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UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL

Estudio del plasma seminal y la espermadhesina PSP-I/PSP-II sobre la funcionalidad de los espermatozoides de verraco

Study of seminal plasma and the spermadhesin PSP-I/PSP-II on the functionality of boar sperm

Ignacio Caballero Posadas

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I. – Introducción

La incorporación de nuevas biotecnologías, como la preselección del sexo mediante la separación de espermatozoides X e Y por citometría de flujo o la criopreservación espermática, a la producción animal presenta actualmente un especial interés en producción porcina, sobre todo cuando van dirigidas a grupos de alto valor económico y/o genético. La aplicación de estas tecnologías permitiría, en el caso del semen criopreservado, la creación de bancos y el comercio internacional de dosis seminales, ayudaría a una mejor gestión, planificación y operatividad de los centros de inseminación artificial y minimizaría el problema de la estacionalidad reproductiva, con dosis seminales de calidad uniforme a lo largo del año (Roca y cols., 2006a). En el caso de la preselección del sexo, su aplicación permitiría producir una aceleración de los programas de mejora genética, un incremento en la eficiencia de la producción, y una mayor flexibilidad en los sistemas de manejo de las explotaciones (Maxwell y cols., 2004).

A pesar de las mejoras realizadas en los últimos años en el desarrollo de estas biotecnologías, la eficiencia de estos procedimientos sigue siendo insuficiente para ser aplicadas de manera rutinaria en la industria porcina. Las lesiones en los espermatozoides como consecuencia implícita a la aplicación de estas tecnologías

conllevan alteraciones en la viabilidad, integridad de membrana, estado acrosomal y patrones de motilidad de los espermatozoides (Maxwell y cols., 1997; Maxwell y Johnson, 1997; Maxwell y Johnson, 1999), además de la aparición de una subpoblación de espermatozoides en un estado de capacitación o precapacitación que culmina en una exocitosis prematura del contenido acrosomal provocando una disminución de la capacidad fecundante de los espermatozoides procesados (Maxwell y Johnson, 1997; Centurión y cols., 2003; Parrilla y cols., 2004). Entre los factores causantes de esta situación se ha incluido la pérdida de sustancias protectoras procedentes del plasma seminal que cubren la membrana plasmática del espermatozoide (Maxwell y Jonson, 1997; Parrilla y cols., 2004).

La adición de cierta cantidad de plasma seminal completo (entre un 1% y un 10% v/v) a los diluyentes utilizados tanto en el proceso de criopreservación, como en el de separación espermática por citometría de flujo, ha sido la base de diversos estudios cuyo objetivo era minimizar el daño que los espermatozoides pudieran sufrir tras la aplicación de estas biotecnologías (Maxwell y Johnson, 1999). Sin embargo, el efecto de la adición de plasma seminal a los espermatozoides en las distintas especies domesticas es controvertido, presentando efectos tanto estimuladores como inhibidores de la función espermática y la fertilidad. Algunos autores han observado un efecto perjudicial sobre la supervivencia espermática de algunas fracciones del plasma seminal, como son la fracción prostática de los perros (England y Allen, 1992), la fracción gelatinosa en caballos o verracos (Mann y Lutwak-Mann, 1981), o la secreción de las vesículas seminales (carnero, Ashworth y cols., 1994; toro, Way y cols., 2000; macho cabrío, Azerêdo y cols., 2000).

Sin embargo, otros autores han hallado efectos beneficiosos del plasma seminal sobre los espermatozoides, como sería el aumento de resistencia al choque frío cuando los espermatozoides son incubados con plasma seminal o ciertas porciones proteicas del plasma seminal (Barrios y cols., 2000; Pursel y cols., 1973). Además, la adición de un 10% de plasma seminal a los medios de recogida de los espermatozoides de verraco que han sido sometidos a un proceso de separación mediante citometría de flujo mejora su viabilidad espermática y motilidad (García y cols., 2006), previniendo procesos de capacitación prematura y alargando por lo tanto su capacidad fertilizante (Maxwell y cols., 1997; Maxwell y cols., 1998).

Una explicación posible para esta variabilidad encontrada en los efectos del plasma seminal podría ser la presencia o ausencia de determinados componentes del plasma seminal, así como la variación existente en la concentración de los mismos, más probablemente en el caso de componentes proteicos (Fournier-Delpech y Thibault, 1993). Diversos estudios han mostrado una relación entre ciertas proteínas del plasma seminal del toro y los índices de fertilidad presentados por dichos animales (Killian y cols., 1993; Moura y cols., 2006), así como diferencias en los efectos del plasma seminal sobre los espermatozoides dependiendo de si la fuente de plasma seminal eran machos de alta o baja fertilidad, o machos con alta o baja motilidad postdescongelación (Aurich y cols., 1996; Henault y Killian, 1996).

La alta variabilidad observada en los efectos del plasma seminal sobre los espermatozoides en las diferentes especies, tal y como se ha descrito anteriormente, determinó que el objetivo de nuestro primer estudio fuera evaluar el efecto del plasma seminal sobre espermatozoides sometidos a un grado de dilución extremo, similar al

producido mediante la separación espermática por citometría de flujo, y si este efecto era dependiente o no del verraco donante de plasma seminal.

Una vez analizado el efecto de los diferentes plasmas seminales, tanto autólogos como homólogos, sobre los espermatozoides altamente diluidos, se trató de estudiar el posible mecanismo de acción. En verracos, las espermadhesinas son el mayor componente proteico del plasma seminal, un grupo de glicoproteínas de las cuales se sospecha una doble función en la fecundación porcina. Por un lado podrían recubrir la membrana acrosomal, previniendo capacitaciones prematuras siendo la mayor parte de ellas liberadas de la cabeza del espermatozoide conforme avanza por el tracto genital femenino, y por otro lado constituirían parte del sistema de reconocimiento entre gametos (Töpfer-Petersen y cols., 1998).Esta familia de proteínas podría ser clasificada dependiendo de que presenten afinidad por la heparina (AQN-1, AQN-3 y AWN) o no (heterodímero PSP-I/PSP-II) (Centurión y cols., 2003). Entre las proteínas del plasma seminal, se ha demostrado que la espermadhesina PSP-I/PSP-II tiene un efecto protector sobre los espermatozoides sometidos a altas diluciones, similar al hallado cuando se adiciona plasma seminal de machos selectos, preservando su integridad de membrana, motilidad y actividad mitocondrial (Centurión y cols., 2003). Este efecto protector apunta al heterodímero PSP-I/PSP-II como un potencial candidato para el pre- o post-tratamiento de espermatozoides que van a ser manipulados, con la ventaja de que el uso de una proteína aislada evitaría la alta variabilidad encontrada con el uso de plasma seminal de distintos machos, eyaculados de un mismo macho e incluso entre fracciones de un mismo eyaculado (Killian y cols., 1993; Asworth y cols., 1994; Zhu y cols., 2000; Peña y cols., 2006). Debido a que se desconocen los patrones de unión del heterodímero al espermatozoide, en una segunda experiencia se estudió mediante microscopía

electrónica el patrón de unión de esta proteína al espermatozoide y la relación existente entre este patrón y su capacidad para preservar la funcionalidad espermática.

Otro de los aspectos a evaluar sería el efecto del heterodímero PSP-I/PSP-II sobre la funcionalidad espermática. El papel de este heterodímero durante la unión entre gametos no ha sido determinado todavía pero teniendo en cuenta los resultados descritos por Töpfer-Petersen y cols. (1998) podría pensarse que tuviera cierta influencia sobre la capacidad fecundante de los espermatozoides. Por ello, en una tercera experiencia se evaluó el efecto de la presencia del heterodímero en el medio de cocultivo sobre la fecundación.

Por último, aunque se piensa que la unión del heterodímero PSP-I/PSP-II al espermatozoide es lábil, y por lo tanto sería eliminado de la cabeza del espermatozoide a lo largo de su desplazamiento por el tracto genital femenino y en la capacitación, no participando en el reconocimiento entre gametos (Calvete y cols., 1995a; Dostálová y cols., 1995a), los resultados obtenidos en los ensayos de afinidad de esta proteína con la zona pelúcida (ZP) han sido contradictorios (Calvete y cols., 1995a; Jonáková y cols., 2000). Estos datos, junto a los resultados de nuestra experiencia previa, sugieren que la incubación de los espermatozoides con el heterodímero podría bloquear la penetración del ovocito por parte del espermatozoide, al menos *in vitro.* Por lo tanto, considerando el efecto beneficioso sobre los espermatozoides tratados que presenta el heterodímero PSP-I/PSP-II, en una cuarta experiencia se realizaron diversos ensayos para discernir como afectaría esta proteína sobre el reconocimiento e interacción de gametos y sobre la capacidad fecundante de los espermatozoides tratados con la misma.

Por todo lo anteriormente expuesto, los objetivos fundamentales de este trabajo fueron:

1. Evaluar si la adición de plasma seminal proveniente del mismo macho (autólogo) o de un macho distinto (homólogo) mejora la viabilidad espermática, así como su actividad mitocondrial y su motilidad.

2. Estudiar la relación existente entre el efecto protector de la espermadhesina PSP-I/PSP-II y su patrón de unión sobre la superficie de la membrana plasmática de espermatozoides de verraco sometidos a una dilución similar a la que se produce tras preselección del sexo mediante la separación de espermatozoides X e Y por citometría de flujo.

3. Examinar el efecto del heterodímero PSP-I/PSP-II durante el cocultivo espermatozoide-ovocito tanto en ovocitos inmaduros como madurados *in vitro.*

4. Examina el efecto del pretratamiento con PSP-I/PSP-II sobre la capacidad fecundante de espermatozoides de verraco tanto en ovocitos inmaduros como madurados *in vitro.*

II. – Revisión Bibliográfica

2.1. Plasma Seminal. Generalidades

El plasma seminal es definido como el medio líquido en el cual los espermatozoides se encuentran inmersos tras la eyaculación, producido por una mezcla de las secreciones procedentes de los túbulos seminíferos, el epidídimo y las glándulas sexuales accesorias (Mann y Lutwak-Mann, 1981). Debido al conocimiento que se tiene en numerosas especies (incluyendo el porcino) de que los espermatozoides epididimales son capaces de fertilizar ovocitos *in vitro* (Iritani A y cols., 1978; Nagai y cols., 1984), se podría asumir que la función del plasma seminal secretado por las glándulas sexuales accesorias sería de menor importancia. Sin embargo, actualmente esta fuera de toda duda el valor del plasma seminal como regulador en todos los procesos que ocurren tanto a nivel espermático, como a nivel del tracto genital de la hembra y fecundación. Estas funciones se refieren principalmente a la nutrición, protección, regulación de la motilidad y capacitación de los espermatozoides, reconocimiento y unión entre gametos y de una manera mas indirecta a su acción sobre el tracto genital de la hembra produciendo un incremento de las contracciones uterinas, una modulación de la respuesta inmune y una relajación del istmo oviductal (Johnson y cols., 2000).

El plasma seminal es un fluido muy complejo donde encontramos un alto contenido en agua, iones inorgánicos, ácido cítrico, azucares, sales orgánicas, prostaglandinas y un número variado de proteínas que servirán de sustancias tampón, manteniendo una osmolaridad adecuada y un pH cercano a 7, además de proporcionar fuentes de energías para su metabolismo tanto aeróbico como anaeróbico (Mann y Lutwak-Mann, 1981). Otras sustancias halladas en el plasma seminal son los prostasomas, los cuales han sido caracterizados en verraco recientemente (Piehl y cols., 2006), y que han sido definidos como orgánulos redondeados delimitados por una, dos o múltiples membranas conteniendo gran cantidad de colesterol y fosfolípidos y de un tamaño entre 21 y 100 nm, estos prostasomas han sido relacionados con procesos de estabilización de membranas, previniendo la capacitación y reacción acrosómica espontánea (Arienti y cols., 1997). Sin embargo, son las proteínas, secretadas más de un 80% por las glándulas vesicales (Lavon y Bournsell, 1971), las que contribuyen de forma más relevante a la regulación de la mayor parte de las funciones espermáticas siendo objeto de numerosos estudios por parte de diferentes grupos de investigación.

2.1.1. Componentes epididimarios

El contacto de los espermatozoides con las distintas sustancias que componen el plasma seminal ocurre de forma secuencial. Los espermatozoides viajan a través del epidídimo donde adquirirán su capacidad fecundante y motilidad, este tránsito tendrá una duración variable dependiendo de la especie, yendo desde los 5'5 días en la especie humana hasta los 16 días del carnero (verraco 9-11 días) (França y cols., 2005). Conforme el espermatozoide entra en el epidídimo, la membrana plasmática sufrirá una gran remodelación, tanto a nivel de composición proteica y fosfolipídica, como de

localización de los diferentes dominios del espermatozoide. Mientras que la mayor parte de las proteínas testiculares serán eliminadas o sufrirán degradación mediante diversos enzimas proteolíticos, como ocurre con diferentes miembros de la familia ADAMs (Blobel, 2000), la hialorunidasa (pH20/2B1) (Seaton y cols., 2000) y la forma germinal de la enzima conversora de la angiotensina (Gatti y cols., 1999), un gran número de nuevas proteínas secretadas por el epidídimo (principalmente en la región de la cabeza y el cuerpo) serán integradas en la membrana espermática durante su tránsito por el epidídimo (Gatti y cols., 2004; Dacheux y cols., 2005).

2.1.2. Componentes de las glándulas sexuales accesorias

Tras la eyaculación, los espermatozoides procedentes del epidídimo entran en contacto con las distintas secreciones procedentes de las glándulas vesicales, próstata y bulbouretrales. El conjunto de sustancias producidas por estas glándulas es especie-específico y altamente variable entre individuos de la misma especie, así como entre eyaculados de un mismo individuo, pudiendo variar por diferentes procesos patológicos, estación del año o estado fisiológico del animal (Pérez-Pé y cols., 2001a; Cardozo y cols., 2006). Variaciones en la composición del plasma seminal de diferentes machos ha sido relacionada con diferentes índices de fertilidad (Killian y cols., 1993; Moura y cols., 2006). Se ha observado, que la incubación de espermatozoides de toros de alta fertilidad con plasma seminal de toros de baja fertilidad disminuía la capacidad de penetración de ovocitos *in vitro* (Henault y Killian, 1996). Otro aspecto influenciado por la variabilidad en la composición del plasma es la motilidad espermática, observándose que la adición de plasma seminal heterólogo puede afectar positiva o

negativamente a este parámetro, dependiendo de cual sea la fuente de plasma seminal (Kneuppel y cols., 2000).

Además de variaciones entre individuos y entre eyaculados, se han demostrado variaciones entre fracciones de plasma seminal dentro de un eyaculado. Zhu y cols. (2000) demostraron una mejor tasa de penetración *in vitro* en aquellos espermatozoides incubados con plasma seminal proveniente de la fracción rica del eyaculado. Más aún, se han encontrado diferencias en la capacidad de los espermatozoides de sobrevivir al proceso de criopreservación según la fracción del eyaculado a la que perteneciesen. Todas estas variaciones se ha sugerido que podrían estar relacionadas con diferencias en los perfiles proteicos que presentan las distintas fracciones del eyaculado (Peña y cols., 2006).

Las glándulas sexuales accesorias serán las encargadas de producir la mayor parte del volumen del eyaculado. Entre los compuestos secretados por estas se encuentran una serie de componentes minerales como son el zinc (Zn^{2+}) con propiedades de estabilizador de macromoléculas y antibacteriano (Strzezek y cols., 1987), el ión calcio (Ca^{2+}) que participa en los fenómenos de capacitación espermática y reacción acrosómica. Los azucares como fructosa, inositol ácido cítrico y ácido ascórbico que proporcionan energía para el metabolismo de la célula (Voglmayr y Amann, 1973; Frei y cols., 1990), aminoácidos (ácido glutámico, carnitina, taurina, hipotaurina) que servirán como fuente de energía y de protección contra sustancias oxigeno reactivas y enzimas (proteasas, acrosina, nucleasas, fosfatasa ácida y alcalina y superóxido dismutasa) que intervendrán en la licuefacción seminal, penetración del ovocito por

parte del espermatozoide, digestión de espermatozoides muertos y dañados y protección contra sustancias oxigeno reactivas (Harrison, 1975; Zini y cols., 1993).

Como se ha mencionado anteriormente, los componentes del plasma seminal que influyen de forma más importante en la fertilidad y función espermática serán las proteínas, de las cuales tenemos 3 familias principales (Tabla 1).

- Proteínas secretadas ricas en cisteína (CRISP).
- Proteínas que contienen el dominio fibronectina tipo II (Fn-II).
- La familia de las espermadhesinas, en las cuales se centrará esta revisión.

En el plasma seminal se han descrito además otras proteínas como son, la Gp54 (complejo glicoproteíco de 54 kDa) el cual es precursor de la proteína SMIF (peptido inhibidor de la motilidad espermática), la proteína fosfotirosina fosfatasa ácida, envuelta en el control de la proliferación y diferenciación celular, la acetilhidrolasa del factor activador de plaquetas (PAF-AH), encargada de regular los niveles de PAF, (revisado en Strezek, 2002) y la ubiquitina cuya función es la degradación de proteínas dañadas y parece estar implicada en el procesamiento de espermatozoides epididimarios anormales (revisado en Sutovsky, 2003)

2.2. Proteínas del plasma seminal

2.2.1. Proteínas secretadas ricas en cisteína (CRISP)

Los miembros de la familia de proteínas CRISP (CRISP1, CRISP2, CRISP3) se caracterizan por la presencia de 16 residuos de cisteína que formarán 8 puentes de

Tabla 1. Principales componentes proteícos del plasma seminal en las diferentes especies.

Familia de proteínas	Especie	Nombre	Referencia
Proteínas CRISP	Humanos	CRISP1, CRISP2, CRISP3	Udby y cols., 2005
	Caballos	CRISP1, CRISP2, CRISP3	Töpfer-Petersen y cols., 2005
	Ratones	CRISP1, CRISP2, CRISP4	Jalkanen y cols., 2005 Udby y cols., 2005
	Ratas	CRISP1, CRISP2	Brooks y Tiver 1983 Udby y cols., 2005
Proteínas con el dominio Fn-II	Verraco	pB1	Calvete y cols., 1997a
	Caballo	HSP-1, HSP-2, EQ-12	Calvete y cols., 1995b Töpfer-Petersen y cols., 2005 Saalman y cols., 2001
	Carnero	RSVP-14, RSVP-20, RSP-15, RSP-22, RSP-24	Barrios y cols., 2005 Bergeron y cols., 2005
	Toro	BSP-A1, BSP-A2, PDC-109, BSP-30 kDa	Manjunath y Sairam 1987 Manjunath y Therien 2002
	Caprino	GSP-14, GSP-15, GSP-20, GSP-22 kDa	Villemure y cols., 2003
	Bisonte	BISV-16, BISV-17, BISV-18, BISV-28 kDa	Boisvert y cols., 2004
Espermadhesinas	Verraco	AWN, AQN-1, AQN-3, PSP-I/PSP-II	Calvete y cols., 1995c Varela y cols., 1997
	Caballo	HSP-7	Reinert y cols., 1996
	Carnero	Espermadhesina de 15’5 kDa	Bergeron y cols., 2005
	Toro	aSFP, Z13	Wempe y cols., 1992 Tedeschi y cols., 2000

disulfuro, subdividiendo la molécula en 3 dominios (Töpfer-Petersen y cols., 2005). Estas 3 proteínas han sido identificadas en el tracto genital de caballos y humanos,

mientras que solamente CRISP1 y CRISP 2 se localizaron en el tracto genital en roedores (Udby y cols., 2005). Recientemente, se ha identificado una nueva proteína (CRISP4) en el epidídimo de ratón (Jalkanen y cols., 2005). La función de estas proteínas en la reproducción parece estar relacionadas con los procesos de espermiogénesis, maduración del espermatozoide, capacitación espermática y en la unión del espermatozoide con el ovocito.

Estas proteínas han sido localizadas en las regiones post-acrosomal, ecuatorial y pieza intermedia de los espermatozoides, incluso tras procesos de capacitación *in vitro* y reacción acrosómica, lo que da una idea de su posible relación con procesos de fusión entre el espermatozoide y el ovocito. La proteína CRISP1 se expresa fundamentalmente en el epidídimo, donde es secretada y se une a la superficie espermática, esta proteína parece estar relacionada con los procesos de maduración e inhibición de una prematura capacitación espermática, además de ser necesaria para la fusión del espermatozoide con el ovocito (Udby y cols., 2005). CRISP2 es sin embargo específica del testículo, y parece estar envuelta en el desarrollo de las espermátidas, siendo una de las responsables de la adherencia de estas células a las células de Sertoli (Maeda y cols., 1999). Dentro de la familia de las proteínas CRISP, CRISP3 es la que presenta mayor distribución a lo largo de los diferentes tejidos del cuerpo, siendo en equino una de las proteínas con mayor concentración en el plasma seminal (1 mg/ml). Esta proteína presenta la particularidad de que puede ser liberada (al menos parcialmente) tras lavado de los espermatozoides, por lo que ha sido sugerido que podría cumplir alguna función en el tracto genital de la hembra (Töpfer-Petersen y cols., 2005).

2.2.2 Proteínas que contienen el dominio fibronectina tipo II (Fn-II)

La familia de proteínas Fn-II, esta caracterizada por la presencia de dos modulos Fn-II y ha sido hallada en numerosas especies domésticas, siendo la mas abundante en vacuno (BSP; Manjunath y Sairam, 1987), caprino (GSP; Villemure y cols., 2003), bisonte (BISV; Boisvert y cols., 2004), carneros (RSP; Barrios y cols., 2005; Bergeron y cols., 2005) y en el equino (HSP; Calvete y cols., 1995b; Töpfer-Petersen y cols., 2005). Además, ha sido descrita en otras especies como en el porcino donde parece ser un componente menor en el plasma seminal del verraco (pB1; Calvete y cols., 1997a). Mas recientemente, se ha identificado un grupo de proteínas Fn-II de cadena larga formadas por 4 módulos Fn-II, originadas en el epidídimo y que parecen tener relación con la maduración espermática, en el equino esta proteína ha sido denominada EQ-12 (Saalman y cols., 2001).

Tras la eyaculación, estas proteínas Fn-II se unen al espermatozoide mediante interacciones específicas con los residuos de colina de los fofolípidos de la membrana del espermatozoide (Desnoyers y Manjunath, 1992; Müller y cols., 1998). Al mismo tiempo se produce una eliminación de colesterol y fosfolípidos de la membrana plasmática produciéndose una reorganización estructural de la misma. La unión de estas proteínas podría prevenir el movimiento de fosfolípidos estabilizando la membrana plasmática. Estas proteínas presentan además la capacidad de unión con lipoproteínas de alta y baja densidad, así como con heparina, la unión con estas sustancias presentes en el oviducto de la hembra podría provocar una segunda salida de colesterol y la disminución del ratio colesterol/fofolípido. El colesterol tiene un efecto estabilizador sobre la membrana plasmática del espermatozoide (Yeagle, 1985), esta segunda

eliminación de colesterol causaría la subsiguiente reorganización o desestabilización de la membrana que terminaría en la capacitación espermática (Manjunath y Thérien 2002; Fernández-Juan y cols.,2006). Estas proteínas también pueden modular los efectos de otros agentes capacitantes como son la heparina, progesterona y angiotensina-II (Fiol de Cuneo y cols., 2004).

2.2.3. Espermadhesinas

Las espermadhesinas son una familia de glicoproteínas de bajo peso molecular (12-16 kDa) compuestas por entre 109 y 133 aminoácidos y constituidas estructuralmente por un único dominio CUB que sirve como soporte estructural y al cual se le pueden atribuir diferentes funcionalidades (Romero y cols., 1997).

Estas proteínas han sido identificadas hasta el momento en porcino, bovino, caprino y equino, siendo el principal componente proteico del plasma seminal en la especie porcina. En el verraco, la familia de las espermadhesinas representan mas del 90% de las proteínas del plasma seminal y esta compuesta por cinco miembros (AQN-1, AQN-3, AWN, PSP-I y PSP-II), de los cuales se han descrito diferentes isoformas glicosiladas para PSP-I, PSP-II, AWN y AQN-3, además de la isoforma acetilada en el extremo amino-terminal de AWN. Los monómeros PSP-I y PSP-II representan aproximadamente el 50% del total del contenido proteico del plasma seminal y forman el heterodímero no covalente PSP-I/PSP-II (Sanz y cols., 1992a; Calvete y cols., 1995a) (Figura 1).

En el resto de especies domesticas han sido descritas las espermadhesinas aSFP y Z13 en el ganado bovino (Wempe y cols., 1992; Tedeschi y cols., 2000), la espermadhesina HSP-7 en equinos (Reinert y cols., 1996) y por último, se ha identificado recientemente una espermadhesina de 15’5 kDa, mayoritaria en el plasma seminal de carnero (Bergeron y cols., 2005).

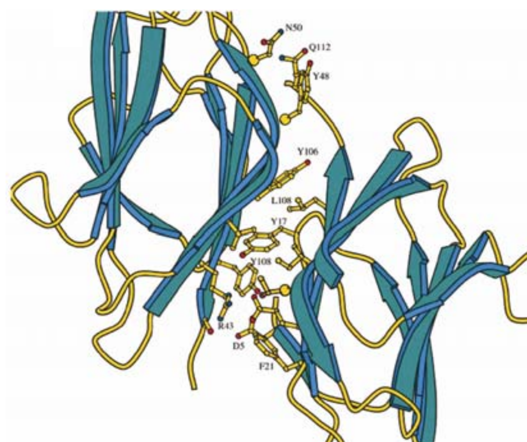

Figura 1. Representación tridimensional del heterodímero PSP-I/PSP-II (Varela y cols., 1997).

2.2.3.1. Biosíntesis y secreción de las espermadhesinas

La expresión y síntesis de todas las espermadhesinas en los distintos tejidos y glándulas del aparato genital masculino ha sido estudiado en el verraco mediante transcripción inversa-reacción en cadena de la polimerasa (RT-PCR), observando la expresión de ARNm para todas las espermadhesinas en las vesículas seminales, próstata y cola del epidídimo, además también se detectó tanto PSP-I como PSP-II en el testículo

(Ekhlasi-Hundrieser y cols., 2002). Anteriormente se habían realizado ensayos tanto inmunológicos (western-blot) como inmunohistoquímicos, donde se detecto la presencia de AWN y PSP-1 en vesícula seminal, además de AWN en algunas células del epidídimo y en la rete testis (Kwok y cols., 1993; Sinowatz y cols., 1995).

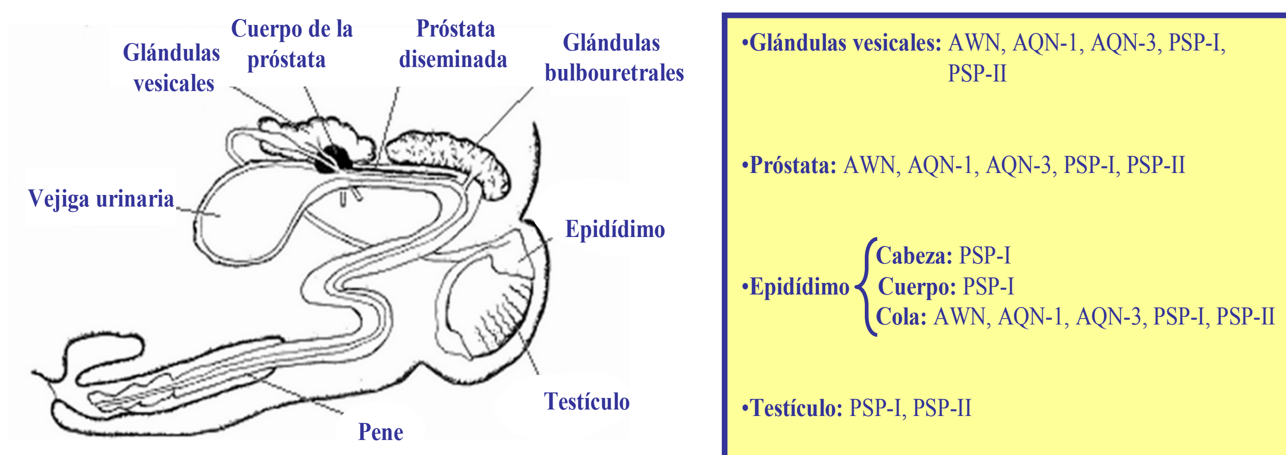

Figura 2. Esquema del tracto genital del cerdo y expresión de las espermadhesinas en los diferentes tejidos estudiados mediante RT-PCR (Ekhlasi-Hundrieser y cols., 2002).

Las espermadhesinas presentan la característica de unirse a la superficie de la membrana plasmática del espermatozoide durante la eyaculación (Sanz y cols., 1992a; Töpfer-Petersen y cols., 1998). El eyaculado del verraco es un fluido heterogéneo compuesto de una serie de fracciones, habitualmente llamadas pre-espermática, con predominio de las secreciones provenientes de las glándulas uretrales, bulbouretrales y próstata, fracción rica del eyaculado, en la cual se encuentran la gran mayoría de los espermatozoides bañados en plasma seminal procedente de la próstata y las glándulas vesicales, y la fracción post-espermática, con presencia de pocos espermatozoides

inmersos en un medio formado por las secreciones crecientes de las glándulas vesicales, la próstata y al final de la eyaculación de las glándulas bulbouretrales. Por lo tanto, como es de esperar, la composición proteica del plasma seminal, así como la concentración relativa de proteínas presente en el mismo varía significativamente con las diferentes fracciones del eyaculado. En esta línea, Rodríguez-Martínez y cols. (2005) han descrito recientemente la presencia a bajas concentraciones de todas las espermadhesinas en la fracción pre-espermática y primeras porciones de la fracción rica del eyaculado, produciendose un aumento de la concentración proteica en la fracción post-espermática a medida que disminuye la concentración de espermatozoides.

2.2.3.2. Capacidad de unión a ligandos de las espermadhesinas

Se ha observado que las espermadhesinas de verraco son proteínas multifuncionales presentando afinidad por una gran variedad de ligandos. Habitualmente las espermadhesinas han sido clasificadas dependiendo de que presentaran afinidad por la heparina (AQN-1; AQN-3 y AWN) o no (heterodímero PSP-I/PSP-II). Sin embargo, a pesar de que el heterodímero PSP-I/PSP-II no se une a la heparina, sus monómeros (PSP-I y PSP-II) si presentan esta capacidad de unión (Calvete y cols., 1995a). Otros ligandos por los cuales presentan afinidad son: fosfolípidos, inhibidores de la serín-proteasa y carbohidratos.

AWN y AQN-3 presentan afinidad por la fosforiletanolamina, sugiriendo una unión directa de estas espermadhesinas con los fosfolípidos de la membrana espermática (Dostàlovà y cols., 1995a; Ensslin y cols., 1995). Con respecto a la afinidad por los inhibidores de la serin-proteasa, se ha demostrado que AQN-1 y las 2 isoformas

de la AWN son capaces de unirse al inhibidor de la acrosina así como al inhibidor de la tripsina procedente de soja, al cual se une también el heterodímero PSP-I/PSP-II (Calvete y cols., 1995a). Esta unión parece estar modulada por el grado de glicosilación de estas proteínas, ya que aumenta al deglicosilar las mismas (Sanz y cols., 1992b; Calvete y cols., 1993).

Por último, Las espermadhesinas aisladas presentan la capacidad de unirse a carbohidratos, AWN, AQN-1 y AQN-3 reconocen β-galactosidos de la zona pelúcida (ZP) de ovocitos homólogos, sugiriendo una posible función de estas espermadhesinas en el reconocimiento de gametos. Al igual que ocurre con los inhibidores de la serin-proteasa, la glicosilación interfiere en la capacidad de las espermadhesinas para unir carbohidratos (Dostàlovà y cols., 1995b; Calvete y cols., 1994; Calvete y cols., 1996a). La capacidad de unión del heterodímero PSP-I/PSP-II y sus monómeros a glicoproteínas de la ZP provoca ciertas discrepancias, mientras que algunos autores encontraron afinidad del heterodímero PSP-I/PSP-II, así como del monómero PSP-II por la ZP (Calvete y cols., 1995a), Jonákova y cols. (2000) encontraron esa misma interacción entre el monómero PSP-II y la ZP, pero esta unión se encontraba inhibida en el heterodímero.

En condiciones fisiológicas, los componentes proteicos del plasma seminal se encuentran en su mayoría formando agregados. Estos agregados han sido separados en cinco fracciones dependiendo de tamaño y de su peso molecular (>100, 55, 45, 30 y 5-15 kDa). Se han observado algunas diferencias en la capacidad de unión a ligandos de los agregados con respecto a las proteínas purificadas, como es la capacidad de interactuar con el colesterol por parte de los agregados (Jonáková y cols., 2000).

2.2.3.3. Funciones biológicas de las espermadhesinas

Como se ha mencionado anteriormente, las espermadhesinas presentan la capacidad de unirse a una gran variedad de ligandos. Esta variedad de ligandos nos sugiere su posible implicación en diferentes etapas de la fertilización.

2.2.3.3.1. Función moduladora de la capacitación y reacción acrosómica

Hace ya más de 50 años que se descubrió el concepto de capacitación espermática, sin embargo, este proceso sigue sin ser comprendido en su totalidad. Fueron Chang (1951) y Austin (1952) quienes en trabajos independientes descubrieron que el espermatozoide necesita un periodo de residencia en el tracto genital femenino antes de adquirir la capacidad de penetrar un ovocito. Actualmente, es sabido que la capacitación espermática conlleva una cascada de reacciones (salida de colesterol, fosforilación de proteínas, incremento intracelular de ión Ca^{2+}) que llevan a una desestabilización y reorganización de los lípidos y proteínas de la membrana plasmática, que terminará con la exocitosis del acrosoma cuando el espermatozoide entre en contacto con la ZP (Flesch y Gazella, 2000).

Se ha observado que la incubación continua de los espermatozoides con el plasma seminal previene los fenómenos de capacitación espermática (Davis y Niwa, 1974; Kanwar y cols., 1979). Además, el proceso de capacitación espermática es reversible, pudiendo ser frenado mediante el contacto de los espermatozoides con sustancias estabilizadoras presentes en el plasma seminal que podrían mediar en los procesos de

capacitación (Chang, 1957). Estos factores decapacitantes deben de ser eliminados de la superficie de la membrana plasmática del espermatozoide para poder iniciar la capacitación. Algunas de estos factores probablemente pertenecerían a la familia de las espermadhesinas (Calvete y cols., 1996b).

Tras la eyaculación, un espermatozoide presentara aproximadamente entre 10 y 60 millones de moléculas de espermadhesinas recubriendo su superficie. Sin embargo, la mayoría de esta moléculas son eliminadas tras procesos de capacitación *in vitro* (Dostàlovà y cols., 1994). Teniendo en consideración la biosíntesis y afinidad por los ligandos de las diferentes espermadhesinas y sus agregados, se ha descrito un modelo, por el cual se sugiere que ya en el epidídimo el espermatozoide presentaría algunas moléculas de AWN-1 adherida a la membrana plasmática, a las cuales se le unirían tras la eyaculación moléculas no agregadas de AWN-1 y AQN-3, y finalmente serían recubiertas por el resto de las espermadhesinas en forma de agregados (Dostàlovà y cols., 1995a). Esta cubierta de espermadhesinas tendría la función de prevenir reacciones acrosomales prematuras, siendo la capa de agregados liberada tras la capacitación (Töpfer-Petersen y Calvete, 1996; Jansen y cols., 2001) (Figura 3).

Sin embargo, se ha visto que los agregados de las espermadhesinas presentan afinidad por el colesterol, pudiendo actuar como receptores de las moléculas de colesterol liberadas del espermatozoide, disminuyendo así el ratio colesterol/fosfolípidos, el cual, como hemos señalado anteriormente, es un fenómeno asociado a la capacitación espermática (Jonáková y cols., 2000). Además, la afinidad de algunas de las espermadhesinas por la heparina y otros glicosaminoglicanos, los cuales se han visto que promueven la capacitación y están presentes en el oviducto de la cerda

(Therien y cols., 1995; Therien y cols., 1997; Tienthai y cols., 2000), indica que estas espermadhesinas estarían envueltas en la regulación de la capacitación, presentando una función bimodal, ya que mantendrían estable la célula espermática hasta su llegada al oviducto donde promovería la capacitación. Una función similar se les ha otorgado a las proteínas del plasma seminal bovino (BSP), las cuales se unen a los fosfolípidos de membrana tras la eyaculación, estabilizando así la membrana plasmática, pero que cuando son expuestos a lipoproteínas de alta densidad y glicosaminoglicanos como la heparina inducen la capacitación (Therien y cols., 1997; Manjunath y Therien, 2002).

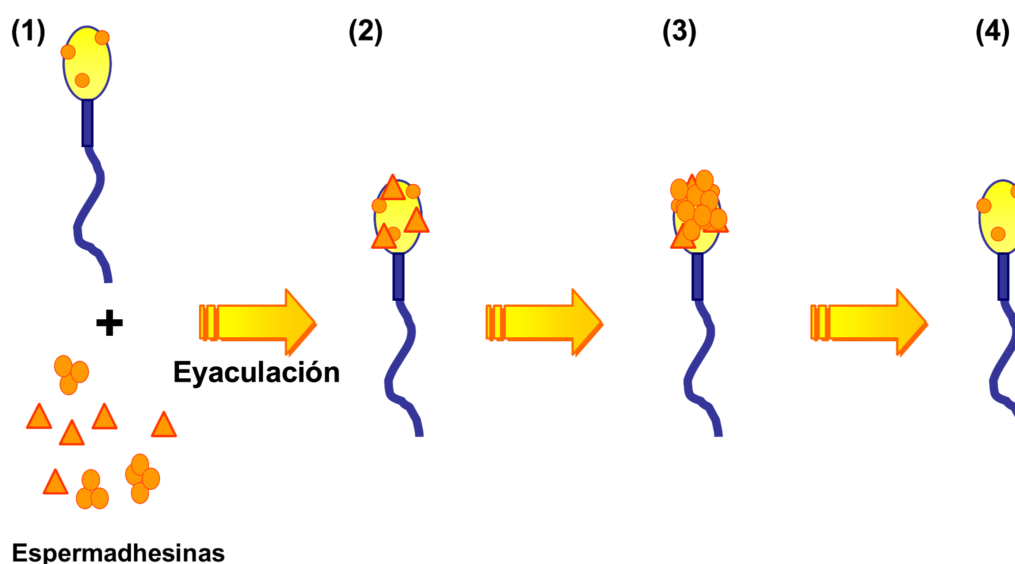

Figura 3. Modelo propuesto para la unión y posterior liberación de las espermadhesinas de la cabeza del espermatozoide. En el epidídimo nos encontramos los espermatozoides cubiertos por algunas partículas de AWN (1). Tras la eyaculación, monómeros aislados de AWN-1 y AQN-3 se unirán al espermatozoide (2) para ser cubierto posteriormente por los diferentes agregados de espermadhesinas (3), los cuales se liberaran tras el paso por el aparato genital de la cerda (4).

2.2.3.3.2. Establecimiento del reservorio oviductal

La formación del reservorio oviductal en la especie porcina ocurre a nivel del la unión útero-tubárica y segmentos adyacentes del istmo en un periodo de entre 5 minutas y 3 horas post-inseminación (Rodríguez-Martínez y cols., 2005) y parece cumplir varias funciones, como son prevención de la poliespermia, modulación de la capacitación, y mantenimiento de la capacidad fecundante de los espermatozoides (Töpfer-Petersen, 1999). La población espermática que es capaz de alcanzar este reservorio parece estar libre de plasma seminal, manteniendo únicamente aquellas proteínas que recubren la superficie espermática (Calvete y cols., 1997b). En el reservorio, los espermatozoides aparecen tanto retenidos en un fluido viscoso (compuesto por glicosaminoglicanos sulfatados y no sulfatados) en la luz del túbulo, como pegados al epitelio oviductal (Mburu y cols., 1997; Tienthai y cols., 2000).

Diversos ensayos basados en la inhibición de la unión espermatozoide-oviducto mediante azúcares sugirieron que la formación del reservorio oviductal podría estar mediada por carbohidratos y ser específica de la especie (Tópfer-Petersen, 1999; Green y cols., 2001). En ganado bovino, esta unión parece estar mediada por residuos de fucosa, los cuales se pierden tras la capacitación (Lefebvre y cols., 1997; Revah y cols., 2000). En el porcino, la formación del reservorio oviductal parece implicar residuos de D-manosa (Green y cols., 2001). En esta línea, recientemente se ha observado que las espermadhesinas y sus agregados que unen heparina presentan afinidad por la manosa, demostrandose que esta afinidad por la manosa esta correlacionada positivamente con su habilidad para interactuar con el epitelio oviductal (Jelínkova y cols., 2004; Liberda y cols., 2006). Por ultimo, AQN-1 ha sido sugerido como posible candidato para la

formación del reservorio, ya que reconoce un amplio espectro de ligandos, entre ellos manosa, y su adición a explantes oviductales inhibió la unión de espermatozoides de una manera dosis dependiente. Tras ser capacitados *in vitro*, los espermatozoides perdieron gran cantidad de AQN-1, sufriendo consecuentemente una reducción de su afinidad por el epitelio oviductal (Ekhlasi-Hundrieser y cols., 2005).

2.2.3.3.3. Reconocimiento y unión entre gametos

La unión y fusión entre gametos es un paso crítico en el proceso de fertilización en el cual se ven implicadas un gran número de moléculas y reacciones bioquímicas hasta la formación de un nuevo zigoto. En las especies domesticas, la unión entre el espermatozoide y el ovocito parece estar mediada en parte por la interacción de las cadenas de carbohidratos de la ZP con ciertas lectinas asociadas a la membrana plasmática de los espermatozoides capaces de reconocer esos carbohidratos (Jansen y cols., 2001).

El hecho de que las espermadhesinas AQN-1, AQN-3 y AWN y sus agregados presentan afinidad por la ZP, ha sugerido que estas proteínas pudieran estar envueltas en la interacción entre gametos (Jonáková y cols., 2000). Asimismo, el uso de anticuerpos policlonales frente a AWN y AQN disminuyó la unión de los espermatozoides a los ovocitos en experiencias realizadas *in vitro* (Veselsky y cols., 1999). Mediante inmunomicroscopía electrónica de barrido se ha demostrado que una pequeña cantidad de AWN sigue presente en los espermatozoides unidos a zona pelúcida. Estas moléculas de AWN corresponderían a aquellas que se encuentran fuertemente adheridos a los fosfolípidos de la membrana plasmática del espermatozoide (Rodríguez-Martínez y

cols., 1998). Resulta interesante que AWN-1 haya sido la única espermadhesina hallada en la superficie de los espermatozoides provenientes del epidídimo, sugiriéndose que podría ser uno de los factores contribuyentes a la capacidad fecundante de los espermatozoides epididimarios (Dostálová y cols., 1994; Calvete y cols., 1995c).

Existe cierta controversia con respecto a la posible función que pudiera presentar el heterodímero PSP-I/PSP-II o sus monómeros en la interacción entre gametos. Mientras que en algunos estudios, el heterodímero mostró afinidad por las glicoproteinas de la ZP (Calvete y cols., 1995a), en otros, esta afinidad fue hallada en el monómero PSP-II, pero inhibida cuando se formaba el heterodímero PSP-I/PSP-II (Jonáková y cols., 2000).

2.2.3.3.4. Inmunomodulación del útero

Es bien conocido que la entrada de semen en el útero provoca una masiva migración de leucocitos, especialmente polimorfonucleares (PMN), desde la lámina propia del útero hacia la luz uterina (Lovell y Getty 1968; Rozeboom y cols., 2000). Los PMN no alcanzan inmediatamente la luz del útero tras la deposición del semen, sino que comienzan a llegar aproximadamente 10 minutos después de la inseminación, observándose una gran presencia de PMN a los 30 minutos, alcanzando el máximo entre las 6 y las 12 horas post-inseminación y manteniéndose hasta 24 horas tras la realización de la misma (Rozeboom y cols., 1998). Se ha observado que el plasma seminal podría ser capaz de modular la respuesta inflamatoria producida tras la inseminación y proteger a los espermatozoides de esa respuesta inflamatoria (Rozeboom y cols., 2001).

Diversas evidencias apuntan al heterodímero PSP-I/PSP-II y sus subunidades como uno de los componentes del plasma seminal que ejercen un efecto modulador de la actividad inmune del útero. Así, se ha demostrado que PSP-I y PSP-II son capaces de estimular la actividad linfocítica *in vitro* (Leshin y cols., 1998). En la misma línea, experiencias realizadas *in vivo* demostraron que el heterodímero PSP-I/PSP-II provoca una invasión de neutrofilos tanto en cavidad abdominal en ratas (Assreuy y cols., 2002) como en la luz uterina de cerdas (Rodríguez-Martínez y cols., 2005).

La migración leucocitaria inducida por el heterodímero PSP-I/PSP-II y su subunidad PSP-II es mediada a través de los macrófagos residentes en el útero, mientras que la subunidad PSP-I parece ejercer un efecto directo sobre los neutrófilos (Assreuy y cols., 2002; Assreuy y cols., 2003). Recientemente, se ha sugerido que la entrada del semen en la cavidad uterina podría provoca la disociación del heterodímero en sus subunidades, lo cual tiene una gran relevancia a nivel fisiológica ya que podría explicar los diferentes mecanismos de acción del heterodímero y sus subunidades en la modulación de la inflamación uterina (Campanero-Rhodes y cols., 2005).

Por lo tanto, podríamos decir que ese periodo de latencia existente hasta la aparición de PMN en la luz uterina y la presencia de diferentes cantidades de proteínas según la fracción del eyaculado daría la oportunidad a una determinada subpoblación espermática de alcanzar el oviducto sin riesgo de ser fagocitada (Rodríguez-Martínez y cols., 2005).

2.3. Aplicaciones del plasma seminal en las biotecnologías reproductivas

A lo largo de los últimos años se esta despertando un creciente interés en la utilización de plasma seminal o alguno de sus componentes (fundamentalmente proteínas) como aditivo de los diluyentes espermáticos durante el procesamiento de los espermatozoides en diferentes procesos biotecnológicos como son la criopreservación espermática (Graham 1994; Aurich y cols., 1996) y la separación espermática X e Y mediante citometría de flujo (Maxwell y Johnson, 1999). Estos procedimientos envuelven la dilución y/o eliminación del plasma seminal, pudiendo producir una eliminación, modificación y reestructuración de las glicoproteínas que rodean la superficie de la membrana plasmática del espermatozoide resultando en una disminución de la motilidad y en una perdida de la capacidad fecundante (Maxwell y Johnson, 1999). Diferentes estudios realizados en este sentido (Asworth y cols 1994; Aurich y cols., 1996; Maxwell y cols., 1998) han demostrado que la adición de plasma seminal, o ciertas proteínas del plasma seminal a los medios en los que se diluyen los espermatozoides puede revertir el efecto deletéreo provocado por la manipulación espermática durante la aplicación de las diferentes biotecnologías.

El continuo progreso de los conocimientos sobre la funcionalidad biológica, la composición y las posibles aplicaciones en biotecnología del plasma seminal, ha propiciado la evolución de un campo como la proteómica del plasma seminal. Estos avances están dirigiendo a los investigadores en la búsqueda de un marcador bioquímica del plasma seminal que pudiera servir como indicador del potencial fértil de un macho (Strzezek y cols., 2005).

2.3.1. Adición de plasma seminal a los espermatozoides congelados-descongelados

La criopreservación de los espermatozoides produce diferentes daños en la célula espermática como consecuencia del choque frío, el estrés osmótico y la formación de cristales de hielo intracelular durante la congelación y posterior descongelación de los mismos (Watson, 2000). Estos daños provocan una disminución (aproximadamente del 50%) del número de espermatozoides viables (Roca y cols., 2005). Dentro de la población de espermatozoides viables, encontraremos una subpoblación espermática con una vida media reducida que presenta unos cambios similares a los producidos en las últimas etapas de la capacitación espermática denominados "criocapacitación" (Watson, 1995). Recientemente se ha observado que aunque la capacitación espermática y la "criocapacitación" presentan similitudes, el mecanismo por el cual se producen estos cambios es diferente (Cormier y Bailey, 2003).

Diversos intentos para optimizar los protocolos de criopreservación incluyen nuevos sistemas de envasado (Eriksson y Rodríguez-Martínez, 2000), desarrollo de nuevas tecnologías de inseminación como la inseminación intrauterina profunda (Vázquez y cols., 2005) y la adición de ciertos aditivos a los diluyentes de congelación espermática (Peña y cols., 2003a; Roca y cols., 2005), entre los que se puede incluir el plasma seminal y sus proteínas como posibles estabilizadores de las membranas plasmáticas de los espermatozoides. Esto podría ser de gran importancia, porque podría amortiguar ese estado de "criocapacitación" que una vez alcanzado acorta la vida media del espermatozoide considerablemente, teniendo por lo tanto un tiempo muy limitado para fecundar al ovocito.

En general, la preincubación de los espermatozoides con su plasma seminal durante un periodo de tiempo (2-7 horas) antes de la congelación, así como la presencia de una cierta proporción de plasma seminal (10-40%) en los medios de dilución de espermatozoides es capaz de aumentar la resistencia de los espermatozoides al choque frío y reducir e incluso revertir el estado de capacitación espermática (Pursel y cols., 1973; Vadnais y cols., 2005a). Sin embargo, el efecto del plasma seminal sobre la congelabilidad espermática ha dado resultados contradictorios. Mientras que algunos autores han mostrado un efecto beneficioso sobre la funcionalidad espermática al adicionar plasma seminal a los medios de congelación y descongelación (Aurich y cols., 1996; Garner y cols., 2001; Maxwell y cols., 1999; Vadnais y cols., 2005b), otros autores han observado un efecto perjudicial de este plasma seminal sobre la calidad espermática (Kawano y cols., 2003).

Se ha descrito la existencia de machos "buenos" y "malos" congeladores, dependiendo de la capacidad de sus espermatozoides para resistir los procesos de criopreservación (Roca y cols., 2006b). La adición de plasma seminal afecta de manera distinta a la congelabilidad espermática según la fuente de procedencia de ese plasma seminal sean "buenos" o "malos" congeladores (Aurich y cols., 1996). Además, es de resaltar que los espermatozoides pertenecientes a diferentes fracciones del eyaculado de un verraco varían en su capacidad de sobrevivir a la congelación, y que esta habilidad varía entre verracos (Peña y cols., 2006). Estas diferencias pueden ser explicadas debido a las variabilidad encontrada en la composición del plasma seminal entre machos, entre eyaculados e incluso entre porciones de un mismo eyaculado.

Estudios recientes han mostrado diferencias en el perfil proteico del plasma seminal antes y después de la congelación, además de entre toros que presentaron una buena o mala calidad espermática a la descongelación (Nauc y Manjunath, 2000; Jobim y cols., 2004). En carneros, se ha observado que los espermatozoides sometidos a un choque frío pueden volver a un estado funcional normal mediante la adsorción de proteínas del plasma seminal. Mas aún, la adición de proteínas del plasma seminal mejoró significativamente la supervivencia de los espermatozoides al choque frío (Barrios y cols., 2000; Pérez Pé y cols., 2001b).

Por lo tanto, y por todo lo anteriormente expuesto, es posible pensar que si añadimos plasma seminal o alguno de los factores decapacitantes del plasma seminal (como pueden ser algunas proteínas) a los espermatozoides congelados-descongelados, en algunos de los pasos del proceso de criopreservación, se podría alargar la longevidad de los espermatozoides mediante la unión de estos factores a la membrana plasmática inhibiendo, en cierto grado, cambios estructurales y fisiológicos (Fraser y cols., 1990; Maxwell y Johnson 1999; Barrios y cols., 2000).

2.3.2. Mejora del proceso de separación de espermatozoides X e Y por citometría de flujo mediante la adición de plasma seminal

La posibilidad de predeterminar el sexo de la descendencia antes de la concepción ha sido y sigue siendo hoy en día uno de los objetivos más importantes de las biotecnologías reproductivas debido a los evidentes beneficios que supone la aplicación de la misman, tanto en la mejora del progreso genético como en la optimización de la producción ganadera. Actualmente la única técnica que ha mostrado una eficiencia

suficiente junto con una potencial rentabilidad es la preselección del sexo mediante la separación de espermatozoides X e Y por citometría de flujo, en función de la diferencia en el contenido de ADN de los mismos. Sin embargo, esta técnica presenta unas limitaciones, ya que requiere de una tinción del material nuclear mediante el fluorocromo Hoechst 33342, exposición al impacto de un láser ultravioleta (UV; 351, 364 nm), además de someter a los espermatozoides a una alta dilución y a diferentes fuerzas mecánicas como son el paso por el citómetro separador y la proyección en el tubo de recogida a altas presiones, lo cual daña al espermatozoide reduciendo su tiempo de vida media (Maxwell y cols., 2004). Otra de las grandes limitaciones de la técnica, especialmente en el caso de la especie porcina, es el rendimiento de los citómetros actuales, capaces de separarar hasta 15 millones de espermatozoide por hora (Johnson y cols., 2005). A pesar del desarrollo de nuevas técnicas de inseminación intrauterina profunda en el ganado porcino, las cuales han sido capaces de disminuir el número de espermatozoides separados por dosis de inseminación a 70 millones (Vázquez y cols., 2003), el rendimiento continua siendo demasiado bajo, siendo necesario un tiempo de entre 5 y 10 horas para conseguir una dosis de inseminación. Por lo tanto, la población final para la inseminación será heterogénea ya que estará compuesta tanto por espermatozoides recién separados como por espermatozoides almacenados hasta 10 horas (Parrilla y cols., 2005; Martínez y cols., 2005).

La adición de un determinado porcentaje de plasma seminal (entre un 1 y un 10%) a los medios de recogida de los espermatozoides separados es una práctica habitual tanto en porcino como ovino, para mejorar la viabilidad de los espermatozoides que han sufrido el proceso de separación Este efecto beneficioso puede ser debido a una disminución del “efecto dilución” y a la estabilización de la membrana plasmática de

los espermatozoides por parte del plasma seminal (Maxwell y cols., 1997; Maxwell y Johnson, 1999).

El Proceso de separación espermática por citometría de flujo produce un incremento en la proporción de espermatozoides capacitados, que puede ser reducida mediante la incubación de los espermatozoides en medios conteniendo plasma seminal (Maxwell y Johnson, 1997). En relación con este efecto decapacitante del plasma seminal, se ha observado una disminución en la proporción de ovocitos poliespérmicos, penetrados y divididos, además de reducir la proporción de blastocistos producidos *in vitro* cuando se añadió plasma seminal a los medios de recogida (Maxwell y cols., 1998). Sin embargo, cuando los espermatozoides separados se dejaron incubar por 2 horas en el medio de recogida con plasma seminal, se alcanzaron tasas de penetración *in vitro* muy similares a los obtenidos en el grupo control formado por espermatozoides sin separar (Parrilla y cols., 2005).

Los factores del plasma seminal que modulan su efecto sobre los espermatozoides separados parecen ser de origen proteico. La utilización de plasma seminal artificial, el cual no presenta proteínas en su composición, como medio en el que se diluirán los espermatozoides a su paso por el citómetro de flujo produjo una disminución general de la calidad de los espermatozoides separados (de Graaf y cols., 2004). En el verraco, la espermadhesina PSP-I/PSP-II y su subunidad PSP-II presentan un efecto protector sobre los espermatozoides altamente diluidos (Centurión y cols., 2003; García y cols., 2006), en el caso del carnero, estas proteínas beneficiosas podría ser la RSVP-14/20 (Barrios y cols., 2005).

En resumen, el proceso de separación produce un daño en el espermatozoide debido a las fuerzas mecánicas a las que es sometido y al alto grado de dilución, el cual provoca una eliminación y reestructuramiento de las proteínas de la membrana plasmática, llevando la célula espermática a un estado similar al de capacitación que puede ser prevenido mediante la adición de plasma seminal o alguno de sus componentes, principalmente proteínas.

2.3.3. Uso de proteínas del plasma seminal como marcadores de la calidad seminal

Los efectos biológicos que presentan las proteínas del plasma seminal sobre la funcionalidad espermática son muy complejos y no totalmente entendidos. Es sabido que determinadas proteínas son capaces de estabilizar la membrana plasmática de los espermatozoides previniendo reacciones acrosómicas prematuras, regulando la respuesta inmune en el útero e incluso desempeñando una función en el desarrollo embrionario temprano (Strzezek y cols., 2005). Todos estos efectos que tienen las proteínas sobre la función espermática, fertilización y desarrollo embrionario han llevado a los investigadores a la búsqueda de marcadores bioquímicos del plasma seminal que pudieran estar relacionados con el potencial fértil del macho.

En el verraco, se ha observado como una elevada cantidad de 2 proteínas diferentes, de 26 kDa y 55 kDa cada una, en el eyaculado del verraco se correspondían con una alta tasa de partos (superiores al 86%) y un elevado número de lechones vivos (más de 11 por camada) (Flowers, 2001). En toros, Killian y cols. (1993) encontraron una asociación entre los índices de fertilidad que presentaban diferentes toros y la composición de sus proteínas del plasma seminal. En ese estudio, se observó como dos

proteínas (26 kDa/6’2 pI y 55 kDa/4,5 pI) predominaban en los toros de alta fertilidad, mientras que otras dos proteínas (16 kDa/4’1 pI y 16 kDa/6’7 pI) prevalecían en los toros de baja fertilidad. En el caballo, se han descubierto otras 4 proteínas relacionadas con la fertilidad, denominadas SP-1, SP-2, SP-3 y SP-4, de las cuales, la SP-1 presentó una correlación positiva con la fertilidad y era homóloga a la proteína asociada a la fertilidad en toros de 55 kDa. El resto de proteínas del plasma seminal equino (SP-2, SP-3 y SP-4) presentaron una correlación negativa con la fertilidad (Brandon y cols., 1999).

Un estudio mas reciente ha relacionado cuatro proteínas provenientes de las glándulas accesorias (espermadhesina Z13, osteopontina, fosfolipasa A_2 y BSP 30 kDa) con los índices de fertilidad presentados por diferentes toros (Moura y cols., 2006).

III. – Material y Métodos

3.1. Animales y obtención del semen

Los animales utilizados en las diferentes experiencias fueron verracos de probada fertilidad con descendencia previa. Los animales se encontraban situados en parques individuales con temperatura controlada y alimentación adecuada, sometidos a un ritmo de extracción de 2 veces por semana.

El eyaculado fue obtenido mediante el método manual y solo aquellos eyaculados que presentaron una motilidad superior al 80% fueron usados en el estudio. El tiempo transcurrido desde la recogida del semen hasta su posterior utilización fue inferior a 90 minutos.

3.2. Criopreservación Espermática

El protocolo de criopreservación usado en nuestras experiencias fue el descrito por Westendorf y cols. (1975) y modificado por Thurston y cols. (1999). Brevemente, la fracción rica del eyaculado diluida en BTS (1:1 v/v) se llevo a una vitrina térmica en la cual se realizo un descenso de temperatura suave y paulatino durante aproximadamente 3 horas hasta 17º C. A esta temperatura, se centrifugó a 800 x g durante 10 minutos

(Megafuge 1.0 R, Heraeus, Alemania) con el fin de eliminar el sobrenadante. El pellet resultante fue diluido en un diluyente lactosa-yema de huevo (LEY, Tabla 2) hasta una concentración de 1’5 x 10^9 espermatozoides/ml. Seguidamente, se realizó un nuevo descenso de temperatura suave y paulatina durante aproximadamente 90 minutos hasta alcanzar los 5º C. Una vez a esta temperatura, se rediluyó hasta 1 x 10^9 espermatozoides/ml con el diluyente LEY-Glicerol-Orvus Es Paste (LEYGO, Tabla 3).

Tabla 2. Composición del medio Lactosa-yema de huevo (LEY).

Componentes	Concentración
Yema de huevo (%)	20
Solución de 310 mM de β-Lactosa (%)	80
Kanamicina (gr/L)	0’1
Osmolaridad 325-335 mOsm/Kg	
pH 6’2-6’5	

Tabla 3. Composición del medio LEY-Glicerol-Orvus Es Paste (LEYGO).

Componentes	Concentración
Yema de huevo (%)	18’5
Solución de 310 mM de β-Lactosa (%)	74
Kanamicina (gr/L)	0’1
Glicerol (%)	6
Orvus Es Paste (%)	1’5
Osmolaridad 1600-1700 mOsm/Kg	
pH 6’2-6’5	

El semen diluido y enfriado a 5º C fue envasado en pajuelas de PVC de 0’5 ml (Minitüb, Alemania) y congeladas bajo condiciones controladas usando un

biocongelador (IceCube 1810, Minitüb, Alemania) con las siguientes velocidades de congelación: de +5º C a -5º C a 6º C/min, de -5º C a -80º C a 40º C/min, mantenidas durante 30 segundos a -80º C y posteriormente enfriadas a 70º C/min hasta alcanzar los -150º C. Una vez congelado, el semen envasado fue almacenado en tanques de nitrógeno líquido hasta el momento de su utilización.

3.3. Obtención del plasma seminal y aislamiento del heterodímero PSP-I/PSP-II

El plasma seminal se obtuvo mediante centrifugación del semen a 3800 x g durante 15 minutos a 20º C en una centrífuga refrigerada (Heraeus Sepatech Megafuge 1.0 R; Osterode, Germany). Posteriormente el sobrenadante fue filtrado sucesivamente a través de filtros de nylon de baja adsorción de solutos de 10 y 1’2 µm. Una vez filtrado el plasma fue utilizado en fresco o almacenado a -20º C y descongelado a temperatura ambiente antes de su uso.

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 (Figura 3), 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).

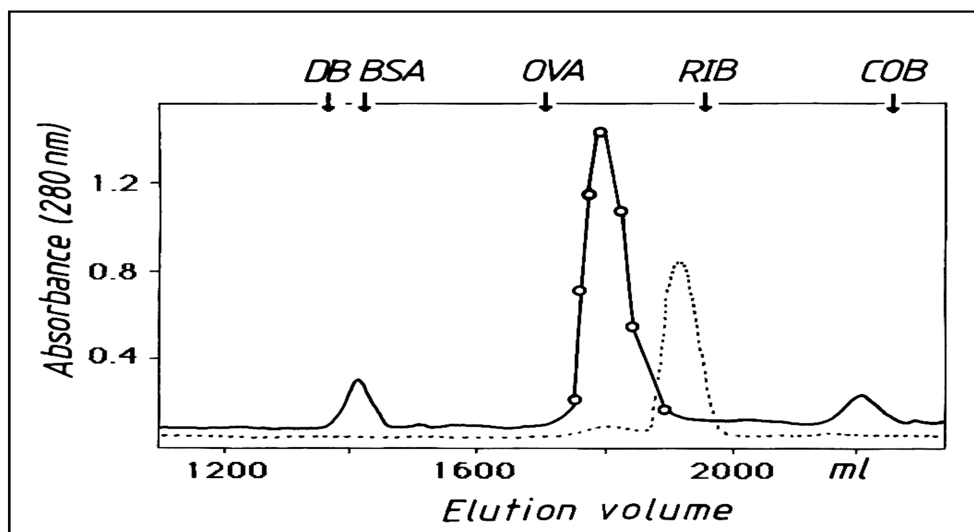

Figura 3. Aislamiento mediante HPLC del heterodímero PSP-I/PSP-II.

La identidad y pureza de la muestra proteica fue valorada mediante análisis de la secuencia N-terminal (usando secuenciador automatizado de proteinas 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 habia sido determinado previamente por Menedez 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.

3.4. Obtención de anticuerpos Anti-PSP-II

Las subunidades PSP-I y PSP-II se purificaron por cromatografía de alta resolución utilizando una columna (250 x 10 mm) de fase reversa Lichrocart (Merk) de 10 µm de tamaño de partícula. La columna se eluyó a 3 ml/minuto con una mezcla de 0,1% de ácido trifluoroacético en agua (solución A) y acetonitrilo (solución B), isocráticamente (25% B) durante 5 minutos, seguidos de 25-45% B por 5 minutos, 45-60% B por 15 minutos y 60-70% B por 5 minutos.

Los anticuerpos policlonales monoespecífico anti-PSP-II fueron obtenidos mediante inmunización de conejas por inyecciones subcutaneas de 0’5 mg del monómero PSP-II en 0’5 ml de PBS emulsificado en 1’5 ml de adyuvante de Freund completo. Los animales fueron inoculados por 2 veces a intervalos de 5 semanas después de la primera inmunización con 0’25 mg del antígeno. 2 semanas después de la última administración, las conejas fueron sangradas a través de la vena auricular. La actividad anti-PSP-II del suero sanguíneo fue testada mediante técnicas de ELISA y Western Blot.

3.5. Reactivos químicos y medios

Todos los reactivos, medios, hormonas y suplementos utilizados en este trabajo fueron de la marca Sigma-Aldrich Co (Alcobendas, M. España), si no se indica lo contrario.

Todos los medios se prepararon con agua bidestilada y purificada (purificador Elgastat UHQ ps, Elga) en un ambiente controlado (cámara de flujo laminar horizontal,

BH-100 de Telstar) con filtros de membrana de diámetro de poro de 0’22 µm (Sterivex GP, Millipore) mediante la ayuda de un equipo de filtración Steritop (Millipore). El pH (pH Crison 2000) y la osmolaridad (Microosmómetro Model 2-MO plus; Advanced Instruments Inc., USA) de cada medio fueron controlados en todos y cada uno de los ensayos realizados en las diferentes experiencias. Una vez preparados los medios fueron almacenados a 5º C y utilizados antes de 15 días. Todos los medios fueron vueltos a filtrar por un filtro de membrana de diámetro de poro de 0’22 µm (Sterivex GP, Millipore) tras ser suplementados en el momento de su uso.

3.6. Técnicas de evaluación de la funcionalidad espermática

3.6.1. Viabilidad espermática

3.6.1.1. Diacetato de carboxifluoresceina (DCF) (experiencia 1)

La determinación de la viabilidad espermática mediante la técnica de DCF descrita por Harrison y Vickers (1990) y modificada por Vázquez y cols. (1997) se basa en la detección de la actividad esterasa intracitoplasmática. Brevemente, 20 µl de 0’46 mg/ml de DCF en dimetilsulfóxido (DMSO) y 20 µl de formaldehído (2’5 mg/ml en agua) fueron añadidos a 1 ml de la suspensión espermática correspondiente y se incubo a 37º C durante 10 minutos. Transcurrido este tiempo la muestra fue observada a 400 aumentos usando un microscopio Nikon Eclipse 800 (Nikon, Chiba, Japón) equipado con epifluorescencia. Se contaron un mínimo de 200 espermatozoides por muestra, considerando viables aquellos que presentaron fluorescencia verde a lo largo de toda su longitud (Figura 4).

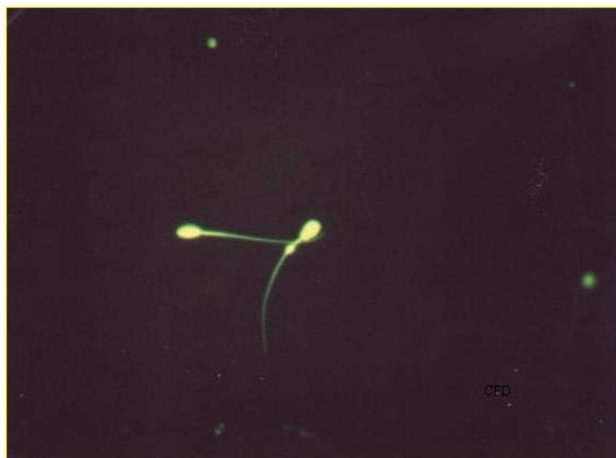

Figura 4. Espermatozoides vivos teñidos con DCF.

3.6.1.2. Triple tinción SYBR-14/IP/PE-PNA (experiencias 2 y 4)

Esta combinación de fluorocromos diseñada por Nagy y cols. (2003) permitió medir simultáneamente tanto la integridad de la membrana plasmática como la integridad acrosomal mediante análisis por citometría de flujo. Brevemente, 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. Todos los análisis se realizaron mediante un citómetro de flujo analizador EPICS XL (Coulter Corporation Inc, Miami, Flo) equipado con ópticas estándar, un láser de argón (Cyonics, Coherent, Santa Clara, Calif) con 15 mW de potencia a 488 nM de longitud

de onda, y el software EXPO 2000 (Coulter Corporation). Se analizaron entre 15000 y 25000 eventos por muestra; solo aquellos eventos que fueron específicos de espermatozoides, los cuales aparecieron mostrando la forma de L habitual de una muestra espermática fueron seleccionados para el análisis. 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 (Figura 5).

3.6.2. Actividad Mitocondrial

3.6.2.1. Rodamina 123 (experiencia 1)

La actividad metabólica de las mitocondrias fue evaluada con Rodamina 123 (R-123; Sigma Chem. Co., Alcobendas, España) (Cuello y cols., 2001). A una muestra espermática de 0’3 x 10^6 espermatozoides/ml en BTS se incubó a 37 º C durante 15 minutos con 1 µg/ml de R-123 y 4 µg/ml de IP. Los espermatozoides fueron examinados con el microscopio epifluorescente con un triple filtro D-F-R (DAPI/FICT/Rodamina).Se contaron 200 espermatozoides por muestra y solo aquellos que mostraron fluorescencia verde en su pieza intermedia fueron considerados vivos con actividad mitocondrial (Figura 6).

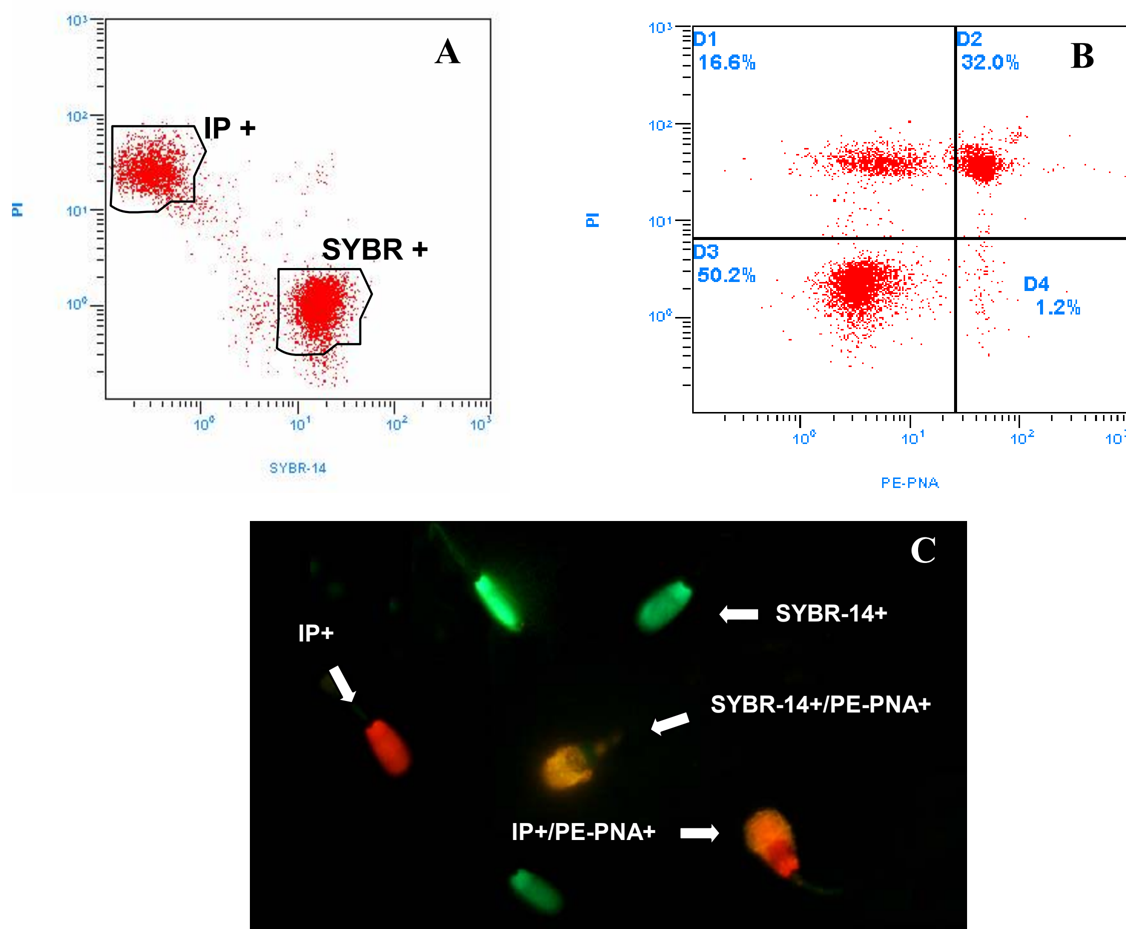

Figura 5. Evaluación de la viabilidad espermática mediante triple tinción SYBR-14/IP/PE-PNA. A) Representación gráfica correspondiente al análisis por citometría de flujo mostrando la población de espermatozoides viables (SYBR-14+) y de espermatozoides con las membranas alteradas (IP+). B) Representación gráfica correspondiente al análisis por citometría de flujo mostrando las 4 poblaciones espermáticas obtenidas. D1 (IP+/PE-PNA-): espermatozoides muertos con el acrosoma intacto; D2 (IP+/PE-PNA+): espermatozoides muertos con el acrosoma alterado; D3 (IP-/PE-PNA-) espermatozoides vivos con el acrosoma intacto; D4 (IP-/PE-PNA+) espermatozoides vivos con el acrosoma alterado. C) Imagen de microscopía de fluorescencia en la cual se observan las 4 poblaciones espermáticas.

Figura 6. Espermatozoides positivos a la Rodamina 123 presentando actividad mitocondrial (fluorescencia verde en su pieza intermedia).

3.6.2.2. JC-1 (experiencia 2)

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 (Figura 7).

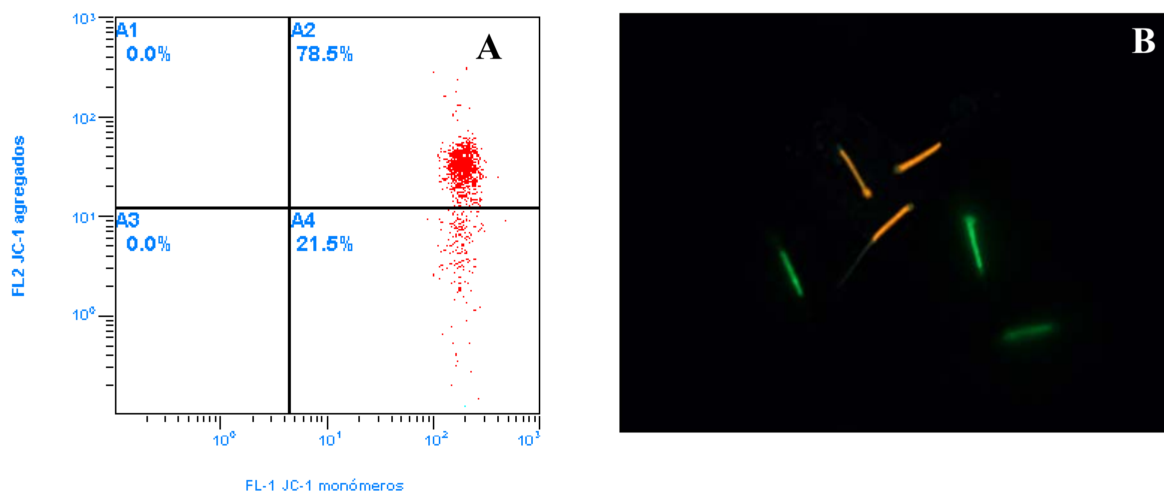

Figura 7. Evaluación de la actividad mitocondrial mediante el fluorocromo JC-1. A) Representación gráfica correspondiente al análisis por citometría de flujo donde se enfrentan la fluorescencia naranja (FL2) y la fluorescencia verde (FL1) mostrando las poblaciones de espermatozoides con alta actividad mitocondrial (cuadrante A2) y espermatozoides con baja actividad mitocondrial (cuadrante A4). B) Imagen de microscopía de fluorescencia en la cual se observan espermatozoides con alta actividad mitocondrial (fluorescencia naranja en su pieza intermedia) y aquellos con baja actividad mitocondrial (fluorescencia verde en su pieza intermedia).

3.6.3. Motilidad espermática (experiencias 1, 2 y 4)

El porcentaje de motilidad espermática fue estimado con un sistema CASA de análisis objetivo de la motilidad (Sperm Class Analyzer; SCA, Microptic, Barcelona, España). Las muestras (5-10 µL según la experiencia) fueron colocados en una cámara de Makler (Haifa, Israel) acondicionada a 38ºC y observada en un microscopio de contraste de fases (Nikon Labophot, Kanagawa, Japón) a 100 aumentos y una cámara de video monocroma (Hitachi CCD, Tokio, Japón) conectada a un ordenador. Se usaron 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); linealidad (LIN, linealidad de la trayectoria curvilínea, VSL/VCL x 100, %) y amplitud del desplazamiento lateral de la cabeza (ALH, amplitud de las variaciones de la trayectoria real de la cabeza espermática respecto a la tayectoria media).

3.6.4. Capacidad fecundante de los espermatozoides (experiencias 3 y 4)

Para la evaluación de la capacidad fecundante de los espermatozoides de verraco *in vitro,* fueron usados tanto ovocitos inmaduros (Martínez y cols., 1993) como madurados *in vitro* (Abeydeera y Day, 1997).

3.6.4.1. Obtención y selección de los ovocitos

Los ovocitos se obtuvieron de ovarios procedentes de cerdas híbridas prepuberales de 90-100 Kg de peso vivo. Una vez obtenidos, se transportaron al laboratorio en un termo con solución salina conteniendo 0’9% de NaCl y 70 µg/ml de kanamicina y atemperada a 34-37º C. en un plazo máximo de 1 hora. Los ovocitos fueron recogidos por aspiración con una aguja de 18G unida a una jeringa de 10 ml. El líquido folicular con los ovocitos se introdujo en tubos de 15 ml. Tras sedimentación del fluido folicular, se eliminó el sobrenadante, y el sedimento resultante fue resuspendido en medio de lavado de ovocitos (PBS de Dulbecco modificado; PBSDm) suplementada con 4 mg/ml de albúmina sérica bovina (BSA, fracción V), 0’34 mM de piruvato sódico, 5’4 mM de glucosa y 70 µg/ml de kanamicina (Tabla 4).

Tabla 4: Composición del medio PBSDm.

Componentes	Concentración
NaCl (mM)	136’89
KCl (mM)	2’68
Na_2HPO_4 (mM)	8’1
KH_2PO_4 (mM)	1’46
$CaCl_2.2H_2$ (mM)	0’88
$MgCl_2.6H_2$ (mM)	1’865
Rojo Fenol (mM)	0’03
Osmolaridad 270-290 mOsml/l	
pH 7’3-7’4	

Los ovocitos resuspendidos fueron depositados en placas de Petri atemperado a 39º C, donde se procedió a la identificación y selección de los ovocitos mediante un estereomicroscopio (SMZ-2T Nikon). Solo aquellos ovocitos que presentaron un mínimo de 6 o 7 capas de células del cúmulus y un citoplasma uniformemente negro y granulado fueron seleccionados para las diferentes experiencias.

3.6.4.2. Fecundación de los ovocitos inmaduros

El medio base para evaluar la capacidad de penetración *in vitro* de los ovocitos inmaduros fue el TCMm (TCM-199 con sales de Earle suplementado como se indica en la tabla 5) (Cheng y cols., 1986). El medio de coincubación en el que se depositaron los ovocitos se realizó suplementando el medio base con 2 mM de cafeína y 5'4 mM de lactato cálcico y ajustando a un pH 7'4. Aquellos ovocitos seleccionados se traspasaron directamente a placas de Petri con 2 ml de medio de coincubación en un número de 30 ovocitos por placa o fueron lavados en PBSDm eliminando de forma mecánica por pipeteados sucesivos las células del cúmulus y posteriormente pasados a las placas de Petri según la experiencia lo requiriese. Los ovocitos fueron mantenidos bajo una atmósfera de 5% de CO_2 en aire, 39º C de temperatura y 95-100% de humedad relativa durante 30 minutos aproximadamente antes de que los espermatozoides fueran añadidos para los ensayos de penetración.

La preparación de los espermatozoides se realizó siguiendo la metodología descrita por Martínez y cols., (1996), y modificado según el experimento. Sobre las placas que contenían los ovocitos se colocaron 2 x 10^6 espermatozoides, alcanzando un ratio ovocito:espermatozoide de 1:66000. Los ovocitos se mantuvieron en coincubación

con los espermatozoides durante 16 horas bajo una atmósfera de 5% de CO_2 en aire, 39º C de temperatura y 95-100% de humedad relativa. Posteriormente, aquellos ovocitos que no habían sido denudados previamente se lavaron en PBSDm y las células del cúmulus retiradas mecánicamente mediante pipeteado.

Tabla 5. Suplementación del medio TCM-199 (Cheng y cols., 1986).

Componentes	**Concentración**
Lactato Cálcico (mM)	2’92
Glucosa (mM)	3’05
Piruvato Sódico (mM)	0’91
Penicilina G (UI)	50
Sulfato de Estreptomicina (µg/ml)	30
Suero Fetal Bovino inactivado (v/v)	12%

Osmolaridad 300-310 mOsml/l
pH 7’8

3.6.4.3. Maduración in vitro de los ovocitos

El medio usado para la maduración *in vitro* de los ovocitos fue NCSU-23 libre de BSA (North Carolina State; Peter y Wells, 1993) (Tabla 6) suplementado con un 10% (v/v) de fluido folicular porcino, 0’1 mg/ml de cisteina, y 10 ng/ml de factor de crecimiento epidérmico.

Los ovocitos seleccionados fueron lavados tres veces en medio de maduración y madurados en placas multidish de 4 pocillos (Nunc, Roskilde, Denmark) conteniendo 50-100 ovocitos por pocillo en 500 µL de medio de maduración cubierto con aceite

mineral y suplementado con 10 UI/ml de eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) y 10 UI/ml de hCG (Chorulon, Intervet International). Este medio fue previamente equilibrado en una atmósfera de 5% de CO_2 en aire, 39º C de temperatura y 95-100% de humedad relativa y los ovocitos cultivados durante 20-22 h y posteriormente otras 20-22 h en medio de maduración sin hormonas.

Tabla 6. Composición del medio NCSU 23 (Petters y Wells, 1993).

Componentes	Concentración
NaCl (mM)	108’73
KCl (mM)	4’784
KH_2PO_4 (mM)	1’19
$MgSO_4$-7H_2O (mM)	1’19
$CaCl_2$.2H_2O (mM)	1’70
$NaHCO_3$ (mM)	25’07
Glucosa (mM)	5’55
L-Glutamina (mM)	1
Taurina (mM)	7
Hipotaurina (mM)	5
Penicilina G potásica (µg/ml)	75
Sulfato de Estreptomicina (µg/ml)	50

Osmolaridad 270-290 mOsml/l
pH 7’3-7’4

3.6.4.4. Fecundación y cultivo de los ovocitos madurados in vitro

Para la fecundación *in vitro* de los ovocitos madurados *in vitro* el medio utilizado fue el Tris-Buffered modificado (TBMm, Abeydeera y Day, 1997) (Tabla 7), suplementado con 1 mM de cafeína y 0’2% BSA (fracción V).

Tabla 7. Composición del medio TBMm (Abeydeera y Day, 1997).

Componentes	**Concentración**
NaCl (mM)	113’10
KCl (mM)	3
$CaCl_2.2H_2O$ (mM)	7’50
Tris (mM)	20
Glucosa (mM)	11
Piruvato sódico (mM)	5
Osmolaridad 270-290 mOsml/l	
pH 9.8- 10	

Después de la maduración, los ovocitos madurados *in vitro* fueron denudados con 0’1% hialuronidasa en medio de maduración mediante vortex durante 2 min, a 1’660 vueltas/ min. Más tarde, los ovocitos fueron lavados dos veces en medio de maduración y 3 veces en medio de fecundación previamente equilibrados en el incubador. Grupos de 50 ovocitos denudados fueron puestos en gotas de 50 µL de medio de fecundación en una placa Petri de 35 x 10 mm (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) cubierto de aceite mineral y bajo una atmósfera de 5% de CO_2 en aire, y a 39º C en el incubador, durante aproximadamente 30 min hasta la adición de los espermatozoides.

El semen fresco o criopreservado fue preparado según lo requiriese la experiencia para posterior adición a los ovocitos madurados *in vitro.* Por último, el semen fue centrifugado y el sedimento resuspendido en TBMm de tal manera que con 50 µl de esta suspensión espermática el ratio ovocito:espermatozoide fue 1:2000. Los gametos fueron

co-incubados bajo una atmósfera de 5% de CO_2, a 39ºC y con una humedad del 95-100%, durante 6 h.

Tras las 6h de co-cultivo, los posibles cigotos fueron sacados del medio de fecundación y lavados 3 veces en medio de cultivo de embriones (NCSU-23 suplementado con 0'4% de BSA, fracción V) previamente equilibrado. Posteriormente, los cigotos fueron transferidos a una placa de 4 pocillos conteniendo 500 µL del mismo medio cubierto con aceite mineral, y cultivados bajo una atmósfera de 5% CO_2 en aire, a 39º C durante 6 h.

3.6.4.5. Valoración de los parámetros de fecundación

Los ovocitos (tanto inmaduros como madurados *in vitro*) fueron montados en portas y se fijaron en 25% (v/v) de ácido acético en etanol durante 48-72 h a temperatura ambiente. Posteriormente, los ovocitos fueron teñidos con 1% lacmoid en 45% (v/v) de ácido acético en agua purificada y examinados bajo un microscopio de contraste de fase a 400 aumentos. Los ovocitos fueron considerados penetrados cuando en su interior presentaba espermatozoides compactos y refringentes (ovocitos inmaduros) o cabezas descondensadas y/o pronúcleo masculino y sus correspondientes colas espermáticas (ovocitos madurados *in vitro*) (Figura 8).

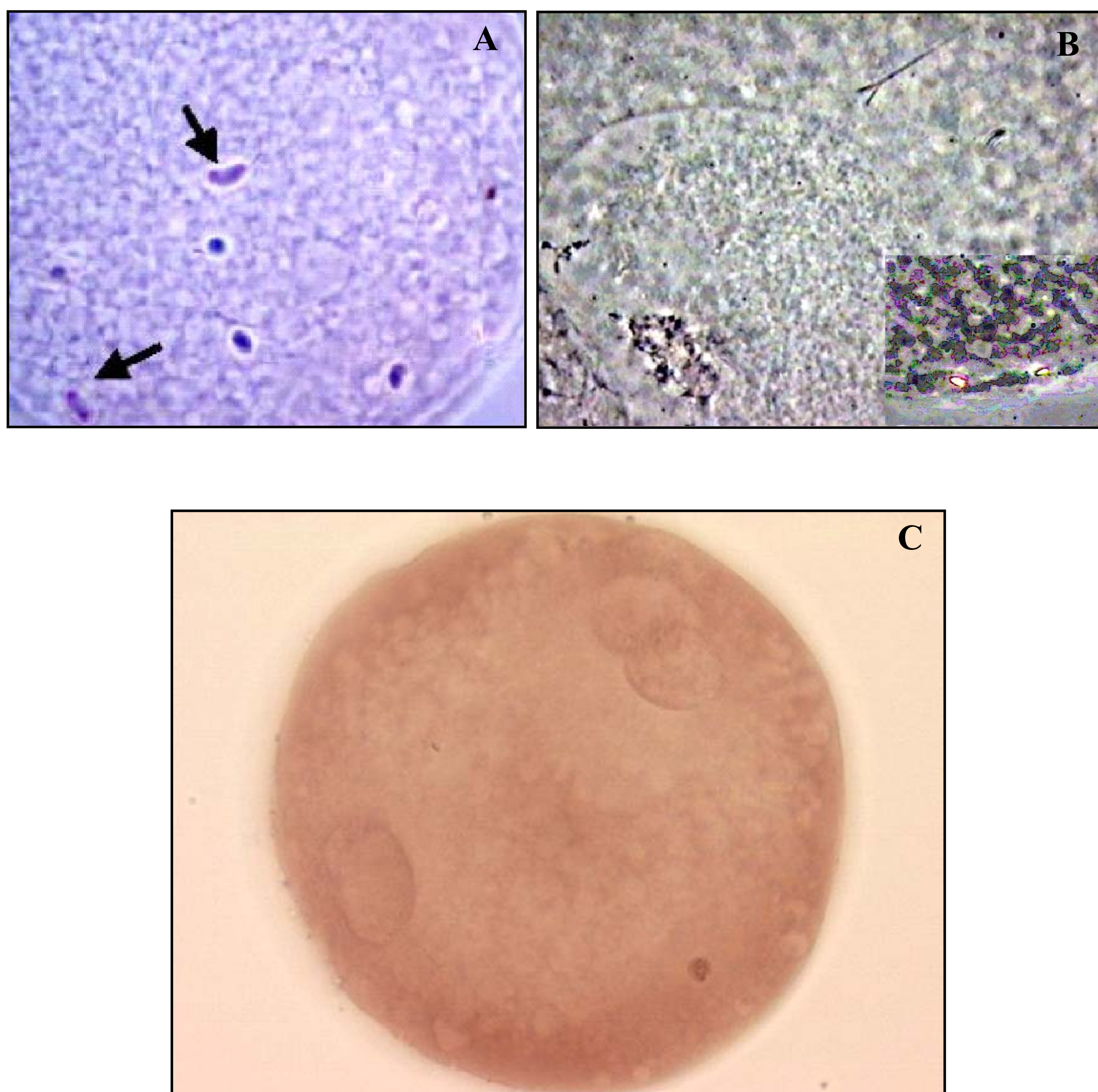

Figura 8. Valoración de los parámetros de fecundación *in vitro*. A) Las flechas indican espermatozoides en un estadío inicial de descondensación de la cabeza. B) Pronúcleo masculino con su correspondiente cabeza espermática. C) Ovocito polispérmico con tres pronúcleos.

3.7. Inmunolocalización del heterodímero PSP-I/PSP-II (experiencia 2)

La inmunolocalización del heterodímero PSP-I/PSP-II sobre la cabeza del espermatozoide fue estudiada mediante microscopía de luz y microscopía electrónica de barrido. Para ello, se realizó un frotis de la muestra espermática sobre portaobjetos (26 x 76 mm) cubiertos por una capa de poli-lisina y secados en aire durante 24 horas. Los frotis fueron fijados en una solución de 1% de paraformaldehido en PBS durante 10 minutos, tras los cuales fueron lavados repetidas veces en PBS e incubados en incubados 120 minutos con el anticuerpo anti-PSP-II (1:500 en PBS) a temperatura ambiente. Las muestras fueron lavadas en PBS e incubadas con un anticuerpo secundario de cabra anti-conejo marcado con partículas de oro de 10 nm (Auroprobe EM GAR G10, Amersham Biosciences, Uppsala, Suecia) durante 100 minutos. Después de varios lavados en PBS y agua destilada, y para una más fácil visualización del heterodímero PSP-I/PSP-II se aplicó un kit comercial de intensificación con plata (RPN 491, Amersham Biosciences, Uppsala, Suecia) durante 10 minutos. Finalmente, los frotis fueron lavados en agua destilada y secados en aire.

Para evaluación de los espermatozoides a nivel del microscopio de luz, las muestras fueron fotografiadas a 400 aumentos con un microscopio de luz Nikon microphot-FXA (Chibe, Japón), siendo contados un mínimo de 100 espermatozoides por muestra. Para la evaluación de los espermatozoides por el microscopio electrónico de barrido (6000 aumentos), una porción del portaobjetos conteniendo espermatozoides marcados con el anticuerpo fue recortada y colocadas en monturas de metal, contrastada durante 15-30 segundos con oro paladio y examinada usando un microscopio electrónico de barrido JEOL JSM-6320F (JEOL LTD, Japón) operando a 5 kV.

Experiencia 1

COMPARATIVE EFFECTS OF AUTOLOGOUS AND HOMOLOGOUS SEMINAL PLASMA ON THE VIABILITY OF LARGELY EXTENDED BOAR SPERMATOZOA

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1.1 Abstract

Sperm handling, associated to artificial reproduction technologies (ART) such as in vitro fertilization (IVF) or the use of flow cytometry for cell analysis or sorting imposes volumetric extension of the sperm suspension and decreases sperm viability, presumably owing to the removal of seminal plasma (SP) components. This study evaluated whether a 10% v/v of autologous SP (retrieved from the same donor boar) or homologous SP (eg from any of the 4 fertile boars included, other than the one providing the spermatozoa) would differently affect the viability of boar spermatozoa subjected to large extension in a simple saline medium (phosphate-buffered saline and 0.1% EDTA, PBSm) to a concentration of 0.3 x 10^6 spermatozoa/ml and incubated for 2 h at 30ºC. Sperm viability was monitored as membrane integrity [using the fluorophore carboxyfluorescein diacetate (C-FDA) and propidium iodide (PI)], mitochondrial function (using the fluorophore R-123) and motility characteristics [using Computer Assisted Sperm Analysis (CASA)]. Substraction of the SP and extension followed by incubation in PBSm significantly ($p<0.05$) decreased sperm viability, which could be restored by addition of autologous SP. Furthermore, exposure of the extended spermatozoa to homologous SP (from any other individual boar) significantly ($P< 0.05$) varied with the source sire; some boars exerting beneficial effects (even surpassing the effects of the autologous SP, $P< 0.05$) while at least one boar negatively ($P< 0.05$) influencing the viability of the incubated spermatozoa. It is concluded that SP should be present when incubating highly extended spermatozoa. Owing to the obvious differences among boars, it would be advantageous to examine the ability of SP to maintain sperm viability prior to the use of SP pools during sperm handling in vitro.

1.2. Introduction

The ejaculated spermatozoa are immersed in seminal plasma (SP), a medium composed by aliquots of the fluid of the testis, epididymal tail and the secretions of the accessory sexual glands of the sire. The SP contains a wide variety of factors that influence the functionality of spermatozoa (Mann and Lutwak-Mann 1981; Rodriguez-Martinez et al. 1984, Rodríguez-Martínez 1991; Iwamoto et al. 1992; Maxwell et al. 1997; Strzezek 2002) although the biological effects of these SP-factors on sperm function are complex and not well understood. A variation in the presence, absence or concentration of some components, most probably proteins (either of epididymal or accessory gland origin, Fournier-Delpech and Thibault 1993), may be responsible for the variability seen on the effects –detrimental or beneficial- on the spermatozoa (Maxwell and Johnson 1999). A high variability exists in the SP composition among species or even males within the same species, as well as between ejaculates of homologous males (Asworth et al. 1993; Killian et al. 1993; Zhu et al. 2000).

Some SP fractions, such as the prostatic one (England and Allen 1992), the gel fraction in stallion, boar or camelids (Mann and Lutwak-Mann 1981), as well as the secretion of the seminal vesicles, are considered by some authors as detrimental for sperm survival *in vitro* (ram, Ashworth et al 1994; bull, Way et al 2000; buck, Azerêdo et al. 2000). Decreases in fertility have been reported following the exposure of ruminant (Dott et al. 1979) or stallion (Corteel et al. 1980) spermatozoa to either autologous or homologous SP. Therefore, when handling spermatozoa in vitro, the removal of SP (by centrifugation or extension with a buffer) is praxis, and considered

critical to ensuring maximal sperm viability in the majority of protocols for semen preservation.

However, the SP has also proven beneficial for spermatozoa. In pigs or bulls, incubation of spermatozoa in autologous or homologous SP at room temperature increased the sperm resistance to cold shock, a process that occurs when recently ejaculated spermatozoa are chilled (Pursel et al. 1973; Ollero et al. 1998; Eriksson et al. 2001). Viability can be restored in cold-shocked ram spermatozoa if incubated with selected portions of SP-proteins, particularly a 20 kDa band (Barrios et al. 2000). Addition of SP to thawed ram spermatozoa reverted changes seen during cryopreservation (capacitation-like, Pursel and Johnson 1975) to such an extent that motility was increased *in vitro* (Gillan and Maxwell 1999; Maxwell et al. 1999), as well as fertility post-Artificial Insemination (Maxwell et al. 1999). Similar changes have been reported in other species (for review, see Rodriguez-Martinez et al. 1998). Furthermore, it is usual the addition of SP to boar spermatozoa subjected to sperm sorting for chromosomal sex through flow-cytometry (Maxwell et al. 1998; Johnson and Welch 1999; Vazquez et al. 2003) where a 2,000-fold extension of the ejaculate is required. Addition of 10% (v/v) of SP to the collection medium of sex-sorted spermatozoa improves their sperm viability and motility as well as stabilizes the plasma membrane preventing premature capacitation (Maxwell et al. 1997), and extending their fertilizing ability (Maxwell et al. 1998).

Addition of homologous SP (eg from different males) can even influence the fertilizing capacity of the spermatozoa (bulls: Killian et al. 1993). For instance, the addition of SP from low fertility bulls decreased the penetration of zona-free oocytes by

spermatozoa from bulls of high fertility (Henault and Killian, 1996). Moreover, addition of SP from stallions with high post-thaw motility to semen from stallions with low post-thaw motility improved membrane integrity and progressive motility (Aurich et al. 1996).

As a result of the variability in results of the addition of SP already described, the present study aimed to evaluate whether the addition of SP improves boar sperm viability, monitored as membrane integrity, mitochondrial function and motility characteristics, when spermatozoa were subjected to similar extreme extension conditions as when sperm sorting by flow cytometry is performed. Furthermore, it intended to disclose whether this effect depends of the SP source (as autologous or homologous).

1.3. Materials and Methods

1.3.1. Preparation of seminal plasma and sperm samples

Sperm-rich ejaculate fractions (SRF, 100 ml) were manually collected from five mature Pietrain boars of proven fertility (hereby coded A-E), once weekly during six consecutives weeks using the gloved-hand method. Shortly after collection, the semen samples were evaluated for motility and only those showing >80% of sperm motility were used. Each SRF was divided in two aliquots of 85 and 15 ml, as sources for SP or spermatozoa, respectively. The 85 ml aliquot of the SRF was centrifuged at 3,800x g for 15 min (20ºC) using a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatant was harvested and filtered twice through a 10 and a 1.2 µm-mesh filter

respectively, before use. The SP was always used fresh in the experiments. The 15 ml-aliquot was centrifuged at 1,900x g for 4 min (20ºC), the supernantant discarded and the pellet used as source for further sub-aliquots as required by the experiments (see below).

1.3.2. Evaluation of sperm viability following exposure of boar spermatozoa to autologous or homologous SP

Spermatozoa obtained from each and every boar were thus split in 6 sub-aliquots (A-E and control). These sub-aliquots were sequentially extended to yield a concentration of 0.3 x 10^6 spermatozoa/ml in phosphate-buffered saline with 0.1% ethylenediaminetetraacetic acid (EDTA) alone (PBSm, control) or PBSm with 10% (v/v) of its own SP (hereby named autologous SP, eg extended in PBSm + 10% v/v of autologous SP) or to the SP from any other of the remaining 4 boars studied (hereby named homologous SP, eg extended in PBSm + 10% v/v of homologous SP).

In either group spermatozoa were incubated in a water bath in the dark for 2 h at 30ºC in capped tubes until evaluated for plasma membrane integrity, mitochondrial membrane potential activity and sperm motility.

Sperm plasma membrane integrity (MI) was determined using the fluorescent probe carboxyfluorescein diacetate (C-FDA, Sigma Chem. Co. Alcobendas, Spain) using a modification of the method described by Harrison and Vickers (1990). Briefly, 20 μL of 0.46 mg/ml of C-FDA in dimethylsulfoxide (DMSO) and 20 μL of formaldehyde (2.5 mg/ml in water) were added to 1 ml of BTS media containing 0.3 x 10^6 spermatozoa to achieve a final concentration of 1.7 mM-formaldehyde and 20 mM-

C-FDA. After 10 min at 30ºC, the stained sperm suspensions were observed at 400x magnification using a Nikon Eclipse 800 microscope (Nikon, Chiba, Japan) equipped with epifluorescence illumination. Spermatozoa were scored for the percentage of those showing green fluorescence along their whole length (spermatozoa with intact plasma membranes).

Rhodamine 123 (R 123, Sigma Chem. Co., Alcobendas, Spain) was used as a probe of mitochondrial membrane potential. Spermatozoa (0.3 x 10^6 spermatozoa/ml) were stained by incubation for 15 min in a BTS solution containing 1 μg/ml of R 123 and 4 μg/ml of propidium iodide (PI, Sigma Chem. Co., Alcobendas, Spain). Spermatozoa were examined as explained above. Only spermatozoa showing green fluorescence in their midpieces were considered live spermatozoa having mitochondrial membrane potential activity.

Sperm motility was estimated with a Computer Assisted Sperm Analysis equipment (CASA). Sperm samples (10 μL at 0.3 x 10^6 spermatozoa/ml) were placed in a warm (38ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38ºC) of a Nikon Laborphot microscope, equipped with a 10x phase objective and a monochrome video camera (Hitachi CCD model), connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyser (SCA, Barcelona, Spain). The program settings were as follows: frames rate: 25Hz; search radius: 11.5 μm; minimum track points: 7 frames; threshold straightness: 75%. The motion variables recorded were: percentage of motile spermatozoa, curvilinear velocity (VCL, time-average velocity of the sperm head along its actual trajectory) and

linearity (LIN, time-average velocity of the sperm head along a straight line from its first position to its last position (VSL)/VCL x 100).

Two replicates per sample were analyzed with at least one hundred spermatozoa recorded per replicate for each parameter evaluated.

1.3.3. Statistical Analysis

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 effects of SP source and of sperm source and the random effect of replicate. To analyze data of sperm viability and sperm motility, 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 $p<0.05$.

1.4. Results

Results of sperm viability are presented in Tables 1 to 5. Table 1 summarizes the results of membrane integrity (as mean percentages ± SEM) following incubation with the fluorophore C-FDA. As it can be seen, incubation of spermatozoa from any particular boar with his own SP (autologous SP) was not detrimental for MI. Moreover, the addition of 10% (v/v) of the SP from most boars was beneficial for MI, showing significantly ($P< 0.05$) higher percentages of MI respect to control incubations in a buffer. Incubation of the spermatozoa of a given boar with SP from other boars could either result in an amelioration of MI, a decrease or a non-significant effect. A similar

picture could be seen when mitochondrial activity was assessed under similar conditions (Table 2) but here a single boar (C) could be identified as the one whose SP negatively ($P< 0.05$) affected the spermatozoa from other boars during incubation.

Table 1. Percentages of boar sperm membrane integrity following exposure to autologous or homologous seminal plasma, assessed by the fluorescent probe carboxyfluorescein diacetate (C-FDA)(mean ± SEM).

Boar	Source of seminal plasma (individual boars)					
	A	B	C	D	E	Control
A	73.3±1.37^{a,1}	67.3±1.23^{ab,12}	46.9±2.33^{a,3}	80.2±1.05^{ab,4}	77.4±1.25^{ab,14}	65.2±1.54^{ab,2}
B	79.4±1.43^{b,1}	72.6±1.19^{a,2}	54.5±2.65^{b,3}	81.2±1.15^{a,1}	75.7±1.93^{ac,12}	68.3±1.23^{a,2}
C	72.5±1.44^{a,1}	67.8±1.43^{ab,12}	62.4±1.93^{c,2}	79.3±1.51^{ab,3}	72.2±1.83^{ac,1}	62.2±1.82^{ab,2}
D	71.3±2.17^{a,1}	64.3±1.54^{b,12}	61.5±2.05^{c,23}	77.2±1.72^{ab,4}	70.3±2.23^{c,1}	57.2±2.89^{b,3}
E	77.8±1.93^{ab,12}	72.5±1.34^{a,2}	52.5±2.83^{b,3}	74.3±1.32^{b,12}	79.6±1.93^{b,1}	63.2±1.95^{ab,4}

Values (a-c) in a column that do not share a common superscript are significantly different ($p<0.05$).

Values (1-4) in a row that do not share a common superscript are significantly different ($p<0.05$).

Table 2. Percentages of live boar spermatozoa with mitochondrial membrane activity following exposure to autologous or homologous seminal plasma, assessed by fluorescent probes propidium iodide and Rhodamine 123 (mean ± SEM).

Boar	Source of seminal plasma (individual boars)					
	A	B	C	D	E	Control
A	68.4±1.24^{a,12}	63.1±1.12^{a,13}	40.9±2.12^{a,4}	77.3±1.05^{a,5}	72.5±1.25^{ab,15}	60.3±1.25^{a,3}
B	75.9±1.54^{a,1}	72.8±1.24^{b,1}	46.7±1.45^{ab,2}	77.1 ±1.32^{a,1}	71.3±1.92^{ab,1}	61.4±1.85^{a,3}
C	71.4±1.65^{a,1}	65.5±2.32^{ab,2}	55.4±2.21^{c,3}	71.5±1.82^{ab,1}	69.6 ±2.32^{ab,12}	58.5±2.01^{a,3}
D	72.4±2.09^{a,1}	61.5±1.04^{a,2}	50.3±1.53^{b,3}	75.9±1.81^{a,1}	68.6±1.92^{a,1}	55.7±2.76^{a,3}
E	73.4±1.81^{a,1}	71.3±1.24^{b,1}	45.3±1.89^{ab,2}	69.6±1.83^{b,1}	75.2±2.18^{b,1}	57.3±1.62^{a,3}

Values (a-c) in a column that do not share a common superscript are significantly different ($p<0.05$).

Values (1-3) in a row that do not share a common superscript are significantly different ($p<0.05$).

The CASA analysis of sperm motility followed the same pattern of effects; incubation with autologous SP improved most CASA-variables, such as motility (Table 3), curvilinear velocity (VCL, Table 4) or linearity (LIN, Table 5), while the exposure to homologous SP could either significantly improve, decrease (see once again boar C) or show no effect.

Table 3. Percentages of motile boar spermatozoa following exposure to autologous or homologous seminal plasma, assessed with the Sperm Class Analyzer (CASA system, mean ± SEM).

Boar	Source of seminal plasma (individual boars)					
	A	B	C	D	E	Control
A	67.3±1.19^{a,1}	60.3±1.42^{a,1}	35.3±2.43^{a,2}	75.5±1.12^{a,3}	70.1±1.12^{a,13}	50.3±1.32^{a,4}
B	72.4±1.76^{a,1}	70.2±1.33^{b,1}	39.7±1.32^{ab,2}	73.2 ±1.41^{a,1}	69.2±1.87^{a,1}	49.2±2.13^{a,3}
C	72.3±1.52^{a,1}	60.2±2.39^{a,2}	49.2±2.07^{c,3}	68.5±1.56^{ab,1}	67.1 ±2.05^{a,12}	45.2±1.89^{ab,3}
D	70.1±1.94^{a,1}	59.9±1.52^{a,2}	43.7±1.21^{bc,3}	75.3±1.76^{a,1}	64.3±1.76^{a,2}	41.4±1.32^{b,3}
E	71.3±1.51^{a,1}	68.3±1.43^{b,1}	40.2±1.65^{ab,2}	65.2±1.87^{b,1}	70.7±2.04^{a,1}	45.2±1.78^{ab,2}

Values (a-c) in a column that do not share a common superscript are significantly different ($p<0.05$).

Values (1-3) in a row that do not share a common superscript are significantly different ($p<0.05$).

1.5. Discussion

Sperm handling includes very often the extension of the sperm suspension to levels where SP components are washed away or, at least, significantly diluted. Such is the case when preparing spermatozoa for cooling and deep freezing, for IVF or for flow cytometrical analyses or cell sorting by chromosomal sex. Under the latter conditions (cell sorting by chromosomal sex), sperm viability decreases and the use of additives

has been rather widespread, albeit empirically used. The SP has been postulated among these additives (Maxwell and Johnson 1999).

Table 4. Curvilinear velocity (VCL, in µm/seconds) of boar spermatozoa assessed with the Sperm Class Analyzer, following exposure to autologous or homologous seminal plasma (mean ± SEM).

Boar	Source of seminal plasma (individual boars)					
	A	B	C	D	E	Control
A	86.8±0.76^{a,1}	86.0±1.13^{abc,1}	56.7±2.89^{a,2}	70.4±1.22^{a,3}	65.3±1.52^{a,3}	42.3±1.22^{ab,4}
B	89.4±0.94^{ab,1}	88.7±1.37^{ac,1}	59.3±2.33^{a,2}	72.1±1.34^{a,3}	65.6±1.41^{a,23}	47.5±2.43^{a,4}
C	85.2±1.10^{a,1}	79.8±1.16^{ab,1}	62.4±2.01^{a,2}	67.4±1.66^{a,2}	67.7±1.34^{a,2}	35.7±1.67^{b,3}
D	90.7±1.26^{ab,1}	81.2±1.30^{ab,2}	57.3±1.99^{a,3}	70.3±1.79^{a,4}	70.3±1.67^{ab,4}	41.5±1.56^{ab,5}
E	95.0±1.17^{b,1}	90.2±1.08^{c,1}	49.3±2.34^{b,2}	61.2±1.51^{b,3}	75.5±1.88^{ab,4}	45.1±1.69^{a,5}

Values (a-c) in a column that do not share a common superscript are significantly different ($p<0.05$).

Values (1-5) in a row that do not share a common superscript are significantly different ($p<0.05$).

Table 5. Linearity (LIN, as percentages) of boar spermatozoa assessed with the Sperm Class Analyzer, following exposure to autologous or homologous seminal plasma (mean ± SEM).

Boar	Source of seminal plasma (individual boars)					
	A	B	C	D	E	Control
A	46.3±0.76^{a,12}	50.0±1.13^{a,1}	35.3±2.89^{ab,3}	42.6±1.22^{a,2}	40.9±1.52^{a,23}	41.4±1.34^{a,2}
B	47.3±0.94^{a,1}	48.6±1.37^{a,1}	32.9±2.33^{a,2}	43.9±1.34^{a,1}	43.7±1.41^{ab,1}	42.4±2.32^{a,1}
C	50.3±1.10^{a,1}	48.8±1.16^{a,1}	40.1±2.01^{b,2}	44.9±1.66^{a,12}	44.6±1.34^{ab,12}	39.4±1.45^{a,2}
D	51.9±1.26^{a,1}	45.2±1.30^{a,12}	39.4±1.99^{b,3}	42.9±1.79^{a,2}	42.7±1.67^{ab,2}	42.7±1.76^{a,2}
E	52.0±1.17^{a,1}	50.2±1.08^{a,1}	32.6±2.34^{a,2}	40.8±1.51^{a,2}	47.8±1.88^{b,1}	38.4±1.81^{a,2}

Values (a-b) in a column that do not share a common superscript are significantly different ($p<0.05$).

Values (1-3) in a row that do not share a common superscript are significantly different ($p<0.05$).

The results obtained in the present study clearly show that the subtraction of the SP and the extension followed by incubation in a simple saline medium decreases sperm viability (hereby assessed in terms of membrane integrity, mitochondrial function and sperm motility). These results confirm previous studies (reviewed by Maxwell and Johnson 1999). Moreover, the present results clearly show that restoration of a certain amount (10% v/v) of SP could, in the majority of the cases, restore or even enhance the viability of the largely PBSm-extended boar spermatozoa from most boars included. Such beneficial effect of 10% SP has been previously reported in bulls, rams and boars (Ashworth et al. 1994; Maxwell et al. 1997; Garner et al. 2001), leading to the definition, albeit performed empirically, of 10% as a threshold SP concentration (Ashworth et al. 1994; Maxwell et al. 1997; 1998; Maxwell and Johnson 1999).

There has been a tendency for the use of pools of SP, a clear advantage when practical solutions are searched for. The results of the present study showed, however, that sperm viability, and perhaps even function, can be influenced by the exposure to the SP of a given sire, either negatively (here illustrated by boar C) or positively (here represented, for example, by boar A).

It is noteworthy that spermatozoa from different boars differed as to their response to SP from the one and the same male. Obviously, an in vitro examination of the effects of a given SP on sperm viability should be advisable before the use of homologous SP (individuals or pooled) for semen handling. In the same way, Killian et al (1993) in bulls and Van der Ven et al (1983) in humans, found that the effect of SP on spermatozoa could be greater or less evident depending on the SP source (sire). Moreover, exposure of spermatozoa from high fertile bulls to SP from bulls of low

fertility negatively affected their penetrating ability in vitro (Henault and Killian 1996). This finding and previous studies by this group has suggested that fertility-enhancing factors may be present in accessory sex gland fluid of sires with higher-fertility, or that fertility-inhibiting factors are presented in the accessory sex gland fluids from lower-fertility individuals (Henault and Killian 1995). Results from our laboratory and others have shown that the addition of SP from "good freezer" sires to a freezing extender improves post-thaw sperm survival when compared SP from "bad freezer" sires, either in stallions (Aurich et al. 1996) or boars (Cremades et al. 2004).

The evaluation of the component(s) of the porcine SP responsible for the beneficial effect on spermatozoa is ongoing in our laboratory. Evidence is now provided that some proteins in the boar SP, such as the non-heparin binding spermadhesins PSP-I/PSP-II heterodimer, can exert beneficial effects on boar spermatozoa in vitro (Centurión et al. 2003). Exposure to heparin-binding proteins of the SP has shown detrimental to sperm viability (Centurión et al. 2003). While differences in heparin-binding proteins have been correlated with low fertility in bulls (Kandel et al. 1992); a relationship has been shown between the fertility of the sires and the SP-protein profiles (Killian et al. 1993). A 26-kDa protein, identified as lipocalin-type prostaglandin D synthase, has later been associated with the fertility of the sires (Gerena et al. 1998).

Although interesting for comparisons, it should be bewared in mind that the present study was carried out with SP from the sperm-rich fraction (SRF) where the presence of heparin-binding proteins is much lower than in SP from the post sperm-rich fraction (PSRF) (Centurión 2002). Such comment is pertinent in the light of the results from Zhu et al (2000), who observed that boar spermatozoa incubated with SP from the

SRF had higher penetration rates than the same spermatozoa incubated with SP from the PSRF. The SP is a heterogeneous biologic fluid the composition of which includes not only the above mentioned proteins, but also aminoacids, peptides and enzymes that, apparently, influence spermatozoa in a variety of ways. A variation in the presence, absence or concentration of some components, but most probably their concerted action, may be responsible for the variability seen on the effect on the spermatozoa. Such biological effects of the interacting SP-factors on sperm function are complex and not yet well understood. Further research is obviously needed to disclose whether a single SP component could be strongly related to these effects, a matter of utmost importance for the practical handling of spermatozoa intended for ART.

In conclusion, the results of the present study indicate that addition of 10% (v/v) of SP (from SRF) from certain boars maintain or enhance the viability of largely extended boar spermatozoa in vitro. Owing to significant differences among sires, a screening for “good” boar SP sources is recommended. Further research is necessary to determine which component(s) is(are) responsible for its protective effect.

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Experiencia 2

Immunolocalization and Possible Functional Role of PSP-I/PSP-II Heterodimer in Highly-Extended Boar Spermatozoa

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2.1. Abstract

PSP-I/PSP-II heterodimer is a major protein of boar seminal plasma which is able to preserve, *in vitro*, the viability, motility and mitochondrial activity of highly-extended boar spermatozoa for at least 5 hours. However, little is known about the binding pattern of the heterodimer to the sperm plasma membrane and its eventual relation with the maintenance of the sperm functionality. The present study investigated the effect of exposing highly-extended boar spermatozoa (1 million/mL) to 1.5 mg/mL of PSP-I/PSP-II for 0.5, 5 and 10 hours at 38ºC on sperm characteristics and the changes in PSP-I/PSP-II localization as a result of both the addition of PSP-I/PSP-II to the extender and the incubation time. Exposure of the spermatozoa to PSP-I/PSP-II preserved sperm viability, motility and mitochondrial activity when compared to non-exposed spermatozoa. This protective effect lasted for 10 hours ($p<0.05$). After immunolabelling of highly-extended semen with rabbit monospecific polyclonal antibody against PSP-I/PSP-II, the percentage of immunopositive spermatozoa declines over time from 71% (0.5 h) to 49% (10 h). However, more than 80% of spermatozoa remained labelled during the 10h-incubation period if PSP-I/PSP-II was added. Scanning electron microscopy revealed 4 different binding patterns. The heterodimer was mainly localized to the acrosomal area, being redistributed to the post-acrosomal area or lost during *in vitro* incubation. In conclusion, the protective effect of the heterodimer appears to be related to its adhesion to the acrosomal area, and the loss of this protective effect coincides with a stepwise redistribution of PSP-I/PSP-II during incubation.

2.2. Introduction

In the boar, the majority of seminal plasma (SP) proteins belong to the spermadhesin family, a group of (glyco)proteins built by a single CUB domain architecture (Romero et al., 1997) and thought to play important roles in individual steps of the fertilization process such as capacitation and zona pellucida binding (Töpfer –Petersen et al., 1998). The spermadhesin family comprises five members: AQN-1, AQN-3, AWN, PSP-I, PSP-II. The last two form a glycosylated PSP-I/PSP-II heterodimer under physiological conditions (Calvete et al., 1995). *In vitro,* low doses of PSP-I/PSP-II heterodimer (1.5 mg/mL) appear to preserve membrane integrity, motility, and mitochondrial activity of highly-extended spermatozoa (Centurión et al., 2003). Processes linked to the removal of factors present in the SP that coat the sperm surface and maintain the stability of the plasma membrane, such as high extension of the spermatozoa (Maxwell and Johnson, 1999), trigger a series of phenomena that resemble those occurring during sperm capacitation culminating in premature acrosome exocytosis, thus decreasing the life span of the spermatozoa (Maxwell and Johnson, 1999). Although the addition of homologous seminal plasma (from 1% to 10% v/v) is a possible counter-measure to alleviate the consequences of such an “extension effect” (Asworth et al., 1994; Maxwell et al., 1997), differences in SP-protein profiles have been found between males of different fertility. These differences may be related to the variability between different sources of SP (Fournier-Delpech and Thibault, 1993; Killian et al., 1993; Caballero et al., 2004a) that lead to both beneficial and detrimental effects on the spermatozoa

Either whole SP or specific SP-components of low molecular weight affect survivability of boar spermatozoa, depending on how long they are exposed to the PSP-I/PSP-II (Centurión et al., 2003). However, there is an advantage of using an isolated protein instead of whole SP in order to avoid the inherent variability that exists in SP-composition between males or ejaculates (Killian et al., 1993; Asworth et al., 1994; Zhu et al., 2000). Previous studies, using various in vitro fertilization treatments, indicated that the PSP-I/PSP-II heterodimer does not sustain its influence for long periods during incubation (Caballero et al., 2004b). This suggests that the heterodimer may either lose its action by blockade or degradation or perhaps by attaching in a loose manner to the plasmalemma, thus losing influence over time. In any case, there is a need to establish the pattern of attachment and the influence of incubation on this pattern, and to elucidate whether there is any relationship between the presence of the protein, and its relative concentration, and the protective role *in vitro*.

The present study examined the relationship between the protective effect of the spermadhesin PSP-I/PSP-II and its binding pattern on the membrane surface of boar spermatozoa subjected to extreme dilution conditions similar to those used for flow cytometric high speed sorting is performed for chromosomal sex separation.

2.3. Methods

2.3.1. Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar seminal plasma

All experiments were performed with the SP from sexually mature boars, which had previously sired offspring. Ejaculates were obtained using the gloved hand method and SP was harvested after centrifugation of the sperm suspension at 3,800 x g at 20ºC for 15 min. The supernatants were sequentially filtered through 10 and 1.2 µm diameter filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of SP by size-exclusion chromatography on a 2000 x 5 cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, 0.025% sodium azide, pH 7.4 (Calvete et al., 1995). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 492 automated protein sequencer, Langen, Germany) and MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-Pro mass spectrometer (Langen, Germany). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a molar absorption coefficient (27332 M^{-1} cm^{-1}) as determined by Menéndez et al. (1995), or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 h at 106ºC in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyser (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilised, and stored at -20ºC until used.

2.3.2. Preparation of Anti-PSP-II antibodies

The PSP-I and PSP-II subunits were purified from the isolated PSP-I/PSP-II heterodimer by reverse-phase HPLC as described by Calvete et al. (1995). Polyclonal anti-PSP-II monospecific antibodies were obtained by immunizing female rabbits with sub-cutaneous inoculations of 0.5 mg of highly purified PSP-II heterodimer 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 immunization 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-II heterodimer activity by dot-blot ELISA and Western blot.

2.3.3. Semen handling, incubation with the heterodimer PSP-I/PSP-II, and evaluation of sperm parameters

Sperm-rich ejaculate fractions were collected from three mature Pietrain boars of proven fertility using the gloved hand method. Shortly after collection, the semen was extended in Beltsville Thawing Solution (BTS; [Pursel and Johnson, 1975]) to 30 x 10^6 spermatozoa/mL and spermatozoa evaluated for motility. Only ejaculates showing >80% progressive sperm motility were used. The extended ejaculates were pooled and centrifuged (1,700 x g for 3 minutes), the supernatant discarded, and the final sperm pellet re-extended in PBS to a final concentration of 1 x 10^6 spermatozoa/mL.

One million spermatozoa, extended as described above, were pre-incubated without or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized

proteins) at 38º C. After 0.5, 5 or 10 hours of pre-incubation, the sperm plasma membrane, acrosome integrity, mitochondria membrane potential, motility and binding patterns of PSP-I/PSP-II were evaluated.

Plasma membrane and acrosome integrity were simultaneously assessed by flow cytometry, using the staining protocol described by Nagy et al. (2003). For flow cytometric analysis, 50 nM of SYBR-14 working solution (100 µM stock solution in DMSO; component A of LIVE/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands), 0.5 µg/mL of PE-PNA (PhycoErythrin-conjugated PeaNut Agglutinin, Biomeda Corp. Foster City, CA) solution (1 mg/mL stock solution) and 7.5 µM of PI solution (Propidium Iodide, 1.5 mM stock solution in PBS) were added to 500 µL of sperm suspension. The samples were incubated at 37ºC in the dark for 10 min. All analyses were performed by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc, Miami, Fla) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, Calif) with 15-mW laser power at 488 nM and EXPO 2000 software (Coulter Corporation). Forward and side scatter were recorded for a total of 15,000 to 25,000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. The SYBR-14 fluorescence was measured through a 525-nm band pass filter, PI fluorescence was collected through a 635-nm band pass filter and PE-PNA fluorescence was detected through a 575-nm band pass filter. Membrane intact spermatozoa with intact acrosomes were defined as those stained only with SYBR-14.

The JC-1 probe (5,5',6'6-tetrachloro-1,1',3,3'tetraethylbenzymidazolcarbocianyne iodide, Molecular Probes Europe, Leiden, The Netherlands) was used to assess

mitochondrial membrane potential of spermatozoa as described by Martinez-Pastor et al. (2004), with slight modifications. Briefly, 0.2 µM of JC-1 working solution (3.8 mM stock solution) was added to 500 µL of sperm suspension. Then, samples were incubated at 37ºC in darkness for 10 min and analyzed immediately on the flow cytometer (see above). Fluorescence emission of JC-1 monomers and JC-1 aggregates were detected, respectively, in FL1 and FL2 using 520-nm or 590-nm band pass filters.

Sperm motility was estimated with a computer-assisted motility analysis system. Samples (5 µL containing 1 x 10^6/mL spermatozoa) were placed in a warm (38ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38ºC) of a Nikon Labophot microscope (Kanagawa, Japan) equipped with a 10x contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the Sperm Class Analyzer software (Microptic, Barcelona, Spain). The program settings were as follows: frame rate, 25 HZ; search radius, 11.5 µm; minimum track points, 7 frames; and threshold straightness, 75%. Two replicates per sample were examined with at least 100 spermatozoa recorded per replicate for each parameter evaluated. Motion parameters were: percentage of motile spermatozoa, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and amplitude of lateral head displacement (ALH, µm).

2.3.4. PSP-I/PSP-II heterodimer Immunocytochemistry

Immunolocalization of the PSP-I/PSP-II heterodimer was studied by both light and scanning electron microscopy (SEM) levels. Sperm suspensions pre-incubated either without or with PSP-I/PSP-II as described above were smeared onto poly-lysine

coated glass slides and air dried for 24 hours. Smears were fixed in 1% paraformaldheyde in PBS for 10 min., rinsed in PBS and incubated with a rabbit monospecific polyclonal antibody against PSP-II (1:500 in PBS) for 120 min at room temperature. Samples were rinsed several times in PBS and incubated with Auroprobe EM GAR G10 (10 nm gold labelled goat anti-rabbit IgG, Amersham Biosciences, Uppsala, Sweden) for 100 min. After washing in PBS and distilled water, a silver enhancement kit (RPN 491, Amersham Biosciences, Uppsala, Sweden) was applied for 10 min. Finally, the smears were washed in distilled water and air dried. To provide positive controls, samples of epididymal spermatozoa were smeared, air dried and fixed as above, incubated with the PSP-I/PSP-II heterodimer for 30 min and subjected to the same protocol as above. Negative controls were obtained by omission of the primary antibody.

For evaluation by light microscopy level, incubated samples were photographed with a Nikon microphot-FXA light microscope (Chibe, Japan) with at least 100 spermatozoa being evaluated per sample at 400x magnification. For evaluation by SEM level (6,000x magnification), portions of the glass slides with labelled spermatozoa were cut out, mounted onto metal chucks, critical-point coated with gold-palladium for 15-30 s, and examined using a JEOL JSM-6320F SEM (JEOL LTD, Japan), operated at 5 kV.

2.3.5. Statistical analysis

All data editing and statistical analyses were performed in SPSS version 11.5 (SPSS Inc, Chicago, Il, USA). Data were subjected to arcsine transformation and analysed by ANOVA using the MIXED-procedure according to a statistical model

including the fixed effects of heterodimer presence and the random effect of replicate. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered significant at $p<0.05$.

2.4. Results

Figure 1A-C shows the protective effect of PSP-I/PSP-II heterodimer on the spermatozoa exposed to high extension. Addition of the PSP-I/PSP-II heterodimer to a final concentration of 1.5 mg/mL resulted in a greater ($p<0.05$) percentage of membrane intact cells at each incubation time period compared with the controls (>81% of membrane intact spermatozoa at 10 h incubation) (Fig 1A). In the absence of PSP-I/PSP-II heterodimer, only 60% of highly-extended spermatozoa were viable after 0.5 h of incubation at 38ºC, and this value significantly decreased to 43% at 10 h ($p<0.05$). The same trend was observed regarding the mitochondrial activity of highly-extended spermatozoa (Fig. 1 B). After 10 h of incubation at 38ºC, 1.5 mg/mL of PSP-I/PSP-II preserved the mitochondrial activity in up to 79% of spermatozoa compared with only 37% in the controls ($p<0.05$).

Sperm motility was better maintained in presence of the PSP-I/PSP-II heterodimer than in controls ($p<0.05$), even considering a significant ($p<0.05$) decrease of motility being observed after 10 hours of incubation (Fig. 1C).

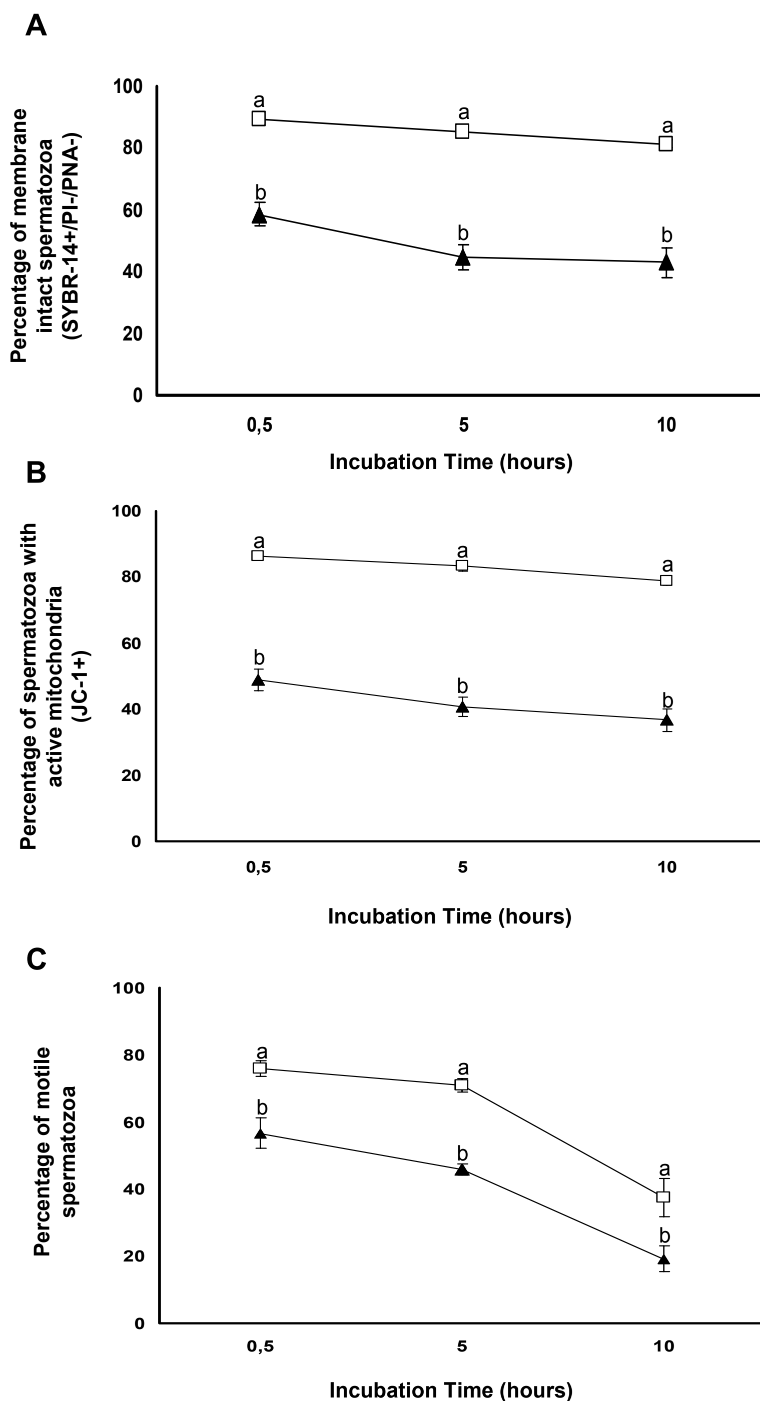

Fig.1. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the percentage of membrane intact spermatozoa with intact acrosome (A), mitochondrial activity (B) and progressive motility (C) of ejaculated boar spermatozoa diluted to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5 and 10 hours of incubation at 38ºC. Controls (▲) were sourced from the same spermatozoa but incubated in the absence of PSP-I/PSP-II heterodimer. Data are the mean ± SEM of three independent experiments. Different letters indicate significantly different values ($p<0.05$).

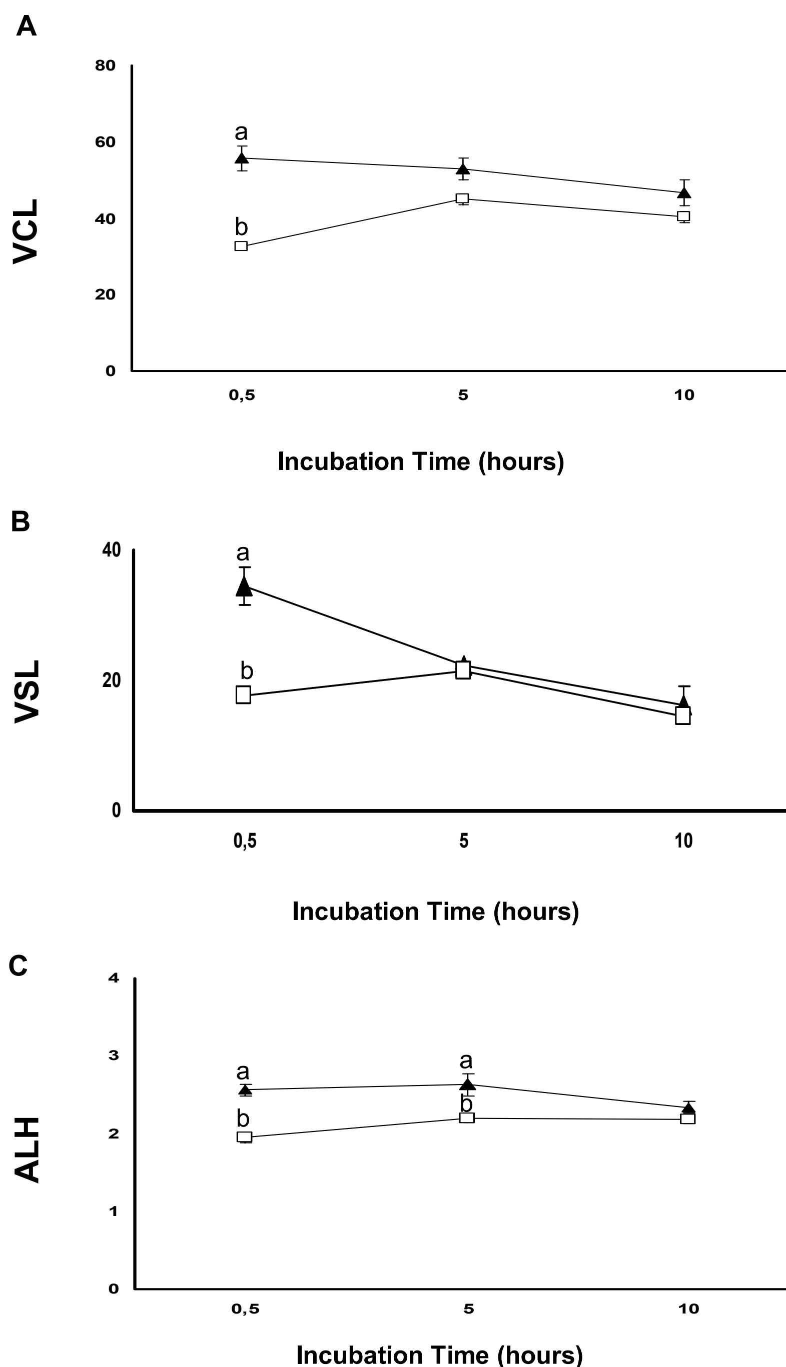

Fig.2. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the curvilinear velocity (VCL, µm/s) (A), straight-line velocity (VSL, µm/s) (B), and amplitude of lateral head displacement (ALH, µm) (C) of ejaculated boar spermatozoa diluted to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5 and 10 hours of incubation at 38ºC. Controls (▲) were sourced from the same spermatozoa but incubated in the absence of PSP-I/PSP-II heterodimer. Data are the mean ± SEM of three independent experiments. Different letters indicate significantly different values ($p<0.05$).

The displacement speed of the incubated spermatozoa (as VCL or VSL) as well as the ALH showed changes over time and following the addition of PSP-I/PSP-II to the incubation medium (Fig. 2A-C). The addition of PSP-I/PSP-II heterodimer reduced all three variables after 0.5 hours of incubation when compared to controls ($p<0.05$). However, from 5 hours of incubation onwards, the variables equilibrated to levels similar to controls.

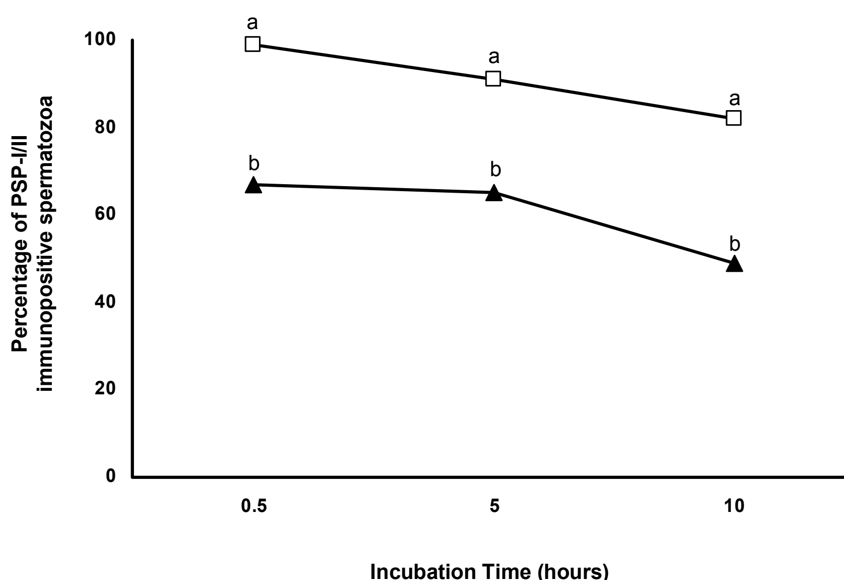

Fig.3. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the percentage of immunolabelled ejaculated boar spermatozoa extended to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5 and 10 hours of incubation at 38ºC evaluated by light microscope. Controls (▲) were incubated in the absence of PSP-I/PSP-II heterodimer. Different letters indicate significantly different values ($p<0.05$).

Regarding the immunolocalization of PSP-I/PSP-II heterodimer on the surface of the highly-extended spermatozoa, two populations could be discerned by light microscope. These were visible either with or without silver aggregates on the sperm head, independently of the addition of the heterodimer. Approximately 71% of the

ejaculated spermatozoa were immunolabelled after 30 minutes of incubation. In the controls, immunolabelling decreased from 71% at 0.5 h to 49% by 10 h (Fig. 3, $p<0.05$). By contrast, a 30-minute exposure to PSP-I/PSP-II resulted in an increase in the proportion of immunopositive spermatozoa compared to controls with almost 100% of the cells immunolabelled ($p<0.05$). This effect lasted during the whole incubation, maintaining around 82% of immunolabelled spermatozoa after 10 hours of pre-incubation with the heterodimer (Fig. 3).

The SEM evaluation was performed to confirm the immunolabelling and its temporal localization on the plasma membrane head domains. The SEM disclosed several binding patterns which are presented in figure 4: Pattern **a**: PSP-I/PSP-II heterodimer overlying the entire acrosome domain (Fig. 4a); pattern **b**: PSP-I/PSP-II heterodimer covering the acrosome domain with additional labelling on the postacrosomal area and a negative equatorial segment (Fig. 4b); pattern **c**: Slight immunolabelling of the postacrosomal area (Fig. 4c) and; pattern **d**: no immunolabelling of spermatozoa (Fig. 4d). As expected, negative controls were totally immunonegative (Fig. 4e) while positive controls had silver aggregates over the whole spermatozoon (Fig. 4f). The PSP-I/PSP-II heterodimer was mainly located in the acrosomal area (>90%) of highly-extended spermatozoa incubated either with or without heterodimer at 0.5 hours. During the incubation, there was a temporal redistribution of the pattern of immunolabelling over time, from Pattern **a** to Pattern **d**. While most of the spermatozoa incubated without PSP-I/PSP-II (66%) showed Pattern **b** after 5 hours, an increase in Patterns **c** and **d** (26 and 30%, respectively) was observed after 10 hours of incubation. However, most spermatozoa incubated with PSP-I/PSP-II displayed pattern a throughout the 10 h incubation period (Table 1).

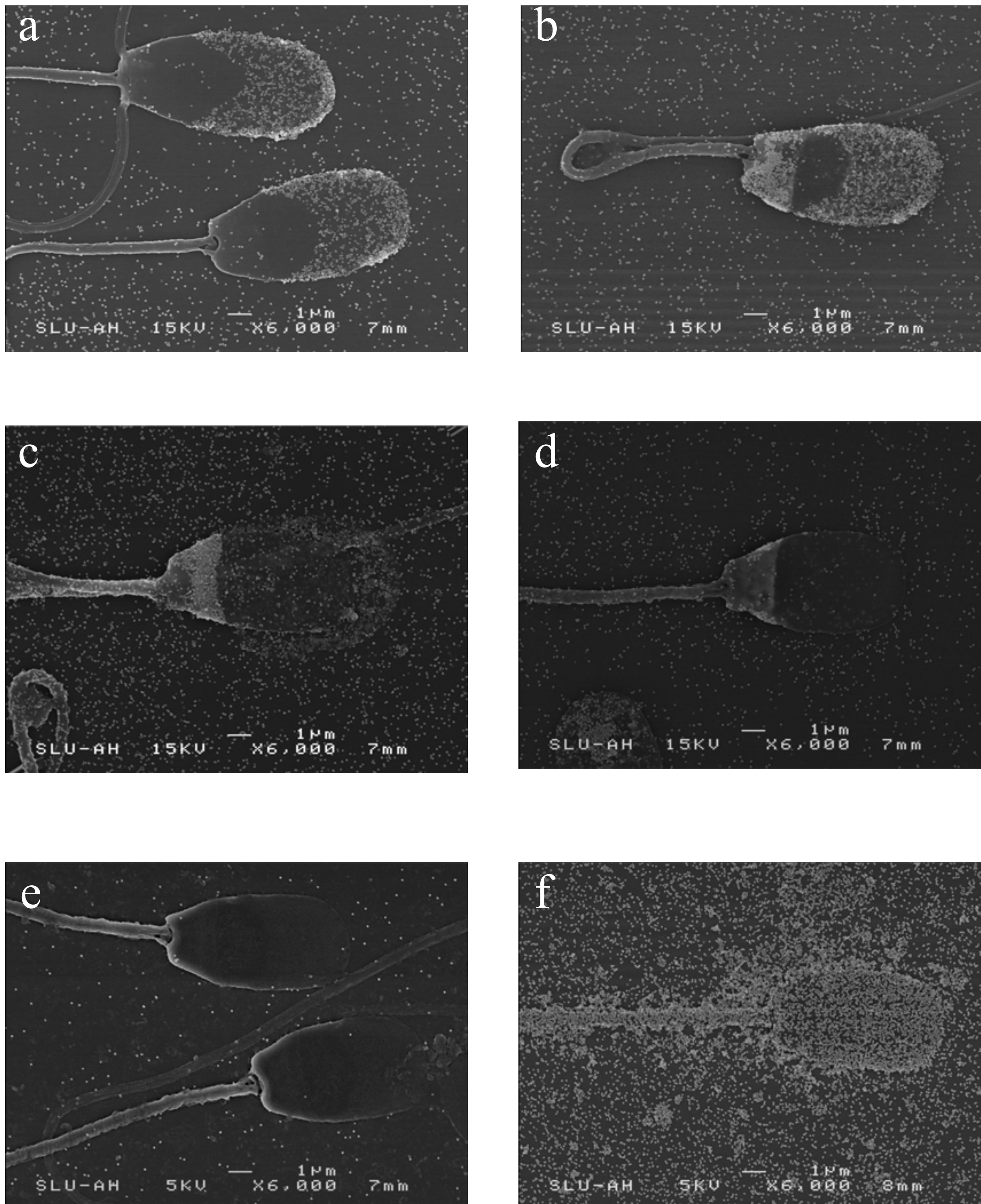

Fig.4a-f. Scanning electron micrographs of silver-enhanced immunogold-labelled highly-extended, boar spermatozoa incubated with a rabbit monoespecific polyclonal antibody against PSP-II. The sperm population displayed four distinctive staining patterns. Pattern **a**: PSP-I/PSP-II heterodimer labelling overlying the acrosomal sperm head domain. Pattern **b**: PSP-I/II heterodimer labelling covering the acrosomal domain with additional labelling on the postacrosomal area. Pattern **c**: Immunonegative acrosomal domain and a slight staining of the postacrosomal area. Pattern **d**: Immunonegative sperm head. Negative and positive controls are shown in figures 4e and f, respectively.

2.5. Discussion

High dilution rates remain an inevitable consequence of some new biotechnologies such as high-speed flow sorting of spermatozoa for chromosomal sex. Such high extension rates have been repeatedly documented as detrimental for spermatozoa, inducing changes in viability and motility patterns all of which are primarily associated with the removal of adsorbed seminal plasma components (Maxwell and Johnson, 1999; Centurión et al., 2003; Caballero et al., 2004a). To avoid this undesirable effect, attempts to maintain the functionality of highly-extended spermatozoa have included the addition of 10% (v/v) of SP or SP-components of low molecular weight to the sperm suspensions (Asworth et al., 1994; Maxwell et al., 1997).

Table 1. Redistribution of the different patterns of immunolabelling of PSP-I/PSP-II heterodimer on the surface of highly-extended spermatozoa (1 x 10^6 spermatozoa/mL) after incubation either with or without PSP-I/PSP-II (exposed and non-exposed, respectively) during 0.5, 5 and 10 hours at 38ºC.

Incubation time (hours)	PSP-I/PSP-II	Percentage of spermatozoa with pattern[1]			
		a	**b**	**c**	**d**
0.5	Non-exposed	97	3	0	0
	Exposed	98	1	1	0
5	Non-exposed	29	66	3	2
	Exposed	90	7	3	0
10	Non-exposed	32	12	26	30
	Exposed	84	11	5	0

[1]Pattern **a**: labelled acrosome; Pattern **b**: labelled acrosome with additional labelling on the postacrosomal area; Pattern **c**: Slight staining of the postacrosomal area; Pattern **d**: Immunonegative sperm head

Recently, we have identified the non-heparin-binding spermadhesin PSP-I/PSP-II heterodimer as one of the SP-components responsible for such beneficial effect on boar spermatozoa (Centurión et al., 2003; Caballero et al., 2004b). However, to the best of our knowledge, whether this effect relates to the association of the heterodimer with the sperm plasma membrane has not been studied.

To evaluate the binding pattern of the PSP-I/PSP-II heterodimer on the spermatozoa, a rabbit monospecific polyclonal antibody against PSP-II was produced. In boar seminal plasma, spermadhesin PSP-II has been found exclusively associated into a heterodimeric complex with PSP-I, whereas PSP-I, which exists in excess over PSP-II, is present also as a non-complexed molecular species (Calvete et al., 1995). Thus, the anti-PSP-II antiserum represents a specific immunochemical to detect the PSP-I/PSP-II complex. The immunocytochemistry clearly revealed that PSP-I/PSP-II heterodimer is located mainly on the acrosomal region of the head sperm and that the immunolabelling was being lost over time. Addition of exogenous PSP-I/PSP-II heterodimer caused, as expected, that almost 100% of the spermatozoa became immunolabelled. Contrary to a previous report suggesting that the PSP-I/PSP-II complex lacked sperm-binding activity (Calvete et al., 1995), the present results show that a high subpopulation of highly-extended boar spermatozoa (71%) did not entirely lose their adsorbed PSP-I/PSP-II coating in samples of highly-extended ejaculated spermatozoa where no extra PSP-I/PSP-II was added (e.g. controls). Similar results have previously been reported in boar spermatozoa in relation to the spermadhesins AQNs and AWN which coat the sperm membrane after ejaculation in almost 100% of the spermatozoa but were progressively lost during incubation under capacitation conditions (Dostálová et al., 1994). In our experiment, the proportion of

immunolabelled control spermatozoa dropped below 50% after 10 hours of incubation. However, the proportion of spermatozoa incubated in the presence of PSP-I/PSP-II heterodimer remained in about 80% positively labelled with the immuno-stain ($p<0.05$). This indicates that (i) the heterodimer binds to the sperm membrane head domains before or at ejaculation and (ii) that the binding is intense enough to last for several hours in a large proportion of spermatozoa, even following a extreme dilution of the sperm suspension. However, the binding is probably not strong enough to remain unless an additional source of the heterodimer is present. Lastly, the highly-extended, long-incubated spermatozoa are able to bind additional heterodimer and maintain functionality. Regarding functionality, while the percentage of membrane intact spermatozoa as well as the percentage of spermatozoa presenting appropriate mitochondrial activity were maintained during the incubation process, the percentage of progressively motile spermatozoa was always lower than that of membrane intact spermatozoa (and also below the proportions of immunolabelled spermatozoa). Differences among motility and mitochondrial activity have been reported earlier and probably reflect differences in sperm activity or the ability of the measuring methods (Windsor, 1997; Centurión et al., 2003). It has been recently hypothesised that mitochondria are thought to provide the sperm head and the midpiece with ATP (by oxidative phosphorylation) in order to maintain different gradient processes over the plasma membrane that are important for sperm survival. However, the ATP needed for sperm motility seem to be produced by glycolisis (anaerobic ATP production) by enzymes located in the fibrous sheath of the tail (Silva and Gadella, 2006). Although interesting as hypothesis , there is a need for experimentally-based proof for these assumptions.

Although a higher percentage of motile cells was seen in the exogenous PSP-I/PSP-II group than in controls, the addition of PSP-I/PSP-II during incubation decreased their ALH when compared with control group. These results do not suggest that spermatozoa in PBS were hyperactivated, but indicate that the width of the sperm heads movement were significantly higher than those of spermatozoa in PSP-I/PSP-II. Consequently, the sperm motility should be more preserved in spermatozoa incubated in presence of PSP-I/PSP-II as showed the percentage of motile cells.

The percentages of acrosome-reacted and immunonegative spermatozoa were similar during the first stages of incubation. These results might indicate that the PSP-I/PSP-II heterodimer is linked to the acrosome while it remains intact. Nevertheless, it should be noted that when spermatozoa were incubated for 10 hours, a higher number of immunonegative spermatozoa than of acrosome-reacted spermatozoa was found. Moreover, in some of the spermatozoa, the PSP-I/PSP-II was partially (Pattern b) or totally (Patterns c and d) removed from the acrosome-intact sperm head membrane. These findings suggest that the removal of the heterodimer from the sperm acrosome domain occurs earlier than the disruption of the acrosome and could, therefore, be related to remodelling of the sperm surface, as it occurs during the destabilization of the plasma membrane (Tulsiani et al., 1997). This hypothesis is in agreement with the fact that PSP-I/PSP-II seems to be removed from the sperm surface when spermatozoa are subjected to *in vitro* capacitation treatments (Caballero et al., 2004b). These data would suggest that, at least *in vivo*, PSP-I/PSP-II is released from the spermatozoa before reaching the ovulated oocytes and may not have the chance to play a role in gamete interaction (as other SP proteins, such as the AWN; [Rodríguez-Martínez et al., 1998]). However, the *in vivo* role of the heterodimer is yet to be determined.

The SEM study showed that PSP-I/PSP-II is a surface-adsorbed protein located principally on the acrosomal sperm head domain of ejaculated boar spermatozoa (Pattern **a**). The PSP-I/PSP-II seems to migrate to the post-acrosomal domain during the incubation process (Patterns b and c) being finally lost from the sperm surface (Pattern **d**). This redistribution is consistent with previous results in several species where it has been related to the processes of sperm capacitation and acrosome reaction (Kamaruddin et al., 2004; Barrios et al., 2005). However, before establishing a causal relationship between surface localization and temporal distribution to sperm capacitation, more studies are required. In any case, the electron microscopy showed an overwhelming population of spermatozoa (97%) with Pattern **a** after 0.5 h of incubation, which corresponded to samples showing the best sperm viability, motility and mitochondrial activity. Likewise, there was a clear trend toward redistribution to Pattern **d** after 10 hours of incubation in a spermatozoa population (30%) whose viability, mitochondrial activity and particularly their motility were lowest.

Related to the protective effect of the heterodimer on boar spermatozoa, it could be argued that the PSP-I/PSP-II heterodimer would be responsible for the stabilization of the sperm membrane, thus counteracting destabilizing phenomena that lead to membrane deterioration, opening of the acrosome and, eventually, cell death. Obviously, identifying such an additive for sperm suspensions resulting from flow cytometry should help to improve the efficiency of new biotechnological processes. Moreover, there is no negative influence of the heterodimer on the capability of fresh-extended boar spermatozoa to penetrate *in vitro* the homologous oocytes pre-exposed to low doses of PSP-I/PSP-II (Caballero et al., 2004b).

In conclusion, the protective effect of the PSP-I/PSP-II heterodimer on highly-extended spermatozoa seems to last for at least 10 hours and could be related to the adhesion of the heterodimer to the acrosome domain of the sperm head plasma membrane. Although these results may indicate a stabilizing effect of the heterodimer on the fluidity of the sperm membrane and perhaps a decapacitating post-ejaculation role, further research is needed in order to clarify the causal link between the binding patterns of the PSP-I/PSP-II and the capacitation status of the spermatozoa.

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Experiencia 3

Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction

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3.1. Abstract

The seminal plasma PSP-I/PSP-II spermadhesin is able to preserve, *in vitro,* the viability of highly extended boar spermatozoa, suggesting it might be used as a suitable ameliorator for the damaging effects of sperm handling, including in vitro fertilisation. However, little is known about the ligand capability of PSP-I/PSP-II to the zona pellucida (ZP) or its possible role in gamete interaction. The present study evaluated the effect of the presence of PSP-I/PSP-II (1.5 mg/mL) during *in vitro* oocyte maturation and also during co-incubation of frozen-thawed boar spermatozoa with either immature (IM) or *in vitro* matured (IVM) oocytes, either enclosed by cumulus cells or denuded. Exposure of the gametes to the heterodimer during *in vitro* gamete co-incubation showed a significantly blocking effect of sperm penetration rates and a decreased number of spermatozoa per oocyte in both IM- and IVM- denuded oocytes. Such an effect was not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP-receptors. Besides, when PSP-I/PSP-II was added to the IVM–medium, oocyte maturation rates were significantly reduced. In conclusion, the results suggest that PSP-I/PSP-II, when present *in vitro,* block sperm-ZP binding.

3.2. Introduction

Gamete interaction is an early critical step in mammalian fertilization, where at least three morphologically disparate cell types, the capacitated acrosome-intact spermatozoon, the mature oocyte and the surrounding cumulus cells, are involved (Yanagimachi, 1994). The events of gamete recognition, binding and fusion are highly regulated processes that imply a number of biochemical reactions until a new zygote is formed. In domestic species, this mechanism of cell-to-cell adhesion seems mediated by protein-carbohydrate interactions between sperm-associated lectins and glycan structures of the oocyte zona pellucida (ZP), the latter synthesised by the concerted action of the oocyte and the granulosa cells during oocyte maturation (Sinowatz et al., 2001).

One of these sperm lectins constitutes the spermadhesin family (Sinowatz et al., 1997). Boar spermadhesins are a group of (glyco)proteins built by a single CUB domain architecture (Romero et al., 1997), coating the sperm surface (Dostálová et al., 1994). They play a role in several biological functions, including sperm capacitation and, as already mentioned, gamete recognition and binding (Calvete et al., 1994). According to their binding properties, spermadhesins can be divided into two groups, depending on their ability to either bind heparin (AQN-1, AQN-3, AWN) or not (PSP-I/PSP-II heterodimer).

The PSP-I/PSP-II heterodimer appears to preserve -*in vitro*- the membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa for as long these are exposed to the heterodimer (Centurión et al., 2003). Spermadhesin PSP-

I/PSP-II reproduces the protective effect that has been reported upon the addition to spermatozoa of either bulk seminal plasma (SP) from selected males (Maxwell et al., 1997; Maxwell and Johnson, 1999; Caballero et al., 2004) or SP-components of low molecular weight (Ashworth et al., 1994). These properties highlight PSP-I/PSP-II as a potential candidate for pre-treatment of manipulated spermatozoa (extended, stored, deep-frozen or sex sorted) aiming at promoting sperm survival and performance *in vitro*. However, the competence of PSP-I/PSP-II to interact during gamete binding is still unclear. While the heparin-binding spermadhesin AWN is able to reach the ovulated oocytes(s) *in vivo* (Rodriguez-Martinez et al., 1998), the PSP-I/PSP-II heterodimer binds loosely to the sperm surface and does not seem to maintain any attachment to the spermatozoa either following in vitro capacitation or sperm transport in the female genital tract, as seen to be the case with the AWNs (Dostálová et al., 1994; Calvete et al., 1997; Rodriguez-Martinez et al., 1998). In some studies, PSP-I/PSP-II heterodimer showed affinity for ZP glycoproteins and this activity was located in the isolated PSP-II monomer and was reported to be cryptic in the heterodimer (Calvete et al., 1995a). In contrast, Jonáková et al. (2000) also found an interaction between the ZP and the isolated PSP-II, but this binding was inhibited by the aggregated PSP-I/PSP-II heterodimer. Therefore, although an *in vivo* functional role of this spermadhesin during gamete binding is unexpected, the fact that the ZP-binding properties of PSP-I/PSP-II heterodimer are still a matter of controversy in the literature calls for further research in this area, if the purpose is to use these spermadhesins during *in vitro* gamete handling, for instance as a preparatory step for IVF.

Consideration of the beneficial effect on sperm viability already assessed *in vitro* and the divergent results in ZP-binding ability of PSP-I/PSP-II, has prompted us to

examine the effect the heterodimer PSP-I/PSP-II during boar sperm-oocyte co-culture using both immature (IM) and *in vitro* matured (IVM) homologous oocytes.

3.3. Materials and methods

All chemicals used in the preparation of the culture media were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

3.3.1. Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar seminal plasma

All experiments were performed with the SP from sexually mature boars, which had previously sired offspring. Ejaculates were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at 3,800 x g for 15 min, at 20ºC. The supernatants were sequentially filtered through 10 and 1.2 µm filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of SP by size-exclusion chromatography on a 2,000 x 5 cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, 0.025% sodium azide, pH 7.4 (Calvete et al., 1995a). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 472 automated protein sequencer, Langen, Germany) and MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-Pro mass spectrometer (Langen,Germany). A

saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a 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 106ºC in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyser (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilised, and stored at -20ºC until used.

3.3.2. Culture Media

The basic medium used to in *vitro* assess sperm penetration ability of immature oocytes was TCM-199 with Earle´s salts supplemented with 12% heated fetal calf serum (v/v), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 UI penicillin G, and 30 µg/mL streptomycin sulphate (Cheng, 1986).

The basic medium used for *in vitro* maturation was Bovine serum albumin (BSA)-free North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). This medium shall be named "IVM medium" hereafter.

The *in vitro* fertilisation (IVF) medium used (Abeydeera and Day, 1997) consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1mM caffeine and 0.2% BSA (fraction V, Cat nr A 7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was North

Carolina State University (NCSU) 23 containing 0.4% BSA (fraction V, Cat nr A 8022, initial fractionation by cold alcohol precipitation).

3.3.3. Recovery, *in vitro* maturation, penetration and evaluation of oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martinez et al., 1993; Abeydeera and Day, 1997).

Ovaries were obtained from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 µg/mL kanamycin, maintained at 34-37ºC. Cumulus-oocyte-complexes (COCs) were aspirated from medium-sized follicles (3-6 mm in diameter) with an 18-gauge needle fixed to a 10 mL disposable syringe. Only COC´s having a compact cumulus mass (with at least six or seven layers) an intact ZP and an oocyte with an evenly granulated cytoplasm were selected for the different experiments.

For preparation of IM-oocytes, oocytes were divided in two groups for cumulus enclosed (CE) and denuded oocytes, respectively. Both, CE oocytes and those denuded by repeated pipetted to mechanically remove the cumulus cells, were placed directly in 2 mL of pre-equilibrated modified TCM-199 medium supplemented with 2 mM caffeine and 5.4 mM calcium lactate (Cheng, 1986) in batches of 30 IM-oocytes and kept in the incubator at 39ºC in 5% CO_2 in air for about 30 minutes before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed three times in IVM-medium. Thereafter, COCs were transferred to a Nunc 4- well multidish plate (50-100 COCs/well) submerged in 500 µL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV, Boxmeer, The Netherlands), and cultured at 39ºC in 5% CO_2 in air for 22 h. The medium was then changed for maturation medium without hormone supplementation, and incubated at 39ºC in 5% CO_2 in air for another 22 h. After *in vitro* maturation, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed three times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera and Day, 1997). Batches of 50 IVM oocytes were placed in 50 µL drops of IVF medium covered with warm mineral oil in a 35 x 10 mm Petri dish. The dishes were kept in the incubator for about 30 minutes before spermatozoa were added for sperm penetration assays.

Frozen-thawed spermatozoa were cryopreserved from a fertile Pietrain boar as described by Roca et al. (2003) in a plastic medium-straw (0.5 mL) and thawed in circulating water at 37ºC for 20 s. Thawed spermatozoa were re-suspended in modified TCM-199 for co-incubation with IM oocytes. For IVM oocytes, thawed semen was washed three times by centrifugation at 1, 900 x g for 3 min in Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/mL BSA (Fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 µg/mL kanamycin (mDPBS). Spermatozoa were co-incubated with IM or IVM oocytes at a oocyte:spermatozoa ratio of 1:66,000 (Martinez et al., 1993) or 1:2,000 (Gil et al., 2004), respectively. The oocytes were co-incubated with the spermatozoa at 39ºC in an atmosphere of 5% CO_2 in air, the IM

oocytes for 16 h and the IVM oocytes for a primary period of 6 h. The IVM oocytes were thereafter washed three times in pre-equilibrated embryo culture medium (NCSU-23 containing 0.4% BSA), transferred to a Nunc 4-well multi-dish containing 500µL of the same medium per well (covered by mineral oil) and cultured for another 6 h at 39ºC and 5% CO_2 in air.

Following the co-incubation, IM-oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells (group of CE oocytes) and those spermatozoa attached to the surface of the ZP (groups of CE and denuded oocytes). The IM (16 h post insemination) and the IVM oocytes (12 h post insemination) were mounted on slides, fixed and stored in 25% (v/v) acetic acid in ethanol at room temperature for 48-72 hours, stained with 1% lacmoid in 45% (v/v) acetic acid, and examined under a phase contrast microscope at 400x magnification. Oocytes were considered penetrated when spermatozoa with unswollen (IM-oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM-oocytes) were found in the ooplasm.

3.3.4. Experimental design

Experiments were designed to disclose the effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/mL) during sperm-oocyte co-incubation on penetration rates and number of spermatozoa per oocyte in IM (experiment 1, including a total of 673 IM-oocytes) or IVM oocytes (experiment 2, including a total of 730 IVM-oocytes). All experiments were carried out with the same batch of lyophilized proteins. In addition, frozen-thawed spermatozoa from a single ejaculate were used to avoid inter-ejaculate

variability. In order to rule out confounding effects related to the presence of cumulus cells, CE and denuded IM-oocytes were exposed to the heterodimer during co-incubation with frozen-thawed boar spermatozoa (single batch) in a 2 x 2 factorial design. Controls were provided by PSP-I/PSP-II unexposed, co-incubated gametes. All experiments were replicated 3 times.

3.3.5. Data analysis

All the data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analysed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effect of presence of PSP-I/PSP-II heterodimer and the random effect of replicate. For experiment 1, presence or absence of CE was included as fixed effect and interaction with the heterodimer PSP-I/PSP-II was considered. Data of percentage of penetration were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p<0.05$.

3.4. Results

3.4.1. Experiment 1: Effect of adding 1.5 mg/mL of PSP-I/PSP-II heterodimer to the sperm-IM-oocyte co-culture medium on sperm penetration ability

Results are presented in Table 1. The presence of the cumulus cells affected the number of spermatozoa per oocyte ($p<0.05$) while the presence of the heterodimer PSP-

I/PSP-II affected both penetration rates and number of spermatozoa per oocyte ($p<0.05$). The interaction between the presence of cumulus cells and the heterodimer was significant ($p<0.05$) for both, penetration rates and number of spermatozoa per oocyte. In control groups (unexposed), a significant increase in sperm penetration rates and number of spermatozoa per oocyte was found in denuded IM oocytes compared with CE IM oocytes ($p<0.05$). When both types of IM oocytes were exposed to PSP-I/PSP-II, only the denuded oocytes showed a significant difference with controls, the sperm penetration rates and the sperm number per oocyte becoming significantly lower ($p<0.05$).

Table 1. Effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/mL) during co-culture of sperm with either cumulus enclosed (CE) or cumulus denuded (IM) pig oocytes on the percentage of oocyte penetrated and the number of spermatozoa per oocytes.

PSP-I PSP-II	Type of immature oocyte	Number of oocytes	Percentage of oocytes penetrated	Number of spermatozoa per oocyte
Unexposed	CE	196	79.0±2.9[a]	8.5±0.5[a]
	Denuded	151	95.0±1.8[b]	20.4±0.9[b]
Exposed	CE	194	72.0±3.3[a]	7.4±0.6[a]
	Denuded	132	67.0±4.3[a]	12.9±1.1[c]
Probability				
PSP-I/PSP-II			0.001	0.001
Type of immature oocyte			NS	0.001
PSP-IPSP-II x Type of immature oocyte			0.001	0.004

Values within columns with different superscripts differ ($p<0.05$).

Values are the mean ± SEM.

3.4.2. Experiment 2: Effect of adding 1.5 mg/mL of PSP-I/PSP-II heterodimer to the sperm-IVM oocyte co-culture medium on sperm penetration ability

Results are presented in Table 2. Co-culture of sperm-IVM oocytes in IVF medium supplemented with 1.5 mg/mL of PSP-I/PSP-II heterodimer blocked the penetration of spermatozoa into the oocytes, resulting in a significant decrease in both penetration rates and number of spermatozoa per oocyte compared with controls ($p<0.05$).

Table 2. Effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/mL) during co-culture of sperm and IVM oocytes in IVF medium on the percentage of oocytes penetrated and the number of spermatozoa per oocyte.

Group	Number of oocytes	Penetration Rate (%)	Number of spermatozoa per oocyte (mean)
Control	348	98 ±0.7[a]	6.7 ±0.2[a]
PSP-I/II-exposed	382	46 ±2.8[b]	1.9±0.8[b]

Values within columns with different superscripts differ ($p<0.05$).

Values are the mean ± SEM.

3.5. Discussion

The addition of low amounts of the spermadhesin PSP-I/PSP-II (1.5 mg/mL), which have proven protective for boar sperm viability (Centurión et al., 2003), to denuded-IM oocytes or IVM oocytes co-cultured with frozen-thawed boar spermatozoa

induced a significant decrease of both in vitro penetration rates and mean number of spermatozoa per oocyte. Boar SP contains many proteins that have affinity for the ZP (Peterson et al., 1989; Jonáková et al., 2000), inhibiting the binding of the spermatozoa. The ability of the PSP-I/PSP-II heterodimer and its isolated subunits (PSP-I and PSP-II) to bind known ligands has been previously reported with contradictory results. While PSP-I does not bind to ZP using a biotinylated ligand assay (Calvete et al., 1995a), the PSP-II subunit shows affinity for ZP glycoproteins (Calvete et al., 1995a; Jonáková et al., 2000). Furthermore, some the discrepancies include the affinity of the aggregated PSP-I/PSP-II heterodimer for the ZP (Calvete et al., 1995a; Jonáková et al., 2000). In other words, the degree of interaction between the oocyte vestment and the PSP-I/PSP-II is far from clear.

Variations in the amount and distribution of the ZP (glyco)components of many species during oocyte growth have been reported (Parillo and Verini Supplizi 1999; Parillo et al., 2001). In a previous experiment (data not shown), exposure of cumulus oocyte complexes to PSP-I/PSP-II inhibited nuclear maturation, with only 32% of the oocytes exposed to PSP-I/PSP-II reaching metaphase II while 84% of the unexposed oocytes matured normally. Moreover, the expansion of the cumulus cells was virtually inhibited in a large proportion of these PSP-I/PSP-II-exposed COCs. The fact that the IM oocyte population collected from abattoir ovaries is heterogeneous in quality and size (Roca et al., 1998; Lucas et al. 2002), could explain why PSP-I/PSP-II exposure did not affect the entire oocyte population, since the oocytes could have been in different growth stages. Growing oocytes would then be surrounded by different glycoprotein domains, as these are sequentially synthesized and added by the follicular cells and the oocyte or are modified in the ZP along with follicular growth (Lucas et al., 2003), either

one of these factors could result in modifications of the affinity of the ZP for the heterodimer PSP-I/PSP-II.

The mechanism by which the heterodimer PSP-I/PSP-II mediates *in vitro* maturation might be different from the effect it exerts during *in vitro* sperm penetration of porcine oocytes. Therefore, the effect of PSP-I/PSP-II on the penetration ability of the spermatozoa could be unrelated to the blockade of the ZP binding by the heterodimer, since whole seminal plasma and some of their components have already shown inhibitory effects on sperm-oocyte binding by deterring the functionality of the spermatozoa before they bind to the ZP (Peterson et al., 1984; Capková and Peknicová 1997).

While the presence of cumulus cells exerted a detrimental effect on sperm penetration rates in co-culture of sperm and IM oocytes not exposed to PSP-I/PSP-II, the cumulus cells surrounding the oocytes elicited a beneficial effect on the in vitro penetration ability of the spermatozoa when heterodimer PSP-I/PSP-II was present. Although an easy removal of the heterodimer from the sperm surface was expected to occur in vitro (Calvete et al., 1995b), similar rates between control and treatment arms of the experiment were seen only when IM oocytes surrounded by cumulus cells were co-incubated with the spermatozoa in the presence of PSP-I/PSP-II. Penetration of the spermatozoa through the cumulus oophorus cloud, a structure composed not only of the cumulus cells but primarily by the glycosaminoglycan-based fibrous matrix they secrete, made up of hyaluronic acid and proteoglycans, is considered to facilitate sperm capacitation and penetration (Fléchon et al., 2003). Whether this passage through the cumulus cloud implies that the carbohydrate of the proteoglycan matrix would trap

PSP-I/PSP-II heterodimer, thus cleansing the sperm surface from attached seminal plasma proteins or whether the heterodimer is removed during sperm plasma membrane redistribution during the ongoing sperm capacitation in presence of cumulus cells is as yet unknown. However, these are tempting hypotheses to explain why higher oocyte penetration rates following exposure to PSP-I/PSP-II were found in CE IM oocytes.

In conclusion, while there is no obvious influence of the PSP-I/PSP-II heterodimer on the co-culture of CE oocytes and spermatozoa, a deleterious effect is observed in the absence of cumulus cells in both IM and IVM oocytes. Whether this inhibitory effect is exerted directly on the spermatozoa or by blocking ZP receptors is unknown. Further research is therefore needed to clarify the mechanisms by which PSP-I/PSP-II heterodimer affects oocyte-sperm interaction.

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Experiencia 4

Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to fertilize homologous oocytes *in vitro*?

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4.1. Abstract

Low concentration (0.15 mg per million of spermatozoa) of seminal plasma-derived PSP-I/PSP-II spermadhesin heterodimer is able to preserve the viability of highly diluted boar spermatozoa. Whether spermatozoa also keep their fertilizing capacity is not yet known. The present study evaluated the effect of exposing freshly-extended and frozen-thawed boar spermatozoa (10 million/mL) to PSP-I/PSP-II (1.5 mg/mL) for 30 or 120 minutes, on sperm characteristcs and the outcome of in vitro penetration of immature (IM) and in vitro matured (IVM) homologous oocytes, aiming to identify this spermadhesin as a suitable modulator for sperm handling protocols. Although exposure to the heterodimer improved sperm viability and motility without increasing the sperm acrosome reaction level of both fresh-extended and frozen-thawed spermatozoa, this pre-treatment did not affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes, compared with controls. When cryopreserved spermatozoa were tested, however, on IVM oocytes, already a 30 minute preincubation exposure to PSP-I/PSP-II showed a significant blocking effect on penetration rate (from 90% to 32%, $p<0.05$) and on mean sperm numbers per oocyte (2.9 to 1.6, $p<0.05$). In order to disclose the nature of this paradox, frozen-thawed spermatozoa were cleansed (by centrifugation in saline-BSA or through Percoll density gradient separation) and the procedure repeated. Penetration (but not number of spermatozoa/oocyte) increased ($p<0.05$) when spermatozoa were filtered through Percoll compared with either washed or unwashed-controls (53% vs. 13% vs. 31%, respectively). In addition, the percentages of polyspermic oocytes remained lower than control (38.5% vs. 68.7%, respectively; $p<0.05$). In conclusion, the results confirm that exposure of fresh or frozen-thawed boar

spermatozoa to a low dose of seminal PSP-I/PSP-II spermadhesin preserves sperm viability and motility in vitro. While there was no obvious influence of the heterodimer on the capability of freshly extended boar spermatozoa to penetrate homologous oocytes (either IM or IVM), PSP-I/PSP-II exerted a deleterious effect when frozen-thawed spermatozoa were used to penetrate IVM oocytes. Such an effect of cryopreservation seems to a certain extent reversible, since cleansing of the sperm surface decreased, at least partially, this blocking effect, increasing both, the penetration and the monospermic rates.

4.2. Introduction

Seminal plasma (SP), the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions that originate from the testes, epididymides, and male accessory sexual glands. The SP contains factors that influence both spermatozoa and the female genital tract during sperm transport (Shivaji et al, 1990; Yanagimachi, 1994; Waberski et al, 1995). In particular, SP proteins play a role in the modulation of sperm function before they reach the oocyte(s) at the tubal site of fertilization, during gamete recognition, and when spermatozoa and oocytes bind at fertilization (Calvete et al, 1995a). In boars, the major protein component of the SP is the spermadhesin family (Calvete et al, 1995a; Töpfer-Petersen et al, 1998), a group of (glyco)proteins built by a single CUB domain architecture (Romero et al, 1997), coating the sperm surface (Dostálová et al, 1994). According to their binding properties, spermadhesins can be divided into 2 groups, depending on their ability to either bind heparin (AQN-1, AQN-3, AWN) or not (PSP-I/PSP-II heterodimer). The PSP-I/PSP-II heterodimer appears to preserve in vitro membrane integrity, motility, and

mitochondrial activity of highly extended spermatozoa for as long as these are exposed to the heterodimer (Centurión et al, 2003). Spermadhesin PSP-I/PSP-II has a similar protective effect as that reported for boar spermatozoa on the addition of either bulk SP from selected males (Maxwell et al, 1997; Maxwell and Johnson, 1999) or SP components of low molecular weight (Ashworth et al, 1994). The use of an isolated heterodimer, the PSP-I/PSP-II, has the advantage of avoiding the inherent variability shown by bulk SP among males or ejaculates from one and the same male (Killian et al, 1993; Ashworth et al, 1994; Rozeboom et al, 2000).

Porcine in vitro fertilization (IVF) is still considered suboptimal compared with the outcome of this reproductive biotechnology on other species, with unacceptable levels of polyspermia associated with high penetration rates. Although ejaculated boar spermatozoa, surrounded by SP, are able to produce high penetration levels when coincubated with homologous oocytes (Martinez et al, 1996), the level of fertilization is lower with ejaculated than with epididymal spermatozoa (Rath and Niemman, 1997), indicating that use of bulk SP on IVF is not beneficial (Bonilla et al, 1996; Rath and Niemman, 1997; Maxwell et al, 1998). Coincubation of immature (IM) or in vitro matured (IVM) oocytes with 1.5 mg/mL of PSP-I/PSP-II in the IVF medium significantly decreased penetration rates by untreated boar spermatozoa (Caballero et al, unpublished data). These data, together with the fact that the PSP-II subunit of the PSP-I/PSP-II heterodimer exhibits binding affinity for zona pellucida (ZP) receptors (Calvete et al, 1995b), suggest that the heterodimer can block penetration, at least in vitro. However, the PSP-I/PSP-II spermadhesin binds loosely to the sperm surface (Calvete et al, 1995b), and, consequently, an easy removal should be expected in vitro and during sperm coincubation with the oocytes. Therefore, preincubation of spermatozoa with

PSP-I/PSP-II should not affect the interaction between spermatozoa and the oocyte. These properties, along with the sperm protective effect, point to PSP-I/PSP-II as a potential candidate for pretreatment of manipulated spermatozoa (extended, stored, or deep frozen) aimed at promoting sperm survival and performance in vitro. This is especially important when technologies such as sex selection of spermatozoa by flow cytometric sorting or sperm cryopreservation are used, procedures known to induce deleterious changes in boar spermatozoa.

Owing to such a potential beneficial effect on sperm viability and since the effect of sperm pretreatment with the PSP-I/PSP-II heterodimer on IVF has not been investigated, the present study examined the in vitro penetration ability (penetration rate and number of spermatozoa per oocyte) of fresh and frozen-thawed boar spermatozoa (10 x 106/mL) on IM and IVM homologous oocytes following preincubation with low doses of the heterodimer (1.5 mg/mL) for 30 or 120 minutes. In addition, the rate of polyspermia was determined when IVM oocytes were penetrated.

4.3. Methods

4.3.1. Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar SP

All experiments were performed with the SP from 4 sexually mature boars, which had previously sired offspring. Sperm-rich ejaculate fractions were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at 3800 x g for 15 minutes at 20°C. The supernatants were sequentially filtered through 10- and 1.2-μm filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non–heparin-binding fraction of SP by size exclusion chromatography on a 2000 x 5-cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, and 0.025% sodium azide (pH 7.4) (Calvete et al, 1995b). The identity and purity of the protein preparation were assessed by N-terminal sequence analysis (using a 472 automated protein sequencer, Applied Biosystems, Langen, Germany) and MALDI-TOF mass spectrometry using a Voyager DE-Pro mass spectrometer (Applied Biosystems). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a molar absorption coefficient (27 332 M-1 cm-1) determined by Menendez et al. (1995) or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 hours at 106°C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilized, and stored at -20°C until used.

4.3.2. Handling, incubation with the heterodimer PSP-I/PSP-II and evaluation of spermatozoa

Fresh semen was received in commercial doses for artificial insemination (collected from different boars of proven fertility and extended with Beltsville Thawing Solution; Pursel and Johnson, 1975) at a concentration of 30 x 10^6/mL of spermatozoa. Semen from 4 different boars and doses was pooled and centrifuged (1900 x g for 3 minutes), the supernatant discarded, and the pellet resuspended to adjust the sperm concentration with phosphate-buffered saline (PBS) to 10 x 106/mL of spermatozoa.

Spermatozoa from a fertile Pietrain boar were cryopreserved as described by Roca et al (2003) in a plastic medium straw (0.5 mL). Spermatozoa were thawed in circulating water at 37°C for 20 seconds and washed 3 times by centrifugation at 1900 x g for 3 minutes in Dulbecco PBS supplemented with 4 mg/mL of bovine serum albumin (BSA) (fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose, and 70 µg/mL of kanamycin. After washing, the sperm pellet was resuspended in PBS to a final concentration of 10 x 10^6/mL of spermatozoa.

Ten million spermatozoa (fresh or frozen-thawed) extended as described above were preincubated without (control) or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized proteins) at 38°C. After 30 and 120 minutes of preincubation, sperm viability, motility, and acrosome status were evaluated.

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes Europe, Molecular Probes Europe, Leiden, The Netherlands). Briefly, 0.5 mL of sperm suspension containing 5 x 10^6 of fresh or frozen-thawed spermatozoa was stained with 25 nM SYBR-14 solution and 12 µM propidium iodide (PI) solution. Samples were incubated at room temperature in the dark for 10 minutes before cytometric analysis. All analyses were performed by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc, Miami, Fla) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, Calif) with 15-mW laser power at 488 nm, and the software EXPO 2000 (Coulter Corporation). Forward and sideward light scatter were recorded for a total of 15 000 to 25 000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile,

were positively gated for the analysis. SYBR-14 was measured through a 525-nm band pass filter, whereas PI was collected through a 635-nm band pass filter. Viable spermatozoa were defined as those stained with SYBR-14 and not stained with PI.

The percentage of motile spermatozoa was estimated with a computer-assisted motility analysis system. Sperm samples (5 µL of 10 x 10^6/mL of spermatozoa) were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot (Kanagawa, Japan) equipped with a 10x contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyzer (Barcelona, Spain). The program settings were as follow: frames rate, 25 Hz; search radius, 11.5 µm; minimum track points, 7 frames; and threshold straightness, 75%. Two samples were examined with at least 100 spermatozoa being analyzed per sample.

Live spermatozoa showing acrosome exocytosis were evaluated using simultaneously PI to stain dead cells and the lectin FITC-PNA to evaluate disrupted acrosome. Briefly, 0.5 mL of sperm suspension containing 5 x 10^6 of fresh or frozen-thawed spermatozoa was stained with 12 µM of PI and 5 µL of FITC-PNA stock solution (1 mg/mL in bidistilled water). Spermatozoa were incubated for 5 minutes in the dark and analyzed immediately on the flow cytometer (see above). FITC-PNA was measured through a 530-nm band pass filter, whereas PI was measured through a 635-nm band pass filter. Spermatozoa were identified in 1 of the 3 following populations: PI positive, nonviable cells; PI negative and FITC-PNA negative, live spermatozoa with

intact acrosome; and PI negative and FITC-PNA positive, corresponding to live spermatozoa with exocytosed acrosome.

4.3.3. Recovery, IVM, sperm penetration and evaluation of oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martinez et al, 1993; Abeydeera and Day, 1997). Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 µg/mL of kanamycin maintained at 34°C to 37°C. Cumulus-oocyte complexes (COCs) were aspirated from medium-sized follicles (3 to 6 mm in diameter) with an 18-gauge needle fixed to a 10-mL disposable syringe. Only COCs with a compact cumulus mass (with at least 6 or 7 layers), an intact ZP, and an oocyte with an evenly granulated cytoplasm were selected for the different trials.

For preparation of IM oocytes, batches of 30 IM oocytes were placed directly in 2 mL of pre-equilibrated modified TCM-199 medium (Cheng, 1985) and kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed 3 times in BSA-free North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL of cysteine, and 10 ng/mL of epidermal growth factor (IVM medium). Thereafter, COCs were transferred to a Nunc 4-well multidish plate (50 to 100 COCs per well) submerged in 500 µL of pre-equilibrated maturation medium (previously covered with warm mineral

oil), supplemented with 10 IU/mL of eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV), and cultured at 39°C in 5% CO2 in air for 22 hours. The medium was then changed for maturation medium without hormone supplementation and incubated at 39°C in 5% CO2 in air for another 22 hours. After IVM, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed 3 times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera and Day, 1997). The latter consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2 · 2H2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.2% BSA (fraction V; A 7888, initial fractionation by precipitation with cold alcohol). Batches of 50 IVM oocytes were placed in 50-µL drops of IVF medium covered with warm mineral oil in a 35 x 10-mm Petri dish. The dishes were kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

Spermatozoa exposed or not exposed to the PSP-I/PSP-II heterodimer, as described above, were centrifuged at 1000 x g for 3 minutes, and the sperm pellet was resuspended in modified TCM-199 medium for coincubation with IM oocytes or resuspended in IVF medium for coincubation with IVM oocytes. Spermatozoa were coincubated with IM or IVM oocytes at a oocyte-spermatozoa ratio of 1:66 000 (Martinez et al, 1993) or 1:2000 (Gil et al, 2004), respectively. The oocytes were coincubated with the spermatozoa at 39°C in an atmosphere of 5% CO2 in air, the IM oocytes for 16 hours and the IVM oocytes for a primary period of 6 hours. The IVM oocytes were thereafter washed 3 times in pre-equilibrated embryo culture medium (NCSU-23 containing 0.4% BSA), transferred to a Nunc 4-well multidish containing

500 µL of the same medium per well (covered by mineral oil), and cultured for another 6 hours at 39°C and 5% CO2 in air.

Following the coincubation, the IM oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells, and those spermatozoa attached to the surface of the ZP. The IM (16 hours post insemination) and IVM oocytes (12 hours post insemination) were mounted on slides, fixed, and stored in 25% (vol/vol) acetic acid in ethanol at room temperature for 48 to 72 hours, stained with 1% lacmoid in 45% (vol/vol) acetic acid, and examined under a phase contrast microscope at 400x magnification. Oocytes were considered penetrated when spermatozoa with intact (IM oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM oocytes) were found in the ooplasm.

The trials attempted to disclose the effect of the sperm exposition to PSP-I/PSP-II heterodimer on the ability of freshly diluted or frozen-thawed spermatozoa to penetrate pig oocytes (IM or IVM), being distributed as follows: 1) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IM oocytes, a total of 175 IM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 minutes at 38°C, whereas 182 IM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); 2) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 400 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 415 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); 3) To study the penetrating

ability of frozen-thawed boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 792 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 773 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); and 4) Finally, an experiment was designed, based on the results of experiment 3 with frozen-thawed spermatozoa, to evaluate the effect of sperm washing (by centrifugation in saline BSA or through Percoll gradient separation) to cleanse the sperm surface of frozen-thawed spermatozoa and, consequently, on the penetration capability of these spermatozoa on IVM oocytes. For this purpose, aliquots of 3 mL of re-extended postthaw semen (10 x 106/mL of spermatozoa in PBS) were preincubated for 2 hours at 38°C with 1.5 mg/mL of PSP-I/PSP-II heterodimer in PBS. Thereafter, spermatozoa were either washed 3 times by centrifugation at 1200 x g for 3 minutes in 0.9% saline solution containing 1 mg/mL of BSA, with the final pellet being resuspended in fertilization medium at 2 x 106/mL of spermatoza, or centrifuged through a 35%/70% Percoll gradient (900 x g for 20 minutes), the lowest pellet being recovered and resuspended with fertilization medium at 2 x 106/mL of spermatozoa. Control groups were built by 1) spermatozoa preincubated in the presence of PSP-I/PSDP-II heterodimer as described above but pelleted without washing or 2) spermatozoa preincubated in the absence of PSP-I/PSP-II heterodimer. A total of 1592 IVM oocytes were cocultured with the spermatozoa as above described.

4.3.4. Statistical analysis

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) using

the MIXED procedure according to a statistical model that included the fixed effects of presence of PSP-I/PSP-II heterodimer and incubation time and the random effect of replicate. In the last experiment, a washing procedure was included as a fixed effect. To analyze data of sperm viability, motility, and acrosome status, percentages were subjected to arcsine transformation before analysis. Data on the percentage of penetration and polyspermia were modeled according to the binomial model of parameters as described by Fisz (1980) before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be statistically significant when $P < .05$. Experiments were replicated 4 times.

4.4. Results

4.4.1. PSP-I/PSP-II heterodimer preserves the viability and motility of freshly extended and frozen-thawed spermatozoa

Viability and motility percentages for freshly diluted spermatozoa before re-extension in PBS until 10 x 106/mL of spermatozoa were 95.2% ± 2.8% and 85.3% ± 2.8%, respectively. Likewise, viability and motility percentages for frozen-thawed spermatozoa before re-extension in PBS were 56.7% ± 3.2% and 45.7% ± 2.4%, respectively. The percentages of viable spermatozoa with an exocytosed acrosome were 2.1% ± 0.8% and 4.7% ± 1.4% for freshly extended and frozen-thawed spermatozoa, respectively. Exposition of spermatozoa to the PSP-I/PSP-II heterodimer had a significantly positive influence on sperm viability and motility for both freshly diluted and frozen-thawed spermatozoa (Figure 1A and B). Sperm viability and motility percentages were significantly higher for spermatozoa incubated with the PSP-I/PSP-II

heterodimer than for spermatozoa incubated in the absence of the heterodimer. In relation to the spermatozoa with acrosome exocytosis, we have not noticed differences between spermatozoa exposed or unexposed to the PSP-I/PSP-II heterodimer (Figure 1C).

4.4.2. Pre-incubation of freshly extended boar spermatozoa with PSP-I/PSP-II heterodimer does not affect their ability to penetrate IM pig oocytes

Results are presented in Table 1. Exposure of freshly diluted boar spermatozoa to PSP-I/PSP-II heterodimer did not influence either penetration or the number of spermatozoa per oocyte compared with controls.

4.4.3. Pre-incubation of freshly extended boar spermatozoa with PSP-I/PSP-II heterodimer does not affect their ability to penetrate IVM pig oocytes

Results are presented in Table 2. Preincubation of freshly extended boar spermatozoa with 1.5 mg/mL of PSP-I/PSP-II heterodimer for 30 or 120 minutes did not significantly affect either penetration and polyspermy rates or the number of spermatozoa present in the ooplasm compared either with controls or over time.

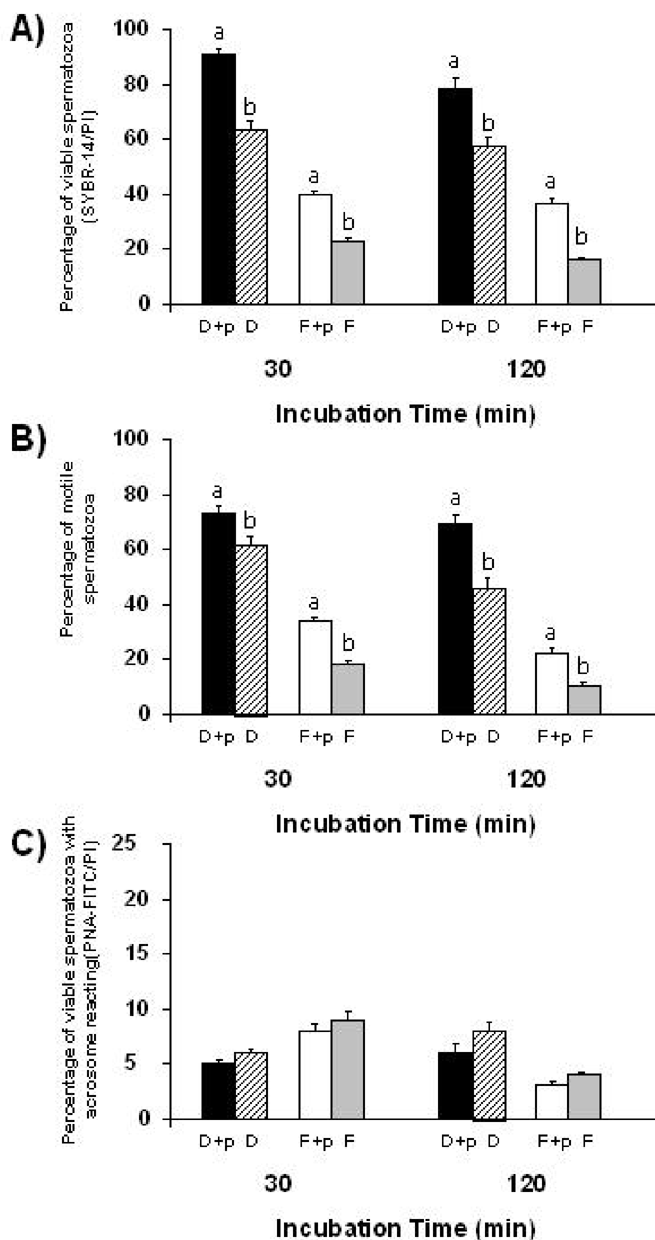

Figure 1. Effect of PSP-I/PSP-II heterodimer (1.5 mg/mL in phosphate-buffered saline) on the viability (A), motility (B), and acrosome exocytosis (C) of freshly extended (E+p) or frozen-thawed (F+p) boar spermatozoa extended until 10 x 106/mL of spermatozoa after 30 or 120 minutes of incubation at 38°C. Controls were treated with the same spermatozoa source but incubated in the absence of PSP-I/PSP-II heterodimer (E for extended and F for frozen-thawed spermatozoa). Columns represent the mean ± SEM (error bars) of 4 separate experiments. Different letters between columns per time and type of spermatozoa (freshly extended or frozen-thawed) indicate significantly different values ($P < .05$).

4.4.4. Pre-incubation of frozen-thawed spermatozoa with PSP-I/PSP-II heterodimer decreases their ability to penetrate IVM-pig oocytes

Results are presented in Table 3. Control spermatozoa (preincubated in the absence of the PSP-I/PSP-II heterodimer) penetrated approximately 90% and 85% of oocytes at 30 and 120 minutes, respectively. The addition of PSP-I/PSP-II heterodimer at a final concentration of 1.5 mg/mL had a detrimental effect on the penetration rate at both 30 ($P < .05$) and 120 minutes ($P < .05$) compared with controls, indicating an immediate inhibitory effect that was maintained over time. Moreover, PSP-I/PSP-II also had an effect on the number of spermatozoa per oocyte, decreasing significantly ($P < .05$) with respect to controls and, consequently, increasing the percentages of monospermic oocytes.

Table 1. Effect of pre-incubation of fresh boar spermatozoa with PSP-I/PSP-II heterodimer (1.5 mg/mL) on their ability to in vitro penetrate immature pig oocytes

Group	Oocytes Penetrated (%)	Number of spermatozoa per oocyte (nº)
Control (n=182)	74.5 ± 3.5	6.4 ± 0.4
PSP-I/II-exposed (n=175)	72.8 ± 3.3	6.2 ± 0.2

Data are expressed as mean ± SEM.

Table 2. Effect of preincubation of fresh boar spermatozoa with PSP-I/PSP-II heterodimer (1.5 mg/mL) for 30 or 120 minutes on their ability to *in vitro* penetrate IVM pig oocytes.

Group	Incubation time (minutes)					
	30			120		
	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean sperm number/oocyte (nº)	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean sperm number/oocyte (nº)
Control (n=202)	96.5 ± 1.2	95.3 ± 1.4	6.2 ± 0.4	98.4 ± 1.6	91.4± 1.1	7.4 ± 0.5
PSP-I/II exposed (n=210)	97.1 ± 1.3	89.4 ± 1.2	5.3 ± 0.2	98.0 ± 1.4	90.5 ± 1.4	6.1 ± 0.3

Data are expressed as mean ± SEM.

4.4.5. Sperm cleansing (by centrifugation in saline-BSA and through Percoll discontinuous gradient separation) of frozen-thawed spermatozoa previously incubated with PSP-I/PSP-II heterodimer modified their capability to penetrate IVM oocytes in vitro

Table 4 displays the results from this experiment, showing that exposure of frozen-thawed boar spermatozoa to PSP-I/PSP-II significantly decreased penetration rates, polyspermy rates, and the number of spermatozoa per penetrated oocyte ($P < .05$). Washing (by centrifugation and re-extension in saline BSA) of pre-exposed spermatozoa further decreased penetration rates ($P < .05$) but did not influence the number of spermatozoa penetrated per oocyte, although the percentage of monospermic oocytes was high. On the other hand, the rate of oocyte penetration by spermatozoa

cleansed by Percoll discontinuous gradient separation, albeit still lower than controls (P < .05), increased significantly (P < .05) compared with the internal control (exposed to PSP-I/PSP-II heterodimer but unwashed) or washing by centrifugation. No significant differences were seen for the number of spermatozoa per penetrated oocyte, which continued being fewer than in controls (P < .05), and the percentage of monospermic fertilization was, consequently, higher than in controls (P < .05).

Table 3. Effect of preincubation of frozen-thawed boar spermatozoa (single batch) with PSP-I/PSP-II heterodimer (1.5 mg/mL) for 30 or 120 minutes at 38ºC on their ability to *in vitro* penetrate IVM pig oocytes

Group	Incubation time (minutes)					
	30			120		
	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean sperm number/oocyte (nº)	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean sperm number/oocyte (nº)
Control (n=390)	90.5±1.5[a]	73.6±1.9[a]	2.9±0.2[a]	85.3±1.8[a]	64.2±1.9[a]	2.6±0.3[a]
PSP-I/II exposed (n=402)	32.3±2.3[b]	41.2±2.8[b]	1.6±0.1[b]	25.6±2.2[b]	35.7±2.3[b]	1.4±0.1[b]

Data are expressed as mean ± SEM.

Different superscripts in the same column indicate significantly different values (p<0.05).

5. Discussion

Biotechnological manipulation of semen (ie, flow cytometric sorting for chromosomal sex or cryopreservation) invariably induces changes in the viability, membrane integrity, acrosome status, and motility patterns of the spermatozoa (Maxwell et al, 1997; Maxwell and Johnson, 1997; Maxwell and Johnson, 1999). Attempts to minimize these effects include the addition of a certain proportion of whole (homologous or autologous) SP in the medium where spermatozoa are extended or simply bathe in. However, the high variability in the composition of the SP among males of the same species and among ejaculates of a single male prompted us to evaluate a specific protein, the spermadhesin PSP-I/PSP-II heterodimer, as an additive to protect the spermatozoa against the detrimental effect of the above mentioned biotechnological manipulations. To avoid variability among samples, the experiments were performed using either pools of ejaculates from several artificially inseminated boars or frozenthawed spermatozoa from a single ejaculate from a fertile artificially inseminated boar.

The experiments were performed at a concentration of 1.5 mg/mL of PSP-I/PSP-II, which represents the concentration of this particular spermadhesin in 10% of bulk SP (Dostálová et al, 1994; Calvete et al, 1995a). At this concentration, the PSP-I/PSP-II has the sperm protective effect of SP concentration (10%) (Maxwell et al, 1997) as observed previously (Centurión et al, 2003).

Table 4. Effect of sperm cleansing (by centrifugation in saline-BSA and through discontinous Percoll gradient separation) of frozen-thawed spermatozoa (single batch) previously incubated for 120 minutes at 38°C with PSP-I/PSP-II heterodimer (1.5 mg/mL) , on their capability to in vitro penetrate IVM oocytes

Group	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean sperm number/oocyte
Control (n=402)	91.1±2.2^a	68.7±1.9^a	2.6±0.2^a
Exposed to PSP-I/II (n=395)	30.8±3.6^b	36.4±2.7^b	1.4±0.1^b
Exposed to PSP-I/II and washed by centrifugation (n=390)	12.8±1.8^c	25.1±2.3^c	1.6±0.1^b
Exposed to PSP-I/II and cleansed by Percoll gradient centrifugation (n=405)	53.1±4.2^d	38.5±2.9^b	1.4±0.2^b

Data are expressed as mean ± SEM.

Different superscripts in the same column indicate significantly different values ($p<0.05$).

Under the present experimental conditions, exposure to the heterodimer dramatically improved sperm viability in vitro, thus confirming the above mentioned previous results. This pretreatment did not, however, affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes; that is, no particular beneficial effect was seen in fertilization rates. Rates of polyspermy were not affected when IVM oocytes were evaluated. Polyspermy rate was not evaluated in IM oocytes, because they lack the mechanism for sperm penetration block.

By contrast, when cryopreserved spermatozoa were tested for penetration of IVM oocytes, a 30-minute exposure to PSP-I/PSP-II during pre-IVF incubation showed a significant blocking effect on penetration rate and on mean sperm numbers per oocyte and decreasing polyspermy. This effect lasted for 120 minutes before oocyte exposure. This unexpected result motivated a cleansing of the spermatozoa by centrifugation in saline BSA or through Percoll density discontinuous gradient separation before repeating the IVF procedure. Penetration rate (but not the number of spermatozoa per oocyte) significantly increased when spermatozoa were filtered through Percoll compared with either washed spermatozoa or unwashed controls. The Percoll treatment yielded a significant decrease in polyspermy.

The blockade of sperm penetration by incubation with the heterodimer seemed to be a rather rapid phenomenon, since the number of oocytes penetrated with spermatozoa decreased from 90% to 33% of oocytes when the PSP-I/PSP-II heterodimer was added to the sperm suspension just before coincubation with the oocytes (30 minutes). Cleansing seemed to improve this situation, suggesting that the cryopreservation procedure might cause a coating effect by the extender components on the surface of the spermatozoa.

Such results are puzzling for several reasons. PSP-I/PSP-II heterodimer is the major protein in the sperm-rich fraction of the ejaculate (Centurión et al, 2003). Boar spermatozoa preincubated with SP from selected portions of the sperm-rich fraction, where low doses of the PSP-I/PSP-II heterodimer (in the range of the ones used hereby) are present, sustained in vitro manipulation (such as extension, storage, or cryopreservation) better (Peña et al, 2003). Also, boar spermatozoa preincubated with

SP from the sperm-rich fraction produced better IVF results than when spermatozoa were incubated with SP from other fractions of the ejaculate (Zhu et al, 2000).

The PSP-I/PSP-II heterodimer seems to influence the ZP and decrease sperm penetration in vitro. Exposure of pig oocytes to 1.5 mg/mL of PSP-I/PSP-II heterodimer in the IVF medium was able to significantly decrease penetration rates by untreated boar spermatozoa (Caballero et al, unpublished data) either by blocking sperm ZP receptors or acting on the surrounding spermatozoa during IVF. Such a response is unlikely to occur in vivo, since PSP-I/PSP-II does not seem to maintain attachment to the ZP following sperm transport in the female genital tract (as other SP proteins, such as the AWN, do; Rodriguez-Martinez et al, 1998). The PSP-I/PSP-II heterodimer seems to be easily removed from the sperm surface by in vitro capacitation treatments (Calvete et al, 1995a). Ejaculated spermatozoa (surrounded by SP) can undergo capacitation and acrosome reaction when coincubated with oocytes, even when not washed or preincubated in capacitating media (Martinez et al, 1996).

Pre-exposure of spermatozoa to the heterodimer should not affect the interaction between spermatozoa and oocytes, thus explaining why no positive effects were seen when freshly extended spermatozoa were tested with IM or IVM oocytes. On the other hand, there is no easy explanation as to why frozen-thawed spermatozoa could be influenced so negatively by the heterodimer when no detrimental effects on sperm viability, motility, and acrosome were recorded (experiment 1). To the best of our knowledge, no data concerning interactions between PSP-I/PSP-II and cryopreserved spermatozoa are available. This calls for further studies, particularly in light of a lack of

relationship among normal levels of sperm viability, their ability to acrosome react in vitro, and their penetrating capacity on in vivo matured oocytes (Vazquez et al, 1993).

Several experiments have shown that inseminating boar spermatozoa, either cryopreserved or sex selected (by flow cytometry sorting), results in lower farrowing rates and decreased litter size when compared with freshly ejaculated and liquid extended spermatozoa (Roca et al, 2003; Vazquez et al, 2003). This decrease in pregnancy rates is considered to be due to "capacitation-like" alterations in the plasma membrane and changes in motility patterns (similar to hyperactivated motility) caused by cooling or flow cytometry sorting. These alterations shorten the life span of those spermatozoa that survive these manipulations by provoking premature spontaneous acrosome exocytoses and cell death (Maxwell and Johnson, 1997; Green and Watson, 2001; Kaneto et al, 2002).

The status of the plasma membrane is altered in manipulated spermatozoa, and the exposure to PSP-I/PSP-II heterodimer increases the life span of highly extended boar spermatozoa (Centurión et al, 2003). We argue that the PSP-I/PSP-II protective effect on the spermatozoa may be linked to its interacting capability with the plasma membrane of "capacitated-like" spermatozoa, which in turn impairs the ability of the spermatozoa to penetrate the oocyte vestments.

As mentioned above, removal of SP spermadhesin occurs during in vivo sperm transport through the female genital tract (Calvete et al, 1997; Mortimer, 2000), and only very small concentrations of some spermadhesins (such as the AWN) are present on the sperm plasmalemma when reaching the ZP (Rodriguez-Martinez et al, 1998).

This removal of SP components could be the reason for the partial restoration of the ability to penetrate oocytes by Percoll cleansed frozen-thawed spermatozoa. This procedure is more effective for sperm cleansing that any other available procedure (Rodriguez-Martinez et al, 1997).

Our results are also in line with previous investigations that show that sperm washings by centrifugation in saline BSA can damage the spermatozoa (Harrison and White, 1972) and, consequently, lead to a decrease in penetration rates when used for IVF (Martinez et al, 1996). This effect may be amplified when "weaker" spermatozoa, such as those cryopreserved or flow cytometry–sorted spermatozoa, are washed by centrifugation in saline BSA. Consequently, washing by centrifugation in saline BSA may not be the most appropriate method to restore the fertilizing ability of spermatozoa preincubated with PSP-I/PSP-II. In contrast, discontinuous density gradient centrifugation with Percoll seemed effective to restore the fertilizing capability of some of the spermatozoa. Our results are in agreement with reports of the enhancement of penetration rates of Percoll-washed boar spermatozoa (Grant et al, 1994; Jeong and Yang, 2001). Moreover, using this cleansing method, at higher monospermic penetration was observed, although further studies are necessary, particularly in light of the correlation established between the absolute number of spermatozoa penetrated per oocyte and the degree of oocytes penetrated and polyspermy (Rath, 1992; Gil et al, 2004). Although washing by centrifugation in saline removes bulk SP from spermatozoa more rapidly than Percoll separation (Levay et al, 1995), the latter cleanses the surface without damaging the plasmalemma structure, thus maintaining sperm viability (Rodriguez-Martinez et al, 1997). Most spermatozoa were recovered in the soft pellet at the bottom, and only a very low percentage of the spermatozoa were recovered

at the interface of the 35% and 70% Percoll layers, which may indicate that the action of Percoll on the spermatozoa was more associated with a "cleansing effect" than with an effect related to selection and enrichment of the population recovered. However, whether the effect of Percoll procedure is only due to a cleansing effect remains to be clarified.

In conclusion, the results confirm that short-time exposure of fresh or frozen-thawed boar spermatozoa to low doses of the seminal heterodimer PSP-I/PSP-II preserves (or improves) sperm viability and motility in vitro without affecting the sperm acrosome. Although there is no obvious influence of the heterodimer on the capability of fresh extended boar spermatozoa to penetrate homologous oocytes (either IM or IVM), PSP-I/II exerts a deleterious effect when frozen-thawed spermatozoa are used to penetrate IVM oocytes. However, this inhibition did not seem permanent, since a subsequent washing through a Percoll gradient restored sperm function in some of the cells. We are currently investigating the mechanism by which purified PSP-I/PSP-II modulates sperm viability and oocyte penetration ability in vitro and the effect of this protein on in vivo fertilizing capacity in pigs.

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IV. – Discusión General

Durante los últimos años existe un creciente interés por el desarrollo y mejora de las nuevas biotecnologías asociadas a la reproducción con el fin de incrementar la eficiencia productiva de la industria porcina. Dentro de estos procedimientos, destacan la separación de espermatozoides X e Y por citometría de flujo, la criopreservación espermática, y la transferencia no quirúrgica de embriones porcinos (Martínez y cols., 2005).

A pesar del desarrollo de la nueva técnica de inseminación artificial, como es la inseminación intrauterina profunda que permite una deposición de los espermatozoides cercana al lugar de la fecundación, con una considerable reducción del número de espermatozoides necesarios en cada inseminación, y la posibilidad de inseminar cerca del momento de la ovulación mediante control ecográfico, el uso de de espermatozoides tanto criopreservados como sexados mediante citometría de flujo se encuentra muy limitado en la industria porcina ya que, mientras el semen criopreservado no alcanza todavía resultados similares al semen fresco, la separación de espermatozoides X e Y mediante citometría de flujo continua presentando una baja eficiencia de separación además de producir una disminución de la viabilidad, capacidad de conservación y capacidad fecundante, siendo económicamente inviable (Johnson y cols., 2000; Martínez y cols., 2005).

Esta disminución es debida en parte al lavado que se produce de las sustancias estabilizadoras del plasma seminal adsorbidas en el espermatozoide por la alta dilución requerida para la separación de espermatozoides X e Y mediante citometría de flujo (Maxwell y Johnson, 1997), habiéndose observado como con la adición a los medios de recogida de una cierta proporción de plasma seminal o proteínas específicas del plasma seminal, los espermatozoides son capaces de sufrir una reestabilización de su membrana plasmática que lleva a una mejora de su viabilidad (Maxwell y Johnson, 1999; Centurión y cols., 2003).

Sin embargo, existe una gran variabilidad en la literatura sobre los efectos de la adición del plasma seminal a una muestra espermática, por lo cual en nuestro primer experimento evaluamos el efecto sobre la viabilidad, función mitocondrial y motilidad de espermatozoides sometidos a diluciones extremas (de forma similar a las condiciones encontrados para la separación de espermatozoides X e Y mediante citometría de flujo) que produciría la adición de una cierta proporción de plasma seminal (10%) tanto autólogo como homólogo.

Los resultados obtenidos en el estudio mostraron claramente como la substracción del plasma seminal y la dilución en un medio salino simple disminuyó la viabilidad espermática (evaluada como integridad de membrana, función mitocondrial y motilidad espermática). Estos resultados confirmaron estudios anteriores (revisado por Maxwell y Johnson, 1999). Además, los presentes resultados muestran como la restitución de una cierta cantidad de plasma seminal (10% v/v) podría, en la mayoría de los casos, restaurar e incluso aumentar la viabilidad de los espermatozoides altamente diluidos en

PBS. Este efecto beneficioso del plasma seminal ha sido previamente descrito en toros, carneros y verracos (Asworth y cols., 1994; Maxwell y cols., 1997, 1998; Maxwell y Johnson, 1999).

Habitualmente, ha existido la tendencia de usar mezclas de plasma seminal de distintos machos a la hora de buscar soluciones prácticas para la adición del plasma seminal. Sin embargo, los resultados mostrados en este estudio indican que la viabilidad y funcionalidad espermática pueden ser influenciados por la exposición al plasma seminal tanto negativa (verraco C) como positivamente (por ejemplo, verraco A), según la fuente de plasma seminal provenga de uno u otro macho.

Es digno de mención que los espermatozoides procedentes de diferentes verracos respondieron de manera distinta al plasma seminal de un mismo macho. Obviamente, estos resultados indican que sería aconsejable un examen *in vitro* de los efectos de un determinado plasma seminal sobre la viabilidad espermática previo a su uso en el manejo espermático. Del mismo modo, Killian y cols., (1993) en toros y Van der Ven y cols., (1983) en humanos, encontraron que el efecto beneficioso del plasma seminal sobre los espermatozoides podría ser mayor o menor evidente dependiendo de la fuente de plasma seminal. Más aún, la exposición de espermatozoides provenientes de toros de alta fertilidad con plasma seminal procedente de toros de baja fertilidad afectó negativamente su capacidad de penetración *in vitro* (Henault y Killian, 1996).

Estos hallazgos y estudios previos realizados por el mismo grupo de investigación han sugerido la presencia de factores beneficiosos para la fertilidad en el fluido de las glándulas accesorias de machos de alta fertilidad, o de factores inhibidores de la

fertilidad en aquellos machos con baja fertilidad individual (Henault y Killian, 1995).En concordancia con lo anterior, los resultados presentados por nuestro laboratorio y otros grupos de investigación han mostrado como la adición de plasma seminal proveniente de machos "buenos congeladores" a los medios de congelación mejoraba la supervivencia espermática post-descongelación en comparación con la adición de plasma seminal procedente de machos "malos congeladores", tanto en caballos (Aurich y cols., 1996) como en verracos (Cremades y cols., 2004).

Actualmente, se están desarrollando en nuestro laboratorio diversas experiencias para la evaluación de los componentes del plasma seminal que ejercen un efecto beneficioso sobre los espermatozoides. Las evidencias apuntan a que algunas proteínas del plasma seminal, como el heterodímero PSP-I/PSP-II, podrían ejercer un efecto protector sobre los espermatozoides de verraco, al menos *in vitro* (Centurión y cols., 2003). Además, la exposición de los espermatozoides a la fracción proteica del plasma seminal con afinidad por la heparina se mostró perjudicial para la viabilidad espermática (Centurión y cols., 2003).

Se ha descrito una relación entre las proteínas del plasma seminal con la fertilidad. De este modo, mientras que diferencias en las proteínas con afinidad por la heparina se han correlacionado con una baja fertilidad en toros (Kandel y cols., 1992), Killian y cols. (1993) describieron como el perfil proteico del plasma seminal se relacionaba con la fertilidad presentada por los toros estudiados. Posteriormente, se han identificado diversas proteínas como son, la proteína de 26 kDa prostaglandina D sintasa tipo lipocalina (Gerena y cols., 1998), espermadhesina Z13, osteopontina, fosfolipasa A_2 y la

BSP 30 kDa (Moura y cols., 2006), las cuales se asocian con la fertilidad de los distintos machos.

Aunque lo descrito anteriormente resulta interesante, debe ser tenido en cuenta que el presente estudio fue llevado a cabo con plasmas seminales provenientes de la fracción rica del eyaculado, donde la presencia de proteínas con afinidad por la heparina es mucho menor que la hallada en la fracción post-espermática (Centurión, 2002). Este comentario es pertinente a la luz de los resultados de Zhu y cols. (2000), los cuales observaron como los espermatozoides de verraco incubados con plasma seminal procedentes de la fracción rica del eyaculado tuvieron una mayor tasa de penetración de ovocitos *in vitro* que esos mismos espermatozoides incubados con plasma seminal de la fracción post-espermática.

Hay que tener en cuenta que el plasma seminal es un fluido biológico de composición heterogénea, la cual incluye no solo las proteínas mencionadas anteriormente, sino también aminoácidos, péptidos, y enzimas que, aparentemente, tienen un efecto sobre el espermatozoide de diverso tipo. Por lo tanto, la presencia, ausencia o concentración de alguno de esos componentes, pero más probablemente la acción concertada de varios de ellos, podría ser responsable de la variabilidad de los efectos que el plasma seminal ejerce sobre los espermatozoides. Tales efectos biológicos del plasma seminal son muy complejos y están lejos de ser totalmente entendidos en la actualidad y por lo tanto, es necesario seguir investigando para conocer si un único componente del plasma seminal pudiera estar fuertemente relacionado con estos efectos, lo cual a efectos prácticos sería de gran importancia para el manejo espermático en las diferentes biotecnologías reproductivas.

En conclusión, los resultados de nuestra primera experiencia indican que la adición de un 10% (v/v) de plasma seminal (de la fracción rica del eyaculado) de ciertos verracos selectos mantienen o incrementan la viabilidad de los espermatozoides altamente diluidos *in vitro*.

Debido a la variabilidad encontrada en el efecto del plasma seminal sobre los espermatozoides, nuestra investigación se encaminó hacia la identificación de sustancias del plasma seminal que pudieran estar relacionadas con su efecto beneficioso. La identificación de la espermadhesina PSP-I/PSP-II como uno de los componentes del plasma seminal responsable de este efecto beneficioso del plasma seminal sobre los espermatozoides de verraco (Centurión y cols., 2003) apuntan al heterodímero PSP-I/PSP-II como un perfecto aditivo para los espermatozoides tratados, evitando así la variabilidad del plasma seminal. Sin embargo, si este efecto protector esta relacionado de alguna manera con la asociación del heterodímero con la membrana plasmática del espermatozoide no ha sido estudiado.

Para evaluar el efecto protector del heterodímero PSP-I/PSP-II y su posible relación con el patrón de unión de este sobre la superficie del espermatozoide de verraco sometido a altas diluciones, se incubaron durante 10 horas los espermatozoides altamente diluidos en un medio salino simple (PBS) en presencia o ausencia del heterodímero PSP-I/PSP-II, a los cuales se les fue monitorizando su viabilidad y funcionalidad espermática (como integridad de membrana, actividad mitocondrial y motilidad), además de estudiar su patrón de unión a la cabeza del espermatozoide mediante técnicas inmunocitoquímicas.

Para esta experiencia se produjo un anticuerpo policlonal monoespecífico de conejo contra PSP-II. La razón por la cual se produjo un anticuerpo anti-PSP-II fue debido a que en el plasma seminal del verraco, la espermadhesina PSP-II ha sido encontrada exclusivamente asociada a la espermadhesina PSP-I formando el heterodímero PSP-I/PSP-II, mientras que en el plasma seminal existe un exceso de espermadhesina PSP-I pudiendo ser encontrada libre en el plasma seminal (Calvete y cols., 1995a). Por lo tanto, el antisuero anti-PSP-II se mostrará específico para detectar el complejo PSP-I/PSP-II.

La inmunocitoquímica reveló claramente como el heterodímero PSP-I/PSP-II se localiza principalmente en la región acrosomal de la cabeza del espermatozoide, y como esta unión se va perdiendo a lo largo del tiempo de incubación. La adición de PSP-I/PSP-II exógena provocó que casi el 100% de los espermatozoides fueran inmunopositivos. A diferencia de trabajos previos en los cuales se sugería una falta de unión al espermatozoide del complejo PSP-I/PSP-II (Calvete y cols., 1995a), los resultados obtenidos en nuestro estudio mostraron como una gran población de espermatozoides altamente diluidos (71%) no perdían enteramente la cubierta proteica adsorbida a la superficie de la membrana incluso cuando no se añadió PSP-I/PSP-II extra. Estos resultados son similares a los hallados para las espermadhesinas AQN y AWN, las cuales cubren el espermatozoide tras la eyaculación pero se pierden progresivamente durante la incubación bajo condiciones capacitantes *in vitro* (Dostálová y cols., 1994). En nuestro experimento, observamos que la proporción de espermatozoides marcados por el anticuerpo disminuía hasta el 50% tras 10 horas de incubación, mientras que a aquellas muestras a las cuales se les suplementó PSP-I/PSP-

II exógena, aproximadamente el 80% de los espermatozoides permanecieron positivos ($p<0'05$). Esto nos indica que, el heterodímero se une a la membrana del espermatozoide antes o durante la eyaculación y que la unión es suficientemente intensa para que la proteína permanezca unida varias horas en un gran porcentaje de espermatozoides, incluso cuando estos son sometidos a diluciones extremas. Sin embargo, para que esta unión permanezca parece ser necesaria la presencia de una fuente adicional de heterodímero. Por último, los espermatozoides altamente diluidos son capaces de unir ese heterodímero adicional y mantener su funcionalidad.

Con respecto a la funcionalidad espermática, mientras que el porcentaje de espermatozoides que presentaban la membrana intacta, así como el porcentaje de espermatozoides con una actividad mitocondrial apropiada se mantuvo durante todo el proceso de incubación, el porcentaje de espermatozoides mótiles fue siempre menor que el de aquellos espermatozoides con membrana intacta (y también menor que la proporción de inmunopositivos). Las diferencias encontradas en nuestro estudio entre la motilidad y la actividad mitocondrial han sido descritas con anterioridad y probablemente reflejen diferencias en la actividad espermática o sean debidas a la metodología empleada (Windsor 1997; Centurión y cols., 2003). Una nueva hipótesis sugiere que la función de las mitocondrias sería proveer de ATP a la cabeza espermática y la pieza intermedia (por fosforilación oxidativa) para mantener activos los diferentes gradientes de difusión de la membrana plasmática que permiten la supervivencia del espermatozoide. Además, el ATP necesario para la motilidad espermática parece ser producido mediante glicólisis (producción anaeróbica de ATP) por enzimas localizados en la vaina fibrosa de la cola del espermatozoide (Silva y Gazella, 2006). Aunque esto

resulta muy interesante como hipótesis, son necesarias mas pruebas experimentales para corroborar estos supuestos.

Otro hecho que resulto llamativo en el estudio por microscopía óptica fue que los porcentajes de espermatozoides inmunonegativos y con el acrosoma reaccionado fueron similares en las primeras etapas de la incubación. Esto podría indicar que el heterodímero PSP-I/PSP-II esta ligado al acrosoma mientras este permanece intacto. Sin embargo, hay que hacer notar que cuando los espermatozoides fueron incubados durante 10 horas, se encontró un mayor porcentaje de espermatozoides inmunonegativos que de espermatozoides con el acrosoma reaccionado. Más aún, en algunos de los casos, el heterodímero PSP-I/PSP-II fue parcialmente (Patrón b) o totalmente (Patrones c y d) eliminado de la cabeza de espermatozoides con acrosoma intacto. Estos hallazgos sugieren que la eliminación del heterodímero del dominio acrosomal ocurre previa a la rotura del acrosoma, y podría estar relacionado con una reestructuración de la superficie espermática ocurrida durante procesos que conllevan una desestabilización de la membrana plasmática, como podría ser la capacitación espermática (Tulsiani y cols., 1997). Esta hipótesis está en concordancia con el hecho de que el heterodímero PSP-I/PSP-II parece ser eliminado de la superficie espermática tras tratamientos de capacitación *in vitro*, como se observa en una experiencia posterior de nuestro trabajo. Todos estos datos sugerirían que el heterodímero PSP-I/PSP-II es liberado de la superficie del espermatozoide previamente a alcanzar los ovocitos ovulados, no pareciendo que ejerza ningún papel en el reconocimiento y unión entre gametos, mientras otras espermadhesinas como la AWN si llegan a alcanzar el ovocito (Rodríguez-Martínez y cols., 1998).

El estudio mediante microscopía electrónica de transmisión mostró claramente que el heterodímero PSP-I/PSP-II es una proteína de superficie adsorbida principalmente al dominio acrosomal de los espermatozoides eyaculados (Patrón a). Posteriormente, el heterodímero parece migrar durante el proceso de incubación a la región post-acrosomal del espermatozoide (Patrones b y c), siendo finalmente liberado de la superficie espermática (Patrón d). Esta redistribución es consistente con los resultados obtenidos previamente en distintas especies, donde se ha relacionado con procesos de capacitación y reacción acrosómica (Kamaruddin y cols., 2004; Barrios y cols., 2005). Sin embargo, para poder establecer una relación casual entre la localización del heterodímero y su distribución con la capacitación espermática se requerirían más estudios. En cualquier caso, el microscopio electrónico de transmisión mostró una abrumadora población de espermatozoides (97%) presentando el Patrón a tras 0’5 horas de incubación, lo cual corresponde con aquellas muestras que presentaban una mejor viabilidad, motilidad y actividad mitocondrial. Igualmente, hubo una clara tendencia hacia la redistribución hacia el Patrón d tras 10 horas de incubación en una subpoblación espermática (30%), en el cual la viabilidad, motilidad y actividad mitocondrial fue la menor de nuestro estudio.

En relación al efecto protector del heterodímero sobre los espermatozoides de verraco, se podría argumentar que el heterodímero PSP-I/PSP-II sería en parte responsable de la estabilización de la membrana espermática, contrarrestando aquellos fenómenos que terminasen en la desestabilización de la membrana plasmática, ruptura del acrosoma y muerte celular. Por lo tanto, se podría pensar en la utilización de este aditivo para suspensiones espermáticas resultantes de la citometría de flujo como una manera de mejorar la eficiencia de estas nuevas biotecnologías.

En conclusión, el efecto protector del heterodímero PSP-I/PSP-II sobre los espermatozoides altamente diluidos parece prolongarse hasta 10 horas, y podría estar relacionado con la adhesión del heterodímero al dominio acrosomal de la cabeza del espermatozoide. Aunque estos resultados sugieren un posible papel estabilizador del heterodímero sobre la fluidez de la membrana plasmática y un posible efecto decapacitante, serían necesarias más investigaciones para aclarar la posible conexión entre los patrones de unión de esta proteína con el estado de capacitación del espermatozoide.

Es conocido que las espermadhesinas de verraco son glicoproteínas multifuncionales con gran afinidad por diversidad de ligandos. Algunos de los miembros de esta familia de proteínas (AQN-1, AQN-3 y AWN) presentan la capacidad de reconocer β-galactósidos de la ZP, lo cual hace imaginar que puedan presentar un papel en el reconocimiento y unión entre gametos, un paso crítico en la fertilización que parece ser mediado por interacciones entre proteínas y carbohidratos (Sinowatz y cols., 2001). Sin embargo, si el heterodímero presenta alguna función en la interacción entre gametos continúa sin estar claro. Los estudios realizados hasta el momento han presentado contradicciones. Mientras que algunos autores han descrito la existencia de afinidad de del heterodímero PSP-I/PSP-II por las glicoproteínas de la ZP, siendo localizada esta actividad en la subunidad PSP-II (Calvete y cols., 1995a). Jonáková y cols. (2000) encontraron que la afinidad de la subunidad PSP-II por la ZP se encontraba inhibida cuando se formaba el heterodímero. Por lo tanto, considerando el efecto beneficioso del heterodímero sobre los espermatozoides de verraco *in vitro*, y los diferentes resultados hallados en lo que respecta a su capacidad de unión a ZP,

diseñamos esta tercera experiencia para discernir el posible efecto que pudiera tener el heterodímero PSP-I/PSP-II durante el cocultivo de los espermatozoides de verraco con ovocitos de cerda tanto inmaduros (IM) como madurados *in vitro* (MIV).

Nuestros resultados mostraron como la adición de pequeña cantidades del heterodímero PSP-I/PSP-II (1’5 mg/ml), cantidad que ha sido probada beneficiosa para los espermatozoides de verraco (Centurión y cols., 2003), a ovocitos IM a los cuales se les había eliminado las células del cúmulus (ovocitos denudados) o a ovocitos MIV disminuyó tanto las tasas de penetración *in vitro* como el número de espermatozoides por ovocito.

Anteriormente, se había observado como el plasma seminal de verraco contiene una variedad de proteínas capaces de inhibir la unión del espermatozoide debido a que presentan afinidad por la ZP (Peterson y cols., 1989; Jonáková y cols., 2000). Sin embargo, el grado de interacción entre el ovocito y el heterodímero PSP-I/PSP-II esta lejos de ser clara, debido a las discrepancias mencionadas anteriormente sobre la capacidad del heterodímero para unir ZP (Calvete y cols., 1995a; Jonáková y cols., 2000).

La ZP es una cubierta glicoproteica que sufre variaciones en la cantidad y distribución de sus componentes durante la fase de maduración y crecimiento del ovocito (Parillo y Verini Supplizi, 1999; Parillo y cols., 2001). En un experimento previo, se observó como la exposición de ovocitos inmaduros al heterodímero PSP-I/PSP-II inhibió la maduración nuclear de los ovocitos, alcanzando la metafase II solamente el 32% de los ovocitos expuestos al heterodímero, mientras que el 84% de los

no expuestos maduró con normalidad. Más aún, la expansión de las células del cúmulus fue virtualmente inhibida en aquellos ovocitos expuestos al heterodímero. La razón por la cual el heterodímero PSP-I/PSP-II no afectó al total de la población pudo ser debido a la heterogeneidad en calidad y tamaño de la población de ovocitos recogidos de ovarios procedentes de matadero (Roca y cols., 1998; Lucas y cols., 2002), pudiendo encontrarse los ovocitos en diferentes estadios de crecimiento. Los ovocitos en desarrollo podrían estar rodeados por diferentes dominios glicoproteicos, ya que estos son sintetizados secuencialmente y añadidos por las células foliculares y el ovocito o son modificados en la ZP junto con el crecimiento folicular (Lucas y cols., 2003). Cualquiera de estos factores podría resultar en una modificación de la afinidad del heterodímero PSP-I/PSP-II por la ZP.

El mecanismo por el cual el heterodímero PSP-I/PSP-II interviene en la maduración *in vitro* podría ser diferente del efecto que ejerce durante la penetración *in vitro* de los ovocitos porcinos. Además, el efecto que ejerce el heterodímero sobre la penetración *in vitro* de los ovocitos porcinos podría no presentar relación con el bloqueo de los receptores de la ZP por parte del heterodímero, ya que se ha comprobado como el plasma seminal completo y algunos de sus componentes son capaces de ejercer un efecto inhibitorio sobre la unión espermatozoide-ovocito debido a un deterioro de la funcionalidad del espermatozoide previo a la unión a la ZP (Peterson y cols., 1984; Capková y Peknicová, 1997).

Mientras que la presencia de las células del cúmulus ejerció un efecto deletéreo sobre la penetración cuando se cocultivaron los espermatozoides con ovocitos IM en ausencia de PSP-I/PSP-II, la cubierta de células del cúmulus produjo un efecto

beneficioso en la capacidad de penetración *in vitro* de los espermatozoides en presencia del heterodímero PSP-I/PSP-II. Aunque podría esperarse una fácil eliminación del heterodímero tras los procesos de capacitación *in vitro* (Calvete y cols., 1995c), solamente se vieron porcentajes similares de penetración entre los grupos control y expuestos al heterodímero cuando los espermatozoides tratados con el heterodímero fueron coincubados con ovocitos IM rodeados por células del cúmulus. El paso de los espermatozoides a través del cúmulus oophorus, una estructura compuesta no solo de las células del cúmulus sino que contiene una densa matriz de glicosaminoglicanos compuesta principalmente por ácido hialurónico y proteoglicanos, se considera que es capaz de facilitar la capacitación espermática y penetración del ovocito por parte del espermatozoide (Flechón y cols., 2003). Si el heterodímero PSP-I/PSP-II es atrapado por los carbohidratos de la matriz de proteoglicanos del cúmulus oophorus cuando es atravesada por el espermatozoide, limpiando de esta forma la superficie espermática, o si el heterodímero es liberado durante la redistribución de los dominios de la membrana plasmática sucedidos durante la capacitación espermática nos es desconocido. Sin embargo, estas dos hipótesis podrían explicar el porque nos encontramos un mayor porcentaje de penetración de los espermatozoides expuestos al heterodímero PSP-I/PSP-II cuando estos fueron coincubados con ovocitos IM cubiertos por células del cúmulus.

En conclusión, mientras que no parece haber una influencia obvia del heterodímero PSP-I/PSP-II en el cocultivo de los ovocitos rodeados por células del cúmulus y los espermatozoides, se observa un efecto negativo en ausencia de esta cubierta de células del cúmulus tanto en ovocitos IM como ovocitos MIV.

Por último, debido al potencial efecto beneficioso que tendría el pretratamiento de los espermatozoides de verraco con la espermadhesina PSP-I/PSP-II, y a la posibilidad de que este pretratamiento pudiera disminuir la capacidad fecundante del espermatozoide (al menos *in vitro*), se realizó una experiencia en la cual se trató de estudiar la capacidad de penetración *in vitro* de espermatozoides de verraco tanto frescos como congelados preincubados con el heterodímero PSP-I/PSP-II sobre ovocitos homólogos IM y MIV.

Los experimentos se realizaron usando una concentración de 1’5 mg/ml de PSP-I/PSP-II, la cual representa la concentración de esta espermadhesina en el 10% del plasma seminal (Dóstalová y cols., 1994; Calvete y cols., 1995c). A esta concentración, el efecto protector de la PSP-I/PSP-II es similar al descrito con la adición de un 10% de plasma seminal (Maxwell y cols., 1998) como ha sido observado previamente (Centurión y cols., 2003).

Bajo las presentes condiciones experimentales, la exposición al heterodímero mejoró la viabilidad in vitro de los espermatozoides, confirmando los resultados previos mencionados anteriormente. Este pretratamiento no afectó sin embargo los porcentajes de penetración o el número de espermatozoides por ovocito cuando se coincubaron los espermatozoides frescos con ovocitos IM o MIV. Los porcentajes de polispermia no fueron evaluados con los ovocitos IM debido a que en ellos falta el mecanismo para bloquear la penetración espermática.

Por el contrario, cuando se probaron espermatozoides criopreservados con ovocitos MIV, ya a los 30 minutos se observó un efecto perjudicial significativo en el

porcentaje de penetración y el número de espermatozoides por ovocito cuando los espermatozoides fueron preincubados con el heterodímero PSP-I/PSP-II. Este efecto se prolongo durante 120 minutos antes de la exposición a los ovocitos. Lo inesperado de este resultado motivó una limpieza del espermatozoide por centrifugación en solución salina con BSA o a través de un gradiente de Percoll antes de la fecundación in vitro. Los porcentajes de penetración (pero no el número de espermatozoides por ovocito) incrementó significativamente cuando los espermatozoides se filtraron a través del Percoll comparado con aquellos lavados en solución salina con BSA o aquellos preexpuestos al heterodímero y no lavados.

El bloqueo de la penetración espermática por la incubación con el heterodímero parece ser un fenómeno bastante rápido, ya que el número de ovocitos penetrados disminuyó desde un 90% hasta un 33% cuando la PSP-I/PSP-II fue añadida justo antes de la coincubación con los ovocitos (30 minutos). La limpieza parece mejorar esta situación, sugiriendo que el proceso de criopreservación podría originar un recubrimiento de la superficie del espermatozoide por los componentes del diluyente.

Estos resultados son desconcertantes por varias razones. PSP-I/PSP-II es el componente proteico mayoritario en la fracción rica del eyaculado (Centurión y cols., 2003); los espermatozoides de verraco preincubados con porciones seleccionadas de plasma seminal de la fracción rica, en donde hay presente un bajo nivel del heterodímero PSP-I/PSP-II (similar al usado en nuestros experimentos), soportan mejor las manipulaciones in vitro (como son, dilución, almacenamiento y criopreservación) (Peña y cols., 2003b); además, los espermatozoides de verraco preincubados con plasma seminal de la fracción rica produce mejores resultados de fecundación in vitro que

cuando son incubados con plasma seminal procedente de otras fracciones del eyaculado (Zhu y cols., 2000).

El heterodímero PSP-I/PSP-II parece influenciar la ZP y disminuir la penetración in vitro. Como se observó en la experiencia anterior, la exposición de ovocitos de cerda a 1’5 mg/ml de PSP-I/PSP-II en el medio de fecundación disminuye significativamente los porcentajes de penetración in vitro de espermatozoides sin tratar, mediante el bloqueo de receptores de ZP o actuando sobre los espermatozoides circundantes durante la fecundación in vitro. Tal respuesta no parece ocurrir in vivo, ya que la PSP-I/PSP-II no parece mantenerse unida a la ZP después del transporte espermática a través del tracto genital femenino (mientras que otras espermadhesinas como la AWN si; Rodríguez-Martínez y cols, 1998). El heterodímero PSP-I/PSP-II parece ser eliminado fácilmente de la superficie espermática mediante tratamientos de capacitación in vitro (Calvete y cols, 1995c). Además, es sabido que los espermatozoides eyaculados (y por tanto rodeados por plasma seminal) pueden sufrir la capacitación y la reacción acrosómica cuando se coincuban con los ovocitos, incluso cuando no han sido lavados o preincubados en medio capacitante (Martínez y cols., 1996).

La preexposición de los espermatozoides al heterodímero no debería afectar la interacción entre el espermatozoide y el ovocito, explicando el por qué no se vio ningún efecto cuando los espermatozoides frescos fueron testados con ovocitos IM y MIV. Por otra parte, no hay una fácil explicación a por qué los espermatozoides congelados son influenciados tan negativamente por el heterodímero cuando no se observan efectos negativos sobre la viabilidad, motilidad o acrosomas reaccionados. Hasta el momento,

no hay datos disponibles en lo que se refiere a las interacciones entre la PSP-I/PSP-II y los espermatozoides criopreservados.

Varios experimentos han mostrado que la inseminación con semen de verraco, criopreservado o con el sexo seleccionado (mediante citometría de flujo), resulta en una menor tasa de partos y una camada mas pequeña comparado con la inseminación con semen fresco o diluido (Roca y cols., 2003; Vázquez y cols., 2003). Este descenso se considera que es debido a alteraciones similares a la capacitación que ocurren en la membrana plasmática y en los patrones de motilidad causados por la congelación o la separación por citometría de flujo. Estas alteraciones disminuyen la vida media de los espermatozoides que sobreviven a estas manipulaciones debido a que provocan reacciones acrosómicas espontaneas prematuras y muerte celular (Maxwell y Johnson 1997; Green y Watson 2001; Kaneto y cols., 2002).

Se ha visto que el heterodímero PSP-I/PSP-II incrementa la vida media de los espermatozoides altamente diluidos (Centurión y cols., 2003). Nosotros sugerimos que el efecto protector de la PSP-I/PSP-II podría estar unido a una capacidad para interaccionar con la membrana plasmática de aquellos espermatozoides que han sufrido cambios similares a la capacitación, lo que de hecho disminuye la habilidad del espermatozoide para atravesar las envolturas del ovocito.

Como mencionamos anteriormente, durante el transporte de los espermatozoides por el tracto genital femenino se produce una eliminación de la mayoría de las espermadhesinas del plasma seminal (Calvete y cols., 1997b; Mortimer 2000), y solo una pequeña proporción de estas (como las AWN) están presentes sobre la membrana

plasmática del espermatozoide cuando este alcanza la ZP (Rodríguez-Martínez y cols., 1998). Esta eliminación de los componentes del plasma seminal podría ser la razón para ese restablecimiento parcial de la capacidad de penetración de los ovocitos cuando se realiza el lavado a través del gradiente de Percoll de los espermatozoides congelados. Este procedimiento ha sido visto como uno de los más efectivos para la limpieza de los espermatozoides (Rodríguez-Martínez y cols., 1997).

Nuestros resultados coinciden con investigaciones previas que muestran como el lavado de los espermatozoides mediante centrifugación en solución salina con BSA puede dañar el espermatozoide (Harrison y White, 1972) y, consecuentemente, conducir a un descenso en los porcentajes de penetración cuando es usada para fecundación in vitro (Martínez y cols., 1996). Este efecto puede ser amplificado cuando espermatozoides "debiles", como los criopreservados o separados mediante citometría de flujo, son lavados mediante centrifugación en solución salina con BSA. Por tanto, este lavado puede no ser el método mas apropiado para restaurar la capacidad fecundante de los espermatozoides preincubados con PSP-I/PSP-II. Por el contrario, una centrifugación en un gradiente discontinuo de Percoll parece ser efectivo para recuperar la capacidad fecundante de algunos de los espermatozoides. Nuestros resultados coinciden con otras publicaciones que mencionan el incremento de los porcentajes de penetración tras un lavado con Percoll de los espermatozoides de verraco (Grant y cols., 1994; Jeong y Yang, 2001). Más aún, cuando usamos este método de limpieza, se observó un aumento de las penetraciones monospérmicas. No obstante, se necesitan estudios más en detalle, particularmente para establecer una correlación entre el número absoluto de espermatozoides por ovocito y el número de ovocitos penetrados y la polispermia (Rath, 1992; Gil y cols., 2004). Aunque el lavado por centrifugación en

solución salina elimina el plasma seminal de los espermatozoides mas rápidamente que la separación por Percoll (Levay y cols., 1995), este último procedimiento limpia la superficie sin dañar la membrana plasmática manteniendo por tanto la viabilidad espermática (Rodríguez-Martínez y cols., 1997). En nuestros experimentos, la mayoría de los espermatozoides fueron recogidos en el pellet, y solo un bajo porcentaje se recogió de la interfase entre los dos gradientes de densidad (35% y 70%) de la columna de Percoll, lo cual podría indicar que la acción del Percoll esta mas asociado a una limpieza de la cabeza del espermatozoide que a una selección y enriquecimiento de la población recuperada. Sin embargo, si el efecto del lavado por Percoll se debe solo a la limpieza de la superficie espermática necesita ser clarificado.

En resumen, nuestros resultados demuestran que un tiempo de exposición corto de espermatozoides frescos o congelados a bajas dosis del heterodímero PSP-I/PSP-II preserva (o mejora) la viabilidad espermática y la motilidad in vitro sin afectar al acrosoma. Aunque no se observe una influencia del heterodímero en la capacidad de los espermatozoides frescos de verraco para penetrar ovocitos homólogos (tanto IM como MIV), la PSP-I/PSP-II ejerce un efecto perjudicial sobre los espermatozoides congelados cuando se usaron para penetrar ovocitos MIV. Sin embargo, esta inhibición no fue permanente, ya que un lavado a través de un gradiente de Percoll recuperó la función espermática en algunas de las células.

V. – Conclusiones/Conclusions

5.1. Conclusiones

1. La adición de una cierta proporción de plasma seminal de verracos selectos a los medios de incubación de los espermatozoides altamente diluidos ejerce un efecto beneficioso sobre su viabilidad. Como consecuencia de las diferencias halladas entre verracos debería evaluarse la capacidad de los diferentes plasmas seminales para mantener la viabilidad espermática antes de su uso.

2. El heterodímero PSP-I/PSP-II ejerce su efecto protector sobre los espermatozoides altamente diluidos mediante su adhesión al dominio acrosomal del espermatozoide. La incubación de los espermatozoides altamente diluidos produce la migración del heterodímero a la región post-acrosomal, siendo eliminado de la superficie espermática.

3. La adición del heterodímero PSP-I/PSP-II a los medios de cocultivo entre gametos disminuye significativamente el número de espermatozoides por ovocito y la tasa de penetración de los ovocitos denudados tanto IM como MIV. Este efecto no se presenta en aquellos ovocitos cubiertos por las

células del cúmulus, sugiriendo que la inhibición de la penetración pudiera ser debida a un bloqueo de los receptores de la zona pelúcida.

4. La exposición de los espermatozoides de verraco tanto frescos como congelados preserva la viabilidad y motilidad espermáticas. Mientras que esta adición no afecta a la capacidad fecundante de los espermatozoides frescos de verraco, el heterodímero PSP-I/PSP-II disminuye significativamente la capacidad fecundante de los espermatozoides congelados, la cual puede ser restaurada parcialmente mediante un lavado a través de un gradiente de Percoll.

5.2. Conclusions

1. The addition of 10% of seminal plasma from certain boars maintains or enhances the viability of largely extended boar spermatozoa *in vitro*. As a result of significant differences among sires, a screening for "good boar" seminal plasma sources is recommended.

2. The protective effect of the PSP-I/PSP-II heterodimer on highly-extended boar spermatozoa could be related to the adhesion of the heterodimer to the acrosome domain of the sperm head plasma membrane. These results may indicate a stabilizing effect of the heterodimer on the fluidity of the sperm membrane.

3. Exposures of the gametes to the heterodimer during *in vitro* gamete co-incubation significantly decrease the sperm penetration rates and number of spermatozoa per oocytes in both IM and IVM denuded oocytes. Such an effect is not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP receptors.

4. Short-time exposure of fresh or frozen-thawed boar spermatozoa to PSP-I/PSP-II preserves sperm viability and motility. Although there is no obvious influence of the heterodimer on the capability of fresh diluted boar spermatozoa to penetrate homologous oocytes, PSP-I/II exerts a deleterious effect when frozen-thawed spermatozoa are used to penetrate IVM-oocytes. However, this inhibition did not seem permanent since a

subsequent washing through a Percoll gradient restored sperm function in some of the cells.

VI. – Resumen/Summary

6.1. Resumen

El desarrollo de nuevas biotecnologías, como la criopreservación espermática y el sexaje de espermatozoides por citometría de flujo, aplicadas a la producción animal suscitan cada día un mayor interés ya que permitirían un aumento importante de la eficiencia de la producción y de los programas de mejora genética. Sin embargo, a pesar de las mejoras realizadas en los últimos años la eficiencia de los procedimientos continua siendo insuficiente para ser aplicadas de manera rutinaria en la industria porcina, debido a las alteraciones que estos procedimientos producen en los espermatozoides.

Entre los diversos intentos que se han realizado para paliar estas alteraciones ocurridas en los espermatozoides se encuentra la adición de una cierta proporción de plasma seminal. Esta adición ha producido sin embargo resultados contradictorios entre los distintos estudios realizados. Por ello, en nuestra primera experiencia evaluamos si la adición de un 10% de plasma seminal tanto autólogo como homólogo ejercería un efecto beneficioso sobre espermatozoides que habían sido sometidos a diluciones extremas (0'3 x 10^6 espermatozoides/ml) similares a las encontradas durante el proceso de sexaje de espermatozoides mediante citometría de flujo.

Los resultados de nuestra experiencia indican que la eliminación del plasma seminal y posterior dilución en un medio salino simple como el PBS disminuyó la viabilidad y motilidad espermática, la cual pudo ser restaurada mediante la adición de plasma seminal antólogo. Además, la adición de plasma seminal homólogo a los espermatozoides altamente diluidos ejerció un efecto variado tanto positivo como negativo dependiendo de la fuente de plasma seminal, siendo recomendable por tanto examinar la capacidad del plasma seminal para mantener la viabilidad espermática previa a su uso.

Una vez evaluado el efecto de los diferentes plasmas seminales, se trato de examinar el posible mecanismo de acción. Recientemente, se ha visto que el heterodímero PSP-I/PSP-II ejerce una función protectora sobre los espermatozoides de verraco altamente diluidos similar al hallado con la adición de plasma seminal de machos selectos. Sin embargo, se desconocen los patrones de unión de esta proteína al espermatozoide y su posible relación con el mantenimiento de la funcionalidad espermática.

Por lo tanto, en nuestra segunda experiencia investigamos el efecto de la adición de 1’5 mg/ml de PSP-I/PSP-II a los espermatozoides altamente diluidos e incubados por un periodo de 10 horas sobre las características espermáticas y los cambios producidos en la inmunolocalización del heterodímero. La exposición de los espermatozoides al heterodímero PSP-I/PSP-II preservó la funcionalidad espermática durante las 10 horas de incubación. La inmunolocalización del heterodímero mostró como el porcentaje de espermatozoides inmunopositivos disminuía con el tiempo de incubación desde un 71%

(0’5 h) a un 49% (10 h) (p<0’05), mientras que la adición de PSP-I/PSP-II exógena mantenía estos niveles por encima del 80%. El estudio mediante microscopía electrónica de transmisión reveló 4 patrones de unión del heterodímero a la membrana plasmática del espermatozoide, siendo localizado principalmente sobre la región acrosomal para ser redistribuido durante la incubación al área post-acrosomal siendo finalmente liberado.

La capacidad del heterodímero para interactuar en la unión entre gametos no ha sido determinada todavía, habiéndose encontrado resultados contradictorios en lo referente a su capacidad para unirse a la ZP. En nuestra tercera experiencia evaluamos el efecto de la presencia del heterodímero (1’5 mg/ml) durante la maduración *in vitro* de los ovocitos así como durante el cocultivo de los espermatozoides congelados de verraco con ovocitos IM y MIV, cubiertos por las células del cúmulus o denudados. La exposición de los gametos al heterodímero durante el cocultivo provocó una disminución significativa de la tasa de penetración y el número de espermatozoides por ovocito tanto en ovocitos IM y MIV que habían sido previamente denudados. Este efecto no se presentó cuando los ovocitos se encontraron cubiertos por células del cúmulus, sugiriendo que el efecto pudiera ser mediado por la exposición de los receptores de la ZP. Además, la adición del heterodímero al medio de maduración *in vitro* redujo la tasa de ovocitos maduros significativamente.

Por último, debido al efecto beneficioso que ejerce el heterodímero PSP-/PSP-II sobre los espermatozoides y a la posibilidad de que este pretratamiento pudiera disminuir la capacidad fecundante de los espermatozoides, nuestra cuarta experiencia estudió el efecto que tendría la preincubación de los espermatozoides con el

heterodímero PSP-I/PSP-II sobre la capacidad de penetración *in vitro* tanto ovocitos IM como MIV.

La preincubación de los espermatozoides con el heterodímero PSP-I/PSP-II mejoró la viabilidad y motilidad de los espermatozoides de verraco tanto frescos como congelados. Aunque esta preincubación no afectó la capacidad de los espermatozoides frescos para penetrar *in vitro* tanto ovocitos IM como MIV, los espermatozoides congelados sufrieron una disminución significativa de su tasa de penetración comparado con el control (de 90% a 32%, p<0’05) y en el número de espermatozoides por ovocito (de 2’9 a 1’6, p<0’05). Para discernir el motivo de este efecto paradojico, los espermatozoides congelados fueron lavados (por centrifugación en solución salina con BSA o a través de un gradiente de Percoll), observando un incremento de la penetración en los espermatozoides lavados a través del gradiente de Percoll, así como una disminución de la polispermia comparado con los espermatozoides que no habían sido lavados o se lavaron por centrifugación en un medio salino con BSA. Por lo tanto, este efecto negativo que produce el heterodímero PSP-I/PSP-II sobre los espermatozoides congelados parece ser parcialmente reversible, incrementando tanto la tasa de penetración como de monospermia.

6.2. Summary

Sperm handling associated to artificial reproduction technologies (ART) such as cryopreservation or the use of flow cytometry for cell analysis or sorting decreases sperm viability, presumably owing to the removal of seminal plasma (SP) components. The addition of SP has been proposed as a suitable ameliorator for the damaging effects of sperm handling. However, reports on the benefits of addition of SP are somewhat equivocal.

Therefore, in our first study we evaluated whether a 10% v/v of autologous SP or homologous SP would differently affect the viability of boar spermatozoa subjected to large extension in a simple saline medium (PBSm) to a concentration of 0.3 x 10^6 spermatozoa/ml and incubated for 2 h at 30ºC. Sperm viability was monitored as membrane integrity [using the fluorophore carboxyfluorescein diacetate (C-FDA) and propidium iodide (PI)], mitochondrial function (using the fluorophore R-123) and motility characteristics [using Computer Assisted Sperm Analysis (CASA)]. Substraction of the SP and extension followed by incubation in PBSm significantly ($p<0.05$) decreased sperm viability, which could be restored by addition of autologous SP. Furthermore, exposure of the extended spermatozoa to homologous SP significantly ($P< 0.05$) varied with the source sire; some boars exerting beneficial effects while at least one boar negatively ($P< 0.05$) influencing the viability of the incubated spermatozoa. It is concluded that SP should be present when incubating highly extended spermatozoa. Owing to the obvious differences among boars, it would be advantageous to examine the ability of SP to maintain sperm viability prior to the use of SP pools during sperm handling in vitro.

It has been recently reported that PSP-I/PSP-II heterodimer has a similar protective effect as that reported for boar spermatozoa on the addition of SP from selected males. However, little is known about the binding pattern of the heterodimer to the sperm plasma membrane and its eventual relation with the maintenance of the sperm functionality.

Our second experience investigated the effect of exposing highly-extended boar spermatozoa (1 million/mL) to 1.5 mg/mL of PSP-I/PSP-II for 0.5, 5 and 10 hours at 38ºC on sperm characteristics and the changes in PSP-I/PSP-II localization as a result of both the addition of PSP-I/PSP-II to the extender and the incubation time. Exposure of the spermatozoa to PSP-I/PSP-II preserved sperm viability, motility and mitochondrial activity when compared to non-exposed spermatozoa. This protective effect lasted for 10 hours ($p<0.05$). After immunolabelling of highly-extended semen with rabbit monospecific polyclonal antibody against PSP-I/PSP-II, the percentage of immunopositive spermatozoa declines over time from 71% (0.5 h) to 49% (10 h). However, more than 80% of spermatozoa remained labelled during the 10h-incubation period if PSP-I/PSP-II was added. Scanning electron microscopy revealed 4 different binding patterns. The heterodimer was mainly localized to the acrosomal area, being redistributed to the post-acrosomal area or lost during *in vitro* incubation.

Controversial results are reported about the ligand capability of PSP-I/PSP-II to the zona pellucida (ZP) or its possible role in gamete interaction. Therefore, our third experience evaluated the effect of the presence of PSP-I/PSP-II (1.5 mg/mL) during *in vitro* oocyte maturation and also during co-incubation of frozen-thawed boar

spermatozoa with either immature (IM) or *in vitro* matured (IVM) oocytes, either enclosed by cumulus cells or denuded. Exposure of the gametes to the heterodimer during *in vitro* gamete co-incubation showed a significantly blocking effect of sperm penetration rates and a decreased number of spermatozoa per oocyte in both IM- and IVM- denuded oocytes. Such an effect was not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP-receptors. Besides, when PSP-I/PSP-II was added to the IVM–medium, oocyte maturation rates were significantly reduced. In conclusion, the results suggest that PSP-I/PSP-II, when present *in vitro,* block sperm-ZP binding.

Owing to the potential beneficial effect of PSP-I/PSP-II on sperm handling and since the effect of sperm pretreatment on sperm fertilizing ability is not yet known. Our forth experience evaluated the effect of exposing freshly-extended and frozen-thawed boar spermatozoa (10 million/mL) to PSP-I/PSP-II (1.5 mg/mL) for 30 or 120 minutes, on sperm characteristcs and the outcome of in vitro penetration of immature (IM) and in vitro matured (IVM) homologous oocytes.

Although exposure to the heterodimer improved sperm viability and motility without increasing the sperm acrosome reaction level of both fresh-extended and frozen-thawed spermatozoa, this pre-treatment did not affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes, compared with controls. When cryopreserved spermatozoa were tested, however, on IVM oocytes, already a 30 minute preincubation exposure to PSP-I/PSP-II showed a significant blocking effect on penetration rate (from 90% to 32%, $p<0.05$) and on mean sperm numbers per oocyte (2.9 to 1.6, $p<0.05$). In order to

disclose the nature of this paradox, frozen-thawed spermatozoa were cleansed (by centrifugation in saline-BSA or through Percoll density gradient separation) and the procedure repeated. Penetration (but not number of spermatozoa/oocyte) increased ($p<0.05$) when spermatozoa were filtered through Percoll compared with either washed or unwashed-controls. In addition, the percentages of polyspermic oocytes remained lower than control. Such an effect of cryopreservation seems to a certain extent reversible, since cleansing of the sperm surface decreased, at least partially, this blocking effect, increasing both, the penetration and the monospermic rates.

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VIII. – Abreviaturas

ADAMs	Metalloprotease-disintegrin protein family	
ALH	Amplitude of lateral-head displacement	Amplitud del desplazamiento lateral de la cabeza
AQN-1	Boar spermadhesin	Espermadhesina porcina
AQN-3	Boar spermadhesins	Espermadhesina porcina
ARNm/mRNA	Messenger ribonucleic acid	Ácido ribonucleico Mensajero
ART	Artificial reproduction technologies	Tecnologías usadas en reproducción artificial
ATP	Adenosine triphosphate	Adenosina trifosfato
aSFP	Acidic seminal fluid protein	Proteína acídica del plasma seminal
AWN	Boar spermadhesins	Espermadhesina porcina
BSA	Bovine serum albumin	Albúmina sérica bovina
BISV	Bison seminal vesicle protein	Proteínas del plasma seminal de bisonte
BSP	Bovine seminal plasma proteins	Proteínas plasma seminal Bovino

BTS	Betsville thawing solution	
Ca^{2+}	Calcium	Calcio
CASA	Computer assisted semen analysis system	Análisis computarizado de la motilidad espermática
CRISP	Cysteine-rich secretory proteins	Proteínas secretadas ricas en cisteína
DCF	Carboxyfluorescein diacetate	Diacetato de Carboxifluoresceina
DMSO	Dimethyl sulfoxide	Dimetilsulfoxido
ELISA	Enzyme Linked Inmunoabsorvent Assay	Ensayo enzima inmuno-absorbente
Fn-II	Fibronectin type II protein	Proteínas que contienen el dominio fibronectina tipo II
Gp54	Glycoprotein complex 54 kDa	Complejo glicoproteíco de 54 kDa
HSP	Horse seminal plasma protein	Proteínas del plasma seminal de caballo
IM	Immature oocytes	Ovocitos inmaduros
IP	Propidium iodide	Ioduro de propidio
IU/ml	International unit/milliliter	Unidades internacionales/mililitro
kDa	Kilodalton	Kilodalton
LEY	Lactose-egg yolk extender	Diluyente lactosa-yema de huevo
LEYGO	LEY-glycerol-orvus ES paste	LEY-glicerol-orvus ES paste

LIN	Linearity	Linealidad
mg/ml	milligram/milliliter	miligramo/mililitro
ng/ml	nanogram/milliliter	nanogramo/mililitro
MIV	In vitro matured oocytes	Ovocitos madurados in vitro
mM	Millimolar	Milimolar
mW	Milliwat	Milivatio
nM	Nanometers	Nanometros
PAF	Platelet activating factor	Factor activador de plaquetas
PAF-AH	Platelet activating factor acetylhydrolase	Acetilhidrolasa del factor activador de plaquetas
PBS	Phosphate-buffered saline	Solución tamponada de fosfato
PE-PNA	phycoerythrin-conjugated peanut agglutinin	Ficoeritrina conjugada con lectina de Arachis hypogaea
pI	Isolectric point	Punto isoeléctrico
PMN	Polymorphonuclear	Polimorfonucleares
PSP-I	Porcine seminal plasma protein 1	Espermadhesina porcina
PSP-II	Porcine seminal plasma protein 2	Espermadhesina porcina
R-123	Rhodamine 123	Rodamina 123
RSP	Ram seminal plasma proteins	Proteínas del plasma seminal caprino
RSVP-14	14 kDa ram seminal plasma protein	Proteínas del plasma seminal caprino de 14 kDa

RSVP-20	20 kDa ram seminal plasma protein	Proteínas del plasma seminal caprino de 20 kDa
RT-PCR	Reverse transcriptase polimerase chain reaction	Trascripción reversa de la reacción en cadena de la polimerasa
SMIF	Sperm motility inhibiting peptide	Factor inhibidor de la motilidad espermática
VCL	Curvilinear velocity	Velocidad curvilínea
VSL	Mean straight-line velocity	Velocidad rectilínea
v/v	volume/volume	volumen/volumen
Zn^{2+}	Zinc	Cinc
ZP	Zona pellucida	Zona pelúcida

Apéndice I. – Extended Summary

INTRODUCTION

The ejaculated spermatozoa are immersed in seminal plasma (SP), a medium composed by aliquots of the fluid of the testis, epididymal tail and the secretions of the accessory sexual glands of the sire. The SP contains a wide variety of factors that influence the functionality of spermatozoa (Mann and Lutwak-Mann 1981; Rodriguez-Martinez et al. 1984, Rodríguez-Martínez 1991; Iwamoto et al. 1992; Maxwell et al. 1997; Strzezek 2002) although the biological effects of these SP-factors on sperm function are complex and not well understood. A variation in the presence, absence or concentration of some components, most probably proteins (either of epididymal or accessory gland origin, Fournier-Delpech and Thibault 1993), may be responsible for the variability seen on the effects –detrimental or beneficial- on the spermatozoa (Maxwell and Johnson 1999). A high variability exists in the SP composition among species or even males within the same species, as well as between ejaculates of homologous males (Asworth et al. 1993; Killian et al. 1993; Zhu et al. 2000).

Some SP fractions, such as the prostatic one (England and Allen 1992), the gel fraction in stallion, boar or camelids (Mann and Lutwak-Mann 1981), as well as the secretion of the seminal vesicles, are considered by some authors as detrimental for

sperm survival *in vitro* (ram, Ashworth et al 1994; bull, Way et al 2000; buck, Azerêdo et al. 2000). Decreases in fertility have been reported following the exposure of ruminant (Dott et al. 1979) or stallion (Corteel et al. 1980) spermatozoa to either autologous or homologous SP. Therefore, when handling spermatozoa in vitro, the removal of SP (by centrifugation or extension with a buffer) is praxis, and considered critical to ensuring maximal sperm viability in the majority of protocols for semen preservation.

However, the SP has also proven beneficial for spermatozoa. In pigs or bulls, incubation of spermatozoa in autologous or homologous SP at room temperature increased the sperm resistance to cold shock, a process that occurs when recently ejaculated spermatozoa are chilled (Pursel et al. 1973; Ollero et al. 1998; Eriksson et al. 2001). Viability can be restored in cold-shocked ram spermatozoa if incubated with selected portions of SP-proteins, particularly a 20 kDa band (Barrios et al. 2000). Addition of SP to thawed ram spermatozoa reverted changes seen during cryopreservation (capacitation-like, Pursel and Johnson 1975) to such an extent that motility was increased *in vitro* (Gillan and Maxwell 1999; Maxwell et al. 1999), as well as fertility post-Artificial Insemination (Maxwell et al. 1999). Similar changes have been reported in other species (for review, see Rodriguez-Martinez et al. 1998). Furthermore, it is usual the addition of SP to boar spermatozoa subjected to sperm sorting for chromosomal sex through flow-cytometry (Maxwell et al. 1998; Johnson and Welch 1999; Vazquez et al. 2003) where a 2,000-fold extension of the ejaculate is required. Addition of 10% (v/v) of SP to the collection medium of sex-sorted spermatozoa improves their sperm viability and motility as well as stabilizes the plasma

membrane preventing premature capacitation (Maxwell et al. 1997), and extending their fertilizing ability (Maxwell et al. 1998).

Addition of homologous SP (eg from different males) can even influence the fertilizing capacity of the spermatozoa (bulls: Killian et al. 1993). For instance, the addition of SP from low fertility bulls decreased the penetration of zona-free oocytes by spermatozoa from bulls of high fertility (Henault and Killian, 1996). Moreover, addition of SP from stallions with high post-thaw motility to semen from stallions with low post-thaw motility improved membrane integrity and progressive motility (Aurich et al. 1996).

As a result of the variability in results of the addition of SP already described, our first study aimed to evaluate whether the addition of SP improves boar sperm viability, monitored as membrane integrity, mitochondrial function and motility characteristics, when spermatozoa were subjected to similar extreme extension conditions as when sperm sorting by flow cytometry is performed. Furthermore, it intended to disclose whether this effect depends of the SP source (as autologous or homologous).

In the boar, the majority of seminal plasma (SP) proteins belong to the spermadhesin family, a group of (glyco)proteins built by a single CUB domain architecture (Romero et al., 1997) and thought to play important roles in individual steps of the fertilization process such as capacitation and zona pellucida binding (Töpfer –Petersen et al., 1998). The spermadhesin family comprises five members: AQN-1, AQN-3, AWN, PSP-I, PSP-II. The last two form a glycosylated PSP-I/PSP-II heterodimer under physiological conditions (Calvete et al., 1995). *In vitro,* low doses of

PSP-I/PSP-II heterodimer (1.5 mg/mL) appear to preserve membrane integrity, motility, and mitochondrial activity of highly-extended spermatozoa (Centurión et al., 2003). Processes linked to the removal of factors present in the SP that coat the sperm surface and maintain the stability of the plasma membrane, such as high extension of the spermatozoa (Maxwell and Johnson, 1999), trigger a series of phenomena that resemble those occurring during sperm capacitation culminating in premature acrosome exocytosis, thus decreasing the life span of the spermatozoa (Maxwell and Johnson, 1999). Although the addition of homologous seminal plasma (from 1% to 10% v/v) is a possible counter-measure to alleviate the consequences of such an “extension effect” (Asworth et al., 1994; Maxwell et al., 1997), differences in SP-protein profiles have been found between males of different fertility. These differences may be related to the variability between different sources of SP (Fournier-Delpech and Thibault, 1993; Killian et al., 1993; Caballero et al., 2004a) that lead to both beneficial and detrimental effects on the spermatozoa

Either whole SP or specific SP-components of low molecular weight affect survivability of boar spermatozoa, depending on how long they are exposed to the PSP-I/PSP-II (Centurión et al., 2003). However, there is an advantage of using an isolated protein instead of whole SP in order to avoid the inherent variability that exists in SP-composition between males or ejaculates (Killian et al., 1993; Asworth et al., 1994; Zhu et al., 2000). Previous studies, using various in vitro fertilization treatments, indicated that the PSP-I/PSP-II heterodimer does not sustain its influence for long periods during incubation (data not shown). This suggests that the heterodimer may either lose its action by blockade or degradation or perhaps by attaching in a loose manner to the plasmalemma, thus losing influence over time. In any case, there is a need to establish

the pattern of attachment and the influence of incubation on this pattern, and to elucidate whether there is any relationship between the presence of the protein, and its relative concentration, and the protective role *in vitro*.

Therefore, our second study examined the relationship between the protective effect of the spermadhesin PSP-I/PSP-II and its binding pattern on the membrane surface of boar spermatozoa subjected to extreme dilution conditions similar to those used for flow cytometric high speed sorting is performed for chromosomal sex separation.

Gamete interaction is an early critical step in mammalian fertilization, where at least three morphologically disparate cell types, the capacitated acrosome-intact spermatozoon, the mature oocyte and the surrounding cumulus cells, are involved (Yanagimachi, 1994). The events of gamete recognition, binding and fusion are highly regulated processes that imply a number of biochemical reactions until a new zygote is formed. In domestic species, this mechanism of cell-to-cell adhesion seems mediated by protein-carbohydrate interactions between sperm-associated lectins and glycan structures of the oocyte zona pellucida (ZP), the latter synthesised by the concerted action of the oocyte and the granulosa cells during oocyte maturation (Sinowatz et al., 2001).

One of these sperm lectins constitutes the spermadhesin family (Sinowatz et al., 1997). They play a role in several biological functions, including sperm capacitation and, as already mentioned, gamete recognition and binding (Calvete et al., 1994).

As mentioned above, the PSP-I/PSP-II heterodimer appears to preserve -*in vitro*- the membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa for as long these are exposed to the heterodimer (Centurión et al., 2003). These properties highlight PSP-I/PSP-II as a potential candidate for pre-treatment of manipulated spermatozoa (extended, stored, deep-frozen or sex sorted) aiming at promoting sperm survival and performance *in vitro*. However, the competence of PSP-I/PSP-II to interact during gamete binding is still unclear. While the heparin-binding spermadhesin AWN is able to reach the ovulated oocytes(s) *in vivo* (Rodriguez-Martinez et al., 1998), the PSP-I/PSP-II heterodimer binds loosely to the sperm surface and does not seem to maintain any attachment to the spermatozoa either following in vitro capacitation or sperm transport in the female genital tract, as seen to be the case with the AWNs (Dostálová et al., 1994; Calvete et al., 1997; Rodriguez-Martinez et al., 1998). In some studies, PSP-I/PSP-II heterodimer showed affinity for ZP glycoproteins and this activity was located in the isolated PSP-II monomer and was reported to be cryptic in the heterodimer (Calvete et al., 1995a). In contrast, Jonáková et al. (2000) also found an interaction between the ZP and the isolated PSP-II, but this binding was inhibited by the aggregated PSP-I/PSP-II heterodimer. Therefore, although an *in vivo* functional role of this spermadhesin during gamete binding is unexpected, the fact that the ZP-binding properties of PSP-I/PSP-II heterodimer are still a matter of controversy in the literature calls for further research in this area, if the purpose is to use these spermadhesins during *in vitro* gamete handling, for instance as a preparatory step for IVF.

Thus, in our third experience and taking into account the beneficial effect on sperm viability already assessed *in vitro* and the divergent results in ZP-binding ability

of PSP-I/PSP-II, has prompted us to examine the effect the heterodimer PSP-I/PSP-II during boar sperm-oocyte co-culture using both immature (IM) and *in vitro* matured (IVM) homologous oocytes.

It is known that the use on the ART of an isolated heterodimer, the PSP-I/PSP-II, has the advantage of avoiding the inherent variability shown by bulk SP among males or ejaculates from one and the same male (Killian et al, 1993; Ashworth et al, 1994; Rozeboom et al, 2000).

Porcine in vitro fertilization (IVF) is still considered suboptimal compared with the outcome of this reproductive biotechnology on other species, with unacceptable levels of polyspermia associated with high penetration rates. Although ejaculated boar spermatozoa, surrounded by SP, are able to produce high penetration levels when coincubated with homologous oocytes (Martinez et al, 1996), the level of fertilization is lower with ejaculated than with epididymal spermatozoa (Rath and Niemman, 1997), indicating that use of bulk SP on IVF is not beneficial (Bonilla et al, 1996; Rath and Niemman, 1997; Maxwell et al, 1998). Coincubation of immature (IM) or in vitro matured (IVM) oocytes with 1.5 mg/mL of PSP-I/PSP-II in the IVF medium significantly decreased penetration rates by untreated boar spermatozoa (Caballero et al, unpublished data). These data, together with the fact that the PSP-II subunit of the PSP-I/PSP-II heterodimer exhibits binding affinity for zona pellucida (ZP) receptors (Calvete et al, 1995b), suggest that the heterodimer can block penetration, at least in vitro. However, the PSP-I/PSP-II spermadhesin binds loosely to the sperm surface (Calvete et al, 1995b), and, consequently, an easy removal should be expected in vitro and during sperm coincubation with the oocytes. Therefore, preincubation of spermatozoa with

PSP-I/PSP-II should not affect the interaction between spermatozoa and the oocyte. These properties, along with the sperm protective effect, point to PSP-I/PSP-II as a potential candidate for pretreatment of manipulated spermatozoa (extended, stored, or deep frozen) aimed at promoting sperm survival and performance in vitro. This is especially important when technologies such as sex selection of spermatozoa by flow cytometric sorting or sperm cryopreservation are used, procedures known to induce deleterious changes in boar spermatozoa.

Owing to such a potential beneficial effect on sperm viability and since the effect of sperm pretreatment with the PSP-I/PSP-II heterodimer on IVF has not been investigated, the forth study examined the in vitro penetration ability (penetration rate and number of spermatozoa per oocyte) of fresh and frozen-thawed boar spermatozoa (10 x 106/mL) on IM and IVM homologous oocytes following preincubation with low doses of the heterodimer (1.5 mg/mL) for 30 or 120 minutes. In addition, the rate of polyspermia was determined when IVM oocytes were penetrated.

MATERIAL AND METHODS

1.- Experience 1: Comparative effect of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa.

Sperm-rich ejaculate fractions (SRF, 100 ml) were manually collected from five mature Pietrain boars of proven fertility (hereby coded A-E), once weekly during six consecutives weeks using the gloved-hand method. Shortly after collection, the semen samples were evaluated for motility and only those showing >80% of sperm motility

was used. Each SRF was divided in two aliquots of 85 and 15 ml, as sources for SP or spermatozoa, respectively. The 85 ml aliquot of the SRF was centrifuged at 3,800x g for 15 min (20ºC) using a Heraeus Sepatech Megafuge (Osterode, Germany). The supernatant was harvested and filtered twice through a 10 and a 1.2 µm-mesh filter respectively, before use. The SP was always used fresh in the experiments. The 15 ml-aliquot was centrifuged at 1,900x g for 4 min (20ºC), the supernantant discarded and the pellet used as source for further sub-aliquots as required by the experiments (see below).

1.1. Evaluation of sperm viability following exposure of boar spermatozoa to autologous or homologous SP

Spermatozoa obtained from each and every boar were thus split in 6 sub-aliquots (A-E and control). These sub-aliquots were sequentially extended to yield a concentration of 0.3 x 10^6 spermatozoa/ml in phosphate-buffered saline with 0.1% ethylenediaminetetraacetic acid (EDTA) alone (PBSm, control) or PBSm with 10% (v/v) of its own SP (hereby named autologous SP, eg extended in PBSm + 10% v/v of autologous SP) or to the SP from any other of the remaining 4 boars studied (hereby named homologous SP, eg extended in PBSm + 10% v/v of homologous SP).

In either group spermatozoa were incubated in a water bath in the dark for 2 h at 30ºC in capped tubes until evaluated for plasma membrane integrity, mitochondrial membrane potential activity and sperm motility.

Sperm plasma membrane integrity (MI) was determined using the fluorescent probe carboxyfluorescein diacetate (C-FDA, Sigma Chem. Co. Alcobendas, Spain)

using a modification of the method described by Harrison and Vickers (1990). Briefly, 20 µL of 0.46 mg/ml of C-FDA in dimethylsulfoxide (DMSO) and 20 µL of formaldehyde (2.5 mg/ml in water) were added to 1 ml of BTS media containing 0.3 x 10^6 spermatozoa to achieve a final concentration of 1.7 mM-formaldehyde and 20 mM-C-FDA. After 10 min at 30ºC, the stained sperm suspensions were observed at 400x magnification using a Nikon Eclipse 800 microscope (Nikon, Chiba, Japan) equipped with epifluorescence illumination. Spermatozoa were scored for the percentage of those showing green fluorescence along their whole length (spermatozoa with intact plasma membranes).

Rhodamine 123 (R 123, Sigma Chem. Co., Alcobendas, Spain) was used as a probe of mitochondrial membrane potential. Spermatozoa (0.3 x 10^6 spermatozoa/ml) were stained by incubation for 15 min in a BTS solution containing 1 µg/ml of R 123 and 4 µg/ml of propidium iodide (PI, Sigma Chem. Co., Alcobendas, Spain). Spermatozoa were examined as explained above. Only spermatozoa showing green fluorescence in their midpieces were considered live spermatozoa having mitochondrial membrane potential activity.

Sperm motility was estimated with a Computer Assisted Sperm Analysis equipment (CASA). Sperm samples (10 µL at 0.3 x 10^6 spermatozoa/ml) were placed in a warm (38ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38ºC) of a Nikon Laborphot microscope, equipped with a 10x phase objective and a monochrome video camera (Hitachi CCD model), connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyser (SCA, Barcelona, Spain). The program settings were as follows: frames rate: 25Hz;

search radius: 11.5 µm; minimum track points: 7 frames; threshold straightness: 75%. The motion variables recorded were: percentage of motile spermatozoa, curvilinear velocity (VCL, time-average velocity of the sperm head along its actual trajectory) and linearity (LIN, time-average velocity of the sperm head along a straight line from its first position to its last position (VSL)/VCL x 100).

Two replicates per sample were analyzed with at least one hundred spermatozoa recorded per replicate for each parameter evaluated.

1.2 Statistical Analysis

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 effects of SP source and of sperm source and the random effect of replicate. To analyze data of sperm viability and sperm motility, 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 $p<0.05$.

2.- Experience 2: Immunolocalization and Possible Functional Role of PSP-I/PSP-II Heterodimer in Highly Extended Boar Spermatozoa.

2.1 Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar seminal plasma

All experiments were performed with the SP from sexually mature boars, which had previously sired offspring. Ejaculates were obtained using the gloved hand method and SP was harvested after centrifugation of the sperm suspension at 3,800 x g at 20ºC for 15 min. The supernatants were sequentially filtered through 10 and 1.2 µm diameter filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of SP by size-exclusion chromatography on a 2000 x 5 cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, 0.025% sodium azide, pH 7.4 (Calvete et al., 1995). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 492 automated protein sequencer, Langen, Germany) and MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-Pro mass spectrometer (Langen, Germany). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a molar absorption coefficient (27332 M^{-1} cm^{-1}) as determined by Menéndez et al. (1995), or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 h at 106ºC in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyser (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilised, and stored at -20ºC until used.

2.2 Preparation of Anti-PSP-II antibodies

The PSP-I and PSP-II subunits were purified from the isolated PSP-I/PSP-II heterodimer by reverse-phase HPLC as described by Calvete et al. (1995). Polyclonal anti-PSP-II monospecific antibodies were obtained by immunizing female rabbits with sub-cutaneous inoculations of 0.5 mg of highly purified PSP-II heterodimer 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 immunization 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-II heterodimer activity by dot-blot ELISA and Western blot.

2.3 Semen handling, incubation with the heterodimer PSP-I/PSP-II, and evaluation of sperm parameters

Sperm-rich ejaculate fractions were collected from three mature Pietrain boars of proven fertility using the gloved hand method. Shortly after collection, the semen was extended in Beltsville Thawing Solution (BTS; [Pursel and Johnson, 1975]) to 30 x 10^6 spermatozoa/mL and spermatozoa evaluated for motility. Only ejaculates showing >80% progressive sperm motility were used. The extended ejaculates were pooled and centrifuged (1,700 x g for 3 minutes), the supernatant discarded, and the final sperm pellet re-extended in PBS to a final concentration of 1 x 10^6 spermatozoa/mL.

One million spermatozoa, extended as described above, were pre-incubated without or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized

proteins) at 38º C. After 0.5, 5 or 10 hours of pre-incubation, the sperm plasma membrane, acrosome integrity, mitochondria membrane potential, motility and binding patterns of PSP-I/PSP-II were evaluated.

Plasma membrane and acrosome integrity were simultaneously assessed by flow cytometry, using the staining protocol described by Nagy et al. (2003). For flow cytometric analysis, 50 nM of SYBR-14 working solution (100 µM stock solution in DMSO; component A of LIVE/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands), 0.5 µg/mL of PE-PNA (PhycoErythrin-conjugated PeaNut Agglutinin, Biomeda Corp. Foster City, CA) solution (1 mg/mL stock solution) and 7.5 µM of PI solution (Propidium Iodide, 1.5 mM stock solution in PBS) were added to 500 µL of sperm suspension. The samples were incubated at 37ºC in the dark for 10 min. All analyses were performed by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc, Miami, Fla) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, Calif) with 15-mW laser power at 488 nM and EXPO 2000 software (Coulter Corporation). Forward and side scatter were recorded for a total of 15,000 to 25,000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. The SYBR-14 fluorescence was measured through a 525-nm band pass filter, PI fluorescence was collected through a 635-nm band pass filter and PE-PNA fluorescence was detected through a 575-nm band pass filter. Membrane intact spermatozoa with intact acrosomes were defined as those stained only with SYBR-14.

The JC-1 probe (5,5',6'6-tetrachloro-1,1',3,3'tetraethylbenzymidazolcarbocianyne iodide, Molecular Probes Europe, Leiden, The Netherlands) was used to assess

mitochondrial membrane potential of spermatozoa as described by Martinez-Pastor et al. (2004), with slight modifications. Briefly, 0.2 µM of JC-1 working solution (3.8 mM stock solution) was added to 500 µL of sperm suspension. Then, samples were incubated at 37ºC in darkness for 10 min and analyzed immediately on the flow cytometer (see above). Fluorescence emission of JC-1 monomers and JC-1 aggregates were detected, respectively, in FL1 and FL2 using 520-nm or 590-nm band pass filters.

Sperm motility was estimated with a computer-assisted motility analysis system. Samples (5 µL containing 1 x 10^6/mL spermatozoa) were placed in a warm (38ºC) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38ºC) of a Nikon Labophot microscope (Kanagawa, Japan) equipped with a 10x contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the Sperm Class Analyzer software (Microptic, Barcelona, Spain). The program settings were as follows: frame rate, 25 HZ; search radius, 11.5 µm; minimum track points, 7 frames; and threshold straightness, 75%. Two replicates per sample were examined with at least 100 spermatozoa recorded per replicate for each parameter evaluated. Motion parameters were: percentage of motile spermatozoa, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and amplitude of lateral head displacement (ALH, µm).

2.4 PSP-I/PSP-II heterodimer Immunocytochemistry

Immunolocalization of the PSP-I/PSP-II heterodimer was studied by both light and scanning electron microscopy (SEM) levels. Sperm suspensions pre-incubated either without or with PSP-I/PSP-II as described above were smeared onto poly-lysine

coated glass slides and air dried for 24 hours. Smears were fixed in 1% paraformaldheyde in PBS for 10 min., rinsed in PBS and incubated with a rabbit monospecific polyclonal antibody against PSP-II (1:500 in PBS) for 120 min at room temperature. Samples were rinsed several times in PBS and incubated with Auroprobe EM GAR G10 (10 nm gold labelled goat anti-rabbit IgG, Amersham Biosciences, Uppsala, Sweden) for 100 min. After washing in PBS and distilled water, a silver enhancement kit (RPN 491, Amersham Biosciences, Uppsala, Sweden) was applied for 10 min. Finally, the smears were washed in distilled water and air dried. To provide positive controls, samples of epididymal spermatozoa were smeared, air dried and fixed as above, incubated with the PSP-I/PSP-II heterodimer for 30 min and subjected to the same protocol as above. Negative controls were obtained by omission of the primary antibody.

For evaluation by light microscopy level, incubated samples were photographed with a Nikon microphot-FXA light microscope (Chibe, Japan) with at least 100 spermatozoa being evaluated per sample at 400x magnification. For evaluation by SEM level (6,000x magnification), portions of the glass slides with labelled spermatozoa were cut out, mounted onto metal chucks, critical-point coated with gold-palladium for 15-30 s, and examined using a JEOL JSM-6320F SEM (JEOL LTD, Japan), operated at 5 kV.

2.5 Statistical analysis

All data editing and statistical analyses were performed in SPSS version 11.5 (SPSS Inc, Chicago, Il, USA). Data were subjected to arcsine transformation and

analysed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effects of heterodimer presence and the random effect of replicate. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered significant at $p<0.05$.

3.- Experience 3: Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction.

All chemicals used in the preparation of the culture media were purchased from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain) unless otherwise stated.

3.1 Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar seminal plasma

All experiments were performed with the SP from sexually mature boars, which had previously sired offspring. Ejaculates were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at 3,800 x g for 15 min, at 20ºC. The supernatants were sequentially filtered through 10 and 1.2 µm filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of SP by size-exclusion chromatography on a 2,000 x 5 cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, 0.025% sodium azide, pH 7.4 (Calvete et al., 1995a). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 472 automated

protein sequencer, Langen, Germany) and MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-Pro mass spectrometer (Langen,Germany). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a 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 106ºC in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyser (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilised, and stored at -20ºC until used.

3.2 Culture Media

The basic medium used to in *vitro* assess sperm penetration ability of immature oocytes was TCM-199 with Earle´s salts supplemented with 12% heated fetal calf serum (v/v), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 UI penicillin G, and 30 µg/mL streptomycin sulphate (Cheng, 1986).

The basic medium used for *in vitro* maturation was Bovine serum albumin (BSA)-free North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). This medium shall be named "IVM medium" hereafter.

The *in vitro* fertilisation (IVF) medium used (Abeydeera and Day, 1997) consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2·2H_2O, 20 mM Tris, 11 mM glucose, 5

mM sodium pyruvate, 1mM caffeine and 0.2% BSA (fraction V, Cat nr A 7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was North Carolina State University (NCSU) 23 containing 0.4% BSA (fraction V, Cat nr A 8022, initial fractionation by cold alcohol precipitation).

3.3 Recovery, *in vitro* maturation, penetration and evaluation of oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martinez et al., 1993; Abeydeera and Day, 1997).

Ovaries were obtained from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 µg/mL kanamycin, maintained at 34-37ºC. Cumulus-oocyte-complexes (COCs) were aspirated from medium-sized follicles (3-6 mm in diameter) with an 18-gauge needle fixed to a 10 mL disposable syringe. Only COC´s having a compact cumulus mass (with at least six or seven layers) an intact ZP and an oocyte with an evenly granulated cytoplasm were selected for the different experiments.

For preparation of IM-oocytes, oocytes were divided in two groups for cumulus enclosed (CE) and denuded oocytes, respectively. Both, CE oocytes and those denuded by repeated pipetted to mechanically remove the cumulus cells, were placed directly in 2 mL of pre-equilibrated modified TCM-199 medium supplemented with 2 mM caffeine and 5.4 mM calcium lactate (Cheng, 1986) in batches of 30 IM-oocytes and kept in the incubator at 39ºC in 5% CO_2 in air for about 30 minutes before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed three times in IVM-medium. Thereafter, COCs were transferred to a Nunc 4- well multidish plate (50-100 COCs/well) submerged in 500 µL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV, Boxmeer, The Netherlands), and cultured at 39ºC in 5% CO_2 in air for 22 h. The medium was then changed for maturation medium without hormone supplementation, and incubated at 39ºC in 5% CO_2 in air for another 22 h. After *in vitro* maturation, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed three times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera and Day, 1997). Batches of 50 IVM oocytes were placed in 50 µL drops of IVF medium covered with warm mineral oil in a 35 x 10 mm Petri dish. The dishes were kept in the incubator for about 30 minutes before spermatozoa were added for sperm penetration assays.

Frozen-thawed spermatozoa were cryopreserved from a fertile Pietrain boar as described by Roca et al. (2003) in a plastic medium-straw (0.5 mL) and thawed in circulating water at 37ºC for 20 s. Thawed spermatozoa were re-suspended in modified TCM-199 for co-incubation with IM oocytes. For IVM oocytes, thawed semen was washed three times by centrifugation at 1, 900 x g for 3 min in Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/mL BSA (Fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 µg/mL kanamycin (mDPBS). Spermatozoa were co-incubated with IM or IVM oocytes at a oocyte:spermatozoa ratio of 1:66,000 (Martinez et al., 1993) or 1:2,000 (Gil et al., 2004), respectively. The oocytes were co-

incubated with the spermatozoa at 39ºC in an atmosphere of 5% CO_2 in air, the IM oocytes for 16 h and the IVM oocytes for a primary period of 6 h. The IVM oocytes were thereafter washed three times in pre-equilibrated embryo culture medium (NCSU-23 containing 0.4% BSA), transferred to a Nunc 4-well multi-dish containing 500µL of the same medium per well (covered by mineral oil) and cultured for another 6 h at 39ºC and 5% CO_2 in air.

Following the co-incubation, IM-oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells (group of CE oocytes) and those spermatozoa attached to the surface of the ZP (groups of CE and denuded oocytes). The IM (16 h post insemination) and the IVM oocytes (12 h post insemination) were mounted on slides, fixed and stored in 25% (v/v) acetic acid in ethanol at room temperature for 48-72 hours, stained with 1% lacmoid in 45% (v/v) acetic acid, and examined under a phase contrast microscope at 400x magnification. Oocytes were considered penetrated when spermatozoa with unswollen (IM-oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM-oocytes) were found in the ooplasm.

3.4 Experimental design

Experiments were designed to disclose the effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/mL) during sperm-oocyte co-incubation on penetration rates and number of spermatozoa per oocyte in IM (experiment 1, including a total of 673 IM-oocytes) or IVM oocytes (experiment 2, including a total of 730 IVM-oocytes). All experiments were carried out with the same batch of lyophilized proteins. In addition,

frozen-thawed spermatozoa from a single ejaculate were used to avoid inter-ejaculate variability. In order to rule out confounding effects related to the presence of cumulus cells, CE and denuded IM-oocytes were exposed to the heterodimer during co-incubation with frozen-thawed boar spermatozoa (single batch) in a 2 x 2 factorial design. Controls were provided by PSP-I/PSP-II unexposed, co-incubated gametes. All experiments were replicated 3 times.

3.5 Data analysis

All the data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analysed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effect of presence of PSP-I/PSP-II heterodimer and the random effect of replicate. For experiment 1, presence or absence of CE was included as fixed effect and interaction with the heterodimer PSP-I/PSP-II was considered. Data of percentage of penetration were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p<0.05$.

4.- Experience 4: Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to penetrate homologous oocytes in vitro?

4.1. Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar SP

All experiments were performed with the SP from 4 sexually mature boars, which had previously sired offspring. Sperm-rich ejaculate fractions were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at 3800 x g for 15 minutes at 20°C. The supernatants were sequentially filtered through 10- and 1.2-µm filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non–heparin-binding fraction of SP by size exclusion chromatography on a 2000 x 5-cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, and 0.025% sodium azide (pH 7.4) (Calvete et al, 1995b). The identity and purity of the protein preparation were assessed by N-terminal sequence analysis (using a 472 automated protein sequencer, Applied Biosystems, Langen, Germany) and MALDI-TOF mass spectrometry using a Voyager DE-Pro mass spectrometer (Applied Biosystems). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a molar absorption coefficient (27 332 M-1 cm-1) determined by Menendez et al. (1995) or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 hours at 106°C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilized, and stored at -20°C until used.

4.2 Handling, incubation with the heterodimer PSP-I/PSP-II and evaluation of spermatozoa

Fresh semen was received in commercial doses for artificial insemination (collected from different boars of proven fertility and extended with Beltsville Thawing Solution; Pursel and Johnson, 1975) at a concentration of 30 x 10^6/mL of spermatozoa. Semen from 4 different boars and doses was pooled and centrifuged (1900 x g for 3 minutes), the supernatant discarded, and the pellet resuspended to adjust the sperm concentration with phosphate-buffered saline (PBS) to 10 x 106/mL of spermatozoa.

Spermatozoa from a fertile Pietrain boar were cryopreserved as described by Roca et al (2003) in a plastic medium straw (0.5 mL). Spermatozoa were thawed in circulating water at 37°C for 20 seconds and washed 3 times by centrifugation at 1900 x g for 3 minutes in Dulbecco PBS supplemented with 4 mg/mL of bovine serum albumin (BSA) (fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose, and 70 µg/mL of kanamycin. After washing, the sperm pellet was resuspended in PBS to a final concentration of 10 x 10^6/mL of spermatozoa.

Ten million spermatozoa (fresh or frozen-thawed) extended as described above were preincubated without (control) or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized proteins) at 38°C. After 30 and 120 minutes of preincubation, sperm viability, motility, and acrosome status were evaluated.

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes Europe, Molecular Probes Europe, Leiden, The Netherlands). Briefly, 0.5 mL of sperm suspension containing 5 x 10^6 of fresh or frozen-thawed spermatozoa was stained with 25 nM SYBR-14 solution and 12 µM propidium iodide (PI) solution. Samples were incubated at room temperature in the dark for 10 minutes

before cytometric analysis. All analyses were performed by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc, Miami, Fla) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, Calif) with 15-mW laser power at 488 nm, and the software EXPO 2000 (Coulter Corporation). Forward and sideward light scatter were recorded for a total of 15 000 to 25 000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. SYBR-14 was measured through a 525-nm band pass filter, whereas PI was collected through a 635-nm band pass filter. Viable spermatozoa were defined as those stained with SYBR-14 and not stained with PI.

The percentage of motile spermatozoa was estimated with a computer-assisted motility analysis system. Sperm samples (5 µL of 10 x 10^6/mL of spermatozoa) were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot (Kanagawa, Japan) equipped with a 10x contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyzer (Barcelona, Spain). The program settings were as follow: frames rate, 25 Hz; search radius, 11.5 µm; minimum track points, 7 frames; and threshold straightness, 75%. Two samples were examined with at least 100 spermatozoa being analyzed per sample.

Live spermatozoa showing acrosome exocytosis were evaluated using simultaneously PI to stain dead cells and the lectin FITC-PNA to evaluate disrupted acrosome. Briefly, 0.5 mL of sperm suspension containing 5 x 10^6 of fresh or frozen-thawed spermatozoa was stained with 12 µM of PI and 5 µL of FITC-PNA stock

solution (1 mg/mL in bidistilled water). Spermatozoa were incubated for 5 minutes in the dark and analyzed immediately on the flow cytometer (see above). FITC-PNA was measured through a 530-nm band pass filter, whereas PI was measured through a 635-nm band pass filter. Spermatozoa were identified in 1 of the 3 following populations: PI positive, nonviable cells; PI negative and FITC-PNA negative, live spermatozoa with intact acrosome; and PI negative and FITC-PNA positive, corresponding to live spermatozoa with exocytosed acrosome.

4.3 Recovery, IVM, sperm penetration and evaluation of oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martinez et al, 1993; Abeydeera and Day, 1997). Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 µg/mL of kanamycin maintained at 34°C to 37°C. Cumulus-oocyte complexes (COCs) were aspirated from medium-sized follicles (3 to 6 mm in diameter) with an 18-gauge needle fixed to a 10-mL disposable syringe. Only COCs with a compact cumulus mass (with at least 6 or 7 layers), an intact ZP, and an oocyte with an evenly granulated cytoplasm were selected for the different trials.

For preparation of IM oocytes, batches of 30 IM oocytes were placed directly in 2 mL of pre-equilibrated modified TCM-199 medium (Cheng, 1985) and kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed 3 times in BSA-free North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL of cysteine, and 10 ng/mL of epidermal growth factor (IVM medium). Thereafter, COCs were transferred to a Nunc 4-well multidish plate (50 to 100 COCs per well) submerged in 500 μL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL of eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV), and cultured at 39°C in 5% CO2 in air for 22 hours. The medium was then changed for maturation medium without hormone supplementation and incubated at 39°C in 5% CO2 in air for another 22 hours. After IVM, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed 3 times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera and Day, 1997). The latter consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2 · 2H2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.2% BSA (fraction V; A 7888, initial fractionation by precipitation with cold alcohol). Batches of 50 IVM oocytes were placed in 50-μL drops of IVF medium covered with warm mineral oil in a 35 x 10-mm Petri dish. The dishes were kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

Spermatozoa exposed or not exposed to the PSP-I/PSP-II heterodimer, as described above, were centrifuged at 1000 x g for 3 minutes, and the sperm pellet was resuspended in modified TCM-199 medium for coincubation with IM oocytes or resuspended in IVF medium for coincubation with IVM oocytes. Spermatozoa were coincubated with IM or IVM oocytes at a oocyte-spermatozoa ratio of 1:66 000

(Martinez et al, 1993) or 1:2000 (Gil et al, 2004), respectively. The oocytes were coincubated with the spermatozoa at 39°C in an atmosphere of 5% CO2 in air, the IM oocytes for 16 hours and the IVM oocytes for a primary period of 6 hours. The IVM oocytes were thereafter washed 3 times in pre-equilibrated embryo culture medium (NCSU-23 containing 0.4% BSA), transferred to a Nunc 4-well multidish containing 500 µL of the same medium per well (covered by mineral oil), and cultured for another 6 hours at 39°C and 5% CO2 in air.

Following the coincubation, the IM oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells, and those spermatozoa attached to the surface of the ZP. The IM (16 hours post insemination) and IVM oocytes (12 hours post insemination) were mounted on slides, fixed, and stored in 25% (vol/vol) acetic acid in ethanol at room temperature for 48 to 72 hours, stained with 1% lacmoid in 45% (vol/vol) acetic acid, and examined under a phase contrast microscope at 400x magnification. Oocytes were considered penetrated when spermatozoa with intact (IM oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM oocytes) were found in the ooplasm.

The trials attempted to disclose the effect of the sperm exposition to PSP-I/PSP-II heterodimer on the ability of freshly diluted or frozen-thawed spermatozoa to penetrate pig oocytes (IM or IVM), being distributed as follows: 1) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IM oocytes, a total of 175 IM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 minutes at 38°C, whereas 182 IM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer

(control); 2) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 400 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 415 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); 3) To study the penetrating ability of frozen-thawed boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 792 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 773 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); and 4) Finally, an experiment was designed, based on the results of experiment 3 with frozen-thawed spermatozoa, to evaluate the effect of sperm washing (by centrifugation in saline BSA or through Percoll gradient separation) to cleanse the sperm surface of frozen-thawed spermatozoa and, consequently, on the penetration capability of these spermatozoa on IVM oocytes. For this purpose, aliquots of 3 mL of re-extended postthaw semen (10 x 106/mL of spermatozoa in PBS) were preincubated for 2 hours at 38°C with 1.5 mg/mL of PSP-I/PSP-II heterodimer in PBS. Thereafter, spermatozoa were either washed 3 times by centrifugation at 1200 x g for 3 minutes in 0.9% saline solution containing 1 mg/mL of BSA, with the final pellet being resuspended in fertilization medium at 2 x 106/mL of spermatoza, or centrifuged through a 35%/70% Percoll gradient (900 x g for 20 minutes), the lowest pellet being recovered and resuspended with fertilization medium at 2 x 106/mL of spermatozoa. Control groups were built by 1) spermatozoa preincubated in the presence of PSP-I/PSDP-II heterodimer as described above but pelleted without washing or 2) spermatozoa preincubated in the absence of PSP-I/PSP-II heterodimer. A total of 1592 IVM oocytes were cocultured with the spermatozoa as above described.

4.4 Statistical analysis

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) using the MIXED procedure according to a statistical model that included the fixed effects of presence of PSP-I/PSP-II heterodimer and incubation time and the random effect of replicate. In the last experiment, a washing procedure was included as a fixed effect. To analyze data of sperm viability, motility, and acrosome status, percentages were subjected to arcsine transformation before analysis. Data on the percentage of penetration and polyspermia were modeled according to the binomial model of parameters as described by Fisz (1980) before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be statistically significant when $P < .05$. Experiments were replicated 4 times.

CONCLUSIONS

1. The addition of 10% of seminal plasma from certain boars maintains or enhances the viability of largely extended boar spermatozoa *in vitro*. As a result of significant differences among sires, a screening for “good boar” seminal plasma sources is recommended.

2. The protective effect of the PSP-I/PSP-II heterodimer on highly-extended boar spermatozoa could be related to the adhesion of the heterodimer to the acrosome domain of the sperm head plasma membrane. These results may

indicate a stabilizing effect of the heterodimer on the fluidity of the sperm membrane.

3. Exposures of the gametes to the heterodimer during *in vitro* gamete co-incubation significantly decrease the sperm penetration rates and number of spermatozoa per oocytes in both IM and IVM denuded oocytes. Such an effect is not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP receptors.

4. Short-time exposure of fresh or frozen-thawed boar spermatozoa to PSP-I/PSP-II preserves sperm viability and motility. Although there is no obvious influence of the heterodimer on the capability of fresh diluted boar spermatozoa to penetrate homologous oocytes, PSP-I/II exerts a deleterious effect when frozen-thawed spermatozoa are used to penetrate IVM-oocytes. However, this inhibition did not seem permanent since a subsequent washing through a Percoll gradient restored sperm function in some of the cells.