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Criopreservación Espermática en la Especie Porcina: Variabilidad Individual

Boar Sperm Cryopreservation: individual variability

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1 Factors influencing boar sperm cryosurvival. Roca J, **Hernández M**, Carvajal G, Vazquez JM, Martinez EA. *Journal of Animal Science* 2006;84:2692-2699.

2 Cryo-scanning electron microscopy (Cryo-SEM) of semen frozen in medium-straws from good and sub-standard freezer AI-boars. **Hernández M**, Ekwall H, Roca J, Vázquez JM, Martínez E, Rodríguez-Martínez H. *Cryobiology* 2007;54:63-70.

3 Differences in SCSA outcome among boars with different sperm freezability **Hernández M**, Roca J, Ballester J, Vázquez JM, Martínez EA, Johannisson A, Saravia F, Rodríguez-Martínez H. *International Journal of Andrology* 2006;29:583-91.

4 Adjustments on the cryopreservation conditions reduce the Incidence of boar ejaculates with poor sperm freezability. **Hernández M**, Roca J, Gil MA, Vázquez JM, Martínez EA. *Theriogenology* 2007;67:1436-1445.

5 Cryo-survival and in vitro fertilizing capacity post-thaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. **Hernández M**, Roca J, Calvete JJ, Sanz L, Muiño-Blanco T, Cebrián-Perez JA, Vázquez JM, Martínez EA. *Journal of Andrology* 2007, en prensa

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Contenidos

Introducción

Introducción

La inseminación artificial (IA) es la tecnología reproductiva más empleada en la especie porcina, siendo utilizada de manera rutinaria por todas las grandes empresas del sector. Así, a finales de los años 90, más del 50% de las cerdas en producción a nivel mundial eran inseminadas [1], porcentaje que no ha dejado de crecer en los últimos años. En algunos países europeos, tales como Bélgica, España, Holanda, Italia o Noruega, más del 80% de las cerdas están incluidas en programas de IA. Actualmente, se realizan en el mundo alrededor de 25 millones de inseminaciones cada año. De ellas, más del 99% se llevan a cabo utilizando semen diluido y conservado a 15-20ºC durante 1-3 días, con una concentración de 2'5-3 x 10^9 espermatozoides por dosis de inseminación [2]. La fertilidad y prolificidad alcanzadas son altas, similares o incluso mejores que las obtenidas con monta natural, situándose el porcentaje de cerdas que llegan a parto entre el 80 y 90% en la mayoría de las granjas. En el restante 1% de las inseminaciones se utiliza semen criopreservado, con dosis de 5 a 6 x 10^9 de espermatozoides. A pesar del elevado número de espermatozoides por dosis, la fertilidad y prolificidad logradas con semen criopreservado son sensiblemente menores que las obtenidas con semen refrigerado, siendo la tasa media de partos aproximadamente del 70% en el mejor de los casos (revisado por [3]). Esta menor fertilidad, junto al elevado número de espermatozoides utilizado, representan las principales razones del uso limitado del semen criopreservado en los actuales programas de IA en la especie porcina [4].

En la actualidad, la criopreservación es el único procedimiento que existe para conservar espermatozoides durante largos periodos de tiempo, probablemente incluso por periodos ilimitados. Por lo tanto, el empleo de semen congelado en programas comerciales de IA está ampliamente justificado dadas las ventajas que ofrece frente al semen fresco o refrigerado. Su utilización permitiría maximizar las posibilidades que nos ofrece la IA, mejorando los rendimientos productivos, tanto en las granjas de producción como en aquellas destinadas a la mejora genética. Así, se podría rentabilizar en mayor medida a los reproductores de elevado valor genético, importar-exportar dosis espermáticas y crear "bancos de semen" que permitan abastecer y gestionar las necesidades de las

explotaciones. Más aún, la congelación espermática juega hoy en día un papel fundamental en el desarrollo de diferentes biotecnologías emergentes, tales como la selección del sexo, o transgénesis [5].

En los últimos años los protocolos de criopreservación e inseminación han sido mejorados sustancialmente. Se han desarrollado nuevos sistemas de envasado (Flatpack® [6], Mini-Flatpack® [7], optimizado el protocolo de congelación [8,9] y demostrado la necesidad de complementar los diluyentes de congelación con compuestos con propiedades antioxidantes [10-13]. Todo ello ha repercutido positivamente en una mejor calidad espermática tras la descongelación. Actualmente, pueden obtenerse dosis espermáticas post-descongelación con más del 50% de espermatozoides mótiles, porcentajes similares a los conseguidos en vacuno de leche, donde la IA con semen criopreservado es utilizada con éxito. Además, la incorporación de nuevos procedimientos de inseminación, tales como la inseminación intrauterina profunda (DUI) [14], ha contribuido de manera notable a que el semen criopreservado pueda ser aplicado de manera eficiente en los programas comerciales de IA. La DUI permite obtener más que aceptables tasas de fertilidad y prolificidad empleando dosis de inseminación con tan sólo 1 x 10^9 espermatozoides congelados-descongelados [15]. Dosis que, aplicadas próximas al momento de ovulación, pueden ofrecer resultados similares a los obtenidos con semen refrigerado, con tasas de parto superiores al 80% [16].

Sin embargo, estos prometedores resultados se ven empañados por la existencia de una elevada población de verracos cuyos espermatozoides muestran una permanente mala congelabilidad, que cuestiona su empleo efectivo en cualquier programa de IA [3,17].

Hasta ahora la selección de verracos en los centros de IA ha estado basada en los rendimientos productivos que éstos ofrecen en la descendencia y no en la selección de reproductores por su criopreservabilidad espermática, como sería de interés para crear bancos de semen criopreservado, y que se viene haciendo durante años en el vacuno de leche [18]. Una diferencia esencial entre la refrigeración y la criopreservación es que mientras la gran mayoría de los eyaculados responden bien al proceso de refrigeración, la respuesta de sus espermatozoides es muy variable a la criopreservación. Dicha variabilidad es un hecho constatado en la especie porcina desde hace tiempo [19-21]. Las diversas mejoras introducidas en los protocolos de criopreservación no han modificado esta situación. Obviamente para mejorar la eficiencia productiva del semen criopreservado es necesario conocer con la mayor exactitud los posibles factores extrínsecos e intrínsecos al individuo, que condicionan esta variabilidad. Sin embargo, existen muchos aspectos desconocidos con respecto a la variabilidad existente en la criopreservación espermática y pocos los estudios que hayan abordado dicha problemática en la especie porcina [22]. Por

todo lo expuesto, nos propusimos como primer objetivo de la presente tesis, estudiar diferentes factores que pudieran explicar dicha variabilidad, y así, su correcto control poder minimizarla, reduciendo el elevado porcentaje de eyaculados que deben ser eliminados de los bancos de semen por su mala calidad post-descongelación. Entre los factores estudiados se encuentran la edad, raza, características del eyaculado antes de la congelación como factores intrínsecos y la frecuencia de recolección del eyaculado, estación y temperatura de transporte del semen, como factores extrínsecos (Artículo 1). Otro posible factor de variación, es el propio proceso de congelación. El desarrollo de biocongeladores capaces de controlar la velocidad de congelación ha supuesto un importante avance para la incorporación comercial del semen criopreservado. Éstos permiten mantener velocidades constantes de congelación y controlar la temperatura en el interior y exterior de las muestras. Sin embargo, con la mayoría de los sistemas actuales, incluidos el nuestro, el inicio de la cristalización en cada muestra se produce de manera aleatoria y no controlada [23]. Esto podría traducirse en variaciones a la descongelación debidas a una irregular distribución de los cristales de hielo durante la congelación, factor que estudiamos en el Artículo 2.

Tradicionalmente la calidad espermática tras la descongelación se evalúa en base a dos importantes características espermáticas, la integridad de la membrana plasmática y la calidad de movimiento. Sin embargo, se ha demostrado que estos parámetros, siendo importantes, no son suficientes para definir la capacidad fecundante de los espermatozoides. Así, Gil y cols. [24] encuentran que no todos los verracos clasificados como “buenos” congeladores, en función de estas características, muestran altos porcentajes de fecundidad *in vitro,* mientras que algunos clasificados como “malos” pueden llegar a alcanzar tasas aceptables. Esto pone de manifiesto, las importantes limitaciones de las pruebas convencionales para evaluar la funcionalidad espermática y la necesidad de definir, con un mejor criterio, qué consideramos como “buen” o “mal” congelador. Un tipo de análisis, relativamente reciente, y que presenta grandes posibilidades, es el análisis de la integridad del ADN nuclear. El interés en este tipo de análisis reside en la posibilidad de que la fertilidad de una muestra espermática pueda depender del grado de estabilidad de su cromatina y no tanto de otras características comúnmente evaluadas como la motilidad o la viabilidad [24]. Además, se ha demostrado que la integridad del ADN de espermatozoides porcinos puede verse afectada durante el proceso de congelación-descongelación [26-28]. Por ello, en el Artículo 3, evaluamos la integridad del ADN de espermatozoides congelados-descongelados, en particular la de aquellos procedentes de eyaculados de verracos clasificados como “buenos” y “malos” congeladores.

Dadas estas importantes diferencias entre individuos, tradicionalmente los verracos se han clasificado como “buenos”, “moderados” o “malos” congeladores, definiendo como

“malos” congeladores, aquellos verracos cuyos espermatozoides tienen una permanente mala tolerancia a la congelación [29]. Estos verracos, pueden representar incluso un tercio de la población, lo que es un serio problema para la producción rentable de dosis espermáticas criopreservadas en la especie porcina (revisado [3]). Tratando de reducir estas diferencias, Watson [29] propone una “criopreservación a la carta”, es decir, el empleo de protocolos de criopreservación optimizados para cada verraco, lo cual debería ser contemplado como una solución factible para mejorar la congelabilidad espermática de verracos con un apreciable valor. Por ello, en el artículo 4, evaluamos el efecto de ligeras modificaciones en el protocolo de criopreservación, siempre dentro de un rango de seguridad, sobre sensibilidad espermática al proceso de congelación-descongelación y, de este modo, reducir la incidencia de verracos clasificados como “malos” congeladores.

Por último, es interesante señalar que la distinta susceptibilidad entre individuos a la congelabilidad espermática parece tener un origen genético. Así, se han encontrado diferencias entre verracos buenos y malos congeladores [30] utilizando marcadores moleculares AFLP (Amplified Fragment Length Polymorphism). Sin embargo, los mecanismos por los que diferencias genéticas se relacionan con diferente susceptibilidad al proceso de criopreservación se desconocen. Estas diferencias podrían estar representadas tanto por una distinta composición lipídica o proteica de la membrana espermática así como por variaciones en la composición del plasma seminal o funcionalidad de las glándulas accesorias [31]. Asimismo, Rath y Niemann [32] señalan que las diferencias entre verracos en la calidad espermática post-descongelación, sólo son observadas en espermatozoides procedentes del eyaculado, no cuando provienen de la cola del epidídimo. Una de las principales diferencias existentes entre los espermatozoides de la cola del epidídimo y los eyaculados, es la presencia de plasma seminal entre éstos últimos. En equino se ha observado que la adición de plasma seminal afecta de manera distinta a la congelabilidad espermática según la fuente de procedencia sea de “buenos” o “malos” congeladores [33]. Además, diferentes porciones del eyaculado de un verraco varían en su capacidad de sobrevivir a la congelación, y esta habilidad varía entre verracos [34]. Estas diferencias pueden ser explicadas debido a que la composición del plasma seminal varía ampliamente entre especies e individuos de la misma especie [35]. Estudios recientes muestran la existencia de diferencias en el perfil proteico del plasma seminal entre eyaculados que presentan una buena o mala calidad espermática a la descongelación (bovino [36], equino [37]). Por lo tanto, es posible pensar que puedan existir diferencias en dicha composición entre verracos con buena y mala congelabilidad y que éstas, puedan condicionar la mayor o menor susceptibilidad espermática a soportar el proceso de criopreservación. Así, en el Artículo 5, decidimos determinar el posible efecto de la adición de plasma seminal en la

congelabilidad espermática, según si éste procedía de verracos clasificados como “buenos” o “malos” congeladores.

[1] Weitze KF. Update on the worldwide application of swine AI. In: Boar semen preservation IV, Johnson LA and Guthrie HD (Ed.), Allen Press, Lawrence, KS, 2000, pp 141-146.

[2] Wagner HG and Thibier M. World statistics for artificial insemination in small ruminant and swine. Proceedings of the 14th International Congress on Animal Reproduction, Stockholm, Sweden, 2002, Abstracts vol. 2, 15: 3.

[3] Roca J, Rodríguez-Martínez H, Vázquez JM, Bolarín A, Hernández M, Saravia F, et al. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Control of pig reproduction VII, Ashworth CJ, Kraeling RR (Eds), Nottingham: Nottingham University Press; 2006, pp. 261–275.

[4] Eriksson BM, Petersson H, Rodríguez-Martínez H. Field fertility with exported boar semen frozen in the new flatpack container. Theriogenology 2002;58:1065–1079.

[5] Gerrits RJ, Lunney JK, Johnson LA, Pursel VG, Kraeling RR, Rohrer GA, Dobrinsky JR. Perspectives for artificial insemination and genomics to improve global swine populations. Theriogenology 2005;63:283-299.

[6] Eriksson BM, Rodríguez-Martínez H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. Anim Reprod Sci 2000;63:205-220.

[7] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodríguez-Martínez H. Deep freezing of concentrated boar semen for intra-uterine insemination: effect on sperm viability. Theriogenology 2005;63:1320-1333.

[8] Carvajal G, Cuello C, Ruiz M, Vázquez JM, Martínez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389-396.

[9] Devireddy RV, Fahrig B, Godke RA, Leibo SP. Subzero water transport characteristics of boar spermatozoa confirm observed optimal cooling rates. Mol Reprod Dev 2004;67:446-457.

[10] Peña FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 2003;78:85-98.

[11] Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397-405.

[12] Roca J, Rodríguez MJ, Gil MA, Carvajal G, García EM, Cuello C, Vázquez JM, Martínez EA. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15-24.

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

[14] Martínez EA, Vázquez JM, Roca J, Lucas X, Gil MA, Parrilla I, Vázquez JL, Day BN. Minimum number of spermatozoa required for normal fertility after deep intrauterine insemination in non-sedated sows. Reproduction 2002;123:163-170

[15] Roca J, Carvajal G, Lucas X, Vázquez JM, Martínez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. Theriogenology 2003;60:77-87.

[16] Bolarín A, Roca J, Rodríguez-Martínez H, Hernández M, Vázquez JM, Martínez EA. Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used. Theriogenology 2006; 65:669-680.

[17] Medrano A, Watson PF, Holt WV. Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope, Reproduction 2002; 123:315–322.

[18] Vishwanath, R. Artificial insemination: the state of art Theriogenology 2003;59:571-584.

[19] Larsson K, Einarsson S. Fertility of deep frozen boar spermatozoa: influence of thawing diluents and of boars. Acta Vet Scand 1976; 17:43–62.

[20] Johnson LA. Fertility results using frozen boar spermatozoa. In: Deep freezing of boar semen, Johnson LA and Larsson K (Eds), 1985, pp 199-224.

[21] Reed HCB. Current use of frozen boar semen – Future need of frozen boar semen. In: Deep freezing of boar semen, Johnson LA and Larsson K (Eds), 1985, pp 225-238.

[22] Joyal SM, Kennedy BW, Wilkins JN. Boar, breed and environmental effects on motility of frozen-thawed spermatozoa. Can J Anim Sci 1986;66:663–668.

[23] Woelders H, Matthijs A, Zuidberg CA, Chaveiro AE. Cryopreservation of boar semen: equilibrium freezing in the cryomicroscope and in straws. Theriogenology 2005;63:383–395.

[24] Gil MA, Roca J, Cremades T, Hernández M, Vázquez JM, Rodríguez-Martínez H, Martínez EA. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates?. Theriogenology 2005;64:305–316.

[25] Evenson DP, Larsson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25–43.

[26] Córdova A., Pérez-Gutiérrez JF, Lleo B, Garcia-Artiga C, Alvarez A, Drobchak V, Martin-Rillo S. In vitro fertilizing capacity and chromatin condensation of deep frozen boar semen packaged in 0.5 and 5 ml straws. Theriogenology 2002;57: 2119–2128.

[27] Fraser L, Strzezek J. Effects of freezing–thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. Reprod Dom Anim 2005; 40: 1–7.

[28] Fraser L, Strzezek J. Effects of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing–thawing. Anim Reprod Sci 2007; 99:317-329.

[29] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their postthawing function. Reprod Fertil Dev 1995;7:871–891.

[30] Thurston LM, Siggins K, Mileham, AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002;66: 545–554.

[31] Holt WV, Medrano A, Thurston LM and Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope Theriogenology 2005;63:370-382

[32] Rath D, Niemann H. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen thawed epididymal semen obtained from identical boars. Theriogenology 1997;47:785–793.

[33] Aurich JE, Kühne A, Hoppe H, Aurich C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. Theriogenology 1996;46:791-797.

[34] Peña FJ, Saravia F, Nuñez-Martínez I, Johannisson A, Wallgren M, Rodríguez Martínez, H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Anim Reprod Sci 2006;93:101-113.

[35] Strzezek J, Wysocki P, Kordan W, Kuklinska M, Mogielnicka M, Soliwoda D, Fraser L. Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. Reprod Biol 2005;5:279-290.

[36] Jobim MI, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC. Two dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 2004;61:255-266.

[37] Zahn FS, Papa FO, Melo CM, Brisola ML. Protein profile of equine seminal plasma: correlation to semen freezability. Anim Reprod Sci 2005;89:313-315.

Objetivos

Basándonos en lo anteriormente expuesto, el objetivo fundamental de la presente tesis fue mejorar la calidad espermática tras la descongelación del semen porcino, minimizando la recurrente variabilidad entre eyaculados existente en los bancos de semen criopreservado. Para ello propusimos los siguientes objetivos particulares:

1 Identificar factores que afectan a la variabilidad en la supervivencia espermática tras la descongelación y estimar la importancia de la variación entre verracos o entre eyaculados dentro de un mismo verraco (Artículos 1 y 2).

2 Evaluar el grado de integridad del ADN de los espermatozoides porcinos congelados-descongelados y su relación con verracos “buenos” y “malos” congeladores (Artículo 3).

3 Optimizar diferentes parámetros del proceso de criopreservación con el fin de mejorar la congelabilidad espermática, especialmente en eyaculados procedentes de verracos “malos” congeladores (Artículo 4).

4 Examinar el efecto de la suplementación del medio de congelación con plasma seminal procedente de individuos con distinta congelabilidad, sobre la calidad espermática a la descongelación (Artículo 5).

Artículo 1

Factors influencing boar sperm cryosurvival[1]

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ABSTRACT: Optimal sperm cryopreservation is a prerequisite for the sustainable commercial application of frozen-thawed boar semen for AI. Three experiments were performed to identify factors influencing variability of postthaw sperm survival among 464 boar ejaculates. Sperm-rich ejaculate fractions were cryopreserved using a standard freezing-thawing procedure for 0.5-mL plastic straws and computer-controlled freezing equipment. Postthaw sperm motility (assessed with a computer-assisted semen analysis system) and viability (simultaneously probed by flow cytometry analysis after triple-fluorescent stain), evaluated 30 and 150 min postthaw, were used to estimate the success of cryopreservation. In the first experiment, 168 unselected ejaculates (1 ejaculate/boar), from boars of 6 breeds with a wide age range (8 to 48 mo), were cryopreserved over a 12-mo period to evaluate the predictive value of boar (breed and age), semen collection, transport variables (season of ejaculate collection, interval between collections, and ejaculate temperature exposure), initial semen traits, and sperm quality before freezing on sperm survival after freezing-thawing. In Exp. 2, 4 ejaculates from each of 29 boars, preselected according to their initial semen traits and sperm quality before freezing, were collected and frozen over a 6-mo period to evaluate the influence of interboar and intraboar ejaculate variability in the survival of sperm after cryopreservation. In Exp. 3, 12 ejaculates preselected as for Exp. 2, from each of 15 boars with known good sperm cryosurvival, were collected and frozen over a 12-mo period to estimate the sustainability of sperm cryosurvival between ejaculates over time. Boar and semen collection and transport variables were not predictive of sperm cryosurvival among ejaculates. Initial semen traits and sperm quality variables observed before freezing explained 23.2 and 10.9%, respectively, of the variation in postthaw sperm motility and viability. However, more that 70% of total variance observed in postthaw sperm quality variables among ejaculates was explained by boar. This indicates that boar is the most important ($P < 0.001$) factor explaining the variability among ejaculates in sperm cryosurvival, with most (14 of the 15 boars in Exp. 3) showing consistent ($P > 0.05$) sperm cryosurvival over time.

Key words: cryopreservation, pig, semen, variability

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INTRODUCTION

Efficient application of frozen-thawed (**FT**) boar semen in commercial AI programs is now feasible due to improvements in cryopreservation protocols and the development of new insemination procedures (reviewed by Roca et al., 2006). The promising fertility results achieved by Eriksson et al. (2002) and Roca et al. (2003) have revived the interest of commercial pig companies in establishing sperm cryobanks. Obviously, these companies require cryobanks that are based on frozen semen with high and consistent postthaw sperm quality. Unfortunately, it is not easy to achieve this goal because ejaculated boar sperm show great variability in their survival of the cooling and thawing processes.

To minimize this variability, an accurate knowledge of the factors influencing sperm cryosurvival is necessary, including boar variability, ejaculate quality, and semen handling conditions. Very few studies have evaluated the factors influencing sperm cryosurvival, apart from the evident difference between males (Larsson and Einarsson, 1976, among others).

Therefore, the objective of the current study was to identify factors responsible for variability among ejacu-

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lates in their ability to survive cryopreservation. Three experiments were carried out to evaluate the predictive value of boar breed and age, semen collection and transport variables, conventional semen measurements of fresh ejaculates and sperm quality before freezing on sperm survival after freezing and thawing, and to estimate the importance of inter- and intraboar variability on the sustainability of sperm quality after cryopreservation.

MATERIALS AND METHODS

Reagents and Media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO) and were made up under sterile conditions in a laminar-flow hood (HH48, Holten LaminAir, Denmark) with purified water (18 MΩ -cm; Elgastat U0HQPS, Elga Ltd., Buckinghamshire, UK).

The basic medium used for sperm extension was Beltsville Thawing Solution (**BTS**, composed of 205 m*M* glucose, 20.39 m*M* NaCl, 5.4 m*M* KCl, 15.01 m*M* $NaHCO_3$, and 3.35 m*M* EDTA, pH 7.2; Pursel and Johnson, 1975), containing kanamycin sulfate (50 μg/mL). The basic medium used for sperm cryopreservation was a lactose-egg yolk (**LEY**) extender composed of 80% (vol/vol) β-lactose solution (310 m*M* in water), 20% (vol/vol) egg yolk, and 100 μg/mL of kanamicyn sulfate.

Animals, and Source, Handling, Evaluation, and Processing of Ejaculates

Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of Murcia University.

Ejaculates from healthy boars housed at 3 commercial insemination stations were used in this study. Boars were housed in individual pens in environmentally controlled buildings. They were given ad libitum access to water and were fed commercial diets according to the nutritional requirements for adult boars.

Sperm-rich ejaculate fractions were collected using the gloved-hand method, extended (1:1, vol/vol) in BTS, and evaluated for conventional semen characteristics. Volume was assessed from a graduated collection test tube. Sperm concentration was evaluated by hemocytometer after extending (1:10 vol/vol) an aliquot of semen with a 0.3% solution of formaldehyde in PBS. The percentage of motile sperm was evaluated from 3 samples of the extended semen (1:1 vol/vol in BTS) placed under a coverslip in the center of a prewarmed (37°C) microscope slide and transferred to a heated microscope stage set at 37°C. Sperm morphology was assessed according to the proportions of sperm with normal morphology by viewing wet mounts of extended semen fixed in buffered 2% glutaraldehyde solution (Pursel and Johnson, 1974) under a phase contrast microscope at a magnification of 400×. Two hundred sperm were counted per preparation.

After evaluation, the extended semen was transferred to 50-mL tubes, cooled to 17°C, and sent by mail, packaged in insulated containers under conditions of monitored temperature (miniature data logger, Gemini Data Loggers, Ltd., Chichester, UK), to the sperm cryopreservation laboratory of the Faculty of Veterinary Medicine, University of Murcia. The extended semen arrived at the laboratory 14 to 15 h after collection of the ejaculate. At the laboratory, the semen was reevaluated (see Sperm Quality Assessment) and centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) for 3 min at 2,400 $\times$ *g* (Carvajal et al., 2004). After centrifugation, the supernatant, containing mostly seminal plasma, was removed by aspiration.

Sperm Cryopreservation

Sperm pellets obtained after centrifugation of extended sperm-rich fractions were cryopreserved using the straw freezing procedure described by Westendorf et al. (1975), as modified by Thurston et al. (2001). Briefly, sperm pellets were reextended in LEY (pH 6.2 and 330 ± 5 mOsm/kg) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5°C in 90 min, the sperm were resuspended with LEY-Glycerol-Orvus ES Paste extender [92.5% LEY + 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA) and 6% glycerol, vol/vol; pH 6.2 and 1,650 ± 15 mOsm/kg] to a final concentration of 1×10^9 sperm/mL.

The resuspended and cooled sperm were packed into 0.5-mL, PVC, French straws (Minitüb, Tiefenbach, Germany) and frozen using a controlled-rate freezing instrument (IceCube 1810, Minitüb, Tiefenbach, Germany) as follows: cooled to –5°C at 6°C/min, cooled from –5°C to –80°C at 40°C/min, held for 30 s at –80°C, then cooled at 70°C/min to –150°C, and finally plunged into liquid N. The straws remained in the liquid N tank for at least 2 wk before thawing. Thawing of straws was done in circulating water at 37°C for 20 s. Thawed sperm from 2 straws per ejaculate were resuspended in BTS (1:2, vol/vol; 37°C) and incubated in a waterbath at 37°C for 150 min.

Sperm Quality Assessment

Each ejaculate was assessed for sperm motility and viability before freezing (immediately after arrival at the laboratory) and after thawing (at 30 and 150 min postthaw).

Sperm motility (proportion of total motile sperm) was objectively evaluated using a computer-aided sperm analysis system (Sperm Class Analyzer, SCA, Microptic, Barcelona, Spain). The extended and incubated FT sperm were reextended in BTS to a concentration of 20 $\times 10^6$ sperm/mL. For each evaluation, a 4-μL sperm sample was placed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were

analyzed at 39°C, assessing a minimum of 100 sperm/sample.

Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity. These characteristics were analyzed simultaneously using a modification of a triple-fluorescent procedure, described by Graham et al. (1990) and adapted for boar sperm by Carvajal et al. (2004), which includes the DNA-specific fluorochrome propidium iodide, the mitochondria-specific fluorochrome rhodamine-123 (R123), and the acrosome-specific fluorochrome fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin. Three hundred sperm were counted under 1,000× magnification (Eclipse E800, Nikon, Tokyo, Japan), using a BV-2A filter (excitation filter 400–440 nm, barrier filter 470 nm, dichroic 455 nm combination). Sperm showing only green fluorescence over their midpiece (R123-positive) were considered viable with an intact acrosome (viable sperm), and the values were expressed as a percentage of viable sperm.

Experimental Design

Experiment 1: Predictive Value of Boar, Semen Collection, and Transport Variables, and Initial Semen Traits, and Sperm Quality before Freezing on Sperm Survival after Cryopreservation. Ejaculates (n = 168) were collected over a 12-mo period from 168 boars (1 ejaculate per boar). The boars varied in breed and age (8 to 48 mo) and ejaculate collection frequency (interval between ejaculations), and initial semen traits also differed. All ejaculates collected were sent to the laboratory and cryopreserved, regardless of the initial semen traits recorded, or sperm quality assessed, before freezing.

Experiment 2: Inter- and Intraboar Variability of Sperm Survival after Cryopreservation. Four ejaculates were cryopreserved from each of 29 boars over a 6-mo period (1 ejaculate/boar every 6 to 10 wk). All boars were 1 to 3 yr of age, known to be fertile, and were undergoing regular semen collection (1 or 2 times per wk) for commercial artificial insemination. The ejaculates to be cryopreserved were preselected according to their initial semen traits and sperm quality before freezing. Therefore, only ejaculates with $\geq 200 \times 10^6$ sperm/mL, ≥ 85% sperm with normal morphology, and ≥ 75% and ≥ 80% of motile and viable sperm, respectively, before freezing, were retained for cryopreservation.

Experiment 3: Sustainability of Sperm Survival after Cryopreservation between Ejaculates over Time. Twelve ejaculates were cryopreserved from each of 15 boars over a 12-mo period (1 ejaculate·boar^{-1}·mo^{-1}). All boars were 2 to 3 yr of age, known to be fertile, and undergoing regular semen collection for AI. The boars were selected, on the basis of their consistent good sperm cryosurvival, from those used for Exp. 2. As for Exp. 2, the ejaculates to be cryopreserved were preselected according to their initial semen traits and sperm quality before freezing. Therefore, only ejaculates with $\geq 200 \times 10^6$ sperm/mL, ≥ 85% sperm with normal morphology, and ≥ 75% and ≥ 80% of motile and viable sperm, respectively, before freezing, were retained for cryopreservation.

Statistical Analysis

For data analysis, SPSS (version 13.0, SPSS Inc., Chicago, IL) and PATN (CSIRO, Canberra, Australia) software packages were used. Descriptive analyses of boar, semen collection, and transport variables, and initial semen traits and sperm quality before freezing and after thawing were made in Exp. 1. In Exp. 1 and 2, multiple regression analyses were performed using boar, semen collection, and transport variables, and initial semen traits and sperm quality before freezing to predict postthaw sperm quality variables. For these analyses, breed (in Exp. 1) and age (in Exp. 2) of individual boars, ejaculate collection frequency, temperature of semen exposure, initial semen traits (ejaculate volume, sperm concentration, total sperm output, sperm morphology, and total sperm motility), and sperm quality before freezing (sperm motility and viability) were treated as the independent variables. Breed of boar and individual boars were included in the models as dummy variables. The percentages of total sperm motility and viability at 30 and 150 min after thawing, analyzed at first separately and then combined for the 2 times, were treated as the dependent variables.

In Exp. 1, analysis was performed using the PATN pattern analysis package to identify naturally occurring subgroups within the ejaculate data set (Abaigar et al., 1999). The PATN analysis classifies the ejaculates, using postthaw sperm variables, into a small number of groups. Three groups were finally obtained from the nonhierarchical classification of the 168 ejaculates. The data for boar and semen collection and transport variables, and initial semen traits and sperm quality before freezing and after thawing of the 3 groups of ejaculates were calculated and compared using 1-way ANOVA.

In Exp. 2 and 3, 2 separate ANOVA were carried out to investigate interboar (among boars) and intraboar (among ejaculates within the same boar) variability on postthaw sperm quality. Initially, the fixed effects of boar and ejaculate within boar were included in the statistical model. When the latter was significant, a second ANOVA was carried out to assess the effect of ejaculate within boar (intraboar variability) for each of boars tested. In Exp. 3, intraboar variability was also estimated by calculating the CV. When means for the 2 postthaw evaluation times (30 and 150 min) did not differ, they were averaged across the 2 times. Statistical significance was defined as $P < 0.05$.

Table 1. Distribution of ejaculates among breeds of boars, and initial semen traits and sperm quality variables observed before freezing and after thawing (Exp. 1)[1]

Item	Mean ± SEM	Range
Initial semen trait[2]		
Volume, mL	116.15 ± 2.41	46 to 185
Sperm concentration, millions/mL	371.76 ± 8.87	147 to 680
Total sperm output, billions	43.26 ± 1.32	10 to 93
Sperm morphology, normal morphology, %	90.72 ± 0.72	54 to 100
Total sperm motility, %	82.13 ± 1.17	65 to 95
Sperm quality before freezing, 17°C		
Total sperm motility	76.31 ± 0.88	42 to 96
Sperm viability	83.94 ± 0.58	49 to 96
Sperm quality after thawing, 37°C		
Total sperm motility at 30 min	46.85 ± 1.14	11 to 77
Sperm viability at 30 min	51.86 ± 1.04	11 to 78
Total sperm motility at 150 min	37.88 ± 1.09	6 to 68
Sperm viability at 150 min	44.41 ± 1.06	9 to 69
Mean sperm motility[3]	42.37 ± 1.07	9 to 70
Mean sperm viability[3]	48.14 ± 1.02	10 to 71

[1]Breeds of boars represented included Landrace (n = 20; 11.9%), Large White (n = 42; 24.7%), Duroc (n = 20 (11.9%), Pietrain (n = 35; 20.6%), Yorkshire (n = 10; 5.9%), and Crossbred (n = 43; 25.3%). n = 168 ejaculates.
[2]Sperm-rich fraction.
[3]Mean values of 30 and 150 min assessment.

RESULTS

Experiment 1: Predictive Value of Boar, Semen Collection, and Transport Variables, and Initial Semen Traits, and Sperm Quality before Freezing on Sperm Survival after Cryopreservation

Age of the boars ranged from 8 to 48 mo (mean ± SEM: 19.03 ± 0.64), and frequencies of ejaculate collection ranged from 4 to 15 d (7.86 ± 0.14). Ejaculates (n = 168; 1 per boar) were collected during 1 yr with the following distribution among the 4 seasons: 31, 49, 22, and 66 for winter, spring, summer, and autumn, respectively. The temperature of diluted semen during transport from the AI center to the cryobiology laboratory ranged from 12 to 21°C (mean ± SEM: 16.69 ± 0.14). The distribution of ejaculates among breeds, the initial semen traits of ejaculates, the sperm quality variables assessed before freezing, and those assessed after thawing are summarized in Table 1.

To determine the predictive value of boar and semen collection and transport variables, initial semen traits and sperm quality before freezing on sperm quality assessed at 30 and 150 min postthawing, multiple regression models were generated for all possible combinations. The statistical models generated were similar for the 2 postthaw times. For simplicity of presentation, only statistical models generated from the combination of the 2 times were presented in detail. When all independent variables were included in the model, only 25 and 16.5% (adjusted R^2), respectively, of the variation in postthaw sperm motility and viability was accounted for. Three models derived by forward stepwise regression showed statistical results. There was a weak predictive value for both postthaw sperm variables. The best predictive value (23.2%) for postthaw sperm motility was achieved with a model including motility before freezing, morphology and concentration. For postthaw sperm viability, only one variable (sperm morphology) was significant and had a very low predictive value (10.9%).

After the PATN analysis of FT sperm quality variables, 3 groups of ejaculates (boars) were clearly identified. The ejaculates with best postthaw sperm quality were classified as good, whereas those that showed moderate and reduced sperm cryosurvival were classified as moderate or poor, respectively. A summary of the data for these 3 groups of ejaculates is shown in Table 2. Most ejaculates (66.7%) were classified as good. Differences between groups ($P < 0.05$ to 0.001) were achieved for 2 initial semen traits (sperm concentration and proportion of morphologically normal sperm) and the 2 sperm quality variables (motility and viability) assessed before freezing. Ejaculates classified as good showed the greatest percentages of normal morphology and sperm motility before freezing. Likewise, they showed a high sperm concentration and viability before freezing.

Experiment 2: Inter- and Intraboar Variability of Sperm Survival after Cryopreservation

Multiple regression analyses revealed one significant ($P < 0.01$) model, which included individual boars as the only variable. This model had a high predictive value for postthaw sperm quality, with postthaw sperm motility and viability accounting for 70.8 and 80.4% of total variance, respectively.

Variability among boars (interboar variability) was significant ($P < 0.001$) for postthaw sperm motility and viability. Variation between ejaculates within each boar (intraboar variability) was also significant ($P < 0.001$). However, interboar variability (F-values = 71.85 and 102.21, df = 28) was considerably greater than intraboar variability (F-values = 4.74 and 2.52, df = 87, for motility and viability, respectively). Moreover, intraboar variability was only significant ($P < 0.05$) in 4 of the 29 boars. Fifteen of the 25 boars with no statistical intraboar variability had consistently high values for motility and viability of sperm after thawing (over to 50% in all ejaculates at 30 min after thawing). Another 4 boars had very low postthaw sperm quality (below 35% in all ejaculates at 30 min after thawing).

Experiment 3: Sustainability of Sperm Survival after Cryopreservation between Ejaculates over Time

Figure 1 summarizes the data for the 15 boars in a series of box-whisker plots. Although high overall postthaw sperm motility and viability was obtained for all boars, interboar (boar differences) and intraboar

Table 2. Differences on boar, semen collection and handling conditions, and semen traits before cryopreservation among the 3 groups of ejaculates classified according their freezability (Exp. 1)[1]

Item	Sperm freezability group[2]			*P*-value
	Good	Moderate	Poor	
Number of boars, %	112 (66.7)	49 (29.2)	7 (4.2)	
Boar age, mo	18.47 ± 0.73 (10.1 to 34.3)	19.83 ± 1.39 (9 to 37)	23.13 ± 2.71 (10.9 to 32)	0.270
Ejaculate collection frequency, d	7.78 ± 0.17 (6 to 12)	7.88 ± 0.25 (5.5 to 15)	9.14 ± 0.71 (7 to 12)	0.146
Semen transport temperature, °C	16.74 ± 0.16 (13 to 19.5)	16.68 ± 0.31 (11.6 to 19.5)	15.93 ± 0.76 (11.5 to 17)	0.528
Initial semen trait				
Volume, mL	114.94 ± 2.82 (62.9 to 170.7)	118.10 ± 5.08 (48 to 167.5)	121.14 ± 13.11 (76 to 168)	0.772
Sperm concentration, millions/mL	374.28 ± 11.06[a] (203 to 640)	378.71 ± 16.11[a] (192.5 to 600)	279.99 ± 28.78[b] (147.5 to 375)	0.047
Total sperm output, billions	42.93 ± 1.50 (17.2 to 70.8)	45.17 ± 2.84 (14.2 to 84.4)	35.12 ± 5.64 (11.2 to 52.1)	0.331
Normal sperm morphology, %	93.10 ± 0.66[a] (79 to 100)	86.00 ± 1.63[b] (60.5 to 99)	84.57 ± 4.82[b] (68 to 100)	0.001
Total sperm motility, %	83.33 ± 0.95 (75 to 95)	81.66 ± 1.77 (70 to 90)	79.66 ± 4.48 (70 to 90)	0.156
Sperm quality before freezing, %				
Total sperm motility, %	78.62 ± 0.92[a] (59.1 to 90.79)	72.66 ± 1.82[b] (50.9 to 91.6)	64.79 ± 5.18[b] (44.5 to 79.5)	0.001
Sperm viability, %	85.11 ± 0.61[a] (73.3 to 93.4)	82.24 ± 1.19[ab] (69 to 93.5)	77.14 ± 4.47[b] (60 to 95)	0.004
Sperm quality postthawing, %				
Total sperm motility at 30 min	54.86 ± 0.92[a] (40.9 to 71.7)	32.81 ± 1.09[b] (20.8 to 44.8)	17.01 ± 2.36[c] (11.4 to 26.1)	0.001
Sperm viability at 30 min	59.37 ± 0.62[a] (48.3 to 70.4)	39.80 ± 1.18[b] (25.5 to 54)	18.29 ± 1.95[c] (11 to 25)	0.001
Total sperm motility at 150 min	45.27 ± 0.98[a] (27.5 to 63.5)	25.02 ± 0.83[b] (15.2 to 35.4)	9.81 ± 1.12[c] (5.1 to 12.8)	0.001
Sperm viability at 150 min	51.89 ± 0.76[a] (37 to 65)	31.90 ± 1.13[b] (20 to 46.5)	12.29 ± 1.77[c] (6 to 17)	0.001

[a–c]Different letters within rows denote significant differences ($P < 0.05$).
[1]Values are means (± SEM), with ranges in parentheses.
[2]The ejaculates with best, moderate, and reduced sperm cryosurvival were classified as good, moderate, or poor, respectively, using PATN analysis (Abaigar et al., 1999).

(among ejaculate of a same boar) variability was found for both postthaw sperm characteristics evaluated ($P < 0.001$). However, intraboar variability was less (F-values = 3.77 and 2.08, df = 165) than interboar variability (F-values = 17.48 and 5.50, df = 14, for motility and viability, respectively), with only 1 boar (the number 14 in Figure 1) having ejaculate differences in postthaw sperm motility ($P = 0.001$) and viability ($P = 0.003$). Therefore, in 14 of the 15 boars tested, a consistently high postthaw sperm motility and viability was achieved in the 12 ejaculates cryopreserved during the year. Indeed, the low coefficients of variation (below 15%) observed in 13 of the 15 boars in both postthaw sperm characteristics confirmed the consistent sperm cryosurvival achieved.

DISCUSSION

Optimum sperm cryopreservation is of primary importance if improvements are to be made in the efficiency of utilization of frozen-thawed semen for pig AI, particularly for commercial use. Obviously, there is a need to accurately determine which factors influence sperm survival after cryopreservation of ejaculated boar sperm. The results from Exp. 1, designed to evaluate the influence of boar, semen collection and transport variables, and conventional seminal measurements, demonstrated that semen collection and transport variables were not useful predictors of sperm quality postthaw. Especially noticeable was the lack of predictive value for boar breed, previously reported as important (Almlid and Hofmo, 1996; Thurston et al., 2001; Park and Yi, 2002). Although a significant influence of breed on postthaw sperm quality was observed in the current study, with ejaculates collected from Landrace and Pietrain boars having the greatest percentage of postthaw sperm motility and viability (results not shown), the variance in both postthaw sperm quality measurements was not explained by breed or any other boar and management variables. This suggests that the best mean

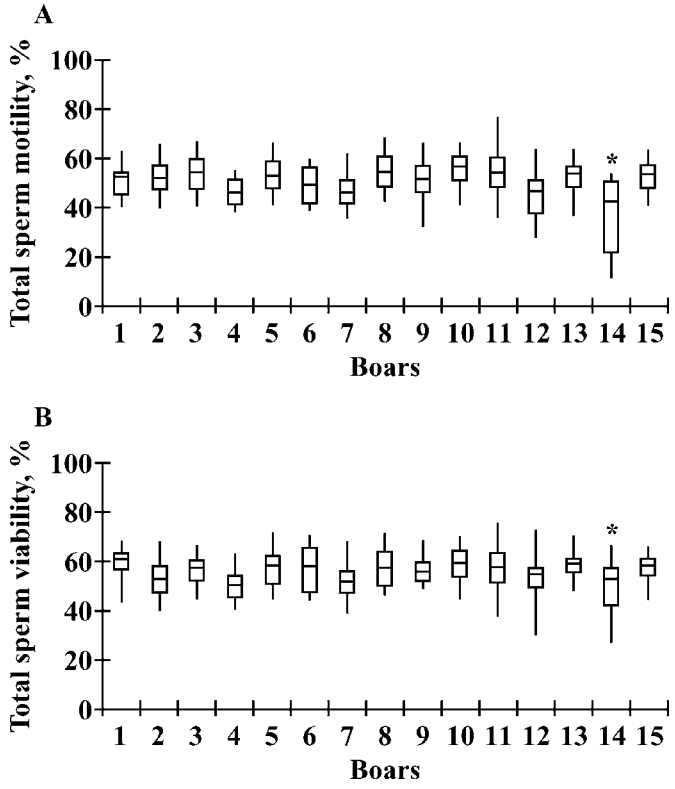

Figure 1. Box-whisker plots showing variation in total sperm motility (A) and viability (B) postthawing of cryopreserved semen of 15 boars (12 ejaculates per boar; Exp. 3). Boxes enclose the 25th and 75th percentiles, the line is the median, and whiskers extend to the 5th and 95th percentiles. Data are means of 2 postthaw evaluation times (30 and 150 min). *Boars with differences ($P < 0.05$) between ejaculates (intraboar variability).

postthaw sperm quality values could be expected from certain breeds, but there is likely to be considerable variability among ejaculates within those breeds. The same circumstance could occur with the other factors, such as age of the boar. For example, significant differences in postthaw sperm motility have been reported between ejaculates collected from boars grouped in different age ranges (Joyal et al., 1986). However, according to the multiple regression analysis in Exp. 1, age of the boar does not explain the variance observed in postthaw sperm quality.

Traditionally, great importance has been given to conventional measurements of semen quality for the selection of ejaculates suitable for cryopreservation. Commonly, only ejaculates exceeding certain quality limits are selected. However, to the best of our knowledge, there have been no specific studies correlating conventional semen traits or sperm quality assessments before freezing with sperm quality postthaw. In Exp. 1, multiple linear regression analysis indicated that sperm concentration together with sperm morphology and sperm motility before freezing were the only variables that should be considered for the selection of boar ejaculates for cryopreservation. However, only 23.2 and 10.9% of total variance in postthaw sperm motility and viability, respectively, could be explained by these variables. This weak predictive value of conventional semen measurements and sperm quality assessments before freezing was not surprising because we and others have observed previously, using individual ejaculates from the same boars, that sperm quality and the in vitro fertilizing ability of cooled semen are not statistically related to the values obtained postthaw (Rath and Niemann, 1997; Roca et al., 2000).

Despite having a weak predictive value, a positive correlation was found between ejaculate measurements and postthaw sperm quality. This suggested that those ejaculates with increased sperm concentration, percentage of normal morphology and motility before freezing should have the best sperm recovery after cryopreservation. In an attempt to establish possible limiting values for sperm measurements and determine which ejaculates should be selected for cryopreservation, a PATN analysis was performed to classify the ejaculates into groups according to postthaw sperm quality (Thurston et al., 2001; Gil et al., 2005). Focusing on the ejaculates classified as good, the PATN analysis confirmed the results of the multiple regression analysis, in that the good ejaculates were those that showed the greatest mean values for sperm concentration, normal morphology, and motility before freezing. However, with a careful evaluation of the data, it was clear that ejaculates showing good sperm cryosurvival had a wide range of sperm concentration, normal morphology, and motility before freezing. Moreover, a similar range was observed also in the ejaculates classified as moderate and poor according to sperm cryosurvival. Therefore, increased sperm concentration, together with the greatest proportions of sperm with normal morphology and motility before freezing, did not necessarily guarantee good sperm survival after freezing and thawing. Moreover, ejaculates with low sperm concentration and moderate sperm quality immediately after collection or before freezing could have good sperm quality postthaw. Overall, these data demonstrated that conventional semen measurements and sperm quality assessments before freezing were quantitative rather than qualitative with regard to their relationship with sperm survival after cryopreservation. Thus, they could be used to reduce the variability among ejaculates in postthaw sperm quality, but they did not provide an accurate prediction of the survival of sperm in an ejaculate after cryopreservation.

In Exp. 2, we attempted to reduce the variability in sperm survival postthaw attributed to ejaculate and sperm variables by preselection of ejaculates before cryopreservation. Hence, only ejaculates with more than 200×10^6 sperm per mL and with percentages of normal morphology, sperm motility, and viability before freezing above 85, 75, and 80%, respectively, were cryopreserved (4 ejaculates from each of 29 boars). Under these conditions, the ejaculate and sperm variables did not have any predictive value for sperm cryosurvival. More-

over, despite the rigorous ejaculate selection criteria, considerable variability of sperm cryosurvival among ejaculates was still observed, especially among boars. The interboar variability in postthaw sperm quality found in the current study was not surprising because it has been extensively demonstrated by others (Larsson and Einarsson, 1976; Thurston et al., 2001, 2002; Medrano et al., 2002; Saravia et al., 2005). Moreover, individual male differences in sperm cryosurvival are not exclusive to pigs because they have also been observed in horses (Janett et al., 2003) and sheep (D'Alessandro and Martemucci, 2003). However, this is the first study, evaluating several factors, that has demonstrated the importance of boar in explaining variability between ejaculates in sperm cryosurvival. More that 70% of total variance among ejaculates in postthaw sperm quality was explained by boar. This suggests that boar is the primary factor influencing ejaculate variability in sperm cryosurvival and that it should be the most important criterion for selecting ejaculates for cryopreservation.

The reason for boar variability in cryosurvival of sperm is unknown at present, although it may have a genetic origin. Differences in specific DNA sequences have been identified between boars in which postthaw sperm quality was classified as poor or good (Thurston et al., 2002). However, such individual variations may be minimized when epididymal sperm are cryopreserved. For example, in a limited study involving 3 boars from which epididymal and ejaculated sperm were cryopreserved, Rath and Niemann (1997) observed that boar differences in postthaw motility were only significant for ejaculated sperm, with epididymal sperm having a consistently high postthaw motility. Although no clear explanation was given by these researchers because it was not the aim of their study, 2 of the 3 boars had differences in postthaw survival between epididymal and ejaculated sperm. This could be related to variation in exposure of sperm to the complex secretions of the accessory sex glands during ejaculation, which could modify the cryosurvival of the sperm. In this case, differences in seminal plasma composition between good and poor boars could explain the variation in sperm cryosurvival. This hypothesis remains to be investigated.

As boar was found to be the primary factor explaining variability in sperm cryosurvival between ejaculates, it seemed logical to select boars with good postthaw sperm survival as cryobank founders. However, there was no evidence that boars could maintain good sperm cryosurvival in ejaculates over time. In Exp. 2, 25 of 29 boars were found to maintain consistent postthaw sperm quality from 4 ejaculates collected and cryopreserved at intervals over a period of 6 mo. This finding was confirmed in Exp. 3, where postthaw sperm quality was consistently high in all ejaculates cryopreserved from 14 of the 15 boars, indicating that boars with semen classified as having good freezability could maintain this condition over time. This suggests that the assessment of a single ejaculate after cryopreservation may be sufficient to identify those with good freezability scores for selection as cryobank founders. Further investigation is required to confirm this finding.

Intraboar variability in sperm cryosurvival was also observed in the current study, although it was less important than interboar variation. However, from a practical and commercial viewpoint, intraboar variability should be taken into account, as it was observed in 4 of the 29 boars in Exp. 2 and in 1 of the 15 boars in Exp. 3. The reasons provided by various authors for this variation are not consistent, but it may be related to poor sustainability of the cryopreservation process (Thurston et al., 2001). This was not the case in the current study because the ejaculates from all boars, with or without significant intraboar variability, were processed at the same time. Transitory changes in boar health and inappropriate ejaculate manipulation before freezing have been also suggested as possible causes of this variation (Roca et al., 2006). Therefore, good knowledge of boar health and careful manipulation of ejaculates before freezing are of practical importance to minimize intraboar variability in sperm cryosurvival between ejaculates.

In conclusion, the results of the current study demonstrate that the boar is the most important factor explaining the variability in sperm cryosurvival among ejaculates. Although a certain degree of intraboar variability was observed, most boars showed consistent sperm cryosurvival over time. Standard semen measurements in fresh ejaculates and sperm quality assessments before freezing were shown to have limited value in explaining variation in postthaw sperm quality between ejaculates. Nevertheless, those ejaculates containing a high sperm concentration and a high proportion of motile sperm with normal morphology before freezing are the best to select for cryopreservation.

LITERATURE CITED

Abaigar, T., W. V. Holt, R. A. P. Harrison, and G. Delbarrio. 1999. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessment. Biol. Reprod. 60:32–41.

Almlid, T., and P. O. Hofmo. 1996. A brief review of frozen semen application under Norwegian AI service conditions. Reprod. Domest. Anim. 31:169–173.

Carvajal, G., C. Cuello, M. Ruiz, J. M. Vazquez, E. A. Martinez, and J. Roca. 2004. Effect of centrifugation before freezing on boar sperm cryosurvival. J. Androl. 25:389–396.

D'Alessandro, A. G., and G. Martemucci. 2003. Evaluation of seasonal variations of semen freezability in Leccese ram. Anim. Reprod. Sci. 79:93–102.

Eriksson, B. M., H. Petersson, and H. Rodriguez-Martinez. 2002. Field fertility with exported boar semen frozen in the new flat-pack container. Theriogenology 58:1065–1079.

Gil, M. A., J. Roca, T. Cremades, M. Hernandez, J. M. Vazquez, H. Rodriguez-Martinez, and E. A. Martinez. 2005. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 64:305–316.

Graham, J. K., E. Kunze, and R. H. Hammerstedt. 1990. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biol. Reprod. 43:55–64.

Janett, F., R. Thun, S. Bettschen, D. Burger, and M. Hassig. 2003. Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. Anim. Reprod. Sci. 77:213–221.

Joyal, S. M., B. W. Kennedy, and J. N. Wilkins. 1986. Boar, breed and environmental effects on motility of frozen-thawed spermatozoa. Can. J. Anim. Sci. 66:663–668.

Larsson, K., and S. Einarsson. 1976. Influence of boars on the relationship between fertility and post thawing sperm quality of deep frozen boar spermatozoa. Acta Vet. Scand. 17:74–82.

Medrano, A., J. Anderson, J. D. Millar, W. V. Holt, and P. F. Watson. 2002. A custom-built controlled-rate freezer for small sample cryopreservation studies. Cryo Lett. 23:397–404.

Park, C. S., and Y. J. Yi. 2002. Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. Anim. Reprod. Sci. 73:53–61.

Pursel, V. G., and L. A. Johnson. 1974. Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. Theriogenology 1:63–68.

Pursel, V. G., and L. A. Johnson. 1975. Freezing of boar spermatozoa: Fertilizing capacity with concentrated semen and a new thawing procedure. J. Anim. Sci. 40:99–102.

Rath, D., and H. Niemann. 1997. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology 47:785–793.

Roca, J., G. Carvajal, X. Lucas, J. M. Vazquez, and E. A. Martinez. 2003. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. Theriogenology 60:77–87.

Roca, J., X. Lucas, M. A. Gil, J. M. Vazquez, G. Carvajal, and E. A. Martinez. 2000. Motility and in vitro penetrating ability of cooled and frozen-thawed spermatozoa from identical boars. Page 260 in Boar Semen Preservation IV. L. A. Johnson and H. D. Guthrie, ed. Allen Press, Lawrence, KS.

Roca, J., H. Rodriguez-Martinez, J. M. Vazquez, A. Bolarin, M. Hernandez, F. Saravia, M. Wallgren, and E. A. Martínez. 2006. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. Pages 261–275 in Control of Pig Reproduction VII. C. J. Ashworth and R. R. Kraeling, ed. Nottingham Univ. Press, Nottingham, UK.

Saravia, F., M. Wallgren, S. Nagy, A. Johannisson, and H. Rodriguez-Martinez. 2005. Deep freezing of concentrated boar semen for intra-uterine insemination: Effects on sperm viability. Theriogenology 63:1320–1333.

Thurston, L. M., K. Siggins, A. J. Mileham, P. F. Watson, and W. V. Holt. 2002. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol. Reprod. 66:545–554.

Thurston, L. M., P. F. Watson, A. J. Mileham, and W. V. Holt. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with variation in boar semen quality following cryopreservation. J. Androl. 22:382–394.

Westendorf, P., L. Richter, and H. Treu. 1975. Zur Tierfgefrierung von Ebersperma: Labor und besamungsergebnisse mit dem Hülsenberger Pailleten Verfahren. Dtch. Tierárztl Wschr. 82:261–267.

Artículo 2

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Cryo-scanning electron microscopy (Cryo-SEM) of semen frozen in medium-straws from good and sub-standard freezer AI-boars ☆

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Abstract

A major limiting factor for commercial cryopreservation of boar semen for artificial insemination (AI) is the large individual variation to cooling, where the degree of cell dehydration during ice (re)shaping seems to play a major role. This study investigated, in the frozen state, the degree of dehydration and ice crystal distribution in boar semen doses whose spermatozoa displayed different viability after thawing. Cross-sectioned medium-straws (0.5 mL, $n = 10$) from a total of 10 stud boars classified as "good" ($n = 5$) or sub-standard (e.g., "bad" freezers, $n = 5$) by conventional analyses (computer assisted motility and sperm viability) were examined by Cryo-scanning electron microscopy (Cryo-SEM) to determine whether differences between groups could be already distinguishable prior to thawing. The degree of hydration was monitored in relation to the areas of ice crystal formed extracellularly (lakes), the areas of frozen, concentrated extender (veins) where spermatozoa were located and the degree of compartmentalization (number of lakes) present. Irrespectively of the region studied, the gradient of main dehydration (as lakes) observed along the cross-section area of the straws was very irregular. Most spermatozoa were enclosed in the freezing extender matrix and no obvious signs of external membrane damage were observed. None of the Cryo-SEM variables significantly correlated with post-thaw sperm parameters ($p > 0.05$). However, we identified significant differences ($p < 0.0001$) among boars for all ultrastructure variables studied, including the size of the veins, where differences in solute concentration is expected. We concluded that despite the large variability in ice crystal formation during the conventional freezing process among boars, this is unrelated to inter-boar post-thaw sperm differences.

Keywords: Pig; Spermatozoa; Ultrastructure; Ice crystal; Cryopreservation

Boar semen for artificial insemination (AI) was successfully frozen more than 35 years ago [6,32,44]. Already then, variation was noticed among boars in the ability of their semen so sustain cryopreservation [23,34,46]. Despite the process of cryopreservation has improved over this period, this inter-sire variation is still present [21]. This variation hampers the application of this technology to all boars, ultimately conspiring against a wider use of AI.

Attempts have been done in order to minimise variability among boars and, indirectly, enhance the overall post-thaw sperm quality of boar semen. Optimization of the cryopreservation protocol, including use of novel packaging systems for freezing and thawing [14,37], different cooling rates [7], inclusion of different additives [16,30,33] or the use of particular ejaculate portions [29] has reduced,

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but not eliminated, inter-boar variation for sperm cryosurvival. The reason behind such variability is yet to be elucidated [21].

The low survival post-thaw that characterizes boar semen has been classically attributed to unsustainable osmotic changes and water flow during freezing and thawing that disrupt the plasma membrane with subsequent cell-death [47]. During freezing of semen extended with glycerol as cryoprotectant, ice nucleation starts in the external medium (between −5 and −10 °C) with subsequent physical events in the cells and the supercooled medium, supposedly dependant on the speed of cooling. An optimal rate of cooling is necessary to avoid both an extensive loss of intracellular water leading to severe cell dehydration or the formation of excessive intracellular ice which could, consequently, lead to re-crystallisation at thawing (see [17] for review). Recent theoretical simulations have set the optimal rate of cooling after ice nucleation at around 30 °C/min for boar spermatozoa [7]. However, after re-warming or thawing, boar sperm quality assessment still reveals important male-to-male differences [25], which could indicate that males differ in their sperm membrane permeability characteristics [47]. Such differences could lead to different degrees of dehydration and, therefore, to larger amounts of solid-state water in the extracellular, outer–extender areas of a frozen sample.

Cryo-scanning electron microscopy (Cryo-SEM) is a technique that allows the study of the fully hydrated ultrastructure of sperm samples in the frozen state without the introduction of artefacts caused by fixation or processing [36]. Cryo-SEM allows visualization of the topography of the sample, including the distribution and size of the frozen water, which are dictated by the freezing steps [38]. This method has been employed to investigate the fine structure of frozen samples and the effects of different cooling rates on post-thaw sperm survival [26], using different packaging systems [9] or glycerol concentrations [27].

The objective of the present study was to investigate, using Cryo-SEM, the levels of *in situ* dehydration and levels of homogeneity of semen frozen in medium-straws from good and sub-standard freezer boars used for artificial insemination (AI). We hypothesized that inter-boar differences in post-thaw sperm survival could be related to individual dehydration differences during the freezing process.

Materials and methods

Sperm-rich ejaculate fractions were collected, using the gloved-hand method, from sexually mature boars of various commercial hybrid lines undergoing regular semen collection for commercial AI. Semen was evaluated and extended (1:1, v/v) with Beltsville Thawing Solution (BTS, 205 mmol/L glucose, 20.39 mmol/L NaCl, 5.4 mmol/L KCl, 15.01 mmol/L NaHCO3 and 3.35 mmol/L EDTA) containing kanamycin sulfate (50 μg/mL), transferred into 50 mL tubes, packaged in insulated containers at 17 °C, and delivered 14–16 h later to the Laboratory of Andrology of the Veterinary Teaching Hospital of the University of Murcia, Spain. All ejaculates had $\geqslant 200 \times 10^6$ spermatozoa/mL, ⩾85% spermatozoa with normal morphology, ⩾75% of them progressively motile and ⩾80% displaying intact membranes, before cooling and freezing. At the laboratory, the ejaculates were frozen following the straw freezing procedure described by [43] and modified by [41] and [4]. In brief, the extended semen was centrifuged at 2400*g* for 3 min at 17 °C. The resulting pellets were re-extended in lactose egg-yolk (LEY) extender (80% [v/v] 310 mM β-lactose, 20% [v/v] egg yolk and 100 μg/mL kanamicyn sulfate, pH 6.2 and 330 ± 5 mOsmol kg^{-1}) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5 °C within 90 min, the extended spermatozoa were re-suspended with LEY-Glycerol-OrvusESPaste (LEYGO) extender (92.5% LEY, 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass] and 6% [v/v] glycerol; pH 6.2 and 1650 ± 15 mOsmol kg^{-1}) to yield a final concentration of 1×10^9 cells/mL. The cooled spermatozoa were packed into 0.5 mL medium plastic straws (Minitüb, Tiefenbach, Germany), and frozen using a controlled-rate freezer (IceCube 1810, Minitüb, Tiefenbach, Germany), as follows: from 5 to −5 °C at a rate of 6 °C/min, from −5 to −80 °C at 40 °C/min, held for 30 s at −80 °C, then cooled at 70 °C/min to −150 °C and plunged into liquid nitrogen for storage until analysis.

Sperm quality assessment post-thaw

Two straws from each ejaculate were thawed at 37 °C for 20 s, pooled and re-extended at 37 °C with BTS (1/1, v/v) and kept in the water-bath (37 °C) during sperm evaluation.

Sperm motility (as total sperm motility, TSM) was evaluated using a computer assisted sperm analysis (CASA) system (Sperm Class Analyzer® Microptic, Barcelona, Spain) in thawed semen samples further re-suspended in BTS to reach a concentration of 30×10^6/mL. For each evaluation, 4 μL of the sperm sample were placed in a pre-warmed (39 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and three fields were analysed to assess a minimum of 100 spermatozoa/sample.

Sperm viability (VIA) was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity of sperm samples re-suspended in BTS to yield a concentration of 15×10^6/mL. Sperm viability was analysed simultaneously using a modification of a triple fluorescent staining method described by [18], and adapted for boar spermatozoa by [4], which included the DNA-specific fluorochrome propidium iodide (PI), the mitochondria-specific fluorochrome rhodamine-123 (R123) and the acrosome-specific fluorochrome fluorescein isothiocyanate labelled peanut (Arachis hypogaea) agglutinin (FITC-PNA) (Sigma Chemical Co, St Louis, Mo). Two hundred spermatozoa per sample were examined under 1000× magnification (Eclipse E800, Nikon, Tokio, Japan), using BV-2A filter (400–440 nm excitation filter, 455 nm

emission, 470 barrier filter). Spermatozoa showing only green fluorescence over the mid-piece (R123 positive) were considered viable and with an intact acrosome (VIA).

Following these evaluations of post-thaw sperm quality, cryopreserved ejaculates were objectively classified by multivariate pattern analysis [1] into three groups, as described in detail by [19]. The classification of ejaculates was highly consistent within boar. Those boars with the best frozen-thawed sperm characteristics (>60% of motile spermatozoa [TSM] and VIA) were identified as "good" freezers whereas boars showing reduced frozen-thawed sperm characteristics were considered as "moderate" freezers (40–60% TSM and VIA) or "bad" (<40% TSM and VIA). Only straws from boars classified as "good" ($n = 5$) and sub-standard (e.g, "poor", $n = 5$) freezers were used in the current study.

Cryo-scanning electron microscopy (Cryo-SEM)

Cryo-SEM was used to examine the ultrastructure of the samples and determine whether the amounts of solid-state water in the extracellular, outer–extender areas of frozen straws differed between straws from boars with different sperm cryosurvival post-thaw. Working inside liquid nitrogen, the straw was manually broken, the plastic removed, and a cylinder of the frozen semen mounted in a mechanical grip holder. The grip holder was transferred into an Oxford HT 1500F Cryo System chamber attached to the microscope (JEOL Scanning Microscope 6320F, Tokyo, Japan). Once the sample was inside the chamber, a fracture of the sample was made to get a fresh clean surface to be examined. The temperature of the sample was raised by heating the holder to −92 °C for 30 s in order to sublimate free water in the solid state lakes, followed by a temperature decrease to −130 °C to stabilise the sample. The surface of the frozen preparation was then coated with platinum/palladium to obtain a good relation between signal and noise. The coated sample was thereafter transferred into the microscope chamber where it was analysed at a temperature range of −125 to −135 °C. Digital images (7–10 per straw at 600× for image analyses and 3000× or 4000× for fine structural examinations) were collected at 5 KV and computer stored (JEOL SemAfore software, Sollentuna, Sweden) before being further assessed by image analysis (EasyImage 3000 software, version 1.3.0, Tekno Optik, Huddinge, Sweden). Three variables were retrieved per image: the area of the freezing extender matrix (containing spermatozoa, e.g., veins), the area of the spaces occupied by the partially sublimated solid-state water (extracellular, outside the extender, e.g., lakes) and the degree of compartmentalization in the sample (number of lakes per image). The area of each variable was expressed as the relative percentage out of the total image area.

Statistics

Data were expressed as ranges and percentages (mean ± SD). The multivariate pattern analysis into three groups was performed using the PATN pattern analysis package to identify naturally occurring subgroups within the ejaculate data set, as described in detail by [1]. The PATN analysis classified the ejaculates, using post-thaw sperm variables (sperm motility, including motion characteristics and sperm viability), into a small number of groups. Three groups were finally obtained from the non-hierarchical classification of the ejaculates tested. The data for thawing of the three groups of ejaculates were calculated and compared using one-way ANOVA. Statistical analyses were performed by SPSS, version 14 (SPSS Inc, Chicago, Il, USA). The non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests were used to evaluate differences on Cryo-SEM variables between boars and groups ("good" and sub-standard ["bad"] boars). Spearman correlations were calculated to disclose relationships between post-thaw sperm quality variables and Cryo-SEM measurements. The level of significance was set at $p < 0.05$.

Results

Representative Cryo-SEM micrographs are depicted in Figs. 1 and 2. The images clearly show that neither the number nor the distribution of ice lakes (or their counterpart veins) followed a uniform pattern (compare Figs. 1a–d), irrespective of the considered group of boars or the area of the section of the straw (peripheral *vs* central). The relative size and shape of the lakes varied within the same straw. The complementary relative surface (as %) of the veins (frozen concentrated extender and included spermatozoa) showed a similar and homogeneous width in both groups of boars but without a linear trend of solidification. Spermatozoa were, largely without exception, entrapped within the veins of concentrated extender (Fig. 2, 3000×) and only very few spermatozoa or sperm structures were occasionally apparent in the lakes. At high magnification (4000×), the majority of the spermatozoa appeared intact, without obvious signs of plasma membrane disruption (Fig. 2).

The overall relative mean percentage of the lake fraction (e.g., of solid-state free water in the frozen straws) occupied ~80% of the total surface while that of the veins was ~20%. There were no significant differences ($p > 0.05$) between the two groups of boars (Table 1). In the sub-standard ("bad") sperm freezers the area of the freezing extender matrix (veins) averaged 17.7% (range, 10.5–21.3%), being 82.3% (range, 78.7–89.6%) for the area of the spaces occupied by ice crystal (lakes), the number of the latter averaging 35.6 (range, 16–64). The corresponding values measured in "good" sperm freezers were 18.5% (range, 11.6–22.7%), 81.5% (range, 77.7–88.4%) and 47.3 (range, 12–111), for the area of veins, area of lakes and number of lakes, respectively. None of the variables correlated significantly ($p > 0.05$) with the post-thaw sperm

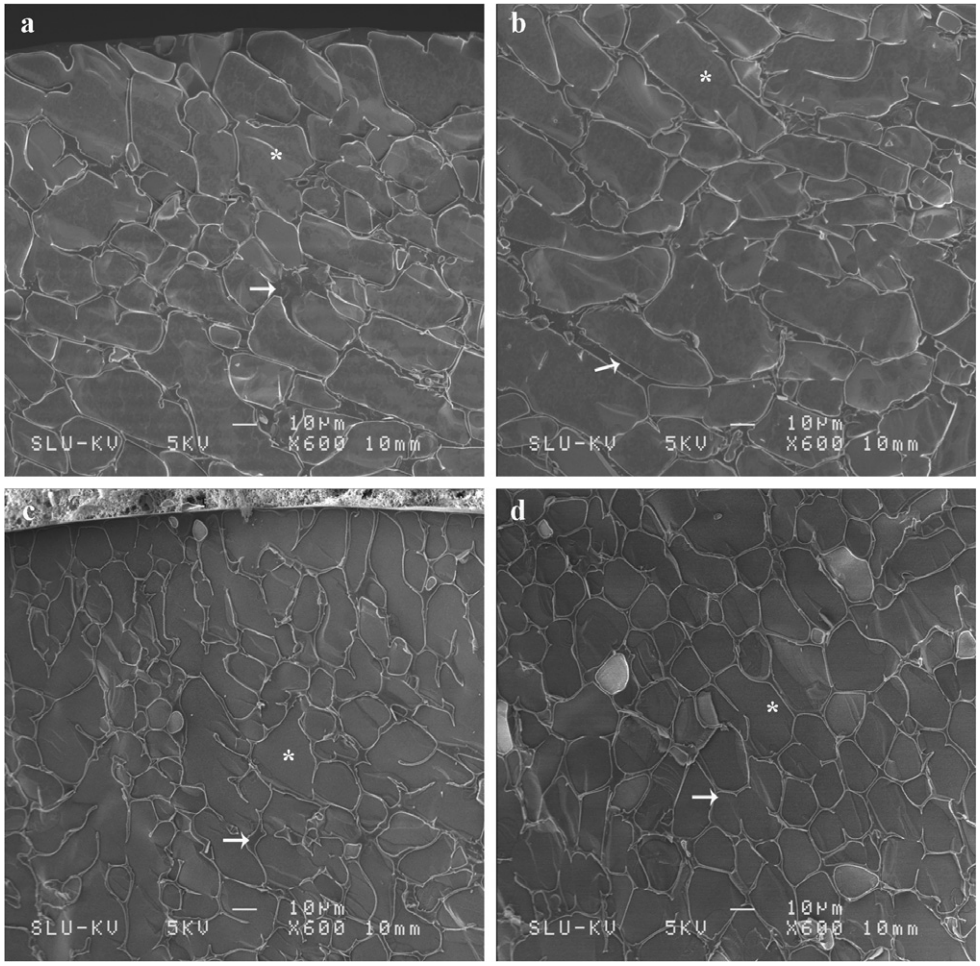

Fig. 1. (a–d) Cryo-SEM micrographs of boar sperm samples cryopreserved in 0.5 mL straws from boars classified as (a–b) "bad" freezer or (c–d) "good" freezer according to their post-thaw sperm quality (motility and viability). (a, c) Image of the periphery of the straw (600×). (b, d) Image of the centre of the straw (600×). Note the distribution of the external ice crystals (lakes∗) and the freezing extender matrix (veins, arrows).

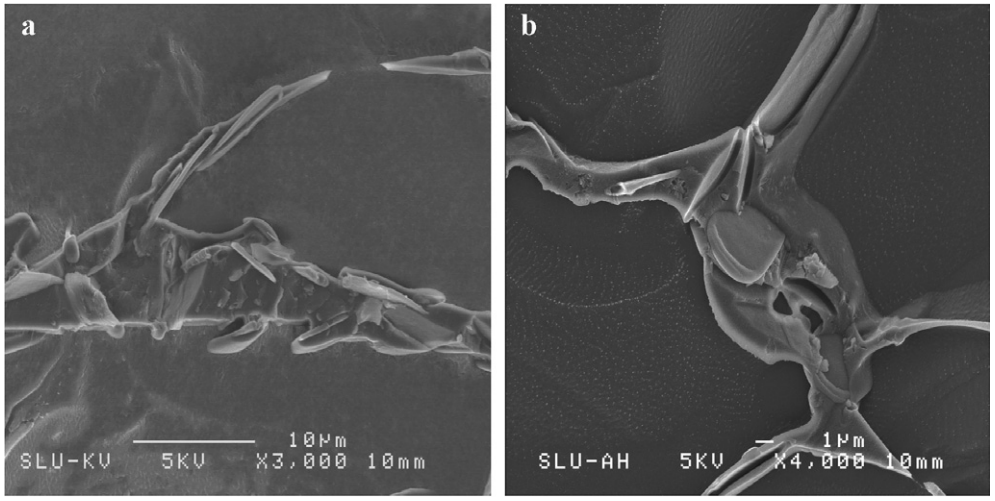

Fig. 2. (a–b) Cryo-SEM micrographs of boar sperm samples cryopreserved in 0.5 mL straws. (a) Most spermatozoa are located in veins of concentrated extender that separate large ice lakes (3000×). (b) Detail image of a representative spermatozoon entrapped in the freezing extender matrix (4000×), where there was no obvious evidence of external damage.

quality parameters (Table 2). However, significant inter-boar differences in all the Cryo-SEM measures were detected ($p < 0.0001$), showing one of the boars much more compartmentalization of veins, leading to an increased number of lakes per area (86.4 ± 17.7, mean $\pm$ SD).

Table 1
Ultrastructural analysis by Cryo-scanning electron microscopy (Cryo-SEM) of frozen medium-straws containing processed semen from boars classified as "good" or sub-standard (e.g., "bad") sperm freezers according to their post-thaw sperm quality

Images variables	Boar groups[a]		Probability
	"Good" ($n = 5$)	Sub-standard ("Bad", $n = 5$)	
Area of veins[b] (%)	17.7 ± 2.27	18.5 ± 2.34	0.069
Area of lakes[c] (%)	82.3 ± 2.27	81.4 ± 2.34	0.069
Number of lakes	38.6 ± 11.61	47.3 ± 24.70	0.284

Descriptive data are given as mean ± SD. A total of 83 images were compared using the non-parametric Mann–Whitney *U*-test for eventual differences.

[a] Boars (one ejaculate per boar; one straw per ejaculate).

[b] The area of the freezing extender matrix.

[c] The area of the spaces occupied by extracellular, outer from extender ice lakes. The area was expressed as the relative proportion of the total image area.

Discussion

In our experience, cryopreservation of a single boar ejaculate, using it as a simple freezability test, has shown to be useful to identify sub-standard ("bad") boar sperm freezers, mostly owing to the little intra-boar variability we have found over time [13,35,45] and the prevalence of an inter-boar variation for survival post-thaw [14,41,47]. However, little is known about the inter-boar differences that could exist during the rest of the freezing steps, besides what has been found when studying different packages [2]. Clearly, allocation of ice and degree of dehydration follows particular patterns [3] that relate to theoretical predictions [15,20,24].

During conventional cryopreservation, with a rather slow speed of cooling, the movement of water and of cryoprotectant across the plasma membrane and of organelles affects the survival of boar spermatozoa [28]. At slow cooling rates, the degree of sperm dehydration is higher, and the intracellular water permeates across the cell and joins the extracellular water, than at high cooling rates, where the water freezes inside the cell [3,7,8,12,24,26]. The current cryopreservation protocols are built on the use of rather slow cooling rates, and therefore most of the free water is removed from the cells and the immediate adjacent concentrated extender to freeze in extracellular lakes [9]. The present study confirms these data. Hereby, more than 80% of the free water was present in the extracellular lakes and pressed the highly dehydrated spermatozoa and adjacent extender to narrow veins of varying dimensions. Since a certain proportion of water remains in the veins, and this proportion dictates how high the concentration of the solutes is around the spermatozoa; the variation in vein size might relate to the concentration of the solute. Most evidence links the degree of dehydration to membrane damages post-thaw [5,28] and opened the question behind the hypothesis of the present study; e.g., the source of individual variability on sperm cryosurvival, definitory of freezability, could be detected by assessing the size of the frozen free water in the straws of semen from boars considered as sub-standard ("bad") freezers or "good" freezers, as deriving from different dehydration grades while running a conventional single, well controlled, freezing protocol. Since the variation was clearly present among boars for vein size and aspect, it might be possible that the boars differed just in their membrane's ability to sustain cooling and thus dehydrate differently. Such studies would require of a much detail study of the veins size (area and volume) a matter not deprived of difficulties since the border of the veins is always difficult to measure in a precise way.

The structure of the ice crystals is essentially fixed by the temperature of ice nucleation in the under-cooled straws [26]. In samples where ice nucleation was manually induced (e.g., seeding at −7 °C), followed by rapid (1.000 °C/min) cooling, the surface of the veins, as registered by Cryo-SEM, occupied a much larger area and contained numerous ice crystals than when slow cooling (10 °C/min) was applied [27]. At a cooling of 10 °C/min (to −100 °C), the surface area of the veins was smaller (∼15%) [27]. The present results, using a cryopreservation protocol without seeding and with a cooling rate of 40 °C/min, yielded a relative vein surface of approx 18%, which confirms the effect of the cooling rate on the exit of water from the cells and the immediate extender, to the lakes. The lakes contain, almost without exception, free water in a solid state, their size

Table 2
Spearman correlation coefficients between sperm quality variables of the assessed post-thaw boar semen and the Cryo-SEM variables of frozen medium-straws containing processed semen from boars classified as "good" or sub-standard (e.g., "bad") sperm freezers according to their post-thaw sperm quality

Variable	TSM (%)	VIA(%)	Area of veins (%)	Area of lakes (%)	Number of lakes
TSM (%)		0.945**	−0.042	0.042	−0.115
VIA (%)			0.430	−0.430	0.248
Area of veins				−1.000**	0.713*
Area of lakes					0.713*
Number of lakes					

TSM: % of total motile spermatozoa determined by CASA analyses, VIA: % of viable spermatozoa with intact acrosomes as assessed by a triple stain. Area of veins: the area of the freezing extender matrix, area of lakes: the area of the spaces occupied by extracellular, outer from extender ice lakes, as assessed by Cryo-SEM and expressed as the relative proportion of the total image area.

* $p < 0.05$.

** $p < 0.01$.

being directly related to the degree of dehydration. Despite the fact that all the straws were frozen under the same condition, in the same extender, with identical cryoprotectant concentration and cooling rates, there were apparent fluctuations in the movement of water and cryoprotectant across the plasma membrane among samples, since the size and aspect of the veins differed among boars. Whereas most straws presented the same percentage in relative areas of veins and lakes, the distribution in size was completely arbitrary. This would indicate the presence of an irregular ice diffusion across the straw, not bound to the post-thaw sperm quality registered in complementary samples of the same ejaculates (e.g., freezing batches).

During conventional methods used for freezing semen, ice nucleation is a stochastic event [38]. It may occur slightly below the freezing point or more than 10–20 °C below that temperature [39]. Moreover, if ice nucleation is not taken into control, the different samples within the same controlled rate freezer will have different nucleation temperatures and different ice crystal structures [38]. This will modify the recovery on thawing and it is considered a major factor in sample-to-sample variation during cryopreservation [47]. It has been suggested that the induction of extracellular ice crystals slightly below the freezing point (seeding) eliminates the arbitrariness of ice nucleation and the cooling conditions become more controllable [39,47]. Under this condition, the structure of the veins usually follows linear cooling and linear ice fraction solidification, leading to a uniform arrangement of lakes and veins [26]. Seeding of supercooled semen samples appears beneficial for cryosurvival. For instance, human semen frozen in a conventional programmable freezer and where ice nucleation was induced by seeding, resulted in a significantly higher sperm motility post-thaw compared to non-seeded samples [26]. This beneficial effect of seeding on post-thaw sperm survival has been clearly manifested on boar spermatozoa frozen flattened using a cryomicroscope [47] or in 0.5 mL straws [22]. Seeding is, however, not routinely included in most sperm freezing protocols for boar semen and neither in our current protocol, which might have accounted for the frozen patterns found in our samples. Another factor that might accounted for the patterns found, could be the use of glycerol as cryoprotectant since freezing in glycerol solutions, increasing supercooling shifts ice morphology from hexagonal to irregular shapes [38]. Also the customary addition of sodium dodecylsulphate (laurylsulphate), (Orvus Es Paste) to the freezing extender increases the irregularity of the ice crystals, which tend to become large [11,36]. It is possible that the lack of seeding and the subsequent ice nucleation answers for the lacking of linear ice fraction solidification, and the presence of individual variations in the size of the veins and lakes hereby shown. Interestingly enough, those variations were more pronounced in the group of boars considered ''good'' freezers.

Cryo-SEM does not allow visualization of intracellular ice, a matter that can only be solved with freeze-substitution and transmission electron microscopy [5,8]. Neither can it determine the proportion of water in the veins, the size of which obviously relates to a given concentration of solutes. Cryo-SEM is, however, accurate to disclose the external structure of the frozen material following partial sublimation of water in the electron microscope. In the present study, the size of the extracellular ice appeared heterogeneous in most samples (different size and distribution of the lakes), their distribution being heterogeneous even across the medium-straw. Irregular ice distribution, with larger lakes in the periphery compared with the centre of the container has been found in Maxi-straws [36]. Similar observations concerning temperature differences through different parts of the Maxi-straw during freezing have also been done [3] which has been proposed as the major cause of their lower post-thaw sperm quality compared with other packages [2,14].

When observing the frozen medium-straw samples at higher magnification, neither the internal nor external surface of the straw revealed an increase in damaged spermatozoa or sperm structures enclosed in the frozen free water (lakes). Previous investigations, using ice granulometry, have found such damages more prevalent in maxi-straws compared to Flat-packs [10]. When ram or boar spermatozoa were frozen in medium-straws, most acrosomes and plasma membranes were destroyed in spermatozoa located in the centre of the straw while no intracellular ice was observed in cells placed close to the straw wall [5]. These varying percentages of membrane-intact spermatozoa have been explained by irregularities in the spreading of ice and temperature throughout the medium-straw because of the high surface area respect to volume ratio [31,47]. These somewhat conflicting results might depend on differences in the cryopreservation protocol used.

Up to now, the mechanisms related to the source of variation in sperm freezability have not been clearly elucidated. Medrano et al. [25], using direct freezing of small boar semen samples on a light cryomicroscope observed that sperm variability in cell damage was caused by plasma membrane instability during the rewarming process, confirming data from Ortman and Rodriguez-Martinez [28]. It has been suggested that this inter-boar variation could also have a genetic basis [40]. Recently, individual variability in sperm cryosurvival appeared to be partly related on intrinsic properties of the spermatozoa, such as membrane fatty acid composition [42]. If this variability could also be related with differences in some other sperm characteristics such as some specific protein content or some extrinsic features, remain unknown. In any case, instability of the plasma membrane may cause differences in dehydration rates and thus be linked to differences in solute concentration around the spermatozoa, a matter of concern at thawing, if thinking of sperm cryosurvival.

In conclusion, the present study indicates that there is a large individual variation in the degree of freezing of extracellular bound- and free water as measured by the Cryo-SEM, but this is apparently not linked to the post-thaw survival rate, that defined the apparent semen ''freezability'' of the tested boars. The results call for more detailed

studies of the amount of intracellular frozen water, the concentration of solutes and the ability of these spermatozoa, particularly at the plasma membrane level, to sustain rewarming, since this is probably where the major differences among individuals are registered.

References

[1] T. Abaigar, W.V. Holt, R.A. Harrison, G. del Barrio, Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments, Biol. Reprod. 60 (1999) 32–41.

[2] C.O. Bwanga, S. Einarsson, H. Rodriguez-Martinez, Cryopreservation of boar semen. II: Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags, Acta Vet. Scand. 32 (1991) 455–461.

[3] C.O. Bwanga, H. Ekwall, H. Rodriguez-Martinez, Cryopreservation of boar semen. III: Ultrastructure of boar spermatozoa frozen ultrarapidly at various stages of conventional freezing and thawing, Acta Vet. Scand. 32 (1991) 463–471.

[4] G. Carvajal, C. Cuello, M. Ruiz, J.M. Vazquez, E.A. Martinez, J. Roca, Effects of centrifugation before freezing on boar sperm cryosurvival, J. Androl. 25 (2004) 389–396.

[5] J.L. Courtens, J.M. Rety, Numerical simulation for freezing and thawing mammalian spermatozoa. Evaluation of cell injuries at different depths in bags or straws during all steps of the technique, Genet. Sel. Evol. 33 (2001) 83–104.

[6] B. Crabo, S. Einarsson, Fertility of deep frozen boar spermatozoa, Acta Vet. Scand. 12 (1971) 125–127.

[7] R.V. Devireddy, B. Fahrig, R.A. Godke, S.P. Leibo, Subzero water transport characteristics of boar spermatozoa confirm observed optimal cooling rates, Mol. Reprod. Dev. 67 (2004) 446–457.

[8] H. Ekwall, B. Eriksson, J.L. Courtens, H. Rodriguez-Martinez, Assessment of ice content and morphological alterations in boar spermatozoa frozen in maxi-straws, Proc. of the 13th ICAR Sydney 2 (1996) P2–P5.

[9] H. Ekwall, B.M. Eriksson, H. Rodriguez-Martinez, Cryo-EM of frozen boar semen, Proc. of the Annual Meeting of the Royal Microscopy Society, "Low temperature microscopy and analysis, York, UK, 1997 pp. P-2.

[10] H. Ekwall, B.M. Eriksson, J.L. Courtens, H. Rodriguez-Martinez, Ice granulometry and cell damages in boar semen frozen in two types of containers, Infus. Ther. Transfus. Med. 245 (1997) 29.

[11] B.M. Eriksson, H. Ekwall, J.L. Courtens, H. Rodriguez-Martinez, Effect of sodium dodecylsulphate on the frozen status and post-thaw viability of boar spermatozoa frozen in maxi-straws and plastic bags, Proc. of the 13th ICAR, Sydney 2 (1996) P2–P6.

[12] B.M. Eriksson, H. Ekwall, H. Rodriguez-Martinez, Effect of freezing rates on the ultrastructure of the frozen state and the postthaw viability of boar semen, Cryobiology 35 (1997) 368.

[13] B.M. Eriksson, H. Petersson, H. Rodriguez-Martinez, Field fertility with exported boar semen frozen in the new flatpack container, Theriogenology 58 (2002) 1065–1079.

[14] B.M. Eriksson, H. Rodriguez-Martinez, Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws, Anim. Reprod. Sci. 63 (2000) 205–220.

[15] P.S. Fiser, R.W. Fairfull, Combined effect of glycerol concentration and cooling velocity on motility and acrosomal integrity of boar spermatozoa frozen in 0.5 ml straws, Mol. Reprod. Dev. 25 (1990) 123–129.

[16] J. Gadea, D. Gumbao, C. Matas, R. Romar, Supplementation of the thawing media with reduced glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after cryopreservation, J. Androl. 26 (2005) 749–756.

[17] D.Y. Gao, P. Mazur, J.K. Critser, Fundamental cryobiology of mammalian spermatozoa, in: A.M. Karow, J.K Critser, San Diego, CA, 1997, pp. 263–328.

[18] J.K. Graham, E. Kunze, R.H. Hammerstedt, Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry, Biol. Reprod. 43 (1990) 55–64.

[19] M. Hernandez, J. Roca, J. Ballester, J.M. Vazquez, E.A. Martinez, A. Johannisson, F. Saravia, H. Rodriguez-Martinez, Differences in SCSA outcome among boars with different sperm freezability, Int. J. Androl. 29 (2006) 583–591.

[20] W.V. Holt, Fundamental aspects of sperm cryobiology: the importance of species and individual differences, Theriogenology 53 (2000) 47–58.

[21] W.V. Holt, A. Medrano, L.M. Thurston, P.F. Watson, The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope, Theriogenology 63 (2005) 370–382.

[22] S. Kumar, J.D. Millar, P.F. Watson, The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines, Cryobiology 46 (2003) 246–253.

[23] K. Larsson, S. Einarsson, Fertility of deep frozen boar spermatozoa: influence of thawing diluents and of boars, Acta Vet. Scand. 17 (1976) 43–62.

[24] P. Mazur, The role of intracellular freezing in the death of cells cooled at supraoptimal rates, Cryobiology 14 (1977) 251–272.

[25] A. Medrano, P.F. Watson, W.V. Holt, Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope, Reproduction 123 (2002) 315–322.

[26] G.J. Morris, E. Acton, S. Avery, A novel approach to sperm cryopreservation, Hum. Reprod. 14 (1999) 1013–1021.

[27] G.J. Morris, M. Goodrich, E. Acton, F. Fonseca, The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation, Cryobiology 52 (2006) 323–334.

[28] K. Ortman, H. Rodriguez-Martinez, Membrane damage during dilution, cooling and freezing–thawing of boar spermatozoa packaged in plastic bags, Zentralbl Veterinarmed A 41 (1994) 37–47.

[29] F.J. Pena, A. Johannisson, M. Wallgren, H. Rodriguez-Martinez, Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity, Theriogenology 60 (2003) 677–689.

[30] F.J. Pena, A. Johannisson, M. Wallgren, H. Rodriguez Martinez, Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate, Anim. Reprod. Sci. 78 (2003) 85–98.

[31] L.M. Penfold, D.L. Garner, A.M. Donoghue, L.A. Johnson, Comparative viability of bovine sperm frozen on a cryomicroscope or in straws, Theriogenology 47 (1997) 521–530.

[32] V.G. Pursel, L.A. Johnson, Procedure for the preservation of boar spermatozoa by freezing, USDA ARS Bull 44–227 (1971) 1–5.

[33] J. Roca, M.A. Gil, M. Hernandez, I. Parrilla, J.M. Vazquez, E.A. Martinez, Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene, J. Androl. 25 (2004) 397–405.

[34] J. Roca, M. Hernández, G. Carvajal, J. Vázquez, E. Martínez, and Factors influencing boar sperm cryosurvival, J. Anim. Sci. 84 (2006) 2692–2699.

[35] J. Roca, H. Rodríguez-Martínez, J.M. Vázquez, A. Bolarín, M. Hernández, F. Saravia, M. Wallgren, E. Martínez, Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination, in: C.J. Ashworth, R.R. Kraeling (Eds.), Control of Pig Reproduction VII, University Press, Nottingham, 2006, pp. 261–275.

[36] H. Rodriguez Martinez, H. Ekwall, Electron microscopy in the assessment of cryopreserved spermatozoa viability, Microsc. Anal. 65 (1998) 11–13.

[37] F. Saravia, M. Wallgren, S. Nagy, A. Johannisson, H. Rodriguez-Martinez, Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability, Theriogenology 63 (2005) 1320–1333.
[38] J.A. Searles, J.F. Carpenter, T.W. Randolph, The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf, J. Pharm. Sci. 90 (2001) 860–871.
[39] N. Songsasen, S.P. Leibo, The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf, Cryobiology 35 (1997) 240–254.
[40] L.M. Thurston, K. Siggins, A.J. Mileham, P.F. Watson, W.V. Holt, Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation, Biol. Reprod. 66 (2002) 545–554.
[41] L.M. Thurston, P.F. Watson, W.V. Holt, Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system, J. Reprod. Fertil. 117 (1999) 271–280.
[42] K.E. Waterhouse, P.O. Hofmo, A. Tverdal, R.R. Miller Jr., Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm, Reproduction 131 (2006) 887–894.
[43] P. Westendorf, L. Richter, H. Treu, Zur Tierfgefrierung von Ebersperma: Labor und besamungsergebnisse mit dem Hülsenberger Pailleten Verfahren, Dtch. Tierárztl. Wschr. 82 (1975) 261–267.
[44] I. Wilmut, C. Polge, Recent progress in the deep-freeze preservation of boar semen, J. Reprod. Fertil. 25 (1971) 301–302.
[45] H. Woelders, M. Den Besten, Cryopreservation of boar semen with small between-boar variation of post-thaw sperm survival, Cryobiology 30 (1993) 645.
[46] H. Woelders, A. Matthijs, M. Den Besten, Boar variation in "freezability" of the semen, Reprod. Dom. Anim. 31 (1996) 153–159.
[47] H. Woelders, A. Matthijs, C.A. Zuidberg, A.E. Chaveiro, Cryopreservation of boar semen: equilibrium freezing in the cryomicroscope and in straws, Theriogenology 63 (2005) 383–395.

Artículo 3

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ORIGINAL ARTICLE

Differences in SCSA outcome among boars with different sperm freezability

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Summary

Spermatozoa from some boars sustain the process of cryopreservation poorly and yield poor fertility after artificial insemination. Poor freezability has not been disclosed using conventional semen analyses. A defective chromatin can, if present in a substantial number of spermatozoa, affect the fertilizing ability of spermatozoa. Here we tested the hypothesis that nuclear DNA instability could explain differences in freezability among boars, and complement or supersede conventional tests for sperm quality such as sperm motility or membrane assessments. Frozen–thawed (FT) spermatozoa from a total of 44 stud boars were assessed by the sperm chromatin structure assay (SCSA), in relation to computer-assisted sperm analysis-derived sperm motility variables and sperm viability (triple fluorescent microscopic staining), including three experiments. The first trial, including 24 boars, evaluated the relationship between the sperm motility and viability with levels of DNA integrity. The SCSA showed that most spermatozoa had intact DNA [levels of DNA fragmentation index (%DFI) ranging from 0.63% to 11.85%] significantly correlated (albeit weakly) with current sperm quality variables. The second trial, on 15 boars, assessed the influence of two different thawing rates (20 s at 37 °C vs. 8 s at 70 °C) and the post-thaw incubation times (0, 30, 150 and 300 min) at 37 °C on FT-boar sperm quality. The highest sperm survival ($p < 0.05$) and the lowest DNA damage ($p < 0.01$) were achieved when thawing was carried out at 70 °C for 8 s, without any change during the first 150 min of incubation. Finally, the third experiment studied if differences in sperm freezability showed by stud boar semen, as 'good' or 'bad' freezers by conventional analyses, could be attributed to differences in chromatin structure. All SCSA parameters were low, but significantly ($p < 0.05$–0.001) higher for 'bad' freezers, showing they had less homogeneous sperm chromatin than the 'good' freezers. The results indicate that SCSA outcome complements conventional assessment of FT-boar spermatozoa, disclosing differences in their ability to sustain freezing and thawing. However, the low overall DNA damage observed in FT spermatozoa seems to have poor biological significance.

Introduction

Although semen cryopreservation offers considerable advantages for the further development of porcine reproductive technologies, frozen–thawed (FT) semen is still seldom used by the pig industry worldwide, largely due to its low fertilizing ability after cryopreservation (Roca *et al.*, 2006). The main reasons for this low fertility are

the low overall sperm quality after the freezing–thawing process and the diminished lifespan of the surviving spermatozoa (Watson, 2000).

Considerable variation in post-thaw boar sperm viability exists, irrespective of the sperm quality the semen shows before freezing (Roca *et al.*, 2006) or the cryopreservation protocols used (Woelders *et al.*, 1996; Holt *et al.*, 2005). Therefore, the identification and pre-selection of boars according to their capacity to sustain freezability of their semen is one of the major goals to maximize the use of FT spermatozoa. Current measurements of the post-thaw sperm characteristics such as the percentages of spermatozoa depicting motility [using computer-assisted sperm analysis (CASA) instruments] and/or membrane integrity (using specific probes) have been used to objectively classify boars as 'good', 'moderate' or 'bad' freezers (Thurston *et al.*, 2002; Holt *et al.*, 2005; Roca *et al.*, 2006). However, it is interesting to point out that not all those boars classified as 'good' freezers have been able to achieve high rates of in vitro fertility, whereas some classified as 'bad' offered acceptable fertility rates, highlighting the limitations of the sperm evaluation tests currently available (Gil *et al.*, 2005). Moreover, it seems clear that others sperm features related to fertility could also be negatively affected during the freezing–thawing procedure. One of those attributes might be the integrity of the nuclear DNA, whose stability largely depends on the integrity of the chromatin. It is therefore recommended that chromatin integrity should be studied as an independent complementary parameter for the better assessment of sperm quality (Evenson *et al.*, 2002). Furthermore, changes in sperm chromatin structure and DNA integrity have been widely related to infertility in several mammalian species (boar: Evenson *et al.*, 1994; humans: Evenson *et al.*, 2002; bull: Januskauskas *et al.*, 2001; stallion: Love, 2005). In boars, previous studies have shown that sperm chromatin can undergo important changes after the freezing–thawing procedure (Cordova *et al.*, 2002; Fraser & Strzezek, 2005), calling for the analyses of FT spermatozoa as part of the determination of their resistance to freezability. In addition, different changes in porcine sperm chromatin structure could occur depending on the thawing procedure (Cordova *et al.*, 2005), the freezing package system (Cordova *et al.*, 2002) or by an increase in the storage time (Boe-Hansen *et al.*, 2005a).

A variety of methods have been developed for detecting different changes in the chromatin structure or DNA integrity including the neutral single-cell gel electrophoresis assay (neutral comet assay), terminal deoxynucleotidyl transferase-mediated nick end labelling, in situ nick translation, sperm chromatin structure assay (SCSA) and more recently the sperm chromatin dispersion test (reviewed by Chohan *et al.*, 2006). The SCSA measures the increased susceptibility of sperm DNA to acid denaturation in situ using flow cytometry. It has been widely used and described as a highly reproducible, precise and cost-effective diagnostic method (Evenson & Wixon, 2006).

The main goal of the present study was to test the hypothesis that nuclear DNA instability could explain differences in freezability among boars. It also aimed at evaluating the degree of DNA integrity of FT-boar spermatozoa using the SCSA protocol and to determine its possible relationship with the outcome of classical sperm quality tests (sperm motility and viability). Furthermore, the influence of different thawing rates and post-thaw incubation times on the chromatin structure was also tested.

Materials and methods

Semen source

A total of 280 ejaculates from 44 fertile, mature boars of various commercial hybrid lines (3–10 ejaculates per boar) undergoing regular semen collection for commercial artificial insemination (AI) and included in a sperm cryobank were used in the experiments. Sperm-rich fractions were collected by the gloved-hand method, extended (1 : 1, v/v) in Beltsville thawing solution (BTS, 205 mmol/L glucose, 20.39 mmol/L NaCl, 5.4 mmol/L KCl, 15.01 mmol/L $NaHCO_3$ and 3.35 mmol/L EDTA) and evaluated by standard laboratory techniques (Martin Rillo *et al.*, 1996). Only those ejaculates with more than 75% motile spermatozoa, 80% normal acrosome and abnormal morphology less than 15% were packed for shipping at 17 °C, and delivered 14–16 h later to the Laboratory of Andrology of the Veterinary Teaching Hospital of the University of Murcia, Spain.

Sperm cryopreservation

At the laboratory, the ejaculates were frozen following the straw freezing procedure described by Westendorf *et al.* (1975) and modified by Thurston *et al.* (1999) and Carvajal *et al.* (2004). Briefly, extended sperm-rich fractions were centrifuged at 2400 ***g*** for 3 min at 17 °C, the pellets were re-extended in lactose egg yolk (LEY) extender [80% (v/v) 310 mM β-lactose, 20% (v/v) egg yolk and 100 μg/mL kanamycin sulphate, pH 6.2 and 330 ± 5 mOsmol/kg] to yield a concentration of 1.5×10^9 cells/mL. After further cooling to 5 °C within 90 min, the extended spermatozoa were re-suspended with LEY-Glycerol-OrvusESPaste (LEYGO) extender [92.5% LEY, 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA, USA) and 6% (v/v) glycerol; pH 6.2 and 1650 ± 15 mOsmol/kg] to yield a final concentration of 1×10^9 cells/mL. The cooled spermatozoa were packed

into 0.5 PVC-French straws (Minitüb, Tiefenbach, Germany) which were frozen using a controlled-rate freezer (IceCube 1810; Minitüb), as follows: from 5 to −5 °C at a rate of 6 °C/min, from −5 to −80 °C at 40 °C/min, held for 30 s at −80 °C, then cooled at 70 °C/min to −150 °C and plunged into liquid nitrogen for storage until analyses. The straws were then stored in liquid nitrogen for at least 1 week before thawing.

Thawing and post-thaw sperm evaluation

Straws were thawed in a circulating water bath, re-extended at 37 °C in BTS (1 : 1, v/v) and kept in the water bath (37 °C) during sperm evaluation. Spermatozoa were assessed according to motility and motion characteristics, sperm viability (plasma membrane integrity, mitochondrial membrane potential and acrosome integrity) and sperm chromatin stability.

Sperm motility and motion characteristics were objectively evaluated using the CASA system (Sperm Class Analyzer; Microptic, Barcelona, Spain). Thawed spermatozoa were further re-suspended in BTS at a concentration of 30×10^6/mL. For each evaluation, a 4-μL sperm sample was placed in a pre-warmed (39 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and three fields were analysed to assess a minimum of 100 spermatozoa. The following motility parameters were measured: the percentage of total motile spermatozoa (% TSM); percentage of spermatozoa with motility up to 50 μm/s and straightness up to 75% (rapid progressive spermatozoa, % RPS); straight-line velocity (VSL, μm/s) and linearity (% LIN).

Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential and acrosomal integrity. Diluted–thawed spermatozoa were further re-suspended in BTS at a concentration of 15×10^6/mL. Sperm viability was analysed simultaneously using the modification of a triple fluorescent staining described by Graham *et al.* (1990), adapted for boar spermatozoa by Carvajal *et al.* (2004), which included the DNA-specific fluorochrome propidium iodide, the mitochondria-specific fluorochrome rhodamine-123 (R123) and the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin from Sigma Chemical Co (St Louis, MO, USA). Two hundred spermatozoa per sample were examined under 1000× magnification (Eclipse E800; Nikon, Tokyo, Japan), using BV-2A filter (400–440 nm excitation filter, 455 nm emission, 470 barrier filter). Spermatozoa showing only green fluorescence over the middle piece (R123 positive) were considered viable with an intact acrosome (VIA). Values were expressed as percentages.

Assessment of sperm DNA integrity

The SCSA was applied according to the procedure described by Evenson *et al.* (2002) to determine the degree of increased susceptibility of spermatozoa following acid-induced denaturation in situ, and after quantification by flow cytometric measurement of the metachromatic shift from green (double-stranded DNA, dsDNA) to red (single-stranded DNA, ssDNA) acridine orange (AO) fluorescence, thus representing the amount of denatured, ssDNA over the total cellular DNA. The thawed semen samples were re-extended with TNE buffer (0.15 mol/L NaCl, 0.01 mol/L Tris–HCl and 1 mmol/L EDTA, pH 7.4) into a final sperm concentration of approximately 2×10^6/mL. An aliquot of 0.2 mL was subjected to partial DNA denaturation in situ by mixing with 0.4 mL of a low pH detergent solution (0.17% Triton X-100, 0.15 mol/L NaCl and 0.08 N HCl, pH 1.4), followed 30 s later by staining with 1.2 mL of AO (6 μg/mL in 0.1 mol/L citric acid, 0.2 mol/L Na_2HPO_4, 1 mmol/L EDTA and 0.15 mol/L NaCl, pH 6.0). The stained samples were split into two halves and analysed within 3 min after AO staining.

Flow cytometry analyses

Assessment of sperm chromatin integrity was carried out on a BD FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with standard optics. AO was excited with an Ar ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm and running at 200 mW. In association with dsDNA, AO fluoresces green (530 ± 30 nm, as detected with the FL1 detector), but in the presence of ssDNA, the resulting fluorescence is red (>630 nm, as detected with FL3). The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0 μM; Polysciences Inc., Warrington, PA, USA). Equivalent instrument settings were used for all samples. From each sample, a total of 10 000 events were measured at a flow rate of ∼200 cells/s and recorded using CellQuest, version 3.1 software (Becton Dickinson). Data were stored as list mode files and further analyses of the parameters were carried out by using the FCSExpress software, version 2 (DeNovo Software; Thornhill, ON, Canada).

The extent of DNA denaturation, expressed as DNA fragmentation index (DFI), was calculated based on the ratio of red/total (red + green) fluorescence for each sperm in the sample. (Evenson *et al.*, 2002). For each sample, four terms of DFI were evaluated: mean DFI and standard deviation of the DFI (SD-DFI), expressed in channels of fluorescence; the percentage of sperm with detectable DFI (%DFI) and the percentage of sperm with

high-DNA stainability (HDS). The mean DFI (formerly termed as $X\alpha_t$) indicates shifts and trends in the whole population of cells. The SD-DFI (formerly termed as $SD\alpha_t$) shows the extent of chromatin structure abnormality within a population. The %DFI (formerly known as $COMP\alpha_t$, cells outside the main peak of α_t) indicates the percentage of cells with denatured DNA. Finally, HDS (formerly termed HIGRN, high green fluorescence) represents the percentage of immature cells in a sample.

Experimental design

All ejaculates used in the present study were objectively evaluated immediately after arrival at the laboratory (17 °C) just before freezing and only those with more than 75% viable spermatozoa were cryopreserved ($n = 280$). The overall percentage (mean ± SD) of total sperm motility and viability was 78.51 ± 21.25 and 86.25 ± 9.53, respectively, not showing differences between boars ($p > 0.05$).

Experiment 1: Relationship between outcomes of sperm motility and membrane integrity with DNA integrity post-thaw

For this experiment, two straws from each one of the 24 randomly selected ejaculates (24 boars), were thawed at 37 °C for 20 s, pooled and re-extended at 37 °C with BTS (1 : 1, v/v). The percentage of total sperm motility, viability and chromatin integrity were evaluated immediately after thawing. The assay was replicated four times.

Experiment 2: Effect of thawing rate and post-thaw incubation time in sperm DNA integrity

Two randomly selected straws from each one of the 15 different ejaculates (15 boars) were thawed and pooled for each of two thawing rate (20 s at 37 °C or 8 s at 70 °C) and re-extended at 37 °C with BTS (1 : 1, v/v). The percentage of total sperm motility, viability and chromatin integrity were evaluated at 0, 30, 150 and 300 min post-thaw.

Experiment 3: Differences in sperm DNA integrity among 'good' and 'bad' boar sperm freezers

In the first approach (data not shown), cryopreserved ejaculates ($n = 280$) were objectively classified by multivariate pattern analysis into three groups according to their post-thaw sperm quality (motility and viability). Those boars with the best FT sperm characteristics (>60% TSM and VIA) were identified as 'good' freezers whereas boars showing reduced FT sperm characteristics were considered as 'moderate' freezers (40–60% TSM and VIA) or 'bad' freezers (<40% TSM and VIA). The classification of ejaculates was highly consistent within boars.

For this experiment, only straws from boars classified as 'good' ($n = 10$) and 'poor' ($n = 10$) freezers were used. Two straws (randomly selected within a single ejaculate from each one of the 10 boars classified as 'good' or 'bad') were thawed, pooled and re-extended at 37 °C with BTS (1 : 1, v/v). Samples were immediately evaluated after thawing for their motility, viability and chromatin integrity.

Statistical analysis

Statistical analyses were performed by using SPSS, version 13 (SPSS Inc., Chicago, IL, USA). Boar and ejaculate (within boar) effects on sperm quality before and after cryopreservation were assessed by using analysis of variance (ANOVA). In experiment 1, the nonparametric Kruskal–Wallis test was performed to evaluate differences on the post-thaw sperm quality and DNA integrity between boars. The Spearman nonparametric correlation test was used to calculate the relationships between post-thaw sperm quality and SCSA parameters. In experiment 2, for those variables that were non-normally distributed, data were rank transformed prior to further analysis. Data were evaluated by a mixed model ANOVA, including thawing temperature and incubation time as fixed effects and boar as random effect. In experiment 3, the multivariate pattern analysis (PATN; CSIRO, Canberra, Australia) was used to classify the 280 ejaculates cryopreserved, using all the measured current sperm variables (post-thaw sperm motility, including motion characteristics and sperm viability) within the data set, on a small number of groups as described by Abaigar *et al.* (1999). Three groups were finally obtained ('good', 'moderate' and 'bad' freezers) from the non-hierarchical classification of the ejaculates tested. The statistics of each SCSA parameter of 'good' and 'bad' boar sperm freezers were calculated, and compared using the non-parametric Mann–Whitney *U*-test. When ANOVA revealed a significant effect, values were compared using the Bonferroni test. The level of significance was set at $p < 0.05$. The range was presented as the highest and lowest values observed in a data set.

Results

Experiment 1: Relationship between outcomes of sperm motility and membrane integrity with DNA integrity post-thaw

The overall percentage (mean ± SD) of total sperm motility and viability was 41.8 ± 16.6 and 48.6 ± 12.5 respectively. The SCSA values on FT samples were 285.6 ± 9.0

Table 1 Correlation (Spearman correlation coefficients) between sperm chromatin structure assay (SCSA) parameters and current semen quality parameters for post-thaw boar semen

Sperm quality parameter	Mean DFI	SD-DFI	%DFI	HDS
TSM (%)	−0.09	−0.34**	−0.24*	−0.84
RPS (%)	−0.14	−0.47**	−0.34**	0.24*
VSL (μm/s)	−0.16	−0.38**	−0.33**	0.18
LIN (%)	−0.06	−0.03	−0.07	0.12
VIA (%)	−0.27**	−0.23*	−0.18*	−0.61

Motility characteristics obtained from CASA analysis: TSM, % total motile spermatozoa; RPS, rapid progressive spermatozoa; VSL, straight-line velocity; LIN, linearity; VIA, % viable spermatozoa with intact acrosome assessed by triple stain. SCSA parameters: mean DFI, mean DNA fragmentation index; SD-DFI, standard deviation of DFI; %DFI, percentage of cells with detectable DFI; HDS, percentage spermatozoa with high DNA stainability.
*$p < 0.05$; **$p < 0.01$.

Table 3 Statistics of post-thaw sperm motility and viability (mean ± SD) in 'good' and 'bad' sperm freezers defined after multivariate pattern analysis

Sperm characteristics (mean ± SD)	Boar groups[a] 'Good' ($n = 10$)	'Bad' ($n = 10$)	p-value
CASA			
TSM (%)	58.3 ± 4.8	25.8 ± 5.2	0.001
RPS (%)	19.0 ± 2.2	6.8 ± 1.8	0.001
VSL (μm/s)	50.6 ± 3.2	35.7 ± 4.7	0.001
LIN (%)	61.9 ± 2.8	61.2 ± 3.6	NS
VIA (%)	63.6 ± 3.2	35.5 ± 3.5	0.001

Motility characteristics obtained from CASA analysis: TSM, % total motile spermatozoa; RPS, rapid progressive spermatozoa; VSL, straight-line velocity; LIN, linearity. VIA, % viable spermatozoa with intact acrosome assessed by triple stain.
[a]Boars (one ejaculate per boar; two pooled straws per ejaculate).

(range 261.9–312.4) for mean DFI, 27.9 ± 5.8 (range 20.1–50.3) for SD-DFI, 2.2 ± 2.1 (range 0.6–11.8) for %DFI and 0.34 ± 0.1 (0.09–0.8) for HDS. Significant differences ($p < 0.001$) were found among boars for SD-DFI,%DFI and HDS. Most of the SCSA parameters studied were significantly correlated (albeit weakly) with current sperm quality parameters (Table 1).

Experiment 2: Effect of thawing rate and post-thaw incubation time in sperm DNA integrity of FT boar spermatozoa

The effects of thawing rate (20 s at 37 °C vs. 8 s at 70 °C) and incubation time (0, 30, 150 and 300 min) on the post-thaw sperm quality and DNA integrity of FT spermatozoa are shown in Table 2. No significant ($p > 0.05$) interaction between both effects was found. The sperm samples thawed for 8 s at 70 °C showed the highest percentages of TSM and viability ($p < 0.05$) together with the lowest values of %DFI and HDS ($p < 0.01$). The results also indicated that incubation time had a significant effect on TSM, viability ($p < 0.05$) and %DFI ($p < 0.01$), the overall post-thaw sperm quality being significantly decreased at 300 min, whereas the differences between 0, 30 and 150 min were not significant.

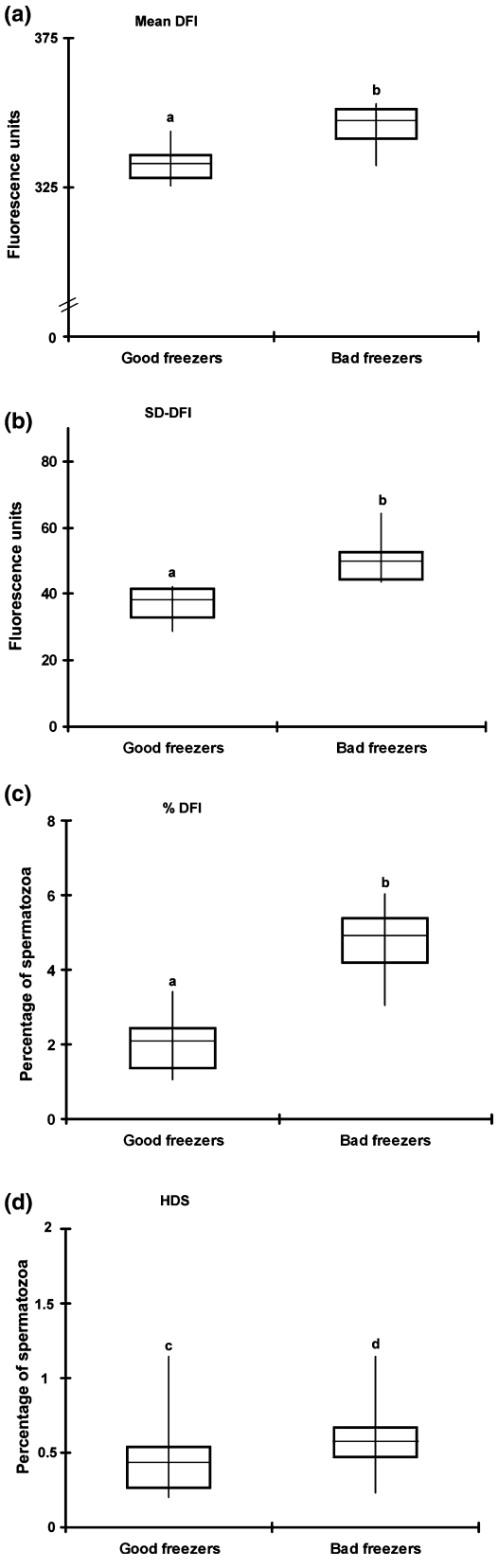
Experiment 3: Differences in sperm DNA integrity among 'good' and 'bad' boar sperm freezers

Summary statistics of post-thaw sperm quality parameters for 'good' and 'bad' freezers are shown in Table 3. The significant differences ($p < 0.001$) present between these two groups for all sperm parameters are evaluated, except for LIN. The frequency distributions for 'good' or 'bad' freezers in each of the four SCSA parameters assessed are represented in Fig. 1. Differences were statistically

Table 2 Statistics of the different post-thaw boar ($n = 15$) sperm quality parameters and the sperm chromatin structure assay (SCSA) given as mean ± SD, according to thawing rate and post-thaw sperm incubation time

	Mean DFI	SD-DFI	%DFI	HDS	TSM	RPS	VSL	LIN	VIA
Thawing rate									
20 s at 37 °C	285.3 ± 8.1	27.6 ± 3.3	2.1 ± 1.0 a	0.4 ± 0.1 a	40.3 ± 12.0 c	9.4 ± 6.6	42.4 ± 9.6	68.8 ± 4.9	46.5 ± 7.0 c
8 s at 70 °C	282.7 ± 6.4	26.9 ± 3.0	1.8 ± 0.6 b	0.3 ± 0.1 b	43.8 ± 14.9 d	11.1 ± 7.8	43.5 ± 10.3	68.1 ± 4.3	52.1 ± 10.8 d
Incubation time (min)									
0	283.2 ± 8.8	26.5 ± 5.8	1.7 ± 0.6 a	0.4 ± 0.2	48.6 ± 13.1 c	12.5 ± 6.7	44.8 ± 8.9	66.3 ± 4.0	48.8 ± 8.9 c
30	285.1 ± 6.1	26.7 ± 3.1	1.9 ± 0.6 a	0.4 ± 0.1	47.9 ± 14.4 c	9.7 ± 6.3	43.2 ± 8.7	67.6 ± 3.8	52.9 ± 9.1 c
150	281.5 ± 7.6	27.2 ± 2.3	1.9 ± 0.5 a	0.3 ± 0.1	41.9 ± 12.2 c	10.9 ± 7.9	44.6 ± 11.4	69.1 ± 4.9	50.2 ± 9.6 c
300	286.4 ± 6.2	27.7 ± 3.9	2.3 ± 1.3 b	0.3 ± 0.2	35.8 ± 12.0 d	9.9 ± 7.8	41.1 ± 9.8	68.8 ± 5.1	43.5 ± 8.6 d

SCSA parameters: mean DNA fragmentation index, mean DFI; standard deviation of DFI, SD-DFI; percentage of cells with detectable DFI, %DFI; percentage of spermatozoa with high-DNA stainability, HDS. TSM, % total motile spermatozoa; VIA, % viable spermatozoa with intact acrosome assessed by triple stain. Different letters denote a significant difference within the procedures (a, b, $p < 0.01$; c, d, $p < 0.05$).



significant for all the SCSA parameters, mean DFI, SD-DFI, % DFI ($p < 0.001$) and HDS ($p < 0.05$). The mean SCSA parameters for 'good' sperm freezers were 333.8 (range 325.5–343.9) for mean DFI, 36.2 (range 28.8–42.1) for SD-DFI, 2% (range 1.1–3.4%) for %DFI and 0.4% (range 0.2–0.6%) for HDS. The values for 'bad' sperm freezers were 345.6 (range 332.3–352.9), 50.07 (range 43.8–64.3), 4.7% (range 3.1–6%) and 0.5% (0.2–1.2%) for mean DFI, SD-DFI, %DFI and HDS respectively.

Discussion

Cryopreservation produces physical and chemical stress on numerous sperm structures and reduces post-thaw sperm viability and fertilizing ability (Watson, 2000), variables not easily revealed by current sperm evaluation tests. The integrity of DNA is of vital importance for the ability of spermatozoa to contribute to a normal embryo development after fertilization (reviewed by Evenson & Wixon, 2006). In humans, percentages of fragmented DNA (%DFI) around 30% or higher have been established as a threshold for low fertility prognosis when the SCSA method was applied (Evenson *et al.*, 2002). Similar observations have been made for bulls and stallions where chromatin abnormalities close to 25% were also related to sires with low fertility (Bochenek *et al.*, 2001; Love, 2005).

Fraser & Strzezek (2005), using the neutral comet assay that detects double-stranded DNA breaks, found that the cryopreservation process affects the DNA integrity of boar spermatozoa, irrespective of the extender type and packaging material used. The percentage of spermatozoa with damaged DNA for fresh semen found was very low (~3.5%) compared with that reported for post-thaw spermatozoa (~23%). Furthermore, an increase in the chromatin condensation and instability of boar spermatozoa after the freezing–thawing procedure has also been described (Cordova *et al.*, 2002).

The above results are not in accordance with those obtained in our study, where a relatively low proportion of cells with fragmented DNA (%DFI ~2.2%) were found in FT boar spermatozoa, similar to those observed in fresh boar spermatozoa by SCSA (Evenson *et al.*, 1994). Furthermore, these authors did not find altered boar sperm chromatin structure after freezing directly on dry ice or

Figure 1 Whisker and Box plots showing the sperm chromatin structure assay (SCSA) parameters: (a) mean DNA fragmentation index (mean DFI), (b) standard deviation of the DFI (SD-DFI), (c) percentage of cells with detectable DFI (%DFI) and (d) percentage of cells with high-DNA stainability (HDS). Boxes enclose the 25th and 75th percentiles, the dot is the median and the whiskers extend to the 5th and 95th percentiles. Different letters (a, b, $p < 0.001$; c, d, $p < 0.05$) denote a significant difference between 'good' ($n = 10$, one ejaculate per boar) and 'bad' ($n = 10$, one ejaculate per boar) sperm freezers.

liquid nitrogen. For this reason, ultrafreezing sperm cells directly into liquid nitrogen or an ultracold freezer, without any cryoprotectants, has been recommended as a storage method, before further analysis by the SCSA (Evenson *et al.*, 2002; Boe-Hansen *et al.*, 2005c). Under these conditions, the mean percentage of fresh spermatozoa with damaged chromatin structure observed was around 5–10% (Evenson *et al.*, 1994; Rybar *et al.*, 2004; Boe-Hansen *et al.*, 2005a). Then, it could be hypothesized that chromatin structure of boar spermatozoa in samples evaluated in the present study were poorly affected by the freezing–thawing procedure. However, several techniques other than the SCSA method can be used to analyse different types of sperm DNA alterations, and the results obtained are not always directly comparable among them (Boe-Hansen *et al.*, 2005b; Chohan *et al.*, 2006). Therefore, further studies are needed to clarify how cryopreservation affects DNA integrity of boar spermatozoa, so as to select the most adequate method to evaluate FT boar sperm.

Despite the low percentage of DNA sperm damage registered in our study, significant differences among ejaculates (boars) were found. Differences in sperm chromatin abnormalities among boars and ejaculates within the same boar have been described previously (Boe-Hansen *et al.*, 2005a; Enciso *et al.*, 2005). Moreover, the sperm DNA resistance to the freezing–thawing process also varied among boars (Fraser & Strzezek, 2005). Although sperm chromatin abnormalities have been attributed to defective spermatogenesis and abnormal apoptotic degeneration, the reason for this individual variation in chromatin stability is not clear. Current reports suggest that oxidative stress is one of the main contributors to abnormal sperm chromatin structure (reviewed by Evenson & Wixon, 2006). Reactive oxygen species (ROS) can be generated by the freezing–thawing cycle (Bilodeau *et al.*, 2000) and ROS can also induce sperm DNA damage (reviewed by Aitken & Baker, 2004). Recently, Kasimanickam *et al.* (2006) observed that ram sperm quality is negatively related to the extent of DNA fragmentation and lipid peroxidation in sperm and seminal plasma (SP). Love (2005) also showed that increasing concentrations of SP in the cooling extender decreased the sperm DNA quality in the stallion. A high variability exists in the SP composition among males within the same species, as well as among ejaculates (Strzezek, 2002). Consequently, excessive ROS production or a lack in some antioxidant defence system could explain the variability in DNA damage among boars.

Classically, cooling and re-warming are known to be the most critical factors influencing sperm cryosurvival (reviewed by Johnson *et al.*, 2000). In the second experiment, we tested how two different thawing rates, a rapid (20 s 37 °C, ~1200 °C/min) and an ultra-rapid one (8 s 70 °C, ~1800 °C/min), influenced sperm DNA integrity. Our results revealed that DNA integrity was significantly improved by the highest rewarming velocity, together with motility and viability. Therefore, ultra-rapid velocities (e.g. 8 s 70 °C) should be the recommended method for thawing boar spermatozoa packaged in 0.5 mL straws to obtain a higher survival sperm yield. However, both high temperature and reduced time are extremely critical, as a longer period would raise the temperature in the straw to non-physiological limits. Then, as the beneficial effect has little biological relevance, it does not seem to be a very practical method under field conditions.

Irrespective of the thawing rate, handling of FT boar sperm during thawing-to-insemination must be carried out in the minimal period to avoid a drastic fall in fertility levels. However, under commercial conditions, thawing is not currently performed in the same farm where inseminations are undergone, so a reasonable thawing-to-insemination interval is required to adequately perform the AI. Thus, in the second experiment, FT spermatozoa were maintained to physiological temperatures (37 °C) during an extremely long period of 300 min. No changes in sperm motility, viability or DNA integrity were observed within the 150 min post-thawing ($p > 0.05$). A slightly significant increase in %DFI together with a significant decrease in sperm motility and viability were observed after 300 min of incubation. To our knowledge, no previous studies have assayed as to how post-thaw incubation time affects boar DNA integrity. Similarly, Peris *et al.* (2004) found in FT ram sperm that the chromatin structure was not altered immediately or 3 h after thawing. Moreover, differences in SCSA parameters between fresh and cryopreserved sperm were only observed after 20 h post-thawing. It shows that the holding time does not seem to produce a great deleterious effect on DNA integrity measured with the SCSA protocol. Thus, it seems clear from our results that no higher DNA sperm damage should be expected in cryopreserved semen doses within 150 min of thawing; this being a much more suitable period to perform AI without loss in post-thaw sperm quality.

Controversial results concerning a relationship between sperm chromatin integrity and current sperm quality parameters have been reported. In humans, some authors (Larson *et al.*, 2000; Evenson *et al.*, 2002) have largely concluded that DNA integrity parameters are independent of other sperm quality variables studied by using current assessment methods. However, poor semen quality has been strongly associated with DNA damage using different evaluation methods by other investigators (Irvine *et al.*, 2000; Zini *et al.*, 2001). In our study, SCSA parameters were weakly but significantly correlated with current post-thaw sperm characteristics, in agreement with

results obtained in other different species (ram: Peris *et al.*, 2004; Kasimanickam *et al.*, 2006; bull: Januskauskas *et al.*, 2001; Hallap *et al.*, 2005; humans: Giwercman *et al.*, 2003). This weak correlation might be explained by the narrow range of the SCSA parameters and due to the low percentages of DNA damage registered. However, it could also indicate that ejaculates with the best post-thaw sperm quality had less DNA damage, but not all the ejaculates would show the same trend. To test this hypothesis, the third experiment was carried out to determine potential differences in DNA damage between two distinct populations of boars, selected according to post-thaw sperm quality parameters: 'good' or 'bad' sperm freezers. The 'bad' freezers had a significantly higher level of mean DFI, SD-DFI, %DFI and HDS than the 'good' freezers, showing more damaged DNA in the 'bad' group. Although the extent to which the process of cryopreservation (including cooling and thawing) would be responsible for this difference in DNA integrity is unknown, the percentages of affected spermatozoa were so low that they cannot explain the great differences in post-thaw sperm survival seen by the two categories of sires.

In conclusion, SCSA results indicated that the susceptibility of FT boar spermatozoa to denaturation was relatively low even when different thawing rates and large incubation times were applied. FT spermatozoa from boars classified as 'good' freezers were less susceptible to chromatin denaturation than 'bad' freezers. Based on the results of this and other studies, it is apparent that SCSA can be used as an effective and sensible procedure to determine small differences in DNA integrity of FT-boar semen. However, the low overall DNA damage observed in FT spermatozoa seems to have poor biological significance.

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References

Abaigar, T., Holt, W. V., Harrison, R. A. P. & del Barrio, G. (1999) Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessment. *Biology of Reproduction* 60, 32–41.

Aitken, R. J. & Baker, M. A. (2004) Oxidative stress and male reproductive biology. *Reproduction, Fertility and Development* 16, 581–588.

Bilodeau, J. F., Chatterjee, S., Sirard, M. A. & Gagnon, C. (2000) Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development* 55, 282–288.

Bochenek, M., Smorag, Z. & Pilch, J. (2001) Sperm chromatin structure assay of bulls qualified for artificial insemination. *Theriogenology* 56, 557–567.

Boe-Hansen, G. B., Ersboll, A. K., Greve, T. & Christensen, P. (2005a) Increasing storage time of extended boar semen reduces sperm DNA integrity. *Theriogenology* 63, 2006–2019.

Boe-Hansen, G. B., Morris, I. D., Ersboll, A. K., Greve, T. & Christensen, P. (2005b) DNA integrity in sexed bull sperm assessed by neutral comet assay and sperm chromatin structure assay. *Theriogenology* 63, 1789–1802.

Boe-Hansen, G. B., Ersboll, A. K. & Christensen, P. (2005c) Variability and laboratory factors affecting the sperm chromatin structure assay in human semen. *Journal of Andrology* 26, 360–368.

Carvajal, G., Cuello, C., Ruiz, M., Vaázquez, J. M., Martinez, E. A. & Roca, J. (2004) Effect of centrifugation before freezing on boar sperm cryosurvival. *Journal of Andrology* 25, 389–396.

Chohan, K. R., Griffin, J. T., Lafromboise, M., De Jonge, C. J. & Carrell, D. T. (2006) Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *Journal of Andrology* 27, 53–59.

Cordova, A., Pérez-Gutiérrez, J. F., Lleo, B., Garcia-Artiga, C., Alvarez, A., Drobchak, V. & Martin-Rillo, S. (2002) In vitro fertilizing capacity and chromatin condensation of deep frozen boar semen packaged in 0.5 and 5 ml straws. *Theriogenology* 57, 2119–2128.

Cordova, A., Oliva, J. H., Lleó, B., García-Artiga, C., Corcuera, B. D. & Pérez-Gutiérrez, J. F. (2005) Effect of different thawing temperatures on the viability, in vitro fertilizing capacity and chromatin condensation of frozen boar semen packaged in 5 ml straws. *Animal Reproduction Science* 92, 145–154.

Enciso, M., López-Fernández, C., Fernández, J. L., García, P., Gosálbez, A. & Gosálvez, J. (2005) A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. *Theriogenology* 65, 308–316.

Evenson, D. P. & Wixon, R. (2006) Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 65, 979–991.

Evenson, D. P., Thompson, L. & Jost, L. (1994) Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* 41, 637–651.

Evenson, D. P., Larson, K. L. & Jost, L. K. (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *Journal of Andrology* 23, 25–43.

Fraser, L. & Strzezek, J. (2005) Effects of freezing–thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. *Reproduction in Domestic Animals* 40, 1–7.

Gil, M. A., Roca, J., Cremades, T., Hernández, M., Vázquez, J. M., Rodríguez-Martínez, H. & Martínez, E. A. (2005) Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? *Theriogenology* 64, 305–316.

Giwercman, A., Richthoff, J., Hjollund, H., Bonde, J. P., Jepson, K., Frohm, B. & Spano, M. (2003) Correlation between sperm motility and sperm chromatin structure assay parameters. *Fertility and Sterility* 80, 1404–1412.

Graham, J. K., Kunze, E. & Hammerstedt, R. H. (1990) Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biology of Reproduction* 43, 55–64.

Hallap, T., Nagy, S. z., Håård, M., Jaakma, Ü., Johannisson, A. & Rodriguez-Martinez, H. (2005) Sperm chromatin stability in frozen-thawed semen is maintained over age in AI bulls. *Theriogenology* 63, 1752–1763.

Holt, W. V., Medrano, A., Thurston, L. M. & Watson, P. F. (2005) The significance of cooling rates and animal variability for boar sperm cryopreservation insights from the cryomicroscope. *Theriogenology* 63, 370–382.

Irvine, D. S., Twigg, J. P., Gordon, E. L., Fulton, N., Milne, P. A. & Aitken, R. J. (2000) DNA integrity in human spermatozoa: relationships with semen quality. *Journal of Andrology* 21, 33–44.

Januskauskas, A., Johannisson, A. & Rodríguez-Martínez, H. (2001) Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology* 55, 947–961.

Johnson, L. A., Weitze, K. F., Fiser, P. & Maxwell, W. M. (2000) Storage of boar semen. *Animal Reproduction Science* 62, 143–172.

Kasimanickam, R., Pelzer, K. D., Kasimanickam, V., Swecker, W. S. & Thatcher, C. D. (2006) Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. *Theriogenology* 65, 1407–1421.

Larson, K. L., DeJonge, C. J., Barnes, A. M., Jost, L. K. & Evenson, D. P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Human Reproduction* 15, 1717–1722.

Love, C. C. (2005) The sperm chromatin structure assay: a review of clinical applications. *Animal Reproduction Science* 89, 39–45.

Martin Rillo, S., Martínez, E., Garcia Artiga, C. & De Alba, C. (1996) Boar semen evaluation in practice. *Reproduction in Domestic Animals* 31, 519–526.

Peris, S. I., Morrier, A., Dufour, M. & Bailey, J. L. (2004) Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *Journal of Andrology* 25, 224–233.

Roca, J., Rodríguez-Martínez, H., Vázquez, J. M., Bolarín, A., Hernández, M., Saravia, F., Wallgren, M. & Martínez, E. A. (2006) Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: *Control of Pig Reproduction*, Vol. 7. (eds. C. J. Ashworth & R. R. Kraeling), pp 261–275. Nottingham University Press, Nottingham.

Rybar, R., Faldikova, L., Faldyna, M., Machatkova, M. & Rubes, J. (2004) Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic. *Czech Republic Veterinary Medicine* 49, 1–8.

Strzezek, J. (2002) Secretory activity of boar seminal vesicle glands. *Reproductive Biology* 2, 243–266.

Thurston, L. M., Watson, P. F. & Holt, W. V. (1999) Sources of variation in boar spermatozoa fertility following cryopreservation. *Cryobiology* 39, 355.

Thurston, L. M., Siggins, K., Mileham, A. J., Watson, P. F. & Holt, W. V. (2002) Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. *Biology of Reproduction* 66, 545–554.

Watson, P. F. (2000) The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science* 60/61, 481–492.

Westendorf, P., Richter, L. & Treu, H. (1975) Zur Tierfgefrierung von Ebersperma: Labor und besamungsergebnisse mit dem Hülsenberger Pailleten Verfahren. *Dtch Tierärztl Wschr* 82, 261–267.

Woelders, H., Matthijs, A. & Den Besten, M. (1996) Boar variation in ''freezability'' of the semen. *Reproduction in Domestic Animals* 31, 153–159.

Zini, A., Bielecki, R., Phang, D. & Zenzes, T. (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertility and Sterility* 75, 674–677.

Artículo 4

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Adjustments on the cryopreservation conditions reduce the incidence of boar ejaculates with poor sperm freezability

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Abstract

The objective of the present study was to evaluate the effectiveness of different cryopreservation conditions (CCs) for freezing and thawing boar ejaculates, focusing on those having sub-optimal sperm freezability. Using a split-ejaculate technique, single ejaculates from 53 boars were diluted in lactose-egg yolk extender, containing a final glycerol concentration (GLY) of 2 or 3%, packaged in 0.5 mL straws and were cooled at rates of −10, −40 or −60 °C/min (cooling rate: CR). Thereafter, the frozen sperm samples were thawed by warming them at rates of ∼1200 or ∼1800 °C/min (warming rate: WR). Frozen-thawed sperm samples were assessed for the sperm motility (CASA system) and flow cytometric analysis of plasma and acrosomal membranes integrity. Cooling rate had no influence ($P > 0.05$) on sperm quality parameters, however GLY and WR independently affected ($P < 0.05$) all assessed sperm parameters. Evaluating the combined effect of GLY and WR (four different CCs resulting of a 2 × 2 factorial design), the best post-thaw quality results were achieved for sperm samples frozen with 3% glycerol and thawed at 1800 °C/min (CC4). However, there was a significant interaction ($P < 0.001$) between CC and ejaculate for all post-thaw sperm quality assessments. Therefore, ejaculates were classified in three different populations according to the post-thaw sperm quality achieved using control CC (CC1: 2% of glycerol and ∼1200 °C/min of warming). The effectiveness of CCs was different ($P < 0.05$) in the three ejaculate populations. Spermatozoa from ejaculates considered as ''good'' freezers were relatively unaffected ($P > 0.05$) by the modifications in the CCs, whereas those from ''moderate'' and, mainly, ''bad'' freezers were very sensitive ($P < 0.05$). In conclusion, optimization of the CCs – GLY and WR – can improve the cryosurvival of spermatozoa in some ejaculates, particularly in those having poor sperm freezing ability.

Keywords: Semen; Cryopreservation; Ejaculate variability; Boar

1. Introduction

Poor survival of spermatozoa after thawing has been one of the major limitations to the successful application of frozen-thawed (F-T) boar semen in commercial AI programmes. Therefore, the primary objective of any strategy to enhance the fertility after AI with F-T spermatozoa must be to improve cryopreservation conditions in order to maximize sperm cryosurvival. During the past few years, cryobiological studies focussing on the adaptation of cooling rates to biophysical properties of spermatozoa, changes of sperm packaging systems as well as the accurate and consistent freezing of large numbers of samples have led to the improvement of cryopreservation protocols [1]. Nowadays it is possible to achieve post-thaw sperm survival rates above 50% in a high proportion of boar sires. This is a similar threshold to that used for bull semen. However, these promising results are overshadowed by important and consistent variations between boars in their sperm

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freezability. The magnitude of these differences is so large that it allows the boars to be classified into ''good'', ''moderate'' and ''bad'' sperm freezers [2]. The hypothesis underlying the present study was that the magnitude of individual differences between the boars in their freezing ability can be minimized by adapting specific cryopreservation conditions for ejaculates of either an individual boar or groups of boars. In support of our hypothesis others [3] have reported a considerable improvement in sperm cryosurvival in bulls with intrinsic low sperm freezability by optimizing freezing conditions.

At present, medium sized straws (0.5 mL) provide an effective packaging system for freezing boar spermatozoa worldwide. These straws allow uniform ice crystallization [4] and enable the storage of a relatively high number of spermatozoa [5], achieving good post-thaw sperm survival [6] and acceptable fertility after AI [7]. A safe equilibrium among cooling rates, glycerol concentrations and warming rates is a prerequisite to the achievement of good sperm freezability. A cooling rate of 30 °C/min together with a glycerol concentration of 2 to 4% and a warming rate of approximately 1200 °C/min were recommended for frozen and thawed boar spermatozoa packaged in 0.5 mL straws [8,9]. Surprisingly in their investigations, Fiser et al. [9] did not considered individual boar differences in their sperm freezability as a source of variation. We have observed significant differences in sperm survival among boars in response to the above mentioned cryopreservation conditions [10].

The objective of the present study was to evaluate the effectiveness of different cryopreservation conditions for freezing and thawing boar ejaculates, and check their suitability for individual ejaculates, focusing on those having sub-optimal sperm freezability. The differences in the cryopreservation conditions were limited to slight variations – always within a safety window – in cooling rate, glycerol concentration and warming rate, but otherwise under optimal cryopreservation conditions previously derived for 0.5 mL straws.

2. Material and methods

2.1. Reagents

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO), and made up under sterile conditions in a laminar-flow hood (HH48, Holten LaminAir, Allerod, Denmark) with purified water (18 MΩ-cm; Elgastat UHQPS, Elga Ltd. Lane End, Buckinghamshire, England).

2.2. Source, handling, evaluation and processing of ejaculates

Fifty-three healthy mature (2–4 years of age) and fertile crossbreed boars housed at two different commercial insemination stations were used as ejaculate donors. The management conditions of boars in both insemination stations were similar. Boars were housed in individual pens in an environmentally controlled (15–25 °C of air temperature) building with windows exposed to a natural daylength and supplementary light for 16 h daily. They were given *ad libitum* access to water and fed a commercial diet according to the nutritional requirements for adult boars.

Sperm-rich ejaculate fractions were collected using the gloved-hand method following the standard operating procedure, extended (1:1, v:v) in Beltsville Thawing Solution (BTS, composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$ and 3.35 mM EDTA, pH 7.2; [11]) and evaluated for conventional semen characteristics using standard laboratory techniques. Only ejaculates with $\geq 85\%$ sperm with normal morphology, and with $\geq 75\%$ and $\geq 80\%$ of motile and viable spermatozoa, respectively, were selected for cryopreservation. The extended semen was transferred to 50 mL plastic tubes, cooled to 17 °C, and sent by mail, packaged in insulated containers under conditions of monitored temperature (miniature data logger, Gemini Data Loggers Ltd., Chichester, England), to the sperm cryopreservation laboratory of the Veterinary Teaching Hospital (VTH), University of Murcia, Spain. The extended semen arrived at the laboratory 14 to 15 h after collection of the ejaculate. The temperature of extended semen during transport from the AI center to the laboratory ranged from 15 to 21 °C. At the laboratory, the semen was stored at 17 °C (60 min) and the sperm quality re-evaluated (see sperm quality assessment). Only ejaculates with $\geq 75\%$ and $\geq 80\%$ of motility and viability (plasma membrane integrity), respectively, were retained for cryopreservation.

2.3. Sperm cryopreservation

The ejaculates were frozen in straws using the procedure described by Westendorf et al. [12], as modified by Thurston et al. [13] and Carvajal et al. [14]. Briefly, extended semen was centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) at 17 °C for 3 min

at 2400 × g. After centrifugation, the supernatant was removed by aspiration and the pellet was re-extended with lactose-egg yolk (LEY) extender (80%, v:v 310 mM β-lactose, 20%, v:v egg yolk and 100 μg/mL kanamycin sulfate, pH 6.2 and 330 ± 5 mOsmol kg^{-1}) to a concentration of 1500 × 10^6 cells/mL. After cooling to 5 °C in 90 to 120 min, the extended spermatozoa were resuspended with LEY-Glycerol-Orvus ES Paste (LEYGO) extender [89.5 or 92.5% LEY, 9 or 6% glycerol and 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA, USA) v:v; pH 6.2 and 1650 ± 15 or 1145 ± 17 mOsmol kg^{-1}] to a final concentration of 1000 × 10^6 cells/mL. Then, the resuspended and cooled sperm were packed into 0.5 mL polyvinyl chloride-French straws (Minitüb, Tiefenbach, Germany) and frozen using a horizontal controlled-rate freezing instrument (IceCube 1810, Minitüb, Tiefenbach, Germany) as follows: 5 to −5 °C at 6 °C/min, from −5 to −80 °C at 10, 40 or 60 °C/min (see Section 2.5), held for 30 s at −80 °C, cooled at 70 °C/min to −150 °C, and finally plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for at least 2 weeks before thawing. Thawing of straws was achieved in a circulating water bath, set at two different temperatures to achieve the desired thawing rate (see Section 2.5). Thawed sperm samples from each straw were resuspended in BTS (1:2, v:v; 37 °C) and incubated in a waterbath at 37 °C for 150 min.

2.4. Sperm quality assessment

Sperm quality was determined by assessing motility, plasma membrane integrity and acrosomal status of spermatozoa before freezing (immediately after arrival at VTH laboratory) and after thawing (at 30 and 150 min).

Motility was objectively evaluated using a computer-aided sperm analysis system (Sperm Class Analyzer®, SCA, Microptic, Barcelona, Spain) following the procedure described by Cremades et al. [15]. Briefly, semen samples were re-extended in BTS to a concentration of 20 to 30 × 10^6 sperm/mL. For each evaluation, a 4 μL aliquot of sperm sample was placed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and three fields were analyzed at 39 °C, assessing a minimum of 100 sperm/sample. The proportions of total motile spermatozoa (% TMS) and rapid progressive motile (% RPM, VAP > 50 μm/s and STR > 75%) spermatozoa were recorded.

Plasma membrane integrity (PMI) was evaluated using the fluorescent probes SYBR-14 (SY) and propidium iodide (PI) according to the manufacturer's instructions (L-7011; Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands). Briefly, 100 μL of semen sample (30 × 10^6 sperm/mL in Hepes buffer: 10 mM HEPES/NaOH pH 7.5, containing 0.14 M NaCl and 2.5 mM $CaCl_2$) were rediluted with 25 nM SY solution and 12 μM PI solution. Rediluted semen samples were incubated at room temperature in dark for 10 min before flow cytometric analysis [16]. Spermatozoa were allocated to ''intact'' plasma membrane, ''dying'' and ''dead'' classifications if they exhibited SY+/PI−, SY+/PI+ and SY−/PI+ staining, respectively. Only spermatozoa with intact plasma membranes (plasma membrane integrity: PMI) were recorded.

Sperm acrosomal status was assessed by FITC-PNA (PNA) and PI staining as described by Nagy et al. [17], with slight modifications. Briefly, 100 μL of semen sample (30 × 10^6 cells/mL in Hepes buffer) was rediluted with 10 μL of PNA solution (1 μg/mL in bidistilled water) and 12 μM PI solution. Samples were incubated at room temperature in the dark for 5 min before flow cytometric analysis. Spermatozoa were allocated to ''live with intact acrosome'', ''live with damaged acrosome'', ''dead with intact acrosome'' and ''dead with damaged acrosome'' classifications if they exhibited PNA-/PI−, PNA+/PI−, PNA−/PI+ and PNA+/PI+ staining, respectively. Only live sperm with intact acrosomes (live with intact acrosome: LIA) were recorded.

Immediately prior to analysis by flow cytometry, stained sperm samples were rediluted to 6 × 10^6 sperm/mL in Hepes buffer. Analyses were carried out on a Becton Dickinson FACSort flow cytometer (San Jose, California, USA) equipped with an argon-ion laser (488 nm, 15 mW). The green fluorescence (FITC) was measured using a 530/30 nm band pass filter and the red fluorescence (PI) by a 650 nm LP filter except in the plasma membrane integrity assessment, where a 585/30 band pass filter was used. Debris was gated out based on the forward scatter and side scatter dot plot by drawing a region gating the sperm population only. On top of this, events with scatter characteristics similar to sperm cells but without reasonable DNA content (very weak SY or PI staining) were also gated out. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. A minimum of 10,000 spermatozoa were collected and analyzed at flow rate of ∼800 cells/s.

2.5. Experimental design

Using a split-ejaculate technique, single ejaculates from 53 boars were diluted in extender with either a 2 or

3% final glycerol concentration. Once packaged, 10–15 straws semen samples from each glycerol concentration were randomly assigned to one of the three following cooling rates: −10, −40 and −60 °C/min (from −5 to −80 °C, to overcome the critical temperature zone between −10 and −30 °C). For thawing, two straws from each glycerol concentration × cooling rate combination were randomly assigned to one of the following warming rates: ∼1200 °C/min (37 °C water bath for 20 s) or ∼1800 °C/min (70 °C for 8 s). Then, post-thaw sperm quality of two independent straws for each one of the 12 different cryopreservation protocols and ejaculate was assessed at two different times: 30 and 150 min.

As cooling rate did not show a significant statistical difference (see Section 3), the initial factorial design 2 (glycerol concentration) × 3 (cooling rate) × 2 (warming rate) was simplified to one 2 (glycerol concentration) × 2 (warming rate). Four different defined cryopreservation conditions (CCs) were then established: semen samples frozen with 2% glycerol and thawed at ∼1200 °C/min (CC1); frozen with 3% glycerol and thawed at ∼1200 °C/min (CC2); frozen with 2% glycerol and thawed at ∼1800 °C/min (CC3); and frozen with 3% glycerol and thawed at ∼1800 °C/min (CC4). The CC1 was considered as control because it was the cryopreservation condition usually used in our laboratory, achieving good post-thaw sperm quality [14,18,19] and acceptable fertility after AI [7,20].

2.6. Statistical analysis

For data analysis, SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) and PATN software package (CSIRO, Canberra, Australia) were used. Analyses of variance were performed using General Linear Mixed Models (GLMM). Percentage data (as square root of the proportion) of post-thaw sperm quality parameters were arc-sin transformed before ANOVA. When ANOVA revealed a significant effect, means were compared using the Bonferroni test and were considered to be significant when $P < 0.05$. Initially, a first model including the main effects of glycerol concentration, cooling rates, warming rates and their interactions, with ejaculate as random effect, was used. Subsequently, to investigate the combined effect of glycerol concentration and warming rate (four different cryopreservation conditions), data were reanalyzed including only the main effects of cryopreservation conditions (CC1 to CC4), ejaculate and their interaction. To further investigate ejaculate differences, a multivariate pattern analysis (PATN analysis) was carried out to identify naturally occurring groups within the ejaculates data set [21]. Pattern analysis classifies the ejaculates into a small number of groups according to the four post-thaw sperm assessments (total and rapid progressive sperm motility, plasma membrane integrity and live sperm with intact acrosome). Three groups (detailed in Section 3) were finally obtained from the non-hierarchical classification of the ejaculates samples. The hypothetical differences among PATN groups were assessed using one-way ANOVA. Finally, the relative frequencies of ejaculates belonging to each group (defined by PATN analysis) were compared among CCs by Chi square test. Results are presented as means ± S.E.M.

3. Results

Table 1 shows the statistical significance of the treatments and interactions evaluated on post-thaw quality of spermatozoa. While cooling rate had no influence ($P > 0.05$), glycerol concentration and warming rate, both independently, affected ($P < 0.05$) all post-thaw sperm quality parameters assessed. There were no interactions ($P > 0.05$) between treatments for any of the sperm parameters. Post-thaw sperm quality was highest ($P < 0.05$) in semen samples frozen with 3% glycerol or when the straws were thawed with a warming rate of ∼1800 °C/min (Table 2).

Evaluating the combined effect of glycerol concentration and warming rate (four different cryopreservation conditions -CCs- resulting of a 2 × 2 factorial design), the highest post-thaw sperm quality was achieved in the semen samples frozen with 3% glycerol and thawed at 1800 °C/min (Table 3). However, there was a significant interaction ($P < 0.001$) between CCs and ejaculate for all the post-thaw sperm quality parameters assessed, indicating differences among ejaculates in the response of their spermatozoa to the different cryopreservation conditions.

Taking into account the mentioned interaction, the ejaculates were grouped by PATN according to their post-thaw sperm quality. For this purpose, the data set included four descriptors of sperm quality (TMS, RPM, PMI and LIA) to each one of the ejaculate samples. Initially PATN was applied to the 53 ejaculate samples cryopreserved using the cryopreservation conditions that were defined as control (CC1). Three groups of ejaculates were identified. The ejaculates with best post-thaw sperm quality (21 ejaculates, 39.6%) were identified as ‘‘good’’ whereas those having moderate (18 ejaculates, 34%) and low (14 ejaculates, 26.4%) post-thaw sperm quality were considered as ‘‘moderate’’ and ‘‘bad’’ sperm freezers, respectively. Summary statistics of pre-freezing and

Table 1
ANOVA for influence of different treatments on post-thaw sperm quality parameters (53 cryopreserved ejaculates)

Parameter	Source	d.f.	MS	*F*	*P*-value
Total sperm motility	Cooling rate	2	279.5	1.4	NS
	Glycerol	1	1835.2	9.1	0.003
	Warming rate	1	16358.3	81.3	0.001
	Interactions				NS
Rapid progressive motility	Cooling rate	2	284.0	2.4	NS
	Glycerol	1	525.7	4.5	0.034
	Warming rate	1	3929.3	33.7	0.001
	Interactions				NS
Plasma membrane integrity	Cooling rate	2	32.7	0.3	NS
	Glycerol	1	4477.4	36.1	0.001
	Warming rate	1	5593.7	45.2	0.001
	Interactions				NS
Live sperm with intact acrosome	Cooling rate	2	111.9	1.0	NS
	Glycerol	1	4658.2	41.2	0.001
	Warming rate	1	3081.7	27.3	0.001
	Interactions				NS

ANOVA was calculated using ejaculate as random effect. As any interaction was significant, specific statistic data of them were not included. MS, mean square.

Table 2
Post-thaw quality (Mean ± S.E.M.) of boar spermatozoa cryopreserved using two glycerol concentrations and two warming rates (overall means across individual ejaculates collected from 53 boars)

Sources of variation	Post-thaw sperm quality assessments (%)*			
	TMS	RPM	PMI	LIA
Glycerol concentration (%):				
2	50.1 ± 0.6^{a}	16.4 ± 0.3^{a}	52.9 ± 0.5^{a}	52.2 ± 0.5^{a}
3	52.5 ± 0.5^{b}	17.3 ± 0.3^{b}	56.6 ± 0.5^{b}	56.0 ± 0.5^{b}
Warming rate (°C/min):				
~1200	47.7 ± 0.6^{a}	16.2 ± 0.3^{a}	52.7 ± 0.5^{a}	52.6 ± 0.5^{a}
~1800	54.8 ± 0.5^{b}	17.6 ± 0.3^{b}	56.8 ± 0.5^{b}	55.7 ± 0.4^{b}

Different superscripts (a, b) in the same column denote significant differences ($P < 0.05$).
* TMS, total sperm motility; RPM, spermatozoa with rapid and progressive motility (VAP > 50 μm/s and STR > 75%); PMI, spermatozoa with plasma membrane integrity; LIA, live sperm with intact acrosome.

Table 3
Post-thaw quality (Mean ± S.E.M.) of boar spermatozoa cryopreserved using four different cryopreservation conditions (overall means across individual ejaculates collected from 53 boars)

Cryopreservation conditions*	Post-thaw sperm quality assessments (%)**			
	TMS	RPM	PMI	LIA
CC1	46.3 ± 0.9^{a}	15.7 ± 0.4^{a}	50.6 ± 0.7^{a}	50.4 ± 0.7^{a}
CC2	49.0 ± 0.8^{b}	$16.7 \pm 0.4^{a,b}$	54.7 ± 0.7^{b}	54.0 ± 0.7^{b}
CC3	53.8 ± 0.7^{c}	$17.2 \pm 0.4^{b,c}$	55.2 ± 0.6^{b}	54.7 ± 0.6^{b}
CC4	55.9 ± 0.7^{d}	18.0 ± 0.4^{c}	58.5 ± 0.6^{c}	57.3 ± 0.6^{c}

Different superscripts (a–d) in the same column denote significant differences ($P < 0.05$).
* CC1: 2% of glycerol and ~1200 °C/min of warming rate; CC2: 3% and ~1200 °C/min; CC3: 2% and ~1800 °C/min; and CC4: 3% and ~1800 °C/min.
** TMS, total sperm motility; RPM, spermatozoa with rapid and progressive motility (VAP > 50 μm/s and STR > 75%); PMI, spermatozoa with plasma membrane integrity; LIA, live sperm with intact acrosome.

Table 4
Pre-freeze and post-thaw sperm quality parameters for each group of ejaculates, classified according to PATN analysis of post-thaw sperm assessments of semen samples cryopreserved following the control cryopreservation conditions (CC1: 2% of glycerol and ~1200 °C/min of warming rate)

Sperm quality parameters (%)	PATN freezer groups			*P*-value
	Good (no. = 21)	Moderate (no. = 18)	Bad (no. = 14)	
Pre-freeze:				
Total sperm motility	83.9 ± 1.0 (75.6–95.8)	82.9 ± 1.2 (75.1–93.7)	86.0 ± 1.9 (78.1–93.3)	0.343
Rapid progressive motility	18.3 ± 1.1 (10.6–30.8)	20.0 ± 1.3 (11.2–33.4)	19.2 ± 1.6 (11.9–25.4)	0.571
Plasma membrane integrity	87.9 ± 1.0 (81.9–97.6)	87.7 ± 0.9 (80.6–97.3)	86.6 ± 1.4 (80.5–93.6)	0.747
Live sperm with intact acrosome	86.6 ± 0.8 (80.4–96.7)	87.4 ± 0.9 (80.6–96.5)	86.1 ± 1.4 (80.1–92.9)	0.775
Post-thawing:				
Total sperm motility	58.0 ± 0.9[a] (42.4–68.2)	41.6 ± 1.1[b] (30.8–49.6)	25.7 ± 1.5[c] (9.7–34.7)	0.001
Rapid progressive motility	20.3 ± 0.8[a] (15.1–31.2)	13.9 ± 0.7[b] (9.4–18.6)	7.7 ± 0.6[c] (3.8–12.2)	0.001
Plasma membrane integrity	59.0 ± 0.6[a] (48.7–72.1)	47.7 ± 0.9[b] (36.2–56.5)	36.7 ± 1.5[c] (18.1–44.2)	0.001
Live sperm with intact acrosome	57.8 ± 0.7[a] (43.2–70.7)	47.4 ± 0.9[b] (30.8–52.2)	35.2 ± 1.6[c] (17.0–42.7)	0.001

Values are mean (±S.E.M.) with ranges in parentheses. Within rows, values with different superscripts (a–c) are different ($P < 0.05$).

post-thawing sperm quality assessments for these three ejaculate groups are shown in Table 4. There was no significant variation ($P > 0.05$) for any of the sperm quality assessments between ejaculate groups before freezing. However, there were significant differences ($P < 0.001$) for all of them between the three groups of ejaculates after thawing.

From the groups of ejaculates achieved after the PATN analysis of the ejaculate samples cryopreserved using CC1, the influence of each CC was evaluated within each ejaculate group: ''good'', ''moderate'' and ''bad''. The influence of CCs was different according to the ejaculate group. No significant differences ($P > 0.05$) for any of sperm quality assessments between CCs were found within ejaculates classified as ''good'', however the differences were significant ($P < 0.05$) for all sperm assessments within ''moderate'' and ''bad'' ejaculates. The best post-thaw sperm quality was achieved in semen samples frozen and thawed following CC4 (3% of glycerol and thawed at ~1800 °C/min) within ejaculates classified as ''moderate'' and in those semen samples thawed at ~1800 °C/min (CC3 and CC4) within ejaculates classified as ''bad'' (Fig. 1a and b).

A second PATN analysis was applied to the 212 ejaculate samples cryopreserved (53 for each one of the 4 CCs). The proportion of ejaculates classified as ''good'', ''moderate'' or ''bad'' varied between CCs (Fig. 2). The proportion of ''moderate'' ejaculates remained constant ($P > 0.05$) to the four cryopreservation conditions, whereas those of ''good'' and ''bad'' groups varied ($P < 0.01$), showing an inverse trend. The proportion of ''good'' ejaculates was highest in CC4 whereas that of ''bad'' ejaculates was highest in CC1.

4. Discussion

In the present study sperm samples packaged in 0.5 mL straws from 53 boars were cryopreserved using 12 different CCs. Overall, the spermatozoa tolerated, without significant differences, the three cooling rates evaluated (−10, −40 and −60 °C/min). However, there were significant differences in post-thaw sperm quality in response to glycerol concentration (2 versus 3%) and/or warming rate (~1200 versus ~1800 °C/min). The overall best CCs were those including a 3% final glycerol concentration in the freezing extender and a warming rate of ~1800 °C/min. These results are not surprising, especially those related to cooling rate and glycerol concentration, because other researchers obtained similar results (22–25). Medrano et al. [22] reported that boar spermatozoa tolerate cooling rates in the range −15 to −60 °C/min through the critical temperature zone of −15 to −25 °C (where the cell damage is most likely to occur) without or with only marginal differences in cryosurvival. Several systematic studies have also been carried out to assess the optimum concentration of glycerol for freezing of boar spermatozoa, which was generally recommended to be in the range of 2 to 4% [8,23–25]. The latter authors [25] considered that 3% glycerol would be the most effective when spermatozoa are packaged in 0.5 mL straws. In relation to more suitable warming rates for thawing, Fiser et al. [9] claimed that ~1200 °C/min should be the most appropriate to thaw boar spermatozoa packaged in 0.5 mL straws. However, these authors reported that the optimum warming rate between ~1200 and ~1800 °C/min differed depending on the criterion used to assess post-thaw sperm quality. It should be noted that post-

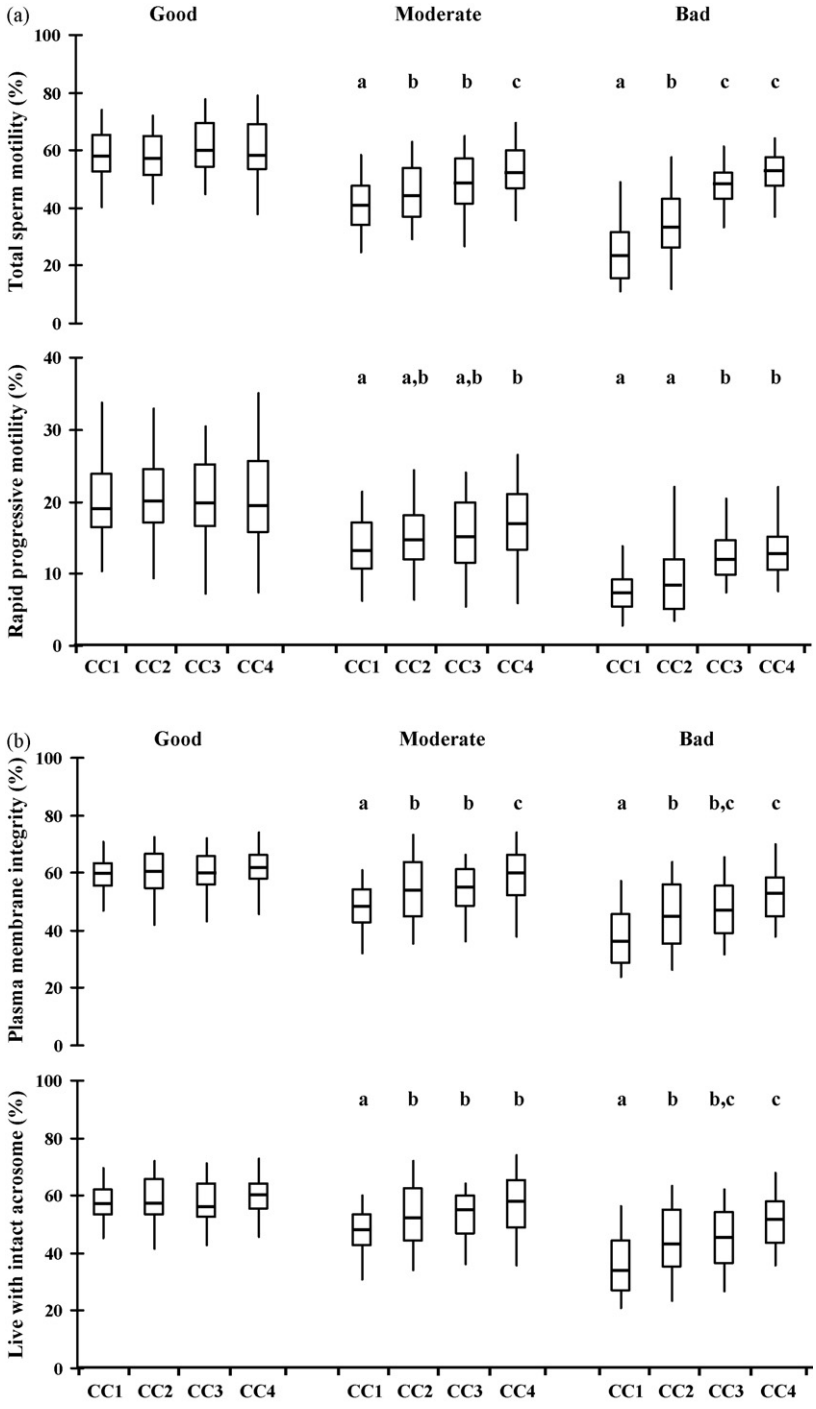

Fig. 1. (a) Whisker-box plots showing variation in the post-thawing percentages of total sperm motility and spermatozoa with rapid progressive motility in the four cryopreservation conditions (CC) for each of the three groups of ejaculates (good, moderate and bad sperm freezers). CC1: 2% glycerol and ∼1200 °C of warming rate (as control); CC2: 3% and ∼1200 °C; CC3: 2% and ∼1800 °C; CC4: 3% and ∼1800 °C. Boxes enclose the 25th and 75th percentiles, the line is the median, and whiskers extending to the 5th and 95th percentiles. a–c, indicate significant differences ($P < 0.05$) among cryopreservation conditions in each sperm freezer group. (b) Whisker-box plots showing variation in the post-thawing percentages of spermatozoa with plasma membrane integrity and live with intact acrosome in the four cryopreservation conditions (CC) for each of the three groups of ejaculates (good, moderate and bad sperm freezers). CC1: 2% glycerol and ∼1200 °C of warming rate (as control); CC2: 3% and ∼1200 °C; CC3: 2% and ∼1800 °C; CC4: 3% and ∼1800 °C. Boxes enclose the 25th and 75th percentiles, the line is the median, and whiskers extending to the 5th and 95th percentiles. a–c, indicate significant differences ($P < 0.05$) among cryopreservation conditions in each sperm freezer group.

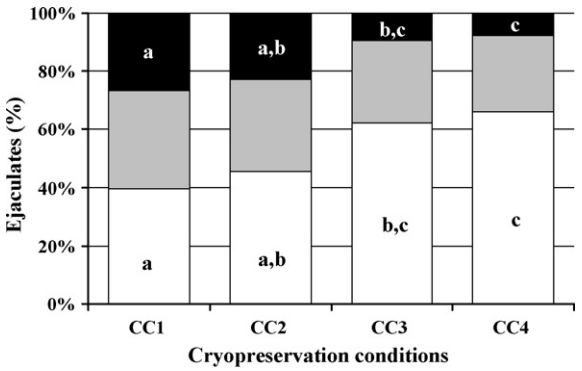

Fig. 2. Frequency distribution of ejaculates in ''good'' (white), ''moderate'' (grey) and ''bad'' (black) sperm freezer groups according to their post-thaw sperm quality in each cryopreservation condition. a–c, indicate significant differences ($P < 0.05$).

thaw sperm assessments in the present study were different from those checked by Fiser et al. Recently, Hernandez et al. [26] found better nuclear DNA stability in boar sperm samples thawed at ∼1800 °C/min than at ∼1200 °C/min.

One of the most important findings of our experiment was the clear demonstration that a fast warming rate (∼1800 °C/min) improves sperm cryosurvival. To achieve this rate, the straws must be submersed in a very warm water bath (70 °C) for a very short period of time (8 s). Both temperature and timing seem to be extremely critical for this technique to be transferable to commercial situations. Since a longer period in the water bath would raise the temperature inside the straw to non-physiological limits. However, it is interesting to point out that slight variation (±1 s) in the time that the straw is kept in the water bath at 70 °C should still be safe as the temperature of the semen within the straw is raised to around 23 (7 s) or 37 °C (9 s) [27]. Moreover, commercial thawing machines are now available to carry out the above warming protocol accurately and under controlled conditions.

As expected, there were significant differences among ejaculates in the cryosurvival of spermatozoa under the different CCs evaluated. This is not surprising taking into account that each ejaculate was from a different boar. The fact that spermatozoa from different individuals may exhibit different responses to the same freezing and thawing treatment has been previously reported in cattle [3], horses [28], sheep [29], dogs [30] and pigs [2,22,31]. Moreover, as the degree of sperm cryosensitivity seems to be consistent over time for each male [2,10], they can be classified as ''good'', ''moderate'' or ''bad'' sperm freezers [22,32]. This phenomenon, that is independent of male breed or genetic background, is poorly related with prior sperm quality of ejaculates [10]. However, it may have a genetic origin. Differences in specific DNA sequences have been identified between boars classified as ''bad'' or ''good'' sperm freezers [33]. However, the present study demonstrated that a certain degree of intra-ejaculate variation in sperm cryosurvival can be attributed to the CC used; and therefore it can be attenuated by choosing a much more appropriate frozen and thawed protocol.

The 53 ejaculates frozen-thawed following the CC considered as control (2% of glycerol and ∼1,200 °C of warming rate) were objectively classified (PATN analysis) as ''good'', ''moderate'' or ''bad'' according to the post-thaw sperm quality. The percentage of ''bad'' ejaculates (26.4%) was within the range recorded in previous studies (25 to 35%; reviewed by 1). This percentage is sufficiently high to allow some boars with genetic merit to be eliminated as sperm cryobank founders. From a practical viewpoint, it is important to the sperm cryopreservation industry to minimize the incidence of ''bad'' sperm freezers. To achieve this, Watson [32] suggested the concept of individually-tailored frozen-thawed protocol. Following this suggestion in the present study, 12 different CCs were initially used for freezing and thawing each of the 53 ejaculates. Finally, four of them influenced on the percentage of ejaculates classified as ''bad'' freezers, which was reduced to only 7.5% using CC4 (3% glycerol and ∼1800 °C of warming rate). Similar observations have also been reported with bovine spermatozoa [3]. These observations support the view that the proportion of ejaculates with poor sperm cryosurvival could be minimized by optimizing the CCs within a small range. This can be regarded as ''safety range''. Moreover, as intra-boar differences in sperm cryosurvival are considerably lower than inter-boar differences [10], it seems clear that some boars considered as ''bad'' freezers could become ''good'' freezers by adjustment of the CCs, mainly the warming rate. The CCs × ejaculate interaction tend to imply that optimum sperm cryosurvival is likely to be achieved when freezing and thawing protocol is tailored to each individual boar. However, this approach is impractical in a commercial setting. But in exceptional cases, tailoring the protocol for a certain highly valuable boar with intrinsic low sperm cryosurvival could be a way to use these boars as ejaculate donors.

An important observation in the present study was the different behaviour of spermatozoa to support the different CCs between the ejaculates classified as ''good'', ''moderate'' and ''bad'' freezers. Spermatozoa

from ejaculates considered as ''good'' have a major tolerance to the adjustments on the CCs, whereas those from ''moderate'' and, mainly, ''bad'' were very sensitive to those adjustments, even when they were made always within a safe range. This finding would be in agreement with those of Parkinson and Whitfield [3] and Medrano et al. [22], for bull and boar semen, respectively, who reported differences in tolerance of spermatozoa to sub-optimal cooling rates between males classified as ''bad'' or ''good'' sperm freezers, with ''bad'' freezers having the lowest tolerance. Therefore, our result demonstrated clearly that adjustments of CCs may be suitable for both minimizing the variability between ejaculates and enhancing the overall post-thaw sperm quality of cryopreserved ejaculates. This finding has an important practical implication because the variability between ejaculates on post-thaw sperm quality is a recurrent problem of commercial boar sperm cryobanks.

In conclusion, this study demonstrated that slight modifications in the freezing and thawing protocol – glycerol concentration and warming rate for thawing – can improve the sperm cryosurvival of some ejaculates. This point was particularly true for those ejaculates showing intrinsic poor sperm freezability.

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References

[1] Roca J, Rodriguez-Martinez H, Vazquez JM, Bolarin A, Hernandez M, Saravia F, et al. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Ashworth CJ, Kraeling RR, editors. Control of pig reproduction VII. Nottingham: Nottingham University Press; 2006. p. 261–75.

[2] Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22: 382–94.

[3] Parkinson TJ, Whitfield CH. Optimisation of freezing conditions for bovine spermatozoa. Theriogenology 1987;27:781–97.

[4] Weitze KF, Rath D, Baron G. Neue Aspekte der Tiefgefrierkonservierung von Ebersperma in Plastikröhren Dtsch Tierärztl Wschr 1987;82:261–7.

[5] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodríguez-Martinez H. Deep freezing of concentrated boar semen for intrauterine insemination: effects on sperm viability. Theriogenology 2005;63:1320–33.

[6] Eriksson BM, Vazquez JM, Martinez EA, Roca J, Lucas X, Rodriguez-Martinez H. Effects of holding time during cooling and of type of package on plasma membrane integrity, motility and in vitro oocyte penetration ability of frozen-thawed boar spermatozoa. Theriogenology 2001;55:1593–605.

[7] Roca J, Carvajal G, Lucas X, Vázquez JM, Martínez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. Theriogenology 2003;60:77–87.

[8] Fiser PS, Fairfull RW. Combined effect of glycerol concentration and cooling velocity on motility and acrosomal integrity of boar spermatozoa frozen in 0.5 ml straws. Mol Reprod Dev 1990;25:123–9.

[9] Fiser PS, Fairfull RW, Hansen C, Panich PL, Shrestha JN, Underhill L. The effect of warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of freezing and glycerol level. Mol Reprod Dev 1993;34:190–5.

[10] Roca J, Hernandez M, Carvajal G, Vazquez JM, Martinez EA. Factors influencing boar sperm cryosurvival. J Anim Sci 2006;84:2692–9.

[11] Pursel VG, Johnson LA. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. J Anim Sci 1975;40:99–102.

[12] Westendorf P, Ritcher L, Treu H. Zur Tiefgefrierung von Ebersperma. Labor-und Besamungsergebnisse mit dem Hülsenberger Pailletten-verfahren. Dtsch Tierärztl Wschr 1975;82: 261–7.

[13] Thurston LM, Watson PF, Holt WV. Sources of variation in boar spermatozoa fertility following cryopreservation. Cryobiology 1999;39:355.

[14] Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J. Effect of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389–96.

[15] Cremades T, Roca J, Rodriguez-Martinez H, Abaigar T, Vazquez JM, Martinez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610–8.

[16] Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276–84.

[17] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68: 1828–35.

[18] Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397–405.

[19] Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, et al. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15–24.

[20] Bolarín A, Roca J, Rodríguez-Martínez H, Hernández M, Vázquez JM, Martínez EA. Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility

between farms when frozen-thawed semen is used. Theriogenology 2006;65:669–80.
[21] Abaigar T, Holt WV, Harrison RAP, del Barrio G. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod 1999;60:32–41.
[22] Medrano A, Watson PF, Holt WV. Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Reproduction 2002;123:315–22.
[23] Almlid T, Clarke RN, Pursel VG, Johnson LA. Effectiveness of in vitro methods for predicting in vivo fertilizing capacity of boar spermatozoa cryopreserved with 2% or 4% glycerol. Zuchthyg 1989;24:8–15.
[24] Almlid T, Johnson LA. Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws. J Anim Sci 1988;66:2899–905.
[25] Buhr MM, Fiser P, Bailey JL, Curtis EF. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. J Androl 2001;22:961–9.
[26] Hernandez M, Roca J, Ballester J, Vazquez JM, Martinez EA, Johannisson A, et al. Differences in SCSA outcome among boars with different sperm freezability. Int J Androl 2006;29:583–91.
[27] Nöthling JO, Shuttleworth R. The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. Theriogenology 2005;63:1469–80.
[28] Janett F, Thun R, Bettschen S, Burger D, Hassig M. Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. Anim Reprod Sci 2003;77:213–21.
[29] D'Alessandro AG, Martemucci G. Evaluation of seasonal variations of semen freezability in Leccese ram. Anim Reprod Sci 2003;79:93–102.
[30] Yu I, Songsasen N, Godke RA, Leibo SP. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. Cryobiology 2002;44: 62–78.
[31] Larsson K, Einarsson S. Influence of boars on the relationship between fertility and post thawing sperm quality of deep frozen boar spermatozoa. Acta Vet Scand 1976;17:74–82.
[32] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–91.
[33] Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of of amplified restriction fragment length polymorphism markers linked to genes controlling boar spermviability following cryopreservation. Biol Reprod 2002;66:545–54.

Artículo 5

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Cryosurvival and In Vitro Fertilizing Capacity After Thaw is Improved When Boar Spermatozoa Are Frozen in the Presence of Seminal Plasma From Good Freezer Boars

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ABSTRACT: The study evaluated the protective effect of seminal plasma (SP) added to freezing extender against cryopreservation injuries to boar spermatozoa. Pooled sperm-rich fractions collected from 9 fertile boars were frozen in 0.5-mL straws after being extended in a conventional freezing extender either alone or supplemented with 5% of SPs (SP1–SP4) collected from the sperm-rich fractions (diluted 1:1, vol/vol, in Beltsville Thawing Solution extender) from 4 boars (1–4) with known sperm cryosurvival (poor, moderate, and good sperm freezers). Cryopreservation injuries were assessed in terms of post-thaw sperm motility (assessed by computer-assisted sperm analysis), viability (plasma membrane and acrosome integrity assessed simultaneously by flow cytometry), membrane lipid peroxidation (malondialdehyde [MDA] production), and the ability of thawed spermatozoa to fertilize in vitro–matured homologous oocytes. The addition of SP from good sperm freezers (SP3 and SP4) improved ($P < .01$) the motility and viability of thawed spermatozoa without any influence on MDA production. Moreover, SP from good sperm freezers also increased ($P < .05$) the percentage of penetrated (SP3) and polyspermic oocytes (SP4) with respect to the control. Neither the total amount of SP proteins, protein profiles, nor antioxidant capacity of the different SPs were related to the various cryosurvival/fertilizing capacities of the processed spermatozoa.

Key words: Cryopreservation, pig.
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It is well known that processing of spermatozoa such as with incubation, flow cytometric sorting, cooling, or cryopreservation affects sperm structures including membrane destabilization, which might ultimately result in cell death (reviewed by Maxwell and Johnson, 1999). In this respect, hardly 50% of the spermatozoa from an ejaculate survive the freezing and thawing process (reviewed by Roca et al, 2006b). Seminal plasma (SP) apparently minimizes these effects when a certain proportion of whole SP or particular SP components are added to the medium in which spermatozoa are suspended, including thawing media (Larsson and Einarsson, 1976; Vadnais et al, 2005a). Using this rationale, SP has proven beneficial to protect bull (Garner et al, 2001) and stallion (Aurich et al, 1996) spermatozoa during the cryopreservation process when added to the freezing extender. However, to the best of our knowledge, observations about the potential benefits of SP added to the freezing extender on post-thaw sperm cryosurvival have not been reported in pigs mostly owing to the customary removal of the bulk of SP before cooling in boar sperm cryopreservation protocols.

The SP composition varies among species as well as among males within the same species (reviewed by Strzezek et al, 2005). Thus, different SP profiles have been found between boars of different in vivo fertility (Flowers and Turner, 2001). Moreover, it has been suggested that the important variability among boars to sustain sperm cryopreservation could be related to differences in SP composition (Roca et al, 2006a), which may have a genetic origin (Thurston et al, 2002). In this way, as seen in other species, differences in SP protein profiles have been related to low and high sperm freezability in bulls (Jobim et al, 2004) and stallions (Zahn et al, 2005). Then, a logical hypothesis is that the supplementation of freezing extender with SP from boars with good sperm freezability could eventually improve the ability of boar spermatozoa to sustain freezing-thawing processes.

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Therefore, the present study examined the possible protective effects of the addition of homologous SP to cooling extender on boar sperm cryosurvival. In addition, because the SPs were collected from ejaculates of boars with different sperm freezability, the amounts of the most relevant SP components were quantified to relate these to the encountered effect.

3 Material and Methods

Reagents

Unless otherwise stated, all media components were purchased from Sigma Chemical Co (St Louis, Mo) and made up under sterile conditions in a laminar flow hood (HH48; Holten LaminAir, Allerod, Denmark) with 4 purified water (18 MΩ -cm; Elgastat UHQPS; Elga 5 Ltd, Lane End, Buckinghamshire, England).

Boars and Ejaculate Collection and Processing

Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of Murcia University. Healthy and fertile mature boars of various commercial hybrid lines undergoing regular semen collection for commercial artificial insemination and included in a sperm cryobank program were used. Boars were housed in individual pens in environmentally controlled buildings (commercial insemination station of PROINSERGA SA, Segovia, Spain). They were given ad libitum access to water and were fed commercial diets according to the nutritional requirements for adult boars.

Sperm-rich fractions were collected by the gloved-hand method, extended (1:1, vol/vol) in Beltsville Thawing Solution ([BTS] 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$, and 3.35 mM EDTA), and evaluated by standard laboratory techniques (subjective analysis). Only ejaculates with at least 200×10^6 sperm per milliliter, at least 85% sperm with normal morphology, and at least 75% and at least 80% of motile and viable sperm, respectively, were selected for cryopreservation. The selected ejaculates were packed for shipping at 17°C and delivered 14–16 hours later to the Laboratory of Andrology of the Veterinary Teaching Hospital of the University of Murcia, Spain.

Collection and Storage of SP Samples

In a preliminary study in which 116 ejaculates from 29 mature fertile hybrid boars (4 ejaculates per boar) were cryopreserved, the boars were classified into 3 groups according to the post-thaw sperm survival assessed at 30 and 150 minutes after thawing as good, moderate, and poor freezers (Roca et al, 2006a). Four boars were selected among them as SP donors: SP1 was a poor sperm freezer (less than 40% of motile and viable spermatozoa), SP2 was a moderate sperm freezer (40%–60%), and SP3 and SP4 were good sperm freezers (more than 60%). SP was obtained during the cryopreservation process. After centrifugation ($2400 \times g$ for 3 minutes) of sperm-rich fractions diluted 1:1 (vol/vol) in BTS, the supernatant was collected and recentrifuged, filtered through a 10-μm nylon mesh filter to remove debris or clumped spermatozoa, and examined using phase microscopy to ensure no spermatozoa remained. Samples containing spermatozoa were refiltered. The supernatant (SP diluted in BTS) was stored frozen at −20°C until further use. Before using, SP was thawed at room temperature.

Sperm Cryopreservation

Ejaculates were cryopreserved using the straw freezing procedure described by Westendorf et al (1975) as modified by Thurston et al (2001) and Carvajal et al (2004). Briefly, pooled extended sperm-rich fractions were centrifuged at $2400 \times g$ for 3 minutes at 17°C, and sperm pellet was reextended in lactose egg yolk (LEY) extender (80% [vol/vol] 310 mM β-lactose, 20% [vol/vol] egg yolk, 100 μg/mL kanamycin sulphate [pH 6.2], and 330 ± 5 mOsmol/kg) to yield to a concentration of 1.5 6 $\times 10^9$ cells per milliliter. After further cooling to 5°C in 90–120 minutes, the spermatozoa were resuspended with LEY-Glycerol-Orvus ES Paste (LEYGO) extender (92.5% LEY + 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass], 6% glycerol [vol/vol] [pH 6.2], and 1145 ± 17 mOsm/kg) to a final concentration of 1×10^9/mL. The resuspended and cooled spermatozoa were 7 packed into 0.5-mL PVC-French straws (Minitüb, Tiefenbach, Germany) and frozen using a controlled-rate freezing instrument (IceCube 1810; Minitüb) as follows: cooled to −5°C at 6°C/min, from −5°C to −80°C at 40°C/min, held for 30 seconds at −80°C, cooled at 70°C/min to −150°C, and finally plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for at least 2 weeks before thawing, which was done in circulating water at 37°C for 20 seconds. Thawed spermatozoa from 2 straws per ejaculate were resuspended in BTS (1:2, vol/vol; 37°C) and incubated in a water bath at 37°C for 150 minutes.

Evaluation of Post-Thaw Sperm Quality

Measurements were done 30 minutes and 150 minutes after thawing. Post-thaw spermatozoa were assessed for motility and viability (plasma membrane and acrosome integrity). Sperm motility was objectively evaluated using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer; Microptic, Barcelona,

Spain) following the procedure described by Cremades et al (2005). Briefly, BTS-diluted thawed spermatozoa were further resuspended in BTS at a concentration of 30×10^6/mL. For each evaluation, a 4-µL sperm sample was placed in a prewarmed (39°C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were analyzed to assess a minimum of 100 spermatozoa. The analysis yielded the following motility parameters: TSM (total motile spermatozoa, %), VCL (curvilinear velocity, µm/s), VSL (straight linear velocity, µm/s), VAP (average path velocity, µm/s); LIN (linearity, %), and ALH (amplitude of lateral head displacement, µm).

Plasma membrane and acrosome integrity was evaluated simultaneously using a triple fluorescent procedure described by Nagy et al (2003). Aliquots (100 µL each, about 3×10^6 cells) were transferred into culture tubes, and dyes were added at a final concentration of 25 nM SYBR-14 (100 µM stock solution in DMSO; Component A of LIVE/DEAD Sperm Viability Kit, L-7011; Molecular Probes, Europe BV, Leiden, The Netherlands), 1 µg/mL of peanut agglutinin conjugated with phycoerythrin (PE)–PNA solution (1 mg/mL stock solution Phycoprobe R-PE-PNA, P44; Biomeda Corp, Foster City, Calif), and 12 µM PI (1.5 mM in phosphate buffer [PBS]; Component B of Sperm Viability Kit). Samples were mixed and incubated at room temperature (20°C–22°C) in the dark for 10 minutes. Just before analysis, 400-µL PBS was added to each sample and remixed before run through a flow cytometer. Flow cytometer analysis was performed by using a Coulter Epics XLTM (Coulter Corp, Miami, Fla). All dyes were excited by an argon ion 488-nm laser. SYBR-14 fluorescence (particles containing DNA, living cells) was detected with a 525 nm band-pass (BP) filter, PI signal (nonviable cells) was detected by a 620 nm BP filter, and PE-PNA fluorescence (cells with damaged acrosomes) was detected using a 575 BP filter. Acquisition and analysis were done using the EXPO 2000 (Coulter) software. Debris was gated out based on scatter properties and double gated out based on SYBR-14 or PI fluorescence. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Acquisition was stopped after recording 10 000 gated events, and event rates were kept around 800 cells per second. Only the percentage of live spermatozoa with intact acrosome was considered in results.

Measurement of Membrane Lipid Peroxidation

Membrane lipid peroxidation was estimated by the endpoint generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test following the procedure described by Carvajal et al (2004). Briefly, extended spermatozoa (250×10^6 cells in 1 mL) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate proteins. The precipitate was pelleted by centrifugation ($1500 \times g$ for 10 minutes), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was determined by spectrophotometry (UNICAM PU 8610 kinetics spectrophotometer; Philips, Eindhoven, Holland) at 534 nm. The results were expressed as a simple concentration of MDA (picomoles per 10^8 cells).

In Vitro Oocyte Maturation and Fertilization

The protocol used was that previously described by Gil et al (2004). Briefly, oocytes were obtained from ovaries of prepubertal gilts collected at a local slaughterhouse. The oocytes, surrounded by a compact cumulus mass and having evenly granulated cytoplasm, were matured (45–50 oocytes in 500 µL per well) in BSA-free NCSU-23 medium supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL cysteine, and 10 ng/mL epidermal growth factor for 44 hours in 5% CO_2 in air at 39°C (22 hours with added hormones, 10 IU/mL eCG and 10 IU/mL hCG [Intervet International BV, Boxmeer, The Netherlands], and then for another 22 hours without hormones). After the completion of in vitro maturation, batches of 30 denudated oocytes (0.1% hyaluronidase) were placed in 50-µL drops of preequilibrated in vitro fertilization (IVF) medium (mTBM; Abeydeera and Day, 1997). The dishes with the oocytes were kept in the incubator for about 30 minutes until spermatozoa were added for fertilization. A total of 100 µL of extended spermatozoa from 1 pool of 3 frozen-thawed (FT) straws was washed 3 times by centrifugation at $1900 \times g$ for 3 minutes in DPBS medium supplemented with 0.1% BSA, 75 µg/mL potassium penicillin G, and 50 µg/mL streptomycin sulfate (pH 7.2). At the end of the washing procedure, the sperm pellet was resuspended in IVF medium, and then 50 µL of this sperm suspension was added to the medium that contained oocytes so that each oocyte was exposed to 2000 spermatozoa. At 6 hours after insemination, oocytes were washed and transferred (30–35 oocytes per well) to a Nunc 4-well multidish containing 500 µL of NCSU-23 with 0.4% BSA and cultured at 39°C, 5% CO_2 in air for 10–12 hours. Then oocytes were mounted on slides, fixed in 25% (vol/vol) acetic acid in ethanol (24 hours at least), stained with 1% lacmoid in 45% (vol/vol) acetic acid, and examined under a phase contrast microscope at magnifications of 400×. The fertilization parameters evaluated were percentage of penetrated ([number of oocytes penetrated/total insem-

inated oocytes] × 100) and monospermic oocytes [(number of monospermic oocytes/total penetrated oocytes) x 100] and the number of spermatozoa per oocytes (mean number of spermatozoa in penetrated oocytes). Four replicates were done, and at least 120 oocytes per each FT sperm sample were evaluated.

Determination of SP Protein Profiles

Total protein concentration of SPs was estimated following the procedure described by Bradford (1976). SP proteins were isolated by reverse-phase high-performance liquid chromatography (RP-HPLC). Briefly, SP proteins were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrospher RP100 C18 column (250 × 4 mm, 5-μm particle size; 13 Merck, Darmstadt, Germany) eluted at 1 mL/min with a linear gradient of 0.1% trifluoroacetic acid in water (solution A) and acetonitrile (solution B), isocratically (5% solution B) for 5 minutes, followed by 5%–25% B for 10 minutes, 25%–60% B for 50 minutes, and 60%–70% B for 10 minutes). Protein detection was at 215 nm.

Assessment of the Antioxidant Components of SP

Total Nonenzymatic Antioxidant Capacity—Measurements were performed on deproteinized SP (adding 5% trichloroacetic acid on ice). Samples were subsequently centrifuged at 10 000 × *g,* the pellet was discarded, and measurements were performed on the supernatant. Total nonenzymatic antioxidant capacity (TAC) was measured using the ABTS (2,2′-Azino-di-(3-ethyl-14 benzthiazoline sulphonate)/HRP decoloration method (Cano et al, 1998). This method is based on the capacity of different components to scavenge the ABTS radical cations ($ABTS^{\cdot+}$) compared with a standard antioxidant (Trolox) in a dose-response curve. Preformed $ABTS^{\cdot+}$ was obtained by mixing 1 mmol/L ATBS, 60 μmol/L H_2O_2, and 0.25 μmol/L HRP in 50 mM sodium phosphate buffer (pH 7.5). Then, 10 μL of SP was added to the reaction medium (200 μL) and the decrease in absorbance, directly proportional to the $ABTS^{\cdot+}$ quenched, was determined at 730 nm at 5 and 30 minutes in a 96-well microplate reader (Anthos 201, Rosy 15 Anthos, Germany) Data were expressed as micromoles of Trolox equivalents.

Enzymatic Antioxidant Activity—For estimation of the enzymatic antioxidant activity, SP samples (1 mL) were centrifuged at 10 000 × *g* for 5 minutes. Then the supernatant was separated and kept in ice until the analysis. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) activities were measured as described by Martí et al 16 (2003) for ram spermatozoa. The absorbance changes were monitored with an UV-Vis Hitachi spectrophotometer (U-2000; Hitachi Ltd, Tokyo, Japan) measuring the NADPH oxidation or XTT reduction at 340 or 17 470 nm, respectively. One enzyme unit (IU) was defined for GPx and GR as the oxidation of 1.0 μmol/min of NADPH at 25°C at pH 7.5 or as the amount of SOD capable of transforming 1.0 μmol/min of $O_2^{\cdot-}$. The enzyme activity was expressed in units per milliliter per milligram (U/mL/mg) of protein.

Experimental Design

Extended sperm-rich fractions (1:1, vol/vol in BTS) from 9 boars were pooled and centrifuged. After centrifugation, the pooled sperm pellet was split into 5 aliquots and then extended with LEY alone (control) or LEY supplemented with 5% (vol/vol) of SP from poor (SP1), moderate, (SP2), and good sperm freezers (SP3–SP4). Eight replicates were done. The SP inclusion level was chosen based on its demonstrated efficacy in a preliminary experiment where other concentrations were also tested (unpublished data).

Statistical Analysis

Statistical analyses were performed by SPSS, version 14 (SPSS Inc, Chicago, Ill). Data were analyzed as a split plot design using a mixed-model analysis of variance (ANOVA). To fulfill the assumption of a normal distribution, percentage data of post-thaw sperm quality were log-transformed before statistical analysis. The effect of SP on the post-thaw sperm survival was examined according to a statistical model that includes the fixed effects of post-thaw evaluation time (30 and 150 minutes), SP donor (0 to 4), and the random effect of replicate. There were no interactions between evaluation time and SP donor. Therefore, data of the post-thaw evaluation times were combined and analyzed as a complete data set. The antioxidant composition of SP was analyzed by 1-way ANOVA. Data of IVF were analyzed according to statistical model, including the fixed effect of SP donor and the random effect of replicate. Data in percentages of penetrated and monospermic oocytes were modeled according to the binomial model of parameters before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferrroni or Turkey's post-hoc test and were considered to be significant when the *P* value was less than .05. Results are presented as mean ± SEM.

Results

Effects of the addition to the freezing extender of SP from poor, moderate, or good sperm freezers on the

Table 1. *Post-thaw sperm motility and viability of frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability**

Sperm Parameters†	Source of Seminal Plasma					SEM	Probability
	Control	SP1	SP2	SP3	SP4		
TSM, %	50.3a‡	54.4ab	54.9ab	56.7b	59.5b	2.5	.001
VCL, μm/s	64.6	61.8	63.1	66.1	64.8	2.4	NS§
VSL, μm/s	43.7	40.6	41.1	44.8	43.2	2.0	NS
VAP, μm/s	50.1	47.5	48.1	51.3	50.2	2.2	NS
LIN, %	66.6	65.4	65.8	66.7	66.7	1.4	NS
ALH, μm	2.0	2.0	2.1	2.1	2.1	0.7	NS
VIA, %	49.5a	52.3ab	52.4ab	57.2bc	60.0c	3.4	.001

* Values are mean ± SEM of 2 separate measurements at 30 and 150 minutes after thawing in sperm samples incubated during 150 minutes in a water bath at 37°C. The sperm sample indicated as control was frozen in extender without additional seminal plasma. Data are from 8 replicates.

† Motility characteristics obtained from computer-assisted sperm analysis: TSM indicates total motile spermatozoa; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and VIA, live sperm with intact acrosome assessed by a fluorescent triple stain.

‡ Letters a–c denote a significant difference within treatments ($P < .05$).

§ NS indicates nonsignificant.

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motility and viability of post-thawed spermatozoa are summarized in Table 1. Irrespective of the source, the addition of SP to the freezing extender increased the percentage of motile sperm in all samples, being the improvement statistically significant ($P < .01$) in samples frozen in the presence of SP from good sperm freezers (SP3 and SP4). The same trend was observed for post-thaw sperm viability. The percentage of live sperm with intact acrosome (VIA) was also significantly ($P < .01$) higher in sperm samples frozen in the presence of SP from good sperm freezers (SP3 and SP4). The presence of SP in the freezing extender did not modify ($P > .05$) the kinematic pattern of post-thaw motile spermatozoa.

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The addition of SP on the freezing extender had no effect ($P > .05$) on MDA production of FT spermatozoa (Figure 1). Furthermore, the maximum and minimum levels of MDA were observed in sperm samples frozen with SP from good freezers (119.4 and 86.0 pmol MDA per 10^8 for SP3 and SP4, respectively).

The addition of SP to the freezing extender influenced ($P < .01$) the IVF parameters evaluated (Figure 2). Sperm samples frozen with SP3 (good freezer) showed the highest penetration rate. Moreover, SP4 (good freezer) also increased the number of spermatozoa per oocyte ($P < .01$) compared with control and consequently decreased ($P < .01$) the percentages of mono-

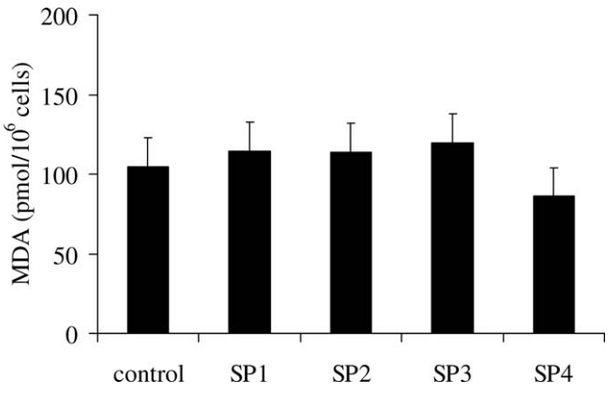

Figure 1. Concentration of malondialdehyde (MDA) in frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (SP) (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability. Values are mean ± SEM of 2 separate measurements done at 30 and 150 minutes after thawing in sperm samples incubated during 150 minutes in a water bath at 37°C. The sperm sample indicated as control was frozen in extender without additional SP.

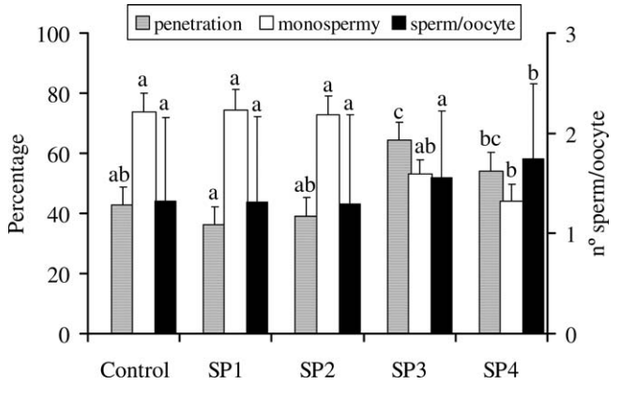

Figure 2. Parameters during in vitro homologous oocyte penetration testing of frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (SP) (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability. The sperm sample indicated as control was frozen in extender without additional SP. The parameters evaluated include penetration and monospermic rates and the mean number of spermatozoa per oocyte (number e/o). Data are means ± SEM of 4 replicates. Letters a–c denote a significant difference within treatment. ($P < .05$)

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Table 2. *Mean ± SEM enzymatic (superoxide dismutase [SOD], glutathione peroxidase [GPx], and glutathione reductase [GR]) and nonenzymatic (TAC) antioxidant activity levels of the seminal plasma (SP1–SP4) collected from 4 boars with different sperm freezability (poor to good) and used in the present study as exogenous supplement during cooling of boar spermatozoa*

Seminal Plasma Sources*	SOD, U × 10^{-3}/mL/mg protein	GPx, U × 10^{-3}/mL/mg protein	GR, U × 10^{-3}/mL/mg protein	TAC, μmol/L of Trolox Equivalents
SP1	54.9 ± 1.3a†	10.3 ± 8.5a	0.5 ± 0.2a	726.66 ± 12.13a
SP2	37.5 ± 0.3b	13.7 ± 7.5b	0.5 ± 0.1a	866.00 ± 25.32b
SP3	25.8 ± 1.6c	6.9 ± 3.7c	0.1 ± 0.2a	640.33 ± 30.07a
SP4	96.9 ± 0.4d	14.3 ± 6.0b	0.6 ± 0.0a	636.00 ± 27.78a

* SP1 was collected from a boar poor sperm freezer, SP2 from moderate, and SP3 and SP4 from good sperm freezers.
† Letters a–d denote significant differences among seminal plasma sources ($P < .05$).

spermic oocytes. In contrast, no significant ($P > .05$) differences on IVF parameters were found between sperm samples frozen in the presence of SP from moderate or poor freezers and control (sperm samples frozen without SP).

The activities levels of SOD, GPx, GR, and TAC in the 4 SP sources are shown in Table 2. The enzymatic activity levels of SOD, GPx, and TAC were highly variable ($P < .01$) among SPs, without any definite pattern related to sperm freezability of SP donors. Moreover, GR concentration was relatively inappreciable in all SPs, without a significant difference ($P > .05$) among them.

The total protein content varied among SPs, showing SP3 and SP4, both collected from boars with good sperm freezability, the highest and lowest quantity, respectively (Table 3). Among individual proteins, PSP-I and PSP-II were, in this order, the more abundant proteins in the 4 SPs evaluated. The reverse-phase separated PSP proteins followed the same pattern as the total protein profile, showing the highest (PSP-I) and lowest (PSP-II) quantity of these proteins in the SPs from the boars considered good sperm freezers (Figure 3).

Discussion

Removing the bulk SP by centrifugation is a necessary step in boar sperm cryopreservation protocols to concentrate the sperm population so that it can be resuspended in adequate proportions of freezing extender (Carvajal et al, 2004). Therefore, boar spermatozoa are exposed to bulk and diluted SP until centrifugation and thereafter deprived from large quantities of the SP. Because exposure to autologous SP before (Tamuli and Watson, 1994) or to homologous SP after processing (eg, after thaw; Larsson and Einarsson, 1976) has beneficial effects on viability, it is of utmost importance to know if such benefits are also issued during cooling and freezing, a matter largely unknown thus far. In others species, such as bovine (Garner et al, 2001), caprine (Azerêdo et al, 2001), equine (Katila et al, 2002; Aurich et al, 1996), or red deer (Martinez-Pastor et al, 2006), SP has improved sperm cryosurvival. Although SP has never been used as a freezing extender additive in pigs, the possible role in sperm freezability seems controversial. In fact, Kawano et al (2003) showed an improvement in boar sperm cryosurvival when SP was removed immediately after collection (within 20 minutes) whereas Pursel et al (1973) and Tamuli and Watson (1994) showed that the incubation of spermatozoa in its own SP at room temperature during a prefreezing holding time allowed spermatozoa to resist better eventual cold shock. Differences among boars in the composition of SP could explain the above disparity between results. In this respect, the present study shows that the effect of SP added in the freezing extender on sperm cryosurvival is SP-donor dependent. Hence, the percentages of motile and viable FT

Table 3. *Total protein content and contents of individual proteins (mg/mL) in the seminal plasma of individual boars (n = 4; SP1–SP4) with different sperm freezability; each seminal plasma was used in the present study as exogenous supplement during cooling of boar spermatozoa*

Seminal Plasma Sources	Seminal Plasma Proteins							
	Total Protein	AQN1	AQN3	PSP-I	PSP-II	AWN1	AWN2	Others
SP1	11.0	0.5	0.3	5.2	1.6	0.2	0.7	2.4
SP2	9.5	0	0.1	4.2	2.8	0.2	0.4	1.8
SP3	14.2	0.3	0.4	6.2	3.8	0.1	0.7	2.6
SP4	5.1	0.1	0.1	2.1	1.5	0.2	0.2	0.8

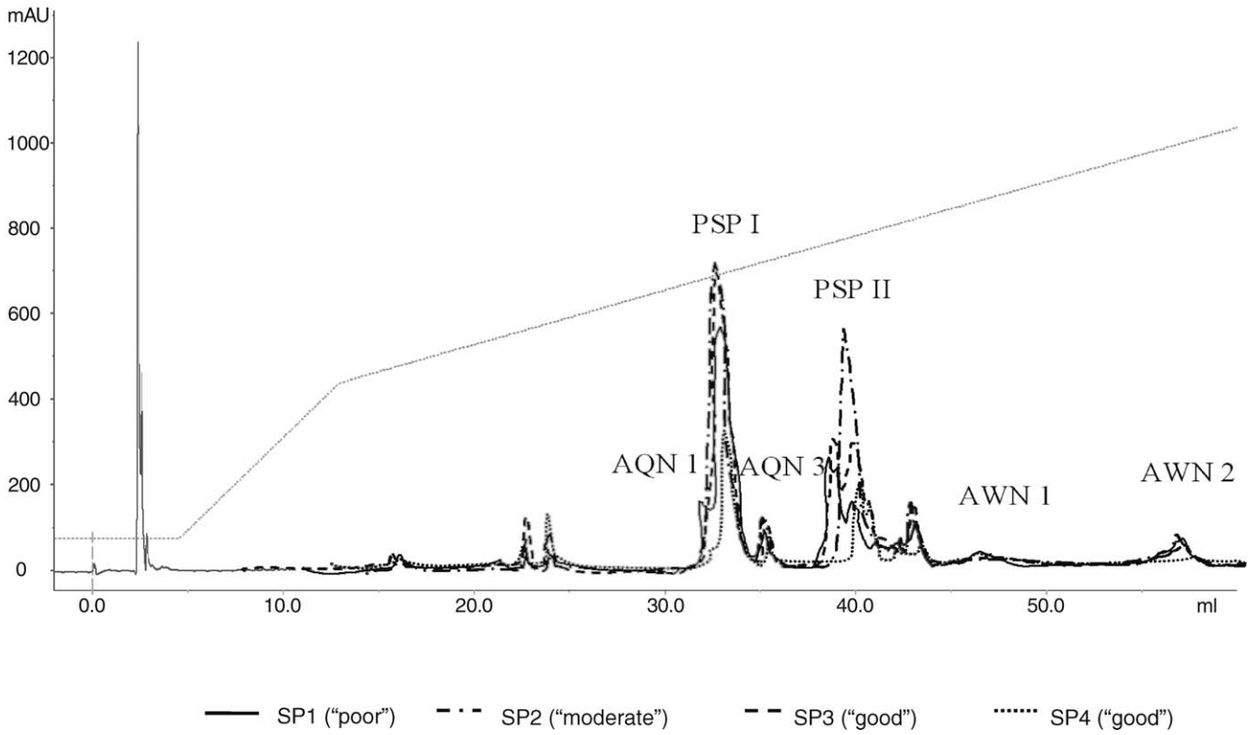

Figure 3. Reverse-phase high-performance liquid chromatography protein profiles of the seminal plasma (SP1–SP4) from the 4 boars exhibiting distinct sperm freezability (poor to good).

spermatozoa were only significantly higher in the sperm samples frozen in the presence of SP from good sperm freezers. The different effectiveness of these SPs, clearly donor-related, has already been reported. Caballero et al (2004) showed that SP can exert a positive or negative effect on highly diluted boar spermatozoa depending on the boar. In the same way, Henault and Killian (1996) showed different in vitro penetration abilities of SP-incubated bull spermatozoa according to SP donors. Moreover, Aurich et al (1996) achieved similar results to ours, showing that the addition of SP from good sperm freezers to freezing extender improved the post-thaw sperm survival in stallions.

It is clear that the composition of SP varies among species, among males within the same species, and among ejaculates within the male (reviewed by Strzezek et al, 2005). Thus, an adequate knowledge of the composition of SPs used in the present study should help to understand the different response of spermatozoa to cryopreservation. Proteins are among the major components of SP, and they affect sperm functionality. For instance, Barrios et al (2005) have recently shown that SP proteins are capable of protecting ram spermatozoa against cold shock. On the other hand, heparin-binding SP proteins (HBPs) have shown a dose-dependent adverse effect on buffalo epididymal spermatozoa during cryopreservation (Harshan et al, 2006). In the boar, spermadhesins represent about 75% of the total protein content of SP, with PSP-I and PSP-II being the most abundant proteins, amounting to up to 50% of the total proteins (Calvete et al, 1995). We have reported that these proteins can preserve sperm quality of highly diluted fresh and FT spermatozoa (Centurión et al, 2003; Caballero et al, 2004; García et al, 2006). However, although the SP proteinogram differs among SPs, those differences seemed unrelated to the different protective effects observed. Moreover, this approach had been assessed in a previous experiment in which we showed that the supplementation of the freezing extender with PSP-I/PSP-II or HBP did not affect post-thaw sperm survival positively or negatively (Cremades et al, 2004). Confounding results were also found by Peña et al (2006) in relation to portions of the ejaculate with clear differences in SP contents. The lack of any effect should be due to either the presence of other substances in SP or in the freezing extender, which limit or mask the action of those proteins, avoiding its adsorption onto the sperm membranes or suppressing its activity (Vadnais et al, 2005a). In fact, it has been shown that the activity of bovine SP proteins on sperm membranes is inhibited by the formation of stable complexes with egg yolk lipoproteins (Manjunath et al,

2002; Bergeron and Manjunath, 2006). In this respect, it should be taken into account that freezing extenders used for cryopreserving boar spermatozoa contain 20% egg yolk.

Besides the above-mentioned effects of certain proteins, boar SP contains a wide variety of organic and inorganic components capable of modulating different sperm functions, including protection from the negative effects of reactive oxygen species (Strzezek, 1999; Strzezek et al, 2005). Lipid peroxidation, originated by raeactive oxygen species, has been claimed as one of the main causes of sperm damage during freezing and thawing processes (Bilodeau et al, 2000). In addition, supplementation of freezing extenders with antioxidant components has been demonstrated to be effective for the improvement of post-thaw boar sperm quality (ie, vitamin E [Peña et al, 2003] and catalase, SOD, and butylated hydroxytoluene [Roca et al, 2004; 2005]). Thus, qualitative and/or quantitative differences in SP components with antioxidant properties could explain the distinct effectiveness of different SPs. To test this possibility, we have determined the concentrations of the more relevant antioxidants components present in boar SP (Strzezek et al, 2005). As expected, the concentration of the different antioxidants showed significant differences among the 4 boars providing SP, although always within the normal range of boar SP according to the values previously showed by Strzezek (2002). However, it is noteworthy that the antioxidant properties of the SPs sources were not related to post-thaw sperm quality. Moreover, no differences in lipid peroxidation, indirectly measured as MDA production, were found among sperm samples. Therefore, we conclude that the antioxidant property of SPs was not a major factor responsible for the distinct influence of SP on post-thaw sperm survival.

Among the major protective effects attributed to SP are preventing spermatozoa from undergoing capacitation and to reverse capacitation in a sperm population that has apparently already undergone this process (Vadnais et al, 2005a,b; Kaneto et al, 2002). Fresh or FT boar spermatozoa, incubated with whole SP or with specific SP proteins, have a limited ability to penetrate oocytes under in vitro conditions (Suzuki et al, 2002; Caballero et al, 2004). In the present study, although without a post-thaw sperm quality-related pattern, SP added to the freezing extender did not decrease the ability of thawed spermatozoa to penetrate oocytes under in vitro conditions. Moreover, the highest rates of penetrated oocytes were obtained from sperm samples frozen in the presence of SP. This observation could be related to the contact time of SP with the spermatozoa. In the present experiments, SP was added to the freezing extender and FT spermatozoa were thoroughly washed before IVF. Thus, the IVF results achieved seem to be related primarily to the higher proportion of spermatozoa surviving the cryopreservation process, in the presence of some sources of SP, rather than to the possible role of SP on the sperm capacitation process. Further studies are needed to clarify any other eventual relation.

In conclusion, the addition of SP to the freezing extender can improve boar sperm cryosurvival. However, the beneficial effect depends on SP source, being only significant when SP was collected from boar with good sperm freezability. Looking at the SP composition, it is likely that neither protein profiles nor the antioxidant capacity of SP can explain per se the differences on effectiveness among SPs. Therefore, further investigations are necessary to elucidate which components of SP and mechanisms linked to the SP are responsible for the potential protective effect on boar sperm freezability.

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References

Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology*. 1997;48:537–544.

Arezêdo GA, Esper CR, Resende KT. Evaluation of plasma membrana integrity of frozen-thawed goat spermatozoa with or without seminal plasma. *Small Ruminant Res*. 2001;41:257–263.

Aurich JE, Kühne A, Hoppe H, Aurich C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenology*. 1996;46:791–797.

Barrios B, Fernández-Juan M, Muiño-Blanco T, Cebrián-Pérez P. Imunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. *J Androl*. 2005;26:539–549.

Bergeron A, Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Mol Reprod Dev*. 2006;73:1338–1344.

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev*. 2000;55:282–288.

Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding. *Anal Biochem*. 1976;72:248–254.

Caballero I, Vázquez JM, Centurión F, Rodríguez-Martínez H, Parrilla I, Roca J, Cuello C, Martínez EA. Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. *Reprod Domest Anim*. 2004;39:370–375.

Calvete JJ, Mann K, Schäfer W, Raida M, Sanz L, Töpfer-Petersen E. Boar spermadhesin PSP-II: location of posttranslational modifica-

tions, heterodimer formation with psp-i glycoforms and effect of dimerization on the ligand-binding capabilities of the subunits. *FEBS Lett*. 1995;365:179–182.

Cano A, Hernández-Ruiz J, García-Cánovas F, Acosta M, Arnao MB. An end-point method for estimation of the total antioxidant activity in plant material. *Phytochem Anal*. 1998;9:196–202.

Carvajal G, Cuello C, Ruiz M, Vázquez JM, Martínez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. *J Androl*. 2004;25:389–396.

Centurión F, Vázquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, García E, Martínez EA. Influence of porcine spermadhesins on the susceptibility of boar spermatozoa to high dilution. *Biol Reprod*. 2003;69:640–646.

Cremades T, Roca J, Rodríguez-Martínez H, Abaigar T, Vázquez JM, Martínez EA. Kinematic changes during the cryopreservation of boar spermatozoa. *J Androl*. 2005;26:610–618.

Fisz M. Some probability distributions. In: De Krieger R. ed. *Probability Theory and Mathematical Statistics*. Malabar: John Wiley & Sons; 1980:129–174.

Flowers WL, Turner ZA. Relationships between seminal plasma protein profiles and estimates of fertility for boars. In: *VIth International Conference on Pig Reproduction*. City, State: University of Missouri; 2001:65.

Garcia EM, Vázquez JM, Calvete JJ, Sanz L, Caballero I, Parrilla I, Gil MA, Roca J, Martínez EA. Dissecting the protective effect of the seminal plasma spermadhesin PSP-I/PSP-II on boar sperm functionality. *J Androl*. 2006;27:434–443.

Garner DL, Thomas CA, Gravance CG, Marshall CE, DeJarnette JM, Allen CH. Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology*. 2001;56:31–40.

Gil MA, Ruiz M, Cuello C, Vázquez JM, Roca J, Martínez EA. Influence of sperm/oocyte ratio during in vitro fertilization of in vitro matured cumulus-intact pig oocytes on fertilization parameter and embryo development. *Theriogenology*. 2004;61:551–560.

Harshan HM, Singh LP, Arangasamy A, Ansari MR, Kumar S. Effect of buffalo seminal plasma heparin binding protein (HBP) on freezability and in vitro fertility of buffalo cauda spermatozoa. *Anim Reprod Sci*. 2006;93:124–133.

Henault MA, Killian GJ. Effect of homologous and heterologous seminal plasma on the fertilizing ability of ejaculated bull spermatozoa assessed by penetration of zona-free bovine oocytes. *J Reprod Fertil*. 1996;108:199–204.

Jobim MI, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. *Theriogenology*. 2004;61:255–266.

Kaneto M, Harayama H, Miyake M, Kato S. Capacitation-like alterations in cooled boar spermatozoa: assessment by the chlortetracycline staining assay and immunodetection of tyrosine-phosphorylated sperm proteins. *Anim Reprod Sci*. 2002;73:197–209.

Katila T, Andersson M, Reilas T, Koskinen E. Post-thaw motility and viability of fractionated and frozen stallion ejaculates. *Theriogenology*. 2002;58:241–244.

Kawano N, Shimada M, Terada T. Motility and penetration competence of frozen-thawed miniature pig spermatozoa are substantially altered by exposure to seminal plasma before freezing. *Theriogenology*. 2004;61:351–364.

Larsson K, Einarsson S. Influence of boars on the relationship between fertility and post thawing sperm quality of deep frozen boar spermatozoa. *Acta Vet Scand*. 1976;17:74–82.

Manjunath P, Nauc V, Bergeron A, Ménard M. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. *Biol Reprod*. 2002;67:1250–1258.

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

Maxwell WMC, Johnson LA. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. *Theriogenology*. 1999;52:1353–1362.

Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol Reprod*. 2003;68:1828–1835.

Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim Reprod Sci*. 2003;78:85–98.

Peña FJ, Saravia F, Nuñez-Martínez I, Johannisson A, Wallgren M, Rodríguez Martínez H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? *Anim Reprod Sci*. 2006;93:101–113.

Pursel VG, Johnson LA, Schulman LL. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. *J Anim Sci*. 1973;37:528–531.

Roca J, Gil MA, Hernández M, Parrilla I, Vázquez JM, Martínez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. *J Androl*. 2004;25:397–405.

Roca J, Hernández M, Carvajal G, Vázquez JM, Martínez EA. Factors influencing boar sperm cryosurvival. *J Anim Sci*. 2006a;84:2692–2699.

Roca J, Rodríguez MJ, Gil MA, Carvajal G, García EM, Cuello C, Vázquez JM, Martínez EA. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J Androl*. 2005;26:15–24.

Roca J, Rodríguez-Martínez H, Vázquez JM, Bolarín A, Hernández M, Saravia F, Wallgren M, Martínez EA. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Ashworth CJ, & Kraeling RR. eds. *Control of Pig Reproduction VII*. Nottingham, Location: University Press, 2006b:261–275.

Strzezek J. Secretory activity of boar seminal vesicle glands. *Reprod Biol*. 2002;2:243–266.

Strzezek J, Lapkiewicz S, Lecewics M. A note on antioxidant capacity of boar seminal plasma. *Anim Sci Pap Rep*. 1999;4:181–188.

Strzezek J, Wysocki P, Kordan W, Kuklinska M, Mogielnicka M, Soliwoda D, Fraser L. Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. *Reprod Biol*. 2005;5:279–290.

Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodríguez-Martínez H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int J Androl*. 2002;25:84–93.

Tamuli MK, Watson PF. Cold resistance of live boar spermatozoa during incubation after ejaculation. *Vet Rec*. 1994;135:160–162.

Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. *Biol Reprod*. 2002;66:545–554.

Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm subpopulations defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J Androl*. 2001;22:382–394.

Vadnais ML, Kirkwood RN, Specher DJ, Chou K. Effects of extender, incubation temperature, and added seminal plasma on

capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim Reprod Sci.* 2005a;90:347–354.

Vadnais ML, Kirkwood RN, Tempelman RJ, Sprecher DJ, Chou K. Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. *Anim Reprod Sci.* 2005b;87:121–132.

Westendorf P, Ritcher L, Treu H. Zur tiefgefrierung von ebersperma. Labor-und besamungsergebnisse mit dem hülsenberger pailletten-verfahren [in Language]. DtschTierärztl Wschr. 1975;82:261–267.

Zahn FS, Papa FO, Melo CM, Brisola ML. Protein profile of equine seminal plasma: correlation to semen freezability. *Anim Reprod Sci.* 2005;89:313–315.

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Discusión General

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Tal como se señala en la introducción, la existencia de una amplia variabilidad entre eyaculados en la congelabilidad espermática, supone un importante factor limitante para la creación y correcta gestión de bancos de semen congelado en la especie porcina. La importancia del verraco, como individuo, para explicar estas diferencias, ya había sido propuesta con anterioridad [1,2]. Sin embargo, nunca había sido evaluada en el contexto de otros potenciales factores de variación y por lo tanto no se conocía, con precisión, que nivel de la variabilidad existente podía ser explicada por el verraco. En el primer artículo de la presente Tesis Doctoral se evaluaron un número importante de factores que podrían explicar parte de dicha variabilidad. Hasta el momento en los centros de IA no se ha realizado una verdadera selección de reproductores por su capacidad de congelación. Tradicionalmente los eyaculados que van a ser o no criopreservados son seleccionados en función de las características convencionales de su espermiograma en semen fresco. A pesar de que este proceder está ampliamente difundido, en nuestro primer artículo demostramos que tales características, como la concentración, morfoanomalías o motilidad espermática antes de la congelación, presentan limitado valor predictivo sobre la calidad espermática post-descongelación y aún más este valor predictivo desaparece al realizar una selección previa de los eyaculados antes de la criopreservación. Más aún, observamos la clara ausencia de valor predictivo de otras variables como la edad y raza del verraco, el ritmo de recogida o la temperatura a la que se transporta el semen hasta el momento de su procesado. A pesar de que se ha demostrado la existencia de importantes diferencias tras la descongelación en factores como la raza [3-5], la ausencia de predictibilidad es atribuible al amplio rango de congelabilidad entre individuos dentro de la misma. Todo esto indicaría en términos generales, que si bien estos parámetros pueden ser tenidos en cuenta para mejorar la calidad global de un banco de semen, éstos no pueden utilizarse para predecir la congelabilidad espermática de un eyaculado. En este sentido, en este artículo se pone de manifiesto la gran relevancia del individuo como factor determinante de la variabilidad entre eyaculados, siendo responsable de más del 70% de la varianza observada en la calidad espermática post-descongelación. Según nuestro estudio, ésta variabilidad no debe

atribuirse a diferencias entre eyaculados dentro de un mismo verraco, ya que la mayoría de aquellos verracos que muestran una buena o mala congelabilidad son capaces de mantenerla relativamente estable a lo largo de su vida productiva. Estos resultados, confirman los obtenidos previamente en nuestro laboratorio [6] y por otros autores [7,8], demostrando que se pueden identificar y seleccionar verracos "buenos" congeladores a partir de la criopreservación de unos pocos eyaculados, e incluso podría llegar a realizarse a partir de tan sólo un eyaculado. De este modo, en ausencia de técnicas de laboratorio capaces de identificar, verracos "buenos" y "malos" congeladores, la congelación de un sólo eyaculado o "test de congelabilidad" [6], es una buena estrategia a nivel práctico para discriminar aquellos individuos aptos para entrar a formar parte del banco de semen criopreservado.

El movimiento de agua y crioprotectores a través de la membrana plasmática del espermatozoide, condiciona la deshidratación celular y ésta a su vez, se relaciona directamente con los daños observados tras la descongelación [9,10]. En el artículo 2 intentamos determinar si las diferencias observadas entre individuos tras la descongelación, podrían deberse a una deshidratación irregular durante el proceso de criopreservación, motivado por diferencias individuales en la permeabilidad de la membrana plasmática. Al mismo tiempo, esta experiencia nos permitiría verificar la adecuada clasificación a la que se habían sometidos los eyaculados, no atribuyendo la variabilidad a una inconsistente reproducibilidad del proceso de criopreservación, tal y como han sugerido ciertos autores [4]. El empleo de la Crio-microscopía electrónica, nos permitió estudiar tanto la distribución cómo el área de hielo extracelular (lagos) y el área de solutos congelada donde se encuentran los espermatozoides formando estructuras similares a venas [11]. De esta manera, cuanto mayor es el grado de deshidratación celular, mayor es el tamaño de los lagos de hielo extracelular y paralelamente menor el área de la venas [12]. Así, se observó, que bajo las mismas condiciones de criopreservación, tanto el área de los lagos y venas, como su distribución, difirió entre los distintos eyaculados estudiados e incluso su distribución entre la periferia y el centro de la pajuela, no existiendo un patrón lineal y homogéneo de solidificación. Dicha heterogeneidad puede deberse a la ausencia de un inicio de la cristalización controlado [13] y/o a un envase criogénicamente inadecuado que no permite la propagación regular del hielo y temperatura a lo largo del envase, hecho que ha sido ampliamente relacionado con una disminución de la calidad espermática tras la descongelación [9,12-15]. Sin embargo, a pesar de la existencia de esta difusión irregular del hielo y por ende una heterogénea deshidratación, no se encontró ninguna relación entre el grado de deshidratación y la supervivencia espermática. Es decir, el proceso de congelación, no repercute en la variabilidad espermática a la descongelación exhibida entre eyaculados y por lo tanto la variabilidad intra-verraco observada podría estar más

relacionada con cambios transitorios en el estado de salud de los animales, o con incorrectas manipulaciones de los eyaculados durante su recogida y posterior envío al laboratorio.

Tras demostrar la importancia del individuo en la variabilidad existente entre eyaculados en la calidad espermática post-descongelación, decidimos agrupar los verracos en función de su congelabilidad en tres grupos: "buenos", "moderados" y "malos" congeladores, siguiendo el concepto propuesto por Thurston y cols. [2]. Para realizar objetivamente la distribución de los verracos entre dichas categorías, utilizamos el software estadístico denominado PATN®, empleado previamente por diversos autores para el análisis de subpoblaciones espermáticas dentro de un eyaculado [4,16,17], así como para discriminar entre machos dependiendo de su calidad espermática [2, 18]. Para realizar la distribución consideramos principalmente como parámetros de calidad, los porcentajes de espermatozoides mótiles y viables con el acrosoma intacto, ambos valorados a los 30 y 150 minutos post-descongelación. Probablemente, la inclusión de más parámetros de calidad espermática en el análisis, hubiera podido contribuir a un mayor rigor en la clasificación. Sin embargo, es importante destacar que para diferenciar eficazmente a los verracos, no es necesario incluir todos los parámetros de movilidad y calidad de movimiento que proporciona el sistema CASA o los derivados del análisis de viabilidad [2, 18], siendo dos de las características más discriminativas entre grupos el porcentaje total de espermatózoides mótiles, y el de viables [8]. Una vez clasificados en "buenos", "moderados" y "malos" congeladores, decidimos averiguar si dicha clasificación afectaría a otras características espermáticas además de la motilidad e integridad de la membrana plasmática, que son las tradicionalmente evaluadas. En el artículo 3, valoramos el estado de la cromatina en espermatozoides congelados-descongelados mediante el *Sperm Chromatin Structure Assay* (SCSA). Ésta es la técnica más utilizada y estandarizada en espermatozoides de mamíferos, ya que el empleo de la citometría de flujo permite un análisis de las muestras rápido, objetivo y repetible [19]. Además la alta correlación que presenta con la fertilidad, ha permitido definir valores umbrales para el diagnóstico clínico de infertilidad en la especie humana y bovina [revisado por 19]. De forma general, observamos que el porcentaje de espermatozoides congelados-descongelados que mostraron el ADN fragmentado (%DFI) fue muy bajo, independientemente, de si los eyaculados procedían de verracos clasificados como "buenos" o "malos" congeladores, habían estado sometidos a distintos protocolos de descongelación o largos periodos de incubación. A pesar de que detectamos diferencias significativas para todos los parámetros del SCSA entre "buenos" y "malos" congeladores, existiendo una mayor estabilidad de ADN en los "buenos", los niveles medios de %DFI fueron siempre inferiores al 5%. Estos valores aunque similares a los obtenidos en semen fresco mediante ésta [19-22] y otras técnicas, tales como, *Sperm-Sus-Halomax* [23,24] o

Comet Assay [25,26], son mucho más bajos que los descritos en espermatozoides descongelados por Frazer y Strzezek [25,26] mediante el *Comet Assay*. Las distintas metodologías aplicadas y la falta de correlación entre las técnicas [27,28] ponen de manifiesto la necesidad de esclarecer cómo afecta el proceso de criopreservación al ADN porcino y por lo tanto qué métodos deben ser incorporados para un análisis más informativo del estado de la cromatina espermática.

La existencia de verraco "malos" congeladores limita el potencial de dichos verracos como reproductores, al no ser incluidos en bancos de semen criopreservado. Este problema se agrava si son precisamente éstos, por sus características, principalmente genéticas, los más interesantes para el productor. Un requisito fundamental para garantizar el éxito de la criopreservación espermática es encontrar un adecuado equilibrio entre la velocidad de enfriamiento, la concentración del crioprotector y la velocidad de descongelación [revisado por 29]. En el artículo 4, comprobamos como ligeras modificaciones en los mencionados puntos del protocolo de criopreservación, se traducían en importantes diferencias entre eyaculados en la calidad espermática post-descongelación, tal como han descrito otros autores en bovino [30]. Al igual que Parkinson y Whitfield [30] observamos que dichos cambios afectan principalmente a los eyaculados de verracos considerados como "malos" congeladores. La utilización de un 3% de glicerol en el diluyente de congelación junto a una velocidad de descongelación próxima a 1800 ºC/min resultaron ser las más idóneas para la criopreservación de espermatozoides de porcino envasados en pajuelas de 0'5 mL. Condiciones particularmente adecuadas para mejorar la congelabilidad de la mayoría de los verracos considerados como "malos" congeladores, que permitieron reducir la incidencia de éstos de un 26'4% hasta tan sólo un 7'6%. Por lo tanto, antes de descartar definitivamente un verraco como donante, deberíamos probar diferentes protocolos de congelación-descongelación.

Uno de los aspectos más contradictorios ha sido definir la velocidad más adecuada para descongelar las pajuelas de 0'5 mL. En las dos experiencias en las que dicho aspecto se evalúa, se demuestra estadísticamente, que velocidades próximas a 1800 ºC/min son más adecuadas, siendo mayores tanto los porcentajes de espermatozoides mótiles y viables (artículo 3 y 4), como el de aquellos con integridad del ADN (artículo 3). Nuestras dudas se centran en sí a pesar de las significativas diferencias estadísticas, éstas lo son también desde una perspectiva biológica, sobre todo teniendo en cuenta que para alcanzar velocidades de descongelación próximas a 1800 ºC/min, las pajuelas deben someterse a elevadas temperaturas (70 ºC) por un corto periodo de tiempo (8 s), parámetros difíciles de controlar con exactitud. En el artículo 3, centrado principalmente en el análisis de ADN, si bien las diferencias apreciadas en el porcentaje de espermatozoides con el ADN fragmentado (1'8 ± 0'6 y 2'1 ± 1'0, respectivamente para 1800 y 1200 ºC/min) son

estadísticamente significativas, no lo son tanto desde una perspectiva biológica. Por ello, concluíamos que velocidades de descongelación próximas a 1800 ºC/min no se justificaban teniendo en cuenta el riesgo que implica el proceso de descongelación. Sin embargo, en el artículo 4, éstas velocidades permiten alcanzar una mayor supervivencia espermática en general, y de manera más notoria en los eyaculados procedentes de verracos clasificados como “moderados” o “malos” congeladores espermáticos. Estos resultados nos hicieron finalmente sugerir velocidades de descongelación próximas a 1800 ºC/min como las más adecuadas. No obstante, debido al riesgo que puede entrañar realizar la descongelación a tan alta velocidad, aconsejamos que ésta se realice empleando instrumentos apropiados para tal fin, que existen en el mercado y garantizan tanto la temperatura como el tiempo con elevada exactitud.

En el artículo 5, nos propusimos determinar si el plasma seminal (SP), en función de su composición podría influir, positiva o negativamente, en la congelabilidad espermática y, por lo tanto, ser el responsable, al menos en parte, de la existencia de verracos “buenos” y “malos” congeladores. Los resultados demuestran dicha hipótesis, puesto que la calidad post-descongelación de espermatozoides procedentes de una mezcla de varios eyaculados, es mejor cuando son congelados con un diluyente que contiene un 5% de plasma seminal procedente de eyaculados recogidos de verracos catalogados como “buenos” congeladores. Sin embargo, ¿qué componente o componentes del plasma seminal son responsables de dicha propiedad?. Los antioxidantes, tanto hidrosolubles como liposolubles, han demostrado ser necesarios para un buena congelabilidad espermática [31-33]. También, el complejo proteico PSP I–PSP II, que representa más del 50% de la fracción proteica del plasma seminal [34], contribuye a mantener la calidad espermática en espermatozoides de verraco altamente diluidos [35-37]. Sin embargo, las diferencias encontradas en los niveles de proteínas y de los más importantes antioxidantes presentes en el plasma seminal entre los plasmas procedentes de “buenos” y “malos” congeladores, no justifican el efecto beneficioso que ejerce el plasma seminal procedente de verracos catalogados como “buenos” congeladores. Por lo tanto, a la vista de su potencial efecto protector, es necesario profundizar en un futuro en los posibles factores del SP que afectan a la congelabilidad.

[1] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their postthawing function. Reprod Fertil Dev 1995;7:871–891.

[2] Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002;66:545–554.

[3] Almlid T, Hofmo PO. A brief review of frozen semen application under norwegian AI service conditions. Reprod Dom Anim 1996;31:169-173.

[4] Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382-394.

[5] Park CS, Yi YJ. Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. Anim Reprod Sci 2002;73:53-61

[6] Roca J, Rodríguez-Martínez H, Vázquez JM, Bolarín A, Hernández M, Saravia F, et al. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Control of pig reproduction VII, Ashworth CJ, Kraeling RR (Eds), Nottingham: Nottingham University Press; 2006, pp. 261–275.

[7] Medrano A, Watson PF, Holt WV. Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Reproduction 2002;123:315–322.

[8] Holt WV, Medrano A, Thurston LM and Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 2005;63:370-382.

[9] Courtens JL, Rety JM. Numerical simulation for freezing and thawing mammalian spermatozoa. Evaluation of cell injuries at different depths in bags or straws during all steps of the technique, Genet Sel Evol 2001;33:83–104.

[10] Ortman K, Rodríguez-Martínez H. Membrane damage during dilution, cooling and freezing–thawing of boar spermatozoa packaged in plastic bags. Zentralbl Veterinarmed A 1994;41:37–47.

[11] Rodríguez-Martínez H, Ekwall H. Electron microscopy in the assessment of cryopreserved spermatozoa viability. Microsc Anal 1998;65:11–13.

[12] Ekwall H, Eriksson BM, Rodriguez-Martinez H. Cryo-EM of frozen boar semen. Proc. of the Annual Meeting of the Royal Microscopy Society, ‘‘Low temperature microscopy and analysis, York, UK, 1997 pp. P-2.

[13] Searles JA, Carpenter JF, Randolph TW. The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf. J Pharm Sci 2001;90:860–871.

[14] Penfold LM, Garner DL, Donoghue AM, Johnson LA. Comparative viability of bovine sperm frozen on a cryomicroscope or in straws. Theriogenology 1997; 47:521–530.

[15] Woelders H, Matthijs A, Zuidberg CA, Chaveiro AE. Cryopreservation of boar semen: equilibrium freezing in the cryomicroscope and in straws. Theriogenology 2005;63:383–395.

[16] Abaigar T, Holt WV, Harrison RAP, del Barrio G. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessment. Biol Reprod 1999;60:32–41.

[17] Thurston LM, Watson PF, Holt WV. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. J Reprod Fert 1999;117:271-280.

[18] Gil MA, Roca J, Cremades T, Hernández M, Vázquez JM, Rodríguez-Martínez H, Martínez EA. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates?. Theriogenology 2005;64:305–316.

[19] Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 2006;65:979–991.

[20] Evenson DP, Thompson L, Jost L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 1994;41:637–651.

[21] Rybar R, Faldikova L, Faldyna M, Machatkova M, Rubes J. Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic. Czech Republic Vet Med 2004;49:1–8.

[22] Boe-Hansen GB, Ersboll AK, Greve,T, Christensen P. Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology 2005;63:2006–2019.

[23] Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez A, Gosálvez, J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology 2005;65:308–316.

[24] López-Fernandez C, Pérez-Llano B, García-Casado P, Sala R, Gosalbez A, Arroyo F, Fernandez JL, Gosalvez J. Sperm DNA fragmentation in a random sample of the Spanish boar livestock. Anim Reprod Sci. 2006, in press

[25] Fraser L, Strzezek J. Effects of freezing–thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. Reprod Dom Anim 2005;40:1–7.

[26] Fraser L, Strzezek J. Effects of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing–thawing. Anim Reprod Sci 2007;99:317-329.

[27] Boe-Hansen GB, Morris ID, Ersboll AK, Greve T, Christensen P. DNA integrity in sexed bull sperm assessed by neutral comet assay and sperm chromatin structure assay. Theriogenology 2005;63:1789–1802.

[28] Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl 2006;27:53–59.

[29] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. Anim Reprod Sci 2000;62:143–172.

[30] Parkinson TJ, Whitfield CH. Optimisation of freezing conditions for bovine spermatozoa. Theriogenology 1987;27:781-797.

[31] Peña FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 2003;78:85-98.

[32] Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397-405.

[33] Roca J, Rodríguez MJ, Gil MA, Carvajal G, García EM, Cuello C, Vázquez JM, Martínez EA. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15-24.

[34] Calvete JJ, Mann K, Schäfer W, Raida M, Sanz L, Töpfer-Petersen E. Boar spermadhesin PSP-II: Location of posttranslational modifications, heterodimer formation with psp-i glycoforms and effect of dimerization on the ligand-binding capabilities of the subunits. FEBS Letters 1995;365:179–182.

[35] Centurión F, Vázquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, García E, Martinez EA. Influence of porcine spermadhesins on he susceptibility of boar spermatozoa to high dilution. Biol Reprod 2003;69:640-646.

[36] Caballero I, Vázquez JM, Centurión F, Rodríguez-Martínez H, Parrilla I, Roca J, Cuello C, Martínez EA. Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. Reprod Dom Anim 2004;39:370-375.

[37] García EM, Vázquez JM, Calvete JJ, Sanz L, Caballero I, Parrilla I, Gil MA, Roca J, Martinez EA. Dissecting the protective effect of the seminal plasma spermadhesin PSP-I/PSP-II on boar sperm functionality. Journal of Andrology 2006;27:434-443.

Conclusiones

A continuación se enumeran las conclusiones obtenidas a partir de los resultados de los trabajos presentados en la presente Tesis doctoral:

1 El verraco es el factor más importante capaz de explicar la variabilidad existente entre eyaculados tras la descongelación, siendo responsable de más del 70% de ella.

2 La calidad espermática pre-congelación no limita el éxito de la criopreservación. Sin embargo para mejorar las características de un banco de dosis seminales debemos descartar aquellos eyaculados con una menor concentración espermática y morfologia normal antes de la congelación.

3 Aunque existe cierto grado de variación entre eyaculados dentro de un mismo verraco, ésta es poco relevante comparada con la variación existente entre verracos.

4 La formación y distribución irregular de los cristales de hielo durante el proceso de criopreservación utilizado en el presente estudio, no justifica la variabilidad entre verracos y/o eyaculados tras la descongelación.

5 Los espermatozoides descongelados procedentes de eyaculados de verracos clasificados como “buenos” congeladores, son menos susceptibles a la desnaturalización que los “malos” congeladores. Sin embargo, el daño en la integridad del ADN observado en general en los espermatozoides congelados-descongelados tiene escasa significancia biológica.

6 La incidencia de malos congeladores se puede reducir mediante ligeras modificaciones en el protocolo de criopreservación, principalmente la concentración de glicerol y velocidad de descongelación.

7 La incorporación de bajas concentraciones de plasma seminal al medio de congelación procedente de verracos “buenos congeladores” mejora la supervivencia espermática tras la descongelación. Sin embargo, ni el perfil proteico ni la composición antioxidante, pueden explicar los efectos positivos del plasma seminal.

Resumen General

Resumen General

Resumen

Para incrementar el empleo de espermatozoides criopreservados en los programas de inseminación artificial porcina, es necesario minimizar la variabilidad existente en la congelabilidad espermática post-descongelación entre eyaculados, un serio problema para la producción rentable de dosis espermáticas criopreservadas en la especie porcina. El objetivo de la presente tesis fue estudiar i) que factores pueden explicar parte de dicha variabilidad, ii) la integridad del ADN en los espermatozoides congelados-descongelados, iii) la posibilidad de adaptar el protocolo de criopreservación a las exigencias o necesidades de los espermatozoides de cada verraco y, iv) el posible papel del plasma seminal en la congelabilidad espermática. Los resultados revelaron que el verraco es el factor más importante capaz de explicar la diferente susceptibilidad entre eyaculados para soportar el proceso de criopreservación y que la variabilidad dentro de un mismo verraco es relativamente baja. Además, la calidad espermática antes de la congelación tiene limitada influencia sobre la congelabilidad espermática. El grado de fragmentación del ADN de los espermatozoides porcinos congelados-descongelados es relativamente bajo, pero los "malos" congeladores muestran menor homogeneidad en la cromatina espermática que los "buenos" congeladores. Por otro lado, la realización de ligeros ajustes en la concentración de glicerol y la velocidad de descongelación, permiten mejorar la supervivencia espermática, especialmente de aquellos eyaculados considerados como "malos congeladores. Finalmente, la suplementación del medio de congelación con plasma seminal de individuos con buena congelabilidad tiene un efecto beneficioso sobre la congelabilidad espermática. Sin embargo debemos indagar en los mecanismos responsables de dicho beneficio. La información recogida en la presente tesis ha resultado de gran ayuda para mejorar la calidad espermática global de los bancos de semen criopreservado porcino, especialmente a la hora de seleccionar a los individuos que van a formar parte de él.

Material y métodos

Animal

Todos los eyaculados empleados en la presente tesis procedieron de verracos de fertilidad contrastada, de diversas líneas híbridas comerciales, utilizados de manera rutinaria en los programas de inseminación artificial habituales en las distintas empresas. Los eyaculados fueron proporcionados por las compañías Batallé SA, Proinserga SA, y UVE SA para la creación de distintos bancos de dosis seminales criopreservadas. Cada eyaculado se recogió por el método manual descartando las fracciones pre y post- espermáticas, recogiendo la fracción rica en un recipiente atemperado. Inmediatamente después de su obtención se colocó en un baño maría a 37ºC y se diluyó 1:2 en Beltsville Thawing Solution (BTS) a 37ºC y se evaluó mediante técnicas estándar de laboratorio. Tras depositar la mezcla en tubos de 50 mL, precalentados y protegidos de la acción directa de la luz, se refrigeraron a 17ºC. Los eyaculados se enviaron por correo en cajas de poliespan debidamente selladas, monitorizando la temperatura interior del envase (miniature data logger, Gemini Data Loggers, Ltd., Chichester, UK), al laboratorio de Andrología del Departamento de Medicina y Cirugía Animal (Universidad de Murcia) para comenzar el proceso de congelación. Las muestras seminales llegaron al laboratorio entre 14 y 16 horas después de su recogida.

Reactivos químicos y medios utilizados

Todos los reactivos utilizados fueron obtenidos de Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA), salvo para los que se indique lo contrario. Los reactivos empleados en la preparación de medios y diluyentes fueron pesados en una balanza de precisión (Gram Precision Serie ST Mod. ST-120, A.R.W.T.) y diluidos con agua bidestilada y purificada (Elgastat UHQ ps, Elga). Las diluciones fueron homogeneizadas con un agitador magnético (Mod. 234 P Agimatic, Selecta). Todos los medios y diluyentes se prepararon en un ambiente regulado (cámara de flujo laminar horizontal, Micro- R, Telstar). El pH (Mod. GLP 22, Crison) y la osmolaridad (Mod. 3300, Advanced Instruments Inc.) fueron comprobados tras la preparación de cada medio.

Criopreservación espermática

Para las experiencias desarrolladas en los **Artículos 1-4** se emplearon eyaculados individualizados, mientras que para el **Artículo 5** se realizó la mezcla de 9 eyaculados distintos (pool). Una vez en el laboratorio, la fracción rica diluida fue re-evaluada y centrifugada a 2400 x g durante 3 minutos utilizando para ello una centrífuga refrigerada (Heraeus Sepatech Megafuge 1.0R; Hanau, Alemania). El sobrenadante obtenido tras la

centrifugación fue descartado. Los espermatozoides sedimentados en el fondo de los tubos fueron rediluidos en diluyente lactosa-yema de huevo (LEY) hasta alcanzar una concentración de 1'5 x 10^9espz/mL. A continuación, se procedió al enfriado de los espermatozoides rediluidos a 5ºC a lo largo de 90-120 min; este descenso de temperatura debe ser gradual, por lo que los tubos fueron sumergidos en agua a 17ºC, en recipientes colocados en el interior de la vitrina térmica. Una vez alcanzados los 5ºC, se añadió el diluyente LEY-Orvus ES Paste extender [92'5%-89'5% LEY + 1'5% Equex STM (Nova Chemical Sales Inc., Scituate, MA) y el 6% (**Artículos 1-5**) o 9% (**Artículo 4**) de glicerol, v/v; pH 6'2 y 1145 $\pm$ 17 mOsm/Kg o 1650 ± 15 mOsm/kg, respectivamente] hasta alcanzar una concentración de 1 x 10^9 espz/mL. Inmediatamente fueron envasados y sellados automáticamente (Verschißgerät MF 0'5, Minitüb; Tiefenbach, Alemania) en pajuelas francesas de 0.5 mL (Minitüb; Tiefenbach, Alemania). Estas pajuelas fueron colocadas en bandejas adecuadas para su introducción en un biocongelador (IceCube 1810, Minitüb; Tiefenbach, Alemania) mediante el cual se congelaron siguiendo la rampa propuesta.

Curvas de congelación y descongelación

La curva de congelación seguida en los diferentes estudios fue: en los **Artículos 1, 2, 3** y **5**, de 5ºC a –5ºC a una velocidad de 6ºC/min, de –5ºC to –80ºC a 40ºC/min, mantenimiento durante 30 segundos a –80ºC, posteriormente a 70ºC/min hasta –150ºC; en el **Artículo 4**, de 5ºC a –5ºC a 6ºC/min y de –5ºC a –80ºC a diferentes velocidades de congelación: 10, 40 ó 60ºC/min, posteriormente a 70ºC/min hasta –150ºC. Tras finalizar el programa de congelación, se sumergieron en nitrógeno líquido (-196ºC) y almacenaron en el interior de tanques de nitrógeno hasta su evaluación. En todas las ocasiones se procedió a la descongelación de las pajuelas tras 7-15 días después de su congelación.

Las descongelaciones se realizaron en un baño termostatizado a una temperatura de 37ºC (**Artículos 1-5**) durante 20 s o a 70ºC durante 8 s (**Artículos 3-4**). Transcurrido este tiempo, las pajuelas se retiraron del baño y se procedió al secado de las mismas con papel para eliminar la totalidad del agua superficial. Posteriormente, tras cortar el extremo de la pajuela, su contenido fue vertido a un tubo graduado de cristal. Para cada análisis se descongelaron simultáneamente 2 pajuelas de cada eyaculado, que fueron mezcladas y diluidas inmediatamente en BTS (1:2, v/v; 37ºC). Las muestras espermáticas se mantuvieron incubadas en el baño a 37ºC durante 150 min.

Análisis de la calidad espermática

Se incluyeron en el programa de criopreservación espermática los eyaculados con una concentración igual o superior a 200 x 10^6 sperm/mL, al menos un 85% de espermatozoides con morfología normal y porcentaje de espermatozoides mótiles y viables igual o superior a 75% y 80% respectivamente (**Artículos 2-5**), excepto en el **Artículo 1** (experimento 1) dónde los eyaculados no fueron preseleccionados antes de la congelación.

Análisis objetivo de la motilidad espermática

Las valoraciones se realizaron a los 30 y 150 minutos tras la descongelación. Para el análisis de la motilidad y la cinética del movimiento espermático se utilizó un sistema de análisis objetivo de la motilidad (Sperm Class Analyzer®, SCA, Microptic, Barcelona, España). Las muestras espermáticas se diluyeron de forma seriada hasta una concentración final de 30 x 10^6 espz/mL. De cada una de las muestras a analizar se depositó una alícuota de 4 μL en una cámara de Makler (Sefi-Medical Instruments; Haifa, Israel) atemperada a 39ºC. Dicha muestra se visualizó en un microscopio de contraste de fases (Labophot, Nikon; Tokio, Japón) equipado con una placa térmica (HT400, Minitüb; Tiefenbach, Alemania) con el objetivo de 10x. Para la captura o adquisición de las imágenes por el sistema SCA se incorporó al microscopio una cámara de video (CCD Camera, Hitachi; Tokio, Japón) conectada a un ordenador (Pentium 150) dotado del programa informático SCA 5.1 v. Por último, para la visualización de la imagen se utilizó un monitor de televisión (Sony, modelo Trinitron, Tokio, Japón). Cada una de las muestras fue sometida a nueve capturas de series de imágenes, siendo el número mínimo de espermatozoides analizados por muestra de 100. Una vez capturadas, se procedió al análisis de las mismas, valorando los siguientes parámetros de motilidad: **TSM** (% espermatozoides mótiles totales), rápidos progresivos (% RPM, VAP > 50 μm/s y STR > 75%), **VCL** (velocidad curvilínea, velocidad media de la cabeza espermática a lo largo de su trayectoria real, μm/s), **VSL** (velocidad rectilínea, velocidad media de la cabeza espermática a lo largo de la línea recta que une la primera posición en el análisis y la última. μm/s), **VAP** (velocidad de la trayectoria media, velocidad media de la cabeza espermática a lo largo de su trayectoria media, μm/s); **LIN** (linealidad, linealidad de la trayectoria curvilínea, %) y **ALH** (amplitud del desplazamiento lateral de la cabeza, amplitud de las variaciones de la trayectoria real de la cabeza espermática respecto a la trayectoria media, μm).

Análisis de la viabilidad espermática

La viabilidad fue expresada como el porcentaje de espermatozoides con la membrana plasmática y acrosomal intacta (**Artículos 1-5**), así como con funcionalidad mitocontrial (**Artículos 1-3**). En los **Artículos 1-3**, éstas características fueron evaluadas simultáneamente utilizando una triple tinción fluorescente, adaptada para espermatozoides de porcino y microscopia de fluorescencia. Esta tinción incluye la utilización de los fluorocromos, yoduro de propidio, marcador del ADN, tiñe de rojo la cabeza de los espermatozoides con pérdida de integridad de membrana (PI), rodamina 123 (R123), reactivo específico para detectar la actividad mitocondrial, tiñe la porción intermedia de los espermatozoides metabólicamente activos de verde brillante y por último el conjugado de lectina de *arachis hypogaea* con fluoresceína- isotiocianato (PNA-FITC), que marca de verde el interior y la membrana acrosómica de aquellos espermatozoides con daño acrosomal. Los espermatozoides se diluyeron gradualmente en BTS hasta una concentración de 15-20 x 10^6 espz/mL. A 1 mL de esta dilución se le añadieron 2 µL de PI y 3 µL de R123. Las muestras se incubaron 5 min en una estufa (MIR 153 incubator, Sanyo; Gunma, Japón) a 39ºC. Posteriormente, tras la incubación, se añadieron 5 µL de la solución fijadora de formaldehído (25 µL de formaldehído al 37% en 2 mL de agua purificada), y se incubó otros 5 min. Por último, se adicionaron 10 µL de PNA-FITC y pasados 5 min de incubación en la estufa se procedió a su valoración. Los espermatozoides fueron examinados con un microscopio de fluorescencia (Nikon VFM, modelo ECLIPSE E800; Tokio, Japón), utilizando un objetivo de 100x en aceite de inmersión, usando un triple filtro D-F-R (DAPI/FITC/Rhodamine). Se evaluaron 300 espermatozoides de cada muestra, considerando espermatozoides viables aquellos con fluorescencia verde en la pieza intermedia (R123 positivos) y el acrosoma intacto (FITC-PNA negativos). El resultado fue expresado como porcentaje (%). En el **Artículo 4**, la integridad de la membrana plasmática (PMI) fue evaluada utilizando los fluorocromos SYBR-14 y PI siguiendo las instrucciones del fabricante (L-7011; kit de viabilidad espermática Live/Dead; Molecular Probes Europe, Leiden, Holanda) y la integridad del acrosoma utilizando la combinación FITC-PNA y PI. Las muestras se incubaron a temperatura ambiente en la oscuridad durante 5-10 min antes del análisis mediante citometría de flujo. Los análisis se realizaron mediante un citómetro Becton Dickinson FACSort (San Jose, California, USA) equipado con un láser de argón (488nm, 15 mW). Sólo los espermatozoides con membrana plasmática y acrosomal intacta fueron procesados. En el **Artículo 5,** la integridad de la membrana plasmática y la acrosomal fueron evaluadas simultáneamente utilizando una triple tinción fluorescente mediante citometría de flujo. Se tomaron alícuotas de 100 µL (~3 x 10^6 células), que fueron teñidas con una concentración final de 25 nM SYBR-14 (100 µM de la solución stock en DMSO; Component A del kit de viabilidad espermática LIVE/DEAD, L-7011; Molecular

Probes, Europe BV, Leiden, Holanda), 1 μg/mL de ficoeritrina-PNA (PE-PNA, 1 mg/mL solución stock P44, Biomeda Corp. Foster City, CA) y 12 μM de PI (1'5 mM en PBS, Component B del kit de viabilidad espermática). Las muestras se incubaron a temperatura ambiente y oscuridad durante 10 min, justo antes del análisis fueron rediluidas con 400 μL de PBS. Para el análisis de las muestras se utilizó un citómetro de flujo Coulter Epics XLTM (Coulter Corporation., Miami, Florida) equipado con un láser de argón (488nm). El debris fue eliminado del forward scatter y el side scatter englobando la población de interés en una región y además eliminado los eventos con características similares a los espermatozoides pero sin contenido razonable de ADN (tinción muy débil de SYBR-14 o PI). Se realizaron subpoblaciones mediante cuadrantes y se contabilizó el porcentaje de cada subpoblación. Se adquirieron y analizaron un mínimo de 10000 eventos a una velocidad de ~800 eventos/seg.

Análisis de la integridad del ADN espermático (**Artículo 3**)

El análisis de la cromatina espermática fue realizado mediante la técnica SCSA (Sperm chromatin structure assay). Se basa en el principio de que la cromatina anormal presenta una mayor suseptibilidad *in situ* a desnaturalizarse parcialmente. El grado de desnaturalización resultante como consecuencia de su tratamiento con ácido se determina midiendo el cambio metacromático del colorante naranja de acridina (AO) de fluorescencia verde (AO intercalado, cadena doble, ADN nativo) a fluorescencia roja (AO sin intercalar, cadena sencilla, ADN desnaturalizado). Los resultados se expresan como el índice de fragmentación de ADN (DFI). Las muestras descongeladas fueron re-diluidas conel medio TNE (0'15 mol/L NaCl, 0'01 mol/L Tris–HCl y 1 mmol/L EDTA, pH 7'4) has una concentración final de aproximadamente 2 x 10^6/mL. Una alícuota de 0'2 mL fue sometida a desnaturalización parcial del ADN mezclando 0'4 mL de una solución detergente de pH ácido (0'17% Triton X-100, 0'15 M NaCl y 0'08 N HCl, pH 1.4), siendo teñida justo 30 s después con 1'2 mL de AO (6 μg/mL en 0'1 M de ácido cítrico, 0'2 M Na_2HPO_4, 1 mM EDTA y 0'15 M NaCl, pH 6'0). Las muestras teñidas se dividieron en 2 mitades y se analizaron de forma consecutiva tras 3 minutos de incubación con AO. El análisis de la cromatina espermática fue realizado mediante un citómetro de flujo BD FACStar Plus (Becton Dickinson, San Jose, CA, USA) equipados con óptica estándar. El AO fue excitado con un láser de Argón (Innova 90; Coherent, Santa Clara, CA, USA) a 488 nm y 200 mW. La fluorescencia verde fue detectada con el detector FL1 (530 ± 30 nm) y la roja con el FL3 (>630 nm). Los datos fueron analizados con el software FCSExpress, version 2 (DeNovo Software; Thornhill, ON, Canada).

Criomicroscopio electrónico de barrido (Cryo-SEM) **(Artículo 2)**

El Cryo-SEM fue utilizado para examinar la ultraestructura de las pajuelas en estado congelado y determinar si la cantidad de agua en estado sólido del medio extracelular, que rodea a la matriz de solutos donde se encuentran los espermatozoides, difería entre individuos con distinta supervivencia espermática a la descongelación. En el interior de un envase con nitrógeno líquido, las pajuelas se rompieron manualmente, eliminando el envoltorio plástico, y una porción cilíndrica de semen congelado fue colocada sobre un soporte metálico. El soporte metálico fue transferido al interior de una crio-cámara (Oxford HT 1500F Cryo System) unida al microscopio (JEOL Scanning Microscope 6320F, Tokyo, Japan), donde se fracturó la muestra para obtener una superficie limpia y clara durante el análisis. La temperatura de la muestra se elevó a -92ºC durante 30 s para sublimar el agua libre en estado sólido. Seguidamente la temperatura volvió a descender a -130 ºC para estabilizar la muestra. La superficie de la preparación congelada se cubrió con platino/paladio para obtener una buena relación entre señal y ruido. Las muestras fueron transferidas a la cámara del microscopio y se analizaron a una temperatura de -125 a -135 ºC. Las imágenes digitales (7–10 por pajuela a 600x para el análisis de imagen y 3000x o 4000x para el examen detallado) fueron tomadas y guardadas en el ordenador (JEOL SemAfore software, Sollentuna, Sweden) hasta su posterior examen mediante el programa específico de analisis de imágen (EasyImage 3000 software, versión 1.3.0, Tekno Optik, Huddinge, Sweden). Se evaluaron 3 variables por imagen: el área donde se encuentran los solutos congelados (contiene a los espermatozoides, denominada “venas”), el área de los espacios ocupados por el agua en estado sólido, parcialmente sublimada (extracelular, rodeando al medio, denominada “lagos”) y el grado de compartimentalización de la muestra (número de lagos por imagen). El área de cada variable fue expresada como porcentaje (%) relativo al total del área de la imagen.

Test homólogo de penetración “in vitro” **(Artículo 5)**

Para determinar la capacidad fecundante de los espermatozoides se utilizaron ovocitos homólogos madurados *in vitro*. Después de la maduración, los ovocitos madurados *in vitro* se denudaron, y grupos de 30 ovocitos fueron colocados en placas en gotas de 50 µL de medio de fecundación, cubiertos de aceite mineral y mantenidos en el incubador, durante aproximadamente 30 min hasta la adición de los espermatozoides. Para cada grupo experimental se descongelaron tres pajuelas que fueron mezcladas, centrifugadas y el sedimento resuspendido en medio de fecundación de tal manera que con 50 µL de esta suspensión espermática el ratio ovocito:espermatozoide fue 1:2000. Los gametos fueron co-incubados bajo una atmósfera de 5% de CO_2, a 39ºC y con una humedad del 95-100%,

durante 6 h y posteriormente transferidos y cultivados bajo una atmósfera de 5% CO_2 en aire, a 39º C durante 10-12 h. Finalmente, los ovocitos fueron montados en portas, fijados y teñidos para ser examinados bajo un microscopio de contraste de fase a 40 aumentos. Los parámetros de fecundación evaluados fueron: el porcentaje de penetración (% de ovocitos penetrados/número total ovocitos), porcentaje de monospermia (% ovocitos monospérmicos/total ovocitos penetrados) y el número de espermatozoides por ovocito. Se realizaron 4 replicados y se evaluaron al menos 120 ovocitos por grupo experimental.

Obtención del plasma seminal (Artículo 5)

En un estudio preliminar, se criopreservaron 116 eyaculados de 29 verracos híbridos de fertilidad comprobada (4 eyaculados por individuo). Tras su descongelación se realizó la clasificación de los verracos en 3 grupos: “buenos” “moderados” y “malos” congeladores, según su calidad espermática, valorada a los 30 y 150 min post-descongelacion. Se seleccionaron 4 individuos como donantes de plasma seminal (SP): el donante SP1, como mal congelador (<40% de espermatozoides mótiles y viables), SP2 congelador moderado (40-60%), y SP3-SP4 clasificados como buenos congeladores (>60%). El plasma seminal se obtuvo durante el proceso de criopreservación, tras la centrifugación (2,400 g durante 3 min) de la fracción rica. Posteriormente el sobrenadante fue re-centrifugado y filtrado sucesivamente a través de filtros de nylon de baja adsorción de solutos de 10 µm, hasta asegurarse de la ausencia de espermatozoides. Una vez filtrado el plasma fue almacenado a -20ºC y descongelado a temperatura ambiente antes de su uso.

Determinación del perfil proteico del plasma seminal

Las proteinas del plasma seminal se aislaron mediante cromatografía de altavresolución utilizando una columna (250 x 4 mm) de fase reversa Lichrospher RP100 C18, de 5 µm de tamaño de partícula. La columna se eluyó a 1 mL/min con una mezcla de 0,1% de ácido trifluoroacético en agua (solución A) y acetonitrilo (solución B), isocráticamente (5% B) durante 5 minutos, seguidos de 5-25% B por 10 minutos, 24-60% B por 50 minutos y 60-70% B por 10 minutos. La detección proteica se realizó a 215 nm.

Determinación de los componentes antioxidantes del plasma seminal

Con el fin de evitar interacciones con otras sustancias antioxidantes, la determinación de la capacidad antioxidante total no enzimática del plasma seminal (TAC) se realizó sobre muestras desproteinizadas (añadiendo 5% de ácido tricloroácetico, v/v). Las muestras se centrifugaron a 10,000 g, el sedimento fue descartado y las mediciones se

realizaron sobre el sobrenadante. La capacidad antioxidante total se cuantificó utilizando el método de decoloración ABTS/HRP. Este método se basa en la capacidad de distintos componentes en captar o secuestrar al radical ABTS (2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS·+) comparándolo contra una curva de calibración estándar del antioxidante Trolox (análogo soluble de la vitamina E). El radical ABTS·+ se obtuvo mezclando 1 mmol/L ATBS, 60 µmol/L H_2O_2 y 0'25 µmol/L HRP en 50 mM tampón fosfato (pH 7'5). A la mezcla de reacción (200 µL) se adicionaron 10 µL de SP y se determinó el descenso de absorbancia, directamente proporcional a la reducción de ABTS +, a 730 nm durante 5 y 30 min en un lector de microplacas de 96 pocillos (Anthos 201, Rosy Anthos, Germany). Los resultaron se expresaron como de capacidad antioxidante equivalente a µmoL de Trolox.

Para la estimación de la actividad antioxidante enzimática, las muestras de SP (1 mL) se centrifugaron a 10,000 g 5 min. El sobrenadante se separó y mantuvo en hielo hasta la determinación de la actividad de las enzimas Superóxido Dismutasa (SOD), Glutatión Peroxidasa (GPx) y Glutatión Reductasa (GR). La unidad de actividad enzimática (UI) se definió como la cantidad de enzima necesaria para catalizar la formación de un µmol de producto por minuto, o la desaparición de un µmol de sustrato por minuto, en las condiciones del ensayo. Las determinaciones enzimáticas se realizan midiendo para GPx y GR, la velocidad de oxidación del NADPH a 25ºC a pH 7'5 o bien en la SOD, la reducción del XTT, mediante la variación de la absorbancia a 340 y 470 nm, respectivamente, en un espectrofotómetro (U-2000; Hitachi Ltd., Tokyo, Japan) de doble haz (Vis-UV) utilizando cubetas de 1 cm de paso de luz. La actividad enzimática se expresó en U/mL/mg proteina.

Análisis estadístico

El análisis estadístico se realizó mediante el paquete estadístico SPSS (SPSS Inc, Chicago, IL, USA, versión 13 (**Artículos 1, 3** y **4**) y versión 14 (**Artículos 2** y **5**). Los análisis multivariantes sobre los parámetros de motilidad y viabilidad espermática se realizaron utilizando el programa estadístico PATN (CSIRO, Caberra, Australia), para identificar sub-grupos entre los eyaculados en función de su calidad espermática a la descongelación. La identificación de los sub-grupos y su clasificación jerárquica es llevada a cabo por el programa, independientemente del investigador, sobre los datos sin transformar. En todos los experimentos descritos en la presente tesis, el análisis PATN identificó tres grupos finales de eyaculados. Tras este análisis, cada eyaculado fue incluido en uno de los grupos descritos previamente, catalogándose como "buenos", "moderados" y "malos" según su calidad espermática post-descongelación. Es importante destacar, que los valores específicos de motilidad y viabilidad espermática pueden diferir entre experimentos, sin

embargo, el objetivo principal es la identificación de sub-grupos y sus características distintivas esenciales. La existencia de diferencias entre los grupos resultantes fue evaluada mediante un ANOVA de una vía. Las frecuencias relativas de eyaculados pertenecientes a cada grupo se compararon entre condiciones de criopreservación (**Artículo 4)** mediante la prueba no paramétrica de Chi-cuadrado. En el **Artículo 1,** se realizaron análisis de regresión lineal multivariante para determinar el valor predictivo del verraco, manejo reproductivo, parámetros seminales y calidad espermática antes de la congelación en la calidad espermática post-descongelación. Las pruebas no paramétricas de Kruskal-Wallis y de Mann-Whitney se utilizaron para evaluar diferencias entre verracos y grupos de verracos, respectivamente, en los parámetros determinados en los análisis Cryo-SEM (**Artículo 2**) y SCSA (**Artículo 3**). Correlaciones de Spearman se utilizaron para calcular la posible relación entre la calidad espermática a la descongelación y los parámetros obtenidos en análisis Cryo-SEM y SCSA. Para el resto de los diseños experimentales se utilizaron ANOVA's de modelo mixto o de una vía, seguidos de la prueba *post hoc* de Bonferroni. Las variables que no cumplían los criterios de normalidad fueron transformadas apropiadamente. Los resultados se expresaron como media ± SEM (**Artículos 1, 4 y 5**) o media ± SD (**Artículos 2-3**). Se consideraron diferencias estadísticamente significativas para $P < 0'05$.

Resultados y Conclusiones

Factores que influyen en la congelabilidad de los espermatozoides de porcino (**Artículo 1**)

En este artículo se estudió el valor predictivo del verraco, manejo reproductivo, parámetros seminales del eyaculado y calidad espermática antes de la congelación sobre la congelabilidad espermática, así como la variabilidad inter- e intra- verraco en la congelabilidad espermática. Destacar el amplio rango tanto en la edad de los verracos como en los parámetros seminales de los eyaculados y en la calidad espermática pre-congelación y post-descongelación, lo que indica la heterogeneidad de los animales y de la calidad de los eyaculados utilizados. Para determinar el valor predictivo de los dichos parámetros sobre la calidad espermática post-descongelación, se crearon diferentes modelos multivariantes de regresión lineal contemplando todas las posibles combinaciones. El número de combinaciones con resultados significativos fue reducido, al igual que su valor predictivo. Los resultados demostraron que la raza o la edad del verraco, así como el manejo reproductivo carecían de valor predictivo sobre la calidad espermática a la descongelación. Algunos parámetros seminales y la calidad espermática antes de la congelación mostraron un limitado valor predictivo de la congelabilidad espermática de los eyaculados, explicando un 23'2% y 10'9% del total de la varianza observada en la motilidad y viabilidad espermática

post-descongelación. Para la motilidad post-descongelación, el valor predictivo más alto (23'2%) fue obtenido para con un modelo que incluía como variables predictoras, a la motilidad pre-congelación, la morfología y concentración espermática. Para la viabilidad espermática post-descongelación, sólo un modelo, con una única variable, la morfología, fue significativo teniendo un valor predictivo muy reducido (10'9%). Sin embargo, más del 70% de la varianza total observada en la motilidad y viabilidad espermática post-descongelación, fue explicada por el verraco, indicando que el individuo es el factor determinante de las importantes diferencias observadas entre eyaculados en la congelabilidad espermática. El análisis PATN realizado a partir de la calidad espermática post-descongelación, reveló la existencia de tres grupos de eyaculados claramente definidos, observándose eyaculados con buena, moderada y mala congelabilidadad. La mayoría de los eyaculados (66'7%) se clasificaron como buenos, siendo 29'2% y 4'7%, el porcentaje de eyaculados con moderada y mala calidad espermática a la descongelación, respectivamente. Se detectaron diferencias significativas entre grupos (P < 0'05-0'001) en dos de los parámetros seminales, la concentración espermática y el porcentaje de espermatozoides con morfología normal, y en dos parámetros de calidad espermática evaluados antes de la congelación, la motilidad y la viabilidad. Así, los eyaculados con buena congelabilidad mostraron los porcentajes más altos de espermatozoides normales y la mejor motilidad antes de la congelación. Igualmente, dichos eyaculados también mostraron una elevada concentración espermática y buena viabilidad pre-congelación. Finalmente, auqnue un cierto grado de variabilidad intra-verraco fue observado (experimento 3), la mayoría de los verracos (14 de 15) mantuvieron una buena congelabilidad espermática a lo largo del tiempo.

Diferencias entre verracos durante el proceso de criopreservación (**Artículo 2**)

La homogeneidad de la deshidratación se estimó evaluando las áreas de formación de hielo extracelular (*lagos*), las áreas de medio concentrado (*venas*) donde quedan englobados los espermatozoides durante la congelación y el grado de compartimentalización (número de lagos). Independientemente de la región estudiada, la distribución y la forma de los *lagos* a lo largo de la sección de la pajuela fueron muy irregulares. La mayoría de los espermatozoides se encontraban dentro de las *venas*, sin signos obvios de daño celular externo. Ninguno de los parámetros evaluados con el Cryo-SEM estuvo correlacionado con la calidad espermática a la descongelación. (P > 0'05). Sin embargo, existieron diferencias significativas (P < 0'0001) entre verracos para todas las características ultraestructurales evaluadas, incluyendo el tamaño de las venas, por lo que pone se ponen de manifiesto diferencias en la concentración de solutos. Sin embargo, a pesar de la clara existencia de una difusión irregular de hielo durante el proceso de

criopreservación, ésta no esta relacionada con las diferencias encontradas en la supervivencia espermática a la descongelación.

Variabilidad en la integridad del ADN en espermatozoides porcinos congelados-descongelados (**Artículo 3**)

La técnica SCSA mostró que la mayoría de los espermatozoides congelados-descongelados evaluados presentaban bajos niveles de ADN fragmentado (% DFI), con valores entre 0'63% y 11'85%. Además, existía una correlación débil entre el estatus de la cromatina y los parámetros de viabilidad y motilidad espermática post-descongelación. La mayor supervivencia espermática (P < 0'05) y menor fragmentación del ADN (P < 0'01) se obtuvieron durante la descongelación a 70ºC 8 s, sin ningún cambio durante los primeros 150 min de incubación. Todos los parámetros del SCSA fueron significativamente mayores para los "malos" congeladores (P < 0'05–0'001), mostrando que éstos presentan una cromatina menos homogénea que los "buenos" congeladores. Los resultados indican, que la técnica SCSA complementa los análisis realizados de manera convencional para evaluar a los espermatozoides congelados-descongelados. Sin embargo, debido a su bajo nivel, el daño de ADN observado parece tener escasa significancia biológica.

Variabilidad entre verracos frente a distintos protocolos de criopreservación (**Artículo 4**)

Mientras que la curva de congelación no influyó, (P > 0'05), la concentración de glicerol y la curva de descongelación afectaron de manera independiente a todos los parámetros espermáticos evaluados a la descongelación (P < 0'05). De la evaluación combinada de ambos efectos, se deduce que la mayor supervivencia espermática es obtenida con un 3% de glicerol y una velocidad de descongelación de 1,800ºC/min. Se observaron diferencias significativas (P < 0'05) en la supervivencia espermática, según la condiciones de criopreservación entre eyaculados (entre verracos). Basándose en la condiciones de criopreservación control (2% glicerol y ~1,200ºC/min de velocidad de descongelación), los eyaculados se distribuyeron según su calidad espermática a la descongelación en 3 sub-poblaciones, mediante el análisis PATN. Estas sub-poblaciones respondieron de manera significativamente distinta (P < 0'05) a las diferentes condiciones de criopreservación. Mientras que los espermatozoides de eyaculados considerados como "buenos" congeladores apenas se veían afectados (P > 0'05), los de "moderados" y principalmente "malos" congeladores se mostraban muy sensibles a estos cambios (P < 0'05). En definitiva, ajustes en el protocolo de criopreservación, principalmente la concentración de glicerol y velocidad de descongelación pueden mejorar la calidad

espermática a la descongelación de ciertos eyaculados, principalmente de aquellos con pobre supervivencia espermática a la descongelación.

*Efecto del plasma seminal sobre la supervivencia espermática a la descongelación (***Artículo 5***)*

La adición de PS de "buenos" congeladores (SP3 and 4) mejoró (P < 0'01) la motilidad y viabilidad de los espermatozoides congelados-descongelados, sin observase ningún efecto sobre la peroxidación lipídica (niveles de malondialdehido). Además, el SP de los "buenos" congeladores, incrementó (P < 0'05) el porcentaje de penetración (SP3) y ovocitos polispérmicos (SP4) respecto al control. Ni la cantidad total de proteínas presentes en el SP, ni el perfil proteico o la capacidad antioxidante de los distintos SP utilizados, estuvo relacionada con el efecto observado de los distintos SP sobre la criopreservación espermática.

Extended Summary

Extended Summary

Abstract

In order to further increase the use of frozen-thawed (FT) spermatozoa in commercial pig breeding it is necessary to minimise the existing ejaculate variation in sperm freezability, a constraining issue when creating a sperm cryobank. The aim of this thesis was to study i) which factors could explain part of this variability, ii) the incidence of DNA fragmentation in frozen-thawed boar spermatozoa, iii) whether different boars require modifications in the freezing and thawing protocol to enhance sperm cryosurvival and, iv) the possible role of seminal plasma in boar sperm freezability. The results revealed that boar is the most important factor explaining the different susceptibility among ejaculates to sustain cryopreservation and that the intra-boar variability is relatively low. The sperm quality before freezing has limited influence on sperm cryosurvival variability. The overall DNA damage in frozen-thawed boar sperm was low, but "bad" freezers showed less homogeneous sperm chromatin than "good" freezers. Slight adjustment of the glycerol concentration and warming rate provides a better sperm cryosurvival, particularly in those ejaculates which are considered as "bad" freezers. Supplementation of freezing media with seminal plasma from males with "good" freezability, has a beneficial effect on post-thaw sperm cryosurvival, however we still have to determine the underlying mechanisms involved in this protection. These findings have rendered very helpful information to improve the management and the overall quality of boar sperm cryobanks, especially when selecting samples to be included as founders.

Introduction

Artificial insemination (AI) is the most widely used biological technology in developed and developing countries in livestock farming. Today, AI is applied in several species, such as cattle, sheep, goats, turkeys, pigs, chickens or rabbits. Frozen semen allows genetic progress to be disseminated worldwide and represents the basis for the recent biotechnological and genomic advances such as sexed semen or transgenesis [1]. It has been most extensively applied in dairy cattle production, where progeny testing is well

established [2]. However, cryopreservation of boar semen is still considered to be a suboptimal methodology, technically demanding and basically limited to research, genetic banking or export of semen for selected nuclei lines [3]. Poor sperm survival after thawing has traditionally represented the major limitation to the successful application of cryopreserved boar semen in commercial AI programmes, since it largely explained the low fertility results achieved [4]. During the last years, continuing efforts have being made to refine the freezing procedures and to improve the insemination technique itself. Incorporation of novel types of containers for freezing and thawing, FlatPack® and MiniFlatPack® [5,6], different centrigufation [7] and cooling rates [8] or inclusion of various additives [10-12], have led to optimize the cryopreservation protocol. Moreover, newer catheters to surpass the cervix and even to inseminate deep into the uterine horns are nowadays available, so that a higher degree of effectiveness can be obtained after AI [13,14]. However, the great variability among boars and even differences between ejaculate portions to sustain sperm cryopreservation has led to controversies among results. As a consequence, frozen semen of some genetically interesting breeds or males may not be suitable as a gene bank resource, or can be used only with a poor efficiency [reviewed by 15]. Therefore, to increase the survival of frozen-thawed spermatozoa, some studies are required to improve the methods for cryopreservation, as well as minimizing the existing intra- and inter-boar variation in sperm freezability, present in a population unselected for this purpose. An accurate knowledge of the factors influencing sperm cryosurvival, including an assessment of boar variability, ejaculate quality and reproductive management strategies, may help to minimize this variability. Studies of how the freezing procedure itself contributes to these differences in sperm freezability are also necessary. Nowadays, a large number of programmable freezers are commercially available, which allows the control of the cooling rate curve, and both the internal and external temperatures of straws being frozen. However, in most of them, the initial ice nucleation of the samples, is generally not taken into control and occurs as a stochastic event. Subsequently, the differences observed in sperm cryosurvival after thawing could be, at least in part, due to the detrimental effects of fluctuations during ice crystal formation [16].

The sperm characteristics usually evaluated to classify boars as ‘good’, ‘moderate’ or ‘bad’ freezers include traits such as motility or viability [3,17,18]. However, not all those boars classified as ‘good’ freezers have been able to achieve high rates of in vitro fertility, whereas some classified as ‘bad’ offered acceptable fertility rates. A sperm feature that has been the focus of recent studies is the chromatin structure stability. Assessment of sperm DNA integrity is required for a detailed knowledge of spermatozoa ability for fertilization and embryo development [19]. Thus, DNA integrity evaluation could be an important tool for frozen-thawed sperm quality determination.

Numerous studies have been carried out to define optimal conditions for freezing boar spermatozoa, leading to considerable improvement of sperm survival after freezing and thawing. However, most of them have not considered individual boar differences in sperm freezability as a source of variation. As a consequence, the important incidence of "bad" sperm freezers could be in part, a response to sub-optimal freezing protocols. In relation to this, it has been claimed as a possible alternative, the concept of individually-tailored frozen-thawed protocol, which implies the adaptation of specific cryopreservation conditions depending of every individual boar [20]. It would have important practical implications when creating a sperm cryobank, if the percentage of boars with low sperm freezability is minimized.

Finally, it is interesting to point out, that some genetic studies have indicated that sperm freezability is likely to be an intrinsic property of boar semen [17], dependent perhaps on components of the sperm membranes or even seminal plasma [18]. In this way, it has been shown that epididymal boar spermatozoa have better cryoresistance than ejaculated spermatozoa [21]. It could be due to that epididymal spermatozoa have not been exposed to the complex secretions of the accessory sex glands. In stallion, the effect observed after the addition of seminal plasma to the freezing medium, was directly proportional to the potential sperm freezability of seminal plasma donors [22]. Moreover, boar ejaculates fractions have different ability to sustain cryopreservation, and this condition appears to vary among boars [23]. These differences could be attributed to the complex composition of seminal plasma, which differs among males within the same species, as well as between ejaculates or different fractions of the ejaculate [24]. Furthermore, differences in SP-protein profiles in bull and stallion have been related with low and high semen freezability [25,26]. Therefore, "good" and "bad" sperm freezers could differ in their seminal plasma composition and this feature influences boar sperm cryosurvival.

Objectives

The main objective of this study was to enhance the overall post-thaw sperm quality of boar sperm, by minimizing the recurring ejaculate variability existing in a sperm cryobank. The specific aims of the present study were:

1. To identify factors responsible for variability among ejaculates in their ability to survive cryopreservation and estimate the importance of inter- and intra-boar variability on the sustainability of sperm quality after cryopreservation. (**Paper I and II**)

2. To evaluate the degree of DNA integrity in frozen-thawed boar spermatozoa and its relationship with "good" and "bad" sperm freezers. (**Paper III**)

3. To assess the effectiveness of different cryopreservation conditions for freezing and thawing boar ejaculates, focusing on those having sub-optimal sperm freezability. (**Paper IV**)

4. To examine the effect of supplementation the freezing extender with SP, from ejaculates with different sperm freezability, on boar sperm cryosurvival (**Paper V)**.

Materials and methods

Animals

All the ejaculates cryopreserved in the present thesis belonged to healthy and fertile mature boars of various commercial hybrid lines undergoing regular semen collection for commercial artificial insemination. They were supplied by three commercial insemination stations, Batallé SA, Proinserga SA, and UVE SA for cryobanking purposes. After collection (gloved-hand method), sperm rich fractions were extended (1:1, v/v) in Beltsville Thawing Solution (BTS) and evaluated by standard laboratory techniques. The extended semen was transferred to 50-mL tubes, cooled to 17°C, and sent by mail, packaged in insulated containers under conditions of monitored temperature (miniature data logger, Gemini Data Loggers, Ltd., Chichester, UK), to the sperm cryopreservation laboratory of the Faculty of Veterinary Medicine, University of Murcia. The extended semen arrived at the laboratory 14 to 16 h after collection of the ejaculate.

Semen processing

Sperm pellets obtained after centrifugation of extended sperm-rich fractions were cryopreserved using the straw freezing procedure described by [27], as modified by Thurston et al. [28] and Carvajal et al. [7]. Single ejaculates were used in **Papers 1-4**, while in **Paper 5**; the semen from nine boars was pooled. At the laboratory, the semen was re-evaluated and centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) for 3 min at 2,400 × *g*. After centrifugation, the supernatant was removed by aspiration and sperm pellets were re-extended in lactose-egg yolk (LEY) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5°C in 90-120 min, the sperm were re-suspended with the LEY-Orvus ES Paste

extender [92.5%-89.5% LEY + 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA) and 6% (**Paper 1-5**) or 9% (**5**) glycerol, v/v; pH 6.2 and 1145 ± 17 mOsm/Kg or 1650 ± 15 mOsm/kg, respectively] to a final concentration of 1×10^9 sperm/mL. The re-suspended and cooled sperm were packed into 0.5-mL, PVC, French straws (Minitüb, Tiefenbach, Germany) and frozen using a controlled-rate freezing instrument (IceCube 1810, Minitüb, Tiefenbach, Germany).

Freezing and thawing rates

The freezing programme used in the different studies were as follows: in **Papers 1, 2, 3** and **5**, cooled to –5ºC at 6ºC/min, from –5ºC to –80ºC at 40ºC/min, held for 30 sec at –80ºC, then cooled at 70ºC/min to –150ºC; in **Paper 4**, from 5 ºC to –5 ºC at 6 ºC/min and from –5 ºC to –80 ºC at different freezing rates: 10, 40 or 60 ºC/min, then cooled at 70ºC/min to –150ºC, and finally plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for 1-2 wk before thawing. Thawing of straws was done in circulating water at 37°C for 20 s (**Paper 1-5**) or at 70ºC for 8 sec (**Paper 3-4**). Thawed sperm from 2 straws per ejaculate were re-diluted in BTS (1:2, v/v; 37°C) and incubated in a waterbath at 37°C for 150 min.

Sperm quality assessment

The inclusion criteria for semen quality were at least 200 x 10^6 sperm/mL, 85% sperm with normal morphology, and ≥75% and ≥80% of motile and viable sperm, respectively (**Papers 2-5**), except for **Paper 1** (experiment 1) where ejaculated were no preselected before freezing.

Sperm motility

Measurements were done 30 min and 150 min after thawing. Sperm motility was objectively evaluated using a computer assisted sperm analysis (CASA) system (Sperm Class Analyzer® Microptic, Barcelona, Spain). Before analysis, BTS-diluted thawed spermatozoa were always further re-diluted in BTS to obtain about 30 x 10^6/mL. For each evaluation, a 4 µL sperm sample was placed in a pre-warmed (39ºC) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were analyzed to assess a minimum of 100 spermatozoa. The analysis yielded the following motility parameters: TSM (% of total motile spermatozoa), rapid progressive motile (% RPM, VAP > 50 µm/s and STR > 75%), VCL (curvilinear velocity, µm/s), VSL (straight linear velocity, µm/s), VAP (average path velocity, µm/s); LIN (linearity, %) and ALH (amplitude of lateral head displacement, µm).

Sperm viability

Sperm viability was expressed as the percentage of spermatozoa with intact plasma and acrosomal membrane (**Paper 1-5**) and mitochondrial membrane potential (**Paper 1-3**). In **Paper 1-3**, these characteristics were analyzed simultaneously using a modification of a triple fluorescent procedure, adapted for boar sperm by [7] for fluorescence microscopy. It included the fluorochrome propidium iodide (PI), the mitochondria specific fluorochrome rhodamine-123 (R123) and the acrosome specific fluorochrome fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA). Three hundred sperm were counted under 1000× magnification (Eclipse E800, Nikon, Tokyo, Japan), using a BV-2A filter (400 to 440 nm excitation filter, 455 nm emission, 470 nm barrier filter). Sperm showing only green fluorescence over their mid-piece (R123 positive) were considered viable with an intact acrosome (viable sperm) and the values were expressed as a percentage (%). In **Paper 4**, plasma membrane integrity (PMI) was evaluated using the fluorescent probes SYBR-14 and PI as manufacturer's instructions (L-7011; Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands) and the acrosomal intactness was assessed by FITC-PNA and PI. Samples were incubated at room temperature in the dark for 5-10 min before flow cytometric analysis. Analyses were carried out on a Becton Dickinson FACSort flow cytometer (San Jose, California, USA) equipped with an argon-ion laser (488nm, 15 mW). Only spermatozoa with intact plasma and acrosomes membranes were recorded. In **Paper 5**, plasma membrane and acrosome integrity were evaluated simultaneously using a triple fluorescent procedure described by [29]. Aliquots (100µL each, ~3 x 10^6 cells) were transferred into culture tubes and dyes were added at a final concentration of 25 nM SYBR-14 (100 µM stock solution in DMSO; Component A of LIVE/DEAD Sperm Viability Kit, L-7011; Molecular Probes, Europe BV, Leiden, The Netherlands), 1 µg/mL of peanut agglutinin conjugated with phycoerythrin (PE-PNA solution (1 mg/mL stock solution Phycoprobe R-PE-PNA, P44, Biomeda Corp. Foster City, CA) and 12 µM PI (1.5 mM in PBS, Component B of Sperm Viability Kit). Flow cytometer acquisition and analysis were performed by using a Coulter Epics XLTM (Coulter Corporation, Miami, FL, USA). Debris was double gated out based on the forward and side scatter and DNA content (events negative to SYBR 14 or PI fluorescence were considered non sperm). Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified.

Assessment of sperm DNA integrity (**Paper 3**)

The sperm chromatin structure assay (SCSA) was applied according to the procedure described by [19] to determine the degree of increased susceptibility of

spermatozoa following acid induced denaturation in situ, and after quantification by flow cytometric measurement of the metachromatic shift from green (double-stranded DNA, dsDNA) to red (single-stranded DNA, ssDNA) acridine orange (AO) fluorescence, thus representing the amount of denatured, ssDNA over the total cellular DNA. The thawed semen samples were re-extended with TNE buffer (0.15 mol/L NaCl, 0.01 mol/L Tris–HCl and 1 mmol/L EDTA, pH 7.4) into a final sperm concentration of approximately 2 x 10^6/mL. An aliquot of 0.2 mL was subjected to partial DNA denaturation in situ by mixing with 0.4 mL of a low pH detergent solution (0.17% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.4), followed 30 s later by staining with 1.2 mL of AO (6 µg/mL in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA and 0.15 M NaCl, pH 6.0). The stained samples were split into two halves and analysed within 3 min after AO staining. Assessment of sperm chromatin integrity was carried out on a BD FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with standard optics. AO was excited with an Ar ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm and running at 200 mW. In association with dsDNA, AO fluoresces green (530 ± 30 nm, as detected with the FL1 detector), but in the presence of ssDNA, the resulting fluorescence is red (>630 nm, as detected with FL3). Data were stored as list mode files and further analyses of the parameters were carried out by using the FCSExpress software, version 2 (DeNovo Software; Thornhill, ON, Canada).

Cryo-scanning electron microscopy (Cryo-SEM) **(Paper 2)**

Cryo-SEM was used to examine the ultrastructure of the samples and determine whether the amounts of solid state water in the extracellular, outer–extender areas of frozen straws differed between straws from boars with different sperm cryosurvival post-thaw. Working inside liquid nitrogen, the straw was manually broken, the plastic removed, and a cylinder of the frozen semen mounted in a mechanical grip holder. The grip holder was transferred into an Oxford HT 1500F Cryo System chamber attached to the microscope (JEOL Scanning Microscope 6320F, Tokyo, Japan). Once the sample was inside the chamber, a fracture of the sample was made to get a fresh clean surface to be examined. The temperature of the sample was raised by heating the holder to -92 ºC for 30 s in order to sublimate free water in the solid state lakes, followed by a temperature decrease to -130 ºC to stabilise the sample. The surface of the frozen preparation was then coated with platinum/palladium to obtain a good relation between signal and noise. The coated sample was thereafter transferred into the microscope chamber where it was analysed at a temperature range of -125 to -135 ºC. Digital images (7–10 per straw at 600x for image analyses and 3000x or 4000x for fine structural examinations) were collected at 5 KV and computer stored (JEOL SemAfore software, Sollentuna, Sweden) before being further assessed by image analysis (EasyImage 3000 software, version 1.3.0, Tekno Optik,

Huddinge, Sweden). Three variables were retrieved per image: the area of the freezing extender matrix (containing spermatozoa, e.g., veins), the area of the spaces occupied by the partially sublimated solid-state water (extracellular, outside the extender, e.g., lakes) and the degree of compartmentalization in the sample (number of lakes per image). The area of each variable was expressed as the relative percentage out of the total image area.

Homologous "in vitro" penetration assay **(Paper 5)**

The ability of spermatozoa to penetrate, *in vitro*, homologous IVM-oocytes was assessed as described by [30]. Cumulus-oocyte complexes (COCs) were matured *in vitro* and prepared according to the protocol before semen was added. Three semen straws were thawed, pooled, washed and re-suspended in *in vitro* fertilization (IVF) medium before being added to the medium containing the oocytes. Each oocyte was exposed to 2,000 spermatozoa and after 6 hours of co-incubation, oocytes were transferred and cultured at 39ºC in 5% CO_2 in air for 10-12 hours. The oocytes were mounted on slides, fixed and then stained and examined under a phase contrast microscope. The fertilization variables evaluated were sperm penetration rates (% of the number of oocytes penetrated/total number inseminated) and the number of spermatozoa per oocytes (mean number of spermatozoa in penetrated oocytes). At least 120 oocytes per each frozen-thawed sperm sample were evaluated.

Collection and storage of seminal plasma samples (Paper 5)

In a preliminary study in which 116 ejaculates from 29 mature fertile hybrid boars (4 ejaculates per boar) were cryopreserved, the boars were classified into 3 groups according to the post-thaw sperm survival assessed at 30 and 150 min post-thawing as "good", "moderate" and "poor" freezers. Four boars were selected among them as SP donors: SP1 was a poor sperm freezer (<40% of motile and viable spermatozoa), SP2 was a moderate sperm freezer (40-60%), and SP3-SP4 were good sperm freezers (>60%). Seminal plasma was obtained during the cryopreservation process. After centrifugation (2,400 g for 3 min) of sperm-rich fractions diluted 1:1 (v/v) in BTS, the supernatant was collected and re-centrifuged, filtered through a 10 μm nylon mesh filter to remove debris or clumped spermatozoa and examined using phase microscopy to ensure no spermatozoa remained. Samples containing spermatozoa were re-filtered. The supernatant (SP diluted in BTS) was stored frozen at -20ºC until further use. Before using, SP was thawed at room temperature.

Determination of seminal plasma protein profiles

Seminal plasma proteins were isolated by reverse-phase high performance liquid chromatography (RP-HPLC). Briefly, SP proteins were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrospher RP100 C18 column (250 x 4 mm, 5µm particle size; Merk, Darmstadt, Germany) eluted at 1 mL/min with a linear gradient of 0.1% trifluoroacetic acid in water (solution A) and acetonitrile (solution B), isocratically (5% solution B) for 5 min, followed by 5-25% B for 10 min, 25-60% B for 50 min, and 60-70% B for 10 min). Protein detection was at 215 nm.

Assessment of the antioxidant components of seminal plasma.

Determination of total non-enzymatic antioxidant capacity (TAC) was performed on deproteinized SP (adding 5% trichloroacetic acid on ice). Samples were subsequently centrifuged at 10,000 g, the pellet was discarded and measurements were performed on the supernatant. Total non-enzymatic antioxidant capacity was measured using the ABTS/HRP decolouration method. This method is based on the capacity of different components to scavenge the ABTS (2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) radical cations (ABTS·+) compared to a standard antioxidant (Trolox) in a dose-response curve. Pre-formed ABTS·+ was obtained by mixing 1 mmol/L ATBS, 60 µmol/L H_2O_2 and 0.25 µmol/L HRP in 50 mM sodium phosphate buffer (pH 7.5). Then, 10 µL of SP was added to the reaction medium (200 µL) and the decrease in absorbance, directly proportional to the ABTS·+ quenched, was determined at 730 nm at 5 and 30 min in a 96-well microplate reader (Anthos 201, Rosy Anthos, Germany) Data were expressed as µmoL of Trolox equivalents.

For estimation of the enzymatic antioxidant activity, SP samples (1 mL) were centrifuged at 10,000 g for 5 min. Then the supernatant was separated and kept in ice until the analysis. Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Glutathione reductase (GR) activities were measured as described by [31] for ram spermatozoa. The absorbance changes were monitored with an UV-Vis Hitachi spectrophotometer (U-2000; Hitachi Ltd., Tokyo, Japan) measuring the NADPH oxidation or XTT reduction at 340 or 470 nm respectively. One enzyme unit (IU) was defined for GPx and GR as the oxidation of 1.0 µmoL/min of NADPH at 25ºC at pH 7.5 or as the amount of SOD capable of transforming 1.0 µmoL/min of O2-. The enzyme activity was expressed in U/mL/mg protein.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (SPSS Inc, Chicago, IL, USA, version 13 (**Papers 1, 3** and **4**) and version 14 (**Papers 2** and **5**). Multivariate analysis on sperm motility parameters and sperm viability were carried out using

the computer program PATN (CSIRO, Caberra, Australia) to identify naturally occurring sub-groups within the data set. The identification of the sub-groups and their hierarchical classification is carried out by the program independently of the investigator, based on untransformed data. In all of the experiments described in the present thesis the PATN software identified three final ejaculate groups. Upon completion of the PATN analysis, each individual ejaculate was categorised as belonging to one of the small number of groups described above (note that the specific parameter values (sperm motility and viability) inevitably differed slightly between experiments, however, the main task was the identification of sub-groups and their basic characteristics). Once the populations had been identified, differences among PATN groups within each experimental sample were compared by one-way ANOVA. The relative frequencies of ejaculates belonging to each group were compared among cryopreservation conditions (**Paper 4**) by Chi square test. The ejaculates with best, moderate, and reduced sperm cryosurvival were classified as "good", "moderate", or "poor", respectively. In **Paper 1,** multiple regression analyses were performed to disclose the predictive value of some variables on postthaw sperm quality. The non-parametric Kruskal-Wallis and Mann-Whitney *U*-tests were used to evaluate differences on Cryo-SEM variables (**Paper 2**) and DNA integrity (**Paper 3**) between boars and groups ("good" and "poor" boars) respectively. The Spearman non-parametric correlation test was used to calculate the relationships between the post-thaw sperm quality and Cryo-SEM and SCSA parameters. The one-way or mixed model ANOVA was further used to determine differences in the rest of experimental data. For those variables that were non-normally distributed, data were properly transformed. When ANOVA revealed a significant effect, values were compared using the Bonferroni test. Values are presented as means ± SEM (**Papers 1, 4 and 5**) or means ± SD (**Papers 2-3**). Probability values of < 0.05 were considered to be statistically significant.

Results

Factors influencing boar sperm cryosurvival (**Paper 1**)

Boar and reproductive management variables were not predictive of sperm cryosurvival among ejaculates. Of the total variance observed in post-thaw sperm motility and viability, 23.2% or 10.9% could be explained by sperm concentration, morphology and motility before freezing, or sperm morphology, respectively. However, more that 70% of total variance observed in post-thaw sperm quality variables among ejaculates was explained by boar. This indicates that boars are the most important factor explaining the variability among ejaculates in sperm cryosurvival, with most (14 of the 15 boars in experiment 3) showing consistent sperm cryosurvival over time.

Inter-boar differences during the freezing process (**Paper 2**)

The degree of hydration was monitored in relation to the areas of ice crystal formed extracellularly (lakes), the areas of frozen, concentrated extender (veins) where spermatozoa were located and the degree of compartmentalization (number of lakes) present. Irrespectively of the region studied, the gradient of main dehydration (as lakes) observed along the cross-section area of the straws was very irregular. Most spermatozoa were enclosed in the freezing extender matrix and no obvious signs of external membrane damage were observed. None of the Cryo-SEM variables significantly correlated with post-thaw sperm parameters ($P > 0.05$). However, there were significant differences ($P < 0.0001$) among boars for all ultrastructure variables studied, including the size of the veins, where differences in solute concentration are expected. We concluded that despite the large variability in ice crystal formation during the conventional freezing process among boars; this is unrelated to inter-boar post-thaw sperm differences.

Inter-boar DNA-integrity differences in frozen-thawed boar spermatozoa (**Paper 3**)

The SCSA showed that most spermatozoa had intact DNA [levels of DNA fragmentation index (%DFI) ranging from 0.63% to 11.85%] and significantly correlated (albeit weakly) with current sperm quality variables. The highest sperm survival ($P < 0.05$) and the lowest DNA damage ($P < 0.01$) were achieved when sperm thawing was carried out at 70ºC for 8 s, without any change during the first 150 min of incubation. Finally, the third experiment studied if differences in sperm freezability showed by stud boar semen, as 'good' or 'bad' freezers by conventional analyses, could be attributed to differences in chromatin structure. All SCSA parameters were low, but significantly ($P < 0.05$–0.001) higher for 'bad' freezers, showing they had less homogeneous sperm chromatin than the 'good' freezers. The results indicate that SCSA outcome complements conventional assessment of FT-boar spermatozoa, disclosing differences in their ability to sustain freezing and thawing. However, the low overall DNA damage observed in FT spermatozoa seems to have poor biological significance.

Inter-boar variability to different cryopreservation conditions (**Paper 4**)

Whereas CR had no influence ($P > 0.05$), GLY and WR independently affected ($P < 0.05$) all sperm parameters assessed. Evaluating the combined effect of GLY and WR, the highest post-thaw quality of spermatozoa was achieved in semen frozen with 3% glycerol and thawed at 1,800ºC/min. Inter-ejaculate (boar) differences supporting survival of

spermatozoa under the different CCs were significant ($P < 0.05$). Three different ($P < 0.05$) ejaculate (boar) populations, defined by PATN analysis, were identified according post-thawing sperm assessments in semen frozen and thawed using control CC (2% of glycerol and ~1,200ºC/min of warming). Different ($P < 0.05$) susceptibility in the tolerance of spermatozoa to the different CCs was found among the ejaculate populations. Whereas spermatozoa from ejaculates considered as "good" freezers were relatively unaffected ($P > 0.05$), those from "moderate" and, mainly, "bad" freezers were very sensitive ($P < 0.05$). In conclusion, adjustments in the CCs - GLY and WR - can improve the cryosurvival of spermatozoa from some ejaculates (boars), particularly those showing poor sperm freezability.

*Effect of seminal plasma in boar sperm cryosurvival (***Paper 5***)*

The addition of SP from "good" sperm freezers (SP3 and 4) improved ($P < 0.01$) the motility and viability of thawed spermatozoa, without any influence on MDA production. Moreover, SP from "good" sperm freezers also increased ($P < 0.05$) the percentage of penetrated (SP3) and polyspermic oocytes (SP4) respect to the control. Neither the total amount of SP proteins, protein profiles, nor antioxidant capacity of the different SPs were related to the various cryosurvival/fertilizing capacity of the processed spermatozoa.

Conclusions

1. The boar is the most important factor explaining the variability in sperm cryosurvival among ejaculates, being responsible for more than 70% of variation.

2. Standard semen measurements in fresh ejaculates and sperm quality assessments before freezing, do not limit the success of sperm cryopreservation. Nevertheless it is recommendable to select those ejaculates containing a high sperm concentration and a high proportion of sperm with normal morphology before freezing, to improve the overall quality of a sperm cryobank.

3. Although a certain degree of intra-boar variability is observed, it is not relevant compared to the inter-boar differences.

4. The large variability in ice crystal formation observed among boars during the conventional freezing process, is unrelated to post-thaw sperm differences.

5. Frozen-thawed (FT) spermatozoa from boars classified as ‘good’ freezers, were less susceptible to chromatin denaturation than ‘bad’ freezers. However, the low overall DNA damage observed in FT spermatozoa has poor biological significance.

6. The incidence of “bad” sperm freezers is reduced by slight modifications in the freezing and thawing protocol, particularly the glycerol concentration and warming rate for thawing.

7. The addition of seminal plasma (SP) to the freezing extender can improve boar sperm cryosurvival. However, the beneficial effect depends on SP source, being only significant when SP was collected from boar with “good” sperm freezability. Looking at the SP composition, it is likely that neither protein profiles nor the antioxidant capacity of SP can explain per se the differences in effectiveness among SPs. components.

References

[1] Gerrits RJ, Lunney JK, Johnson LA, Pursel VG, Kraeling RR, Rohrer GA, Dobrinsky JR. Perspectives for artificial insemination and genomics to improve global swine populations. Theriogenology 2005;63:283-299.

[2] Vishwanath, R. Artificial insemination: the state of art Theriogenology 2003;59:571-584.

[3] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. Anim Reprod Sci 2000;62:143–172.

[4] Eriksson BM, Petersson H, Rodríguez-Martínez H. Field fertility with exported boar semen frozen in the new flatpack container. Theriogenology 2002;58:1065–1079.

[5] Eriksson BM, Rodríguez-Martínez H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. Anim Reprod Sci 2000;63:205-220.

[6] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodríguez-Martínez H. Deep freezing of concentrated boar semen for intra-uterine insemination: effect on sperm viability. Theriogenology 2005;63:1320-1333.

[7] Carvajal G, Cuello C, Ruiz M, Vázquez JM, Martínez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389-396.

[8] Devireddy RV, Fahrig B, Godke RA, Leibo SP. Subzero water transport characteristics of boar spermatozoa confirm observed optimal cooling rates. Mol Reprod Dev 2004;67:446-457.

[9] Peña FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 2003;78:85-98.

[10] Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397-405.

[11] Roca J, Rodríguez MJ, Gil MA, Carvajal G, García EM, Cuello C, Vázquez JM, Martínez EA. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15-24.

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

[13] Roca J, Carvajal G, Lucas X, Vázquez JM, Martínez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. Theriogenology 2003;60:77-87.

[14] Bolarín A, Roca J, Rodríguez-Martínez H, Hernández M, Vázquez JM, Martínez EA. Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used. Theriogenology 2006; 65:669-680.

[15] Roca J, Rodríguez-Martínez H, Vázquez JM, Bolarín A, Hernández M, Saravia F, et al. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Control of pig reproduction VII, Ashworth CJ, Kraeling RR (Eds), Nottingham: Nottingham University Press; 2006, pp. 261–275.

[16] Woelders H, Matthijs A, Zuidberg CA, Chaveiro AE. Cryopreservation of boar semen: equilibrium freezing in the cryomicroscope and in straws. Theriogenology 2005;63:383–395.

[17] Thurston LM, Siggins K, Mileham, AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002;66: 545–554.

[18] Holt WV, Medrano A, Thurston LM, Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope Theriogenology 2005;63:370-382.

[19] Evenson DP, Larsson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25–43.

[20] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their postthawing function. Reprod Fertil Dev 1995;7:871–891.

[21] Rath D, Niemann H. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen thawed epididymal semen obtained from identical boars. Theriogenology 1997;47:785–793.

[22] Aurich JE, Kühne A, Hoppe H, Aurich C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. Theriogenology 1996;46:791-797.

[23] Peña FJ, Saravia F, Nuñez-Martínez I, Johannisson A, Wallgren M, Rodríguez Martínez, H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Anim Reprod Sci 2006;93:101-113.

[24] Strzezek J, Wysocki P, Kordan W, Kuklinska M, Mogielnicka M, Soliwoda D, Fraser L. Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. Reprod Biol 2005;5:279-290.

[25] Jobim MI, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC. Two dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 2004;61:255-266.

[26] Zahn FS, Papa FO, Melo CM, Brisola ML. Protein profile of equine seminal plasma: correlation to semen freezability. Anim Reprod Sci 2005;89:313-315.

[27] Westendorf P, Richter L, Treu H. Zur Tierfgefrierung von Ebersperma: Labor und besamungsergebnisse mit dem Hülsenberger Pailleten Verfahren. Dtch Tierárztl Wschr 1975;82:261–267.

[28] Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with variation in boar semen quality following cryopreservation. J Androl 2001; 22:382-394.

[29] Nagy Sz, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol. Reprod 2003;68:1828–1835.

[30] Gil MA, Ruiz M, Cuello C, Vázquez JM, Roca J, Martínez EA. Influence of sperm/oocyte ratio during in vitro fertilization of in vitro matured cumulus-intact pig oocytes on fertilization parameter and embryo development. Theriogenology 2004; 61: 551-560.

[31] Marti JI, Marti E, Cebrian-Perez JA, Muiño-Blanco T. Survival rate and antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. Theriogenology 2003;60:1025-1037.

Anexo Gráfico

Figura 1. Registrador de temperatura (Tynitag Plus, Gemini, UK) utilizado para monitorizar la temperatura de las muestras espermáticas durante el transporte.

Figura 3. a y b) Descongelación de pajuelas a 37 y 70ºC.

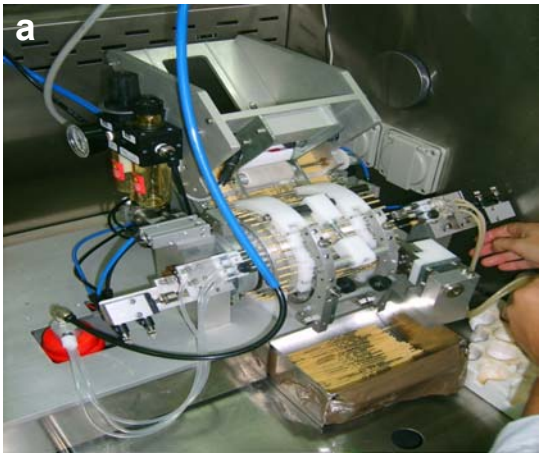

Figura 2. a) Envasadora de pajuelas automática, b) Biocongelador (Icecube 1810, Minitüb) y c) curva de congelación de referencia.

Figura 4. Evaluación de la motilidad mediante Sistema SCA (Sperm Class Analyzer, v. 5.1, Microptic)

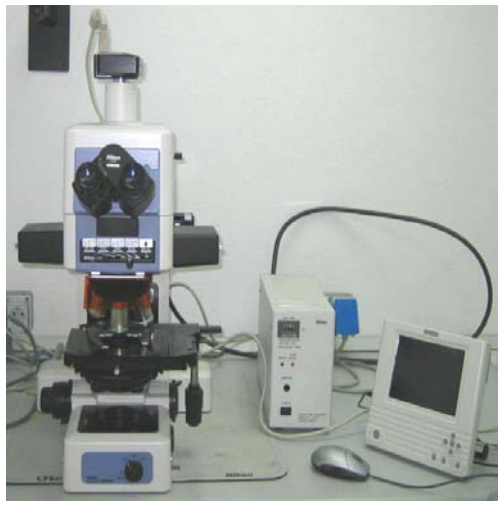

Figura 5. Microscopio de fluorescencia (Eclipse E-800, Nikon)

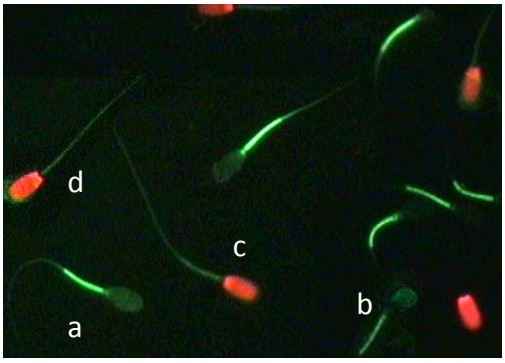

Figura 6. Evaluación de la viabilidad espermática mediante triple tinción para microscopía (R123/PI/FITC-PNA). Se distinguen cuatro poblaciones espermáticas: a) vivos (R123+/PI-), b) vivos acrosoma dañado (R123+/FITC-PNA+), c) muertos (PI+) y d) muertos acrosoma dañado PI+/FITC-PNA+).

Figura 7. Citómetro de flujo (Epics XL, Beckman Coulter)

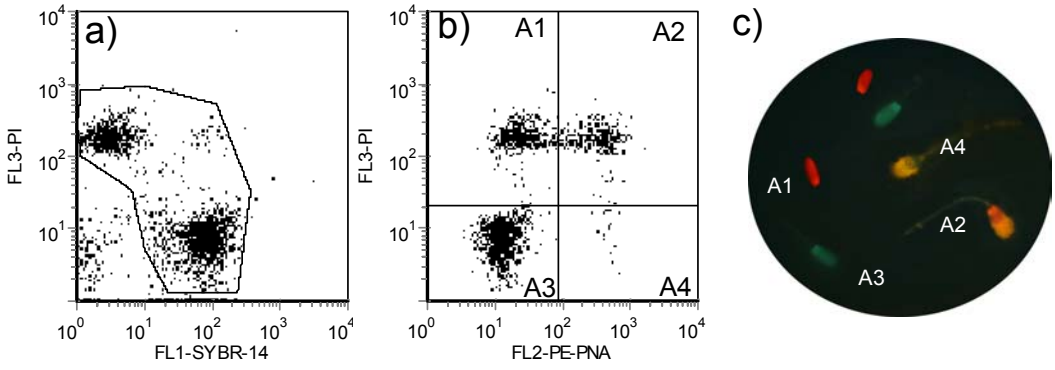

Figura 8. a y b) Representación gráfica correspondiente al análisis por citometría de flujo de la combinación SYBR-14/PI/PE-PNA. c) Imagen de microscopía de fluorescencia en la cual se observan las 4 poblaciones espermáticas. A1 (PI+/PE-PNA-): muertos acrosoma intacto; A2 (PI+/PE-PNA+): muertos acrosoma dañado; A3 (PI-/PE-PNA-) vivos acrosoma intacto; A4 (PI-/PE-PNA+) vivos acrosoma dañado.

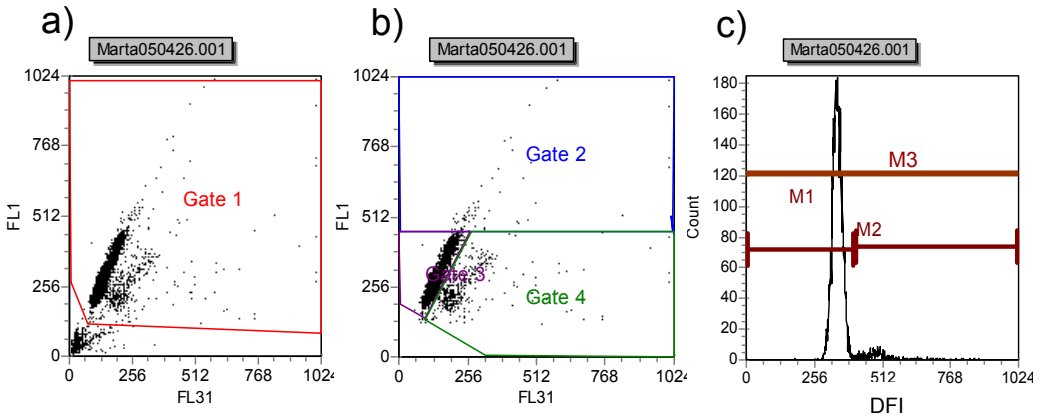

Figura 9. Representación gráfica del análisis de la integridad del ADN mediante la técnica SCSA utilizando el software FCSExpress, v. 2 (DeNovo Software). Diagramas de puntos (a y b) representan la fluorescencia roja (FL3, ADN desnaturalizado) *vs* fluorescencia verde (FL1, ADN no desnaturalizado). (c) Histograma del DFI index (fluorescencia roja/total fluorescencia). La población M2 representa el porcentaje espermático con estructura anormal de la cromatina (%DFI).

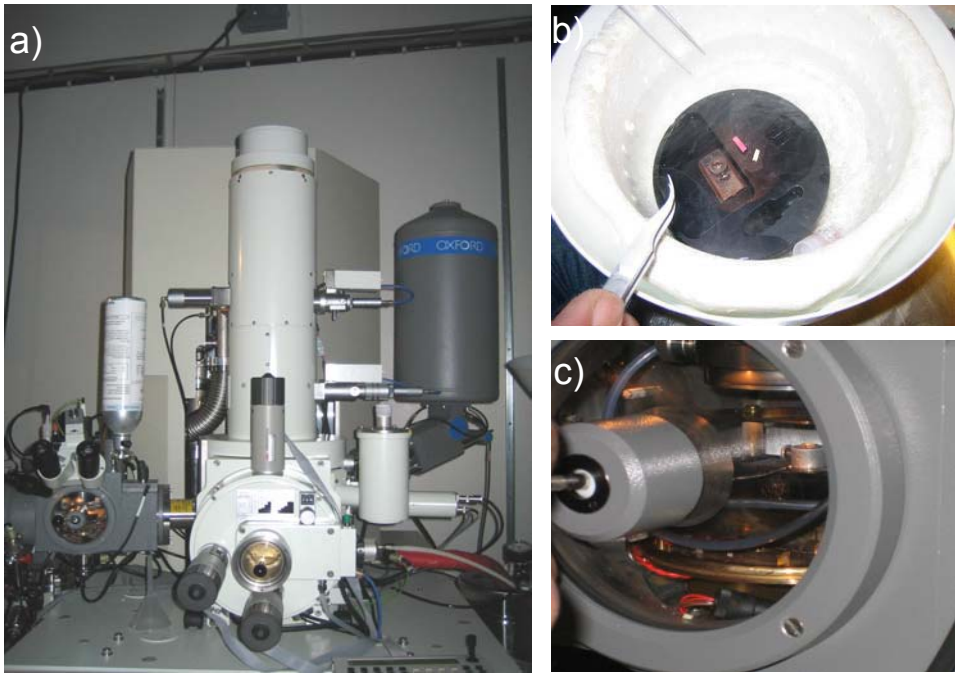

Figura 10. (a) Crio-microscopio electrónico de barrido. Rotura de la pajuela, extracción de la porción de semen congelado y (b) montaje sobre soporte metálico. (c) Fractura de la muestra mediante bisturí dentro de la cámara de vacío para la obtención de la superficie a analizar.

Apéndice

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Rank	Abbreviated Journal Title *(linked to journal information)*	ISSN	Total Cites	Impact Factor	Immediacy Index	Articles	Cited Half-life
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2	CRYOBIOLOGY	0011-2240	2013	1.765	0.174	69	9.
3	INT J ANDROL	0105-6263	1367	2.308	0.211	71	7.9
4	THERIOGENOLOGY	0093-691X	8357	2.161	0.399	351	6.8
5	J ANDROL	0196-3635	2949	2.035	0.510	96	6.1