



**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**  
**POSGRADO EN CIENCIAS BIOLÓGICAS**

FACULTAD DE MEDICINA  
BIOLOGÍA EXPERIMENTAL

**PARTICIPACIÓN DE PROTEÍNAS ASOCIADAS A LA MEMBRANA PLASMÁTICA DEL  
ESPERMATOZOIDE HUMANO DURANTE LA CAPACITACIÓN Y LA INTERACCIÓN DE  
GAMETOS**

**TESIS**

QUE PARA OPTAR POR EL GRADO DE:  
**DOCTORA EN CIENCIAS**

PRESENTA:

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CD. MX., ENERO, 2020



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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

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**M. en C. Ivonne Ramírez Wence**  
Directora General de Administración Escolar, UNAM  
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, celebrada el día **07 de octubre de 2019** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **HERNÁNDEZ SILVA GABRIELA** con número de cuenta **406013949** con la tesis titulada **“PARTICIPACIÓN DE PROTEÍNAS ASOCIADAS A LA MEMBRANA PLASMÁTICA DEL ESPERMATOZOIDE HUMANO DURANTE LA CAPACITACIÓN Y LA INTERACCIÓN DE GAMETOS”**, realizada bajo la dirección de la **DRA. MAYEL DEL VALLE CHIRINOS ESPÍN**, quedando integrado de la siguiente manera:

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Suplente: DRA. ROCÍO ÁNGELES GARCÍA BECERRA

Sin otro particular, me es grato enviarle un cordial saludo.

**A T E N T A M E N T E**  
**“POR MI RAZA HABLARA EL ESPÍRITU”**  
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## **DEDICATORIA**

A mis papás

A mi hermana

A mi familia

A mis amigos



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## ABREVIATURAS

2-DE	Electroforesis en geles bidimensionales
BCA	Ácido bicinconínico
BSA	Albúmina sérica bovina
Cal	Ionóforo de calcio (A23187)
CAP	Espermatozoides capacitados
FITC	Isotiocianato de fluoresceína
FS	Fluido seminal
HSA	Albúmina sérica humana
HRP	Enzima peroxidasa de rábano
HTF	Fluido tubárico humano
IEF	Isoelectroenfoque
LTF	Lactotransferrina
NC	Espermatozoides no capacitados
O.M.S.	Organización Mundial de la Salud
PAMP	Proteínas Asociadas a la Membrana Plasmática
PBS	Solución salina amortiguadora de fosfatos
pl	Punto isoeléctrico
PIP	Proteína inducible por prolactina
PM	Peso molecular
PSA	Antígeno prostático específico
PVA	Polivinil alcohol
RA	Reacción acrosomal
SDS-PAGE	Electroforesis en condiciones desnaturalizantes
Spm	Espermatozoides
TBS	Solución salina amortiguadora con Tris
ZAG	Glicoproteína zinc alfa 2
ZP	Zona pelúcida

## RESUMEN

Los espermatozoides en el eyaculado se encuentran recubiertos por proteínas de superficie adquiridas a su paso por el epidídimo y al combinarse con el fluido seminal, las cuales mantienen al espermatozoide en un estado no capacitado. Durante la residencia en el tracto reproductor femenino, estas proteínas deben removerse para que los espermatozoides puedan capacitarse y fecundar. El objetivo de esta tesis fue caracterizar las proteínas asociadas a la membrana plasmática del espermatozoide (PAMP) humano que se liberan durante la capacitación *in vitro* y evaluar sus efectos sobre la función espermática. Para ello, se obtuvieron las PAMP a partir de muestras de semen de donadores normozoospermicos y se caracterizaron por medio de electroforesis en geles de poliacrilamida de una y dos dimensiones. Adicionalmente, las proteínas se identificaron por medio de espectrometría de masas y se emplearon para evaluar su efecto en la movilidad, la fosforilación en tirosinas de proteínas espermáticas, la reacción acrosomal y la interacción con la zona pelúcida. Los resultados mostraron que se liberan al menos 29 proteínas durante la capacitación *in vitro*, destacando la proteína inducible por prolactina, la lactoferrina y el antígeno prostático específico. La incubación de espermatozoides humanos no capacitados con las PAMP (1.6 y 3.2 mg/ml) disminuyó el porcentaje de espermatozoides hiperactivados, la fosforilación en tirosinas y la reacción acrosomal inducida por ionóforo de calcio. En cambio, cuando espermatozoides capacitados se incubaron con las mismas concentraciones de PAMP, sólo se inhibió la hiperactivación y la interacción con la zona pelúcida. Los resultados indican que las PAMP de humano pueden disociarse y re-asociarse a la membrana plasmática y tienen un efecto descapacitante. El presente trabajo contribuye a comprender mejor los mecanismos que regulan la capacitación espermática en humano y podría ayudar a mejorar el diagnóstico de algunas patologías asociadas con la infertilidad masculina.

## ABSTRACT

Ejaculated spermatozoa are covered by surface proteins acquired during epididymal transit and when combined with the seminal fluid that keeps the sperm uncapacitated. During female reproductive tract residency, these proteins must be removed in order to allow sperm capacitation and fertilization. The objective of this thesis was to characterize the sperm plasma membrane-associated proteins (SMAP) released during *in vitro* human capacitation and to study their effects on sperm function. For this purpose, SMAP were obtained from semen samples of normozoospermic donors and characterized by one and two dimensional electrophoresis. Additionally, proteins were identified by mass spectrometry and used to evaluate their effect on motility, protein tyrosine phosphorylation, acrosome reaction and interaction with zona pellucida. Results showed that at least 29 were released during *in vitro* capacitation, highlighting the prolactin-inducible protein, the lactoferrin and the prostate-specific antigen. Incubation of human non-capacitated spermatozoa with the SMAP (1.6 and 3.2 mg/ml) decreased the percentage of hyperactivated sperm, the tyrosine phosphorylation and the acrosome reaction induced by calcium ionophore. On the other hand, when capacitated sperm were incubated with same concentrations of SMAP, only hyperactivation and interaction with zona pellucida were inhibited. These results indicate that human SMAP can dissociate and re-associate to the sperm plasma membrane and have a decapacitating effect. The present work contributes to a better understanding of the mechanisms that regulate human sperm capacitation and may help to improve the diagnosis of some pathologies related to male infertility.

# 1. INTRODUCCIÓN

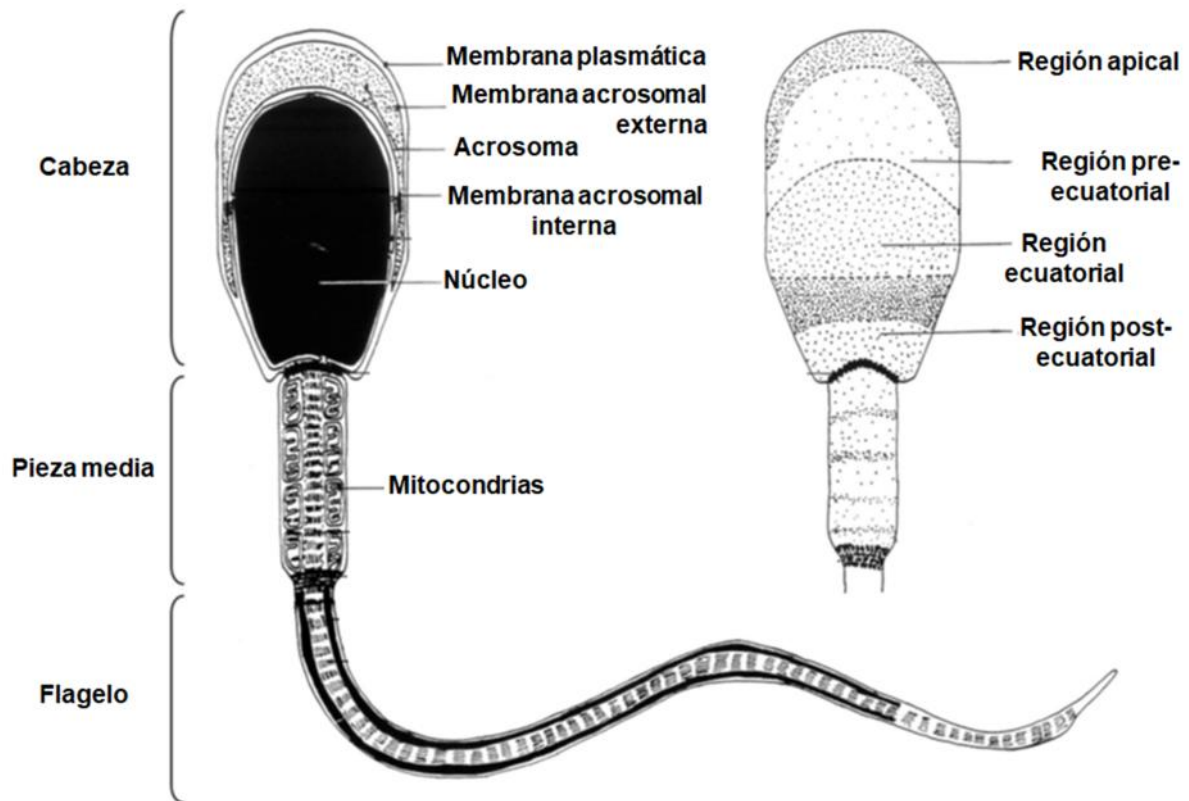
## 1.1. Generalidades sobre la fecundación

La fecundación es el proceso mediante el cual se fusionan los gametos masculino y femenino para formar el cigoto y dar origen a un nuevo individuo. En mamíferos, para que el espermatozoide recién eyaculado pueda fecundar al ovocito, es necesario que migre a través del tracto reproductor femenino en donde adquiere la capacidad de fecundar, proceso conocido como capacitación. Únicamente los espermatozoides capacitados pueden atravesar el cúmulo oóforo, penetrar la zona pelúcida y fusionarse con el oolema del ovocito [1].

## 1.2. Estructura del espermatozoide

El espermatozoide se compone de la cabeza y el flagelo, los cuales se encuentran unidos por la pieza de conexión. En la cabeza, la membrana plasmática tiene una morfología altamente polarizada en la que se distinguen 4 regiones, que son la región apical, la pre-ecuatorial, la ecuatorial y la post-ecuatorial [2]. Por debajo de la membrana plasmática, se localiza el acrosoma y el núcleo haploide que contiene la cromatina altamente condensada. El acrosoma es una vesícula derivada del aparato de Golgi que está compuesto por la membrana acrosomal interna, la membrana acrosomal externa y el segmento ecuatorial que une estos dominios. El lumen del acrosoma contiene componentes solubles y la matriz acrosomal, entre los que se encuentran proteasas, glicosidasas y proteínas de unión a la zona pelúcida [3]. Por su parte, el flagelo está constituido por el axonema, el cual consiste de un par de microtúbulos centrales rodeado por nueve pares de microtúbulos, y se divide en cuatro regiones denominadas pieza de conexión, pieza media, pieza principal y pieza terminal [4]. El movimiento del flagelo es mediado por las dineínas, que son las proteínas motoras localizadas en los microtúbulos. Estas proteínas utilizan la energía que se libera de la hidrólisis del ATP, el cual se genera a partir de la respiración celular en las mitocondrias ubicadas de la pieza media y por la glucólisis en la cabeza y la pieza principal del flagelo (Fig. 1.1) [5].



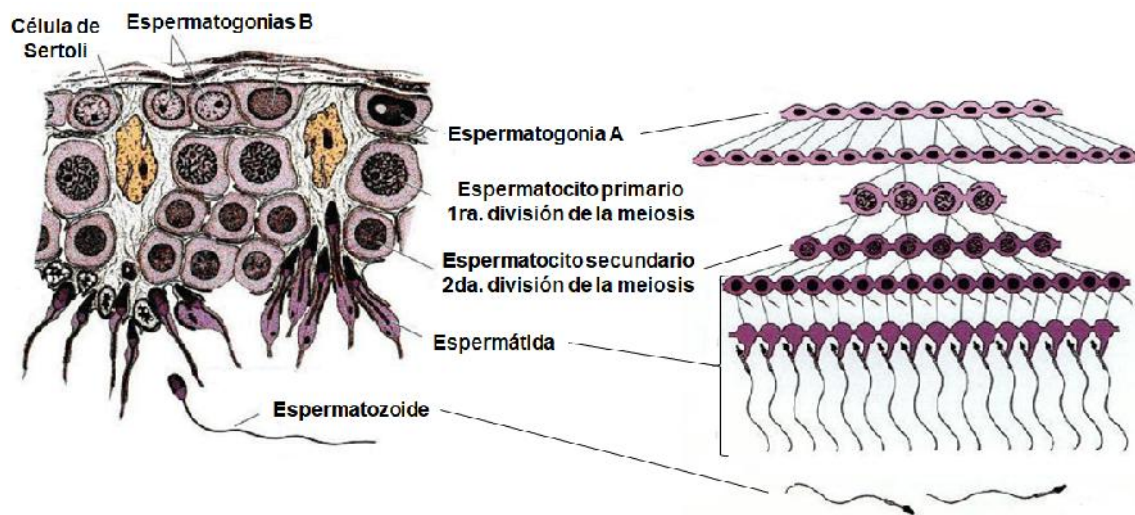


**Fig. 1.1. Estructura del espermatozoide humano.** A la izquierda se muestra la estructura de un espermatozoide humano, el cual se compone de cabeza, pieza media y flagelo. A la derecha se muestra una ampliación de la cabeza y sus subdominios. Modificado de Gadella (2008) [2].

### 1.3. Espermatogénesis

La espermatogénesis es el proceso mediante el cual las células germinales masculinas denominadas espermatogonias se diferencian dentro de los túbulos seminíferos de los testículos para dar lugar a espermatozoides. Las espermatogonias A son células diploides ubicadas en la base de los túbulos seminíferos que se dividen por mitosis para generar espermatogonias tipo B, las cuales atraviesan la barrera hemato-testicular para formar los espermatocitos primarios. Estas células inician la primera división de la meiosis, en la cual hay intercambio de DNA entre cromátidas homólogas, y como resultado se producen dos células haploides duplicadas a partir de cada espermatocito primario llamadas

espermatoцитos secundarios. Estos permanecen en interfase hasta que se inicia la segunda división de la meiosis para producir 4 espermátidas con número haploide de cromosomas. Posteriormente, las espermátidas pasan por una serie de transformaciones conocidas como fase de Golgi, de cubierta, acrosómica y de maduración. Algunos de los cambios que se producen son la pérdida de citoplasma, la formación del acrosoma a partir del aparato de Golgi, la migración de los centriolos hacia el polo contrario del acrosoma, la reorganización y compactación de la cromatina, la elongación del núcleo, la acumulación del citoplasma residual y de la membrana celular en un extremo de la célula para formar el flagelo y la localización de las mitocondrias alrededor de la parte proximal del flagelo, formando la pieza media. Por último, los espermatozoides se liberan a la luz de los túbulos seminíferos (Fig. 1.2) [1, 4].



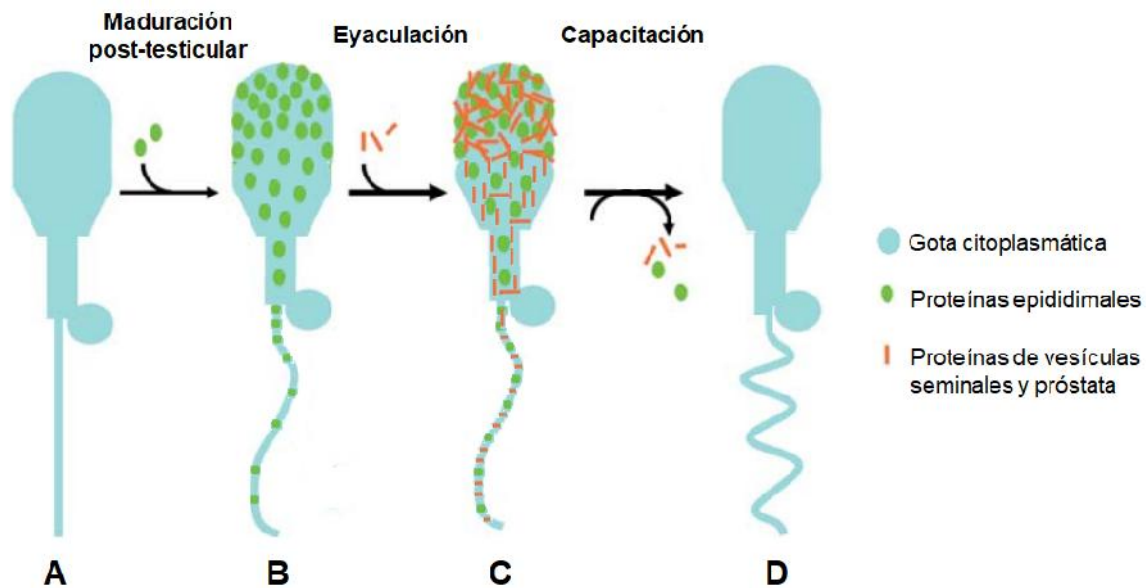
**Fig. 1.2. Espermatogénesis.** En el epitelio de los túbulos seminíferos, las espermatogonias se diferencian para dar origen a los espermatozoides. A la izquierda se muestra un corte transversal del túbulo seminífero que muestra las relaciones entre las células de Sertoli y las células germinales en desarrollo. A la derecha se muestran las fases de la espermatogénesis. Modificado de Gilbert (2013) [6].

#### **1.4. Maduración post-testicular del espermatozoide**

Los espermatozoides liberados de los testículos están completamente diferenciados pero aún no tienen capacidad para fecundar (Fig. 1.3.A). Para convertirse en células competentes, es necesario que pasen por dos procesos subsiguientes de maduración funcional. El primero de ellos es la maduración post-testicular, la cual ocurre durante su tránsito por el epidídimo y durante la eyaculación. A medida que los espermatozoides transitan por el epidídimo y durante su almacenamiento, experimentan una serie de cambios morfológicos, bioquímicos y fisiológicos que incluyen modificaciones en el tamaño y la apariencia del acrosoma y el núcleo, la migración de la gota citoplasmática a lo largo del flagelo, la asociación de proteínas epididimales a la membrana plasmática y la adquisición de movilidad progresiva (Fig. 1.3.B) [7]. Durante la eyaculación, los espermatozoides almacenados en el epidídimo se transportan por el conducto deferente hacia la base de la próstata y el conducto de la vesícula seminal. Allí se combinan con las secreciones de las vesículas seminales, la próstata y las glándulas bulbouretrales, las cuales constituyen el fluido seminal (FS) en el que los espermatozoides están suspendidos para su deposición en el tracto reproductor femenino (Fig. 1.3.C) [8].

La mayor contribución al FS proviene de las secreciones de las vesículas seminales, mismas que son ricas en fructosa, aminoácidos, ácido cítrico, ácido ascórbico y proteínas como las prostaglandinas, las cuales estimulan las contracciones uterinas que ayudan al transporte de los espermatozoides a través del tracto reproductor femenino [9]. Por su parte, la próstata produce una secreción rica en calcio, potasio, zinc, ácido cítrico, fosfatasa ácida y fibrinolisisina, mientras que las glándulas bulbouretrales secretan fundamentalmente mucina [4]. El FS le proporciona a los espermatozoides las condiciones adecuadas para mantener su viabilidad [10, 11]. Además, sirve como fuente de nutrientes, los protege contra infecciones y permite que superen el entorno ácido de la vagina [12]. Adicionalmente, las secreciones que conforman el eyaculado contienen glicoproteínas que se asocian a la membrana plasmática de los espermatozoides,

las cuales contribuyen a inhibir el progreso de la capacitación y por tanto se conocen como factores descapacitantes [13].



**Fig. 1.3. Cambios en la membrana plasmática del espermatozoide.** (A) Los espermatozoides que salen del testículo se encuentran morfológicamente maduros. (B) En el epidídimo sufren cambios fisiológicos como la unión de proteínas que se asocian a su membrana plasmática y la adquisición de movilidad progresiva. (C) Durante la eyacuación, la vesícula seminal y la próstata liberan proteínas que se unen a la membrana plasmática del espermatozoide, las cuales actúan como factores descapacitantes. (D) Los factores descapacitantes se remueven de la superficie de los espermatozoides en el tracto reproductor femenino. Modificado de Leahy y Gadella (2011) [14].

## 1.5. Capacitación espermática

El segundo proceso de maduración se conoce como capacitación espermática [15, 16]. Este proceso inicia cuando el semen se deposita en el tracto reproductor femenino, el cual pasa por un proceso de licuefacción que permite la salida de los espermatozoides para entrar en contacto con el moco cervical y atravesarlo. Posteriormente, en su viaje hacia el ámpula para encontrarse con el ovocito, los

espermatozoides interactúan con moléculas de superficie y secreciones del útero y del oviducto, mismas que modulan su función [1].

Entre los cambios tempranos que ocurren durante la capacitación espermática está la remoción y redistribución del colesterol y de los factores descapacitantes (Fig. 1.3.D). Para ello, la albúmina presente en las secreciones del tracto reproductor femenino actúa como aceptor del colesterol. Posteriormente, se produce un influjo del ion bicarbonato ( $\text{HCO}^{-3}$ ), el cual promueve la redistribución de colesterol remanente en la membrana e induce el reordenamiento de los fosfolípidos [17], todo lo cual incrementa la fluidez y permeabilidad de la membrana plasmática. Paralelamente, se produce un aumento en la concentración intracelular de calcio, que proviene de pozas intracelulares localizadas en el acrosoma y en las mitocondrias y del medio extracelular, el cual ingresa a través de canales iónicos como Catsper [18], TRPM8 [19] y TRPV4 [20].

El aumento en las concentraciones iónicas intracelulares afecta el potencial de membrana y el pH intracelular. En ratones, los gametos pasan de tener un potencial de membrana entre -35 y -45 mV en espermatozoides no capacitados a valores de aproximadamente -65 mV luego de la capacitación [21]. En cuanto al pH intracelular, éste se alcaliniza pasando de 6.7 a 6.85 en humano [22], lo cual está regulado por intercambiadores  $\text{Na}^+/\text{H}^+$ ,  $\text{Cl}^-/\text{HCO}^{-3}$  y por canales de protones dependientes de voltaje [23, 24]. Este aumento de pH es importante debido a que regula la activación de algunos canales dependientes de pH como Slo3 [25] y Catsper [18].

Los altos niveles de  $\text{HCO}^{-3}$  y calcio intracelular activan la enzima adenilato ciclasa soluble, la cual incrementa los niveles de AMPc que actúa como segundo mensajero para activar a la proteína cinasa A (PKA). La PKA activa la cinasa de tirosinas rica en prolina 2 (PYK2) [26], misma que participa en la fosforilación en tirosinas de proteínas como la proteína de anclaje de la cinasa A 82 (AKAP82) y su precursor la pro-AKAP82 [27]. En modelo murino, se ha sugerido que la proteína cinasa Fer podría igualmente participar en este proceso [28]. El incremento en la fosforilación en tirosinas de algunas proteínas espermáticas

constituye uno de los rasgos característicos del proceso de capacitación, tanto en humano como en ratón [29, 30].

Otro evento asociado con la capacitación son los cambios en la movilidad del espermatozoide, que pasa de un movimiento mayormente progresivo a otro vigoroso no progresivo con amplio batido flagelar y amplio desplazamiento lateral de la cabeza, denominado hiperactivación [31]. La hiperactivación es necesaria para que el espermatozoide se libere del reservorio en las criptas del oviducto y atraviese el cúmulo oóforo y la zona pelúcida del ovocito [32, 33]. La hiperactivación está regulada por la vía AMPc/PKA, y por factores como el calcio y el  $\text{HCO}^{-3}$ . Para que este proceso ocurra, los brazos de dineína de los axonemas se fosforilan, activando la ATPasa de la dineína para hidrolizar el ATP y generar la energía necesaria para que los microtúbulos se deslicen. La desfosforilación de la dineína por fosfatasas dependientes de calmodulina revierte este proceso. Debido a que la dineína produce una fuerza unidireccional, la movilidad del flagelo requiere que la fosforilación/desfosforilación y por tanto la activación e inactivación de los brazos de dineína ocurra de manera asincrónica [34].

Hacia el final de la capacitación, en las proximidades del complejo cúmulo oóforo-ovocito, los espermatozoides pasan por un proceso conocido como la reacción acrosomal, en el cual se produce la exocitosis del acrosoma. Este proceso inicia con el influjo de calcio que permite la fusión de la membrana plasmática y la membrana acrosomal externa en múltiples sitios, lo cual provoca la formación de vesículas cuya dispersión permite la liberación del contenido enzimático del acrosoma al medio extracelular. Este proceso requiere de la remodelación de los haces de F-actina del citoesqueleto del espermatozoide [35]. Este proceso puede estimularse *in vitro* por la progesterona [36], el ATP [37] y la zona pelúcida, específicamente las proteínas ZP1 [38], ZP3 y ZP4 [39] que conforman la zona pelúcida humana. La reacción acrosomal es imprescindible para la fecundación, ya que sólo los espermatozoides reaccionados pueden atravesar la zona pelúcida y fusionarse con el ovocito [36, 40].

## 1.6. Proteínas descapacitantes del FS

Las proteínas descapacitantes inhiben el progreso de la capacitación [13, 41], por lo que para que el espermatozoide pueda fecundar es necesaria su remoción, la cual permite exposición de receptores en la membrana plasmática que interactúan con diferentes moléculas presentes en las secreciones y superficie del tracto reproductor femenino.

Las proteínas descapacitantes de origen epididimal más estudiadas son las del ratón, donde se han identificado la proteína DF y la proteína de unión a fosfatidiletanolamina 1 (PEBP1) [41]. Durante la capacitación, DF se libera de la membrana plasmática permitiendo el aumento en la concentración de calcio mediante la activación de una ATPasa de calcio sensible a calmodulina [42]. Se ha descrito que la eliminación de esta proteína de la membrana plasmática de espermatozoides no capacitados aumenta su fertilidad, mientras que cuando se incuban con espermatozoides capacitados inhibe la reacción acrosomal [43]. DF se une a la región post-acrosomal de la cabeza de espermatozoides de ratón [44]. Por otra parte, PEBP1 inhibe la actividad de la cinasa de serinas-treoninas Raf1, la cual está implicada en la fosforilación de tirosinas en ratón [45] e inhibe la interacción del espermatozoide con la zona pelúcida así como la reacción acrosomal [41]. Esta proteína se une a la región post-acrosomal y al flagelo de espermatozoides de ratón y humano y forma complejos con la proteína DF [46]. En el modelo de rata [47] y cerdo [48] se ha identificado la proteína descapacitante HongrES1, la cual pertenece a la familia de inhibidores de serinas proteasas. Esta proteína se secreta en el epidídimo y se une a la región acrosomal de la cabeza de los espermatozoides. Posteriormente, se libera de forma parcial durante la capacitación y desaparece completamente durante la reacción acrosomal. HongrES1 se considera un factor descapacitante debido a que inhibe el influjo de calcio así como la hiperactivación [48].

Igualmente se han descrito algunas proteínas descapacitantes secretadas por las vesículas seminales. La proteína SPINK3 (también conocida como P12) se une a la región anterior del acrosoma de espermatozoides de ratón y actúa como

inhibidor del transporte de calcio durante la capacitación [49]. Adicionalmente, inhibe la unión a la zona pelúcida y la inducción de la reacción acrosomal por las proteínas de la zona pelúcida [50]. Por su parte, la SVS2 se une a la región post-acrosomal de espermatozoides de ratón, inhibiendo la fosforilación en tirosinas, la reacción acrosomal inducida por progesterona y por lo tanto la capacidad de fertilizar de los espermatozoides [51].

Las proteínas descapacitantes anteriormente descritas tienen su origen en las secreciones que conforman el FS. Sin embargo, se ha identificado en humano una proteína que actúa como inhibidor intrínseco de la capacitación. La proteína NYD-SP27, la cual es una isoforma de la fosfolipasa C, se localiza en el acrosoma y se libera durante la capacitación y la reacción acrosomal, pero de no hacerlo disminuye el número de espermatozoides capacitados e inhibe la reacción acrosomal inducida por ATP y progesterona [52].



## **2. JUSTIFICACIÓN**

Para que la capacitación espermática se lleve a cabo es necesaria la remoción de proteínas asociadas a la membrana plasmática del espermatozoide, mismas que se adquieren durante su paso por el epidídimo y la eyaculación. Estudios previos en diferentes modelos de mamífero han mostrado que algunas de estas proteínas mantienen al gameto en un estado no capacitado. Por lo tanto, los espermatozoides de humano presentes en el semen también podrían estar asociados a proteínas de superficie que inhiban su capacitación. Sin embargo, las proteínas descapacitantes del semen humano no se han caracterizado. Por lo tanto, su identificación y estudio podría contribuir a entender los mecanismos que regulan la capacidad fecundante del gameto masculino.

### **3. HIPÓTESIS**

Si se remueven las Proteínas Asociadas a la Membrana Plasmática (PAMP) del espermatozoide humano eyaculado, entonces podrá capacitarse e interactuar con la zona pelúcida del ovocito.

### **4. OBJETIVO GENERAL**

Caracterizar las PAMP del espermatozoide humano que se liberan durante la capacitación *in vitro* y estudiar sus efectos sobre la fisiología espermática.

#### **4.1. Objetivos específicos**

- Aislar, identificar y caracterizar las PAMP del espermatozoide que se liberan durante la capacitación *in vitro*.
- Estudiar la participación de las PAMP en la regulación de la capacitación del espermatozoide.
- Determinar si las PAMP inhiben la interacción del espermatozoide con la zona pelúcida.

## 5. METODOLOGÍA

Esta investigación fue aprobada por el Comité Científico y de Ética en Investigación Humana del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Todos los participantes dieron por escrito su consentimiento informado.

### 5.1. Procesamiento de las muestras de semen

Las muestras de semen utilizadas fueron donadas por voluntarios sanos con edades comprendidas entre 18-40 años de edad. Las muestras se obtuvieron mediante masturbación luego de un período de abstinencia de 3 a 5 días y se incubaron durante un mínimo de 30 min a 37°C para permitir su licuefacción. Posteriormente, se evaluaron de acuerdo a los parámetros y valores de referencia del Manual de la Organización Mundial de la Salud (O.M.S.) [53]. Sólo se emplearon en este estudio aquellas muestras que se clasificaron como normozoospermicas.

Los espermatozoides móviles se separaron del FS por medio de la técnica de *swim-up* en medio Fluido Tubárico Humano (HTF) (Ver anexo 4), suplementado con piruvato de sodio [0.33 mM] y polivinil alcohol (PVA) (1 mg/ml) [37] o albúmina sérica humana (HSA) al 0.3%. Para ello, 250 µl de semen se colocaron en el fondo de tubos cónicos de 15 ml y encima se colocaron 800 µl de medio HTF con cuidado de no mezclar las dos fases. Después de 1 h de incubación a 37°C con una inclinación de los tubos de 45°, se recuperaron 750 µl de la fracción superior conteniendo los espermatozoides móviles que migraron hacia el medio, los cuales se re-evaluaron y ajustaron a una concentración de  $15 \times 10^3$  espermatozoides/ml.

Alternativamente, las muestras se procesaron mediante centrifugación a través de gradientes discontinuos de densidad (ISolate™; Irvine Scientific, Santa Ana CA, EE.UU.). Para ello, en un tubo cónico se colocó 1 ml de la muestra de semen sobre un gradiente formado por dos fases de diferentes densidades (50% y 90% de sílice), y se centrifugó a 2,800 rpm durante 30 min. El FS se recuperó a partir

de la fase superior a los gradientes y fue re-centrifugado a 10,000 rpm durante 20 min a 4°C para eliminar contaminantes celulares. Por su parte, los botones celulares resultantes se colectaron y lavaron con solución amortiguadora de fosfatos (PBS) para luego evaluar su densidad y el porcentaje de células móviles totales. En todos los casos, sólo se emplearon las muestras de espermatozoides con al menos 75% de células móviles.

## **5.2. Obtención de las PAMP**

Los espermatozoides móviles recuperados por medio de *swim-up* o gradientes de densidad se incubaron durante 6 h en medio HTF suplementado con PVA bajo condiciones capacitantes (37°C, 5% CO<sub>2</sub>, 95% de humedad). Los espermatozoides se separaron del medio de capacitación mediante centrifugación a 2,800 rpm durante 8 min. Posteriormente, se colectaron los sobrenadantes y se centrifugaron a 10,000 rpm durante 8 min para eliminar contaminantes celulares. La concentración de proteínas en los medios que contenían las PAMP se cuantificó por medio del método del ácido bicinconínico (BCA; Pierce, Rockford IL, EE.UU.). Para los diferentes estudios, se prepararon mezclas de PAMP y de FS a partir de muestras de 6 donadores diferentes, en donde cada muestra aportó la misma cantidad de proteínas. La ausencia de contaminación celular en las PAMP se verificó bajo el microscopio y por medio de Western blot empleando anticuerpos monoclonales anti- $\alpha$ -tubulina (Sigma-Aldrich; St. Louis MO, EE.UU.), siguiendo los procedimientos descritos más adelante (Apartado 5.6.2).

## **5.3. Caracterización de las PAMP**

### **5.3.1. Electroforesis en geles desnaturizantes de una dimensión (SDS-PAGE)**

Las PAMP se caracterizaron por medio de electroforesis en condiciones desnaturizantes (SDS-PAGE). Para ello, 25  $\mu$ g de las PAMP se re-suspendieron en solución desnaturizante para muestra (ver anexo 4) y se separaron mediante SDS-PAGE en geles de gradiente de poliacrilamida del 4-20% (Bio-Rad; Hercules

CA, EE.UU.). A continuación, los geles se tiñeron con el colorante azul de Coomassie para observar las proteínas [54].

### **5.3.2. Electroforesis en geles bidimensionales (2-DE)**

Adicionalmente, las PAMP se analizaron mediante electroforesis en geles bidimensionales (2-DE). La primera dimensión consistió en separar las proteínas según su punto isoeléctrico mediante isoelectroenfoque (IEF). Para ello, 169 µg de las PAMP se diluyeron en buffer de rehidratación (Ver anexo 4) a un volumen final de 125 µl y la mezcla se utilizó para rehidratar tiras de gel de 7 cm con un rango de pH de 3-10 (ReadyStrip IPG; Bio-Rad) durante 12 h. Los IEF se llevaron a cabo en un equipo PROTEAN IEF Cell (Bio-Rad), siguiendo el protocolo de condiciones eléctricas sugerido en el manual que lo acompaña (Ver anexo1). Posteriormente, las tiras con las muestras se incubaron sucesivamente con buffer de equilibrio I y II (ver anexo 4) durante 10 min y 15 min respectivamente, y a continuación las proteínas se separaron mediante SDS-PAGE en geles de gradiente de poliacrilamida del 4-20%. Finalmente, los geles se tiñeron con azul de Coomassie y se fotodocumentaron en un equipo Chemidoc XRS+ Imager (Bio-Rad). Los análisis de los mapas proteínicos obtenidos se llevaron a cabo utilizando el software PD Quest (Bio-Rad).

### **5.4. Identificación de las PAMP**

La composición de las PAMP se analizó mediante espectrometría de masas de acuerdo a los protocolos establecidos en la Unidad de Servicios de Apoyo a la Investigación y la Industria (USAI) de la Facultad de Química de la UNAM [55]. Brevemente, las mezclas de proteínas se inmovilizaron por medio de SDS-PAGE en geles al 7% de poliacrilamida, seguido de su tinción con azul de Coomassie. La sección de gel conteniendo la mezcla de las PAMP se cortó en fragmentos más pequeños que se destiñeron con metanol al 50% (v/v) y ácido acético al 5% (v/v) durante 12 h. Los fragmentos resultantes se lavaron con agua destilada, seguido de dos lavados con bicarbonato de amonio 100 mM durante 15 min, para después reducirlos con DTT 50 mM durante 45 min y alquilarlos con iodoacetamida 30 mM

durante 2 h. Finalmente, las muestras se lavaron tres veces con bicarbonato de amonio 100 mM y se deshidrataron con acetonitrilo al 100%.

Para la digestión de las proteínas en gel, se adicionaron 30  $\mu$ l de una solución de tripsina de porcino modificada 20 ng/ $\mu$ l (Sigma-Aldrich) y se incubó durante 18 h a 37°C. Los péptidos resultantes se extrajeron del gel por sonicación en presencia de acetonitrilo al 50% (v/v) y ácido fórmico al 5% (v/v), y la fracción soluble se recuperó y llevó a sequedad total. Posteriormente, las muestras se resuspendieron en 20  $\mu$ l de ácido fórmico al 1% (v/v), se desalaron en columnas Zip Tip C18 (Millipore, Temecula CA, EE.UU.) y se eluyeron con 12  $\mu$ l de acetonitrilo al 3% y ácido fórmico al 0.1%.

Los péptidos resultantes se analizaron en un cromatógrafo de líquidos modelo nanoACQUITY (Waters; Cerdaniola de Valles, España) acoplado a un espectrómetro de masas de tiempo de vuelo modelo Synapt G2S (Waters) con sistema de ionización tipo electrospray. Los datos espectrométricos obtenidos se procesaron con el programa ProteinLynx Global (PLGS versión 2.5.1, Waters) para reconstruir los espectros MS/MS, utilizando una tasa máxima de falsos positivos de 4. Finalmente, los péptidos se contrastaron contra la base de datos UNIPROT (UniprotKB, <http://www.uniprot.org/>) utilizando tripsina como proteasa específica. Sólo los péptidos con un PLGS > 95% se aceptaron como correctos.

Posteriormente, la identidad de algunas de las proteínas identificadas se validó por medio de Western blot. Para ello, se investigó la presencia en las PAMP de las proteínas identificadas con más alto PLGS, específicamente la proteína inducible por prolactina (PIP), la lactotransferrina (LTF), el antígeno prostático específico (PSA) y la glicoproteína zinc alfa 2 (ZAG), empleando anticuerpos comerciales monoclonales específicos para estas proteínas (Santa Cruz Biotechnologies; Santa Cruz CA, EE.UU.), siguiendo los procedimientos descritos en el apartado 5.6.2. Adicionalmente, los números de acceso correspondientes a las proteínas identificadas fueron contrastados con la base de datos UniProtKB para realizar un análisis de ontología génica de las mismas y crear un panel con las 13 funciones

moleculares más representativas entre las PAMP, como se ha descrito anteriormente [56].

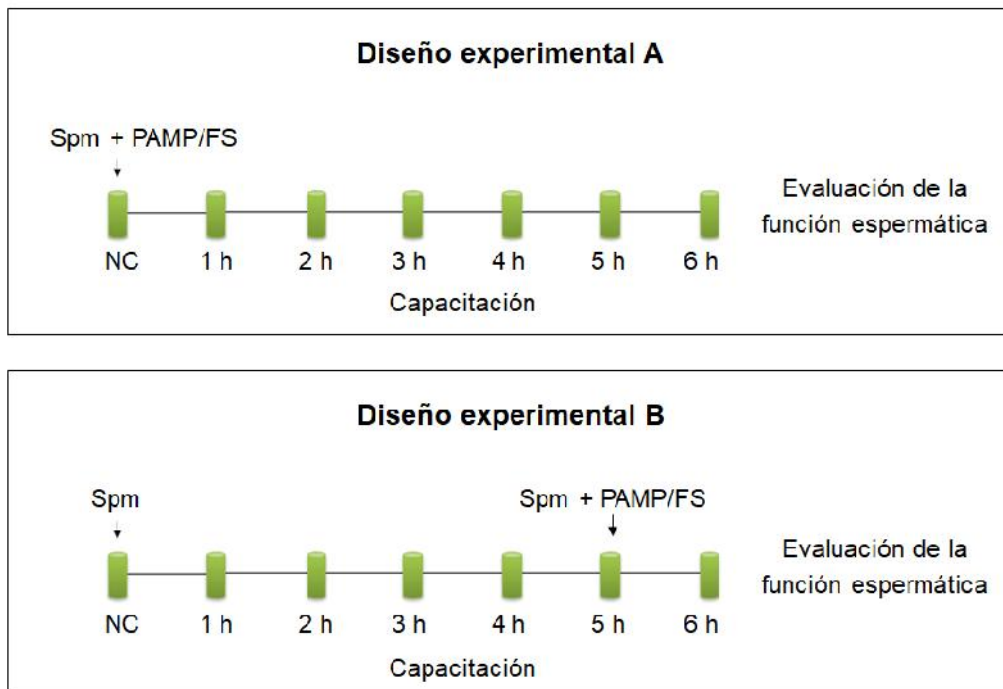
### **5.5. Marcaje de las PAMP con fluoresceína y estudios de unión a espermatozoides**

Para estudiar la capacidad de re-asociación de las PAMP a los espermatozoides, las PAMP se conjugaron a fluoresceína. Para ello, las proteínas se diluyeron 1:10 (v/v) en solución amortiguadora de carbonatos e se incubaron con isotiocianato de fluoresceína (FITC; Sigma-Aldrich) en una relación molar de 1:20 (proteína:FITC) durante 6 h a 4°C con agitación constante en oscuridad. Luego, las proteínas conjugadas a fluoresceína (PAMP-FITC) se dializaron contra PBS durante 24 h para eliminar la fluoresceína libre y subsiguientemente se cuantificaron utilizando el método del BCA.

Posteriormente, 150 mil espermatozoides obtenidos mediante *swim-up* se incubaron bajo condiciones capacitantes en ausencia o presencia de diferentes concentraciones de las PAMP-FITC durante 1 h. Para los ensayos de competencia de unión de las PAMP a espermatozoides, 150 mil espermatozoides se incubaron con 3.2 mg/ml de PAMP-FITC en ausencia o presencia de 3.2 ó 6.4 mg/ml de PAMP sin marcaje con FITC. Luego de la incubaciones, las células se lavaron y fijaron con etanol al 70% para realizar frotis en laminillas pre-tratadas con poli-L-lisina, las cuales se montaron con el medio de montaje Vectashield (Vector Laboratories; Burlingame CA, EE.UU.) para su observación en un microscopio de fluorescencia (Olympus BX51; Olympus Inc., Tokyo, Japón). Las imágenes se capturaron con una cámara Olympus modelo DP72 y procesadas con el programa DP2-BSW (Olympus). La intensidad de fluorescencia se cuantificó utilizando el programa ImageJ (National Institutes of Health; MD, EE.UU.) para medir la densitometría del área total de 5 espermatozoides por condición experimental. A los valores de intensidad de fluorescencia obtenidos se les sustrajo el valor del fondo observado en cada campo y se expresaron como unidades arbitrarias de fluorescencia (U.A.).

## 5.6. Evaluación de efectos de las PAMP sobre la función espermática

Con el propósito de evaluar el efecto de las PAMP en la función espermática, espermatozoides obtenidos mediante *swim-up* se incubaron bajo condiciones capacitantes durante 6 h en ausencia o presencia de diferentes concentraciones de las PAMP (0.8, 1.6 y 3.2 mg/ml) (Diseño experimental A). Por otra parte, para estudiar los efectos sobre espermatozoides capacitados, las muestras se incubaron en condiciones capacitantes durante 5 h para permitir la liberación de las PAMP endógenas y posteriormente se incubaron durante 1 h adicional en ausencia o presencia de las PAMP (Diseño experimental B) (Fig. 5.1). Como control, paralelamente se incluyeron muestras de espermatozoides incubados con FS (16 mg/ml). Al final de las incubaciones, los espermatozoides se procesaron para estudiar posibles cambios en la movilidad, la fosforilación de tirosinas y la reacción acrosomal.



**Fig. 5.1. Diseños experimentales utilizados para evaluar los efectos de las PAMP en la función espermática.** Spm: espermatozoides; NC: espermatozoides no capacitados; PAMP: proteínas asociadas a la membrana plasmática; FS: fluido seminal.



### **5.6.1. Movilidad espermática**

Los cambios en la movilidad espermática se evaluaron mediante observación directa en un microscopio de contraste de fases, en donde se contaron al menos 100 células cuya movilidad se clasificó como progresiva rápida (A), progresiva lenta (B), no progresiva (C) o inmóvil (D), siguiendo los criterios del Manual de la O.M.S. [53]. Los datos obtenidos se expresaron como el porcentaje de células móviles de cada categoría.

Por otra parte, las variables cinéticas de movilidad (Ver anexo 2) se evaluaron mediante un Analizador Espermático (CASA; Hamilton Thorne IVOS versión 14.0, Beverly MA, EE.UU.), ubicado en el laboratorio del Dr. Víctor Torres en el Departamento de Biomembranas, UNAM. Para ello, 5  $\mu$ l de las muestras de espermatozoides se colocaron en cámaras de recuento de células (Leja; Nieuw-Vennep, Holanda). Una vez introducidas las cámaras en el equipo, para cada condición experimental se evaluaron 10 campos aleatorios para un mínimo de 300 células. Para estimar el porcentaje de células hiperactivadas, los valores de corte utilizados fueron VCL 150, LIN 0.5 y ALH 3.5 [14]. Los valores de hiperactivación obtenidos se normalizaron equiparando a 1 el porcentaje de hiperactivación observado en los espermatozoides no capacitados (NC) (control) y los resultados de los tratamientos se expresaron como veces de cambio respecto al control.

### **5.6.2. Fosforilación en tirosinas de proteínas espermáticas**

Al final de las incubaciones, los espermatozoides se lavaron con PBS suplementado con los inhibidores ortovanadato de sodio [1mM] y genisteína [0.2 mM] y se resuspendieron en solución desnaturante para muestra (ver anexo 4) con la finalidad de analizar cambios en la abundancia de la fosforilación en tirosinas mediante Western blot. Para ello, las proteínas separadas mediante SDS-PAGE se electrotransfirieron a membranas de nitrocelulosa, las cuales a continuación se bloquearon durante 1 h con albúmina sérica bovina (BSA) al 3% en solución amortiguadora TBS-T (TBS + Tween 20 0.1%). Seguidamente, las

membranas se incubaron durante toda la noche con anticuerpos monoclonales anti-fosfotirosina (clon 4G10; Millipore) diluidos 1:5,000 en TBS-T. Posteriormente, las membranas se lavaron 3 veces con TBS-T para continuar con una incubación con anticuerpos secundarios anti-IgG de ratón acoplados peroxidasa de rábano (Zymed, San Francisco, CA, EE.UU.). Luego de lavar las membranas, la unión específica se reveló mediante quimioluminiscencia (ECL Prime; GE Healthcare, Reino Unido) y la señal se detectó utilizando el fotodocumentador Chemi Doc XRS+ Imaging System (Bio-Rad). Para verificar la carga homogénea de proteínas en las membranas, estas se re-incubaron con anticuerpos monoclonales anti- $\beta$ -tubulina (Sigma-Aldrich) diluidos 1:30,000, la cual se consideró como proteína constitutiva del espermatozoide, y se procesaron del mismo modo que para la detección de fosfotirosinas. El análisis densitométrico de cada condición experimental se realizó con el programa informático ImageLab™ (Bio-Rad) y la abundancia relativa de fosfotirosinas se obtuvo normalizando la intensidad de señal de las fosfotirosinas por la señal de  $\beta$ -tubulina en cada carril (pY/tub). A los valores de abundancia relativa obtenidos con los espermatozoides capacitados a las 6 h en ausencia de las PAMP se les asignó el valor de 1 para normalizar los otros valores obtenidos.

### **5.6.3. Reacción acrosomal**

Para evaluar los efectos sobre la reacción acrosomal espontánea, 150 mil espermatozoides se lavaron con PBS y se fijaron con etanol al 70%. Paralelamente, otra alícuota de 150 mil células de la misma incubación se incubó con el ionóforo de calcio A23187 (CaI) [10 $\mu$ M] (Sigma-Aldrich) durante 20 min a 37°C, al final de lo cual se lavaron y fijaron con etanol al 70%. Posteriormente, las células fijadas se utilizaron para hacer frotis en laminillas pre-tratadas con poli-L-lisina y se tiñeron con lectina *Pisum sativum* acoplada a FITC durante 30 min, como se ha descrito previamente [39]. El estatus del acrosoma se evaluó al microscopio de fluorescencia, donde las células que mostraron una tinción completa del acrosoma se consideraron intactas, mientras que las que presentaron tinción en la región ecuatorial o ausencia de tinción se clasificaron

como reaccionadas. Los datos obtenidos se expresaron como el índice ARIC (*Acrosome Reaction to Ionophore Challenge*), definido como el porcentaje de espermatozoides con reacción acrosomal inducida por Cal menos la reacción acrosomal espontánea observada en las diferentes condiciones experimentales [57].

### 5.7. Ensayos de hemizona

Los ensayos de hemizona se realizaron en la Clínica de Fertilidad CITMER, con asesoría del Biól. Israel Maldonado Rosas y el Biól. Israel Jiménez, siguiendo procedimientos ya descritos [58]. Se utilizaron ovocitos humanos provenientes de donadoras sanas, a los cuales se les removi6 el c6mulo o6foro con hialuronidasa y se mantuvieron en un medio de alta sal para preservaci6n de la zona pel6cida (ver anexo 4) a 4°C por un m6ximo de 2 meses. El d6a del ensayo, los ovocitos con las zonas pel6cidas se lavaron con HTF y se procesaron bajo el microscopio invertido con un micromanipulador para aislar las zonas pel6cidas. Los ovocitos se desecharon y las zonas pel6cidas se cortaron a la mitad para obtener 2 hemizonas (HZ) a partir de cada ovocito que se colocaron en gotas separadas de medio HTF. A la HZ control se le adicionaron 100 mil espermatozoides capacitados toda la noche (control), mientras que a la HZ hom6loga se le agregaron 100 mil espermatozoides capacitados toda la noche y pre-incubados durante 15 min con 3.2 mg/ml de las PAMP (tratamiento), para ser co-incubadas durante 4 h a 37°C, 5% de CO<sub>2</sub> y 95% de humedad. Posteriormente, las HZ's se pasaron a medio fresco y pipetearon varias veces para remover los espermatozoides que no se encontraban fuertemente unidos. A continuaci6n, con ayuda de un microscopio, se cont6 el n6mero de espermatozoides unidos a cada HZ y se estim6 el 6ndice de HZ (HZI) seg6n la relaci6n:

$$\text{HZI} = \left( \frac{\text{N6mero de espermatozoides unidos a la HZ con tratamiento}}{\text{N6mero de espermatozoides unidos a la HZ control}} \right) \times 100$$

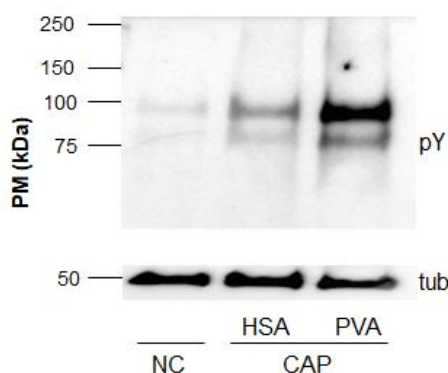
## **5.8. Análisis de resultados**

Los resultados se presentan como la media  $\pm$  EE. Adicionalmente, se analizaron utilizando el programa de análisis estadístico GraphPad Prism (GraphPad Software Inc., versión 5.01) aplicando la prueba de análisis de varianza de una vía (ANOVA) seguida de Tukey para comparaciones múltiples. Se consideraron significativas las diferencias entre los grupos con valores de  $P < 0.05$ .

## 6. RESULTADOS

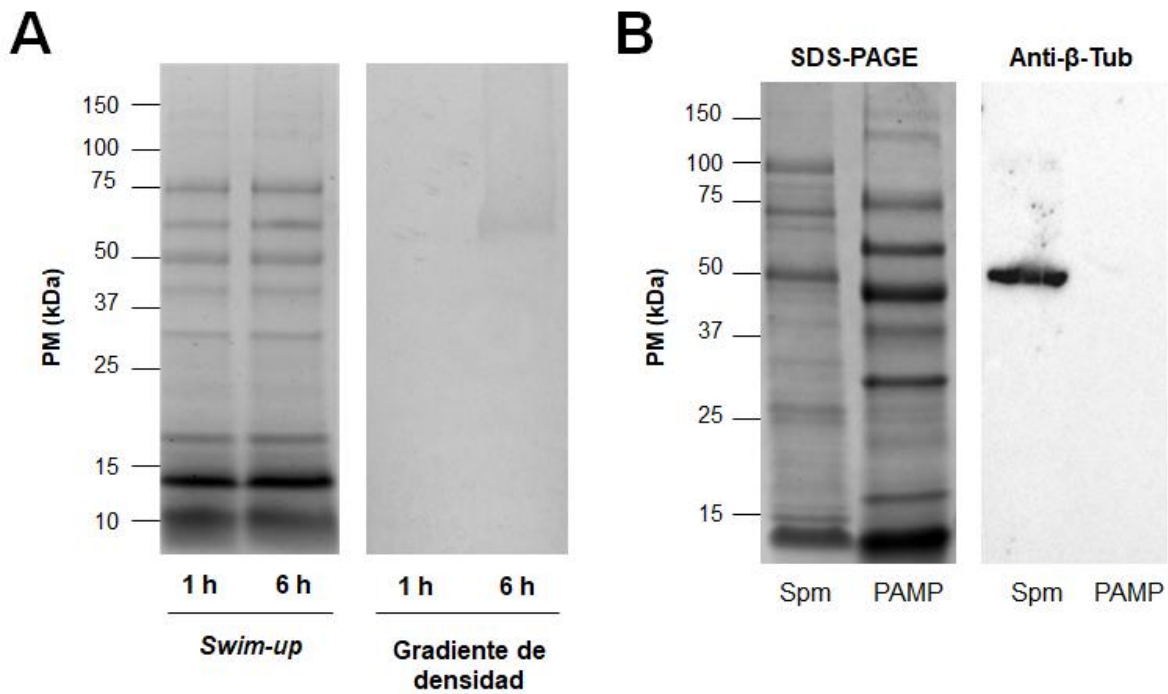
### 6.1. Obtención y caracterización de las PAMP

Para el estudio de las PAMP liberadas por el espermatozoide durante la capacitación *in vitro* fue necesario optimizar su obtención. Generalmente, los medios para capacitación se suplementan con albúmina al 3% [53], pero el exceso de proteína exógena obstaculiza la detección y caracterización de las PAMP. Otro aceptor de colesterol que se ha empleado previamente para la capacitación *in vitro* es el polivinil alcohol (PVA) [41, 59]. Por tanto, se comparó la eficiencia de la capacitación espermática en medio HTF suplementado con PVA y HSA a través de los cambios en la fosforilación en tirosinas de las proteínas espermáticas mediante Western blot. En la Fig. 6.1 se observa que tanto los espermatozoides incubados con HSA como con PVA muestran un incremento en la fosforilación en tirosinas de 2 proteínas de pesos moleculares aparentes de 82 y 110 kDa, cuyo patrón de fosforilación durante la capacitación se ha descrito previamente [27, 60]. Adicionalmente, se observó que la fosforilación en tirosinas en los espermatozoides incubados con PVA es mayor a la observada en presencia de HSA.



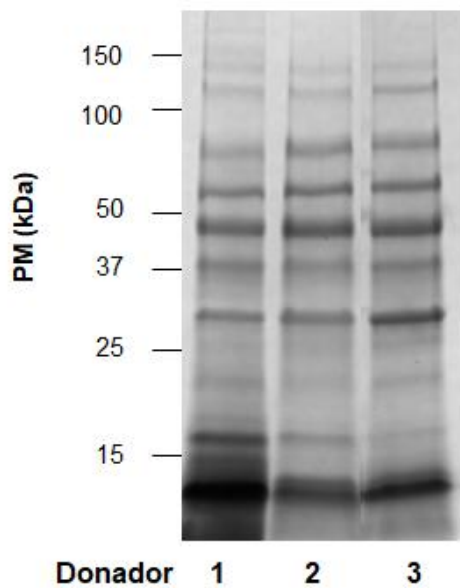
**Fig. 6.1. Espermatozoides capacitados en medio suplementado con PVA presentan mayor fosforilación en tirosinas que en medio con HSA.** Western blot representativo de espermatozoides no capacitados (NC) y capacitados (CAP) en medio HTF suplementado con HSA y con PVA. La membrana se incubó con el anticuerpo 4G10 para la detección de fosfotirosinas (pY). Se muestra la señal de  $\alpha$ -tubulina (tub) del mismo experimento como control de la carga total de proteína. n=3.

Con el propósito de evaluar el método más eficiente para la obtención de las PAMP, espermatozoides procesados mediante *swim-up* y centrifugación en gradientes de densidad (que son los dos métodos más comunes para recuperar espermatozoides del semen) se incubaron en HTF suplementado con PVA durante 1 h y 6 h y posteriormente se analizaron las proteínas presentes en el medio de incubación. En la Fig. 6.2.A se muestra que a partir de los espermatozoides obtenidos mediante *swim-up* se obtuvieron 12 proteínas, mientras que de los espermatozoides procesados mediante centrifugación en gradientes de densidad sólo se observó 1 proteína. Esto sugiere que la fricción generada durante la centrifugación en gradientes de densidad remueve la mayor parte de las proteínas asociadas a la superficie del espermatozoide, por lo que consideramos que la técnica más adecuada para estudiar las PAMP era la de *swim-up*. Adicionalmente, se observó que el patrón y abundancia de proteínas liberadas a 1 h y 6 h es similar. Para verificar que las PAMP obtenidas no estuvieran contaminadas con células, se evaluó la presencia de  $\alpha$ -tubulina en las preparaciones, la cual es una proteína que forma parte de los microtúbulos y normalmente está ausente en el FS. En la Fig. 6.2.B se observa que la  $\alpha$ -tubulina está presente en el control de espermatozoides pero no en las PAMP, demostrando la pureza de las muestras.



**Fig. 6.2.** La técnica de *swim-up* permite recuperar las PAMP de manera más eficiente que los gradientes de densidad y sin contaminantes celulares. (A) Comparación de técnicas de obtención de las PAMP. Las PAMP se recuperaron a diferentes tiempos a partir de espermatozoides procesados mediante *swim-up* o gradiente de densidad y se analizaron mediante SDS-PAGE. (B) Análisis de la pureza de las PAMP. La ausencia de contaminantes celulares en las PAMP se evaluó mediante Western blot con anticuerpos anti- $\beta$ -tubulina. Se incluyeron espermatozoides (Spm) como control positivo y se muestra el SDS-PAGE correspondiente como control de carga similar de proteínas.

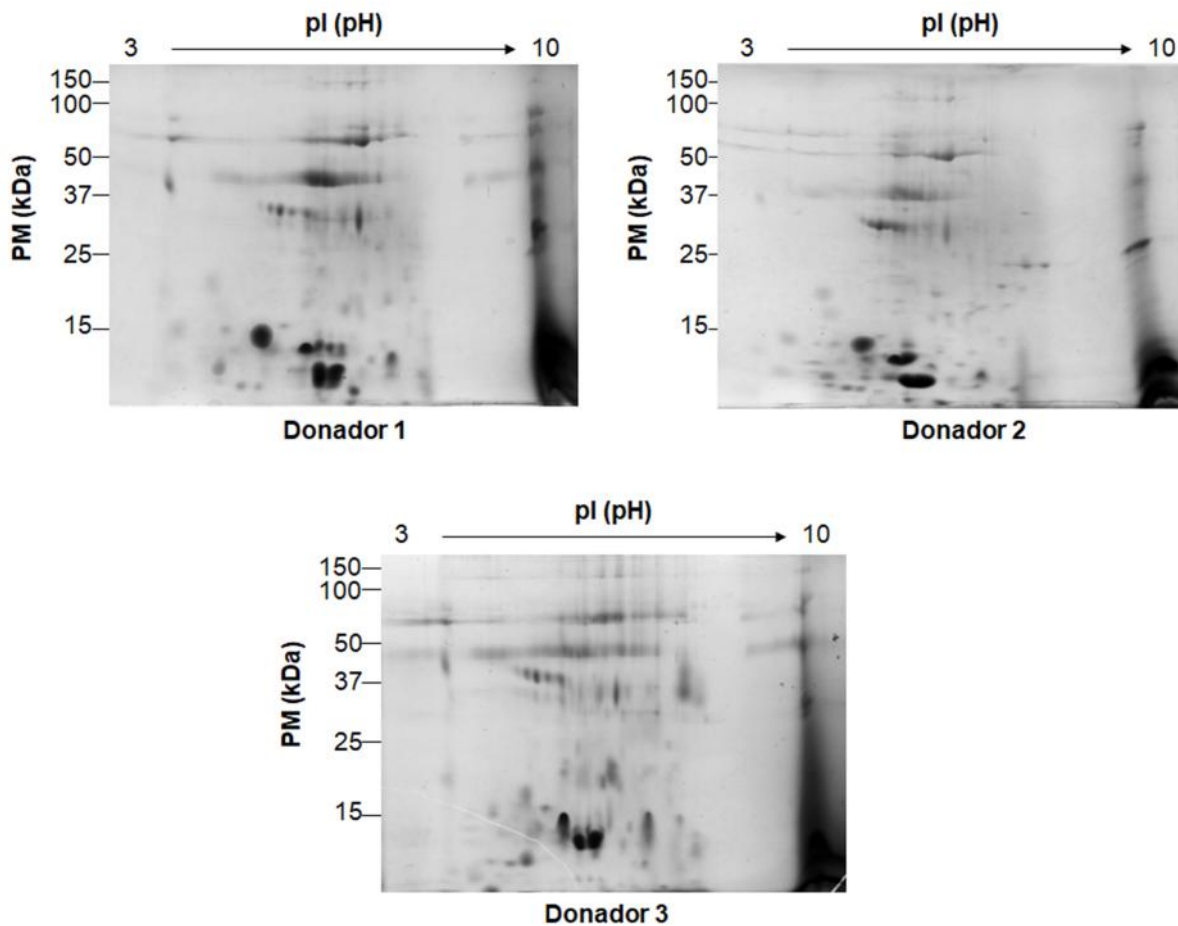
Por lo tanto, los espermatozoides móviles se recuperaron mediante *swim-up* y se incubaron durante 6 h en medio HTF con PVA para la obtención y caracterización de las PAMP de diferentes donadores. La cuantificación de las PAMP a partir de muestras de semen de 19 donadores dio una recuperación promedio de  $0.55 \pm 0.06$  mg de PAMP/ $10^6$  de espermatozoides. En la Fig. 6.3 se muestra el patrón de las PAMP obtenidas a partir de tres muestras de donadores diferentes separadas mediante SDS-PAGE, observándose que el patrón de proteínas liberadas es muy semejante entre ellas.



**Fig. 6.3. Las PAMP de diferentes donadores son similares y están compuestas por 12 bandas de proteínas en SDS-PAGE.** Las PAMP de 3 donadores diferentes se recuperaron a partir de espermatozoides procesados mediante *swim-up* y se analizaron mediante SDS-PAGE.

Al analizar las PAMP de los mismos tres donadores mediante 2-DE, se detectaron 59 proteínas que consistentemente estaban presentes en las tres muestras (Fig. 6.4), cuyos puntos isoeléctricos y pesos moleculares aparentes se muestran en el anexo 3. Estas 59 proteínas corresponden al 97% de las proteínas detectadas en cada muestra, lo que corrobora que la composición de proteína es muy similar en estas 3 muestras.





**Fig. 6.4. Las PAMP de diferentes donadores son similares y están compuestas por 59 proteínas en 2-DE.** Las PAMP de 3 donadores diferentes se recuperaron de espermatozoides procesados mediante *swim-up* y se analizaron mediante 2-DE.

Posteriormente, se procedió a la identificación de las proteínas contenidas en una mezcla de PAMP provenientes de 5 donadores mediante espectrometría de masas. Dicho análisis permitió la identificación de 29 proteínas, mismas que se detallan en la tabla 6.1.

**Tabla 6.1. PAMP identificadas mediante espectrometría de masas.**

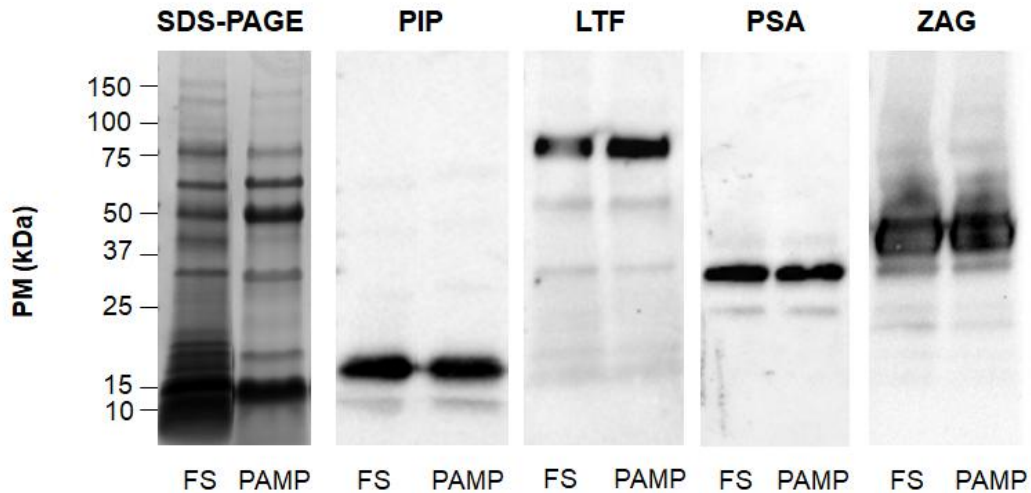
Nombre	Número de acceso en Uniprot	PM (kDa)	pI (pH)	PLGS Score	Péptidos	Péptidos teóricos	Cobertura (%)
Proteína inducible por prolactina (PIP)	P12273	16.6	8.1	83452.9	48	12	71.9
Lactotransferrina (LTF)	P02788	78.1	8.0	63999.4	161	74	70.7
Isoforma delta de la lactotransferrina	P02788	73.1	7.7	60323.5	157	71	72.4
Isoforma 3 del antígeno específico de próstata (PSA)	P07288	23.7	7.1	45684.8	31	15	72.0
Isoforma 5 del antígeno específico de próstata	P07288	25.1	6.6	45580.7	30	14	57.7
Glicoproteína zinc alfa 2 (ZAG)	P25311	34.2	5.6	30551.8	46	27	43.6
Albúmina sérica	P02768	69.3	5.9	25358.3	102	55	40.4
Isoforma 3 de la albúmina sérica	P02768	45.1	5.7	25224.1	85	36	45.9
Fosfatasa ácida prostática	P15309	44.5	5.8	23654.8	51	27	44.3
Isoforma 4 de la clusterina	P10909	48.8	6.3	18858.9	24	36	26.9
Isoforma 10 de la fibronectina	P02751	239.5	5.5	18053.6	103	138	35.9
Isoforma 4 de la fibronectina	P02751	222.8	5.3	18023.9	100	122	36.8
Inhibidor plasmático de serina proteasa	P05154	45.6	9.6	7636.9	27	30	32.3
Aminopeptidasa N	P15144	109.5	5.2	7297.9	52	73	28.3
Isoforma corta de la proteína de Crisp 1	P54107	20.4	6.7	6892.6	4	12	11.2
Proteína de unión a la galectina 3	Q08380	65.3	4.9	5285.6	34	39	23.1

Isoforma 2 de antitripsina alfa 1	P01009	40.2	5.1	3849.8	13	29	30.6
Región C Ig alfa 1	P01876	37.6	6.1	2927.6	7	23	31.4
Isoforma 2 de la semenogelina 1	P04279	45.3	9.8	2626.9	4	35	8.5
Cistatina S	P01036	16.2	4.8	2329.5	8	14	53.9
Antileucoproteinasa	P03973	14.3	8.7	2251.0	2	13	9.1
Isoforma 2 de la Carboxipeptidasa	P16870	49.8	4.9	1803.1	16	39	37.7
Región C Ig gamma	P01857	36.1	8.2	1765.9	7	21	20.3
Prosaposina	P07602	58.1	4.9	1376.3	12	47	14.3
Antiquimotripsina alfa 1	P01011	47.6	5.2	882.4	6	31	17.5
Proteína 1 de la matriz extracelular	Q16610	60.6	6.2	855.4	6	47	14.4
Isoforma 2 de sulfhidril oxidasa 1	O00391	66.8	8.6	644.6	7	53	14.9
Isoforma 2 de la glicodelina	P09466	18.3	4.9	637.7	2	11	12.7
Región C Ig gamma	P01860	41.3	7.8	618.1	4	24	9.5

El puntaje PLGS se estimó con el programa Protein Lynx Global Server (Waters) a partir de los datos espectrométricos y es una medida estadística de la precisión de la identificación de los péptidos. Una puntuación alta implica una mayor confianza en la identidad de la proteína [61].

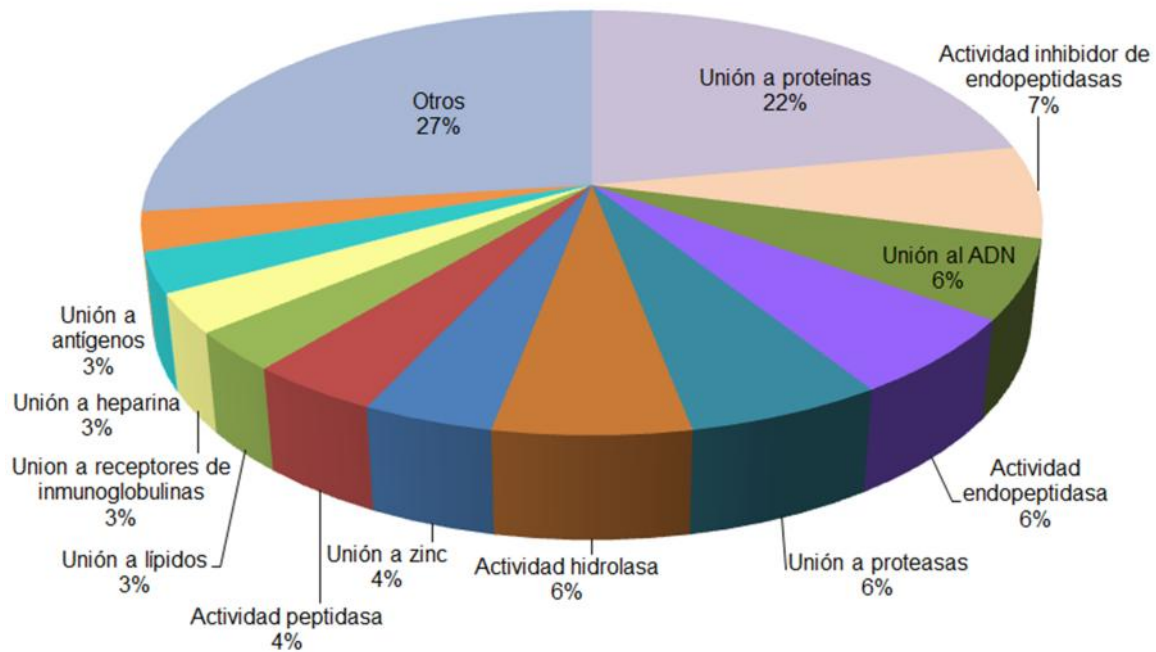
Para corroborar la confiabilidad de los datos obtenidos mediante espectrometría de masas, la identidad de varias de las proteínas identificadas se validó mediante Western blot en muestras de PAMP y FS. Para ello, se seleccionaron las proteínas que presentaron valores más altos de PLGS, que fueron PIP, LTF, PSA y ZAG. En la Fig. 6.5 se muestra el patrón de proteínas del FS y las PAMP separadas por SDS-PAGE y al analizar estas mismas proteínas mediante Western blot se

demuestra la presencia de PIP, LTF, PSA y ZAG tanto en el FS como en las PAMP, con pesos moleculares aproximados de 16, 77, 30 y 37 kDa, respectivamente, los cuales son similares a los esperados para esas proteínas (ver Tabla 6.1).



**Fig. 6.5. Validación de la identidad de varias PAMP mediante Western blot.** SDS-PAGE y Western blots para corroborar la identidad de las proteínas PIP, LTF, PSA y ZAG, utilizando anticuerpos específicos para cada una de las proteínas. Como control positivo se utilizó FS.

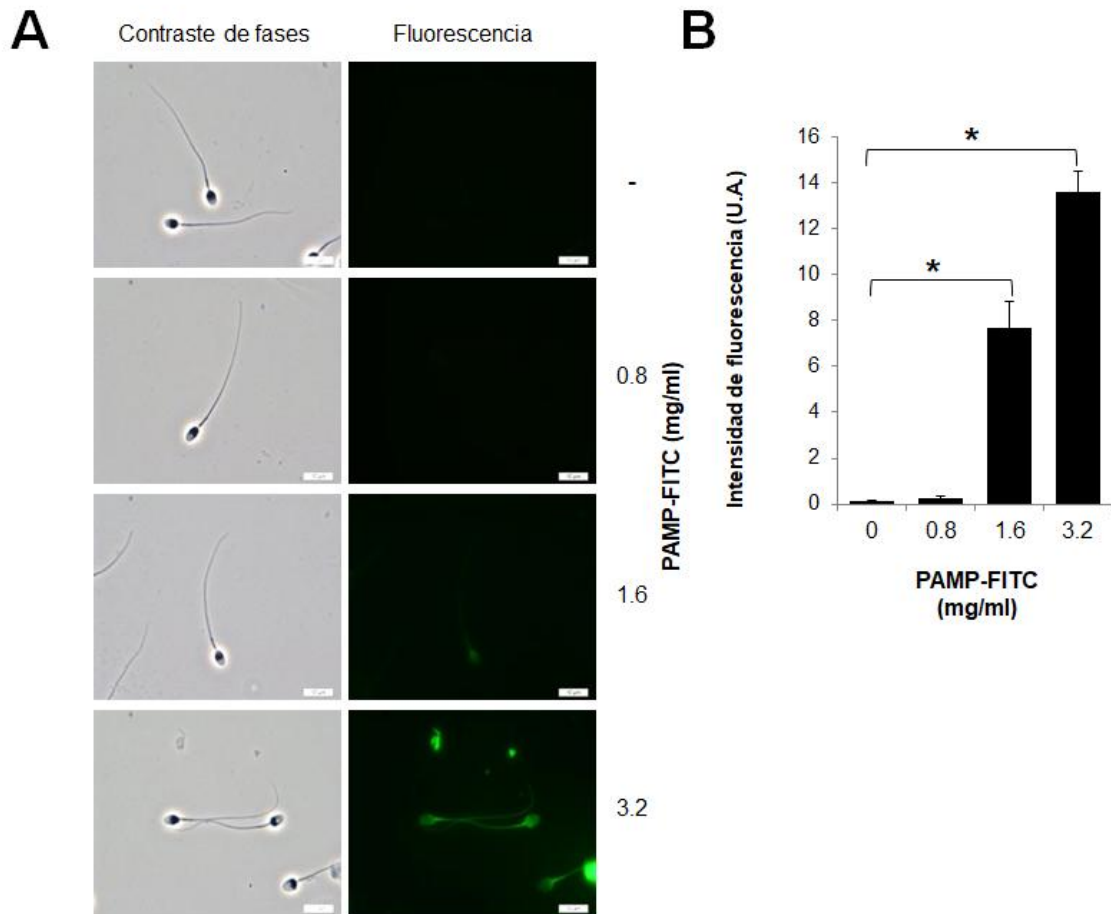
Adicionalmente, la distribución de las principales funciones moleculares de las proteínas identificadas se analizó utilizando las funciones moleculares descritas en la base de datos UniprotKB para cada una de las proteínas. En la Fig. 6.6 se muestra que las PAMP identificadas poseen funciones moleculares variadas, con predominancia de proteínas involucradas en la unión a proteínas (22%) y en la actividad inhibidora de endopeptidasa (7%).



**Fig. 6.6. Funciones moleculares de las PAMP.** Distribución de las funciones moleculares de las PAMP identificadas por espectrometría de masas, según las definiciones de ontología génica publicadas en la base de datos UniprotKB.

## 6.2. Unión de las PAMP a los espermatozoides

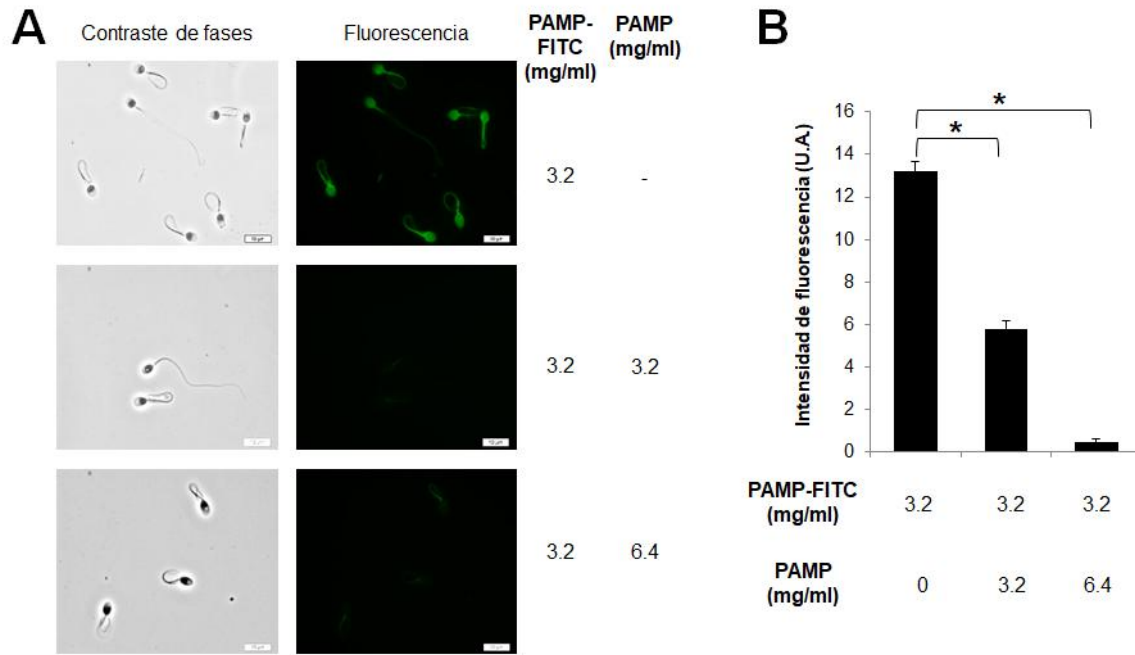
Antes de estudiar el efecto de las PAMP en la función espermática, evaluamos si las proteínas obtenidas podían re-asociarse a la membrana plasmática de los espermatozoides *in vitro*. Para ello, los espermatozoides se incubaron con diferentes concentraciones de las PAMP conjugadas a fluoresceína (PAMP-FITC) en condiciones capacitantes y se evaluó al microscopio su asociación a las células. En la Fig. 6.7.A se muestra que la intensidad de fluorescencia en las células aumentó en función de la concentración de las PAMP-FITC en el medio. Al hacer un análisis densitométrico de la intensidad de señal observada en 7 experimentos independientes, encontramos que la fluorescencia en los espermatozoides fue significativamente mayor para las concentraciones de 1.6 y 3.2 mg/ml de PAMP-FITC en comparación con el control en ausencia de PAMP-FITC (Fig. 6.7.B).



**Fig. 6.7. Las PAMP se re-asocian a espermatozoides *in vitro*.** (A) Espermatozoides capacitados en ausencia o presencia de diferentes concentraciones de PAMP acopladas a fluoresceína (PAMP-FITC) durante 1 h. Se muestran las imágenes de contraste de fases y de fluorescencia. Aumento: 100 x. Barra blanca en parte inferior de las imágenes equivale a 10 micras. (B) Análisis densitométrico de la intensidad de fluorescencia evaluada utilizando el programa ImageJ. Se muestran las medias  $\pm$  EE.  $n=7$ . (\*)  $P < 0.05$ .

Para evaluar la especificidad de la unión de las PAMP-FITC a los espermatozoides, se realizaron ensayos de competencia por la unión a espermatozoides con PAMP sin marcaje fluorescente. En la Fig. 6.8.A se muestra que la fluorescencia observada en espermatozoides incubados con PAMP-FITC (3.2.mg/ml) disminuyó a medida que aumentaba la concentración de las PAMP sin fluoresceína en el medio. El análisis densitométrico correspondiente demostró que

hubo una disminución significativa de la intensidad de señal en presencia de 3.2 y 6.4 mg/ml de las PAMP sin fluoresceína en comparación con el control (Fig. 6.8.B), indicando que su unión al espermatozoide es específica.



**Fig. 6.8. La unión de las PAMP a espermatozoides *in vitro* es específica.** (A) Intensidad de fluorescencia de espermatozoides incubados con 3.2 mg/ml de PAMP-FITC en presencia de concentraciones crecientes de PAMP no fluorescentes durante 1 h. Se muestran el contraste de fases y la fluorescencia. Aumento: 100 x. Barra blanca en la parte inferior de las imágenes equivale a 10 micras. (B) Análisis densitométrico de la intensidad de fluorescencia. Se muestran las medias  $\pm$  EE.  $n=5$ . (\*)  $P < 0.05$ .

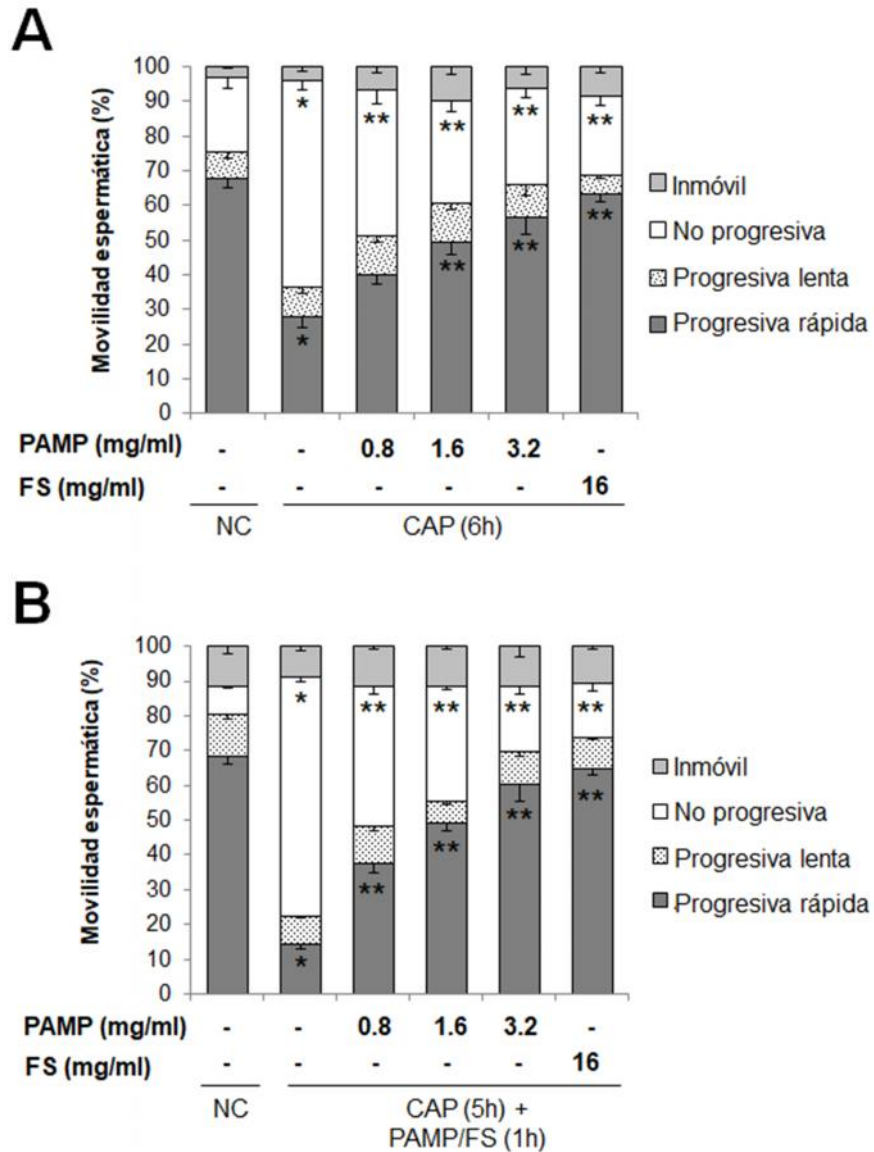
### 6.3. Efecto de las PAMP sobre la función espermática

Con el objeto de estudiar los posibles efectos de las PAMP sobre diferentes variables de la función espermática, se emplearon dos diseños experimentales con el propósito de evaluar sus efectos sobre espermatozoides no capacitados y capacitados: el diseño experimental A, en el que los espermatozoides se incubaron en condiciones capacitantes durante 6 h en presencia de las PAMP, y el diseño experimental B donde los espermatozoides se capacitaron durante 5 h y

luego se les adicionaron las PAMP para una incubación adicional de 1 h (ver Fig. 5.1).

Al evaluar de forma manual los efectos de las PAMP sobre la movilidad espermática (Fig. 6.9.A y B), encontramos que al capacitar los espermatozoides en ausencia de las PAMP (CAP) la movilidad progresiva rápida disminuyó mientras que la movilidad no progresiva aumentó en comparación con el control no capacitado (NC) evaluado al tiempo 0 h, pero al capacitar los espermatozoides en presencia de concentraciones crecientes de PAMP (diseño experimental A), aumentó la movilidad progresiva rápida y disminuyó la no progresiva (Fig. 6.9.A). Bajo el diseño experimental B, se observaron efectos similares (Fig. 6.9.B). Los resultados indican que las PAMP promueven la movilidad progresiva e inhiben el aumento de la movilidad no progresiva, sin importar si los espermatozoides están capacitados o no. Este efecto es semejante al observado cuando los espermatozoides se incubaron en presencia de FS, el cual se empleó como control de los efectos descapacitantes del semen.





**Fig. 6.9. Las PAMP inhiben la movilidad no progresiva y estimulan la progresiva rápida en espermatozoides no capacitados y capacitados.** Distribución de los tipos de movilidad espermática de acuerdo a los valores de referencia de la O.M.S. (A) Espermatozoides capacitados en ausencia o presencia de diferentes concentraciones de PAMP durante 6 h; (B) Espermatozoides capacitados durante 5 h y posteriormente incubados 1 h en ausencia o presencia de diferentes concentraciones de PAMP. Se utilizó FS como control. Se muestran las medias  $\pm$  EE.  $n=6$ . (\*)  $P < 0.05$  vs NC, (\*\*)  $P < 0.05$  vs CAP en ausencia de PAMP. NC: No capacitado; CAP: capacitado.

Debido a que estos resultados sugieren que las PAMP modulan la movilidad espermática, evaluamos el efecto de estas proteínas en las variables cinéticas de movilidad utilizando un analizador automático de espermatozoides (CASA). En la tabla 6.2, correspondiente a los valores cinéticos de espermatozoides incubados con PAMP bajo el diseño experimental A, se muestra que en presencia de 0.8 mg/ml de las PAMP disminuyó la VCL, mientras que con las concentraciones de 1.6 y 3.2 mg/ml de las PAMP así como con el FS disminuyeron la VAP, la VCL, la VSL y la ALH en comparación con los espermatozoides capacitados durante 6 h en ausencia de las PAMP. Por su parte, la tabla 6.3 muestra los resultados correspondientes a los espermatozoides incubados con las PAMP bajo el diseño experimental B, donde se observan resultados similares con una disminución de las mismas variables de movilidad con todas las concentraciones evaluadas.

**Tabla 6.2.** Efecto de las PAMP y el FS sobre las variables cinéticas de movilidad de espermatozoides no capacitados (Diseño experimental A).

	NC	CAP (6 h)				
PAMP (mg/ml)	-	-	0.8	1.6	3.2	FS
VAP ( $\mu\text{m/s}$ )	40.9 $\pm$ 3.9	60.0 $\pm$ 4.8*	46.2 $\pm$ 3.1	40.8 $\pm$ 2.3**	33.2 $\pm$ 3.4**	27.5 $\pm$ 3.3**
VSL ( $\mu\text{m/s}$ )	32.7 $\pm$ 3.6	49.1 $\pm$ 5.3*	37.0 $\pm$ 3.5	31.8 $\pm$ 2.5**	25.2 $\pm$ 2.9**	20.9 $\pm$ 2.6**
VCL ( $\mu\text{m/s}$ )	67.5 $\pm$ 5.8	96.4 $\pm$ 5.1*	74.3 $\pm$ 3.5**	65.9 $\pm$ 3.0**	55.1 $\pm$ 4.7**	44.8 $\pm$ 4.5**
ALH ( $\mu\text{m}$ )	3.5 $\pm$ 0.3	4.6 $\pm$ 0.1*	4.1 $\pm$ 0.2	3.5 $\pm$ 0.2**	2.9 $\pm$ 0.3**	2.6 $\pm$ 0.2**
BCF (Hz)	24.9 $\pm$ 1.8	27.8 $\pm$ 1.5	24.7 $\pm$ 1.4	23.3 $\pm$ 1.2	26.5 $\pm$ 1.4	23.6 $\pm$ 1.2
STR (%)	76.9 $\pm$ 2.1	76.7 $\pm$ 1.9	76.3 $\pm$ 2.1	73.9 $\pm$ 1.7	74.0 $\pm$ 2.6	73.9 $\pm$ 0.9
LIN (%)	47.6 $\pm$ 1.9	48.3 $\pm$ 2.2	48.3 $\pm$ 2.6	45.9 $\pm$ 1.6	45.4 $\pm$ 1.9	47.0 $\pm$ 0.9

Se muestran las medias  $\pm$  EE. n=6. (\*) P < 0.05 vs NC, (\*\*) P < 0.05 vs CAP en ausencia de PAMP.

NC: No capacitado; CAP: capacitado.

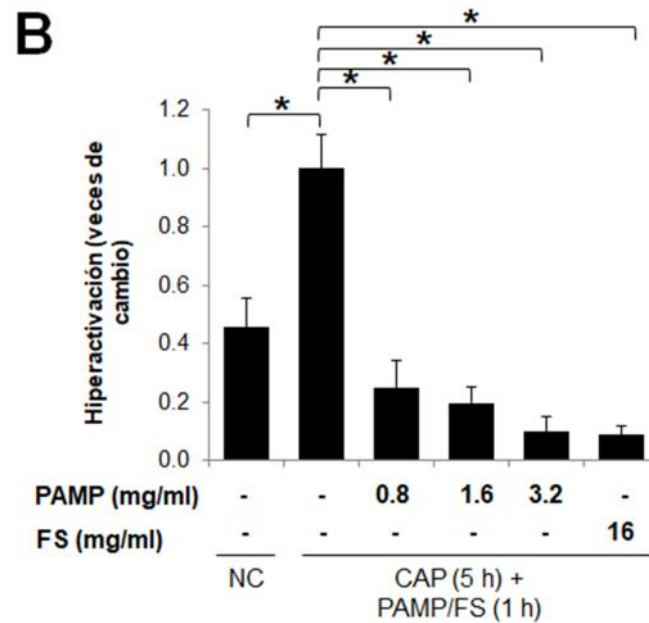
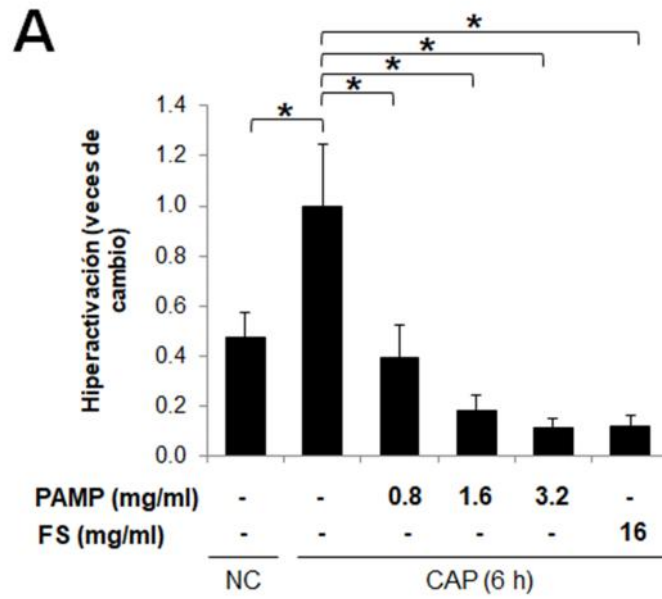
**Tabla 6.3.** Efecto de las PAMP y el FS sobre las variables cinéticas de movilidad en espermatozoides capacitados (Diseño experimental B).

	NC	CAP (5 h) + PAMP/FS (1 h)				
PAMP (mg/ml)	-	-	0.8	1.6	3.2	FS
VAP ( $\mu\text{m/s}$ )	38.8 $\pm$ 1.9	63.3 $\pm$ 1.7*	43.3 $\pm$ 6.2**	42.8 $\pm$ 5.3**	38.4 $\pm$ 3.7**	36.9 $\pm$ 4.5**
VSL ( $\mu\text{m/s}$ )	28.6 $\pm$ 1.4	50.4 $\pm$ 3.4*	35.0 $\pm$ 5.9	34.3 $\pm$ 5.1	30.3 $\pm$ 3.8**	28.5 $\pm$ 4.0**
VCL ( $\mu\text{m/s}$ )	69.4 $\pm$ 4.6	100.2 $\pm$ 2.6*	71.1 $\pm$ 7.5**	71.0 $\pm$ 6.4**	64.6 $\pm$ 5.0**	64.2 $\pm$ 6.2**
ALH ( $\mu\text{m}$ )	3.2 $\pm$ 0.2	4.8 $\pm$ 0.1*	3.5 $\pm$ 0.3**	2.9 $\pm$ 0.2**	2.9 $\pm$ 0.3**	3.0 $\pm$ 0.3**
BCF (Hz)	23.9 $\pm$ 1.5	27.0 $\pm$ 1.3	23.4 $\pm$ 1.9	22.1 $\pm$ 1.5	22.7 $\pm$ 1.5	22.5 $\pm$ 1.7
STR (%)	74.3 $\pm$ 3.3	79.2 $\pm$ 3.3	79.0 $\pm$ 3.2	78.5 $\pm$ 3.3	77.7 $\pm$ 3.5	76.3 $\pm$ 2.2
LIN (%)	41.9 $\pm$ 2.7	50.4 $\pm$ 3.6	47.5 $\pm$ 4.5	47.0 $\pm$ 3.9	46.2 $\pm$ 3.6	43.5 $\pm$ 2.5

Se muestran las medias  $\pm$  EE. n=6. (\*)  $P < 0.05$  vs NC (0 h), (\*\*)  $P < 0.05$  vs CAP en ausencia de PAMP.

NC: No capacitado; CAP: capacitado

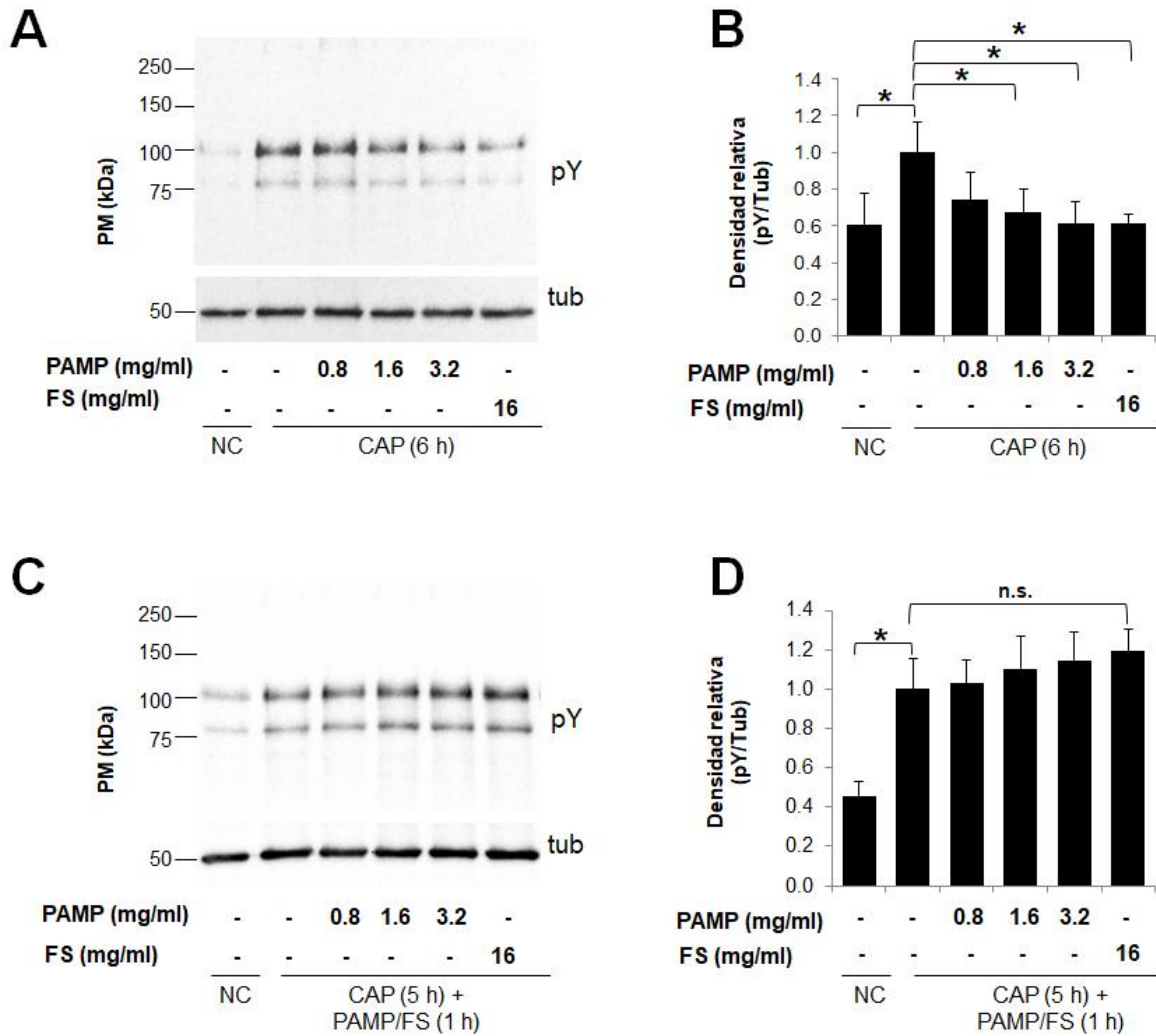
En vista de que las PAMP promovieron cambios en la VCL y la ALH, variables que caracterizan la hiperactivación, se decidió analizar su efecto sobre este parámetro. En la Fig. 6.10.A se muestra que cuando los espermatozoides se procesaron bajo el diseño experimental A, la hiperactivación aumentó cuando los espermatozoides se incubaron durante 6 h en ausencia de las PAMP, pero al hacer las incubaciones en presencia de concentraciones crecientes de las PAMP y FS la hiperactivación disminuyó (Fig. 6.10.A). Por su parte, bajo el diseño experimental B, se observaron resultados similares donde la adición de las PAMP o FS a espermatozoides capacitados inhibió la hiperactivación (Fig. 6.10.B).



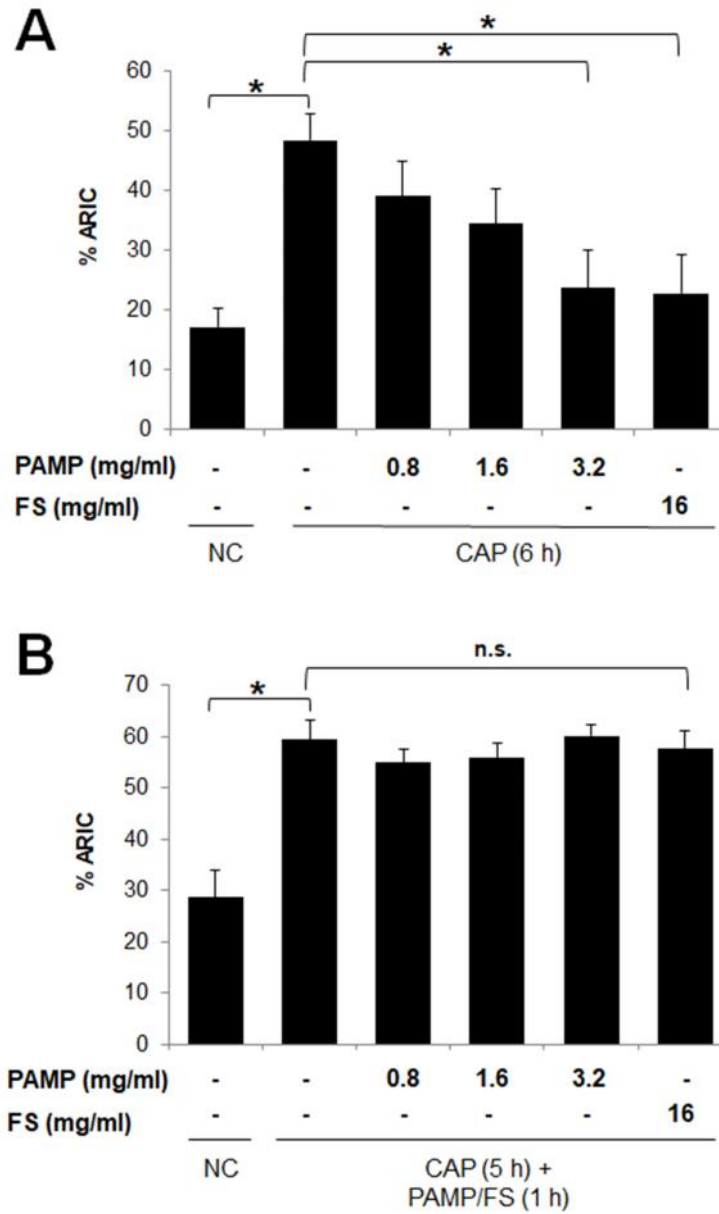
**Fig. 6.10. Las PAMP inhiben la hiperactivación en espermatozoides no capacitados y capacitados.** (A) Espermatozoides capacitados en ausencia o presencia de diferentes concentraciones de PAMP durante 6 h; (B) Espermatozoides capacitados durante 5 h y posteriormente incubados en ausencia o presencia de diferentes concentraciones de PAMP durante 1 h. Los valores se presentan como veces de cambio sobre el porcentaje de células hiperactivadas en ausencia de las PAMP a las 6 h de incubación. Se utilizó FS como control. Se muestran las medias  $\pm$  EE.  $n=6$ . (\*)  $P < 0.05$ . NC: No capacitado; CAP: capacitado.

Paralelamente, se evaluó el efecto de las PAMP sobre la fosforilación en tirosinas de proteínas espermáticas. En la Fig. 6.11 se muestran Western blots representativos para la detección de fosfotirosinas en espermatozoides procesados e incubados con las PAMP. Cuando los espermatozoides se procesaron siguiendo el diseño experimental A (Fig. 6.11.A), la fosforilación en tirosinas de los CAP fue mayor a la de espermatozoides NC, como era de esperarse. Sin embargo, este efecto disminuyó a medida que aumentaba la concentración de PAMP en el medio de capacitación y en presencia de FS. Al analizar las densitometrías de siete Western blots (Fig. 6.11.B), se observó una disminución significativa de la fosforilación en tirosinas en presencia de 1.6 y 3.2  $\mu\text{g/ml}$  de las PAMP y el FS. En cambio, bajo el diseño experimental B, no se observaron efectos de las PAMP (Fig. 6.11.C y D). Estos resultados sugieren que cuando los espermatozoides se capacitan en presencia de las PAMP las proteínas inhiben la fosforilación de proteínas, pero cuando las PAMP son adicionadas a espermatozoides capacitados no es posible revertir este proceso.

Por otra parte, dado que el ionóforo de calcio A23187 (Cal) sólo induce la reacción acrosomal en espermatozoides capacitados [62], se evaluó el efecto de las PAMP sobre la reacción acrosomal espontánea e inducida por Cal y los resultados obtenidos se expresaron como el índice ARIC. En el diseño experimental A (Fig. 6.12.A), se observa el aumento de espermatozoides reaccionados después de 6 h de capacitación, pero cuando los espermatozoides se incubaron con 3.2 mg/ml de las PAMP y con FS disminuyó la proporción de espermatozoides reaccionados (Fig. 6.12.A). Sin embargo, bajo el diseño experimental B (Fig. 6.12.B) se observó que las PAMP y el FS no afectaron este parámetro, indicando que las PAMP inhiben la capacitación pero no pueden revertirla en espermatozoides capacitados, en concordancia con los resultados obtenidos para fosforilación en tirosinas (Fig. 6.11).



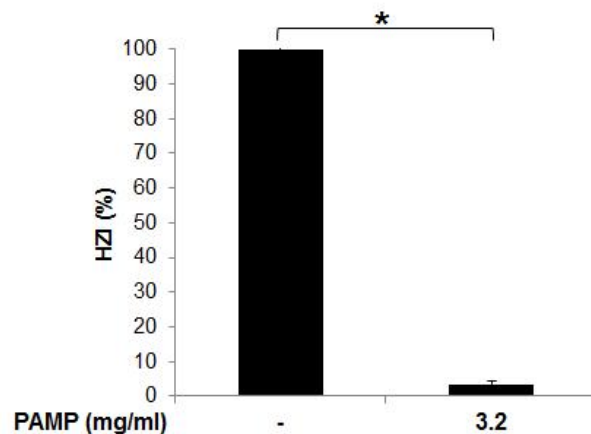
**Fig. 6.11. Las PAMP inhiben la fosforilación en tirosinas en espermatozoides no capacitados pero no en espermatozoides capacitados.** Los cambios en la fosforilación en tirosinas fueron evaluados mediante Western blot, utilizando el anticuerpo 4G10 para la detección de fosfotirosinas. (A y B) Espermatozoides capacitados en ausencia o presencia de diferentes concentraciones de PAMP durante 6 h; (C y D) Espermatozoides capacitados durante 5 h y posteriormente incubados en ausencia o presencia de diferentes concentraciones de PAMP. (A y C) Western blots representativos; (B y D) Análisis densitométrico de la abundancia de fosfotirosinas (pY) normalizada por la señal de  $\alpha$ -tubulina (tub). Se utilizó el FS como control. Se muestran las medias  $\pm$  EE.  $n=7$ . (\*)  $P < 0.05$ ; n.s.: no significativo. NC: No capacitado; CAP: capacitado.



**Fig. 6.12. Las PAMP inhiben la reacción acrosomal inducida por Cal en espermatozoides no capacitados pero no en espermatozoides capacitados.** Se evaluaron los efectos de las PAMP sobre la reacción acrosomal espontánea e inducida por Cal y se estimó el índice ARIC. (A) Espermatozoides capacitados en ausencia o presencia de diferentes concentraciones de PAMP durante 6 h; (B) Espermatozoides capacitados durante 5 h y posteriormente incubados en ausencia o presencia de diferentes concentraciones de PAMP. Se utilizó FS como control. Se muestran las medias  $\pm$  EE.  $n=6$ . (\*)  $P < 0.05$ ; n.s.: no significativo. NC: No capacitado; CAP: capacitado.

#### 6.4. Efecto de las PAMP en la interacción de los gametos

Para estudiar si las PAMP afectaban la capacidad de los espermatozoides de interactuar con el gameto femenino, se evaluó la unión de espermatozoides a la zona pelúcida mediante ensayos de hemizona. Se observó que en comparación al 100% de espermatozoides unidos a las HZ control, la unión a la HZ homólogas co-incubadas con espermatozoides pre-incubados con 3.2 mg/ml de las PAMP disminuyó a 3.4% (HZI:  $3.4 \pm 2.4$ ,  $n=17$ ,  $P < 0.05$ ), sugiriendo que las PAMP asociadas a la membrana plasmática de los espermatozoides interfieren con su capacidad de unirse a la zona pelúcida.



**Fig. 6.13. Las PAMP inhiben la interacción de espermatozoides con la zona pelúcida.** La capacidad de unión de los espermatozoides con la zona pelúcida se evaluó mediante ensayos de hemizona. Espermatozoides capacitados fueron pre-incubados en ausencia o presencia de 3.2 mg/ml de PAMP para co-incubarlos con la HZ control y la HZ del tratamiento, respectivamente. Los datos se expresaron como el índice de hemizona (HZI) y se muestran las medias  $\pm$  EE.  $n=17$ . (\*)  $P < 0.05$ .



## 7. DISCUSIÓN

Uno de los primeros cambios que ocurren durante la capacitación espermática es la remoción de las proteínas de superficie adquiridas durante la residencia en el epidídimo y al mezclarse con el FS durante la eyaculación. Este evento es fundamental para que el espermatozoide adquiera la capacidad de fecundar, ya que estas proteínas actúan como factores descapacitantes en diferentes modelos mamíferos. En ratón, se ha mostrado que las proteínas epididimales que se liberan durante la capacitación *in vitro* inhiben la reacción acrosomal inducida por progesterona y la interacción con la zona pelúcida [41]. Por su parte, en cerdo se ha descrito que la incubación de espermatozoides con FS disminuye la movilidad [63] y la tasa de fertilización *in vitro* [64], mientras que al incubar espermatozoides descongelados de toro con proteínas del FS no se observaron cambios en el porcentaje de espermatozoides con movilidad progresiva, pero sí un retraso en la inducción de la reacción acrosomal inducida por Cal [65]. Igualmente, en el modelo caprino se ha mostrado que la incubación de espermatozoides con proteínas del FS disminuye algunos parámetros cinéticos de movilidad, el porcentaje de espermatozoides capacitados y la fosforilación de tirosinas de algunas proteínas [66]. Adicionalmente, en este modelo se ha mostrado que dos proteínas del FS denominadas RSVP14 y RSVP22 tienen afinidad por la membrana plasmática de los espermatozoides y pueden reparar el daño estructural y mejorar la movilidad de espermatozoides descongelados [67]. Además, estas proteínas mantienen los espermatozoides en un estado no capacitado al inhibir la fosforilación de tirosinas [68], si bien se ha descrito que puede aumentar la unión de los espermatozoides a la zona pelúcida [69]. En este estudio, caracterizamos y aislamos 59 proteínas consistentemente presentes en las PAMP obtenidas a partir de espermatozoides de 3 donadores normozoospermicos, de las cuales 29 se identificaron mediante espectrometría de masas. Todas las proteínas identificadas previamente se describieron como componentes del FS, pero sólo algunas de ellas se evaluaron en cuanto a sus efectos sobre la función espermática, entre las cuales se encuentran LTF, semenogelina, CRISP1 y glicodelina.

La LTF es una glicoproteína que pertenece a la familia de las serotransferrinas, las cuales se componen de dos dominios en los cuales se unen el ión  $\text{Fe}^{3+}$  y una cadena glicano [70]. Esta proteína participa en la absorción del  $\text{Fe}^{3+}$  y en la modulación del sistema inmune [71] y en humano se secreta por el epidídimo [72] y la próstata [73]. Se considera un factor discapacitante debido a que disminuye la fosforilación de proteínas espermáticas de manera dependiente de la dosis [74], así como la interacción del espermatozoide con la zona pelúcida [75] al disminuir el porcentaje de receptores a D-manosa en la cabeza del gameto [76]. LTF tiene un receptor que se localiza en la cabeza y pieza media de los espermatozoides, el cual podría mediar sus efectos biológicos [77].

Los espermatozoides suspendidos en el semen se encuentran restringidos físicamente en el coágulo del plasma seminal, el cual es una red formada principalmente por semenogelina, una proteína secretada por las vesículas seminales [78]. Se ha propuesto que durante la eyaculación, componentes prostáticos como el zinc provocan la apertura de dímeros de fibronectina en el FS, lo que expone sitios de afinidad a la semenogelina para conformar una red a la que también se unen moléculas de fructosa, sialoglicoproteínas e iones metálicos [79, 80]. Después de la eyaculación, la serina proteasa del FS conocida como antígeno prostático específico (PSA) hidroliza la semenogelina, lo que permite la licuefacción del semen y la adquisición de la movilidad progresiva de los espermatozoides [79, 81]. Previamente, se ha demostrado que la incubación de espermatozoides con semenogelina provoca la disminución de algunos parámetros espermáticos de la movilidad [78] e inhibe otros parámetros de la capacitación como la fosforilación en tirosinas de proteínas y la producción del anión superóxido generado durante la capacitación [82].

La proteína CRISP1 forma parte de la familia de proteínas CRISP que se caracteriza por la presencia de 16 cisteínas altamente conservadas. En humanos, se han identificado las proteínas CRISP1, CRISP2 y CRISP3, todas localizadas en el tracto reproductor masculino. CRISP1 se secreta por el epidídimo y se une a la membrana plasmática del espermatozoide durante su almacenamiento en este

órgano [83]. Existen dos poblaciones de CRISP1 asociadas al espermatozoide de rata, una en mayor proporción que se encuentra débilmente unida al espermatozoide y que se libera durante la capacitación, y otra más pequeña que permanece fuertemente unida al gameto que migra al segmento ecuatorial durante la reacción acrosomal y participa durante la fusión de gametos [84]. En humano, también se ha mostrado que esta proteína interviene en la interacción del espermatozoide con la zona pelúcida [85]. Se desconoce el mecanismo de acción de esta proteína en el espermatozoide, pero su secuencia de aminoácidos es altamente similar a la de la toxina peptídica *helothermina*, la cual actúa como un inhibidor del influjo de calcio a través de un receptor de rianodina en las células musculares [86], mismo que ya ha sido localizado en el espermatozoide [87]. Por lo tanto, se ha sugerido que CRISP1 podría interactuar con el receptor de rianodina en el espermatozoide e inhibir el influjo de calcio [88]. Experimentos realizados en rata, mostraron que la incubación de espermatozoides con CRISP1 inhibe la fosforilación de tirosinas y la reacción acrosomal inducida por progesterona, por lo que se considera una proteína descapacitante [88].

La glicodelina S es una de las proteínas más abundantes en el semen [89]. Pertenece a la familia de las lipocalinas, las cuales son pequeñas proteínas extracelulares que se unen a pequeños compuestos lipófilos y a receptores de membrana [90]. Existen 4 isoformas de glicodelina denominadas S, F, A y C de acuerdo a su origen (Semen, fluido Folicular, líquido Amniónico y Cúmulo oóforo, respectivamente), las cuales comparten el mismo esqueleto aminoacídico y se diferencian en los patrones de glicosilación [91]. La glicodelina S es secretada por las vesículas seminales y se une a la cabeza de los espermatozoides, donde se ha mostrado que inhibe el eflujo de colesterol y la fosforilación de proteínas manteniendo al espermatozoide en un estado no capacitado [92]. Existen algunas hipótesis sobre el mecanismo de acción de la glicodelina S como factor descapacitante. Una de ellas sugiere que se une al glicocálix de la membrana plasmática del espermatozoide previniendo el ingreso de los aceptores de colesterol como la albúmina [92]. Otra hipótesis postula que, dado que la glicodelina tiene alta homología con otra lipocalina conocida como  $\alpha$ -lactoglobulina

que se une al colesterol [93], es posible que la glicodelina S también se una al colesterol reduciendo así su salida de la membrana plasmática. Por último, es posible que la unión de la glicodelina S al colesterol promueva la redistribución de este compuesto, reduciendo el acceso de los aceptores de colesterol [92].

Por otra parte, en este estudio se identificaron proteínas cuya posible participación en la regulación de la función espermática se desconoce. La isoforma 2 de antitripsina alfa 1, la antiqumotripsina alfa 1 y el inhibidor plasmático de serina proteasas que se identificaron en este estudio forman parte de la familia de inhibidores de serina proteasas conocida como serpina. En ratón, la serpina2, también conocida como proteasa nexina-1, se expresa en el epitelio del epidídimo, vías deferentes, glándulas coagulantes y en mayor proporción en las vesículas seminales [94]. Esta proteína se localiza en la región acrosomal de espermatozoides sin capacitar, lo que indica que se libera durante la capacitación. Serpina2 inhibe el eflujo de colesterol impidiendo la fosforilación en tirosinas y la unión de los espermatozoides a los ovocitos [95]. Otra de las proteínas identificadas en este estudio cuya función en el espermatozoide se desconoce es la PIP. Sin embargo, el gen que codifica para la PIP humana comparte una homología de secuencia significativa con el gen del autoantígeno de la vesícula seminal (SVA) de ratón [96] y ambas pertenecen a la misma familia de proteínas. La SVA es una glicoproteína que se une a los fosfolípidos que contienen colina de la membrana plasmática del gameto. Cuando los espermatozoides murinos llegan al tracto reproductor femenino esta proteína debe ser removida, ya que estudios previos han mostrado que inhibe algunas variables cinéticas de movilidad como VAP, VCL y VSL [97]. Asimismo, SVA inhibe la remoción de zinc, la fosforilación en tirosinas de proteínas y dificulta la unión de la albúmina, todo lo cual es necesario para la capacitación [98]. Sin embargo, se desconoce si PIP puede reproducir en espermatozoide humano algunos de los efectos descritos para la SVA en ratón.

Cabe mencionar que algunas de las proteínas identificadas en este estudio forman parte de varios complejos multiméricos que podrían regular la función

espermática. La semenogelina se degrada por PSA, el cual forma complejos con el inhibidor de serina proteasas (conocido como inhibidor de la proteína C) [99], una proteína que protege al espermatozoide contra una reacción acrosomal prematura y participa en la interacción del espermatozoide con la zona pelúcida [100]. Por otra parte, la semenogelina se une a la proteína epididimal Eppin [101], a la LTF y a la clusterina para formar otro complejo heteromultimérico [102] que inhibe la movilidad progresiva de los espermatozoides eyaculados al promover la pérdida de calcio intracelular [103, 104]. Estas evidencias sugieren que estos u otros complejos de proteínas aún no descritos podrían participar en la regulación de la capacitación espermática en humanos. Sin embargo, los mecanismos ejercidos por tales complejos en los espermatozoides son desconocidos y deben ser objeto de nuevas investigaciones.

Este estudio mostró que la asociación de las PAMP a la membrana plasmática de los espermatozoides no capacitados inhibe la fosforilación de tirosinas y la aparición de la hiperactivación, procesos asociados a la capacitación espermática. Estos resultados concuerdan con estudios previos realizados en ratón, en donde la incubación de espermatozoides con proteínas secretadas por el epidídimo inhibe la reacción acrosomal inducida por progesterona y la unión a la zona pelúcida [41]. Por otra parte, se evaluó el efecto de las PAMP sobre espermatozoides capacitados, en donde se observó que las proteínas fueron capaces de modificar el patrón de movilidad promoviendo la movilidad progresiva, similar al de espermatozoides no capacitados. Sin embargo, las PAMP no fueron capaces de revertir la fosforilación en tirosinas ni la reacción acrosomal inducida por Cal en espermatozoides capacitados. Este resultado es consistente con observaciones previas que mostraron que la incubación de espermatozoides capacitados de conejo y ratón con plasma seminal provocó una inhibición de la fertilización *in vitro* [16, 43].

Debido a que las PAMP fueron capaces de disociarse y reasociarse a la membrana plasmática de los espermatozoides, es posible que una desregulación de estos procesos conduzca a una capacitación espermática defectuosa. A este

respecto, estudios previos han mostrado que PIP y la proteína de unión a la galectina 3 se encuentran diferencialmente expresadas en el FS de donadores con oligozoospermia en comparación con donadores normozoospermicos [105] y que PIP se encuentra altamente expresada en la superficie de espermatozoides de donadores con astenozoospermia y oligozoospermia [106]. Adicionalmente, la clusterina y PSA muestran cambios de abundancia en el FS en pacientes con infertilidad idiopática en comparación con muestras de semen de donadores fértiles utilizadas para fertilizaciones *in vitro* [107]. Recientemente, se ha mostrado que existen diferencias en la abundancia relativa de algunas proteínas del FS de pacientes normozoospermicos que tuvieron fallas en tratamientos de fecundación *in vitro* en comparación con donadores fértiles, donde LTF, fibronectina, antileucoproteinasas, fosfatasa ácida prostática, aminopeptidasa N, proteína 1 de la matriz extracelular estaban altamente expresadas, mientras que el inhibidor plasmático de serina proteasa, isoforma 2 de antitripsina alfa 1, PIP, semenogelina, glicodelina y cistatina mostraron una baja abundancia [108]. Estas observaciones son congruentes con la participación de algunas de las PAMP en la patogénesis de ciertos tipos de infertilidad masculina, y por tanto el estudio de los mecanismos de acción de dichas proteínas sobre el espermatozoide podría contribuir no sólo al entendimiento de los mecanismos de regulación de la capacitación espermática sino que también podría tener implicaciones clínicas.

Con base en nuestros resultados, proponemos que la unión de las PAMP a la membrana plasmática del espermatozoide inhibe el eflujo de colesterol y por tanto los cambios subsecuentes que ocurren durante la capacitación, tales como la hiperactivación, la fosforilación de tirosinas y la reacción acrosomal inducida por Cal. Por otra parte, en vista de que las PAMP modificaron el patrón de movilidad de los espermatozoides ya capacitados, proponemos que la unión de uno o varios de los componentes de las PAMP a la membrana plasmática del espermatozoide inducen cambios en la célula que disminuyen la movilidad hiperactivada. Adicionalmente, la asociación de las PAMP a la membrana de los espermatozoides interfiere en el acceso de los receptores de membrana que reconocen a las proteínas de la zona pelúcida.

En síntesis, el presente trabajo permitió la identificación de proteínas asociadas a la membrana plasmática del espermatozoide humano eyaculado y postular algunos mecanismos de cómo podrían inhibir el progreso de la capacitación. Por lo tanto, estos resultados contribuyen a una mejor comprensión de los mecanismos que regulan la capacidad de fecundar de los espermatozoides y podrían ayudar a identificar nuevos biomarcadores para el diagnóstico de infertilidad.

## 8. CONCLUSIONES

- Se identificaron 29 proteínas liberadas durante la capacitación *in vitro* de espermatozoides humanos, todas previamente descritas en el FS humano.
- La incubación de espermatozoides no capacitados con las PAMP inhibió la hiperactivación, la fosforilación en tirosinas de proteínas espermáticas y la reacción acrosomal, cambios que son necesarios para que el espermatozoide se capacite y fecunde al ovocito.
- La incubación de espermatozoides capacitados con las PAMP produjo modificaciones en el patrón de movilidad asociado con la capacitación y la interacción con la zona pelúcida, pero no tuvieron efecto sobre la fosforilación en tirosinas ni la reacción acrosomal.
- La caracterización de las PAMP permitió la identificación de LTF, semenogelina, CRISP1 y glicodelina, mismas que han mostrado efectos inhibitorios sobre la función espermática en humano.
- Asimismo, identificamos la isoforma 2 de antripsina alfa 1, la antiquimotripsina alfa 1 y el inhibidor plasmático de serina proteasas, las cuales pertenecen a la familia de las serpinas que en ratón tienen efectos descapacitantes.
- Otra proteína identificada fue PIP, la cual es homóloga a la proteína SVA que en ratón tiene un efecto inhibitor de la capacitación. Además, se ha descrito que se expresa a la alta en la superficie de espermatozoides de donadores infértiles.
- Las proteínas clusterina, proteína de unión a la galectina 3, fibronectina, antileucoproteínasa, fosfatasa ácida prostática, aminopeptidasa N, proteína 1 de la matriz extracelular y cistatina, identificadas en este estudio, previamente han mostrado una expresión diferencial entre pacientes que fallan en tratamientos de fecundación *in vitro* y donadores fértiles.
- El estudio de los efectos de las PAMP identificadas en el presente trabajo sobre la función espermática en humano podría contribuir a entender los mecanismos que regulan el progreso de la capacitación.



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## 10. APÉNDICE 1

### **ARTÍCULO DE REQUISITO PARA LA OBTENCIÓN DEL GRADO**

Hernández-Silva, G., Fabián López-Araiza, J.E., López-Torres, A.S., Larrea, F., Torres-Flores, V., Chirinos M., Proteomic characterization of human sperm plasma membrane-associated proteins and their role in capacitation. *Andrology*, 2019. (*en imprenta*). doi: 10.1111/andr.12627

## ORIGINAL ARTICLE

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# Proteomic characterization of human sperm plasma membrane-associated proteins and their role in capacitation

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**ABSTRACT**

**Background:** Plasma membranes of ejaculated sperm are covered by epididymal and accessory glands secreted proteins that must be released from sperm surface during the female reproductive tract passage in order to capacitate and fertilize the oocyte.

**Objectives:** As human sperm plasma membrane-associated proteins (SMAP) have not yet been investigated, the aim of this study was to characterize the SMAP released during *in vitro* human capacitation and to study their possible role as decapacitation factors.

**Materials and Methods:** SMAP were characterized by 2-dimensional electrophoresis and mass spectrometry analysis. Besides, we explored SMAP effects on motility, protein tyrosine phosphorylation, and calcium ionophore-induced acrosome reaction of spermatozoa either incubated for 6 h in capacitating medium  $\pm$  SMAP or for 5 h in capacitating medium alone followed by incubation for 1 h  $\pm$  SMAP.

**Results:** Mass spectrometry analysis allowed the identification of 29 proteins, all of which have previously been identified in the human seminal fluid. Spermatozoa incubated for 6 h under capacitating conditions in the presence of the SMAP showed a significant decrease in the incidence of non-progressive motility, hyperactivation, protein tyrosine phosphorylation, and calcium ionophore-induced acrosome reaction. However, spermatozoa incubated for 5 h in capacitating medium and further incubated for 1 h with the SMAP showed a lower percentage of spermatozoa with non-progressive motility and hyperactivated cells but no effects on protein tyrosine phosphorylation were detected.

**Discussion and Conclusions:** Our results indicate that SMAP inhibit the progress of human sperm capacitation, but only motility changes related to capacitation may be reversed by these proteins. The study of the identified proteins on sperm function and their mechanisms of action on this cell may contribute to the understanding of their role during capacitation.

**INTRODUCTION**

In order to fertilize the oocyte, mammalian spermatozoa must migrate through the female reproductive tract from the cervix to the ampulla of the fallopian tube. Along this journey, the spermatozoa experience a series of biochemical and functional changes that enable their capacity to fertilize, a process known as capacitation (Chang, 1951; Austin, 1952). Capacitation begins with removal of sperm plasma membrane stabilizing factors acquired during their passage through the male genital tract, which promotes cholesterol efflux from the plasma membrane (Davis, 1979; Abou-haila & Tulsiani, 2009) and calcium influx by activation of sperm membrane ion channels (Costello *et al.*, 2009). These events result in the increase in protein tyrosine

phosphorylation (Visconti *et al.*, 1995a,b) and sperm hyperactivation (Morales *et al.*, 1988). It is generally accepted that the process of capacitation is accomplished when the spermatozoa are able to undergo the acrosome reaction, an exocytotic event required to penetrate the zona pellucida and fuse with the oocyte (De Jonge, 2005; Abou-haila & Tulsiani, 2009).

Some of the sperm plasma membrane-associated proteins (SMAP) acquired during epididymal storage and when mixed with accessory sex gland secretions during ejaculation keep the spermatozoa in an uncapacitated state, and hence have been named decapacitation factors (Fraser *et al.*, 2006; Fraser, 2010; Hernández-Silva & Chirinos, 2019). Although decapacitation factors are required to protect sperm plasma membrane and extend

cell viability, their removal is required for spermatozoa to interact with female reproductive tract molecules that regulate capacitation progress. Previous studies in mouse have identified several SMAP that have shown to decrease calcium influx (Adeoya-Osiguwa & Fraser, 1996; Chen *et al.*, 1998), inhibit sperm tyrosine phosphorylation (Huang *et al.*, 2000; Kawano and Yoshida, 2007), and prevent the acrosome reaction (Nixon *et al.*, 2006; Lin *et al.*, 2008). As human SMAP have not yet been investigated, the aim of the present study was to characterize the SMAP released during *in vitro* human capacitation and to study their possible role as decapacitation factors.

## MATERIALS AND METHODS

### Semen samples processing

This study was approved by the Human Ethical and Scientific Review Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, and all participants gave written informed consent. Semen samples were obtained by masturbation from normozoospermic volunteer donors after 3–5 days of sexual abstinence (see Table S1 for demographics and initial semen information). Samples were incubated for 30 min at 37 °C to allow its liquefaction and evaluated following standard procedures (World Health Organization, 1999). Spermatozoa were separated from seminal fluid (SF) by the swim-up technique in human tubal fluid (HTF) medium at pH 7.4 supplemented with 1 mg/mL polyvinyl alcohol and sodium pyruvate [0.33 mM]. For this purpose, 250 µL of semen was placed at the bottom of 15-mL tubes and 800 µL of HTF was placed on top. After 1-h incubation at 37 °C, the uppermost fraction with the motile cells was collected, reevaluated, and adjusted to a concentration of 15,000 sperm/mL. Only samples with at least 75% motile cells were used. Alternatively, semen samples were centrifuged through discontinuous density gradients (Isolate; Irvine Scientific, Irvine, CA, USA) as previously described (Lopez-Torres *et al.*, 2017). Seminal fluid samples were recovered from the top of the gradients and centrifuged at 3000 *g* for 20 min at 4 °C to eliminate cell debris, and the supernatants were kept at –70 °C until use.

### Isolation and characterization of SMAP

Motile spermatozoa recovered by either swim-up or density gradients centrifugation were incubated for 6 h in HTF medium under capacitating conditions (37 °C, 5% CO<sub>2</sub>) and centrifuged at 800 *g* for 8 min. To isolate the SMAP fractions, supernatants were re-centrifuged at 3000 *g* for 15 min to eliminate cell debris. Protein concentration was determined by the bicinchoninic acid method (BCA, Pierce, Rockford, IL, USA), and SMAP and SF samples from 6 different donors were pooled. The absence of cell contamination in SMAP samples was assessed under the microscope and additionally by Western blots employing anti-β-tubulin antibodies, as described below.

For characterization of SMAP, pooled samples were analyzed by SDS-PAGE and two-dimensional electrophoresis (2-DE) as previously described (Hernandez-Silva *et al.*, 2018). In SDS-PAGE analyses, 25 µg of protein samples was boiled in denaturing buffer (0.6 M Tris/HCl, 2% SDS, 10% glycerol, bromophenol blue, and 2% β-mercaptoethanol, pH 6.8) and resolved on 4–20% polyacrylamide gradient gels. For 2-DE, each sample was analyzed in triplicate by loading 169 µg of proteins solubilized in

rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte, and bromophenol blue) on linear 3–10 pH gradient strips of 7 cm length (Ready Strip IPG; Bio-Rad, Hercules, CA, USA) for rehydration during 12 h. Afterward, isoelectrofocusing was performed at 20 °C in a Protean IEF Cell System (Bio-Rad) with the following electrical conditions: 20 min with a linear ramp (0–250 V), 2 h with a linear ramp (250–4000 V), and 4000–10,000 V-h with rapid ramp. After focusing, strips were equilibrated for 10 min in 6 M urea, 2% SDS, 20% glycerol, 2% DTT, 0.05 M Tris/HCl, pH 8.8, followed by equilibration for 15 min in 6 M urea, 2% SDS, 20% glycerol, 2.5% iodoacetamide, and 0.05 M Tris/HCl, pH 8.8. Subsequently, strips were placed on top of 4–20% SDS-PAGE and electrophoresed. Gels were stained with Coomassie brilliant blue for 40 min, digitalized with a ChemiDoc XRS+ imager (Bio-Rad), and analyzed using the PDQuest software (Bio-Rad).

### Protein identification by mass spectrometry

SMAP protein composition was analyzed by mass spectrometry, according to the protocol developed in the Research and Industry Support Services Unit (Unidad de Servicios de Apoyo a la Investigación y la Industria-USAI) of the School of Chemistry at the National Autonomous University of Mexico (Duarte Escalante *et al.*, 2018). Briefly, shotgun proteomic was used to analyze total SMAP that were immobilized by electrophoresing the sample in SDS-PAGE gels, followed by staining with Coomassie brilliant blue. Gel section containing the mix of proteins was cut into smaller fragments and destained in 50% methanol (v/v) and 5% acetic acid (v/v). After washing with distilled water and incubated twice in 100 mM of ammonium bicarbonate for 15 min, proteins were reduced in 50 mM DTT for 45 min and alkylated with 30 mM iodoacetamide for 2 h. Subsequently, samples were washed three times with 100 mM of ammonium bicarbonate and dehydrated with 100% acetonitrile. Gel digestion was carried out with 30 µL of modified porcine trypsin from a solution containing 20 ng/µL at a protein:trypsin relation of 30:0.6 µg and incubated for 18 h at 37 °C. The resulting peptide soup was extracted from the gel by sonication in 50% acetonitrile (v/v) and 5% formic acid (v/v), and the soluble fraction was recovered, brought to dryness, and resuspended in 20 µL of 1% formic acid (v/v). Afterward, samples were desalted and concentrated by ZipTip C18 columns (Millipore, Temecula CA, USA) and eluted with 12 µL of 3% acetonitrile, 0.1% formic acid. Peptides were subjected to liquid chromatography (nanoACQUITY, Waters Corporation, Cerdanyola del Vallès, Spain) and mass spectrometry (SYNAPT G2S with time of flight, Waters Corporation) with an electrospray ionization system. The retrieved spectrometric data were processed with ProteinLynx Global Server (PLGS version 2.5.1, Waters, Milford, MA, USA) to reconstruct MS/MS spectra (Rosenegger *et al.*, 2010), using a maximum false positive rate of 4. Only peptides with a PLGS score > 95% of confidence were accepted as correct. Afterward, peptides were contrasted against the UNIPROT Knowledgebase database (UniprotKB, 2016; <http://www.uniprot.org>) for protein identification, considering only matches from the human database. Selected protein identification was validated by Western blot employing commercial specific antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Additionally, accession numbers were submitted to the UniProtKB for human gene ontology analysis and a panel of 13 main molecular functions was used to categorize the proteins and generate a pie graph as described before (Soleilhavoup *et al.*, 2016).

### SMAP binding to sperm plasma membrane

To ascertain the SMAP ability to re-associate to sperm plasma membrane, proteins were fluorescence labeled in carbonate buffer and incubated in the presence of 1:20 (protein: FITC) isothiocyanate of fluorescein (FITC; Sigma-Aldrich, St Louis, MO, USA) for 6 h at 4 °C in darkness. The FITC-labeled SMAP (FITC-SMAP) were dialyzed against PBS for 24 h and the concentration determined by the BCA method. In order to assess the FITC-SMAP ability to bind to spermatozoa, cells were incubated for 1 h in the absence or presence of increasing concentrations of FITC-labeled proteins under capacitating conditions, fixed with 70% ethanol, and smeared onto poly-L-lysine pretreated slides. Binding of proteins to spermatozoa was evaluated under a fluorescence microscope (Olympus model BX51; Olympus Inc., Tokyo, Japan), and images were captured with an Olympus camera, model DP72. Images were analyzed with the ImageJ free software (National Institutes of Health, Bethesda, MD, USA) to quantify the fluorescence intensity of the total area from 5 spermatozoa per experimental condition. Intensity values of fluorescence were normalized after subtraction of the background fluorescence observed in each field and expressed as the average of arbitrary units (A.U.) of intensity for each experimental condition.

### Effect of SMAP on sperm function

Sperm samples obtained by swim-up ( $N = 6$ , from equal number of donors) were incubated for 6 h under capacitating conditions in the absence or presence of 0.8, 1.6, 3.2 mg/mL of SMAP. In a different set of experiments, spermatozoa were incubated in HTF medium during 5 h to allow the release of endogenous SMAP and then further incubated for 1 h in the absence or presence of same concentrations of exogenous SMAP. As a positive control, incubations with 16 mg/mL of SF proteins were also included. At the end of the incubations, sperm samples were processed to evaluate effects on sperm functions.

### Evaluation of motility

Sperm motility was analyzed under a phase contrast microscope. At least two hundred spermatozoa were analyzed per sample and graded as rapid progressive (A), slow progressive (B), non-progressive (C), or immotile (D) according to the W.H.O. criteria (World Health Organization, 1999). The percentage of progressive motility was defined as the sum of A + B, as previously described (Caballero-Campo *et al.*, 2006). Additionally, motility kinetic parameters were analyzed by a computer-assisted sperm analyzer (CASA; Hamilton Thorne IVOS version 14.0, Hamilton Thorne Research Inc., Beverly, MA, USA), operating at a frame rate of 30/sec and a frequency of 60 Hz. Sperm samples were placed in 20  $\mu\text{m}$  deep cell counting chambers (Leja Products BV, Nieuw-Vennep, the Netherlands), and 10 random fields were evaluated for a minimum of 300 cells per experimental condition. Kinetic variables values were obtained, and the percentages of hyperactivated cells were estimated, using  $\text{VCL} \geq 150$ ,  $\text{LIN} \leq 50$  and  $\text{ALH} \geq 3.5$  as the cutoff values.

### Analysis of tyrosine phosphorylation

Changes in sperm protein tyrosine phosphorylation were analyzed by Western blots as described before (Lopez-Torres *et al.*, 2017). Briefly, at the end of incubations spermatozoa were washed with PBS supplemented with sodium orthovanadate

1 mM and genistein 0.2 mM and resuspended in denaturing buffer. After SDS-PAGE separation, proteins were electrotransferred onto nitrocellulose membranes and blocked with BSA 3% in TBS-Tween 20 (0.05%). Afterward, membranes were probed with 1 : 5000 (v/v) monoclonal mouse-anti-phosphotyrosine antibodies (4G10; Millipore) in TBS-Tween 20, followed by incubation in the presence of 1 : 4000 goat-anti-mouse IgG- coupled to HRP (Millipore). For homogeneous loading checking, membranes were re-probed with anti- $\beta$ -tubulin antibodies (Sigma-Aldrich). Immunocomplexes were visualized by chemiluminescence (ECL Prime; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analyses were performed using the Image Lab Software (version 4, Bio-Rad), and the phosphotyrosine intensities were normalized against  $\beta$ -tubulin signal intensity.

### Analysis of acrosome reaction

Sperm spontaneous and calcium ionophore-induced acrosome reactions were evaluated using the *Pisum sativum* agglutinin conjugated to fluorescein staining technique (Caballero-Campo *et al.*, 2006). At the end of incubations with or without the SMAP, two sperm aliquots of 150,000 cells were taken for either fixation or further incubation with the calcium ionophore A23187 (CaI) [10  $\mu\text{M}$ ] (Sigma-Aldrich) for 20 min at 37 °C. After fixing with 70% ethanol, sperm samples were smeared onto poly-L-lysine pretreated slides and stained with 20  $\mu\text{g}/\text{mL}$  of *Pisum sativum* agglutinin conjugated to fluorescein (Sigma-Aldrich). A minimum of two hundred spermatozoa were evaluated under a fluorescence microscope. All spermatozoa showing fluorescence in the acrosomal region were considered as acrosome-intact, whereas those showing absence of fluorescence in the acrosome, staining at the equatorial region, or patchy staining over the acrosome region were considered as acrosome reacted. Data were expressed as the ARIC score (Acrosome Reaction to Ionophore Challenge) defined as the percentage of calcium ionophore-induced acrosome reaction minus the percentage of spontaneous acrosome reaction (Quintero *et al.*, 2005).

### Statistical analysis

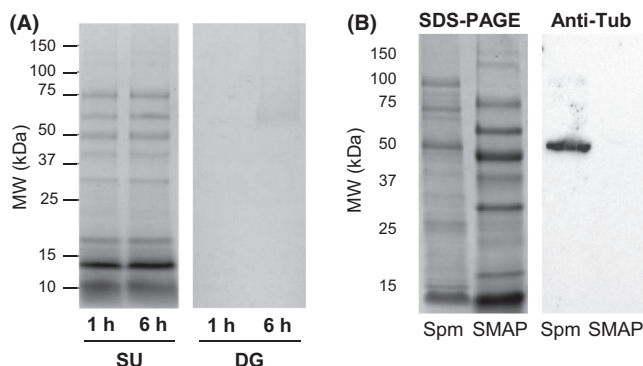
Data are presented as the mean  $\pm$  SEM. Experimental groups were compared using the GraphPad Prism 5.01 software, using ANOVA and Tukey multicomparison for post hoc test, considering significant a  $p$  value  $\leq 0.05$ .

## RESULTS

### Characterization of SMAP

In order to characterize the human SMAP, we first optimized the conditions for their isolation. SMAP were recovered after 1-h incubations in HTF medium of spermatozoa obtained by swim-up and density gradient centrifugation, and protein patterns were compared after SDS-PAGE. As shown in Fig. 1A, when spermatozoa were separated from SF by swim-up, a total of 12 protein bands were obtained, whereas only one protein was detected after density gradient centrifugation. These data suggested that the friction generated during centrifugation removes most of the proteins associated with the surface of the spermatozoa. Moreover, the time course analysis of the proteins released during sperm capacitation for 1 and 6 h showed that the amount of SMAP released slightly improved at longer incubation times,

**Figure 1** Isolation of SMAP. (A) Spermatozoa were separated from seminal fluid by either swim-up (SU) or density gradient (DG) centrifugation and incubated under capacitating conditions for 1 and 6 h. After capacitation, released sperm plasma membrane-associated proteins (SMAP) were separated from spermatozoa by centrifugation and analyzed by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining. (B) Purity of the SMAP. Proteins from spermatozoa (Spm) and SMAP were separated by SDS-PAGE and stained with CBB. A sample set duplicate was analyzed by Western blots with anti- $\beta$ -tubulin antibodies to discard the presence of cellular contaminants.



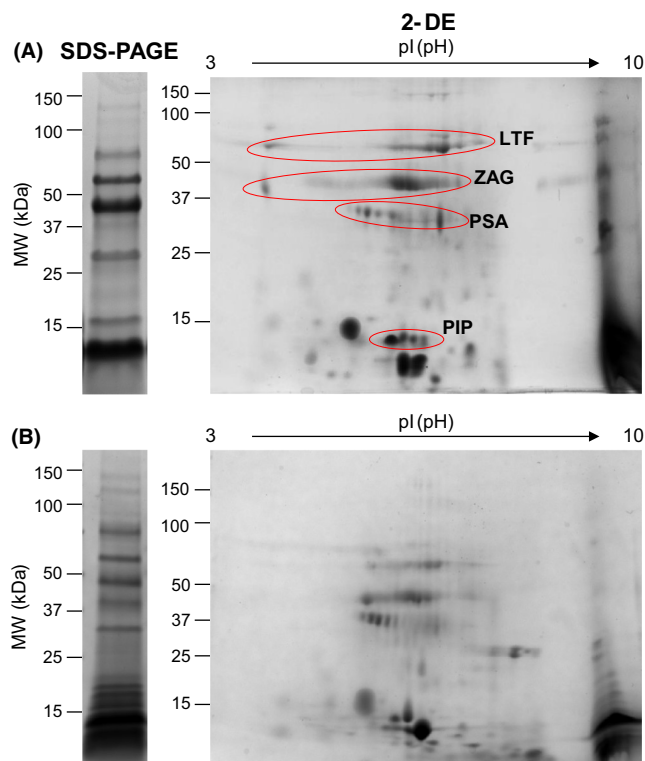
but the protein pattern remained the same. On the other hand, potential cell contamination of SMAP samples obtained after swim-up was checked by Western blot with anti- $\beta$ -tubulin antibodies, a constitutive protein of cell microtubules that was detected in the sperm proteins extract used as control but was absent in the SMAP preparations (Fig. 1B).

The protein pattern of human SMAP and SF is shown in Fig. 2A and B, respectively. Densitometric analysis of SDS-PAGE showed that SMAP were composed by 12 protein bands while 18 were detected in SF. Further analysis by 2-DE gels indicated that SMAP protein maps contained 59 spots vs. 136 detected in SF. These results suggest that not all proteins existing in the SF associate with sperm plasma membrane. Therefore, SMAP were analyzed by mass spectrometry allowing the identification of 29 proteins that were released during *in vitro* capacitation (Table 1). Positive MS/MS identification of the proteins prolactin-induced protein (PIP), lactotransferrin (LTF), prostate-specific antigen (PSA), and zinc alpha 2 glycoprotein (ZAG) was validated by Western blot (Figure S1). Moreover, the functional classification of these proteins according to their GO molecular functions showed a high prevalence of proteins involved in protein binding (22%) and endopeptidase inhibitor activity (7%), as depicted in Fig. 3.

#### SMAP binding to spermatozoa

In order to evaluate the effects of SMAP on sperm function, we first investigated whether the SMAP had the ability to re-associate with the sperm plasma membrane. When spermatozoa were incubated with increasing concentrations of FITC-SMAP, we observed a dose-dependent increase in fluorescent intensities in all cells that were significant at 1.6, 3.2, and 6.4 mg/mL of proteins when compared to the negative controls in the absence of FITC-SMAP (Fig. 4A and B). When re-association experiments were performed with 6.4 mg/mL of SMAP, only a subtle increase in the sperm fluorescence intensities was detected compared to 3.2 mg/mL, suggesting that the re-association dynamic was close to the plateau. Moreover, it should be noted that FITC-

**Figure 2** Proteomic maps of SMAP and seminal fluid. Protein composition of pool samples of SMAP (A) and seminal fluid (B) from normozoospermic donors were separated by SDS-PAGE and two-dimensional electrophoresis (2-DE) and stained with CBB. Representative gels are shown. Approximate location of 4 proteins validated by Western blot is indicated.



SMAP bound to the plasma membrane of all spermatozoa, showing higher fluorescence intensities in the neck and middle piece of the cell. Specific binding of FITC-SMAP to spermatozoa was evaluated by competition assays by incubating spermatozoa with 3.2 mg/mL of FITC-SMAP in the presence of increasing concentrations of non-fluorescent SMAP, which resulted in a concentration-dependent decrease in the fluorescence signal (Fig. 4C).

#### Effect of SMAP on sperm function

Effects of incubating non-capacitated spermatozoa with 0.8, 1.6, and 3.2 mg/mL of SMAP or 16 mg/mL of SF for 6 h on motility, protein tyrosine phosphorylation, and ionophore-induced acrosome reaction were evaluated. When sperm manual motility was analyzed (Fig. 5A), the motility of non-capacitated (0 h) spermatozoa was predominantly progressive. After 6-h incubations under capacitating conditions in the absence of SMAP, the progressive motility decreased concomitantly with an increase in non-progressive motility. However, when incubations were performed in the presence of increasing concentrations of SMAP, the motility patterns of capacitated spermatozoa resulted in a dose-dependent reversion toward a non-capacitated progressive motility pattern. Same effect was observed when spermatozoa were incubated in the presence of SF. Further analysis of sperm motility by CASA showed a SMAP dose-dependent decrease in the kinetic parameters VAP, VSL, VCL, and ALH (Fig. S2A), while other kinetic variables exhibited no changes (Table S2). As VCL and ALH are variables related to

**Table 1** Human SMAP identified by mass spectrometry

Name	UniProt accession number	mW (kDa)	pI (pH)	PLGS Score	Peptides per protein	Theoretical peptides	Coverage (%)	False positive rate
Prolactin inducible protein	P12273	16.6	8.1	83,452.9	48	12	71.9	0
Lactotransferrin	P02788	78.1	8.0	63,999.4	161	74	70.7	0
Isoform delta of lactotransferrin	P02788	73.1	7.7	60,323.5	157	71	72.4	0
Isoform 3 of Prostate-specific antigen	P07288	23.7	7.1	45,684.8	31	15	72.0	0
Isoform 5 of prostate-specific antigen	P07288	25.1	6.6	45,580.7	30	14	57.7	0
Zinc alpha 2 glycoprotein	P25311	34.2	5.6	30,551.8	46	27	43.6	0
Serum albumin	P02768	69.3	5.9	25,358.3	102	55	40.4	0
Isoform 3 of serum albumin	P02768	45.1	5.7	25,224.1	85	36	45.9	0
Prostatic acid phosphatase	P15309	44.5	5.8	23,654.8	51	27	44.3	0
Isoform 4 of clusterin	P10909	48.8	6.3	18,858.9	24	36	26.9	0
Isoform 10 of fibronectin	P02751	239.5	5.5	18,053.6	103	138	35.9	0
Isoform 4 of fibronectin	P02751	222.8	5.3	18,023.9	100	122	36.8	0
Plasma serine protease inhibitor	P05154	45.6	9.6	7636.9	27	30	32.3	0
Aminopeptidase N	P15144	109.5	5.2	7297.9	52	73	28.3	0
Isoform short of cysteine-rich secretory protein 1	P54107	20.4	6.7	6892.6	4	12	11.2	0
Galectin 3 binding protein	Q08380	65.3	4.9	5285.6	34	39	23.1	0
Isoform 2 of alpha 1 antitrypsin	P01009	40.2	5.1	3849.8	13	29	30.6	0
Ig alpha 1 chain C region	P01876	37.6	6.1	2927.6	7	23	31.4	0
Isoform 2 of semenogelin 1	P04279	45.3	9.8	2626.9	4	35	8.5	0
Cystatin S	P01036	16.2	4.8	2329.5	8	14	53.9	0
Antileukoprotease	P03973	14.3	8.7	2251.0	2	13	9.1	0
Isoform 2 of carboxypeptidase	P16870	49.8	4.9	1803.1	16	39	37.7	0
Ig gamma 1 chain C region	P01857	36.1	8.2	1765.9	7	21	20.3	0
Prosaposin	P07602	58.1	4.9	1376.3	12	47	14.3	0
Alpha 1 antichymotrypsin	P01011	47.6	5.2	882.4	6	31	17.5	0
Extracellular matrix protein 1	Q16610	60.6	6.2	855.4	6	47	14.4	0
Isoform 2 of sulfhydryl oxidase 1	O00391	66.8	8.6	644.6	7	53	14.9	0
Isoform 2 of glycodeilin	P09466	18.3	4.9	637.7	2	11	12.7	0
Ig gamma 3 chain C region	P01860	41.3	7.8	618.1	4	24	9.5	0

PLGS score is calculated by the Protein Lynx Global Server (Waters) from all available mass spectrometry data and is a statistical measure of peptide assignment accuracy. A higher score implies greater confidence of protein identity (Rosenegger *et al.*, 2010). Only peptides with a PLGS score > 95% confidence were accepted as correct.

hyperactivation (Morales *et al.*, 1988), we also evaluated the effect of the SMAP on the incidence of this capacitation-related type of motility. As shown in Fig. 5B, hyperactivation increased under capacitating conditions after 6-h incubations, but the presence of increasing concentrations of SMAP significantly reduced the incidence of hyperactivated cells.

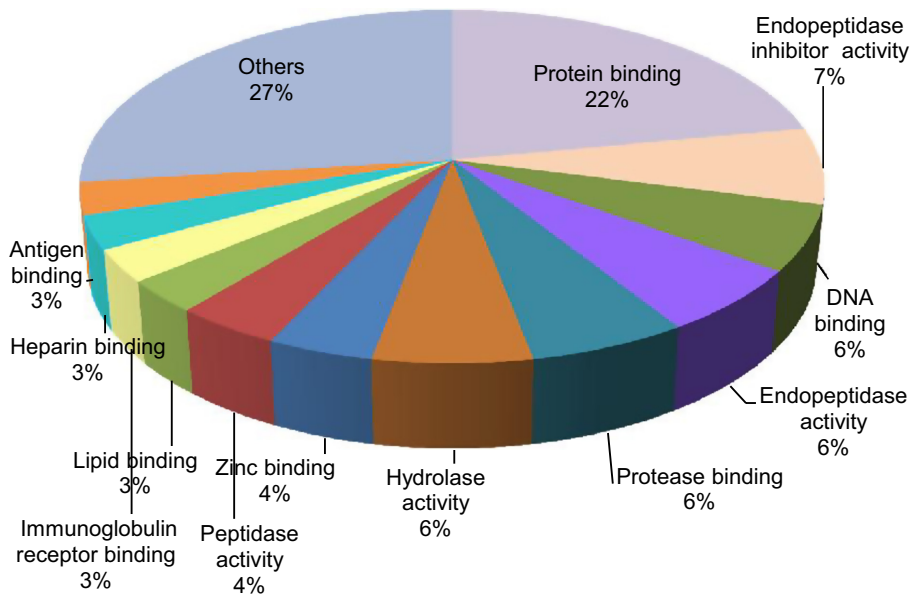
With the purpose of studying SMAP effects on sperm capacitation, changes in the abundance of protein tyrosine phosphorylation were evaluated. Figure 6A shows a representative Western blot of protein tyrosine phosphorylation in non-capacitated spermatozoa (0 h) and after 6-h capacitation in the absence or presence of increasing concentrations of SMAP and SF used as control. The intensity of two proteins of approximately 80 and 105 kDa increased after capacitation, as has been described before (Chirinos *et al.*, 2017). However, sperm incubation with 0.8, 1.6, and 3.2 mg/mL of the SMAP and 16 mg/mL of SF significantly decreased tyrosine phosphorylation. This observation was corroborated after densitometric analysis of 7 independent experiments (Fig. 6B). To further corroborate the SMAP effect on sperm capacitation, calcium ionophore-induced acrosome reaction was also assessed and the ARIC index was estimated. The results showed a significant decrease in the ability of spermatozoa to undergo calcium-induced acrosome reaction with 3.2 mg/mL of SMAP and SF, indicating that these proteins interfere with the process of sperm capacitation (Fig. 6C).

As the SMAP inhibited the progress of sperm capacitation, we also assessed the effects of these proteins on capacitated spermatozoa. For this purpose, in a different set of experiments spermatozoa were capacitated for 5 h in HTF medium alone and

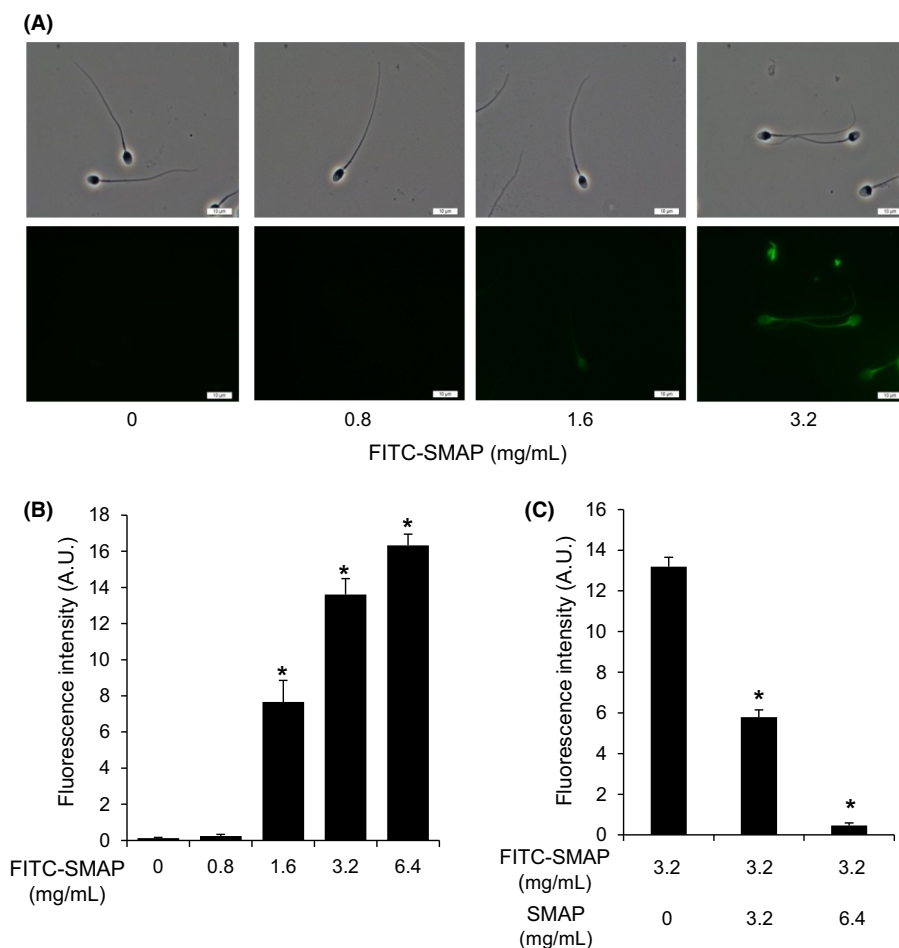
then incubated for one additional hour in the absence and presence of SMAP. The manual motility evaluation showed that when capacitated spermatozoa were exposed to increasing concentrations of SMAP (0.8, 1.6, 3.2 mg/mL) or SF, there was a dose-dependent increase in progressive motility that was accompanied by a decrease in non-progressive motility, similar to the non-capacitated motility pattern (Fig. 7A). Likewise, CASA analysis of kinetics parameters showed changes in several motility variables when capacitated spermatozoa were incubated in the presence of increasing concentrations of SMAP (Fig. S2B and Table S3) and hence hyperactivation decreased as a consequence of sperm exposure to SMAP and SF (Fig. 7B). It should be noted that the effects of SMAP on motility of capacitated spermatozoa were similar to those observed in non-capacitated spermatozoa (see Fig. 5). However, when the effect of SMAP on tyrosine phosphorylation of capacitated sperm was evaluated, no changes were observed at any of the concentrations used (Fig. 7C and D).

## DISCUSSION

During ejaculation, spermatozoa are suspended in the SF containing molecules that stabilizes sperm plasma membrane to prevent premature capacitation. Different molecular entities have been described to be involved in this process, such as cholesterol (Davis, 1981). However, several proteins from the SF have also shown to participate in keeping the spermatozoa in a decapacitation state (reviewed in Hernández-Silva & Chirinos, 2019). Most of the identified proteins have already been identified as components of the SF, either in the soluble fraction



**Figure 3** Molecular functions of SMAP. Distribution of the molecular functions of the SMAP identified by mass spectrometry according to Gene Ontology definitions published in UniprotKB database.

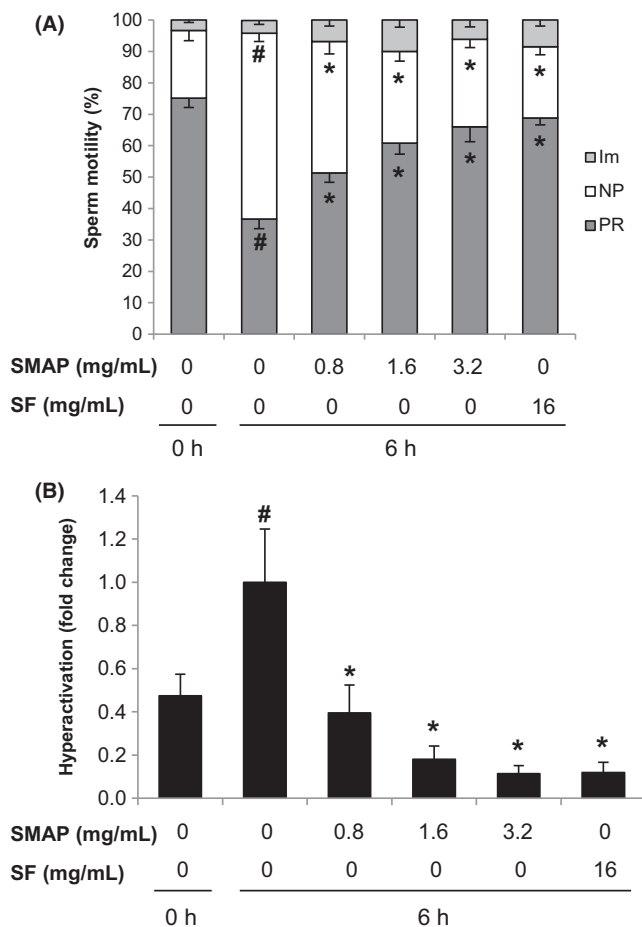


**Figure 4** SMAP binding to spermatozoa. (A) Spermatozoa were capacitated in the absence or presence of increasing concentrations of FITC-conjugated SMAP (FITC-SMAP) for 1 h and evaluated at the microscope. Phase contrast and fluorescent images of the same field are shown. Magnification: 100 $\times$ . (B) Densitometric analysis of fluorescence intensities of spermatozoa incubated with increasing concentrations of FITC-SMAP. The fluorescence intensities from 5 spermatozoa per experimental condition were quantified with the ImageJ software, normalized after subtraction of the background, and expressed as the average of arbitrary units (A.U.). Mean  $\pm$  SEM.  $N = 7$ , from equal number of donors. (\*)  $p < 0.05$  vs. FITC-SMAP 0 mg/mL. (C) Densitometric analysis of fluorescence intensities of spermatozoa incubated with 3.2 mg/mL of FITC-SMAP in the presence of increasing concentrations of non-fluorescent SMAP. Mean  $\pm$  SEM.  $N = 5$ . (\*)  $p < 0.05$  vs. non-fluorescent SMAP 0 mg/mL.

(Bianchi *et al.*, 2018) or contained in the seminal plasma exosomes (Yang *et al.*, 2017), including cystatin S and antileukoprotease whose association to spermatozoa has not been investigated. Contrariwise, other proteins herein identified have previously been implicated as sperm functional regulators. For instance, human semenogelins (Sg1 and Sg2), components of

the human semen coagulum, suppress sperm protein tyrosine phosphorylation (de Lamirande *et al.*, 2001) and motility (Robert & Gagnon, 1996) and therefore act as regulators of sperm capacitation by preventing its premature occurrence. The mouse homologue of semenogelins, named SVS2, is a decapacitation factor that regulates sperm fertilizing ability *in vivo* and its

**Figure 5** Effect of SMAP on motility of non-capacitated spermatozoa. Spermatozoa were capacitated for 6 h in the absence or presence of increasing concentrations of SMAP or seminal fluid (SF) and motility changes were evaluated. (A) Manual motility analysis, classified according to W.H.O. criteria. Im, Immotile; NP, Non-progressive; PR, Progressive. (B) Percentage of hyperactivated spermatozoa. Values on the Y-axis represent the fold change, considering the values obtained in the absence of SMAP at 6-h incubations used as the control. Mean  $\pm$  SEM, from 6 sperm samples from equal number of donors. (#)  $p < 0.05$  vs. SMAP 0 mg/mL (0 h); (\*)  $p < 0.05$  vs. SMAP 0 mg/mL (6 h).



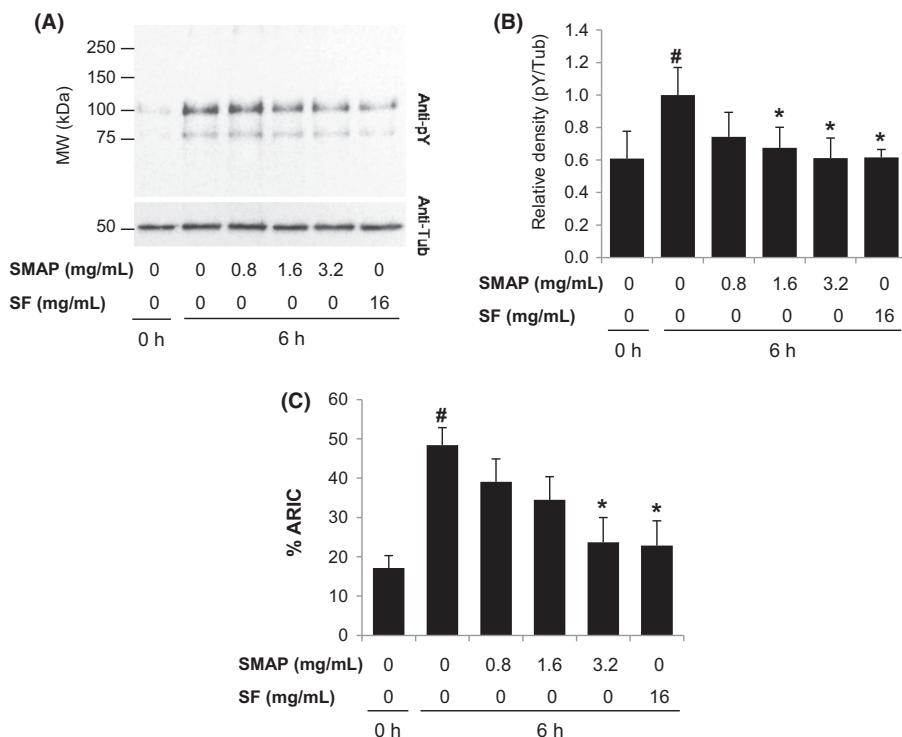
interaction with the spermatozoa is mediated by the ganglioside GM1 located in the sperm membrane (Kawano & Yoshida, 2007; Kawano *et al.*, 2008). Semenogelins binding to GM1 have not yet been reported in humans, but prosaposin, a ganglioside GM1 binding protein (Hiraiwa *et al.*, 1992), was identified in this study and therefore is a potential decapacitation factor. In addition, Sg1 is part of several multimeric complexes involved in sperm function regulation. On one hand, during semen coagulum liquefaction, Sg1 is degraded by the PSA (de Lamirande *et al.*, 2001), which form complexes with the plasma serine protease inhibitor (also known as the protein C inhibitor) (España *et al.*, 1991; Suzuki *et al.*, 2007), a protein that has been involved in sperm protection against premature acrosome reaction and modulation of spermatozoa–oocyte interactions (Elisen *et al.*, 1998). Additionally, in the surface of human spermatozoa Sg1 also binds to the protein Eppin (Wang *et al.*, 2005) that, along with LTF and clusterin, forms a heteromultimer complex (Wang *et al.*, 2007) that inhibits the progressive motility of ejaculated spermatozoa (Mitra *et al.*, 2010). Interestingly, there are

evidences that PIP interacts with serum albumin, ZAG, prostatic acid phosphatase, and Sg1 fragments (Yadav *et al.*, 2011; Tomar *et al.*, 2013), suggesting that this protein complex may also participate in the regulation of human sperm capacitation. Along with Sg1, we identified in the human SMAP several of the proteins involved in Sg1 heteromultimers (specifically PSA, plasma serine protease inhibitor, LTF, clusterin, PIP, serum albumin, and ZAG), and therefore, we hypothesize that such protein complexes may be involved in the regulation of the capacitation progress. Nevertheless, the mechanisms exerted by such protein complexes on spermatozoa are unknown and must be subject of further investigations.

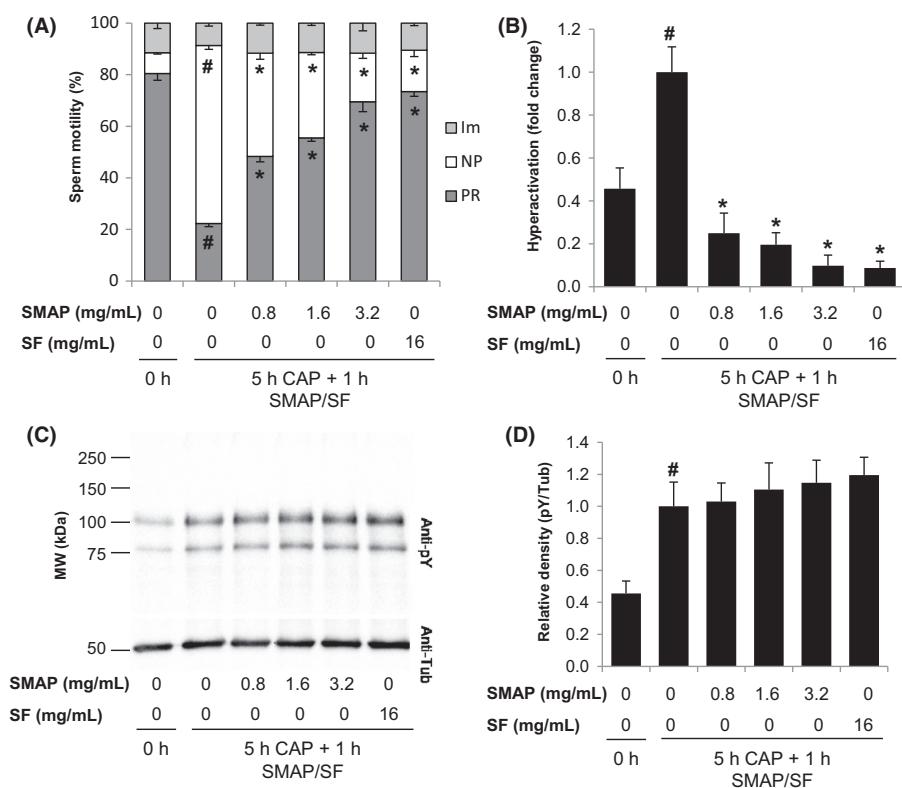
This study showed that association of human SMAP with sperm plasma membranes affects protein tyrosine phosphorylation and sperm hyperactivated motility, which normally occur during the capacitation process, indicating their role as decapacitation factors. Moreover, since only fully capacitated spermatozoa are able to undergo acrosome reaction (Jaiswal *et al.*, 1998), the reduction on the ARIC index herein described further support this observation. In contrast, mouse SMAP obtained from epididymal spermatozoa have shown to inhibit sperm ability to undergo progesterone-induced acrosome reaction and spermatozoa binding to the zona pellucida *in vitro* but had no effects on the tyrosine phosphorylation of internal proteins (Nixon *et al.*, 2006), suggesting that mouse and human SMAP may have different protein composition and/or regulate the capacitation progress by different mechanisms. Supporting potential differences between human and mouse SMAP content, phosphatidylethanolamine binding protein 1 (PEBP1) and decapacitation factor 10 (DF10) described in mouse SMAP (Nixon *et al.*, 2006) are apparently absent in human model, according to our observations. On the other hand, cysteine-rich secretory protein 1 (Crisp-1) has been identified in mouse SMAP (Nixon *et al.*, 2006) and in the present work. Nonetheless, although previous investigations indicated that rat and mouse Crisp-1 inhibited sperm tyrosine phosphorylation (Roberts *et al.*, 2003; Da Ros *et al.*, 2008), human recombinant hCrisp-1 had no effects on protein tyrosine phosphorylation or motility parameters (Maldera *et al.*, 2014), suggesting that functional activity of Crisp-1 on spermatozoa is species specific.

As the SMAP were able to dissociate and re-associate to the sperm plasma membrane, a misregulation of these mechanisms may account for some *in vitro* sperm capacitation defects and therefore has clinical implications. Indeed, an early proteomic approach to study sperm proteins from infertile men detected that Sg2 and clusterin were predominantly present in the semen of healthy men but absent in the semen of infertile men, suggesting unique differences in the sperm protein profiles of fertile and infertile men (Thacker *et al.*, 2011). Likewise, it has been described that PIP is highly expressed in the surface of spermatozoa from donors with asthenozoospermia and oligoasthenoteratozoospermia (Capkova *et al.*, 2007), while Sg1 (Martinez-Heredia *et al.*, 2008) and clusterin (Hashemitabar *et al.*, 2015) are differentially expressed in spermatozoa from asthenozoospermic semen samples. Moreover, PIP and galectin 3 binding protein (Gal3BP) seem to be differentially expressed in SF of oligoasthenozoospermic when compared to normozoospermic donors (Giacomini *et al.*, 2015). Recently, it has been shown that clusterin and PSA are differentially expressed in SF from unexplained male infertility patients between successful





**Figure 6** Effect of SMAP on protein tyrosine phosphorylation and calcium ionophore-induced acrosome reaction of non-capacitated spermatozoa. Spermatozoa were capacitated in the absence or presence of increasing concentrations of SMAP or SF for 6 h. (A) Western blot of sperm samples probed with the 4G10 antibodies for detection of phosphotyrosine residues. A representative experiment is shown. (B) Densitometric analysis of the phosphotyrosine (pY) abundance, normalized by the  $\beta$ -tubulin (tub) signal (pY/tub). Mean  $\pm$  SEM, from 7 sperm samples from equal number of donors. (C) % ARIC (Acrosome Reaction to Ionophore Challenge = % of calcium ionophore-induced acrosome reaction – spontaneous acrosome reaction). Mean  $\pm$  SEM, from 6 sperm samples from equal number of donors. (#)  $p < 0.05$  vs. SMAP 0 mg/mL (0 h); (\*)  $p < 0.05$  vs. SMAP 0 mg/mL (6 h).



**Figure 7** Effect of SMAP on motility and protein tyrosine phosphorylation of capacitated spermatozoa. Spermatozoa were capacitated for 5 h in the absence of SMAP (5 h CAP) and subsequently incubated for 1 h in the absence or presence of increasing concentrations of SMAP or SF. (A) Manual motility analysis classified according to W.H.O. criteria. Im, Immotile; NP, Non-progressive; PR, Progressive. (B) Percentage of hyperactivated spermatozoa evaluated by CASA. Values on the Y-axis represent the fold change, considering the values obtained in SMAP 0 mg/mL (5 h CAP + 1 h SMAP/SF), used as the control. (C) Western blot of sperm samples probed with the 4G10 antibodies for detection of phosphotyrosine residues. A representative experiment is shown. (D) Densitometric analysis of phosphotyrosine abundance, normalized by the  $\beta$ -tubulin signal (pY/tub). Mean  $\pm$  SEM, from 6 sperm samples from equal number of donors. (#)  $p < 0.05$  vs. SMAP 0 mg/mL (0 h); (\*)  $p < 0.05$  vs. SMAP 0 mg/mL (5 h CAP + 1 h SMAP/SF).

and unsuccessful samples used for *in vitro* fertilization, and therefore may be considered as success biomarkers for this technique (Kanannejad & Ghareh-Si-Fard, 2019). In conclusion, this investigation describes for the first time the extracellular proteins associated with human ejaculated spermatozoa that are

released during *in vitro* capacitation and their participation in the regulation of sperm function. Therefore, we believe that presented results may contribute to the understanding of the mechanisms regulating sperm functionality and to identify potential novel markers for the diagnosis and treatment of male infertility.

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## DISCLOSURES

The authors have no conflict of interest to disclose.

## AUTHOR'S CONTRIBUTIONS

GHS performed or participated in all experiments. JEFLA and ASLT contributed to sperm function analyses. VTF and FL contributed to data interpretation and critically revised the manuscript. MC designed the research study and wrote the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Identification of PIP, LTF, PSA and ZAG in the SMAP. The identity of the proteins in the SMAP was validated by Western blots. Seminal fluid (SF) was used as positive control.

**Figure S2.** Effect of increasing concentrations of SMAP on sperm motility kinetic parameters of (A) non-capacitated and (B) capacitated spermatozoa.  $N = 6$ . (\*)  $p < 0.05$  vs. SMAP 0 mg/mL.

**Table S1.** Initial semen parameters of each donor.

**Table S2.** Effect of SMAP on sperm motility kinetic parameters during capacitation.

**Table S3.** Effect of SMAP on capacitated sperm motility kinetic parameters.

## 11. APÉNDICE 2

### ARTÍCULO DE REVISIÓN

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# Proteins from male and female reproductive tracts involved in sperm function regulation

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## Review

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## Summary

Spermatogenesis is a dynamic process that culminates in the production of mature spermatozoa in the seminiferous tubules of sexually mature animals. Although sperm leaving the testis are fully differentiated, they must further undergo two additional maturation steps before acquiring the capability to fertilize the egg. Such processes take place during the epididymal residency and transport in the seminal fluid during ejaculation and, after delivery into the female reproductive tract, during the journey aiming the encountering the egg in the oviduct. Throughout this trip, spermatozoa are exposed to different reproductive fluids whose molecular compositions regulate the progress towards obtaining a fertilized competent cell. This review summarizes the evidence obtained so far supporting the participation of male and female reproductive tract-derived proteins in the modulation of sperm fertilizing ability and discusses the mechanisms by which such regulation may be accomplished.

## Introduction

Spermatozoa released from the seminiferous tubules are morphologically mature cells, but must undergo two additional processes of functional maturation that allow them to fertilize. The first of such processes occurs during epididymal transit and ejaculation. As spermatozoa migrate from the proximal to the distal region of the epididymis, they experience a series of morphological, biochemical and physiological changes, including modifications in size and appearance of the acrosome and nucleus, migration of the cytoplasmic droplet along the tail, as well as structural changes in several intracellular organelles, all of which result in spermatozoa with progressive motility (Cornwall, 2009). After leaving the epididymis, spermatozoa are transported to the vas deferens towards the base of the prostate and the excretory duct of the seminal vesicle. Secretions from the seminal vesicle, prostate, and bulbourethral glands constitute the seminal plasma in which spermatozoa are suspended during delivery to the female reproductive tract and are responsible for the changes that occur in the spermatozoa plasma membrane at ejaculation (Juyena and Stelletta, 2012).

After semen deposition in the female reproductive tract, the second sperm maturation process takes place. Ejaculated semen contains spermatozoa that need to experience changes to acquire the capacity to fertilize oocytes, a process known as capacitation (Austin, 1952; Chang, 1951). This process begins soon after the removal of membrane stabilizing factors originated in the seminal plasma by the cervical mucus, proceeds throughout sperm transit along the female reproductive tract and is considered to be complete when spermatozoa are able to undergo the acrosome reaction (De Jonge, 2005). Such membrane stabilizing factors comprise several epididymal and accessory glands secreted proteins associated to sperm membrane surface denoted as decapacitation factors (Nixon *et al.*, 2006; Aitken *et al.*, 2007). The coordinated loss of decapacitation factors is associated with cholesterol efflux from the sperm plasma membrane (Davis, 1979) that increases its fluidity and permeability, decreases the membrane potential (Abou-haila and Tulsiani, 2009) and initiates signal transduction mechanisms that allow spermatozoa to become capacitated. In addition, an increase in the intracellular calcium concentration from the extracellular medium takes place (Costello *et al.*, 2009). This calcium increase is accompanied by changes in motility, from a progressive and rapid pattern to a non-progressive movement known as hyperactivation (Suarez, 2008), and the increase in tyrosine phosphorylation of various spermatozoa proteins (Visconti *et al.*, 1995a, 1995b).

Decapacitation factors may be provided via the secretory pathway or by extracellular membrane vesicles, such as the epididymosomes (Martin-DeLeon, 2015) and the prostasomes (Ronquist, 2015) which transport proteins (Thimon *et al.*, 2008; Akintayo *et al.*, 2015) as well

as lipids, DNA, microRNAs and mRNA (Ronquist *et al.*, 2009; Belleanne, 2015; Zijlstra and Stoorvogel, 2016). Similarly, the sperm capacitation progress is modulated by its interaction with molecules from the female reproductive tract that are either secreted into the lumen (Chirinos *et al.*, 2017), delivered by extracellular vesicles (Al-Dossary *et al.*, 2015; Martin-DeLeon, 2016), or in the surface of oviductal epithelial cells (Ghersevich *et al.*, 2015). Therefore, the regulation of capacitation appears to involve dynamic interactions between the decapacitation factors from the male reproductive tract and the stimulatory/regulatory factors released by the female reproductive tract (Nixon *et al.*, 2006). The aim of the present work was to review the role of mammalian proteins secreted from male and female reproductive tracts that have been characterized by their ability to interact with spermatozoa and modulate their competence to fertilize.

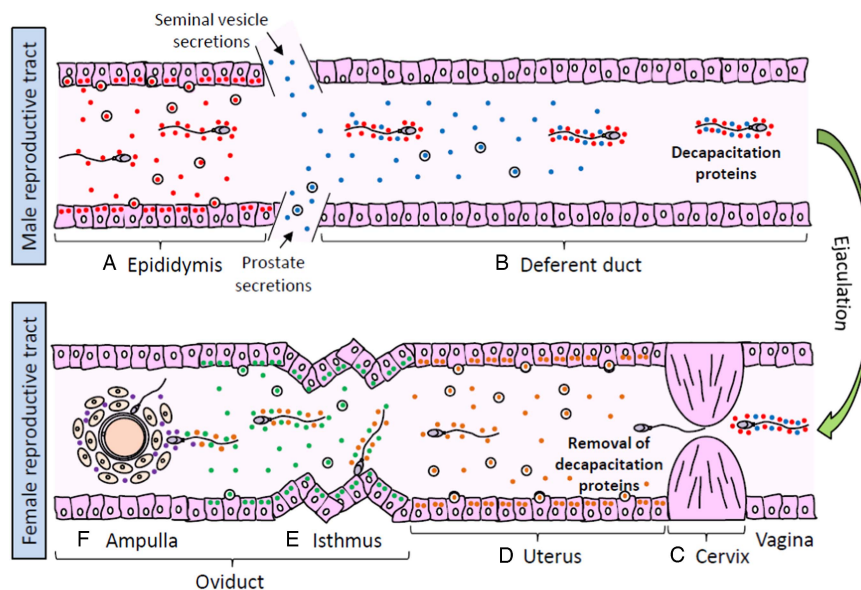
### Proteins derived from male reproductive tract that interact with the sperm

Decapacitation factors on sperm plasma membrane surface keep the gamete protected from premature capacitation. Such factors associate with the sperm plasma membrane surface during epididymal transit and ejaculation (see Fig. 1A, B). Early investigations in mouse have shown that removal of surface-associated proteins from uncapacitated spermatozoa plasma membrane lead to an immediate increase in fertilizing ability, as determined by a rise in the proportion of fertilized eggs (Fraser, 1984). In the following pages, evidence is presented of some male reproductive tract proteins that have been identified as decapacitation factors.

#### Epididymal proteins

One of the first decapacitation factors described is a 40 kDa glycoprotein acquired during mouse sperm epididymal transit

known as DF (Fraser *et al.*, 1990). This protein binds to spermatozoa via a glycosylphosphatidylinositol (GPI)-anchored membrane receptor called the DF receptor, mainly located in the post-acrosomal region (Fraser, 1998). Its elimination from the uncapacitated spermatozoa surface resulted in highly fertile gametes and, when added back to capacitated spermatozoa produced poorly fertile cells in which the acrosome reaction was blocked (Fraser *et al.*, 1990). DF stimulates calmodulin-sensitive calcium ATPase activity, thereby ensuring the maintenance of low intracellular calcium concentrations. As capacitation proceeds, DF is lost and calcium ATPase activity declines, allowing intracellular calcium to rise and hence promoting capacitation-related changes (Adeoya-Osiguwa and Fraser, 1996). In human, the addition of mouse DF to capacitated sperm suspensions causes a significant reversal in the capacitation state of the cells, suggesting that a similar mechanism could take place in both species (DasGupta *et al.*, 1994). The DF receptor on sperm is the phosphatidylethanolamine-binding protein 1 (PEBP1), a 23 kDa protein located extracellularly on the acrosome, the post-acrosomal region and the flagellum of mouse and human spermatozoa (Gibbons *et al.*, 2005). Investigations carried out in the mouse model showed that PEBP1 along with other three proteins, identified as the plasma membrane fatty acid binding protein, the cysteine-rich secretory protein 1 (CRISP1), and a decapacitation factor named DF10, were able to inhibit sperm-zona pellucida (ZP) interaction as well as the sperm ability to acrosome react in response to progesterone (Nixon *et al.*, 2006). CRISPs are a family of proteins closely involved in the process of fertilization. In human and rat, there are three CRISPs (CRISP1, CRISP2 and CRISP3 for human; CRISP1, CRISP2 and CRISP4 for rat), while mice produce four CRISPs named CRISP1, CRISP2, CRISP3 and CRISP4 (Koppers *et al.*, 2011). CRISP2 (originally known as Tpx-1) is expressed exclusively in the mammalian testicle and is incorporated into the developing sperm acrosome and flagellum (O'Bryan *et al.*, 2001), while CRISP1 and CRISP4



**Figure 1.** Sperm interaction with proteins from male and female reproductive tracts. (A) Spermatozoa released from the seminiferous tubules interact with epididymal proteins (red circles) that promote post-testicular maturation. (B) During ejaculation, spermatozoa are transported to the vas deferens and mixed up with seminal vesicle and prostate secretions, containing proteins that interact with the sperm surface and act as decapacitation factors (blue circles). (C) When semen is deposited in the female reproductive tract, decapacitation proteins are removed from spermatozoa during their passage through the cervical mucus in the cervix. (D) Spermatozoa migrating towards the uterus are exposed local secreted proteins (orange circles) that regulate the progress of sperm capacitation. (E) Once in the oviduct, spermatozoa may either move forward to the ampulla stimulated by secreted proteins (green circles) or held on the epithelium surface of mucosal folds in the isthmus to create a sperm reservoir. When ovulation draws near, local secretions stimulate the sperm release from the reservoir and the resumption of capacitation. (F) Spermatozoa that successfully reach the cumulus-oocyte complex are exposed to follicular fluid proteins (purple circles) which promote sperm penetration of the oocyte vestments and fertilization.

are secreted by the epididymis and incorporated onto the spermatozoa during epididymal storage (Reddy *et al.*, 2008). Experiments performed in the rat model have shown that the addition of CRISP1 to the sperm inhibits tyrosine phosphorylation in a dose-dependent manner, therefore inhibiting capacitation and ultimately the acrosome reaction and that such inhibition occurs upstream the production of cAMP by the sperm (Roberts *et al.*, 2003). However, there are two populations of CRISP1 in spermatozoa: a loosely bound that is released during capacitation and the form strongly associated with the sperm that remains on cells after capacitation (Cohen *et al.*, 2000b). It has been shown that CRISP1 that remain associated with the sperm head after capacitation, migrates to the equatorial segment during the acrosome reaction and is involved in sperm–ZP interaction and subsequent gamete fusion (Rochwerger *et al.*, 1992; Cohen *et al.*, 2000a, 2011). Similarly, human CRISP1 also participates in sperm binding to the ZP and in gamete fusion (Maldera *et al.*, 2014; Da Ros *et al.*, 2015). Recent investigations with *Crisp1* and *Crisp4* knockout mice indicated that these proteins may act redundantly or autonomously on sperm function. CRISP1 is essential for the establishment of normally motile sperm, but CRISP4 enhances capacitation-associated tyrosine phosphorylation, and both are required for normal acrosome function and sperm–egg interaction (Hu *et al.*, 2018). In contrast, CRISP3 shows a wider tissue distribution and is secreted by the prostate and seminal vesicles into the seminal plasma (Koppers *et al.*, 2011), but its participation in sperm function regulation is still unknown.

Another protein expressed and secreted in the adult rat epididymis that binds to sperm head plasma membrane is HongrES1, a member of the serine proteinase inhibitor (SERPIN) protein family (Hu *et al.*, 2002). The co-culture of caudal spermatozoa with anti-HongrES1 antibodies resulted in an increased proportion of capacitated spermatozoa. Moreover, the percentage of capacitated spermatozoa increased when HongrES1 was downregulated by RNAi *in vivo* (Zhou *et al.*, 2008). This protein has also been found in the cauda epididymis of guinea pig, where it is gradually exfoliated during capacitation and redistributed to patches on the sperm head and tail, and disappears after acrosome reaction. Removal of HongrES1 from the sperm surface facilitates extracellular calcium influx, thereby allowing the progress of capacitation and hyperactivation (Ni *et al.*, 2009). Moreover, it has been demonstrated that human and equine epididymal secretomes contain lactotransferrin (LTF) (Dacheux *et al.*, 2006, 2009), a protein that has been shown to decrease sperm ability to interact with ZP (Zumoffen *et al.*, 2013) and to have dose-dependent effects on sperm proteins tyrosine phosphorylation (Zumoffen *et al.*, 2015; Hernández-Silva *et al.*, 2018).

### Seminal vesicle proteins

Seminal vesicles secretions contribute with most of the proteins existing in the seminal fluid and semenogelin-1 (Sg1) is the most abundant protein secreted by seminal vesicles and the main component of semen coagulum. After ejaculation, it is degraded by the serine protease prostate-specific antigen (PSA) to generate peptides of various biological activities and allow coagulum liquefaction (de Lamirande *et al.*, 2001). Previous studies have shown that Sg1 inhibits human sperm motility (Robert and Gagnon, 1996), protein tyrosine phosphorylation and the amount of superoxide anion generated during capacitation (de Lamirande *et al.*, 2001), which is related to the progress of hyperactivation (de Lamirande and Gagnon, 1995). Interestingly, during ejaculation, Sg1 binds to the Eppin protein complex in the surface of human spermatozoa (Wang *et al.*, 2005), a heteromultimer containing the

epididymal protease inhibitor (Eppin), LTF and clusterin (Wang *et al.*, 2007), to inhibit the progressive motility of ejaculated spermatozoa (Mitra *et al.*, 2010) by promoting the loss of intracellular calcium (O'Rand and Widgren, 2012). However, during Sg1 digestion after ejaculation, Eppin modulates PSA hydrolytic activity on Sg1 resulting in spermatozoa with forward motility (O'Rand *et al.*, 2006).

Another protein from seminal vesicles that interacts with sperm plasma membrane is the mouse seminal vesicle autoantigen (SVA), which binds to sperm membrane phospholipids and suppresses sperm motility, BSA-stimulated sperm hyperactivation (Huang *et al.*, 1999) and capacitation-related protein tyrosine phosphorylation (Huang *et al.*, 2000). SVA has two orthologues genes in rat (Yoshida *et al.*, 2001) and the human homologue is prolactin induced protein (PIP), a protein highly expressed on the surface of spermatozoa from donors with asthenozoospermia and oligoasthenoteratozoospermia (Capkova *et al.*, 2007). Interestingly, PIP interacts with serum albumin, zinc- $\alpha$ -2 glycoprotein and Sg1 (Tomar *et al.*, 2013), suggesting that it may participate in human sperm capacitation regulation. In addition, it seems that the protein family known as seminal vesicle secretory proteins (SVS) may also regulate sperm capacitation. Murine SVS II, the major component of the copulatory plug (Lundwall, 1996), binds to ganglioside GM1 on the post-acrosomal region of the head sperm (Kawano *et al.*, 2008) and decreases sperm protein tyrosine phosphorylation and the progesterone-induced acrosome reaction (Kawano and Yoshida, 2007).

Glycodelin (Gd) is a glycoprotein that has four well defined isoforms named S, A, F and C, according to their origin (semen, amniotic fluid, follicular fluid and cumulus oophorus, respectively) (Seppala *et al.*, 2007). GdS is one of the most abundant glycoproteins in the seminal plasma (Julkunen *et al.*, 1984) and is able to reduce the cyclodextrin-induced cholesterol efflux and down-regulate the adenylyl cyclase/protein kinase A/tyrosine kinase signalling pathway, resulting in suppression of human sperm capacitation (Chiu *et al.*, 2005). A different group of seminal vesicle proteins that binds to sperm are spermadhesins, a protein family comprised by five polypeptides called AQN-1, AQN-3, AWN (isoforms 1 and 2), PSP-I, and PSP-II. These proteins have been identified in pig, in which a large subpopulation of spermadhesins is loosely associated with the sperm surface and functions as decapacitation factors (Dostalova *et al.*, 1994). Nonetheless, it seems that each spermadhesin plays specific roles in sperm function regulation, given that a PSP-I/PSP-II heterodimer exerts a decapacitation effect by decreasing sperm intracellular calcium (Caballero *et al.*, 2009) while AQN-3 has been described as a ZP-binding protein (Calvete *et al.*, 1996).

Protease inhibitors are widely distributed in nearly all species and are crucial in balancing protease activities. In mouse, the serine protease inhibitor Kazal-type 3 (SPINK3), also called P12, has been found in the seminal vesicle, coagulating gland and prostate of adults. The P12-binding sites are on the anterior region of the acrosome and the protein inhibits sperm binding to the ZP and the ZP-induced acrosome reaction (Boettger-Tong *et al.*, 1992). Furthermore, the protein suppresses calcium uptake by spermatozoa during capacitation (Chen *et al.*, 1998). Additionally, a related serine protease inhibitor known as Kazal-type-like (SPINKL) is secreted by mouse seminal vesicle and has been found to bind to sperm, enhance sperm progressive motility, suppress BSA-stimulated sperm capacitation and block sperm–oocyte interactions *in vitro* (Lin *et al.*, 2008).

Binder of sperm proteins (BSP, previously known as bovine seminal plasma proteins) are part of a family of structurally related proteins characterized by the presence of tandem fibronectin type II domains. They are highly expressed by seminal vesicles, but

BSP-related genes have been found to be also expressed in the epididymis (Fan *et al.*, 2006; Plante and Manjunath, 2015a). BSPs are involved in sperm binding to oviductal epithelium assisting in the formation of the sperm storage reservoir (Gwathmey *et al.*, 2003). Bovine seminal vesicles secrete three BSP (BSP1, BSP3 and BSP5) that are adsorbed onto sperm (Manjunath *et al.*, 1994), but after *in vitro* capacitation BSP5 is almost completely released from sperm surface while BSP1 and a small molecular mass isoform of BSP3 remain associated to sperm, suggesting that BSP3 undergoes proteolytic modifications on the sperm surface that may regulate sperm release from the storage reservoir (Hung and Suarez, 2012). Moreover, when BSPs interact with the sperm surface remove cholesterol from the plasma membrane (Therien *et al.*, 1999) and bind to choline phospholipids (Desnoyers and Manjunath, 1992), preventing free movement of phospholipids and therefore stabilizing sperm membrane. However, they have also been found to promote sperm capacitation in bulls, boars, humans and mice and such effects on capacitation are mediated by stimulation of sperm membrane cholesterol and phospholipid efflux in the presence of heparin or HDL, both components of follicular and oviductal fluids (for review see Manjunath and Thérien, 2002). Recently, it has been shown that antibodies against murine BSPH1 could block capacitation induced by HDLs, suggesting a specific interaction between HDL and BSPH1 (Plante and Manjunath, 2015b). Nevertheless, as the capacitation promoting effect is observed under conditions resembling the milieu of the female reproductive tract while in seminal fluid BSPs stabilize sperm membrane and prevent premature capacitation, it has been proposed that they are multifunctional proteins that employ different mechanisms to exert such effects (Plante *et al.*, 2016).

### Prostate proteins

The major protein secreted by the prostate is the serine protease known as PSA (or human kallikrein) that plays a well known role in seminal coagulum liquefaction that allows the release of motile spermatozoa (Malm *et al.*, 2000), but there is no evidence that it may directly interact with the sperm membrane. Instead, the boar prostate-derived seminal plasma protein identified as WGA16 (wheat germ agglutinin) has been proven to bind to the sperm surface during ejaculation and is later removed during capacitation. It has two functional sites that allow sperm surface association through interaction between its N-glycans and the surface galactosyltransferase, whereas the heparin-binding domain may be involved in binding to sulfated glycosaminoglycans from the female tract, enabling removal of WGA16 from the sperm surface during capacitation (Garenaux *et al.*, 2015), but its involvement in sperm function regulation has not been examined.

Prostasomes are small vesicles secreted by prostatic epithelial cells that fuse with sperm plasma membranes and are the main source of cholesterol in seminal fluid. Previous studies have shown that incubation of sperm with prostasomes decreases the tyrosine phosphorylation intensity and induces motility changes (Bechoua *et al.*, 2011; Pons-Rejraji *et al.*, 2011). The prostasome molecular entities responsible for those effects are unknown, but among other proteins human prostasomes enclose LTF (Utleg *et al.*, 2003), supporting the idea that proteins transferred by prostasomes to sperm may regulate its capability to fertilize.

### Proteins derived from female reproductive tract that interact with the sperm

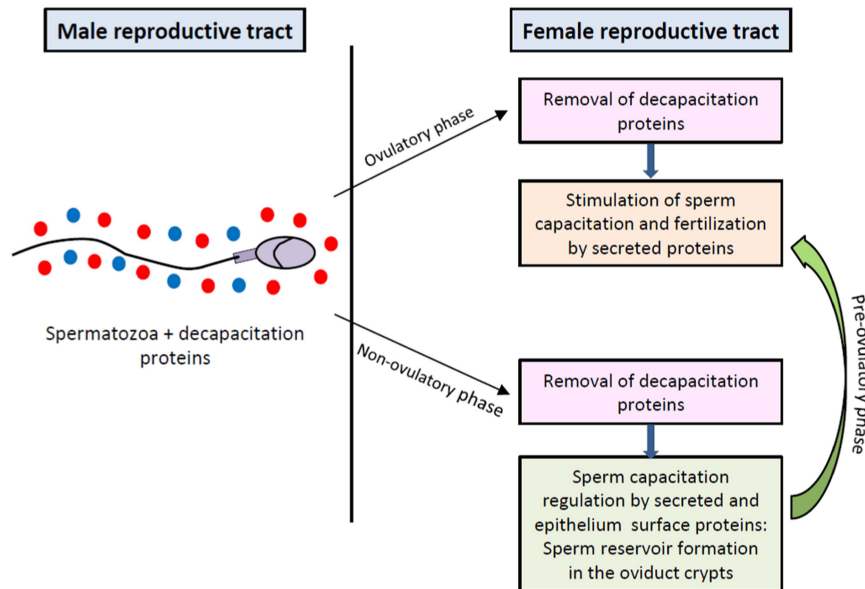
Sperm plasma membranes from ejaculated spermatozoa are scrubbed by the ultrastructural elements in the vagina, uterus and

oviduct (De Jonge, 2005). The cervical mucus is the first selective fluid encountered by sperm after entering the female genital tract and modulates sperm capacitation in ruminants (Wergin, 1985) and human (Gould *et al.*, 1984; Perry *et al.*, 1996) (see Fig. 1C). It has been shown that the sperm cholesterol content is markedly decreased after migration into the mucus (Feki *et al.*, 2004). For this purpose, albumin in the cervical mucus plays a key role during the capacitation, acting as a cholesterol acceptor and contributing to the dispersion of the released proteins (De Jonge, 2005), but no other specific protein from this fluid has been characterized as sperm function modulator. After the cervix, spermatozoa must cross the uterus towards the fallopian tubes, a journey in which the sperm plasma membrane also undergoes modifications by interacting with molecules secreted or exposed on the surface of utero-tubal epithelium that are relevant for their ability to fertilize (see Fig. 1D, E). Finally, when spermatozoa meet the cumulus–oocyte complex in the ampulla of the oviduct, proteins contained in the follicular fluid promote the ultimate changes in the male gamete that allow ZP penetration and fusion with the oocyte (see Fig. 1F). Contrasting with proteins secreted by the male reproductive tract, those from the female reproductive tract are supposed to stimulate capacitation and sperm–ZP interaction. However, the progress of capacitation should be controlled to synchronize capacitation with ovulation, and therefore spermatozoa placed in the female reproductive tract during the pre-ovulatory phase are compelled to move towards the ampulla, but when placed out of this period of the cycle they may be held back in the oviduct sperm reservoir waiting for ovulation signals that should reinitiate the capacitation process (see Fig. 2). Consequently, the secretome of the female reproductive fluids must change according to the different phases of the menstrual/estrous cycle.

### Uterine proteins

Although GdA is abundant in the amniotic fluid, it is also present in the endometrial secretions of non-pregnant females. It has immunosuppressive properties that protect the spermatozoa from immune attack in the female reproductive tract (Bolton *et al.*, 1987), but is also able to inhibit sperm binding to the ZP (Oehninger *et al.*, 1995). However, as it is a progesterone-dependent protein, it is mainly secreted during the luteal phase of the menstrual cycle and therefore spermatozoa are not exposed to significant concentrations of GdA during the fertile window (Durand *et al.*, 2010). Another progesterone-dependent protein expressed and secreted in the human uterus and cervix is the cysteine protease inhibitor cystatin C (CST3), which binds to sperm enhancing motility but inhibits cholesterol efflux and sperm protein tyrosine phosphorylation, indicating CST3's ability to inhibit sperm capacitation (Lee *et al.*, 2018). Interestingly, this protein is also prominently expressed in the male reproductive tract and has been associated with human prostasomes (Carlsson *et al.*, 2011), but its potential role as a decapacitation factor has not been investigated. Other proteins of uterine origin that have been shown to inhibit sperm–ZP interaction are LTF (Teng *et al.*, 2002; Zumoffen *et al.*, 2013) and glucose-regulated protein 78 (Grp78) (Marin-Briggiler *et al.*, 2010), and it has been proposed that such inhibition may contribute to regulate the number of sperm with the ability to interact and fertilize the oocyte (Zumoffen *et al.*, 2013). Conversely, heat shock protein 60 (Hsp60), a chaperone protein secreted by uterus and oviduct epithelial cells from human and bovine





**Figure 2.** Regulation of sperm capacitation by proteins from male and female reproductive tracts. Proteins from male reproductive tract that associate to sperm plasma surface act as decapacitation factors. After removal of decapacitation factors in the female reproductive tract, secreted and oviductal epithelium surface proteins differentially regulate the progress of capacitation in accordance to the menstrual/estrous cycle stage to synchronize sperm maturation with ovulation.

(Boilard *et al.*, 2004; Lachance *et al.*, 2007), binds to spermatozoa and increases the intracellular calcium concentration with no apparent effects on sperm motility or acrosome reaction (Lachance *et al.*, 2007).

Endometrial cells also secrete interleukin-6 (IL-6), a mediator of the inflammatory response that increases during the periovulatory phase (Tabibzadeh and Sun, 1992). IL-6 induces sperm capacitation by increasing protein tyrosine phosphorylation and enhances spontaneous as well as calcium ionophore-induced acrosome reaction (Naz and Kaplan, 1994; Laflamme *et al.*, 2005). Moreover, uterine secretions contain the sialic acid binding protein (SABP), a protein that binds to spermatozoa head plasma membrane (Banerjee and Chowdhury, 1994) and stimulates *in vitro* sperm capacitation, increases the percentage of motile cells and the subsequent acrosome reaction (Banerjee and Chowdhury, 1995). Furthermore, SABP induces the exposure of mannose ligand receptors on the sperm surface and increases the production of the superoxide anion (Banerjee and Chowdhury, 1997). Furthermore, recent investigations indicate a widespread presence of fibroblast growth factor 2 (FGF2) in the mouse uterus and oviduct, and that this protein is able to increase sperm motility, intracellular calcium levels and acrosomal loss *in vitro* (Saucedo *et al.*, 2018).

In addition to uterine secreted proteins, uterosomes may also contribute to sperm function regulation, as they carry proteins that may promote sperm capacitation (Martin-DeLeon, 2016). Moreover, it has been shown that uterosome-like vesicles secreted by endometrial epithelial cells are able to fuse with human spermatozoa, prompting their fertilizing capacity by increasing protein tyrosine phosphorylation and the acrosome reaction (Franchi *et al.*, 2016). In mouse, uterosomes contain sperm adhesion molecule 1 (SPAM1 or PH-20) (Griffiths *et al.*, 2008a), a protein that binds to spermatozoa on the acrosome and the midpiece of the flagella (Griffiths *et al.*, 2008b). SPAM1 increases sperm intracellular calcium in macaque (Cherr *et al.*, 1999) and enables sperm penetration of the cumulus oophorus surrounding

the oocyte in mouse (Lin *et al.*, 1994). Moreover, SPAM1 is required for hyaluronic acid enhancement of progesterone-induced acrosome reaction in human sperm (Sabeur *et al.*, 1998).

### Oviduct proteins

When spermatozoa reach the oviduct, they either migrate to the ampulla region to meet the oocyte or are retained in the isthmus region by oviduct epithelial cells to give rise to the sperm reservoir (Jeulin *et al.*, 1985). Sperm interactions with the oviductal epithelial cells increase the intracellular superoxide dismutase and glutathione peroxidase activities as protective mechanisms against reactive oxygen species (Huang *et al.*, 2013). It has been shown that spermatozoa bound to oviductal epithelial cells show normal morphology and lower DNA fragmentation (Ellington *et al.*, 1999), but the role of the oviductal epithelium on sperm capacitation has been a controversial issue. It has been shown that incubation of sperm with oviductal cells enhances sperm viability and hyperactivated motility (Kervancioglu *et al.*, 1994), but other investigations have indicated that, although there is enhancement in sperm motility, there is a delay in capacitation (Murray and Smith, 1997) by reducing protein tyrosine phosphorylation and avoiding follicular fluid-induced acrosome reaction (Morales *et al.*, 1996; Zumoffen *et al.*, 2010). These observations are in agreement with oviduct cells' role as a sperm reservoir, where oviduct cells must stimulate some early capacitation-associated features but delay capacitation late events that are expected to happen in the oocyte vicinity.

As the time of ovulation approaches, the sperm in the reservoir must reassume capacitation. In mouse, hyperactivation assists the sperm to pull off the epithelium and escape out of mucosal pockets (Demott and Suarez, 1992). At this stage of the journey, oviductosomes transfer proteins to the head and midpiece of spermatozoa (Al-Dossary *et al.*, 2015), delivering calcium ATPase 4 (PMCA4), a membrane protein whose deletion leads to severe loss of hyperactivated motility and male infertility (Al-Dossary *et al.*, 2013).

**Table 1.** Proteins from male reproductive tract and their participation in sperm capacitation and gamete interaction

Origin	Protein name	Effect on capacitation				Acrosome reaction	Effect on sperm-ZP interaction	Effect on gamete fusion
		Efflux of cholesterol	Influx of calcium	Tyrosine phosphorylation	Motility			
Epididymis	DF		↓ mouse (Adeoya-Osiguwa and Fraser, 1996)			↓ mouse (Fraser <i>et al.</i> , 1990)		
	PEBP1					↓ mouse (Nixon <i>et al.</i> , 2006)	↓ mouse (Nixon <i>et al.</i> , 2006)	
	CRISP1			↓ rat (Roberts, 2003)		↓ rat (Roberts, 2003)	↑ human (Maldera <i>et al.</i> , 2014)	↑ rat (Rochwerger <i>et al.</i> , 1992), mouse (Cohen <i>et al.</i> , 2000a)
	HongrES1		↓ pig (Ni <i>et al.</i> , 2009)		↓ HA, pig (Ni <i>et al.</i> , 2009)	↓ pig (Ni <i>et al.</i> , 2009)		
	LTF			↑ human (Zumoffen <i>et al.</i> , 2015), ↓ human (Hernández-Silva <i>et al.</i> , 2018)			↓ human (Zumoffen <i>et al.</i> , 2013)	
Seminal vesicle	Sg1			↓ human (de Lamirande <i>et al.</i> , 2001)	↓ human (Robert and Gagnon, 1996)			
	SVA			↓ mouse (Huang <i>et al.</i> , 2000)	↓ mouse (Huang <i>et al.</i> , 1999)			
	SVS			↓ mouse (Kawano and Yoshida, 2007)		↓ mouse (Kawano and Yoshida, 2007)		
	SPINK3		↓ mouse (Chen <i>et al.</i> , 1998)			↓ mouse (Boettger-Tong <i>et al.</i> , 1992)	↓ mouse (Boettger-Tong <i>et al.</i> , 1992)	
	SPINKL			↓ mouse (Lin <i>et al.</i> , 2008)	↑ mouse (Lin <i>et al.</i> , 2008)	↓ mouse (Lin <i>et al.</i> , 2008)	↓ mouse (Lin <i>et al.</i> , 2008)	
	GdS	↓ human (Chiu <i>et al.</i> , 2005)		↓ human (Chiu <i>et al.</i> , 2005)				
	PSP-I/ PSP-II		↓ pig (Caballero <i>et al.</i> , 2009)					
	AQN-3						? pig (Calvete <i>et al.</i> , 1996)	
	BSP	↑ bovine (Manjunath, 2002)						
	Prostate	LTF			↑ human (Zumoffen <i>et al.</i> , 2015), ↓ human (Hernández-Silva <i>et al.</i> , 2018)			↓ human (Zumoffen <i>et al.</i> , 2013)

↓: inhibition; ↑: stimulation; ?: unknown; HA = hyperactivation.

**Table 2.** Proteins from mammalian female reproductive tract and their participation in sperm capacitation and gamete interaction.

Origin	Protein name	Effect on capacitation						
		Efflux of cholesterol	Influx of calcium	Tyrosine phosphorylation	Motility	Acrosome reaction	Effect on sperm-ZP interaction	Effect on gamete fusion
Uterus	SABP		↑ human (Banerjee and Chowdhury, 1995)		↑ human (Banerjee and Chowdhury, 1995)	↑ human (Banerjee and Chowdhury, 1995)		
	GdA						↓ human (Oehninger <i>et al.</i> , 1995)	
	LTF						↓ human (Zumoffen <i>et al.</i> , 2013)	
	Grp78		↑ human (Lachance <i>et al.</i> , 2007)		= human (Lachance <i>et al.</i> , 2007)	= human (Lachance <i>et al.</i> , 2007)	↓ human (Marin-Briggiler <i>et al.</i> , 2010)	
	Hsp60		↑ human (Lachance <i>et al.</i> , 2007)		= human (Lachance <i>et al.</i> , 2007)	= human (Lachance <i>et al.</i> , 2007)		
	IL-6			↑ human (Laflamme <i>et al.</i> , 2005)		↑ human (Laflamme <i>et al.</i> , 2005)		
	CST3	↓ human (Lee <i>et al.</i> , 2018)		↑ human (Lee <i>et al.</i> , 2018)	↑ human (Lee <i>et al.</i> , 2018)			
	SPAM1		↑ macaque (Cherr <i>et al.</i> , 1999)			↑ human (Sabeur <i>et al.</i> , 1998)	↑ mouse (Lin <i>et al.</i> , 1994)	
Oviduct	FGF2		↑ mouse (Saucedo <i>et al.</i> , 2018)		↑ mouse (Saucedo <i>et al.</i> , 2018)	↑ mouse (Saucedo <i>et al.</i> , 2018)		
	PMCA4				↑ HA, mouse (Al-Dossary <i>et al.</i> , 2013)			
	Oviductin			↑ human (Zhao <i>et al.</i> , 2016)	↑ bovine (Abe <i>et al.</i> , 1995)	↑ human (Zhao <i>et al.</i> , 2016)	↑ bovine (Martus <i>et al.</i> , 1998), ↑ mouse (Ensslin <i>et al.</i> , 2007), ↓ pig (Kouba <i>et al.</i> , 2000), ↓ hamster (Kimura <i>et al.</i> , 1994)	
	SBG			↑ pig (Teijeiro <i>et al.</i> , 2008)	↓ pig (Teijeiro <i>et al.</i> , 2008)			
	ANP					↑ pig (Zhang <i>et al.</i> , 2006)	↑ pig (Zhang <i>et al.</i> , 2006)	
Follicular fluid	GdF						↓ human (Chiu <i>et al.</i> , 2003)	
	GdC						↑ human (Chiu <i>et al.</i> , 2007)	
	Fn						↓ bovine (Thys <i>et al.</i> , 2009; Tanghe <i>et al.</i> , 2004)	↓ bovine (Thys <i>et al.</i> , 2009; Tanghe <i>et al.</i> , 2004)
	CRISP1				↓ HA, mouse (Ernesto <i>et al.</i> , 2015)			

↓: inhibition; ↑: stimulation; =: no effect; HA= hyperactivation.

Oviduct also secretes oviductin, a high-molecular-weight protein that binds to the head and middle piece of the sperm (King and Killian, 1994; Lyng and Shur, 2009). Bovine oviductin promotes *in vitro* sperm viability and total motility (Abe *et al.*, 1995). Moreover, this protein stimulates sperm–ZP interaction in bovine (Martus *et al.*, 1998) and mice (Ensslin *et al.*, 2007), but has an inhibiting effect in pig (Kouba *et al.*, 2000) and hamster (Kimura *et al.*, 1994), indicating that its function may not be conserved among mammalian species. In human, the expression of oviductin at the time of ovulation has been demonstrated (Briton-Jones *et al.*, 2001; Lok *et al.*, 2002). Recently, a secretory form of human oviductin expressed in HEK293 cells has been shown to enhance tyrosine phosphorylation and the incidence of acrosome-reacted sperm induced by calcium ionophore (Zhao *et al.*, 2016). Finally, sperm binding glycoprotein (SBG) that is synthesized at the apical surface of pig isthmic and ampullar epithelial cells (Perez *et al.*, 2006), binds to carbohydrates on the sperm plasma membrane (Marini and Cabada, 2003) to increase tyrosine phosphorylation of a polypeptide with an apparent molecular mass of 97 kDa, although suppressing motility (Teijeiro *et al.*, 2008).

### Follicular fluid proteins

In the ampulla of the oviduct, sperm should meet the cumulus–oocyte complex containing the oocyte surrounded by the hyaluronic acid matrix, cumulus cells and follicular fluid. The hyaluronic acid matrix works as a mechanical filter for the elimination of sperm peripheral proteins that are no longer necessary for ZP penetration and fusion with the oocyte. In addition, follicular fluid stimulates the acrosome reaction rate (Calvo *et al.*, 1989), as a consequence of its high concentrations of progesterone (De Jonge, 2005) that stimulates a biphasic calcium influx (Kirkman-Brown *et al.*, 2000) and acrosome reaction in capacitated sperm (Blackmore, 1993).

There are also secreted proteins in the follicular fluid content that interact with the sperm. Atrial natriuretic peptide (ANP) has been identified in human follicular fluid (Sundsford *et al.*, 1989) and pig oviductal fluid in which it increases the acrosome reaction, the oocyte penetration rate and decreases polyspermy (Zhang *et al.*, 2006). Another follicular fluid protein that interacts with the sperm is Gd, existing as two isoforms: GdF that prevents premature acrosomal reaction and interferes with sperm–ZP interaction (Chiu *et al.*, 2003), and GdC that promotes sperm–ZP interaction (Chiu *et al.*, 2007). It has been proposed that cumulus cells may use GdF as a substrate in the production of GdC, which is then released during cumulus expansion and displaces sperm previously bound Gd isoforms (GdS and/or GdA) to promote sperm–ZP interaction (Yeung *et al.*, 2009).

Glycoprotein fibronectin (Fn) is secreted during cumulus expansion (Sutovsky *et al.*, 1995) and in the fallopian tube (Makrigiannakis *et al.*, 2009). Its receptor, the integrin  $\alpha_5\beta_1$ , has been detected on both male and female human (Fusi *et al.*, 1993, 1996) and bovine gametes (Thys *et al.*, 2009). By contrast, it has been demonstrated that Fn strongly inhibits sperm penetration during bovine IVF by diminishing sperm binding to ZP and oolemma as well as sperm–oocyte fusion (Tanghe *et al.*, 2004; Thys *et al.*, 2009), although its mechanism of action under physiological conditions remains unknown. Recent investigations have indicated that Fn is present in the oviductal fluid as well as in the extracellular matrix of oviductal epithelial cells and that it is

a key regulator of sperm binding to the epithelium when forming the sperm reservoir (Osycka-Salut *et al.*, 2017).

Interestingly, despite CRISP1 being considered as a male reproductive tract protein, recent studies have shown that is also expressed by mouse cumulus cells and that fertilization of CRISP1 knockout females is impaired because of sperm failure to penetrate the cumulus. Apparently, CRISP1 produced in the cumulus cells may regulate sperm orientation by modulating sperm hyperactivation through a mechanism that involves inhibition of CatSper and TRPM8 calcium channels, indicating a novel role for this protein in mammalian fertilization (Ernesto *et al.*, 2015).

### Conclusion

In order to fertilize, sperm must undergo changes that are sequentially regulated by the male and the female reproductive tracts that allow them to meet the oocyte and fuse with it. During that journey, the glycoprotein calyx surrounding the sperm plasma membrane is constantly remodelling to modulate the progress of capacitation. Years of investigation carried out in different models have indicated that mammalian sperm interaction with proteins from the male reproductive tract mostly inhibits sperm capacitation (see Table 1), while those from the female reproductive tract stimulate this process (see Table 2), but not all proteins characterized so far behave as expected. In addition, it is evident that there is still much work ahead to be carried out to fill the gaps that would allow an understanding of the mechanisms that transform the ejaculated spermatozoa into a fully competent cell able to fertilize.

The capacitation progress is regulated by inputs received from the extracellular milieu. An adequate balance between decapacitating and capacitating factors is required and therefore plasma membrane-associated proteins acquired during sperm transit along the male reproductive tract need to be removed to allow interaction with molecules of female origin that must modulate the progress of capacitation. However, some proteins have been found to be expressed in both male and female reproductive tracts, suggesting that there are multifunctional pieces in the capacitation machinery. In addition, protein post-translational modifications such as changes in the glycosylation pattern could be responsible for differential effects of a protein on capacitation, as has been described for Gd. Moreover, the variations in the abundance of certain proteins in each segment of the road and their particular affinity for sperm surface receptors may also contribute to regulate the evolution of sperm capacitation.

The evidence presented here indicates that there are several proteins that clearly regulate the sperm's ability to fertilize. Some of these such as Eppin and CRISP1 are of great interest due to their potential as male contraceptive targets because of its specificity and location on the human sperm surface. Nonetheless their participation during *in vivo* fertilization has not been sufficiently investigated. Furthermore, the *in vitro* studies reviewed evaluated the effects of single proteins on sperm, but the simultaneous or sequential interaction with several proteins competing for sperm membrane receptors, the involvement of non-protein molecules contained in the reproductive fluids and the presence of protein complexes (as in the case of Eppin/LTF/clusterin) are elements that should be considered in the future to fully understand spermatozoa changes that culminate in a successful gamete encounter and fusion. The findings currently revised shall contribute to improve understanding of the mechanisms that regulate sperm function, delineate novel approaches to study sperm physiology and identify potential biomarkers for the diagnosis of infertility.

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## 12. ANEXOS

### Anexo 1. Protocolo eléctrico del IEF para separación de muestras analizadas mediante 2-DE.

	<b>Voltaje</b>	<b>Tiempo</b>	<b>Volt/h</b>	<b>Rampa</b>
Paso 1	250	20 min	-	Lineal
Paso 2	4,000	2 h	-	Lineal
Paso 3	4,000	-	10,000 V-h	Rápida
Total		5 h	14,000 V-h	

### Anexo 2. Variables cinéticas de movilidad evaluadas mediante CASA [53].

<b>Variables cinéticas de movilidad</b>	
VAP	Velocidad media ( $\mu\text{m/s}$ )
VSL	Velocidad rectilínea ( $\mu\text{m/s}$ )
VCL	Velocidad curvilínea ( $\mu\text{m/s}$ )
ALH	Amplitud del desplazamiento lateral de la cabeza ( $\mu\text{m}$ )
BCF	Frecuencia de cruces (Hz)
STR	Índice de rectitud (VSL/VAP, %)
LIN	Índice de linealidad (VSL/VCL, %)

**Anexo 3. Pesos moleculares y puntos isoelectricos aproximados de los 59 spots separados por 2-DE y analizados con el programa PDQest.**

No. de proteína	PM (kDa)	pl (pH)	No. de proteína	PM (kDa)	pl (pH)
1	12.9	4.4	31	17.6	6.2
2	15.0	4.5	32	17.7	5.9
3	15.4	4.0	33	52.9	5.9
4	20.0	4.4	34	55.8	6.2
5	35.3	4.0	35	37.0	8.4
6	59.6	4.0	36	64.5	8.4
7	77.6	4.0	37	28.7	5.1
8	12.0	4.5	38	29.7	4.9
9	11.9	4.7	39	28.8	5.4
10	11.8	4.9	40	28.2	5.8
11	12.7	4.9	41	28.4	5.5
12	13.7	4.9	42	28.3	6.3
13	14.9	4.9	43	28.3	5.9
14	12.6	5.2	44	34.6	5.3
15	13.8	5.3	45	54.3	5.3
16	15.6	5.1	46	34.0	5.81
17	35.1	5.2	47	33.7	5.7
18	29.1	5.2	48	34.4	5.4
19	12.3	5.7	49	33.9	6.0
20	12.4	5.5	50	72.9	5.5
21	11.2	5.9	51	75.6	5.8
22	13.9	5.6	52	75.5	5.6
23	13.8	5.7	53	75.5	5.9
24	13.9	5.5	54	134.4	5.6
25	55.1	5.6	55	136.0	5.8
26	53.7	5.8	56	134.5	5.9
27	11.9	6.3	57	23.5	6.7
28	12.4	6.0	58	23.4	6.9
29	12.7	6.4	59	23.4	7.2
30	13.4	6.1			

N=3. PM: peso molecular, pl: punto isoelectrico.

#### **Anexo 4. Composición de medios y soluciones empleadas**

- **Medio Fluido Tubárico Humano (HTF):** KCl 4.69 mM, NaCl 97.8 mM, D-Glucosa 2.78 mM, CaCl<sub>2</sub> 2.04 mM, MgSO<sub>4</sub> 0.20 mM, KH<sub>2</sub>PO<sub>4</sub> 0.37 mM, lactato de sodio 21.4 mM, piruvato de sodio 0.33 mM, NaHCO<sub>3</sub> 4.0 mM, HEPES 21.0 mM, gentamicina 10 µg/ml, Penicilina/Estreptomicina 0.25%, pH 7.3.
- **Solución desnaturalizante para muestra:** SDS 2%, glicerol 10%, azul de bromofenol, β-mercaptoetanol 2%, Tris-HCl 0.6 M, pH 6.8.
- **Buffer de rehidratación para IEF:** Urea 8 M, CHAPS 2%, DTT 50 mM, Bio-Lyte 0.2%, azul de bromofenol.
- **Buffer de equilibrio I para 2-DE:** Urea 6 M, SDS 2%, glicerol 20%, DTT 2%, Tris-HCl 0.05 M, pH 8.8.
- **Buffer de equilibrio II para 2-DE:** Urea 6 M, SDS 2%, glicerol 20%, iodoacetamida 2.5%, Tris-HCl 0.05 M, pH 8.8.
- **Medio de alta sal para preservación de la zona pelúcida:** Dextran 0.05%, sulfato de amonio 2 M, Tris-HCl 20 mM, pH 7.2-7.6.

## **Anexo 5. Anticuerpos utilizados**

- Anti-fosfotirosina clon 4G10: 05-321; Millipore; Temecula CA, EE.UU.
- R-anti- $\beta$ -tubulina: T0198; Sigma-Aldrich, St. Louis MO, EE.UU.
- R-anti-PIP: HPA009177; Sigma-Aldrich, St. Louis MO, EE.UU.
- R-anti-LTF (H-65): sc-25622; Santa Cruz Biotechnologies, Santa Cruz CA, EE.UU.
- M-anti-PSA (A67-B/E3): sc-7316; Santa Cruz Biotechnologies, Santa Cruz CA, EE.UU.
- M-anti-ZAG (1D4): sc-13585; Santa Cruz Biotechnologies, Santa Cruz CA, EE.UU.