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DEPARTAMENTO DE ZOOLOGÍA Y ANTROPOLOGÍA FÍSICA

Evaluación de la capacidad fecundante de espermatozoides porcinos refrigerados y congelados

Fertilizing capacity evaluation of refrigerated and frozen-thawed boar spermatozoa

Elena Sellés Soriano

2008

Tesis Doctoral como compendio de publicaciones

Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility.

Sellés E, Gadea J, Romar R, Matás C, Ruiz S.

Reproduction in Domestic Animals. 38:66-72. 2003.

Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders.

Gadea J, **Sellés E**, Marco MA, Coy P, Matás C, Romar R, Ruiz S. Theriogenology. 62:690-701. 2004.

The predictive value of porcine seminal parameters on fertility outcome under commercial conditions

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Reproduction in Domestic Animals. 39: 303–308. 2004.

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D. Joaquín Gadea Mateos, Profesor Titular de Universidad del Área de Fisiología Veterinaria en el Departamento de Fisiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Evaluación de la capacidad fecundante de espermatozoides porcinos refrigerados y congelados", realizada por Dª. Elena Sellés Soriano, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 2 de septiembre de 2008

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D. JUAN JOSE PRESA ASENSIO, Catedrático de Universidad del Área de ZOOLOGIA en el Departamento de ZOOLOGIA Y ANTROPOLOGÍA FÍSICA, RATIFICA como tutor:

La presentación de la Tesis Doctoral titulada "Evaluación de la capacidad fecundante de espermatozoides porcinos refrigerados y congelados ", realizada por Dª. Elena Sellés Soriano, bajo la dirección y supervisión de D. Joaquín Gadea Mateos y D. Salvador Ruiz López, en el Departamento de Fisiología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia, por las siguientes razones:

Por acuerdo de la Comisión Académica del Consejo de Universidades de 3 de abril de 2000, se modificó el Catálogo áreas de conocimiento, extinguiéndose el área de Biología Animal, lo que produjo la desaparición del departamento de Biología Animal de la Universidad de Murcia.

Que en virtud de la Disposición adicional única del Real Decreto 371/2001 de 6 de abril (B.O.E. del 27), los profesores de Fisiología de la Facultad de Veterinaria de Murcia, solicitaron su adscripción al área de Fisiología. Dicha adscripción fue aceptada, y en Febrero de 2002 pasaron a formar parte del Departamento de Fisiología, dentro del Área propia de Fisiología Veterinaria.

En Murcia, a 17 de septiembre de 2008

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Dª. Eulalia Clemente Espinosa, Profesora Titular de Universidad del Área de ZOOLOGIA y Directora del Departamento de ZOOLOGIA Y ANTROPOLOGÍA FÍSICA , INFORMA:

Que la Tesis Doctoral titulada "Evaluación de la capacidad fecundante de espermatozoides porcinos refrigerados y congelados ", ha sido realizada por Dª Elena Sellés Soriano, bajo la inmediata dirección y supervisión de D. Joaquín Gadea Mateos y D. Salvador Ruiz López, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado.

Murcia, a 17 de septiembre de 2008

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Departamento de Zoología y Antropología Física

A mi marido y a mis niños

A mi madre, a mi hermano y a mi tía

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ÍNDICE

1

INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN

El sector porcino ha venido evolucionando en las últimas décadas hasta convertirse en una de las principales fuentes de proteínas de origen animal en los países desarrollados y con una gran proyección en los países emergentes. El merecido puesto que esta industria ha alcanzado actualmente en los mercados internacionales está estrechamente relacionado con la evolución que este sector ha sufrido. Este desarrollo no habría sido posible sin una amplia visión de futuro por parte de los productores que han sabido adaptarse a los avances tecnológicos y aplicar a tiempo las mejoras que permiten rentabilizar o mejorar las explotaciones. Dada la importancia económica y social del sector porcino, las investigaciones que faciliten o mejoren las producciones en esta especie tienen una gran repercusión económica y social, tanto en nuestro país como en el marco de la Unión Europea y en general en un mundo con una economía globalizada.

En los últimos años se ha producido un gran desarrollo en el campo de la reproducción porcina en las técnicas de gestión y control reproductivo, que han ido íntimamente ligadas a la aplicación de la inseminación artificial (IA) (Levis, 2000). Esta técnica ha permitido la máxima utilización del potencial genético de reproductores de alto valor, ha sido una herramienta fundamental en la prevención y lucha contra las enfermedades porcinas (Phillpott, 1993) y ha supuesto, en definitiva, un mejor control de todo el proceso reproductivo (Hurtgen, 1986).

En la aplicación de las técnicas de IA porcina se han realizado numerosos e importantes avances, lo que ha permitido alcanzar una amplia difusión en las explotaciones con unos resultados equiparables o superiores a los obtenidos con la monta natural (Colenbrander et al., 1993). La IA como técnica reproductiva aporta una serie de ventajas, entre las que se encuentran (Gadea, 2004):

- La amplia difusión del material genético del verraco seleccionado que permite inseminar un mayor número de hembras.
- Mejoras sanitarias en la explotación, al evitar el contacto directo macho-hembras, por lo que se impide la transmisión de enfermedades por vía venérea y por contacto.
- Evaluación continua de la producción y de la calidad espermática lo que permite monitorizar la fertilidad de los verracos a lo largo del tiempo productivo.
- Mejora del control de los resultados reproductivos de la explotación de forma indirecta
- La reducción en el número de verracos por hembra, con la consiguiente reducción en costes de adquisición, alojamiento, alimentación, etc.

La IA porcina es una técnica reproductiva de amplia aplicación en los países desarrollados, aunque el grado de utilización es muy variable (Levis, 2000). En el contexto europeo, la tasa de aplicación de la IA es muy elevada, llegando a porcentajes superiores al 80% en algunos países como Holanda, Francia, Alemania, España, Noruega, Finlandia, etc. Mientras que, por el contrario, en los Estados Unidos el porcentaje de utilización de la IA es aún reducido (del orden del 50%), aunque en los últimos años se ha producido un incremento muy destacable. Según algunas estimaciones, en el mundo se realizan unos 19 millones de inseminaciones por año, de las cuales la práctica totalidad (99%), se realiza con semen refrigerado a 15–20ºC (Johnson et al., 2000). De estas inseminaciones, más del 85% se realizan en el mismo día de recogida o al día siguiente.

La utilización de la IA con semen refrigerado se ha extendido gracias al desarrollo de diluyentes que permiten la conservación del semen con excelentes resultados. Pero su principal limitación radica en la vida útil del semen que varía entre 2-5 días. Actualmente, se consigue una conservación del semen refrigerado a 15ºC de hasta 7 días con diluyentes denominados de larga duración (Johnson et al., 2000; Gadea, 2003).

La utilización de la IA con semen congelado queda hoy día limitada a casos muy específicos, bien asociada a la introducción en las explotaciones de nuevo material genético de alto valor para inseminar determinados animales de razas puras en las granjas de selección, o bien asociada a labores de investigación (Johnson, 1985; Waberski et al., 1994). Sin embargo, el uso de semen congelado puede aportar ciertas ventajas sobre el semen refrigerado, como son (Gadea, 2004):

- El intercambio de material genético a larga distancia y durante un periodo muy largo (años). Este periodo de tiempo puede ser crucial para efectuar un control sanitario o genético del semen/verraco antes de su uso. Eso es posible hoy con el diagnóstico de enfermedades infecciosas basado en el estudio de la presencia de ADN del agente infeccioso mediante técnicas de PCR (Reacción de la polimerasa en cadena) y además, el desarrollo de marcadores genéticos asociados a la producción es un proceso creciente.

- La creación de bancos genéticos. De evidente interés en el caso de la conservación de razas en peligro de extinción y de grandes posibilidades para la conservación de líneas o estirpes de especial interés. Estos bancos de genes pueden ser realmente importantes a nivel comercial, por ejemplo, para asegurar la conservación de líneas genéticas valiosas ante posibles situaciones desfavorables (epizootía, incapacidad para recogida, infertilidad/subfertilidad por altas temperaturas, etc.).

- La introducción de material genético de alto valor sin los riesgos derivados de la incorporación de nuevos animales en la explotación. Especialmente aplicable a las líneas genéticas puras que se utilizan en las zonas altas de la pirámide de selección (“abuelas”).

La reducción en el rendimiento reproductivo ha sido el gran limitante del uso del semen congelado (Johnson, 1985; Waberski et al., 1994). Sin

embargo, en los últimos años se ha realizado un gran esfuerzo para mejorar la fertilidad del semen congelado en inseminación artificial obteniéndose resultados a nivel experimental muy prometedores, que suponen tasas de partos por encima del 70% y para algunos machos por encima del 80% (Thilmant, 1998; Eriksson et al., 2002; Sellés et al., 2003; Roca et al., 2003; Bolarin et al., 2006). Por tanto, hoy en día con estos resultados sería conveniente evaluar si es rentable comercialmente el uso del semen congelado para determinadas aplicaciones específicas. En cualquier caso se debe desterrar la idea muy difundida entre el sector que afirma que los espermatozoides porcinos no se pueden congelar de forma adecuada y su uso no permite unos rendimientos reproductivos óptimos. Esta misma idea incierta se difunde en sentido contrario cuando hacemos referencia al semen congelado en el ganado vacuno de leche, donde la mayor parte de la inseminación artificial se realiza con semen congelado, cuando la fertilidad media obtenida no supera en el mejor de los casos el 70% (Kastelic y Thundathil, 2008).

El éxito en el proceso de congelación de los espermatozoides porcinos dependen de un gran número de factores que, de forma conjunta, determinan la calidad del semen descongelado (Roca et al., 2006; Mazur et al., 2008). En numerosos estudios se han descrito grandes diferencias en la capacidad de congelación que presentan los espermatozoides de distintos machos (Johnson, 1985; Medrano et al., 2002; Hernández et al., 2006), que afectan tanto a la viabilidad de los espermatozoides tras la descongelación como a la fertilidad *in vivo* (Johnson et al., 1981; Bwanga, 1991; Roca et al., 2006). De manera tradicional, los machos han sido clasificados como “buenos” o “malos congeladores” (Medrano et al., 2002). Hasta hace muy poco tiempo se desconocían las posibles causas ciertas de esta variabilidad, de manera que el único planteamiento viable era la de optimizar los procesos de congelación para reducir al máximo la variabilidad y descartar aquellos machos realmente malos congeladores. Recientemente, se han encontrado los principios de una base genética que justifica estas diferencias (Thurston et al.,

2002) y que abre nuevas posibilidades en la selección de reproductores de acuerdo a su capacidad de congelación.

Se han estudiado diversos factores que pueden mejorar la eficiencia de la técnica: la curva de congelación (con sistemas automatizados o manuales) así como las condiciones óptimas para la descongelación (Fiser et al., 1993; Hernández et al., 2007), las diferencias en la capacidad de congelación de las diversas fracciones del eyaculado (Sellés et al., 2001; Peña et al., 2006), el método de preparación de las muestras seminales (Matás et al., 2007), las condiciones en las que se produce la inseminación (Wabersky et al., 1994; Bertani et al., 1997; Bolarín et al., 2006), así como el efecto de la estación, la línea genética y el verraco en la capacidad de congelación (Gadea et al., 2003, Roca et al., 2006).

En los últimos años, los estudios se han centrado en estudiar las alteraciones que produce la congelación en la célula espermática, lo que permite conocer y comprender el proceso lesivo y la manera práctica de reducir o minimizar los cambios en la función de la célula espermática. En este sentido, se conoce desde hace décadas que el choque frío induce severas alteraciones en la estructura de la membrana espermática que llevan a la muerte celular (Pursel et al., 1973). Estas alteraciones de membrana están relacionadas con la formación de cristales durante el proceso de congelación (Mazur et al., 1997). El daño que ocasionan los cristales es proporcional a la cantidad y el tamaño de los mismos (Watson, 1995).

Posteriormente, se puso de manifiesto que el proceso de congelación además de producir una alteración sobre la estructura de la membrana espermática producía una alteración en sus componentes que podrían llevar a modificaciones severas de la funcionalidad espermática. Uno de los mayores efectos de la congelación se produce sobre la composición, distribución y estabilidad de los lípidos de la membrana espermática que se

alteran de forma similar a los cambios producidos durante la capacitación espermática (Cerolini et al., 2001). Es por ello que a estas modificaciones se le han denominado "tipo capacitación ("capacitation like changes", Watson, 1995; Green & Watson, 2001; Kaneto et al., 2002; Bravo et al., 2005). Del mismo modo recientemente se han relacionado los cambios que se producen en las membranas espermáticas durante la congelación a los que se producen en el proceso de apoptosis (Peña et al., 2003; Trzcinska et al., 2006). Esta información sobre los procesos básicos puede ser vital para que posteriormente pueda ser aplicada en el diseño de nuevos protocolos y diluyentes que permitan minimizar el daño espermático.

Por último mencionaremos el especial interés que tiene el estudio de la desestabilización del sistema antioxidante del espermatozoide durante la congelación. En este sentido, se conoce que los procesos de refrigeración y congelación producen una alteración física y química de las membranas espermáticas que tiene como consecuencia la reducción de la viabilidad celular y de su capacidad fecundante. Las alteraciones producidas por la reducción de la temperatura están asociadas con el denominado estrés oxidativo, estando inducido por la generación de agentes oxidantes (ROS) (Chatterjee et al., 2001). La consecuencia final es la peroxidación de los lípidos y una alteración grave de la funcionalidad espermática.

El semen representa un complejo sistema redox que combina el potencial antioxidante del plasma seminal y de los espermatozoides con el potencial pro-oxidante del espermatozoide a través de la generación de ROS. El sistema defensivo antioxidante incluye una actividad enzimática (superóxido dismutasa, glutatión reductasa, glutatión peroxidasa y catalasa), así como la presencia de diversas sustancias con actividad antioxidante (glutatión reducido (GSH), urato, ácido ascórbico, vitamina E, taurina, hipotaurina, carotenoides y ubiquinonas). El glutatión (L-g-glutamil-L-cisteinilglicina) es un tri-péptido distribuido en todas las células del organismo y juega un papel decisivo en el mecanismo de defensa

intracelular frente el estrés oxidativo. La enzima glutatión peroxidasa usa GSH como agente para reducir el peróxido de hidrógeno hasta agua y el lipoperóxido hasta alquil-alcohol. Por otro lado, la forma oxidada del glutatión (GSSG) se reduce hasta GSH mediante la enzima glutatión reductasa usando NADPH como cofactor. El contenido de glutatión (principal agente antioxidante no enzimático) se reduce durante el proceso de congelación (Bilodeau et al., 2000, Gadea et al., 2004; Molla et al., 2004), así como se alteran las proteínas de la membrana espermática (Gadea et al., 2004).

Para evitar el proceso de oxidación producido durante la congelación se ha estudiado la adición de antioxidantes en los medios de congelación y descongelación con relativo éxito. Así se ha ensayado el uso de diversas sustancias como el tocoferol y análogos (Peña et al., 2003; Breinenger et al., 2005; Satorre et al., 2007), superóxido dismutasa (Roca et al., 2005) y el glutatión reducido (Funahashi y Sano, 2005; Gadea et al., 2005).

Los resultados de estas investigaciones podrán permitir en un futuro próximo conocer con profundidad los daños que se producen en la célula espermática durante la congelación-descongelación. Con esta información básica se podrá diseñar sistemas de congelación (curvas de congelación, diluyentes, crioprotectores, envases, etc…) que mejoren la calida seminal tras la descongelación y mejoren los rendimientos reproductivos.

En todo este proceso de mejora en la congelación de espermatozoides porcinos es necesario el empleo de determinadas técnicas analíticas que miden parámetros de calidad seminal. Por calidad seminal se entiende el conjunto de parámetros que caracterizan la viabilidad de la célula espermática. En un principio se hacía referencia a los caracteres que definen la morfología y el movimiento de los espermatozoides (Larsson, 1986), pero posteriormente se le han añadido otra serie de parámetros que tienen por objetivo cuantificar de algún modo la funcionalidad del espermatozoide (Berger et al., 1996).

Mucho se ha avanzado en las últimas décadas en el campo de la evaluación de la calidad seminal y sobre el valor predictivo de la fertilidad que presentan las pruebas de análisis espermático. Sin embargo, es una tarea aún no resuelta. En este sentido, la calidad del eyaculado ha sido tradicionalmente evaluada con el espermiograma clásico, basado en la aplicación de una serie de pruebas de una ejecución relativamente simple y que pueden ser realizadas con un coste moderado (Gadea, 2005). En el análisis rutinario se incluye un examen macroscópico y microscópico del eyaculado en los que se mide el volumen, la concentración, la motilidad, el estado del acrosoma y las morfoanomalías espermáticas.

En el trabajo diario en los centros de inseminación artificial se detectan animales que tienen una fertilidad reducida pero que al realizar un análisis de rutina presentan un espermiograma normal. Por ello, podemos deducir que ninguno de los parámetros del espermiograma clásico por sí solo parece ser suficiente para predecir adecuadamente la fertilidad, aunque la información combinada de todos ellos ofrece una buena estimación de la calidad seminal (Woelders, 1991).

Para dar una solución a este problema se han desarrollado nuevas técnicas que pretenden alcanzar un mejor conocimiento de la célula espermática (Gadea, 2005; Silva y Gadella, 2006; Rodríguez-Martínez y Barth, 2007). Con este objetivo se puede evaluar la estructura y funcionalidad del espermatozoide. Entre estas nuevas técnicas, el estudio de la membrana parece ser un buen procedimiento para evaluar la funcionalidad del gameto masculino, ya que ésta interviene activamente en la mayoría de las fases del proceso reproductivo. La membrana puede ser estudiada desde el punto de vista estructural mediante la utilización de tinciones, o bien valorando su funcionalidad, para lo que se ha aplicado el test hipoosmótico (Jeyendran et al., 1984; Vázquez et al., 1997) y distintas técnicas con fluorocromos que permiten evaluar otros aspectos de la funcionalidad espermática como son los procesos de capacitación espermática y reacción

acrosómica, la actividad mitocondrial, la permeabilidad de membrana, la estructura y estabilidad del núcleo y la cromatina, etc… (Harrison y Vickers, 1990; Garner y Johnson, 1995; Silva y Gadella, 2006).

Los estudios bioquímicos se desarrollaron con la intención de tener una medición objetiva y fácilmente reproducible de la calidad seminal y que fueran reflejo de su actividad funcional. Se han realizado estudios metabólicos y enzimáticos, cuantificándose los diferentes componentes químicos presentes en el eyaculado y que pueden condicionar la actividad del espermatozoide como son el contenido en iones, la liberación de enzimas o el contenido de ATP (Strzezek y Skaweta, 1984; Ciereszko et al., 1994; Gadea et al., 1998). Posteriormente, los estudios de la composición lipídica de la membrana espermática porcina han puesto de manifiesto que tanto los procesos de capacitación (Shadan et al., 2004) como los de congelación (Cerolini et al., 2001; Maldjian et al., 2005; Waterhouse et al., 2006) están íntimamente relacionados con los cambios que se producen en el componente lipídico de la membrana.

Por otra parte, el estudio del núcleo del espermatozoide permite valorar la madurez y la estabilidad del mismo, condiciones necesarias para que pueda llegar a producirse la descondensación cromosómica y la singamia. Estos estudios se desarrollaron cuando se asociaron problemas de fertilidad con alteraciones de la estructura del núcleo (Evenson et al., 1994). Estas alteraciones del núcleo han sido clasificadas como factores incompensables de la fertilidad (Saacke et al., 1994), ya que no es posible mejorar la fertilidad aumentando el número de espermatozoides por dosis de inseminación.

Los procesos de capacitación y reacción acrosómica son pasos fundamentales para que se lleve a cabo la fecundación (Flesh y Gadella, 2000). Por ello, se ha realizado un importante esfuerzo en estudiar los

mecanismos íntimos que regulan estos procesos y que aún son en parte poco conocidos (Barboni, 1994; Harrison, 1996; Harrison y Gadella, 2005).

Hasta la fecha, el método más preciso para predecir la fertilidad consistiría en determinar la capacidad de los espermatozoides para penetrar ovocitos en un sistema *in vitro* (Bavister, 1990). Primeramente, se desarrollaron sistemas heterólogos basados en la penetración de ovocitos de hámster libres de zona pelúcida, principalmente diseñados para evaluar la capacidad de penetración de los espermatozoides humanos (Yanagimachi et al., 1976). Este método se ha convertido en una buena herramienta para valorar la capacidad fecundante en la mayoría de las especies. Sin embargo, esta prueba no valora fases fundamentales del proceso de penetración del ovocito como son el reconocimiento de los gametos, la unión y la penetración de la zona pelúcida y plantea algunos problemas operativos para su aplicación clínica. Por tanto, parece lógico pensar que con la utilización de un test de penetración de ovocitos homólogos se obtendrían unos resultados más ajustados a la realidad, ya que permitiría el estudio de todas las fases del proceso de fecundación.

En los últimos años se han llevado a cabo diversos estudios utilizando ovocitos homólogos para evaluar la capacidad fecundante del eyaculado porcino. La posibilidad de utilizar ovocitos inmaduros en los sistemas de fecundación con unas tasas de penetración equivalentes a las de ovocitos maduros (Martínez et al., 1993) supone facilitar en gran medida este estudio, ya que se puede disponer de un gran número de ovocitos a partir de hembras prepúberes sacrificadas en los mataderos comerciales. Por otra parte, también se han utilizado sistemas de fecundación de ovocitos madurados *in vitro* que permiten evaluar otros pasos del proceso como la formación del pronúcleo o las tasas de división embrionaria y formación de blastocistos (revisado por Larsson y Rodríguez-Martínez, 2000).

La predicción de la capacidad fertilizante del semen tiene una gran importancia en las producciones ganaderas cuando se hace uso de la IA, así como para la selección de machos con mejor valor reproductivo (Hammerstedt, 1996). Este hecho es de especial importancia cuando se hace uso de semen congelado, cuya viabilidad y funcionalidad está modificada por el proceso de conservación. De momento, sólo unos pocos estudios correlacionan de manera significativa los factores espermáticos con la fertilidad. Esto es debido a que hay un gran número de factores que modulan esta relación y que están asociados con el potencial fértil de las hembras (Gadea, 2005). Algunos autores proponen como otra causa de la inconsistencia de los resultados conseguidos, la evaluación de un número relativamente reducido de células, la gran influencia de la subjetividad del observador y la alta variabilidad que presentan muchos análisis seminales (Evenson et al., 1994; Saacke et al., 1994). Además, el número de espermatozoides aplicados en cada inseminación es un factor muy importante a la hora de evaluar la fertilidad y su relación con los parámetros de calidad seminal (Fearon y Wegener, 2000).

La mayoría de los análisis *in vitro* utilizados hasta el momento ofrecen información sobre la calidad seminal, que es fundamental para los estudios en general de la fisiología del espermatozoide y en particular para la conservación del semen. Sin embargo, lo realmente importante para el sector productivo porcino es el estudio de los caracteres relacionados con el proceso de fecundación, es decir la determinación *in vitro* de la capacidad fecundante de los eyaculados *in vivo* (Woelders, 1991).

La evaluación de la calidad seminal es una parte importante y un punto crítico en el proceso de la inseminación artificial, ya que en muchos casos, verracos asociados con una fertilidad reducida presentan alteraciones detectables mediante un examen rutinario del semen. No obstante, aunque es necesaria una buena calidad seminal para alcanzar unos niveles de fertilidad aceptables, no todos los eyaculados con buena calidad seminal

mantienen niveles de fertilidad dentro de la normalidad (Berger y Parker, 1989; Martínez et al., 1993).

En resumen, podemos afirmar que la IA porcina se ha desarrollado de manera muy importante pero sin duda quedan aún importantes temas en los que es posible trabajar para mejorar los resultados productivos. Esta problemática nos obliga a profundizar en el estudio de técnicas que permitan avances en la crioconservación espermática desarrollando investigaciones en la mejora de los sistemas de congelación-descongelación y en los sistemas de evaluación de la calidad del semen tanto refrigerado como congelado-descongelado en la eficacia del uso en condiciones de campo.

2

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Analysis of *In vitro* Fertilizing Capacity to Evaluate the Freezing Procedures of Boar Semen and to Predict the Subsequent Fertility

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Contents

A porcine *in vitro* fertilization (IVF) system and seminal quality parameters of frozen–thawed boar semen were used to assess the effectiveness of two different thawing rates of frozen boar semen, and to address the question of whether differences between fertility of ejaculates could be predicted in a limited field trial.

In the first experiment, two thawing procedures were analysed (37°C, 30 s; 50°C, 12 s) and no differences in sperm quality were found. However, when the procedure was 50°C, 12 s the IVF results showed a higher number of sperm per penetrated oocyte and a near 10 points higher rate of pronuclear formation.

In the second experiment, the fertility results obtained in the limited field trial show to be efficient enough for application in a commercial use, especially for three of the employed boars (fertility ≥80%). In this limited study, the conventional seminal parameters are not accurate enough to discriminate good and bad boars in relation to fertility. On the contrary, parameters of *in vitro* penetrability are more precise to predict subsequent fertilities.

As conclusion, the IVF fertilization system seems to be a good tool to evaluate the quality of frozen–thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm freezing procedures, as it is the more precise evaluating method in estimating the potential fertilizing ability.

Introduction

Despite almost 40 years of research in freezing of boar semen, the fertility results are not satisfactory enough for commercial use as it is in other domestic animals. However boar frozen–thawed semen is still a valuable tool as a complement to artificial insemination (AI) with fresh semen in some conditions. As it can be stored for a long time, it facilitates the supply of genetic material, as well as building up gene banks to encourage breeds or valuable individuals. In the last years, an increasing effort has been made to improve the fertility results mainly by to ways: first the design of better freezing methods in order to obtain acceptable semen quality (freezing procedures, diluents and cryoprotectants, packages, etc.) (reviewed by Bwanga 1991; Johnson et al. 2000), secondly with optimal routines for heat detection and timing of insemination close to the ovulation (Waberski et al. 1994).

Evaluation of the quality of frozen–thawed semen is an important goal and great deals of assays have been developed (Johnson et al. 1996). Besides, assays including the study of gamete interaction might lead to a better way of predicting male fertility than routine laboratory evaluation of semen (Gadea et al. 1998; Larsson and Rodriguez-Martinez 2000). Some of these assays have been shown to be good tools for evaluating the fertilizing capacity of diluted boar semen (Ivanova and Mollova 1993; Gadea et al. 1998; Xu et al., 1998). However, little information is available about frozen boar semen (Hammitt et al. 1989; Gadea et al. 2001; Pelaez et al. 2001), but it would be very useful to evaluate freezing procedures (Eriksson et al. 2000).

One of the most important factors related with the success of freezing procedures seems to be the thawing rate of semen (Johnson et al. 2000). So, the critical temperature range during thawing is an important factor affecting spermatozoan viability (Fiser et al. 1993). However, it has been demonstrated that the effectiveness of thawing rate also depends on the original rate of freezing (Mazur 1985). Different studies have previously described that when thawing rate is increased (in an optimum range), the motility and acrosome integrity are improved (Pursel and Johnson 1975; Fiser et al. 1993).

For these reasons the objectives of this study were to study the application of the *in vitro* fertilization (IVF) systems: (1) to assess the effectiveness of two different thawing rates of frozen boar semen, and (2) to address the question of whether differences between fertility of ejaculates could be predicted with semen quality parameters and with IVF fertilization systems in a limited field trial.

Material and Methods

Semen collection and freezing

Semen was regularly collected from five mature fertile boars (one Belgium Landrace and four Pietrain 18–30 month-old boars) using the hand method and a dummy. Sperm-rich fraction was collected in a pre-warmed thermos flask and the gel-fraction was held on a gauze tissue covering the thermos opening. The volume of the sperm-rich fraction of the ejaculate was measured in a graduated cylinder and sperm concentration measured with a haemacytometer (Neubauer, Brand, Wertheim, Germany) within 20 min after collection and prior to extension of the semen with isothermal Betsville thawing solution (BTS; Minitüb, Tiefenbach, Germany) extender at a ratio of 1 : 1. Semen was stored at 22°C for 2 h, and processed according to the straw-freezing procedure described by Westendorf et al. (1975) and Almlid and Johnson (1988). Briefly, diluted semen was placed at 15°C for 150 min (in a water bath placed into a freezer) and later centrifuged at 800 × ***g*** for 10 min. The supernatant was discarded and the semen pellet was re-suspended with lactose–egg yolk extender (LEY; 80 ml of 11% lactose and 20 ml egg yolk) to provide

1.5×10^9 spermatozoa per ml. Then, it was cooled at 5°C for 90 min (in a water bath placed into a freezer) and two parts of LEY extender semen were mixed with LEY extender with 9% glycerol and 1.5% Orvus Es Paste (Equex-Paste; Minitüb, Tiefenbach, Germany). The final concentration of semen to be frozen was 1×10^9 spermatozoa per ml and 3% glycerol. The cooled semen was leaded into 0.5 ml. straws (Minitüb, Tiefenbach, Germany) and sealed with polyvinyl alcohol. Straws were wiped dry and the air bubble was brought to the centre of the straw. The straws were placed in contact with nitrogen vapour about 3 cm above the liquid nitrogen level for 20 min in an expandable polystyrene box, plunged into the nitrogen tank and stored until use (1–3 months later). Straws were thawed in a circulating water bath at 50°C for 12 s or at 37°C for 30 s and immediately diluted in 10 ml BTS at 37°C.

Seminal parameters

Sperm motility and movement quality were determined placing two sub-samples on warm glass slides (39°C) and examined under a light microscope (100 × magnification). The percentage of motile sperm cells was subjectively estimated to the nearest 5% and the forward progressive motility (FPM) using an arbitrary scale of 0–5.

The proportion of spermatozoa with a normal apical ridge (NAR) was evaluated after fixed in buffered 2% glutaraldehyde solution and examined under a phase-contrast microscope (Leica DMR, Wetzlar, Germany) (1000 × magnification) to analyse acrosomes (Pursel et al. 1972). The NAR was determined on two slides per sample and a total of 300 spermatozoa per sample.

Eosin–nigrosin (EN) viability staining of sperm was also studied. It was diluted at a ratio of 1 : 1 a semen sample with staining solution (5% yellow eosin, 10% nigrosin in a citrate solution pH = 7.4) and smeared. After air-fixed stained spermatozoa were observed and evaluated 200 sperm per sample (Bamba 1988).

Sperm membrane integrity was evaluated applying a combination of the fluorophores carboxyfluorescein diacetate (DCF) and propidium iodide (Harrison and Vickers 1990) on at least 200 cells per sample using an epifluorescence microscope.

In vitro maturation and *in vitro* fertilization

Ovaries from pre-puberal gilts were obtained at a local slaughterhouse, and transported to the laboratory in saline (0.9% w/v NaCl) with 100 mg/l kanamycin at 35°C. Oocytes surrounded by cumulus cells, were slicing from 3 to 6 mm diameter follicles and washed twice in 35 mm plastic Petri dishes containing Dulbecco's phosphate-buffered saline modified supplemented with 1 mg/ml polyvinyl alcohol and 0.005 mg/ml red phenol. They were washed twice again in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO_2 in maximally humidified air.

The medium used for oocyte maturation was Waymouth-supplemented as previously described (Coy et al. 1999) with 10 UI/ml pregnant mare serum gonadotrophin (PMSG), 10 UI/ml human chorionic gonadotropin (hCG), 1 μg/ml oestradiol-17b, 10% (v/v) foetal calf serum and 10% porcine follicular fluid (v/v). The maturation medium was disposed in three droplets of 100 μl covered with paraffin oil per dish, 20 oocytes per droplet and kept at 38°C under 5% CO_2 in air. After 20–22 h of culture in maturation medium the oocytes were transferred to fresh maturation medium without hormonal supplements, washed twice and cultured for an additional 20–22 h (Funahashi and Day 1993).

The *in vitro* fertilization medium was TCM199-supplemented as previously described (Coy et al. 1999) with 12% heat-inactivated foetal calf serum, 0.9 mM sodium pyruvate, 3.05 mM D-glucose, 8.75 mM calcium lactate, 0.68 mM penicillin G, 3.6 mM caffeine and 0.068 mM streptomycin sulphate at pH = 7.4.

After thawing, the sperm samples were centrifuged at 50 × ***g*** for 3 min and the supernatants at 1200 × ***g*** for 3 min. Resulting pellets of spermatozoa were diluted in TCM199-supplemented medium but without calcium lactate nor caffeine. A semen volume of 100 μl was introduced into Petri dishes containing 2 ml of fertilization medium (final concentration of 1×10^6 spermatoza/ml) and 20 *in vitro* matured oocytes previously washed twice in equilibrated fertilization medium. After 18 h the cultured oocytes were fixed in 3 : 1 ethanol : acetic acid for 24 h, stained with 1% lacmoid and examined under a phase-contrast microscope to asses penetration (PEN) rate, mean number of sperm per penetrated oocyte (S/O), monospermy (MON) rate and rate of male pronuclear formation (MPF).

Fertility trial

The fertility study was conducted on a commercial farm, using a total of 45 multiparous (two to seven pregnancies) crossbred sows. Oestrus was checked daily in the presence of a mature teaser boar. Occurrence of oestrus was defined by the standing reflex in front of a boar (back pressure test) and reddening and swelling of the vulva. The sows were inseminated immediately after thawing the semen at 50°C for 12 s and diluted with BTS to prepare insemination doses containing at least 5×10^9 spermatozoa in 80 ml. Insemination took place on 12 h after the diagnosis of oestrus and was repeated 12 h later, using disposable AI catheters.

Pregnancy diagnosis was performed 23–25 days after AI by ultrasonography. Fertility was measured for every ejaculate as the percentage of sows farrowing to AI. For each sow that farrowed, the number of dead and live piglets was counted and the sum was defined as the total number of piglets born.

Experimental design

Experiment 1. Thawing process

Five ejaculates from the same boar were frozen according to the methods described before and were thawed at each of the following test velocities resulting from immersion in water to 37°C for 30 s or at 50°C for 12 s. Seminal parameters and IVF capacity were assayed.

Experiment 2. Fertility trial

To evaluate the capacity for fertility prediction of different assays and to verify the quality of the frozen–thawed semen, ejaculates from five fertile boars were frozen–thawed and they were used both in an IVF system and in a field assay by AI with at least 5×10^9 sperm per dose. Seminal parameters were evaluated and differences between boars were investigated in blind fashion.

Statistical analysis

Data are presented as mean ± SEM. Data for all rates were modelled according to the binomial model of parameters and were analysed by two-way ANOVA; considering the thawing procedure and sperm batch as main effects in experience 1 and one-way ANOVA in experience 2. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered statistically different at $p < 0.05$.

Linear regression was used to further investigate relationships between litter size and measured semen parameters (Pearson correlation and multiple regression), and logistic regression was used to relate the dichotomous farrowing rate data to the sperm parameters, as previously described by Holt et al. (1997).

Results

Experiment 1

The studied thawing velocities had no effect on the sperm quality (Table 1), but a significant difference on the motility and NAR ($p < 0.001$) was detected between batches. For both treatments the motility and membrane integrity (measured with EN or carboxifluorescein staining) were over 60%.

In relation to the *in vitro* fertilizing capacity of the sperm, thawed under two different procedures, the results obtained showed a higher number of S/O (3.91 vs 3.06, $p < 0.001$) (Table 2) and a near 10 points higher rate of MPF (75.47 vs 65.73, $p = 0.020$) when the procedure was 50°C for 12 s. Besides, the sperm batch had a significant effect on the PEN, number of S/O and MON rate ($p < 0.001$).

Experiment 2

The result of sperm assays showed a significant lower number of intact membrane (EN and DCF) and acrosomal integrity (NAR) for the frozen–thawed sperm from boar PI4 ($p < 0.001$, Table 3). However, all the four IVF parameters were significantly affected by the boar studied and showed a significant lower values for penetrability (PEN and S/O) in PI4 and PI779 boars against the other three (Table 4). In the same way, the MON was affected by boar and showed the highest values for the boars with less penetrability. The MPF was higher than 82% in all the boars studied and this parameter was not related with fertility (Tables 4 and 5).

The *in vitro* penetrability results are consistent with the limited data from the fertility field trial, as fertility was significantly affected by boar ($p = 0.019$), being those with lower penetrability (boars PI4 and PI779) these are with lower fertility (33%) than the other three

Table 1. Seminal parameters measured in boar semen thawed under two different procedures (mean ± SEM)

	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Thawing velocity					
50°C for 12 s	62.27 ± 3.53	3.64 ± 0.15	76.55 ± 1.74	49.36 ± 4.34	66.64 ± 2.53
37°C for 30 s	60.45 ± 4.01	3.73 ± 0.14	72.36 ± 1.86	48.91 ± 3.57	63.45 ± 2.41
Source of variability					
Thawing velocity	0.462	0.671	0.058	0.961	0.117
Sperm batch	< 0.001	0.125	0.221	< 0.001	0.082
Interaction	0.459	0.658	0.132	0.850	0.053

FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate.

Table 2. The IVF results (mean ± SEM) for mature oocytes fertilized with frozen–thawed boar semen under two different procedures: rate of penetration (% PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (% MON) and rate of male pronuclear formation (% MPF)

	n	% PEN	S/O*	% MON*	% MPF*
Thawing velocity					
50°C for 12 s	230	69.13 ± 3.05	3.91 ± 0.27[a]	33.96 ± 3.77	75.47 ± 3.42[a]
37°C for 30 s	254	70.08 ± 2.88	3.06 ± 0.19[b]	34.83 ± 3.58	65.73 ± 3.57[b]
Source of variability					
Thawing velocity	0.840	< 0.001	0.641	0.020	
Sperm batch	< 0.001	< 0.001	< 0.001	0.654	
Interaction	0.949	< 0.001	0.519	0.019	

* Based on penetrated oocytes.
[a,b] Numbers within columns with different superscripts differ ($p < 0.05$).
n: number of oocytes.

Table 3. Seminal parameters measured in sperm thawed from five different boars used in the fertility trial (mean ± SEM)

	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Boar					
BB	62.00 ± 2.00	3.95 ± 0.05	72.50 ± 2.88[a]	42.50 ± 3.49[a]	69.75 ± 3.17[a]
PI4	59.37 ± 2.17	3.75 ± 0.06	53.69 ± 2.51[b]	33.34 ± 2.48[b]	41.71 ± 3.78[b]
PI67	63.70 ± 1.55	3.88 ± 0.04	66.31 ± 1.40[a]	39.97 ± 1.44[a]	53.69 ± 3.48[ab]
PI779	58.92 ± 0.82	3.85 ± 0.04	70.73 ± 1.61[a]	46.76 ± 1.38[a]	57.41 ± 2.52[a]
PI89	59.30 ± 0.80	3.86 ± 0.03	69.20 ± 1.18[a]	51.16 ± 1.30[a]	56.47 ± 2.41[a]
Source of variability					
Boar	0.053	0.208	< 0.001	< 0.001	< 0.001

FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate.
[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 4. The IVF and *in vivo* fertility results (mean ± SEM) for mature oocytes fertilized with frozen–thawed semen from five different boars: rate of penetration (PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (% MON) and rate of male pronuclear formation (MPF)

	n	PEN (%)	S/O*	MON* (%)	MPF* (%)	Fertility (%)	Litter size
Boar							
BB	199	62.81 ± 3.43[cd]	2.78 ± 0.28[a]	48.80 ± 4.49[ab]	88.80 ± 2.83[ab]	80 (4/5)	11.5 ± 1.19
PI4	212	44.34 ± 3.42[b]	1.65 ± 0.13[b]	61.70 ± 5.04[ab]	82.98 ± 3.90[b]	33.33 (2/6)	6 ± 0
PI67	329	67.17 ± 2.59[d]	2.21 ± 0.11[ab]	40.72 ± 3.31[b]	87.33 ± 2.24[b]	84.62 (11/13)	9.18 ± 1.54
PI779	320	23.13 ± 2.36[a]	1.63 ± 0.13[b]	63.51 ± 5.63[a]	94.59 ± 2.65[ab]	33.33 (3/9)	10.67 ± 0.67
PI89	308	52.27 ± 2.85[bc]	2.54 ± 0.18[a]	50.93 ± 3.95[b]	98.14 ± 1.07[a]	83.33 (10/12)	7.3 ± 1.46
Source of variability							
Boar	< 0.001	< 0.001	0.001	< 0.001	0.019	0.405	

*Based on penetrated oocytes.
[a,b,c,d] Numbers within columns with different superscripts differ ($p < 0.05$).
n: number of oocytes.

Table 5. Logistic regression of seminal parameters with fertility result (farrowing rate)

Variable	Coefficient *B*	SE	*t*	Significance (p)
PEN	0.0208	0.0071	2.9402	0.0033
S/O	0.4384	0.1758	2.4935	0.0127
MPF	0.0063	0.0035	1.8046	0.0711
MON	0.0043	0.0050	0.8577	0.3910
Motility	0.0115	0.0051	2.2360	0.0253
FPM	0.1842	0.0821	2.2445	0.0248
EN	0.0107	0.0047	2.2654	0.0235
NAR	0.0164	0.0072	2.2870	0.0222
DCF	0.0135	0.0058	2.3482	0.0189

PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte, MPF: rate of male pronuclear formation, MON: monospermy rate, FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate, SE: standard error.

Table 6. Multiple logistic regression of seminal measurements with fertility result (farrowing rate)

Variable	Coefficient	SE	*t*	Significance (p)
Constant	−46.1846	24.9668	−1.8498	0.0643
PEN	0.3747	0.2228	1.6813	0.0927
MPF	0.8055	0.4882	1.6499	0.0990
Motility	−0.6857	0.5216	−1.3145	0.1887

McFadden's $R^2 = 0.492$; log likelihood: −27.373; SE: standard error.
Significance of statistical model $p < 0.001$.

with fertility rates over 80% (Table 4). No differences between boars were detected for litter size.

When the logistic regression was analysed between *in vitro* penetration and seminal parameters with fertility results, significant regression coefficients were found for PEN rate and S/O, and all the quality seminal parameters (motility, FPM, EN, NAR, DCF; Table 5, $p < 0.05$). Later, when studying all semen parameters through stepwise on multiple logistic regression forward, only three parameters (PEN, MPF and motility) were included (Table 6, $R^2 = 0.492$; $p < 0.001$).

In relation with litter size, significant Pearson correlation was found for all the IVF parameters (except MPF) and two quality parameters (FPM and DCF, Table 7). When a stepwise multivariate analysis was made a significant model ($p < 0.0001$) constructed only with fertilization parameters of penetrability (PEN and S/O) that may explain the nearly 80% of variability (Table 8).

Discussion

To improve the viability and fertilizing capacity of the boar frozen–thawed semen is necessary to optimize all the factors that have an effect in the freezing procedure. In this way we have focused our attention on one of them, the thawing process, and in the assessment methods for frozen semen evaluation.

Table 7. Linear regression of seminal measurements with average litter size[a]

Variable	Pearson correlation coefficient	Significance (p)
PEN	0.4461	0.0031
S/O	0.4356	0.0039
MPF	−0.1421	0.3692
MON	−0.4014	0.0084
Motility	0.2789	0.0635
FPM	0.3317	0.0260
EN	0.1968	0.1951
NAR	0.1181	0.4399
DCF	0.3450	0.0203

[a] Litter sizes included failed conceptions as zero values.
PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte, MPF: rate of male pronuclear formation, MON: monospermy rate, FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxy-fluorescein diacetate.

Table 8. Multiple linear regression of seminal measurements with litter size[a]

Variable	Coefficient	SE	*t*	p
PEN	0.0609	0.0356	1.7089	0.0952
S/O	1.4749	0.9276	1.5900	0.1197

[a] Litter sizes included failed conceptions as zero values.
$R^2 = 79.54\%$; $p < 0.0001$.
PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte.

In the first experiment, we have found no differences in sperm quality with two thawing procedures near to the optimal conditions and previously accepted to thaw frozen boar semen in ministraws (Maxwell and Johnson, 1997; Erkisson et al. 2000). However, Fiser et al. (1993) found that after freezing at optimal rate, the percentage of motile spermatozoa and sperm with NAR increased gradually while increasing warming velocity. They studied a wide range of velocities, some of them out of optimal conditions and those results were related to cryoinjuries made during a slow thawing process by the re-growth of ice crystals (Mazur 1985). Besides this, another problem would be present in the rapid thawing process, as Bamba and Cran (1985) showed that boar spermatozoa might be also severely damaged by warm shock.

Concerning the *in vitro* PEN capacity only the number of S/O and the rate of MPF were significantly affected by the thawing temperature. The capacity of spermatozoa to penetrate oocyte might be assessed by PEN rate as well as S/O (Matttioli et al. 1988). In this study, we have obtained a higher number of S/O in the thawing method with the higher temperature. This situation might be caused by a better sperm function not correctly evaluated by classical sperm analysis (Gadea and Matás 2000). Apart from this, it has been reported that a fast velocity is associated to decrease the cellular damage, so that the effect of a fast velocity could minimize the damages associated to structure and DNA stability and a significant decrease on the growth of ice crystals (Mazur 1984). In the same way, the higher rate of MPF obtained with warmer temperature might be in relation with a less intense alteration of sperm chromatin decondensation (White 1993), or the alteration in the number of thiol groups during freezing process (Chatterjee et al. 2001). However, MPF has also been related with others factors such as the content of glutathione in oocyte (Funahashi et al. 1994), the maturation system (Coy et al. 1999) and boar (Xu et al. 1996).

Significant differences associated to batches (motility, NAR, PEN, S/O and MON) would be related to the use of manual methods (nitrogen vapour) difficulty repeatable. This trouble could be solved with the use of programmable freezing procedures and the optimal curve of freezing (Ruiz et al. 2002). Finally, it is obvious that to obtain the best viability after thawing is extremely important to adapt the thawing velocity to the freezing velocity and to the straw volume (Hofmo and Almlid 1990).

The fertility results obtained in the limited field trial (total 66.67%, 30/45) are efficient enough for application in a commercial use, specially for three of the employed boars (80, 83.33 and 84.32%). These results are comparable with those obtained by other authors (Almlid and Hofmo 1996; Bertani et al. 1997; Hofmo and Grevle 1999; Erkisson, 2000). However, the question of whether differences between fertility of ejaculates could be predicted with semen quality parameters and with IVF systems is not yet answered. A wide variability among boars in fertility rates obtained from their frozen semen have been demonstrated in several studies (reviewed by Johnson 1985), and recently a genetic basis of boar semen freezability have been demonstrated (Thurston et al. 2002). However, till now, no method of evaluating the quality of frozen–thawed semen is yet available when attempting to predict fertility.

In this limited study, the conventional seminal parameters are not enough efficient to discriminate good and bad boars in relation to fertility. Only one of the two bad freezer boars with a low fertility was detected by acrosome and membrane integrity assessment. On the contrary, parameters of penetrability (PEN and S/O), measured in an IVF system, are more precise to predict subsequent fertilities. The S/O does not reflect the normal fertilization events *in vivo*, but may provide a useful estimate of spermatozoa with high fertilizing ability.

The results obtained in the IVF system are consistent with *in vivo* fertility and significant logistic regression was found for PEN and this parameter was included in a multiple model. In this way, in a previous work using diluted fresh semen and a higher number of sows inseminated, a significant relationship was found between *in vitro* PEN rate and S/O with *in vivo* fertility and with a similar multiple logistic regression to fertility, where PEN rate and motility were included (Gadea et al. 1998). In this model the motility also appears, but surprisingly the motility change the sign from logistic regression (positive 0.0115, Table 5) to negative sign in multiple logistic regression (−0.6857, Table 6). These non-sense signs must be related with co-linearity among decisive variables as previously we detected in applications of multiple discriminant analysis model for prognosis of *in vitro* fertility (Gadea and Matás, 2000). The

logistic regression is a robust method to analyse categorical data such as fertility rate. It is better than lineal regression of transformed data, but the former is difficult to manage with the odds ratio.

On the other hand, a greater difficulty is presented to predict the litter size, probably related with the maternal and environment importance effects (ovulation rate, fertilization rate, relation insemination–ovulation, and so on). So, a fewer number of classical sperm parameters are related and only PEN and S/O are necessary to explain near 80% of variability.

In the present study the IVF is shown as a precise technique to assess freezing procedures of boar semen. In this way, a limited number of factors related with the freezing process have been evaluated by the use of an IVF system (holding time and type of package (Eriksson et al. 2000) or volume of straw (Cordova et al. 2001, 2002). As conclusion, we can determine that a fast thawing speed had a more positive effect on the fertilizing capacity *in vitro* of the frozen boar semen. Moreover, the IVF seems to be a good tool to evaluate the quality of frozen–thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm-freezing procedures, as it is the evaluating method more precise in estimating the potential fertilizing ability.

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Decrease in glutathione content in boar sperm after cryopreservation Effect of the addition of reduced glutathione to the freezing and thawing extenders

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Abstract

Although glutathione content in boar spermatozoa has been previously reported, the effect of reduced glutathione (GSH) on semen parameters and the fertilizing ability of boar spermatozoa after cryopreservation has never been evaluated. In this study, GSH content was determined in ejaculated boar spermatozoa before and after cryopreservation. Semen samples were centrifuged and GSH content in the resulting pellet monitored spectrophotometrically. The fertilizing ability of frozen–thawed boar sperm was also tested in vitro by incubating sperm with in vitro matured oocytes obtained from gilts. GSH content in fresh semen was 3.84 ± 0.21 nM GSH/10^8 sperm. Following semen cryopreservation, there was a 32% decrease in GSH content ($P < 0.0001$). There were significant differences in sperm GSH content between different boars and after various preservation protocols ($P = 0.0102$). The effect of addition of GSH to the freezing and thawing extenders was also evaluated. Addition of 5 mM GSH to the freezing extender did not have a significant effect on standard semen parameters or sperm fertilizing ability after thawing. In contrast, when GSH was added to the thawing extender, a dose-dependent tendency to increase in sperm fertilizing ability was observed, although no differences were observed in standard semen parameters. In summary, (i) there was a loss in GSH content after cryopreservation of boar semen; (ii) addition of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability; and (iii) addition of GSH to the thawing extender resulted in a significant increase in sperm fertilizing ability. Nevertheless, future studies must conclude if this is the case for all boars. Furthermore, since addition of GSH to the thawing extender did not result in an improvement in standard semen parameters, this suggests that during the thawing process, GSH prevents damage of a

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sperm property that is critical in the fertilization process but that is not measured in the routine semen analysis.

Keywords: Pig spermatozoa; Glutathione; Freezing; IVF

1. Introduction

The process of cooling and freeze–thaw produces physical and chemical stress on the sperm membrane that reduces sperm viability and fertilizing ability. Cold shock of spermatozoa is associated with oxidative stress and reactive oxygen species (ROS) generation [1].

ROS-induced damage to sperm is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids, leading to lipid peroxidation [2]. Since boar sperm have a high polyunsaturated fatty acid content they are very susceptible to lipid peroxidation [3,4]. The effects of lipid peroxidation include irreversible loss of motility, inhibition of respiration, leakage of intracellular enzymes, damage to sperm DNA [5], or deficiencies in oocyte penetration and sperm–oocyte fusion [6]. Semen represents a complex redox system that combines the antioxidant potential of seminal plasma and spermatozoa with the pro-oxidant potential of sperm through the production of ROS. Enzymatic antioxidant defense mechanisms in seminal plasma and spermatozoa include superoxide dismutase, glutathione reductase, gluthathione peroxidase and catalase. Non-enzymatic antioxidants include reduced glutathione (GSH), urate, ascorbic acid, Vitamin E, taurine, hypotaurine, carotenoids, and ubiquinones. The interplay of antioxidant and pro-oxidant mechanisms in semen determines the overall rate of lipid peroxidation in sperm.

Glutahione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide ubiquitously distributed in living cells. It plays an important role in the intracellular defense mechanism against oxidative stress [2]. Glutathione peroxidase uses GSH as the reducing equivalent to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor.

GSH content has been reported in mammalian sperm [7,8], including boar sperm [9]. However, GSH content in boar sperm before and after cryopreservation has never been evaluated.

The main objectives of this study were (i) to determine GSH content in boar sperm before and after cryopreservation; and (ii) to assess the effect of GSH supplementation of freezing and thawing extenders on standard semen parameters and sperm fertilizing ability in IVF.

2. Material and methods

Semen was routinely collected from mature fertile boars using the manual method and a dummy. The sperm-rich fraction was collected in a pre-warmed thermo flask and the gel-fraction was held on a gauze tissue covering the thermo opening. The semen was then diluted with isothermal Beltsville thawing solution (BTS) extender at a ratio of 1:1 (v/v).

2.1. Freezing and thawing protocol

Semen samples were processed using the straw freezing procedure described by Westendorf et al. [10] with minor modifications indicated in the following. Diluted semen was placed at 15 °C for 2 h and later centrifuged at 800 × *g* for 10 min. The supernatant was discarded and the semen pellet was re-suspended with lactose–egg yolk extender (LEY, 80 ml of 11% lactose and 20 ml egg yolk) to provide 1.5×10^9 spermatozoa/ml. After further cooling to 5 °C over a 90-min period, two parts of LEY–extender semen were mixed with LEY extender with 1.5% Orvus Es Paste (Equex-Paste, Minitüb, Tiefenbach, Germany) and 9% glycerol. The final concentration of semen to be frozen was 1×10^9 spermatozoa/ml and 3% glycerol. The diluted and cooled semen was loaded into 0.5 ml straws (Minitüb) and placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min. The straws were then stored in liquid nitrogen until thawing.

Thawing was achieved by immersing the straws in a circulating water bath at 50 °C for 12 s [11]. Immediately after thawing, the semen was diluted in BTS.

2.2. Determination of GSH content in spermatozoa

Semen samples were centrifuged at 1000 × *g* for 5 min at room temperature and the resulting pellet resuspended in BTS and centrifuged again. The supernatant was discarded, the pellet resuspended in BTS, and sperm concentration adjusted to $1–5 \times 10^8$ sperm/ml. To release intracellular GSH, the sperm cells were lysed following three cycles of rapid cooling in liquid nitrogen and thawing at 37 °C. The resulting suspensions were centrifuged at 7000 × *g* for 10 min in order to remove membrane fragments.

Glutathione content was determined using a modified coupled optical test system [12]. In this system glutathione is oxidized by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and then reduced by glutathione reductase with NADPH as hydrogen donor. During the oxidation of glutathione by DTNB, 2-nitro-5 thiobenzoeic acid is formed, which can be detected photometrically by a change of absorption at 412 nm. The total glutathione content (oxidized glutathione (GSSG) and reduced glutathione (GSH)) is calculated according to a standard curve.

2.3. Analysis of standard semen parameters

Percent motility and progression were determined by placing two sample aliquots on warm glass slides (39 °C) and examined under light microscopy (magnification 100×). The percentage of motile sperm was estimated to the nearest 5% and the forward progressive motility (FPM) using an arbitrary scale from 0 to 5.

The proportion of spermatozoa with a normal apical ridge (NAR) was evaluated after fixation in a buffered 2% glutaraldehyde solution and examined under phase-contrast microscopy (magnification 1000×) to analyse acrosomes [13]. NAR was determined on two slides per sample and a total of 200 spermatozoa per sample.

Eosin–nigrosin viability staining of sperm was also performed (EN). Semen was diluted 1:1 (v/v), with the staining solution (5% yellow eosin, 10% nigrosin in a citrate solution,

pH 7.4) and smeared onto slides. After being air-fixed, the stained spermatozoa were observed under brightfield microscopy and 200 sperms per sample were evaluated [14].

Sperm membrane integrity was evaluated applying a combination of the fluorophores carboxyfluorescein diacetate (DCF) and propidium iodide [15] on at least 200 cells per sample using an epifluorescence microscope.

2.4. In vitro fertilization protocol

Ovaries from prepuberal gilts were obtained at a local slaughterhouse and transported to the laboratory in saline solution (0.9%, w/v, NaCl) with 100 mg/l kanamicyn at 35 °C. Oocytes surrounded by cumulus cells, were obtained from 3 to 6 mm diameter follicles and washed twice in 35 mm plastic Petri-dishes containing modified Dulbecco's phosphate buffered saline (mPBSD) supplemented with 1 mg/ml polyvinyl alcohol and 0.005 mg/ml phenol red. They were washed twice again in maturation medium previously equilibrated for a minimum of 3 h at 38.5 °C under 5% CO_2 in 95% humidified air.

The culture media used for oocyte maturation was Waymouth medium supplemented with 10 UI/ml PMSG, 10 UI/ml hCG, 1 μg/ml estradiol-17b, 10% (v/v) foetal calf serum and 10% porcine follicular fluid (v/v), as previously described by Coy et al. [16]. The maturation medium was added to the Petri-dish in 3×100 μl droplets covered with paraffin oil and 20 oocytes introduced in each droplet and incubated at 38 °C under 5% CO_2 in air.

The in vitro fertilisation medium was TCM199 supplemented with 12% heat inactivated foetal calf serum, 0.9 mM sodium pyruvate, 3.05 mM D-glucose, 8.75 mM calcium lactate, 0.68 mM penicillin G, 3.6 mM caffeine and 0.068 mM streptomicyn sulphate at pH 7.4, as previously described [16].

After thawing, the sperm samples were centrifuged at $50 \times g$ for 3 min and the supernatants centrifuged at $1200 \times g$ for 3 min. The resulting pellets were diluted in supplemented TCM199 without calcium lactate and caffeine. Aliquots of 100 μl of semen were placed on Petri-dishes containing 2 ml of fertilization medium (final concentration of 1×10^6 spermatozoa/ml) and 20 in vitro matured oocytes previously washed twice in equilibrated fertilization medium. After 18 h, the cultured oocytes were fixed in 3:1 (v/v) ethanol:acetic acid for 24 h, stained with 1% lacmoid and examined under a phase contrast microscope to assess penetration rate (PEN), mean number of sperm per penetrated oocyte (S/O), monospermy rate (MON) and rate of male pronuclear formation (MPF).

2.5. Experimental design

2.5.1. Experiment 1: evaluation of GSH content in ejaculated boar spermatozoa

Semen parameters and GSH content in boar ejaculated spermatozoa were determined in 44 ejaculates from 27 boars.

2.5.2. Experiment 2: determination of GSH content in fresh, refrigerated or cryopreserved boar spermatozoa

Semen parameters and GSH content were determined in boar spermatozoa from 25 ejaculates from 10 boars, after refrigeration for 24 h at 15 °C and after cryopreservation.

2.5.3. Experiment 3: effect of GSH supplementation of the freezing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

Ejaculates from four boars (four per boar) were processed with or without addition of 5 mM GSH to the freezing extender. Standard semen parameters and in vitro sperm fertilizing ability were evaluated.

2.5.4. Experiment 4: effect of GSH supplementation of the thawing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

To examine the effect of GSH supplementation during the thawing process, frozen straws from two different boars (four ejaculates per boar) were diluted immediately after thawing in BTS (control), BTS + 1 mM GSH and BTS + 5 mM of GSH. After 30 min of equilibration, standard semen parameters were evaluated and the sperm fertilizing ability tested in the IVF system.

2.6. Statistical analysis

Data are expressed as the mean ± S.E.M. and analysed by two-way ANOVA, considering the specific sperm treatment and boar samples as the main variables in experiments 2–4. IVF data (experiments 3 and 4) were modelled according to the binomial model of parameters and were analysed by two-way ANOVA. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered statistically significant at $P < 0.05$.

Linear regression analysis was used to further investigate relationships between semen parameters (Pearson correlation) in experiment 2.

3. Results

3.1. Experiment 1: evaluation of GSH content in ejaculated boar spermatozoa

GSH content in ejaculated boar spermatozoa was determined in 44 ejaculates from 27 boars. The mean value was 3.84 ± 0.21 nM GSH/10^8 cells. There were significant differences in GSH content between different boars ($P < 0.001$) ranging from 1.05 to 6.16 nM GSH/10^8 cells.

3.2. Experiment 2: determination of GSH content in fresh, refrigerated or cryopreserved boar spermatozoa

Standard semen parameters and GSH content were determined in freshly ejaculated spermatozoa, refrigerated sperm suspensions for 24 h at 15 °C, and frozen–thawed spermatozoa. GSH content was significantly lower in cryopreserved sperm as compared to fresh ejaculated spermatozoa ($P < 0.0001$, Table 1), with a 32% decrease after cryopreservation. Significant differences were also observed between boars, and preservation protocols (interaction $P = 0.0102$).

Table 1
Glutathione content (nM GSH/10^8 cells) and sperm quality parameters in fresh, stored and cryopreserved spermatozoa (ejaculates = 25) (mean ± S.E.M.)

	GSH nM/10^8 cells	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Treatment						
Fresh	4.99 ± 0.23[a]	74.61 ± 1.27[a]	3.21 ± 0.07[a]	84.42 ± 1.39[a]	92.53 ± 0.59[a]	90.42 ± 1.31[a]
Stored for 24 h at 15 °C	4.72 ± 0.18[a]	69.03 ± 1.59[a]	3.08 ± 0.04[a]	83.15 ± 1.99[a]	91.23 ± 1.08[a]	84.08 ± 1.68[a]
Frozen–thawed	3.36 ± 0.34[b]	50.00 ± 2.31[b]	3.74 ± 0.07[b]	56.68 ± 2.98[b]	33.24 ± 2.13[b]	43.36 ± 3.47[b]
Source of variability						
Boar	0.0319	0.4351	0.0157	0.1041	0.0399	0.0095
Treatment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Boar × treatment	0.0102	0.2339	0.1552	0.7794	0.1059	0.3911

Numbers within columns with different superscripts (a and b) differ ($P < 0.05$). FPM: forward progressive motility (0–5). EN: eosine–nigrosine stain. NAR: normal apical ridge. DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate. ANOVA, *P*-values.

All semen parameters were affected by preservation method with significant decrease in frozen–thawed semen, except forward motility, which showed an increased value in frozen semen. Although there were differences in semen parameters between different boars (FPM, NAR and DCF), the interaction value was not significant (Table 1).

No significant correlation was observed between semen parameters and GSH content after different preservation treatments. Pearson correlation coefficient analysis indicated an inverse correlation between GSH and NAR in ejaculated spermatozoa ($r = -0.4263$, $P = 0.0002$) and a direct correlation between GSH and forward progressive motility in cryopreserved sperm ($r = 0.2960$, $P = 0.0411$, Table 2).

3.3. Experiment 3: effect of GSH supplementation to the freezing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

Addition of 5 mM GSH to the freezing extender had no a significant effect on semen parameters (data not shown) or sperm fertilizing ability (Table 3). However, significant

Table 2
Relationships between seminal parameters and GSH content in fresh, stored and cryopreserved spermatozoa (*r*: Pearson correlation coefficient; *P*: significance)

	Fresh		Stored for 24 h at 15 °C		Frozen–thawed	
	r	*P*	*r*	*P*	*r*	*P*
Motility	0.2006	0.0911	0.0949	0.5121	−0.1135	0.4425
FPM	0.0923	0.4404	−0.1284	0.3741	0.2960	0.0411
EN	0.1457	0.2221	0.2124	0.1386	−0.1439	0.3293
NAR	−0.4263	0.0002	0.1807	0.2091	−0.1197	0.4179
DCF	0.0031	0.9792	0.2842	0.0502	0.1006	0.4962
Morphology	0.0784	0.5124				

FPM: forward progressive motility (0–5). EN: eosine–nigrosine stain. NAR: normal apical ridge. DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate.

Table 3
IVF results for mature oocytes fertilized with frozen–thawed boar sperm in two different treatments, with (GSH) or without (control) addition of GSH to the freezing extender: rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (%MON) and rate of male pronuclear formation (%MPN)

Boar	Treatment	Number of oocytes	%PEN	S/O[a]	%MON[a]	%MPN[a]
67	Control	91	87.91 ± 3.44[a]	2.41 ± 0.17[a]	36.25 ± 5.41[a]	72.50 ± 5.02
67	GSH	97	69.07 ± 4.72[a,b]	1.83 ± 0.12[a,b]	43.28 ± 6.10[a,b]	73.13 ± 5.46
A	Control	87	56.32 ± 5.35[b]	1.81 ± 0.15[a,b]	53.06 ± 7.20[a,b]	83.67 ± 5.33
A	GSH	96	66.67 ± 4.84[b]	2.25 ± 0.21[a,b]	43.75 ± 6.25[a,b]	79.69 ± 5.07
BB	Control	117	75.94 ± 3.72[a]	2.08 ± 0.17[a,b]	52.48 ± 4.99[a,b]	71.29 ± 4.52
BB	GSH	115	72.06 ± 3.86[a]	2.25 ± 0.17[a]	47.96 ± 5.07[a,b]	73.40 ± 4.58
PI774	Control	124	33.06 ± 4.24[c]	1.75 ± 0.16[a,b]	56.10 ± 7.85[a,b]	65.85 ± 7.50
PI774	GSH	123	31.71 ± 4.21[c]	1.36 ± 0.09[b]	69.23 ± 7.49[b]	74.36 ± 7.08
Source of variability						
Treatment			0.264	0.479	0.727	0.743
Boar			<0.001	0.012	0.011	0.190
Interaction			0.020	0.017	0.320	0.815

Numbers within columns with different superscripts (a–c) differ ($P < 0.05$).
[a] Based on penetrated oocytes.

differences between boars were observed for each IVF parameter ($P < 0.015$, Table 3), except for %MPN. Concerning penetration rate and sperm number per penetrated oocyte, ANOVA indicated that there were significant differences between boars, as also shown by the significant value obtained for the interaction of preservation treatment and individual boars ($P = 0.020$ and $P = 0.017$, respectively).

Table 4
IVF results for mature oocytes fertilized with frozen–thawed boar sperm in three different treatments, with (1 and 5 mM GSH) or without (control) addition of GSH to the thawing extender: rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (%MON) and rate of male pronuclear formation (%MPN)

Boar	Treatment	Number of oocytes	%PEN	S/O[a]	%MON[a]	%MPN[a]
PI774	Control	135	18.52 ± 3.36[a]	1.20 ± 0.08[a]	80.00 ± 8.16[a]	48.00 ± 10.20[a]
PI774	1 mM GSH	154	25.97 ± 3.54[a,b]	1.32 ± 0.10[a]	77.50 ± 6.69[a]	60.00 ± 7.84[a,b]
PI774	5 mM GSH	148	31.76 ± 3.84[b]	1.36 ± 0.10[a]	74.47 ± 6.43[a]	72.34 ± 6.60[a,c]
BB	Control	96	83.33 ± 3.82[c]	3.16 ± 0.29[b]	31.25 ± 5.21[b]	81.25 ± 4.39[b,c]
BB	1 mM GSH	107	85.05 ± 3.46[c]	3.44 ± 0.17[b]	14.29 ± 3.69[b]	84.62 ± 3.80[c]
BB	5 mM GSH	118	97.46 ± 1.46[c]	4.01 ± 0.28[b]	23.48 ± 3.97[b]	84.35 ± 3.40[c]
Source of variability						
Treatment			<0.001	0.254	0.292	0.062
Boar			<0.001	<0.001	<0.001	<0.001
Interaction			0.578	0.480	0.409	0.167

Numbers within columns with different superscripts (a–c) differ ($P < 0.05$). ANOVA, P-values.
[a] Based on penetrated oocytes.

3.4. Experiment 4: effect of GSH supplementation to the thawing extender on the viability and in vitro fertilizing ability of cryopreserved boar spermatozoa

No significant differences in semen parameters were found between control and GSH-supplemented thawing extender (data not shown). In contrast, GSH addition to the thawing extender resulted in an significant increase in sperm in %PEN ($P < 0.001$) and also an increase in %MPN, although it did not reach statistical significance ($P = 0.062$) (Table 4). For all the studied IVF variables a significant boar effect was detected ($P < 0.001$).

4. Discussion

The main findings emerging from this study are the decrease in GSH content observed in boar sperm after cryopreservation and a tendency to increase in boar sperm fertilizing ability after addition of GSH to the thawing extender.

Glutathione is the major non-protein thiol compound in mammalian cells. GSH participates in a number of cell functions including amino acid transport, DNA and protein synthesis, reduction of disulfide bonds and protection against oxidative stress. The sulphydryl groups of GSH have been shown to confer protection against cell damage by oxidants, electrophiles and free radicals (reviewed by Irvine [2]).

The results obtained for GSH content in ejaculated boar semen (3.84 ± 0.21 nM/10^8 cells) are in good agreement with previous reports (3 nM GSH/10^8 cells [9]) and with the results obtained in other mammalian species including human sperm (6.7 ± 0.4 nM GSH/10^8 cells [7]; 6.2 ± 0.6 nM GSH/10^8 cells [17]; 3.49 ± 0.87 nM GSH/10^8 cells [8]), and bull sperm (2.73 ± 0.42 nM GSH/10^8 cells [18]). As previously reported, GSH values in mouse sperm are significantly higher than in other mammalian species (90 nM GSH/10^8 cells [7]). It is noteworthy that comparisons between different studies are often difficult to interpret due to differences in the method of analysis [19] and sperm processing protocols [20].

The results of experiment 1 indicated significant differences in GSH content between different boars. These differences could be related to differences in the fertility potential of these boars [21]. A relationship between GSH content in sperm and seminal plasma and infertility has been previously found in human sperm [8,22] and also between GSH content in sperm and their ability to penetrate bovine cervical mucus [8].

The results also show that cryopreservation of boar sperm is associated with a significant decrease in GSH content (Table 1), as previously reported in bovine [20] and human sperm (Molla and Gadea, data not published). This would be expected to result in changes in the sulphydryl content of proteins from the sperm membranes and nucleus [23]. Similarly, cryopreservation of boar sperm is associated with a reduction in the sperm motility and viability. Interestingly, forward progressive motility was increased in cryopreserved spermatozoa. This could be related to changes brought about by the media used in boar sperm cryopreservation named capacitation like alterations [1,24]. However, these preliminary results must be confirmed in future studies where sperm motility will be monitored by computer-assisted semen analysis (CASA). In

contrast, preservation of boar spermatozoa at 15 °C did not result in a decrease in GSH content or sperm viability. This suggests that the effects of cryopreservation on GSH content and sperm motility and viability observed are specific to the process of freezing and thawing at low temperatures.

Once we detected a decrease in the GSH content in the boar frozen–thawed spermatozoa, we assessed the effect of GSH supplementation of freezing and thawing extenders on standard semen parameters and sperm fertilizing ability in IVF (experiments 3 and 4). One question that remains to be answered is why addition of GSH to the thawing extender and not to the freezing extender increases boar sperm fertilizing ability in vitro. A plausible explanation for this question would be that cryodamage of antioxidant enzymes during the freezing process results in an overall decrease of enzymatic antioxidant defenses in boar sperm. This damage could selectively affect superoxide dismutase (SOD), glutathione reductase (GRD) and glutathione peroxidase (GPX) to a lesser extent. During thawing, there is an increase in the production of ROS [1], with an increase in the superoxide anion production due to a decrease in SOD activity and a decrease in GSH content due to a decrease in GRD activity and to an increase in GSH oxidation by hydrogen peroxide. A decrease in intracellular GSH and an increase in ROS production during thawing would lead to an increase in lipid peroxidation. GSH provided exogenously after thawing (experiment 4) would increase the intracellular levels of GSH which will be used by GPX to prevent hydrogen and lipid peroxide-induced damage. Addition of GSH to the freezing extender, on the other hand, would result in a high rate of ROS-induced GSH oxidation during rewarming and thawing. This is supported by our results that show that there is a decrease in GSH content during cryopreservation. In contrast, addition of GSH after thawing would allow any residual antioxidant enzymatic activity still remaining in the sperm cell to recover, to decrease steady-state ROS levels, and, therefore, to decrease GSH oxidation. This hypothesis is supported by previous studies which show that there is a decrease in SOD activity during cryopreservation and an increase in lipid peroxidation of human and bovine spermatozoa after thawing [20,25]. Sadly, this information is not well known yet in the boar spermatozoa [26,27]. However, if GSH would be acting in concert with GPX to decrease the steady-state level of hydrogen peroxide and membrane lipid peroxides in boar sperm during thawing, an increase in sperm motility and viability should also be expected.

It has also been reported that damage to the plasma membrane overlaying the flagellum or head during cryopreservation of human sperm can occur independently [28] and Holt and North [29] reported that sperm head plasma membranes were preferentially damaged by freezing and thawing. Although acrosomes became swollen after freezing and thawing, the incidence of outer acrosomal membrane vesiculation remained at control (unfrozen) levels with all treatments. However, the results indicate that these semen parameters are not affected by GSH addition. The only effect that is observed is an increase in boar sperm fertilizing ability. Therefore, cryopreservation-induced oxidative stress in boar sperm affects a sperm property that is related to both sperm binding and penetration of the zona pellucida, sperm–oocyte membrane fusion, oocyte activation, or pronuclear formation. In this sense, the membrane fusion events involved in binding with the oolema and in the acrosome reaction appear to be more vulnerable to ROS-induced damage than is overall motility in human spermatozoa [30].

Also, we have previously demonstrated that the IVF is the most precise tool for evaluating boar semen capacities, in both refrigerated and frozen–thawed semen [11,31]. So the use of IVF systems could help us to detect differences that classical assays are not able to do [32].

Other reports indicate that cryopreservation also results in ROS-induced DNA fragmentation [33]. However, this would only partially explain the results since a decrease in DNA fragmentation by GSH supplementation during thawing would explain the observed increase in pronuclear formation rate, but it would not explain the increase in penetration rate. Nevertheless, it has been shown the relationship between the ability to form a male pronucleus in pig oocytes with intracellular concentration of GSH [34,35]. Also the addition of GSH to the insemination medium in an IVF system improves the developmental competence of in vitro matured pig oocytes in a dose-dependent manner [36].

In summary, there was a loss in GSH content after cryopreservation of boar semen. Addition of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability; while addition of GSH to the thawing extender resulted in a significant increase in sperm fertilizing ability. Nevertheless, future studies must conclude if this is the case for all boars. Furthermore, since addition of GSH to the thawing extender did not result in an improvement in standard semen parameters, this suggests that during the thawing process, GSH prevents damage of a sperm property that is critical in the fertilization process but that is not measured in the routine semen analysis.

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The predictive value of porcine seminal parameters on fertility outcome under commercial conditions

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The Predictive Value of Porcine Seminal Parameters on Fertility Outcome under Commercial Conditions

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Contents

The aim of this study was to address the question of whether differences in farrowing rate and litter size after the use of different ejaculates could be predicted using the standard semen parameters under commercial conditions. In this study, a total of 1818 sows were used to evaluate the fertility predictive value of different sperm parameters. Logistic regression analysis (univariate and multivariate) was used to correlate the dichotomous farrowing rate data to the sperm parameters. Linear regression was also used to determine the relationship between litter size and semen parameters (Pearson correlation and multiple regression). Receiver-operating curves (ROC) were used to determine the overall performance characteristics of each semen variable in the logistic regression model. Semen analysis, under commercial conditions, allows to identify ejaculates with very low fertility potential but the pre-selection of the samples, the high number of sperm per doses and the high quality of the semen used in artificial insemination (AI) programmes reduces the variability. Therefore, it is unlikely to detect fertility differences associated with seminal parameters.

Introduction

The prediction of sperm fertilizing ability has a great economic importance to breeding herds when artificial insemination is used, as it leads to the selection of those boars with a good reproductive performance. However, at the moment, only a few trials that correlate sperm factors and fertility have been reported (reviewed by Gadea 2003). In most of them, a reduced or poor significant relationship has been observed (reviewed by Woelders 1991; Flowers 2002), in part, because of the many factors involved in fertility in sows (Foote 2003).

Classical methods of semen evaluation generally measure sperm concentration, progressive motility, the percentage of viable cells and acrosome morphology. These assays are poor in predicting fertility outcome, because only those samples with markedly poor quality can be identified (Gadea et al. 1998). To solve this problem, new procedures of seminal analysis *in vitro* (Braundmeier and Miller 2001), or multiple analysis of the same sample have been evaluated (Woelders 1991) and new assays to study the oocyte–sperm interaction as the *in vitro* fertilisation (IVF) system have been developed (Gadea et al. 1998; Xu et al. 1998; Selles et al. 2003). However, these analyses are expensive and time-consuming, and cannot be applied under field commercial conditions.

Under field commercial conditions, the use of simple, non-expensive and accurate assays is required. Such goal has been pursued over decades but no definitive solution has ever been reported.

The aim of this study was to address the question of whether farrowing rate and litter size after the use of different ejaculates could be predicted with classical semen-quality parameters under commercial conditions. Different analytical strategies have been developed to determine the possible relationship between sperm parameters and farrowing rates and litter size. The first is the exploration of diffences in seminal parameters between ejaculates classified in two categories according to farrowing rate, with a target value for fertility under commercial conditions (85% farrowing rate). Secondly, logistic and linear regression analyses were applied to detect any significant relationship between semen quality and farrowing rate and litter size.

Materials and Methods

Spermatozoa from 273 ejaculates from 57 boars (four Dalland and 53 Pietrain) were evaluated for semen-quality parameters *in vitro*. Sperm-rich fractions were collected by an experienced operator using the gloved-hand technique. At the time of collection, the ejaculate was filtered through a gauze to remove gel and immediately placed at 37°C in a temperature-controlled water bath. Each ejaculate was later diluted to a final concentration of 3×10^7 spermatozoa/ml in a commercial extender for refrigerated semen (MR-A©, Kubus, Madrid, Spain) and stored at 17°C until assay.

Routine laboratory evaluation of semen

The volume of the sperm-rich fraction of the ejaculate was determined in a graduated cylinder and sperm concentration measured with a haemacytometer. To evaluate the motility and the forward progressive motility (FPM), two subsamples were placed on warm glass slides (39°C) and examined under a light microscope (×400 magnification). The percentage of motile sperm cells was estimated subjectively to the nearest 5% and the FPM using an arbitrary scale of 0–5.

Wet mounts of semen fixed in buffered 2% glutaraldehyde solution were examined under a phase-contrast microscope (×1000 magnification) to analyse the morphology and acrosomes (Pursel et al. 1972). The proportion of spermatozoa with a normal apical ridge (NAR) was determined on two slides per sample and a total of 200 spermatozoa per sample. Two hundred spermatozoa were categorized according to sperm morphology into those with normal morphology, cells with attached proximal cytoplasmic droplets (PCD), distal

cytoplasmic droplets (DCD), folded tail, coiled tail and others (abnormal heads) etc.

Eosin–nigrosin (EN) viability staining of sperm was applied. A semen sample was diluted 1 : 1 with staining solution (5% yellow eosin, 10% nigrosin in a citrate solution pH 7.4) and smeared. Air-fixed stained spermatozoa were observed and 200 spermatozoa were evaluated per slide (Bamba 1988) and the percentage of non-stained spermatozoa was registered.

Sperm membrane integrity was assessed by incubation with carboxyfluorescein diacetate (DCF) (Harrison and Vickers 1990) on at least 200 cells/sample using a microscope equipped with epi-fluorescence.

Fertility trial

The fertility study was conducted on a commercial farm, using multiparous (two to six pregnancies) crossbred sows (Landrace × Large White). Oestrus was checked daily in the presence of a mature teaser boar. Occurrence of oestrus was defined by the standing reflex in front of a boar (back pressure test) and reddening and swelling of the vulva. The sows were inseminated with diluted semen, containing 3×10^9 sperm in 100 ml MR-A extender, using disposable artificial insemination (AI) catheters. Insemination of all sows showing oestrus signs took place on immediately after the diagnosis of oestrus and was repeated 24 h later under the same conditions with semen preserved at 17°C.

Fertility was measured for every ejaculate as the percentage of sows farrowing to AI. For each sow that farrowed, the number of dead and live piglets was counted and the sum was defined as the total number of piglets born (TPB).

Experimental design

In this study, a total of 1818 sows were used to evaluate the capacity for fertility prediction of the different sperm assays. Ejaculate was consider to be the experimental unit. First, the ejaculates were grouped into two categories according to fertility rate (low, farrowing rate ≤ 85%; high, farrowing rate > 85%). Seminal parameters were evaluated and differences between fertility groups were investigated. Secondly, logistic and linear regression analyses were applied to detect any significant relationship between semen quality and farrowing rate and litter size.

Statistical analysis

The results are expressed as mean ± SEM and were analysed by one-way ANOVA, using the multivariate general linear models of Systat. When ANOVA revealed a significant effect, values were compared by Tukey's test.

Logistic regression (univariate and multivariate) was used to relate the dichotomous fertility rate data to the sperm parameters. Linear regression was used to further investigate relationships between litter size and measured semen parameters (Pearson's correlation and multiple regression). Receiver-operating curves (ROC) were used to determine the overall discriminant power of each semen variable in the logistic regression model. A ROC was also used to calculate the elective breaking point (cut-off value) for each semen variable in relation to the fertility trial. The breaking point is in relation with the point that maximized specificity and sensitivity.

Results

Relationship between sperm characteristics and farrowing rate

Semen parameters values and the fertility outcome were not significantly different in the two genetic lines of the boars used in this study (data not shown). Therefore, all ejaculates from these boars can be considered as a homogeneous group. Statistical analysis of sperm parameters values and fertility outcome obtained in the field trial are shown in Table 1 for the two groups of ejaculates according to farrowing rate (more or less than

Table 1. Semen measurements and reproduction results determined for 273 ejaculates from 57 boars allocated into two groups according to farrowing rate (mean ± SEM)

	Low fertility (farrowing rate ≤85%)	High fertility (farrowing rate >85%)	Significance (p-value)
Number of ejaculates	138	135	
Volume (ml)	103.20 ± 2.03	107.13 ± 2.09	0.1792
Concentration (10^3 sperm/mm^3)	77.91 ± 2.48	82.20 ± 2.63	0.2358
Total cells (10^9)	78.20 ± 2.71	86.10 ± 2.82	0.0443
Motility	74.09 ± 0.63	75.31 ± 0.63	0.1760
FPM	3.24 ± 0.03	3.20 ± 0.03	0.4669
EN (%)	84.72 ± 0.70	84.82 ± 0.68	0.9147
NAR (%)	93.46 ± 0.46	93.56 ± 0.43	0.8755
DCF (%)	84.23 ± 0.71	84.30 ± 0.87	0.9510
Morphoanomalies (%)	11.94 ± 0.78	12.54 ± 0.97	0.6307
PCD (%)	2.73 ± 0.27	3.41 ± 0.37	0.1376
DCD (%)	4.22 ± 0.38	4.00 ± 0.33	0.6583
Folded tail (%)	4.05 ± 0.49	3.73 ± 0.69	0.7002
Swollen tail (%)	0.30 ± 0.07	0.75 ± 0.30	0.1325
Other anomalies (%)	0.63 ± 0.12	0.64 ± 0.12	0.9691
Number of sows	915	903	
Farrowing rate (%)	645/915 (70.49%)	868/903 (96.12%)	<0.0001
Live piglets born	9.64 ± 0.11	9.84 ± 0.10	0.1689
Total piglets born	10.25 ± 0.12	10.45 ± 0.11	0.2191

FPM, forward progressive motility (0–5); EN, eosin–nigrosin stain; NAR, normal apical ridge; DCF, sperm membrane integrity assessed with carboxyfluorescein diacetate; PCD, proximal cytoplasmic droplets; DCD, distal cytoplasmic droplets.

Table 2. Univariate logistic and linear regression of seminal measurements with farrowing rate

	Logistic regression		Linear regression	
Parameter	Odd ratio	Significance (p-value)	Pearson correlation coefficient (r)	Significance (p-value)
Volume	1.0017	0.5076	0.0157	0.5073
Concentration	1.0016	0.4803	0.0166	0.4828
Total cells	1.0029	0.1499	0.0338	0.1530
Motility	1.0195	0.0237	0.0539	0.0214
FPM	0.8764	0.4337	−0.0184	0.4322
EN	1.0013	0.8674	0.0039	0.8673
NAR	1.0079	0.4891	0.0164	0.4845
DCF	0.9993	0.9273	−0.0021	0.9274
Morphoanomalies	1.0009	0.8892	0.0033	0.8896
PCD	1.0346	0.0621	0.0421	0.0723
DCD	0.9986	0.9252	−0.0022	0.9252
Folded tail	0.9885	0.2300	−0.0291	0.2138
Swollen tail	1.0768	0.0849	0.0324	0.1666
Other anomalies	0.9671	0.4862	−0.0166	0.4784

FPM, forward progressive motility (0–5); EN, eosin–nigrosin stain; NAR, normal apical ridge; DCF, sperm membrane integrity assessed with carboxyfluorescein diacetate; PCD, proximal cytoplasmic droplets; DCD, distal cytoplasmic droplets.

Table 3. Multiple logistic regression of semen measurements with farrowing rate (forward stepwise model)

Parameter	Estimate	SE	Odds ratio	t-ratio	p-value
Constant	−0.0873	0.6511	–	−0.1341	0.8934
Motility	0.0209	0.0086	1.0211	2.4172	0.0156
PCD	0.0385	0.0194	1.0392	1.9843	0.0472
Swollen tail	0.0679	0.0516	1.0702	1.3147	0.1886

$p = 0.0069$; McFadden's $\rho^2 = 0.0075$.
PCD, proximal cytoplasmic droplets.

Table 4. Multiple linear regression of semen measurements with farrowing rate (forward stepwise model)

Parameter	Coefficient	SE	t-ratio	p-value (two-tail)
Constant	0.5867	0.0931	6.3032	0.0000
Motility	0.0031	0.0012	2.5035	0.0124
PCD	0.0046	0.0023	1.9622	0.0499
Swollen tail	0.0056	0.0034	1.6385	0.1015

$p = 0.0090$; multiple $R^2 = 0.0063$.
PCD, proximal cytoplasmic droplets.

Table 5. Cut-off values (breaking points) of semen parameters significantly related to farrowing rate calculated from receiver-operating curves (ROC)

Variable	Cut-off value	Sensitivity	Specificity	Area ROC
Volume	100	52.9	49.2	0.508 ± 0.018
Concentration	77	51.5	51.8	0.509 ± 0.018
Total cells	84	50.7	50.5	0.518 ± 0.018
Motility	70	71.9	33.8	0.531 ± 0.018
FPM	3.5	87.9	13.9	0.510 ± 0.018
EN	86	53.0	45.4	0.499 ± 0.018
NAR	94	51.9	51.0	0.509 ± 0.018
DCF	85	51.8	49.7	0.504 ± 0.018
Morphoanomalies	10	53.8	48.6	0.501 ± 0.018
PCD	1	55.4	51.0	0.534 ± 0.018
DCD	3	54.3	44.0	0.501 ± 0.018
Folded tail	2	67.0	41.7	0.541 ± 0.018
Swollen tail	1	23.2	79.1	0.512 ± 0.018
Other anomalies	1	71.6	28.1	0.500 ± 0.018

FPM, forward progressive motility (0–5); EN, eosin–nigrosin stain; NAR, normal apical ridge; DCF, sperm membrane integrity assessed with carboxyfluorescein diacetate; PCD, proximal cytoplasmic droplets; DCD, distal cytoplasmic droplets.

85%). Ejaculates were distributed equally in both groups (n = 138 vs 135). The mean values of farrowing rate obtained in the two groups of sows (n = 915 and 903) were significantly different (70.49% vs 96.12%, $p < 0.0001$). No differences in semen parameters were found between both groups. Only total cell count in the ejaculate was significantly higher in the high fertility group ($p = 0.0443$).

Linear and logistic regression analyses were used to correlate the farrowing rate data to the semen parameters. In both analyses, a poor correlation with fertility outcome was obtained. Only motility (logistic and linear regression) was significantly correlated to farrowing rate (Table 2). When all conventional semen parameters were analysed through a stepwise, multiple logistic or linear regression analysis, only three parameters (motility, PCD and swollen tail) are necessary to reach statistical significant model (Tables 3 and 4). However, in both models a low percentage of total variance of farrowing rate could be predicted. It was surprising to find that there was a positive correlation between PCD and swollen tail with fertility (Table 3).

The use of ROC led us to calculate the cut-off values for the semen parameters (Table 5) in these experimental conditions. Cut-off values obtained in this study were highly restricted, for example EN (86), NAR (94), normal morphology (90) and DCF (85). However, they were less restricted for motility (70). The sensitivity and specificity of these parameters to identify better ejaculates in terms of fertility outcome are shown in Table 5. Lowering the threshold reduces the sensitivity of the measures (the likelihood of correctly identifying infertile–subfertile ejaculates) but increases their specificity (the likelihood of correctly identifying fertile ejaculates).

On the basis of the area under ROC (area ROC), each seminal parameter did not provide information that was helpful in discriminating fertility outcome, because it was not significantly > 0.500. However, motility, PCD and folded tail are near to this value, although there were not significant differences between area ROC corresponding to the different seminal parameters.

Relationship between sperm characteristics and litter size

Concerning litter size, the total number of piglets born showed a significant positive correlation with motility ($r = 0.1223$, $p = 0.0435$; Table 6), while folded tail showed a negative correlation ($p = 0.0681$) but did not reach statistical significance. However, no correlation was found between the average live piglets born and semen parameters (Table 6). Only a weak positive correlation was found for motility and a negative correlation for folded tail, but neither reach statistical significance.

Table 6. Linear regression of seminal measurements with average litter size[a]

	Live piglets born		Total piglets born	
	Correlation coefficient (r)	p-value	Correlation coefficient (r)	p-value
Volume	−0.0398	0.5149	−0.0650	0.2872
Concentration	0.0945	0.1213	0.1027	0.0921
Total cells	0.0798	0.1909	0.0716	0.2406
Motility	0.1143	0.0592	0.1223	0.0435
FPM	−0.0557	0.3594	−0.0541	0.3728
EN	0.0578	0.3413	0.0705	0.2454
NAR	−0.0044	0.9419	−0.0062	0.9193
DCF	0.0637	0.2959	0.0513	0.4001
Morphoanomalies	−0.0610	0.3150	−0.0673	0.2679
PCD	0.0531	0.3819	0.0522	0.3905
DCD	−0.0388	0.5232	−0.0565	0.3528
Folded tail	−0.1179	0.0516	−0.1106	0.0681
Swollen tail	0.0574	0.3444	0.0502	0.4085
Other anomalies	0.0065	0.9149	−0.0075	0.9021

[a]Litter sizes used included failed conceptions as zero values.
FPM, forward progressive motility (0–5); EN, eosin–nigrosin stain; NAR, normal apical ridge; DCF, sperm membrane integrity assessed with carboxyfluorescein diacetate; PCD, proximal cytoplasmic droplets; DCD; distal cytoplasmic droplets.

Table 7. Multiple linear regression (stepwise forward) of seminal measurements with average number of piglets live[a]

Variable	Coefficient	SE	t-ratio	p-value
Constant	7.9670	1.5577	5.1146	0.0000
Motility	0.0302	0.0174	1.7378	0.0834
FPM	−0.6110	0.3517	−1.7376	0.0834
Folded tail	−0.0317	0.0184	−1.7275	0.0852

Multiple R^2: 0.0318, p = 0.0335.
[a]Litter sizes used included failed conceptions as zero values.

Table 8. Multiple linear regression (stepwise forward) of seminal measurements with average total number of piglets[a]

Variable	Coefficient	SE	t-ratio	p-value
Constant	7.9637	1.6906	4.7107	0.0000
Motility	0.0367	0.0186	1.9707	0.0498
FPM	−0.6109	0.3755	−1.6268	0.1050
PCD	0.0645	0.0392	1.6452	0.1011
Swollen tail	0.0921	0.0548	1.6814	0.0938
Morphoanomalies	−0.0286	0.0159	−1.8007	0.0729

Multiple R^2: 0.0417, p = 0.0435.
[a]Litter sizes used included failed conceptions as zero values.

Significant models were found with multiple linear regression (stepwise forward). For the live piglets born, the model was constructed with three variables including motility, FPM and folded tail, confirming the tendency previously detected (Table 7). For total number of piglets born, a significant model was constructed with five parameters (motility, FPM, PCD, swollen tail and total morpho-anomalies (Table 8). In both models, a low percentage of total variance of the litter size could be predicted.

Discussion

Fertilizing ability is commonly measured as the percentage of sows or gilts conceiving or farrowing after AI (Foote 2003). In general, both measures are indicative of the efficiency with which eggs are fertilized and capable of sustaining embryonic development (Watson 1996). The best way to assess boar fertility is to obtain viable pregnancies and normal offspring following *in vivo* insemination. However, field trials of semen fertility are imprecise (Clark et al. 1989; Foote 2003). Consequently, one of the main goals in spermatology should be to investigate new markers of sperm function that would allow the better prediction of fertility outcome (Hammerstedt 1996; Braundmeier and Miller 2001).

Several studies have previously assessed the predictive value of seminal parameters under different experimental conditions, including insemination with low number of sperm per dose (Tardif et al. 1999); no previous selection of ejaculates (Gadea et al. 1998), one or two AI doses, different time application, etc. However, this study was designed to provide sound scientific information that could be directly applied in the field, under commercial conditions (2 artificial insemination (AI), 3×10^9 sperm/dose, pre-selection of ejaculates). So, we grouped the ejaculates according to the farrowing rate, and selected the threshold value of 85% because it is a common target value for a great numbers of commercial farms.

In this study a poor correlation was found between semen characteristics and fertility outcome. Of all parameters studied, sperm motility is the most significant one because it is significantly correlated with farrowing rate and total number of piglets born, and it appears as a significant component in all multivariate models. However, the correlation coefficient is relatively low. Reports of a correlation between motility and fertility are contradictory (Pursel et al. 1984; Galli and Bosisio 1988; Berger and Parker 1989; Holt et al. 1997; Gadea et al. 1998; Tardif et al. 1999; Selles et al. 2003). This could be due to differences in experimental conditions. In fact, sperm motility assessment is subject to great intra- and inter-observer variability. Although computer-assisted sperm analyses (CASA) would appear as the logical choice to solve this question (Holt et al. 1997), CASA is under other technical biases.

Sperm morphology is another parameter that appears to be correlated with fertility outcome (at least as a tendency). Sperm defects have been related to infertility (Bonet and Briz 1991). In a standard semen analysis, morphology provides information about the efficiency of spermatogenesis and it can facilitate selection of boars for AI programmes (Waberski et al. 1990). It also allows workers to establish the intensity of stress produced by a high frequency of semen collection better (Briz et al. 1995). An inverse correlation has been reported between the number of morphological abnormalities and fertility (Martinez et al. 1986; Galli and Bosisio 1988; Waberski et al. 1990; Zeuner 1992; Waberski et al. 1994a; Gadea et al. 1998). However, in this study under commercial conditions, only a weak correlation was found for some sperm defects (PCD, folded tail) but did not reach statistical significance.

The intact nature of the spermatozoa plasma membrane is a prerequisite for suitable sperm metabolism and function. The methods used to assess sperm viability included EN staining and DCF. However, this information is not much related to fertility, perhaps because it informs about the viability of this sperm but not about its functionality, (capacitation process, acrosome reaction, sperm binding, etc.). Several authors have described how high numbers of altered acrosomes are related to fertilizing problems. However, the correlation rates observed between NAR and fertility were not high (Pursel et al. 1984; Galli and Bosisio 1988; Hammitt et al. 1989).

The use of multivariate analysis would help to discriminate fertility potential, because it combines information of different sperm properties. The combination of selected semen tests provides a higher fertility predictive value compared with single tests (Waberski et al. 1999). However, the results obtained in this study could only explain the low variance observed. Likewise, the use of logistic regression analysis (more adequate to dichotomous variables as farrowing rate) is equally efficient in correlating seminal parameters. As expected, there was a significant correlation between the different methods used confirming that 'biological evidence is related with facts, no with any peculiar statistical test' (A. Romar, personal communication).

The determination of cut-off values for seminal parameters has been described in the literature more as the result of an empirical than a scientific approach to the problem. A statistical discriminant analysis of the data could give some valuable information. However, because of the poor correlation between seminal parameters and fertility, the sensibility and specificity of this cut-off is very limited.

Some possible causes of the lack of correlation between fertility and seminal characteristic could be based on: (1) certain sperm characteristics could not be analysed by the standard spermiogram. In this sense, Saacke et al. (1994) define the uncompensable sperm traits as sperm characteristics or deficiencies associated with incompetent fertilizing sperm (those sperm that can initiate but not complete the fertilization process or sustain early embryogenesis). The compensable sperm traits precluded availability of sperm to fertilization (reviewed by Evenson 1999), and they could be measured by classical spermiogram. These uncompensable sperm traits would be in relation with alteration in DNA and nucleoproteins in sperm chromatin (Evenson et al. 1994).

(2) As the contradictory results would have been due to the experimental differences including low number of ejaculates or sows analysed, a high number of sperm per dose could compensate any infertility factor in the boar that could mask the relationship with sperm quality (Woelders 1991; Tardif et al. 1999; Johnson et al. 2000). Another factor could be the pre-selection of the ejaculates. As, under commercial conditions, the ejaculates were selected usually by motility, only those with motility over a reference value were used. This would tend to reduce the variability in semen parameters. In a previous study, in which no pre-selection was performed, higher correlations were found (Gadea et al. 1998), but the pre-selection carried out on commercial conditions could reduce this correlation, as demonstrated for *in vitro* fertility (Gadea and Matas 2000).

(3) A high variance associated with the sow was not related to sperm quality and was affected by many different factors (Clark et al. 1989). The higher is the sperm quality, the more homogeneous the sow effect is. One way of eliminating this variable would be to improve those factors that impinge on the sows such as the synchrony between inseminations and ovulation with the use of ultrasonography (Waberski et al. 1994b; Nissen et al. 1997).

In conclusion, sperm analysis conducted under commercial conditions lead to the detection of ejaculates with very poor quality (associated with poor fertility), but the pre-selection of the samples, the high number of sperm per dose and the high quality of the semen used in the AI programmes reduces the variability. So, there is low probability of detecting fertility differences associated with seminal parameters.

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3

SUMARIO

3. SUMARIO

En el campo de la inseminación artificial porcina quedan aún importantes temas en los que es posible mejorar para incrementar los resultados reproductivos. Entre ellos, se incluye el desarrollo de la crioconservación espermática que permita el uso eficiente de muestras procedentes de animales de alto valor genético conservadas durante largos periodos de tiempo. Por otra parte, se hace evidente que los métodos de evaluación del semen que en la actualidad se utilizan son muy eficientes para descartar animales o eyaculados de mala calidad. Sin embargo, estas técnicas analíticas no son precisas para discriminar animales de alta fertilidad dentro de una población con valores normales. Por tanto la mejora de los métodos de evaluación del semen es un reto que el sector debe abordar.

En esta línea, el objetivo de esta tesis doctoral se ha centrado en el estudio de diferentes factores que afectan a la calidad y capacidad fecundante del semen congelado, como la velocidad de descongelación (trabajo 1) y el sistema antioxidante del semen porcino (trabajo 2).

Por otra parte, se ha estudiado la capacidad predictiva de la fertilidad *in vivo* que tienen las diferentes técnicas de análisis seminal tanto para el semen congelado (trabajo 1) como en condiciones de campo para el semen refrigerado (trabajo 3).

En cuanto a los resultados obtenidos en el primer trabajo se pudo determinar que la velocidad de descongelación más rápida tiene un efecto positivo sobre la funcionalidad espermática de las muestras evaluado mediante un sistema de FIV. Igualmente se concluyó también que el sistema de FIV parece ser la mejor herramienta disponible para evaluar la calidad del semen congelado-descongelado. Este control de calidad seminal debe ser un requisito previo a la salida comercial de las muestras de un banco de semen.

Respecto a los resultados obtenidos en el segundo trabajo se evidenció que el proceso de crioconservación del semen supuso una pérdida siginificativa en el contenido de GSH intracelular. La adición de GSH a los medios de congelación y descongelación no supuso una mejora significativa de los parámetros seminales, pero si una mejora de los resultados de FIV cuando se añadía GSH al medio de descongelación. Estos resultados nos sugieren que durante el proceso de descongelación el GSH previene del daño espermático que es crítico en los procesos de fecundación. Por otra parte, estos resultados ponen de manifiesto que los daños que se producen en la célula espermática durante la congelación no se evidencian con los análisis espermáticos de rutina.

Finalmente, en el tercer trabajo se demostró que el análisis seminal sólo puede identificar eyaculados con bajo potencial fértil. La preselección de muestras, el alto número de espermatozoides por dosis y la alta calidad del semen usado en IA pueden ser factores que reducen la variabilidad en los resultados de fertilidad bajo condiciones comerciales y en consecuencia limitan la capacidad predictiva del análisis seminal. Por lo que se concluye que es poco probable detectar diferencias de fertilidad asociadas a los parámetros seminales aún cuando la realización del espermiograma es necesaria para eliminar eyaculados de muy baja calidad y asegurar niveles de fertilidad aceptables productivamente.

4

RESUMEN GENERAL

4.1. OBJETIVOS

Basándonos en los antecedentes anteriormente descritos, el objetivo general de la presente tesis doctoral fue evaluar la capacidad fecundante de los espermatozoides porcinos refrigerados y congelados tanto *in vivo* como *in vitro*.

Para ello se plantearon los siguientes objetivos específicos:

1. Evaluación de diferentes factores que afectan a la calidad y capacidad fecundante del semen congelado, como la velocidad de descongelación (trabajo 1) y el sistema antioxidante del semen porcino (trabajo 2).

2. Conocer la capacidad predictiva de la fertilidad *in vivo* que tienen las diferentes técnicas de análisis seminal tanto para el semen congelado (trabajo 1) como en condiciones de campo para el semen refrigerado (trabajo 3).

4.2. MATERIAL Y MÉTODOS

Para llevar a cabo estos trabajos se emplearon diferentes reactivos suministrados por *Sigma Chemical (Madrid, España)*, salvo que se especifique lo contrario.

4.2.1. OBTENCIÓN SEMINAL Y PREPARACIÓN DE DOSIS SEMINALES

Se emplearon eyaculados procedentes de un total de 105 verracos de razas Pietrain y Blanco Belga, con edades comprendidas entre 12 y 30 meses. Los machos reproductores pertenecían a los centros de IA “ADS Cordillera” y “Lo Navarro de Murcia S.A.”

Los animales estuvieron sujetos a un programa higiénico-sanitario y a una alimentación habitual en este tipo de animales y explotaciones comerciales. Los verracos estaban sometidos a un programa de obtención de las muestras seminales con una frecuencia de 1-2 veces a la semana.

La recogida de semen se practicó mediante el método manual, donde el operario seleccionó la fracción rica del eyaculado, que fue recogida en un termo a 37ºC después de filtrarlo a través de una gasa estéril para eliminar los gránulos derivados de la glándula de Cowper y las partículas contaminantes.

En el laboratorio del centro de IA se realizó una segunda filtración a través de una gasa para eliminar totalmente los gránulos o partículas que podían haber quedado en suspensión. Una vez filtrado, el semen se depositó en una probeta graduada situada en el interior de un baño termostatizado a 37ºC donde se valoró el volumen y el color como primer análisis macroscópico. También se observó la motilidad y calidad de movimiento bajo microscopio con objetivo 10x. Se calculó la concentración espermática mediante una cámara de recuento celular, determinándose así

el número total de espermatozoides del eyaculado. Se prepararon dosis de semen a razón de 3 x 10^9 células por dosis en un volumen de 80 ml de diluyente comercial (MRA, Kubus, Madrid).

Cuando fue necesario, el semen se diluyó en una proporción 1:1 sobre el diluyente comercial BTS ("Beltsville thawing solution", Pursel y Johnson, 1975; *Minitüb, Tiefenbach, Alemania*). Este diluyente está compuesto por glucosa 0'2 M, Na_2-EDTA.$2H_2O$ 3'36 mM, CO_3HNa 15 mM, citrato-Na_3.$2H_2O$ 20 mM y ClK 5 mM, y había sido mantenido a la misma temperatura que el semen. El semen se transportó hasta la Facultad de Veterinaria de Murcia en menos de una hora desde su recogida y a una temperatura de 22ºC. En nuestro laboratorio se procedió a procesar y evaluar la calidad seminal con las pruebas pertinentes.

4.2.2. EVALUACIÓN DE LA CALIDAD SEMINAL

4.2.2.1 Motilidad y calidad de movimiento

Se depositó un volumen de 10 μl de semen sobre un portaobjetos precalentado a 39ºC y se observó bajo el microscopio con objetivo 10x. Se valoró el porcentaje de espermatozoides mótiles. La calidad de movimiento también fue determinada al mismo tiempo en una escala de 0-5, basándose en el tipo de movimiento que presentaban los espermatozoides a través del campo microscópico (Gadea y Matás, 2000).

4.2.2.2 Concentración espermática

Se empleó para su determinación una cámara de recuento celular de Neubauer. Para ello el semen fue diluido en una proporción 1:100 en solución salina formolada (SSF) al 0'3% que permitió la fijación de los espermatozoides. La concentración se expresó como el número de espermatozoides por ml.

4.2.2.3 Pruebas de integridad y funcionalidad de membrana espermática

Se trata de pruebas de integridad estructural que determinan el porcentaje de espermatozoides con membrana íntegra. Por una parte, se utilizó la tinción vital eosina-nigrosina cuyo fundamento se basa en que los espermatozoides con membrana alterada permiten la entrada del colorante (Eliasson y Treichl, 1971). Para la preparación de la solución eosina-nigrosina se mezclaron 2'5 g de eosina amarilla y 5 g de nigrosina hidrosoluble en 100 ml de solución citrato trisódico al 3'9 % en agua y posteriormente se procedió a su filtración (Bamba, 1988).

Para cada muestra, se mezclaron 50 μl de semen y 50 μl de la solución de tinción en un portaobjetos y se procedió a realizar la extensión, la cual, una vez seca, se observó al microscopio con el objetivo de 40x. Los espermatozoides con membrana dañada aparecen de color rojo-pardo, mientras que los que tienen la membrana íntegra no resultan teñidos.

Por otra parte, se utilizó una técnica fluorescente con el empleo combinado de diacetato de carboxifluoresceína e ioduro de propidio (Harrison y Vickers, 1990). Primeramente se prepararon las soluciones madre de diacetato de carboxifluoresceína (DCF) de 0'46 mg/ml de DCF en dimetilsulfóxido (DMSO) y de 500 μg/ml de ioduro de propidio (IP) en solución salina. Estas soluciones se conservaron congeladas (-20ºC) hasta su uso. En el momento de su utilización se preparó la siguiente suspensión:

20 μl solución DCF + 20 μl solución IP + 10μl SSF + 1 ml suspensión de espermatozoides con una concentración espermática de 3 x 10^7 células/ml.

Esta suspensión fue incubada durante 10 min a 38ºC y se procedió a la valoración microscópica. Se depositaron 5 μl de la suspensión sobre un

portaobjetos y se valoró en un microscopio de fluorescencia (Leica DMR) a 400 aumentos (40x), con filtro B2 y 495 nm de excitación. Los espermatozoides se clasificaron en dos categorías, en aquellos que emitían fluorescencia de color verde (funcionales) y en espermatozoides que emitían fluorescencia roja por la entrada en la célula de IP (membrana dañada). Se contaron al menos 200 espermatozoides por muestra para determinar el porcentaje de espermatozoides funcionales.

4.2.2.4 Morfoanomalías espermáticas

Se evaluó el número de espermatozoides que son morfológicamente anormales. Las anormalidades en la morfología espermática fueron clasificadas de acuerdo con Howard y Pace (1988) y Bonet et al. (2000) en las siguientes categorías:

- Gotas citoplasmáticas: su presencia se ha tomado como una forma de valorar la madurez espermática. Se han clasificado según su localización en:

    - proximales: la gota se encuentra situada entre la cabeza y el cuello del espermatozoide.
    - distales: la gota se encuentra situada en la mitad de la porción intermedia.

- Anormalidades en la cola: este tipo de alteración implica un impedimento para el movimiento de los espermatozoides. Las clasificamos en:

    - colas en látigo: el flagelo se encuentra plegado o flexionado.
    - colas en ovillo: el flagelo está totalmente enrollado sobre sí mismo.

- Alteraciones de la cabeza: entre éstas podemos destacar la presencia de macrocefalia, microcefalia, cabezas sueltas, cabezas piriformes, cabezas dobles, etc.

- Otras alteraciones: implantaciones anormales de la cola, colas dobles, etc.

Para hacer el análisis de morfoanomalías se fijaron los espermatozoides en SSF. Se valoró en un microscopio de contraste de fases con objetivo de 40x (Leica DMR). Se admitieron un máximo de 25% de formas anormales totales.

4.2.2.5 Estado del acrosomas

Para determinar el porcentaje de espermatozoides con acrosoma normal se diluyó el semen en SSF, se depositaron 10 µl de la suspensión sobre el portaobjetos y se evaluó a 1000 aumentos en contraste de fases (100x). El acrosoma normal se observó en el borde apical de la cabeza del espermatozoide perfectamente definido. Cualquier irregularidad del borde apical correspondería a espermatozoides con el acrosoma dañado o alterado (Pursel et al., 1973).

Según las consideraciones anteriores se dividieron los espermatozoides en dos grupos, normales y alterados en cualquier grado (Pursel et al., 1973), contabilizándose el porcentaje de espermatozoides con acrosoma normal (NAR).

4.2.3 PROCESO DE CONGELACIÓN-DESCONGELACIÓN

El semen fue procesado según el procedimiento descrito por Westendorf et al. (1975) con pequeñas modificaciones. Partiendo de la fracción rica del eyaculado en una dilución 1:1 con el diluyente BTS (Pursel

y Johnson, 1975) se conservó a una temperatura de unos 20-22ºC entre 90-120 min. Pasado este tiempo se llevaron las muestras a una temperatura de 15ºC, durante 150 min. Posteriormente, se centrifugaron a 800 *g* durante 10 min para eliminar el sobrenadante. El pellet resultante se resuspendió con el diluyente lactosa-yema (LEY; Westendorf et al., 1975) compuesto por 80 ml de lactosa al 11% y 20 ml de yema de huevo, hasta una concentración de 1'5 x 10^9 espermatozoides/ml. Esta supensión se mantuvo a 5ºC durante 90 min. Pasado este tiempo se rediluyó hasta una concentración de 1 x 10^9 espermatozoides/ml con el medio LEY + 9% glicerol + 1'5% del detergente *orvus es paste* (OEP, Equex-Paste, *Minitüb, Tiefenbach, Alemania*) quedando una concentración final del 3% de glicerol y 0'5% de OEP.

Posteriormente, se procedió al llenado de las pajuelas de 0'5 ml mediante un sistema de aspiración manual y a su sellado con alcohol de polivinilo. Las pajuelas se depositaron a unos 4 cm aproximadamente sobre el nivel del nitrógeno líquido durante 20 min y posteriormente se dispusieron en el tanque de nitrógeno líquido para su almacenamiento.

El proceso de descongelación se llevó a cabo de forma rutinaria mediante la inmersión de la pajuela en un baño a 50ºC durante 10-12 seg, a menos que se refiera lo contrario según el diseño experimental; después se resuspendió el semen en el diluyente de descongelación BTS mantenido a una temperatura de 37ºC.

4.2.4 DETERMINACIÓN DEL CONTENDIO DE GLUTATIÓN (GSH) EN LOS ESPERMATOZOIDES

Las muestras de semen fueron centrifugadas a 1000 *g* durante 5 min a temperatura ambiente y el pellet resultante fue resuspendido en BTS y centrifugado de nuevo. El proceso de lavado se repitió y finalmente el pellet fue resuspendido en BTS ajustando la concentración espermática a 1-5x10^8

espermatozoides/ml. Para liberar el GSH intracelular, las células espermáticas fueron sometidas a 3 ciclos rápidos de congelación en nitrógeno líquido e inmediatamente descongeladas, sumergiendo la muestra en un baño atemperado a 37ºC, con el objetivo final de producir la lisis de las células espermáticas. La suspensión resultante fue centrifugada a 7000 *g* durante 10 min con el fin de eliminar los fragmentos de membrana.

El contenido de glutatión fue determinado usando un sistema óptico. En este sistema el glutatión es oxidado por el compuesto 5,5-dithiobis-(2-ácido nitrobenzoico) (DTNB) y posteriormente reducido por la enzima glutatión reductasa con NADPH como donante de hidrógeno. Durante la oxidación se forma GSH con DTNB, 2-nitro-5 ácido tiobenzoico, el cual puede detectarse espectrofotométricamente por cambios de absorción a 412 nm. El contenido total de glutatión oxidado (GSSG) y glutatión reducido (GSH) se calcula de acuerdo a una curva estándar.

4.2.5. MADURACIÓN Y FECUNDACIÓN *IN VITRO*

En este apartado describiremos las técnicas de maduración y fecundación de ovocitos porcinos empleados en este trabajo, así como la valoración de resultados.

4.2.5.1. Medios de cultivo empleados

Los medios empleados para el transporte y lavado de ovarios, así como para la manipulación de ovocitos fueron:

- Solución salina (SS): 0’9 % de NaCl con 100 mg/l de sulfato de kanamicina, que se conservó a temperatura ambiente hasta su uso.

- Solución de cetrimida (Cetab): bromuro de hexadecil-trimetilamonio al 0'04 %, que también fue conservado a temperatura ambiente hasta su uso.

- Tampón fosfato salino de Dulbecco modificado (PBSDm) suplementado con 1 mg/ml de alcohol polivinílico y 0'005 mg/ml de rojo fenol. El pH se ajustó a 7'4 y el medio fue esterilizado a través de un filtro de membrana con un diámetro de poro de 0'22 µm en una cabina de flujo laminar horizontal (Telstar). El medio fue preparado con agua ultrapura (Milli-Q) y conservado en frascos estériles a 4ºC hasta su utilización.

4.1.5.2 Obtención de los ovocitos

Los ovarios pertenecían a cerdas prepúberes sacrificadas en el matadero "El Pozo" que fueron transportados al laboratorio en un termo con SS a 38ºC en menos de dos hora desde el sacrificio de los animales.

Ya en el laboratorio los ovarios se lavaron en dos pases en Cetab y luego en dos pases en SS, ambos medios atemperados a 39ºC.

Sobre placa calefactora a 39ºC se seccionaron los ovarios con un bisturí seleccionando los folículos entre 3-6 mm de diámetro y se recogió el contenido folicular en PBSDm sobre placas de Petri.

Los ovocitos se visualizaron bajo estereomicroscopio (Nikon) sobre una placa termostatizada a 39ºC en cabina de flujo laminar, donde se procedió a lavarlos en dos pases de PBSDm. Finalmente, los ovocitos que presentaban un citoplasma homogéneo y estaban rodeados por varias capas de células del *cumulus oophorus* se recogieron a través de una pipeta Pasteur adelgazada, con el extremo romo y conectada a una cánula de silicona para posteriormente agruparlos en placas de Petri de 35 mm de diámetro.

4.2.5.3 Maduración *in vitro* (MIV)

El medio de cultivo empleado para la maduración fue el medio Waymouth (MB 752/1) con L-glutamina, 2'2 mg/ml de bicarbonato sódico y 100 μg/ml de sulfato de kanamicina que fue filtrado (0'22 μm) y conservado en frascos estériles a 4ºC hasta su utilización. El pH fue ajustado a 7'4.

En el momento de la MIV el medio fue suplementado con 10% de suero fetal bovino, 10% de fluido folicular porcino, 10 UI/ml de PMSG, 10 UI de HCG y 1 μg/ml de estradiol-17β, tal como lo describen Yoshida et al. (1992).

El sistema de MIV consistió en la utilización de microgotas de 100 μl de medio cubiertas con aceite de parafina. Se dispusieron 20 ovocitos rodeados por sus células del cúmulus por microgota, a 38'5ºC y un 5% de CO_2 en aire saturado de humedad. Después de 20-22 h en el medio de maduración los ovocitos fueron transferidos posteriormente al mismo medio de maduración sin suplemento hormonal donde fueron lavados dos veces y cultivados 20-22 h (Funahashi et al., 1997).

4.2.5.4 Fecundación *in vitro* (FIV)

Las muestras de semen descongeladas, posteriormente diluidas en BTS, fueron centrifugadas a 50 *g* durante 3 min y el sobrenadante recuperado fue de nuevo centrifugado a 1200 *g* otros 3 min. El *pellet* resultante fue resuspendido hasta alcanzar la concentración final deseada con medio 199 suplementado con 12% de suero fetal bovino inactivado, 0'91 mM piruvato sódico, 3'05 mM D-glucosa, 2'92 mM lactato cálcico, 0'168 mM de penicilina y 0'068 mM de estreptomicina, a un pH de 7'8 y a 39ºC.

Para la FIV se utilizó el medio TCM-199 con L-glutamina y sales de Earle con 2'2 mg/ml de bicarbonato sódico y 75 μg/ml de sulfato de kanamicina (199 stock) y suplementado con 12% de suero fetal bovino, 0'9 mM de piruvato sódico, 8'75 mM de lactato cálcico, 3'05 mM de glucosa, 0'068 mM de estreptomicina, 0'68 mM de penicilina y 3'6 mM de cafeína (199 FIV), que fue filtrado y equilibrado a un pH de 7'4. Cada placa de FIV contenía 2 ml de medio 199 FIV, previamente preincubado, con 20 ovocitos procedentes de MIV, al que se le añadieron aproximadamente una hora más tarde 100 μl de la solución espermática para alcanzar una concentración espermática final en la placa de 1 x 10^6 espermatozoides/ml.

Los ovocitos y los espermatozoides se coincubaron durante 16-18 h en condiciones de 38'5ºC de temperatura y a 5% de CO_2 en aire saturado de humedad.

4.2.5.5 Fijación y tinción

Después de 16-18 h de coincubación se lavaron los ovocitos en PBSDm atemperado eliminando mecánicamente con una pipeta automática los espermatozoides adheridos a la superficie del ovocito y las células del cúmulus, quedando así denudados.

Los ovocitos fueron fijados y permanecieron en la solución fijadora a base de ácido acético:etanol (1:3, v/v) a temperatura ambiente durante 24 h (Coy et al., 1999), tras las cuales se tiñeron con colorante Lacmoid al 1% (Chang, 1952).

4.2.5.6 Valoración microscópica de resultados

Los ovocitos fueron examinados bajo microscopio (Leica DMR) de contraste de fases (40x), donde se valoraron los siguientes parámetros:

- Los ovocitos en estadío nuclear de vesícula germinal se consideraron inmaduros y fueron descartados del estudio.

- Los ovocitos en estadío de metafase II con corpúsculo polar se consideraron maduros.

- Se consideró un ovocito degenerado cuando su citoplasma ofrecía un aspecto granuloso y no se podía visualizar material cromosómico en su interior. Éstos fueron descartados del estudio.

- Se consideraron ovocitos penetrados aquellos que presentaban espermatozoides en el interior del citoplasma, ya fuera con la cabeza en forma compacta, descondensada o en forma de pronúcleo masculino.

- Número de espermatozoides por ovocito: teniéndose en cuenta los pronúcleos masculinos al igual que las cabezas espermáticas en descondensación y compactas.

- Ovocitos monospérmicos: aquellos que sólo habían sido penetrados por un único espermatozoide, apareciendo éste de forma compacta, descondensada o como pronúcleo.

4.2.6 FERTILIDAD *IN VIVO*

En el ensayo con semen congelado (trabajo 1) se utilizaron 45 cerdas reproductoras multíparas. Se llevó a cabo una doble inseminación de cada una de las reproductoras, transcurridas 12 y 24 h después de la detección del celo. Las hembras fueron inseminadas con semen descongelado a 50ºC/12 seg y posteriormente diluido en BTS atemperado a 37ºC. La concentración de las dosis para la inseminación fue aproximadamente de 5 x 10^9 esp en 100 ml de BTS.

En el ensayo con semen refrigerado (trabajo 3) se emplearon un total de 1818 cerdas cruzadas (Landrace x Large White) multíparas. Se llevó a cabo una doble inseminación a las 0 y 24 h después de la detección del celo. La concentración de las dosis para la inseminación fue aproximadamente de 3 x 10^9 células en 100 ml de diluyente MR-A (Kubus, Madrid).

El diagnóstico de gestación se realizó a los 23-25 días después de la IA mediante ultrasonografía. Finalmente, se determinó el porcentaje de fertilidad y el tamaño de camada (número de lechones nacidos totales)

4.2.7 ANÁLISIS ESTADÍSTICO

Los resultados se muestran como medias ± SEM (error estándar de la media). En todos los casos los porcentajes fueron transformados según el modelo binomial de parámetros.

Los valores de calidad seminal, el número de espermatozoides por ovocito penetrado y los porcentajes de penetración, de monospermia y de formación de pronúcleo masculino fueron analizados mediante un ANOVA. También se realizaron estudios de regresión logística y de correlación de Pearson entre los parámetros seminales *in vivo* e *in vitro* y los resultados de fertilidad y tamaño de camada.

Para todo el análisis estadístico se utilizó el “General Linear Models” del programa informático SYSTAT 9.0 y cuando el ANOVA reveló un efecto significativo los valores fueron comparados por el test de Tukey. Se consideraron diferencias estadísticamente significativas aquellas que alcanzaron niveles de probabilidad de $p<0’05$.

4.3. RESULTADOS

Publicación 1.

Analysis of In vitro Fertilizing Capacity to Evaluate the Freezing Procedures of Boar Semen and to Predict the Subsequent Fertility

Sellés E, Gadea J, Romar R, Matás C, Ruiz S.

Reproduction in Domestic Animals. 38:66-72. 2003.

Un primer objetivo de este trabajo fue la evaluación del efecto de la velocidad de descongelación sobre los parámetros de calidad seminal y la capacidad de fecundar ovocitos madurados *in vitro*. Para ello, se emplearon dos sistemas de descongelación, uno de velocidad rápida sumergiendo las pajuelas en un baño a 50ºC durante 12 seg frente a una velocidad control utilizada habitualmente que consiste en descongelar en un baño a 37ºC durante 30 seg.

Se pudo observar que la velocidad de descongelación no ejercía ningún efecto estadísticamente significativo sobre los parámetros seminales estudiados. Sin embargo, los resultados de fecundación *in vitro* mostraron valores significativamente mayores tanto para el número medio de espermatozoides por ovocito (3’91 *vs.* 3’06, p<0’001) como para el porcentaje de formación del pronúcleo masculino (75’47 *vs.* 65’73, p=0’020) cuando se empleó el proceso rápido de descongelación (50ºC durante 12 seg).

En la segunda experiencia, se realizó una prueba de campo para estudiar la fertilidad que se podría obtener al utilizar semen congelado. Al mismo tiempo, se evaluó la capacidad que tienen las diferentes técnicas de análisis del semen congelado para predecir la fertilidad y la prolificidad.

Los análisis seminales fueron capaces de diferenciar a uno de los verracos cuyo semen congelado-descongelado presentaba un porcentaje

significativamente más bajo de espermatozoides viables (EN y DCF) e integridad del acrosoma (NAR) que el resto de los verracos estudiados. Por el contrario, los 4 parámetros analizados en el sistema de FIV estuvieron significativamente afectados por el verraco. Tanto la tasa de penetración como el número medio de espermatozoides por ovocito permitieron diferenciar dos tipos de reproductores los de alta capacidad fecundante frente a los de capacidad inferior. Igualmente, el porcentaje de monospermia estuvo afectado por el verraco y mostró los valores más altos para los machos con menor penetrabilidad.

Al relacionar los resultados de fertilidad y los resultados del sistema FIV observamos que la penetrabilidad *in vitro* estuvo significativamente afectada por el verraco ($p=0'019$), presentando una penetrabilidad más baja en los verracos menos fértiles (tasas de fertilidad 33%).

Al aplicar el análisis de regresión logística entre el porcentaje de fertilidad obtenido en la prueba de campo y los distintos análisis seminales se encontró un coeficiente de regresión significativo para el porcentaje de penetración y el número medio de espermatozoides por ovocito, y para todos los parámetros seminales ($p<0'05$). Cuando se hizo un estudio múltiple de regresión logística con la fertilidad, tres parámetros seminales (porcentaje de penetración, formación de pronúcleo masculino y motilidad) estuvieron incluidos en el modelo ($R^2= 0'492$; $p<0'001$).

En relación con el tamaño de camada se encontró una correlación de Pearson significativa para todos los parámetros de FIV (excepto formación del pronúclo masculino) y dos de los parámetros de calidad seminal (molidad progresiva y funcionalidad de membrana). Mediante un análisis multivariante se consiguió un modelo con significación estadística ($p<0'0001$), que podría explicar cerca de un 80% de variabilidad del tamaño de camada, construido únicamente con los parámetros de tasa de penetración y número medio de espermatozoides por ovocito.

Publicación 2.

Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders.
Gadea J, **Sellés E**, Marco MA, Coy P, Matás C, Romar R, Ruiz S. Theriogenology. 62:690-701. 2004.

En esta segunda publicación se midió el contenido de GSH intracelular en espermatozoides porcinos eyaculados, que ofreció un valor medio de 3'84 ± 0'21 nmol GSH/10^8 células. Se encontraron diferencias significativas sobre el contenido de GSH entre los diferentes verracos ($p<0'001$) dentro de un rango de 1'05 a 6'16 nmol GSH/10^8 células.

Cuando se determinó el contenido de GSH en los espermatozoides procedentes del eyaculado, en refrigeración durante 24 h a 15ºC y tras el proceso de congelación-descongelación, se observó que la cantidad de GSH fue significativamente más baja en los espermatozoides crioconservados que en los frescos ($p<0'0001$), lo que supone una disminución del 32%.

Todos los parámetros seminales presentaron una disminución significativa en el semen congelado-descongelado, excepto la motilidad progresiva. Un análisis de la relación entre los parámetros seminales del semen fresco y congelado y el contenido en GSH permitió poner de manifiesto una correlación inversa y significativa entre GSH y porcentaje de esprmatozoides con acrosoma normal (NAR) en el semen eyaculado, mientras que en el semen criopreservado se encontró una correlación directa entre GSH y la motilidad progresiva.

Posteriormente, se evaluó el efecto de la adición de GSH en el medio de congelación sobre la posterior viabilidad y la capacidad fertilizante del semen crioconservado. La adición de 5 mM GSH en el medio de congelación no tuvo un efecto significativo en términos globales sobre los parámetros

seminales ni sobre la capacidad fecundante de los espermatozoides. Sin embargo, los resultados para las tasas de penetración y el número medio de espermatozoides por ovocito no fueron homogéneos para todos los verracos estudiados.

Finalmente, se evaluó el efecto de la adición de GSH en el medio de descongelación sobre la viabilidad y la capacidad fertilizante de los espermatozoides congelados de porcino. No se encontraron diferencias significativas en los parámetros seminales entre las muestras control y las suplementadas con GSH. Y en contraste, la adición de GSH en el medio de descongelación supuso un aumento significativo en la tasa de penetración ($p<0'001$). Para todos los parámetros de FIV estudiados se detectó un efecto significativo por del verraco ($p<0'001$).

Publicación 3.

The predictive value of porcine seminal parameters on fertility outcome under commercial conditions.
Gadea J, **Sellés E**, Marco MA.

Reproduction in Domestic Animals. 39: 303–308. 2004.

El objetivo de este último trabajo fue estudiar la capacidad predictiva de la fertilidad *in vivo* que tienen las diferentes técnicas de análisis seminal en condiciones de campo usando semen refrigerado.

Los 273 eyaculados estudiados fueron agrupados en dos categorías de acuerdo a la fertilidad a la que dieron lugar (bajo nivel cuando la tasa de partos fue inferior al 85% y alto nivel cuando superó este porcentaje). Los valores medios de fertilidad obtenidos en los 2 grupos de hembras (n = 915 y 903) fueron significativamente diferentes (70'49% *vs.* 96'12%, p<0'0001). No se encontraron diferencias en los parámetros seminales entre ambos grupos de fertilidad. Únicamente, el número total de espermatozoides en el eyaculado fue significativamente más alto en el grupo de mayor fertilidad (78'20 ± 2'71 *vs.* 86'10 ± 2'82 10^9 espermatozoides por eyaculado, p=0'0443).

Posteriormente, se utilizaron diversos métodos de análisis de regresión lineal y logístico para analizar la relación existente entre el porcentaje de fertilidad y los parámetros seminales. Con ambos tipos de análisis, se obtuvo una pobre correlación con los resultados de fertilidad. Solamente la motilidad estuvo significativamente correlacionada con el porcentaje de fertilidad tanto en el análisis de regresión lineal como en el de regresión logística. Cuando se aplicaron análisis de regresión múltiple logística y lineal se pudo encontrar un modelo estadísticamente significativo que incluía los parámetros de motilidad, gota citoplásmica proximal y cola

en ovillo. En cualquier caso y en ambos modelos sólo fue posible explicar un bajo porcentaje de la varianza total de la fertilidad.

Mediante el empleo de curvas de respuesta (ROC) pudimos calcular el valor de corte para los distintos parámetros seminales en nuestras condiciones experimentales. El valor de corte obtenido en este estudio fue muy elevado y restrictivo para algunos parámetros como por ejemplo EN (86), NAR (94), morfología normal (90) y DCF (85). Sin embargo, fue menos elevado y restrictivo para la motilidad (70).

Por último, analizamos la relación entre los parámetros seminales y el tamaño de camada. El número total de lechones nacidos mostró una correlación significativamente positiva con la motilidad ($r=0.1223$, $p=0.0435$), mientras que para el parámetro porcentaje de colas en forma de látigo la correlación fue negativa, aunque no alcanzaron significación estadística ($r=-0.1106$, $p=0.0681$). No se encontró correlación significativa con el resto de los parámetros seminales.

También se estudiaron modelos multivariantes con significación estadística para relacionar los parámetros seminales con el número de lechones nacidos vivos y nacidos totales. En un primer modelo se incluyeron los mismos tres parámetros (motilidad, gota citoplásmica proximal y cola en ovillo) en los modelos antes descritos para la tasa de fertilidad. Para el número total de lechones el modelo es más complejo e incluye un total de cinco parámetros seminales (motilidad total, motilidad progresiva, gota citoplásmica proximal, cola en ovillo y morfoanomalías totales). En ambos casos la capacidad predictiva de la prolificidad que ofrecen los modelos es muy limitada con valores de Multiple R^2 de 0.0318 y 0.0417, respectivamente.

4.5. DISCUSIÓN

Publicación 1.

Analysis of In vitro Fertilizing Capacity to Evaluate the Freezing Procedures of Boar Semen and to Predict the Subsequent Fertility
Sellés E, Gadea J, Romar R, Matás C, Ruiz S.
Reproduction in Domestic Animals. 38:66-72. 2003.

Para aumentar la viabilidad y capacidad fecundante del semen congelado-descongelado de verraco es necesario optimizar todos los factores que tengan un efecto en los procedimientos de congelación. Por este motivo, en este trabajo se centró la atención en el proceso de descongelación, así como en el análisis de métodos de evaluación del semen congelado que permita determinar con precisión la calidad resultante del proceso.

No se encontraron diferencias de calidad seminal en los 2 procedimientos de descongelación utilizados ya que ambos están cerca de las condiciones óptimas. Sin embargo, Fiser et al. (1993) encontraron al estudiar el proceso de congelación-descongelación que el porcentaje de espermatozoides móviles y espermatozoides con acrosoma normal aumentaba gradualmente cuando aumentaba la velocidad de descongelación. Respecto a la capacidad de penetración *in vitro* solamente el número de espermatozoides penetrados por ovocito y el porcentaje de formación de pronúcleo masculino estuvieron significativamente afectados por la velocidad de descongelación, obteniéndose mejores resultados cuando se utilizó la velocidad más rápida. Estos datos confirman resultados previos que apuntan a que la velocidad rápida disminuye las lesiones celulares y las alteraciones en la estabilidad del ADN (Mazur, 1984).

Los resultados de fertilidad obtenidos en condiciones de campo (total 66’67%, 30/45) son superiores a los descritos en la bilbliografía (Johnson et

al 1985; 2000) y podrían ser tasas de fertilidad rentables en el caso de su aplicación en el ámbito comercial. Este hecho sería especialmente interesante para tres de los verracos empleados que presentan tasas de fertilidad por encima del 80% (80, 83'33 y 84'32 %) aunque el tamaño de camada es reducido en algunos casos.

En este estudio, los parámetros seminales convencionales no son suficientes para discriminar entre buenos y malos verracos por su fertilidad. Solamente uno de los dos verracos con baja fertilidad fue detectado por presentar un número mayor de acrosomas alterados y una reducción en la viabilidad. Por el contrario, los parámetros obtenidos en un sistema de fecundación *in vitro* son más precisos para predecir la fertilidad.

Por otro lado, se presenta una gran dificultad para predecir el tamaño de camada, probablemente relacionado con los efectos que ejerce la hembra (ovulación, porcentaje de fertilidad, inseminación…) y que pueden distorsionar la relación entre los parámetros seminales y la prolificidad. Sin embargo, los parámetros de FIV (porcentaje de penetración y número de espermatozoides por ovocito penetrado) son suficientes para explicar cerca del 80% de la variabilidad.

En el presente estudio la FIV se muestra como una técnica precisa para evaluar los procedimientos de congelación en semen de verraco. Hasta ahora sólo se han evaluado con el uso de un sistema de FIV un número reducido de factores relacionados con el proceso de congelación. Entre ellos se incluye el tiempo de mantenimiento y tipo de envase (Eriksson et al., 2000) y el volumen de las pajuelas empleadas (Cordova et al., 2001; 2002).

Como conclusiones, hemos determinado que la velocidad rápida de descongelación tiene un efecto positivo sobre la capacidad de fecundación *in vitro* en el semen congelado de verraco. Por otra parte la FIV parece ser

una buena herramienta para evaluar la calidad del semen congelado-descongelado de verraco previo a su uso comercial, para verificar la calidad del banco de semen y para analizar nuevos procedimientos de congelación, ya que es el método de evaluación más preciso de los disponibles para estimar la capacidad fecundante.

Publicación 2.

Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders.
Gadea J, **Sellés E**, Marco MA, Coy P, Matás C, Romar R, Ruiz S. Theriogenology. 62:690-701. 2004.

Los resultados obtenidos mostraron diferencias significativas en el contenido de GSH entre los diversos verracos estudiados. Este efecto individual podría estar relacionado con las diferencias en el potencial de fertilidad de los diferentes verracos (Gadea, 2003). Previamente, se ha descrito en semen humano una relación entre el contenido de GSH en espermatozoides y plasma seminal y la infertilidad (Ochsendorf et al., 1998, Raijmakers et al., 2003) y también entre el contenido de GSH y la capacidad de penetración en el moco cervical bovino (Ochsendorf et al., 1998).

Estos resultados muestran que la crioconservación de espermatozoides porcinos está asociada con una disminución significativa del contenido de GSH, como previamente ha sido descrito en bovino (Bilodeau et al., 2000) y humano (Molla et al., 2004).

Una vez detectada la disminución del contenido de GSH en el semen congelado-descongelado pasamos a la evaluación de la adición de GSH en el medio de congelación y descongelación sobre los parámetros seminales y la capacidad de fecundación en un sistema de FIV. Durante la descongelación hay un incremento en la producción de ROS (Chatterjee et al., 2001), con un incremento de la producción de anión superóxido debido a la disminución de la actividad SOD, y una disminución del contenido de GSH debido a la disminución de la actividad GRD y al aumento de la oxidación de GSH por el peróxido de hidrógeno. Una disminución del GSH intracelular y un aumento en la producción de ROS durante la descongelación producen daños

oxidativos como un aumento de la peroxidación lipídica o de la fragmentación del ADN.

Estos daños pueden ser corregidos en parte con la adición de antioxidantes a los medios de congelación y descongelación. Con lo que se consigue mejorar la funcionalidad de los espermatozoides congelados porcinos.

Las principales conclusiones resultantes de este estudio fueron el demostrar la disminución del contenido de GSH observado en espermatozoides de verraco cuando son sometidos al proceso de criopreservación y por otra parte, el aumento de la capacidad de fecundación que presentan los espermatozoides congelados porcinos cuando se adiciona GSH en el medio de descongelación.

Publicación 3.

The predictive value of porcine seminal parameters on fertility outcome under commercial conditions.
Gadea J, **Sellés E**, Marco MA.

Reproduction in Domestic Animals. 39: 303–308. 2004.

En este estudio se encontró una baja correlación entre los parámetros seminales y los resultados de fertilidad. De todos los parámetros estudiados, la motilidad espermática fue el único parámetro que se correlacionó significativamente con el porcentaje de fertilidad y el número total de lechones nacidos y aparece como un componente importante en los modelos multivariantes. Sin embargo, el coeficiente de correlación es significativamente bajo. En la literatura científica encontramos estudios contradictorios al estudiar la relación entre la fertilidad y la motilidad (Pursel et al., 1984; Galli y Bosisio, 1988; Berger y Parker 1989; Holt et al., 1997; Gadea et al., 1998; Tardif et al., 1999; Sellés et al., 2003). Este hecho puede ser debido a las diferencias en las condiciones experimentales en las que se desarrollaron los estudios.

Las alteraciones de la morfología espermática están inversamente relacionada con los resultados de fertilidad (al menos como una tendencia). Anteriormente, se ha asociado la presencia de defectos en la morfología espermática con la infertilidad (Bonet y Briz, 1991). La morfología ofrece información acerca de la eficiencia de la espermatogénesis y puede facilitar la selección de verracos en los programas de inseminación artificial (Waberski et al., 1990). Otros autores han descrito una correlación inversa entre el número de formas anormales y la fertilidad (Martínez et al., 1986; Galli and Bosisio, 1988; Waberski et al., 1990; Zeuner 1992; Waberskiet al., 1994a; Gadea et al., 1998).

Algunas posibles causas de la pobre correlación entre la fertilidad y los parámetros seminales pueden estar basadas en: *i)* Ciertas características espermáticas que no son analizadas en los espermiogramas estándares como alteraciones del ADN o daños en la cromatina (Evenson et al., 1994). *ii)* El bajo número de eyaculados o hembras empleados en el estudio, un alto número de espermatozoides por dosis que puedan compensar una infertilidad asociada al factor masculino (Woelders, 1991; Tardif et al., 1999; Jonson et al., 2000). Y, *iii)* un último factor podría ser la preselección de los eyaculados con alta motilidad que tiende a reducir la variabilidad entre los parámetros seminales.

En conclusión, los análisis espermáticos bajo condiciones comerciales sirven para detectar eyaculados con pobre calidad seminal (asociados con pobre fertilidad), pero la preselección de muestras, el alto número de espermatozoides por dosis y la alta calidad del semen utilizado en los programas de IA reducen la variabilidad, por lo que resulta difícil detectar diferencias de fertilidad asociadas a los parámetros seminales.

4.5. CONCLUSIONES

1. La velocidad rápida de descongelación del semen porcino tiene un efecto positivo sobre la capacidad de fecundación *in vitro* de ovocitos.
2. El uso de los sistemas de fecundación *in vitro* es el método de evaluación más preciso, de los disponibles, para estimar la capacidad fecundante del semen porcino congelado.
3. El proceso de congelación produce una disminución del contenido intracelular de GSH en el espermatozoide porcino.
4. La adición de GSH en el medio de descongelación mejora la capacidad de fecundación *in Vitro* de ovocitos porcinos.
5. Los análisis seminales que se llevan a cabo en condiciones comerciales permiten la detección de muestras seminales de baja calidad que están asociadas a baja fertilidad.
6. Bajo estas condiciones comerciales existe una baja correlación de los parámetros seminales con la fertilidad y la prolificidad.

5

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6

ABSTRACT

6. ABSTRACT

Despite almost 40 years of research in freezing of boar semen, the fertility results are not satisfactory enough for commercial use as it is in other domestic animals. However boar frozen-thawed semen is still a valuable tool as a complement to artificial insemination with fresh semen in some conditions. Since it can be stored for a long time, it facilities the supply of genetic material, as well as building up gene banks to encourage breeds or valuable individuals. In the last years, an increasing effort has been made to improve the fertility results mainly by to ways: firstly the design of better freezing methods in order to obtain acceptable semen quality (freezing procedures, diluents and cryoprotectans, packages, etc.), secondly with optimal routines for heat detection and timing of insemination close to the ovulation.

Evaluation of the quality of frozen-thawed semen is an important goal and great deals of assays have been developed. Besides, assays including the study of gamete interaction might lead to a better way of predicting male fertility than routine laboratory evaluation of semen. Some of these assays have been shown to be good tools for evaluating the fertilising capacity of diluted boar semen. However, little information is available about frozen boar semen, but it would be very useful to evaluate freezing procedures.

One of the most important factors related with the success of freezing procedures seems to be the thawing rate of semen. So, the critical temperature range during thawing is an important factor affecting spermatozoa viability. However, it has been demonstrated that the effectiveness of thawing rate also depends on the original rate of freezing. Different studies have previously described when thawing rate is increased (in an optimum range) the motility and acrosome integrity are improved.

In this study, a porcine in vitro fertilization (IVF) system and seminal quality parameters of frozen–thawed boar semen were used to assess the effectiveness of two different thawing rates of frozen boar semen and to address the question of whether differences between fertility of ejaculates could be predicted in a limited field trial.

In the first experiment, two thawing procedures were analysed (37ºC, 30 s; 50ºC, 12 s) and no differences in sperm quality were found. In relation to the in vitro fertilising capacity of the sperm, thawed under two different procedures, the results obtained showed a higher number of sperm per penetrated oocyte (3.91 vs. 3.06, $p<0.001$) and a near ten points higher rate of pronuclear formation (MPF) (75.47 vs. 65.73, $p=0.020$) when the procedure was 50ºC x 12 sec.

In the second experiment, the fertility results obtained in the limited field trial show to be efficient enough for application in a commercial use, especially for three of the employed boars (fertility >80%). The conventional seminal parameters are not accurate enough to discriminate good and bad boars in relation to fertility. On the contrary, parameters of in vitro penetrability are more precise to predict subsequent fertilities.

The in vitro penetrability results are consistent with the limited data from the fertility field trial, since fertility was significantly affected by boar ($p=0.019$), being those with lower penetrability (boars PI4 and PI779) these are with lower fertility (33%) than the other three with fertility rates over 80%. No differences between boars were detected for litter size.

When the logistic regression was analysed between in vitro penetration and seminal parameters with fertility results, significant regression coefficients were found for penetration rate and S/O, and all the quality seminal parameters (Motility, FPM, EN, NAR DCF. $p<0.05$). Later, when studying all semen parameters through stepwise on multiple logistic

regression forward, only three parameters (penetration rate, male pronuclear formation and motility) were included (R^2= 0.492; p<0.001).

As conclusion, the IVF fertilization system seems to be a good tool to evaluate the quality of frozen–thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm freezing procedures, as it is the more precise evaluating method in estimating the potential fertilizing ability.

Cold shock of spermatozoa is associated with oxidative stress and reactive oxygen species (ROS) generation. ROS-induced damage to sperm is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids, leading to lipid peroxidation. The effects of lipid peroxidation include irreversible loss of motility, inhibition of respiration, leakage of intracellular enzymes, damage to sperm DNA, or deficiencies in oocyte penetration and sperm–oocyte fusion.

Semen represents a complex redox system that combines the antioxidant potential of seminal plasma and spermatozoa with the pro-oxidant potential of sperm through the production of ROS. Enzymatic antioxidant defense mechanisms in seminal plasma and spermatozoa include superoxide dismutase, glutathione reductase, gluthathione peroxidase and catalase. Nonenzymatic antioxidants include reduced glutathione (GSH), urate, ascorbic acid, Vitamin E, taurine, hypotaurine, carotenoids, and ubiquinones. The interplay of antioxidant and prooxidant mechanisms in semen determines the overall rate of lipid peroxidation in sperm.

Glutahione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide ubiquotuosly distributed in living cells. It plays an important role in the intracellular defense mechanism against oxidative stress. Glutathione peroxidase uses GSH as the reducing equivalent to reduce hydrogen

peroxide to H2O and lipoperoxides to alkyl alcohols. The resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor.

Glutathione content in boar spermatozoa has been previously reported. However, GSH content in boar sperm before and after cryopreservation has never been evaluated. In this study, GSH content was determined in ejaculated boar spermatozoa before and after cryopreservation. Semen parameters and GSH content were determined in boar spermatozoa from 25 ejaculates from 10 boars, after refrigeration for 24 hours at 15ºC and after cryopreservation. Semen samples were processed using the straw freezing procedure described by Westendorf et al. (1975). Thawing was achieved by immersing the straws in a circulating water bath at 50ºC for 12 seconds. Immediately after thawing the semen was diluted in BTS.

Semen samples (fresh and frozen-thawed) were centrifuged and GSH content in the resulting pellet monitored spectrophotometrically. In this system glutathione is oxydized by 5,5-dithiobis-(2-nitrobenzoic acid (DTNB) and then reduced by glutathione-reductase with NADPH as hydrogen donor. During the oxidation of glutathione by DTNB 2-nitro- 5 thiobenzoeic acid is formed, which can be detected photometrically by a change of absorption at 412 nm. The total glutathione content (oxidized glutathione (GSSG) and reduced glutathione (GSH)) is calculated according to a standard curve.

GSH content in ejaculated boar spermatozoa was determined in 44 ejaculates from 27 boars. It was 3.84 ± 0.21 nmol GSH/108 sperm. There were significant differences in GSH content between different boars ($p<0.001$) ranging from 1.05 to 6.16 nmol GSH/108 cells.

GSH content was significantly lower in cryopreserved sperm as compared to fresh ejaculated spermatozoa ($p<0.0001$, Table 1), with a 32 % decrease after cryopreservation. Significant differences were also

observed between boars, and preservation protocols (interaction p= 0.0102). All semen parameters were affected by preservation method with significant decrease in frozen-thawed semen, except forward motility, which showed an increased value in frozen semen. No significant correlation was observed between semen parameters and GSH content after different preservation treatments.

The effect of addition of GSH to the freezing and thawing extenders was also evaluated. The fertilizing ability of frozen–thawed boar sperm was also tested in vitro by incubating sperm with in vitro matured oocytes obtained from gilts. Addition of 5 mM GSH to the freezing extender did not have a significant effect on standard semen parameters or sperm fertilizing ability after thawing. In contrast, when GSH was added to the thawing extender, a dose-dependent tendency to increase in sperm fertilizing ability was observed, although no differences were observed in standard semen parameters.

In summary, (i) there was a loss in GSH content after cryopreservation of boar semen; (ii) addition of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability; and (iii) addition of GSH to the thawing extender resulted in a significant increase in sperm fertilizing ability. Nevertheless, future studies must conclude if this is the case for all boars. Furthermore, since addition of GSH to the thawing extender did not result in an improvement in standard semen parameters, this suggests that during the thawing process, GSH prevents damage of a sperm property that is critical in the fertilization process but that is not measured in the routine semen analysis.

The prediction of sperm fertilizing ability has a great economic importance to breeding herds when artificial insemination is used, as it leads to the selection of those boars with a good reproductive performance. However, at the moment, only a few trials that correlate sperm factors and fertility have

been reported. In most of them a reduced or poor significant relationship has been observed, in part due to many factors involved in fertility in sows.

Classical methods of semen evaluation generally measure the sperm cell concentration, the progressive motility, the percentage of viable cells and acrosome morphology. These assays are poor in predicting the subsequent sperm fertility, because only the samples with markedly poor quality can be detected. To solve this situation new procedures of seminal analysis *in vitro*, or multiple analysis of the same sample have been evaluated and new assays to study the oocyte-sperm interaction as the IVF system have been developed. However, these analyses are expensive and time consuming so being far from field commercial conditions.

In the third manuscript, the aim was to address the question of whether differences in farrowing rate and litter size after the use of different ejaculates could be predicted using the standard semen parameters under commercial conditions.

In this study, spermatozoa from 273 ejaculates from 57 boars (4 Dalland and 53 Pietrain) were evaluated by in vitro semen quality parameters and a total of 1818 sows were used to evaluate the fertility predictive value of different sperm parameters. The values of the semen parameters and the reproductive result were not affected by the two genetic lines of the boar used in the experiments Then, we consider all the ejaculates as a homogeneous group.

Logistic regression analysis (univariate and multivariate) was used to correlate the dichotomous farrowing rate data to the sperm parameters. Linear regression was also used to determine the relationship between litter size and semen parameters (Pearson correlation and multiple regressions). In both analyses a poor relation with fertility was obtained. Only motility (logistic and linear regression) was significantly related to fertility results. When studying all conventional semen parameters through a stepwise on

multiple logistic or linear regression analysis of semen measurements, only three parameters (motility, PCD and swollen tail) are necessary to obtain a significant model. However, in both models a low percentage of total variance of farrowing rate could be predicted.

Receiver-operating curves (ROC) were used to determine the overall performance characteristics of each semen variable in the logistic regression model. Also, ROC led us to calculate the cut-off values for the semen parameters in these experimental conditions. Cut-off values obtained in this study are high restricted, for example EN (86), NAR (94), normal morphology (90) and DCF (85), however it is less restricted for motility (70).

In relation with litter size, the total number of piglets born was only significantly related with motility in a positive way with a low coefficient ($r = 0.1223$, $P=0.0435$), and folded tail showed a tendency ($P=0.0681$) to be related in an inverse way. On the other hand, correlation was not found between average live piglets born and semen parameters, only a tendency was found in the positive relation for motility and a negative relation for folded tail.

Significant models were found with multiple linear regression (stepwise forward). For live piglets born the model was constructed with 3 variables including motility, FPM and folded tail, confirming the tendency previously detected. For total number of piglets born a significant model was constructed with 5 parameters (motility, FPM, PCD, Swollen Tail and total morpho-anomalies. In both models a low percentage of total variance of the litter size could be predicted.

In conclusion, semen analysis, under commercial conditions, allows to identify ejaculates with very low fertility potential but the pre-selection of the samples, the high number of sperm per doses and the high quality of the semen used in artificial insemination (AI) programmes reduces the

variability. Therefore, it is unlikely to detect fertility differences associated with seminal parameters.

7

ANEXOS

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THERIOGENOLOGY	0093-691X	8704	1.911	0.362	356	7.3

THOMSON

Los coautores de los artículos publicados abajo señalados declaran su conformidad con la presentación de los artículos por parte de Dª Elena Sellés Soriano en su *Tesis Doctoral como compendio de publicaciones*, que la parte fundamental del trabajo recogido en los artículos ha sido realizado por el autor de la tesis, y que no se incluirán los artículos presentados en ninguna otra tesis.

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