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Análisis, función y aplicaciones biotecnológicas de las proteínas del plasma seminal de porcino PSP-I y PSP-II

Analysis, function and biotechnological applications of boar seminal plasma proteins PSP-I and PSP-II

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

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1 Introducción

Introducción

En los últimos años se han producido grandes avances en el desarrollo de nuevas biotecnologías asociadas con la reproducción animal. En el caso concreto de la especie porcina, la aplicación de biotecnologías sobre células espermáticas resultaría extremadamente relevante debido a la repercusión que, tanto en términos económicos como de optimización del manejo de las explotaciones, podría tener en el sector productivo porcino.

Entre estas técnicas destacan la criopreservación espermática, la separación de espermatozoides X e Y mediante citometría de flujo para preseleccionar el sexo de la descendencia y la conservación de los espermatozoides en medios que aseguren su viabilidad a lago plazo. La aplicación de estos procesos biotecnológicos permite incrementar de forma considerable la eficiencia de los programas de mejora genética, además de permitir una optimización del rendimiento de las explotaciones porcinas. La preservación de razas en peligro de extinción, y la creación de bancos de dosis seminales que permitan la exportación del semen, así como la posibilidad de disponer de semen de individuos genéticamente valiosos, son otros de los beneficios que ofrece la aplicación de estas nuevas tecnologías. Sin embargo, hay que destacar que, a pesar de los avances producidos en el desarrollo de estas biotecnologías espermáticas, la aplicación práctica de alguna de ellas se ve limitada por la baja eficiencia que actualmente presentan, quedando su uso restringido al ámbito experimental.

Asimismo, la aplicación de estos procedimientos biotecnológicos sobre los espermatozoides implica una serie de manipulaciones que pueden generar alteraciones en las células provocando así un estado prematuro de capacitación espermática (Maxwell y Johnson, 1997). El proceso de capacitación, que conduce a la reacción acrosómica, genera una disminución en la vida fértil de los espermatozoides (Maxwell y cols., 1998). Por lo tanto, para garantizar el éxito en la fecundación es necesario intentar estabilizar los espermatozoides sometidos a tratamientos tecnológicos. Esta estabilización permitiría alargar la

vida fértil de los espermatozoides (Vázquez y cols., 2001), aumentando así la capacidad fecundante de los mismos.

Este estado prematuro de capacitación es debido, principalmente, al grado de dilución al que se ven sometidos los espermatozoides durante los diferentes tratamientos biotecnológicos que provoca la eliminación de proteínas adsorbidas y otros componentes presentes en el plasma seminal necesarios para mantener la viabilidad espermática. En este sentido, se ha observado que la adición de plasma seminal, en cantidades adecuadas, protege a los espermatozoides cuando estos son sometidos a procesos de criopreservación (Berger y Clegg, 1985; Ollero y cols., 1997), a altas diluciones (Maxwell y Johnson, 1999; Centurión y cols., 2003) o a procesos de separación espermática por citometría de flujo (Maxwell y Johnson, 1998; Caballero y cols., 2004) al permitir la estabilización de las membranas espermáticas.

El plasma seminal es el fluido en el cual se encuentran inmersos los espermatozoides tras la eyaculación. Está constituido principalmente por compuestos inorgánicos y orgánicos entre los que se encuentran carbohidratos, lípidos, aminoácidos y proteínas de bajo y alto peso molecular (Pursel y cols., 1973; Mann y Lutwak-Mann, 1981). El plasma seminal se caracteriza, no sólo por ser el vehículo en el que se encuentran los espermatozoides, sino que también les proporciona soporte metabólico además de actuar como fuente de energía desde su formación hasta su posterior transporte por el tracto genital de la hembra (Maxwell y cols., 2007). Diferentes estudios realizados en referencia al efecto que ejerce el plasma seminal sobre la funcionalidad y fertilidad de los espermatozoides ofrecen resultados contradictorios. Así, mientras algunos estudios han demostrado que la adición de plasma seminal al medio que rodea a los espermatozoides puede reducir la funcionalidad y fertilidad espermática (Dott y cols., 1979; Kawano y cols., 2003; Moore y cols., 2005; Akcay y cols., 2006), otros muchos muestran que la adición del plasma seminal en determinadas etapas de la manipulación espermática, estabiliza la célula evitando que se produzca el proceso de capacitación, mejorando así la viabilidad y capacidad fecundante de estas células (Ashworth y cols., 1994; Henault y Killian, 1996; Maxwell y Johnson, 1999; Barrios y cols., 2000; Garner y cols., 2001; Vadnais y cols., 2005; 2007). Estas diferencias podrían ser debidas a una variabilidad en la proporción entre factores capacitantes y decapacitantes presentes en el plasma seminal, o posiblemente, al efecto beneficioso o perjudicial de determinadas

proteínas del plasma seminal y a la variación que existe en la concentración de las mismas entre individuos de la misma especie e incluso entre eyaculados del mismo individuo (Caballero y cols., 2004; Strzezek y cols., 2005; Maxwell et al., 2007). En este sentido, diversos estudios demuestran que el plasma seminal contiene factores proteicos específicos que influyen en la funcionalidad y capacidad fertilizante del espermatozoide en varias especies de mamíferos domésticos (Barrios y cols., 2000; Killian y cols., 1993; Centurión y cols., 2003; Moura y cols., 2006; Fernández-Juan y cols., 2006), y que, además, parecen interaccionar con el entorno del tracto genital de la hembra durante el transporte de los espermatozoides hasta el lugar de fecundación (Maxwell y Johnson, 1999; Rozeboom y cols., 2000; Strezezek y cols., 2005).

En el caso de la especie porcina estudios previos han demostrado que el plasma seminal de verraco presenta un heterodímero proteico de bajo peso molecular compuesto de los monómeros PSP-I y PSP-II que, añadido a los medios de dilución cuando los espermatozoides están altamente diluidos genera, *in vitro,* un efecto beneficioso sobre la viabilidad, motilidad y actividad mitocondrial de los espermatozoides (Centurión y cols., 2003). Este heterodímero pertenece a la familia de las espermadhesinas, proteínas presentes en el plasma seminal, que representan aproximadamente el 75% del contenido proteico total del plasma seminal de verraco (Calvete y cols., 1995; Töpfer-Petersen y cols., 1998). Estas glicoproteínas están compuestas por 109-113 aminoácidos, presentan un 40-60% de identidad en la secuencia primaria y están constituidas estructuralmente por un único dominio CUB que ejerce, principalmente, de soporte estructural (Romero y cols., 1997). La especie porcina es la que presenta mayor diversidad de miembros de esta familia. En función de su capacidad de unión a la heparina, las espermadhesinas han sido clasificadas en proteínas que unen heparina (AQN-1, AQN-3, AWN) y proteínas que no unen heparina (heterodímero PSP-I/PSP-II).

Las espermadhesinas con capacidad de unión a la heparina, también llamadas HBPs (Heparin Binding Proteins), se caracterizan por ser moléculas con capacidad de unión a las glicoproteínas de la zona pelúcida del ovocito (Sanz y cols., 1992), además de estar involucradas en el reconocimiento espermatozoide-ovocito (Rodríguez-Martínez y cols., 1998; Jonakova y cols., 2000). También existen evidencias de que estas proteínas estabilizan *in vivo* la membrana plasmática sobre la región acrosomal del espermatozoide, y que

están principalmente relacionadas con el proceso de capacitación (Dostalova y cols., 1994; Calvete y cols., 1997).

Las otras dos espermadhesinas, PSP-I y PSP-II (Porcine Seminal Plasma Proteins: PSPs), se encuentran formando un heterodímero no covalente que constituye aproximadamente más del 50% de las proteínas totales (Calvete y cols., 1995; Calvete y cols., 1996). Dicho heterodímero muestra *in vitro* un efecto modulador de la actividad inmune del útero (Yang y cols., 1998), así como la capacidad de inducir *in vivo* migración de polimorfonucleares (PMN) en la luz uterina de la cerda (Assreury y cols., 2002, 2003; Rodríguez-Martínez et al., 2005).

Además de las funciones fisiológicas sobre la hembra, las HBPs y las PSPs ejercen efectos opuestos sobre los espermatozoides expuestos a altos grados de dilución, como es el caso de los espermatozoides separados por citometría de flujo (Centurión y cols., 2003). De este modo, cuando los espermatozoides altamente diluidos son incubados en presencia de las proteínas que unen heparina (HBP), se produce un descenso en la integridad de membrana y motilidad espermática. Sin embargo, cuando los espermatozoides se incuban solamente en presencia del heterodímero PSP-I/PSP-II, se produce un mantenimiento de la viabilidad, motilidad y actividad mitocondrial de los espermatozoides (Centurión y cols., 2003).
Aunque estos resultados muestran claramente el efecto beneficioso que ejerce el heterodímero PSP-I/PSP-II sobre los espermatozoides de verraco, se desconoce su mecanismo de acción. Así, el objetivo de la primera experiencia fue realizar un estudio exhaustivo de la acción de esta espermadhesina sobre la célula espermática. El trabajo fue diseñado para conocer si la actividad biológica protectora que presenta el heterodímero corresponde a la subunidad PSP-I o a la subunidad PSP-II así como si esta actividad está asociada a, la fracción peptídica o a la glicídica de los monómeros.

Es necesario conocer, además, dónde se producen la subunidad PSP-I y la PSP-II, es decir, qué estructuras dentro del tracto genital del verraco son las más relevantes en la síntesis de las mismas. En nuestra opinión, esto nos permitiría conocer en qué momento y en qué secuencia el espermatozoide entra en contacto con las proteínas PSP-I y PSP-II a lo largo del trayecto que realizan desde el testículo hasta la eyaculación, y quizás a obtener un mayor conocimiento de las funciones de estas proteínas. Para el desarrollo de una

segunda experiencia se partió del conocimiento previo de que la mayoría de las proteínas que se encuentran en el plasma seminal son producidas y secretadas por las vesículas seminales, por la próstata y por las glándulas bulbouretrales, aunque una importante fracción también es originada en el epidídimo y los testículos contribuyendo así en el volumen final del eyaculado (Dostalova y cols., 1994; Sinowatz y cols., 1995).

La expresión y localización de las diferentes espermadhesinas, a lo largo del tracto genital del verraco, han sido estudiadas por diversos grupos de investigación (Kwok y cols., 1993; Ekhlasi-Hundrieser y cols., 2002) utilizando diferentes procedimientos tales como técnicas inmunológicas (western-blotting), técnicas inmunohistoquímicas y técnicas de transcripción inversa de la polimerasa (RT-PCR). Sin embargo, estos estudios han proporcionado escasos resultados y las conclusiones sobre la localización y expresión de las proteínas PSP-I y PSP-II a nivel de algunas de las estructuras que conforman el tracto genital del verraco son contradictorias. Estas diferencias podrían ser debidas a las distintas condiciones experimentales utilizadas en cada uno de los estudios. Por lo tanto, la segunda experiencia tuvo como objetivo estudiar la localización y expresión de las espermadhesinas PSP-I y PSP-II y de sus ARN mensajeros (ARNm) en testículos, epidídimo (cabeza, cuerpo y cola) y en las principales glándulas sexuales (vesícula seminal y glándula bulbouretral) de verracos adultos de fertilidad probada.

Se han observado diferencias en el efecto que tiene el plasma seminal sobre los espermatozoides. Estas diferencias en cuanto al efecto beneficioso o perjudicial del plasma seminal parecen estar relacionadas con las variaciones existentes en la composición y concentración de ciertas proteínas del plasma seminal entre machos de diferentes especies, individuos de una misma especie, eyaculados de un mismo macho e, incluso, entre las distintas fracciones de un mismo eyaculado (Killian y cols., 1993; Maxwell y Johnson, 1998; Zhu y cols., 2000; Dacheaux y cols., 2003; Caballero y cols., 2004).

En la especie porcina el eyaculado está dividido en tres fracciones, que presentan una gran heterogeneidad en cuanto a la composición y distribución de los componentes del plasma seminal. Estas tres fracciones se caracterizan por presentar en la primera fracción (fracción pre-espermática), escaso volumen, carente de espermatozoides y con baja presencia de proteínas; una segunda

fracción (fracción rica) de alta concentración espermática y baja concentración proteica, y una tercera (fracción post-espermática) de gran volumen y alta concentración de proteínas donde el número de espermatozoides presentes va disminuyendo hasta prácticamente desaparecer (Ghaoui y cols.., 2004; Rodríguez-Martínez y cols., 2005).

Recientemente se han realizado experiencias cuyos resultados indican la existencia de diferencias en el efecto que producen distintas fracciones del plasma seminal sobre la motilidad y viabilidad de los espermatozoides (Peña y cols., 2006; Kereskoski y cols., 2006; Akcay y cols., 2006), lo que podría estar relacionado con diferencias existentes en el perfil proteico de dichas fracciones.

La búsqueda de una relación entre componentes del plasma seminal fragmentado en función del tiempo de eyaculación y la capacidad estabilizadora y conservadora del mismo sobre los espermatozoides, es de gran interés ya que permite buscar factores que ayuden a incrementar los rendimientos de los espermatozoides procesados, así como desarrollar estrategias que aconsejen desechar determinadas fracciones del plasma seminal. En la tercera experiencia se plantearon como objetivos estudiar en primer lugar, cómo afecta el perfil proteico de cada una de las fracciones del plasma seminal a las características funcionales de espermatozoides sometidos a altas diluciones y, en segundo lugar, evaluar la presencia de la espermadhesina PSP-I/PSP-II a lo largo de las fracciones, debido al efecto protector que ejerce el heterodímero PSP-I/PSP-II sobre espermatozoides altamente diluidos.

Sin lugar a dudas, es necesario un mayor conocimiento de la forma en que estas proteínas beneficiosas del plasma seminal ejercen su acción, de igual modo que lo es conocer su lugar de expresión y, por supuesto, su localización dentro de las diferentes fracciones en las que podemos dividir un eyaculado. No obstante, uno de los aspectos de mayor importancia sea quizás el estudio de la aplicabilidad de estas proteínas, mediante la inclusión de las mismas en los medios que envuelven a los espermatozoides en los diferentes procesos biotecnológicos a los que pueden ser sometidos, como la separación espermática.

La posibilidad de determinar y seleccionar el sexo de la progenie es uno de los objetivos más perseguidos dentro del campo de la biotecnología de la

reproducción. El hecho de saber con antelación el sexo del futuro individuo resulta de gran importancia no sólo en la especie humana, sino también en el resto de mamíferos. En el campo de la reproducción animal la aplicación de esta técnica de predeterminación del sexo sería de gran interés tanto en los programas de recuperación de especies en peligro de extinción, como en programas de cría de animales en cautividad. Sin embargo, es en el campo de la producción animal donde la posibilidad de elegir el sexo de la progenie tendría una mayor repercusión. Una optimización tanto de los programas de mejora genética como de los sistemas productivos o un aumento en la eficiencia biológica de las explotaciones ganaderas son algunas de las ventajas que supondría poder obtener únicamente animales del sexo deseado (Johnson y cols., 2005). En la actualidad el único método exacto, eficaz, fiable y rentable para predeterminar el sexo de la descendencia en las diferentes especies de mamíferos domésticos es el de separación espermática mediante citometría de flujo (Johnson, 2000; Maxwell y cols., 2004). Esta técnica se basa en la separación de los espermatozoides en función de la diferencia que existe en el contenido de ADN entre los espermatozoides portadores del cromosoma X y los portadores del cromosoma Y. Sin embargo, y a pesar de que el proceso de separación espermática mediante esta técnica se encuentra en constante avance tanto en lo que respecta a su aspecto metodológico como al aplicativo, y que la eficiencia de esta tecnología ha aumentado considerablemente durante los últimos años, su uso en la especie porcina presenta ciertas limitaciones. Estas limitaciones son debidas principalmente a que el número de espermatozoides separados por unidad de tiempo es, en la actualidad, de unos 15 millones por hora, lo cual, si bien puede ser útil en otras especies, resulta insuficiente en el ganado porcino si se tiene en cuenta que son necesarios 3000 millones de espermatozoides para realizar una inseminación artificial tradicional (Maxwell y cols., 2004; Johnson y cols., 2005).

Además, las diferentes etapas a las que se someten los espermatozoides durante el proceso de separación por citometría de flujo (la tinción con el fluorocromo Hoechst 33342, la exposición a altas presiones y al impacto del láser UV, la proyección sobre el tubo de recogida), y la dilución extrema de estas células afectan directamente a las membranas espermáticas (Maxwell y Johnson, 1999), provocando una disminución de la viabilidad, capacidad de conservación y capacidad fecundante de los espermatozoides separados. Las altas diluciones facilitan la eliminación de componentes del plasma seminal, principalmente

proteínas, que son beneficiosas para la los espermatozoides y cuya ausencia favorece la aparición de los estadios de precapacitación (Maxwell y cols., 1997; Maxwell y Johnson, 1999; Maxwell y cols., 2007).

El desarrollo de medios de dilución y/o recogida y de protocolos de manipulación post-separación que permitan prolongar la vida útil de los espermatozoides separados y, por tanto, su capacidad fecundante, es una de las principales estrategias para incrementar la eficiencia de la técnica de separación espermática por citometría de flujo.

La adición de plasma seminal al medio de recogida es una práctica habitual en diferentes especies de interés productivo, incluida la especie porcina, ya que mejora la viabilidad de los espermatozoides separados por citometría de flujo. De esta manera se consigue amortiguar los efectos del proceso de separación, ya que se estabilizan las membranas plasmáticas de los espermatozoides y se revierte el proceso de precapacitación que experimentan las células espermáticas justo después de la separación (Maxwell y cols., 1997; Maxwell y Johnson, 1997; Maxwell y Johnson, 1999; Parrilla y cols., 2004). Sin embargo, debido a la variabilidad existente en el plasma seminal entre eyaculados de distintos machos e incluso entre eyaculados del mismo macho, es aconsejable realizar una selección previa de los verracos donantes. Teniendo en cuenta los efectos beneficiosos que el heterodímero PSP-I/PSP-II tiene sobre los espermatozoides, su adición a los medios de recogida podría ser una alternativa eficaz al uso de plasma seminal ya que permite evitar esa variabilidad producida por el plasma seminal.

Tras la separación espermática es necesario concentrar los espermatozoides, siendo la centrifugación el método más utilizado. Sin embargo, debido a que esta técnica puede produce efectos nocivos sobre la funcionalidad espermática (Álvarez y cols., 1993) nos planteamos la opción de utilizar técnicas alternativas de concentración espermática como la sedimentación utilizada en la especie humana con el fin de concentrar espermatozoides en muestras de baja calidad ((Henkel y cols., 2003).

Así, el objetivo de la última experiencia fue evaluar el efecto de la presencia del heterodímero sobre las características funcionales además de su

capacidad fecundante *in vivo,* de espermatozoides de verraco separados por citometría de flujo y sometidos a diferentes protocolos post-separación.

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2 objetivos

objetivos

Los objetivos de la presente Tesis doctoral son:

1.- Determinar si el la actividad biológica que presenta el heterodímero PSP-I/PSP-II de preservar la viabilidad, motilidad y actividad mitocondrial en espermatozoides de verrraco altamente diluidos, reside en la subunidad PSP-I o por el contrario, en la subunidad PSP-II y más específicamente, en su fracción peptídica o glicídica.

2.- Revelar la localización y expresión de las espermadhesinas PSP-I y PSP-II y de sus ARN mensajeros a lo largo del tracto genital del verraco, concretamente en testículo, epidídimo (cabeza, cuerpo y cola) y en las principales glándulas sexuales (vesículas seminales y glándula bulbouretral) utilizando para ello machos adultos de fertilidad probada mediante el uso de inmunohistoquímica, western-blott y RT-PCR.

3.- Estudiar el efecto de las fracciones del plasma seminal (pre-espermática, rica y post-espermática) sobre las características funcionales de los espermatozoides altamente diluidos, así como la presencia del heterodímero PSP-I/PSP-II en dichas fracciones.

4.- Evaluar la funcionalidad, así como la fertilidad in vivo, de espermatozoides de verraco separados por citometría de flujo tras el proceso de concentración espermática mediante sedimentación y centrifugación en presencia de plasma seminal o del heterodímero PSP-I/PSP-II.

3 Experiencia 1

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Dissecting the Protective Effect of the Seminal Plasma Spermadhesin PSP-I/PSP-II on Boar Sperm Functionality

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ABSTRACT: To dissect the protective activity of PSP-I/PSP-II, the effect of the isolated subunits PSP-I and PSP-II and their affinity-purified tryptic peptide and glycan fractions on the viability, mitochondrial activity, and motility of highly diluted boar spermatozoa was investigated. High dilution exerted a negative effect on control spermatozoa. Incubation of spermatozoa with PSP-I/PSP-II or with its PSP-II subunit had a protective effect on sperm functionality, high mitochondrial membrane potential, and sperm motility. These effects were less pronounced when spermatozoa were incubated with the PSP-I subunit. It was noteworthy that motility was abolished by incubation of spermatozoa with isolated PSP-I. Trypsin-degraded PSP-I/PSP-II, PSP-I, and PSP-II reproduced the effects of the native proteins. Incubating spermatozoa with the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II for 5 hours preserved a higher percentage of viable spermatozoa than when sperm was incubated for the same time with the native heterodimer, trypsin-digested PSP-I/PSP-II, the glycan fraction or without added proteins. However, sperm motility decreased as the concentration of added peptide fraction increased. On the other hand, spermatozoa incubated with the glycan fraction showed lower values than spermatozoa incubated with the peptide fraction. We concluded that the subunits of the PSP-I/PSP-II heterodimeric spermadhesin exert different activities on sperm functions. The finding that the beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require the glycan moiety points to a peptide moiety as a potential sperm function-preserving additive of highly diluted boar spermatozoa.

Key words: Boar seminal plasma, spermadhesin PSP-I/PSP-II, protective effect on sperm function, sperm survival, highly diluted spermatozoa.

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The seminal plasma, consisting of secretions from the testes, epididymis, and the accessory sex glands, contains a variety of factors (amino acids, lipids, fatty acids, osmolytes, peptides, and proteins) that influence the viability and fertilizing capacity of ejaculated spermatozoa (Mann and Lutwak-Mann, 1981; Shivaji et al, 1990; Yanagimachi, 1994). Thus, the seminal plasmas of a variety of mammalian species contain both factors that prevent inappropriate acrosome reactions and proteins that upon binding to the sperm surface enhance the fertilizing potential of spermatozoa (Killian et al, 1993; Thérien et al, 1997; Rodríguez-Martínez et al, 2005). The concerted action of these regulatory seminal plasma factors modulates the capacitation state of spermatozoa. However, the precise role of most of the seminal plasma proteins on sperm physiology remains obscure. In addition, the effect of seminal plasma on spermatozoa is variable among species, males of the same species, and ejaculates from a single male. The distinct effects exerted by different seminal plasmas on sperm functionality have been in part ascribed to variability of the composition and concentration of some proteins (Maxwell and Johnson, 1999; Centurión et al, 2003).

Spermadhesins are male secretory proteins detected so far in ungulates (pig, cattle, and horse) (Haase et al, 2005). In the pig, this family of proteins consists of 5 members—AQN-1, AQN-3, AWN, PSP-I, and PSP-II (Töpfer-Petersen et al, 1998)—and together they represent over 90% of the total boar seminal plasma proteins (Dostàlovà et al, 1994). The porcine spermadhesin genes are clustered on SCC 14q28–q29 (Haase et al, 2005). Porcine spermadhesins, 110- to 133-residue polypeptides built by a single CUB domain architecture (Romero et al, 1997), are synthesized by the epididymis and accessory glands (Ekhlasi-Hundrieser et al, 2002) and exhibit distinct sperm-coating and ligand-binding capabilities. Sequence variation, glycosylation, and their aggregation state of spermadhesins contribute to their specific pattern of biological activities (Calvete et al,

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1993a,b; Dostàlovà et al, 1995a). AQN-1, AQN-3, and AWN coat the sperm surface at ejaculation and display zona pellucida glycoprotein-binding capability and affinity for β-galactosides and heparin (Sanz et al, 1993; Dostàlovà et al, 1995b; Calvete et al, 1996a,b; Rodríguez-Martínez et al, 1998). These spermadhesins are thought to stabilize the plasma membrane over the acrosomal vesicle and are mainly released from the spermatozoal surface during capacitation (Sanz et al, 1993; Dostàlovà et al, 1994; Calvete et al, 1997). Moreover, AQN-3 has been reported to form part of a sperm motility-inhibitor factor complex (Iwamoto et al, 1995), and AQN-1 and AWN are sperm-associated acrosin-inhibitor acceptor proteins (Sanz et al, 1992). On the other hand, PSP-I and PSP-II are the most abundant boar seminal plasma proteins and form a non–heparin-binding heterodimer (Calvete et al, 1995) of glycosylated spermadhesins (Nimtz et al, 1999). Accumulating evidence points to a role for the PSP-I/PSP-II spermadhesin complex as an exogenous modulator of the uterine immune activity (Leshin et al, 1998; Yang et al, 1998), thus ensuring reproductive success. Hence, PSP-I and PSP-II are immunostimulatory for lymphocyte activity in vitro (Nimtz et al, 1999), and binding of PSP-I to lymphocyte has been demonstrated (Yang et al, 1998). Furthermore, the PSP-I/PSP-II heterodimer and its isolated subunits induce the recruitment of neutrophils into the peritoneal cavity of rats (Assreuy et al, 2002) and pigs (Rodríguez-Martínez et al, 2005). The neutrophil migration-inducing activity of PSP-I/PSP-II, and possibly of the PSP-II subunit, is mediated by the stimulation of resident macrophages, which release a neutrophil chemotactic substance (Assreuy et al, 2002), whereas PSP-I appears to act directly on neutrophils (Assreuy et al, 2003). The purpose of these proinflammatory and immunostimulatory activities would be to prevent possible infections of the lower reproductive tract and to provide a foreign cell-free uterine environment for the descending early embryos.

Besides their physiological functions in porcine reproduction, the heparin-binding and the non–heparin-binding spermadhesins exert opposite effects on spermatozoa that have been subjected to high dilution to mimic the conditions of sex sorting by flow cytometry (Centurión et al, 2003). Thus, whereas the pooled heparin-binding spermadhesins (AQN-1, AQN-3, and AWN) cause a concentration-dependent sperm membrane damage, the purified non–heparin-binding PSP-I/PSP-II spermadhesin complex contributes to maintaining sperm with high viability, motility, and mitochondrial activity for at least 5 hours at physiological temperature (Centurión et al, 2003). The beneficial effect of the PSP-I/PSP-II heterodimer points to this spermadhesin as a candidate for an additive to improve the viability of highly diluted porcine spermatozoa (ie, flow cytometric sorting for chromosomal sex). In the present study, we sought to dissect the structural basis of the protective effect of boar spermadhesin PSP-I/PSP-II, its isolated PSP-I and PSP-II subunits, and their derived peptidic and glycan fractions on the sperm functionality by a time-course evaluation of the viability, the acrosomal status, the mitochondrial activity, and the motility of highly diluted boar spermatozoa.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich Co (Alcobendas, Madrid, Spain) unless otherwise stated.

Preparation of Seminal Plasma

All experiments were performed with the seminal plasma from mature boars that had previously sired offspring. Ejaculates were collected by the gloved-hand method (Larsen, 1986). The seminal plasma was separated from spermatozoa by centrifugation at 3800 × *g* for 15 minutes at room temperature with a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatants were filtered sequentially through 10- and 1.2-µm filters and pooled.

Isolation of the Boar Seminal Plasma PSP-II PSPII Heterodimer

The PSP-I/PSP-II heterodimer was isolated from the non–heparin-binding fraction of boar seminal plasma by affinity chromatography on a heparin-Sepharose column, equilibrated in 100 mM Tris-HCl; 150 mM NaCl; 5 mM EDTA; and 0.025% sodium azide, pH 7.4, as previously described (Calvete et al, 1995). The identity and purity of the protein was assessed by N-terminal sequence analysis with an Applied Biosystems 472 automated protein sequencer (Applied Biosystems, Langen, Germany) and by MALDI-TOF mass spectrometry with an Applied Biosystems Voyager DE-Pro mass spectrometer (Applied Biosystems) and a saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) as the matrix. Protein concentration was determined spectrophotometrically with the molar absorption coefficient (27 332 M^{-1} cm^{-1}) determined by Menéndez et al (1995) or by amino acid analysis (after sample hydrolysis in 6 M chloride acid (HCl) for 24 hours at 106°C in evacuated and sealed ampoules) with a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water and lyophilized.

Isolation of Boar Seminal Plasma PSP-I and PSP-II Subunits

The PSP-I and PSP-II subunits were purified from the heterodimer by reverse-phase high-performance liquid chromatography (HPLC) on a Lichrocart column (250 × 10 mm, RP-18, 7-µm particle size) (Merck, Germany) eluted at 2 mL/min with a mixture of 0.1% TFA in water (solution A) and

0.1% TFA in acetonitrile (solution B), first isocratically (10% B) for 5 minutes, followed by 30% B for 10 minutes, 45% B for 45 minutes, 70% B for 15 minutes, and 10% B for 15 minutes. The purified PSP-I and PSP-II subunits were dialysed against distilled water and lyophilized. Purity and protein concentration were determined as above.

Isolation of Peptides and Glycopeptides

To separate the peptidic and the glycan moieties, the 100 mg of the PSP-I/PSP-II heterodimer were digested overnight at 37°C with trypsin by using a 1:100 (wt/wt) enzyme:substrate ratio. Thereafter, the enzyme was inactivated by heating at 100°C for 2 minutes, and the reaction mixture was lyophilized. Completion of proteolysis was checked by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, reversed-phase HPLC, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Nonglycosylated tryptic peptides were separated from glycopeptides by affinity chromatography on a 5-mL Sepharose-Concanavalin A column (Amersham Biosciences, Uppsala, Sweden) equilibrated and eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (phosphate-buffered saline [PBS]). ConA-bound glycopeptides were eluted with equilibration buffer containing 100 mM methyl-α-D-mannopyranoside. The glycopeptide fraction was desalted on a C-18 Lichrosphere column (250 × 4 mm, 5-μm particle size) (Merck, Germany), equilibrated, and washed with 0.1% TFA until the absorbance at 214 reached baseline level, followed by elution with 0.1% TFA and 50% acetonitrile. Peptide concentration was determined by amino acid analysis.

Collection of Semen and Evaluation of Sperm Parameters

Sperm-rich fractions from fertile mature boars were collected by gloved-hand method and extended to 30×10^6 sperm/mL in Beltsville Thawing Solution (Pursel and Johnson, 1975). Diluted sperm-rich fractions from 3 boars were pooled and spermatozoa were separated from seminal plasma by centrifugation at 1200 × *g* (Megafuge 1.0 R, Heraeus, Germany) during 3 minutes. To avoid sperm membrane damage caused by pipetting spermatozoa directly into dilution medium (Maxwell and Johnson, 1999), the pellet was serially diluted in PBS to a final cell count of 1×10^6 sperm/mL and incubated at 38°C (Steri-Cult 200 incubator, Marietta, Ohio). Samples were taken at 0.5, 2, and 5 hours and analyzed for viability (membrane integrity), acrosomal status, mitochondrial membrane potential, and motility.

Flow Cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc, Miami, Fla) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, Calif) performing 15 mW at 488 nm, and the EXPO 2000 software (Coulter Corporation). Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Nonsperm events (debris) were gated out based on the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to sperm cells but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10 000 events per sample. Samples were measured at flow rate of ≈300 cells/s.

Flow Cytometric Assessment of Sperm Viability and Acrosomal Exocytosis

For an accurate estimation of the spermatozoa, the membrane and the acrosome integrity was assessed simultaneously by flow cytometry using the triple staining protocol described by Nagy et al (2003). Briefly, 500 μL of sperm samples (~500 000 cells) were incubated with 50 nM SYBR-14 (using a 100-μM stock solution in DMSO; component A of LIVED/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands), 0.5 μg/mL PE-PNA (peanut agglutinin conjugated with phycoerythrin; Biomeda Corp, Foster City, Calif), and 7.5 μM propidium iodide (PI). The samples were mixed and incubated at 37°C in dark for 10 minutes before flow cytometric analysis. SYBR-14 was measured with a 525-nm band pass filter, PI was collected with a 620-nm band pass filter, and PE-PNA was detected with a 575-band pass filter. Viable spermatozoa with intact acrosome were defined as those stained only with SYBR-14. Acrosome-reacted spermatozoa were defined as those stained with SYBR-14 and PE-PNA. Spermatozoa stained with PI were classified as dead cells.

Flow Cytometric Assessment of Sperm Mitochondrial Membrane Potential

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzymidazolyl carbocianyne iodide; Molecular Probes Europe), a stain that differentiates cells exhibiting high and low mitochondrial membrane potential (Peña et al, 2003), was used to assess the mitochondrial membrane potential of spermatozoa as described in Martínez-Pastor et al (2004) with slight modifications. A total of 500 μL of sperm samples were incubated in a water bath at 37°C in dark for 20 minutes with 0.2 μm JC-1, followed by flow cytometric measurement through a 590-nm band pass filter. At low membrane potential JC-1 exists as a green fluorescent monomer, whereas at higher potentials JC-1 forms "J-aggregates" after accumulation in mitochondria that emit a red-orange florescence at 590 nm (Garner and Thomas, 1999; Gravance et al, 2000).

Sperm Motility

Sperm motility was estimated by a computer-assisted sperm motility analysis system with the software Sperm Class Analyzer (Microptic 2002, Barcelona, Spain). Aliquots of 10 μL of semen samples (at 10^6 spermatozoa/mL) were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot positive-phase contrast light microscope (Tokyo, Japan) equipped with a 10× objective and a monochrome video camera (Hitachi CCD model, Chiba, Japan) connected to a personal computer. The program settings were as in Centurión et al (2003). Objective percentage of motile spermatozoa was analyzed in at least 100 spermatozoa per sample.

Effect of Isolated PSP-I and PSP-II and Their Proteolytic Products on Sperm Functions

Diluted spermatozoa (1 × 10^6 sperm/mL PBS) were incubated at 38°C for 5 hours with 0.75 mg/mL of either 1) native PSP-I, 2) native PSP-II, 3) trypsin-digested PSP-I, 4) trypsin-digested PSP-II, or 5) 1.5 mg/mL of trypsin-digested PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Controls were sperm incubated with 1.5 mg/mL of heterodimer PSP-I/PSP-II or without added proteins.

Effect of the Peptidic and Glycan Fractions of PSP-II PSP-II on Sperm Functions

Diluted spermatozoa (1 × 10^6 sperm/mL PBS) were incubated at 38°C for 5 hours with either 1) 1.05 mg/mL of tryptic peptide fraction depleted from glycopeptides by affinity chromatography on ConA-Sepharose (see above) or 2) 0.5 mg/mL of ConA-retained glycopeptides. These amounts of peptides and glycopeptides are equivalent to those released by proteolytic digestion of 1.5 mg/mL of the PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Samples incubated with intact or trypsin-digested PSP-I/PSP-II heterodimer as well as without proteins were used as control.

Dose-Dependent Effect of the Peptidic Fraction of PSP-II PSP-II on Sperm Functions

Samples of 1 × 10^6 sperm/mL in PBS were incubated at 38°C for 5 hours with decreasing concentrations of the peptidic fraction of trypsinized PSP-I/PSP-II depleted from glycopeptides by ConA-Sepharose chromatography. The following final concentrations were used: 1.05, 0.52, 0.23, and 0.10 mg/mL, which correspond to 1.5, 0.75, 0.33, and 0.15 mg/mL of the native PSP-I/PSP-II heterodimer. Viability, acrosomal exocytosis, mitochondrial membrane potential, and motility were assessed at 0.5, 2, and 5 hours of incubation. Sperm incubated with 1.5 mg/mL of native heterodimer or without added proteins were used as controls.

For each experiment, the effects of the same batch of lyophilized proteins and their derived proteolytic products were assessed on 4 pools of spermatozoa collected on 4 different days. Each pool was made by mixing spermatozoa from 4 different boars. The same group of 4 boars was used on each day. Duplicate samples were performed for each treatment.

Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc, Chicago, Ill). Data were analyzed by analysis of variance (ANOVA) by using the Hierarchical Linear Mix Model (MIXED) procedure according to a statistical model including the fixed effect of treatment and of incubation time and the random effect of replicate. To analyze data of sperm viability, motility, mitochondrial activity, and acrosome status, percentages were subjected to arcsine transformation before analysis. When ANOVA revealed a significant effect, values were compared by using the Bonferroni test and were considered to be significant when $P < .05$. Experiments were replicated 4 times.

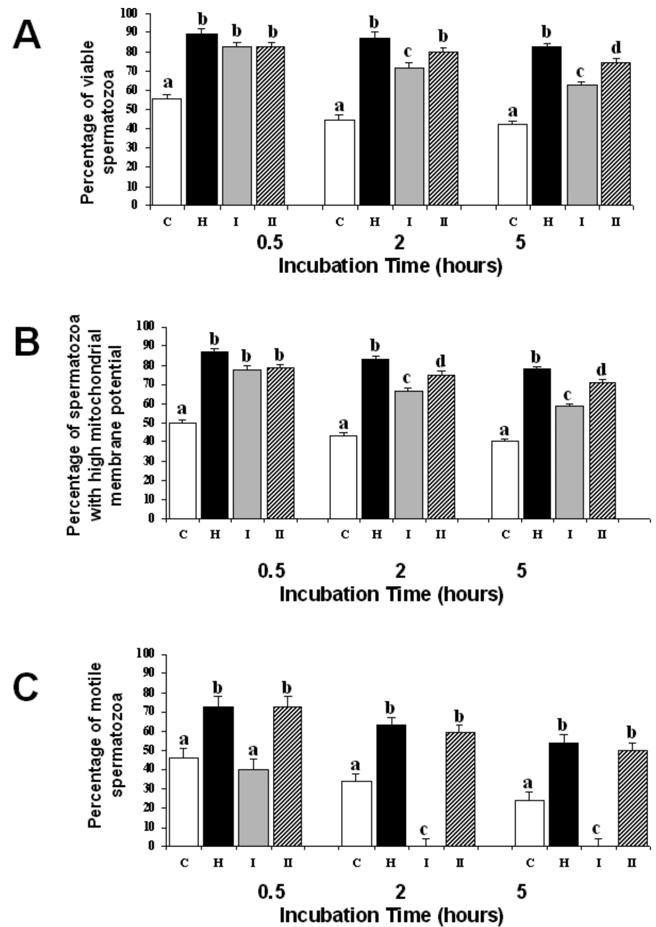

Figure 1. Effect of the PSP-I and the PSP-II subunits on the viability (sperm with intact acrosome) **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted boar spermatozoa. Spermatozoa were diluted to 1 × 10^6 sperm/mL phosphate-buffered saline and incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of native PSP-I/PSP-II (H), 0.75 mg/mL of isolated PSP-I subunit (I), and 0.75 mg/mL of purified PSP-II subunit (II). Control was incubated in the absence of added protein (c). Viability and acrosomal status were assessed by triple staining (PI/SYBR-14/PE), the mitochondrial membrane potential was determined by using JC-1, and the percentages of motile spermatozoa were evaluated by a computer-assisted motility analysis system. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

Results

Effect of the Isolated PSP-I and PSP-II Subunits on Highly Diluted Spermatozoa

Figure 1A shows the percentage of viable spermatozoa that have been exposed to the PSP-I/PSP-II heterodimer

Table 1. *Percentage (mean ± SEM) of viable acrosome-reacted spermatozoa in samples of highly diluted boar spermatozoa (1 × 10^6 sperm/mL) as a function of the incubation time at 38°C and of the presence or absence (control) of native PSP-I/PSP-II and its isolated PSP-I and PSP-II subunits**

Incubation Time, h	Protein				
	Control	Heterodimer	PSP-I	PSP-II	SEM
0.5	1.96[a]	0.16a	1.06a	4.61b	0.65
2	2.2[a]	0.15a	1.76a	4.75b	0.74
5	1.36[ab]	0.16b	3.48ac	5.68c	0.71

* Different superscript letters in the same file indicate significantly different values ($P < .05$).

(H) and its isolated subunits, PSP-I (I) and PSP-II (II). In line with previous reports (Centurión et al, 2003, and references therein), in the absence of added proteins, a significant decrease in the viability of the highly diluted control sample was already shown at 0.5 hours compared with spermatozoa exposed to PSP-I/PSP-II heterodimer and its subunits. The dilution-caused decrease in sperm viability of control sperm continued with the incubation time (reaching 38.2% at 5 hours), whereas the native PSP-I/PSP-II heterodimer and its isolated PSP-I and PSP-II subunits exerted a protective effect on sperm viability (78.3%, 71.6%, and 57.7%, respectively). However, after 2 hours, lower values ($P < .05$) of viability were recorded in samples incubated with PSP-I compared with those containing PSP-I/PSP-II or PSP-II (Figure 1A).

The same trend was observed regarding the percentages of spermatozoa with high mitochondrial membrane potential (Figure 1B). After 5 hours of incubation, the native PSP-I/PSP-II heterodimer, PSP-I, and PSP-II all preserved the mitochondrial activity in 75.5%, 68.1%, and 53.8% of the spermatozoa, though the PSP-I subunit showed a lower value ($P < .05$). For comparison, only 40.1% of spermatozoa retained this activity in the absence of added proteins.

Regardless of incubation time, the percentage of motile spermatozoa was higher ($P < .05$) in spermatozoa that were exposed to the native heterodimer (72.8% at 0.5 hours) or to the PSP-II subunit (75.5% at 0.5 hours) than that of control sperm (46.7% at 0.5 hours) and of spermatozoa incubated with the PSP-I subunit (39.6% at 0.5 hours) (Figure 1C). Moreover, after 2 hours of incubation, PSP-I had a marked detrimental effect on sperm motility, which dropped to undetectable values. This effect was not attributed to a membrane damage because the percentages of acrosome-reacted highly diluted spermatozoa at 0.5, 2, and 5 hours of incubation with the heterodimer or with PSP-I were not significantly different from that of control sperm (Table 1). On the other hand, the exposition of spermatozoa to PSP-II resulted in higher values ($P < .05$) of viable spermatozoa showing a reacted acrosome (Table 1).

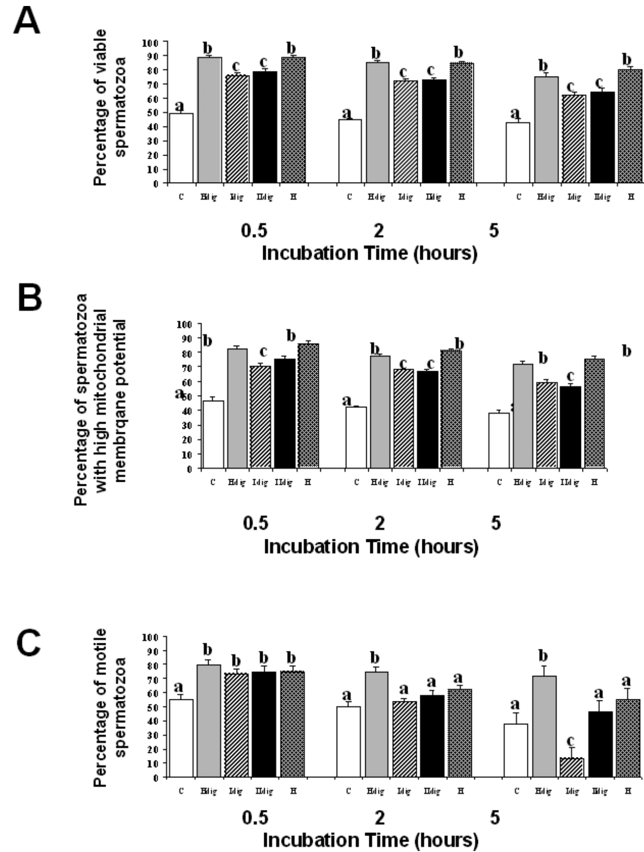

Figure 2. Effect of trypsin-digested PSP-I (Idig) and PSP-II (IIdig) subunits on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted boar spermatozoa. Spermatozoa were diluted to 1 × 10^6 sperm/mL in phosphate-buffered saline and incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of intact PSP-I/PSP-II (H), 1.5 mg/mL of trypsin-digested heterodimer (Hdig), 0.75 mg/mL of trypsin-digested PSP-I subunit (Idig), and 0.75 mg/mL of trypsin-digested PSP-II subunit (IIdig). Control samples were incubated in the absence of added protein (c). Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

Effect of Trypsin-Digested PSP-I/PSP-II, PSP-I, and PSP-II on Highly Diluted Spermatozoa

Figure 2A shows the time-course effect of incubating highly diluted boar spermatozoa with trypsin-degradation mixtures of PSP-I/PSP-II (Hdig), PSP-I (Idig), and PSP-II (IIdig). The percentages of viable spermatozoa were significantly higher ($P < .05$) in sperm exposed to trypsin-digested heterodimer than to either of its trypsin-digested subunits or the control. It is worth nothing that there were no major differences when these results were compared with those obtained with the

Table 2. *Percentage (mean ± SEM) of viable acrosome-reacted spermatozoa in samples of highly diluted boar spermatozoa (1 × 10^6 sperm/ml) as a function of incubation time at 38°C and of the presence or absence of trypsin-digestion mixtures of PSP-I/PSP-II (Hdig), PSP-I (Idig), and PSP-II (IIdig) subunits; sperm samples incubated without added proteins and with native PSP-I/PSP-II represent controls**

Incubation Time, h	Protein					
	Control	PSP-I/PSP-II	Hdig	Idig	IIdig	SEM
0.5	4.43^a	0.15b	1.06b	1.8b	5.8a	0.82
2	1.88^a	0.21a	0.98a	0.76a	4.8b	0.56
5	0.98^a	0.18a	0.73a	0.46a	5.1b	0.42

* Different superscript letters in the same file indicate significantly different values ($P < .05$).

native proteins, and the same was observed upon analysis of the percentage of spermatozoa displaying high mitochondrial membrane potential (Figure 2B). However, after 2 hours of incubation, the percentage of motile spermatozoa remained higher ($P < .05$) in the samples incubated with trypsin-digested PSP-I/PSP-II heterodimer (72.1%) than in any other condition (Figure 2C). Moreover, trypsin-digested PSP-I subunit exerted a marked negative effect on sperm motility, thus mimicking the effect observed with the native PSP-I subunit (Figure 1C). Similar to what had been noticed when intact proteins were tested, there were no differences ($P > .05$) in the percentages of acrosome-reacted spermatozoa between controls and samples incubated in the presence of intact or trypsin-digested PSP-I/PSP-II and PSP-I subunit (Table 2). However, viable spermatozoa exposed to digested PSP-II subunit showed higher values ($P < .05$) of acrosome-reacted spermatozoa (Table 2).

Effect of Glycopeptide and Peptide Fractions on Highly Diluted Boar Spermatozoa

The presence in the incubation medium of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), the glycopeptide fraction of PSP-I/PSP-II (Glyc), and the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II improved ($P < .05$) the sperm viability compared with the control at all times tested (Figure 3A). However, the percentages of viable spermatozoa were significantly different among samples. Thus, spermatozoa exposed to Hdig or Pept exhibited the highest values ($P < .05$) of viable spermatozoa (72.4% and 82.2% at 5 hours, respectively), which were not different from the percentage of spermatozoa incubated with the intact heterodimer (76.6%). By contrast, spermatozoa incubated with the glycopeptide fraction showed lower values during the 5 hours of incubation (51.3% at 5 hours), though they were significantly higher ($P < .05$) than in the control by about 12%–15%. The same trend was observed in the percentages of spermatozoa showing high mitochondrial membrane potential (Figure 3B).

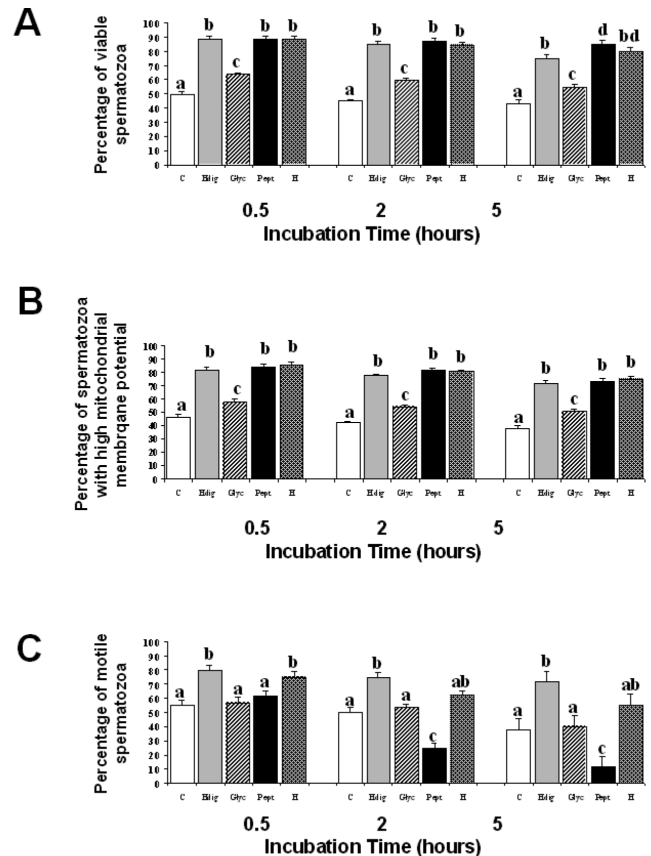

Figure 3. Effect of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), the affinity-purified peptide (Pept), and the glycopeptide (Glyc) fractions on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted (1 × 10^6 sperm/mL) boar spermatozoa. Spermatozoa were incubated for 0.5, 2, and 5 hours at 38°C with 1.5 mg/mL of trypsin-digested PSP-I/PSP-II heterodimer (Hdig), 0.5 mg/mL of glycopeptides (Glyc), and 1.05 mg/mL of tryptic peptides (Pept). Controls were sperm incubated without added proteins (c) and spermatozoa incubated with 1.5 mg/mL of native PSP-I/PSP-II heterodimer (H). Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

At all incubation times, the percentage of motile spermatozoa was significantly higher in samples exposed to either native or trypsin-digested PSP-I/PSP-II heterodimer than in any other condition tested, including the control (Figure 3C). The time-dependent effect of incubating spermatozoa without added proteins (c) and in the presence of the PSP-I/PSP-II glycopeptide fraction (Glyc) were indistinguishable (Figure 3C). On the other hand, the percentage of motile spermatozoa after incubation in the presence of 1.05 mg/mL of the

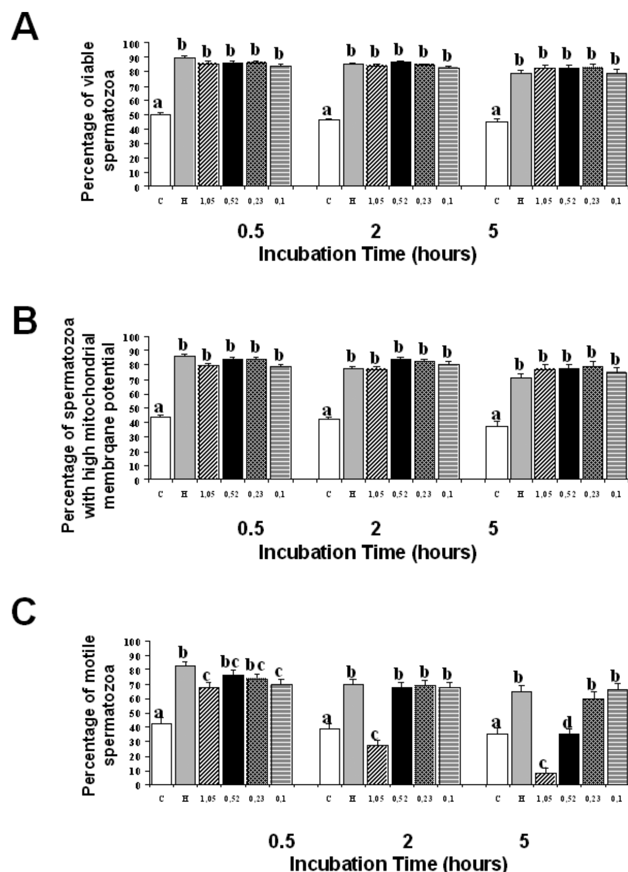

Figure 4. Effect of decreasing concentrations of the affinity-purified (glycopeptide-depleted) tryptic peptide mixture of the PSP-I/PSP-II heterodimer on the viability **(A)**, mitochondrial membrane potential **(B)**, and motility **(C)** of highly diluted (1×10^6 sperm/mL) boar spermatozoa. Spermatozoa were incubated for 0.5, 2, and 5 hours at 38°C with 1.05, 0.52, 0.23, and 0.10 mg/mL of the peptide mixture. Samples incubated with 1.5 mg/mL of PSP-I/PSP-II heterodimer (H) and without added proteins (c) acted as controls. Viability and acrosomal status, mitochondrial membrane potential, and the percentage of motile spermatozoa were evaluated as in Figure 1. Columns represent the mean ± SEM (error bars) of 4 samples per treatment evaluated in duplicate. Bars per time not sharing a letter are significantly different ($P < .05$).

glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II (Pept) markedly decreased with the incubation time from 68.7% at 0.5 hours to 29.4% at 2 hours and 4.1% at 5 hours (Figure 3C).

Dose-Dependent Effect of the Peptidic Fraction of PSP-I/PSP-II on Highly Diluted Boar Spermatozoa

Decreasing the concentration of glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II in the incubation medium of diluted spermatozoa from 1.05 to 0.1 mg/mL did not change the percentage of viable spermatozoa, which were always significantly higher (>35%–40%) than in the controls (Figure 4A). The effect produced by different concentrations of peptide backbone on the percentage of spermatozoa with high mitochondrial membrane potential showed the same trend (Figure 4B). However, increasing concentrations of the peptidic fraction had an incubation time-dependent detrimental effect on the motility of the highly diluted spermatozoa (Figure 4C). Thus, sperm motility decreased from 65.8% to 8.5% as the concentration of added peptide fraction increased from 0.1 to 1.05 mg/mL. However, the percentages of acrosome-reacted spermatozoa at 0.5, 2, and 5 hours of incubation remained below 3% in any condition, and no significant differences between groups were detected (data not shown).

Discussion

Biotechnological manipulations of semen, such as cooling and deep freezing, in vitro fertilization, or for sexing by flow cytometry or cell-sorting procedures, requires extending the viability of diluted spermatozoa. Sperm extension is linked with the wash away, or high dilution of seminal plasma components (Maxwell and Johnson, 1999), despite the fact that the seminal plasma contributes to preserving the integrity and the fertilizing potential of sperm and also provides metabolic support to spermatozoa. A detrimental effect on spermatozoa highlighted by an increase of the percentage of cell death, changes in the metabolic activity, and a decrease in their fertilizing activity has been extensively documented (reviewed in Maxwell and Johnson, 1999). Although the composition of diluents has been improved by more-or-less empirical studies, the formulation of species-specific extenders to overcome the detrimental effects of sperm dilution remains a subject of current discussion in reproduction technology (Levis, 2000). Restoring whole seminal plasma has been proposed for improving sperm functionality after dilution (Garner et al, 2001; Caballero et al, 2004). However, the large, inherent variability of seminal plasmas from homologous males as well as between ejaculates from the same male advises against its use (Killian et al, 1993; Zhu et al, 2000; Garner et al, 2001; Caballero et al, 2004). On the other hand, the use of isolated seminal plasma proteins for sperm extension has the advantage of avoiding the inherent variability of the whole seminal plasma. In a previous work, we have shown that different members of the boar spermadhesin family, the purified glycoprotein PSP-I/PSP-II spermadhesin and the pooled heparin-binding spermadhesins exert antagonistic effects on the functionality of highly diluted boar spermatozoa (Centurión et al, 2003). The finding that PSP-I/PSP-II contributes to maintaining sperm with high viability, motility, and mitochondrial activity

for at least 5 hours at physiological temperature points to its potential use as an additive for sperm preservation (Centurión et al, 2003) and prompted us to investigate the nature of the sperm protecting epitopes.

First, we studied the effects of the isolated PSP-I and PSP-II subunits on sperm functions. The PSP-II subunit mimicked the effect of the native heterodimer on maintaining sperm viable, motile, and with a high mitochondrial membrane potential (Figure 1). The effect of the PSP-I subunit was beneficial, though less pronounced than that of PSP-II on sperm viability and mitochondrial potential. However, it almost completely abolished sperm motility after 2 hours of incubation (Figure 1). Whether PSP-I might be affecting the glycolytic pathway of the spermatozoa is as yet unknown. This detrimental effect of PSP-I follows a similar time course and magnitude to the previously noticed sperm-immobilizing activity exerted by the pooled heparin-binding spermadhesins (Centurión et al, 2003). PSP-I is a glycosylated spermadhesin, and glycosylation indirectly modulates its ligand-binding properties. Thus, mannose-rich PSP-I glycoforms have been reported in the heparin-binding fraction of spermadhesins (Calvete et al, 1993b), whereas complex-type PSP-I glycoforms specifically associate with PSP-II into a noncovalent heterodimer (Calvete et al, 1995; Nimtz et al, 1999). The fact that the PSP-I glycoprotein isolated from PSP-I/PSP-II abolished the motility of highly diluted spermatozoa whereas the native heterodimer did not strongly suggested that this activity might be suppressed upon dimerization. Other binding activities impaired by complex formation are the heparin-binding capability and the mannose-6-phosphate recognition by PSP-II (Solis et al, 1998). Taken together, these results suggest a mechanism for the modulation of the ligand-binding properties of PSP-I and PSP-II.

To further dissect the effects of the PSP-I/PSP-II subunits on sperm functions, the native heterodimer and the isolated subunit were degraded with trypsin and the resulting peptide mixtures were checked for their activities on sperm parameters. The protective effect of the PSP-II on viability, mitochondrial membrane potential, and motility of highly diluted spermatozoa were not abolished by trypsin digestion, indicating that the conformation of the active epitope or epitopes was not destroyed by proteolysis. Similar results were observed when the PSP-I/PSP-II digestion mixture was analyzed. Moreover, the biological activity of the heterodimer was amplified after trypsin digestion compared with the native protein. In contrast, the negative effect of PSP-I on sperm motility was clearly attenuated. Hence, we may conclude that degradation of the native PSP-I/PSP-II exposes active epitopes, which remained hidden in the quaternary structure of the heterodimer or in the tertiary structure of its constituent subunits.

To evaluate the activity of the peptide and the glycan moieties of PSP-I/PSP-II, the tryptic peptide mixture of the heterodimer was fractionated by affinity chromatography on ConA-Sepharose. The nonbound (nonglycosylated peptides) and the bound (glycopeptides) fractions were used at 1.05 mg/mL and 0.5 mg/mL, respectively, which correspond to their calculated concentrations for a 1.5 mg/mL solution of native PSP-I/PSP-II heterodimer. This PSP-I/PSP-II concentration represents the optimal protective dose determined in previously reported experiments (Centurión et al, 2003). The data displayed in Figure 3 show that the peptide fraction retained the biological activity of PSP-I/PSP-II on the viability and mitochondrial activity of highly diluted boar spermatozoa, whereas the glycopeptide fraction exhibited a light positive effect, which was not different from the control. In addition, the glycopeptide fraction had no effect on sperm motility, whereas the peptide fraction abolished in a time-dependent manner the motility of viable spermatozoa (Figure 3C). This net detrimental activity may represent a composite of actions of the motility-impairing activities of native and trypsinized PSP-I and the positive effect of native and trypsin-degraded PSP-II (Figures 1C and 2C).

The deleterious effect of the tryptic peptide fraction of the PSP-I/PSP-II heterodimer followed a marked concentration-dependent pattern. Thus, decreasing the total peptide concentration from 1.05 to 0.1 mg/mL restored sperm motility to the same level observed with the intact heterodimer, and this effect lasted for at least 5 hours.

In conclusion, the subunits of the PSP-I/PSP-II heterodimeric disintegrin exert different activities on sperm functions. The beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require its glycan moiety.

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ORIGINAL ARTICLE

Localization and expression of spermadhesin PSP-I/PSP-II subunits in the reproductive organs of the boar

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Summary

The epithelial localization and expression of the spermadhesin PSP-I and PSP-II subunits were determined in the testis, ductus epididymes (caput, corpus and cauda), seminal vesicles and bulbourethral glands of mature boars, using immunohistochemical, western blotting and RT-PCR methods. Immunohistochemistry showed positive labelling for PSP-I and PSP-II antibodies in the epithelium of seminal vesicles in all males tested. Positive immunolabelling, but with variable intensity, was also present in the epididymal epithelium (caput, corpus and cauda), although varying largely among segments and boars. Immunoreactivity was nearly or completely absent in the seminiferous epithelium and the bulbourethral gland, although SDS-PAGE and western blotting revealed the presence of PSP-I and PSP-II immunoreactive bands in all the tissue extracts, including the testis and the bulbourethral gland. mRNA amplification by RT-PCR using primers specific for PSP-I and PSP-II showed a trend similar to that observed for western blotting, i.e. intensity variation between tissues (even between segments of the same epididymis) and among boars. Our results indicate that the seminal vesicles are the main source of PSP-I and PSP-II spermadhesins, although epididymal segments, testis and the bulbourethral gland also participate in the expression of both proteins.

Introduction

The seminal plasma (SP), the fluid surrounding mammalian spermatozoa at ejaculation, comprises a variety of organic and inorganic components (amino acids, lipids, fatty acids, peptides and proteins secreted by the testis, epididymis and the accessory sexual glands, that influence the functionality of spermatozoa, both in vivo and in vitro (Mann & Lutwak-Mann, 1981; Shivaji *et al.*, 1990; Yanagimachi, 1994; Matas *et al.*, 1996; Caballero *et al.*, 2005). In particular, SP proteins play an important role in the control of molecular mechanisms underlying sperm transport in the female reproductive tract, suppression of the immune response against sperm antigens, gamete interaction following egg fertilization and preventing premature acrosome exocytosis (Strzezek *et al.*, 2005). The loss of these proteins may contribute to the development of a physiological unstable state for the spermatozoa (Roldan & Vázquez, 1996).

The major protein components of the boar SP are the spermadhesins (Calvete *et al.*, 1995; Töpfer-Petersen *et al.*, 1998). These (glyco)proteins represent over 90% of the total boar SP proteins (Dostàlovà *et al.*, 1994) and are built by a single CUB domain (Romero *et al.*, 1997). Depending on their binding capability, spermadhesins can be conveniently classified into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding (PSP-I/PSP-II heterodimer; Calvete *et al.*, 1994). The heparin-binding spermadhesins (HBS) bind zona pellucida glycoconjugates (Sanz *et al.*, 1992) and are involved in sperm-egg recognition (Rodríguez-Martínez *et al.*, 1999), and, in addition, AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins and may act as decapacitation factors preventing premature acrosome reactions (Sanz *et al.*,

1992). Their binding properties appear to be modulated by the aggregation state and glycosylation status of spermadhesins (Töpfer-Petersen & Calvete, 1995; Rodríguez-Martínez *et al.*, 2005; Campanero-Rhodes *et al.*, 2006). On the other hand, the non-heparin-binding PSP-I/PSP-II non-covalent heterodimer represents more than 50% of the total boar SP proteins (Calvete *et al.*, 1995, 1996a,b; Nimtz *et al.*, 1999) and display pro-inflammatory and immunostimulatory activities in the uterus of the rat (Assreury *et al.*, 2002, 2003, Assreury *et al.*, 2003) and pig (Rodríguez-Martínez *et al.*, 2005). Moreover, the heterodimer preserves in vitro the membrane integrity, motility and mitochondrial activity of highly extended boar spermatozoa, and these effects are mainly associated with the polypeptide moiety of the PSP-II subunit (Centurión *et al.*, 2003; García *et al.*, 2006).

Both PSP-I and PSP-II proteins have been reported to be synthesized by the epithelium of the seminal vesicles, the caudal epididymis and the prostate of pigs (Ekhlasi-Hundrieser *et al.*, 2002). The PSP-I, in addition, has been found in caput epididymis and in the rete testis (Ekhlasi-Hundrieser *et al.*, 2002). In contrast, Kwok Simon *et al.* (1993) showed immunolabelling for PSP-I only in the epithelium of the seminal vesicles of the pig. These differences may be related to the different techniques used as well as the age, breed or fertility of the boars used. In the present study, we sought to disclose the epithelial localization and expression of spermadhesins PSP-I and PSP-II and their mRNAs in the testis, epididymis and main accessory sexual glands of mature, fertile boars by immunohistochemistry, SDS-PAGE and western blot and RT-PCR.

Materials and methods

Animals

Testes, epididymis (caput, corpus and cauda), seminal vesicles and bulbourethral glands from 10 fertile mature boars of proven fertility aged between 2 and 3 years were collected immediately post-mortem and used for immunohistochemistry, western blotting and RT-PCR.

The use of the animals in the present study was approved by the corresponding Committee for Experimentation with Animals of Swedish University of Agricultural Sciences (Uppsala, Sweden) and the University of Murcia (Spain).

All reagents were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

Experimental procedures

Isolation of boar SP spermadhesin PSP-I/PSPII heterodimer

The PSP-I/PSP-II heterodimer was isolated from the non-heparin binding fraction of boar SP by affinity chromatography on a heparin-Sepharose column, equilibrated in 100 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA and 0.025% sodium azide, pH 7.4, as previously described (Calvete *et al.*, 1995). The identity and purity of the protein was assessed by N-terminal sequence analysis using an Applied Biosystems 472 automated protein sequencer (Langen, Germany) and MALDI-TOF mass spectrometry using and Applied Biosystems Voyager DE-Pro mass spectrometer. A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetric acid was employed as the matrix. Protein concentration was spectrophotometrically determined using the molar absorption coefficient (27332 M^{-1} cm^{-1}) determined by Menéndez *et al.* (1995) or by amino acid analysis (after sample hydrolysis in 6 mol/L HCl for 24 h at 106 °C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialysed against distilled water and lyophilized.

Isolation of boar SP PSP-I and PSP-II subunits

The PSP-I and PSP-II subunits were purified from the heterodimer by reverse-phase HPLC on a Lichrocart column (250 × 10 mm, RP-18, 7 μm particle size) (Merck, Darmstadt, Germany) eluted at 2 mL/min with a mixture of 0.1% trifluoroacetic acid in water (solution A) and 0.1% trifluoroacetic (TFA) in acetonitrile (solution B), first isocratically (10% B) for 5 min, followed by 30% B 10 min, 45% B for 45 min, 70% B for 15 min and 10% B for 15 min. The purified PSP-I and PSP-II subunits were dialysed against distilled water and lyophilized. Purity and protein concentration were determined as above.

Preparation of PSP-I and PSP-II antibodies

Polyclonal anti-PSP-I anti-PSP-II monospecific antibodies were obtained by immunizing female rabbits with subcutaneous injections of 0.5 mg of purified PSP-I and PSP-II subunits in 0.5 mL of PBS emulsified with 1.5 mL of Freund's complete adjuvant. The animals were inoculated twice at intervals of 5 weeks after the first injection with 0.25 mg of the antigen. Two weeks after the last administration, the rabbits were bled through the ear vein and the blood sera were tested for anti PSP-I and PSP-II activity by dot-blot ELISA and western blot.

Inmunohistochemistry

The tissues were fixed in 1% paraformaldehyde solution in PBS, dehydrated in a graded series of ethanol and embedded in paraffin. Immunolocalization of PSP-I and PSP-II proteins in male boar tissues was made using an Elite ABC-staining procedure (Vectastain Elite ABC Kit; Vector Laboratories; Burlingame, CA, USA). Briefly, 5 μm tissue sections were prepared from the paraffin-embedded mater-

ial using a Leitz microtome and mounted on poly-L-lysine-coated glass slides (Sigma). Thereafter, the tissue sections were deparaffinized, rinsed in PBS for 5 min and incubated with 3% of H_2O_2 in PBS for 20 min, in order to block endogenous peroxidase activity. Nonspecific protein binding was prevented by incubation in 10% normal goat serum for 30 min at 20 °C. After this step, and without rising, samples were incubated overnight at 4 °C with the primary polyclonal antibodies PSP-I or PSP-II (1 : 1000 v/v in PBS). The sections were then rinsed in PBS (5 min) before incubation with biotinylated goat anti-rabbit IgG antibody (1 : 1000) for 30 min. All sections were rinsed again in PBS for 5 min prior to application of the ABC-Elite complex. Visualization of antibody–antigen complexes was done using diaminobenzidine tetrahydrochloride with 3% H_2O_2 (DAKO® DAB Chromogen tablets; Mannheim, Germany) for 5–6 min. Sections were mounted with glycerine-gelatin. Negative controls were run by omission of the primary antibody. As positive controls, samples of freshly ejaculated boar spermatozoa were subjected to the same protocol as above with polyclonal antibody against PSP-II. Because of the reported weak association of PSP-I/PSP-II with the sperm surface (Calvete *et al.*, 1995), the fresh ejaculated spermatozoa were not subject to any treatment, including washing or removal of SP. A liver tissue extract was used as a negative (background) control. All incubations were performed in a humidified chamber. Selected sections were photographed with a Nikon microphot-FXA photomicroscope (Nikon, Tokyo, Japan).

RNA extraction

Samples were quick-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. RNA was isolated from the following tissues: testis, seminal vesicles, bulbourethral gland and epididymis (caput, corpus and caudal epididymis) of 10 boars using TRIzol Reagent (Invitrogen, Paisley, Scotland) according to the Chomczynski & Sacchi (1987) protocol. To this end, 50 mg of each tissue was homogenized in 1 mL TRIzol Reagent (mixture of acid phenol and guanidine isothiocyanate) using polypropylene mini-pestles (Sigma). The homogenates were stored for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. For total RNA extractions from the homogenates, 0.2 mL chloroform were added followed by centrifugation at 4 °C, and the aqueous phase was recovered. RNA was precipitated from the aqueous phase by addition of isopropanol, incubation for 15 min at −20 °C, followed by centrifugation at 4 °C. Pellets were successively washed once with 1 mL of ethanol 75% and once with 1 mL absolute ethanol, air dried and solubilized in 20 μL DEPC-treated water.

DNAase treatment (Ambion, Houston, TX, USA) of the total RNA fraction was carried out before first-strand cDNA synthesis. Total RNA was quantified spectrophotometrically at OD_{260nm} and the purity was assessed by the OD_{260}/OD_{280} ratio. All samples had OD_{260}/OD_{280} ratios higher than 1.8. RNA integrity was checked by electrophoresis of 1 μg of sample on a 2% agarose gel containing formamide (RNA sample loading buffer). Gels were stained with ethidium bromide.

Reverse transcription

Reverse transcriptions and PCRs were performed with a termocycler (Personal Master Cycler; Eppendorf, Forster City, CA, USA) with hot-lid. Reverse transcription of RNA into cDNA was carried out in a total volume of 20 μL. Briefly, 5 μg of DNAase-treated RNA was mixed with 1 μL of oligo $(dT)_{20}$ (50 μmol/L), heated for 5 min at 65 °C and then placed on ice. Thereafter, 4 μL of 5× RT buffer, SuperScript™ III Reverse Transcriptase (200 U/μL), dNTPs 25 μmol/L, 1 μl of DTT (0.1 mol/L) and 1 μL of Rnase out (40 U/μL) were added and incubated for 55 min at 50 °C. The reaction mixtures were then heated for 15 min at 70 °C to deactive the enzyme, and then cooled at 4 °C.

PCR amplification of PSP-I and PSP-II

PSP-I and PSP-II primers were those designed by Ekhlasi-Hundrieser *et al.* (2002), according to cDNA sequences obtained by Kwok Simon *et al.* (1993):

PSP-I → forward: 5′-TTCAACAGGATGGGGCTTGG-3′

Reverse: 5′-GAAGGAAAATGATCTCATAGGG-3′

PSP-II → forward: 5′-GCACGGATCAATGGCCCTG-3′

Reverse: 5′-TTCGGATCCTGGTGAACTAC-3′

The master mix contained 0.4 μL of 4 dNTPs (dTTP, dATP, dGTP and dCTP each at 2 mmol/L), 2.5 μL of primers (10 pmol/μL stock solution), 5 μL of reverse-transcribed cDNA, 5 μL of 10× PCR buffer (100 mmol/L Tris-HCl, 15 mmol/L MgCl2, 500 mmol/L KCl, pH 8.3 at 20 °C) and 0.5 μL of 5 U/μL Taq DNA polymerase. Five microlitres of cDNA was added to the PCR reaction mixture to yield a total volume of 50 μL. The mixture was denatured at 95 °C for 5 min and amplified by 35 cycles of 30 sec at 94 °C (denaturing), 30 sec at 58 °C (annealing) and 30 sec at 72 °C (elongation). Following 5 min at 72 °C, the reaction mixture was cooled at 4 °C. Reactions products were visualized on 1% ethidium bromide-stained agarose gels.

β-Actin gene (233 bp) was used as a positive control to confirm the presence of cDNA, and negative controls were set up using RNA instead of cDNA to role out genomic DNA contamination. The β-actin primers were: forward, 5′-GAGAAGCTCTGCTACGTCGC-3′; reverse: 5′-CCAGACAGCACCGTGTTGGC-3′ (Ponsuksili *et al.*,

2001). PCR-amplified band intensities were normalized to β-actin mRNA content and scored from 0 (negative) to +++ (highest expression).

Western blotting

Boar tissues in PBS buffer containing a cocktail of protease inhibitors (Complete Mina EDTA-free; Roche, Mannheim, Germany) were homogenized on ice using a Polytron (Polytron Kinematica AG, Litlan-Luzern, Switzerland). The homogenates were centrifuged at 3939 ***g*** for 10 min at 4 °C in a Heraeus Labofugue 400R centrifuge. Protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, MI, USA) according to the manufacturer's instructions.

Protein extracts from homogenated samples (5 μg) were mixed with SDS sample buffer, boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel using 25 mmol/L Tris/0.2 mol/L glycine buffer, pH 8.3, 0.1% SDS for 1.5 h at 150 V at room temperature. After SDS-PAGE, proteins were electrotransferred at 100 V for 1 h at 4 °C to 0.45 μm-pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon™-P membrane; Millipore, Billerica, MA, USA) using 25 mmol/L Tris/192 mmol/L glycine in 20% (v/v) methanol as transfer buffer. Membranes were then blocked overnight at 4 °C with Tris-buffered saline (50 mmol/L Tris buffer, pH 7.0, 150 mmol/L NaCl) containing 1% Tween 20 (TBS-T), and 1% bovine serum albumin. Membranes were then incubated with anti-PSP-I or anti-PSP-II rabbit antiserum (1 : 2000) for 45 min. After washing three times in TBST, each membrane was incubated for 45 min at room temperature with the secondary antibody (anti-rabbit-IgG conjugated to horseradish peroxidase A-257 (1 : 20 000). Detection of immunoreactive signals was accomplished using the ECL plus detection system (Amersham Biosciences, Uppsala, Sweden) following the manufacturer's instructions. Five micrograms of purified PSP-I and PSP-II proteins was used as positive control. Liver tissue protein extract was used as negative control. Anti-PSP-I and anti-PSP-II reactive bands were scored according to their relative intensity as +++ (highest, strong), ++ (moderate) and + (lowest, weak). The negative (background) control was scored '0'.

Results

Immunocytochemical localization of PSP-I and PSP-II in sections of porcine testis, epididymis, seminal vesicle and bulbourethral gland

Both polyclonal antibodies generated against boar PSP-I/PSP-II spermadhesin subunits showed clear positive immunolabelling in the epithelium of the seminal vesicles of all males examined ($n = 10$) (Figs 1 and 2). Immunolabelling for PSP-I was also detected in the epithelium of the ductus epididymis (caput, corpus and caudal; Fig. 1, a4–a6), although a high variability among segments and boars was noticed. PSP-I immunolabelling was nearly or totally absent from the seminiferous epithelium (Fig. 1, a1) and from the epithelium of the bulbourethral glands (Fig. 1, a3) of most boars (85%). Immunolabelling for PSP-II was detected in epididymal segments (caput, corpus and cauda; Fig 2, b1–b6), although the signal was less intense than for PSP-I. Spermatozoa from freshly ejaculated boar spermatozoa, without any washing, were used as positive control (Fig. 2).

SDS/PAGE and western blot detection of PSP-I and PSP-II proteins in extracts of porcine testis, epididymis, seminal vesicle and bulbourethral gland

Extracts from tissue samples used for immunohistochemistry were assayed to confirm the presence of the PSP-I and/or PSP-II proteins by western blot. Immunoreactive bands were evidenced in all tissue extracts (Fig. 3). The highest intensity for each PSP subunit

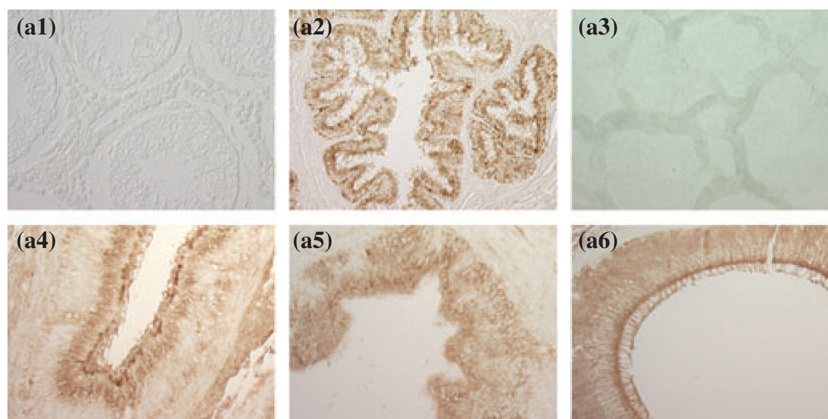
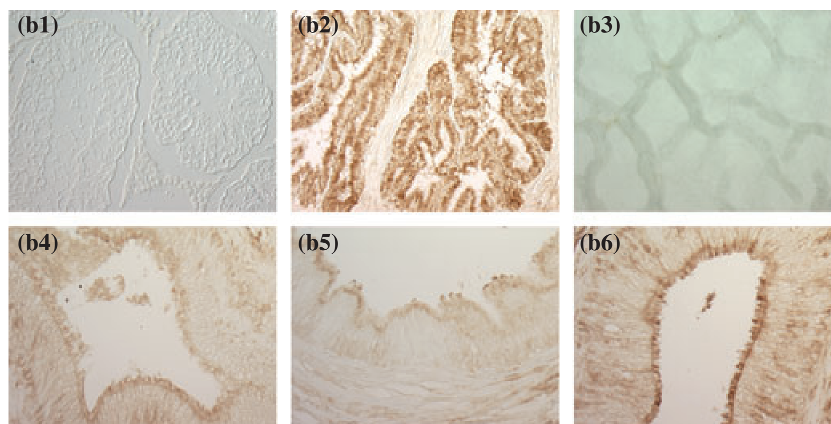


Figure 1 Immunocytochemical localization of PSP-I protein in sections of testis, epididymal segments (caput, corpus and caudal), seminal vesicles and bulbourethral gland from fertile, mature boars. The spermadhesin was detected using a rabbit anti-PSP-I monospecific polyclonal antiserum. Sections were stained using a Vectastain Elite ABC Kit for rabbit IgG. Photographs (100×) show immunostaining (brown colour) for PSP-I at: a1 (testis), a2 (seminal vesicles), a3 (bulbourethral gland), a4 (caput), a5 (corpus) and a6 (cauda).



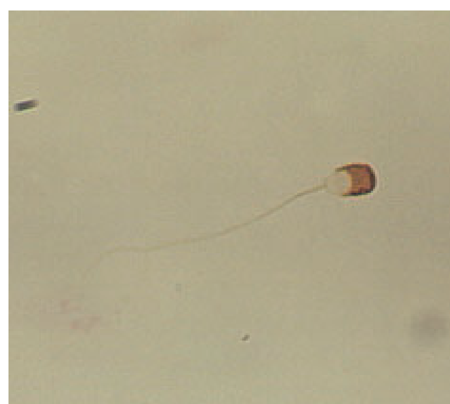


Figure 2 Immunocytochemical localization of PSP-II protein in sections of testis, epididymal segments (caput, corpus and caudal), seminal vesicles and bulbourethral gland from fertile, mature boars. The spermadhesin was detected using a rabbit anti-PSP-II monospecific polyclonal antiserum. Sections were stained using a Vectastain Elite ABC Kit for rabbit IgG. Photographs (100×) show immunostaining (brown colour) for PSP-II at: b1 (testis), b2 (seminal vesicles), b3 (bulbourethral gland), b4 (caput), b5 (corpus) and b6 (cauda). Ejaculated spermatozoa were used as positive control.

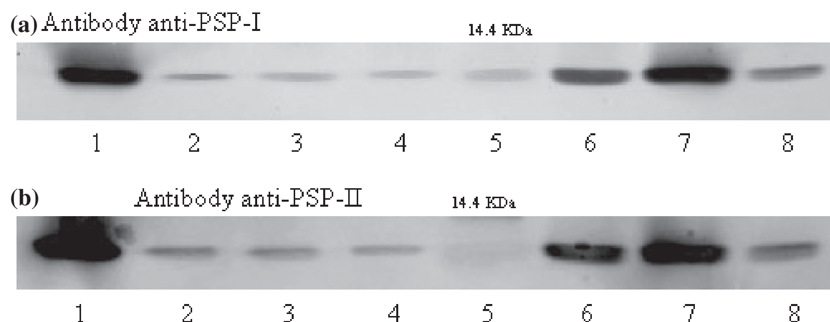


Figure 3 Western blot analysis for PSP-I and PSP-II spermadhesins in the male reproductive organs of the boar. Briefly, 50 mg from testis (lane 8), seminal vesicles (lane 7), bulbourethral gland (lane 6) epididymis (caput (lane 4), corpus (lane 3) and caudal (lane 2) were used for protein extraction. Extracted proteins were boiled in SDS sample buffer for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel. Proteins were transferred to a 0.45 μm PVDF membrane. Spermadhesins were detected using monospecific polyclonal anti-PSP-I (a) and anti-PSP-II (b) antibodies. In lane 1, purified PSP-I and PSP-II proteins used as positive controls. Liver tissue was used as a negative control (data not shown). Lane 5 corresponds to the molecular mass marker lysozyme (14.4 kDa).

corresponded to the seminal vesicles (lane 7). PSP-I and PSP-II were also detected in the ductus epididymes segments, including caput (lane 4), corpus (lane 3) and cauda (lane 2), bulbourethral gland (lane 6) and testis (lane 8), although exhibiting variable band intensities. Intensity variability was observed between tissues and also between the same tissue from different boars. Five micrograms of PSP-I or PSP-II were used as positive control (lane 1). No protein band was detected in the liver extract (not shown).

RT-PCR amplification of porcine PSP-I and PSP-II mRNA transcripts

Using RT-PCR, PSP-I and PSP-II cDNAs were amplified at high levels from seminal vesicle mRNA (Fig. 4, lanes 3: PSP-I and lane 9: PSP-II). Epididymal segments (caput, corpus and caudal; lanes 5–7 for PSP-I and lanes 11–13 for PSP-II), testis (Fig.4, lane 2 for PSP-I and lane 8 for PSP-II) and bulbourethral gland (Fig. 4, lane 4 for PSP-I and lane 10 for PSP-II) epithelia also showed specific PSP-I and PSP-II cDNA bands. In each case, bands were less intense than in the seminal vesicles. Different band intensities were found between tissues and also between the same tissue type of different males, supporting the results obtained by western blot.

The β-actin message was used as positive control (Fig. 4b). No contamination of genomic DNA in any of the samples tested could be detected, and all negative controls failed to yield amplification products.

Table 1 summarizes the semiquantitative results obtained for the expression of spermadhesins PSP-I and PSP-II in the 10 individual boars investigated, as determined by immunohistochemistry, RT-PCR and western blot. The relative intensities of the signals were scored as 0 (negative), + (weak), ++ (moderate) and +++ (strong).

Discussion

Seminal plasma contains a variety of factors that influence the viability and fertilizing capacity of ejaculated sperma-

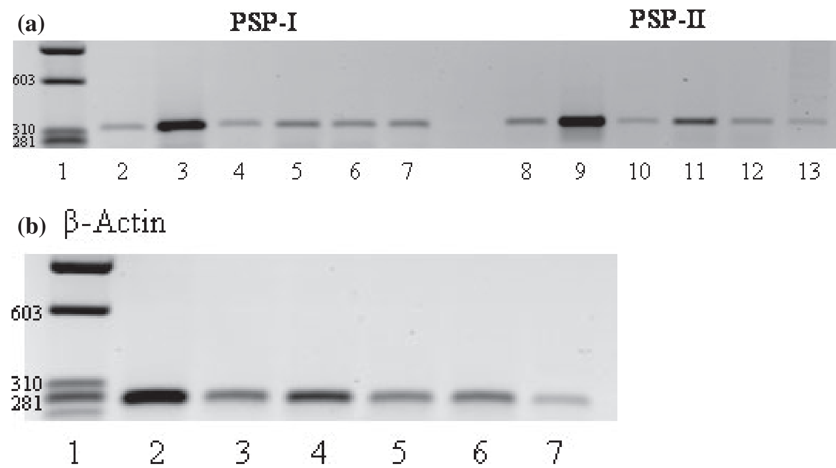


Figure 4 Gel electrophoretic analysis of the RT-PCR product for PSP-I and PSP-II in porcine reproductive organs. Total RNA was isolated from 50 mg of seminal vesicles (lanes 3 and 9 for PSP-I and PSP-II respectively), epididymis (caput, corpus and caudal: lanes 5–7 for PSP-I and lanes 11–13 for PSP-II), testis (lanes 2 and 8 for PSP-I and PSP-II respectively) and bulbourethral gland (lanes 4 and 10 for PSP-I and PSP-II respectively) tissues. Following RT-PCR with primers specific for PSP-I, PSP-II (a) and β-actin (as positive control; b) amplification products were separated in a 1% agarose gel. RNA, instead of cDNA, was used as negative control to rule out genomic DNA contamination. DNA size markers (in bp) are shown on the left-hand side.

Table 1 Semiquantitative expression of PSP-I and PSP-II spermadhesins assessed by immunohistochemistry, RT-PCR and western blot of testis, epididymis (caput, corpus, caudal), seminal vesicles and bulbourethral gland tissues of 10 fertile mature boars. Intensity was graded as: negative (0), weak (+), moderate (++) and strong (+++)

	Immunochemistry		PCR amplification		Western blotting	
Tissue	PSP-I	PSP-II	PSP-I	PSP-II	PSP-I	PSP-II
Seminal vesicles	+++ (10)	+++ (10)	+++ (10)	+++ (10)	+++ (10)	+++ (10)
Caput of epididymis	++ (6) + (4)	++ (3) + (7)	++ (10)	++ (2) + (8)	++ (5) + (5)	++ (5) + (5)
Corpus of epididymis	++ (7) + (3)	++ (4) + (6)	++ (8) + (2)	++ (4) + (6)	++ (3) + (7)	++ (3) + (7)
Caudal of epididymis	+++ (2) ++ (7) + (1)	++ (5) + (5)	+++ (1) ++ (7) + (2)	++ (2) + (6)	++ (7) + (3)	++ (4) + (6)
Testis	0 (10)	0 (10)	++ (4) + (6)	++ (4) + (6)	++ (4) + (6)	++ (4) + (6)
Bulbourethral gland	0 (10)	0 (10)	++ (2) + (8)	++ (2) + (8)	++ (4) + (6)	++ (4) + (6)

tozoa. The effects exerted by SP on sperm functionality have been, in part, ascribed to the variability in the composition and concentration of certain species-specific or widely distributed proteins (Maxwell & Johnson, 1999; Centurión *et al.*, 2003; Dacheux *et al.*, 2003). The non-heparin-binding PSP-I/PSP-II heterodimeric spermadhesin complex, the most abundant boar SP protein (Calvete *et al.*, 1995), binds to the acrosomal head region of ejaculated spermatozoa (Caballero *et al.*, 2006) and preserves sperm membrane integrity, motility and mitochondrial activity in vitro (Centurión *et al.*, 2003). The expression of the PSP-I/PSP-II subunits along the porcine reproductive tract tissues is unclear and was thus the aim of this work, using immuno(histo)chemical detection and PCR amplification of PSP-I and PSP-II spermadhesins in testis (seminiferous epithelium), epididymis (caput, corpus and caudal), seminal vesicles and bulbourethral gland of fertile, mature boars.

Both PSP-I and PSP-II (protein and mRNA) were detected in all tissues examined, although staining intensity varied between tissues and also between the same tissue of different boars (Table 1). Despite immunoreactivity being nearly or completely absent for both antisera (PSP-I and PSP-II) in the seminiferous epithelium, PSP-I and PSP-II were clearly detected by western blot (Fig.3), and single bands of approximately 330 base pairs (PSP-I) and 344 base pairs (PSP-II) were amplified by RT-PCR using PSP-I- and PSP-II-specific primers respectively (Fig.4). In contrast, Ekhlasi-Hundrieser *et al.* (2002) found expression of PSP-I, but not of PSP-II, in rete testis but did not investigate the seminiferous epithelium. It has been reported that certain proteins entering the epididymis from the rete testis disappear in the proximal caput epididymis (Gatti *et al.*, 2004). However, this might not be the general case as spermadhesin AWN, which is synthesized in the rete testis and caudal epididymis (Ekhlasi-Hundrieser *et al.*, 2002), has been also detected in epididymal sperm extracts (Dostàlovà *et al.*, 1994).

Testicular fluid has been shown to be more than just a diluent and a vehicle of spermatozoa, and secretory compounds present in it affect sperm function (Hedger *et al.*, 1998) as well as in the epididymal fluid (Dacheux *et al.*, 2003) . Hence, during its passage through the epididymis, spermatozoa develop motility, undergo continuous plasma membrane remodelling, and acquire fertilizing capability (Dacheux *et al.*, 2003; Tulsiani, 2003). The most active regions of the epididymis are the caput and the corpus, which synthesize about 80% of the compounds in the fluid. In addition, there is a high degree of regionalization along the epididymis. Thus, proximal secretions have been shown to be involved in sperm–egg fusion whereas sperm–zona binding proteins and decapacitation factor appear to be secreted more distally in the ductus epididymes (Gatti *et al.*, 2004). Regarding PSP-I and PSP-II synthesis, such regionalization is not observed as both proteins were consistently detected in the epithelium of caput, corpus and cauda epididymis by immunohistochemistry, western blotting and by RT-PCR analysis. The fact that PSP-I/PSP-II was not detected on the epididymal sperm surface (Dostàlovà *et al.*, 1994) suggests that this spermadhesin complex may interact rather loosely with the sperm surface. Alternatively, the absence of PSP-I/PSP-II on epididymal sperm might also be interpreted in the light of reports showing that proteins produced in the epididymis are rapidly degraded or reabsorbed (Syntin *et al.*, 1996; Castella *et al.*, 2003).

On the other hand, PSP-I/PSP-II heterodimers are clearly visible on the plasma membrane surface of spermatozoa at ejaculation (Caballero *et al.*, 2006), when spermatozoa become mixed with the fluids of the accessory sexual glands. Accordingly, our results showing strong positive signals for both PSP-I and PSP-II mRNA and protein support studies from other groups reporting that seminal vesicles are the major producers of SP proteins (Kwok Simon *et al.*, 1993; Ekhlasi-Hundrieser *et al.*, 2002; Fernández-Juan *et al.*, 2006). The seminal vesicles secrete around 80–90% of the proteins of the SP (Lavon & Boursnell, 1971) and, in particular, porcine spermadhesins account for about 75% of the total protein content of the SP (Ekhlasi-Hundrieser *et al.*, 2002).

Spermatozoa from different boars vary in their response to the addition of SP from the same male. Caballero *et al.* (2004) showed that, depending on the source, SP can exert a positive or a negative effect on highly extended boar spermatozoa. This observation is not specific for the pig as Killian *et al.* (1993) and Van der Ven *et al.* (1983) have demonstrated the same effect in bulls and humans respectively. Moreover, the high variability existing among SP of ejaculates from homologous males, as well as among different fractions of the same ejaculate, has been reported (Killian *et al.*, 1993; Zhu *et al.*, 2000), and different SP protein profiles have been found between boars of different fertility (Flowers & Turner, 2001). Although variability in the staining intensity of the bands corresponding to the PSP-I and PSP-II proteins among tissues and between the same tissue from different boars is reported in this study, whether this difference is related to variable production of the PSP-I/PSP-II heterodimer deserves a detailed investigation. In a previous paper, we showed that the non-heparin-binding PSP-I/PSP-II heterodimer exerted a concentration- and time-dependent sperm function-preserving activity, whereas the pool of the HBS had a deleterious effect on sperm physiology (Centurión *et al.*, 2003). Moreover, different molar ratios of PSP-I/PSP-II-to-HBS displayed

intermediate effects. Thus, it seems plausible to hypothesize that variability in the relative concentrations of sperm-protecting (PSP-I/PSP-II) versus sperm-detrimental (HBS) SP proteins would be responsible for the distinct response of spermatozoa upon mixing with the accessory gland fluids.

In conclusion, our results provide evidence that the spermadhesins PSP-I and PSP-II are expressed by different tissues along the male reproductive tract of the boar. The physiological post-ejaculation function of the PSP-I/PSP-II heterodimer has been investigated in certain detail. Further research is needed to clarify the physiological effect of these proteins on sperm cells during sperm epididymal transit, storage and maturation.

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5 Experiencia 3

Distinct effects of boar seminal plasma fractions exhibiting different protein profiles on the functionality of highly diluted boar sperm.

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ABSTRACT

The aim of this study was to evaluate how different protein profiles of seminal plasma fractions affect in vitro sperm functionality. Ejaculates from three boars were separated into six fractions. The fractions differed from each other in their sperm content, in their total seminal plasma protein content, and their spermadhesin PSP-I/PSP-II and heparin binding protein (HBP) concentrations. Spermatozoa were mainly recovered in fraction 2 (sperm-rich fraction, > 1800 x 10^6 spermatozoa/ml), whereas the pre-sperm fraction 1 and the post-sperm fractions 4-6 contained low numbers of spermatozoa (< 500 x 10^6/ml). Except in fraction 2, the total seminal plasma protein concentration and the concentration of both, spermadhesin PSP-I/PSP-II and the HBPs increased with fraction order. Distinct time-dependent effects were observed on motility characteristics and membrane integrity of highly diluted boar spermatozoa upon incubation with a 10% dilution of the seminal plasma from each fraction. The highest sperm viability was recorded after exposure for 5 hours to fraction 2, followed by fractions 1 and 3. The percentages of motile spermatozoa also differed significantly among fractions after 5 hours of incubation. Spermatozoa incubated with seminal plasma of fractions 1-3 showed the highest percentage motility. We conclude that different seminal plasma fractions exert distinct effects on the functionality of highly diluted boar spermatozoa. Fractions 1-3 appear to promote sperm survival, whereas fractions 4-6 seem to be harmful for preserving the physiological functions of highly diluted boar spermatozoa.

Key words: highly diluted boar spermatozoa, boar seminal plasma fractions, effect on sperm function, PSP-I/PSP-II heterodimer.

INTRODUCTION

Mammalian seminal plasma (SP), a physiological secretion produced in rete testis, epididymis and the male accessory sex glands, is the natural medium where sperm are suspended. Seminal plasma contains a wide variety of factors that provide not only metabolic support as an energy source for the sperm cells, but also influence their functionality (Maxwell et al., 2007; Rodríguez-Martínez, 2005; Centurión et al., 2003; Strzezek, 2002; Maxwell et al., 1997). Seminal plasma proteins appear to play active roles during sperm cell maturation and development in the male reproductive tract, as well as during transportation and survival of viable spermatozoa in the female reproductive tract (Troedsson et al., 2005). Proteins isolated from seminal plasma have been reported to exert both beneficial and/or deletereous effects on sperm physiology and fertility (Centurión et al., 2003; Caballero et al., 2004; Kraus, 2005; Maxwell et al., 2007). *In vivo*, the concerted action of these regulatory seminal plasma factors modulates the capacitation state of spermatozoa. *In vitro*, the viability of spermatozoa can be compromised by manipulation, such as cryopreservation, high dilution or sorting techniques, which remove seminal plasma components from spermatozoa (Ashworth et al., 1994; Maxwell et al., 1997; Garner et al., 2001). This deletereous effect can be counteracted by the addition of around 10%

homologous seminal plasma (Maxwell and Johnson, 1999; Barrios et al., 2000; Caballero et al., 2004).

Although under physiologycal conditions the whole boar ejaculate is deposited into the female genital tract, boar semen is homogeneously emitted in fractions with different characteristics and sperm concentrations, called *pre-sperm-rich fraction*, *sperm-rich fraction* and finally *post-sperm-rich* fraction (Ghaoui et al., 2004). Traditionally, the sperm-rich fraction is the only one used for artificial insemination. The pre-sperm rich fraction may have high bacterial and low sperm counts (Colenbrander et al., 1993), and the nature of the effect exerted by the *post-sperm-rich* fraction on sperm functionality remains controversial (Fraser and Strzezek, 2006).

The precise role of most of the seminal plasma proteins in sperm physiology remains obscure. Spermadhesins are male secretory proteins detected so far in ungulates (pig, cattle, horse and ram) (Calvete and Sanz, 2007). In the pig, this family of proteins comprises five members, AQN-1, AQN-3, AWN, PSP-I and PSP-II, together representing over 90% of the total boar seminal plasma proteins, which are thought to participate in different aspects of porcine fertilization. Thus, immunoelectronmicroscopic studies have revealed spermadhesin AWN epitopes on plasmalemmal remnants of boar spermatozoa bound *in vivo* to the zona pellucida of oocyte-sperm complexes recovered by surgery from naturally mated sows (Rodríguez-Martínez et al., 1999). This evidence clearly showed that at least spermadhesin AWN fulfils the requirements expected for a *bona-fide* zona pellucida-binding protein acting at the site of *in vivo* fertilisation. On the other hand, accumulating evidence points to a role for the heterodimeric PSP-I/PSP-II spermadhesin, the most abundant boar seminal plasma protein constituting around 50% of the total seminal plasma proteins, as an exogenous modulator of uterine immune activity (Leshin et al., 1998; Yang et al., 1998; Assreuy et al., 2002; Assreuy et al., 2003). The immunological action of this protein ensures reproductive success by preventing possible infections of the lower reproductive tract and providing a foreign cell-free uterine environment for the descending early embryos (Rodríguez-Martínez et al., 2005).

Besides their physiological functions in porcine reproduction, the heparin-binding (AQN-1, AQN-3, and AWN) and the non-heparin-binding (PSP-I/PSP-II) spermadhesins exert opposite effects on spermatozoa which have been subjected to high dilution to mimic the conditions of sex sorting by flow cytometry. Thus, while the pooled heparin-binding spermadhesins cause concentration-dependent sperm membrane damage, the spermadhesin PSP-I/PSP-II heterodimer (Calvete et al., 1995; 1996; Nimtz et al., 1999) mimicks the sperm-protective effect of whole seminal plasma (Centurión et al., 2003; Caballero et al., 2006; García et al., 2006). In particular, PSP-I/PSP-II heterodimer, and its isolated PSP-II subunit, are able to preserve the viability, acrosome integrity and mitochondrial activity of highly diluted boar spermatozoa. Hence, regardless of their *bona fide* physiological function, seminal plasma proteins, like PSP-I/PSP-II, that preserve the functionality of sperm cells *in vitro*, may represent valuable tools in preservation technologies for boar semen (Centurion et al., 2003; Parrilla et al., 2004; Garcia et al., 2006). The present study investigated the protein composition and effect of different seminal plasma fractions on boar sperm functionality, as assessed by integrity and motility characteristics, after spermatozoa were subjected to similar dilution as occurs in the process of sex sorting by flow cytometry.

MATERIAL AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

Collection and Preparation of Seminal Plasma

The ejaculation profiles

(ejaculation time and volume) from 20 fertile mature boars, which had previously sired offspring, were first evaluated. From these 20 boars, three showing similar ejaculation profiles were selected as seminal plasma donors for all the experiments. Whole ejaculates from the selected boars were collected using the gloved-hand method (Larsen, 1986), into a series of 50 ml sterile tubes in ejaculation-time depending fractions. The spermatozoa in each ejaculate fraction were counted using a Burker Chamber (Superior, Marienfeld, Germany) at a 1:100 dilution in saline solution as recommended by the manufacturer.

The seminal plasma was separated from the spermatozoa by centrifugation at 3,800 x g for 15 min at room temperature using a Heraeus Sepatech Megafuge (1.0 R, Heraeus, Germany). Supernatants were filtered sequentially through 10 and 1.2 µm filters and stored at -20ºC, and the sperm pellets were discarded. Total protein concentration was determined by the Lowry method (Lowry et al., 1951) in each seminal plasma fraction.

Quantification of PSP-I/PSP-II heterodimer and HBP proteins by HPLC

The proteins PSP-I/PSP-II and HBPs in the seminal plasma from the different ejaculate fractions were determined as described by Calvete et al (1995). Briefly, proteins were separated by reverse-phase HPLC using an ETTAN LC system (Amersham Biosciences, Uppsala, Sweden) and a Lichrospher RP100 C18 column (250x4 mm, 5µm particle size; Merk, Darmstadt, Germany) eluted at 1 ml/min with a linear gradient of 0.1% of TFA in water (solution A) and ACN (solution B) (isocratically (5%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 done at 215 nm. Since PSP-I occurs in both, the PSP-I/PSP-II heterodimer and the HBP pool, the relative amount of the PSP-I/PSP-II heterodimer in the different fractions was calculated from the area of the chromatographic peak of the PSP-II subunit (% PSP-I/PSP-II = [PSP-II area] x 2 / [total chromatogram area]). The relative amount of HBP was calculated from the sum of the areas of the heparin binding proteins (% HBP= [AQN-1 area + AQN-3 area + AWN area] + [PSP-I area - PSP-II area] / [total chromatogram area], where the term [PSP-I area - PSP-II area] represents the non-PSP-II-complexed PSP-I protein.

Semen collection and dilution

Sperm-rich fractions from three fertile mature boars were collected by the gloved-hand method and extended to $30x10^6$ sperm/ml in Beltsville Thawing Solution (BTS) (Pursel and Johnson, 1975). After collection, samples were evaluated for normality (motility >80%, viability >85%, total sperm count per ejaculate $>20\times10^9$, acrosomal abnormalities <10%, abnormal sperm morphology <15%). Only samples that met these minimum criteria were included in the study (Vazquez et al., 1997). Semen from these boars were pooled (in order to minimize individual boar variation), centrifuged at 1,200 x g (Megafuge 1.0 R, Heraeus, Germany) for 3 min, and the supernatant was discarded. Spermatozoa were sequentially extended to yield a concentration of $3x10^6$ sperm/ml in PBS containing 10% (v/v) of each seminal plasma fraction. In an additional tube, spermatozoa were resuspended in PBS buffer without seminal plasma as a control. Spermatozoa were analyzed for viability (membrana integrity), acrosomal status and motility after incubation at 38 ºC (Steri-Cult 200 incubator, Ohio, U.S.A.) for 0.5, 2 and 5 hours.

Flow Cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm and the EXPO 2000 software. Subpopulations were divided by quadrants and the frequency of each subpopulation was quantified. Non-sperm events (debris) were gated out

based on the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to sperm cells but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10,000 events per sample. Samples were measured at a flow rate of ≈300 cells/sec.

Flow Cytometric Assessment of Sperm Viability and Acrosomal Exocytosis

For an accurate estimation of the spermatozoa, the membrane and the acrosome integrity were assessed simultaneously by flow cytometry using the triple staining protocol described by Nagy et al. (Nagy et al, 2003; Garcia et al., 2006). Briefly, 500 μl of sperm sample (approximately 500,000 cells) were incubated with 50 nM SYBR-14 (using a 100 μM stock solution in DMSO; component A of LIVED/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, Netherlands), 0.5 μg/ml PE-PNA (peanut agglutinin conjugated with phycoerythrin; Biomeda Corp. Foster City, CA) and 7.5 μM propidium iodide (PI). The samples were mixed and incubated at 37 ºC in the dark for 10 min before flow cytometric analysis. SYBR-14 was measured using a 525 nm band pass filter, PI was collected through a 620 nm band pass filter and PE-PNA was detected through a 575 band pass filter. Viable spermatozoa with intact acrosomes were defined as those stained only with SYBR-14. Acrosome reacted spermatozoa were defined as those stained with SYBR-14 and PE-PNA. Spermatozoa stained with PI were regarded as dead cells.

Sperm Motility

Sperm motility was estimated using a CASA system (ISAS® Proiser, Valencia, Spain). Analysis was based on the examination of 25 consecutive, digitalized images obtained from a single field. Aliquots of 10 μl of semen sample (at a concentration of 10^6 spermatozoa/ml) were placed in a warm (38 ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38 ºC) of a Nikon Labophot positive-phase contrast light microscope (Tokio, Japan) equipped with a 10x objective and a monochrome video camera (Basler Vision technologies A 312f, Gemany) connected to a personal computer. The parameters evaluated were VCL (curvilinear velocity, µm/s) , VSL (straight linear velocity, µm/s), and ALH (lateral head displacement, µm). Objective percentage of motile spermatozoa was analyzed in at least 100 spermatozoa per sample.

Statistical Analysis

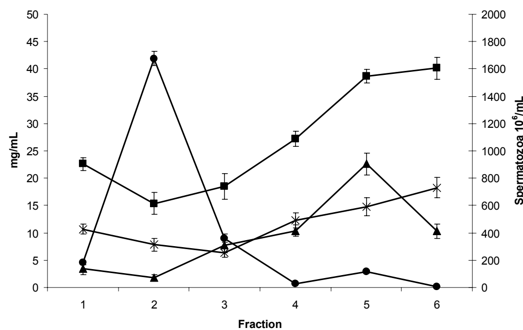
All data editing and statistical analyses were performed in SPSS, version 14.0 (SPSS Inc., Chicago, IL). Data from four replicates from each experiment were analyzed by analysis of variance (ANOVA) using the mixed-procedure including the fixed effects of incubation time, boar and seminal plasma fraction and the random effect of replicate. The effect of boar was subsequently excluded from the analysis because none of the parameters were influenced by boar. Therefore, the results pooled for boar are presented as means±SEM. When the ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p<0.05$.

RESULTS AND DISCUSSION

Figure 1 summarizes the mean total protein concentrations (mg/mL), sperm concentration (10^6/mL) and the amounts of PSP-I/PSP-II (mg/mL) and HBPs (mg/mL) for the six ejaculate fractions collected. As expected, sperm count varied in the different fractions. The first fraction (called *pre-sperm-rich fraction*) contained mainly seminal plasma and very low sperm numbers. Fraction 2 was characterized by the highest number of spermatozoa (*sperm-rich fraction*). The concentration of spermatozoa drastically decreased in *post-sperm rich fractions* 3-6 along with an increase in the total protein concentration. PSP-I/PSP-II and HBP concentrations also increased in parallel to total proteins in fractions 3-5 but the concentration of PSP-I/PSP-II

heterodimer dropped in fraction 6, whereas that of the HBPs and the total proteins remained high.

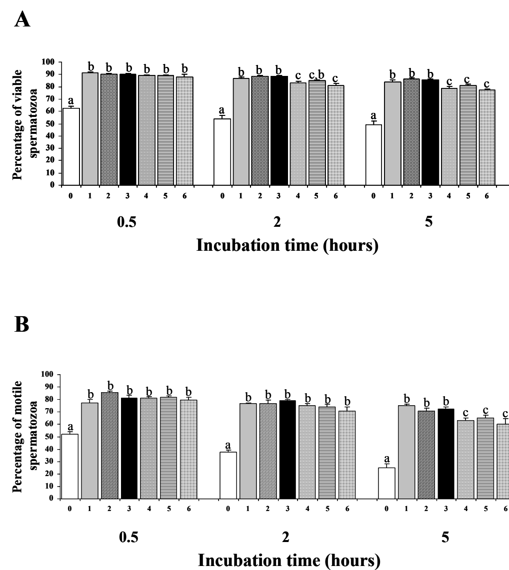
Figure 1. Profiles of seminal plasma protein and sperm concentrations in the different boar ejaculate fractions. The curves represent the mean ± SEM (errors bars) of the sperm concentration (●: expressed in sperm x 10^6/mL), total protein concentration (■: expressed in mg/mL) and the relative amounts of PSP-I/PSP-II heterodimer (▲: expressed in mg/mL) and HBP (**x**: expressed in mg/mL). * Indicates statistically significant differences compared to the previous point.



Viability, acrosome integrity and motility were evaluated to study the effect of the six seminal plasma fractions on the sperm cell function. Figure 2A shows the time-course effect of incubating diluted spermatozoa with the different seminal plasma fractions. The presence in the incubation medium of seminal plasma from all six fractions improved ($p<0.05$) the percentage of viable acrosome-intact spermatozoa compared with the control at all times evaluated. The effect of 10% of each seminal plasma fraction on sperm membrane integrity was significantly different among fractions after 2 hours of incubation. Fractions 1, 2, 3 and 5 resulted in the highest percentages of viable acrosome-intact spermatozoa (87.5±2.5, 87.8±0.5, 88.3±0.49 and 84.7±0.74, respectively). After 5 hours, the percentages of viable acrosome-intact spermatozoa were lower ($p<0.05$) in samples exposed to fractions 4-6 (78.9±1.33, 80.9±1.25 and 77.3±0.9, respectively) than those exposed to fractions 1-3 (83.6±1.9, 86.3±0.7 and 85.7±0.4, respectively). Thus, spermatozoa exposed to fractions 1-3 exhibited the highest values ($p<0.05$) of viable acrosome-intact spermatozoa after 5 hours of incubation, with fraction 2 (sperm-rich fraction) the one showing the best values.

There were no differences in the percentages of motile spermatozoa (Figure 2B) in the samples incubated with each of the six fractions of seminal plasma between 0.5 and 2 hours of incubation (Figure 2B). However, motility of spermatozoa was significantly better for all samples exposed to seminal plasma fractions compared with the control. After 5 hours of incubation the percentage of motile spermatozoa exposed to fractions 1 (74.1±1.1), 2 (69.5±2.9) and 3 (72±1.74) was significantly higher ($p<0.05$) than for fractions 4, 5 and 6 (64.4±2.7, 66.2±1.2 and 60.2±4.41, respectively).

Figure 2. Effect of the six seminal plasma fractions on the viability (sperm with intact membrane and acrosome) **(A)** and motility **(B)** of highly diluted boar spermatozoa. Spermatozoa were diluted to $3x10^6$ sperm/mL in phosphate-buffered saline and incubated for 0.5, 2 and 5 hours at 38 ºC with 10% of each seminal plasma fraction. Control (0) was incubated in the absence of seminal plasma. Viability was assessed by triple staining (PI/SYBR-14/PE) and the percentages of motile spermatozoa were evaluated by a computer-assisted motility analysis system. Columns represent the mean ± SEM (errors bars). Columns labelled with different letter are significantly ($p<0.05$) different.



The mean motility parameters assessed by CASA for spermatozoa

exposed to the six fractions and control after 0.5, 2 and 5 hours of incubation are displayed in Table 1. Significant differences were recorded among the seminal plasma fractions and control for VCL and VSL during incubation. Sperm velocities were significantly ($p<0.05$) higher in the samples containing seminal plasma from fraction 2, compared with all other fractions and the control after 0.5 h of incubation.

In the sample containing seminal plasma from fraction 2, ALH was higher ($p<0.05$) than in the rest of the samples and control, but ALH did not differ between any sample and the control after 2 hours of incubation. After 5 hours, ALH increased significantly for spermatozoa incubated with seminal plasma from fractions 2-6 compared with both the control and fraction 1.

The results of this study showed that the PSP-I/PSP-II heterodimer was present in all seminal plasma fractions, although its concentration increased steadily and in parallel with the HBPs and the total proteins in the post-sperm rich fractions 3-5. Conversely, sperm-rich fraction 2 was characterized by the lowest levels of both total protein (15.37 mg/ml) and PSP-I/PSP-II heterodimer (1.8 mg/ml), and by a low amount of HBP protein. In line with Peña et al. (2003, 2006), the addition of seminal plasma from the sperm rich fraction induced the best results in this study. The detrimental effects on highly diluted boar spermatozoa of the post-sperm rich fractions, in which PSP-I/PSP-II reached a concentration of up to 10 mg/ml, is also in line with our previous results showing that PSP-I/PSP-II exhibits an optimal sperm protective effect at 1.5 mg/ml, whereas this effect is reversed at concentrations higher than 7.5 mg/ml (Centurion et al., 2003). In addition, similarly to the effects exerted by the *post-sperm-rich* fractions in this study, incubation of highly diluted boar spermatozoa in the presence of mixtures of heparin-binding and non-heparin-binding spermadhesins produced a markedly detrimental effect on their viability and motility as the relative concentration of the heparin-binding spermadhesin pool (AQN-1, AQN-3 and AWN) increased in the mixture (Centurión et al., 2003). We conclude that the different effects that seminal plasma fractions exert on the functionality of highly diluted boar spermatozoa may be due to the distinct protein profiles of the seminal plasma fractions. Our findings further suggest that *post-sperm-rich* seminal plasma fractions should not be used as additives in sperm preservation technologies.

Table 1. VCL, VSL and ALH of highly diluted boar spermatozoa (3×10^6 spermatozoa/ml) as a function of the incubation time at 38ºC and of the presence or absence (control) of 10% of seminal plasma fractions. Motility paremeters are reported as mean ± SEM of four samples per treatment evaluated in duplicate.

	0,5 h			**2 h**			**5 h**		
Fraction	**VCL**	**VSL**	**ALH**	**VCL**	**VSL**	**ALH**	**VCL**	**VSL**	**ALH**
F0	56.16±4,06	21.95±3,23	2.85±0,1	49.53±5,49	13.6±1,43	2.56±0,23	42.28±6,75	11±0,94	2.18±0,21
F1	30.5±1,2[a]	17.85±0,85	1.75±0,15[a]	47.33±1,45	23.8±0,86[a]	2.8±0,2	47.6±1,8	20.25±0,75[a]	2.35±0,05
F2	72.84±4[ab]	39.76±4,25[ab]	3.15±0,13[b]	63.63±3,55[ab]	36.58±4,4[ab]	2.64±0,1	60.06±2,72[ab]	28.68±1,75[ab]	2.77±0,11[ab]
F3	63.21±2,84	30.82±2,73	2.76±0,11	61.95±2[a]	31.99±2,25[a]	2.82±0,06	60.44±1,92[a]	25.77±1,84[a]	2.85±0,07[a]
F4	52.46±2,48[b]	25.78±1,64	2.42±0,12	57.57±2,41	26.82±1,82[a]	2.74±0,08	54.79±1,41[a]	22.80±1,05[a]	2.66±0,05[a]
F5	54.52±2,3	26.11±1,46	2.55±0,11	59±1,95	25.55±0,83[a]	2.82±0,1	55.28±1,28[a]	20.35±0,76[a]	2.68±0,07[a]
F6	42.2±1,92[a]	19.6±1,21	1.92±0,02[ab]	50.6±2,12	20.81±1,16	2.56±0,07	50.85±1,67	18.05±1,17[a]	2.63±0,07[a]

a $p<0.05$ compared with control (F0)
b $p<0.05$ compared to the previous point within the same incubation time series

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Improving the fertilizing ability of sex sorted boar spermatozoa

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Abstract

The sex sorting of spermatozoa by flow cytometry induces damage, since sperm cells are highly diluted, affecting their functionality and fertilizing ability. In this work it was investigated whether the concentration of sex sorted spermatozoa by the sedimentation method, rather than centrifugation, in combination with the presence of the seminal plasma protein PSP-I/PSP-II heterodimer may improve their fertilizing ability.

Spermatozoa were sorted by flow cytometry and collected in BTS with 10% of seminal plasma (group C: control) or with 1.5 mg/mL of PSP-I/PSP-II heterodimer (group H). Collected spermatozoa from each medium were split into two aliquots. One aliquot of each group was centrifuged ($800 \times g$/5 min) just after sorting and stored 16–18 h at 17 °C (groups Cc and Hc) at 6×10^6 sperm/mL. The second aliquot was directly stored at 17 °C for 16–18 °C (group Cs and Hs). After storage the supernatant was discarded and the sedimented pellet adjusted to 6×10^6 sperm/mL. Membrane integrity, acrosome status and motility characteristics of spermatozoa from all groups were assessed. Post-weaning pre-ovulatory sows were inseminated by laparoscopy into the oviduct with 0.3×10^6 sex sorted spermatozoa to assess their ability to penetrate oocytes in vivo. Putative zygotes were collected 18 h after insemination by washing the oviduct. Penetration and monospermic rates were evaluated.

After 16–18 h of storage, centrifuged spermatozoa collected with 10% seminal plasma or 1.5 mg/mL PSP-I/PSP-II heterodimer after sex sorting showed lower ($p < 0.05$) percentages of membrane integrity, motility and fertilization than sedimented spermatozoa. Overall, the presence of 10% seminal plasma or PSP-I/PSP-II heterodimer did not affect the results. However, a positive effect of PSP-I/PSP-II heterodimer ($p < 0.05$) was observed in sedimented spermatozoa. Hence, our results indicate that the sedimentation method in the presence of PSP-I/PSP-II heterodimer improves the in vivo fertilizing ability of sex sorted boar spermatozoa.
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Keywords: Sex sorted boar spermatozoa; PSP-I/PSP-II heterodimer; Intraoviductal laparoscopic insemination; Sedimentation

1. Introduction

The development of semen sexing represents a major advance in reproductive technology. The preselection of the sex in swine improves the efficiency in the production of male or female crossbred lines. Moreover, animal welfare laws in some countries could forbid the use of castration and alternatives for producing higher numbers of females will need to be found. However, the success in the implementation and wide spread of this technology depends on economics, efficiency, safety and ease of use.

The major problem with the preselection of the sex by flow cytometric sorting, the only procedure available to produce sex sorted spermatozoa, is related

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to the low number of spermatozoa obtained, since the number of sexed spermatozoa produced per unit time is limited. The yield is relatively slow and long times are required to obtain the adequate number of sorted boar spermatozoa [1]. Moreover, it is known that the sperm membrane is adversely affected by the flow cytometry and sorting processes, which limits the viability, storage capability and fertilization ability of spermatozoa [2,3].

The presence of seminal plasma in the collection medium seems to stabilize the sperm plasma membrane and to reverse the capacitation status after sorting [3,4]. Similar results have been found with highly diluted boar spermatozoa when the PSP-I/PSP-II heterodimer spermadhesin from seminal plasma was added [5,6]. These properties highlight PSP-I/PSP-II as a potential candidate for treatment of sex sorted spermatozoa before insemination, with the aim of promoting sperm survival and performance in vitro.

On the other hand, the highly diluted sex sorted spermatozoa must be concentrated before insemination. Centrifugation has been the routine procedure used for sperm concentration. However, in several species, such as the rat [7], human [8], mouse [9] and boar [10], centrifugation is a potentially damaging step during the processing of spermatozoa. This procedure has a detrimental effect on the lifespan of the spermatozoa due to a direct effect on the sperm membranes [11]. Moreover, the damage appears to be greatest when ''weaker'' spermatozoa are used, such as those that have been sex sorted. Consequently, alternative procedures to concentrate sex sorted spermatozoa should be evaluated. Sedimentation procedure has been occasionally used to concentrate human spermatozoa with poor quality [12].

The aim of the present study was: (i) to evaluate the function of the flow cytometrically sex sorted spermatozoa after concentration by either sedimentation or centrifugation in presence of the seminal plasma or the spermadhesin PSP-I/PSP-II heterodimer and (ii) to assess the in vivo fertilizing ability of sex sorted spermatozoa after concentration by sedimentation and centrifugation in presence of the seminal plasma or the PSP-I/PSP-II heterodimer and deposited by laparoscopic intraoviductal insemination.

2. Material and methods

All chemicals were purchased from Sigma–Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

2.1. Preparation of seminal plasma and isolation of the boar seminal plasma PSP-I/PSP-II heterodimer

Seminal plasma was collected from four males with semen containing high percentages of viable and motile spermatozoa [13]. Ejaculates were collected using the gloved-hand method [14].

The seminal plasma was separated from spermatozoa by centrifugation at $3800 \times g$ for 15 min at room temperature [5] using a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatants were filtered sequentially through 10 and 1.2 μm filters, pooled and stored at −80 °C.

The PSP-I/PSP-II heterodimer was isolated from the non-heparin binding fraction of boar seminal plasma by affinity chromatography on a heparin-Sepharose column, equilibrated in 100 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA and 0.025% sodium azide, pH 7.4, as previously described [15]. The identity and purity of the protein was assessed by N-terminal sequence analysis using an Applied Biosystems 472 automated protein sequencer (Langen, Germany) and MALDI-TOF mass spectrometry using and Applied Biosystems Voyager DE-Pro mass spectrometer (Applied Biosystems, Langen, Germany) and a saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid as the matrix. Protein concentration was determined spectrophotometrically using the molar absorption coefficient (27,332 M-1 cm-1) determined previously [16] or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 h at 106 °C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water and lyophilized until use.

2.2. Semen collection and flow cytometric sorting

Sperm-rich fractions from fertile mature boars, that had previously sired offspring, were collected by gloved-hand method and extended in Beltsville Thawing Solution (BTS) to 150×10^6 spermatozoa/mL [17]. After collection, samples were evaluated for normality (motility >80%, membrane integrity >85%, total sperm count per ejaculate $>20 \times 10^9$, acrosomal abnormalities <10%, abnormal sperm morphology <15%). Only samples that met the minimum criteria were used [13].

The extended semen was then processed for sperm sorting following the general procedure as previously described [18]. Briefly, 1 mL of extended semen was stained with Hoechst-33342 fluorophore (0.3 μM per

1×10^6) and incubated for 1 h in darkness at 35 °C. After incubation, samples were filtered through a 30 μm nylon mesh filter to remove debris or clumpled spermatozoa. The stained spermatozoa were passed through a modified EPICS Altra high speed flow sorter (Coulter Corporation, Miami, FL, USA), operating at 3.655 kg/cm^2 and modified with a model 90C-6, 6 W argon laser [19] operated in the ultraviolet wavelength (351, 364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA). Spermatozoa were separated into X- and Y-chromosome bearing populations, and only the X sperm population was recovered.

2.3. Assessment of sperm characteristics

Samples were stored at 16 °C. After 0.5 h and 16–18 h of storage the spermatozoa were analyzed for membrane integrity, acrosomal status and motility.

2.3.1. Flow cytometric assessment of sperm viability and acrosomal exocytosis

The membrane and the acrosome integrity of spermatozoa were assessed simultaneously by flow cytometry using the triple staining protocol described previously [6,20]. Briefly, 500 μl of sperm sample was incubated with 50 nM SYBR-14 (using a 100 μM stock solution in DMSO; component A of LIVED/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, Netherlands), 0.5 μg/mL PE-PNA (peanut agglutinin conjugated with phycoerythrin; Biomeda Corp., Foster City, CA) and 7.5 μM propidium iodide (PI). The samples were mixed and incubated at 37 °C in the dark for 10 min before flow cytometric analysis. SYBR-14 was measured using a 525 nm band pass filter, PI was collected through a 620 nm band pass filter and PE-PNA was detected through a 575-band pass filter. Viable spermatozoa with intact acrosomes were defined as SYBR-14-positive/PI-negative/PE-PNA-negative.
Acrosome reacting spermatozoas were defined as PE-PNA-positive and spermatozoa PI-positive was classified as dead cells.

2.3.2. Sperm motility

Sperm motility [5] was estimated by a computer-assisted sperm motility analysis (CASA) system using the software Sperm Class Analyzer (ISAS® CASMA System, Proiser SL, Valencia, Spain). Ten microliters aliquots of sperm sample (at 6×10^6 spermatozoa/mL) were placed in a warm (38 °C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38 °C) of a Nikon Labophot positive-phase contrast light microscope (Tokyo, Japan) equipped with a 10× objective and a monochrome video camera (Basler Vision technologies A 312f, Germany), connected to a personal computer. The objective percentage of motile spermatozoa was analyzed in at least 100 spermatozoa per sample. The program settings were VCL (curvilinear velocity, μm/s), VSL (straight linear velocity, μm/s) and ALH (lateral head displacement, μm).

2.4. Intraoviductal laparoscopic insemination

Sows were allocated individually to crates in a mechanically ventilated confinement facility under field conditions on a commercial pig farm in Murcia (Spain). Animals were fed a commercial ration twice a day and water was provided ad libitum. A total of 140 crossbred sows (2–6 parity) were weaned at 21.01 ± 0.04 days. Oestrus was induced by injecting each female intramuscularly with 1250 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning, followed 72 h later with 750 IU human chorionic gonadotrophin (hCG; Veterin Corion, Divasa, Farmavic S.A., Barcelona, Spain). Oestrus detection was performed once a day (7:00 a.m.), beginning 2 days after eCG injection, by allowing females nose to nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in oestrus and the ovaries scanned. The ovaries were examined at intervals of 4 h from 30 h after hCG injection by transrectal ultrasonography using a 5 MHz multiple scan angle transducer for the presence of preovulatory follicles. Only sows showing multiple preovulatory follicles (diameter of antrum >6 mm) were selected for insemination [21]. Inseminations were carried out within 2–3 h after ultrasonography.

Laparoscopic inseminations were performed on sows sedated by azaperone (Stresnil, Lab. Dr. Esteve, Barcelona, Spain) administration (2 mg/kg body weight, i.m.). General anesthesia was induced with sodium thiopenthal (Lab. Abbot, Madrid, Spain; 7 mg/kg body weight, i.v.) and maintained with halothane (3.5–5%). The sow was placed in the supine position, on her back in a laparoscopy cradle. The cradle was placed in a Trendelemburg position at an angle of approximately 20° above horizontal.

A 1.5-cm incision was made close to the umbilicus. The edges of the incision were then pulled up with countertraction and a 12-mm Optiview trocar (Ethicon Endo-surgery Cincinnati OH, USA) with an inserted 0° laparoscope was advanced into the wound. At the umbilicus, the subcutaneous fatty tissue, the anterior fascia of the rectus muscles, the rectus muscles, the

posterior fascia of the rectus muscles, the transversalis fascia and the peritoneum were traversed by slight cutting and moderate pressure. The process was controlled via monitor feedback. Although the CO_2 tubing was connected to the trocar, inflation did not begin until the peritoneum was punctured. After the peritoneal cavity was entered and the pneumoperitoneum started, the handpiece of the Optiview was removed and replaced by the 0° laparoscope. The abdominal cavity was inflated to 14 mmHg with CO_2.

Two accessory ports were placed in the right and left part of the hemi abdomen, which provided access for laparoscopic Duval forceps for manipulating the uterine horn and grasping the oviduct for the insemination needle, respectively. The oviduct was grasped with the Duval forceps in the isthmus region. Then the dose-flow (containing 0.3 million of spermatozoa in 0.1 mL) was inserted, and sex sorted spermatozoa was flushed into the oviduct. The procedure was then repeated in the other oviduct. After both oviducts were inseminated, the trocars were removed and minor suturing was required.

2.5. Embryo collection and embryo evaluation

The putative zygotes were obtained surgically by laparotomy 18 h after insemination. Sedation of the sows was performed by azaperone administration (2 mg/kg body weight, i.m.). General anesthesia was induced with sodium thiopenthal (7 mg/kg body weight, i.v.) and maintained with halothane (3.5–5%). The reproductive tract was exposed via mid-line incision and corpora lutea were counted on the ovaries. Putative zygotes were collected by flushing each oviduct and the upper part of the uterine horn with 10 mL of NCSU-23 medium supplemented with 0.4% bovine serum albumin and 10 mL Hepes (flushing medium). Presumptive zygotes recovered 18 h after insemination were mounted on slides, fixed for 48–72 h in 25% (v:v) acetic acid in ethanol at room temperature, stained with 1% lacmoid in 45% (v:v) acetic acid and examined under a phase contrast microscope at 400×. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei with their corresponding sperm tails and two polar bodies. The fertilization parameters evaluated were: penetration rate (number of penetrated oocytes/number of oocytes) and monospermic rate (number of monospermic oocytes/number of penetrated oocytes). Degenerated oocytes, immature oocytes and oocytes with a broken oolemma or abnormal appearance of the cytoplasm were not counted [22].

2.6. Experimental design

2.6.1. Experiment 1

In order to evaluate the effect of the concentration methodology of the spermatozoa after sorting and the presence of the heterodimer PSP-I/PSP-II or seminal plasma on the functionality of the post-sorted spermatozoa, a 2 × 2 factorial experiment was carried out. Samples of semen were sorted for chromosomal sex by flow cytomeric sorting. Spermatozoa were collected in 10 mL BSA-coated tubes with the BTS extender containing 10% of seminal plasma (group C—control) or 1.5 mg/mL of spermadhesin PSP-I/PSP-II heterodimer (group H) at 17 °C. Collected spermatozoa from each medium (control or PSP-I/PSP-II) were split in two aliquots. The first aliquot was centrifuged (c) at $800 \times g$ (Megafuge 1.0 R, Heraeus, Germany) for 5 min just after sorting, supernatants were discarded and pellets were stored for 16–18 h at 17 °C (groups Cc and Hc). After storage the sperm concentration was adjusted to 6×10^6/mL. The second aliquot (sedimentation method) was directly stored (s) at 17 °C for 16–18 h. After storage, the supernatant was discarded and the sedimented pellet adjusted to a concentration of 6×10^6 spermatozoa/mL (groups Cs and Hs). Membrane integrity, acrosome status and motility characteristics of spermatozoa from all groups were assessed. Six replicates were carried out from each group.

2.6.2. Experiment 2

In order to evaluate the effect of the concentration methodology of the spermatozoa after sorting and the presence of the heterodimer PSP-I/PSP-II or seminal plasma on the in vivo fertilizing ability of the sex sorted spermatozoa, a 2 × 2 factorial experiment was carried out. Spermatozoa were distributed in four groups as above (Cc, Cs, Hc and Hs).

During 8 weeks, 140 post-weaning pre-ovulatory sows hormonally tretated as described above were divided in four groups and 126 sows inseminated by laparoscopic into the oviductal ampulla using 0.3×10^6 Cc, Cs, Hc or Hs sex sorted spermatozoa in 0.1 mL. Fourteen sows were not inseminated since they had not follicles with more than 6 mm at insemination time.

Putative zygotes were surgically collected 18 h after insemination by washing the oviducts. Putative zygotes were fixed and stained with lacmoid, and examined by phase contrast microscopy. Penetration and monospermic rates were evaluated.

2.7. Statistical analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analyzed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effect of collection media (Cc, Cs, Hc or Hs) and the random effect of replicate. To analyze data, percentages were subjected to arcsine transformation before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p < 0.05$.

3. Results

3.1. Experiment 1: Effect of the methodology used to concentrate sex sorted spermatozoa in presence of seminal plasma or the heterodimer PSP-I/PSP-II on the sperm functionality

Data on the viability, motility and acrosome integrity of spermatozoa sorted in presence of the PSP-I/PSP-II heterodimer or 10% of seminal plasma and after sedimentation or centrifugation are shown in Table 1. After 16–18 h of storage, there was a significant ($p < 0.05$) decrease in the membrane integrity of sorted spermatozoa concentrated by centrifugation compared to sedimentation. The same trend was observed in the percentages of motile spermatozoa, which were lower ($p < 0.05$) for centrifugation than sedimentation (Table 1). The velocity parameters of the spermatozoa (VCL, VSL) were preserved after 16–18 h of storage only after sedimentation and storage in the presence of PSP-I/PSP-II heterodimer. ALH was not different among groups. However, centrifugation adversely affected the kinematics of spermatozoa ($p < 0.05$) (Table 1).

The percentage of acrosome reacting cells in the samples was not affected neither concentration procedure nor presence of seminal plasma or the heterodimer PSP-I/PSP-II.

The presence of 10% of seminal plasma or PSP-I/PSP-II heterodimer did not show differences with respect to membrane integrity and motility levels. However, spermatozoa stored in presence of PSP-I/PSP-II heterodimer showed better velocity parameters than spermatozoa stored with 10% seminal plasma.

The efficiency of recovery of spermatozoa obtained by sedimentation or centrifugation was also evaluated by counting the total number of sex sorted cells in each sample. The percentage of cells recovered was significantly higher after sedimentation (81.5 ± 2.1%) compared with centrifugation (63.4 ± 2.98%; $p < 0.05$).

3.2. Experiment 2: Effect of the methodology used to concentrate sex sorted spermatozoa in the presence of seminal plasma or PSP-I/PSP-II heterodimer on in vivo fertilizing ability of spermatozoa

The mean ovulation rate (±S.E.M.) in the sows was 20.3 ± 1.8 (oocytes/sow). The ratio of collected putative zygotes to the number of corpora lutea was 95.8%. The total numbers of putative zygotes were 593, 628, 603 and 567 for Cc, Cs, Hc and Hs groups, respectively. The results from this experiment are displayed in Fig. 1. Higher oocyte penetration rates ($p < 0.05$) were obtained for sorted spermatozoa concentrated by sedimentation (Cs and Hs groups: 74.5 and 84.2%, respectively) than for centrifugation (Cc and Hc groups: 55.8 and 58.2%, respectively). Monospermic rates were in all cases close to 90%. There was no difference between 10% seminal plasma and the purified hetero-

Table 1
Percentage (mean ± S.E.M.) of live spermatozoa (intact membrane integrity) with both intact or reacting acrosomes identified with triple fluorescent staining (SYBR-14/PI/PE-PNA), percentage motility, curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s) and lateral head displacement (ALH,μm) of sedimented (s) or centrifuged (c) sex sorted spermatozoa in presence of 10% of seminal plasma (C) or 1.5 mg/mL of PSP-I/PSP-II heterodimer (H), stored 16–18 h at 17 °C

Sperm (%)	Sex sorted (before concentration, 0.5 h after sorting)	Sex sorted (after concentration, 16–18 h after sorting)			
		Cc	Cs	Hc	Hs
Live[a]	85.9 ± 0.6[a]	66.1 ± 2[b]	87.3 ± 1.2[a]	68.1 ± 1.8[b]	83.7 ± 2.6[a]
Motile	78.4 ± 0.9[a]	57.7 ± 1.1[b]	71.3 ± 1.6[a]	57.5 ± 1.9[b]	72.2 ± 1.2[a]
Acrosome reacting[b]	2 ± 0.5[a]	2.3 ± 0.4[a]	2.2 ± 0.3[a]	2 ± 0.6[a]	2 ± 0.2[a]
VCL	121.6 ± 5.8[a]	46.1 ± 3.5[b]	84.4 ± 2.7[c]	50.2 ± 4.4[b]	105.2 ± 4.7[a]
VSL	72.5 ± 4.2[a]	22.7 ± 3.4[c]	42.9 ± 2.9[b]	33.7 ± 3.8[c]	64.4 ± 4.0[a]
ALH	4.2 ± 0.6[a]	2.3 ± 0.2[b]	2.8 ± 0.1[b]	2.3 ± 1.1[b]	2.9 ± 1.2[b]

[a] Live spermatozoa with intact acrosomes: SYBR-14-postive/PI-negative/PE-PNA-negative.
[b] Live spermatozoa with reacting acrosomes: SYBR-14-postive/PI-negative/PE-PNA-positive.Values with different letters (a–c) within the same row differ significantly.

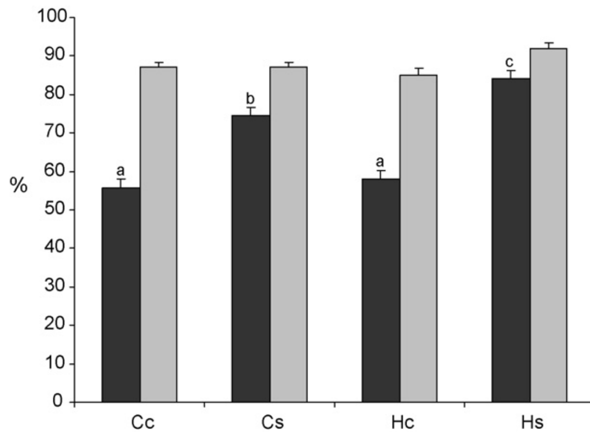

Fig. 1. Percentage (mean ± S.E.M.) of penetration rates (■) and monospermic rates (■) of oocytes from pre-ovulatory sows inseminated with sex sorted spermatozoa 0.3 million per oviduct in a total volume of 0.1 mL of BTS with 10% of seminal plasma (C) or 1.5 mg/mL of PSP-I/PSP-II heterodimer (H) concentrated by sedimentation (s) or centrifugation (c). Different letters indicate a significant difference ($p < 0.05$) between semen groups.

dimer when spermatozoa were concentrated by centrifugation. However, a positive effect of PSP-I/PSP-II heterodimer ($p < 0.05$) was observed when spermatozoa were concentrated by sedimentation.

4. Discussion

Spermatozoa are sensitive to the different steps included in the sex sorting process by flow cytometry such as the staining, high dilution, laser exposure and changes in media composition [4]. These stresses reduce the lifespan of spermatozoa after sorting [23,24]. This short sperm lifespan is a critical ‘‘weak’’ point in the application of the sexed semen in the pig industry, since the farms are not usually located near to the flow cytometry laboratories.

Consequently, strategies to extend the lifespan of sex sorted spermatozoa must be developed before this technology can be applied in the field. In this study, we attempted to minimize the detrimental effects of both high dilution, with the associated cleansing of beneficial seminal plasma proteins from the plasma membrane, and the process of sperm concentration, on sex sorted spermatozoa stored for 18 h.

Centrifugation is the most common technique used for the concentration of sperm cells necessary after sex sorting. However, mammalian spermatozoa are sensitive to centrifugation since this methodology can lead to structural damage to the acrosome, reduced functionality and impaired fertilizing capacity [25,26]. We have previously shown in several experiments with diluted and frozen-thawed boar spermatozoa that the integrity of the plasma membrane and percentage motility were adversely affected by the numbers, time and force of centrifugation [27,10]. The present study showed a significant decrease ($p < 0.05$) in the membrane integrity and motility of flow cytometrically sexed spermatozoa when concentrated by centrifugation after sorting and storage for 16–18 h. The kinematic measurements of VCL and VSL were also reduced in centrifuged sex sorted spermatozoa, whereas they were preserved after sedimentation in the presence of PSP-I/PSP-II heterodimer. Sedimentation has been used as an alternative to centrifugation to obtain a sufficient number of motile and functional human spermatozoa from poor quality samples [12]. In our work, there was a significant increase in sperm membrane integrity and motility when the spermatozoa were concentrated by sedimentation for 18 h, when compared to centrifugation and 18 h storage. Moreover, the percentage of cells recovered by sedimentation was higher than that obtained by centrifugation. This might be due to the extended time (16–18 h) that sex sorted spermatozoa were sedimented at 17 °C. While other studies have reported that higher number of spermatozoa was recovered by centrifugation than sedimentation [28], our results suggest that sedimentation is a better procedure for concentration of spermatozoa after sex sorting, since better recovery efficiency was obtained (in terms of sperm quality and sperm number) in comparison to centrifugation. This is particularly important when there is a necessary time delay between sex sorting and insemination. The use of the sedimentation instead of centrifugation is especially important with boar spermatozoa, since these cells are clearly more susceptible to damage by centrifugation than for other species such as bull spermatozoa [1]. Moreover, the sedimentation of the sex sorted spermatozoa in combination with PSP-I/PSP-II heterodimer maintained sperm functionality during 18 h of incubation, allowing sufficient time for inseminations to be undertaken at farms located a long distance from the laboratory.

The flow cytometric sorting process results in the removal of many seminal plasma components from the sperm surface, mainly proteins that are beneficial for the stability of the plasma membrane. These components may affect the functionality and fertilization capacity of spermatozoa [1,2,29]. Consequently, the addition of 10% seminal plasma to the collection medium of sorted boar spermatozoa has been used to attenuate the removal beneficial seminal plasma components [23,3]. However, the high variability of seminal plasma protein profiles from homologous males, as well as between

ejaculates from the same male [30,31], suggests that males should be screened before selection as seminal plasma donors.

The use of the isolated seminal plasma protein PSP-I/PSP-II heterodimer as an additive to the medium for collection of sex sorted spermatozoa has the advantage of avoiding the variability of protein content in whole seminal plasma, since it has been shown that the addition of 1.5 mg/mL of PSP-I/PSP-II (corresponding to 10% of seminal plasma) contributes to maintaining the functionality (membrane and acrosome integrity, motility) of highly diluted boar spermatozoa. [5,6]. In our experiment, although sex sorted spermatozoa were adversely affected by the concentrating procedure, the effect of collection and concentration of sex sorted boar spermatozoa in presence of 10% of seminal plasma or with 1.5 mg/mL of PSP-I/PSP-II heterodimer only affected the kinematics of the spermatozoa. It is important to take into account that best donors were chosen as the source of seminal plasma used in our experiments and a high variability among donors has been previously observed [31]. The beneficial effect of the heterodimer on the function of sex sorted boar spermatozoa suggests that this spermadhesin protein may be suitable as an additive in the collection medium, since its effect is not dependent on the source (semen donor).

Laparoscopic oviductal insemination is a minor surgical procedure that allows semen to be deposited into the site of fertilization, the oviduct, decreasing the loss of spermatozoa by phagocytosis or backflow and increasing the probability of fertilization [32]. Consequently, this insemination method was chosen as an efficient way to evaluate the methodology used to concentrate or store the sex sorted spermatozoa in the present study.

The highest percentage of the penetration was obtained when 0.3×10^6 sexed spermatozoa, concentrated by sedimentation and stored in the presence of PSP-I/PSP-II heterodimer, were deposited directly into the oviduct 16–18 h after sorting. To our knowledge, this is the first time that sex sorted boar spermatozoa, incubated in the presence of PSP-I/PSP-II heterodimer and stored for an extended period (18 h), has been used to penetrate oocytes in vivo. Our results demonstrate that the oviduct generates, at least during the ovulation period, the necessary conditions to initiate the capacitation and hyperactivation of sex sorted spermatozoa stored in presence of both seminal plasma and the heterodimer PSP-I/PSP-II to allow them to penetrate the oocytes. It has been shown that oviductal fluid contains high density lipoproteins and heparin-like glicosaminoglycans which have been shown as physiological capacitation factors [33].

It is well known that the polyspermy block from the porcine oocytes is low compared with other species [34], and high percentages of polyspermic oocytes were expected in our experiments. Surprisingly, regardless of the presence of a high number of sex sorted spermatozoa in the oviduct and regardless of sperm treatment, the incidence polyspermic penetration was very low, showing the potential effectiveness of the laparoscopic insemination as a method for obtaining pregnancies with sex sorted spermatozoa. The oviductal environment may modulate the penetration of oocytes by spermatozoa deposited directly into the oviduct, as a number of authors have reported normal fertility and litter sizes after surgical oviductal deposition by laparotomy of both frozen-thawed [35] and sex sorted frozen-thawed boar spermatozoa [36].

In conclusion, our results demonstrate the benefits that the combination of the sedimentation method and the presence of PSP-I/PSP-II heterodimer in the collection medium have on the in vitro function and in vivo fertilizing ability of sex sorted spermatozoa stored for 18 h.

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7 Discusión General

Discusión general

El desarrollo e implementación de nuevas biotecnologías espermáticas en los últimos años, como la criopreservación y la separación de espermatozoides X e Y, ofrece grandes expectativas al sector porcino. Sin embargo, la baja eficiencia que presentan actualmente estas técnicas, pone de manifiesto la necesidad de desarrollar estrategias que consigan mantener, de forma óptima la funcionalidad espermática. En este sentido, se está trabajando en el desarrollo de nuevos medios de dilución y de conservación de espermatozoides, en el diseño de protocolos de criopreservación, de separación espermática, de manipulación post-separación de los mismos, y en el desarrollo de nuevos métodos de inseminación. Todo ello tiene como objetivo conseguir mejorar la aplicabilidad de estas técnicas a nivel productivo en la especie porcina.

Una de las mejoras realizadas, y ampliamente utilizadas, en los protocolos de separación espermática por citometría de flujo es la adición de plasma seminal al medio de recogida de espermatozoides separados de porcino. Con la adición de plasma seminal se ha conseguido mejorar la funcionalidad de los espermatozoides tras el proceso de separación (Maxwell y cols., 1997; Parrilla y cols., 2004). Sin embargo, la gran variabilidad existente entre el plasma seminal procedente de machos homólogos así como entre eyaculados del mismo macho y fracciones del mismo eyaculado (Killian y cols., 1993; Zhu y cols., 2000; Garner y cols., 2001; Caballero y cols., 2004) aconseja evitar su uso o realizar previamente una selección de los verracos donantes, lo que nos permitiría utilizar únicamente los plasmas seminales más adecuados. Con el objetivo de evitar la variabilidad intrínseca que posee el plasma seminal, algunos estudios sugieren la adición de determinadas proteínas aisladas del plasma seminal a los medios de dilución (Barrios y cols., 2005; Centurión y cols., 2003). Así, estudios previos realizados en porcino sugieren que la glicoproteína PSP-I/PSP-II podría ser el candidato ideal para ser añadido al medio espermático, ya que contribuye a mantener la viabilidad, motilidad y actividad mitocondrial *in vitro*, en espermatozoides altamente diluidos (Vázquez y cols., 2001; Centurión y cols., 2003).

A pesar del interés que ha despertado la posible adición de esta espermadhesina a los medios espermáticos, es necesario conocer más y mejor los mecanismos de acción del heterodímero PSP-I/PSP-II. Nuestro primer objetivo fue conocer cómo actúa el heterodímero y qué epítopos de la proteína son los responsables del efecto protector sobre los espermatozoides altamente diluidos, y saber si es un aditivo apropiado para estabilizar las membranas y mejorar la viabilidad de espermatozoides sometidos a elevadas diluciones como ocurre durante el proceso de separación espermática. Para ello, estudiamos el efecto *in vitro* de las subunidades PSP-I y PSP-II sobre las características funcionales de espermatozoides altamente diluidos. Observamos que la subunidad PSP-II, parece imitar el efecto descrito para el heterodímero (Centurión y cols., 2003) ya que mantiene la viabilidad, motilidad y actividad mitocondrial de los espermatozoides a lo largo del tiempo. En cambio, la subunidad PSP-I aunque también ejerce un efecto protector sobre la viabilidad y actividad mitocondrial, éste es de menor intensidad que el que muestra la subunidad PSP-II. No obstante, hay que destacar que PSP-I ejerce un efecto perjudicial sobre la motilidad espermática, siguiendo un patrón muy similar al que previamente se observó *in vitro* en las espermadhesinas que unen heparina (HBPs) (Centurión y cols., 2003). Dichas proteínas producían una clara inmovilización cuando los espermatozoides sometidos a altas diluciones eran incubados en presencia de HBPs (Centurión y cols., 2003). Sin embargo, el hecho de que la subunidad PSP-I suprima la motilidad espermática, pero no el heterodímero nativo, nos lleva a sugerir que esta actividad podría estar suprimida por la dimerización que se produce al unirse ambas subunidades para formar el heterodímero PSP-I/PSP-II.

Por otro lado, tanto el heterodímero como las subunidades que lo forman, fueron digeridos mediante tripsina con el fin de evaluar si el efecto protector de la espermadhesina se encuentra vinculado o no a la estructura proteica cuaternaria del heterodímero. Tanto el heterodímero nativo como sus subunidades PSP-I y PSP-II mostraron el mismo efecto beneficioso sobre los espermatozoides que las formas nativas (sin digerir), lo que nos sugiere que la estructura cuaternaria de la proteína no intervine en el efecto que ejercen dichas proteínas sobre las células espermáticas. Por último, los resultados obtenidos al valorar si es la fracción glicídica o peptídica la parte del heterodímero responsable de ejercer ese efecto protector sobre los espermatozoides, sugirieron que es en la fracción peptídica donde reside la capacidad del

heterodímero PSP-I/PSP-II de mantener la funcionalidad espermática. Todo esto indica, en términos generales, que las subunidades PSP-I y PSP-II ejercen diferentes actividades sobre las características funcionales espermáticas. Más aún, la capacidad de proteger a los espermatozoides parece residir en gran medida, en su subunidad PSP-II y, concretamente, en la fracción peptídica del heterodímero.

Además de entender el mecanismo de acción del heterodímero PSP-I/PSP-II, es fundamental conocer de forma exacta en qué tejidos del tracto genital del verraco se localizan y/o expresan las proteínas PSP-I y PSP-II con el fin de determinar en qué momento entran en contacto los espermatozoides con dichas proteínas y dónde y cuándo la espermadhesina PSP-I/PSP-II ejerce su función.

Entre las distintas técnicas que facilitan el estudio de localización y expresión de las proteínas PSP-I y PSP-II en los tejidos del tracto genital del verraco encontramos las técnicas de inmunohistoquímica, western-blott y RT-PCR. Sin embargo, los resultados obtenidos mediante estos procedimientos por diversos grupos de investigación han sido contradictorios (Kwok y cols., 1993; Ekhlasi-Hundrieser y cols., 2002). Con el fin de obtener resultados objetivos, en la segunda experiencia aplicamos las 3 técnicas anteriormente citadas, para localizar las proteínas PSP-I y PSP-II en diferentes tejidos del tracto genital (testículo, epidídimo, vesícula seminal y glándula bulbouretral). En esta experiencia, los resultados mostraron que ambas subunidades (PSP-I y PSP-II) son expresadas en cada uno de los tejidos del tracto genital del verraco estudiados, concretamente en testículo (epitelio seminífero), vesícula seminal, glándula bulbouretral y epidídimo (cabeza, cuerpo y cola). Sin embargo, estos resultados contrastan con los obtenidos anteriormente por otros grupos de investigación que aplicaron distintos procedimientos para el estudio de localización de dichas proteínas. Kwok y cols. (1993) muestran que la espermadhesina PSP-I es sintetizada únicamente en el epitelio de las vesículas seminales, mientras que Ekhlasi-Hundrieser y cols. (2002) describen que además de ser expresadas ambas subunidades en las vesículas seminales, éstas son también localizadas en la cola del epidídimo, próstata y en la red testicular, siendo el nivel de expresión en este último tejido para PSP-II significativamente más bajo que para PSP-I. Además, en este último estudio, la subunidad PSP-I fue localizada también en la cabeza y en el canal del epidídimo, aunque en

niveles de expresión más bajos que en el resto de tejidos (Ekhlasi-Hundrieser y cols., 2002).

Cabe destacar que, a pesar de que ambas subunidades son detectadas a lo largo del epidídimo, el heterodímero PSP-I/PSP-II no ha sido detectado en la superficie de espermatozoides epididimarios (Dostàlovà y cols., 1994). Estos datos podrían sugerir que la espermadhesina PSP-I/PSP-II interaccione de forma lábil con la superficie del espermatozoide, aunque esta ausencia podría ser interpretada, a su vez, en la misma línea que los estudios que muestran que las proteínas producidas en el epidídimo son rápidamente degradadas o reabsorbidas (Syntin y cols., 1996; Castella y cols., 2003). El heterodímero PSP-I/PSP-II, sin embargo, ha sido claramente detectado en la membrana de espermatozoides eyaculados (Caballero y cols., 2006). Este resultado no fue sorprendente, ya que el 75% de las proteínas que componen el plasma seminal pertenecen a la familia de las espermadhesinas, siendo PSP-I y PSP-II las espermadhesinas más abundantes en el verraco (Calvete y cols., 1995). Además, ambas proteínas están principalmente sintetizadas en las vesículas seminales (Kwok y cols., 1993; Ekhlasi-Hundrieser y cols., 2002), que son la principal fuente de proteínas del plasma seminal en el eyaculado. La presencia del heterodímero PSP-I/PSP-II en la membrana de espermatozoides eyaculados podría estar también justificada basándonos en su efecto protector al unirse a la superficie del espermatozoide (Caballero y cols., 2006; Vadnais y cols., 2007) estabilizando las membranas y regulando el proceso de capacitación (Jansen y cols., 2001).

Como hemos comentado anteriormente, hay estudios que establecen que mientras las HBPs ejercen un efecto nocivo sobre la fisiología espermática *in vitro*, la espermadhesina PSP-I/PSP-II ejerce el efecto contrario y que la mezcla de ambos tipos de espermadhesinas manifiestan efectos intermedios sobre espermatozoides altamente diluidos (Centurión y cols., 2003). Por tanto, parece lógico pensar que la variabilidad entre las concentraciones relativas de ambas proteínas del plasma seminal podrían ser las responsables de las diferentes respuestas que muestran los espermatozoides cuando son mezclados con el plasma seminal, ya que son las más abundantes. Para ello en la tercera experiencia nos planteamos como objetivo estudiar cómo afecta el perfil proteico de las diferentes fracciones que componen el eyaculado a los parámetros

seminales de espermatozoides altamente diluidos y así como la distribución del heterodímero PSP-I/PSP-II a lo largo de estas fracciones.

Los resultados obtenidos en esta experiencia muestran que la concentración del heterodímero no fue uniforme ni entre las fracciones en las que se dividió el eyaculado, ni entre las subfracciones en las que se dividió la fracción post-espermática. La fracción post-espermática presentó mayor proporción de la espermadhesina PSP-I/PSP-II, además de los máximos valores de proteínas totales, mientras que la fracción rica fue la fracción con menor concentración proteica. Sin embargo, al evaluar el efecto del plasma seminal procedente de las diferentes fracciones sobre espermatozoides de verraco altamente diluidos, los resultados mostraron que la adición del 10% de plasma seminal de las distintas fracciones mejoró la viabilidad y motilidad comparado con los espermatozoides que habían sido incubados sin plasma seminal. No obstante, la adición del plasma seminal procedente de la fracción rica indujo los mejores resultados de motilidad e integridad de membrana, coincidiendo con los resultados obtenidos por Peña y cols. (2006). Hay que tener en cuenta que la presencia del heterodímero PSP-I/PSP-II en esta fracción fue baja y que, a pesar de la gran cantidad de heterodímero que presenta la fracción post-espermática, el efecto del plasma seminal de dicha fracción sobre los espermatozoides altamente diluidos no fue tan beneficioso como el que produjo la fracción rica. Estas diferencias podrían estar relacionadas con la concentración de PSP-I/PSP-II presente en cada fracción. Se conoce el efecto que protector que ejerce el heterodímero sobre espermatozoides altamente diluidos, se corresponde con una concentración específica de la espermadhesina (1’5 mg/mL). Concentraciones superiores a 7’5 mg/mL, provocan que el efecto positivo aparentemente desaparezca (Centurión y cols., 2003). Sin embargo, aunque la fracción post-espermática es la fracción que produce los peores porcentajes de integridad de membrana y motilidad sobre los espermatozoides comparado con el resto de fracciones, la subfracción de la fracción post-espermática que causó menos daño sobre las células espermáticas fue aquella en la que el heterodímero era predominante. Asimismo, el efecto negativo que ejerce el plasma seminal de la fracción post-espermática podría ser también debido a la presencia de las proteínas HBPs, ya que su concentración aumenta de forma paralela a la del herterodímero PSP-I/PSP-II en la fracción post-espermática. Estos resultados coinciden con los obtenidos por Centurión y cols. (2003), que mostraron que al incubar los espermatozoides de verraco altamente diluidos en presencia de todas

las espermadhesinas (PSPs y HBPs) la funcionalidad espermática sufría un descenso debido a la presencia de HBPs.

Por lo tanto, los distintos efectos que ejerce el plasma seminal de las diferentes fracciones del eyaculado sobre la funcionalidad de espermatozoides altamente diluidos, podría estar debida a los distintos perfiles proteicos que presentan cada una de las fracciones del plasma seminal. Además, debería evitarse la adición de plasma seminal de la fracción post-espermática al medio espermático con el fin de conservar la funcionalidad de espermatozoides de verraco altamente diluidos a lo largo del tiempo.

Con el objetivo de contribuir a que la separación de espermatozoides X e Y mediante citometría de flujo pueda ser aplicada, de forma eficaz, en el campo de la reproducción porcina, en la última experiencia se desarrollaron estrategias que optimizaran los rendimientos del proceso de separación espermática y de la manipulación post-separación, con el fin de prolongar la vida útil de los espermatozoides separados hasta el momento de la inseminación. En esta experiencia empleamos el uso de la espermadhesina PSP-I/PSP-II como aditivo al medio de recogida de espermatozoides separados, basándonos en los resultados obtenidos previamente que demuestran su capacidad de mantener la funcionalidad de espermatozoides sometidos a altas diluciones utilizada a 1.5 mg/mL (concentración correspondiente al 10% de plasma seminal) (Centurión y cols., 2003). El uso de dicha proteína tiene además la ventaja de evitar la variabilidad proteica contenida en el plasma seminal.

Otro punto crítico del proceso de separación espermática es la necesidad de concentrar células espermáticas tras el proceso de separación, siendo la centrifugación el método más utilizado. Sin embargo, los espermatozoides de mamíferos son susceptibles a esta metodología ya que puede provocar daños en el acrosoma, reducir su funcionalidad y perjudicar a su capacidad fecundante (Maxwell y Johnson, 1997; Garner y cols., 2001). La sedimentación espermática ha sido utilizada como una alternativa a la centrifugación para concentrar espermatozoides mótiles y funcionales en muestras seminales de baja calidad en la especie humana (Henkel y Schill, 2003). Acorde con estos resultados, nuestro estudio mostró que la sedimentación es proceso más adecuado de concentración espermática tras la separación por citometría de flujo que la centrifugación. Mediante sedimentación se obtiene una mayor recuperación espermática en términos tanto de concentración como de calidad espermática. Además, el mejor

porcentaje de penetración in vivo fue obtenido cuando los espermatozoides separados fueron concentrados mediante sedimentación y conservados en presencia del heterodímero PSP-I/PSP-II. Con relación al sistema de inseminación laparoscópica, inseminar directamente en el oviducto no originó ningún problema ya que parece que el oviducto genera, al menos durante el período de ovulación, las condiciones necesarias para iniciar el proceso de capacitación e hiperactivación de los espermatozoides separados en presencia de la espermadhesina PSP-I/PSP-II, lo que les permitirá fecundar los ovocitos.

La sedimentación en combinación con la adición del heterodímero PSP-I/PSP-II al medio de recogida mantiene la funcionalidad espermática durante, al menos, 18 horas. Esto permite llevar a cabo inseminaciones con semen separado en aquellas explotaciones que se encuentran a grandes distancias del laboratorio.

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8 conclusiones

conclusiones

1. Las subunidades PSP-I y PSP-II, que componen el heterodímero nativo PSP-I/PSP-II, ejercen diferentes actividades sobre los parámetros funcionales espermáticos. El efecto beneficioso que ejerce el heterodímero sobre la funcionalidad espermática en espermatozoides altamente diluidos parece estar en gran parte conservada en la subunidad aislada PSP-II, y no parece necesitar para ello la fracción glicídica.

2. Las espermadhesinas PSP-I y PSP-II están expresadas en diferentes tejidos del tracto genital del verraco. Las vesículas seminales son la principal fuente de producción de PSP-I y PSP-II, aunque los segmentos epididimales, los testículos y la glándula bulbouretral participan también en la expresión de dichas proteínas.

3. Las fracciones que componen el plasma seminal ejercen efectos distintos sobre la funcionalidad de espermatozoides de verraco sometidos a altas diluciones, quizás debido a los distintos perfiles proteicos que muestran cada una de las fracciones. Con el fin de conservar la funcionalidad de espermatozoides de verraco altamente diluidos a lo largo del tiempo debe evitarse la adición de plasma seminal de la fracción post-espermática.

4. La utilización de la espermadhesina PSP-I/PSP-II en el medio de recogida de espermatozoides separados en combinación con el método de concentración por sedimentación, beneficia la funcionalidad *in vitro* de los espermatozoides separados, así como su capacidad fecundante *in vivo* tras 18 horas de conservación.

9 Extended Summary

Extended summary

INTRODUCTION

Biotechnological procedures of semen, such as cooling and deep-freezing or sexing using flow cytometry/cell sorting procedures, causes several changes in the sperm structures which involve membrane destabilization and a physiological unstable state for the spermatozoa (Maxwell and Johnson, 1999; Garner et al., 2001). High dilutions, which occurs during sperm manipulation, is linked with the wash away or high dilution of seminal plasma components (Ashworth et al., 1994; Maxwell et al., 1997; Maxwell and Johnson, 1999). Thus, to develop strategies to extend the viability of treated spermatozoa are necessary.

The seminal plasma, the fluid in which mammalian spermatozoa are immersed at the ejaculation, comprises a wide variety of factors (amino acids, lipids, fatty acids, osmolytes, peptides, and proteins) that influence the viability and fertilizing capacity of ejaculated spermatozoa (Mann and Lutwak-Mann, 1981; Shivaji et al, 1990; Yanagimachi, 1994). Particularly, seminal plasma contains specific protein factors that play important roles on the sperm cell as their maturation and development in the male reproductive tract, or on the transportation and survival of viable spermatozoa in the female reproductive tract and gamete interaction following egg fertilization (Troedsson et al., 2005; Strzezek et al., 2005).

In this regard, it is well known that seminal plasma contributes to preserving the integrity and the fertilizing potential of sperm and provides also metabolic support to spermatozoa (Killian et al., 1993). Moreover, several studies have been reported that the addition of whole seminal plasma to the sperm media may attenuate the removal of many beneficial components of seminal plasma improving sperm functionality after dilution (Maxwell and Johnson, 1999; Garner et al., 2001; Centurion et al., 2003; Caballero et al., 2004). Nevertheless, although seminal plasma have been associated with beneficial or deleterious effects on sperm physiology and fertility (Centurión et

al., 2003; Caballero et al., 2004; Kraus, 2005; Maxwell et al., 2007) the beneficial effects of seminal plasma seem to be restricted to specific proteins (Centurión et al., 2003; Barrios et al., 2005).

Previous studies have related this protective effect of the seminal plasma on the sperm cells to a protein called PSP-I/PSP-II heterodimer (Centurión et al., 2003). The PSP-I/PSP-II heterodimer belows to the spermadhesin family which are the major protein components of the boar seminal plasma (Calvete et al, 1995 and Töpfer-Petersen et al., 1998) representing over 90% of the total boar seminal plasma proteins (Dostàlovà et al, 1994). These (glyco)proteins are 14-16 KDa and are built by a single CUB domain (Romero et al., 1997). Depending on their binding capability, spermadhesins can be conveniently classified into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding (PSP-I/PSP-II heterodimer; Calvete et al., 1994). Sequence variation, glycosylation, and the aggregation state of spermadhesins contribute to their specific pattern of biological activities (Calvete et al, 1993a; Calvete et al, 1993b; Dostàlovà et al, 1995). The heparin-binding spermadhesins bind zona pellucida glycoconjugates (Sanz et al, 1992) and have been involved in sperm-egg recognition (Rodríguez-Martínez et al, 1999). In addition, AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins and may act as decapacitation factors preventing premature acrosome reactions (Sanz et al., 1992). These spermadhesins are thought to stabilize the plasma membrane over the acrosomal vesicle and are mainly released from the spermatozoa surface during capacitation (Sanz et al, 1993; Dostàlovà et al, 1994; Calvete et al, 1997).

On the other hand, PSP-I and PSP-II, the most abundant boar seminal plasma proteins, form a non-heparin-binding heterodimer that represents more than 50% of the total boar seminal plasma proteins (Calvete et al., 1995) and display pro-inflammatory and immunostimulatory activities in the uterus of the rat (Assreuy et al., 2002, 2003) and pig (Rodriguez-Martinez et al., 2005). The purpose of these proinflammatory and immunostimulatory activities would be to prevent possible infections of the lower reproductive tract and to provide a foreign cell-free uterine environment for the descending early embryos.

Besides their physiological functions in porcine reproduction, the heparin-binding and the non-heparin-binding spermadhesins exert opposite effects on spermatozoa which have been subjected to high dilution to mimic the conditions

of sex sorting by flow cytometry (Centurión et al., 2003). Moreover, while the pooled heparin-binding spermadhesins (AQN-1, AQN-3, and AWN) cause a concentration-dependent sperm membrane damage, the purified non-heparin-binding PSP-I/PSP-II spermadhesin complex preserves - *in vitro*- the membrane integrity, motility and mitochondrial activity of highly extended boar spermatozoa (Centurion et al., 2003). This beneficial effect points to this spermadhesin as a candidate to improve, by addition in the dilution extenders, the sperm functionality on highly diluted porcine spermatozoa (i.e. sex sorted spermatozoa). Thus, the aim of the first study was to dissect the structural basis of the protective effect of boar spermadhesin PSP-I/PSP-II, its isolated PSP-I and PSP-II subunits, and their derived peptidic and glycan fractions on the sperm functionality by a time-course evaluation of the viability, the acrosomal status, the mitochondrial activity and the motility of highly diluted boar spermatozoa.

It is well known that the proteins that make up seminal plasma are secreted by the testis, epididymis and the accessory sexual glands which influence the viability and fertilizing capacity of ejaculated spermatozoa (Mann and Lutwak-Mann, 1981). However, controversial results have been found about the localization and expression of the PSP-I and PSP-II spermadhesins along the porcine reproductive tract. Both PSP-I and PSP-II proteins have been reported to be synthesized by the epithelium of the seminal vesicles, the caudal epididymis and the prostate in swine. In addition, PSP-I has been found in caput epididymis and in the rete testis (Ekhlasi-Hundrieser et al, 2002). In contrast, Kwok et al. (1993) showed the presence for PSP-I only in the epithelium of the seminal vesicles of the pig. These differences may be related to the different techniques applied as well as the age, breed or fertility of the boars used. Hence, since the results about the expression and localization of the PSP-I/PSP-II subunits are contradictories, in the second study we sought to disclose the epithelial localization and expression of spermadhesins PSP-I and PSP-II and their mRNAs in the testis, epididymis and main accesory sexual glands of mature, fertile boars by immunohistochemistry, SDS-PAGE and Western blot and RT-PCR techniques.

On the other way, although under physiological conditions the whole boar ejaculate is deposited into the female genital tract, it is well known that the boar ejaculate is emitted in fractions with different characteristics, called *pre-sperm rich fraction*, *sperm- rich fraction* and finally *post-sperm rich* fraction (Ghaoui et al., 2004). Moreover, the high variability existing among seminal plasma of

ejaculates from homologous males, among ejaculates from the same male as well as among different fractions of the same ejaculate, has been reported (Killian et al., 1993; Zhu et al., 2000). Even, different seminal plasma protein profiles have been found between boars of different fertility (Flowers and Turner, 2001).

In addition, Caballero et al. (2004) showed that, depending on the source, seminal plasma can exert a positive or a negative effect on highly extended boar spermatozoa. The effects exerted by seminal plasma on sperm functionality have been in part ascribed to the variability in the composition and concentration of certain species-specific or widely distributed proteins (Maxwell and Johnson, 1999; Centurión et al., 2003; Dacheaux et al., 2003). Thus, the high variability of seminal plasma protein profiles suggests that males should be screened before selection as seminal plasma donors.

In spite of the sperm rich fraction is the only ejaculated fraction used for artificial insemination, recent studies have showed that collecting the post-sperm fraction (which has high amounts of seminal plasma proteins; Rodriguez-Martinez y cols., 2005) could be beneficial for the sperm cells, providing a suitable environment for them and even improving DNA fragmentation rate (Fraser and Strzezek, 2006). However, in this respect and according to the variability in the concentration of proteins, in a previous study we showed that the non-heparin-binding PSP-I/PSP-II heterodimer exerted a concentration- and time-dependent sperm function-preserving activity, whereas the pool of the heparin-binding spermadhesins (HBS) had a deleterious effect on sperm physiology (Centurión et al., 2003), as we have described above. Moreover, different molar ratios of PSP-I/PSP-II-to-HBS displayed intermediate effects. Hence, it seems plausible to hypothesize that variability in the relative concentrations of sperm-protecting (PSP-I/PSP-II) versus sperm-detrimental (HBS) seminal plasma proteins would be responsible for the distinct response of spermatozoa upon mixing with the accessory gland fluids.

In the third experience we aimed to evaluate how the protein profile of the different seminal plasma fractions affect to sperm functionality monitored as membrane integrity and motility characteristics, when spermatozoa were subjected to similar dilution as in the process of sex sorted sperm by flow cytometry, in presence of 10% of different seminal plasma fractions. At the

same time, evaluate the presence of the well-known protective PSP-I/PSP-II heterodimer on these fractions was also an objective of this experience.

Finally and from a practical point of view, the major problem of gender sex preselection by flow cytometry sperm sorting is the low number of sex sorted spermatozoa obtained per time unit. This low outputs implies long sorting time in order to get an adequate number of sorted boar spermatozoa (Maxwell et al., 1997). Moreover, it is know that the sperm membrane is adversely affected by the flow cytometric sorting procedure, compromising the viability, storage capability and fertilization ability of spermatozoa. The removal of many seminal plasma components from the sperm surface, mainly proteins, which are beneficial for the stability of sperm membranes, could be the main reason of these changes in sperm cells (Maxwell and Johnson, 1999). In this sense, several authors described that the absence of these components may affect the functionality and fertilization capacity of sex sorted spermatozoa (Maxwell et al., 1997; Maxwell and Johnson, 1999; Maxwell et al., 2007). Consequently, strategies to extend the lifespan of sex sorted spermatozoa should be developed before this technology can be applied in a practical way.

Supplementation of sorted boar spermatozoa collection media with 10% of seminal plasma has been used often used to attenuate the adverse effects of removing the beneficial seminal plasma components, and seems to stabilize the sperm plasma membrane and to reverse the sperm capacitation or pre-capacitacion status after flow sorting (Maxwell et al., 1998; Parrilla et al., 2004). Moreover, similar results have been found with highly diluted boar spermatozoa when the PSP-I/PSP-II heterodomer spermadhesin from porcine seminal plasma was added to the dilution media (Centurion et al., 2003).

The use of the isolated seminal plasma protein PSP-I/PSP-II heterodimer as an additive to the collection medium for sex sorted boar spermatozoa could have the advantage of use just the beneficial protein components of the seminal plasma, avoiding the detrimental effects of use whole seminal plasma. It has been shown that the presence of 1.5 mg/ml of PSP-I/PSP-II (corresponding to 10 % of seminal plasma) in the dilution media, contributes to maintain the functionality of highly diluted boar spermatozoa (Centurion et al., 2003). In this way, we thought that postsorting handling procedures are also an important point to study, in order to maintain optimal viability levels of sex sorted boar

spermatozoa. Previous experiences carried out in our laboratory had shown that sedimentation process instead centrifugation, for concentration of sex sorted boar spermatozoa is more adequate than centrifugation when long storage of sex-sorted boar sperm is required (Parrilla et al., 2006). In the last experience of this work we tried to minimize the detrimental effects of flow cytometric sex sorting technology on boar spermatozoa, using two different collection mediums (containing seminal plasma or PSP-I/PSP-II heterodimer) in combination with two different post-sorting handling procedures (centrifugation vs sedimentation). We evaluated the sperm functionality and also the in vivo fertilizing ability of sex sorted boar spermatozoa collected in presence of seminal plasma or PSP-I/PSP-II heterodimer and subjected to centrifugation or sedimentation as necessary step for sexed sperm concentration before its use for insemination.

MATERIAL AND METHODS

All reagents were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

Preparation of Seminal Plasma (paper 1, 3, 4)

All experiments were performed using the seminal plasma from mature boars, which had previously sired offspring. Ejaculates were collected using the gloved-hand method (Larsen, 1986). The seminal plasma was separated from spermatozoa by centrifugation at 3,800 x g for 15 min at room temperature using a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatants were filtered sequentially through 10 and 1.2 μm filters and pooled.

In paper 3 we previously achieved an experiment regarding the ejaculations profiles (ejaculation time and volume) from 20 fertile mature boars, which had previously sired offspring. From these 20 boars we selected 3 of them with similar ejaculation profile, as seminal plasma donors, for all experiments.

Isolation of the Boar Seminal Plasma PSP-I/PSPII Heterodimer (paper 1, 2, 4)

The PSP-I/PSP-II heterodimer was isolated from the non-heparin binding fraction of boar seminal plasma by affinity chromatography on a heparin-Sepharose column, equilibrated in 100 mM Tris-HCl, 150mM NaCl, 5 mM EDTA and 0.025% sodium azide, pH 7.4, as previously described (Calvete et al, 1995). The identity and purity of the protein was assessed by N-terminal sequence analysis using an Applied Biosystems 472 automated protein sequencer (Langen, Germany) and MALDI-TOF mass spectrometry using and Applied Biosystems Voyager DE-Pro mass spectrometer (Applied Biosystems, Langen, Germany) and a saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid as the matrix. Protein concentration was determined spectrophotometrically using the molar absorption coefficient (27332 $M^{-1}cm^{-1}$) determined by Menéndez *et al*. (1995) or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 h at 106 ºC in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water and lyophilized.

Isolation of Boar Seminal Plasma PSP-I and PSP-II Subunits (paper 1, 2)

The PSP-I and PSP-II subunits were purified from the heterodimer by reverse-phase HPLC on a Lichrocart column (250 x 10 mm, RP-18, 7 μm particle size) (Merck, Germany) eluted at 2 ml/min with a mixture of 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in acetonitrile (solution B), first isocratically (10% B) for 5min, followed by 30% B 10 min, 45% B for 45 min, 70% B for 15 min and 10% B for 15 min. The purified PSP-I and PSP-II subunits were dialysed against distilled water and lyophilized. Purity and protein concentration were determined as above.

Isolation of Peptides and Glycopeptides (paper 1)

To separate the peptidic and the glycan moieties, the 100 mg of the PSP-I/PSP-II heterodimer were digested overnight at 37 ºC with trypsin using a 1:100 (w/w) enzyme: substrate ratio. Thereafter, the enzyme was inactivated by heating at 100 ºC for 2 min, and the reaction mixture was lyophilized. Completion of proteolysis was checked by SDS polyacrylamide gel electrophoresis, reversed-phase HPLC, and MALDI-TOF mass spectrometry. Non-glycosylated tryptic peptides were separated from glycopeptides by affinity chromatography on a 5ml Sepharose-Concanavalin A column (Amersham

Biosciences) equilibrated and eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS). ConA-bound glycopeptides were eluted with equilibration buffer containing 100 mM methyl-α-D-mannopyranoside. The glycopeptide fraction was desalted on a C-18 Lichrosphere column (250 x 4 mm, 5 µm particle size) (Merck, Germany) equilibrated and washed with 0.1% TFA until the absorbance at 214 reached baseline level, followed by elution with 0.1% TFA and 50% acetonitrile. Peptide concentration was determined by amino acid analysis.

Collection of Semen (Paper 1, 3, 4)

Sperm-rich fractions from fertile mature boars were collected by gloved-hand method and extended to 30 x 10^6 sperm/mL in Beltsville Thawing Solution (BTS) (Pursel and Johnson, 1975). Samples were evaluated for normality (motility>80%, viability > 85%, total sperm count per ejaculate >20×10^9, acrosomal abnormalities <10%, abnormal sperm morphology <15%). Only samples that met the minimum criteria were used (Vazquez et al., 1997).

Spermatozoa were separated from seminal plasma by centrifugation at 1,200 x *g* (Megafuge 1.0 R, Heraeus, Germany) during 3 min. To avoid sperm membrane damage caused by pipetting spermatozoa directly into dilution medium (Maxwell and Johnson, 1999), the pellet was serially diluted in PBS to a final cell count of 1 x 10^6 sperm/mL and incubated at 38 ºC (Steri-Cult 200 incubator, Ohio, U.S.A.).

Evaluation of Sperm Parameters by Flow Cytometry (Paper 1, 3, 4)

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm and the EXPO 2000 software. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Non-sperm events (debris) were gated out based on the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to sperm cells but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10,000 events per sample. Samples were measured at flow rate of ≈300 cells/sec.

Flow Cytometric Assessment of Sperm Viability and Acrosomal Exocytosis

The membrane and the acrosome integrity of spermatozoa were assessed simultaneously by flow cytometry using the triple staining protocol described previously [Nagy et al., 2003, García et al., 2006]. Briefly, 500 μL of sperm sample was incubated with 50 nM SYBR-14 (using a 100 μM stock solution in DMSO; component A of LIVED/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, Netherlands), 0.5 μg/mL PE-PNA (peanut agglutinin conjugated with phycoerythrin; Biomeda Corp., Foster City, CA) and 7.5 μM propidium iodide (PI). The samples were mixed and incubated at 37ºC in the dark for 10 min before flow cytometric analysis. SYBR-14 was measured using a 525 nm band pass filter, PI was collected through a 620 nm band pass filter and PE-PNA was detected through a 575 band pass filter. Viable spermatozoa with intact acrosomes were defined as those stained only with SYBR-14. Acrosome reacted spermatozoa were defined as those stained with SYBR-14 and PE-PNA. Spermatozoa stained with PI were classified as dead cells.

Flow Cytometric Assessment of Sperm Mitochondrial Membrane Potential (Paper 1)

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzymidazolyl carbocianyne iodide; Molecular Probes Europe, Leiden, The Netherlands), a stain which differentiate cells exhibiting high and low mitochondrial membrane potential (Peña et al, 2003), was used to assess the mitochondrial membrane potential of spermatozoa as described in (Martínez-Pastor et al, 2004) with slight modifications. 500 μL of sperm samples were incubated in a water bath at 37 ºC in dark for 20 min with 0.2 μM JC-1, followed by flow cytometric measurement through a 590 nm band pass filter. At low membrane potential, JC-1 exists as a green fluorescent monomer, whereas at higher potentials JC-1 forms "J-aggregates" after accumulation in mitochondria that emit a red-orange florescence at 590 nm (Garner and Thomas, 1999; Gravance et al, 2000).

Sperm Motility (Paper 1, 3, 4)

Sperm motility was estimated by a computer-assisted sperm motility analysis (CASA) system using the software Sperm Class Analyzer (S.C.A.®, Microptic, 2002. Barcelona, Spain). Aliquots of 10 μL of semen samples (at 10^6

spermatozoa/ml) were placed in a warm (38 ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38 ºC) of a Nikon Labophot positive-phase contrast light microscope (Tokio, Japan) equipped with a 10x objective and a monochrome video camera (Hitachi CCD model, Chiba, Japan), connected to a personal computer. The program settings were as in (Centurión et al, 2003). Objective percentage of motile spermatozoa was analyzed in at least 100 spermatozoa per sample.

Preparation of PSP-I and PSP-II antibodies (Paper 2)

Polyclonal anti-PSP-I anti-PSP-II monospecific antibodies were obtained by immunizing female rabbits with subcutaneous injections of 0.5 mg of purified PSP-I and PSP-II subunits in 0.5 mL of PBS emulsified with 1.5 mL of Freund's complete adjuvant. The animals were inoculated twice at intervals of 5 weeks after the first injection with 0.25 mg of the antigen. Two weeks after the last administration, the rabbits were bled through the ear vein and the blood sera were tested for anti PSP-I and PSP-II activity by dot-blot ELISA and Western Blot.

Inmunohistochemistry (Paper 2)

The tissues were fixed in 1% paraformaldehyde solution in PBS, dehydrated in a graded series of ethanol and embedded in paraffin. Immunolocalization of PSP-I and PSP-II proteins in male boar tissues was done using an Elite ABC-staining procedure (Vectastain Elite ABC Kit; Vector Laboratories; Burlingame, CA, USA). Briefly, 5 µm tissue sections were prepared from the paraffin-embedded material using a Leitz microtome and mounted on poly-L-lysine-coated glass slides (Sigma). Thereafter, the tissue sections were deparaffinized, rinsed in PBS for 5 min and incubated with 3% of H_2O_2 in PBS for 20 min, in order to block endogenous peroxidase activity. Non-specific protein binding was prevented by incubation in 10% normal goat serum for 30 min at 20ºC. After this step, and without rising, samples were incubated overnight at 4ºC with the primary polyclonal antibodies PSP-I or PSP-II (1:1000 v/v in PBS). The sections were then rinsed in PBS (5 min) before incubation with biotinylated goat anti-rabbit IgG antibody (1 : 1000) for 30 min. All sections were rinsed again in PBS for 5 min prior to application of the ABC-Elite complex. Visualization of antibody-antigen complexes was done using diaminobenzidine

tetrahydrochloride with 3% H_2O_2 (DAKO® DAB Chromogen tablets; CA, USA) for 5-6 min. Sections were mounted with glycerine-gelatin. Negative controls were run by omission of the primary antibody. As positive controls, samples of freshly ejaculated boar spermatozoa were subjected to the same protocol as above with polyclonal antibody against PSP-II. Because of the reported weak association of PSP-I/PSP-II to the sperm surface (Calvete et al., 1995), the fresh ejaculated spermatozoa were not subject to any treatment, including washing or removal of seminal plasma. A liver tissue extract was used as a negative (background) control. All incubations were performed in a humidified chamber. Selected sections were photographed with a Nikon microphot-FXA photomicroscope (Nikon, Tokyo).

RNA extraction (Paper 2)

Samples were quick-frozen in liquid nitrogen and stored at -80ºC until RNA extraction. RNA was isolated from the following tissues: testis, seminal vesicles, bulbourethral gland and epididymis (caput, corpus and caudal epididymis) of 10 boars using TRIzol Reagent (Invitrogen) according to the Chomczynski & Sacchi (1987) protocol. To this end, 50 mg of each tissue were homogenized in 1mL TRIzol Reagent (mixture of acid phenol and guanidine isothiocyanate) using polypropylene mini-pestles (Sigma). The homogenates were stored for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. For total RNA extractions from the homogenates, 0.2 mL chloroform were added followed by centrifugation at 4ºC, and the aqueous phase was recovered. RNA was precipitated from the aqueous phase by addition of isopropanol, incubation for 15 min at -20ºC, followed by centrifugation at 4ºC. Pellets were successively washed once with 1 mL of ethanol 75% and once with 1 mL absolute ethanol, air dried and solubilized in 20 µL DEPC-treated water.

DNAase treatment (Ambion) of the total RNA fraction was carried out before first-strand cDNA synthesis. Total RNA was quantified spectrophotometrically at $O.D._{260\ nm}$ and the purity was assessed by the $O.D._{260}/O.D._{280}$ ratio. All samples had $O.D._{260}/O.D._{280}$ ratios higher than 1.8. RNA integrity was checked by electrophoresis of 1 µg of sample on a 2% agarose gel containing formamide (RNA sample loading buffer). Gels were stained with ethidium bromide.

Reverse Transcription (Paper 2)

Reverse transcriptions and PCRs were performed with a termocycler (Personal Master Cycler, Eppendorf) with hot-lid. Reverse transcription of RNA into cDNA was carried out in a total volume of 20 µL. Briefly, 5 µg of DNAase-treated RNA was mixed with 1 µL of oligo $(dT)_{20}$ (50 µmol/L), heated for 5 min at 65ºC and then placed on ice. Thereafter, 4 µL of 5x RT buffer, SuperScript^{TM} III Reverse Transcriptase (200 U/µL), dNTPs 25 µmol/L, 1 µl of DTT (0.1 mol/L) and 1 µL of Rnase out (40 U/µL) were added and incubated for 55 min at 50ºC. The reaction mixtures were then heated for 15 min at 70ºC to desactive the enzyme, and then cooled at 4ºC.

PCR Amplification of PSP-I and PSP-II (Paper 2)

PSP-I and PSP-II primers were those designed by Ekhlasi-Hundrieser et al (2002), according to cDNA sequences obtained by Kwok et al (1993):

PSP-I → forward: 5´-TTCAACAGGATGGGGCTTGG-3´
Reverse: 5´-GAAGGAAAATGATCTCATAGGG-3´

PSP-II → forward: 5´-GCACGGATCAATGGCCCTG-3´
Reverse: 5´-TTCGGATCCTGGTGAACTAC-3´

The master mix contained 0.4 µL of 4 dNTPs (dTTP, dATP, dGTP and dCTP each at 2 mmol/L), 2.5 µL of primers (10 pmol/µL stock solution), 5 µL of reverse-transcribed cDNA, 5 µL of 10 × PCR buffer (100 mmol/L Tris-HCl, 15 mmol/L MgCl2, 500 mmol/L KCl, pH 8.3 at 20ºC) and 0.5 µL of 5 U/µL Taq DNA polymerase. Five µL of cDNA was added to the PCR reaction mixture to yield a total volumen of 50 µL. The mixture was denatured at 95ºC for 5 min and amplified by 35 cycles of 30s at 94ºC (denature), 30s at 58ºC (annealing) and 30s at 72ºC (elongation). Following 5 min at 72ºC, the reaction mixture was cooled at 4ºC. Reactions products were visualized on 1% ethidium bromide stained agarose gels.

Β-Actin gene (233 pb) was used as a positive control to confirm the presence of cDNA, and negative controls were set up using RNA instead of cDNA to role out genomic DNA contamination. The β-actin primers were: forward, 5´-GAGAAGCTCTGCTACGTCGC-3´; reverse: 5´-CCAGACAGCACCGTGTTGGC-3´ (Ponsuksili et al., 2001). PCR-amplified band intensities were normalized to β-actin mRNA content and scored from 0 (negative) to +++ (highest expression).

Western Blotting (Paper 2)

Boar tissues in PBS buffer containing a cocktail of protease inhibitors (Complete Mina EDTA-free, Roche, Germany) were homogenized on ice using a Polytron (Polytron Kinematica AG, Litlan-Luzern, Switzerland). The homogenates were centrifuged at 3939 × g for 10 min at 4ºC in a Heraeus Labofugue 400R centrifugue. Protein concentration was determined using the BCA^{TM} Protein Assay Kit (Pierced) according to the manufacturer's instructions.

Protein extracts from homogenated samples (5 µg) were mixed with SDS sample buffer, boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel using 25mmol/L Tris/0.2 mol/L glycine buffer, pH 8.3, 0.1% SDS for 1.5h at 150 V at room temperature. After SDS-PAGE, proteins were electrotransferred at 100 V for 1 h at 4ºC to 0.45 µm-pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon^{TM}-P membrane, Millipore) using 25 mmol/L Tris/192 mmol/L glycine in 20% (v/v) methanol as transfer buffer. Membranes were then blocked overnight at 4ºC with Tris buffered saline (50 mmol/L Tris buffer, pH 7.0, 150 mmol/L NaCl) containing 1% Tween 20 (TBS-T), and 1% bovine serum albumin (BSA). Membranes were then incubated with anti-PSP-I or anti-PSP-II rabbit antiserum (1 : 2000) for 45 min. After washing three times in TBST, each membrane was incubated for 45 min at room temperature with the secondary antibody (anti-rabbit-IgG conjugated to horseradish peroxidase A-257 (1 : 20000). Detection of immunoreactive signals was accomplished using the ECL plus detection system (Amersham Biosciences) following the manufacturer's instructions. 5 µg of purified PSP-I and PSP-II proteins were used as positive control. Liver tissue protein extract was used as negative control. Anti-PSP-I and anti-PSP-II reactive bands were scored according to their relative intensity as +++ (highest, strong), ++ (moderate), and + (lowest, weak). The negative (background) control was scored "0".

Determination of total protein concentration (Paper 3)

The protein content in the samples was determined by a colorimetric method using the Folin-Ciocalteu's phenol reagent (Lowry et al., 1951) in each seminal plasma fraction. This assay uses the Biuret reaction in which Cu^{2+} reacts with the peptide bond to give a deep blue colour. Under alkaline conditions the

divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin-Ciocalteu's phenol reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

The Biuret working reagent was freshly prepared by mixing 50 volumes of a solution containing 2% (w/v) sodium carbonate in 0.1 M NaOH with 1 volume of 1% (w/v) sodium potassium tartrate in water and 1 volume of 0.5% (w/v) cupric sulfate in water. Prior to the assay, samples were diluted 100-fold into distiller water. Freshly prepared bovine serum albumin solutions containing from 0 μg/mL to 1000 μg/mL were used as standards. For the assay, 1 mL of the diluted sample or standard was transferred into a tube and mixed thoroughly with 5 mL of Biuret working reagent. The mixture was then stored at room temperature for 15 minutes before the addition of 250 μL of Folin-Ciocalteu's phenol reagent (diluted 1:1 with distiller water just before used), vortexed and incubated at room temperature for 30 minutes. Absorbance readings were taken at 700 nm on a Hitachi spertophotometer model u-200 (Tokyo, Japan).

Quantification of PSP-I/PSP-II heterodimer byHPLC (Paper 3)

The seminal plasma proteins were separated, in each fraction, using an ETTAN LC HPLC system (Amersham Biosciences, Uppsala, Sweden) and a Lichrospher RP100 C18 column (250x4 mm, 5μm particle size; Merk, Darmstadt, Germany) eluted at 1 ml/min with a linear gradient of 0.1% of TFA in water (solution A) and ACN (solution B) (isocratically (5%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 done at 215 nm. The relative amounts of the PSP-I/PSP-II heterodimer were expressed as the area under the curve in chromatogram peaks after HPLC.

Flow cytometric sorting (Paper 4)

Sperm-rich fractions from fertile mature boars, that had previously sired offspring, were collected by gloved-hand method and extended in Beltsville Thawing Solution (BTS) to 150×10^6 spermatozoa/mL (Pursel and Johnson, 1975). After collection, samples were evaluated for normality (motility>80%, viability > 85%, total sperm count per ejaculate $>20\times10^9$, acrosomal abnormalities <10%, abnormal sperm morphology <15%). Only samples that

met the minimum criteria were used (Vazquez et al., 1997).

The extended semen was then processed for sperm sorting following the general procedure as previously described (Johnson et al., 1989). Briefly, 1 mL of extended semen was stained with Hoechst-33342 fluorophore (0.3 µM per 1×10^6) and incubated for 1 hour in darkness at 35ºC. After incubation, samples were filtered through a 30 µm nylon mesh filter to remove debris or clumpled spermatozoa. The stained spermatozoa were passed through a modified EPICS Altra high speed flow sorter (Coulter Corporation, Miami, FL, USA), operating at 3655 Kg/cm^2 and modified with a model 90C-6, 6 W argon laser (Johnson and Pinkel, 1986) operated in the ultraviolet wavelength (351, 364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA). Spermatozoa were separated into X- and Y-chromosome bearing populations, and only the X sperm population was recovered.

Intraoviductal laparoscopic insemination (Paper 4)

Sows were allocated individually to crates in a mechanically ventilated confinement facility under field conditions on a commercial pig farm in Murcia (Spain). Animals were fed a commercial ration twice a day and water was provided ad libitum. A total of 140 crossbred sows (2-6 parity) were weaned at 21.01 ± 0.04 days. Oestrus was induced by injecting each female intramuscularly with 1250 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning, followed 72 h later with 750 IU human chorionic gonadotrophin (hCG; Veterin Corion, Divasa, Farmavic S.A., Barcelona, Spain). Oestrus detection was performed once a day (7:00 am), beginning 2 days after eCG injection, by allowing females nose to nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in oestrus and the ovaries scanned. The ovaries were examined at intervals of 4 h from 30 h after hCG injection by transrectal ultrasonography using a 5.0 MHz multiple scan angle transducer for the presence of pre-ovulatory follicles. Only sows showing multiple pre-ovulatory follicles (diameter of antrum > 6 mm) were selected for insemination (Vazquez et al., 2003). Inseminations were carried out within 2-3 hour after ultrasonography

Laparoscopic inseminations were performed on sows sedated by azaperone (Stresnil, Lab. Dr. Esteve, Barcelona, Spain) administration (2 mg/Kg body weight, i.m.). General anesthesia was induced with sodium thiopenthal (Lab. Abbot, Madrid, Spain; 7 mg/Kg body weight, i.v.) and maintained with halothane (3.5-5%). The sow was placed in the supine position, on her back in a laparoscopy cradle. The cradle was placed in a Trendelemburg position at an angle of approximately 20 degrees above horizontal.

A 1.5-cm incision was made close to the umbilicus. The edges of the incision were then pulled up with countertraction and a 12-mm Optiview trocar (Ethicon Endo-surgery Cincinnati OH, USA) with an inserted 0° laparoscope was advanced into the wound. At the umbilicus, the subcutaneous fatty tissue, the anterior fascia of the rectus muscles, the posterior fascia of the rectus muscles, the transversalis fascia, and the peritoneum were traversed by slight cutting and moderate pressure. The process was controlled via monitor feedback. Although the CO_2 tubing was connected to the trocar, inflation did not begin until the peritoneum was punctured. After the peritoneal cavity was entered and the pneumoperitoneum started, the handpiece of the Optiview was removed and replaced by the 0° laparoscope. The abdominal cavity was inflated to 14 mmHg with CO_2.

Two accessory ports were placed in the right and left part of the hemi abdomen, which provided access for laparoscopic Duval forceps for manipulating the uterine horn and grasping the oviduct for the insemination needle, respectively. The oviduct was grasped with the Duval forceps in the isthmus region. Then the dose-flow (containing 0.3 million of spermatozoa in 0.1 mL) was inserted, and sex sorted spermatozoa was flushed into the oviduct. The procedure was then repeated in the other oviduct. After both oviducts were inseminated, the trocars were removed and minor suturing was required.

Embryo collection and embryo evaluation (Paper 4)

The putative zygotes were obtained surgically by laparotomy 18h after insemination. Sedation of the sows was performed by azaperone administration (2 mg/Kg body weight, i.m.). General anesthesia was induced with sodium thiopenthal (7 mg/Kg body weight, i.v.) and maintained with halothane (3.5-5%). The reproductive tract was exposed via mid-line incision and corpora lutea

were counted on the ovaries. Putative zygotes were collected by flushing each oviduct and the upper part of the uterine horn with 10 mL of NCSU-23 medium supplemented with 0.4% bovine serum albumin and 10 mL Hepes (flushing medium). Presumptive zygotes recovered 18 h after insemination were mounted on slides, fixed for 48-72 h in 25% (v:v) acetic acid in ethanol at room temperature, stained with 1% lacmoid in 45% (v:v) acetic acid and examined under a phase-contrast microscope at 400 magnification. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei with their corresponding sperm tails and two polar bodies. The fertilization parameters evaluated were: penetration rate (number of penetrated oocytes/number of oocytes) and monospermic rate (number of monospermic oocytes/number of penetrated oocytes). Degenerated oocytes, immature oocytes and oocytes with a broken oolemma or abnormal appearance of the cytoplasm were not counted (Gil et al., 2003).

Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analyzed using the MIXED-procedure according to a statistical model including the fixed effect of treatment, incubation time, collection media and the random effect of replicate. To analyze data, percentages were subjected to arcsine transformation before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test (Paper 1, 4) or Duncan test (Paper 3) and were considered to be significant when $p<0.05$.

CONCLUSIONS

1.- The subunits of the PSP-I/PSP-II heterodimeric disintegrin exert different activities on sperm functions. The beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require its glycan moiety. Thus, peptide moiety is a potential sperm function-preserving additive of highly diluted boar spermatozoa.

2.- The spermadhesins PSP-I and PSP-II are expressed by different tissues along the male reproductive tract of the boar. Seminal vesicles are the main source of PSP-I and PSP-II spermadhesins, although epididymal segments, testis and bulbourethral gland also participate in the expression of both proteins. However, much research is needed to clarify the physiological effect of these proteins on sperm cells during sperm epididymal transit, storage, and maturation.

3.- Seminal plasma portions exert different effect on sperm functionality on highly diluted boar spermatozoa, may be due to the different protein profile that shows each seminal plasma portion. The use of the post-sperm rich fraction should be avoid in order to increase the functionality of boar spermatozoa.

4.- The combination of the sedimentation method and the presence of PSP-I/PSP-II heterodimer in the collection medium is beneficial on the in vitro function and in vivo fertilizing ability of sex sorted boar spermatozoa stored for 18 h.

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10 Resumen General

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OBJETIVOS

Para la realización de la presente Tesis doctoral se realizaron 4 experiencias 4 experiencias con los siguientes objetivos:

1. Determinar si el la actividad biológica que presenta el heterodímero PSP-I/PSP-II de preservar la viabilidad, motilidad y actividad mitocondrial en espermatozoides de verrraco altamente diluidos, reside en la subunidad PSP-I o por el contrario, en la subunidad PSP-II y más específicamente, en su fracción peptídica o glicídica.

2. Revelar la localización y expresión de las espermadhesinas PSP-I y PSP-II y de sus ARN mensajeros a lo largo del tracto genital del verraco, concretamente en testículo, epidídimo (cabeza, cuerpo y cola) y en las principales glándulas sexuales (vesículas seminales y glándula bulbouretral) utilizando para ello machos adultos de fertilidad probada mediante el uso de inmunohistoquímica, western-blott y RT-PCR.

3. Estudiar el efecto de las fracciones del plasma seminal (pre-espermática, rica y post-espermática) sobre las características funcionales de los espermatozoides altamente diluidos, así como la presencia del heterodímero PSP-I/PSP-II en dichas fracciones.

4. Evaluar la funcionalidad, así como la fertilidad in vivo, de espermatozoides de verraco separados por citometría de flujo tras el proceso de concentración espermática mediante sedimentación y centrifugación en presencia de plasma seminal o del heterodímero PSP-I/PSP-II.

METODOLOGÍA

Todos los reactivos utilizados en el estudio fueron adquiridos de Sigma-Aldrich Co. (Alcobendas, Madrid) a menos que se indique lo contrario.

Obtención del plasma seminal (Objetivos 1, 3 y 4)

Todos los experimentos fueron realizados utilizando plasma seminal procedente de verracos adultos, de fertilidad probada. Los eyaculados fueron recogidos mediante el método manual (Larsen, 1986) y, posteriormente, el plasma seminal de cada uno de los eyaculados fue obtenido mediante centrifugación a 3800 x g durante 15 minutos a temperatura ambiente, usando para ello una centrífuga Heraeus Sepatech Megafuge (Osterode, Germany). Los sobrenadantes fueron filtrados sucesivamente a través de filtros de 10 y 1’2 µm y, finalmente, mezclados.

En la experiencia 3, se realizó un estudio en cuanto al perfil de eyaculación (tiempo de eyaculación y volumen) entre 20 verracos de fertilidad probada. De estos 20 machos, se escogieron como donantes de plasma seminal 3 verracos con un perfil de eyaculación similar. Lo eyaculados fueron recogidos en función del tiempo y de las fracciones. La concentración espermática de cada una de las fracciones recogidas fue contada mediante el uso de cámaras de Burker (Superior, Marienfeld, Germany).

Aislamiento de la heterodímero PSP-I/PSP-II (Objetivos 1, 2 y 4)

Para la obtención del heterodímero PSP-I/PSP-II, el plasma seminal fue sometido a cromatografía de afinidad en columna de heparina-sepharosa (Calvete y cols., 1995). De esta manera separamos una fracción que no une heparina, correspondiente al heterodímero PSP-I/PSP-II, de otra que si une heparina.

El heterodímero PSP-I/PSP-II fue purificado de la fracción que no une heparina mediante cromatografía de filtración en gel en una columna G-50 de Sephadex de 2000 x 5 cm equilibrada en 50 mM de Tris-HCl, 150 mM de NaCl, 1 mM de EDTA y 0’025% de azida sódica y pH 7’4 (Calvete y cols., 1995).

La identidad y pureza de la muestra proteica fue valorada mediante análisis de la secuencia N-terminal (usando secuenciador automatizado de proteínas Applied Biosystems 492, Langer, Alemania) y espectrometría de masas MALDI-

TOF (Applied Biosystems Voyager DE-Pro mass spectrometer, Lancen, Alemania). Una solución saturada de ácido sinapínico en 50% de acetonitrilo y 0'1% de ácido trifluoroacético (v/v) fue usada como matriz. La concentración proteica fue determinada por medio de espectrofotometría usando un coeficiente de absorción molar de 27332 M^{-1} cm^{-1} como había sido determinado previamente por Menéndez y cols. (1995) o por análisis de aminoácidos tras hidrólisis de la muestra en 6 M de HCl durante 24 h a 106º C en ampollas selladas a las cuales se les había creado el vacío (Beckman Gold Amino Acid Analyser, Beckman, Barcelona, España). Las proteínas fueron dializadas contra agua destilada, liofilizadas y almacenadas a -20º C hasta su uso.

Aislamiento de las subunidades PSP-I y PSP-II (Objetivos 1 y 2)

Las subunidades PSP-I y PSP-II se purificaron por cromatografía de alta resolución utilizando una columna (250 x 10 mm; RP-18) de fase reversa Lichrocart (Merk, Germany) de 7 µm de tamaño de partícula. La columna se eluyó a 2 mL/minuto con una mezcla de 0'1% de ácido trifluoroacético en agua (solución A) y acetonitrilo (solución B), en primer lugar isocráticamente (10% B) durante 5 minutos, seguido de 30% B 10 minutos, 45% B 45 minutos y 70% B por 15 minutos y 10% B 15 minutos. las subunidades purificadas fueron dializadas contra agua destilada y finalmente, liofilizadas. La concentración y pureza fueron determinadas como se describe en el apartado anterior.

Aislamiento de los péptidos y glicopéptidos (Objetivo 1)

Para separar la fracción peptídico de la glicídica, 100 mg de heterodímero PSP-I/PSP-II fueron digeridos a 37ºC con tripsina durante toda la noche utilizando una proporción de 1:100 (v/v) de enzima-substrato. A continuación, se realizó una electroforesis con gel de SDS poliacrilamida, HPLC de fase inversa y espectometría de masas para comprobar si la proteolisis había sido realizada correctamente. La fracción peptídica fue separada de la glicídica por cromatografía de afinidad mediante una columna de Concanavalina A (Amersham Bioscience) equilibrada y eluída con 20 mM de fosfato sódico, pH 7'4, 150 mM de cloruro sódico. Los glicopéptidos, unidos a las Con-A, fueron eluídos con un tampón compuesto por 100mM de metil-α-D-manopiranosa. Los glicopéptidos fueron separados de las sales con una columna C-18 (Lichrosphere,

250x4 mm, 5 µm de tamaño de partícula; Merck, Germany) equilibrada y lavada con 0’1 % de TFA hasta que la absorbancia a 214 alcanza el nivel base, seguida de la elusión con 0’1% de TFA y 50% de acetonitrilo. La concentración de los péptidos obtenida fue determinada por el análisis de aminoácidos.

Obtención del semen y evaluación de los parámetros seminales (Objetivos 1 y 3)

La fracción rica de eyaculados de machos adultos de fertilidad probada fue recogida mediante el método manual y diluidas posteriormente en BTS (Beltsville Thawing Solution) hasta $30x10^6$ de espermatozoides por mililitro (Pursel y Jonson, 1975). Una vez diluidas las fracciones ricas, estas fueron mezcladas y los espermatozoides fueron separados del plasma seminal mediante centrifugación a 1200 x g (Megafuge 1.0 R, Heraeus, Germany) durante 3 minutos. Finalmente, los espermatozoides recogidos tras la centrifugación fueron diluidos seriadamente hasta una concentración final de 1×10^6 spermatozoides /mL e incubados a 38ºC (Steri-Cult 200 incubator, Ohio, USA). La integridad de membrana, integridad acrosomal, la actividad mitocondrial y la motilidad fueron valorados a las 0’5, 2 y 5 horas de incubación. En el caso de la experiencia 4, los espermatozoides fueron diluidos a una concentración de $150x10^6$ espermatozoides /mL.

Análisis de la calidad espermática (Objetivos 1, 3 y 4)

La evaluación de la integridad de membrana y acrosomal de los espermatozoides así como la actividad mitocondrial, se llevó acabo mediante por citometría de flujo con un citómetro Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) equipado con una óptica estándar, un láser de argón con 15 mW de potencia (Cyonics, Coherent, Santa Clara, CA, USA) que excita a 488 nm, y un programa informático EXPO 2000. Todas las partículas que no eran espermatozoides fueron eliminadas del análisis. Se analizaron 10000 eventos por muestra.

La combinación de los fluorocromos SYBR-14/PI/PE-PNA propuesta por Nagy y cols. (2003), permitió medir simultáneamente la integridad de la membrana plasmática así como la integridad acrosomal mediante análisis por citometría de flujo. En primer lugar, 50 nM de solución de trabajo de SYBR-14 (100 µM de solución stock en DMSO; componente A del kit de viabilidad espermática LIVE/DEAD; Molecular Probes Europa, Leiden, Holanda), 0’5 µg/mL

de solución de PE-PNA (1 mg/mL de solución stock; Biomeda Corp, Foster City, CA) y 7’5 µM de solución de IP (ioduro de propidio, 1’5 mM de solución stock en PBS) fueron añadidos a 500 µL de la suspensión espermática. Las muestras fueron incubadas posteriormente a 37º C durante 10 minutos. La fluorescencia para el SYBR-14 fue medida a través de un filtro de paso de banda de 525 nM de longitud de onda, mientras que el IP fue recogido a través de un filtro de paso de banda de 635 nM de longitud de onda. La fluorescencia para la PE-PNA fue detectada a través de un filtro de paso de banda de 575 nM de longitud de onda. Se consideró espermatozoide viable con el acrosoma intacto aquellos que solamente fueron teñidos por el SYBR-14. Los espermatozoides viables con el acrosoma íntegro fueron aquellos teñidos únicamente por el fluorocromo SYBR-14 (SYBR-14 positivos). Los teñidos por PE-PNA se definieron como espermatozoides con el acrosoma alterado y los PI positivos se clasificaron como esprmatozoides muertos.

Valoración de la actividad mitocondrial (Objetivo 1)

El fluorocromo JC-1 (5,5´,6´6-tetracloro-1,1´,3,3´tetraetilbenzimidazolcarbocianina yodada, Molecular Probes Europe, Leiden, Holanda) fue usado para distinguir entre espermatozoides con alta y baja actividad mitocondrial de la manera descrita por Martínez-Pastor y cols. (2004) con ligeras modificaciones. Los monómeros de JC-1 penetran en aquellos espermatozoides con mitocondrias activas dando una fluorescencia verde y solamente cuando presentan una alta actividad en la membrana mitocondrial interna se agregan los monómeros de JC-1 dando una fluorescencia naranja. Brevemente, 0’2 µM de solución de trabajo de JC-1 (3’8 mM de solución stock) fueron añadidos a 500 µL de la suspensión espermática e incubada a 37º C durante 10 minutos para posteriormente ser analizada por citometría de flujo. La fluorescencia de emisión de los monómeros de JC-1 y los agregados de JC-1 fueron detectados en FL1 y FL2 usando filtros de paso de banda de 520 y 590 nm de longitud de onda respectivamente.

Valoración de la motilidad espermática (Objetivos 1, 3 y 4)

El porcentaje de motilidad espermática fue estimado con un sistema CASA de análisis objetivo de la motilidad (ISAS® Proiser, Valencia, Spain). Las

muestras (5-10 µL según la experiencia) fueron colocadas en una cámara de Makler (Haifa, Israel) acondicionada a 38ºC y observadas en un microscopio de contraste de fases (Nikon Labophot, Kanagawa, Japón) a 100 aumentos conectado a una cámara de video monocroma (Hitachi CCD, Tokio, Japón) y a un ordenador. Se utilizaron dos muestras del mismo semen para hacer la lectura y fueron contados un mínimo de 100 espermatozoides por replicado.

Los parámetros de motilidad evaluados fueron los siguientes: Porcentaje de espermatozoides mótiles totales, velocidad curvilínea (VCL, velocidad media de la cabeza del espermatozoide a lo largo de su trayectoria real, µm/s), velocidad rectilínea (VSL, velocidad media de la cabeza del espermatozoide a lo largo de la línea recta que une el primer punto del análisis con el último, µm/s) y amplitud del desplazamiento lateral de la cabeza (ALH, amplitud de las variaciones de la trayectoria real de la cabeza espermática respecto a la trayectoria media).

Preparación de los anticuerpos anti-PSP-I y anti-PSP-II (Objetivo 2)

Los anticuerpos policlonales anti-PSP-I y anti-PSP-II fueron obtenidos mediante la inmunización de conejos hembras por inyección subcutánea de 0'5 mg de la subunidades PSP-I y PSP-II diluidos en 0'5 mL de PBS emulsionado con 1'5 mL de adyuvante de Freund completo. Los animales fueron inoculados por 2 veces a intervalos de 5 semanas después de la primera inyección con 0'25 mg del antígeno. Dos semanas después de la última administración, los conejos fueron sangrados a través de la vena auricular. La actividad anti-PSP-I y anti-PSP-II del suero sanguíneo fue testada mediante técnicas de ELISA y Western Blot.

Inmunohistoquímica (Objetivo 2)

Los tejidos fueron fijados en una solución de PBS con paraformaldehído al 1%, deshidratados en series graduadas de etanol y embebidos en parafina. La inmunolocalización de las proteínas PSP-I y PSP-II en los tejidos del verraco fue realizada mediante el procedimiento de la tinción Elite ABC (Vectastain Elite ABC Kit; Vector Laboratorios; Burlingame, CA, USA). Para ello, de los tejidos embebidos en parafina fueron preparadas secciones de 5 µm utilizando un mocrotomo Leitz y porta objetos con poly-L-lisina (Sigma). A continuación, las secciones fueron desparafinadas, lavadas en PBS durante 5 minutos e incubadas en PBS con H_2O_2 al 3% durante 20 minutos con el fin de bloquear la actividad

endógena de la peroxidasa. La unión no específica de proteínas fue prevenida por la incubación en suero de cabra (10%) durante 30 minutos a 20ºC. Tras este paso, y sin lavar las secciones de los tejidos, las muestras fueron incubadas durante toda la noche a 4ºC con los anticuerpos policlonales primarios anti-PSP-I y anti-PSP-II (1:1000 v/v en PBS). Tras esta incubación, las secciones fueron lavadas en PBS durante 5 minutos antes de la incubación con el anticuerpo de conejo "biotinylated gota anti-rabbit IgG" (1:1000) durante 30 minutos.

A continuación, todas las muestras fueron lavadas de nuevo en PBS durante 5 minutos antes de aplicar el complejo ACB-Elite sobre ellas. La visualización de los complejos antígeno-anticuerpo fue realizada mediante la incubación de las muestras en presencia de "diaminobenzide tetrahydrochloride" con H_2O_2 al 3% (DAKO® DAB Chromogen tablets; CA, USA) durante 5-6 minutos. Finalmente, los portaobjetos las secciones fueron montados con gelatina de glicerina. Como controles negativos se utilizaron los tejidos no incubados en presencia del ancticuerpo primario. En cambio, espermatozoides eyaculados de verraco fueron utilizados como control positivo. Para ello, fueron sometidos al mismo protocolo que los tejidos utilizando los anticuerpos anti-PSP-I y anti-PSP-II. Todas las incubaciones fueron realizadas en una cámara húmeda. Los resultados obtenidos fueron fotografiados con una cámara Nikon microphot-FXA.

Extracción de RNA (Objetivo 2)

Las muestras de tejidos fueron congeladas en nitrógeno líquido y conservadas a -80ºC en criotubos. Se extrajo ARN de testículo, vesícula seminal, glándula bolbouretral y epidídimo de 10 machos, utilizando para ello el reactivo "TRIzol Reagent" (Invitrogen) siguiendo el protocolo descrito por Chomoczynski y Sacchi (1987). Para la extracción de RNA, 50 mg de cada tejido fueron homogeneizados en 1 mL de TRIzol, mediante puntas de polipropileno. Los homogenizados fueron guardados durante 5 minutos a temperatura ambiente para permitir la completa disociación de los complejos nucleoproteicos. A continuación, se añadieron 0'2 mL de cloroformo a cada tejido homogenizado y tras una centrifugación a 4ºC, la fase acuosa obtenida fue recogida. A esta fase acuosa se le añadió isopropanol, se incubó durante 15 min a -20ºC y se centrifugó a 4ºC para poder precipitar el ARN contenido en dicha fase. Los pellets obtenidos fueron sucesivamente lavados en 1 mL de etanol al 75% y posteriormente, en 1 mL de etanol absoluto. Finalmente, los pellets fueron

resuspendidos en 20 µL de agua DEPC.

La cantidad total de ARN obtenido fue cuantificado espectrofotometricalmente a $O.D._{260}$ nm, mientras que la pureza fue valorada mediante el ratio $O.D._{260}/O.D._{280}$. Todas las muestras obtuvieron valores mayores de 1’8. La integridad de ARN fue valorada mediante electroforesis en gel de agarosa (2%).

Tras la extracción de ARN, se realizó un tratamiento para eliminar las posibles ADNasas antes de transformar el ARN en ADNc.

Trascripción inversa (Objetivo 2)

La trascripción inversa, así como la PCR, fue realizada en un termociclador (Personal Master Cycler, Eppendorf) con tapa caliente. La trascripción inversa de ARN a ADNc fue realizada a partir de 20 µL de ARN. En primer lugar, 5 µg de ARN fue mezclado con 1 µL del oligo $(dT)_{20}$. La mezcla se calentó a 65ºC durante 5 minutos y a continuación, fue colocada en contacto con hielo. Después de esto, se añadieron 4 µL del tampón “5x RT buffer, 200 U/µL de la enzima “Superscript^{TM} III Reverse Transcriptase), 1 µL de DTT (0’1 mol/L), 25 µmol/L de dNTPs y 1 µL de ARNasa out (40 U/µL) a la mezcla que posteriormente se incubó durante 55 minutos a 50ºC. Pasado este tiempo, la mezcla de reacción fue calentada 15 minutos a 70ºC para desactivar la enzima. Finalmente, la mezcla se enfrió hasta alcanzar 4ºC.

Amplificación por PCR de PSP-I y PSP-II (Objetivo 2)

Los primers para la amplificación de las proteínas PSP-I y PSP-II fieron descritos por Ekhlasi-Hundrieser y cols. (2002), de acuerdo con las secuencias de ADNc obtenidas por Kwok y cols. (1993).

PSP-I → forward: 5´-TTCAACAGGATGGGGCTTGG-3´
Reverse: 5´-GAAGGAAAATGATCTCATAGGG-3´
PSP-II → forward: 5´-GCACGGATCAATGGCCCTG-3´
Reverse: 5´-TTCGGATCCTGGTGAACTAC-3´

La master para llevar a cabo la PCR contiene: 0’4 µL de los 4 dNTPs (dTTP, dATP, dGTP and dCTP de una concentración 2 mmol/L), 2’5 µL de los primers (10 pmol/µL solución stock), 5 µL de ADNc, 5 µL del 10 × tampón de PCR (100 mmol/L Tris-HCl, 15 mmol/L MgCl2, 500 mmol/L KCl, pH 8.3 at 20ºC)

y 0’5 µL de la enzima Taq ADN polimerasa (5 U/µL). Cinco µL of cDNA fueron añadidos a la mezcla de PCR obteniendo un volumen final de 50 µL. La mezcla fue desnaturalizada a 95ºC durante 5 minutos y amplificada 35 ciclos de 30s a 94ºC (desnaturalización), 30s a 58ºC (annealing) y 30s a72ºC (elongación). Finalmente, la mezcla fue puesta a 72ºC durante 5 minutos y, a continuación, a 4ºC. Los productos obtenidos de las reacciones anteriores fueron visualizados en un gel de agarosa al 1%.

El gen de la β-actina fue utilizado como control positivo para confirmar así, la presencia de ADNc. Como control negativo se añadió a la mezcla de reacción ARN en lugar de ADNc, lo cual permite descartar la presencia de ADN genómico. Los primers de β-actina utilizados fueron: forward, 5´-GAGAAGCTCTGCTACGTCGC-3´; reverse: 5´-CCAGACAGCACCGTGTTGGC-3´ (Ponsuksili *et al.,* 2001).

Western Blott (Objetivo 2)

Los tejidos sometidos a estudio fueron homogeneizados en hielo usando un Politrón (Polytron Kinematica AG, Litlan-Luzern, Switzerland). Los tejidos homogeneizados fueron centrifugados a 3939 x g durante 10 minutos, y el sobrenadante fue recogido. Para determinar la concentración de proteínas presente en casa uno de los extractos, se utilizó “BCA Protein Assay Kit (Pierced)”. Cinco µg de cada extracto proteico fue mezclado con tampón SDS y llevado a ebullición durante 5 minutos. A continuación, se realizó una electroforesis en un gel de poliacrilamida al 12%. Tras la electroforesis las proteínas fueron transferidas a una membrana de polivinilo de 0’45 µM de tamaño de poro (ImmobilonTM –Pmembrane, Millipore). Las membranas fueron incubadas en presencia de albúmina al 1% durante toda la noche a una temperatura de 4ºC con el objetivo de bloquear los lugares de unión inespecíficos. A continuación, las membranas fueron incubadas con los anticuerpos anti-PSP-I y anti-PSP-II (1 : 2000) durante 45 minutos. Tras lavar las membranas 3 veces con el medio TBST, cada membran fue incubada con el anticuerpo secundario “A-257” (anti-rabbit-IgG, 1 : 20000) conjugado con peroxidasa. Las señales inmunoreactivas obtenidas tras la incubación fue detectada mediante el sistema ECL plus (Amersham Biosciences). Como control negativo fue utilizado tejido de hígado y como control positivo PSP-I y PSP-II pura.

Valoración de la concentración de proteínas totales (Objetivo 3)

El contenido proteico de las fracciones del plasma seminal se determinó mediante un método colorimétrico que utiliza el reactivo "Folin-Ciocalteu phenol" (Lowry y cols., 1951). Este método se basa en la reacción de Biuret, donde los iones Cu^{2+} reaccionan con los péptidos presentes en la muestra dando un color azul intenso.

El reactivo Biuret fue preparado mezclando 50 mL de una solución que contiene 2% de carbonato sódico en 0'1 M de NaOH con 1 volumen de potasio sódico tartrato (en agua) al 1% y 1 volumen de sulfato cúprico al 0'5% (en agua). Previo al ensayo, las muestras a valorar fueron diluidas 100 veces en agua destilada. Como curva patrón se utilizó albúmina sérica bovina con concentraciones desde 0 µg/mL a 1000 µg/mL. A continuación, 1 mL de la muestra diluida fue mezclada con 5 mL del reactivo Biuret. La mezcla fue guardada a temperatura ambiente durante 15 minutos antes de la adición de 250 µL del reactivo "Folin-Ciocalteu's phenol", previamente diluido 1:1 en agua destilada. Una vez añadido dicho reactivo, la mezcla se incubó durante 30 minutos a temperatura ambiente. Los valores de absorbancia fueron medidos a 700 nm en un espectofotómetro Hitachi modelo u-200 (Tokio-Japan).

Cuantificación del heterodímero PSP-I/PSP-II mediante HPLC (Objetivo 3)

Las proteínas presentes en cada fracción del plasma seminal fueron separadas utilizando el sistema HPLC ETTAN LC (Amersham Biosciences, Uppsala, Sweden) y una columna Lichrospher RP100 C18 eluída a 1mL/min con un gradiente lineal de TFA al 0'1% en agua (solución A) y ACN (solución B) durante 5 minutos, seguida de 5-25% de B durante 10 minutos, 25-60% de B durante 50 minutos y 60-70% de B durante 10 minutos. La detección del heterodímero fue realizado a 215 nm. La cantidad relativa de PSP-I/PSP-II fue expresada como el área de la curva en el cromatograma tras el HPLC.

Separación espermática mediante citometría de flujo (Objetivo 4)

El semen diluido a $150x10^6$ espermatozoides/mL fue preparado para el proceso de separación espermática siguiendo el protocolo descrito por Johnson y cols., (1989). El semen fue teñido con el flourocromo Hoechst-33342 (0'3 µM) e incubado en oscuridad durante 1 hora a 35ºC. Tras la incubación, el semen fue

filtrado con una malla de nylon de 30 µm para eliminar las partículas no deseadas. Los espermatozoides teñidos con el fluorocromo fueron sometidos al proceso de separación utilizando para ello un citómetro separador de células modificado para espermatozoides (EPICS Altra High Speed Flor Sorter; Coulter Corporation, Miami, FL, USA), que trabaja a una presión de 3’655 Kg/cm^2 y funciona con un láser de argón que trabaja en la longitud de onda del ultravioleta a 175 mW (Coherent Lasers, Inc., Santa Clara, CA). Los espermatozoides fueron separados en espermatozoides X e Y, aunque sólo los que contenían el cromosoma X fueron recogidos y utilizados en el estudio.

Inseminación laparoscópica (Objetivo 4)

Las cerdas se alojaron en jaulas individuales en una nave con ventilación controlada y estuvieron sometidas al manejo rutinario de la granja. Las cerdas fueron alimentadas dos veces la día con una ración comercial y disponían de agua *ad limitum*. Un total de 140 cerdas híbridas fueron destetadas a los 21’01±0’04 días.

Para inducir el estro, se inyectaron en cada una de las cerdas 1250 UI de gonadotropina coriónica equina vía intramuscular (eCG; Folligon, Intervet B.V., Boxmeer, The Netherlands) 24 horas tras el destete. A las 72 horas se inyectó gonadotropina coriónica humana (750 UI; Veterin corion, Divasa, Farmavic S.A., Barcelona, España). La detección del estro se realizó una vez al día con la ayuda de un verraco y aplicando presión en el dorso de la hembra, 2 días después de la inyección de eCG. A las cerdas que mostraron estar en estro se les realizó un examen ovárico transrectal con una sonda de 5 MHz a intervalos de 4 horas desde 30 horas después de la inyección de la hCG, capaz de detectar la presencia de folículos pre-ovulatorios. Sólo aquellas cerdas que mostraron un tamaño de folículo mayor de 6 mm fueron seleccionadas para la inseminación (Vázquez y cols., 2003).

Para llevar a cabo las inseminaciones laparoscópicas, las cerdas fueron sedadas por administración de azaperona (Stresnil, Lab. Dr. Esteve, Barcelona; 2mg/Kg de peso vivo). La anestesia general se indujo con tiopental sódico (Lab. Abbot, Madrid; 7 mg/Kg de peso vivo) y fue mantenida vía inhalatoria con halotano (3’5-5%). Posteriormente, la cerda se colocó de espaldas sobre la camilla en posición supina. La camilla se colocó la posición “Trendelemburg” con un ángulo, aproximadamente, de 20º. A continuación, se realizó una incisión de 1’5 cm cerca del ombligo. Los bordes de la incisión de levantaron y se insertó un

trocar Optiview de 12 mm con un laparoscopio 0º. En el ombligo, el tejido graso subcutáneo, la fascia anterior del músculo rectal, la fascia transversal y el peritoneo fueron atravesados con un ligero corte y presión moderada. El proceso fue controlado por un monitor. Aunque el tubo de CO_2 fue conectado con el trocar, no se procedió a inflar la zona hasta que el peritoneo fue pinchado. Tras entrar en la cavidad peritoneal y comenzar el pneumoperitoneo, se procedió a quitar el Optiview y a sustituirlo por el laparoscopio de 0º. La cavidad abdominal fue inflada con CO_2 a 14 mmHg.

Dos puertos accesorios se colocaron en la parte izquierda y derecha del abdomen medio, permitiendo el acceso de los forceps Duval para manipular el cuerno uterino y sujetar el oviducto respectivamente, para poder insertar la aguja de inseminación. Cuando el oviducto fue sujetado con los forceps Duval en la región del istmo, 0'3 millones de espermatozoides separados fueron depositados dentro del oviducto. El mismo procedimiento fue repetido en el otro oviducto.

Recogida y evaluación de los embriones (Objetivo 4)

La recogida de embriones vía quirúrgica se realizó 18 horas tras la inseminación mediante laparotomía. Las cerdas fueron sedadas y anestesiadas siguiendo el protocolo utilizado en la inseminación laparoscópica. Se realizó una incisión ventromedial para localizar el tracto genital. Una vez exteriorizado el útero y los ovarios, se procedió al recuento de los cuerpos lúteos y al lavado de la región anterior de cada uno de los cuernos uterinos con 10 mL del medio NCSU-23 suplementado con un 0'4% de albúmina sérica bovina y 10 mL de Hepes. Para evaluar los zigotos recogidos, se colocaron en portaobjetos y fueron fijados durante 48-72 horas en ácido acético al 25% en etanol a temperatura ambiente. Más tarde se tiñeron con lacmoid al 1% en ácido acético al 45% y fueron examinados en un microscopio de contraste de fases. Se consideraron ovocitos penetrados a aquellos que contenían una o más cabezas de espermatozoide y/o pronúcleo masculino con su correspondiente cola espermática o 2 cuerpos polares. Los ovocitos degenerados, inmaduros, con su oolema roto o con el citoplasma anormal no se tuvieron en cuenta en el contaje (Gil y cols., 2003).

Análisis estadístico

El análisis estadístico fue realizado en el programa SPSS, versión 11.5 (SPSS, Inc., Chicago, IL). Los datos fueron analizados mediante ANOVA utilizando un modelo mixto de acuerdo incluyendo como efecto fijo el tratamiento (objetivo 1), tiempo de incubación (objetivo 1 y 3) y el medio de recogida (objetivo 4), siendo en todos ellos el replicado el efecto aleatorio. Para analizar los datos, los porcentajes fueron transformados uilizando para ello el arcoseno. Cuando ANOVA mostró un efecto significativo, los valores fueron comparados utilizando el test de Duncan (objetivo 3) o el test de Bonferroni (objetivos 1 y 4), considerando los datos diferentes significativamente cuando $p<0'05$.

RESULTADOS

Análisis del efecto protector del heterodímero PSP-I/PSP-II sobre la funcionalidad espermática (Objetivo 1).

Tras el estudio exhaustivo sobre el efecto que ejercen las subunidades PSP-I y PSP-II sobre la viabilidad, actividad mitocondrial y motilidad en espermatozoides altamente diluidos, los resultados mostraron que la incubación de espermatozoides en presencia de la subunidad PSP-II ejerce un efecto muy similar, sobre los parámetros seminales, al producido por el heterodímero nativo. Sin embargo, estos efectos fueron menos pronunciados cuando los espermatozoides se incubaron en presencia de la subunidad PSP-I. Además es necesario destacar, que la motilidad fue suprimida como consecuencia de la presencia de dicha subunidad en el medio espermático. No obstante, tanto el heterodímero como sus subunidades digeridas mediante tripsina, muestran el mismo efecto que las formas nativas (sin digerir).

Por otro lado, se observan mejores resultados en cuanto a la viabilidad, actividad mitocondrial y motilidad espermática en los espermatozoides incubados en presencia de la fracción peptídica que en aquellos incubados con la fracción glicídica.

Localización y expresión de las espermadhesinas PSP-I y PSP-II en el tracto genital del verraco (Objetivo 2).

La técnica de inmunohistoquímica mostró marcaje para los anticuerpos de las subunidades PSP-I y PSP-II en el epitelio de las vesículas seminales de todos los machos estudiados. A su vez, se produjo marcaje de ambas subunidades en el epitelio del epidídimo (cabeza, cuerpo y cola), aunque con intensidad variable entre tejidos e incluso, machos. Sin embargo, no se produjo marcaje inmunológico en el epitelio seminífero ni en la glándula bulbouretral, aunque el western-blott reveló la presencia de bandas inmunoreactivas en todos los tejidos sometidos a estudio, incluyendo los testículos y la glándula bulbouretral. Además, la amplificación de ARNm por RT-PCR utilizando primers específicos para PSP-I y PSP-II mostró la misma tendencia que se observó tras el western-blott. La intensidad de las bandas obtenidas tras la realización de la RT-PCR fue también variable entre tejidos y machos. Los resultados indican que las vesículas seminales son la mayor fuente de las espermadhesinas PSP-I y PSP-II, aunque los segmentos del epidídimo, testículos y glándulas bulbouretrales participan también en la expresión de ambas proteínas.

Perfil proteico de las fracciones del plasma seminal y su influencia sobre la funcionalidad de espermatozoides de verraco altamente diluidos (Objetivo 3).

Los resultados mostraron que la cantidad de proteínas totales varía entre las diferentes fracciones del plasma seminal y que es la fracción post-espermática la que mostró un elevado contenido proteico comparado con el resto de fracciones. Además, aunque la espermadhesina PSP-I/PSP-II fue detectada en todas las fracciones del plasma seminal, es en la fracción post-espermática en la que se encuentra en mayor proporción.

La adición del 10% de cada fracción del plasma seminal sobre espermatozoides altamente diluidos, mejora la viabilidad espermática comparada con los espermatozoides incubados sin plasma seminal. El efecto de esta adición de plasma seminal de las diferentes fracciones mostró diferencias significativas, en cuanto a la integridad de membrana, tras 2 horas de incubación. Los espermatozoides expuestos a las fracciones pre-espermática y rica presentaron los mejores porcentajes de integridad de membrana y motilidad espermática, siendo la fracción rica la que muestra los valores más altos. Mientras las

fracciones pre-espermática y rica mejoran la funcionalidad espermática, la fracción post-espermática ejerce un efecto nocivo en el mantenimiento de espermatozoides altamente diluidos. Teniendo en cuenta el efecto negativo que ejerce sobre los espermatozoides y que dicha fracción se dividió en 3 subfracciones, la subfracción que menos daño ejerce sobre los espermatozoides es la que contiene una la elevada cantidad del heterodímero PSP-I/PSP-II.

Mejora de la capacidad de fecundación de los espermatozoides de verraco separados (Objetivo 4).

Los espermatozoides separados mediante citometría de flujo fueron recogidos en presencia del 10% de plasma seminal o de la proteína PSP-I/PSP-II y concentrados mediante centrifugación o sedimentación. Tras 16-18 horas de conservación a 17ºC, los espermatozoides recogidos en presencia de plasma seminal o del heterodímero PSP-I/PSP-II y concentrados mediante centrifugación, presentaron porcentajes de integridad de membrana, motilidad y fertilización más bajos que los espermatozoides concentrados por sedimentación. No obstante, la presencia en el medio espermático de plasma seminal o de la espermadhesina no afectó a los resultados obtenidos cuando se aplica el proceso de centrifugación, aunque sí se observa un efecto positivo del heterodímero sobre los espermatozoides cuando es la sedimentación el método de concentración espermática utilizado.

CONCLUSIONES

1. Las subunidades PSP-I y PSP-II, que componen el heterodímero nativo PSP-I/PSP-II, ejercen diferentes actividades sobre los parámetros funcionales espermáticos. El efecto beneficioso que ejerce el heterodímero sobre la funcionalidad espermática en espermatozoides altamente diluidos parece estar en gran parte conservada en la subunidad aislada PSP-II, y no parece necesitar para ello la fracción glicídica.

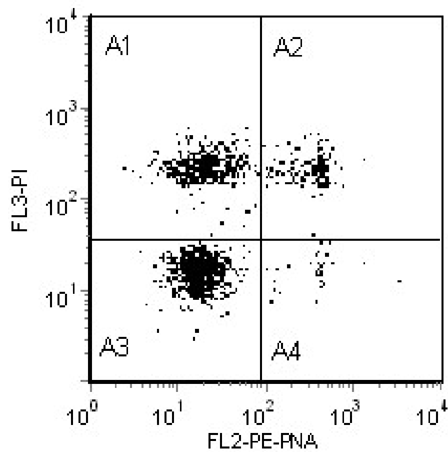
2. Las espermadhesinas PSP-I y PSP-II están expresadas en diferentes tejidos del tracto genital del verraco. Las vesículas seminales son la principal fuente de producción de PSP-I y PSP-II, aunque los segmentos epididimales,

los testículos y la glándula bulbouretral participan también en la expresión de dichas proteínas.

3. Las fracciones que componen el plasma seminal ejercen efectos distintos sobre la funcionalidad de espermatozoides de verraco sometidos a altas diluciones, quizás debido a los distintos perfiles proteicos que muestran cada una de las fracciones. Con el fin de conservar la funcionalidad de espermatozoides de verraco altamente diluidos a lo largo del tiempo debe evitarse la adición de plasma seminal de la fracción post-espermática.

4. La utilización de la espermadhesina PSP-I/PSP-II en el medio de recogida de espermatozoides separados en combinación con el método de concentración por sedimentación, beneficia la funcionalidad *in vitro* de los espermatozoides separados, así como su capacidad fecundante *in vivo* tras 18 horas de conservación.

11 Anexo Gráfico



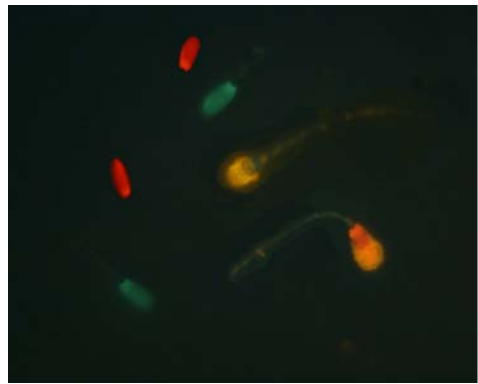
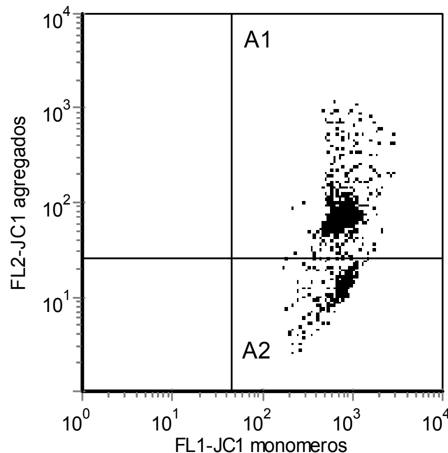


Figura 1. (a) Representación gráfica correspondiente al análisis por citometría de flujo de la tinción SYBR-14/PI/PE-PNA b) Imagen de microscopía de fluorescencia donde se observan las 4 poblaciones espermáticas: A1 (muertos acrosoma intacto); A2 (muertos acrosoma dañado); A3 (vivos acrosoma intacto); A4 (vivos acrosoma dañado).



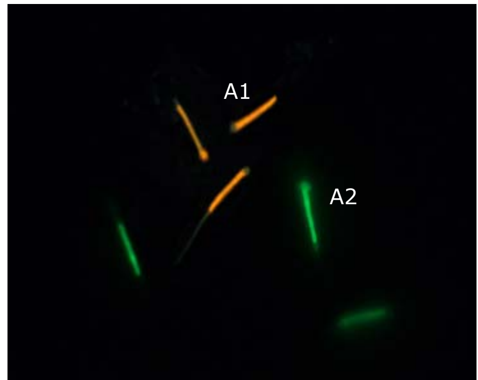


Figura 2. a) Representación gráfica correspondiente al análisis por citometría de flujo de la tinción con JC-1. b) Imagen de microscopía de fluorescencia donde se observan las 2 poblaciones espermáticas: A1 (fluorescencia naranja: espermatozoides con alta actividad mitocondrial; A2 (fluorescencia verde: espermatozoides con baja actividad mitocondrial).

Figura 3. Citómetro de flujo (Epics XL, Beckman Coulter).

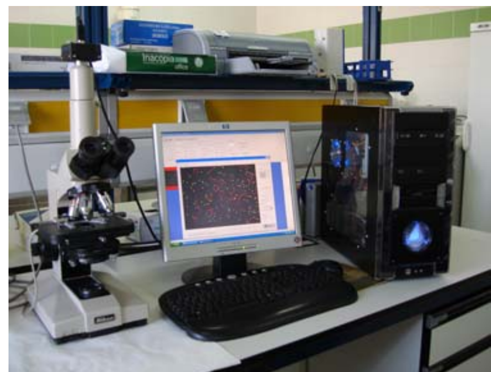


Figura 4. Evaluación de la motilidad mediante Sistema SCA (ISAS® CASMA System, Proiser SL, Valencia, España).

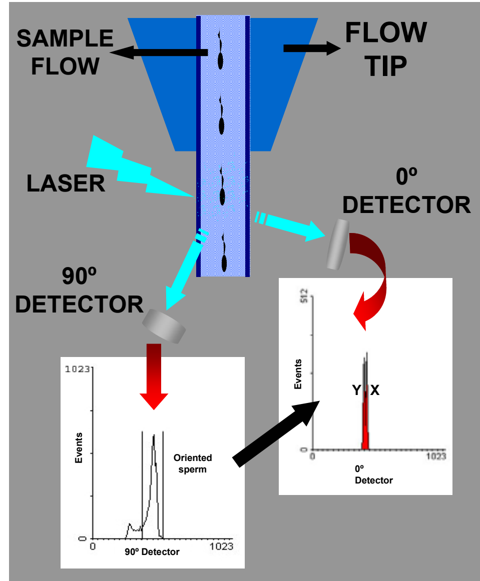
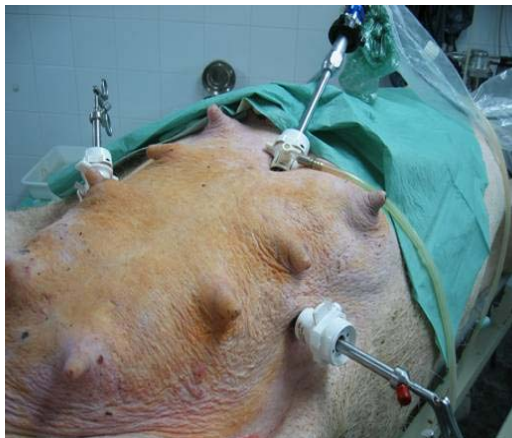


Figura 4. a) Citómetro de flujo EPICS Altra (Coulter corporation) modificado para espermatozoides; b) Esquema del proceso de separación espermática.



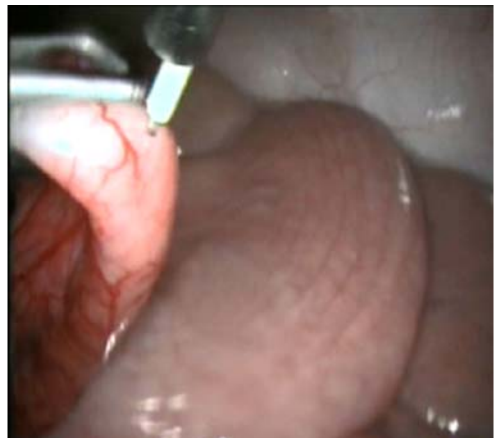
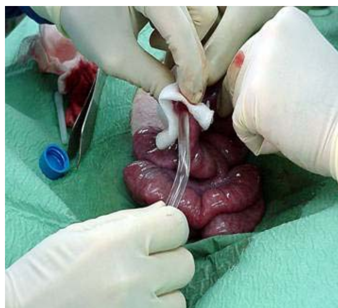
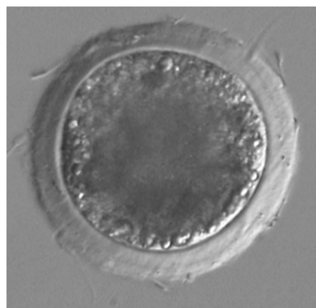


Figura 5. Inseminación del semen separado, en cada oviducto, mediante laparoscopia.





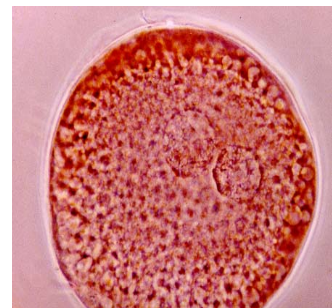


Figura 6. Recogida de embriones por laparotomía y tinción de los mismos con lacmoid al 1%.

12 Apéndice 1