2006) marcan de manera específica fracciones glucídicas ligadas exclusivamente a la membrana acrosomal. Otra lectina que marca en espermatozoides de mamífero de manera específica la membrana acrosomal es el inhibidor de tripsina de la soja (SGTI). La SGTI bloquea la actividad catalítica de la serina proteasa acrosomal. Esta proteasa inhibe directamente la acrosina. Si se utiliza esta lectinas marcada con un fluorocromo, el espermatozoide aparece menos teñido cuando el acrosoma está intacto y un alto nivel de tinción es observado cuando el acrosoma reacciona. (Peña et al., 1999; De Andrade., 2007).

2.2.2.9. Integridad de la vaina mitocondrial

La funcionalidad mitocondrial es básica para el mantenimiento de la funcionalidad espermática. En los últimos años se han desarrollado diversos marcadores que evalúan la actividad mitocondrial como parte del análisis completo seminal. Entre los diversos marcadores desarrollados destacan todo el grupo de los MitoTracker© (Bonet et al 2012), El JC-1 **(**Ramio-Lluch et al., 2012**)** y la Rodamina 123 (Fraser et al., 2001). Estos fluorocromos son catiónicos y lipofilicos, por lo que tienen la capacidad de difusión pasiva a través de la membrana plasmática, acumulándose en la matriz mitocondrial cargada negativamente. Una vez allá, la actividad mitocondrial los oxida, lo que provoca un incremento en la intensidad emitida por el fluorocromo (caso de gran parte de los fluorocromos del grupo MitoTracker©) o bien un cambio en la longitud de onda de emisión del fluorocromo. Este es el caso del JC-1, que al oxidarse vira desde una emisión verde a una naranja-roja. A mayor oxidación de los fluorocromos, mayor actividad mitocondrial. Siguiendo estas técnicas, se ha podido observar que la actividad mitocondrial puede estar correlacionada con la motilidad espermática y la capacidad fecundante del espermatozoide humano y de perro (Bonet el al 2012). Además, la tinción combinada de un marcador de vaina mitocondrial como el MitoTracker© Green y el PI es usada para monitorear la viabilidad espermática conjuntamente con la funcionalidad mitocondrial (Flores et al., 2010).

2.2.2.10. Técnicas de tinción múltiple

Las técnicas de tinción múltiple involucran más de 2 fluorocromos y fueron desarrolladas con el objetivo de evaluar no solo el estado del núcleo si no también otros componentes celulares (Bussalleu et al., 2005). Este método permite una mejor predicción de la habilidad de fecundación del espermatozoide (Nagy et al., 2003). Generalmente, la tinción múltiple determina la viabilidad, la integridad acrosomal y la funcionalidad de la vaina mitocondrial (Bussalleu et al., 2005). En general esta prueba consiste en adicionar a la muestra seminal al principio dos fluorocromos como la Bisbezimida y el PI, determinando la emisión de fluorescencia de acuerdo al estado de la membrana celular y unión con DNA. Posteriormente se añaden otros dos fluorocromos a la suspensión espermática, el MitoTracker© Green y la SBTI conjugada con el fluorocromo Alexa fluor©488. El MitoTracker© Green se pasivamente difunde a través de la membrana plasmática y se une a los lípidos de membrana de las mitocondrias funcionales emitiendo fluorescencia si la membrana mitocondrial no esta alterada. Por otra parte, la SBTI conjugada con Alexa fluor©488 es un marcador de integridad acrosómica, como se ha comentado anteriormente. Siguiendo esta técnica, el espermatozoide puede ser considerado que tiene membrana intacta (núcleo teñido de azul) o membrana dañada (núcleo teñido de rojo). Además se evalúa al mismo tiempo a los espermatozoides dividiéndolos en diferentes categorías de acuerdo a su estado de integridad del acrosoma y de la membrana mitocondrial (Bussalleu et al., 2005; Bonet et al., 2012).

2.2.2.11. Peroxidación de lípidos y compuestos oxigenados reactivos

Normalmente el espermatozoide porcino tras su recolección está suspendido en el plasma seminal, lo que le protege del daño oxidativo. Sin embargo el espermatozoide es muy sensible a la peroxidación lipídica por el alto contenido de ácidos grasos poli-insaturados, el principal sustrato de los ROS (Bathgate, 2011). Los cambios que sufre el espermatozoide con el estrés oxidativo pueden ser medidos por emisión espectral en citometría de flujo (Gadea et al, 2005). Una de las técnicas para estimar el nivel de ROS en el espermatozoide de verraco es la tinción fluorescente diacetato de 2´7´-diclorodihidrofluoresceína (H2DCFDA; ver Awda et al., 2009). Este colorante es permeable a la membrana celular, por lo que se difunde dentro del espermatozoide. Una vez allí, los grupos acetato del

colorante son hidrolizados por la actividad esterasa inducida por peróxido de hidrogeno (H_2O_2). Esta reacción que, por lo tanto, está íntimamente ligada a los niveles intracelulares de H_2O_2, produce 2´7´diclorofluoresceína (DCF), medible por citometría de flujo. De esta forma, la producción de DCF es una estimación directa de la formación de uno de los principales ROS, el H_2O_2. (Matas et al., 2010). También está documentado que los niveles de ROS pueden ser evaluados por la tinción doble Mitotracker Red©-proxilfluorescamina (Flores et al., 2010), si bien en este caso sólo se evalúa el ROS formado específicamente en el entorno mitocondrial.

2.2.2.12. Integridad nuclear y fragmentación de DNA

La cromatina del espermatozoide es extremadamente estable debido a sus uniones con protaminas en una estructura helicoidal (Balhorn, 2007). Durante la maduración nuclear final en el transito epididimario se forman los puentes disulfuro entre protaminas para asegurar la estabilización del DNA (Garcia-Macias et al., 2006; Fraser y Strzezek, 2007). La deposición de las protaminas entre la cromatina espermática y su condensación está controlada por procesos de fosforilación cuando se unen a DNA y desfosforilación durante la maduración (Lewis et al., 2002). Una unión adecuada a las nucleoproteínas estabiliza el DNA y es esencial para expresar la capacidad fecundante del espermatozoide (Silva y Gadella, 2006). De hecho, el DNA espermático puede ser dañado y fragmentado durante la espermatogénesis al igual que durante el procesamiento de semen y almacenamiento, siendo este daño extremadamente perjudicial para la capacidad fecundante la célula (Fraser y Strzezek, 2007), si bien el grado de fragmentación del DNA del espermatozoide porcino es poco notorio en comparación con otras especies (Flores et al., 2008). De esta manera, el daño a la estructura del DNA en el espermatozoide ha sido asociado con bajas tasas de fertilidad y reducido tamaño de camada (Evenson et al., 1994).

El estado del DNA espermático es analizado por técnicas basadas en fluorocromos mostrando interacciones especificas y complejas con la cromatina, tales como el ensayo celular de electroforesis en gel simple (COMET; ver Enciso et al., 2011), el ensayo terminal deoxiribunucleoido transferasa mediada por dTUP marcado (TUNEL; ver Sun et al., 1997) y el test de dispersión de cromatina (SCSA o SCDt; ver Evenson y Jost, 2001; Enciso et al., 2006). Desde un punto de vista práctico, el SCSA es la prueba más utilizada

en los análisis rutinarios. El SCSA presenta dos variantes técnicas dependiendo de si el marcaje es para microscopio de luz ordinaria o para microscopio de fluorescencia. En ambos casos, la fragmentación del DNA provoca la aparición en este test de un halo periférico de dispersión alrededor de la cabeza del espermatozoide. El tamaño e intensidad de los halos observados se valora por observación directa o mediante procesamiento de las imágenes a través de un software específico. Este análisis se sistematiza dando lugar al índice de fragmentación de DNA (IFD). Respecto al valor analítico de esta prueba, se ha publicado que el IFD obtenido mediante SCSA se ha visto correlacionado con la motilidad, la integridad del acrosoma y el porcentaje de morfología normal, así como con el número de espermatozoides positivos al HOST (Pérez-Llano et al., 2006). También el IFD obtenido por SCSA se relaciona con la fertilidad y el tamaño de camada en cerdas multíparas (Evenson et al., 1994: Boe-Hansen et al., 2008), aunque otro estudios no muestran ninguna relación entre el IFD y la fertilidad en verraco (Waberski et al., 2011).

Finalmente, y centrándonos en la citometría de flujo, también se utiliza el fluorocromo naranja de acridina como prueba de evaluación del núcleo en base a la selectividad de los ácidos nucleicos, cuando emite fluorescencia verde unido a DNA no alterado (doble cadena) y fluorescencia naranja cuando se une al RNA o DNA desnaturalizado (cadena simple). En este caso, el grado de emisión del espectro naranja ha sido correlacionado con verracos infértiles (Silva y Gadella, 2006).

2.2.2.13. Marcadores de capacitación

Una causa de deterioro y, por lo tanto, de pérdida de calidad, de un eyaculado consiste en el desencadenamiento prematuro de la capacitación espermática. Por lo tanto, la evaluación de la presencia de capacitación en un eyaculado también se considera como un parámetro del análisis de calidad seminal. En estos momentos, la presencia de espermatozoides capacitados prematuramente se suele evaluar con dos técnicas de fluorescencia utilizando los fluorocromos Merocianina 540 (M540) y fluo-3- acetometoxiéster (Fluo3–AM). El M540 se adhiere a la membrana espermática y emite fluorescencia naranja cuando hay un incremento en la fluidez debido a un desorden lipídico en ella, como ocurre durante la capacitación espermática. En cambio, el Fluo3-AM interactúa con el calcio intracelular y emite fluorescencia verde. Un espermatozoide capacitado contiene niveles altos de calcio

intracelular, por lo que emitirá una gran intensidad de fluorescencia verde (Bonet et al., 2012). En ambos casos, la fluorescencia puede detectarse o bien mediante microscopía de fluorescencia o mediante citometría de flujo, de manera similar a las técnicas descritas anteriormente.

2.2.2.14. Marcadores morfológicos y moleculares de maduración espermática

Las alteraciones ocurridas durante la maduración epididimaria también son una causa frecuente de pérdida de calidad seminal. Uno de los marcadores más fáciles de observar de inmadurez epididimaria es la presencia de gotas citoplasmáticas, tanto proximales como distales. Los espermatozoides con gota citoplasmática tienen menos resistencia osmótica así como menor habilidad de unirse *in vitro* a células del epitelio oviductal (Yeste et al., 2009). También la protuberancia acrosomal en los espermatozoides inmaduros es menos desarrollada y cubre casi la totalidad de la vesícula acrosómica. Asimismo, las alteraciones secundarias como las colas enroscadas en la cabeza asociada a gota proximal también son marcadores morfológicos de la maduración incompleta del espermatozoide (Bonet et al., 2006).

Por otro lado, otros marcadores, en este caso moleculares, del proceso de maduración son algunos residuos de carbohidratos (galactosa, glucosa, manosa, N-acetilglucosamina, fructosa) presentes en las glucoproteínas de la membrana de los espermatozoides situados en diferentes regiones del epidídimo. Estos residuos se identifican con lectinas específicas. Sobre este punto, es interesante reseñar que la maduración epididimaria anormal provoca a su vez distribuciones aberrantes de estos residuos en las glucoproteínas (Bonet et al., 2012).

La fertilina es otro buen marcador de la maduración espermática epididimaria. La fertilina es una proteína localizada en la membrana plasmática apical del espermatozoide. Es sintetizada durante la espermatogénesis como precursor formado por 2 subunidades, la α (ADAM-1) y la β (ADAM-2), La fertilina está involucrada en la interacción con proteínas de membrana del ovocito durante la penetración y su localización difiere en espermatozoides inmaduros, incapaces de llevar a cabo la penetración del ovocito (Fábrega et al., 2011).

La acrosina es otro marcador molecular involucrado en la digestión de la zona pelúcida del ovocito. Es una proteasa que se sintetiza en forma de precursor, la pro-acrosina. La pro-acrosina es sintetizada durante la espermatogénesis, apareciendo en los espermatozoides inmaduros. La pro-acrosina se transforma gradualmente en acrosina durante el proceso de maduración epididimaria, empezando la transformación en la membrana acrosomal apical. En espermatozoides maduros la acrosina cubre toda la vesícula acrosomal (Bonet et al., 2012). La alteración de este patrón de distribución lleva a la incapacidad por parte del espermatozoide de llevar a cabo la digestión enzimática de la zona pelúcida del ovocito, provocando así la correspondiente falla en la fecundación (Bozzola et at., 1991).

Finalmente, existen otros marcadores moleculares que se han propuesto para la evaluación de la maduración epididimaria. Entre éstos, destacarían el mio-inositol y la L-carnitina, con un incremento en su concentración paralelo al grado de maduración epididimaria (Prueda et al., 2006).

2.2.2.15. Pruebas de unión y penetración *in vitro*

En estos momentos existen diversos ensayos *in vitro* utilizables para estimar la capacidad fecundante del espermatozoide porcino. Algunos de estos métodos están enfocados en la habilidad del espermatozoide para desarrollar funciones específicas involucradas en los procesos de fecundación. Otros, en cambio, se centran en el ensayo del reconocimiento satisfactorio entre los gametos (Rodriguez-Martinez et al., 2003). Todos ellos se basan en el hecho que el reconocimiento entre gametos, su unión y la posterior fusión son procesos altamente regulados que involucran un número de mecanismos bioquímicos en los cuales un gran número de moléculas especializadas actúan a través de una función particular. Teniendo en cuenta la complejidad de este proceso, este tipo de ensayos se enfoca en el estudio de moléculas que participan en la interacción entre gametos antes de la penetración y fecundación. Co-cultivos de espermatozoides con ovocitos o extracto oviductal como sustratos permiten la evaluación de indicadores espermáticos que validan el éxito de este proceso (Yeste et al., 2009). Las pruebas de este tipo más utilizadas son las siguientes:

1.- Prueba de unión del espermatozoide con la zona pelúcida.

El contacto inicial entre el espermatozoide y la zona pelúcida induce la reacción acrosomal. Así pues, la prueba de unión a la zona pelúcida evalúa la habilidad de reconocimiento bioquímico entre la membrana espermática y los receptores correspondientes de la zona pelúcida (Yanagimachi, 1994; Rath et al., 2005). El reconocimiento específico y la posterior unión y fusión de los gametos son necesarios antes de la penetración en el ovocito. Así, esta prueba *in vitro* actúa como un indicador de la capacidad fecundante del espermatozoide con variaciones en el número de células espermáticas que se unen a la zona pelúcida entre verracos fértiles y subfértiles (Waberski et al., 2005; Collins et al., 2008).

2.- Prueba de unión del espermatozoide a células oviductales.

Esta prueba valora la capacidad del espermatozoide de unirse al epitelio oviductal (Holt el al., 2006). La población espermática en el reservorio oviductal depende de la calidad inicial espermática de cada eyaculado, el sitio de deposición y el número de espermatozoides introducidos en el tracto genital femenino (Foxcroft et al., 2008). Esta prueba, además de evaluar la interacción del espermatozoide con el oviducto, también predice, aunque de manera débil, la fertilidad y es complemento de otras pruebas como el ensayo de penetración espermática (Wabersky et al., 2005).

3.- Prueba de penetración en el moco cervical

Dado que el moco cervical ejerce una selección cualitativa de espermatozoides que entran al cérvix (Martínez-Rodríguez et al., 2012), esta prueba ha sido propuesta como un ensayo *in vitro* para analizar la fertilidad del eyaculado. Consiste en la valoración visual de la distancia lineal cubierta por las células espermáticas en un tubo capilar con moco natural usando el número de espermatozoides acumulados en diferentes segmentos del tubo capilar como parámetro de análisis (Cox et al., 2002). Existen otras pruebas derivadas de la anterior con pajuelas plásticas transparentes. Sin embargo, el método es difícil de estandarizar dada la gran variabilidad en la calidad de moco cervical y la dificultad de obtener grandes cantidades de moco cervical homólogo. Se ha utilizado en sustitución de este, la acrilamida, la metilcelulosa y el ácido hialurónico en humanos, bovinos y ovinos,

aunque en verracos la prueba aun no está estandarizada (Tas et al., 2007; O'Hara et al., 2010)

4.- Prueba de penetración del ovocito

Esta técnica incluye dos variantes, la prueba de penetración homóloga y la de penetración heteróloga. Ambos ensayos están basados en la capacidad de penetración del espermatozoide porcino en un ovocito, ya sea porcino (prueba homóloga) o de diferente especie (prueba heteróloga). En la especie porcina, la prueba de penetración heteróloga *in vitro* utiliza preferentemente ovocitos de hámster y ha sido correlacionada positivamente con la calidad del semen (Berger y Horton, 1988).

También se ha descrito la prueba de penetración espermática en ovocitos de bovino. En este caso, la prueba requiere la preparación *in vitro* del espermatozoide y ovocito en diferentes pasos incluyendo la capacitación espermática la superovulación de la hembra, la remoción de la zona pelúcida de los ovocitos recuperados, la co-incubación de gametos y la evaluación final del porcentaje de penetración de los espermatozoides (Brown et al., 1990).

En cualquier caso, se ha reportado una alta correlación entre la tasa de penetración homologa y la fertilidad del macho utilizando ovocitos porcinos libre de zona pelúcida, discriminando así verracos fértiles y subfértiles (Martínez et al., 1993). A pesar de ello, autores como Foxcroft et al. (2008) observaron que la habilidad para identificar diferencias en fertilidad entre verracos es baja en este ensayo, lo que limita su uso práctico en el análisis seminal rutinario.

5.- Prueba de fecundación *in vitro*

El ensayo de fecundación *in vitro* FIV permite evaluar el potencial del espermatozoide para completar los cambios bioquímicos y biofísicos necesarios para lleva a cabo la capacitación, la subsiguiente reacción acrosomal, la unión espermatozoide-ovocito, la penetración y, finalmente, la descondensación de la cromatina nuclear como estimadores requeridos para una satisfactoria fecundación (Rath et al., 2005). Esta prueba generalmente usa ovocitos inmaduros colectados de ovarios frescos de cerdas jóvenes y madurados *in vitro*, si bien también puede utilizarse ovocitos inmaduros congelados (Martínez et al.,

1993: Foxcroft et al., 2008). La evaluación se hace 16-18 horas después de la fecundación *in vitro* por tinción de ovocitos con ácido acético y bajo microscopio de contrate de fase o por la tinción de Hoechst 33258 en microscopio de fluorescencia (Coy et al., 2005).

Los parámetros evaluados son el número de espermatozoides por ovocito, la tasa de penetración, el índice de poliespermia, el porcentaje de formación del pronúcleo espermático y el potencial de producción embrionaria. (Rath et al., 2005; Gil et al., 2007).

2. EFECTO DE LA CONGELACION-DESCONGELACION SOBRE EL ESPERMATOZOIDE PORCINO

El semen de verraco difiere en varios aspectos del semen de otros animales domésticos por su gran volumen fraccionado y la gran vulnerabilidad al choque frío o enfriamiento rápido, por lo cual requieren atención ya que la capacidad funcional del espermatozoide está relacionado con el potencial de fecundación (Larsson, 1978). La susceptibilidad del espermatozoide a todas las manipulaciones sufridas desde el inicio del proceso de criopreservación es muy elevada (Watson, 2000). Por ejemplo, la centrifugación tiene un efecto adverso por el efecto mecánico sobre la membrana plasmática con decremento en la motilidad (Salamon, 1973) e incremento de formación de ROS (Aitken et al., 2010). Este último punto se agrava con la eliminación del plasma seminal durante la centrifugación, ya que dicha eliminación, así como su dilución, elimina o diluye en exceso substancias antioxidantes y protectoras para el espermatozoide porcino (Roca et al., 2006). Sin embargo, hay que recordar que el aumento intracelular en los niveles de ROS en los espermatozoides porcinos durante la congelación no es un factor determinante para explicar las lesiones ligadas a la congelación (Flores et al., 2011), lo que minimiza este efecto en la especie porcina.

La resistencia a la criopreservación del semen de verraco depende de varios factores. Estos factores se pueden clasificar en 2 categorías, factores internos o fijos y factores externos. Los factores internos son todas aquellas características inherentes al espermatozoide que marcan diferencias de respuesta entre verracos y eyaculados. Los factores externos destacan la composición del diluyente, la concentración de agentes crioprotectores, las tasas de dilución, enfriamiento, equilibrio y el método concreto de congelación-descongelación. (Johnson et al., 2000; Holt et al., 2000a; Hernández et al., 2006). Seguidamente, haremos un pequeño repaso a las alteraciones más importantes ligadas a la criopreservación.

3.1. Efectos sobre la membrana plasmática

La membrana del espermatozoide se compone de varias clases de lípidos en un arreglo heterogéneo que participan en el mantenimiento de la estructura doble capa. Cada uno de

estos lípidos tiene un punto de fusión diferente. Debido a ésto, durante el enfriamiento los lípidos con el punto de fusión más alto se separan y se acumulan en forma homogénea, alterando así la estructura de la membrana (Parks., 1997). Este tipo de reorientación altera al mismo tiempo la distribución de las proteínas de membrana. En su conjunto, estos cambios provocan una gran inestabilidad estructural de la membrana espermática ligada choque frío (Drobnis et al., 1993). En este sentido, diferentes estudios muestran que el espermatozoide porcino durante la fase de enfriamiento del proceso de congelación altera la fluidez de su membrana por lo menos 2 veces (Drobnis et al., 1993) e incluso puede haber otra alteración al iniciarse la fase de congelación, alrededor de los 0 ºC (Watson., 2000). Este proceso de alteración de membrana ligada al frío es más intenso en el espermatozoide porcino que en otras especies de mamífero. Esta característica se debe a la diferente composición lipídica que presenta la membrana espermática porcina. Así, la membrana espermática porcina tiene una baja relación colesterol/fosfolípidos si la comparamos con otras especies (Watson, 2000; Cerolini et al., 2001), así como un alto contenido de fosfolípidos insaturados (Watherhouse et al., 2006; Chen y Liu, 2007). Estos últimos proporcionan a la membrana celular una alta fluidez y mejoran su permeabilidad (Alberts et al., 2008). Sin embargo, niveles altos de colesterol estabilizan la membrana espermática en otras especies como humanos, caninos y bovinos, por lo que estas especies tienen mejor respuesta la criopreservación (Brouwers et al., 2005). Por lo tanto, la diferente fluidez de membrana del espermatozoide porcino debilitan su capacidad para resistir el estrés del enfriamiento (Rodriguez-Martinez y Barth, 2007; Bailey et al., 2008; Grobfel et al., 2008; Roca et al., 2011). En este contexto, se han descrito cambios celulares que recuerdan, aunque no son iguales, a los observados durante la capacitación en la etapa de enfriamiento del espermatozoide porcino que involucran el incremento de la permeabilidad de la membrana plasmática a los iones, principalmente al calcio (White, 1993). La reorganización de la membrana plasmática y liberación de colesterol por el desorden lipídico causado por el enfriamiento provoca en última instancia alteraciones en las cascadas de señalización intracelular, especialmente en las relacionadas con los mecanismos de fosfo-defosforilación de proteínas, lo que a su vez provoca cambios en toda la funcionalidad espermática, incluyendo la alteración en los parámetros de motilidad. Así, Green y Watson. (2001) observaron que los cambios en la fluidez de la bicapa de lípidos y

en los niveles de fosforilación en residuos tirosina son las principales vías de señalización de las alteraciones funcionales observadas en el espermatozoide de verraco sometido a enfriamiento de 5 ºC y a la descongelación (Green y Watson, 2001). Efectos similares se han observado en otras especies como el toro (Cormier y Bailey, 2003) y el caballo (Thomas et al., 2006).

3.2. Cambios osmóticos ligados a la criopreservación

El efecto del enfriamiento en el daño de la membrana espermática ocasiona que la célula altere su capacidad de regular el movimiento de líquidos a través de la membrana, en gran medida debido al efecto de deshidratación durante la fase de criopreservación (Hernández et al., 2007). Como ya se ha dicho, el daño asociado al choque frío de la membrana celular es el resultado de la rápida reorganización que se producen a medida que cambian las membranas de un estado líquido a sólido (Holt, 2000a). Así, la afectación de la membrana plasmática del espermatozoide producida en primera instancia por los cambios de temperatura asociados a la criopreservación también limitan la capacidad de la célula para controlar la presión osmótica intracelular a través de los mecanismos asociados con el intercambio de iones y agua (Peña et al., 2007).

Por otro lado, la capacidad del espermatozoide de regular la entrada y salida de agua durante el proceso de congelación también depende de la adición del crioprotector y de la tasa de control del cambio de temperatura ya que altos niveles de crioprotectores y una curva de congelación baja ocasionan pérdida de enzimas responsables de la motilidad por la mayor demanda de ATP y potasio, lo que a su vez altera la actividad de los canales iónicos dependientes de potasio (Zeng et al., 2001; Thurston et al., 2003). También durante la criopreservación se produce liberación de calor específico del agua que puede aumentar la temperatura alterando la formación de cristales (Pursel y Park, 1985). En este sentido, el equilibrio en la formación de hielo dentro y fuera de la célula espermática donde aparecen los primeros cristales de hielo en el proceso de nucleación depende de la naturaleza de la solución y de la curva de congelación, ya que después de la nucleación toda el agua puede solidificarse y el calor latente de la fusión de hielo ha de disiparse rompiendo el equilibrio. Esta rotura del equilibrio produce como consecuencia un nuevo descenso de la temperatura, lo que conduce a un subenfriamiento al inicio de la congelación. Este subenfriamiento, así

como la utilización de curvas irregulares no controladas, tienen que evitarse a toda costa, ya que estos eventos determinan la respuesta celular espermática basada en los cambios lipídicos, osmóticos, de solubilización y desnaturalización de las proteínas de membrana (Watson et al., 1981; Dayong y Critser, 2000: Morris, 2007). Finalmente, la sensibilidad de la membrana durante la fase de gelificación por debajo de los 5 ºC lleva a su desestabilización y a la perdida de la selectividad que conjuntamente son el detonante de la alteración del flujo de iones en el citosol, estimulando así cambios en las cascadas funcionales intracelulares conducentes a la aparición de fenómenos parecidos a los observados durante la verdadera capacitación espermática (Green y Watson, 2001; Petrunkina et al., 2005). Estos efectos durante el proceso de criopreservación acortan la vida media del espermatozoide porcino e interfiere su respuesta en los eventos de señalización requeridos para la fecundación y sobrevivencia del espermatozoide en el oviducto (Green y Watson, 2001).

3.3. Estrés oxidativo y producción de ROS durante la criopreservación

El espermatozoide, como las demás células eucariotas, produce ROS originados por la actividad metabólica normal radicada en las mitocondrias. Así, entre el 1 al 2 % del oxígeno utilizado durante la respiración mitocondrial es convertido a ROS (Awda et al., 2009). Los sitios principales productores de ROS son la membrana plasmática y especialmente, como ya se ha dicho, las mitocondrias (Agarval et al., 2005). Los ROS incluyen al anión superóxido ($O_2^{-\bullet}$), el peróxido de hidrogeno (H_2O_2), el radical hidroxilo (OH), los diferentes óxidos de nitrógeno (NO) y el peroxinitrato (ONOO; ver Bathgate, 2011). Durante su conservación, a la temperatura que sea, el espermatozoide es sometido a peroxidación de lípidos siendo común la formación excedente de ROS. Esta formación afecta a la estructura de la membrana del espermatozoide, disminuyendo así su viabilidad (Aitken et al., 2010; Radomil et al., 2011). Por otro lado, algunas anormalidades espermáticas como las gotas citoplásmicas también se han asociado con alta peroxidación lipídica dando lugar a alteraciones estructurales de membrana (Saleh y Agarwal, 2002; Brouwers et al., 2005). La producción de ROS también puede dañar al DNA causando deleción, mutaciones y otros efectos letales (Moustafa et al., 2004). Sin embargo, en el caso del espermatozoide porcino, la producción de ROS vinculado al procedimiento de

congelación-descongelación es muy bajo (Guthrie y Welch., 2006**;** Awda et al 2009) y, de hecho, incluso llega a disminuir (Flores et al., 2009). Este efecto es opuesto al observado en otras especies, como el hombre (Álvarez y Storey, 1992), el caballo (Ball et al., 2001), el toro (Bilodeau et al., 2000) y el perro (Kim et al., 2010), mostrando así las grandes diferencias interespecíficas existentes en la funcionalidad espermática en general.

3.4. Efectos sobre las nucleoproteínas y la integridad del DNA

El proceso de criopreservación en espermatozoides porcinos parece alterar la cromatina nuclear principalmente mediante la desestabilización de la estructura de nucleoproteínas con ruptura de los enlaces disulfuro y, en mucha menor medida, mediante el aumento de la fragmentación de DNA (Balhorn, 2007: Flores et al., 2010). La fragmentación del DNA espermático puede deberse a una deficiente formación de la cromatina durante la espermatogénesis o al efecto deletéreo de la producción de ROS asociado al enfriamiento y congelación-descongelación (Sailer et al., 1995; Aitken et al., 2010). Como cabría esperar, el efecto de la criopreservación sobre la fragmentación de DNA es diferente entre especies. Así, mientras que la criopreservación aumenta claramente la fragmentación del DNA en espermatozoides de toros (Hallap et al 2005) y caballos (Baumber et al 2003), en otras como el carnero (López-Fernández et al 2010) o el verraco (Flores et al 2011) el aumento de fragmentación es mucho más moderado, si bien se ha descrito una correlación negativa entre el grado de fragmentación del DNA y la fecundación del ovocito y desarrollo temprano del embrión porcino *in vitro* (Silva y Gadella, 2006).

3.5. Efectos sobre la actividad mitocondrial

Las mitocondrias espermáticas se suelen considerar como la principal fuente de energía del espermatozoide, aportando el ATP necesario para los procesos de mantenimiento de la membrana tal como la sustentación del gradiente de Na^+/K^+ a la membrana plasmática (Silva y Gadella, 2006). Sin embargo ésta hipótesis parece no ser del todo cierta en la especie porcina. Así, en el espermatozoide porcino en condiciones basales similares a las observadas en muestras en el inicio del proceso de congelación, como mínimo un 95% del ATP producido lo es a través de la glucolisis (Marín et al., 2003). Por lo tanto, la producción de energía no parce ser la principal función de las mitocondrias en el espermatozoide porcino, aunque, curiosamente, si se inhibe la actividad ATP sintasa

mitocondrial sin afectar ni el ciclo de Krebs ni la cadena electrónica se produce una inmovilización total de las células, que no se acompaña ni con una caída de los niveles intracelulares de ATP ni con cambios en el ritmo del ciclo de Krebs ni con un descenso de la viabilidad (Ramió-Lluch et al., 2013). Sea como fuere, las mitocondrias son muy susceptibles al proceso de congelación-descongelación (Cummins et al., 1994), lo que, sin duda provocará alteraciones funcionales ligadas a procesos como el control de la motilidad y de la capacitación espermática, así como en el metabolismo del calcio, si bien son necesarios nuevos estudios para elucidar esta cuestión.

3.6. Efectos sobre los dominios de membrana y proteínas espermáticas ligadas al proceso de criopreservación

Como ya se ha mencionado anteriormente, la célula espermática es sometida a modificación de sus dominios de membrana durante la criopreservación, por lo que decrece su capacidad de fecundación (Casas y Flores, 2013). Esto es debido al hecho que los cambios en los dominios de membrana provocarán alteraciones concomitantes en la localización de determinadas proteínas presentes en estos dominios, lo que a su vez provocará la alteración en la función de dichas proteínas. Una de las proteínas más estudiadas en este aspecto es el transportador específico de glucosa GLUT 3. Antes de la congelación, el GLUT 3 está homogéneamente distribuido sobre la membrana acrosomal y la pieza principal de la cola (Casas et al., 2009). Sin embargo, después de la criopreservación su marcaje solo se observa en la zona apical del acrosoma y en la pieza principal. Esta alteración se inicia ya durante la fase de enfriamiento de 16-17 ºC a 5 ºC del protocolo de congelación, lo que alterará de manera grave la capacidad de metabolización de los azúcares y, por lo tanto, la eficiencia energética de las células.

Otras proteínas que muestran alteraciones importantes de posición durante el proceso de congelación son alguna pertenecientes a la familia de las “heat shock proteins” (HSP), tales como la HSP70/HSPA1A y la HSP90AA1 (Paasch et al., 2004; Casas et al., 2010a). Las proteínas de la familia HSP son chaperonas relacionadas con la respuesta celular al estrés térmico. Además, están involucradas en un amplio rango de procesos fisiológicos, como son la activación de la transcripción de varios genes relacionados con el estrés medio-ambiental y con el control del crecimiento y desarrollo celular (Rajopandi et al., 2000). La

expresión de las HSP en células somáticas puede ser inducida como resultado de la exposición celular a diferentes factores estresantes fisicoquímicos (Burg et al., 2007). La HSP90AA1 se localiza en la gota citoplasmática proximal y distal de los espermatozoides inmaduros que vienen de la cabeza y el cuerpo del epidídimo. Sin embargo las gotas distales de los espermatozoides obtenidos en la cola epididimaria no acumulan HSP90AA1 (Volpe et al., 2008; Bucci et al., 2010). Por lo tanto, la acumulación de esta proteína en gotas distales puede ser un marcador que indica el grado de maduración del espermatozoide. Teniendo en cuenta que la resistencia a la congelación depende del grado de maduración epididimaria, la HSP90AA1 puede usarse como marcador molecular de la congelabilidad (Casas et al., 2010a). Por otro lado, la HSP70 se localiza en la región ecuatorial de la cabeza del espermatozoide (Volpe et al., 2008) y se relaciona con los cambios parecidos a la capacitación observados durante la congelación (Choi et al., 2008). Además se ha descrito el incremento de espermadhesinas asociadas con la HSP70 en el mejoramiento de la calidad de semen congelado de verraco (Huang et al., 2011). Por lo tanto, parece claro que la actividad y/o presencia de la HSP70 está relacionada de alguna manera con la capacidad de resistencia a la congelación de los espermatozoides porcinos.

Se ha observado también la expresión de otras proteínas espermáticas aparte de las de la familia HSP durante el choque frío que parecen jugar un importante papel en la criopreservación del espermatozoide de verraco (Casas, 2010). Dos de ellas son la Bcl-2 y el citocromo C. La Bcl-2 es una proteína ubicua en el espermatozoide cuya expresión va ligada a la del citocromo C de la membrana mitocondrial (Choi et al., 2008). La expresión de ambas proteínas está relacionada con alteraciones de origen mitocondrial ligadas a un estrés ambiental que conducen a alteraciones en la composición lipídica de la membrana citoplasmática y a la integridad estructural del DNA (Saravia et al., 2007; Frazer y Strzezek, 2007). En este sentido, se han observado cambios importantes en la expresión y localización de ambas proteínas durante los procesos de capacitación y congelación que se relacionan con los anteriormente descritos cambios en la composición de membrana y la fragmentación del DNA (Choi et al., 2008). Por lo tanto, la expresión correcta de estas proteínas se relaciona con la resistencia a la congelabilidad espermática.

Otra proteína que podría utilizarse como marcadora de la congelabilidad de una muestra es la calpaína (proteasa cisteína dependiente de calcio). La calpaína es un enzima que regula la capacitación al modular la entrada de iones de calcio en el espermatozoide. La activación de esta enzima además se relaciona con cambios en los niveles intracelulares de ROS a nivel mitocondrial, lo que indica su importancia como regulador de la resistencia la congelabilidad en especies como el toro o el caballo. Sin embargo la posible relación de la calpaína con la resistencia a la congelabilidad del espermatozoide de verraco no ha sido estudiada, y parece poco probable, teniendo en cuenta la falta de formación de ROS durante la congelación en esta especie (Watson, 2000; García-Herreros et al., 2008).

Existen muchas más proteínas que podrían utilizarse como marcadores de la congelabilidad , tales como las espermadhesinas, glucoproteínas de bajo peso molecular multifuncionales que pueden actuar como receptores de colesterol (Wagner et al., 2002; Caballero et al., 2012), la prosaposina (ProSAP), glicoproteína del plasma seminal que está involucrada en la fecundación, mejorando la adhesión del esperma con el ovocito (Gadella et al., 1995) y con un mecanismo de acción relacionado indirectamente con la HSP70 (Matzner et al., 2009), diversas proteínas de control del ciclo celular como la Cdc2 y la CdK1 (Naz et al., 1993) y proteínas de reconocimiento célula-célula tales como la clusterina (Ibrahim et al., 2000), la Caspasa 9 (Martin et al., 2007; Erata et al., 2008). También todo el conjunto de proteínas quinasas y fosfatasas espermáticas podrían utilizarse como marcadores de congelabilidad, teniendo en cuenta la estrecha relación existente entre el estado funcional del espermatozoide y el estado de fosforilación de muchas de sus proteínas constituyentes. Así, proteínas quinasas como la PKA y la PKC (Tardif et al., 2001; Harrison, 2004) y fosfatasas como PPI, PP2A, PP2B, PTP1, PTP2 (Vijayaraghavan et al., 2007; Travert et al., 2009) entrarían dentro de este conjunto. Sin embargo, faltan muchos más estudios para determinar con mayor precisión el posible uso de cualquiera de estas proteínas o incluso de factores no proteicos como los óxidos de nitrógeno, como marcadores predictivos de la congelabilidad del semen porcino.

3.7. Efectos sobre la estructura de poblaciones espermáticas mótiles

En general, tras la descongelación la motilidad total del eyaculado porcino está entre el 40 y el 60 % (Casas et al., 2010a). Flores et al. (2009) observaron que durante el proceso de

criopreservación, la estructura cinética de 4 subpoblaciones espermáticas varía de acuerdo a la congelabilidad propia del eyaculado con cambios de una subpoblación a otra. Asimismo, hallazgos reportados en la cinética de las subpoblaciones espermáticas durante la congelación y descongelación en los parámetros de LIN y STR parecen ser indicativos de hiperactivación de la motilidad asociados a la presencia de daño espermático y/o cambios parecidos a la capacitación temprana (Watson, 1995; Schmidt y Kamp, 2004; Casas et al., 2009). En este sentido, los cambios de la motilidad inducida por el efecto parecido a la capacitación durante la criopreservación están relacionados con cambios específicos en el porcentaje de motilidad de cada subpoblación en el eyaculado sin perder la estructura general de cuatro subpoblaciones específicas. De esta manera, el mantenimiento de una estructura de cuatro subpoblaciones parece ser importante en el control de la función espermática (Ramio et al., 2008). También se ha observado que algunas subpoblaciones espermáticas exhiben incremento a la resistencia osmótica en comparación a otras subpoblaciones debido a cambios de membrana celular inherentes al proceso de la criopreservación que al espermatozoide (Druart et al., 2009). Otro efecto observado es la correlación entre la producción de ROS y el decremento de la motilidad asociado al número de pajuelas descongeladas en presencia de aire durante la descongelación (Casas et al., 2012).

3.8. Efecto de la descongelación

Durante la descongelación el espermatozoide es expuesto una vez más a un cambio de temperatura intenso y brusco, lo que tiene un impacto importante en su funcionalidad celular (Holt et al., 2000a). En este proceso el espermatozoide es rehidratado por el abrupto decremento en la concentración extracelular debido a la fusión de hielo. Ello hace que la sobrevivencia espermática durante la descongelación básicamente dependa de la tolerancia a la toxicidad del crioprotector y la respuesta mecánica de su membrana cuando retorna a condiciones fisiológicas (Mazur, 1984). Para mejorar estas respuestas, durante la descongelación, tasas rápidas de calentamiento son aplicadas para prevenir la recristalización, es decir, la neoformación de pequeños cristales de hielo dentro de los existentes, más grandes, que empiezan a fundirse con la subida de temperatura. De todas maneras, la buena aplicación de una tasa de calentamiento eficaz no garantiza completamente que el espermatozoide se recobre totalmente del daño que puede ocurrir

durante el proceso de criopreservación (Dayong y Critser, 2000). La tasa de descongelación está ligada a la velocidad a que el semen se congeló anteriormente (Fiser et al., 1993), así como a la forma en el semen se haya envasado. Respecto a este último punto, es interesante observar que los envases en pajuelas de 0,25 y 0,7 ml así como la mini-bolsa plástica de 0,5 ml ofrecen mejor superficie de descongelación que envases de volúmenes superiores, por lo que presentan unas tasas superiores de resistencia a la congelación-descongelación (Saravia et al., 2005). En general, un rápido ritmo de descongelación ha demostrado menor daño de la membrana espermática al choque térmico y a la formación de cristales de hielo (Thurston et al., 2003; Hernández et al., 2007). Aunque la temperatura de descongelación es una relevante fuente de variación de la motilidad (Purdy et al., 2010) también existe el efecto de variabilidad individual (Dziekonska et al., 2011). Sin embargo cuando el semen es utilizado para analíticas de laboratorio o inseminación es práctica la descongelación a 37 ^{0}C, 20 segundos (Casas et al., 2012).

4. MEJORAS EN EL PROTOCOLO DE CRIOPRESERVACIÓN

Las mejoras en los procedimientos de criopreservación desarrollados actualmente ofrecen el medio necesario para la protección eficaz durante el enfriamiento y congelación proporcionando al espermatozoide la adaptación a los cambios de temperatura (Saravia et al., 2005) y al medio a utilizar durante de la criopreservación (Spencer, 2010). Seguidamente, haremos un repaso rápido a los principales puntos de optimización que se han desarrollado en los últimos tiempos para la mejora de los resultados de congelación en el semen porcino.

4.1. Crioprotectores

Desde los años 70 existen 2 modelos desarrollados para la criopreservación del semen porcino que hoy en día, con ciertas modificaciones, se siguen implementado (Carvajal et al., 2004; Casas et al., 2010b; Roca et al., 2006a; 2011): el método americano o de Beltsville (Pursel y Johnson, 1975) y el método alemán o de Hülsenberger (Westenford et al., 1975). Las principales diferencias entre ambos métodos radican en el uso de azucares y de sistemas de envasado diferentes. El método de Beltsville adiciona glucosa y la congelación de semen se lleva a cabo en pellets con hielo carbónico mientras que el método

de Hülsenberger usa lactosa y el semen dentro de pajuelas que se congelan en vapores de N_2 líquido (Casas, 2010).

Los diluyentes de criopreservación son usados para la congelación de semen con la inclusión de crioprotectores, los cuales son substancias que minimizan el efecto del choque por frío. Los crioprotectores han sido clasificados como coligativos, que actúan como protectores osmóticos aumentando las partículas de soluto, y como no permeables, que actúan como estabilizadores de membrana (Watson et al., 1981; Storey y Storey, 1991). Los crioprotectores coligativos previenen la expansión de hielo, haciendo difícil la adición de moléculas de agua en el crecimiento de cristales de hielo. Este fenómeno evita el incremento de la concentración de soluto extracelular y la deshidratación. Los más utilizados son la lactosa, el sorbitol, la glucosa y el ficol (Casas y Flores, 2013). Estos crioprotectores son permeables al espermatozoide y presentan una baja toxicidad a las concentraciones utilizadas, de alrededor de 1 molar (Mazur et al., 1972).

En este grupo también el glicerol está incluido, siendo éste el protector coligativo de elección, aunque es toxico cuando es adicionado a una concentración superior al 6 % (Fiser y Fairful et al., 1990; Curry, 2000; Holt, 2000b; Hernández et al., 2007). Por otra parte, junto al glicerol se suele adicionar algún tipo de detergente sintético, como el "Orvus® Paste", que no sólo facilita la entrada del glicerol dentro de las células, ayudando así a disminuir su concentración y, por tanto, su toxicidad, si no que además facilita la emulsión de los lípidos de la yema de huevo a bajas temperaturas favoreciendo su efecto protector del frío (Thurston et al., 2003).

Por otro lado, los crioprotectores no permeables principalmente funcionan estabilizando las proteínas y lípidos en la membrana plasmática previniendo la entrada de hielo. Dentro de este grupo se encuentran la yema de huevo, el dimetil sulfóxido (DMSO), la albúmina sérica bovina (BSA), la polivinilpirrolidona (PVP) y el ácido etilendiaminotretraácetico (EDTA; ver Thurston et al., 2003). Estos crioprotectores no protegen al espermatozoide en ausencia de un protector coligativo pero incrementan su efectividad, evitando así la utilización de concentraciones toxicas de la sustancia coligativa (Johnson et al., 2000; Zeng et al., 2001).

Los diluyentes de congelación a base de yema de huevo contienen principalmente lipoproteínas de baja densidad con propiedades protectoras compensando la falta de colesterol de la membrana espermática y modelando la formación de cristales de hielo (Andreeva et al., 2008). El uso de fosfolípidos sintéticos de yema de huevo como los liposomas es una buena alternativa a la yema de huevo natural. Estos componentes sintéticos modifican la composición de la membrana celular por la transferencia de lípidos saturados y colesterol, moderando así la respuesta del espermatozoide a bajas temperaturas (Zeron et al., 2002; Röpke et al., 2011). Además, sobre la base de yema de huevo numerosos aditivos son usados en forma de azúcares y tampones para el control de la osmolaridad y el pH (Pursel y Park, 1985). La protección ofrecida con esta combinación limita el choque frío al modular la absorción de calcio que tiene un efecto directo sobre las crio-alteraciones (White, 1993). Así, las membranas reforzadas por fosfolípidos serán más capaces de regular el flujo de iones, proteínas y ATP que son necesarios para la viabilidad y motilidad del espermatozoide porcino (Pillet et al., 2012). Finalmente, otros suplementos, como los basados en lípidos como la leche entera y semidescremada ha sido descritos como crioprotectores del espermatozoide porcino con resultados inconsistentes, siempre inferiores a los obtenidos con yema de huevo (Salamon, 1993).

El motivo de la superioridad de la yema de huevo no es completamente conocido, aunque se supone que se basa en el hecho de poseer más lípidos similares a los que se encuentran en membrana de los espermatozoides de verraco que los de otras sustancias como la leche (Buhr et al., 2000). En general los diluyentes de criopreservación para el espermatozoide de verraco contienen un medio protector para el enfriamiento y otro medio protector para la congelación (Kikuchi et al., 1997). El primero se utiliza desde el inicio del enfriamiento de 17 ºC a 5 ºC reduciendo el efecto del enfriamiento.

El componente principal de este diluyente de enfriamiento es la yema de huevo, que representa el 20 % del volumen total del diluyente adicionado de lactosa y kanamicina como antibiótico (LEY o NSF-I). A partir de este diluyente se prepara el segundo medio para congelación (LEYGO o NSF-II) que incorpora el glicerol y el detergente sintético; éstos últimos se agregan al inicio de la fase de congelación propiamente dicha, con una

temperatura de la muestra de 5 ºC (la composición exacta de los diluyentes de enfriamiento y congelación se muestra en la Tabla 1).

COMPOSICION	LEY (NSF-I)	LEYGO (NSF-II)
Yema de huevo (%)	20	-
B-Lactosa (mM)	248	-
Kanamicina (g/L)	0,8	-
LEY (%)	-	92,5
Glicerol (%)	-	6
Equex STM® o Orvus-ES® Pasta (%)	-	1,5
PH	6-6,3	6-6,3
Osmolaridad (mOsm/Kg)	330-390	1650- 1750

Tabla 1. Composición de los diluyentes de criopreservación (Kikuchi et al., 1997; Casas et al., 2010)

4.2. Metodología de criopreservación

Metodológicamente hablando, la criopreservación de los espermatozoides de verraco es un proceso gradual en periodos de tiempo cortos hasta cubrir un tiempo total de 3-5 horas en protocolos cortos (Roca et al., 2011) y de 8-10 horas en protocolos largos (Almlid et al., 1987; Hammerstedt et al., 1990). El procesamiento de semen comienza tras la recogida de semen y del mantenimiento del eyaculado a 17 ºC en diluciones de 1:1 a 1:2 en diluyente comercial de conservación a 16-17 ºC (Holt et al., 1996; Hernández et al., 2007; Flores et al., 2009). El tiempo en el que se almacena el semen desde su obtención hasta el inicio del proceso de congelación parece ser necesario para que las membranas espermáticas se adapten a los medios de dilución (Pursel et al., 1973). En estos momentos, este período es variable según los autores, yendo desde unas 3-4 horas (Eriksson et al., 2001) hasta las 24 horas (Tamuli y Watson 1994). Tras este tiempo, las muestras se centrifugan a 16-17ºC a

600-800 xg durante 5-10 minutos, agregándose al sedimento celular obtenido el medio de enfriamiento. Una vez resuspendidas las células en este medio se procede al enfriamiento de las muestras pasando por una etapa de transición de entre 90 a 150 minutos en la que se lleva a cabo un enfriamiento gradual (0,1 ºC/min de 17 ºC a 5 ºC; ver Fiser et al., 1993: Roca et al., 2011). Una vez pasado este período se inicia la fase de congelación en sí con la adición al medio de congelación con crioprotectores. En el verraco, es indispensable que esta fase se lleve a cabo en una cámara o biocongelador programable, en la cual descenderá la temperatura de las muestras hasta los -150 ºC (Fiser y Fairfull, 1990; Flores et al, 2009: Casas et al., 2010b). Los biocongeladores permiten una precisión prácticamente exacta en las curvas de congelación, con una variación térmica inferior a 1 ºC, lo cual es absolutamente necesario en la especie porcina, al contrario de lo que ocurre en otras especies como el vacuno (Spencer, 2010).

Los actuales programas de curvas de congelación del espermatozoide porcino consisten en periodos de tiempo de 5 a 20 minutos (Thurston et al., 2003; Carbajal et al., 2004). En general hay 3 pasos principales después de agregar el crioprotector a 5 ºC, el primero comienza con una lenta diminución (5 ºC/min) desde 5 ºC hasta los – 5 ºC, permitiendo que el espermatozoide alcance el equilibrio osmótico y elimine la máxima cantidad posible de agua de la célula antes de la formación de cristales de hielo (nucleación uniforme), lo que ocurre entre los -3 ºC y los -20 ºC (Bwanga et al., 1991). A continuación, se lleva a cabo una rápida disminución de temperatura (20 a 40 ºC/min) hasta alcanzar una temperatura que varía según el protocolo utilizado entre los -130 ºC y los -150 ºC para limitar la formación de hielo intracelular evitando la deshidratación de la célula (Ericsson et al., 2002; García et al., 2010). En esta fase se procede a realizar una breve pausa justo antes de su inicio o durante el descenso rápido de la temperatura al alcanzar los, -60 ºC o los -80 ºC. Esta pausa dura entre 30 segundos a 1 minuto y sirve para liberar el calor especifico del agua y permitir la reducción del volumen espermático debido a presión osmótica así como permitir la correcta vitrificación del glicerol (Thurston et al., 2003; Carbajal et al., 2004; Zondervan et al., 2007). Una vez alcanzados los -150 ºC, se procede a almacenar las muestras en tanques de nitrógeno líquido a -196 ºC hasta su descongelación.

Este protocolo general permite variaciones para obtener mejores resultados. Así, Casas. (2010) propone una curva de congelación para el espermatozoide de verraco en biocongelador programable, que consiste en un total de 313 segundos (Fig. 9) con las siguientes fases. La primera, un descenso a velocidad de 6 ºC/minuto de 5 a – 5 ºC durante 100 segundos. Seguidamente, una rampa de descenso de 39,82 ºC/minuto de -5 a -80 ºC durante 113 segundos, manteniendo una pausa de 30 segundos al alcanzar los -80 ºC. Finalmente, un enfriamiento de -60 ºC/min entre los -80 ºC y los -150 ºC de 70 segundos. Con esta rampa, se han conseguido resultados óptimos al compararla con otras rampas más lentas o sin la pausa a los -80 ºC.

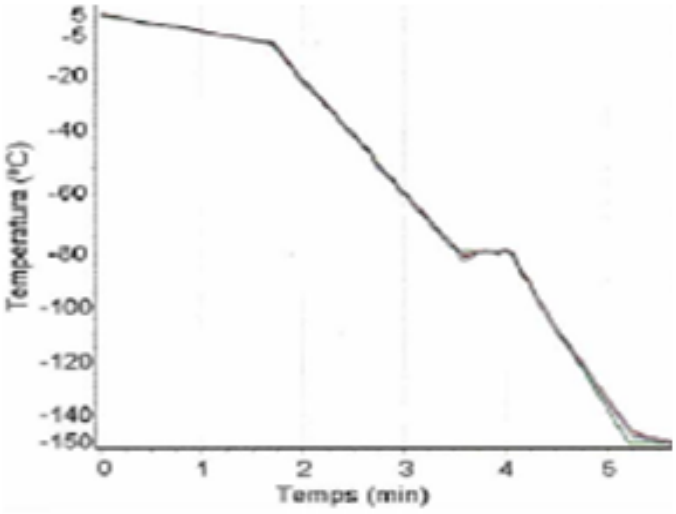


Figura 9. Curva optimizada para la congelación de semen porcino (Casas, 2010)

4. 3. Suplementos para semen congelado-descongelado

Diversos suplementos son usados para combatir el daño de la membrana celular y mejorar la calidad espermática durante el proceso de enfriamiento, congelación y descongelación. Uno de ellos son los antioxidantes, los cuales se clasifican en enzimáticos, como la superóxido dismutasa, la catalasa y la peroxidasa, y no enzimáticos, como el glutatión reducido (GSH), el alfa tocoferol (vitamina E) y el butirato de hidroxitolueno (BHT; ver Bathgate, 2011). Aunque la adición de antioxidantes a estos medios no ha sido ampliamente reportada, existen diversos artículos que describen una mejora de los

resultados obtenidos. Así, Gadea et al. (2004b) señalan efectos positivos al añadir GSH en el medio de descongelación mejorando la viabilidad, la proporción de espermatozoides sin signos de capacitación prematura, la condensación de la cromatina y mejorando la tasa de fecundación *in vitro*. También se ha observado que la adición de GSH al medio de congelación mantiene la distribución normal de los puentes disulfuro y el equilibrio redox intracelular en diversas especies (Chatterjee et al., 2001; Brewer et al., 2003; Jacob et al., 2003). Además, el GSH suplementado al medio de descongelación incrementa la motilidad de los espermatozoides reduciendo los niveles de ROS y mejorando la capacidad del espermatozoide para penetrar el ovocito (Gadea et al., 2004b). También la adición de BTH al medio de congelación ha sido reportada induciendo un alto índice de supervivencia espermática post-descongelación al reducir la peroxidación de lípidos y mejorando los resultados de fecundación *in vitro* (Roca et al., 2004). Finalmente, otro suplemento que se ha estudiado es el propio plasma seminal. Así, el plasma seminal añadido al medio de congelación y descongelación en una proporción del 10% ha demostrado ser beneficioso (Hernández et al., 2007). Este efecto parece ser debido al hecho que el plasma seminal contiene propiedades antioxidantes necesarias para el mantenimiento de la integridad de la membrana espermática (Vadnais et al., 2005) y la protección de la integridad del DNA (Frazer y Strzezek., 2007).

5. DIFERENCIAS EN LA RESISTENCIA CELULAR A LA CRIOPRESERVACION ENTRE EYACULADOS

De manera similar a lo observado en otras especies, no todos los eyaculados de verraco presentan la misma capacidad para resistir la congelación-descongelación (Medrano y Holt, 1998; Hernández et al., 2006; Leahy y Gadella, 2011)**.** A este respecto, se han informado sobre diferencias en la congelabilidad del espermatozoide entre razas (Park et al., 2002; Waterhouse et al., 2006), verracos individuales (Holt et al., 2005; Casas et al., 2009; Druart et al., 2009) e incluso entre las fracciones procedentes del mismo eyaculado (Peña et al., 2006). Basándose en estas diferencias, los verracos y sus eyaculados han sido calificados como buenos congeladores (BC) y malos congeladores (MC; ver Watson, 1995; Hernández et al**.,** 2006; Casas et al., 2009).

Las diferencias en la congelabilidad del eyaculado también afectan a su posterior capacidad fecundante. Así, se ha observado que los espermatozoides criopreservados provenientes de eyaculados BC tienen una tasa de penetración en el ovocito *in vitro* superior a la de los espermatozoides de verracos MC (Gil et al., 2005). Por lo tanto, las diferencias entre eyaculados MC y BC rebasan el ámbito estricto de la capacidad de resistencia a la congelación. El fenómeno de diferenciación entre eyaculados MC y BC parece ponerse en marcha en el momento de la eyaculación, puesto que Rath y Niemann. (1997) observaron que las diferencias de congelabilidad entre verracos se dan en muestras eyaculadas, pero no cuando los espermatozoides son colectados en la cola del epidídimo. Como ya se ha comentado, variaciones individuales en la congelación del semen han sido documentadas también en otras especies como en el caballo (Amann y Pickett, 1987) y el toro (Thomas et al., 1997). En el caso del verraco, varios estudios indican que la variabilidad en la congelación de eyaculados está relacionada con la línea genética de la que proceden los machos (Thurston et al., 2001), si bien la heredabilidad genética para esta característica de congelabilidad es baja (Safranski et al., 2011). A pesar de ello, se ha llegado a la identificación por marcadores moleculares polimórficos de fragmentos amplificados asociados a verracos BC y MC (Thurston et al., 2002). De hecho, las diferencias individuales entre verracos explican el 70 % de la variación en viabilidad y motilidad espermática a la descongelación (Roca et al., 2006a). Este fenómeno es común a otras especies como el toro (Chaveiro et al., 2006), el ratón (Songsasen y Leibo, 1997) y el perro (Yu et al 2002).

La variabilidad entre verracos en parámetros como la viabilidad espermática se ha relacionado con la pobre sostenibilidad durante el proceso de congelación de los eyaculados MC. También se ha observado que la variabilidad es mayor en presencia de alteraciones transitorias en la salud del verraco y con la manipulación inapropiada del eyaculado (Roca et al., 2006a).

Se han llevado a cabo diversos estudios con la finalidad de determinar qué factores hacen posible la distinción entre eyaculados BC y MC. En este aspecto, se ha sugerido que las diferencias entre los eyaculados BC y MC pueden deberse a una defectuosa espermatogénesis o fallas en la maduración en el epidídimo, principalmente en verracos

MC (Calvin y Bedford, 1971). Esto llevaría a la aparición de diferencias entre eyaculados MC y BC en parámetros como la morfología de cabeza del espermatozoide (Thurston et al., 2001), la longitud de las cadenas de ácidos grasos poli-insaturados de la membrana citoplasmática, los niveles de colesterol de dicha membrana (Waterhouse et al., 2006) y la composición de proteínas del plasma seminal (Holt et al., 2005; Hernández et al., 2007). Respecto a este último punto, se ha obtenido mejor la calidad de semen y fertilidad *in vivo* al retirar el plasma seminal después de la colección de semen y agregarlo en un 10 % al momento de la descongelación de semen en muestras MC (Okazaki et al 2009). El origen de las diferencias entre el plasma seminal entre muestras MC y BC no se conoce. Sin embargo Martínez-Alborcia et al. (2012) mostraron que la presencia de espermatozoides no funcionales durante la criopreservación podría estar relacionada con la diferente composición del plasma seminal entre muestras MC y BC. El efecto global de todos estos parámetros, conjuntamente con otros que se desconocen en este momento, provocan que los verracos BC se vean menos afectados por protocolos subóptimos de congelación produciendo una calidad de semen a la descongelación muy superior a la de los eyaculados MC sometidos a protocolos similares (Medrano y Holt. 1998). Esta diferencia evidencia que las modificaciones en los protocolos de congelación serán más o menos efectivas sobre la congelabilidad de los eyaculados en dependencia de si éstos son MC o BC. Sin embargo, a pesar de todo lo descrito hasta ahora, aún falta mucho por conocer sobre las causas que provocan la diferente resistencia a la congelabilidad entre distintos eyaculados.

6. LA INSEMINACIÓN ARTIFICIAL EN LA CERDA

6.1. Recuerdo morfológico-funcional de la función reproductiva en la cerda

El sistema reproductivo de la cerda está constituido por los ovarios, los oviductos, el útero, dividido en cuernos y cuerpo, el cérvix, la vagina y los genitales externos (Edströn, 2009; Galina y Valencia, 2011; ver Fig.10), estando coordinada su función por estímulos neuro-hormonales originados en el cerebro, el hipotálamo y la glándula pituitaria (Mc Donald, 1991; Hafez, 2006).

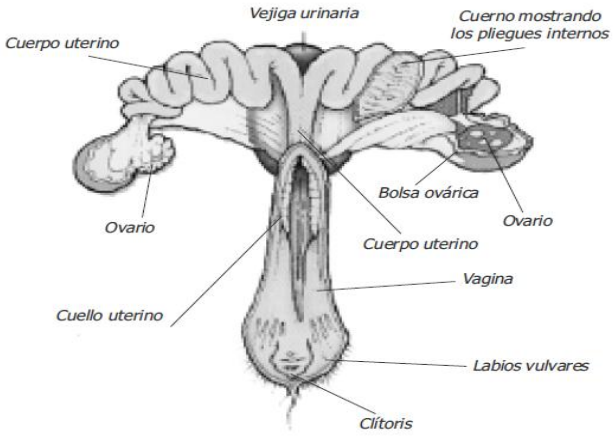


Figura 10. Presentación general del tracto reproductivo de la cerda (Galina y Valencia 2011).

Los ovarios son órganos ovalados de 3-5cm de longitud que se encuentran suspendidos en la cavidad abdominal por un doble pliegue peritoneal, meso-ovario o bolsa ovárica. Presentan forma irregular como resultado de la presencia de numerosos folículos y cuerpos lúteos prominentes en la superficie, variando su estructura y consistencia de acuerdo a la etapa del ciclo estral que se encuentre la hembra (Edströn, 2009).

La estructura del folículo (Fig. 11) está constituida por el ovocito primordial preovulatorio rodeado de las células de la teca externa e interna, las células de la granulosa, la zona pelúcida y las células del cumulus alrededor del ovocito. El folículo contiene en su interior líquido folicular, que va haciéndose más abundante cuanto más cerca de la ovulación se encuentre. Después de la ovulación la estructura del folículo cambia morfológicamente pasando a la luteinización de las células de la teca interna y granulosa que junto con el

incremento de vascularización sanguínea forman el inicio del cuerpo lúteo (Foxcroft et al., 1985; Hafez, 2006).

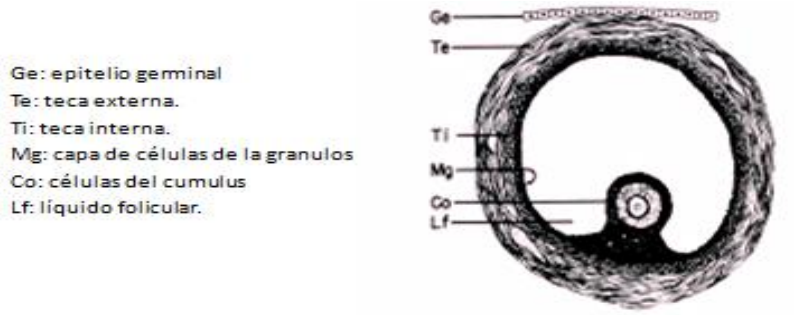


Figura 11. Estructura del folículo (Hafez 2006)

Los oviductos son conductos tubulares pares que ponen en contacto los ovarios con los cuernos del útero. Cada oviducto tiene una longitud de alrededor de 20 cm y la pared del oviducto está constituida por fuertes fibras musculares longitudinales y circulares que le permiten realizar movimientos peristálticos. Está dividido en tres partes: El infundíbulo, la ámpula y el istmo, este último segmento se une al útero a través de la unión útero-tubárica (Thibault et al., 1993; Hafez, 2006).

El útero consta de dos largos cuernos uterinos, con una longitud de alrededor de 60 a 90 cm en hembras no gestantes y de un cuerpo corto de solo 6-10 cm que aumenta según la edad y el número de partos (Barone, 1995; Galina y Valencia, 2011). En la cerda, los cuernos se sostienen dentro de la cavidad abdominal mediante un mesenterio o ligamento ancho compuesto por tejido muscular en sus paredes, que les permite ejercer intensos movimientos peristálticos en dos direcciones, durante la cubrición. Estos movimientos desplazan a su vez el esperma en dirección al oviducto, y durante el parto impulsan el feto en dirección contraria (Thibault et al., 1993; Hafez, 2006).

El cérvix es un conducto muscular fibroso localizado en la cavidad pélvica conectado a la vagina y cuerpo uterino. En la cerda es el sitio de depósito del semen durante la monta natural o durante la inseminación intra-cervical. Su longitud es de alrededor de 25 cm y tiene pliegues de la mucosa internos muy prominentes para enganchar el glande del pene (Hafez, 2006). Además, tiene la capacidad de contracción durante la gestación y el diestro así como de dilatación durante el estro y el parto (Galina y Valencia, 2011).

La vagina mide alrededor de 20-25 cm y presenta algunas variaciones entre razas. Se divide en cuerpo vaginal y vestíbulo que se encuentran separados por el himen en cerdas prepuberes, que siempre se encuentra poco desarrollado. Inmediatamente por detrás del himen desemboca la uretra proveniente de la vejiga. La vagina sirve de pasaje de los lechones al parto y salida de la orina (Thibault et al., 1993).

La vulva es la abertura genital al exterior, conteniendo en su ángulo inferior el clítoris. Los labios vulvares están poco desarrollados y fusionados en una sola estructura, formando la mucosa de los labios vulvares (Hafez, 2006). La vulva sufre cambios dependiendo del ciclo estral, la gestación y el parto. Estos cambios suelen estar relacionados con variaciones en el flujo sanguíneo de la zona, que depende de la fase del ciclo reproductivo en el que esté la cerda (Edström, 2009).

Centrándonos en aspectos más funcionales, la cerda comienza su actividad cíclica a partir de la pubertad, la cual llega entre los 180 y 200 días de edad con un peso promedio de 80 a 120 kilogramos. La entrada en pubertad depende de factores como la raza, la nutrición, el estado de salud, el medio ambiente (temperatura y fotoperíodo) y la interacción social ligada a las condiciones de crianza y manejo (Hugues y Varley, 1984; Galina y Valencia, 2011). La cerda es poliéstrica continua con ciclos regulares a través de todo el año. Así, el ciclo estral solo llega a ser interrumpido cuando la cerda esta gestante, lactante o por anestro patológico (Arthur et al., 1991; Yeste y Castillo-Marín., 2013). El ciclo estral tiene un promedio de 21 días con rango de 18 a 24 días y es definido como el tiempo del inicio de un estro al inicio del próximo, aunque desde el punto de vista fisiológico y endocrino el inicio es considerado desde el desarrollo folicular al termino de la regresión del cuerpo lúteo (Hafez, 2006). El ciclo se divide en 4 etapas: proestro (1-3 días), estro (1-3 días), metaestro (2-3 días) y diestro (13-18 días). Sin embargo, algunos autores también dividen el ciclo estral de acuerdo al estado ovárico en 2 fases: folicular (proestro más estro) y luteal (metaestro más diestro; ver Almond et al., 1994; Galina y Valencia, 2011).

Durante el proestro tiene lugar el crecimiento y maduración folicular y puede alargarse hasta 4 días principalmente en el proestro puberal. De forma paralela tiene lugar una intensa vascularización del aparato genital en su conjunto (Galina y Valencia, 2011., Yeste y Castillo-Marín., 2013). Además, la capa muscular y el endometrio del útero experimentan

cambios iniciales de proliferación celular (Hafez, 2006). Exteriormente, esta fase se caracteriza por el enrojecimiento y tumefacción de los labios vulvares, así como por la variación del comportamiento de la cerda que se vuelve inquieta, nerviosa y deseosa de montar a otras cerdas (Galina y Valencia, 2011).

El estro es el período de receptividad sexual para el macho o para la inseminación y se caracteriza por la gran producción de estrógenos (Yeste y Castillo-Marín., 2013). De acuerdo con la presentación durante la vida productiva de la cerda el estro se clasifica en tres tipos (Gordon, 1997):

1) Puberal. Es el primer estro e indica el inicio de la pubertad.

2) Posparto: Se presenta de uno a tres días después del parto y generalmente es anovulatorio.

3) Pos-destete: Ocurre de 2 a 7 días después del destete

Durante el estro las manifestaciones externas del aparato genital son importantes. Aparece un aumento del espesor de las mucosas del tracto genital, la cerda se vuelve más intranquila y emite gruñidos característicos ante la presencia del verraco (Galina y Valencia, 2011). La manifestación más marcada del celo es el reflejo de inmovilidad previo al apareamiento. Este efecto puede comprobarse también sin la presencia del macho haciendo presión sobre el lomo o flancos de la hembra (Yeste y Castillo-Marín., 2013). La ovulación ocurre de 24-40 horas del comienzo del estro, siendo en las hembras nulíparas o primerizas entre las 24 a 36 horas y en las cerdas destetadas o multíparas entre las 28 a 40 horas (Mc Donald, 1991; Hafez, 2006; Casas et al., 2010b).

El metaestro es el inicio de la fase lútea y se caracteriza por cambios en la mucosa del endometrio y la disminución de la hiperemia de las mucosa vaginal y vulvar (Dziunk, 1991).

Por último, el diestro se caracteriza por la función plena de los cuerpos lúteos que alcanzan su máximo desarrollo recibiendo un considerable aporte sanguíneo. Hacia el final del diestro ocurre la regresión del cuerpo lúteo. Asimismo el útero tiene una consistencia

edematosa y flácida, no existiendo contracciones del miometrio (Yeste y Castillo-Marín., 2013)

El control neuro-endocrino del ciclo estral se lleva a cabo por la asociación de los estímulos externos (fotoperíodo, temperatura ambiental y transporte) y por la presencia y olor del verraco. El conjunto de estos estímulos es el detonante para que los núcleos neuronales del cerebro los traduzcan en señales y conjuntamente con mecanismos internos de retroalimentación hormonal se lleve a cabo el ciclo estral en forma recurrente (Hugues y varley, 1984; Galina y Valencia, 2011). Los eventos endocrinos se inician en el hipotálamo con la secreción de GnRH, la cual estimula a la pituitaria a secretar las hormonas gonadotrópicas FSH y LH (Blödow et al., 2009). Las células de la granulosa presentan receptores específicos para la FSH mientras que los receptores para LH aparecen en estas células en el curso de la maduración del folículo. Las células de la teca interna solo presentan receptores específicos para la LH. (Hafez, 2006), siendo éstas células la principal fuente de la esteroidogénesis durante la fase final de maduración del folículo preovulatorio (Conley et al., 1994). Además, las células de la granulosa también producen inhibina, que regula la secreción de FSH a nivel de hipófisis (Brinkley, 1981). La inhibina modula la esteroidogénesis de los folículos pequeños y medianos conjuntamente con la activina y folistatina (Ciereszko et al., 2001). Cada folículo contiene un ovocito en desarrollo y las células de la granulosa responden a la secreción de estradiol (E2; ver Hafez, 2006). Además, el E2 tiene funciones importantes como la proliferación y diferenciación del endometrio, el aumento del flujo sanguíneo en genitales externos y el cambio de comportamiento de la cerda observado en momentos como el celo y la fase inductiva del parto (Mburu et al., 1998). El incremento de E2 estimula a la hipófisis para la liberación pulsátil de LH de mayor frecuencia y menor amplitud llevando a un pico necesario para la ovulación (Eiler y Nalbandov, 1977; Galina y Valencia, 2011).

Tras la ovulación, la luteinizacion rápida de las células de la granulosa y teca interna dan lugar a células luteínicas grandes y pequeñas formando el cuerpo lúteo e iniciando la secreción de progesterona (P4; ver Noguchi et al., 2010: Soede et al., 2011). El aumento gradual de la secreción de P4 causa retroalimentación negativa sobre el hipotálamo, bloqueando así la producción de GnRH e indirectamente la FSH y en menor medida a la

LH (Hafez, 2006). Otra función de P4 es la de inhibir las contracciones del miometrio y aumentar la secreción glandular del endometrio para preparar la pre-implantación de los embriones (Razdan et al., 2001).

En el caso que no haya habido concepción, la regresión del cuerpo lúteo se lleva a cabo al final del diestro por efecto de la prostaglandina F2α (PGF2α ver Singleton y Diekman, 2009). La PGF2α es secretada en el endometrio y se difunde a través de la vena uterina media y la arteria ovárica hacia el cuerpo lúteo (Yeste y Castillo-Marín., 2013). Una vez allá, provoca luteolisis, lo que a su vez lleva al decremento en los niveles de P4 y al inicio del retorno de la actividad hipotalámica liberando GnRH y comenzando el nuevo ciclo (Fig. 12; ver Mc Donald, 1991). Durante este proceso se desarrollan nuevos folículos en el ovario, produciéndose el fenómeno de las ondas foliculares, con la consiguiente selección y dominancia de folículos de más de 4 mm de diámetro en la cerda (Mirando et al., 1995).

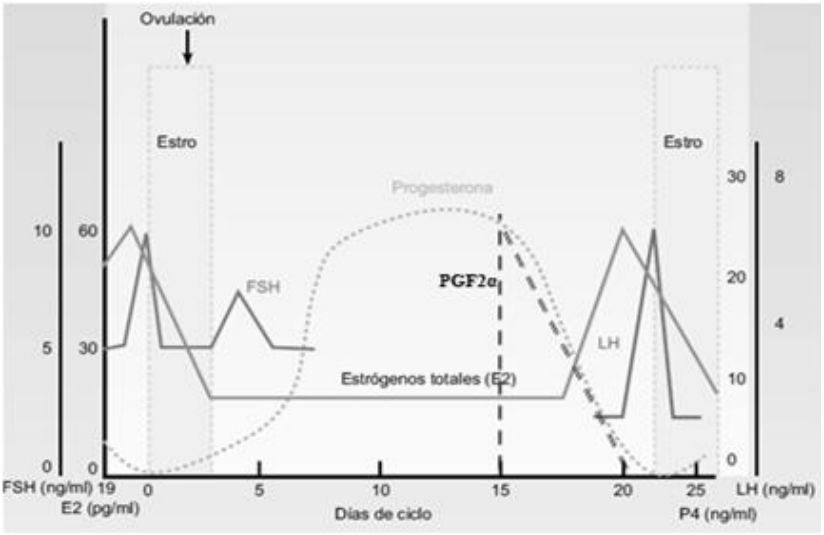


Figura 12. Patrón endocrino durante el ciclo estral de la cerda (Mc Donald, 1991).

6.2. Inseminacion artificial y tecnologías de inseminación

Actualmente la inseminación artificial (IA) tiene un crecimiento exponencial sostenido desde los años 80. De hecho, hoy en día las unidades porcinas intensivas son completamente dependientes en su eficacia. Así, en la practica el 90 % de las hembras son servidas por IA en países europeos y más del 80 % de granjas porcinas en USA y Canadá así mismo el 50 % en el resto del mundo (Gerrits et al., 2005; Vyt et al., 2007; Lowe y Gereffi, 2008; Reporlinker, 2011). Los resultados de la IA en gran medida dependen de la calidad del semen y de los procedimientos de inseminación. En la práctica, el semen fresco diluido es el más usado a nivel producción intensiva (Rodríguez-Gil y Estrada, 2013).

El semen es obtenido de verracos de granjas o centros especializados en IA ofreciendo diversidad de razas y líneas genéticas para cubrir los objetivos de producción de las granjas por la difusión rápida del progreso genético (Knox, 2011; Didion et al., 2013). La IA es un método reproductivo de bajo coste comparado con el apareamiento natural. Este hecho es debido a la disminución del número de verracos con ahorro de espacio y de coste de su mantenimiento. Otras ventajas de la IA son el control de la calidad espermática de los sementales, ya que éstos están sujetos a múltiples efectos ambientales y de manejo. También se reduce el riesgo de transmisión de enfermedades por el control de la entrada de animales portadores de enfermedades del exterior, facilitando así la implementación de medidas de bioseguridad en los centros de recogida de semen (Glossop, 2000; Flowers, 2002; Gadea et al., 2004a; Martínez et al., 2005; Pallas, 2006; Maes et al., 2008).

El correcto momento de la inseminación requiere la detección adecuada del estro a intervalos regulares, ya que el verraco promueve el incremento de la frecuencia de contracciones uterinas y el comportamiento del estro (Langendijk et al., 2006). Sin embargo, la importancia del estímulo de presencia del verraco durante la IA es relativa, ya que la fertilidad es similar sin la presencia de verraco. Este hecho indica la existencia de mecanismos como la estimulación del catéter durante y después de la inseminación así como la presencia del semen en el cérvix y útero, que sustituyen de manera eficaz el efecto estimulador del verraco (Rodríguez-Gil y Estrada, 2013). Estos mecanismos parecen tener una base neuro-endocrina parecida a la que ejerce el feto durante el inicio del parto pero

con contracciones ascendentes que ayudan al espermatozoide llegar a la unión útero-tubárica y acelerar al mismo tiempo el proceso de ovulación (Ludmir y Sehdev, 2000). De manera complementaria, la prueba de presión en el dorso y flancos es otra herramienta básica durante la detección del estro en la hembra (Hafez, 2006; Casas et al., 2010b). La IA debe ser realizada lo más cerca de la ovulación si consideramos que en la cerda la ovulación es espontánea y se lleva a cabo en el último tercio del estro. Durante la aplicación de IA es recomendable proporcionar la primera dosis durante las primeras 24 horas detrás el inicio de la detección del estro e inseminar otra vez a las 12-18 horas de la primera inseminación (Roca et al., 2006b). Este protocolo lleva a índices aceptables de fertilidad y prolificidad con uso de dosis frescas diluidas. Sin embargo, con semen congelado es recomendable la doble inseminación a 32 y 40 horas después de la aparición del estro (Waberski et al., 1994; Soede et al., 1995; Althouse, 1997; Bolarin et al. 2006) Estas diferencias han hecho que se lleven a cabo estrategias de manejo reproductivo durante la realización de la IA, como serían, por ejemplo, el destete de hembras en grupo con buena condición corporal y sin patologías durante la lactancia, la sincronización del estro con hormonas al destete, uso de ultrasonografía ovárica o la utilización de dosis seminales heterólogas (Martínez et al., 2005; Roberts y Bilkei, 2005; Hafez, 2006; Roca et al., 2006b; Galina y Valencia, 2011)

Metodológicamente hablando, la IA en porcinos se lleva a cabo básicamente con semen fresco diluido o semen congelado-descongelado (Roca et al 2006b; Rodríguez-Gil y Estrada 2013). La IA con semen fresco diluido se basa en el uso de diluyentes después de la colección. Estos diluyentes mejoran la conservación del espermatozoide aportando protectores y moduladores del metabolismo espermático. Además, la adición de antibióticos al diluyente ayuda a inhibir el desarrollo de microorganismos (Crabo et al., 1972; Gadea, 2003). Dependiendo del mantenimiento de la fertilidad del espermatozoide, los diluyentes se han clasificado de corta duración, en los que el semen se conserva perfectamente de 3 a 5 días y de larga duración que garantizan una conservación seminal de hasta 15 días (Gottardi et al., 1980; Martin–Rillo, 1984; Gadea, 2003). La fertilidad y el tamaño de camada obtenido con semen fresco diluido ha sido igual o incluso mejor que con servicio de apareamiento natural (Martínez et al., 2002; Bailey et al., 2008; Casas et al., 2010b; Roca et al., 2011). El uso de la IA con semen congelado-descongelado está

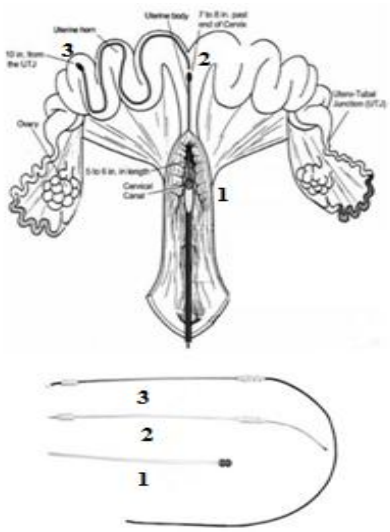
restringido debido a varios factores. Por un lado, el decremento en la fertilidad y prolificidad cuando se compara con el uso del semen diluido refrigerado. Por otro lado, el coste de la dosis y del personal capacitado para su aplicación es mucho mayor que con el semen refrigerado. Por estas razones el protocolo de IA debe ser adoptado de manera específica en cada granja de acuerdo al manejo reproductivo de las cerdas (Roca et al., 2006b). Sin embargo, el decremento de la fertilidad y el tamaño de camada con el semen congelado-descongelado cuando se compara con el semen fresco diluido es considerado el principal obstáculo para su uso extensivo como herramienta reproductiva. De hecho, como ya se ha descrito anteriormente, el espermatozoide porcino es particularmente sensible a la criopreservación y, de media, solo el 40 a 50% de los espermatozoides sobreviven a este proceso (Crabo, 1990; De Leeuw et al., 1990; Holt, 2000a). A pesar de todas las dificultades, hoy en día la utilización de semen criopreservado para granjas de multiplicación genética es la mejor alternativa para la difusión constante del aporte de material genético a granjas comerciales (Rodríguez-Gil y Estrada, 2013).

Actualmente existen 3 principales métodos de IA (Fig.13) que han sido llevados a cabo de acuerdo al sitio de depósito del semen: intra-cervical o convencional (Intra-CIA) (Hafez 2006), post-cervical (post-CIA) (Gil et al., 2000: 2006) e intra-uterina profunda (P-IA) (Bathgate et al., 2005). Todas estas técnicas deben garantizar una concentración mínima de 50 millones de espermatozoides en la unión útero-tubárica para mantener buenas tasas de fertilidad y tamaño de camada (Rath et al., 2009).

En la Intra-CIA el catéter convencional es insertado al final del cérvix o a la entrada del cuerpo uterino. Ésta es una técnica muy aplicada a nivel campo por su facilidad y bajo coste, si bien uno de sus inconvenientes es el reflujo de semen. Generalmente, una concentración de 1,5–3 x 10^9 espermatozoides en un volumen de 80-100 mL proporciona los mejores resultados con semen fresco diluido (Almond et al., 1994; Foote, 2002; Hafez 2006; Roca et al., 2006b). También se ha aplicado semen congelado-descongelado por esta vía de inseminación con resultados variables. Roca et al., (2011) reportan el 85,6 % de fertilidad a parto y un tamaño de camada de 12,6 lechones. Didion et al. (2013) encontraron el 78.8 % de fertilidad y 12.5 lechones y, Thilmant. (2009) indica una tasa de fertilidad de solo 60% y 10 lechones nacidos totales

En la post-CIA el semen es introducido directamente en el cuerpo del útero (Gil, 2000: 2006; Watson y Behan, 2002). Con esta técnica se reduce considerablemente el reflujo de semen y por lo tanto requiere menor cantidad de espermatozoides en un volumen reducido. Aunque esta técnica se aplica para semen fresco diluido es recomendada también para la inseminación con semen congelado-descongelado y semen sexado (Gil, 2006; Roca et al., 2011). La concentración espermática en semen fresco diluido es de 0,5-1 x 10^9 en un volumen de 15 a 50 mL (Rozenbon et al., 2004; Gil, 2006; Roca, 2006b) y de 2-3,5 x 10^9 espermatozoides en un volumen de 50-60 mL para semen congelado-descongelado (Rath, 2002; Casas et al., 2010b). Los resultados de fertilidad y prolificidad encontrados son alrededor del 85 a 90 % y 10-14 lechones por camada con semen fresco diluido (Lewis, 2002; Echegaray et al., 2003; Roberts y Bilkey, 2005; Gil et al., 2006) y para el caso de semen congelado-descongelado de 61-90 % fertilidad y 9,3-12 lechones nacidos (Eriksson et al., 2002; Fraser et al., 2007; Casas et al., 2010b; Roca et al., 2011).

En la P-IA el semen es depositado en el segmento proximal de un cuerno uterino alrededor de 25 cm de la unión útero-tubárica. Este método admite una reducción de 5 a 20 veces el número de espermatozoides (Vázquez et al., 2008). Además, minimiza el reflujo de semen y el efecto de los leucocitos polimorfonucleares, responsables de la muerte del 60% de los espermatozoides. Sin embargo, su utilización requiere personal capacitado y con esta técnica es de esperar que se lleve a cabo la fertilización unilateral, lo que puede ocasionar un decremento en la prolificidad de la hembra (Rodríguez-Gil y Estrada, 2013). Para semen fresco diluido, congelado-descongelado y semen sexado la concentración espermática varía desde 0,15 a 0,6 x 10^9 espermatozoides en un volumen de 7.5 a 20 mL, los resultados en fertilidad y tamaño de camada son de 80-90 % y de 9 a 12 lechones, respectivamente (Lewis et al., 2002; Roca et al., 2003; Vázquez et al., 2008).



1. Intrauterina convencional (Intra-CIA)

2. Post-Cervical (post-CIA)

3. Profunda (P-IA)

Figura 13. Representación esquemática del sitio y catéter utilizado de acuerdo al tipo de inseminación en la cerda (Casas, 2010)

OBJETIVOS

El objetivo general de este estudio fue valorar los efectos del glutatión reducido y el clorhidrato de procaína en la resistencia a la criopreservación de semen porcino a nivel de la estabilidad nuclear y su efecto en la fertilidad *in vivo*. Específicamente, los aspectos estudiados fueron los siguientes:

I. Evaluación del efecto de la adición de sustancias protectoras de los puentes disulfuro, como el glutatión reducido y el clorhidrato de procaína en el medio de congelación sobre la calidad del semen de verraco (Artículo 1).

II. Determinación de la posible relación entre los niveles de residuos de cisteína libres como un indicador de la integridad de los enlaces disulfuro entre nucleoproteínas y la congelabilidad de eyaculados de verraco (Artículo 2).

III. Determinar el efector del mejora del glutatión reducido en la criotolerancia del espermatozoide de acuerdo con la congelabilidad propia del eyaculado porcino (Artículo 3)

IV. Determinación de la posible relación entre los mecanismos de modulación de la función espermática en base al estado de fosforilación en residuos serina de diversas proteínas, el tiempo de conservación en diluyente comercial y la tolerancia a la criopreservación del espermatozoide porcino (Artículo 4).

V. Valoración del efecto del glutatión reducido sobre la capacidad de fecundación *in vivo* en cerdas inseminadas con semen congelado-descongelado (Artículo 5).

RESULTADOS

ARTICULO 1

Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze–thawing by stabilising disulfide bonds.

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Abstract.

One important change the head of boar spermatozoa during freeze–thawing is the destabilisation of its nucleoprotein structure due to a disruption of disulfide bonds. With the aim of better understanding these changes in frozen–thawed spermatozoa, two agents, namely reduced glutathione (GSH) and procaine hydrochloride (ProHCl), were added at different concentrations to the freezing media at different concentrations and combinations over the range 1–2 mM. Then, 30 and 240 min after thawing, cysteine-free residue levels of boar sperm nucleoproteins, DNA fragmentation and other sperm functional parameters were evaluated. Both GSH and ProHCL, at final concentrations of 2 mM, induced a significant (P <0.05) increase in the number of non-disrupted sperm head disulfide bonds 30 and 240 min after thawing compared with the frozen–thawed control. This effect was accompanied by a significant (P<0.05) decrease in DNA fragmentation 240 min after thawing. Concomitantly, 1 and 2mM GSH, but not ProHCL at any of the concentrations tested, partially counteracted the detrimental effects caused by freeze–thawing on sperm peroxide levels, motility patterns and plasma membrane integrity. In conclusion, the results show that both GSH and ProHCL have a stabilising effect on the nucleoprotein structure of frozen–thawed spermatozoa, although only GSH exerts an appreciable effect on sperm viability.

Additional keywords: sperm cryopreservation.

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Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze–thawing by stabilising disulfide bonds

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Introduction

Currently, the cryopreservation of boar spermatozoa in liquid nitrogen is the most efficient method for storing sperm samples for a long period of time, but this procedure induces a wide variety of cell alterations that can reduce sperm fertilising ability (for a review, see Watson 2000).

One of these changes concerns sperm chromatin. In some species, like humans and horses, this damage is related to increased DNA fragmentation immediately after thawing (Baumber *et al.* 2003; Silva and Gadella 2006), whereas in other species, such as the ovine, DNA fragmentation appears 2–6 h after incubation of frozen–thawed spermatozoa at 37°C (López-Fernández *et al.* 2010).

In boars, cryopreservation of the spermatozoa destabilises nucleoprotein structure, disrupting the disulfide bonds of sperm nucleoproteins (Flores *et al.* 2011). It is worth noting that cysteine residues of the nucleoproteins form inter- and intra-protamine cross-links via the formation of disulfide bonds (Fuentes-Mascorro *et al.* 2000) and these bonds are one of the most important stabilising mechanisms of sperm chromatin (Brewer *et al.* 2003). Conversely, there are inconsistent findings in the literature regarding the effects of cryopreservation on DNA fragmentation. Thus, some authors have observed that cryopreservation increases sperm DNA fragmentation (Fraser and Strezeżek 2005), whereas others have not (Hernández *et al.* 2006; Flores *et al.* 2008, 2011).

Because the integrity of sperm chromatin plays a critical role at the time of fertilisation (Didion *et al.* 2009; Oliva 2006; Tsakmakidis *et al.* 2010), the addition of agents to protect the disulfide bonds could counteract the destabilisation of sperm nucleoprotein structure linked to freeze–thawing procedures. Thus, the aim of the present study was to determine whether two different agents that can protect disulfide bonds, namely reduced glutathione (GSH) tripeptide and procaine hydrochloride (ProHCl), were able to protect boar sperm chromatin from the damage induced by cryopreservation.

The antioxidant GSH is the most abundant thiol in cells and, among other functions, is vital for the maintenance of the

intracellular redox balance (for a review, see Jacob *et al.* 2003). Although sperm cryopreservation induces changes in the quantity and distribution of sulfhydryl groups in sperm membrane proteins, the addition of GSH to freezing media has been reported to maintain a normal distribution of these sulfhydryl groups (Chatterjee *et al.* 2001). Furthermore, the addition of GSH to freeze–thawing media has been reported to increase sperm motility, reduce levels of reactive oxygen species (ROS) and increase the ability of spermatozoa to penetrate the oocyte (Gadea *et al.* 2004, 2005, 2011) for spermatozoa from the boar and other mammalian species.

The second agent evaluated in the present study, ProHCl, also protects disulfide bonds (Zhang *et al.* 1992) and exhibits antioxidant activity (Lee *et al.* 2010). It is a well-known local anaesthetic that increases the antitumoural activity of cisplatin (Fenoglio *et al.* 2002) and induces sperm capacitation and hyperactivation in some species (i.e. bull, stallion and guinea-pig; Mújica *et al.* 1994; Márquez and Suárez 2004; McPartlin *et al.* 2009).

Thus, the main aim of the present study was to determine whether the addition of GSH and/or ProHCl to freezing media could protect boar sperm chromatin from the damage induced by cryopreservation without affecting other functional parameters. To this end, different concentrations and combinations of these two agents were added to the freezing extenders used in the present study, namely one containing lactose and egg yolk (LEY) and another (LEYGO) containing LEY with 6% glycerol and 1.5% Orvus ES Paste (OEP; Equex STM; Nova Chemical Sales, Scituate, MA, USA). These two extenders are the most commonly used extenders for the cryopreservation of boar spermatozoa according to the Westendorf method and its modifications (Westendorf *et al.* 1975; Casas *et al.* 2009). After cryopreservation of boar spermatozoa, we assessed the number of free cysteine residues in sperm nucleoproteins (as a direct indication of disulfide bond levels), sperm DNA fragmentation and several functional parameters, such as computer-assisted motility analysis (CASA), ROS and plasma membrane integrity.

Materials and methods

Sperm samples

The experimental protocol was designed according to the guidelines established by the Animal Welfare Directive of the Autonomous Government of Catalonia (Spain) and the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Spain).

Twenty ejaculates from different healthy and adult boars (ages range 18 months–3 years) were used in the present study. Each ejaculate came from a different boar. Boars were housed in climate-controlled buildings, fed an adjusted diet (2.3 kg day^{-1}) consisting of basal diet plus 1% premix for boars (P174N; TecnoVit, Tarragona, Spain) and were provided with water *ad libitum*.

Ejaculates were collected twice a week by the gloved-hand technique with an interval of at least 3 days between collections. After removing the gelatinous fraction by filtration through gauze, the total volume of the sperm-rich fraction was diluted 1 : 5 (v/v) in a long-term extender (Duragen; Magapor, Zaragoza, Spain). These diluted sperm-rich fractions were transported within to the laboratory within 4 h of extraction in an insulated container before being stored at 17°C for 24 h. The quality of the sperm samples was then evaluated to confirm that they satisfied the quality standard (i.e. total sperm motility >80%, morphologically normal spermatozoa; sperm viability >85%; see Casas *et al.* 2009). Because the quality of the 20 ejaculates used in the present study was over the set thresholds, they were frozen according to the experimental design described below.

Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method adapted by Casas *et al.* (2009). All ejaculates diluted in long-term extender were centrifuged at 400*g* for 5 min at 17°C. The pellets were then resuspended in 3–4 mL of the remaining supernatant and diluted to a concentration of 1.5×10^9 spermatozoa mL^{-1} in LEY using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). All spermatozoa diluted in LEY were then cooled down to 5°C for 150 min before being subsequently diluted to 1×10^9 spermatozoa mL^{-1} in LEYGO. The final concentration of glycerol and OEP in LEYGO was 2% and 0.5%, respectively. Spermatozoa were finally packed into 0.5-mL plastic straws (Minitub Ibérica, Tarragona, Spain) and transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica). The freezing program (SY-LABORATORY software; Minitub Ibérica) consisted of 313 s of cooling at: -6°C min^{-1} from 5°C to -5°C for 100 s; -39.82°C min^{-1} from -5°C to -80°C for 113 s; 30 s at -80°C; and -60°C min^{-1} from -80°C to -150°C for 70 s. The straws were then plunged into liquid nitrogen (-196°C) for further storage.

After at least 2 months storage in liquid nitrogen, four straws per ejaculate and treatment were thawed and diluted with three volumes of warmed Beltsville Thawing Solution (BTS) at 37°C (at a final dilution of 1 : 4). To thaw the samples, each straw was shaken for 20 s in a 37°C waterbath.

Experimental design

Each ejaculate was split into 10 fractions consisting of two controls (extended control and frozen–thawed control [FT-C]), and eight treatments in which both freezing media (LEY and LEYGO) were supplemented with different combinations of GSH and ProHCl. The extended control, diluted in a long-term extender, was incubated at 37°C for 30 or 240 min before levels of free cysteine radicals and sperm DNA fragmentation were determined, and sperm motility and other functional parameters were assessed by flow cytometry.

The remaining nine fractions were used in the cryopreservation study. As mentioned above, the eight treatments consisted of supplementation with both LEY and LEYGO cryopreservation extenders with reduced L-glutathione ($C_{10}H_{17}N_3O_6S$; Sigma-Aldrich, St Louis, MO, USA) and/or ProHCl ($C_{13}H_{20}N_2O_2 \cdot HCl$; Fluka; Sigma-Aldrich) and the following concentrations and combinations of test drugs: 1 mM GSH (G1); 2 mM GSH (G2); 1 mM ProHCl (P1); 2 mM ProHCl (P2); 1 mM GSH + 1 mM ProHCl (G1P1); 2 mM GSH + 1 mM ProHCl

(G2P1); 1 mM GSH + 2 mM ProHCl (G1P2); and 2 mM GSH + 2 mM ProHCl (G2P2).

Samples were cryopreserved and stored in liquid nitrogen at −196°C for at least 2 months, for methodological purposes only. After thawing, samples were incubated for 30 or 240 min at 37°C before evaluation of sperm functional parameters and levels of free cysteine radicals and sperm DNA fragmentation. Thus, two time-points (30 and 240 min) were chosen to evaluate spermatozoa after freeze–thawing, the last being set to ensure the survival of frozen–thawed spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses (Casas *et al.* 2010).

The experiments were replicated 20 times, using 20 different ejaculates, each from a different boar.

Determination of free cysteine radicals in the sperm head

The determination of free cysteine radicals in sperm nucleoproteins was performed according to the protocol adapted to boar spermatozoa and described by Flores *et al.* (2011). Briefly, samples were centrifuged at 600*g* for 20 min at 17°C and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1 mM benzamidine, 10 μg mL^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). The homogenates were then centrifuged at 850*g* for 20 min at 4°C. Both the supernatant and the upper layer of the pellet were discarded, and the lower layer of the pellet was resuspended in 500 μL phosphate-buffered saline (PBS). The purity of this separation was determined by observation under a phase contrast microscope (Zeiss Primo Star; Carl Zeiss, Jena, Germany) at ×40 magnifications (Zeiss Plan-Achromat 40×/0.65; Carl Zeiss). The purity of the samples is given as the percentage of loose heads compared with the presence of whole, non-fractioned spermatozoa and separated tails in each sample. In all cases, the mean purity was >95% loose heads compared with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separation of the heads from their respective mid-pieces.

Levels of free cysteine radicals in sperm nucleoproteins were determined using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulfide; Sigma, St Louis, MO, USA), as described by Brocklehurst *et al.* (1979). Briefly, 10-μL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 μL of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine and the mixture was incubated at 37°C for 1 h. Then, levels of free cysteine radicals were determined using spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalised against the total protein content of the samples, determined in parallel by the Bradford method (Bradford 1976) using a commercially available kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules, CA, USA).

Sperm chromatin dispersion test

We assessed DNA fragmentation in the present study using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm-Halomax-Sus for fluorescence microscopy; ChromaCell, Madrid, Spain) according to the manufacturer's instructions. This test is based on the different responses exhibited by intact and fragmented DNA a deproteinisation treatment, and previous studies have reported that the results obtained using the SCDt are strongly correlated with those obtained using other tests, such as the neutral comet assay (Enciso *et al.* 2006).

Briefly, the lysis buffer included in the kit was incubated to 22°C and vials containing low-melting agarose were heated at 100°C for 5 min in a waterbath. Vials were then left in another waterbath at 37°C for 5 min to equilibrate the agarose temperature. Then, 25 μL of each sperm sample (at a final concentration of 10^7 spermatozoa mL^{-1}) was added to a vial and mixed thoroughly. One drop of the 25-μL solution containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid the formation of air bubbles.

Slides were placed on a cooled plate within a fridge and left at 4°C for 5 min. The coverslip was then removed and 50 μL lysis solution was added to each slide. Slides were then incubated at 22°C for 5 min before being washing for 5 min with MilliQ water. The slides were subsequently dehydrated by three steps of 2 min each with ethanol at 70%, 90% and 100%. Finally, sperm samples were stained with propidium iodide (PI; 2.5 μg mL^{-1}) and mounted in DABCO anti-fading medium (Sigma-Aldrich, St Louis, MO, USA). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at ×100 magnification.

Three counts of 250 spermatozoa each using three different slides were carried out per sample, prior to calculating the corresponding mean ± s.e.m. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only a small halo.

Flow cytometric analyses

Flow cytometry analyses were performed according to the recommendations of the International Society for Advancement of Cytometry (ISAC), as described previously (Lee *et al.* 2008). These analyses were conducted to evaluate certain parameters of sperm function, namely sperm viability and membrane permeability, acrosome integrity, disordering of membrane lipids, and ROS, in all treatment groups. After either 30 or 240 min incubation at 37°C after thawing, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa mL^{-1} in a final volume of 0.5 mL. The spermatozoa were then stained with the appropriate combinations of fluorochromes according to the protocols described in Annex A available as Supplementary Material to this paper (i.e. SYBR-14/PI, YO-PRO-1/PI, peanut agglutinin (PNA)-fluorescein isothiocyanate [FITC]/PI, M540/YO-PRO-1, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DFCDA)/PI, hydroethidine (HE)/YO-PRO-1, PI after hypotonic treatment to correct raw data).

Samples were evaluated using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA). This instrument, which had not been altered from the original configuration

provided by the manufacturer (see http://www.beckmancoulter.com, accessed 5 July 2012), was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter and volume was measured directly using a Cell Laboratory Quanta SC cytometer (Beckman Coulter) and the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. Thus, in this system, forward scatter (FS) is replaced by electronic volume (EV). The EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning the beads in Channel 200 on the volume scale.

The optical filters used were also the original ones supplied (FL1, FL2 and FL3). The optical characteristics for these filters were as follows: for FL1 (green fluorescence), Dichroic/Splitter, dichroic longpass (DRLP) 550 nm, band pass (BP) filter 525 nm, detection width 505–545 nm; for FL2 (orange fluorescence), DRLP 600 nm, BP filter 575 nm, detection width: 560–590 nm; and for FL3 (red fluorescence), long pass (LP) filter 670 nm. Signals were amplified logarithmically and photomultiplier settings were adjusted to particular staining methods. The FL1 filter was used to detect green fluorescence (SYBR14, YO-PRO-1, PNA-FITC and dichlorfluorescein-positive (DCF^+)), whereas the FL3 filter was used to detect PI and ethidium-positive (E^+).

Sheath flow rate was set at 4.17 μL min^{-1} in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE vs SS dot plots) for a minimum of 10 000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particles with a diameter <7 μm) and cell aggregates (particles with a diameter >12 μm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described in Annex A, compensation was used to minimise spill-over of green fluorescence into the red channel.

Information on the events was collected in list-mode data files (.LMD). These files were then analysed using Cell Laboratory Quanta SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 *v.* FL3) and to analyse the cytometric histograms. Data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina and Harrison (2010) and Petrunkina *et al.* (2010). (See Annex A for more detailed information regarding the protocol used.) Each assessment for each sample and parameter was repeated three times in independent tubes before calculation of the mean ± s.e.m.

Sperm motility

Sperm motility was analysed using a commercially available CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain) on 15-μL sperm samples placed in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Total and progressive motility, together with other kinetic parameters, were recorded. A more detailed description is available in Annex B available as Supplementary Material to this paper.

Statistical analyses

The present study was developed with 20 ejaculates from 20 different boars. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and data are presented as the mean ± s.e.m. Data obtained from the analysis of all sperm parameters were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. When necessary, data were transformed using arcsine square root before a generalised estimating equation (GEE), an extension of generalised linear model (GLM) for repeated-measures, was performed. Characteristics of the GEE were normal distribution and identity link function. The inter-subject factor was treatment, with an intrasubject factor of incubation time after thawing (i.e. 30 or 240 min). In all cases, each functional parameter was the dependent variable and multiple post hoc comparisons were calculated using Sidak's test.

When transformation did not result in normal data distribution (i.e. geometric mean of fluorescence intensity (GMFI) of viable spermatozoa with a high H_2O_2 content, GMFI of DCF^+-stained spermatozoa, GMFI of viable spermatozoa with a high $\cdot O_2^-$ content, GMFI of E^+-stained spermatozoa, and in the case of three kinetic parameters, namely straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP)), non-parametric procedures were used with raw data. Friedman's test was performed as a non-parametric alternative to the GEE, and the Wilcoxon matched-pairs test was used to evaluate differences among treatments, as well as the effects of thawing time.

In all statistical analyses, the minimal level of significance was set at $P < 0.05$.

Results

Effects of GSH and ProHCl on levels of free cysteine radicals in sperm nucleoproteins after freeze–thawing

Sperm cryopreservation significantly increased ($P < 0.001$) levels of free cysteine radicals, from 3.11 ± 0.32 nmol μg^{-1} protein in extended refrigerated samples to 6.92 ± 0.55 nmol μg^{-1} protein in FT-C after 30 min incubation after thawing, and from 3.29 ± 0.34 nmol μg^{-1} protein in the extended control to 8.41 ± 0.63 nmol μg^{-1} protein in FT-C after 240 min incubation after thawing (Fig. 1).

Both GSH and ProHCl counteracted the increase in free cysteine radicals due to the freeze–thawing protocols. This counteraction was dependent on the concentration of both GSH and ProHCl, the incubation time (i.e. 30 or 240 min at 37°C) after thawing and even on the combined effect of concentration × thawing time ($P < 0.01$ for all factors). In five of the treatments tested (G2, P2, G1P2, G2P1 and G2P2), levels of free cysteine radicals after 30 min thawing were similar to those observed in the extended control. After 240 min incubation after thawing, the addition of 2 mM GSH + 2 mM ProHCl (G2P2) completely abolished the effects of freeze–thawing on levels of free cysteine radicals. In contrast, for all other

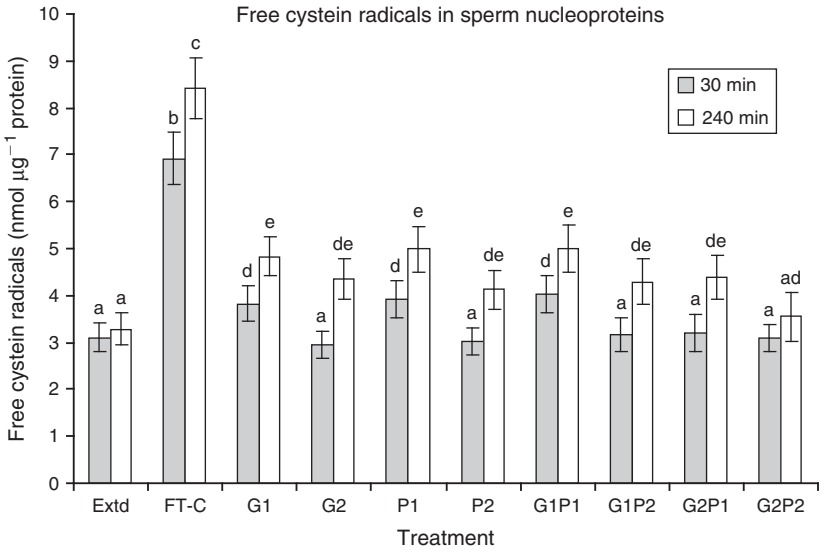

Fig. 1. Free cysteine radicals in sperm head proteins in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing. Extd, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl. Data are the mean ± s.e.m. Columns with different superscript letters differ significantly ($P < 0.05$).

Table 1. Percentage of spermatozoa exhibiting DNA fragmentation, sperm viability and acrosome integrity in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl; PI, propidium iodide; PNA, peanut agglutinin

	% Spermatozoa with fragmented DNA		% Viable spermatozoa ($SYBR14^+/PI^-$)		% Acrosome-intact spermatozoa (PNA^-)	
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	1.4 ± 0.2^{a}	2.7 ± 0.3^{b}	86.1 ± 3.2^{a}	52.6 ± 2.5^{b}	87.0 ± 4.0^{a}	64.7 ± 3.2^{b}
FT-C	1.8 ± 0.3^{a}	6.2 ± 0.8^{c}	46.8 ± 2.0^{c}	32.5 ± 1.4^{d}	47.7 ± 2.2^{c}	21.3 ± 1.0^{d}
G1	1.6 ± 0.3^{a}	4.7 ± 0.7^{d}	50.6 ± 2.3^{bc}	36.7 ± 1.6^{ef}	51.5 ± 2.4^{ch}	26.1 ± 1.2^{i}
G2	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	60.5 ± 2.5^{d}	46.9 ± 2.0^{c}	61.2 ± 2.9^{b}	38.3 ± 1.8^{eg}
P1	1.7 ± 0.3^{a}	4.9 ± 0.6^{d}	48.3 ± 2.2^{bc}	35.0 ± 1.5^{df}	49.8 ± 2.4^{ch}	24.1 ± 1.1^{di}
P2	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	49.5 ± 2.2^{bc}	39.3 ± 1.7^{e}	48.4 ± 2.3^{ch}	29.5 ± 1.3^{f}
G1P1	1.7 ± 0.3^{a}	4.8 ± 0.7^{d}	48.1 ± 2.1^{bc}	35.8 ± 1.6^{ef}	49.3 ± 2.4^{ch}	24.7 ± 1.2^{di}
G1P2	1.6 ± 0.2^{a}	4.1 ± 0.6^{d}	51.1 ± 2.2^{b}	39.1 ± 1.7^{e}	52.9 ± 2.5^{h}	28.8 ± 1.3^{fi}
G2P1	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	59.1 ± 2.5^{d}	46.2 ± 2.0^{c}	61.1 ± 2.9^{b}	37.0 ± 1.8^{e}
G2P2	1.5 ± 0.2^{a}	2.7 ± 0.4^{b}	61.9 ± 2.6^{d}	49.4 ± 2.3^{bc}	62.6 ± 3.0^{b}	41.2 ± 2.0^{g}

conditions tested, these levels remained significantly higher ($P < 0.001$) than in the extended control (e.g. 4.35 ± 0.45 nmol μg^{-1} protein in the G2 group vs 3.29 ± 0.34 nmol μg^{-1} protein in the extended control; see Fig. 1).

Effects of GSH and ProHCl on DNA fragmentation of boar spermatozoa subjected to freeze–thawing

Freshly obtained and diluted boar spermatozoa exhibited very low levels of DNA fragmentation (1.4% ± 0.2%; Table 1). Furthermore, under the present conditions, freeze–thawing did not modify sperm DNA fragmentation when determined after 30 min incubation at 37°C after thawing (Table 1), nor did the addition of either GSH or ProHCl modify this result at any of the concentrations and combinations tested.

In contrast, sperm DNA fragmentation was significantly higher in the FT-C group after 240 min incubation after thawing ($P < 0.05$) compared with levels in extended samples, although the levels remained relatively low in both groups (6.2 ± 0.8% vs 2.7 ± 0.3%, respectively; Table 1). Supplementation of the freezing medium with G2, P2, G2P1 and G2P2 completely

counteracted the increase in DNA fragmentation observed in the FT-C group after 240 min incubation after thawing (e.g. $2.7 \pm 0.3\%$ in the extended control vs $2.9 \pm 0.4\%$ in the G2 group; $P > 0.05$).

Effects of GSH and ProHCl on viability of boar spermatozoa subjected to freeze–thawing (SYBR-14/PI)

Treatment ($P < 0.001$), incubation time ($P < 0.001$) and treatment × incubation time ($P < 0.05$) significantly affected the viability of spermatozoa (SYBR-14^+/PI^-), with extended semen having significantly higher viability than the spermatozoa from the other treatment groups (Table 1). Furthermore, in frozen–thawed samples analysed after 30 min incubation after thawing, the percentage of viable spermatozoa in the G2, G2P1 and G2P2 groups was significantly higher than in the FT-C group. Sperm viability after 240 min incubation after thawing decreased in all treatment groups, but although sperm viability in the FT-C group was significantly lower than in the extended control, that in the G2P2 group was similar to that in the extended control ($49.4 \pm 2.4\%$ vs $52.6 \pm 2.5\%$, respectively; $P > 0.05$; Fig. 2).

Effects of GSH and ProHCl on acrosome integrity of boar spermatozoa subjected to freeze–thawing (PNA-FITC/PI)

The proportion of acrosome-intact spermatozoa (PNA-$FITC^-$) was significantly higher ($P < 0.001$) in the extended control than in all of the frozen–thawed groups after 30 min incubation at 37°C after thawing (Table 1). Three treatments (G2, G2P1 and G2P2) resulted in a significantly higher percentage ($P < 0.01$) of acrosome-intact spermatozoa compared with the FT-C group after 30 min incubation after thawing (e.g. $47.7 \pm 2.2\%$ vs $61.2 \pm 2.9\%$ in the FT-C and G2 groups, respectively) and after 240 min incubation after thawing. In contrast, no significant differences were observed between the FT-C, P1 and G1P1 groups after 240 min incubation after thawing.

Effects of GSH and ProHCl on the plasma membrane of boar spermatozoa subjected to freeze–thawing (YO-PRO-1/PI)

In all cases, freeze–thawing increased the percentage of viable spermatozoa with early changes in membrane permeability (YO-PRO-1^+/PI^-), as well as that of non-viable spermatozoa (PI^+), after both 30 and 240 min incubation after thawing (Table 2). Conversely, the percentage of viable spermatozoa without changes in membrane permeability (YO-PRO-1^-/PI^-) were significantly ($P < 0.05$) higher in the G2, P2, G2P1 and G2P2 groups than in the FT-C group after both 30 and 240 min incubation after thawing, whereas the percentage of viable spermatozoa with early changes in membrane permeability (YO-PRO-1^+/PI^-) was significantly higher in the latter groups than in the former group (Fig. 3).

Effects of GSH and ProHCl on membrane lipids in boar spermatozoa subjected to freeze–thawing

Freeze–thawing significantly ($P < 0.001$) increased the percentage of both viable and non-viable spermatozoa exhibiting increased levels of disordered membrane lipid organisation ($M540^+$; Table 3) after 30 min incubation after thawing. Incubating samples at 37°C for 240 min after thawing also increased the percentage of non-viable spermatozoa with increased membrane disorder in both the extended and frozen–thawed groups. However, the increase in the percentage of non-viable spermatozoa with high membrane disorder after 30 min incubation after thawing was significantly lower after the addition of G2, G2P1 and G2P2 to the freezing extenders (e.g. $41.0 \pm 2.2\%$ vs $24.5 \pm 1.6\%$ in the FT-C and G2P2 groups, respectively; $P < 0.01$). After 240 min incubation after thawing, the percentage of viable spermatozoa exhibiting low levels of disordered membrane lipid organisation was significantly higher ($P < 0.05$) in the G1, G2, P2, G1P1, G1P2, G2P1 and G2P2 groups compared with the FT-C group (Fig. 4).

Effects of GSH and ProHCl on intracellular peroxide and superoxide levels in boar spermatozoa subjected to freeze–thawing

Table 4 lists peroxide levels in the different treatment groups. The percentage of viable spermatozoa with high levels of peroxides (DCF$^+$/PI$^-$) was significantly higher ($P < 0.05$) in the FT-C group after 30 min incubation after thawing than in the extended control and G2, G2P1 and G2P2 groups (e.g. $3.5 \pm 0.3\%$, $2.4 \pm 0.2\%$ and $2.5 \pm 0.2\%$ in the FT-C, extended control and G2P2 groups, respectively; Fig. 5). Concomitantly, after 30 min incubation after thawing, the GMFI was significantly higher ($P < 0.01$) for both total and viable spermatozoa with high levels of H_2O_2 in the FT-C group after 30 min incubation after thawing compared with the G2, G2P1 and G2P2 groups.

After 240 min incubation after thawing, significantly lower ($P < 0.01$) GMFI was observed for viable spermatozoa with high levels of peroxides in the G2, G2P1, and G2P2 groups compared with the extended control and FT-C groups (e.g. 19.2 ± 1.1, 68.6 ± 3.8 and 45.3 ± 2.5 a.u. in the G2, extended control and FT-C groups, respectively). The GMFI of DCF$^+$ in the G2, G2P1 and G2P2 groups was similar to that in the extended control group after 240 min incubation after thawing.

In contrast with peroxide levels, no significant differences were observed in superoxide levels among any of the groups, including the extended control and FT-C groups; specifically, there were no differences in the percentage of viable spermatozoa with high superoxide levels (E^+/YOPRO-1) after either 30 min incubation after thawing (e.g. $3.3 \pm 0.3\%$, $3.4 \pm 0.3\%$ and $3.3 \pm 0.3\%$ in the extended control, FT-C and G2P2 groups, respectively) or after 240 min incubation after thawing (e.g. $3.4 \pm 0.3\%$, $3.5 \pm 0.3\%$ and $3.5 \pm 0.3\%$ in the extended control, FT-C and G2P2 groups, respectively). There were no significant differences in the GMFI of total (E^+) and viable (E^+/YOPRO-1-) spermatozoa with high superoxide levels.

Effects of GSH and ProHCl on motility parameters of boar spermatozoa subjected to freeze–thawing

As expected, the percentage of total motile spermatozoa (TMOT) decreased after cryopreservation in all treatment groups (Table 5). This reduction was observed after both 30 and 240 min incubation after thawing (e.g. $58.0 \pm 2.9\%$ and

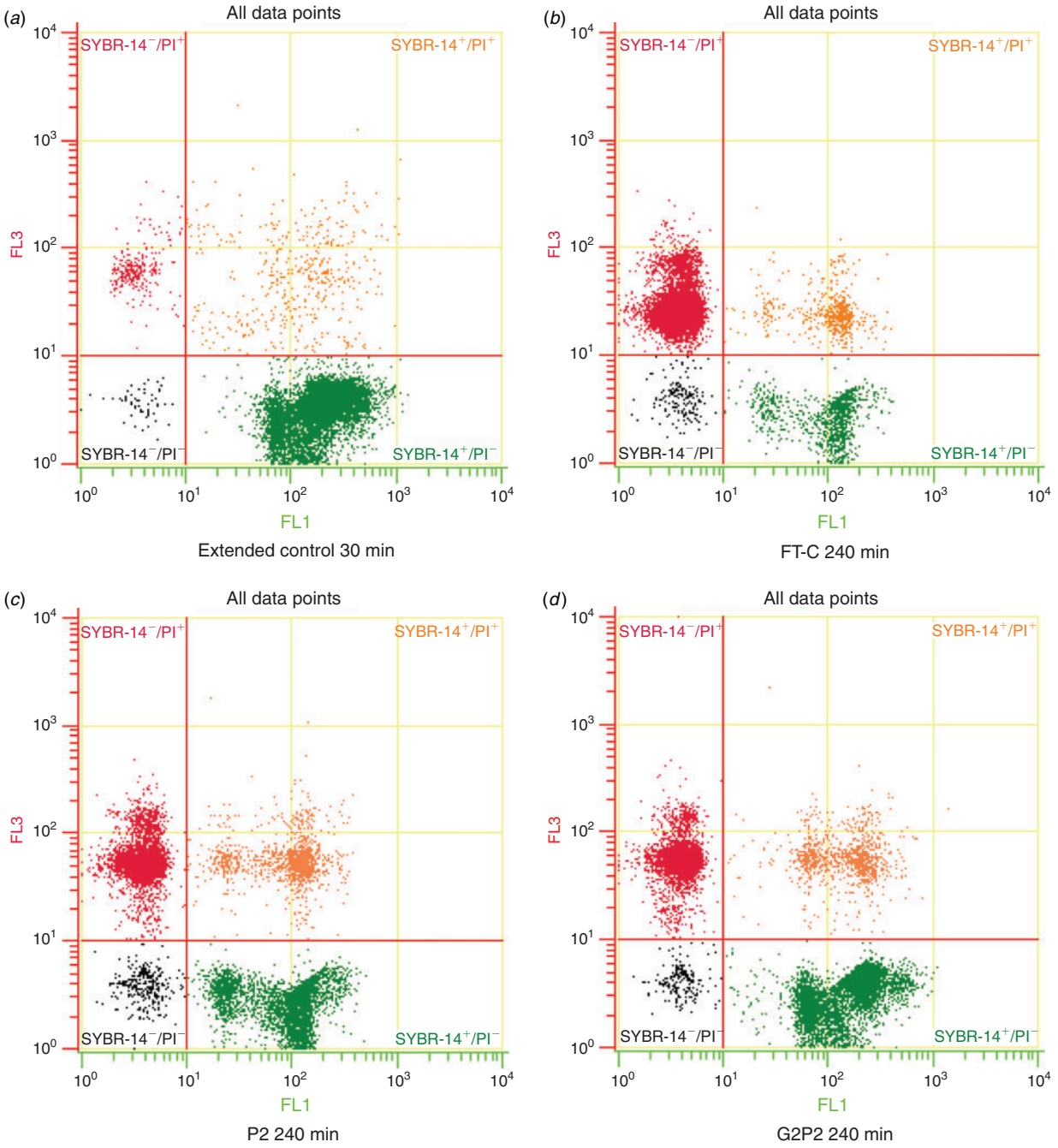

Fig. 2. Representative dot plots from SYBR-14/propidium iodide (PI) staining obtained (*a*) before or (*b–d*) after boar spermatozoa were subjected to different treatments. Samples were incubated at 37°C for 30 or 240 min after thawing, as indicated. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G2, 2 mM reduced glutathione; P2, 2 mM procaine hydrochloride. The upper left quadrants ($SYBR\text{-}14^-/PI^+$) contain non-viable red-stained spermatozoa, the upper right quadrants ($SYBR\text{-}14^+/PI^+$) contain non-viable spermatozoa stained both green and red, the lower left quadrants ($SYBR\text{-}14^-/PI^-$) contain alien particles (debris) and the lower right quadrants ($SYBR\text{-}14^+/PI^-$) contain viable green-stained spermatozoa.

38.7 ± 2.3% in the extended control and FT-C groups after 240 min incubation after thawing).

A significant ($P < 0.01$) increase in TMOT was observed in the G1, G2, G2P1 and G2P2 groups compared with the FT-C group after 30 and 240 min incubation after thawing (e.g. 50.4 ± 2.9% vs 38.7 ± 2.3% in the G2 and FT-C groups after 240 min incubation after thawing). Supplementation of the freezing medium with G1, G2, P2, G1P1, G1P2, G2P1 and

Table 2. Percentage of spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing during the YO-PRO-1/ propidium iodide assay

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. PI, propidium iodide; MP, membrane permeability; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	Viable spermatozoa				Non-viable spermatozoa (PI^+)	
	No changes in MP ($YO\text{-}PRO\text{-}1^-/PI^-$)		Early changes in MP ($YO\text{-}PRO\text{-}1^+/PI^-$)			
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	83.0 ± 4.2^{a}	45.9 ± 2.4^{b}	3.1 ± 0.2^{a}	5.0 ± 0.4^{b}	13.3 ± 0.9^{a}	47.3 ± 2.7^{b}
FT-C	37.0 ± 1.8^{c}	8.9 ± 0.8^{f}	12.9 ± 1.1^{c}	11.7 ± 1.0^{cd}	40.5 ± 2.1^{c}	66.1 ± 3.5^{d}
G1	42.1 ± 2.2^{bc}	16.0 ± 1.0^{e}	11.5 ± 1.0^{cd}	10.6 ± 0.9^{de}	37.7 ± 1.9^{c}	60.7 ± 3.0^{d}
G2	57.3 ± 2.9^{e}	28.3 ± 1.5^{d}	8.9 ± 0.8^{efg}	8.4 ± 0.7^{fg}	27.4 ± 1.5^{e}	52.4 ± 2.5^{b}
P1	39.7 ± 2.0^{bc}	12.6 ± 0.9^{f}	12.2 ± 1.1^{cd}	10.8 ± 1.0^{cd}	39.0 ± 2.0^{c}	63.3 ± 3.3^{d}
P2	45.2 ± 2.3^{b}	18.9 ± 1.1^{e}	10.5 ± 1.0^{de}	9.7 ± 0.9^{eg}	36.0 ± 1.8^{c}	59.2 ± 2.9^{df}
G1P1	40.7 ± 2.0^{bc}	12.5 ± 0.9^{f}	12.1 ± 1.1^{cd}	10.3 ± 1.0^{de}	38.3 ± 1.9^{c}	64.0 ± 3.3^{d}
G1P2	44.1 ± 2.3^{b}	17.6 ± 1.0^{e}	10.9 ± 1.0^{cd}	9.5 ± 0.9^{eg}	36.6 ± 1.8^{c}	60.6 ± 2.9^{d}
G2P1	55.8 ± 2.8^{e}	28.2 ± 1.5^{d}	9.4 ± 0.9^{eg}	8.0 ± 0.8^{fg}	28.1 ± 1.5^{e}	53.1 ± 2.6^{bf}
G2P2	59.5 ± 3.0^{e}	30.7 ± 1.6^{d}	8.1 ± 0.8^{fg}	7.7 ± 0.7^{f}	26.3 ± 1.5^{e}	51.2 ± 2.5^{b}

G2P2 had a significant ($P < 0.05$) positive effect on VCL, VSL, VAP and percentage of linearity (LIN) after 30 and 240 min incubation after thawing compared with values in the FT-C group at the same time-points (see Annex C available as Supplementary Material to this paper).

Discussion

Cryopreservation of mammalian spermatozoa damages sperm chromatin (Flores *et al.* 2011). This damage is related to destabilisation of the nucleoprotein structure, as well as defective packaging, activity of sperm nucleases and/or ROS generation (Agarwal and Said 2003; Chapman and Michael 2003). Previous studies have reported a strong relationship between DNA integrity and the fertilising ability of mammalian spermatozoa (Silva and Gadella 2006). Indeed, although chromatin-damaged spermatozoa can fertilise oocytes (Tesarik *et al.* 2004), this may lead to early embryo death and can affect implantation and post-implantation development (Sakkas *et al.* 1998; Fatehi *et al.* 2006).

In the present study, we showed that GSH and ProHCl protect boar sperm chromatin against damage induced by freeze–thawing because they protect the disulfide bonds between the cysteine residues of protamines, reduce peroxide levels in the case of GSH and diminish DNA fragmentation after thawing. These three effects are discussed separately below.

The addition of GSH and/or ProHCl at final concentrations of 2 mM had a protective effect on the disulfide bonds of sperm nucleoproteins. This is even more evident after 240 min incubation after thawing, when the levels of free cysteine radicals in the G2P2 group were similar to those in the extended control. According to previous reports, freeze–thawing affects the sperm nucleus by inducing changes in the structural interaction between nucleoproteins (protamine 1 and histone 1) and DNA (Flores *et al.* 2008, 2011). These changes are related to the disruption of disulfide bonds between cysteines of the nucleoproteins analysed (Flores *et al.* 2011). Although the mechanism responsible for this disruption remains unknown, these bonds are responsible for the proper packaging, compaction and stabilisation of sperm chromatin (Nasr-Esfahani *et al.* 2004; Balhorn 2007). In addition, protamines protect DNA from nucleases and ROS, and remove transcription factors and proteins to help reset the imprinting code in the oocyte (Oliva 2006).

Disulfide bonds can be weakened and disrupted when osmotic conditions are greatly modified, as is the case during the cryopreservation of boar spermatozoa, or unspecifically formed in the cytosol under oxidising conditions, thereby causing irreversible damage to proteins (Cumming *et al.* 2004; Yang *et al.* 2007). Thus, the changes observed to boar sperm head disulfide bonds may be caused by a combined affect of oxidative and osmotic changes related to freeze–thawing. In the present study, GSH and ProHCl stabilisation of the nucleoprotein structure and, hence, of the disulfide bonds, could be explained by their role in the maintenance of intracellular redox balance (Jacob *et al.* 2003). Glutathione can exist in a reduced (GSH) or oxidised (GSSG) state and the thiol group of cysteine in GSH can donate an electron to unstable molecules, such as ROS. In somatic cells, ProHCl has an antioxidant effect against ROS-induced endothelial damage in the rabbit aorta (Lee *et al.* 2010). Thus, the beneficial effects of GSH and ProHCl in protecting disulfide bonds could be related to their antioxidant activity.

In the present study, ROS levels were also assessed. Freeze–thawing appeared to slightly increase the percentage of viable spermatozoa with high levels of H_2O_2, whereas the extended control and treatments containing 2 mM GSH had similar low percentages of viable spermatozoa with high levels of H_2O_2 after both 30 and 240 min incubation after thawing. In a previous study, Awda *et al.* (2009) did not

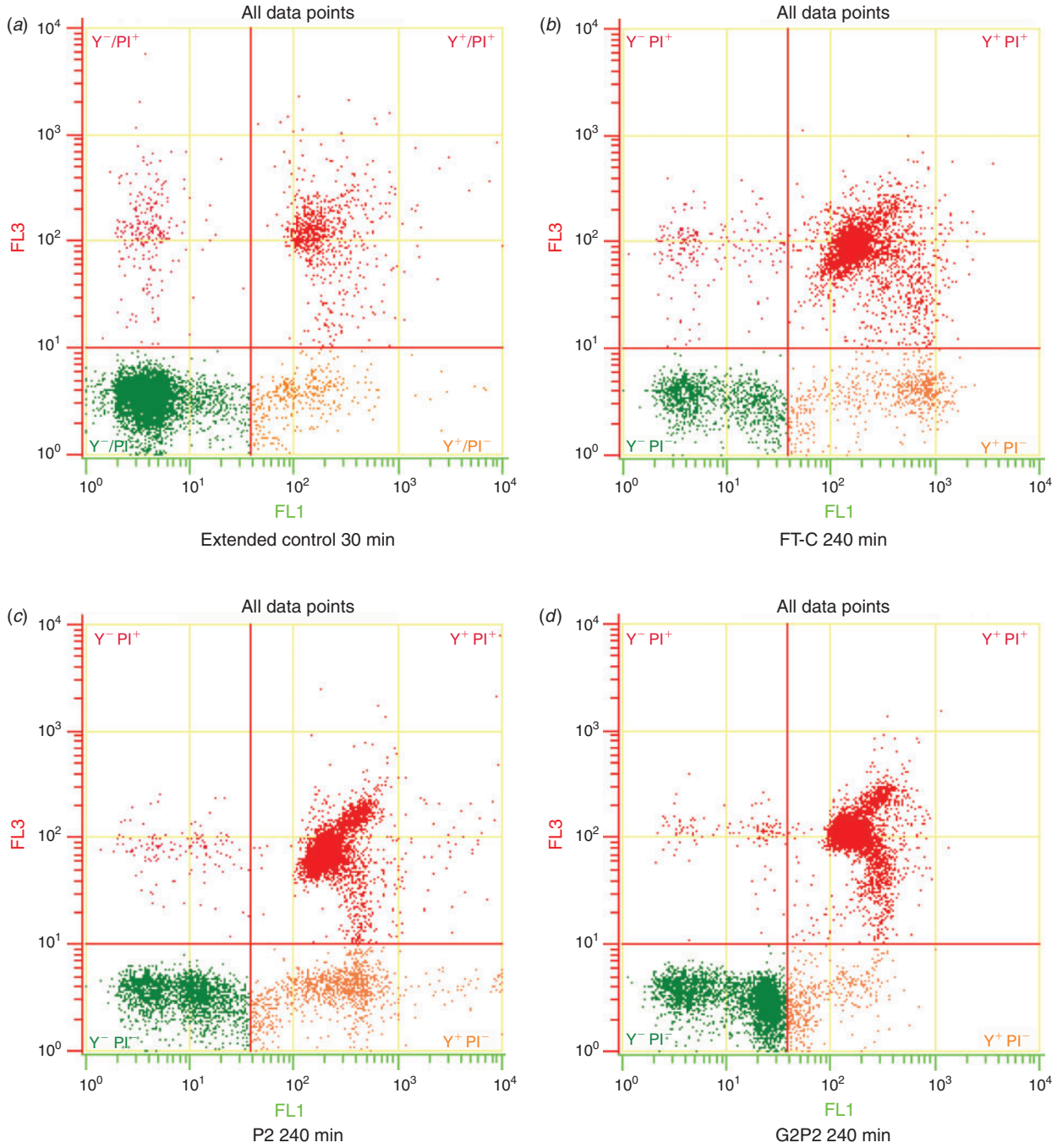

Fig. 3. Representative dot plots from YO-PRO-1/propidium iodide (PI) staining obtained (*a*) before or (*b–d*) after boar spermatozoa were subjected to different treatments. Samples were incubated at 37°C for 30 or 240 min after thawing, as indicated. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G2, 2 mM reduced glutathione; P2, 2 mM procaine hydrochloride. The upper left quadrants (YO-PRO-1^{-}/PI^{+} and YO-PRO-1^{-}/PI^{+}) contain non-viable spermatozoa, the lower left quadrants (YO-PRO-1^{-}/PI^{-}) contain viable spermatozoa without changes in membrane permeability and the lower right quadrants (YO-PRO-1^{+}/PI^{-}) contain viable spermatozoa with early changes in membrane permeability.

observe changes in H_2O_2 levels in viable boar spermatozoa after freeze–thawing protocols, whereas Kim *et al.* (2011) reported a slight increase in the percentage of viable spermatozoa with a high level of H_2O_2.

There were no changes in intracellular levels of $\cdot O_2^-$ in viable spermatozoa either in the FT-C group or after the addition of GSH and ProHCl. This could be related to the chemical properties of $\cdot O_2^-$, which has a very short half-life and is too polar to

Table 3. Levels of disordered membrane lipid organisation (high or low) in sperm plasma membrane after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	% Viable spermatozoa				% Non-viable spermatozoa			
	Highly disordered membrane lipids ($M540^+/YO\text{-}PRO\text{-}1^-$)		Low disordered membrane lipids ($M540^-/YO\text{-}PRO\text{-}1^-$)		Highly disordered membrane lipids ($M540^+/YO\text{-}PRO\text{-}1^+$)		Low disordered membrane lipids ($M540^-/YO\text{-}PRO\text{-}1^+$)	
	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min
Extended	5.0 ± 0.3^{a}	11.1 ± 0.6^{bc}	82.2 ± 4.5^{a}	47.7 ± 2.6^{b}	6.7 ± 0.4^{a}	24.2 ± 1.3^{b}	5.2 ± 0.3^{a}	15.0 ± 1.0^{b}
FT-C	13.8 ± 0.8^{b}	9.2 ± 0.5^{c}	32.2 ± 1.9^{c}	16.9 ± 0.9^{d}	41.0 ± 2.2^{ce}	59.6 ± 3.3^{d}	2.9 ± 0.2^{c}	1.8 ± 0.2^{c}
G1	13.6 ± 0.7^{b}	9.7 ± 0.5^{c}	37.8 ± 2.1^{ef}	21.0 ± 1.2^{gh}	36.5 ± 2.0^{eh}	55.9 ± 3.0^{df}	2.7 ± 0.2^{c}	1.6 ± 0.1^{c}
G2	13.4 ± 0.7^{b}	9.3 ± 0.5^{c}	49.9 ± 2.7^{b}	33.1 ± 2.0^{ce}	26.6 ± 1.6^{bi}	46.2 ± 2.6^{cg}	2.6 ± 0.1^{c}	1.5 ± 0.1^{c}
P1	13.9 ± 0.7^{b}	9.5 ± 0.5^{c}	37.0 ± 2.0^{ef}	18.7 ± 1.1^{dh}	36.0 ± 2.1^{eh}	57.6 ± 3.3^{df}	2.8 ± 0.2^{c}	1.6 ± 0.1^{c}
P2	14.2 ± 0.8^{b}	9.8 ± 0.5^{c}	40.6 ± 2.2^{f}	25.3 ± 1.4^{i}	33.5 ± 2.1^{h}	52.3 ± 2.8^{f}	2.7 ± 0.2^{c}	1.7 ± 0.1^{c}
G1P1	13.5 ± 0.7^{b}	9.5 ± 0.5^{c}	35.6 ± 2.0^{ce}	19.6 ± 1.2^{h}	38.2 ± 2.3^{eh}	57.5 ± 3.1^{df}	2.8 ± 0.2^{c}	1.6 ± 0.1^{c}
G1P2	14.1 ± 0.8^{b}	9.6 ± 0.5^{c}	37.7 ± 2.1^{ef}	23.7 ± 1.3^{gi}	36.2 ± 2.2^{eh}	53.6 ± 3.0^{df}	2.7 ± 0.2^{c}	1.5 ± 0.1^{c}
G2P1	13.5 ± 0.7^{b}	9.7 ± 0.5^{c}	47.2 ± 2.6^{b}	32.0 ± 2.0^{c}	28.6 ± 1.7^{i}	46.4 ± 2.5^{g}	2.9 ± 0.2^{c}	1.7 ± 0.2^{c}
G2P2	13.7 ± 0.7^{b}	9.5 ± 0.5^{c}	51.7 ± 2.8^{b}	36.6 ± 2.1^{ef}	24.5 ± 1.6^{b}	42.8 ± 2.3^{cg}	2.7 ± 0.2^{c}	1.7 ± 0.1^{c}

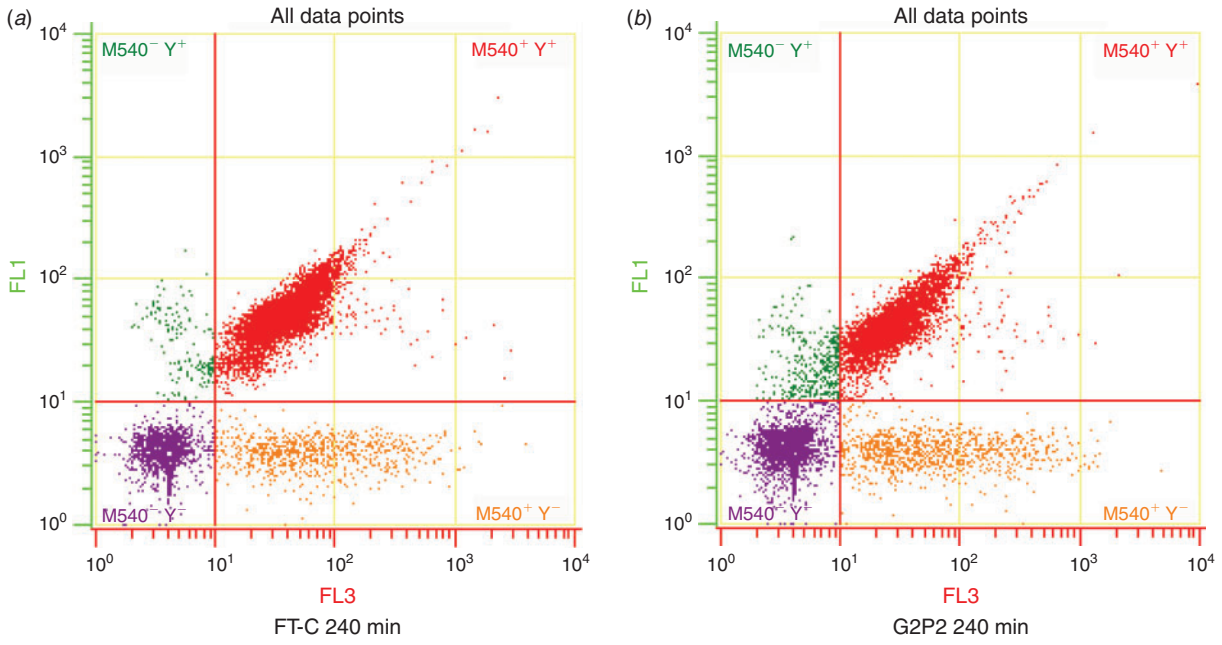

Fig. 4. Representative dot plots from M540/YO-PRO-1 staining after 240 min incubation at 37°C after thawing in the (*a*) frozen–thawed control (FT-C) and (*b*) 2 mM reduced glutathione + 2 mM procaine hydrochloride (G2P2)-treated groups. The upper left quadrants ($M540^-/YO\text{-}PRO\text{-}1^+$) contain non-viable spermatozoa with low levels of disordered membrane lipid organisation, the upper right quadrants ($M540^+/YO\text{-}PRO\text{-}1^+$) contain non-viable spermatozoa with high levels of disordered membrane lipid organisation, the lower left quadrants ($M540^-/YO\text{-}PRO^{®}\text{-}1^-$) contain viable spermatozoa with low levels of disordered membrane lipid organisation and the lower right quadrants ($M540^+/YO\text{-}PRO^{®}\text{-}1^-$) contain viable spermatozoa with high levels of disordered membrane lipid organisation.

pass through intact plasma membranes (Aitken 1995). In this way, the rate of destruction of $\cdot O_2^-$ would be very high, thus maintaining its intracellular values within a narrow range. In fact, conflicting reports exist in the literature regarding $\cdot O_2^-$ levels in cryopreserved boar spermatozoa. Specifically, although Awda *et al.* (2009) observed a decrease in the intracellular $\cdot O_2^-$ content of viable spermatozoa after freeze–thawing, Kim *et al.* (2011) observed a decrease in intracellular $\cdot O_2^-$ levels after the cooling step up to 5°C, but not after freeze–thawing.

Oxidative stress, which is a result of an imbalance between ROS generation and antioxidant scavenging activities (for a

Table 4. Percentage of spermatozoa and peroxide levels in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. GMFI, geometric mean of fluorescence intensity of Optical Filter 1 (FL1); DCF, dichlorodihydrofluorecein-positive; PI, propidium iodide; DCF^+/PI^-, viable spermatozoa with a high H_2O_2 content; DCF^+, total spermatozoa; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

			GMFI (arbitrary units)			
	% DCF^+/PI^- spermatozoa		DCF^+/PI^-		DCF^+	
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	2.4 ± 0.2^{a}	1.8 ± 0.2^{b}	86.4 ± 4.7^{a}	68.6 ± 3.8^{b}	82.0 ± 4.4^{a}	26.4 ± 1.6^{b}
FT-C	3.5 ± 0.3^{ce}	1.3 ± 0.1^{d}	106.0 ± 5.8^{c}	45.3 ± 2.5^{d}	100.3 ± 5.5^{c}	48.2 ± 2.6^{d}
G1	3.1 ± 0.3^{e}	1.4 ± 0.1^{d}	105.3 ± 5.8^{c}	35.7 ± 2.0^{e}	98.5 ± 5.4^{c}	38.7 ± 2.2^{e}
G2	2.4 ± 0.2^{a}	1.8 ± 0.2^{b}	83.1 ± 4.6^{a}	19.2 ± 1.1^{f}	79.8 ± 4.2^{a}	28.5 ± 1.6^{b}
P1	3.7 ± 0.3^{c}	1.2 ± 0.1^{d}	103.5 ± 5.5^{c}	47.1 ± 2.6^{d}	101.1 ± 5.6^{c}	49.8 ± 2.7^{d}
P2	3.7 ± 0.4^{ce}	1.3 ± 0.1^{d}	111.4 ± 5.9^{c}	49.2 ± 2.7^{d}	103.9 ± 5.5^{c}	52.8 ± 2.9^{d}
G1P1	3.2 ± 0.3^{ce}	1.4 ± 0.1^{d}	110.3 ± 6.0^{c}	37.0 ± 2.0^{e}	106.2 ± 5.7^{c}	39.7 ± 2.1^{e}
G1P2	3.2 ± 0.3^{ce}	1.3 ± 0.1^{d}	106.2 ± 5.6^{c}	41.6 ± 2.3^{de}	105.6 ± 5.8^{c}	41.7 ± 2.4^{e}
G2P1	2.6 ± 0.2^{a}	1.8 ± 0.2^{b}	86.0 ± 4.3^{a}	23.1 ± 1.2^{f}	83.5 ± 4.5^{a}	30.1 ± 1.7^{b}
G2P2	2.5 ± 0.2^{a}	1.7 ± 0.1^{b}	83.6 ± 4.5^{a}	21.1 ± 1.2^{f}	78.6 ± 4.3^{a}	29.1 ± 1.7^{b}

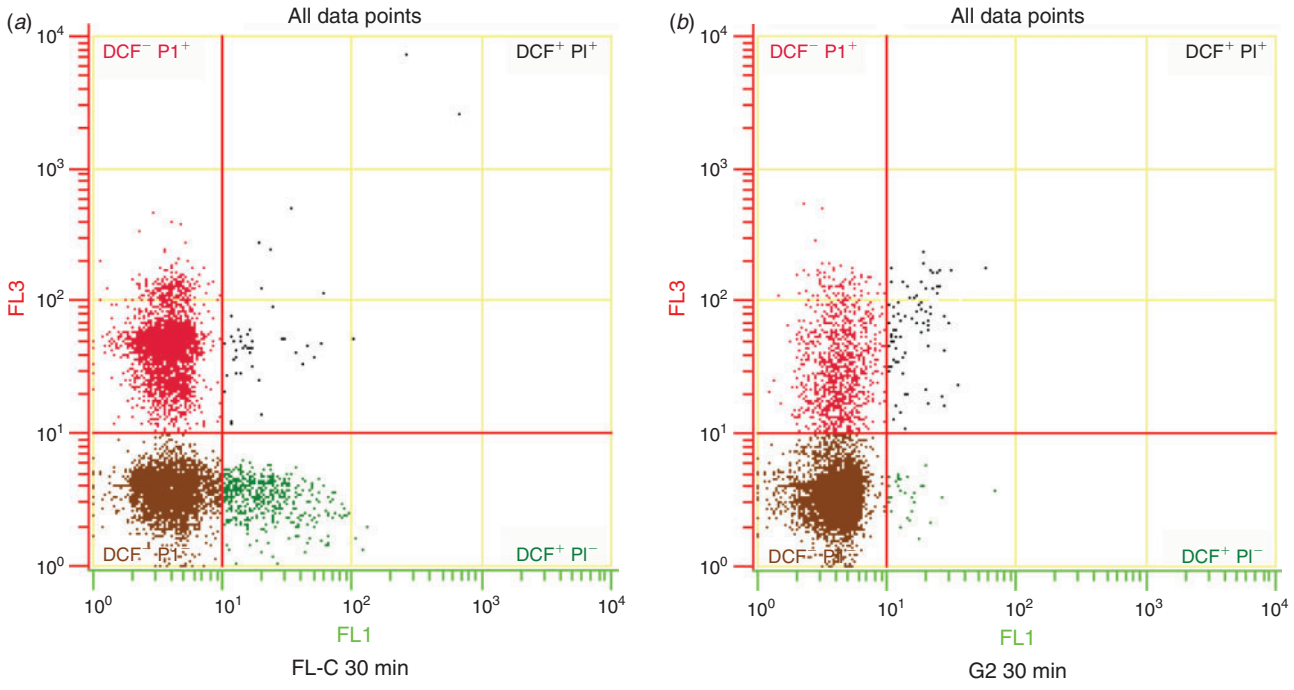

Fig. 5. Representative dot plots from 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA)/propidium iodide (PI) staining (peroxide levels) after 30 min incubation at 37°C after thawing in the (*a*) frozen–thawed control (FT-C) and (*b*) 2 mM reduced glutathione (G2)-treated groups. The upper left quadrants (DCF^-/PI^+) contain non-viable spermatozoa with low levels of intracellular H_2O_2, the upper right quadrants (DCF^+/PI^+) contain non-viable spermatozoa with high levels of intracellular H_2O_2, the lower left quadrants (DCF^-/PI^-) contain viable spermatozoa with low levels of intracellular H_2O_2 and the lower right quadrants (DCF^+/PI^-) contain viable spermatozoa with high levels of intracellular H_2O_2.

review, see Sikka 2001), has been observed in human (Gadea *et al.* 2011), horse (Ball *et al.* 2001), bull (Bilodeau *et al.* 2000) and dog (Kim *et al.* 2010) spermatozoa in response to cryopreservation. However, in the boar, the link between ROS production and freeze–thawing remains unclear. In the boar, H_2O_2 has been identified as being the primary source of ROS damage in viable spermatozoa not only during cryopreservation (Kim *et al.* 2011), but also when using ROS-generating systems

Table 5. Percentage of motile spermatozoa and spermatozoa exhibiting progressive motility in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. TMOT, total motile spermatozoa; PMOT, spermatozoa exhibiting progressive motility; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	TMOT (%)		PMOT (%)	
	30 min	240 min	30 min	240 min
Extended	88.0 ± 4.9^{a}	58.0 ± 2.9^{b}	64.3 ± 3.2^{a}	39.1 ± 2.1^{bf}
FT-C	60.5 ± 3.4^{bg}	38.7 ± 2.3^{c}	35.1 ± 2.0^{bg}	17.6 ± 1.0^{c}
G1	70.2 ± 3.8^{d}	46.1 ± 2.5^{ef}	51.7 ± 2.9^{d}	28.7 ± 1.7^{ei}
G2	74.0 ± 3.8^{d}	50.4 ± 2.9^{f}	53.6 ± 3.0^{d}	36.5 ± 2.0^{b}
P1	59.0 ± 2.9^{b}	40.2 ± 2.3^{c}	40.0 ± 2.2^{bf}	30.6 ± 1.8^{e}
P2	64.9 ± 3.8^{g}	42.9 ± 2.3^{ce}	43.6 ± 2.2^{f}	31.8 ± 1.9^{e}
G1P1	62.4 ± 3.3^{g}	40.5 ± 2.4^{c}	52.6 ± 2.9^{d}	32.5 ± 1.7^{eg}
G1P2	61.0 ± 3.2^{bg}	41.2 ± 2.1^{c}	48.1 ± 2.6^{d}	31.4 ± 1.6^{e}
G2P1	70.3 ± 3.8^{d}	47.8 ± 2.7^{ef}	42.4 ± 2.5^{f}	24.3 ± 1.4^{i}
G2P2	73.0 ± 3.9^{d}	55.1 ± 2.8^{b}	53.1 ± 2.9^{d}	35.8 ± 1.6^{bg}

(Guthrie and Welch 2006). In the present study, a lack of catalase and exhaustion of the other antioxidants may explain why the H_2O_2 levels were slightly higher after the freeze–thawing procedures, in agreement with the findings reported by Kim *et al.* (2011). In this regard, the positive effect of the addition of 2 mM GSH to the reduction of H_2O_2 levels could be related to a mechanism linked to the deactivation of the oxidising agents by reduction (Stenesh 1998), thereby compensating for the lack of other antioxidants and/or catalase.

In somatic cells, ProHCl shows a greater ability to bind purines than do pyrimidines, which is why it appears to bind DNA cytosine–phosphorous–guanine (CpG) islands (Ping *et al.* 2006), and has an antioxidant effect on ROS-induced endothelial damage in the rabbit aorta (Lee *et al.* 2010), potentially via H_2O_2 scavenging. However, although GSH slightly reduced the H_2O_2 content in boar spermatozoa in the present study, ProHCl did not exhibit an H_2O_2-scavenging effect. This leads us to suggest that these two agents have different mechanisms of action. Nonetheless, it is difficult to determine exactly what the extent of ROS impairment is, because $\cdot O_2^-$ levels were not modified by the addition of GSH even though a significant decrease was seen in H_2O_2, although to a lesser extent (from ~105 to 80 a.u. on GMFI). The low production of H_2O_2 in boar spermatozoa due to freeze–thawing procedures observed in the present and other studies (Guthrie and Welch 2006; Kim *et al.* 2011), along with the different effects of GSH and ProHCl on H_2O_2 levels and the accuracy of the detection mechanisms used in the present and other studies, opens reasonable doubts as to the real role of ROS on the function and survival of boar spermatozoa.

The integrity of sperm chromatin is usually assessed through DNA fragmentation tests. However, the results of previous studies in boar investigating DNA fragmentation after freeze–thawing procedures are not clear. Thus, Fraser and Strzeżek (2005), using the neutral comet assay that detects double-strand DNA breaks, observed an increase in DNA fragmentation in boar spermatozoa after freeze–thawing procedures, but Hernández *et al.* (2006), using a sperm chromatin dispersion assay (SCSA), and Flores *et al.* (2008, 2011), using the SCDt, found similar sperm DNA fragmentation indices in extended and frozen–thawed boar spermatozoa. It is worth noting that the SCSA consists of *in situ* DNA denaturation and further staining with acridine orange (Evenson *et al.* 1999), whereas the SCDt assesses the level of DNA damage through chromatin dispersion after controlled DNA denaturation and protein depletion (Enciso *et al.* 2006).

In the present study, differences in sperm DNA fragmentation were not found after 30 min incubation after thawing. These findings are in agreement with other previous reports (Hernández *et al.* 2006; Flores *et al.* 2008, 2011), as well as the results reported by Rybar *et al.* (2004) and Boe-Hansen *et al.* (2005), who found only 5%–10% changes in chromatin structure after direct freezing in liquid nitrogen. Thus, sperm DNA fragmentation appears to be not increased by freeze–thawing procedures if we take into account levels of sperm DNA fragmentation after 30 min incubation after thawing. However, in the FT-C group, the percentage of spermatozoa with fragmented DNA increased from $1.8 \pm 0.3\%$ after 30 min incubation after thawing to $6.2 \pm 0.8\%$ after 240 min incubation after thawing. Despite this being a marginal increase, significantly higher levels of sperm DNA fragmentation were seen in the FT-C group after 240 min incubation after thawing than in the G2, P2, G2P1 and G2P2 groups. In addition, the levels of sperm DNA fragmentation in these four treatment groups were similar to those in the extended control group after 240 min incubation after thawing.

Together, these results allow us to hypothesise that destabilisation of the nucleoprotein structure, due to disruption of disulfide bonds, after 30 min incubation after thawing of boar spermatozoa leads to chromatin decondensation (Flores *et al.* 2008, 2011) and seems to underlie the subsequent increase in DNA fragmentation. In fact, less inter- and intraprotamine interactions make DNA more susceptible to damage (Fuentes-Mascorro *et al.* 2000; Nasr-Esfahani *et al.* 2004), because DNA strands are tightly wrapped around the protamine molecules and reduced chromatin packaging leads to lower resistance against strong acids, proteases, DNases and/or detergents (Chapman and Michael 2003). Thus, after 240 min incubation after thawing, there are higher levels of leakage products from very significant and increasing numbers of dead and dying spermatozoa. These products are likely to include acrosomal lytic enzymes, as well as sperm nuclear, cytoplasmic and mitochondrial components, including nucleases released from spermatozoa with damaged plasma membranes. Endogenous nucleases, found in hamster, mouse and human spermatozoa, can fragment DNA by cleaving it into loop-sized fragments (Ward and Ward 2004) and, in the mouse, the nucleases can be activated during freeze–thawing procedures (Sotolongo *et al.* 2005). In addition, there can be traces of components of the cryopreservation medium, including glycerol and yolk particles, that may be detrimental for spermatozoa. Thus, we suggest that all these

detrimental products may directly damage sperm chromatin and/or accelerate DNA fragmentation upon destabilisation of the nucleoprotein structure already observed after 30 min incubation after thawing. According to our hypothesis, cryopreservation of boar spermatozoa would alter the nucleoprotein structure without the necessity to arrive at the end-point that DNA fragmentation represents, as observed immediately after thawing. However, after 240 min incubation after thawing, this and other factors (e.g. ROS species and particles from dying spermatozoa) concomitantly act to increase levels DNA fragmentation. Our hypothesis also explains the contradictory results reported in the literature thus far. Indeed, it could explain why previous reports (Hernández *et al.* 2006; Flores *et al.* 2011) did not observe increases in DNA fragmentation immediately after thawing. In a similar fashion, DNA fragmentation does not appear in the ovine immediately after thawing, only after a 2–6 h incubation period.

In this context, GSH and ProHCl seem to counteract the increase in sperm DNA fragmentation, having beneficial effects on the spermatozoa not only during the freezing procedures, but also during incubation after thawing. This counteracting effect may be related to their protection of disulfide bonds and to their antioxidant activity, as well as to the lower levels of dying and/or dead spermatozoa observed in the GSH and ProHCl treatment groups. Furthermore, Lopes (1997) have reported that GSH protects DNA against ROS-induced fragmentation in humans.

Finally, the positive effects of 2 mM GSH (G2, G2P1 and G2P2 treatment groups) in the freezing medium were observed on sperm viability (SYBR-14/PI), acrosome integrity (PNA-FITC/PI) and changes in membrane permeability (YO-PRO-1/PI assay), because the results for these parameters were better in these three treatment groups (G2, G2P1 and G2P2) than in the FT-C group after both 30 and 240 min incubation after thawing. Although freeze–thawing increased the percentage of non-viable spermatozoa with high levels of disordered membrane lipid organisation (M540/YO-PRO-1), the addition of 2 mM GSH (G2, G2P1 and G2P2) to the freezing extenders resulted in a lower percentage of non-viable spermatozoa with high levels of disordered membrane lipid organisation. Conversely, despite ProHCl inducing sperm capacitation and hyperactivation in several species (bull, stallion and guinea-pig) without triggering the acrosome reaction (Mújica *et al.* 1994; Márquez and Suárez 2004; McPartlin *et al.* 2009), this effect was not observed in the present study.

Positive effects of GSH were seen on several parameters of sperm motility (TMOT, VSL, VCL, VAP and LIN) after 30 and 240 min incubation after thawing, in agreement with previous studies (Gadea *et al.* 2005). In the present study, we could hypothesise that GSH may compensate for ROS production linked to freeze–thawing procedures, because ROS impairs motility via ATP depletion mediated by the inhibition of oxidative phosphorylation and/or glycolysis by H_2O_2 (de Lamirande and Gagnon 1992).

Together, these findings indicate that the freeze–thawing of boar spermatozoa impairs sperm motility and membrane integrity, destabilises the nucleoprotein structure by disrupting disulfide bonds and increases levels of both DNA fragmentation and intracellular H_2O_2 in viable spermatozoa, although this occurs with a low incidence. During freeze–thawing, both ProHCl and GSH protect disulfide bonds between the cysteines residues of nucleoproteins and sperm chromatin, especially after 240 min incubation after thawing. This provides the rationale for the addition of GSH and ProHCl to the freezing medium, even though more research is needed to determine the exact mechanisms of action of these two agents and how they may affect fertilisation and subsequent embryo development. In addition, our data warrant further investigation to determine whether GSH and/or ProHCl are able to stabilise sperm nucleoprotein structure during freeze–thawing in other mammalian species.

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ARTICULO 2

Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels.

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Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels

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Abstract

The main aim of the present study was to determine whether differences in the amounts of free cysteine residues in sperm nucleoproteins, which are a direct marker of the integrity of the disulfide bonds between nucleoproteins, existed between good (GFE) and poor boar freezability ejaculates (PFE) during the different steps of the freeze-thawing process. The analyzed steps were: (1) immediately before starting cryopreservation (17 ^{0}C), (2) at the end of the cooling step (5 ^{0}C), and (3) 30, and (4) 240 minutes after thawing. In addition, the present study also sought to determine whether GFE and PFE differed in the amounts of peroxides and superoxides generated during freeze-thawing as an overall measure of the boar sperm reactive oxygen species (ROS) accumulation rate. According to our results, PFE present lower resistance than GFE to cryopreservation-induced alterations of disulfide bonds between nucleoproteins, because levels of cysteine free residues were higher in PFE than in GFE at 30 and 240 minutes after thawing. On the other hand, no significant differences were observed between GFE and PFE in ROS levels during freeze-thawing. In conclusion, PFE are less resistant than GFE to cryopreservation not only in terms of sperm motility and membrane integrity, but also in the integrity of nucleoprotein structure. However, this difference between PFE and GFE in the resistance of the nucleoprotein structure to freeze-thawing is not linked with concomitant changes in ROS levels.

Keywords: Boar sperm, Cryopreservation, Freezability, Sperm nucleus, ROS.

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1. Introduction

Sperm cryopreservation is the most efficient method for storing boar spermatozoa for a long period of time [1], even though their fertilizing ability is lower than that of fresh or refrigerated semen (see [2] for a review). In pigs, not all the ejaculates present the same ability to withstand freeze-thawing, but differences in sperm freezability (i.e., the ability of sperm to sustain cryopreservation procedures) have been reported to exist between breeds [3,4], between and within boars [5–7], and even between fractions coming from the same ejaculate [8]. For this reason, boars and their ejaculates have been respectively rated as 'good' or 'bad' freezers [9], and as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) [7].

Although the mechanisms underlying different ejaculate freezability remain to be elucidated, such freezability differences have been related to protein or lipid composition of the sperm membrane [4], and to the composition of the seminal plasma and/or to the functionality of the accessory glands [5]. On the other hand, Thurston et al. [10] demonstrated that a consistent and genetically determined variation between boars exists in frozen-thawed sperm quality. Subsequently, Thurston et al. [11] identified amplified fragment length polymorphism markers associated with 'good' and 'poor' boar freezers. Related to this, Safranski et al. [1] have suggested that direct selection for

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freezability might be successful because heritabilities for freezability are low to moderate but higher than for fresh semen traits.

One of the most studied changes induced by sperm cryopreservation in mammals concerns generation of reactive oxygen species (ROS) and chromatin damage. Regarding ROS, variations of these substances during the boar sperm cryopreservation process are low [12–14], although boar sperm have been suggested to be susceptible to ROS-induced damage during the cryopreservation process [15]. In addition, Gómez-Fernández et al. [16], compared peroxide levels in 'good' and 'bad' boar freezers, and did not find significant differences between both groups either before starting cryopreservation or after thawing. Thus, the role of ROS levels in boar sperm cryodamage is an unresolved question, which merits a more in-depth approximation.

Cryopreservation of boar spermatozoa also appears to alter the sperm nucleus, because it destabilizes the nucleoprotein structure by disrupting disulfide bonds and, to a much less extent, increases DNA fragmentation [14,17]. Although Hernández et al. [6] studied the differences in terms of chromatin integrity in good and bad freezers immediately after thawing, and found low but significant differences between these two groups, no study has been conducted to evaluate whether GFE and PFE differ in the amounts of disulfide bonds disrupted during the cryopreservation process. In addition, it has been suggested that ROS generation during freeze-thawing can increase DNA fragmentation [6,17], but no study has hitherto evaluated ROS generation and changes in nucleoprotein structure and chromatin integrity altogether during freeze-thawing, including the cooling step (5 °C).

Against this background, the present study sought to determine whether differences between GFE and PFE exist in terms of sperm chromatin packaging (assessed as levels of free cysteine radicals in sperm nucleoproteins, as a measure of the amounts of disrupted disulfide bonds, and DNA fragmentation) and ROS species, before starting the cryopreservation procedure (extended semen at 17 °C), after the cooling step (i.e., when sperm has been diluted in lactose and egg yolk (LEY) extender and cooled to 5 °C) and 30 and 240 minutes after thawing. In addition, other sperm function parameters, such as sperm motility, and membrane and acrosome integrity have also been assessed.

2. Materials and methods

2.1. Sperm samples

The experimental protocol was designed following the guidelines established by the Animal Welfare Directive of the Autonomous Government of Catalonia (Spain) and the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain).

Thirty-five ejaculates from different healthy adult boars (ages ranging between 18 months and 3 years old) were used in this study. Each ejaculate came from a different boar. Animals were housed in climate-controlled buildings, fed with an adjusted diet (2.3 kg per day) consisting of a basal diet and 1% premix for boars (P174N; TecnoVit, Tarragona, Spain), and provided with water *ad libitum*. Ejaculates were collected twice per week by the gloved-hand technique at a local farm (Servicios Genéticos Porcinos, S.L., Roda de Ter, Barcelona, Spain) with an interval of at least 3 days between collections. After removing the gelatinous fraction by filtering through gauze, the total volume of the sperm-rich fraction was diluted 1:5 (v:v) in a long-term extender (Duragen; Magapor S.L., Zaragoza, Spain). These diluted sperm-rich fractions were transported within 4 hours after the extraction in an insulated container and stored in our laboratory at 17 °C for 20 hours. The quality of the sperm samples was then evaluated to check that they satisfied the quality standard (i.e., total sperm motility >80%, progressive sperm motility >60%; morphologically normal spermatozoa >85%; sperm viability >85%; see [14]). Because the quality of the 35 ejaculates involved in this study was better than the set thresholds, they were frozen according to the experimental design described in section 2.2.

2.2. Experimental design

In this study, seven parameters (sperm motility, sperm viability, acrosome integrity, levels of peroxides and superoxides, amounts of free cysteine residues in sperm nucleoproteins, and sperm chromatin integrity) were examined for each ejaculate, at each of the four following cryopreservation steps: (1) before starting the cryopreservation procedure (i.e., at 17 °C); (2) at the end of the cooling step (i.e., after sperm had cooled at 5 °C in LEY extender for 120 minutes); (3) at 30 minutes after thawing (frozen-thawed spermatozoa [FT] 30 minutes); and (4) at 240 minutes after thawing (FT 240 minutes). For each ejaculate, a 20-mL aliquot was taken to assess all the mentioned sperm parameters in the first step (before starting the cryopreservation protocol, i.e., at 17 °C). The remaining volume of each ejaculate was cooled to 5 °C for 120 minutes and a 20-mL aliquot was then taken to assess the seven mentioned sperm parameters. Finally, the remaining sperm volume was cryopreserved and stored in liquid nitrogen at −196 °C for at least 2 months, for methodological purposes only. After thawing, samples were incubated for 30 or 240 minutes at 37 °C, before determining all the sperm parameters. These two time points (30 and 240 minutes) corresponded to the third and fourth steps of our experimental design, and were chosen to assess the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses [18]. The experimental design was replicated 35 times, using 35 different ejaculates, and each ejaculate came from a different boar.

Classification of ejaculates as GFE or PFE was performed when the seven parameters (i.e., sperm motility, sperm viability, acrosome integrity, levels of peroxides and superoxides, amounts of free cysteine radicals in sperm nucleoproteins, and sperm chromatin integrity) had been evaluated in all the ejaculates and in all the cryopreservation steps. Then, and on the basis of sperm viability and motility post-thawing assessments, both groups were set and further comparisons were performed to examine whether differences between GFE and PFE existed in terms of sperm motility, and membrane and acrosome integrity,

levels of peroxides and superoxides, amounts of free cysteine radicals in sperm nucleoproteins, and in sperm chromatin integrity throughout the four cryopreservation steps (extended semen [17 °C], cooled semen [5 °C], 30 minutes after thawing, and 240 minutes after thawing).

2.3. Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method [19] adapted by Yeste et al. [14]. All the ejaculates were centrifuged at 17 °C and 400× *g* for 5 minutes. Pellets were recovered with 3 to 4 mL of supernatant and diluted at 1.5×10^9 spermatozoa per mL (using a Makler counting chamber; Sefi-Medical Instruments, Haifa, Israel) in a freezing medium containing LEY. Spermatozoa were then cooled down to 5 °C for 120 minutes in a programmable freezer (Icecube14S-B; Minitüb Ibérica SL) with a cooling ramp of 0.1 °C/min, and an aliquot was taken for the assessment of the seven mentioned sperm parameters. This aliquot corresponded to the second step (i.e., 5 °C as stated in section 2.2.). The remaining volume of LEY solution was subsequently diluted at 1×10^9 spermatozoa/mL in a second medium (LEYGO) containing LEY with 6% glycerol and 1.5% Orvus ES Paste (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA). Final concentrations of glycerol and Orvus ES Paste in LEYGO medium were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitüb Ibérica, SL, Tarragona, Spain) and transferred to a programmable freezer (Icecube14S-B; Minitüb Ibérica, SL). The freezing program (SY-LAB software Version 1.0; Minitüb Ibérica SL) consisted of 313 seconds of cooling at the following rates: −6 °C/min from 5 °C to −5 °C (100 seconds), −39.82 °C/min from −5 °C to −80 °C (113 seconds), maintained for 30 seconds at −80°C, and finally cooled at −60 °C/min from −80 °C to −150 °C (70 seconds). The straws were then plunged into liquid N_2 (−196 °C) for storage.

After being in liquid N_2 for at least 2 months, four straws per ejaculate and treatment were thawed and diluted with three volumes of warmed Beltsville thawing solution at 37 °C (at a final dilution of 1:4). Each straw was shaken for 20 seconds under a water bath at 37 °C. After 30 and 240 minutes of thawing, all the sperm parameters were again assessed, the results corresponding to the third and fourth steps as stated in section 2.2.

2.4. Determination of sperm head free cysteine radicals

The determination of free cysteine radicals in sperm nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. [17]. Briefly, samples were centrifuged at 600× *g* at 17 °C for 20 minutes and resuspended in 50 mM ice-cold Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (wt:vol) sodium deoxycolate, 1 mM benzamidine, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenized using sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). Afterward, homogenates were centrifuged at 850× *g* at 4 °C for 20 minutes. The resultant supernatants and the upper layer of the pellet were discarded, and the pellets were subsequently resuspended in 500 μL of PBS. The purity of this separation was determined by observation using a phase-contrast microscope (Zeiss Primo Star; Karl Zeiss, Jena, Germany) at magnification ×40 (Zeiss Plan-Achromat 40×/0.65; Karl Zeiss). Sample purity was described as the percentage of loose heads in comparison with the presence of whole, nonfractioned sperm and separated tails in the sample. In all cases, the mean purity percentage was >95% of loose heads in comparison with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separating the heads from their respective midpieces.

The levels of free cysteine radicals in sperm nucleoproteins were determined in the samples obtained using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulfide; Sigma, Saint Louis, USA) as described by Brocklehurst et al. [20]. Briefly, the 10-μL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 μL of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine. The mixture was incubated at 37 °C for 60 minutes. Afterward, levels of free cysteine radicals were determined using spectrophotometric analysis at a wavelength of 343 nm. The results were normalized using a parallel determination of the total protein content of samples using the Bradford method [21], and a commercial kit (Quick Start Bradford Protein Assay; BioRad, Hercules, California, USA). Three replicates per sample and cryopreservation step were evaluated, and the corresponding mean ± SEM was subsequently calculated.

2.5. DNA fragmentation

DNA fragmentation was assessed using a sperm chromatin dispersion test specifically designed for boar spermatozoa (Sperm-Halomax-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain) following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this technique strongly correlated with those obtained with other tests, like the neutral comet assay [22].

Briefly, the lysing buffer included in the commercial kit was tempered to 22 °C and vials containing low-melting agarose were incubated at 100 °C for 5 minutes in a water bath. Vials were then left in another water bath at 37 °C for 5 minutes to equilibrate the agarose temperature. Twenty-five microliters of each sperm sample (at a final concentration of 10^7 spermatozoa/mL) were added to a vial before mixing it thoroughly. One 25 μL drop containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid air bubble formation.

Slides were placed on a cooled plate in a refridgerator and left at 4 °C for 5 minutes. The coverslip was then removed and 50 μL of lysis solution per slide was added. An incubation step at 22 °C for 5 minutes was performed, before washing for 5 minutes with milli-Q water (Millipore). The slides were subsequently dehydrated using three steps of 2 minutes each with ethanol at 70%, 90%, and 100%. Finally, sperm samples were stained with propidium iodide

(PI, 2.5 μg/mL; Molecular Probes, Eugene, Oregon, USA) and mounted in DABCO antifading medium (Sigma-Aldrich, St. Louis, MO, USA). Samples were observed using an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at magnification ×1000.

Three counts of 250 spermatozoa each using three different slides were carried out per sample, before calculating the corresponding mean ± SEM. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only a small halo.

2.6. Flow cytometric analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry [23]. These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), acrosome integrity, and ROS levels in GFE and PFE at each of the four mentioned steps. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described herein (i.e., SYBR-14/PI, Peanut agglutinin [PNA]-fluorescein isothiocianate [FITC]/PI, 2′,7′-dichlorodihydrofluorescein diacetate [H_2DFCDA]/PI, hydroethidine [HE]/YO-PRO-1,or PI after hypotonic treatment to correct raw data).

Samples were evaluated using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, California, USA; Serial number AL300087; the technical specifications are available at http://www.beckmancoulter.com). This instrument, which had not been altered from the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter and volume was directly measured with the Cell Lab Quanta SC cytometer using the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by nonconductive particles suspended in an electrolyte solution. This system has thus forward scatter replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2, and FL3. In this system, the optical characteristics for these filters were: FL1 (green fluorescence): Dichroic/Splitter, dichroic long-pass: 550 nm, band-pass filter: 525 nm, detection width 505 to 545 nm; FL2 (orange fluorescence): dichroic long-pass: 600 nm, band-pass filter: 575 nm, detection width: 560 to 590 nm; and FL3 (red fluorescence): long pass filter: 670/630 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL1 was used to detect green fluorescence (SYBR-14, PNA-FITC, YO-PRO-1, and H_2DFCDA), and FL3 was used to detect red (HE and PI) fluorescence.

Sheath flow rate was set at 4.17 μL/min in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EV vs. SS dot plots) for a minimum of 10,000 events per replicate. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter <7 μm) and cell aggregates (particle diameter >12 μm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated based on EV and SS distributions, and the others were gated out. In some protocols, as described below, compensation was used to minimize spillover of green fluorescence into the red channel.

The event information was collected in List-mode Data files (.LMD). These generated files were then analyzed using Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyze the cytometric histograms. In all cases except for the SYBR-14/PI assessment, data obtained from flow cytometry experiments at cooling and postthawing steps were corrected according to the procedure described by Petrunkina et al. [24]. Each assessment for each sample and parameter was repeated three times in independent tubes, before calculating the corresponding mean ± SEM.

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes (Invitrogen; Eugene, Oregon, USA) and were diluted with dimethyl sulfoxide (DMSO; Sigma).

2.6.1. Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner and Johnson [25]. Briefly, sperm samples were incubated at 38 °C for 10 minutes with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 μM for 5 minutes and at the same temperature. FL1 was used for measuring the SYBR-14 fluorescence, and PI fluorescence was detected using FL3. After this assessment, three sperm populations were identified: (1) viable green-stained spermatozoa (SYBR-14^+/PI^-); (2) nonviable red-stained spermatozoa (SYBR-14^-/PI^+); and (3) nonviable spermatozoa that were stained green and red (SYBR-14^+/PI^+). Nonsperm particles (debris) were found in the SYBR-14^-/PI^- quadrant.

Single-stained samples were used for setting the EV gain, FL1 and FL3 photomultiplier tubes (PMT)-voltages, and for compensation of SYBR-14 spillover into the PI channel (2.45%).

2.6.2. Acrosome integrity (PNA-FITC/PI)

Acrosome integrity was assessed by costaining the spermatozoa with the lectin from *Arachis hypogaea* (peanut agglutinin) conjugated with FITC and PI, according to the procedure described by Nagy et al. [26]. Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5 μg/mL) and PI (final concentration: 10 μM) and incubated at 38 °C for 5 minutes. PNA-FITC fluorescence was collected using FL1 and PI fluorescence was detected using FL3. Spermatozoa were identified and placed in one of the four following populations: (1) viable spermatozoa with intact acrosome (PNA-$FITC^-$/PI^-); (2) viable spermatozoa with damaged

(exocytosed) acrosome (PNA-FITC$^+$/PI$^-$); (3) nonviable cells with intact acrosome (PNA-FITC$^-$/PI$^+$); and (4) nonviable cells with damaged acrosome (PNA-FITC$^+$/PI$^+$).

Unstained and single-stained samples were used for setting the EV gain, FL1 and FL3 PMT-voltages, and for compensation of PNA-FITC spillover into the PI channel (2.45%).

2.6.3. Assessment of oxidative stress: Peroxides (H_2DFCDA/PI) and superoxides (HE/YO-PRO-1)

Reactive oxygen species levels were determined using two different oxidation-sensitive fluorescent probes: H_2DFCDA and HE, to analyze the intracellular content of peroxides (H_2O_2) and superoxide anions ($O_2^{-\bullet}$), respectively. Following a procedure modified from Guthrie and Welch [12], a simultaneous differentiation of viable from nonviable spermatozoa was performed by costaining the spermatozoa either with PI or with YO-PRO-1.

In the first case, spermatozoa were stained with H_2DFCDA at a final concentration of 200 μM and PI at a final concentration of 10 μM and incubated at 25 °C for 60 minutes in the dark. 2′,7′-Dichlorodihydrofluorescein diacetate is a stable cell-permeable nonfluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) on oxidation [12]. This DCF fluorescence was collected using FL1, and PI fluorescence was detected using FL3. Measurements were expressed as the geometric mean of green intensity fluorescence units (GMFI; geometric mean in FL1) and this was used as the index of ROS generation. Unstained and single-stained samples were used for setting the EV gain, FL1 and FL3 PMT-voltages, and data were not compensated.

In the second probe, samples were stained with HE (final concentration: 4 μM) and with YO-PRO-1 (final concentration: 40 μM) and incubated at 25 °C for 40 minutes in the dark [12]. Hydroethidine is freely permeable to cells and it is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of E (E^+) was detected using FL3 and that of YO-PRO-1 was collected using FL1. Data were expressed as the percentage of viable sperm with high $O_2^{-\bullet}$ (high E^+) and the geometric mean of red-intensity fluorescence (geometric mean channel in the FL3). Data were not compensated.

2.6.4. Correction of data: Identification of non-DNA-containing particles

The percentage of non-DNA-containing particles (alien particles) was determined because in some flow cytometry assessments, especially when working with cryopreserved spermatozoa, there might be an overestimation of sperm particles. Indeed, alien particles such as cytoplasmic droplets, cell debris, or diluent components (as egg yolk), will often show EV/forward scatter and SS characteristics similar to those of spermatozoa and can not thus be excluded via light scatter [24]. For this reason, 5 μL of each sperm sample from cooling or after thawing steps were diluted with 895 μL of milli-Q distilled water. Samples were then stained with PI at a final concentration of 10 μM and incubated at 38 °C for 3 minutes, according to the procedure described by Petrunkina et al. [24]. Percentages of alien particles (f) were used to correct the percentages of nonstained spermatozoa (q_1) in each sample and dual-staining analysis, except in the SYBR-14/PI assay (i.e., PNA-FITC/PI, H_2DFCDA/PI, and HE/YO-PRO-1), according to the following formula:

$$q_1^{'} = \frac{q_1 - f}{100 - f} \times 100$$

where $q_1^{'}$ is the percentage of nonstained spermatozoa after correction.

2.7. Sperm motility

Sperm motility analysis was performed using a commercial computer-assisted sperm analysis system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system was based on the analysis of 25 consecutive digitalized photographic images obtained from a single field at magnification ×100 in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10x 0.30 PLAN objective lens). These 25 consecutive photographs were taken in a time-lapse of 1 second, which implied a velocity of image capturing of one photograph every 40 msec. Five to six separate fields were taken for each replicate, and three replicates were run per sample.

For each assessment, 15 μL of sperm sample were placed in a Makler counting chamber (Sefi-Medical Instruments), and total and progressive motility together with other kinetic parameters were recorded [14]. Total motility was defined as the percentage of spermatozoa that showed a average path velocity >10 μm/s, and progressive motility was defined as the percentage of spermatozoa that showed a average path velocity >45 μm/s. In the case of extended (17 °C) and cooled (5 °C) sperm, samples were incubated at 37 °C for 15 minutes before evaluation of sperm motility.

2.8. Sperm morphology

As stated, sperm morphology was assessed on arrival of seminal samples to the lab to verify that they satisfied the quality standard (i.e., morphologically normal spermatozoa >85%). For this purpose, 5 μL of each semen sample was placed on a slide and mounted with a cover slip. Slides were then incubated for 30 minutes in 100% humidity at 25 °C to immobilize the spermatozoa. Sperm morphology was assessed subjectively by making three counts of 100 spermatozoa each, before calculating the corresponding mean ± SEM and differentiating between morphologically normal spermatozoa, spermatozoa with cytoplasmic droplets, and aberrant spermatozoa (coiled tails, tails folded at the connecting piece, at the intermediate piece, or at the Jensen's ring). A phase contrast microscope (Olympus BX41) was used, and the samples observed at magnification ×200 (Olympus 20x 0.40 PLAN objective, positive phase-contrast field).

2.9. Statistical analyses

Statistical analyses were performed using IBM SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) and data are presented as mean ± SEM. The data obtained from the analysis of all sperm parameters were tested for normality

and homoscedasticity using the Shapiro-Wilk and Levene tests. The present study was developed with 35 ejaculates from 35 different boars. In all statistical analyses, the minimal level of significance was set at $P < 0.05$.

2.9.1. Obtaining GFE and PFE groups

After evaluating the seven mentioned parameters in all 35 ejaculates and throughout the four cryopreservation steps, GFE and PFE groups were set by running a hierarchical cluster analysis for dissimilarities that used the values obtained for sperm progressive motility (Table 1) and for sperm viability at 30 and 240 minutes after thawing, following the procedure described by Casas et al. [7]. This procedure consisted of calculating the chi-square frequencies from the sperm progressive motility and the sperm viability at 30 and 240 minutes after thawing, and constructed a dissimilarity dendrogram as a result.

2.9.2. Comparisons between GFE and PFE during freeze-thawing

After separating the ejaculates into the two groups, the sperm parameters of GFE and PFE were compared with independent sample *t* tests for repeated measures, where the intersubject factor was the freezability ejaculate group (i.e., GFE vs. PFE) and the intrasubject factor was the cryopreservation step (i.e., before starting cooling, at cooling to 5 °C, FT after 30 minutes, and FT after 240 minutes).

3. Results

3.1. Differences between GFE and PFE in terms of free cysteine radicals in sperm nucleoproteins during freeze-thawing

After evaluating postthaw sperm motility and viability of the 35 ejaculates, 19 were classified as GFE, and the other 16 classified to the PFE group. The classification of ejaculates obtained at 30 minutes after thawing agreed with that obtained at 240 minutes after thawing (data not shown).

Table 1
Percentages of acrosome-intact spermatozoa (PNA^-/PI^-), total (TMOT), and progressive motile (PMOT) spermatozoa during freeze-thawing.

Sperm parameter	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
% Spermatozoa PNA^-/PI^-				
GFE	87.5 ± 3.4^a	81.8 ± 3.2^a	50.9 ± 2.2^b	35.1 ± 1.6^c
PFE	85.4 ± 3.5^a	80.0 ± 3.1^a	36.2 ± 1.8^c	18.7 ± 0.9^d
% Total sperm motility				
GFE	89.3 ± 4.1^a	84.1 ± 3.8^a	62.5 ± 2.9^b	42.8 ± 2.0^c
PFE	87.5 ± 3.9^a	81.7 ± 3.8^a	45.2 ± 2.1^c	27.6 ± 1.3^d
% PMOT				
GFE	68.5 ± 3.1^a	59.7 ± 2.9^b	38.2 ± 1.7^d	25.6 ± 1.2^e
PFE	67.9 ± 3.0^a	50.6 ± 2.4^c	27.8 ± 1.3^e	12.1 ± 0.6^f

Different superscript letters (a–f) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30, and FT 240 minutes) within the same parameter (% spermatozoa PNA^-/PI^-, % TMOT, % PMOT). Abbreviations: FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; PFE, poor freezability ejaculate.

Regarding free cysteine radicals in sperm nucleoproteins during cryopreservation, freeze-thawing increased the levels of free cysteine residues in sperm nucleoproteins, which were 3.02 ± 0.22 nmol/μg protein (mean ± SEM) in GFE before freezing and was 7.05 ± 0.45 nmol/μg protein in GFE at 240 minutes after thawing (Fig. 1). This increase was not observed at the end of the cooling step, but only after 30 and 240 minutes after thawing. When comparing GFE with PFE, no significant differences were found either before starting the cryopreservation procedure (i.e., extended semen at 17 °C) or at the end of the cooling step (5 °C). In contrast, the levels of free cysteine residues in sperm nucleoproteins were significantly ($P < 0.001$) higher in PFE than in GFE, both at 30 and 240 minutes after thawing (Fig. 1).

3.2. Differences in chromatin integrity between GFE and PFE during freeze-thawing

Freeze-thawing increased the percentage of spermatozoa with fragmented DNA after thawing, but GFE and PFE differed in the time point at which this increase was observed (Fig. 2). Thus, whereas the percentage of spermatozoa with fragmented DNA in PFE was significantly higher ($P < 0.05$) than that of extended semen (17 °C) at 30 and 240 minutes after thawing, sperm DNA fragmentation in the GFE group only increased significantly at, but not before, 240 minutes after thawing. In addition, the levels of sperm DNA fragmentation between GFE and PFE did not significantly differ either at 17 °C or after being cooled to 5 °C for 120 minutes (Fig. 2). Nevertheless, the percentages of spermatozoa with fragmented DNA in PFE were significantly higher than those observed in GFE, both at 30 ($P < 0.05$) and 240 minutes ($P < 0.001$) after thawing.

3.3. Differences in sperm viability between GFE and PFE during freeze-thawing

Sperm viability (% of spermatozoa $SYBR\text{-}14^+/PI^-$) dramatically decreased ($P < 0.01$) after thawing (i.e., at FT

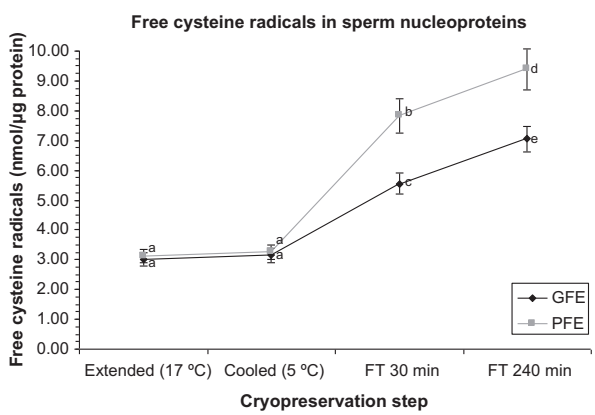

Fig. 1. Free cysteine radicals in sperm head proteins (as an indicator of disrupted disulfide bonds) in GFE and PFE during freeze-thawing. Different letters (a–e) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30 and FT 240 minutes). FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; PFE, poor freezability ejaculate.

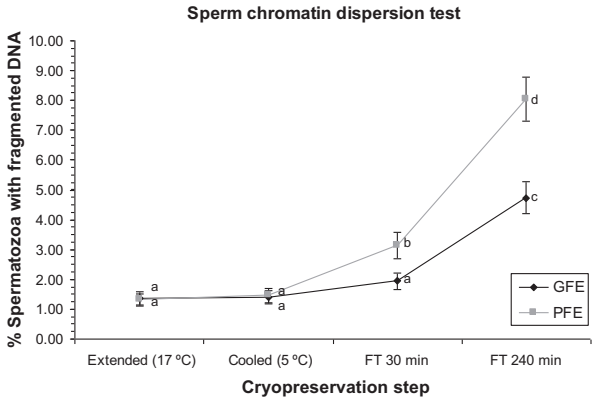

Fig. 2. Percentage of spermatozoa with fragmented DNA in GFE and PFE during freeze-thawing. Different letters (a–d) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30, and FT 240 minutes). FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; PFE, poor freezability ejaculate.

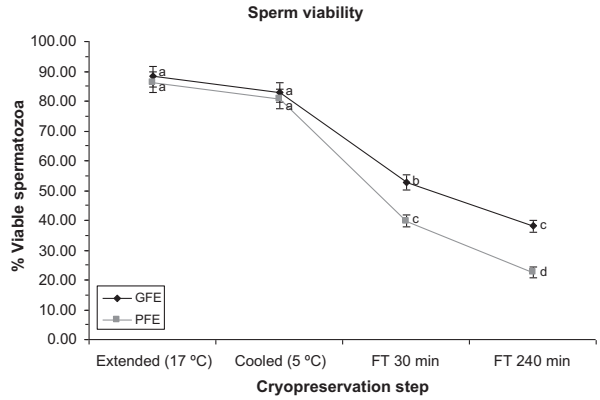

Fig. 3. Percentage of viable spermatozoa (SYBR-14$^+$/PI$^-$) in GFE and PFE during freeze-thawing. Different letters (a–d) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30, and FT 240 minutes). FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; PFE, poor freezability ejaculate.

30 and FT 240 minutes) in GFE and PFE, as shown in Figure 3 (mean ± SEM). Moreover, significant differences ($P < 0.01$) between GFE and PFE were only observed after thawing, but neither before starting cryopreservation (i.e., extended semen at 17 °C) nor at the cooling step (i.e., at 5 °C).

3.4. Differences in sperm motility between GFE and PFE during freeze-thawing

Total sperm motility underwent a significant ($P < 0.01$) decrease at 30 and 240 minutes after thawing in GFE and PFE (Table 1). Furthermore, PFE presented a significant ($P < 0.05$) lower percentage of total motile spermatozoa than GFE at 30 and 240 minutes after thawing.

In contrast, sperm progressive motility gradually decreased after starting the cryopreservation process, because the percentages of progressive motile spermatozoa at the cooling step were significantly ($P < 0.05$) lower than those observed before starting the cryopreservation process, and those observed at 30 and 240 minutes after thawing were significantly lower ($P < 0.05$) than those found at the cooling step (Table 1). In addition, the percentages of progressive motile spermatozoa were not only significantly ($P < 0.05$) higher in GFE than in PFE at 30 and 240 minutes after thawing but also at the cooling step.

3.5. Differences in acrosome integrity of spermatozoa between GFE and PFE during freeze-thawing

Similar to the observed sperm viability assessment, a significant ($P < 0.01$) decrease in the percentage of acrosome-intact spermatozoa was observed after, and not before, thawing (Table 1). In contrast, percentages of spermatozoa with an intact acrosome in extended (17 °C) and cooled (5 °C) semen did not differ between GFE and PFE, and percentages of acrosome-intact spermatozoa in GFE were significantly ($P < 0.05$) higher than those observed in PFE at 30 and 240 minutes after thawing.

3.6. Differences in peroxide and superoxide levels of spermatozoa between GFE and PFE during freeze-thawing

Table 2 shows the percentage of viable spermatozoa with high peroxide levels (DCF$^+$/PI$^-$) and GMFI in the FL1 channel in viable (DCF$^+$/PI$^-$) and total (DCF$^+$) spermatozoa. No significant differences ($P > 0.05$) were observed between GFE and PFE in any of the cryopreservation steps.

Table 3 shows the percentage of viable spermatozoa with high superoxide levels (E$^+$/YO-PRO-1$^-$) and GMFI in the FL3 channel in viable (E$^+$/YO-PRO-1$^-$) and total (E$^+$) spermatozoa. The percentage of viable spermatozoa with high superoxide levels (E$^+$/YO-PRO-1$^-$) did not differ among the four cryopreservation steps. In contrast, the GMFI (FL3) in viable spermatozoa with high superoxide levels (E$^+$/YO-PRO-1$^-$) progressively decreased from the

Table 2
Peroxide (H_2O_2) levels in boar spermatozoa during freeze-thawing.

Sperm parameter	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
% Spermatozoa DCF$^+$/PI$^-$				
GFE	2.5 ± 0.2^a	2.3 ± 0.2^a	3.9 ± 0.3^b	1.5 ± 0.1^c
PFE	2.6 ± 0.2^a	2.3 ± 0.2^a	3.4 ± 0.3^b	1.4 ± 0.1^c
GMFI (FL1) DCF$^+$/PI$^-$				
GFE	89.4 ± 4.7^a	92.7 ± 5.1^a	110.3 ± 5.9^b	49.2 ± 2.8^c
PFE	90.5 ± 4.9^a	94.8 ± 5.3^a	108.4 ± 5.6^b	51.8 ± 3.0^c
GMFI (FL1) DCF$^+$				
GFE	85.1 ± 4.4^a	86.8 ± 4.9^a	102.2 ± 5.4^b	52.9 ± 3.1^c
PFE	83.7 ± 4.3^a	89.4 ± 4.8^a	97.5 ± 5.2^b	47.1 ± 2.5^c

Different superscript letters (a–c) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30, and FT 240 minutes) within the same parameter, i.e., % spermatozoa DCF$^+$/PI$^-$, GMFI (FL1) DCF$^+$/PI$^-$ (viable spermatozoa with high H_2O_2), and GMFI (FL1) DCF$^+$ (total spermatozoa).

Abbreviations: FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; GMFI, geometric mean of fluorescence intensity (arbitrary units); PFE, poor freezability ejaculate.

Table 3
Superoxide ($O_2^{-\bullet}$) levels in boar spermatozoa during freeze-thawing.

Sperm parameter	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
% Spermatozoa E^+/YO-PRO-1^-				
GFE	3.4 ± 0.3[a]	2.9 ± 0.2[a]	3.3 ± 0.3[a]	3.5 ± 0.3[a]
PFE	3.5 ± 0.3[a]	3.1 ± 0.2[a]	3.5 ± 0.3[a]	3.7 ± 0.3[a]
GMFI (FL3) E^+/YO-PRO-1^-				
GFE	106.9 ± 6.0[a]	87.8 ± 4.9[b]	69.4 ± 3.8[c]	71.5 ± 4.2[c]
PFE	110.7 ± 6.3[a]	90.1 ± 5.1[b]	73.2 ± 4.5[c]	76.4 ± 4.3[c]
GMFI (FL3) E^+				
GFE	125.6 ± 7.3[a,b]	121.5 ± 7.1[a]	134.1 ± 7.7[a,b]	136.2 ± 7.5[b]
PFE	130.4 ± 7.4[a,b]	124.8 ± 7.3[a]	140.7 ± 7.9[b]	143.9 ± 8.0[b]

Different superscript letters (a–c) indicate significant differences ($P < 0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e., extended at 17 °C, cooled at 5 °C, FT 30, and FT 240 minutes) within the same parameter, i.e., % spermatozoa E^+/YO-PRO-1^-, GMFI (FL3) E^+/YO-PRO-1^- (viable spermatozoa with high $O_2^{-\bullet}$), GMFI (FL3) E^+ (total spermatozoa).
Abbreviations: FT, frozen-thawed spermatozoa; GFE, good freezability ejaculate; GMFI, geometric mean of fluorescence intensity (arbitrary units); PFE, poor freezability ejaculate.

cooling step (5 °C) to 240 minutes after thawing. The geometric mean of E^+ fluorescence intensity in total spermatozoa was significantly ($P < 0.05$) higher at 240 minutes after thawing than at the first two steps (i.e., extended semen at 17 °C and the cooling step at 5 °C). Despite these changes, no significant differences were found in any of these three parameters between GFE and PFE during the entire cryopreservation procedure (Table 3).

4. Discussion

The results shown in this work clearly indicate that boar sperm cryotolerance is concomitant with the resistance of these cells to sustain alterations in their nucleoprotein structure during freeze-thawing. This is evident when comparing the levels of free cysteine radicals in sperm nucleoproteins between GFE and PFE after thawing. On the contrary, boar sperm resistance to cryopreservation does not appear to be concomitant with differences in the ability of sperm to modulate their ROS intracellular levels, because changes in ROS during freeze-thawing are the same when comparing GFE and PFE. These results could seem to be a paradox, because previous work has suggested that ROS generation during boar sperm cryopreservation can induce damage in sperm chromatin [6,17], in a similar fashion to that which occurs in other species [27–29]. However, levels of DNA fragmentation in boar spermatozoa after freeze-thawing, as a method to evaluate nuclear cryodamage, are very low [6,14,17]. These very low values make it difficult to establish a clear relationship between chromatin damage and ROS levels. In contrast, the disruption of disulfide bonds between nucleoproteins induced by boar sperm cryopreservation is much more intense than the damage inflicted on DNA integrity [14]. For this reason, the disruption of disulfide bonds between boar sperm nucleoproteins can be regarded as a more sensitive parameter to determine nuclear cryodamage than DNA fragmentation. In this regard, we must bear in mind that disulfide bonds play a main role in stabilizing the protein conformation and in the union between protamines [30]. When osmotic conditions are greatly modified, as it is the case of boar sperm cryopreservation, disulfide bonds can be weakened and disrupted, thereby causing irreversible damage to the nucleoprotein structure [31].

Our results strongly suggest that ejaculate freezability is related to the integrity of nucleoprotein structure, because the disruption of disulfide bonds between boar sperm nucleoproteins caused by freeze-thawing is better counteracted in GFE than in PFE. Although the mechanism that explains the higher resistance of such disulfide bonds in GFE than in PFE still remains to be elucidated, we suggest that these differences could be explained by a defective spermatogenesis and/or epididymal maturation in the spermatozoa of PFE, because at these steps histones are replaced by protamines and disulfide bonds between and within protamines are formed [32]. In contrast, another possible explanation would be related to the higher amount of HSP90AA1 and other proteins [33] in GFE than in PFE, which might protect sperm cells from cold and osmotic shock.

Another type of cryopreservation-induced damage that affects the sperm nucleus is DNA fragmentation. However, as indicated above, the extent of this damage differs among species. Thus, whereas cryopreservation clearly increases sperm DNA fragmentation in some species, like bulls [34] and stallions [29], in others, such as rams [35] and boars [14,17], this increase is more moderate and does not appear immediately after thawing but only after 2 to 4 hours of post-thawing incubation at 37 °C. Interestingly, in the present work a significant cryopreservation-induced increase in sperm DNA fragmentation was observed earlier in PFE (at 30 minutes after thawing) than in GFE (at 240 minutes after thawing). These results, together with other studies [14], allow us to hypothesize that the destabilization of nucleoprotein structure, because of the disruption of disulfide bonds observed after freeze-thawing, seems to underlie the subsequent increase of DNA fragmentation in boar spermatozoa. Indeed, the fragility of nucleoprotein structure in the case of PFE, clearly detected in the levels of free cysteine residues at 30 minutes after thawing, seems to underlie the chromatin fragmentation observed at the same time point. In contrast, GFE contained lower levels of free cysteine radicals than PFE at 30 minutes after thawing, and the increase in sperm DNA fragmentation in GFE is only observed at 240 minutes after thawing. In fact, less inter- and intraprotamine interactions make DNA more susceptible to damage [36], because reduced chromatin packaging leads to lower

resistance against strong acids, proteases, DNases and/or detergents [37]. In addition, at 240 minutes after thawing, there are higher levels of leakage products from very significant and increasing numbers of dead and dying spermatozoa, including nucleases released from spermatozoa with damaged plasma membranes, that can fragment DNA [38]. Moreover, there can also be traces of components of the cryopreservation medium, including glycerol and yolk particles, which can be detrimental for spermatozoa. Thus, we suggest that all these detrimental products might directly damage sperm chromatin and/or accelerate DNA fragmentation on destabilization of the nucleoprotein structure, especially in the case of PFE that clearly present a lower resistance to freeze-thawing. Finally, it is worth noting that the observed alterations in the nucleoprotein structure of the boar sperm head might affect the sperm's fertilizing ability, because protamines play a critical role for proper sperm chromatin packaging [30] and protamine-deficient sperm adopt a less stable chromatin structure [36]. Related to this, ejaculates classified as GFE have been reported to present higher rates of oocyte penetration, cleavage, and of blastocyst formation than those classified as PFE [39]. This could also be related to the lower amounts of disrupted disulfide bonds between and within sperm nucleoproteins, and with the lesser degree of chromatin fragmentation in GFE than in PFE observed in our study.

Some previous reports have suggested that ROS could be one of the contributors to cryopreservation-induced DNA fragmentation in boar spermatozoa [6,40]. In fact, in other species like humans, there is a significant positive correlation between DNA fragmentation and ROS [41]. In the present study, however, we have found significant differences in the integrity of nucleoprotein structure and DNA fragmentation between GFE and PFE, but not in ROS levels. Although the main cause of DNA damage in frozen-thawed spermatozoa is still open for discussion [40], our results do not support the hypothesis that ROS is the main cause of cryopreservation-induced DNA fragmentation in boar sperm. Notwithstanding, basal ROS formation and membrane lipid peroxidation in the absence of ROS generators are quite low in fresh and frozen-thawed boar spermatozoa, in contrast to what happens in other species, like human, cattle, and poultry [42]. Our data also match with another previous study from our group [14], in which we observed that even though procaine hydrochloride and reduced glutathione protected the nucleoprotein structure of boar sperm during freeze-thawing to a similar extent, only the latter reduced peroxide generation. This indicates that the beneficial action of procaine hydrochloride on the sperm nucleus is independent from modulating ROS generation, and supports our current observations in GFE and PFE, because both groups differ in the extent of damage in the sperm nucleus but not in the ROS levels.

Previous studies have demonstrated that ROS generation and lipid peroxidation occur during cryopreservation in human [43], horse [28], bull [44], and dog [45] spermatozoa. In boar sperm, however, ROS production linked to freeze-thawing procedures is quite low [12–14,46], and small differences are only observed after thawing. In our study, freeze-thawing slightly increased the percentage of viable spermatozoa with high levels of H_2O_2, but no significant differences were found when comparing GFE with PFE in any of the freeze-thawing steps, matching the results of Gómez-Fernández et al. [16].

With regard to superoxides, there was a significant reduction in the GMFI in viable spermatozoa (E^+/YO-PRO-1^-) after cooling (5 °C) and after thawing, in agreement with Awda et al. [46] and Kim et al. [13]. However, we did not find significant differences between GFE and PFE. These data might support the results of Casas et al. [7] who did not find significant differences in Cu/Zn superoxide dismutase content between GFE and PFE. In fact, previous reports have suggested that the endogenous ROS defense system in boar sperm is either very efficient or unchallenged during cryopreservation [47] and that boar spermatozoa has a substantial amount of intracellular superoxide dismutase for scavenging $O_2^{-\bullet}$ and for rapidly dismutating $O_2^{-\bullet}$ to H_2O_2 [13]. Another possible explanation for our results is that levels of $O_2^{-\bullet}$ would not be affected by cryopreservation, because $O_2^{-\bullet}$ presents a very short life and is too polar to penetrate intact plasma membranes [27].

As expected, GFE and PFE differed in plasma membrane (sperm viability) and acrosome integrity after freeze-thawing but not before starting cryopreservation (i.e., extended semen at 17 °C) and at the cooling step. Thus, sperm viability and acrosome integrity decreased in both groups at 30 and 240 minutes after thawing, but the impairment in PFE was higher than in GFE. In terms of sperm motility, significant differences between GFE and PFE were observed in the percentage of progressive motile spermatozoa at the cooling and post-thawing steps. This matches results of other previous studies that have reported differences in post-thaw sperm motility between breeds [3] and ejaculates [7].

Finally, and as previously mentioned, one of the difficulties of boar sperm preservation is to predict the ejaculate freezability before starting the cryopreservation process. Indeed, several times, the variations in semen freezability cannot be detected by a standard spermiogram of the ejaculate before freezing [7,33]. In this regard, previous reports have found that some motility parameters (such as percentage of linearity, percentage of straightness, and progressive motility) differ between GFE and PFE at the cooling step (5 °C) and at 240 minutes after thawing [7], and that the HSP90AA1 content in sperm can be used as freezability marker before starting cryopreservation [33]. However, and in light of our results, levels of chromatin fragmentation and number of cysteine free residues in sperm nucleoproteins are not good predictors of ejaculate freezability, because GFE and PFE do not differ in these two parameters either before starting cryopreservation (i.e., extended semen at 17 °C) or at the cooling step (5 °C).

4.1. Conclusions

In conclusion, freeze-thawing of boar spermatozoa impaired sperm motility and membrane and acrosome integrity, destabilized nucleoprotein structure by disrupting disulfide bonds, and increased the levels of DNA fragmentation, although the extent of this damage was higher in PFE than in GFE after thawing, but not at the cooling step.

In addition, our results indicate that variations in the sperm nuclear damage in GFE and PFE are not directly related to the generation of peroxides and superoxides during cryopreservation.

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ARTICULO 3

The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability.

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The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability

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Reduced glutathione (GSH) improves boar sperm cryosurvival and fertilising ability when added to freezing extenders. Poor freezability ejaculates (PFE) are known to present lower resistance than good freezability ejaculates (GFE) to cryopreservation procedures. So far, no study has evaluated whether the ability of GSH to counteract the cryopreservation-induced injuries depends on ejaculate freezability (i.e. GFE *vs.* PFE). For this reason, thirty boar ejaculates were divided into three equal volume fractions and cryopreserved with or without GSH at a final concentration of either 2mM or 5mM in freezing media. Before and after freeze-thawing, sperm quality was evaluated by analysis of viability, motility, integrity of outer acrosome membrane, DNA fragmentation, integrity of nucleoprotein structure, and ROS levels. Ejaculates were classified into two groups (GFE or PFE) according to their post-thaw sperm motility and viability assessments. Values of each sperm parameter were then compared between treatments (GSH 0mM, GSH 2mM, GSH 5mM) and freezability groups (GFE, PFE). In the case of GFE, GSH significantly improved boar sperm cryotolerance, without differences between 2mM and 5mM.. In contrast, PFE freezability was significantly increased when supplemented with GSH 5mM when compared with GSH 2mM. In conclusion, PFE semen doses need a higher concentration of GSH than GFE to reach similar protective effects.

Keywords: Boar semen; sperm cryopreservation; good and poor freezability ejaculates; reduced glutathione; cryotolerance.

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The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability ☆

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ABSTRACT

Reduced glutathione (GSH) improves boar sperm cryosurvival and fertilising ability when added to freezing extenders. Poor freezability ejaculates (PFE) are known to present lower resistance than good freezability ejaculates (GFE) to cryopreservation procedures. So far, no study has evaluated whether the ability of GSH to counteract the cryopreservation-induced injuries depends on ejaculate freezability (i.e. GFE *vs.* PFE). For this reason, thirty boar ejaculates were divided into three equal volume fractions and cryopreserved with or without GSH at a final concentration of either 2 or 5 mM in freezing media. Before and after freeze–thawing, sperm quality was evaluated through analysis of viability, motility, integrity of outer acrosome membrane, ROS levels, integrity of nucleoprotein structure, and DNA fragmentation. Ejaculates were classified into two groups (GFE or PFE) according to their post-thaw sperm motility and viability assessments in negative control (GSH 0 mM), after running cluster analyses. Values of each sperm parameter were then compared between treatments (GSH 0 mM, GSH 2 mM, GSH 5 mM) and freezability groups (GFE, PFE). In the case of GFE, GSH significantly improved boar sperm cryotolerance, without differences between 2 and 5 mM. In contrast, PFE freezability was significantly increased when supplemented with 5 mM GSH, but not when supplemented with 2 mM GSH. In conclusion, PFE need a higher concentration of GSH than GFE to improve their cryotolerance.

Introduction

Sperm cryopreservation is currently the most efficient method for long-term storage of boar spermatozoa [11]. However, freeze–thawing protocols are known to affect boar sperm function and survival and reduce sperm fertilising ability [38]. Indeed, sperm cryopreservation damages plasma membrane and acrosome, and reduces sperm motility [15,19,21]. In addition, freeze–thawing of boar spermatozoa also alters the sperm nucleus through destabilization of its nucleoprotein structure by disrupting disulfide bonds rather than increasing DNA fragmentation [41].

As other mammalian species, the sperm ability to sustain cryopreservation procedures, namely freezability, differs between individuals and ejaculates coming from the same boar [22,30,31,37]. In this way, ejaculates can be classified into 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE; see [9]). These two groups have been reported to differ in their resistance to cryopreservation as evaluated through several sperm parameters, like viability, acrosome integrity, motility, or nucleoprotein structure [42].

The addition of antioxidants has been reported to partially counteract the deleterious effects that cryopreservation protocols may inflict upon boar spermatozoa [43]. In this context, reduced glutathione (GSH) has been shown to improve boar sperm cryotolerance, as protects nucleoprotein structure, maintains better sperm motility and viability [17,41], and increases the fertilising ability of frozen–thawed (FT) spermatozoa [14]. It is worth mentioning that this antioxidant is one of the most abundant thiol in live cells and plays a prominent role in maintaining intracellular redox balance [23].

To date, no study has been conducted to evaluate whether the intrinsic freezability of a given boar ejaculate affects the ability

Abbreviations: GFE, good freezability ejaculates; PFE, poor freezability ejaculates; ROS, reactive oxygen species; GSH, reduced glutathione; FT, Frozen–thawed spermatozoa.

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of GSH to improve sperm cryosurvival. Against this background, the main aim of the present study was to evaluate whether the improving effects of supplementing freezing extenders with GSH are of different intensity when comparing GFE with PFE. With this purpose, a total of 30 ejaculates were frozen–thawed in the absence (negative control, 0 mM) or presence of either 2 mM GSH or 5 mM GSH. Ejaculates were classified into GFE and PFE groups according to post-thaw sperm viability and motility of their respective 0 mM GSH aliquots. In addition, other sperm parameters were analysed. These parameters were sperm chromatin packaging, assessed as levels of free cysteine radicals in sperm nucleoproteins and DNA fragmentation, integrity of the outer acrosome membrane, and ROS levels. Sperm analysis of the FT samples was performed at four cryopreservation steps: before starting cryopreservation, after cooling step of the cryopreservation procedure (5 °C), and at 30 and 240 min post-thawing. Results indicate that PFE require higher concentrations of GSH than GFE to increase their resistance against cryoinjuries. This suggests that the beneficial effects of additives used to supplement cryopreservation extenders may depend on the intrinsic freezing ability of a given boar ejaculate.

Materials and methods

Animals and semen collection

A total of 33 ejaculates, each ejaculate coming from a different boar (Pietrain breed), were used in this study (age: 21.6 ± 0.9 months; mean ± standard error of the mean, SEM). Animals were housed in climate-controlled buildings at a local farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain), fed with an adjusted diet (2.3 kg day^{-1}) and provided with water *ad libitum*. Boars were collected by the gloved-hand technique twice per week with an interval of at least three days between collections. The total volume of the sperm-rich fraction was diluted 1:1 (v:v) in a long-term extender (Duragen®; Magapor S.L.; Zaragoza, Spain) after removing the gelatinous fraction by filtering through gauze. Within 2 h after collection, ejaculates were transported in an insulated container to the laboratory of the Autonomous University of Barcelona (UAB) at 17 °C.

Experimental design

Upon arrival to the UAB laboratory, sperm quality of all ejaculates was evaluated to check that they satisfied minimal quality standards, i.e. total sperm motility >80%, progressive sperm motility >60%; morphologically normal spermatozoa >85%; sperm viability >85% (see [42]). From the total of thirty-three ejaculates, three were below these thresholds and were thus discarded. The other thirty ejaculates were stored in our laboratory at 17 °C up to 24 h post-collection, when they were cryopreserved.

Ejaculates were divided into three aliquots of equal volume that were simultaneously subjected to cryopreservation using the Westendorf method [39], as modified in Casas et al. [9]. The main difference among these three aliquots was the presence or absence of GSH ($C_{10}H_{17}N_3O_6S$; Sigma®, St Louis, Missouri, USA) in the cryopreservation media (i.e. LEY and LEYGO). Thus, the first aliquot was processed in the absence of GSH in both media (GSH 0 mM samples). In the second aliquot, GSH was added to cryopreservation media to a final concentration of 2 mM (GSH 2 mM). Finally, GSH was added to cryopreservation media to a final concentration of 5 mM (GSH 5 mM) in the third aliquot. Several sperm parameters (sperm viability and integrity of outer acrosome membrane, levels of peroxides and superoxides, sperm motility, levels of free cysteine radicals in sperm nucleoproteins and sperm DNA fragmentation) were evaluated for each aliquot. The analyses of these parameters were carried out after the four following cryopreservation steps: (1) before starting the cryopreservation procedure (i.e. at 17 °C); (2) at the end of the cooling step (i.e. after sperm being cooled at 5 °C in LEY extender for 120 min); (3) after 30 min post-thawing (FT 30 min); and (4) after 240 min post-thawing (FT 240 min). With this purpose, an aliquot of 20 mL per ejaculate and treatment was taken to assess all the mentioned sperm parameters in the first step (before starting the cryopreservation protocol, i.e. at 17 °C). The remaining volume was cooled to 5 °C for 120 min and an aliquot of 20 mL was then taken to assess the mentioned sperm parameters (second step). Finally, the remaining sperm volume was cryopreserved and stored in liquid nitrogen at −196 °C for at least two months. Samples were thawed and sperm parameters evaluated after 30 and 240 min post-thawing (steps 3 and 4).

Freeze–thawing procedure started with the centrifugation of semen aliquots at 17 °C and 600×*g* for 5 min. Pellets were then recovered with 3–4 mL of the remaining supernatant and diluted to a concentration of 1.5×10^9 spermatozoa mL^{-1} (using a Makler counting chamber; Sefi-Medical Instruments; Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Next, spermatozoa were cooled down to 5 °C for 120 min (cooling ramp: 0.1 °C min^{-1}) using a programmable freezer (Icecube14S-B; Minitub Ibérica, SL; Tarragona, Spain) and subsequently diluted at 1×10^9 spermatozoa mL^{-1} in LEYGO extender that contained 6% glycerol (Sigma®) and 1.5% Orvus ES Paste (OEP, Equex STM; Nova Chemical Sales Inc.; Scituate; MA, USA). Final concentrations of glycerol and OEP in cryopreserved samples were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitub Ibérica, SL) and transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica, SL). The freezing programme (SY-LAB software; Minitub Ibérica, SL) consisted of 313 s of cooling at the following rates: −6 °C min^{-1} from 5 to −5 °C (100 s), −39.82 °C min^{-1} from −5 to −80 °C (113 s), maintained for 30 s at −80 °C, and finally cooled at −60 °C min^{-1} from −80 to −150 °C (70 s). The straws were then plunged into liquid N_2 (−196 °C) for further storage.

After being stored in liquid N_2 for at least 2 months only for schedule reasons, four straws per ejaculate and treatments were thawed by heating at 37 °C for 20 s, pooled, and diluted with three volumes of warmed BTS [33] at 37 °C (final dilution: 1:4, v/v). After thawing, aliquots were incubated for 30 or 240 min at 37 °C, and the same parameters determined in the previous steps were evaluated. These two incubation times (30 and 240 min) were chosen to assess the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses, and as a test to determine the sperm resistance after freeze–thawing [9,11,42].

Data obtained from all sperm parameters in all ejaculates, treatments and cryopreservation steps were statistically analysed as follows. First, ejaculates were classified as good (GFE) or poor freezability ejaculates (PFE) based upon assessments of sperm viability and motility after 30 and 240 min post-thawing in negative control (unsupplemented treatment, GSH 0 mM), as described in Casas et al. [9]. Subsequently, the effects of supplementing freezing media with GSH at final concentrations of 2 or 5 mM on the mentioned sperm parameters of GFE and PFE, and over the four cryopreservation steps (extended semen (17 °C), cooled semen (5 °C), FT 30 and FT 240) were tested. Statistical analyses are described with a much greater detail in Supplementary section 2.6.

Flow cytometric analyses

General information about the flow cytometric analyses performed in this work

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement

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of Cytometry (ISAC) [26]. These analyses were conducted to evaluate sperm viability, integrity of outer acrosome membrane and levels of peroxides and superoxides in extended (step 1), cooled (step 2) or frozen–thawed ejaculates (FT 30 and FT 240; steps 3 and 4) with or without GSH (GSH 0 mM, GSH 2 mM, GSH 5 mM). In all cases, sperm concentration was previously adjusted to 1×10^6 spermatozoa mL^{-1} in a final volume of 0.5 mL, prior to staining with SYBR-14/PI, PNA-FITC/PI, H_2DFCDA/PI, HE/YO-PRO®-1, or PI after hypotonic treatment to correct raw data [32].

Samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, California, USA; Serial Number AL300087, Technical specification at http://www.beckmancoulter.com). This instrument, which had not been altered in the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab Quanta™ SC cytometer employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system has, thus, forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10 μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size of bead at channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. The optical characteristics for these filters were: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width 505−545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560−590 nm); and FL3 (red fluorescence): LP filter: 670/730 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, and H_2DFCDA), while FL3 was used to detect red fluorescence (HE and PI).

Sheath flow rate was set at 4.17 μl min^{-1} in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EV *vs.* SS dot plots) for a minimum of 10,000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 μm) and cell aggregates (particle diameter > 12 μm). Therefore, the sperm-specific events were positively gated on the basis of EV and SS distributions, while the others were gated out. In some protocols, as described below, compensation was used to minimise spill-over of green fluorescence into the red channel.

Information on the events was collected in List-mode Data files (.LMD). These files were then analysed using the Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 *vs.* FL3) and to analyse the cytometric histograms. In all cases except for the SYBR-14/PI assessment, data obtained from flow cytometry experiments were corrected according to the procedure described in Petrunkina et al. [32]. Each assessment for ejaculate, treatment and parameter was repeated thrice in independent tubes, prior to calculating the corresponding mean ± SEM.

Sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI; Molecular Probes®, Eugene, Oregon, USA), according to the protocol described by Garner and Johnson [18]. With this purpose, sperm samples were incubated at 38 °C for 10 min with SYBR-14 at a final concentration of 100 nm, and then with PI at a final concentration of 10 μM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, while PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: (a) viable green-stained spermatozoa (SYBR-14^+/PI^-); (b) non-viable red-stained spermatozoa (SYBR-14^-/PI^+), and (c) non-viable spermatozoa that were stained both green and red (SYBR-14^+/PI^+). Non-sperm particles (debris) were found in SYBR-14^-/PI^- quadrant.

Single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI channel (2.45%).

Integrity of the outer acrosome membrane

Integrity of the outer acrosome membrane was assessed by co-staining of sperm samples with a lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocianate (FITC) and PI, according to the procedure described by Nagy et al. [28]. Spermatozoa were first stained with PNA-FITC (Molecular Probes®; final concentration: 2.5 μg mL^{-1}) at 38 °C for 5 min, and then incubated with PI (final concentration: 10 μM) at the same temperature and for also 5 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa were identified and placed in one of the four following populations: (a) viable spermatozoa with intact outer acrosome membrane (PNA-$FITC^-$/PI^-); (b) viable spermatozoa with altered outer acrosome membrane (PNA-$FITC^+$/PI^-); (c) non-viable cells with intact outer acrosome membrane (PNA-$FITC^-$/PI^+), and (d) non-viable cells with altered outer acrosome membrane (PNA-$FITC^+$/PI^+).

Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC-spill over into the PI channel (2.45%).

Intracellular peroxide and superoxide levels

Levels of peroxides and superoxides were determined through two different oxidation-sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE), used to analyse the intracellular content of peroxides (H_2O_2) and superoxide anions ($O_2^{-\cdot}$). Following a procedure modified from Guthrie and Welch [19], a simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa either with PI or with YO-PRO®-1.

In the first case, spermatozoa were stained with H_2DCFDA (Molecular Probes®) at a final concentration of 200 μM and PI at a final concentration of 10 μM, and incubated at 25 °C for 60 min in the dark. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation. This DCF fluorescence was collected through FL-1, while PI fluorescence was detected through FL-3. Measurements were expressed as geometric means of green intensity fluorescence units (GMFI, geometric mean in FL-1) and this was used as the index of ROS generation. Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

In the second probe, samples were stained with HE (Molecular Probes®; final concentration: 4 μM) and with YO-PRO®-1 (Molecular Probes®; final concentration: 40 μM) and incubated at 25 °C for 40 min in the dark. Hydroethidine is freely permeable to cells and it is oxidised by $O_2^{-\cdot}$ to ethidium (E) and other products. Fluorescence of ethidium (E^+) was detected through FL-3 and that of YO-PRO®-1 was collected through FL-1. Data are expressed as the percentage of viable spermatozoa with high $O_2^{-\cdot}$) (high ethidium fluorescence; E^+) and the geometric mean of red-intensity fluorescence (geometric mean channel in the FL-3). Data were not compensated.

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Method for correcting flow cytometry data: identification of non-DNA containing particles

The percentage of non-DNA-containing particles (alien particles) was determined since in some flow cytometry assessments, especially when working with cryopreserved spermatozoa, there may be an overestimation of sperm particles. Indeed, alien particles such as cytoplasmic droplets, cell debris, or diluent components (as egg yolk), often show EV/SS characteristics similar to those of spermatozoa and can not thus be excluded via light scatter [32]. Therefore, 5 μL of each sperm sample, treatment and cryopreservation step was diluted with 895 μL milliQ®-distilled water. Samples were then stained with PI at a final concentration of 10 μM and incubated at 38 °C for 3 min, according to the procedure described by Petrunkina et al. [32]. Percentages of alien particles (f) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and dual-staining analysis, except in SYBR-14/PI assay (i.e. PNA-FITC/PI, H_2DFCA/PI and HE/YO-PRO®-1), according to the following formula:

$$q_1' = \frac{q_1 - f}{100 - f} \times 100$$

where q_1' is the percentage of non-stained spermatozoa after correction.

Analysis of sperm motility

Sperm motility was also evaluated as a relevant sperm parameter, and it was determined in all the experimental steps described above. This parameter was analysed by utilising a commercial computer assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain). Fifteen microlitre of each sperm sample (at a concentration of $1–3 \times 10^7$ spermatozoa mL^{-1}) were placed onto a Makler counting chamber (Sefi-Medical Instruments) that was maintained at a temperature of 37 °C. Our CASA system was based upon the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image capturing of one photograph every 40 ms. Several fields of view were captured and at least 1000 spermatozoa counted in each analysis. The sperm motility descriptors obtained were those described in Yeste et al. [40] and the settings taken into account for these motility descriptors are shown in Table 1. Total motility (%TMOT) was defined as the percentage of spermatozoa that showed a VAP > 10 μm s^{-1}, whereas progressive motility (%PMOT) was defined as the percentage of spermatozoa that showed a VAP > 45 μm s^{-1}.

Evaluation of boar sperm nucleus

The status of sperm nucleus was analysed in all the aforementioned experimental steps and aliquots, and this evaluation consisted of two separate analyses. On the one hand, levels of free cysteine radicals in sperm nucleoproteins were determined, while on the other, percentages of spermatozoa with fragmented DNA were also evaluated.

Determination of free cysteine radicals in sperm nucleoproteins

Free cysteine radicals in sperm nucleoproteins, as a measure of disrupted disulphide bonds, were determined at each cryopreservation step and in all ejaculates and treatments. The protocol followed was the described in Flores et al. [15]. First, sperm samples were centrifuged at 600×g at 17 °C for 20 min and resuspended in an ice-cold 50 mM Tris buffer (pH = 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1 mM benzamidine, 10 μg mL^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate (Na_2VO_4). Then, samples were homogenised through sonication (Ikasonic U50 sonicator, Ika® Labortechnick; Staufen, Germany) and homogenates were subsequently centrifuged at 850×g and 4 °C for 20 min. Both the resultant supernatants and the upper layer of the pellet were discarded, and the pellets were resuspended in 500 μL of PBS. The purity of this separation was determined by observing samples under a phase-contrast microscope (Zeiss Primo Star, Karl Zeiss; Jena, Germany) at 400× magnifications (Zeiss Plan-Achromat 40×/0.65; Karl Zeiss). Purity of samples was described as the percentage of loose heads in comparison with the presence of whole, non-fractioned sperm and separated tails. In all cases, the mean purity percentage was higher than 95% of loose heads, in comparison with other sperm presentations, such as intact sperm or cells with different types of tail rupture without separating the heads from their respective mid-pieces.

Table 1
Sperm motility settings. Parameters and ranges set in the assessment of sperm motility through a computer assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain).

Parameter	Range
Range of particles area	10–80 μm^2
Curvilinear velocity (VCL)	1–500 μm s^{-1}
Linear velocity (VSL)	1–500 μm s^{-1}
Average pathway velocity (VAP)	1–500 μm s^{-1}
Straightness coefficient (STR)	10–98%
Linearity coefficient (LIN)	10–98%
Wobble coefficient (WOB)	10–98%
Amplitude of lateral head displacement (ALH)	0–100 μm
Beat cross frequency (BCF)	0–100 Hz

The levels of free cysteine radicals in sperm nucleoproteins were determined in all prepared sperm samples by using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma®; Saint Louis, USA) as described in Brocklehurst et al. [8]. With this purpose, 10 μL of the prepared sperm samples obtained as described above were added to 990 μL of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine, prior to incubation at 37 °C for 1 h. Levels of free cysteine radicals in sperm nucleoproteins were then determined through spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalised through a parallel determination of the total protein content of samples, using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules; CA, USA) for the Bradford method [6].

Analysis of DNA fragmentation

Sperm DNA fragmentation was assessed using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm–Halomax®–Sus for fluorescence microscopy; ChromaCell S.L.; Madrid, Spain) and following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a de-proteinisation treatment, and the results obtained with this technique strongly correlate with those obtained with other tests, like the neutral comet assay [16].

First, the lysis buffer included in the kit was tempered to 22 °C and vials containing low-melting agarose were incubated at 100 °C for 5 min in a water bath. Vials were then left in another water bath at 37 °C for 5 min to equilibrate the agarose temperature. Twenty-five microlitre of each sperm sample (at a final concentration of 10^7 spermatozoa mL^{-1}) were added to a vial prior to mixing it thoroughly. One drop of 25 μL containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid air-bubble formation.

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Slides were placed on a cooled plate within a fridge and left at 4 °C for 5 min. The coverslip was then removed and 50 μL of lysis solution per slide were added. An incubation step at 22 °C for 5 min was performed, prior to washing with milliQ®-distilled water for 5 min. Slides were subsequently dehydrated by three steps of 2 min each with ethanol at 70%, 90% and 100%. Finally, sperm samples were stained with 2.5 μg mL^{-1} propidium iodide (PI; Molecular Probes®) and mounted in DABCO (Sigma®) antifading medium. Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 400× magnifications, three counts of 250 spermatozoa each being made for each sample. Spermatozoa with fragmented DNA presented a large and spotty halo of chromatin dispersion, while spermatozoa with non-fragmented DNA showed a small halo.

Statistical analyses

Data were managed with a statistical package (IBM® SPSS® 19.0 for Windows; IBM corp.; Chicago, Illinois) and each combination of ejaculate ($n = 30$) and treatment (GSH 0 mM, GSH 2 mM and GSH 5 mM) was considered as an independent observation. Values obtained from the evaluation of sperm parameters were first tested for normality and homogeneity of variances with Kolmogorov–Smirnov and Levene tests. When required, data were transformed using the arcsine square root (arcsin $\sqrt{x}$) to match with parametric assumptions.

Classification of ejaculates in GFE and PFE groups

As mentioned in experimental design section, the 30 ejaculates included in this study were classified as GFE or PFE based on the assessments of sperm viability and sperm progressive motility of 0 mM aliquots (negative controls) after 30 and 240 min post-thawing. With this purpose, hierarchical cluster analyses for dissimilarities were run following the procedure described by Casas et al. [9]. The thresholds to separate both groups were 45% for viable spermatozoa and 35% for progressive motile spermatozoa at 30 min post-thawing, and 30% for viable spermatozoa and 20% for progressive motile spermatozoa at 240 min post-thawing. The method consisted of calculating the chi-squared frequencies from the sperm progressive motility and the sperm viability after 30 and 240 min post-thawing, and constructed a dissimilarity dendrogram as a result.

Effects of GSH supplementation on sperm parameters in GFE and PFE

As our experimental design consisted of a factorial design, a generalized linear mixed model for repeated measures was conducted followed by a post hoc *t*-test with Bonferroni correction for pair-wise comparisons. All sperm parameters were considered as dependent variables, intrasubject factor was the cryopreservation step (i.e. extended, cooled at 4 °C, FT 30 min and FT 240 min), random-effects factors were the ejaculate, and fixed-effects factors were the treatment (GSH 0 mM, GSH 2 mM and GSH 5 mM) and the freezability group (GFE *vs.* PFE). Data are presented as mean ± standard error of the mean (SEM), and the minimal level of significance was set at $P < 0.05$ in all cases.

Results

First of all, ejaculates were classified into GFE or PFE according to their sperm viability and motility assessments after 30 and 240 min of thawing in negative controls (GSH 0 mM aliquots). From the total of 30 ejaculates included in this study, 17 were classified as GFE and the other 13 were classified as PFE. The ejaculate classification obtained after 30 min post-thawing (% viable spermatozoa (mean ± SEM), GFE: 53.6 ± 2.5, PFE: 38.7 ± 1.9; % progressive motile spermatozoa, GFE: 39.1 ± 2.1, PFE: 27.5 ± 1.4) was the same as the obtained after 240 min post-thawing (% viable spermatozoa, GFE: 37.8 ± 2.0, PFE: 22.0 ± 1.3; % progressive motile spermatozoa, GFE: 25.9 ± 1.5, PFE: 12.6 ± 0.6) (data not shown).

Effects of the addition of GSH on the sperm viability of GFE and PFE

As shown in Fig. 1, the specific cryopreservation step, ejaculate freezability and GSH concentration significantly ($P < 0.05$) affected the percentage of viable spermatozoa. In fact, a statistically significant ($P < 0.05$) interaction was seen between these three factors (cryopreservation step × ejaculate freezability × GSH concentration). Before starting cryopreservation and after the cooling step (5 °C), no significant differences were observed between ejaculate freezability groups and GSH treatments. However, there was a significant reduction of sperm viability after 30 and 240 min post-thawing, and this reduction was more marked ($P < 0.05$) in PFE than in GFE (Fig. 1). As far as the effects of GSH concentration on sperm viability are concerned, 2 mM GSH and 5 mM GSH significantly ($P < 0.05$) improved sperm viability in GFE. In contrast, a similar positive effect was only seen for GSH at 5 mM in the case of PFE. It is finally worth mentioning that percentages of viable spermatozoa in PFE, even when supplemented with GSH 5 mM, were always significantly ($P < 0.05$) lower than those observed in GFE.

Effects of the addition of GSH on the integrity of outer acrosome membrane of GFE and PFE

Similarly to that observed for sperm viability, a significant effect ($P < 0.05$) of cryopreservation step, freezability group, and GSH concentration was observed on the percentage of viable spermatozoa with an intact outer acrosome membrane (PNA-FITC$^-$/PI$^-$; see Fig. 2), and there was also interaction between these three factors ($P < 0.05$). Again, no significant differences in the percentage of PNA-FITC$^-$/PI$^-$ spermatozoa were observed either between GFE and PFE or among GSH concentrations when evaluating extended (step 1) and cooled (step 2) spermatozoa. Notwithstanding, both 2 mM GSH and 5 mM GSH in the case of GFE, and 5 mM GSH in the case of PFE, significantly ($P < 0.05$) increased the percentage of viable spermatozoa with an intact outer acrosome membrane in frozen–thawed spermatozoa after 30 and 240 min post-thawing (Fig. 2). At 240 min post-thawing, there were no significant ($P > 0.05$) differences between 2 mM GSH and 5 mM GSH concentrations in GFE. In contrast, these two concentrations significantly ($P < 0.05$) differed in the case of PFE.

Effects of the addition of GSH on the peroxide and superoxide levels of GFE and PFE

Percentages of the DCF$^+$/PI$^-$ spermatozoa did not differ either between GFE and PFE or between GSH concentrations (Table 2). However, and when compared to their corresponding negative controls, the geometric mean of DCF$^+$ intensity ($GMFI^+_{DCF}$) in viable spermatozoa after both 30 and 240 min post-thawing was significantly ($P < 0.05$) lower in 2 mM GSH and 5 mM GSH treatments in GFE, and in 5 mM GSH treatment in PFE (Table 2). Regarding superoxides (Table 3), there were no significant ($P > 0.05$) different either in the percentage of E$^+$/YO-PRO-1$^-$ spermatozoa or in the geometric mean of DCF$^+$ intensity ($GMFI^+_E$) in viable spermatozoa (E$^+$/YO-PRO-1$^-$) between GSH concentrations and freezability groups (GFE *vs.* PFE).

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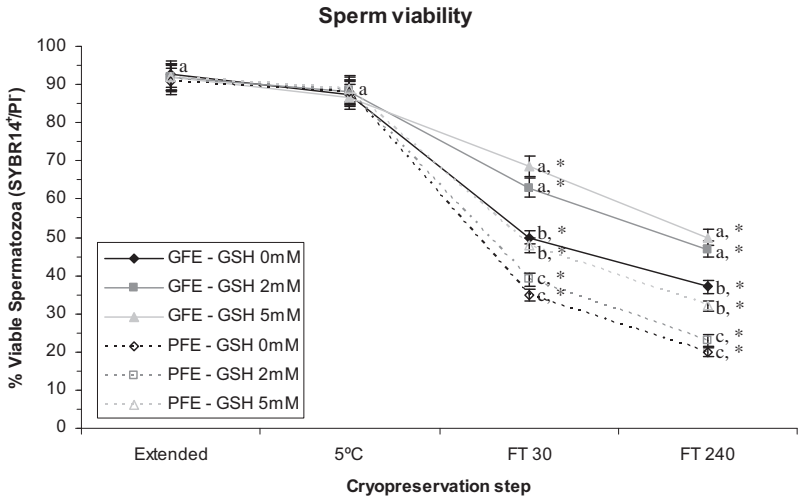

Fig. 1. Percentages of viable spermatozoa (% Spermatozoa SYBR14$^+$/PI$^-$, mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Different letters (*a, b, c*) mean significant differences ($P < 0.05$) between GSH concentrations and freezability groups within a given cryopreservation step, while (*) means significant differences ($P < 0.05$) in the percentage of viable spermatozoa between extended semen and the other three cryopreservation steps (i.e. cooled, FT 30 min, FT 240 min).

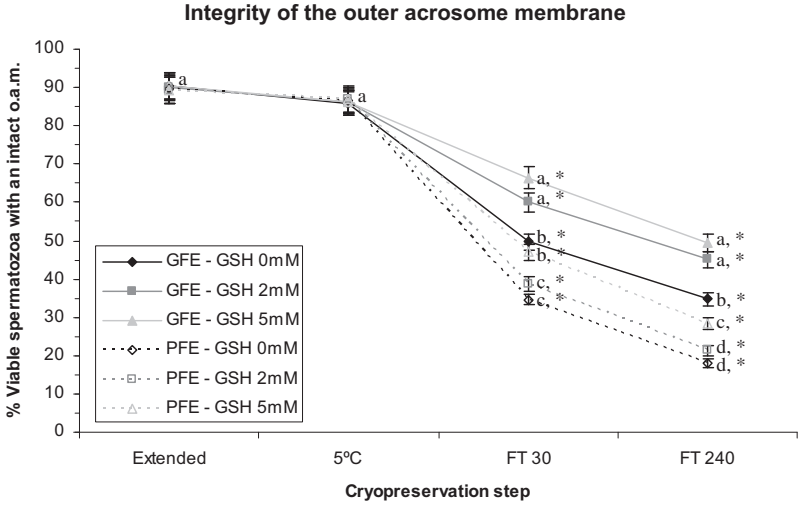

Fig. 2. Percentages of viable spermatozoa with an intact outer acrosome membrane (% Spermatozoa PNA-FITC$^-$/PI$^-$, mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Different letters (*a, b, c, d*) mean significant differences ($P < 0.05$) between GSH concentrations and freezability groups within a given cryopreservation step, while (*) means significant differences ($P < 0.05$) in the percentage of viable spermatozoa between extended semen and the other three cryopreservation steps (i.e. cooled, FT 30 min, FT 240 min). o.a.m: outer acrosome membrane.

Table 2
Peroxide levels (H_2O_2) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Data are shown as mean ± SEM. Different letters (*a, b*) mean significant differences ($P < 0.05$) between rows within a given parameter (% Spermatozoa DCF$^+$/PI$^-$, GMFI (FL1) DCF$^+$/PI$^-$) and column (i.e. GSH concentration and freezability group), while different numbers (1, 2, 3) mean significant differences ($P < 0.05$) between columns (cryopreservation steps) within a given row. GMFI: Geometric mean of fluorescence intensity (arbitrary units).

Parameter	Ejaculate freezability	[GSH] (mM)	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
% Spermatozoa DCF$^+$/PI$^-$	GFE	0	$2.4 \pm 0.2^{a,1}$	$2.5 \pm 0.2^{a,1}$	$3.7 \pm 0.3^{a,2}$	$1.6 \pm 0.1^{a,3}$
		2	$2.4 \pm 0.2^{a,1}$	$2.3 \pm 0.2^{a,1}$	$2.5 \pm 0.2^{b,1}$	$1.7 \pm 0.1^{a,2}$
		5	$2.3 \pm 0.2^{a,1}$	$2.2 \pm 0.2^{a,1}$	$2.3 \pm 0.2^{b,1}$	$1.8 \pm 0.1^{a,2}$
	PFE	0	$2.5 \pm 0.2^{a,1}$	$2.4 \pm 0.2^{a,1}$	$3.5 \pm 0.3^{a,2}$	$1.5 \pm 0.1^{a,3}$
		2	$2.5 \pm 0.2^{a,1}$	$2.3 \pm 0.2^{a,1}$	$2.8 \pm 0.2^{b,1}$	$1.6 \pm 0.1^{a,2}$
		5	$2.3 \pm 0.2^{a,1}$	$2.3 \pm 0.2^{a,1}$	$2.4 \pm 0.2^{b,1}$	$1.7 \pm 0.1^{a,2}$
GMFI (FL1) DCF$^+$/PI$^-$	GFE	0	$87.9 \pm 4.8^{a,1}$	$93.4 \pm 5.2^{a,1}$	$108.7 \pm 5.8^{a,2}$	$46.6 \pm 2.7^{a,3}$
		2	$87.2 \pm 4.8^{a,1}$	$92.8 \pm 5.1^{a,1}$	$84.5 \pm 4.7^{b,1}$	$37.5 \pm 2.2^{b,2}$
		5	$86.9 \pm 4.7^{a,1}$	$92.5 \pm 4.9^{a,1}$	$84.0 \pm 4.8^{b,1}$	$38.0 \pm 2.4^{b,2}$
	PFE	0	$88.3 \pm 4.8^{a,1}$	$94.2 \pm 5.2^{a,1}$	$109.0 \pm 5.8^{a,2}$	$50.3 \pm 2.9^{a,3}$
		2	$88.7 \pm 4.9^{a,1}$	$93.8 \pm 5.0^{a,1}$	$96.9 \pm 5.4^{a,b,1}$	$47.7 \pm 2.8^{a,2}$
		5	$86.6 \pm 4.8^{a,1}$	$93.8 \pm 5.0^{a,1}$	$90.1 \pm 5.0^{b,1}$	$39.4 \pm 2.5^{b,2}$

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Table 3
Superoxide levels ($O_2^{-\cdot}$) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 min and 240 min post-thawing. Data are shown as mean ± SEM. Different letters mean significant differences ($P < 0.05$) between rows within a given parameter (% Spermatozoa E^+/YO-PRO-1$^-$, GMFI (FL3) E^+/YO-PRO-1$^-$ and column (i.e. GSH concentration and freezability group), while different numbers (1, 2, 3) mean significant differences ($P < 0.05$) between columns (cryopreservation steps) within a given row. GMFI: Geometric mean of fluorescence intensity (arbitrary units).

Parameter	Freezability group	[GSH] (mM)	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
% Spermatozoa E^+/YO-PRO-1$^-$	GFE	0	$3.2 \pm 0.3^{a,1}$	$2.9 \pm 0.2^{a,1}$	$3.1 \pm 0.3^{a,1}$	$3.3 \pm 0.3^{a,1}$
		2	$3.3 \pm 0.3^{a,1}$	$3.0 \pm 0.3^{a,1}$	$3.2 \pm 0.3^{a,1}$	$3.4 \pm 0.3^{a,1}$
		5	$3.2 \pm 0.3^{a,1}$	$3.0 \pm 0.3^{a,1}$	$3.1 \pm 0.3^{a,1}$	$3.4 \pm 0.3^{a,1}$
	PFE	0	$3.4 \pm 0.3^{a,1}$	$3.1 \pm 0.3^{a,1}$	$3.4 \pm 0.3^{a,1}$	$3.5 \pm 0.3^{a,1}$
		2	$3.3 \pm 0.3^{a,1}$	$3.0 \pm 0.2^{a,1}$	$3.3 \pm 0.3^{a,1}$	$3.3 \pm 0.3^{a,1}$
		5	$3.3 \pm 0.3^{a,1}$	$3.2 \pm 0.3^{a,1}$	$3.2 \pm 0.3^{a,1}$	$3.5 \pm 0.3^{a,1}$
GMFI (FL3) E^+/YO-PRO-1$^-$	GFE	0	$105.3 \pm 6.1^{a,1}$	$88.5 \pm 4.9^{a,2}$	$70.2 \pm 3.9^{a,3}$	$72.3 \pm 4.2^{a,3}$
		2	$104.8 \pm 6.0^{a,1}$	$88.0 \pm 5.0^{a,2}$	$70.5 \pm 3.9^{a,3}$	$71.1 \pm 4.2^{a,3}$
		5	$105.9 \pm 6.2^{a,1}$	$85.4 \pm 5.0^{a,2}$	$67.8 \pm 3.7^{a,3}$	$69.3 \pm 4.0^{a,3}$
	PFE	0	$108.4 \pm 6.4^{a,1}$	$89.9 \pm 5.2^{a,2}$	$74.9 \pm 4.6^{a,3}$	$75.1 \pm 4.3^{a,3}$
		2	$107.6 \pm 6.2^{a,1}$	$90.5 \pm 5.2^{a,2}$	$72.0 \pm 4.4^{a,3}$	$73.8 \pm 4.2^{a,3}$
		5	$105.7 \pm 6.1^{a,1}$	$87.3 \pm 5.1^{a,2}$	$70.1 \pm 4.5^{a,3}$	$72.4 \pm 4.1^{a,3}$

Effects of the addition of GSH on the sperm motility of GFE and PFE

Percentages of both total and progressive sperm motility were significantly ($P < 0.05$) decreased after freeze–thawing procedures. In the case of total sperm motility (Table 4), this cryopreservation-induced decrease was significantly ($P < 0.05$) lower when freezing media contained GSH, and in GFE than in PFE. However, and even though the reduction in total sperm motility was always lower when GSH was added, GSH concentration required to counteract the decrease in sperm motility was higher in PFE than in GFE. Thus, while 2 mM GSH was enough to significantly ($P < 0.05$) increase the total sperm motility of GFE after freeze–thawing, PFE needed a higher concentration of GSH (5 mM) to observe this counteracting effect (Table 4).

Similar results were observed for sperm progressive motility as Fig. 3 shows (mean ± SEM). Indeed, a significant ($P < 0.05$) reduction in sperm progressive motility was seen as a result of sperm cryopreservation, but this reduction was partially counteracted by GSH when added at final concentrations of 2 and 5 mM in GFE, and when added at a final concentration of 5 mM in PFE. Despite the beneficial effects of GSH supplementation, GFE always presented a significantly ($P < 0.05$) higher percentage of progressive motile spermatozoa than PFE when the same treatments were compared between them (i.e. GFE – GSH 0 mM *vs.* PFE – GSH 0 mM; GFE – GSH 2 mM *vs.* PFE – GSH 2 mM; GFE – GSH 5 mM *vs.* PFE – GSH 5 mM; see Fig. 3).

Effects of adding GSH on the free cysteine radicals in sperm nucleoproteins in GFE and PFE

Overall, the levels of free cysteine radicals in sperm nucleoproteins significantly ($P < 0.05$) differed between cryopreservation steps, ejaculate freezability and GSH treatments (Fig. 4). Additionally, there was a significant ($P < 0.05$) interaction between these three factors (cryopreservation step × ejaculate freezability × GSH concentration). Freeze–thawing also increased the levels of free cysteine radicals in all cases, even though no significant differences between either freezability groups or GSH treatments were observed at the first two cryopreservation steps. However, the increase in levels of free cysteine radicals in sperm nucleoproteins after 30 and 240 min post-thawing was significantly ($P < 0.05$) higher in PFE than in GFE. Similarly, this increase in free cysteine radicals observed after thawing was also significantly ($P < 0.05$) higher in those treatments that contained GSH than in negative controls (Fig. 4). In addition, whereas the cryopreservation-induced increase of free cysteine radicals was significantly ($P < 0.05$) lower in GFE when supplemented with 2 mM GSH and 5 mM GSH, such increase in PFE was only significantly ($P < 0.05$) lower than negative control when supplemented with 5 mM GSH, both after 30 and 240 min post-thawing. Finally, in spite of the improving effect of 5 mM GSH in PFE, levels of free cysteine radicals in PFE were always significantly ($P < 0.05$) higher than in GFE after freeze–thawing, even when the values of this parameter within the same treatment (GSH 0 mM, GSH 2 mM, GSH 5 mM) were compared between GFE and PFE (Fig. 4).

Effects of the addition of GSH in the sperm DNA fragmentation of GFE and PFE

Cryopreservation step, ejaculate freezability and GSH concentration significantly ($P < 0.05$) affected the percentage of spermatozoa with fragmented DNA (Fig. 5). Furthermore, a significant ($P < 0.05$) statistical interaction between these three factors was also detected. Again, an increase in the percentage of spermatozoa with fragmented DNA was observed after freeze–thawing, but the extent of this increase was higher in PFE than in GFE, and in negative controls than in the samples supplemented with GSH. The beneficial effects of GSH supplementation were clearly seen after 240 min post-thawing, when the percentage of spermatozoa with fragmented DNA in both GFE and PFE after supplementation with

Table 4
Percentages of total motile spermatozoa (%TMOT, mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Different letters (*a, b, c*) mean significant differences ($P < 0.05$) between rows within a given column (i.e. GSH concentration and freezability group), and different numbers (1, 2, 3) mean significant differences ($P < 0.05$) between columns (cryopreservation steps) within a given row.

Freezability group	[GSH] (mM)	Extended (17 °C)	Cooled (5 °C)	FT 30 min	FT 240 min
GFE	0	$89.1 \pm 4.8^{a,1}$	$84.8 \pm 4.8^{a,1}$	$48.1 \pm 2.6^{a,2}$	$34.5 \pm 2.3^{a,3}$
	2	$90.4 \pm 5.0^{a,1}$	$84.1 \pm 4.8^{a,1}$	$62.8 \pm 3.5^{b,2}$	$43.6 \pm 2.6^{b,3}$
	5	$89.7 \pm 4.9^{a,1}$	$85.7 \pm 4.9^{a,1}$	$66.2 \pm 3.6^{b,2}$	$45.1 \pm 2.8^{b,3}$
PFE	0	$89.5 \pm 4.9^{a,1}$	$82.2 \pm 4.7^{a,1}$	$32.6 \pm 2.1^{c,2}$	$20.3 \pm 1.5^{c,3}$
	2	$89.2 \pm 4.9^{a,1}$	$84.4 \pm 4.8^{a,1}$	$36.9 \pm 2.3^{c,2}$	$24.0 \pm 1.3^{c,3}$
	5	$89.9 \pm 4.8^{a,1}$	$84.9 \pm 4.8^{a,1}$	$46.4 \pm 2.5^{a,2}$	$31.2 \pm 2.2^{a,3}$

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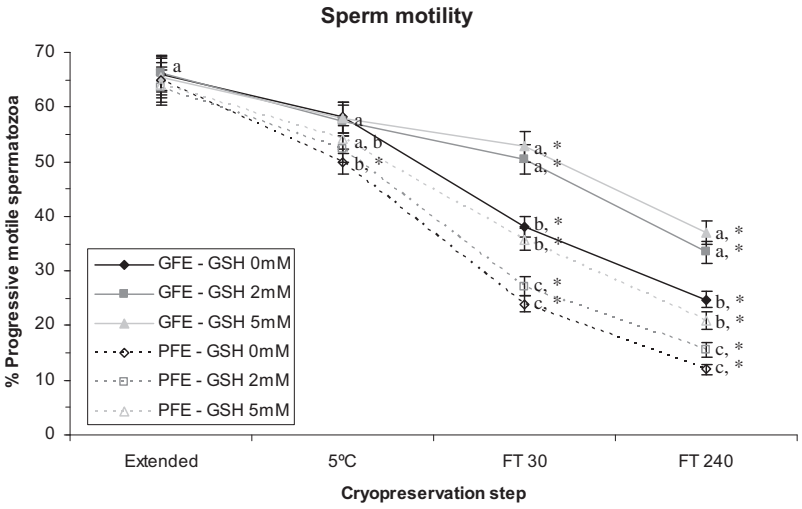

Fig. 3. Percentages of progressive motile spermatozoa (%PMOT, mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Different letters (*a, b, c*) mean significant differences ($P < 0.05$) between GSH concentrations and freezability groups within a given cryopreservation step, while (*) means significant differences ($P < 0.05$) in the percentage of viable spermatozoa between extended semen and the other three cryopreservation steps (i.e. cooled, FT 30 min, FT 240 min).

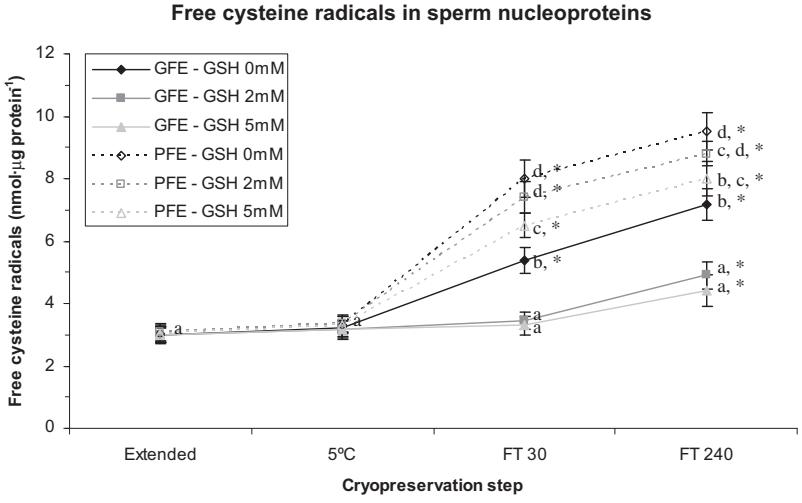

Fig. 4. Levels of free cysteine radicals (FCR) in sperm nucleoproteins (nmol µg protein^{-1}, mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 min and 240 min post-thawing. Different letters (*a, b, c, d*) mean significant differences ($P < 0.05$) between GSH concentrations and freezability groups within a given cryopreservation step, while (*) means significant differences ($P < 0.05$) in the percentage of viable spermatozoa between extended semen and the other three cryopreservation steps (i.e. cooled, FT 30 min, FT 240 min).

2 mM GSH or 5 mM GSH were significantly ($P < 0.05$) lower than those observed in their respective negative controls (i.e. GFE – GSH 0 mM and PFE – GSH 0 mM). However, whereas no significant differences were observed between 2 mM GSH and 5 mM GSH in GFE after 240 min post-thawing, 5 mM GSH in PFE was more effective in counteracting the cryopreservation-induced increase of DNA fragmentation than 2 mM GSH (Fig. 5).

Discussion

Our work clearly establishes that the positive effects of supplementing cryopreservation extenders with GSH depend on the intrinsic ejaculate freezability. In this way, while a final concentration of 2 mM GSH counteracts partially the cryopreservation-induced injuries in good freezability ejaculates, a higher GSH concentration of 5 mM is needed to observe these beneficial effects in poor freezability ejaculates. In fact, all sperm parameters evaluated in this study support the idea that a higher concentration of GSH is required to improve freezability of PFE. Moreover, our data also indicate that the addition of GSH to freezing media never recovers completely a poor freezability ejaculate up to the level of a good freezability one. Indeed, whereas the beneficial effects of GSH on sperm viability and on the integrity of outer acrosome membrane were seen in 2 mM GSH and 5 mM GSH treatments in the case of GFE, they were only observed at 5 mM GSH in PFE, both after 30 min and 240 min post-thawing. It is worth noting that 2 mM GSH and 5 mM GSH did not differ between them in GFE and that, despite GSH effects on function and survival of frozen–thawed boar spermatozoa being dose-dependent [41], at a given concentration of 2 mM GSH there is no longer beneficial effects. Previous studies showed that the impairment seen in sperm viability and integrity of outer acrosome membrane after 30 and 240 post-thawing is higher in PFE than in GFE [42]. In the present work, we have observed that supplementing freezing media with 5 mM GSH improves sperm viability and integrity of outer acrosome membrane after freeze–thawing both in GFE and PFE.

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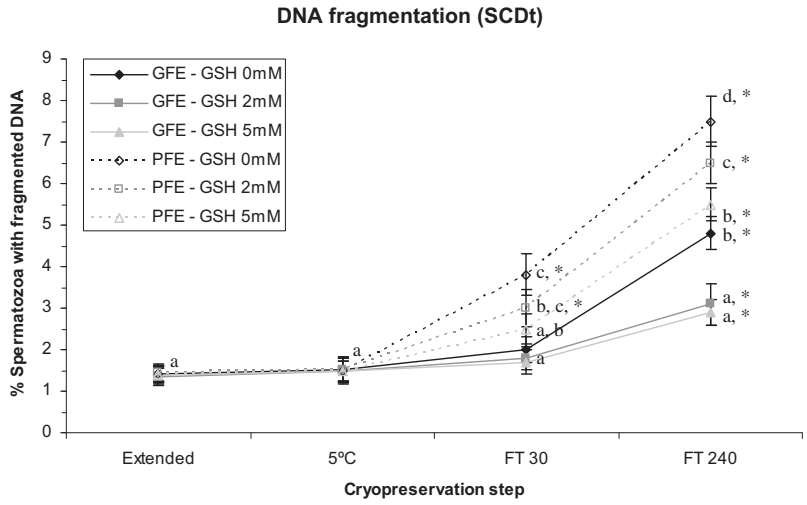

Fig. 5. Percentages of spermatozoa with fragmented DNA (mean ± SEM) after GSH supplementation in extended (17 °C), cooled (5 °C), and frozen–thawed spermatozoa at 30 and 240 min post-thawing. Different letters (*a, b, c, d*) mean significant differences ($P < 0.05$) between GSH concentrations and freezability groups within a given cryopreservation step, while (*) means significant differences ($P < 0.05$) in the percentage of viable spermatozoa between extended semen and the other three cryopreservation steps (i.e. cooled, FT 30 min, FT 240 min).

Regarding sperm motility, it is also known that GFE and PFE differ in the percentages of total and progressive motile spermatozoa after freeze–thawing [9,30,42]. On the basis of the present work, GSH supplementation results in an improving effect on boar sperm motility after freeze–thawing, but the concentration required to observe such positive effect again depends on the intrinsic ejaculate freezability. Therefore, whereas 2 mM GSH was enough to significantly increase the sperm motility of frozen–thawed boar spermatozoa in GFE when compared to negative control, a higher concentration of GSH (5 mM) was required in the case of PFE, in a similar way to that observed for the other sperm parameters. There are no previous reports evaluating the impact of GSH on sperm motility of PFE, but only studies conducted with GFE [14,17,41]. Thus, it is not possible to give an explanation about why GFE and PFE differ in the GSH concentration required to counteract the cryopreservation-induced decrease in sperm motility. However, in the light of our results, we can discard that this positive GSH-effect is due to compensation of ROS production during freeze–thawing protocols. Indeed, although ROS has been suggested to impair sperm motility through an ATP depletion mediated by the H_2O_2 inhibition of oxidative phosphorylation and glycolysis [4,13], there are no differences in ROS production between GFE and PFE, in agreement with our previous study [42]. Thus, it does not seem that the beneficial effects of GSH on sperm motility may be explained by the effect of this molecule in decreasing ROS generation.

The positive effect of GSH supplementation is also observed on the overall sperm nucleus structure. Data shown here indicate that freeze–thawing increases destabilization of DNA–nucleoprotein structure, evaluated by levels of free cysteine radicals in sperm nucleoproteins and DNA fragmentation, which agrees with previous works [15,41,42]. In addition, the present study demonstrates that while 2 mM GSH significantly reduces the cryopreservation-induced damage in sperm nucleus in the case of GFE, the concentration required to obtain a similar effect in PFE is again higher. This finding is of paramount importance as disulphide bonds are one of the most important union mechanisms between nucleoproteins and DNA [2,7], their alteration affects nucleoproteins–DNA structural interaction, and this has been shown to reduce the fertilising ability of boar semen [14]. In this context, one of the most important mechanisms by which GSH supplementation improves boar sperm cryotolerance in both GFE and PFE would be the protection of the sperm nucleoprotein structure by increasing the resistance of this structure against freeze–thawing. The exact molecular mechanism by which the nucleoprotein structure of GFE presents greater resistance to freeze–thawing than that of PFE still remains unknown. In spite of this, we can suggest that the GSH-mediated stabilization of the nucleoprotein structure and, hence, that of its disulphide bonds, could be explained by its role of maintenance of the intracellular redox balance [23]. However, this hypothesis cannot be confirmed, given that the low impact that GSH supplementation has on the ROS levels observed in this study.

In the case of DNA fragmentation, the concentration-dependent effect of GSH on GFE and PFE was mainly observed after 240 min post-thawing in GFE, and after 30 and 240 min post-thawing in PFE. These results agree with previous studies reporting a cryopreservation-induced increase in sperm DNA fragmentation in PFE earlier than in GFE [42], and reinforce the hypothesis that the destabilization of nucleoprotein structure observed after freeze–thawing underlies subsequent DNA fragmentation [41]. On the other hand, as the nucleoprotein structure of PFE is more fragile than that of GFE [42], it appears to require a higher concentration of GSH for counteracting the destabilization of nucleoprotein structure mediated by freeze–thawing. Related to this, we must bear in mind that less inter- and intra-protamine interactions make DNA more susceptible to damage [29]. This is because a reduced chromatin packaging leads to lower resistance against strong acids, proteases, DNases and/or detergents [12]. In addition, after 240 min post-thawing, there are higher levels of leakage products from increasing numbers of dead and dying spermatozoa and traces of components of the cryopreservation medium that can fragment DNA [36]. For this reason, we suggest that a higher GSH concentration could confer higher levels of resistance that are required in the case of PFE.

Good and poor freezability ejaculates do not differ in terms of peroxide and superoxide levels after freeze–thawing protocols. Moreover, the GSH-mediated reduction of peroxide levels observed in this study is marginal, in a similar fashion to that shown in our previous study (Yeste et al. [41]). However, the reduction of DCF^+-fluorescence intensity in viable spermatozoa with high peroxide levels (DCF^+/PI^-) mediated by GSH requires less concentration of this antioxidant in GFE than in PFE. In order to explain these results, it is noteworthy that ROS production linked to freeze–thawing

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procedures has not been clearly established in boar spermatozoa [19,25,41,42]. This is in contrast with other mammalian species, in which ROS production is a main cause of cryodamage [1,3,5,24]. For this reason, it is very difficult to set a clear relationship between the counteracting effect of GSH on ROS levels and on sperm nucleus. This again differs from other mammalian species as humans [27], because percentages of spermatozoa with fragmented DNA are low, and differences between GFE and PFE in the negative control do not allow us to conclude that ROS is the main cause of cryopreservation-induced DNA fragmentation in boar sperm. All these results match with previous reports suggesting that the endogenous ROS defence system in boar sperm is either very efficient or essentially unchallenged during cryopreservation [20]. This may be explained because boar spermatozoa has a substantial amount of intracellular superoxide dismutases for scavenging $O_2^{-\cdot}$ and for rapidly dismutating $O_2^{-\cdot}$ to H_2O_2 [25]. Additionally, superoxide anions have a very short life and are too polar to penetrate the intact plasma membranes of viable cells [1]. A lack of catalase and an exhaustion of other antioxidants could explain the slight increase of peroxide levels after freeze–thawing procedures, in agreement with Kim et al. [25]. For this reason, we could also hypothesise that PFE have lower levels of antioxidants and/or catalase function than GFE, and they would thus require a higher concentration of GSH to compensate the lack of catalase and/or the exhaustion of antioxidant defence system.

At this moment, boar ejaculate freezability cannot be predicted from a conventional spermiogram of fresh/extended semen. This involves that other sperm analyses based on analysis of proteomic [9,10,35] and genomic markers [34] are required. These analyses still remain uncompleted and may be expensive and time-consuming. For this reason, we suggest that freezing media might be supplemented with GSH at a final concentration of 5 mM, as this has a clear positive effect on the cryotolerance of both PFE and GFE.

In conclusion, in this work we have observed that GSH improves boar sperm cryotolerance. However, the concentration required to observe this beneficial effect depends on ejaculate freezability. Apart from confirming other previous studies about the beneficial effects of 2 mM GSH in GFE, the addition of GSH at a final concentration of 5 mM has been shown to improve cryotolerance of PFE. Although in some parameters, as DNA fragmentation, 5 mM GSH in PFE had a counteracting effect that approached PFE to unsupplemented GFE, no GSH treatment added to PFE improved boar sperm cryotolerance to an extent comparable to the same treatment in GFE. In spite of this, GSH supplementation in PFE could partially recover these ejaculates, especially in those cases that a given boar is of interest for its genetic value. Thus, supplementing freezing media with 5 mM GSH may improve the freezability of boar ejaculates in all cases, although the extent of this impact differs between GFE and PFE.

Declaration of interest

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

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ARTICULO 4

The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17^0C is concomitant with a higher cryotolerance of boar spermatozoa.

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The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17ºC is concomitant with a higher cryotolerance of boar spermatozoa

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Abstract

Boar-sperm cryopreservation is not usually performed immediately after semen collection, but rather a holding time (HT) of 4h-30h at 17ºC is spent before starting this procedure. Taking this into account, the aim of this study was to go further in-depth into the mechanisms underlying the improving effects of HT at 17ºC on boar-sperm cryotolerance by evaluating the effects of two different HTs (3h and 24h) on overall boar-sperm function and survival before and after cryopreservation. Given that phospho/dephosphorylation mechanisms are of utmost importance in the overall regulation of sperm function, the phosphorylation levels of serine residues (pSer) in 30 different sperm proteins after a 3h- or 24h-HT period were also assessed. We found that a HT of 24h contributed to a higher sperm resistance to freeze-thawing procedures, whereas mini-array protein analyses showed that a HT of 24h induced a significant (P<0.05) increase in pSer (from 100.0±1.8 arbitrary units in HT 3h to 150.2±5.1 arbitrary units in HT 24h) of HSP70 and, to a lesser extent, in protein kinases GSK3 and total TRK and in the cell-cycle regulatory protein CDC2/CDK1. In the case of HSP70, this increase was confirmed through immunoprecipation analyses. Principal component and multiple regression analyses indicated that a component explaining a percentage of variance higher than 50% in sperm cryotolerance was significantly correlated with pSer levels in HSP70. In addition, from all the parameters evaluated before freeze-thawing, only pSer levels in HSP70 resulted to be able to predict sperm cryotolerance. In conclusion, our results suggest that boar spermatozoa modulate its function during HT, at least partially, by changes in pSer levels of proteins like HSP70, and this is related to a higher cryotolerance.

Keywords**:** Boar sperm; holding time; cryotolerance; serine phosphorylation; HSP70

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Title

The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17ºC is concomitant with a higher cryotolerance of boar spermatozoa

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Abstract

Boar-sperm cryopreservation is not usually performed immediately after semen collection, but rather a holding time (HT) of 4h-30h at 17ºC is spent before starting this procedure. Taking this into account, the aim of this study was to go further in-depth into the mechanisms underlying the improving effects of HT at 17ºC on boar-sperm cryotolerance by evaluating the effects of two different HTs (3h and 24h) on overall boar-sperm function and survival before and after cryopreservation. Given that phospho/dephosphorylation mechanisms are of utmost importance in the overall regulation of sperm function, the phosphorylation levels of serine residues (pSer) in 30 different sperm proteins after a 3h- or 24h-HT period were also assessed. We found that a HT of 24h contributed to a higher sperm resistance to freeze-thawing procedures, whereas mini-array protein analyses showed that a HT of 24h induced a significant (P<0.05) increase in pSer (from 100.0±1.8 arbitrary units in HT 3h to 150.2±5.1 arbitrary units in HT 24h) of HSP70 and, to a lesser extent, in protein kinases GSK3 and total TRK and in the cell-cycle regulatory protein CDC2/CDK1. In the case of HSP70, this increase was confirmed through immunoprecipation analyses. Principal component and multiple regression analyses indicated that a component explaining a percentage of variance higher than 50% in sperm cryotolerance was significantly correlated with pSer levels in HSP70. In addition, from all the parameters evaluated before freeze-thawing, only pSer levels in HSP70 resulted to be able to predict sperm cryotolerance. In conclusion, our results suggest that boar spermatozoa modulate its function during HT, at least partially, by changes in pSer levels of proteins like HSP70, and this is related to a higher cryotolerance.

Keywords: Boar sperm; holding time; cryotolerance; serine phosphorylation; HSP70

Introduction

Mammalian sperm cryopreservation is a stressful event that generates damaged spermatozoa through mechanisms such as oxidative stresses and cold-shock (See Rath et al. [1], for a review). In the case of porcine spermatozoa, cryopreservation is usually performed after sperm has been stored for up to a 24-h period (Holding Time, HT) at 17ºC after collection, as this period in contact with seminal plasma has been reported to yield higher tolerance to low temperatures [2-4]. However, the information about the effects of HT on post-thaw boar-sperm function and survival has been scarce and inconsistent so far. Thus, while Kotzias-Bandeira et al. [5] and Eriksson et al. [6] found that a longer HT, rather than a shorter one, was beneficial for post-thaw sperm viability, Guthrie and Welch [7] found no significant HT effect on post-thaw sperm survival. These discrepancies can be related to the alternate sperm processing procedures utilised by each investigation. In any case, more information is needed to reach a consensus on this point.

Mature spermatozoa are transcriptionally quiescent cells that are not able to regulate gene expression. This implies that sperm cannot modulate gene expression to face stressful environmental conditions and to modulate its physiology. Related to this, post-translation protein modifications like phospho/dephosphorylation are known to play a significant role in some mechanisms regulating sperm function and response to environmental stress [8-11]. In fact, protein phospho/dephosphorylation is a general mechanism present in all cells that plays a major role in a wide array of cellular processes [12]. As indicated above, in the case of mature mammalian spermatozoa, protein phospho/dephosphorylation is of the utmost importance as it is involved in the regulation of processes such as the control of sperm motility [10], [13], sperm capacitation [14-16], response to osmotic stress [17], zona pellucida recognition and acrosome reaction [18-19].

To the best of our knowledge, no previous studies have hitherto investigated whether the differences between shorter and longer HTs are related to changes in phosphorylation patterns of some relevant sperm proteins. For this reason, in this present study we studied the effect of HT on the resistance to cryopreservation of boar spermatozoa. We investigated how two different HTs (3h and 24h) affect nucleoprotein structure, DNA fragmentation, sperm membrane integrity and lipid disorder, and sperm motility, amongst other sperm parameters, before (Ext, extended semen) and after freeze-thawing (FT, frozen-thawed spermatozoa). In addition, an aliquot of these

ejaculates was taken after a HT of 3h and of 24h and immediately before starting sperm cryopreservation to compare the phosphorylation levels of serine residues (pSer) in a set of 30 sperm proteins that may be involved in the modulation of sperm function and that have been studied in a previous report by our group [11]. Within these proteins, there are cell-cycle controlling proteins like cyclins [20]; stress-modulating proteins, like heat-shock protein 70 (HSP70, also known as HSPA1A), and others related to apoptosis like caspase 9 [21-24], and cell-cell adhesion proteins, such as clusterin [25-26]. Additionally, we have also studied specific protein kinases, like PKA and PKC [14], [27-28], and phosphatases, such as PP1, PP2A, PP2B, PTP1 and PTP2, as both are involved in the regulation of sperm function [13], [15-16], [19], [29]. Finally, other protein kinases that also play important roles in the control of capacitation and resistance to oxidative stress in mammalian spermatozoa, such as glycogen synthase kinase-3 (GSK3, see [10]), ERK-1 and ERK-2 [30-31] and the AKT-phosphoinositide 3-kinase (PI3K) system [32-33], have also been included in our study. Given that the results obtained from mini-array analyses, immunoprecipitation-confirming studies were performed on a single protein, HSP70, in order to further clarify the relationship between HT, cryotolerance and specific pSer changes of this protein in boar spermatozoa.

Materials and Methods

Sperm samples

In this study, boars were not handling by us, the semen was obtained from a local farm (the farm - Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain). Thus, such ejaculates were initially dedicated for artificial insemination purposes, and we just bought them for our experimental purposes. Despite all the aforementioned, and even though it was not required as the authors did not manipulate any boar, the experimental protocol was approved by the Ethics Committee of our institution. This ethics committee was known as “Bioethics Commission of the Autonomous University of Barcelona” (Bellaterra, Cerdanyola del Vallès, Spain).

Overall, twelve ejaculates coming from twelve healthy and mature boars of Pietrain pure breed were used (age: 21.5±0.9 months; means±standard error of the mean, SEM). Animals were housed in climate-controlled buildings, fed with an adjusted diet (2.3 $Kg \cdot day^{-1}$) consisting of a basal diet and a 1% premix for boars (P174N; TecnoVit; Tarragona, Spain), and provided with water *ad libitum*. Ejaculates were collected twice

per week by the gloved-hand technique with an interval of at least three days between collections. After removing the gelatinous fraction by filtering through gauze, the total volume of the sperm-rich fraction was diluted 1:1 (v:v) in a short-term Beltsville Thawing Solution (BTS)-based extender (Cidosa, TecnoVit; Tarragona, Spain). The diluted sperm-rich fractions were cooled to 17ºC by placing them in a 17°C mobile-incubator and immediately transported at this temperature in an insulated container to our laboratory within two hours.

Experimental design

Upon arrival, the quality of the ejaculates was evaluated to check that they satisfied the quality standard (total sperm motility>80%, morphologically normal spermatozoa and sperm viability>85%; See [34]). Since the quality of these twelve ejaculates was over the set thresholds, they were all included in our study. Next, each ejaculate was split into two fractions of equal volume. One of these fractions was frozen 3h after collection (HT=3h), while the other was stored at 17ºC up to 24h after collection (HT=24h). Before starting the cryopreservation process, two aliquots of 10 mL each were taken (i.e. after either 3h or 24h of semen collection), one of each for evaluating sperm parameters (Ext, Extended semen) and the other for performing the protein assessment (mini-arrays and immunoprecipation studies). The protein-assessment aliquot was centrifuged at 600×*g* and at 17ºC for 5 min, the supernatant was discarded and the pellet was frozen in liquid nitrogen. The remaining volume of both fractions (i.e. HT=3h and HT=24h) was cryopreserved at each relevant time point, following the procedure described in Section 2.3, and stored in liquid nitrogen for at least two months. After thawing accordingly the procedure described in Section 2.3, samples were diluted with three volumes of warmed (37ºC) BTS ([35]; final dilution: 1/4) and incubated at 37ºC for 30 min and 240 min (FT, Frozen-thawed spermatozoa), prior to determining sperm functional parameters (through motility and flow cytometry assessments), DNA fragmentation and free cysteine radicals in sperm nucleoproteins, as an indication of disrupted disulphide bonds. Two time-points (30 min and 240 min) were chosen to evaluate the sperm cells after freeze-thawing, the last one being set to assure the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses [36] and as a sperm resistance test.

Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method [37], as modified by Yeste et al. [34]. All of the ejaculates were centrifuged at 17ºC and at 600×*g* for 5 min. Pellets were then recovered with 3 mL-4 mL of the remaining supernatant and diluted to a concentration of 1.5×10^9 spermatozoa·mL^{-1} (using a Makler counting chamber; Sefi-Medical Instruments; Haifa, Israel) in a freezing medium (LEY) containing lactose (80%, v:v; 310 mM) and egg yolk (20%, v:v). Next, spermatozoa were cooled down to 5ºC for 120 min (cooling ramp: 0.1°C·min^{-1}) using a programmable freezer (Icecube14S-B; Minitub Ibérica, SL; Tarragona, Spain) and subsequently diluted at 1×10^9 spermatozoa·mL^{-1} in LEYGO extender that contained 6% glycerol (Sigma) and 1.5% Orvus ES Paste (OEP, Equex STM; Nova Chemical Sales Inc.; Scituate; MA, USA). Final concentrations of glycerol and OEP in cryopreserved samples were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitub Ibérica, SL) and transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica SL). The freezing programme (SY-LAB software; Minitub Ibérica SL) consisted of 313 sec of cooling at the following rates: −6ºC·min^{-1} from 5ºC to −5ºC (100 sec), −39.82ºC·min^{-1} from −5ºC to −80ºC (113 sec), maintained for 30 sec at −80ºC, and finally cooled at −60ºC·min^{-1} from −80ºC to −150ºC (70 sec). The straws were then plunged into liquid N_2 (−196ºC) for further storage.

After being stored in liquid N_2 for at least two months only for schedule reasons, samples were thawed and evaluated. With this purpose, four straws per ejaculate were thawed and diluted with three volumes of warmed BTS at 37ºC (at a final dilution of 1/4). Each straw was shaken individually for 20 sec in a water bath at 37ºC.

Flow cytometric analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) [38]. These analyses were conducted to evaluate some functional parameters of spermatozoa, such as plasma membrane integrity and permeability, membrane lipid disorder, intracellular calcium levels, or ROS levels in extended and FT spermatozoa after a HT of 3h or 24h. In each case, sperm concentration was adjusted to 1×10^6 spermatozoa·mL^{-1} in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes. Plasma membrane integrity was assessed through SYBR-14/PI assay according to the protocol described by Garner and Johnson [39], as well as through PNA-FITC/PI co-staining following the procedure described by Nagy et al. [40]. In

addition, changes in the permeability of sperm plasma membrane were evaluated through co-staining with YO-PRO-1 and PI, following Martin et al. [24], and membrane lipid disorder was assessed using the protocol for Merocyanine 540 (M-540) and YO-PRO-1 described by Harrison et al. [41]. Intracellular calcium levels of spermatozoa were determined through Fluo3-AM/PI co-staining [42]. Levels of peroxides and superoxides were evaluated through H_2DCFDA/PI and HE/YO-PRO-1, respectively, according the protocol described by Guthrie and Welch [43]. Finally, data was corrected following Petrunkina et al. [44] by determining the percentage of non-DNA-containing particles, to avoid an overestimation of sperm particles. All protocols are described in detail in Supplementary Information.

In all cases, samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, CA, USA; Serial Number: AL300087) using single-line visible light (488nm) from an argon laser. A minimum of 10,000 events per replicate was evaluated, and data was collected in List-mode Data files (.LMD) and analysed using the Cell Lab Quanta SC MPL Analysis Software (version 1.0; Beckman Coulter). In all cases except for the SYBR-14/PI assessment, data obtained from flow cytometry experiments were corrected according to the procedure set by Petrunkina et al. [44]. Each assessment for each sample and parameter was repeated three times in independent tubes, prior to calculating the corresponding mean±SEM. Technical details are also given in Supplementary Information.

Determination of free cysteine radicals in sperm nucleoproteins

The determination of free cysteine radicals in sperm nucleoproteins, as a measure of disrupted disulphide bonds, was carried out before (extended) and after freeze-thawing (FT) by following the protocol adapted to boar spermatozoa by Flores et al. [45] (See Supplementary Information for details). The results obtained after reading at 343 nm were normalised through a parallel determination of the total protein content of samples, using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules; CA, USA) for the Bradford method [46].

DNA fragmentation analysis

Sperm DNA fragmentation was assessed before (extended) and after freeze-thawing (FT) using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm–Halomax–Sus for fluorescence microscopy; ChromaCell S.L.;

Madrid, Spain). This test is based on the different response that intact and fragmented DNA show after a de-proteinisation treatment, and previous reports have shown that the results obtained with this technique strongly correlate with those obtained with other tests, like the neutral comet assay [47], [48]. Protocol details are provided with a great detail in Supplementary Information. Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 40× magnification and three counts of 250 spermatozoa each were made per sample. Spermatozoa with fragmented DNA presented a large and spotty halo of chromatin dispersion, while spermatozoa with non-fragmented DNA showed a small halo.

Analysis of sperm motility

Sperm motility analysis was performed by utilising a commercial computer assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain), 15µl of each sperm sample (at a concentration of $1\text{-}3\times10^7$ spermatozoa·mL^{-1}) being placed in a Makler counting chamber (Sefi-Medical Instruments). Our CASA system was based upon the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 10× in a negative phase-contrast field. These 25 consecutive photographs were taken in a time lapse of 1sec, which implied a velocity of image capturing of one photograph every 40 msec. Five to six separate fields were taken for each replicate, and three replicates were run per sample. The sperm motility descriptors obtained were those described by Yeste et al. [49], and the settings taken into account for all of the utilised motility parameters are provided as Supplementary Information. Total motility (%TMOT) was defined as the percentage of spermatozoa that showed a VAP>10µm·s^{-1}, whereas progressive motility (%PMOT) was defined as the percentage of spermatozoa that showed a VAP>45µm·s^{-1}.

Mini-array analysis of serine phosphorylation of 30 selected proteins

The phosphorylation levels of serine residues in 30 sperm proteins (Figure 1) in semen samples being at two different HTs (i.e. 3h and 24h) were assessed using customised mini-arrays provided by Hypromatrix Inc. (Worcester, MA, USA). With this aim, twenty-four aliquots coming from twelve different ejaculates (stored after a HT of 3h or of 24h) were used and processed as described in Supplementary Information. As stated

in Introduction, these proteins were chosen following previous results obtained from our laboratory and published in Fernández-Novell et al. [11].

The intensity of the spots was quantified using specific software for image analysis of blots and arrays (Multi Gauge v3.0; Fujifilm Europe; Düsseldorf, Germany), in which the background was previously made uniform for all of the arrays analysed. The values obtained for the HT of 3h were transformed in order to obtain a basal arbitrary value of 100, from which the intensity values for the other samples (i.e. HT 24h) were calculated. For each sperm sample (stored for 3h or 24h), two replicates were assessed prior to calculating the corresponding mean±SEM. Furthermore, two types of negative control were applied. In one, three arrays were incubated with a randomly chosen sample but without further incubation with the antibody. In the other, three arrays were incubated with the antibodies but without samples.

Immunoprecipitation against HSP70 and Western-blot assessments

Results obtained in mini-array analyses were confirmed through immunoprecipitation analyses (immunoprecipitation kit code product 17-6002-35; Healthcare Bio-Sciences AB; Uppsala, Sweden) against human HSP70, using a monoclonal antibody commercially available (ADI-SPA-810, Enzo Life Sciences; New York; NY, USA). With this purpose, twenty-four aliquots (5 mL each) from the same twelve sperm samples used in the other assessments and stored both for 3h (HT=3h) or 24h (HT=24h), were taken at each relevant time point and centrifuged at 600×g and 17ºC. With this method, described with a great detail in Supplementary Information, we isolated HSP70 from sperm protein extracts. Then, samples were evaluated through Western Blot assessment with anti-HSP70 and anti-phosphorylated serines (HM2070, Hypromatrix Inc.; Worcester, MA, USA) antibodies. This allowed us to calculate, per sample, a ratio between the mark intensities of phosphorylated serines and HSP70 (pSer:total HSP70). Two replicates for each sperm sample/HT were evaluated prior to calculating the corresponding mean±SEM per ratio/sperm sample.

Statistical analyses

Statistical analyses were conducted using IBM SPSS 19.0 (IBM corp.; Chicago, Illinois) and SYSTAT 12.0 for Windows statistical packages (SYSTAT Software Inc.; Evanston, IL, USA), and data are presented as mean±SEM. Each sperm sample held at a

given HT was considered as an independent observation, and the minimal level of significance was set at $P<0.05$ in all statistical analyses.

Data obtained from the analysis of all sperm parameters, as well as from the assessments of protein mini-arrays and phosphorylated serine residues in immunoprecipitation studies anti-HSP70 were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene tests. When necessary, data were transformed using the arcsine square root (arcsin $\sqrt{x}$) to match the parametric assumptions.

General linear models

In the case of sperm parameters (i.e. sperm membrane integrity and permeability, intracellular calcium, peroxide and superoxide levels, free cysteine radicals in sperm nucleoproteins, DNA fragmentation, and sperm motility and velocity descriptors), a generalised linear mixed model for repeated measures was run where each sperm parameter was the dependent variable, incubation time at 37ºC (30 min or 240 min) was the intra-subject factor, and the storing method and HT (i.e. Ext 3h, Ext 24h, FT 3h or FT 24h) and the ejaculate were, respectively, the fixed-effect and random-effect factors. A post hoc Bonferroni's test was used for pair-wise comparisons.

In the case of phosphorylated serine residues in mini-array protein and in anti-HSP70 immunoprecipitation analyses, differences in spots-intensity or pSer:total HSP70 ratios were compared through a Student's t-test for related samples, where each protein in the case of mini-arrays or pSer:total HSP70 ratios in the case of immunoprecipitation assessments were the dependent variables, and the HT (3h or 24h) was the factor.

Calculation of cryotolerance indexes

For each sperm sample held at a given HT (*a*; i.e. either 3h or 24h) and sperm parameter (*x*; i.e. sperm membrane integrity and permeability, intracellular calcium, peroxide and superoxide levels, free cysteine radicals in sperm nucleoproteins, DNA fragmentation, and sperm motility and velocity descriptors), a cryotolerance index (C_x) was calculated as the quotient between the value of this parameter (*x*) before (i.e. in extended semen, Ext) and after freeze-thawing (FT). The calculation of this index also took into account the time at 37ºC (*b*), either 30 or 240 min, at which samples were incubated prior to determining such sperm parameter. The mathematic formula was as follows:

$$C_{x,a,b} = \frac{x_{FT,a,b}}{x_{Ext,a,b}} \times 100$$

Thus, for example, the cryotolerance index of viable spermatozoa (SYBR-14$^+$/PI$^-$) evaluated through SYBR-14/PI staining in a given sperm sample held at a HT of 24h and after both extended and frozen-thawed samples were incubated at 37ºC for 240 min was:

$$C_{\%\ SYBR\text{–}14^+/PI^-\ Spermatozoa,\ 3h,\ 240\min} = \frac{\%\ SYBR-14^+/PI^-\ Spermatozoa_{FT,\ 3h,\ 240\min}}{\%\ SYBR-14^+/PI^-\ Spermatozoa_{Ext,\ 3h,\ 240\min}} \times 100$$

Principal Component Analyses

After calculating cryotolerance indexes as described in Section 2.10.2, two factorial analyses were run using the values obtained for the crytolerance indexes after 30 and 240 min of incubation at 37ºC. In each analysis, these indexes were sorted into some components extracted by principal component analysis (PCA) and the obtained data matrix was rotated using the Varimax procedure with Kaiser normalisation. Only those variables with a square factor loading (a_{ij}^2) higher than 0.3 with its respective component, and lower than 0.1 with respect to the other components in the rotated matrix, were selected from the linear combination of *j* variables (*z*) in each component y_i ($y_i = a_{i1}z_1 + a_{i2}z_2 + \ldots + a_{ij}z_j$). Regression factors for each component after PCA were saved and used for multiple regression analyses.

Correlation and multiple regression analyses

Correlations between all the evaluated sperm parameters were calculated using Pearson correlation. In addition, spots-intensity of pSer levels obtained in mini-array protein and pSer:total HSP70 ratios determined in anti-HSP70 immunoprecipitation analyses were correlated with regression factors from PCA using cryotolerance indexes.

Finally, multiple regression analyses were conducted to determine the ability of all the parameters (*x*) evaluated before freeze-thawing (i.e. all sperm parameters, spots intensity for each protein obtained in mini-array protein analyses, and pSer:total HSP70 ratios from anti-HSP70 immunoprecipitation studies) to predict the cryotolerance of a given ejaculate after a given HT. The procedure used (the forward stepwise model) was the same described by Yeste et al. [50] and consisted of optimising the regression equation to increase the determination coefficient (R^2). The dependent variable (*y*) in all

the cases was the regression factor of the first component that resulted from PCA analyses with cryotolerance indexes, as this component explained the highest percentage of variance in each PCA. The significance level for introducing each parameter in the multiple regression model was 0.10 and the significance level (α) for the model was 0.05.

Results

Effects of HT on plasma membrane integrity (SYBR-14/PI and PNA-FITC/PI)

No significant differences were observed in the plasma membrane integrity (SYBR-14/PI) of extended spermatozoa between both HTs (Ext 3h *vs.* Ext 24h; $P>0.05$) before freeze-thawing. Cryopreservation, instead, significantly ($P<0.01$) reduced the percentage of viable (SYBR-14^+/PI^-) spermatozoa (e.g. extended for 3h: 91.5%±3.4% *vs.* FT 24h: 53.1%±2.4%; $P<0.001$; means±SEM; see Figure 2). This decrease was significantly higher when the HT was of 3h than when it was of 24h (FT 3h: 45.3%±1.9% vs. FT 24h: 53.1%±2.4%; $P<0.05$). Incubation of the samples up to 240 min at 37ºC also decreased the percentage of SYBR-14^+/PI^- spermatozoa after freeze-thawing (e.g. extended for 24h: 55.5%±2.3% *vs.* FT 24h: 37.5%±1.7%; $P<0.05$), especially in the case of FT 3h, whose reduction was again significantly higher than that of FT 24h (FT 3h: 22.9%±1.2% *vs*. FT 24h: 37.5%±1.7%; $P<0.05$; see Figure 2).

As far as PNA-FITC/PI staining is concerned, no significant differences between either HT were observed before freeze-thawing (Figure 3). In contrast, the percentages of spermatozoa with an intact plasma membrane (PNA-$FITC^-$/PI^-) were significantly lower in FT spermatozoa than in extended semen after 30 min and 240 min of incubation at 37ºC, whereas PNA-$FITC^+$/PI^+ spermatozoa were significantly higher in the former (FT 3h and FT 24h) than in the latter (extended for 3h and extended for 24h; See Table S1). On the other hand, FT spermatozoa cryopreserved after a HT of 3h presented a significantly ($P<0.05$) higher percentage of PNA-$FITC^+$/PI^+ spermatozoa than that cryopreserved after a HT of 24h (See Table S1).

Effects of HT on sperm membrane permeability (YO-PRO-1/PI)

Figure 4 shows, as means±SEM, the percentages of viable spermatozoa without changes in membrane permeability (YO-PRO-1^-/PI^-), whereas Table S2 also shows the percentages of viable spermatozoa with early changes in membrane permeability (YO-PRO-1^+/PI^-), and those of non-viable spermatozoa (PI^+). Again, no significant

differences were observed in sperm membrane permeability of extended sperm between both HTs before freeze-thawing. Freeze-thawing significantly decreased (P<0.05) the percentages of viable spermatozoa without changes in membrane permeability (Figure 4) and increased those of viable spermatozoa with early changes in membrane permeability and those of non-viable spermatozoa (Table S2). However, the extent of these changes differed in FT spermatozoa between HTs. Indeed, the decrease in viable spermatozoa without changes in membrane permeability observed after a HT of 3h was significantly higher (P<0.05) than that observed after a HT of 24h, both at 30 min and 240 min post-thawing (Figure 4).

Effects of HT on membrane lipid disorder (M540/YO-PRO-1)

As in the previously described parameters, no significant differences were observed in membrane lipid disorder of extended spermatozoa between either HT before freeze-thawing (Figure 5). Freeze-thawing significantly increased (P<0.05) the percentages of non-viable spermatozoa with a high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$) and decreased those of viable ($M540^-/YO\text{-}PRO\text{-}1^-$) and non-viable spermatozoa with a low lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$), after 30 min and 240 min post-thawing (Figure 5; Table S3). Interestingly, the percentages of viable and non-viable spermatozoa with a high lipid disorder after freeze-thawing were significantly higher when the HT was of 3h (FT 3h) than when it was of 24h (FT 24h) (Table S3).

Effects of HT on intracellular calcium levels (Fluo3-AM/PI)

Figure 6 shows, as means±SEM, the percentage of viable spermatozoa with low intracellular calcilum levels ($Fluo3\text{-}AM^-/PI^-$ spermatozoa), whereas Table S4 shows the percentages of viable and non-viable spermatozoa with high/low levels of intracellular calcium and the geometric mean of $Fluo3^+$. In a similar fashion to the previously described parameters, no significant differences were observed in sperm intracellular calcium levels of extended spermatozoa between either HT before freeze-thawing (Figure 6, Table S4). Both after 30 min and 240 min of incubation at 37ºC, the geometric mean of $Fluo3^+$ intensity was significantly lower in FT spermatozoa than in extended semen. Freeze-thawing also decreased the percentages of viable spermatozoa with low and high levels of intracellular calcium ($Fluo3^-/PI^-$ and $Fluo3^+/PI^-$) and increased those of non-viable spermatozoa with low levels of intracellular calcium ($Fluo3^-/PI^+$), but the extent of these changes differed between HTs. Indeed, the

percentages of non-viable spermatozoa with low levels of intracellular calcium were significantly higher (P<0.05) in the sperm cryopreserved after a HT of 3h than in that cryopreserved after a HT of 24h, both after 30 min and 240 min post-thawing (See Table S4)

Effects of HT on ROS levels (H_2DFCDA/PI and HE/YO-PRO-1)

Figure 7 (mean±SEM) shows percentages of viable spermatozoa with high peroxide levels (DCF^+/PI^-) evaluated through H_2DFCDA/PI co-staining, before and after boar-sperm cryopreservation. Other parameters evaluated by H_2DFCDA/PI assay are shown as Supplementary Information (Table S5). Both percentages of spermatozoa DCF^+/PI^- (Figure 7) and the geometric mean of DCF^+-intensity in viable spermatozoa (Table S5) were significantly lower in FT than in extended spermatozoa, although no significant differences between HTs were found either in FT or in extended spermatozoa (Figure 7). In addition, the geometric mean of DCF^+ in total spermatozoa was significantly higher in FT (FT 3h and FT 24h) than in extended spermatozoa (extended for 3h and extended for 24h), but no significant differences between HT were found (Table S5).

As far as the superoxide levels are concerned (HE/YO-PRO-1 co-staining), boar-sperm cryopreservation did not affect either the percentage of E^+/YO-PRO-1^- or the geometric mean of E^+ intensity in total spermatozoa. In contrast, the geometric mean of E^+-intensity in the viable sperm population was significantly higher in the extended rather than in the FT spermatozoa, but significant differences were not observed between either HT (Table S6).

Effects of HT on the amounts of free cysteine residues in sperm nucleoproteins

Before freeze-thawing, the levels of free cysteine radicals in sperm nucleoproteins were similar in both HTs (Figure 8; i.e. extended for 3h: 3.0 nmol·µg $protein^{-1}$±0.3 nmol·µg $protein^{-1}$ *vs.* extended for 24h: 3.1 nmol·µg $protein^{-1}$±0.3 nmol·µg $protein^{-1}$ after 30 min of incubation at 37ºC). In contrast, sperm cryopreservation significantly increased (P<0.01) the levels of free cysteine radicals, as compared to extended semen. This increase was significantly higher when the HT was 3h rather than when it was 24h after both 30 min and 240 min post-thawing (e.g. FT 3h: 9.5 nmol·µg $protein^{-1}$±0.8 nmol·µg $protein^{-1}$ *vs.* FT 24h: 6.3 nmol·µg $protein^{-1}$±0.5 nmol·µg $protein^{-1}$, after 30 min of thawing).

Effects of HT on sperm DNA fragmentation

Figure 9 shows the percentage of spermatozoa with fragmented DNA as means±SEM. No significant differences between HTs were observed in the percentages of spermatozoa with fragmented DNA before freeze-thawing (e.g. extended for 3h: 1.3%±0.2%; extended for 24h: 1.4%±0.2%; $P>0.05$ after incubation at 37ºC for 30 min). Percentages of spermatozoa with fragmented DNA in extended semen did not differ ($P>0.05$) from those obtained in FT spermatozoa cryopreserved after a HT of 24h at 30 min post-thawing (FT 24h: 1.8%±0.2%). Conversely, the percentages of spermatozoa with fragmented DNA in FT spermatozoa cryopreserved after a HT of 3h (FT 3h: 3.2%±0.4%) were significantly higher ($P<0.05$) than those observed in the other cases (i.e. Ext 3h, Ext 24h, FT 24h) at 30 min post-thawing.

After 240 min post-thawing, the percentages of spermatozoa with fragmented DNA in FT spermatozoa (FT 3h and FT 24h) were significantly higher ($P<0.05$) than those observed in extended semen (Ext 3h: 2.0%±0.3%, and Ext 24h: 2.1%±0.3%). Again, the percentages of spermatozoa with fragmented DNA in FT spermatozoa cryopreserved after a shorter HT were significantly higher than those cryopreserved after a longer HT (FT 3h: 9.4%±0.8% vs. FT 24h: 6.2%±0.6%; $P<0.05$).

Effects of HT on sperm motility

Cryopreservation significantly decreased ($P<0.05$) in all cases (extended *vs.* FT) the percentages of total and progressive motile spermatozoa and VCL and VAP (Table 1). This reduction was observed both after 30 min and 240 min post-thawing. On the other hand, and after 240 min post-thawing, FT spermatozoa cryopreserved at a HT of 24h presented significantly ($P<0.05$) higher values of %TMOT, %PMOT, VCL and VAP than FT spermatozoa cryopreserved at a HT of 3h.

Cryotolerance indexes and Principal Component Analyses

Principal component analyses using cryotolerance indexes are shown in Tables 2 and 3. In the case of cryotolerance indexes calculated after 30 min of incubation at 37ºC (Table 2), a total of five components were extracted and the explained variance was 91.23%. The first component explained a variance of 59.49%, and included the most important cryotolerance indexes describing sperm integrity and survival (i.e. viable spermatozoa with no alterations in membrane permeability, %TMOT, %PMOT, levels of free

cysteine radicals in sperm nucleoproteins…). In contrast, cryotolerances indexes of peroxides and superoxide levels were included in the other four components.

In a similar fashion to the last case, five components were also obtained from PCA with cryotolerance indexes evaluated after 240 min of thawing at 37ºC (Table 3). The total explained variance was of 94.20% and, again, the first component represented more than a half of total variance (64.11%) and included the most significant crytolerance indexes describing sperm integrity and survival.

Mini-array analyses of serine-phosphorylation levels of 30 proteins involved in the overall regulation of boar-sperm function

Figure 10 and Table 4 show the changes in pSer levels of 30 different proteins after both HTs (3h and 24h). Storing sperm samples for a longer HT (24h) resulted in a significant increase ($P<0.05$) increase in the phosphorylation levels of serine residues in HSP70, TRK(A, B and C), CDK1/CDC2 and GSK-3α, when compared with the phosphorylation levels of serine residues after a shorter HT (3h). Notwithstanding, from these four proteins, the highest increase was seen in HSP70, which increased pSer levels from 100.0±0.0 arbitrary units (3h) to 150.2±5.1 arbitrary units (24h).

Specific immunopreciptation analysis of pSer changes of HSP70 during HT

The specific immunoprecipitation analysis confirmed that pSer of HSP70 was specifically and significantly higher ($P<0.05$) when the HT was of 24h than when it was of 3h (Figure 11). Indeed, the ratio between serine phosphorylated HSP70 and total content of HSP70 protein (pSer-HSP70:total HSP70) was significantly higher ($P<0.05$) after a HT of 24h (0.68±0.05) than after a HT of 3h (0.35±0.03).

Multiple regression analyses between pSer in HSP70 and cryotolerance indexes

Table 5 shows Pearson correlation coefficients between extracted components from PCA with cryotolerances indexes (calculated after incubation for 30 or 240 min at 37ºC) and values from pSer levels in HSP70 obtained in mini-array analyses and in immuno-precipitation against HSP70 assessment (pSer-HSP70:total HSP70 ratio). Whereas the first components from PCA obtained from cryotolerance indexes, both at 30 and 240 min of incubation at 37ºC, were positively and significantly correlated with pSer levels in HSP70 obtained in mini-array analyses and pSer-HSP70:total HSP70 ratios observed

after immunoprecipitation, no significant correlation was seen for the other four components.

Given that the relevance of the first component in sperm cryotolerance, such component was used in regression equations as an independent variable, whereas dependent variables submitted to the stepwise forward model were all the sperm parameters (not cryotolerance indexes but raw sperm parameters) evaluated before freeze-thawing. As dependent variable, spots intensity for each protein obtained in mini-array protein analyses and pSer-HSP70:total HSP70 ratio were also included and results are shown in Table 6. From all the dependent variables submitted to the model, only the pSer-HSP70:total HSP70 ratio was included, whereas the others were left out. In all the cases, up to four models were run and all included the same dependent variable. Thus, predicting sperm cryotolerance before freeze-thawing resulted to be possible when considering the pSer-HSP70:total HSP70 ratios but not the other sperm parameters.

Discussion

Our results confirm that HT for 24h has an improving effect on boar-sperm cryotolerance. In addition, we have also shown that this improving effect is concomitant with changes in pSer levels of proteins like HSP70. This change suggests that the improving action of HT could be related to post-transcriptional changes in the activity of the anti-stress molecular mechanisms launched by boar spermatozoa during freeze-thawing, taking into account that spermatozoa are not able to up/downregulate their gene expression. In this way, covalent post-translation protein-modification mechanisms like protein phospho/dephosphorylation [9, 11] will be one of the most important post-translational mechanisms involved in the modulation of these anti-stress mechanisms.

In the present study, boar-sperm cryopreservation has been utilised as a model for inducing stressful conditions [1]. However, this application required a previous approach in order to better learn the effects of HT on the success of sperm cryopreservation. Thus, the absence of significant differences between HTs in any of the sperm functional parameters before starting the cryopreservation (i.e. in extended semen) is noteworthy. In contrast, the existence of differences between HTs after freeze-thawing indicates that during this storage period boar spermatozoa increase their cryotolerance. These conclusions are only partially supported by the existing literature, since there are only a few reports regarding the HT effects on boar-sperm post-thaw

602 quality and their results are controversial [5-7]. In addition, these effects have mainly
603 been reported so far on the basis of post-thaw sperm viability and motility assessments,
604 without evaluating other relevant parameters such as the stability of the sperm nucleus
605 or intracellular ROS levels. Against this background, the present work clearly
606 demonstrates that a HT of 24h prior to sperm cryopreservation deteriorates the post-
607 thawed sperm cell less than does a HT of 3h.
608 In the case of the sperm-nucleus structural integrity, our results indicate for the first
609 time that the extent of damages that freeze-thawing infringes upon the boar-sperm
610 nucleus depends on the HT period. It is worth mentioning that previous studies of our
611 group have observed that freeze-thawing increases the number of disrupted disulphide
612 bonds in boar-sperm nucleoproteins [34], [45]. Despite the mechanism which underlies
613 the rupture of these disulphide bonds still remaining unknown, these alterations might
614 affect the sperm's fertilising ability [51-52]. In fact, protamines, the most abundant
615 proteins in a sperm nucleus [53], condense the chromatin, protect DNA from nucleases
616 and other damaging agents, and remove transcription factors and proteins to help reset
617 the imprinting code in the fertilised oocyte [54]. In addition, protamines are cysteine-
618 rich proteins that establish disulphide bonds between them. This, in turn, stabilises
619 protein conformation [55], so that any alteration that disrupts disulfide bonds can
620 negatively affect the protamine functions. Regarding DNA fragmentation, it is not
621 observed immediately after thawing, but mainly after 240 min [34], and in pigs it has a
622 much lower incidence than in other species such as human and horse [51], [56]. In the
623 present work, levels of sperm DNA fragmentation in FT spermatozoa are higher when
624 the HT is of 3h than when it is of 24h. This agrees with our data regarding the levels of
625 free cysteine residues in sperm nucleoproteins (FT spermatozoa) that are higher when
626 the HT is of 3h than when it is of 24h. Thus, it seems reasonable to hypothesise that a
627 longer contact of sperm with seminal plasma factors during HT [2-3] triggers a sperm
628 response that leads to the protection of the sperm nucleus against freeze-thawing.
629 Apart from the protective effects on the boar-sperm nucleus, the benefits of a longer HT
630 in FT spermatozoa have also been observed in sperm membrane integrity (assessed by
631 SYBR-14/PI and PNA-FITC/PI assays), permeability (assessed through YO-PRO-1/PI),
632 and lipid disorder (assessed through M540/YO-PRO-1). Our results match with those
633 obtained by Eriksson et al. [6], who found that post-thaw sperm viability was
634 significantly higher when HT was longer (of 10h or 20h) than when it was shorter (3h),
635 and with those reported by Kotzias-Bandeira et al. [5], who concluded that a longer HT

636 resulted in a higher acrosome integrity after freeze-thawing. In contrast, Guthrie and
637 Welch [7] did not find significant differences between the same two HTs performed in
638 this study (i.e. 3h and 24h) either in sperm plasma membrane integrity or in sperm
639 motility. However, there are two aspects that we must take into account when
640 comparing our results with those obtained by the other authors. First, Kotzias-Bandeira
641 et al. [5], Eriksson et al. [6] and Guthrie and Welch [7] evaluated the differences
642 between HTs within 30 min post-thawing, while in our study we have evaluated the
643 spermatozoa not only after 30 min, but also after 240 min post-thawing, and the highest
644 difference between both HTs has mainly been seen after 240 min rather than after 30
645 min. Second, all of these previous reports only evaluated plasma membrane integrity
646 and motility of spermatozoa, whereas this study has also evaluated HT effects on the
647 sperm nucleus, membrane permeability and lipid disorder. Finally, and even though
648 freeze-thawing slightly increases the percentages of viable spermatozoa with high levels
649 of H_2O_2 without affecting $O_2^{-\bullet}$ levels, HT has no effect on ROS generated during
650 cryopreservation. These results are not surprising in boar sperm. We must remember
651 that, whereas with species such as horse [57], bull [58] and dog [59], sperm
652 cryopreservation increases ROS levels, the production of ROS linked to
653 cryopreservation is lower in pig and has a lesser impact [34], [43], [60-61], minimising
654 thus the role of ROS as deleterious agents in boar-sperm cryopreservation.
655 As indicated above, our results indicate that the higher cryotolerance linked to a HT of
656 24h is concomitant with specific changes in pSer levels of several structural sperm
657 proteins. Specifically, mini-array analyses suggest an increase in pSer levels in four
658 proteins, namely HSP70, GSK3, total TRK and CDK1/CDC2. These results suggest
659 that, at least in part, seminal plasma factors and/or other factors present in extended
660 semen confer higher cryotolerance during HT and act through serine-phosphorylation of
661 some proteins that are involved in stress, such as HSP70. In this sense, we must
662 remember that the presence of seminal factors in extended semen may confer higher
663 resistance to cold shock [3]. This obviously does not preclude the existence of other
664 mechanisms related to the phosphorylation of other residues, such as tyrosines or
665 threonines, and/or phosphorylation/dephosphorylation of other proteins different from
666 the 30 studied in this work. However, the putative effects of HT on pSer of GSK3, total
667 TRK and CDC2/CDK1 are at this moment only hypothetical. This is due to two facts.
668 The first one is that pSer changes observed in mini-arrays were of a much lower
669 intensity than those observed in HSP70. The second fact is that we have not confirmed

the specificity of the obtained results by performing further experiments involving specific immunoprecipitation and subsequent pSer analyses of the specifically immunoprecipitated GSK3, total TRK and CDC2/CDK1. For this reason, further research is warranted to better elucidate the role that the observed changes in pSer of GSK3, total TRK and CDC2/CDK1 plays on boar-sperm cryotolerance.

The interpretation of results regarding HSP70 is different from that of those from GSK3, total TRK and CDC2/CDK1. First, HSP70 presents the highest differences between both HTs. Second, our immunoprecipitation approach confirms the specificity of the anti-HSP70 antibody used in mini-array analyses, since the specific antibody immunoprecipitated a protein with the recorded molecular weight of HSP70. Furthermore, immunoprecipitation analyses have confirmed that differences between levels of phosphorylated serines observed between HTs are due to the phosphorylation of these residues for a longer HT rather than to alterations in HSP70 content. Taking this into consideration, the pSer levels observed after a HT of 24h are half high as those observed after a HT of 3h. Here, we must also point out that although immunoprecipitation yield/effectiveness leads that not all the samples have the same quantity of HSP70, the relevant aspect is the amount of HSP70 that is serine-phosphorylated, and this is rightly assessed through pSer-HSP70:total HSP70 ratio. Finally, it is worth noting that we have correlated sperm cryotolerance (Component 1 from PCA) with pSer-HSP70:total HSP70 ratios, and that the pSer-HSP70:total HSP70 ratio has been the only parameter included in regression analyses as being able to predict boar-sperm cryotolerance before freeze-thawing. Related to this, it is important to keep in mind that, in the case of Component 1, factor loadings (a_{ij}) from cryotolerance indexes were positive in those categories including fully intact spermatozoa (i.e. %SYBR-14$^+$/PI$^-$ spermatozoa, %YO-PRO-1$^-$/PI$^-$ spermatozoa, …) but negative in those categories of non-viable/damaged spermatozoa (e.g. %SYBR-14$^+$/PI$^+$ spermatozoa, %YO-PRO-1$^+$/PI$^+$ spermatozoa, %YO-PRO-1$^+$/PI$^-$ spermatozoa).

The involvement of HSP70 in the modulation of cryotolerance is not surprising if we take into account that heat-shock proteins have been found in mammalian spermatozoa [17], [62-63]. Specifically, HSP70 is a chaperone protein involved in maintaining proper protein conformation [64]. This protein changes its distribution patterns during capacitation and acrosome reaction [65] and is involved in spermatogenesis, fertilisation and post-fertilisation events [66-68]. Up to now, studies relating HSP70 and sperm quality have been focused on the amounts of this protein and its correlation with sperm

quality parameters, heat-stress response and thermotolerance of freshly ejaculated rather than FT spermatozoa [23], [66], [69]. However, the main finding of our work is that the involvement of this protein in boar-sperm cryotolerance seems to occur, at least in part, via signal transduction pathways that involve phosphorylation-related mechanisms, as confirmed by our immunoprecipitation studies and in a similar fashion to that observed in osmotic stress response of rhesus macaque sperm [17]. Nevertheless, the mechanism/s by which HSP70 could exert its protective role against cryopreservation is not clear. Thus, Bohring et al. [70] suggested that HSP70 is hardly been involved in a stress response because spermatozoa have highly condensed chromatin and, in this way, sperm cells are unable to develop a stress response. These authors proposed, instead, that HSP70 might mediate protein folding and the process of translocation across the sperm membrane. However, in our study we have seen that sperm samples stored for a longer HT present higher levels of serine phosphorylation than those stored for a shorter HT. This does not discard the hypothesis that HSP70 intervenes in protein folding, but it does emphasise the possibility that HSP70 also mediates stress response in sperm by activation/inactivation through serine-phosphorylation. Finally, another aspect that remains to be elucidated regards the mechanism by which a protein that, so far, has mainly been found at the equatorial region of boar spermatozoa [68] increases the sperm cryotolerance after serine phosphorylation.

To the best of our knowledge this is the first study demonstrating that a key post-translation mechanism of protein regulation such as phospho/dephosphorylation is involved in sperm cryotolerance. Indeed, previous reports have demonstrated that cryotolerance is related to the content of some proteins involved in stress response, such as HSP90AA1 [71], but no one has previously demonstrated that not only the content but also the phosphorylation status of serine residues is relevant for sperm cryotolerance, as PCA and multiple regression analyses have shown. This suggests that spermatozoa present some regulation mechanism that modulates sperm physiology during storage at 17ºC and confers, among other changes, higher resistance to stressful procedures such as cold-shock and osmotic stress during sperm cryopreservation.

In conclusion, in the present report we have observed, in agreement with other studies [34], [45], [52], that freeze-thawing of boar spermatozoa impairs their plasma membrane, motility, and destabilises their nucleoprotein structure by disrupting disulphide bonds. However, the extent of this deterioration depends on the duration of the previous HT, in a concomitant manner with changes in pSer levels of some sperm

proteins like HSP70. For this reason, we hypothesise that seminal plasma or other factors present in extended semen trigger a signal transduction pathway during the HT period that involves serine-phosphorylation of proteins like HSP70, and this increase, in turn, is involved in the HT-related increase of boar-sperm cryotolerance. Finally, these results open the door to further investigations centred in the study of post-translational protein modifications that may underlie sperm cryotolerance in mammalian species.

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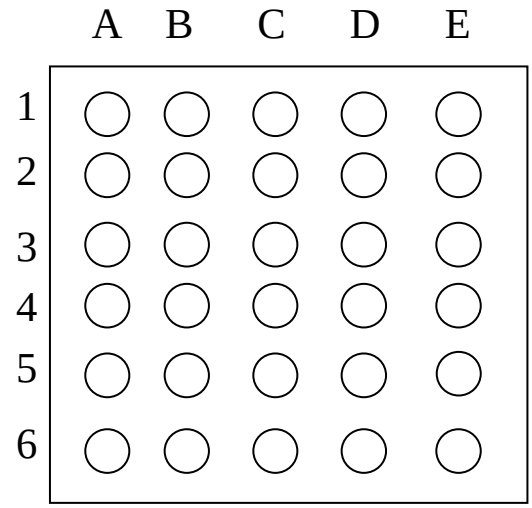
Figure 1 General scheme of the distribution of the proteins for the mini-array analysis.

1A: AKT-1/AKT-2.	4A: CLUSTERIN
1B: CDK6	4B: ERK-2
1C: CYCLIN E	4C: PP1, PP2A, PP2B, PPX
1D: IRAK	4D: TRK A, B, C
1E: PYK2/CAKβ	4E: CDK1/CDC2
2A: CASPASE 9	5A. CYCLIN A
2B: C-KIT	5B: GSK-3α
2C: CYCLIN H	5C: PTP1 (SH)
2D: PI3 KINASE/p85	5D: CDK2
2E: C-RAF-1	5E: CYCLIN B
3A: CDC25	6A: HSP70
3B: ERK-1	6B: PTP1 B
3C: PKC	6C: CDK4
3D: RAS	6D: CYCLIN D3
3E: CDC6	6E: PTP2 (SH)



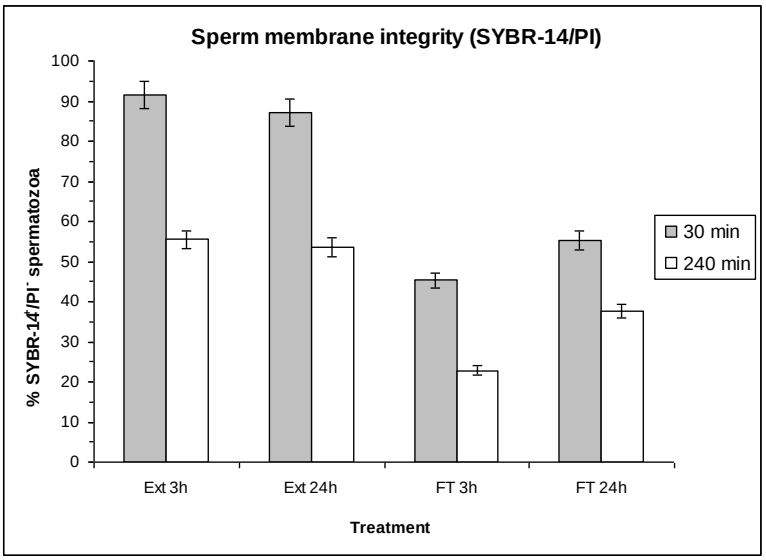


Figure 2 Percentage (as mean±SEM) of spermatozoa with intact plasma membrane (SYBR-14$^+$/PI$^-$, viable spermatozoa), before and after freeze/thawing when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*, *e*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.

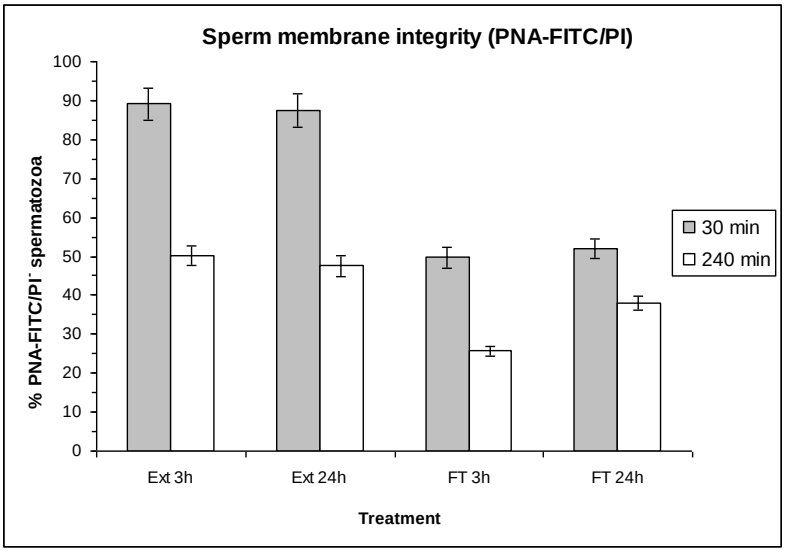
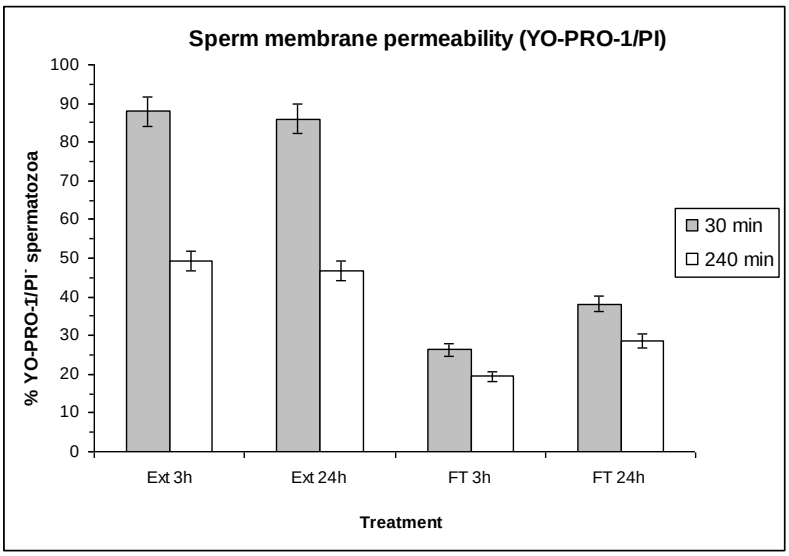


Figure 3 Percentages of spermatozoa with intact plasma membrane evaluated through PNA-FITC/PI assay. Bars show percentages of PNA-FITC$^-$/PI$^-$ spermatozoa (as mean±SEM) before and after freeze/thawing when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext

969 vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-
970 C=Frozen-thawed semen.



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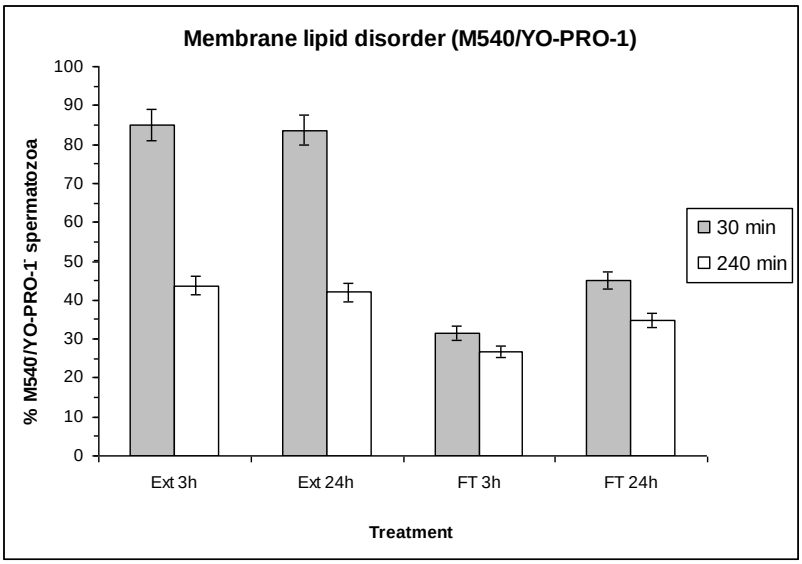
972 **Figure 4** Sperm membrane permeability evaluated through YO-PRO-1/PI assay. Bars
973 show percentages (as mean±SEM) of viable spermatozoa without changes in
974 permeability of plasma membrane (YO-PRO-1$^-$/PI$^-$) before and after freeze/thawing
975 when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*,
976 *e*) mean significant differences (P<0.05) among bars, each bar representing different
977 holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or
978 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.



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980 **Figure 5** Sperm membrane lipid disorder evaluated through M540/YO-PRO-1 co-
981 staining. The figure shows percentages (mean±SEM) of viable spermatozoa with low
982 membrane lipid disorder (M540$^-$/YO-PRO-1$^-$) before and after freeze/thawing when

sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*, *e*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.

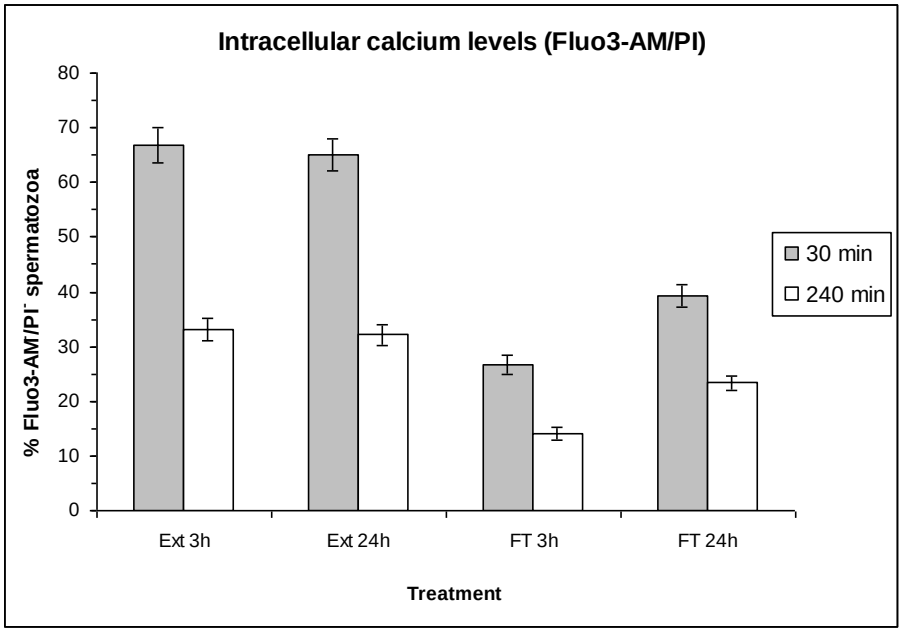

Figure 6 Intracellular calcium levels evaluated through Fluo3-AM/PI assay. Bars show percentages (as mean±SEM) of viable spermatozoa with low levels of intracellular calcium (Fluo3-AM$^-$/PI$^-$) before and after freeze/thawing when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*, *e*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.

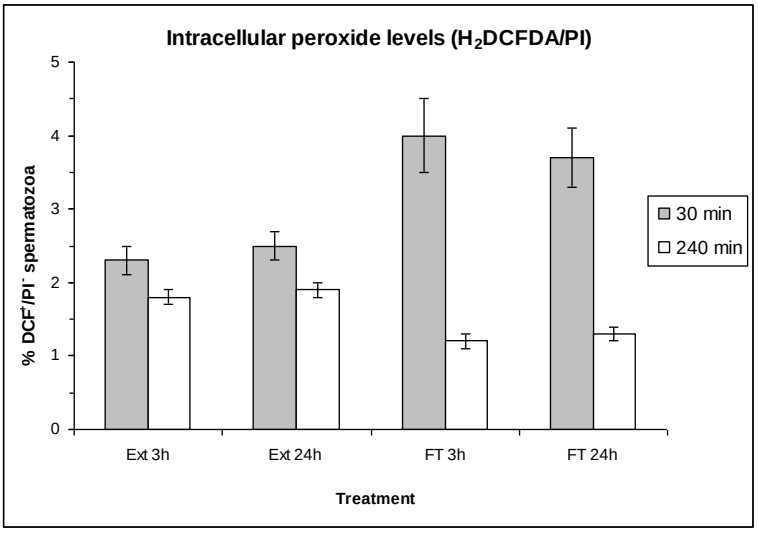

Figure 7 Intracellular peroxide levels (H_2DCFDA/PI staining), as percentages (as mean±SEM) of viable spermatozoa with high levels of intracellular peroxides (DCF $^{+}$/PI^{-}) before and after freeze/thawing when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*, *e*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.

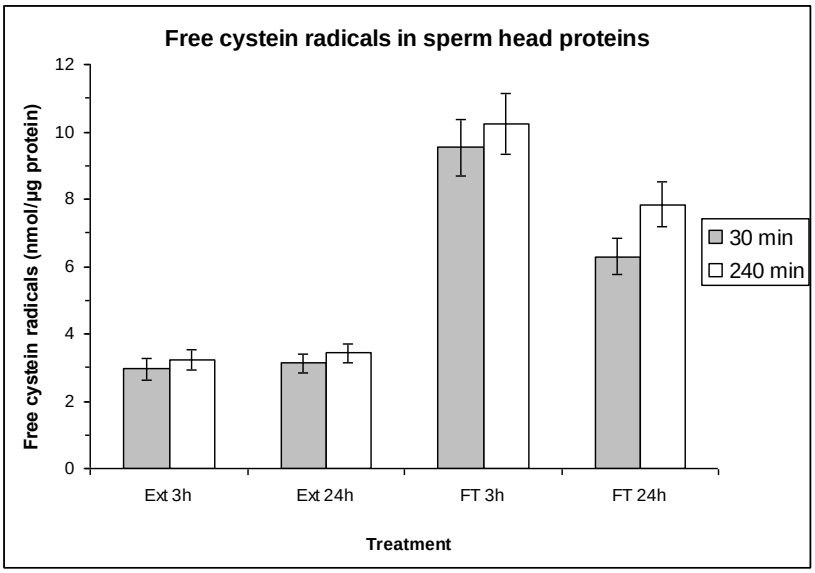

Figure 8 Free cysteine radicals (FCR) in sperm-head proteins (as mean±SEM), before and after freeze-thawing when sperm is stored at 17ºC either for 3h or for 24 hours. Different superscripts (*a*, *b*, *c*, *d*) mean significant differences (P<0.05) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. F-T) and post-thawing times (30 min or 240 min). Ext=Extended semen (17ºC). FT-C=Frozen-thawed semen.

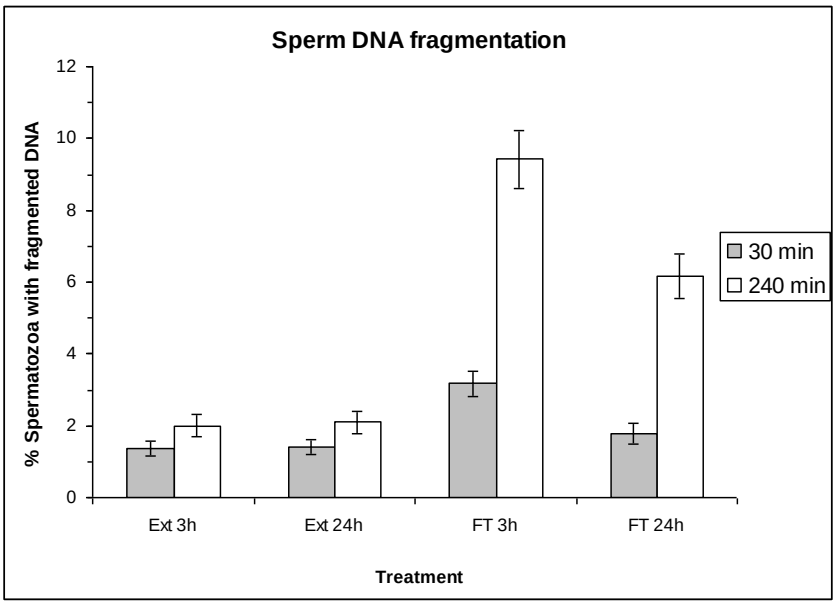

Figure 9 Sperm DNA fragmentation (SDF) as percentage of spermatozoa with fragmented DNA (mean±SEM), before and after freeze/thawing when sperm is stored at 17ºC either for 3h or for 24h. Different superscripts (*a*, *b*, *c*, *d*) mean significant differences ($P<0.05$) among bars, each bar representing different holding times (3h or 24h), preservation (Ext vs. FT) and post-thawing times (30 min or 240 min). Ext=Extended semen (15ºC). FT=Frozen-thawed semen.

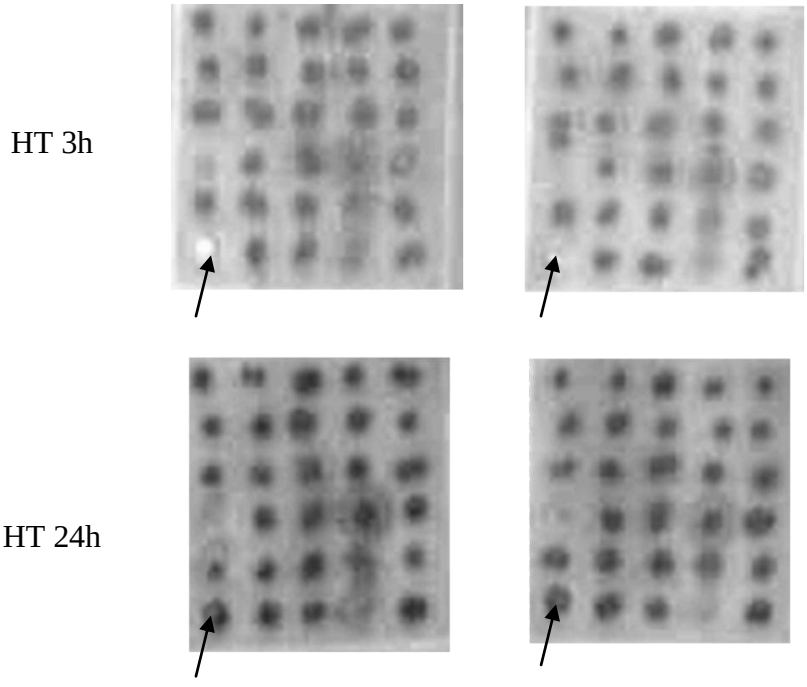

Figure 10 Mini-array analysis of the serine phosphorylation status of 30 sperm proteins after a HT of 3h or 24h. The mini-array analysis and the distribution of the proteins

analysed are described in Figure 1. The figure shows a representative image of two replicates coming from two different sperm samples and is representative of twelve separate experiments. Arrows (→) mark HSP70.

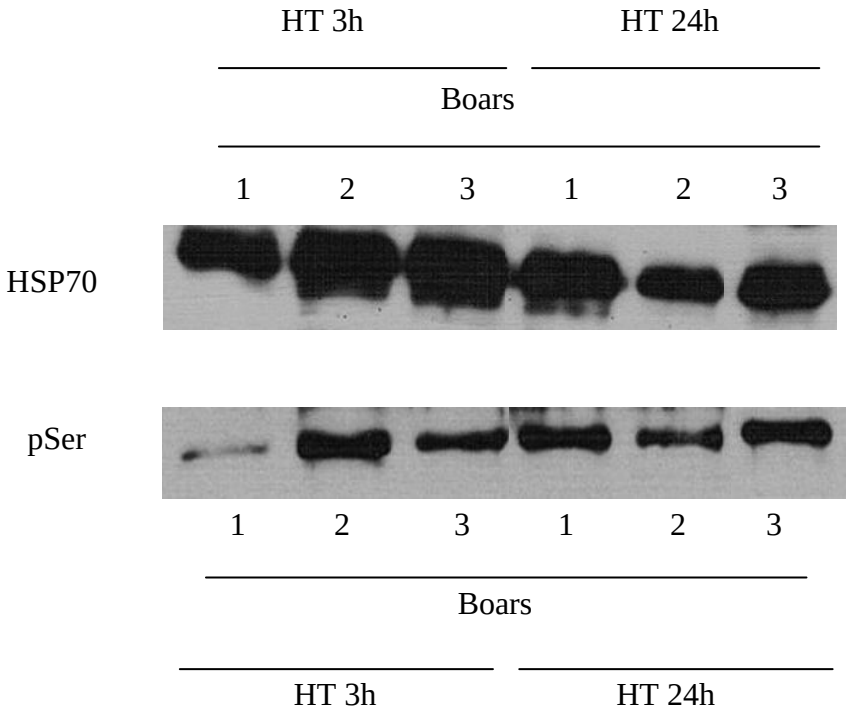

Figure 11 Representative Western-blots against HSP70 and pSer in immunoprecipitation studies after a HT of 3h or 24h. Three replicates coming from three different sperm samples are shown. The figure is representative of twelve different sperm samples stored either for 3h or 24h.

	Extended 3h		*Extended 24h*		*Frozen-Thawed 3h*		*Frozen-Thawed 24h*	
	30 min	***240 min***	***30 min***	***240 min***	***30 min***	***240 min***	***30 min***	***240 min***
% TMOT	87.2 ± 4.8^{a}	58.4 ± 2.9^{b}	86.6 ± 4.9^{a}	57.5 ± 2.8^{b}	48.4 ± 2.9^{b}	26.7 ± 1.6^{e}	55.6 ± 3.0$^{b, c}$	36.9 ± 2.0^{d}
%PMOT	65.3 ± 3.2^{a}	38.6 ± 2.3^{b}	64.2 ± 3.1^{a}	37.8 ± 2.2^{b}	30.1 ± 1.8^{c}	14.8 ± 0.9^{e}	34.5 ± 2.3$^{b, c}$	21.2 ± 1.1^{d}
VSL (µm·s^{-1})	26.6 ± 2.4^{a}	23.8 ± 2.3$^{a, b}$	27.4 ± 2.3^{a}	22.5 ± 2.2$^{a, b}$	19.1 ± 2.0$^{b, c}$	14.1 ± 1.5^{c}	23.1 ± 2.5$^{a, b}$	19.4 ± 1.9$^{b, c}$
VCL (µm·s^{-1})	53.9 ± 3.2^{a}	38.4 ± 2.1^{b}	54.1 ± 3.1^{a}	38.2 ± 2.2^{b}	34.3 ± 2.3$^{b, c}$	23.6 ± 1.8^{d}	40.9 ± 2.4^{b}	31.0 ± 2.4^{c}
VAP (µm·s^{-1})	36.4 ± 2.5^{a}	28.8 ± 1.7^{b}	34.6 ± 2.3^{a}	28.6 ± 1.6$^{b, c}$	25.7 ± 1.8^{c}	17.8 ± 1.4^{d}	31.8 ± 2.5$^{a, b}$	24.2 ± 1.7^{c}
LIN (%)	49.3 ± 2.9^{a}	62.4 ± 3.6^{b}	49.7 ± 2.8^{a}	62.1 ± 3.6^{b}	56.8 ± 3.4^{b}	60.1 ± 3.9^{b}	56.5 ± 3.1^{b}	59.5 ± 3.5^{b}
STR (%)	73.3 ± 3.6^{a}	78.1 ± 3.9^{a}	79.8 ± 3.6^{a}	77.2 ± 3.9^{a}	74.2 ± 3.5^{a}	78.6 ± 3.8^{a}	73.0 ± 3.5^{a}	80.2 ± 3.8^{a}
WOB (%)	68.5 ± 3.8^{a}	75.2 ± 4.1^{a}	67.0 ± 3.7^{a}	74.8 ± 4.3^{a}	74.9 ± 4.1^{a}	75.8 ± 4.2^{a}	76.1 ± 4.3^{a}	75.6 ± 4.0^{a}

Table 1 Effects of holding time prior to freeze-thawing on different sperm motility parameters after 30 and 240 min post-thawing at 37ºC. Data are shown as mean ± SEM. Different superscripts (*a*, *b*, *c*, *d*) mean significant differences (P<0.05) within rows (i.e. for a given motility parameter) among columns (i.e. comparing extended and frozen-thawed semen, and 30 and 240 min of post-thawing incubation at 37ºC).

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	59.49%	% Viable spermatozoa (SYBR-14$^+$/PI$^-$)	0.92	0.85
		Free cysteine radicals in sperm nucleoproteins	-0.91	0.83
		% Spermatozoa with fragmented DNA	-0.89	0.79
		% Non-viable spermatozoa with high lipid disorder (M540$^+$/PI$^+$)	-0.85	0.72
		% Viable spermatozoa without changes in m.p. (YO-PRO-1$^-$/PI$^-$)	0.85	0.72
		% Membrane intact spermatozoa (PNA-FITC$^-$/PI$^-$)	0.84	0.71
		% Viable spermatozoa with low lipid disorder (M540$^-$/PI$^-$)	0.82	0.67
		% TMOT	0.81	0.66
		% Non-viable spermatozoa (YO-PRO-1$^{+/-}$/PI$^+$)	-0.81	0.66
		% Viable spermatozoa with high lipid disorder (M540$^+$/PI$^-$)	-0.80	0.64
		% Membrane damaged spermatozoa that present o.a.m. (PNA-FITC$^+$/PI$^+$)	-0.80	0.64
		VAP	0.79	0.62
		% Viable spermatozoa with low intracellular Ca^{2+} levels (Fluo3-AM$^-$/PI$^-$)	0.79	0.62
		VCL	0.78	0.61
		VSL	0.77	0.59
		% Viable spermatozoa with early changes in m.p. (YO-PRO-1$^+$/PI$^-$)	-0.75	0.56
		% Non-viable spermatozoa with low intracellular Ca^{2+} levels (Fluo3-AM$^-$/PI$^+$)	-0.71	0.50
		% PMOT	0.70	0.49
		GMFI Fluo3-AM$^+$	-0.64	0.41

	2	11.95%	% Membrane damaged spermatozoa with lost o.a.m. (PNA-FITC^{-}/PI^{+})	-0.86	0.74
			% Viable spermatozoa with high intracellular Ca^{2+} levels (Fluo3-AM^{+}/PI^{-})	-0.67	0.45
			% Viable spermatozoa with high intracellular peroxide levels (DCF^{+}/PI^{-})	0.59	0.35
			Non-viable spermatozoa with high intracellular Ca^{2+} levels (Fluo3-AM^{+}/PI^{+})	-0.57	0.32
	3	8.30%	GMFI E^{+}/PI^{-}	0.84	0.71
			GMFI E^{+}/Total	-0.64	0.41
	4	6.78%	GMFI DCF^{+}/PI^{-}	0.98	0.96
			GMFI DCF^{+}/Total	0.68	0.46
	5	4.71%	% LIN	0.87	0.76
			% STR	0.79	0.62
			% WOB	-0.62	0.38
	Total	**91.23%**			

Table 2 Principal component analysis of sperm cryotolerance with parameters (x) evaluated after incubation at 37ºC for 30 min. Each cryotolerance index was calculated as follows: $C_{x,HT,30\text{min}} = \frac{x_{FT,HT,30\text{min}}}{x_{Ext,HT,30\text{min}}} \times 100$

(m.p.: membrane permeability; o.a.m.: outer acrosome membrane; GMFI: Geometric mean of fluorescence intensity; arbitrary units)

Component	Variance	Combinations of variables	a_{ij}	a_{ij}^2
1	64.11%	% Viable spermatozoa without changes in m.p. (YO-PRO-1^-/PI^-)	0.95	0.90
		Free cysteine radicals in sperm nucleoproteins	-0.95	0.90
		% Spermatozoa with fragmented DNA	-0.94	0.88
		% Non-viable spermatozoa (YO-PRO-$1^{+/-}$/PI^+)	-0.93	0.86
		% Viable spermatozoa (SYBR-14^+/PI^-)	0.91	0.83
		% Viable spermatozoa with low intracellular Ca^{2+} levels (Fluo3-AM^-/PI^-)	0.88	0.77
		% Viable spermatozoa with low lipid disorder (M540^-/PI^-)	0.86	0.74
		% TMOT	0.83	0.69
		% Non-viable spermatozoa with low intracellular Ca^{2+} levels (Fluo3-AM^-/PI^+)	-0.82	0.67
		% Viable spermatozoa with high lipid disorder (M540^+/PI^-)	-0.82	0.67
		% Membrane intact spermatozoa (PNA-$FITC^-$/PI^-)	0.81	0.66
		VAP	0.79	0.62
		VCL	0.77	0.59
		% Non-viable spermatozoa with high lipid disorder (M540^+/PI^+)	-0.76	0.58
		% Membrane damaged spermatozoa that present o.a.m. (PNA-$FITC^+$/PI^+)	-0.75	0.56
		VSL	0.75	0.56
		GMFI E^+/ YO-PRO-1^-	0.74	0.55
		% PMOT	0.72	0.52
		% Viable spermatozoa with early changes in m.p. (YO-PRO-1^+/PI^-)	-0.66	0.44

		% Membrane damaged spermatozoa with lost o.a.m. (PNA-FITC$^-$/PI$^+$)	-0.66	0.44
		% Non-viable spermatozoa with low membrane lipid disorder (M540$^-$/PI$^+$)	0.63	0.40
2	11.49%	GMFI E$^+$/Total	-0.90	0.80
		% Viable spermatozoa with high intracellular superoxide levels (E$^+$/YO-PRO-1$^-$)	-0.86	0.73
		% Viable spermatozoa with high intracellular peroxide levels (DCF$^+$/PI$^-$)	-0.75	0.56
		% Viable spermatozoa with high intracellular calcium levels (Fluo3-AM$^+$/PI$^-$)	0.71	0.51
3	9.70%	GMFI DCF$^+$/PI$^-$	0.96	0.92
		GMFI DCF$^+$/Total	0.80	0.64
		% LIN	0.77	0.59
		% STR	0.62	0.38
4	5.24%	GMFI Fluo3-AM$^+$	-0.91	0.83
5	3.66%	% WOB	-0.89	0.79
Total	**94.20%**			

Table 3 Principal component analysis of sperm cryotolerance with parameters (x) evaluated after incubation at 37ºC for 240 min. Each cryotolerance index was calculated as follows: $C_{x,HT,240\text{min}} = \frac{x_{FT,HT,240\text{min}}}{x_{Ext,HT,240\text{min}}} \times 100$

(m.p.: membrane permeability; o.a.m.: outer acrosome membrane; GMFI: Geometric mean of fluorescence intensity; arbitrary units)

Proteins	*Holding time at 17ºC*	
	3h (arbitrary units)	**24h (arbitrary units)**
AKT-1/AKT-2	100.0 ± 0.0[a]	100.5 ± 2.0[a]
CDK6	100.0 ± 0.0[a]	99.8 ± 1.9[a]
CYCLIN E	100.0 ± 0.0[a]	108.6 ± 3.2[a]
IRAK	100.0 ± 0.0[a]	106.3 ± 3.7[a]
PYK2/CAKβ	100.0 ± 0.0[a]	109.4 ± 2.6[a]
CASPASE 9	100.0 ± 0.0[a]	104.8 ± 2.5[a]
c-kit	100.0 ± 0.0[a]	105.3 ± 2.0[a]
CYCLIN H	100.0 ± 0.0[a]	104.2 ± 2.3[a]
PI3 KINASE/p85	100.0 ± 0.0[a]	109.8 ± 3.4[a]
C-RAF-1	100.0 ± 0.0[a]	103.0 ± 4.1[a]
CDC25	100.0 ± 0.0[a]	103.2 ± 2.5[a]
ERK-1	100.0 ± 0.0[a]	110.9 ± 1.4[a]
PKC	100.0 ± 0.0[a]	108.4 ± 2.0[a]
RAS	100.0 ± 0.0[a]	106.9 ± 2.8[a]
CDC6	100.0 ± 0.0[a]	110.3 ± 2.4[a]
CLUSTERIN	100.0 ± 0.0[a]	102.7 ± 2.8[a]
ERK-2	100.0 ± 0.0[a]	112.2 ± 2.9[a]
PP1, PP2A, PP2B, PPX	100.0 ± 0.0[a]	109.5 ± 3.2[a]

TRK Total (A, B, C)	100.0 ± 0.0[a]	120.8 ± 3.4[b]
CDK1/CDC2	100.0 ± 0.0[a]	120.7 ± 2.1[b]
CLYCLIN A	100.0 ± 0.0[a]	109.1 ± 4.1[a]
GSK-3α	100.0 ± 0.0[a]	125.6 ± 0.9[b]
PTP1 (SH)	100.0 ± 0.0[a]	107.5 ± 2.5[a]
CDK2	100.0 ± 0.0[a]	109.9 ± 1.5[a]
CYCLIN B	100.0 ± 0.0[a]	102.4 ± 2.6[a]
HSP70	100.0 ± 0.0[a]	150.2 ± 5.1[b]
PTP1B	100.0 ± 0.0[a]	112.6 ± 2.9[a]
CDK4	100.0 ± 0.0[a]	107.6 ± 2.7[a]
CYCLIN D3	100.0 ± 0.0[a]	105.2 ± 5.5[a]
PTP2 (SH)	100.0 ± 0.0[a]	107.5 ± 1.9[a]

Table 4 Comparison of serine-phosphorylation levels in 30 different sperm proteins after assessment through mini-array analyses. The values obtained for the HT of 3h were transformed in order to obtain a basal arbitrary value of 100, from which the intensity values for the other samples (i.e. HT 24h) were calculated. Results are given as mean ± SEM, and different superscripts (*a*, *b*) mean significant differences ($P<0.05$) within rows (i.e. between the two holding times) for each protein.

		HSP70 (miniarray values)	pSer-HSP70:total HSP70 ratio (immunoprecipitation)
30 min	Regression factor for Component 1	0.77*	0.74*
	Regression factor for Component 2	-0.33	-0.29
	Regression factor for Component 3	-0.02	-0.08
	Regression factor for Component 4	-0.05	-0.08
	Regression factor for Component 5	0.16	0.15
240 min	Regression factor for Component 1	0.75*	0.76*
	Regression factor for Component 2	0.35	0.31
	Regression factor for Component 3	0.06	-0.08
	Regression factor for Component 4	-0.04	0.10
	Regression factor for Component 5	-0.08	0.04

Table 5 Pearson correlation coefficients between the extracted components from PCA with cryotolerances indexes (calculated after incubation for 30 or 240 min at 37ºC) and values from pSer levels in HSP70 obtained in mini-array analyses and in immuno-precipitation against HSP70 assessment (pSer-HSP70:total HSP70 ratio) (*P<0.05)

	Regression equation	R^2	R	*P* value model
30 min				
Lineal	y=4.61x-2.46	0.56	0.75	<0.05
Logarithmic	y=2.34ln(x)+1.60	0.55	0.74	<0.05
Inverse	y=-1.19/x+2.42	0.56	0.75	<0.05
Quadratic	$y=-7.74x^2+11.57x-0.57$	0.59	0.77	<0.05
240 min				
Lineal	y=4.76x-2.54	0.58	0.76	<0.01
Logarithmic	y=2.46ln(x)+1.65	0.58	0.76	<0.01
Inverse	y=-1.23/x+2.51	0.59	0.77	<0.01
Quadratic	$y=-1.65x^2+5.99x-0.97$	0.58	0.76	<0.05

Table 6 Regression equations between regression component 1 of PCA with cryotolerance indexes (*y*) and sperm parameters evaluated before freeze-thawing. From all the dependent variables submitted to the model, only the pSer-HSP70:total HSP70 ratio (*x*) was included, whereas the others were left out.

ARTICULO 5

Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sow inseminated with frozen-thawed boar semen.

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Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen

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Abstract

The main aim of this work was to evaluate how supplementing freezing media with reduced glutathione (GSH) affected the 'in vivo' fertilizing ability of boar semen subjected to cryopreservation procedures. With this purpose, 12 ejaculates coming from 12 boars were cryopreserved in the presence or absence of 2 mM GSH, whereas the same number of extended ejaculates coming from the same boars was used as negative/farm controls. Eight different sperm parameters (levels of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels, and total and progressive sperm motility) were evaluated before freezing and after 30 and 240 min of thawing. In addition, a total of 180 multiparous sows were used in the field fertility trials, the females being randomly divided into three groups and inseminated with extended, frozen-thawed control or frozen-thawed semen supplemented with 2 mM GSH. The presence of GSH in the freezing media significantly ($p < 0.05$) increased farrowing rates and the number of total born piglets and alive born piglets, and partially counteracted the cryopreservation-induced damages inflicted on frozen-thawed spermatozoa. We can thus conclude that supplementing freezing media with 2 mM GSH greatly improves boar sperm cryopreservation technology, as it significantly improves the fertilizing ability of frozen-thawed spermatozoa.

Keywords: AI, boar sperm, cryopreservation, fertility, nucleoprotein structure, prolificacy, reduced glutathione

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Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen

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SUMMARY

The main aim of this work was to evaluate how supplementing freezing media with reduced glutathione (GSH) affected the 'in vivo' fertilizing ability of boar semen subjected to cryopreservation procedures. With this purpose, 12 ejaculates coming from 12 boars were cryopreserved in the presence or absence of 2 mM GSH, whereas the same number of extended ejaculates coming from the same boars was used as negative/farm controls. Eight different sperm parameters (levels of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels, and total and progressive sperm motility) were evaluated before freezing and after 30 and 240 min of thawing. In addition, a total of 180 multiparous sows were used in the field fertility trials, the females being randomly divided into three groups and inseminated with extended, frozen-thawed control or frozen-thawed semen supplemented with 2 mM GSH. The presence of GSH in the freezing media significantly ($p < 0.05$) increased farrowing rates and the number of total born piglets and alive born piglets, and partially counteracted the cryopreservation-induced damages inflicted on frozen-thawed spermatozoa. We can thus conclude that supplementing freezing media with 2 mM GSH greatly improves boar sperm cryopreservation technology, as it significantly improves the fertilizing ability of frozen-thawed spermatozoa.

INTRODUCTION

Artificial insemination (AI) with extended semen offers many benefits to the swine industry, through improving biosecurity and access to high-quality genetic material (Bailey *et al.*, 2008; Knox, 2011). However, AI with frozen-thawed semen only represents 2% of swine inseminations (Roca *et al.*, 2011), as it yields lower fertility rates and litter sizes than those achieved with extended semen (Almlid & Hofmo, 1995; Johnson *et al.*, 2000). New insights into the protocol of boar sperm cryopreservation have previously been made with the aim to increase fertility and prolificacy rates (Spencer *et al.*, 2010). In this regard, it is worth mentioning that the use of post-cervical artificial insemination (post-CAI) with frozen/thawed semen has represented a considerable step forward (Gil *et al.*, 2000; Rath, 2002; Casas *et al.*, 2010).

Previous studies reported that freeze-thawing of boar spermatozoa induces cell alterations that can affect sperm fertilizing ability. Some of these alterations are linked to damage in sperm plasmalemma (Meyers, 2005; Flores *et al.*, 2008; Casas *et al.*, 2009) and osmotic and oxidative stresses (Hernández *et al.*, 2007). Cryopreservation also induces destabilization of nucleoprotein structure, as the increase in the number of disrupted disulphide bonds between sperm nucleoproteins after freeze-thawing manifests (Flores *et al.*, 2011; Yeste *et al.*, 2012). Finally, boar sperm cryopreservation has also been reported to increase DNA fragmentation after 240 min of thawing (Yeste *et al.*, 2012, 2013), even though the extent of such damage is lower than in other mammalian species like horse and human (Baumber *et al.*, 2003; Silva & Gadella, 2006; Fraser *et al.*, 2011).

The antioxidant, reduced glutathione (GSH), is the most abundant thiol in cells and is considered of vital importance, among other functions, for the maintenance of the intracellular redox balance (Jacob *et al.*, 2003). Chatterjee *et al.* (2001) demonstrated that GSH maintains the normal distribution pattern of sulphydryl groups in bull sperm membrane proteins during

freeze-thawing, thereby avoiding the cryopreservation-induced changes in the quantity and distribution pattern of these sulphydryl groups. Furthermore, the addition of GSH to freeze-thawing media has been seen to increase sperm motility, slightly reduce intracellular peroxide levels and enhance the sperm-penetration-to-oocyte ability of boar spermatozoa (Gadea *et al.*, 2004, 2005; Yeste *et al.*, 2012). In this context, the addition of protective agents of disulphide bonds, such as GSH at a final concentration of 2 mM, seems to improve the ability of the nucleoprotein structure to resist cryoinjuries, as previous works of our group have shown (Yeste *et al.*, 2012). However, and despite the relevance of sperm chromatin integrity on fertilizing ability (Silva & Gadella, 2006), no previous study has been conducted to evaluate the actual impact of supplementing freezing extenders with GSH on pig 'in vivo' fertility and prolificacy.

On the basis of this background, this study aimed to determine the effects of adding 2 mM GSH to freeze extenders (LEY and LEYGO) on the field fertility and prolificacy of multiparous sows inseminated with extended, frozen-thawed control or frozen-thawed semen supplemented with 2 mM GSH. In addition, some additional sperm quality parameters (such as the amounts of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, ROS levels and sperm motility) were also assessed in this study and correlated with field fertility and prolificacy data to evaluate their relative importance in explaining boar sperm fertilizing ability 'in vivo' as well as their resistance to cryopreservation.

MATERIALS AND METHODS

The experimental protocol was designed following the guidelines established by the Animal Welfare Directive of the Regional Government of Catalonia (Spain), the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Spain), and according to Spanish welfare and protection standards in swine (R.D. 1392/2012).

Seminal samples

In total, 24 ejaculates coming from 12 healthy boars from the Pietrain breed, 2–3 years of age, were used in this study. These animals were housed in climate-controlled buildings (Swine Genetic Services, S.L., Roda de Ter, Barcelona, Spain), fed with an adjusted diet (2.3 kg/d) and provided with water ad libitum. In addition, all of them were of proven fertility according with the AI station records, and were found to present good ejaculate freezability ('good freezers').

From all of these 24 ejaculates, 12 were used for cryopreservation purposes, whereas the other 12 were used as farm controls (extended semen) for evaluating sperm parameters and performing in vivo fertility trials. In all cases, the sperm-rich fractions were collected manually twice per week using the hand-gloved method, diluted with a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain), and cooled down to 16–17 °C. Ejaculates used as farm controls were diluted 1 : 9 (v:v) with the above-mentioned commercial extender and split into seminal doses of 60 mL and 3×10^9 spermatozoa/dose each, suitable for post-CAI. Two of these seminal doses, stored at 17 °C, were sent to our laboratory to assess the following eight sperm parameters after incubation of the spermatozoa for both 30 and 240 min at 37 °C: levels of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels, and total and progressive sperm motility (PMOT). The other 12 ejaculates were diluted 1 : 2 (v:v) with the same commercial extender, packaged with plastic bags and also sent to our lab at 17 °C. An aliquot from each of these 12 ejaculates was also taken before starting the cryopreservation process to evaluate the eight previously mentioned parameters.

In all cases, the quality of all sperm samples was evaluated upon arrival to check that they satisfied the quality standards previously established as minimal conditions to proceed with the freeze-thawing procedure (i.e. total sperm motility >80%, PMOT >60%; morphologically normal spermatozoa >85%; sperm viability >85% (Yeste *et al.*, 2012).

Cryopreservation and thawing of sperm samples

When stated, semen samples were cryopreserved using the Westendorf method (Westendorf *et al.*, 1975) and adapted by Yeste *et al.* (2012). Briefly, all ejaculates were cryopreserved 24 h after extraction and storage at 17 °C. At that time, the ejaculates were centrifuged at 17 °C and 600***g*** for 5 min. Then, pellets were recovered and diluted at 1.5×10^9 spermatozoa/mL (using a Makler counting chamber; Sefi-Medical Instruments, Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Spermatozoa were next cooled down to 5 °C for 90 min, and subsequently diluted at 1×10^9 spermatozoa/mL in a second medium containing LEY glycerol and ES Paste (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA) (LEYGO), at final concentrations of 2 and 0.5% respectively. During the sperm cryopreservation process, each ejaculate was divided into two equal parts. The first one followed the conventional freezing process, whereas LEY and LEYGO cryopreservation extenders of the other part were supplemented with 2 mM reduced L-glutathione ($C_{10}H_{17}N_3O_6S$; GSH, Sigma-Aldrich®, St Louis, MO, USA). This GSH concentration was chosen because of our previously published results (Yeste *et al.*, 2012). Afterwards, sperm samples were packed in 0.5-mL plastic and labelled straws (Minitub Ibérica, SL) distinguishing between treatments [frozen-thawed control (FT C) vs. frozen-thawed supplemented with 2 mM GSH (FT GSH)], boars and ejaculates. The straws were then transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica, SL). The freezing programme (SY-LAB software; Minitub Ibérica, SL) consisted of 313 sec of cooling at the following rates: −6 °C/min from 5 to −5 °C (100 sec), −39.82 °C/min from −5 to −80 °C (113 sec), maintained for 30 sec at −80 °C, and finally cooled at −60 °C/min from −80 to −150 °C (70 sec). The straws were finally plunged into liquid N_2 (−196 °C) for further storage.

After at least 2 weeks of storage at −196 °C, four straws per ejaculate and treatment (FT C or FT GSH) were taken, thawed at 37 °C for 20 sec and immediately diluted with three volumes of warmed Androstar Plus® at 37 °C at a final dilution of 1/4 (Casas *et al.*, 2012). All the sperm parameters (amounts of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels and sperm motility) were evaluated at 30 and 240 min after thawing at 37 °C.

Evaluation of free-cysteine residues in sperm nucleoproteins before and after freeze-thawing

The determination of free cysteine radicals in sperm nucleoproteins, as an indirect measure of disrupted disulphide bonds

within nucleoproteins, was carried out following the protocol adapted to boar spermatozoa and described by Flores *et al.* (2011). Briefly, samples were centrifuged at 600×***g*** and 17 °C for 20 min and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycolate, 1 mM benzamidine, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator, Ika® Labortechnick; Staufen, Germany), and the homogenates centrifuged at 850***g*** and 4 °C for 20 min. Both the resultant supernatants and the upper layer of the pellet were discarded, and the pellets were subsequently resuspended in 500 μL of PBS. The purity of this separation was determined by observation under a phase-contrast microscope (Zeiss Primo Star, Karl Zeiss; Jena, Germany) at 400× magnifications (Zeiss Plan-Achromat 40×/0.65; Karl Zeiss). Samples purity was described as the percentage of loose heads in comparison with the presence of whole, non-fractioned spermatozoa and separated tails in the sample. In all cases, the mean purity percentage was higher than 95% of loose heads in comparison with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separating the heads from their respective mid-pieces.

The levels of free cysteine radicals in sperm nucleoproteins were determined in the samples obtained by using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma-Aldrich®) as described by Brocklehurst *et al.* (1979). Briefly, the 10-μL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 μL of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine. Ten-μL aliquots of cysteine standards from 0.1 to 5 mM (Sigma-Aldrich®) were also added to 990 μL of 0.4 mM 2,2′-dithiodipyridine for evaluation. In all the cases, mixtures were incubated at 37 °C for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalized through a parallel determination of the total protein content of samples by the Bradford (1976) method, using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules, CA, USA). Three replicates per sample and treatment were evaluated, and the corresponding mean ± SEM (standard error of the mean) was subsequently calculated.

Evaluation of DNA fragmentation before and after freeze-thawing

DNA fragmentation was assessed using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain) and following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this technique strongly correlated with those obtained with other tests, like the neutral comet assay (Enciso *et al.*, 2006).

Briefly, the lysing buffer included in the commercial kit was tempered to 22 °C and vials containing low-melting agarose were incubated at 100 °C for 5 min in a water bath. Vials were then left in another water bath at 37 °C for 5 min to equilibrate the agarose temperature. Twenty-five μL of each sperm sample (at a final concentration of 10^7 spermatozoa/mL) were added to a vial prior to mixing it thoroughly. One drop of 25 μL containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid air-bubble formation.

Slides were placed on a cooled plate within a fridge and left at 4 °C for 5 min. The coverslip was then removed and 50 μL of lysis solution per slide were added. An incubation step at 22 °C for 5 min was performed, prior to washing for 5 min with miliQ® water. The slides were subsequently dehydrated by three steps of 2 min each with ethanol at 70, 90 and 100%. Finally, sperm samples were stained with propidium iodide (PI, 2.5 μg/mL; Molecular Probes®, Eugene, OR, USA) and mounted in DABCO antifading medium (DABCO™ anti-fading medium; Sigma-Aldrich®). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 1000× magnification.

Three counts of 250 spermatozoa each using three different slides were carried out per sample, prior to calculating the corresponding mean ± SEM. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only a small halo.

Flow cytometric analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) (Lee *et al.*, 2008). These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, PNA-FITC/PI, H_2DFCDA/PI, HE/YO-PRO®-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter, Fullerton, CA, USA; Serial Number AL300087, Technical specification at https://www.beckmancoulter.com/wsrportal/ajax/downloadDocument/721742AD.pdf?autonomyId=TP_DOC_32032&documentName=721742AD.pdf). This instrument, which had not been altered in the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab Quanta™ SC cytometer employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. In this system, the optical characteristics for these filters were as follows: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band pass filter: 525 nm, detection width 505–545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter:

575 nm, detection width: 560–590 nm); and FL3 (red fluorescence): long pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, YO-PRO®-1 and H_2DFCDA), whereas FL3 was used to detect red (HE and PI) fluorescence.

Sheath flow rate was set at 4.17 μL/min in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE vs. SS dot plots) for a minimum of 10 000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter <7 μm) and cell aggregates (particle diameter >12 μm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described below, compensation was used to minimize spill-over of green fluorescence into the red channel.

Information on the events was collected in List-mode Data files (LMD), and these generated files were then analysed using Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyse the cytometric histograms. In PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assessments, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina *et al.* (2010). Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± SEM.

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen, Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma).

Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner & Johnson (1995). Briefly, sperm samples were incubated at 38 °C for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 μM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, whereas PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: (i) viable green-stained spermatozoa (SYBR-14$^+$/PI$^-$); (ii) non-viable red-stained spermatozoa (SYBR-14$^-$/PI$^+$); and (iii) non-viable spermatozoa that were stained both green and red (SYBR-14$^+$/PI$^+$). Non-sperm particles (debris) were found in the SYBR-14$^-$/PI$^-$ quadrant.

Single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Acrosome integrity (PNA-FITC/PI)

Acrosome integrity was assessed by costaining the spermatozoa with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocianate (FITC) and PI, according to the procedure described by Nagy *et al.* (2003). Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5 μg/mL) and PI (final concentration: 10 μM) and incubated at 38 °C for 10 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa were identified and placed in one of the four following populations: (i) viable spermatozoa with intact acrosomes (PNA-FITC$^-$/PI$^-$); (ii) viable spermatozoa with damaged (exocytosed) acrosomes (PNA-FITC$^+$/PI$^-$); (iii) non-viable cells with intact acrosomes (PNA-FITC$^-$/PI$^+$); and (iv) non-viable cells with damaged acrosomes (PNA-FITC$^+$/PI$^+$).

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC-spill over into the PI/FL-3 channel (2.45%).

Assessment of intracellular ROS levels

Intracellular peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) levels were determined using two different oxidation-sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed by costaining the spermatozoa either with PI or with YO-PRO®-1.

In the case of peroxides, spermatozoa were stained with H_2DCFDA at a final concentration of 200 μM and PI at a final concentration of 10 μM, and incubated at 25 °C for 60 min in the dark. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie & Welch, 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3. Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

In the case of superoxides, samples were stained with HE (final concentration: 4 μM) and YO-PRO®-1 (final concentration: 40 μM) and incubated at 25 °C for 40 min in the dark (Guthrie & Welch, 2006). Hydroethidine is freely permeable to cells and is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of ethidium (E$^+$) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means ± SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF$^+$ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E$^+$).

Correction of data: identification of non-DNA containing particles

The percentage of non-DNA-containing particles (alien particles) was determined since in some flow cytometry assessments, especially when working with cryopreserved spermatozoa, there may be an overestimation of sperm particles. Indeed, alien particles such as cytoplasmic droplets, cell debris or diluent components (such as egg yolk), will often show EV/FS and SS characteristics similar to those of spermatozoa and cannot thus be excluded via light scatter (Petrunkina *et al.*, 2010). For this reason, 5 μL of each sperm sample coming from cooling or post-thawing steps were diluted with 895 μL of milliQ®-distilled water. Samples were then stained with PI at a final concentration of 10 μM and incubated at 38 °C for 3 min, according to the procedure described by Petrunkina *et al.*, 2010. Percentages of alien particles (f) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and treatment after PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assays, according to the following formula:

$$q_1' = \frac{q_1 - f}{100 - f} \times 100$$

where q_1' is the percentage of non-stained spermatozoa after correction.

Sperm motility

Sperm-motility analysis was performed by utilizing a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10× 0.30 PLAN objective lens, Olympus-Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 ms. Five to six separate fields were taken for each replicate, and three replicates were run per sample.

For each assessment, 15 μL of sperm sample was placed in a Makler counting chamber (Sefi-Medical Instruments), and total and progressive motility together with other kinetic parameters were recorded (Yeste *et al.*, 2008). Total motility was defined as the percentage of spermatozoa that showed a VAP > 10 μM/s, whereas progressive motility was defined as the percentage of spermatozoa that showed a VAP > 45 μm/s.

Sperm morphology

As stated, sperm morphology was assessed upon arrival of the seminal samples to verify that they satisfied the quality standard (i.e. morphologically normal spermatozoa >85%). For this purpose, 5 μL of each semen sample was placed on a slide and mounted with a cover slip. Slides were then incubated for 30 min in 100% humidity at 25 °C to immobilize the spermatozoa. Sperm morphology was assessed subjectively by making three counts of 100 spermatozoa each, prior to calculating the corresponding mean ± SEM and differentiating between morphologically normal spermatozoa, spermatozoa with cytoplasmic droplets and aberrant spermatozoa (coiled tails, tails folded at the connecting piece, at the intermediate piece or at Jensen's ring) (Yeste *et al.*, 2008). A phase-contrast microscope (Olympus BX41) was used, and the samples were observed at 200× magnification (Olympus 20× 0.40 PLAN objective lens, positive phase-contrast field).

Reproductive management and insemination of sows

In this study, a total of 180 multiparous sows from a breeding farm (Swine Genetic Services, S.L., Sant Sadurní d'Osormort, Barcelona, Spain) were used in the fertility trials. These sows, from Landrace and Large White breeds, were also housed in climate-controlled buildings, fed with an adjusted diet (2.2 kg/d) and provided with water ad libitum.

Sows were randomly divided into three groups of 15 animals each, and each group was inseminated with extended, frozen-thawed control (FT C) or frozen-thawed semen supplemented with 2 mM GSH (FT GSH). This experiment was repeated every 2 months and up to four times, according to an insemination programming system of all-in/all-out production followed by the breeding farm. Thus, a total of 60 sows per group (extended, FT C, FT GSH) were inseminated. Each ejaculate was used to inseminate five sows.

The insemination programme was carried out according to the management of sows at weaning. Detection of oestrus was monitored from Day 2 post-weaning by inspection of the vulva for reddening and swelling and response to a male teaser, and confirmed at Days 4 and 5 after pressing on the sow's back and determining for the presence/absence of the standing reflex. (Soede *et al.*, 2011). The time of onset of oestrus was defined as the first time at which a sow revealed a back-pressure response (Roberts & Bilkei, 2005).

Post-cervical insemination (Watson & Behan, 2002; Roberts & Bilkei, 2005; Casas *et al.*, 2010) through a Magaplus S® catheter (Magapor, Zaragoza, Spain) was used in all of the inseminations. This insemination device consists of a conventional foam-type disposable AI catheter that is inserted into the cervix of the female. After waiting for cervix relaxation and engagement, the catheter is inserted up into the uterine body where the semen is deposited. Double insemination was performed in all cases, with an interval of 12 h between both inseminations, thereby covering the insemination-to-ovulation interval recommended for extended and cryopreserved doses (Waberski *et al.*, 1994; Casas *et al.*, 2010).

Extended seminal doses used for AI were prepared as described in Section 2.1 (i.e. 3×10^9 sperm/dose in 60 mL/dose), whereas in the case of frozen-thawed semen, six frozen semen straws (stored at −196 °C) were taken per ejaculate and treatment (FT C or FT GSH), subsequently thawed by shaking for 20 sec into a water bath at 37 °C and diluted with three volumes of Androstar Plus®, also warmed at 37 °C (i.e. a final volume of 12 mL: 3 mL of FT sperm and 9 mL of Androstar Plus®). Finally, 48 mL of the same commercial extender were added to these 12 mL to reach a final volume of 60 mL (i.e. 3×10^9 spermatozoa/dose in doses of 60 mL, as in the case of extended semen).

Assessments of fertility rates and litter sizes

The rate of non-return to oestrus was assessed at 21-Day post-insemination (NRR_{21d}) with a male teaser, and pregnancy diagnosis (PR_{30d}) was verified at 30-Day by ultrasonography (Echoscan T-100; Import-vet SA, Barcelona, Spain). Farrowing rate (FR) was also recorded, together with the litter sizes, that is the total number of piglets born (TP), the number of live-born piglets (AP) and the number of stillborn piglets (SB).

Statistical analyses

Statistical analyses were performed using the IBM® SPSS® 20 (IBM Corp., Chicago, IL, USA) and the SYSTAT 12.0 for Windows statistical packages (SYSTAT Software Inc., Evanston, IL, USA). Data are presented as percentages and mean ± standard error of the mean (SEM). Each ejaculate was considered as an independent observation, and the minimal level of significance was set at $p < 0.05$ in all statistical analyses.

As a first step, fertility rates (i.e. non-return to oestrus rate at 21 days, pregnancy rate at 30 days, farrowing rate at parturition) were transformed using logistic (logit) transformation (e.g. $\text{logit} = \ln(NRR_{21d}/(1 - NRR_{21d}))$, the log-odds then being used for subsequent calculations. Then, both sperm parameters and fertility and prolificacy data were tested for normality and homocedasticity using the Kolmogorov–Smirnov and Levene tests. When needed, data on percentages were recalculated using the arcsine square root (x) transformation to match the parametric assumptions.

General linear models

In the case of sperm parameters (i.e. free cysteine radicals in sperm nucleoproteins, DNA fragmentation, sperm viability and acrosome integrity, intracellular peroxide and superoxide levels and sperm motility), a generalized linear mixed model for repeated measures was run where each sperm parameter was the independent variable, incubation time (30 or 240 min) was the intra-subject factor and the type of semen used (extended, FT C, FT GSH) and the boar were, respectively, the fixed-effect and random-effect factors. A post hoc Bonferroni's test was used for pair-wise comparisons.

The effects of semen type on fertility data, based on non-return to oestrus rates at 21 days, pregnancy rates at 30 days or farrowing rates, and litter sizes were determined through a linear mixed model with treatment as fixed-effects and boar as random-effects factors. A post hoc Bonferroni's test was also run for multiple comparisons.

Correlations and regression analyses

Correlations between the eight sperm parameters (free cysteine radicals in sperm nucleoproteins, DNA fragmentation, sperm viability and acrosome integrity, intracellular peroxide and superoxide levels, and total and PMOT) were calculated using the Pearson correlation. In addition, correlations of the eight mentioned sperm parameters with fertility and prolificacy data were also calculated using the Pearson correlation.

On the other hand, linear regression analyses (the Pearson correlation and multiple regression) were used to determine the ability of these sperm parameters to predict non-return rates at 21 days (NRR_{21d}), pregnancy rates at 30 days (PR_{30d}), farrowing rates (FR), TP and AP. The procedure used (the forward stepwise model) was the same one used in a previous article of our group (Yeste *et al.*, 2010) and consisted of optimizing the regression equation ($y = a + b_1x_1 + b_2x_2 + \ldots + b_kx_k;\ k \leq 8$) to increase the determination coefficient (R^2). The significance level for introducing each parameter in the multiple regression model was 0.10 and the significance level (α) for the model was 0.05.

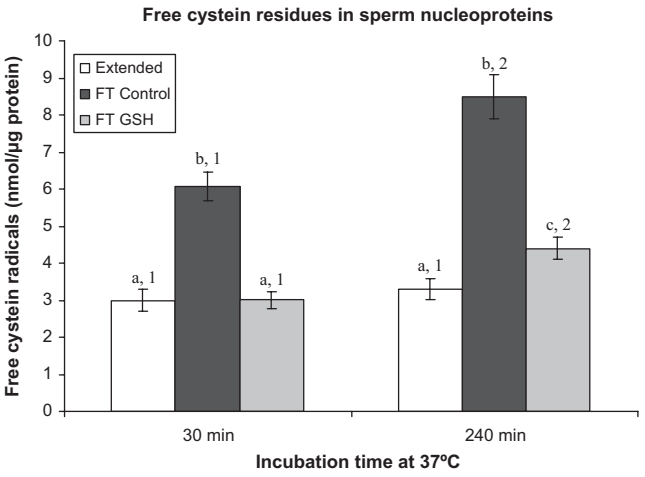
Finally, cross-validation using the Jackknife procedure was performed to estimate the bias of regression equation parameters: slope (b) and intercept (a) (Kott, 2001). In all cases, the values of the Pearson coefficient were transformed using Fisher Z-transform prior to jackknifing. The Jackknife estimation was performed on the resulting Z-transformed values, the final value of the r estimate being obtained by the inverse of the Fisher Z-transformation. The Jackknife estimate of the bias was calculated as the difference between the biased estimator ($\hat{\beta}$) and the unbiased Jackknife estimator ($\tilde{\beta}_J$), and the (1-α) confidence intervals (CI) were calculated as follows: $CI = \tilde{\beta}_J \pm t_{\upsilon,\alpha/2} \cdot \hat{\sigma}_{\tilde{\beta}_J}$, where $\bar{\beta}$ is the mean of the pseudo-values, $\hat{\sigma}_{\tilde{\beta}_J}$ is the standard error of the pseudo-values and $t_{\upsilon,\alpha/2}$ is the α-level critical value of a Student's t distribution with $\upsilon = n - 1$ degrees of freedom (Yeste *et al.*, 2010).

RESULTS

Effects of the addition of 2 mM GSH to cryopreservation extenders on free-cysteine residues of sperm nucleoproteins

Figure 1 shows the effects of treatment (extended, FT C, FT GSH) and post-thawing incubation time on the levels of free cysteine residues in sperm nucleoproteins. After 30 min of incubation at 37 °C, frozen-thawed control sperm presented significantly ($p < 0.05$) higher levels of free cysteine residues in sperm nucleoproteins than extended or frozen-thawed semen supplemented with 2 mM GSH. After 240 min of thawing, levels of free cysteine residues in frozen-thawed control were again significantly ($p < 0.05$) higher than were those observed in extended semen or frozen-thawed spermatozoa supplemented with GSH. However, at that time, frozen-thawed semen supplemented with 2 mM GSH presented significantly higher ($p < 0.05$) levels of free cysteine radicals than did extended semen (FT C: 8.5 ± 0.6 vs. FT GSH: 4.4 ± 0.3).

Figure 1 Free cysteine residues (as mean ± SEM) in sperm nucleoproteins in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.



Effects of the addition of 2 mM GSH to cryopreservation extenders on DNA fragmentation

DNA fragmentation levels were low in all treatments after 30 and 240 min of incubation at 37 °C. However, whereas extended semen and frozen-thawed spermatozoa supplemented with 2 mM GSH did not differ after incubation at 37 °C for either 30 or 240 min, frozen-thawed control semen presented significantly ($p < 0.05$) higher percentages of spermatozoa with fragmented DNA at 240 min post-thawing when compared with extended and frozen-thawed semen supplemented with 2 mM GSH (Fig. 2).

Effects of the addition of 2 mM GSH to cryopreservation extenders on sperm viability

Freeze-thawing of boar spermatozoa significantly ($p < 0.05$) reduced the percentages of viable spermatozoa both after 30 and 240 min post-thawing (Fig. 3). Nevertheless, the extent of this damage differed between frozen-thawed control and frozen-thawed semen supplemented with 2 mM GSH, as the former presented significantly lower percentages of viable spermatozoa than the latter, both after 30 and 240 min post-thawing (FT C: 33.5 ± 1.4 vs. FT GSH: 46.9 ± 1.9).

Figure 2 Percentages of spermatozoa with fragmented DNA (mean ± SEM) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.

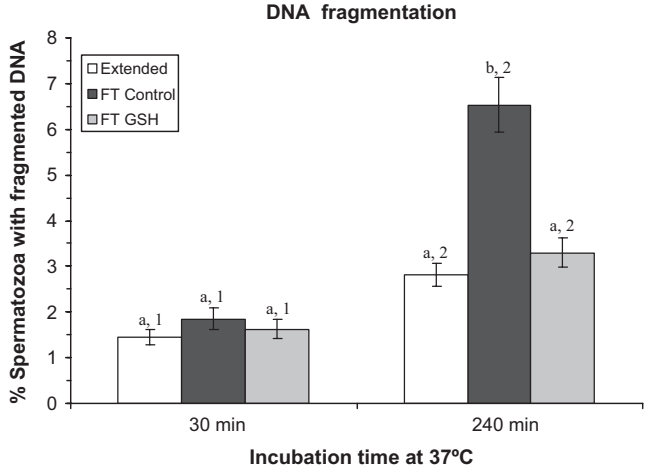
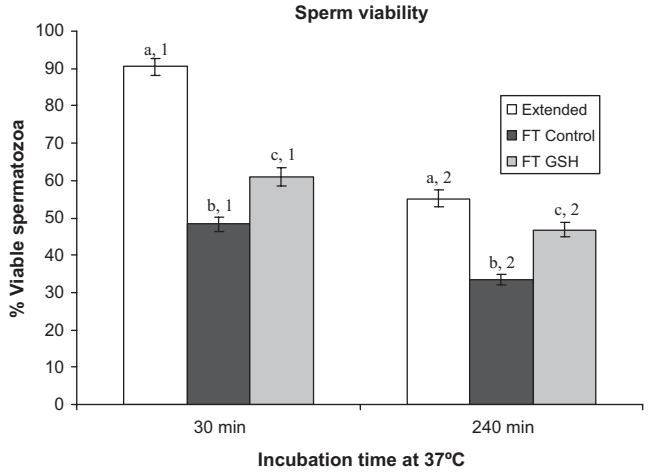


Figure 3 Percentages of viable spermatozoa (SYBR14$^+$/PI$^-$) (mean ± SEM) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.



Effects of the addition of 2 mM GSH to cryopreservation extenders on acrosome integrity

Table 1 shows (as mean ± SEM) the percentages of acrosome-intact spermatozoa in extended semen, frozen-thawed control and frozen-thawed supplemented with GSH after 30 and 240 min of incubation at 37 °C. Again, freeze-thawing of boar spermatozoa was seen to significantly ($p < 0.05$) decrease the percentage of acrosome-intact spermatozoa. Notwithstanding, this reduction was significantly higher ($p < 0.05$) in frozen-thawed control than in frozen-thawed semen supplemented with 2 mM GSH (e.g. at 240 min post-thawing, FT C: 20.9 ± 1.0 vs. FT GSH 38.8 ± 1.6).

Table 1 Percentages of acrosome-intact spermatozoa (PNA-FITC$^-$/PI$^-$), viable spermatozoa with high intracellular peroxide levels (DCF$^+$/PI$^-$), viable spermatozoa with high superoxide levels (E$^+$/ YO-PRO-1$^-$), and total and progressive motile spermatozoa in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min

	% Acrosome intact spermatozoa		% Spermatozoa DCF$^+$/PI$^-$		% Spermatozoa E$^+$/ YO-PRO-1$^-$		Total motility		Progressive motility	
	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min
Extended	85.3 ± 3.2^{a,1}	64.7 ± 2.6^{a,2}	2.2 ± 0.2^{a,1}	1.8 ± 0.2^{a,1}	3.3 ± 0.3^{a,1}	3.4 ± 0.4^{a,1}	90.2 ± 4.5^{a,1}	57.4 ± 2.7^{a,2}	64.8 ± 3.4^{a,1}	41.7 ± 2.2^{a,2}
FT C	47.4 ± 2.0^{b,1}	20.9 ± 1.0^{b,2}	3.3 ± 0.3^{b,1}	1.4 ± 0.1^{b,2}	3.4 ± 0.3^{a,1}	3.5 ± 0.4^{a,1}	57.6 ± 3.3^{b,1}	38.1 ± 1.9^{b,2}	34.0 ± 1.9^{b,1}	18.0 ± 1.1^{b,2}
FT GSH	62.6 ± 2.5^{c,1}	38.8 ± 1.6^{c,2}	2.6 ± 0.2^{a,1}	1.9 ± 0.1^{a,2}	3.2 ± 0.3^{a,1}	3.5 ± 0.3^{a,1}	72.9 ± 3.8^{c,1}	49.5 ± 2.4^{c,2}	52.5 ± 2.8^{c,1}	37.2 ± 2.0^{c,2}

Different letters (a–c) mean significant ($p < 0.05$) differences within a column and different numbers (1, 2) mean significant differences within a row for a given sperm parameter (i.e. % acrosome-intact spermatozoa, % spermatozoa DCF$^+$/PI$^-$, % spermatozoa E$^+$/ YO-PRO-1$^-$, % total motile spermatozoa, or % progressive motile spermatozoa).

Effects of the addition of 2 mM GSH to cryopreservation extenders on ROS levels

After 30 min of incubation at 37 °C, the percentage of viable spermatozoa with high peroxide levels (DCF^+/PI^-) was found to be slightly but significantly higher ($p < 0.05$) in frozen-thawed control than in extended and frozen-thawed semen supplemented with 2 mM GSH (Table 1). In contrast, the percentage of spermatozoa with high intracellular superoxide levels (E^+/YO-PRO-1^-) was not found to be affected by sperm cryopreservation or GSH supplementation.

Effects of the addition of 2 mM GSH to cryopreservation extenders on sperm motility

Table 1 also shows data on total and PMOT in extended semen, frozen-thawed control and frozen-thawed spermatozoa supplemented with 2 mM GSH. As expected, boar sperm cryopreservation significantly decreased total (TMOT) and PMOT. However, in both parameters, the reduction observed in frozen-thawed control was significantly ($p < 0.05$) higher than that observed in frozen-thawed spermatozoa supplemented with 2 mM GSH, both at 30 and 240 min post-thawing (e.g. PMOT at 240 min post-thawing, FT C: 18.0 ± 1.1 vs. FT GSH 37.2 ± 2.0).

Correlations between sperm quality parameters

Table 2a shows the correlation coefficients among all the eight sperm parameters evaluated at 30 min. Apart from the percentages of spermatozoa with fragmented DNA and of viable spermatozoa with high levels of superoxides, the other six sperm parameters were found to be significantly correlated each other. At 240 min post-thawing (Table 2b), all of the sperm parameters except those that evaluated ROS levels, that is percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-) levels, were found to be significantly correlated with each other.

Effects of the addition of 2 mM GSH to cryopreservation extenders on field fertility and prolificacy

The insemination with frozen-thawed control samples yielded significantly ($p < 0.05$) lower fertility results when measured as the non-oestrus return rate after 21 days (NRR_{21d}) of insemination, pregnancy rate after 30 days (PR_{30d}) of insemination and farrowing rate (FR) at parturition than those observed in sows inseminated with extended semen (e.g. FR, extended: 91.4 ± 5.1 vs. FT C: 67.2 ± 3.4, Table 3). Strikingly, the presence of 2 mM GSH counteracted the deleterious effect of freeze-thawing observed in frozen-thawed control samples (e.g. FR, FT C: 67.2 ± 3.4 vs. FT GSH: 92.7 ± 5.5). In this manner, fertility rates of boar semen frozen in the presence of 2 mM GSH were not significantly different to those observed in sows inseminated with extended semen (Table 3).

As far as litter sizes are concerned, both TP and AP per sow were significantly ($p < 0.05$) higher in females inseminated with extended semen or with frozen-thawed spermatozoa supplemented with 2 mM GSH than in sows inseminated with frozen-thawed control (e.g. AP, extended: 13.6 ± 0.5 and FT GSH: 12.4 ± 0.9 vs. FT C: 6.9 ± 2.3; Table 3). Again, no significant differences were observed between extended and frozen-thawed semen supplemented with 2 mM GSH. The number of stillborn piglets did not differ between treatments (Table 3).

Table 2 Correlations (Pearson coefficient) between the eight evaluated sperm parameters (levels of free cysteine residues (FCR) in sperm nucleoproteins, % spermatozoa with fragmented DNA, % viable spermatozoa ($SYBR14^+/PI^-$), % acrosome-intact spermatozoa (PNA-$FITC^-/PI^-$), % viable spermatozoa with high intracellular peroxide levels (DCF^+/PI^-), % viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-), and % total and % progressive motile spermatozoa) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 min (a) or 240 min (b)

	FCR residues in sperm nucleoproteins	% Spermatozoa with fragmented DNA	% Viable spermatozoa	% Acrosome intact spermatozoa	% Spermatozoa DCF^+/PI^-	% Spermatozoa E^+/YO-PRO-1^-	% Total motile spermatozoa	% Progressive motile spermatozoa
(a)								
FCR in sperm nucleoproteins	1	0.131	−0.658**	−0.497*	0.401*	0.107	−0.404*	−0.501**
% Spermatozoa with fragmented DNA	0.131	1	0.047	0.078	0.124	0.054	0.036	0.048
% Viable spermatozoa	−0.658**	0.047	1	0.787**	−0.404*	−0.184	0.788**	0.750**
% Acrosome intact spermatozoa	−0.497*	0.078	0.787**	1	−0.448*	0.043	0.802**	0.733**
% Spermatozoa DCF^+/PI^-	0.401*	0.124	−0.404*	−0.448*	1	0.104	−0.362	−0.395*
% Spermatozoa E^+/YO-PRO-1^-	0.107	0.054	−0.184	0.043	0.104	1	−0.092	−0.051
% Total motile spermatozoa	−0.404*	0.036	0.788**	0.802**	−0.362	−0.092	1	0.935***
% Progressive motile spermatozoa	−0.501**	0.048	0.750**	0.733**	−0.395*	−0.051	0.935***	1
(b)								
FCR in sperm nucleoproteins	1	0.649**	−0.785**	−0.589**	−0.130	0.153	−0.639**	−0.619**
% Spermatozoa with fragmented DNA	0.649**	1	−0.487**	−0.797**	−0.218	−0.008	−0.812**	−0.855***
% Viable spermatozoa	−0.785**	−0.487**	1	0.640**	0.069	−0.082	0.602**	0.499**
% Acrosome intact spermatozoa	−0.589**	−0.797**	0.640**	1	0.282	−0.019	0.896***	0.832**
% Spermatozoa DCF^+/PI^-	−0.130	−0.218	0.069	0.282	1	0.004	0.270	0.203
% Spermatozoa E^+/YO-PRO-1^-	0.153	−0.008	−0.082	−0.019	0.004	1	−0.121	−0.046
% Total motile spermatozoa	−0.639**	−0.812**	0.602**	0.896**	0.270	−0.121	1	0.877***
% Progressive motile spermatozoa	−0.619**	−0.855***	0.499**	0.832**	0.203	−0.046	0.877***	1

Significances of correlation coefficient (r) test are shown as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

Table 3 Fertility performances of sows inseminated with extended, frozen-thawed control (FT C) or frozen-thawed semen supplemented with 2 mM GSH (FT GSH)

	Extended	FT C	FT GSH
Non-return rate to estrus 21 days (NRR_{21d}) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Pregnancy rate at 30 days (PR_{30d}) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Farrowing rate (FR) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Total piglets born (TP) (*n*)	14.3 ± 0.4[a]	7.5 ± 2.4[b]	13.0 ± 1.0[a]
Alive born piglets (AP) (*n*)	13.6 ± 0.5[a]	6.9 ± 2.3[b]	12.4 ± 0.9[a]
Stillborn piglets (SP) (*n*)	0.8 ± 0.1[a]	0.6 ± 0.2[a]	0.6 ± 0.3[a]

Different superscripts within a row (a, b) mean significant differences between treatments (i.e. extended, FT C and FT GSH).

Correlation analyses among sperm parameters, fertility and litter size data

From all of the sperm parameters evaluated after 30 min of incubation at 37 °C, the levels of free cysteine residues in sperm nucleoproteins were found to be significantly ($p < 0.05$) and negatively correlated with non-return at 21 days, pregnancy rates at 30 days and farrowing rates (Table 4). In contrast, the other sperm parameters were not found to be significantly correlated with pregnancy or fertility rates. On the other hand, the levels of free cysteine residues in sperm nucleoproteins and the percentages of viable and acrosome-intact spermatozoa were found to be significantly correlated with both TP and AP (Table 4). Sperm motility data, percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-), and percentages of spermatozoa with fragmented DNA were not significantly correlated with either fertility rates or litter sizes at 30 min post-thawing (Table 4).

In the case of sperm parameters evaluated after 240 min of incubation at 37 °C, levels of free cysteine residues in sperm nucleoproteins, percentages of spermatozoa with fragmented DNA and percentages of viable and acrosome-intact spermatozoa were found to be correlated with non-return at 21 days, pregnancy rate at 30 days and farrowing rate, and with both TP and AP (Table 4). In contrast, percentages of progressive motile spermatozoa were found to be positively and significantly correlated with both TP and AP, but not with fertility rates (i.e. non-return at 21 days, pregnancy rate at 30 days and farrowing rate). Again, percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-) levels were not found to be correlated with fertility rates or litter sizes (Table 4).

Regression analyses among sperm parameters, fertility and litter size data

Following the stepwise forward model, linear regression equations between sperm parameters evaluated at 30 or 240 min post-thawing and fertility rates and litter sizes were worked out and are shown in Table 5.

In the case of fertility rates, determination coefficients (R^2) were lower than in the case of litter size variables. In addition, sperm parameters that were included in the model were only two, namely levels of free cysteine residues in sperm nucleoproteins determined both after 30 and 240 min after thawing, and the percentage of spermatozoa with fragmented DNA at 240 min post-thawing.

As far as the regression equations for litter sizes (TL and AP), two parameters evaluated 30 min after incubation at 37 °C were included in the model (levels of free cysteine residues in sperm nucleoproteins and percentage of acrosome-intact spermatozoa). In the case of 240 min post-thawing, three sperm parameters (levels of free cysteine residues in sperm nucleoproteins, percentage of spermatozoa with fragmented DNA and percentage of viable spermatozoa) rather than two were included in the model.

Cross-validation through the Jackknife test in the 10 regression equations depicted in Table 5 showed that they correctly predicted all of the dependent variables (Logit(NRR_{21d}), Logit(PR_{30d}), Logit(FR), TP and AP), both the β and $\widetilde{\beta}$ falling into 95% confidence intervals calculated with the mean of the pseudo-values and their standard errors (data not shown).

DISCUSSION

Our results strongly indicate that supplementing the freezing media with 2 mM GSH has a very strong improving effect on the 'in vivo' fertilizing ability of boar sperm subjected to freeze-thawing. This is a very important asset, especially if one takes

Table 4 Correlation analyses (Pearson's coefficients) of sperm parameters evaluated either at 30 or 240 min post-thawing with fertility rates [non-return rate at 21 days (NRR_{21d}), pregnancy rate at 30 days (PR_{30d}), farrowing rate (FR)] and litter sizes [total number of piglets born (TP), piglets born alive (AP) and stillborn piglets (SP)]

Incubation time	Sperm parameter	Logit(NRR_{21d})	Logit(PR_{30d})	Logit(FR)	TP	AP	SP
30 min	Free cystein residues in sperm nucleoproteins	−0.339*	−0.339*	−0.339*	−0.471**	−0.474**	0.168
	% Spermatozoa with fragmented DNA	−0.163	−0.163	−0.163	0.054	0.027	−0.139
	% Viable spermatozoa	0.274	0.274	0.274	0.373*	0.375*	−0.052
	Acrosome intact spermatozoa	0.289	0.289	0.289	0.459**	0.459**	−0.199
	% Spermatozoa $DCF^+/PI-$	−0.140	−0.140	−0.140	−0.262	−0.270	0.044
	% Spermatozoa E^+/ YO-PRO-1^-	−0.065	−0.065	−0.065	−0.076	−0.057	0.184
	% Total motile spermatozoa	0.256	0.256	0.256	0.297	0.292	−0.146
	% Progressive motile spermatozoa	0.177	0.177	0.177	0.306	0.307	−0.098
240 min	Free cystein residues in sperm nucleoproteins	−0.427**	−0.427**	−0.427**	−0.580**	−0.590**	0.158
	% Spermatozoa with fragmented DNA	−0.387*	−0.387*	−0.387*	−0.492**	−0.513**	0.029
	% Viable spermatozoa	0.321*	0.321*	0.321*	0.526**	0.529**	−0.188
	Acrosome intact spermatozoa	0.309*	0.309*	0.309*	0.441**	0.445**	−0.151
	% Spermatozoa DCF^+/PI^-	0.118	0.118	0.118	0.045	0.031	−0.126
	% Spermatozoa E^+/ YO-PRO-1^-	−0.079	−0.079	−0.079	−0.052	−0.061	0.091
	% Total motile spermatozoa	0.153	0.153	0.153	0.298	0.296	−0.037
	% Progressive motile spermatozoa	0.262	0.262	0.262	0.330*	0.337*	−0.118

Significances of correlation coefficient (*r*) test are shown as follows: *$p < 0.05$, **$p < 0.01$.

Table 5 Multiple regression analyses of fertility rates (NRR_{21d}, PR_{30d} and FR) or litter sizes (TP, AP and SP) with sperm parameters evaluated either at 30 or 240 min post-thawing. Regression equations, together with determination coefficients (R^2) and *F* and *p* values of the model are depicted

	Regression equation	R^2	*R*	*F*	*p* value model
NRR_{21d}					
30 min	Logit (NRR_{21d}) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (NRR_{21d}) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
PR_{30d}					
30 min	Logit (PR_{30d}) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (PR_{30d}) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
FR					
30 min	Logit (FR) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (FR) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
TP					
30 min	TP = $-0.61(FCR_{30}) + 0.11(ACR_{30}) + 6.79$	0.30	0.55	8.42	0.001
240 min	TP = $-0.46(FCR_{240}) - 0.56(SDF_{240}) + 0.07(VB_{240}) + 12.67$	0.37	0.61	7.58	<0.001
AP					
30 min	AP = $-0.59(FCR_{30}) + 0.10(ACR_{30}) + 6.43$	0.30	0.55	8.49	0.001
240 min	AP = $-0.44(FCR_{240}) - 0.60(SDF_{240}) + 0.06(VB_{240}) + 12.36$	0.39	0.62	8.11	<0.001

NRR_{21d}, non-return rate after 21 days of insemination; PR_{30d}, pregnancy rate after 30 days of insemination; FR, farrowing rate; TP, total piglets born; AP, live-born piglets; FCR, free cysteine radicals in sperm nucleoproteins; SDF, % spermatozoa with fragmented DNA; VB, % viable spermatozoa, ACR, % acrosome-intact spermatozoa.

into account that sperm cryopreservation has been regarded as being the most efficient method for storing boar sperm samples for a long period of time (Holt, 2000). However, a greater utilization of this technique has been hampered so far because of its lower fertility rates and litter sizes when compared with extended semen, despite all of the efforts being made in this regard (Almlid & Hofmo, 1995; Johnson *et al.*, 2000; Casas & Flores, 2013). In this way, our results may be relevant to further develop boar sperm freeze-thawing protocols that could yield 'in vivo' fertility results not far below to those obtained with extended semen. This is clearly stated by our data, in which the addition of 2 mM GSH to the freezing media significantly increased non-return rates at 21 days, pregnancy rates at 30 days and farrowing rates from 67.2 ± 3.4% (FT control) to 92.7 ± 5.5% (FT GSH). In a similar way, adding 2 mM GSH to the freezing media also significantly increased the total number of piglets and the number of piglets born alive. Supporting our results, another study conducted 'in vitro' has demonstrated that the addition of GSH to the thawing extender is beneficial for boar frozen-thawed spermatozoa, as it increases the sperm-penetration-to-oocyte ability (Gadea *et al.*, 2004). Taking all of these results together, and despite this being only a preliminary study, the finding that 2 mM GSH can greatly improve the 'in vivo' fertilizing ability of frozen boar spermatozoa is a very valuable result.

One issue that still remains undetermined is whether the fertility increase mediated by the GSH addition to frozen-thawed boar spermatozoa is specifically based on the improvement of a single, or more than one, sperm parameter. This is one of the aspects that has led us to evaluate a reasonably wide array of separate boar semen quality parameters. Such sperm parameters were evaluated before and after 30 and 240 min of freeze-thawing, the latter post-thawing time being set both to ensure the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses (Waberski *et al.*, 1994; Casas *et al.*, 2010) and as a sperm resistance probe. When considering the results of all of these sperm parameters, cryopreservation, as expected, causes a general impairment of boar sperm function and survival. This impairment includes damage in the nucleoprotein structure, reduction in sperm viability, motility and acrosome integrity, and a slight increase in intracellular peroxide levels. Most of this damage is visible after 30 min of thawing at 37 °C, but the case of DNA fragmentation is particularly noteworthy, as damage is only observed at 240 min post-thawing. Of course, these data are not new and match other previous reports (Casas *et al.*, 2009; Yeste *et al.*, 2012, 2013). In fact, it is widely known that sperm cryopreservation impairs sperm quality not only in porcine but also in other mammalian species (Baumber *et al.*, 2003).

Another question concerns the mechanism/s by which GSH is able to counteract, at least partially, boar sperm cryodamage. On this point, it is well-known that GSH has a strong counteracting effect on the cryopreservation-induced alterations of the sperm nucleoprotein structure and DNA integrity (Tuncer *et al.*, 2010; Yeste *et al.*, 2012). The relationship between the overall sperm nucleoprotein structure and DNA integrity is highlighted by our results. Thus, percentages of spermatozoa with fragmented DNA at 240 min post-thawing were correlated with the levels of free cysteine residues in sperm nucleoproteins. In addition, percentages of spermatozoa with fragmented DNA at 240 min post-thawing were also included in all of the regression equations that were set from the sperm parameters evaluated at this time-point.

From our results, however, it is not possible to know exactly how GSH improves boar sperm fertilizing ability. This is because of the fact that GSH does not only maintain nucleus integrity but also keeps membrane integrity (plasmalemma and acrosome membrane) and sperm motility, even though with a lesser intensity. Despite this, the joint study of data from fertility trials and sperm quality through correlation and regression analyses suggests that the beneficial effects of GSH are mainly related to the stabilization of the sperm nucleoprotein structure. Indeed, Pearson correlation coefficients and their significance tests are the highest when fertility rates (NRR_{21d}, PR_{30d} and FR) or both TP and AP are correlated with the levels of free cysteine residues in boar sperm nucleoproteins, as an indirect measurement of the integrity of the sperm nucleoprotein structure. Furthermore, when linear regression equations are worked out through a stepwise model, the statistical weight of the integrity of the

nucleoprotein structure is the highest. As such, all 10 regression equations included this parameter as an independent variable. This finding emphasizes the relevance of the integrity of the nucleoprotein structure (e.g. free cysteine residues in sperm nucleoproteins at 30 min post-thawing, FT C: 6.1 ± 0.4 vs. FT GSH: 3.0 ± 0.2) when linked to the fertilizing ability of a given ejaculate and to the increase in field fertility and prolificacy (FR, FT C: 67.2 ± 3.4 vs. FT GSH: 92.7 ± 5.5; AP, FT C: 6.9 ± 2.3 vs. FT GSH: 12.4 ± 0.9). On the contrary, the GSH-mediated increases in sperm viability, motility and acrosome-integrity (e.g. Sperm viability at 30 min post-thawing, FT C: 48.3 ± 1.9 vs. FT GSH: 60.9 ± 2.5) are linked to a much lesser extent to the analysed 'in vivo' fertilizing ability parameters. These results suggest that the effects of GSH on the maintenance of the boar sperm nucleoprotein structure after freeze-thawing are, among all of the analysed effects, those most related to the improving action of GSH on boar sperm fertilizing ability. Therefore, we can reasonably hypothesize that one of the most important mechanisms by which the addition of GSH increases fertility and prolificacy of frozen-thawed spermatozoa is the protection of the nucleoprotein structure and subsequent DNA integrity. In this regard, it is worth noting that previous reports have emphasized the relevance of chromatin integrity on sperm fertilizing ability (Silva & Gadella, 2006), and that the oviductal selection mechanisms in the establishment of sperm reservoir, so important in eutherian mammals (Yeste, 2013), have been reported to be endowed with stable and non-fragmented chromatin structure (Ardón *et al.*, 2008). All of these data appear to highlight the importance of a correct nucleoprotein structure to explain boar sperm fertilizing ability.

Nonetheless, this finding does not preclude that other sperm parameters, such as sperm viability or acrosome integrity, were included in regression equations predicting litter sizes (TP and AP), despite not being in those predicting fertility rates. On the other hand, sperm progressive motility after 240 min of incubation was found to be correlated with litter sizes, but not included in any of the regression equations set, and the parameters evaluating intracellular levels of peroxides and superoxides were not correlated with either fertility rates or litter sizes. All of these data also emphasize the relevance of sperm viability and acrosome integrity in sperm fertilizing ability, and at the same time suggest that GSH acts primarily on nucleoprotein structure and, to a lesser extent, on membrane integrity of frozen-thawed boar sperm rather than on ROS levels. The low production of H_2O_2 and $O_2^{-\bullet}$ in boar spermatozoa owing to freeze-thawing procedures observed in this and other studies (Guthrie & Welch, 2006; Kim *et al.*, 2011; Yeste *et al.*, 2012), along with the effects of 2 mM GSH on intracellular ROS levels, raise, in fact, reasonable doubts about the real role of these ROS in the fertilizing ability of boar frozen-thawed spermatozoa.

CONCLUSION

In conclusion, supplementing freezing media with 2 mM GSH improves the fertilizing ability of frozen-thawed boar semen, and it is suggested to be used to improve 'in vivo' fertility performance when utilizing cryopreserved boar spermatozoa. This improvement appears to be related to the protection effect of GSH on the integrity of the nucleoprotein structure and DNA fragmentation and, to a lesser extent, on the general function of the sperm cell.

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AUTHORS' CONTRIBUTIONS

E.E. conducted the experimental analyses in the laboratory and participated in AI. J.E. R.-G. also participated in lab analyses and in AI, and revised the manuscript. L.G.R. and S.B. took part in AI trials. As senior author, M.Y. conducted the experimental analyses in the laboratory, was also involved in AI trials, performed the statistical analyses, and wrote the MS.

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DISCUSIÓN GENERAL

Es evidente el daño sufrido por el espermatozoide porcino durante el proceso de criopreservación, sobre todo a nivel de su membrana celular, su acrosoma y su núcleo. Sin embargo, aunque hay muchos estudios dedicados al daño de membrana y al acrosomal, existen relativamente pocas investigaciones centradas en el daño nuclear creado por la criopreservación en el espermatozoide porcino. En este sentido, estudios previos han informado sobre la fuerte relación entre la integridad del DNA y la capacidad de fecundación del espermatozoide de verraco (Flores et al., 2008; 2011), hipotetizándose que el daño producido en la cromatina nuclear puede desencadenar la inestabilidad de la estructura nucleoproteínica. En este sentido el proceso de congelación-descongelación afecta al núcleo del espermatozoide por los cambios que induce en la interacción de la estructura entre nucleoproteínas (Flores et al., 2011). Estos cambios están relacionados con la ruptura de los enlaces disulfuro entre las cisteínas de la nucleoproteína (Fuentes-Mascorro et al., 2000; Linke y Jacob, 2003; Flores et al., 2011). Estos enlaces son responsables para el empaquetado apropiado, compactación y estabilización de la cromatina espermática (Balhorn, 2007). Además, las protaminas protegen al DNA de las nucleasas y del ROS, así como también eliminan los factores de transcripción y proteínas que ayudan a restablecer el código de impresión en el ovocito (Oliva, 2006).

Nuestros resultados indican claramente que la adición de GSH y ProHCL mostró un efecto protector sobre los enlaces disulfuro de las nucleoproteínas del espermatozoide porcino frente a las alteraciones inducidas por la congelación. El efecto beneficioso de GSH y ProHCL en la protección de los puentes disulfuro puede deberse a su efecto antioxidante (Cumming et al., 2004; Yang et al., 2007). En este sentido, la estabilización de la estructura nucleoproteínica y específicamente de los puentes disulfuro podría ser explicada por la acción de los efectos probados sobre el mantenimiento del equilibrio redox intracelular (Jacob et al., 2003). Sin embargo, podría ser que los efectos del GSH y la ProHCL sobre la estructura nuclear del espermatozoide porcino fueran diferentes a los causados en espermatozoides de otras especies. Esta diferencia vendría dada por la diferente estructura nuclear que presenta el núcleo del espermatozoide porcino cuando se compara con el de especies como el toro (Karoui et al., 2012) y caballo (Baumber et al., 2003), que se pone de manifiesto por las grandes diferencias observadas en el grado de fragmentación del DNA que muestran estas especies entre sí al ser sometidas a un estrés como la congelación-

descongelación. En este sentido, hay que reseñar que la integridad de la cromatina espermática generalmente se evalúa a través de prueba de fragmentación del DNA. En el caso del verraco, los resultados de diferentes estudios que evalúan dicha fragmentación durante el proceso de congelación-descongelación no son claros. Así, por un lado Fraser y Strezez, (2005), utilizando el "neutral comet assay", observaron un aumento significativo de la fragmentación de DNA en el espermatozoide porcino. Sin embargo tanto Hernández et al. (2006) como Flores et al. (2008; 2011) con el uso del test SCDT han encontrado índices de fragmentación del DNA similares en semen fresco y congelado-descongelado. Esta variabilidad de resultados podría estribar en el hecho que, tal y como muestran los resultados de esta Tesis, el porcentaje absoluto de fragmentación de DNA que muestran los espermatozoides porcinos es muy bajo, incluso en las peores condiciones, no superando en ningún caso porcentajes del 8%. Estos bajísimos porcentajes contrastan con los observados en especies como el caballo o el toro, en las cuales los niveles de fragmentación tras la congelación-descongelación pueden llegar al 25% y al 15 %, respectivamente (Baumber et al., 2003; Karoui et al., 2012). Al partir de resultados tan bajos, la presencia o no de diferencias entre tratamientos se convierte en un asunto meramente estadístico que dependerá de cuestiones como el número de experimentos analizados y las desviaciones existentes. Sea como fuere, el conjunto de los resultados expuestos permiten hipotetizar que la desestabilización de la estructura nucleoproteínica debida a la ruptura de los enlaces disulfuro tras 30 minutos de incubación posterior a la descongelación conducirían a una descondensación anómala de la cromatina (Flores et al., 2008; 2011) y este efecto podría ser la causa del posterior incremento, aunque modesto, de la fragmentación de DNA tras 240 minutos de incubación posterior a la descongelación. Este aumento, a su vez, estaría asociado a niveles altos de espermatozoides muertos, que liberan al medio enzimas líticas del acrosoma y nucleasas liberadas de las membranas plasmáticas dañadas, las cuales atacarían la estructura nuclear, tal y como se ha documentado en el espermatozoide de humano, hámster y ratón (Ward y Ward, 2004; Sotolongo et al., 2005).

Respecto al hipotético papel de los ROS en las alteraciones causadas por la congelación-descongelación en el espermatozoide porcino y el efecto protector del GSH y ProHCL, hay que indicar, en primer lugar, que este efecto parece ser secundario en nuestro modelo experimental. Estos resultados son diferentes a los observados en especies como el hombre,

el caballo, el toro y el perro, en las que se ha descrito un incremento en la producción de ROS en respuesta a la criopreservación (Bilodeu et al., 2000;Ball et al., 2010; Kim et al., 2010; Gadea et al., 2011). Sin embargo como ya se ha mencionado anteriormente, en el caso del espermatozoide de verraco no está claro el vínculo de la producción de ROS durante el proceso de criopreservación. A pesar de ello, en el verraco ha sido identificado el peróxido de hidrógeno (H_2O_2) como la fuente primaria de producción de ROS (Kim et al., 2011). Si bien en el presente estudio el GSH redujo significativamente el contenido de H_2O_2, la ProHCL no mostró este efecto contra este tipo de ROS. Esto sugiere que estos dos agentes tienen diferentes mecanismos de acción. Esta hipótesis también viene sugerida por el hecho que los efectos positivos de tanto el GSH como la ProHCL en el medio de congelación sobre la viabilidad del espermatozoide, la integridad del acrosoma y los cambios de permeabilidad de la membrana fueron generalmente acumulativos. A pesar de ello, en este momento no conocemos con exactitud el origen molecular de estas diferencias. A este respecto, el efecto positivo de la adición de 2 mM de GSH en los niveles de ROS podría estar relacionado con un mecanismo de la desactivación de agentes antioxidantes de reducción (Stenesh, 1998), compensando de esta manera la falta de otros antioxidantes. Además, este efecto anti-ROS también ayudaría a explicar en parte los efectos de, al menos el GSH, sobre la estructura nuclear. De hecho, se ha observado en espermatozoides humanos que el incremento de ROS inducido durante la congelación está involucrado en el aumento simultáneo en los porcentajes de fragmentación de DNA, y el GSH protege contra la fragmentación de DNA inducida por ROS (Lopes, 1997).

Sean cuales sean los mecanismos moleculares implicados en la acción protectora de la ProHCL y, especialmente, el GSH, nuestros resultados también indican claramente que la suplementación del medio de congelación con 2 mM de GSH mostró gran efecto mejorante de la capacidad fecundante *in vivo* del espermatozoide porcino sometido al proceso de criopreservación. De hecho, la adición de GSH al medio de congelación aumento significativamente la tasa de no retorno a estro a 21 días, la tasa de gestación a 30 días y la tasa de parto, así como el número total de lechones y el numero de lechones nacidos vivos. En apoyo a estos anteriores resultados un estudio llevado a cabo *in vitro* demostró que la adición de GSH al diluyente de descongelación fue beneficioso para el espermatozoide de verraco ya que aumento la capacidad de penetración del espermatozoide al ovocito (Gadea

et al., 2004b). En nuestro estudio, los resultados de las pruebas de fertilidad *in vivo* y calidad espermática a través de análisis de correlación y regresión sugiere que los efectos benéficos del GSH están relacionados principalmente con la estabilización de la estructura nucleoproteínica del espermatozoide. De hecho, los coeficientes de significación estadística son más altos para las tasas de fertilidad que para las de prolificidad, si bien estos últimos parámetros también están correlacionados significativamente con los niveles de residuos de cisteína libre de la nucleoproteína. De hecho, la ecuación de regresión lineal "step-to-step" muestra que el peso estadístico de las cisteínas libres de la cabeza del espermatozoide es el parámetro con mayor fuerza estadística para explicar los efectos de la fertilidad *in vivo*. Este hallazgo enfatiza la importancia de la integridad de la estructura nucleoproteínica cuando se vincula con la capacidad fecundante del eyaculado y por el aumento de la fertilidad y prolificidad. En este sentido, se ha destacado la importancia de la integridad de la cromatina en la capacidad fecundante del espermatozoide (Silva y Gadella, 2006) y en los mecanismos de selección oviductal en el establecimiento del depósito de espermatozoides en mamíferos (Yeste, 2013). Por otra parte, los aumentos mediados por el GSH de la viabilidad, la motilidad y la integridad del acrosoma de los espermatozoides de verraco, principalmente a los 30 minutos posterior a la descongelación, están vinculados en un grado mucho menor a los parámetros de la capacidad de fertilidad analizados *in vivo*. Estos resultados enfatizan no sólo la importancia del mantenimiento de la estructura nuclear para explicar los resultados *in vivo*, si no también sugiere que los efectos de GSH sobre el mantenimiento de la estructura nucleoproteínica después de la congelación-descongelación son los más importantes para explicar la mejora de acción del GSH en la capacidad de fecundación del espermatozoide porcino.

De todas maneras, a pesar de que el efecto del GSH sobre la unión DNA-nucleoproteínas parece ser el más relevante, no hay que olvidar que la suplementación de GSH en el medio de congelación también mejora otros varios parámetros de calidad del semen, especialmente al determinarse en los 240 minutos posteriores a la descongelación. Este hecho es importante si tenemos en cuenta que este período de 240 minutos post-congelación se establece como una prueba de resistencia del espermatozoide (Waberski et al., 1994; Casas et al., 2010b).Teniendo en cuenta esto, hay que recordar que los resultados muestran que la viabilidad, la integridad del acrosoma y la motilidad progresiva, pero no

los niveles de ROS, a los 240 minutos están correlacionados con el tamaño de camada, aunque sea en menor medida que los niveles de cisteína libre. Estos resultados sugieren que el efecto mejorante de la adición de GSH está relacionado también, aunque sea en menor medida, con la integridad de la membrana, aunque no con una posible reducción de los niveles de ROS. Estos resultados también ponen en relieve el verdadero papel del ROS en la capacidad fecundante *in vivo* de los espermatozoides de verraco durante el proceso de criopreservación. En definitiva, se puede afirmar que el efecto mejorante del GSH sobre otras características espermáticas, tales como la capacidad de resistencia genérica post-descongelación y el mantenimiento de integridad de la membrana citoplasmática y acrosomal, también tendrán importancia en explicar la mejora observada en la capacidad fecundante *in vivo*. Finalmente por lo que hace referencia a los resultados obtenidos *in vivo*, cabe destacar desde un punto de vista práctico que los resultados obtenidos son relevantes para poder desarrollar mejores protocolos de criopreservación que puedan producir, al combinarse con técnica de IA post-cervical, resultados de fertilidad parecidos a los obtenidos con semen fresco diluido.

La comparación de los resultados obtenidos entre eyaculados BC y MC también arroja algunas conclusiones de interés. Así, en primer lugar nuestros resultados con estos eyaculados refuerzan la hipótesis que la resistencia del espermatozoide porcino a la criopreservación es concomitante al sostenimiento de la estructura nucleoproteínica, ya que cuando se compara el nivel de radicales libre de cisteína de las nucleoproteínas entre los eyaculados BC y los MC se observaron diferencias evidentes después de la descongelación. Nuestros resultados también sugieren que el grado de congelabilidad de un eyaculado concreto se relaciona con la integridad concreta de la estructura nucleoproteínica, ya que la ruptura de los enlaces disulfuro es mejor contrarrestada por el GSH en los Eyaculados BC que en los MC. No conocemos con exactitud el/los mecanismo/s que expliquen estas diferencias. A pesar de ello, se puede hipotetizar un origen basado en una defectuosa espermatogénesis y/o maduración del espermatozoide en el epidídimo de los eyaculados MC, ya que es a este nivel en donde se substituyen las histonas por las protaminas y se forman los enlaces disulfuro entre las protaminas, lo que lleva a la condensación óptima de la cromatina espermática (Calvin y Bedford, 1971). Otra causa podría ser diferencias en los niveles intracelulares de proteínas e importancia en la resistencia ambiental del

espermatozoide. En este sentido, se ha descrito una mayor presencia de la proteína HSP90 en los eyaculados BC cuando se comparan con los MC (Casas et al., 2010a). De esta forma, los BC tendrán una mayor protección contra el enfriamiento y choque osmótico producidos durante la congelación-descongelación. Sea como fuere, nuestros resultados indican que las muestras MC presenta una fragilidad mucho mayor en su estructura nuclear que las BC. Este hecho se observa al comparar los resultados de la fragmentación de DNA tras la descongelación. En este caso, se observó un incremento significativo, aunque minino, de la fragmentación del DNA a los 30 minutos posterior a la descongelación en los eyaculados MC, pero sólo a los 240 minutos en los BC. La fragilidad de la estructura nucleoproteínica en el caso de los eyaculados MC detectada en los residuos de cisteína libres después de 30 minutos de incubación se asocia con la fragmentación de la cromatina observada en el mismo periodo de tiempo tras la descongelación. En relación a estos resultados, se ha notificado que las muestras BC presentan mayores tasas de penetración de ovocitos, división y formación de blastocitos que las MC (Lopes et al., 1997). Estos resultados podrían estar relacionado con los niveles más bajos de ruptura de enlaces disulfuro de las nucleoproteínas y con un menor grado de fragmentación de la cromatina en muestras BC cuando se comparan con los eyaculados MC, tal y como se observó en nuestros resultados

También los eyaculados BC y MC difieren en otros parámetros. Así, el deterioro fue mayor en las muestras MC que en las BC en términos de motilidad espermática, puesto que se observaron diferencias significativas entre muestras BC y MC en el porcentaje de motilidad progresiva durante el enfriamiento y descongelación. Esto coincide con otros reportes que han descrito diferencias de motilidad en la descongelación entre razas (Park y Yi, 2002) y eyaculados (Casas et al., 2009). En un estudio reciente, Guthrie y Welch, (2012) determinaron que los efectos inhibidores de ROS sobre la motilidad espermática de eyaculados de verracos se deben básicamente a un mecanismo mitocondrial ligado a la utilización de ATP por parte del aparato contráctil del flagelo. Esto podría explicar las diferencias en la motilidad entre los eyaculados BC y MC, ya que los eyaculados BC podrían tener mejores mecanismos de utilización de ATP y regulación del aparato mitocondrial del flagelo. Esta hipótesis se reforzaría con otros resultados, que muestran que algunos parámetros de motilidad como el coeficiente de linealidad, el coeficiente de

rectitud y la motilidad progresiva difieren entre BC y MC en la etapa de enfriamiento a 5 ^{0}C y a los 240 minutos post- descongelación (Casas et al., 2009). Sea como fuere, la principal diferencia entre los eyaculados BC y MC parece residir no en la existencia de diferentes tipos de respuesta enfrente la agresión ambiental ligada a la congelación-descongelación, sino a una diferente intensidad en esta respuesta, ligada a un mejor o peor funcionamiento de los mecanismos y estructuras espermáticas. Esta diferencia de grado, más que de mecanismo, explicaría por qué el efecto mejorante del GSH sólo se produce en los eyaculados MC cuando se utiliza a unas concentraciones mucho más elevadas (5 mM) que el añadido en las muestras BC (2 mM). Desde un punto de vista práctico, estas diferencias de intensidad de efecto han de tenerse muy en cuenta al diseñar diluyentes optimizados de congelación para semen porcino que contengan GSH, puesto que la concentración de este efecto habrá de variar en consonancia con la capacidad intrínseca de crioresistencia que muestre el eyaculado.

Otro efecto importante que se puede extraer de los resultados obtenidos en esta Tesis es el de que un tiempo de mantenimiento (TM) de 24 horas tras la obtención del semen tiene un efecto de mejora en la criotolerancia del espermatozoide de verraco. Este hecho es especialmente evidente al analizar los resultados obtenidos sobre la integridad del núcleo espermático, puesto que nuestros resultados muestran que este daño durante la congelación-descongelación depende del periodo de TM. De manera concomitante, la fragmentación de DNA es más alta cuando el TM es de 3 horas que cuando es de 24 horas. Resultados parecidos se observan al analizar los datos sobre los residuos de cisteína libre en las nucleoproteínas del espermatozoide. Todo ello hace razonable suponer que un contacto más largo de los espermatozoides con los factores del plasma seminal durante el TM (Johnson et al., 2000) desencadena una respuesta del espermatozoide que conduce a la protección del núcleo contra la criopreservación y descongelación. Curiosamente, el TM no afecto significativamente los parámetros de motilidad y los niveles de ROS post-descongelación. En este contexto se ha observado un incremento de la viabilidad y la integridad del acrosoma al incrementarse el TM hasta las 20 horas de almacenamiento en eyaculados de verraco. En contraste, Guthrie y Welch, (2005) no encontraron diferencias significativas en el TM a 3 y 24 horas en los parámetros de viabilidad, integridad del acrosoma o motilidad. Sin embargo cuando las evaluaciones seminales se midieron a los 240 minutos sí que se

observaron diferencias entre periodos de TM. Por último y a pesar que la congelación-descongelación aumenta ligeramente el porcentaje de espermatozoides viables con altos niveles de H_2O_2 sin afectar los niveles de superóxido ($O_2^{-\bullet}$), el TM tiene ningún efecto sobre la generación de ROS durante la criopreservación del espermatozoide porcino.

En referencia a los mecanismos subyacentes al incremento de la crioresistencia ligado a un mayor TM, nuestros resultados sugieren que este fenómeno parece estar relacionado con cambios post-transcripcionales de proteínas relacionadas con los mecanismos moleculares de resistencia al estrés y, por lo tanto, a la congelación del espermatozoide de cerdo. Hay que tener en cuenta que los espermatozoides no son capaces de regular cambios de su expresión génica, de manera que la actividad de sus proteínas funcionales sólo pueden ser reguladas de manera preeminente por mecanismos post-traduccionales de la modificación covalente de proteínas como son la fosforilación y desfosforilación (Naz y Rajesh, 2004; Fernández- Novell et al., 2011). En nuestro caso, la mayor criotolerancia vinculada a un TM de 24 horas ha sido concomitante con un aumento en los niveles de fosforilación en los residuos serina (pSer) de 4 proteínas, la HSP70, la glucógeno sintasa quinasa 3 (GSK3), la tirosina quinasa (TRK) y la Cdc2, aunque el efecto más notorio ha sido, indudablemente, el observado en la HSP70. Nuevamente, no conocemos el mecanismo exacto que provoca un aumento de pSer en estas proteínas ligado a un TM de 24 horas. A pesar de ello, se puede hipotetizar que algunos factores del plasma seminal y/o factores presentes en el semen fresco diluido confieren mayor criotolerancia durante la TM mediante el incremento en pSer de algunas proteínas que están involucradas en el estrés, tales como la HSP70. La relación de proteínas de resistencia al estrés con el efecto del TM de 24 horas también viene sugerida por la ausencia de diferencias significativas entre las 3 y 24 horas de TM en cualquiera de los parámetros funcionales del espermatozoide antes del inicio de la criopreservación. De hecho, se ha sugerido que el espermatozoide porcino en presencia de factores seminales podrían conferir una mayor resistencia al choque térmico (Johnson et al., 2000), más aún teniendo en cuenta que las proteínas del choque térmico se han encontrado en espermatozoides de mamíferos (Naaby-Hensen y Herr, 2010; Cole y Meyer, 2011). Sea como fuere, parece evidente que proteínas de resistencia al estrés, como la HSP-70 juegan un papel de gran importancia en los mecanismos de crioresistencia desarrollados por los espermatozoides de verraco.

CONCLUSIONES

1. El glutatión reducido y el clorhidrato de procaína, solos o combinados a dosis de 2 mM, protegen los enlaces disulfuro entre protaminas, estabilizando así la estructura nucleoproteínica y contrarrestando un posible incremento en los niveles de fragmentación de DNA del espermatozoide porcino durante el proceso de criopreservación.
2. La suplementación al medio de congelación con glutatión reducido y clorhidrato de procaína mejora la viabilidad espermática, la integridad del acrosoma, la organización de lípidos de membrana, la motilidad total y la producción de ROS.
3. La adición de glutatión reducido a concentraciones de 2mM al medio de congelación incrementa la capacidad fecundante *in vivo*, mejorando la fertilidad y la prolificidad.
4. El efecto de mejora de los resultados de fertilidad y prolificidad *in vivo* inducido por el glutatión reducido parece estar relacionado con el efecto de protección de la integridad de estructura nucleoproteínica y en la fragmentación de DNA y, en menor medida, en la función general del la célula espermática.
5. La congelación–descongelación del espermatozoide de verraco afecta la motilidad, la estabilidad de la membrana, la integridad del acrosoma y la estructura nucleoproteínica en mayor grado en eyaculados malos congeladores que en eyaculados buenos congeladores.
6. Las variaciones de los daños nucleares entre muestras buenas congeladoras y malas congeladoras no están directamente relacionadas con la generación de peróxidos y superóxidos durante la criopreservación.
7. La suplementación de una dosis de 5 mM de glutatión reducido en eyaculados malos congeladores ayuda a mejorar la resistencia a la criopreservación.
8. La conservación del eyaculado porcino durante 24 horas a 16-17ºC en un diluyente comercial mejora de manera significativa la capacidad de resistencia a la congelación-descongelación de dicho eyaculado.
9. El efecto de mejora a la congelación/descongelación mostrado por los espermatozoides porcinos conservados durante 24 horas a 16-17ºc parece estar ligado a cambios en los niveles de fosforilación de residuos serina de proteínas relacionadas con la resistencia al estrés ambiental, como la HSP70.

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