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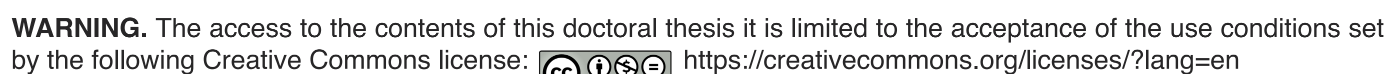
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Universitat Autònoma
de Barcelona

TESIS DOCTORAL

Identificación, localización y función de las acuaporinas en el espermatozoide equino y su relación con la criopreservación

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Universitat Autònoma de Barcelona

Tesis Doctoral

Identificación, localización y función de las acuaporinas en el espermatozoide equino y su relación con la criopreservación

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Dedicada a todas las personas que me han apoyado y acompañado en este proceso y en especial a mi familia que a la distancia siempre estuvo conmigo.

“Considero más valiente al que conquista sus deseos que al que conquista a sus enemigos, ya que la victoria más dura es la victoria sobre uno mismo”.

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ABREVIATURAS

AC: Acetazolamida

AGP: Acuagliceroporinas

ALH: Amplitude of lateral head displacement

AQP: Acuaporinas

ART: Tecnologías en reproducción asistida

ATP: Adenosin tri-fosfato

BCF: Beat cross frequency

CASA: Computer-assisted sperm analysis

CPAs: Cryoprotective agents

DCF+: 2',7'-dichlorofluorescein

DMSO: Dimethyl sulfoxide

E+: Ethidium

EROs: Especies reactivas de oxigeno

EV: **Electronic** volume

FS: Forward scatter

GFE: Good freezability ejaculates

GLPs: Acuaglyceroporins

H2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate

H2O2: Hydrogen peroxide

HE: Hydroethidine

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IA: Inseminación artificial

ISAC: International Society for Advancement of Cytometry

JC-1: 5,5',6,6'-tetrachloro- 1,1',3,3' tetraethyl-benzimidazolylcarbocyanine iodide

JC-1agg: JC-1 aggregates

JC-1mon: JC-1 monomers

kD: Kilodaltons

LIN: Linearity

M540: Merocyanine 540

MMP: Mitochondrial membrane potencial

O2-•: Superoxide

PDO: 1,3-propanediol

PFE: Poor freezability ejaculates

PHL: Floretina

PI: Propidium iodide

PKA: cAMP dependent protein kinase

PMOT: Progressive sperm motility

PNA-FITC: Arachis hypogaea lectin -peanut agglutinin- conjugated with fluorescein isothiocyanate

ROS: Radicales libres de oxigeno

SEM: Standard error of the mean

STR: Straightness

SuperAQP: Superacuaporinas

TMOT: Total sperm motility

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight line velocity

WOB: Motility parameter wobble

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RESUMEN

La criopreservación es una técnica que ha ido avanzando en los últimos años, buscando obtener mejores resultados de fecundación. Esta técnica está asociada a tres factores relevantes para la presente tesis doctoral: en primer lugar, a la aparición de técnicas de inseminación artificial como la IA uterina profunda que solo requiere de una pequeña cantidad de células espermáticas; en segundo lugar a la utilización de crioprotectores distintos al glicerol lo que permite una mejor criosupervivencia del esperma; y finalmente, al establecimiento de nuevas técnicas de selección espermática que aumentan la calidad del semen congelado.

Con todo esto, y sabiendo que el espermatozoide maduro es una célula muy permeable al agua, existen unos canales selectivos altamente permeables al agua y otras sustancias como lo son las Acuaporinas. La función principal de estas proteínas es el transporte y han sido estudiadas en espermatozoides de diferentes mamíferos como el humano, el perro, el cerdo y el toro, pero en caballo su existencia, localización y funciones han sido poco estudiadas.

Por todo esto, el primer objetivo de la presente tesis doctoral fue determinar la presencia de las acuaporinas AQP3, AQP7 y AQP11 en espermatozoide equino mediante los métodos de Western Blot; así como establecer la relación entre estas tres acuaporinas con la calidad espermática y la criotolerancia de eyaculados de caballo; el segundo objetivo fue determinar la localización de AQP3, AQP7 y AQP11 en el espermatozoide de caballo y examinar los cambios en su localización en respuesta a la criopreservación; y finalmente el tercer objetivo de la tesis consistió en evaluar la funcionalidad de estas acuaporinas usando tres diferentes inhibidores (Acetazolamida, Floretina y Propandiol) con el propósito de observar cambios en la motilidad utilizando el sistema CASA y cambios en la viabilidad y actividad de la membrana usando la citometría de flujo.

Frente al primer objetivo, los resultados del método Western blot probaron la presencia de estas tres proteínas en el espermatozoide equino. Las acuaporinas se

presentaron en dos tipos de bandas a pesos moleculares de 60 kD y de 30 kD, siendo más clara la primera que la segunda en todos los casos. Asimismo, se determinó que el contenido relativo de la AQP7 en el espermatozoide del caballo no tenía diferencia significativa entre buenos (GFE) y malos congeladores (PFE). En cambio, para la AQP3 y la AQP11 el contenido relativo en la banda de 60 kD era significativamente diferente entre el grupo de buenos y malos congeladores. Por el contrario, en el caso de la AQP11 en la banda de 30 kD, el contenido relativo de ésta era más alto en los PFE en comparación con los GFE.

En relación al segundo objetivo, se identificó la localización de estas tres acuaporinas encontrando, en el semen fresco, que la AQP3 y la AQP7 están localizadas en la pieza intermedia, mientras que la AQP11 se encuentra en la pieza principal y en la región post-acrosómica de los espermatozoides de caballo. Una vez realizado el proceso de criopreservación, esta localización se modificó en dos de ellas: AQP3 y AQP11.

Ya en cuanto al último objetivo, se pudo observar que la inhibición de las acuaporinas estudiadas depende de la especificidad del inhibidor para cada una, y de sus efectos colaterales en otras proteínas de la membrana del espermatozoide. En este sentido, la Acetazolamida (AC), que inhibe principalmente las AQP ortodoxas, no las gliceroacuapotinas (AGP), parece indicar que estas proteínas no están involucradas en la respuesta a los cambios de osmolaridad producidos durante la criopreservación, mientras que la Floretina (PHL) sugiere que las AGP juegan un papel crucial en la regulación de la osmolaridad durante la criopreservación del espermatozoide equino. Finalmente, el Propandiol (PDO) como suplemento, mejoró la calidad general de los espermatozoides indicando su potencial como agente crioprotector.

Así pues, se puede concluir que las acuaporinas se encuentran en los espermatozoides de caballo, donde juegan un papel fundamental en cuanto a su capacidad de resistencia frente a los protocolos de congelación y descongelación.

Todos estos resultados contribuyen a incrementar nuestro conocimiento acerca del papel de estos canales de agua en la fisiología y criopreservación de los espermatozoides equinos, pudiendo tener así implicaciones prácticas como marcadores de congelabilidad y selección de eyaculados aptos para la fecundación.

Summary

Over recent years, the technique of Cryopreservation has made prominent progress, as part of the research for better fertilization results. This technique is related with three relevant factors for this dissertation: the first is the emergence of new artificial insemination techniques, such as deep uterine AI that only requires a small amount of sperm cells; the second is the use of cryoprotectants other than glycerol, which allows a better cryosurvival of sperm; and finally, the establishment of new sperm selection techniques that increase the quality of frozen semen.

With all this and knowing that the mature sperm is a cell very permeable to water, there are selective channels highly permeable to water and other substances such as the Aquaporins. These substances have a transport function and have been studied in the sperm of different mammals such as humans, dogs, pigs and bulls, but their existence, location and functions have been poorly studied.

For all this, the first objective of the present doctoral thesis was to determine the presence of aquaporins AQP3, AQP7 and AQP11 in equine sperm using the Western Blot method, and also to establish the relationship between these three aquaporins with the sperm quality and the criotolerance of horse ejaculates; the second objective was determine the location of aquaporins AQP3, AQP7 and AQP11 in equine sperm and address whether the localization of these three AQPs occurred in response to cryopreservation; and finally the third objective was to evaluate the functionality of these aquaporins using three different inhibitors (Acetazolamide, Phloretin and Propanediol) to observe changes in motility using the CASA system and changes in the viability and activity of the membrane using flow cytometry.

With respect to the first objective, Western blot results showed the presence of these three proteins in the equine sperm. Aquaporins were presented in two types of bands at molecular weights of 60 kD and 30 kD, the first being clearer than the second in all cases. Likewise, it was determined that the relative content of AQP7 in the horse's

sperm had no significant difference in its relative content between good (GFE) and bad freezers (PFE). Instead for AQP3 and AQP11, the relative content in the 60 kD band was significantly different between the group of good and bad freezers. In contrast to this, in the case of AQP11 in the 30 kd band, the relative content of this aquaporin was higher in PFE compared to GFE.

In relation to the second objective, the location of these three aquaporins was identified by finding in the fresh semen that AQP3 and AQP7 are located in the intermediate part while the AQP11 is found in the main part and in the post-acrosomal region of horse sperm. Once the cryopreservation process was completed, this location was modified in two of them: AQP3 and AQP11.

As for the last objective, it was observed that the inhibition of these aquaporins depends on the specificity of the inhibitor for each one, and on its collateral effects on other sperm membrane proteins. In this respect, Acetazolamide (AC), which mainly inhibits orthodox AQPs, not glyceroacuapotins (AGP), seems to indicate that these proteins are not involved in the response to osmolarity changes produced during cryopreservation, while Floretin (PHL) suggests that AGP play a crucial role in the regulation of osmolarity during cryopreservation of the equine sperm. Finally, Propandiol (PDO) as a supplement, improved the overall quality of sperm by indicating its potential as a cryoprotective agent.

Thus, it can be concluded that aquaporins are found in stallion spermatozoa, where they play a fundamental role in their resistance to freezing and thawing protocols. All these results contribute to increase our knowledge about the role of these water channels in the physiology and cryopreservation of equine sperm, thus having practical implications such as markers of freezing and selection of ejaculates suitable for fertilization.

INTRODUCCIÓN

Una de las tecnologías de reproducción asistida (ART) más importantes en el mundo es la inseminación artificial (IA). Esta biotecnología tiene un gran impacto en la producción equina, abriendo la posibilidad de que un reproductor pueda dejar cantidades de descendientes a lo largo de su vida reproductiva (Canisso et al., 2008).

Por muchos años, el desarrollo y la utilización de la IA en équidos estuvieron limitados por imposiciones de muchas asociaciones de criadores que no permitían su utilización (Canisso et al., 2008). Con el paso del tiempo, asociaciones de diversos países del mundo fueron flexibilizando dichas resistencias, lo que permitió el registro de productos generados por esta biotecnología y causó un gran impacto en la industria equina, especialmente en Estados Unidos (Paul R. Loomis, 2006), y Europa (J. Aurich & Aurich, 2006).

El gran desarrollo de la IA ha incrementado el interés de investigadores y clínicos por las técnicas de procesamiento del semen, creando la necesidad de desarrollar métodos, protocolos o procedimientos que minimicen el daño espermático a la vez que maximicen su viabilidad, supervivencia y capacidad fecundante (Paul R. Loomis, 2006). Los avances en las técnicas y procedimientos han hecho que, en la actualidad, la criopreservación del semen sea uno de los procedimientos más importantes en el desarrollo de la biotecnología de la reproducción asistida en los mamíferos en general y en el equino, en particular (Betancur, Suarez, Páez, Celis, & Henao, 2014).

1. Antecedentes Históricos

1.1 Domesticación de équidos

La historia de la domesticación de caballos y otros équidos todavía se está desentrañando. Durante muchos años, se pensó que los équidos fueron domesticados por primera vez mediante la adopción de potros alrededor de los años 4000–3000 a.C.(Goodall DM. A History of Horse Breeding. London: Robert Hale, 1977). Investigaciones más recientes han sugerido que los équidos también fueron domesticados en China en las mismas fechas. Por lo tanto, no está más allá de los límites de la posibilidad prever que la domesticación de los caballos se produjera casi simultáneamente, alrededor de 3500-3000 aC, en cuatro o cinco lugares diferentes del mundo. Cuando el ser humano se instaló en un estilo de vida sedentario, desarrollando para su subsistencia técnicas basadas en el cultivo de diversas especies hortofrutícolas, el caballo, junto con el buey, se convirtieron en animales más útiles para la labor agraria en las comunidades. Con el tiempo, y como consecuencia de la presión de selección ejercida por humanos de acuerdo a sus necesidades, empezaron a aparecer distintas especies de caballos en ciertas áreas y regiones (Revisado por McKinnon AO; 2011).

En la historia reciente, el caballo ha tenido importancia debido a las diferentes labores que puede brindar para la humanidad, entre las que se destacan su uso como fuente de alimento y su influencia de manera decisiva en el desarrollo de las comunicaciones como medio de transporte. Algunas sociedades lo han utilizado como símbolo religioso y como animal de prestigio y, hasta comienzos del siglo XX, jugó un papel crucial en el desarrollo de los conflictos bélicos.(Lira, 2015)

En la actualidad hay 55 millones de cabezas de caballo repartidas por todo el mundo. El país con mayor número es China, seguido de México, Brasil y EEUU. En Argentina, Colombia y Rusia, entre otros, se registran más de un millón de cabezas. Según la FAO (Food *and Agriculture Organization of the United Nations*), se han contabilizado 633 razas para esta especie animal, dato que indica que representa aproximadamente el 15% de las razas de mamíferos en todo el mundo (Lira, 2015)

1.2 Antecedentes reproductivos

En Europa, alrededor del año 1100 d.C; los monjes irlandeses, que se establecieron en los Países Bajos se pusieron sistemáticamente a mejorar la selección de Caballos en esta área, lo que dio lugar a razas como el Percherón y el Belga. En el siglo XVIII, Robert Bakewell, que junto con otros promovió y lideró la Revolución agrícola británica y contribuyó a incrementar en gran medida la producción ganadera en el Reino Unido, mejoró el caballo de tiro (Bewick T, 1790). Como dato importante para la época el factor que mitigó la fertilidad de los caballos fue el hecho de que los propietarios preferirían utilizar las yeguas para el trabajo, en lugar de para la crianza. Esto significó que muchas veces una yegua útil no era considerada como yegua de cría hasta que habían transcurrido sus años más productivos para el trabajo.

2. Inseminación artificial (IA)

2.1 Antecedentes y Desarrollo

La historia de la inseminación artificial en el género *Equus* ha seguido un camino bastante largo y complicado. El primer incidente registrado fue en 1322, cuando un jeque árabe obtuvo ilícitamente algo de semen del semental de un rival para criar a una de sus yeguas (Perry EJ, 1968). El siguiente hito en este camino histórico fue en 1780, cuando un sacerdote italiano llamado Lazzaro Spallanzani haciendo prácticas de fisiología realizó un experimento con ranas mostrando que los espermatozoides eran el componente esencial del semen requerido para la fecundación (Bowen, 1969). Después de esto, inseminó a una perra y produjo con éxito tres cachorros y más tarde pasó a inseminar con éxito a una yegua (revisado en J. E. Aurich, 2012). También descubrió que el semen de perro se podía conservar con nieve y que éste se inmovilizaba. Sin embargo, la motilidad se restauraba durante varias horas cuando la muestra se calentaba a temperatura ambiente. Estos dos experimentos sentaron las bases para el posterior desarrollo de la inseminación artificial y el uso del semen refrigerado y congelado en la reproducción animal.

El impulso real para desarrollar la inseminación artificial en el caballo provino de Ivanov (revisado en Moore & Hasler, 2017), a quien, en 1909, el Real Stud ruso de San Petersburgo le pidió que investigara el potencial de la inseminación artificial. Se estableció en los laboratorios veterinarios del Ministerio de Agricultura ruso y, a pesar de que los resultados iniciales fueron deficientes, persistió en el empeño de mejorar la técnica. Muchas personas acudieron a los laboratorios de Ivanov para recibir instrucciones sobre la recolección de semen y su uso en la inseminación artificial (Ivanoff, 1922).

A finales de la década de 1930, las técnicas que se utilizan actualmente para la IA con semen refrigerado ya estaban disponibles. Sin embargo, la IA se usó comercialmente solo en Dinamarca y la Unión Soviética. Mientras Dinamarca fue pionera en la IA bovina, en la Unión Soviética se llevó a cabo la IA equina, de modo que en 1938 ya se habían inseminado más de 140,000 yeguas. Posteriormente, los países con programas emergentes de IA equina Estados Unidos, el Reino Unido, Italia, Alemania y Bulgaria (figura 1). Sin embargo, la falta de transporte rápido, el conocimiento limitado sobre la biología de la reproducción y el hecho de que los caballos se criaran principalmente para fines agrícolas dificultaron el uso generalizado de la IA equina durante estos primeros años (revisado en J. E. Aurich, 2012).

En muchos países, se produjo un aumento de la IA equina durante los años ochenta. En Alemania y Francia, la IA equina se puso a disposición de los criadores gracias, en gran parte, al apoyo de los servicios públicos de formación y capacitación. En 1985, más del 98% de las yeguas de razas de caballos deportivos alemanes todavía se criaban con monta natural y solo el 1,4% fueron inseminadas. Por primera vez en 1995, más del 50% de las yeguas de razas de caballos deportivos fueron inseminadas; dicho porcentaje se elevó hasta el 80% en 2004, y se acerca al 90% en la actualidad (revisado en J. E. Aurich, 2012). En América del Norte y del Sur, Escandinavia, Bélgica, los Países Bajos, España, e incluso Gran Bretaña

dominados por la raza pura, existen empresas de cría equina activa con uso a gran escala de la IA. Durante mucho tiempo, el semen congelado no se podía usar para la cría de las razas estadounidenses. Sin embargo, la *American Quarter Horse Association* permitió en 2001 el uso de semen criopreservado para la IA (P. R. Loomis, 2001).

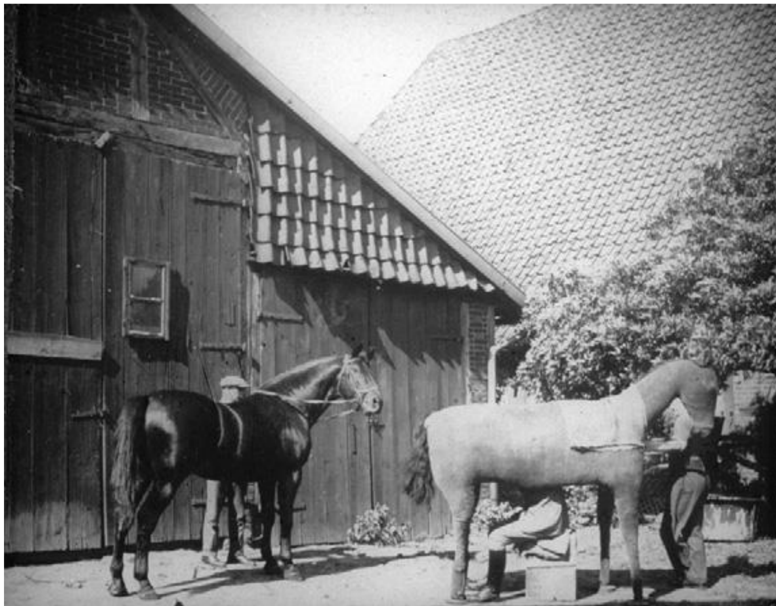

Fig. 1 Recolección de semen en una finca privada de sementales en Alemania a principios de los años 40 en una yegua maniquí con una vagina artificial "integrada". (J. E. Aurich, 2012)

2.2 Ventajas

La inseminación artificial ofrece numerosas ventajas (Juan C. Samper, 2009). Entre otras, cabe destacar las siguientes:

a) Acelera la mejora genética con la mayor difusión de sementales de alto valor.
b) Evita el desplazamiento de las yeguas con los problemas sanitarios que ello puede acarrear.
c) Evita la contracción de enfermedades de transmisión venérea.
d) Disminuye los gastos de cubrición.
e) Evita la sobreutilización de un semental.

f) Permite la utilización de un semental ubicado a miles de kilómetros de la yegua.
g) Además, y en el caso del semen criopreservado, permite el uso del semen procedente de sementales que ya han fallecido.

2.3 Métodos.

El semen equino puede utilizarse para la IA en tres formas distintas.

1) Como semen fresco. El semen es colectado y utilizado inmediatamente en su estado puro, o diluido con un diluyente apropiado. Su uso está restringido dado que la IA debe llevarse a cabo durante las tres horas posteriores a la recolección del semen, y la yegua y el semental deben estar en el mismo lugar.
2) Como semen refrigerado. En este caso, el semen se recolecta del mismo modo que en el caso anterior, pero se diluye con un medio apropiado (p.ej. diluyente de Kenney) y, después de su análisis, se refrigera a 5-8ºC. La IA con este tipo de semen puede llevarse a cabo hasta 48 horas después de su recolección.
3) Como semen criopreservado. El semen es recolectado, diluido, analizado y finalmente congelado (preferiblemente con un biocongelador, o congelador con tasa de enfriamiento controlada) a -196ºC. Las pajuelas resultantes, habitualmente de 0,5 mL, se almacenan en tanques de nitrógeno líquido y, en principio, el tiempo de preservación es ilimitado. Por ello, dicho semen se puede utilizar para la IA después de días, meses o, incluso, años de su recolección (Juan C. Samper, 2009).

3. Criopreservación del semen equino

El uso para la IA del semen congelado de sementales minimiza la propagación de enfermedades, elimina las barreras geográficas y preserva el material genético del animal por tiempo ilimitado. Se ha logrado un progreso significativo en el proceso

de criopreservación de semen equino; durante la última década, se ha mejorado significativamente el poder fecundante de dicho semen. En todo caso, el progreso en el uso de este tipo de semen se ha asociado con la aparición de nuevas técnicas de IA, como la inseminación artificial uterina profunda que solo requiere de una pequeña cantidad de células espermáticas; la utilización de crioprotectores distintos al glicerol, lo que permite una mejor criosupervivencia del esperma; y el establecimiento de nuevas técnicas de selección espermática que aumentan la calidad del semen congelado. También se han hecho esfuerzos tanto para mejorar el conocimiento de los daños asociados al proceso de criopreservación, como para mitigarlos, y se han mejorado las técnicas de análisis de la calidad espermática después de la descongelación (Alvarenga, Papa, & Ramires Neto, 2016).

Los inicios de la criopreservación espermática se remontan a mediados de los años 50 del siglo pasado, cuando Smith y Polge publicaron el primer artículo sobre la congelación del semen equino. Mediante el uso de un solución de glucosa y glicerol, como crioprotectores no permeable y permeable, respectivamente, y una temperatura de conservación de -79ºC, lograron un supervivencia espermática del 25% a la post-descongelación (Barker yGandier,1957). Algunos años más tarde, en 1957, se consiguió la primera gestación de una yegua inseminada con semen congelado-descongelado (Barker y Gandier,1957). Sin embargo, no fue hasta fines de los años 1980 cuando se generalizó su uso en la especie equina (P. R. Loomis, 2001).

Los pasos cruciales del proceso de criopreservación del semen equino (figura 2) incluyen el examen andrológico del semental ; la recolección, evaluación y dilución del semen; la centrifugación y el descarte del sobrenadante; la resuspensión del sedimento celular con el diluyente de congelación; la refrigeración del semen a 4ºC (equilibrado); el envasado; la criopreservación (de 4ºC a -196ºC); y la evaluación de los espermatozoides después de la descongelación (Canisso et al., 2008; J. C. Samper & Morris, 1998).

Fig. 2 Procesamiento del semen congelado (Alvarenga et al., 2016)

La mayoría de los protocolos para la criopreservación de espermatozoides de sementales implican la eliminación de la totalidad o la mayoría (95%) del plasma seminal mediante centrifugación y resuspensión de los sedimentos de esperma en un medio de criopreservación (esto es, con crioprotectores). Los espermatozoides están concentrados, de modo que se empaqueten suficientes células en un pequeño volumen para la congelación. La eliminación de la mayor parte del plasma seminal ayuda a eliminar los efectos perjudiciales que éste tiene para la supervivencia de los espermatozoides durante la congelación (revisado en Paul R. Loomis, 2006).

Hasta el momento no existe un procedimiento estandarizado para cada una de estas etapas por lo que hay una gran variación entre laboratorios y grupos de investigación (J. Aurich & Aurich, 2006; Vidament, 2005).

Otras de las razones por la cuales el uso de semen criopreservado en equinos ha adquirido una gran importancia en las últimas décadas está relacionada con los beneficios que ofrece su utilización (P. R. Loomis, 2001): permite su almacenamiento a largo plazo; el transporte del semen se puede hacer a cualquier destino; facilita la conservación del semen de animales con elevado valor genético

(Pugliesi, Fürst, & Carvalho, 2014) y el desarrollo de un programa de mejora genética. Desde el punto de vista práctico, una de las principales ventajas de la utilización de semen congelado es que permite inseminar un número alto de yeguas con un mismo eyaculado (Davies Morel, 2015).

La reproducción clínica en el caballo es más parecida a la reproducción humana que en otros animales de granja domésticos. Los criadores de caballos rara vez incluyen la fertilidad como criterio de selección al tomar decisiones de apareamiento; En la mayoría de las razas, no hay licencia o aprobación de sementales. Esto ha llevado a que un número significativo de sementales en el grupo de reproducción posean características de rendimiento deseables pero que sean subfertiles por una variedad de razones, algunas de ellas transmitidas genéticamente entre generaciones. Por lo tanto, las características del semen pueden variar mucho entre los sementales dentro de la población reproductora (Paul R. Loomis, 2006). Esta variabilidad también explica que el éxito de la congelación del semen equino sea menor que el de otras especies domésticas y que las tasas de preñez se vean disminuidas cuando se insemina con este tipo de semen (Blottner, Warnke, Tuchscherer, Heinen, & Torner, 2001; Vidament, 2005). Por todo ello, la variabilidad individual existente entre los sementales (P. R. Loomis & Graham, 2008; Sieme, Harrison, & Petrunkina, 2008) e incluso entre los eyaculados de un mismo animal (Dowsett & Knott, 1996) es uno de los factores a tener en cuenta para la criopreservación de los espermatozoides equinos..

Se considera que un semental es "apto para la congelación espermática" si presenta valores de motilidad progresiva ≥50-60% en semen fresco y ≥30-35% en el semen descongelado (revisado en P. R. Loomis & Graham, 2008). Otros autores mencionan que antes de iniciar el proceso de congelación, el semen en fresco tiene que tener como mínimo un 40% de espermatozoides morfológicamente normales, una motilidad progresiva superior al 60% y una concentración mínima de $100x10^6$ de espermatozoides por ml (Betancur et al., 2014). Se han desarrollado, sin embargo, protocolos especiales para procesar eyaculados de bajo volumen y

concentración (Castro & Chacon, 2016). Algunos estudios muestran que solo el 20-30% de los sementales producen un semen con buena capacidad de congelación, llamados “buenos congeladores”; otro 40-60% aproximadamente tiene una capacidad aceptable (aunque se vea afectada negativamente por la criopreservación) y el 20-30% restante son sementales que producen un semen que no es congelable por lo que, reciben el nombre de “malos congeladores” (Betancur et al., 2014; P. R. Loomis & Graham, 2008)

Otro factor que limita la utilización del semen congelado es la baja tolerancia de los espermatozoides equinos a los procesos de congelación y descongelación (Candeias et al., 2012; Fagundes et al., 2015). Los daños en los espermatozoides causados por la criopreservación han sido descritos ampliamente en estudios previos (Ortega-Ferrusola et al., 2008; M. Yeste et al., 2015). En equinos, se ha demostrado que la criopreservación induce a diferentes tipo de daños, entre los que se encuentran la fragmentación del ADN espermático, la disminución del potencial de membrana mitocondrial, daños en el acrosoma y la producción de radicales libres de oxígeno (ROS) (M. Yeste et al., 2015). Por todos estos motivos, es importante conocer los procesos físico-químicos que afectan a los espermatozoides para optimizar los protocolos y tener una mejor fertilidad y viabilidad después de la criopreservación (Pugliesi et al., 2014).

3.1 Daños en el espermatozoide como resultado de la criopreservación

La capacidad de los espermatozoides de soportar los procedimientos de criopreservación sin perder una o varias de sus funciones principales es fundamental para obtener un semen descongelado de buena calidad (Sieme et al., 2008). El principal inconveniente de los procedimientos de congelación y descongelación es el daño causado por la bajas temperaturas que, entre otras causas, se asocia con el cambio de fase del agua intracelular y extracelular (revisado en Yeste et al., 2016). Otros daños que afectan la viabilidad y la integridad de los espermatozoides presentan algunas similitudes con la apoptosis

(spermptosis) y también tienen como consecuencia la reducción de su vida media y de su poder fecundante (Ortega-Ferrusola et al., 2008; Sieme et al., 2008).

3.1.1 Daños por cambios osmóticos

Durante el proceso de criopreservación, el espermatozoide se expone a variaciones de temperatura y osmolaridad, lo cual trae como consecuencia tanto la formación de cristales de hielo como cambios en la composición molecular de los medios intra y extracelular (revisado en Castro & Chacon, 2016). Según Gao y Critser (2000), las células y el medio extracelular permanecen no congelados y sobre enfriados a menos 5ºC. A temperaturas entre -5ºC y -15ºC, se forma hielo en el medio circundante, pero los contenidos intracelulares permanecen no congelados y sobre enfriados. Debido a que el potencial químico del agua es mayor en el estado enfriado (intracelular) que en el estado congelado (extracelular), el agua fluye fuera de la célula y se congela externamente. Relacionado con este fenómeno, hay que destacar la importancia de la velocidad de enfriamiento/congelación que determinará el éxito del proceso (Gao & Critser, 2000). Si la velocidad de enfriamiento es muy alta, el agua intracelular no fluye completamente, las células se congelan intracelularmente, y la formación de cristales de hielo en el citoplasma resulta en lesiones criogénicas (Mazur; P, 1990). Por otra parte, si la velocidad de enfriamiento es muy baja, la mayor parte del agua fluye hacia afuera, los solutos intracelulares se concentran y se elimina el sobreenfriamiento. En lugar de producirse una congelación intracelular, las células se deshidratan, experimentan un encogimiento del volumen de sus orgánulos y membranas, y se exponen a altas concentraciones de solutos antes de que alcancen la temperatura a la que se solidifican todos los componentes de la solución (Figura 3). Esto afecta a los complejos proteínas-lípidos de la membrana, desnaturaliza las macromoléculas, disminuye el tamaño de los canales no congelados, e induce la fusión irreversible de la membrana (Mazur.,et al , 1972).

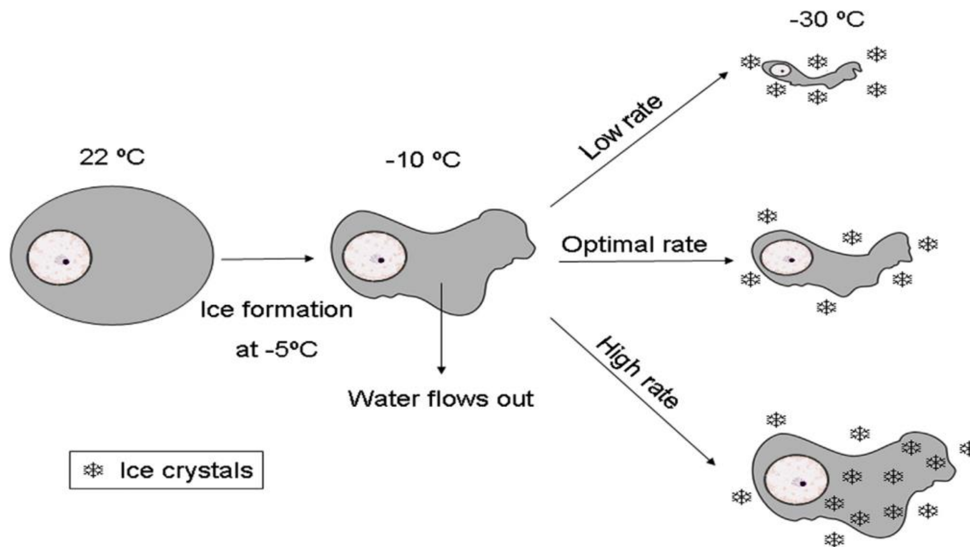

Fig. 3 Velocidades de enfriamiento y eventos físicos durante la congelación. Tomada de (Yeste , 2016).

Por este motivo Mazur y colaboradores propusieron la teoría de los dos factores. Según esta teoría, la lesión criogénica se produce debido:

1) La formación letal de cristales de hielo intracelulares a altas velocidades de enfriamiento

2) La deshidratación celular (concentración de solutos/electrolitos) y la reducción de la fracción no congelada en el espacio extracelular debido a bajas velocidades de enfriamiento.

De este modo, hay una velocidad de enfriamiento óptima para cada tipo de célula y ésta se define como lo suficientemente baja para evitar la formación de cristales de hielo intracelular, pero lo suficientemente alta como para minimizar la lesión por la concentración de soluto / electrolito (Mazur et al., 1972).

3.1.2 Daños causados por cambios de temperatura

El daño causado por el choque térmico ocurre cuando la membrana celular pasa del estado líquido al estado sólido (P. R. Loomis & Graham, 2008), dado que los fosfolípidos presentan diferentes temperaturas de transición y se producen separaciones de fase. Como resultado de esto, estos lípidos se reestructuran, las

proteínas integrales de la membrana se agrupan y se liberan algunas moléculas de colesterol. Esto conduce a una disrupción de las interacciones entre los lípidos y las proteínas, y la pérdida de la función de algunas proteínas esenciales, como los canales iónicos (p.ej. acuaporinas). Estos daños afectan la integridad y función de la membrana plasmática, alterando el tráfico de agua, de iones como el calcio y el bicarbonato, y el de otras moléculas cruciales como los crioprotectores (Leahy & Gadella, 2011; revisado por Yeste, 2016). Como consecuencia de estos cambios que padece la membrana, algunos autores utilizan el término “criocapacitación” “capacitation-like changes”, si bien el vocablo puede inducir a error, dado que, aunque parecidos, los fenómenos observados difieren de la verdadera capacitación (Green & Watson, 2001). En todo caso, los daños en la membrana plasmática hacen que el espermatozoide tenga más posibilidades de padecer una exocitosis acrosómica degenerativa, lo que disminuye tanto su tiempo de vida como su poder fecundante. Por lo tanto, el intervalo entre la inseminación y la ovulación debe ser más corto que el utilizado para la IA con semen fresco/refrigerado, y la detección de la ovulación mediante ecografía es un paso crítico (Thomas, Meyers, & Ball, 2006).

3.1.3 Daños causados por la producción de especies reactivas de oxígeno (EROs)

Durante el metabolismo oxidativo fisiológico de los espermatozoides se producen EROs que desempeñan un papel importante para los procesos de capacitación y reacción acrosómica y, en general, para el mantenimiento de la capacidad fecundante del espermatozoide (Desai, Sharma, Makker, Sabanegh, & Agarwal, 2009). Además, los procedimientos de congelación y descongelación inducen cambios en el potencial de la membrana mitocondrial .De hecho, se ha descrito que el enfriamiento y la congelación reducen la actividad mitocondrial en espermatozoides de equinos (revisado por Peña et al., 2015). Sin embargo, se ha descrito que la criopreservación puede aumentar los niveles intracelulares de EROs, que se producen principalmente en las mitocondrias del espermatozoide equino (Ortega-Ferrusola et al., 2009; Yeste et al., 2015). El problema radica cuando se produce un desequilibrio entre la producción y degradación de estas sustancias,

dado que se generan efectos adversos sobre los espermatozoides. Estos efectos adversos incluyen, entre otros, la peroxidación lipídica de la membrana plasmática del espermatozoide y la fragmentación de cadena simple o doble del ADN (Baumber et al, 2000; Baumber et al, 2003)(revisado por Yeste, 2016).

3.1.4 Otros efectos

Otro efecto deletéreo de la criopreservación espermática es la reducción de la motilidad (revisado por Yeste, 2016). Como se comentó anteriormente, la función mitocondrial es crucial para la motilidad de los espermatozoides. Por ello, la disminución de dicha actividad mitocondrial después de los procedimientos de congelación y descongelación, que se debe al daño en las membranas mitocondriales y a que el ATP generado por la fosforilación oxidativa en las mitocondrias se transfiere a los microtúbulos, explica la reducción de la motilidad espermática (Gao & Critser, 2000).

4. Estructura y transporte de moléculas a través de la membrana plasmática

La membrana plasmática celular es una bicapa lipídica caracterizada esencialmente por la presencia de fosfolípidos, carbohidratos, colesterol y proteínas. Estas proteínas pueden o bien localizarse en una posición periférica o bien atravesar completamente la bicapa lipídica, en cuyo caso se habla de proteínas integrales transmembrana (Cooper, 2000).
La estructura de la membrana plasmática obedece a un modelo de mosaico fluido (Lombard, 2014) que se caracteriza por un movimiento continuo de los componentes que lo constituyen. Las partes externas de la membrana se caracterizan por la presencia de cabezas polares hidrófilas y su interior contiene las cadenas de ácidos grasos insaturados saturados e hidrófobos. El colesterol presente es también uno de los elementos fundamentales de la membrana y le confiere la rigidez necesaria para mantener su arquitectura (Cooper, 2000). La relación entre el número de moléculas de fosfolípidos y el número de moléculas de

colesterol difiere entre especies y es crucial para la criotolerancia del espermatozoide (revisado por Yeste, 2016).

La membrana celular representa una barrera semipermeable entre la célula y el entorno extracelular (Lodish et al., 2000). El transporte de agua y otros solutos a través de la bicapa lipídica puede realizarse con o sin consumo de energía. Se habla de transporte activo cuando éste se lleva a cabo contra el gradiente de concentración y requiere o bien consumo de energía (ATP) o bien la presencia de co-transportadores. En ambos casos, se produce un cambio de la conformación de las proteínas involucradas en dicho transporte para que las moléculas pasen de un lado al otro de la membrana (Cooper, 2000).

El transporte de moléculas también se puede llevar a cabo a favor de gradiente. En este caso, que no requiere consumo de energía, las moléculas son transportadas mediante difusión simple o a través de proteínas canal (difusión facilitada; figura 4). A continuación, se expondrán con más detalle las características cada uno de estos mecanismos de transporte pasivo.

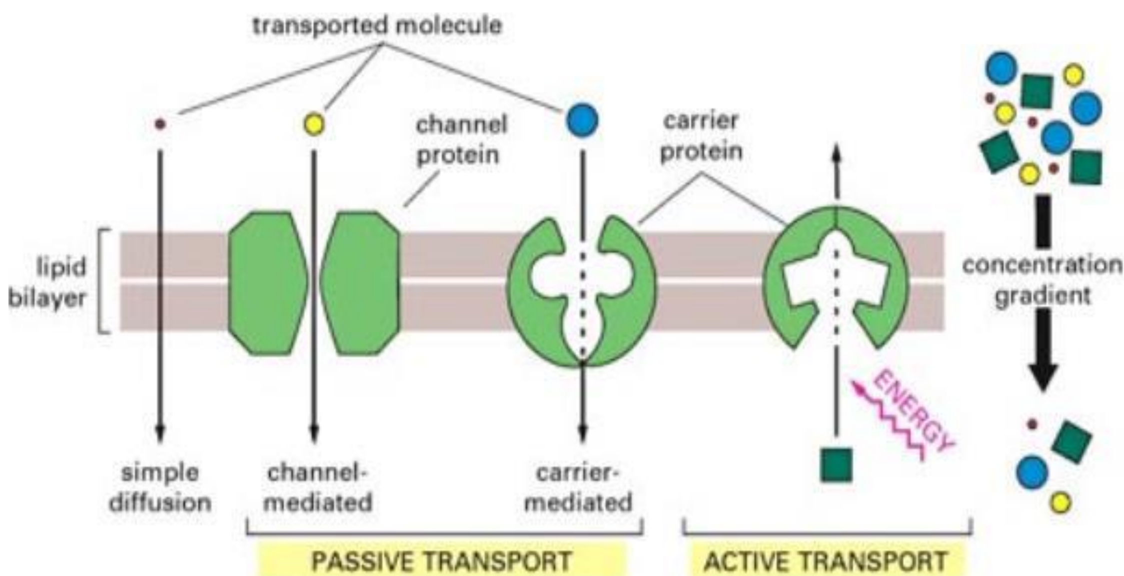

Fig. 4 Diagrama donde se observa la diferencia entre transporte activo y pasivo a través de la membrana. (Tomado de Alberts et al,2002)

En el primer caso, esto es la difusión simple, las moléculas atraviesan directamente la bicapa de fosfolípidos. Para ello, las moléculas tienen que ser pequeñas y no tener carga eléctrica (p.ej. oxígeno, dióxido de carbono y agua). Además, la cantidad de moléculas que pueden cruzar la membrana a través de este mecanismo es pequeña y la velocidad de difusión baja (Cooper, 2000). En el caso de la difusión facilitada, el transporte de moléculas e iones incluye moléculas más grandes y en mayores cantidades, y permite también el paso de cationes y aniones. Éste es, de hecho, el modo más importante de transporte transmembrana (Watson, 2015) (figura 5).

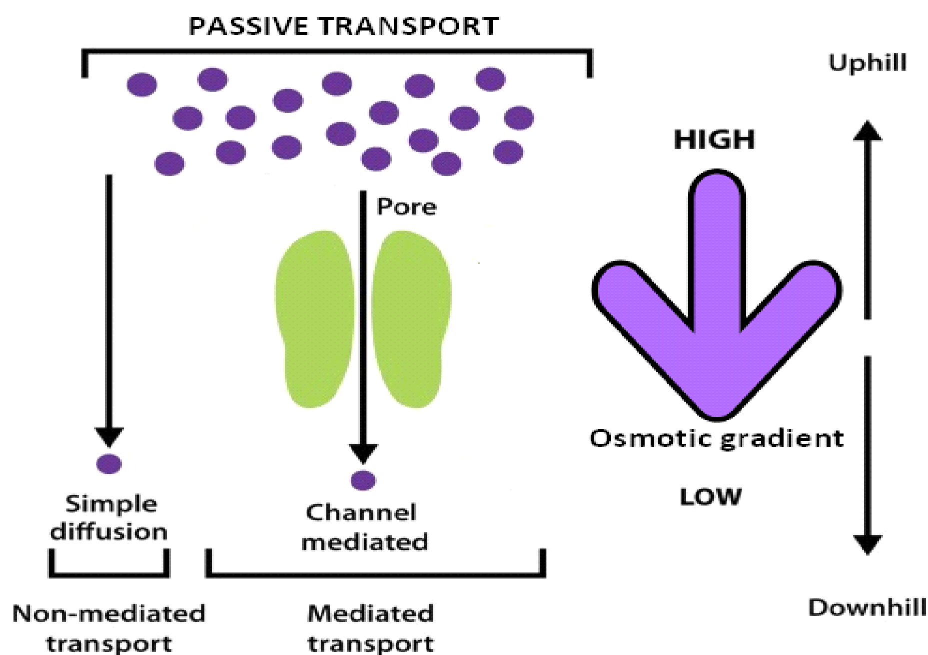

Fig. 5 Representación de los diferentes tipos de transporte pasivo (Modificado de Watson,2015)

5. Las Acuaporinas

Las acuaporinas (AQP), de las palabras latinas aqua = agua y porus = pasaje, son una familia de proteínas integrales transmembrana altamente conservadas que funcionan como canales de agua selectivos (Agre, Sasaki, & Chrispeels, 1993). Las acuaporinas facilitan el transporte de agua y, en algunos casos, ciertos pequeños

solutos no cargados, como el glicerol, la urea, el peróxido de hidrógeno y el arsenito (revisado por Yeste et al, 2017).

El transporte mediado por estas moléculas es pasivo, es decir, no requiere consumo de energía (ATP), y siempre ocurre a favor del gradiente de concentración que se genera entre ambos lados de la membrana (Pérez Di Giorgio et al., 2014). Son proteínas que tienen una estructura tetramérica (figura 6), compuesta por cuatro monómeros, lo que le confiere una mayor estabilidad y se caracterizan por la presencia en la parte central de un poro de paso cuyo tamaño es variable según el tipo de acuaporina.

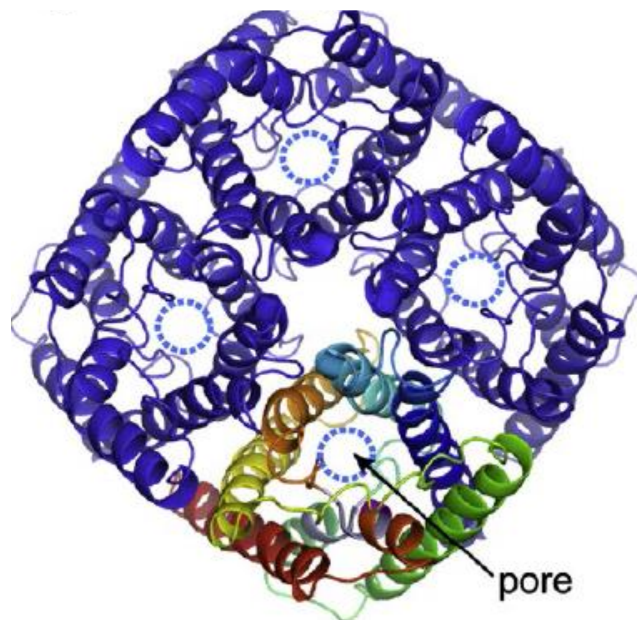

Fig. 6 Estructura tetramérica de las AQPs con cada uno de sus monómeros (tomado de Yeste et al., 2017)

Las AQPs fueron descubiertas por primera vez en 1992 por Peter Agre (figura 7) y sus colegas que, mientras intentaban purificar una proteína de 32 kilodaltons (kD) relacionada con la determinación del grupo sanguíneo Rh, aislaron un polipéptido de menor peso molecular (revisado por Yeste et al., 2017) que estaba involucrado en la regulación del volumen celular. Posteriormente, se investigaron e identificaron hasta 13 AQPs distintas en varios tejidos y órganos.

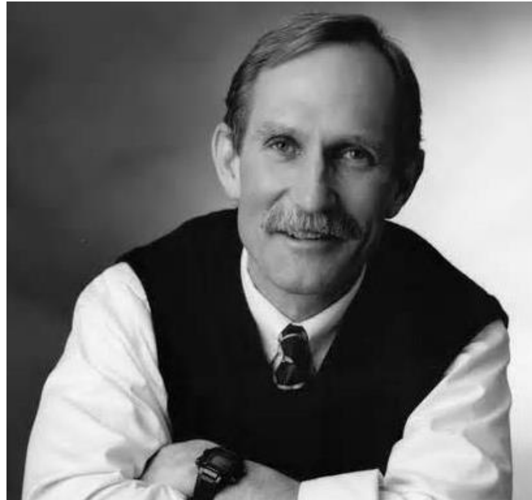

Fig. 7 El profesor Peter Agre, descubridor de las AQPs (tomado de Agre,2004)

5.1 Clasificación de las Acuaporinas

Las acuaporinas están presentes en los tres dominios en los que se clasifican los organismos vivos (Bacteria, Eukarya y Archaea), muestran una alta homología de secuencia y comparten similitudes funcionales y estructurales (revisado por Perez Di Giorgio et al., 2014). Hasta el momento, se han identificado 13 miembros de la familia de las AQPs en células de mamíferos y se clasifican según su similitud de secuencia y selectividad para el sustrato en tres grupos principales: AQP ortodoxas, acuagliceroporinas (AGP) y superacuaporinas (revisado por Yeste et al., 2017)

El primer grupo está compuesto por siete miembros: AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 y AQP8. Son canales selectivos del agua, porque son permeables al agua, pero no a los iones, ni a moléculas orgánicas e inorgánicas pequeñas (Huang et al., 2006).

El segundo grupo, también conocido como acuagliceroporinas (AGP), incluye cuatro miembros: AQP3, AQP7, AQP9 y AQP10, que no solo son permeables al agua sino también a glicerol, urea y otros pequeños electrolitos. Su diferencia con las AQP ortodoxas es el tamaño del poro. Así, mientras que el diámetro del poro es de 2.8 Å

en el caso de los AQP ortodoxas, en las AGP mide 3.4 Å (revisado por Sales, Lobo, Carvalho, Moura, & Rodrigues, 2013).

Finalmente, el tercer grupo, también conocido como superacuaporinas (superAQP), incluye las AQP11 y AQP12. Estas superAQP son permeables al agua, si bien se ha sugerido que la AQP11 también es un canal de glicerol en adipocitos humanos (Madeira et al., 2014). Se expresan dentro de la célula y se localizan específicamente en la membrana de los orgánulos celulares, en lugar de en la membrana plasmática. Las superAQP están involucradas en el transporte del agua intracelular, la regulación del volumen de los orgánulos y la homeostasis intravesicular (revisado por Yeste et al., 2017).

5.2 Las AQPs en el tracto reproductor

Las AQP están presentes en casi todos los tejidos y órganos de los mamíferos. En el caso del tracto genital, se encuentran en el aparato reproductor femenino y en el masculino (Figura 8), lo que indica que tienen un papel fundamental para le producción y función de los gametos (Huang et al., 2006; Thoroddsen et al., 2011).

En cuanto al aparato reproductor femenino, estas proteínas están implicadas en varios procesos fundamentales, como el flujo de agua dentro del útero (Jablonski, McConnell, Hughes, & Huet-Hudson, 2004), la dilatación cervical durante la gestación (Anderson, Brown, Mahendroo, & Reese, 2006), el transporte del oocito a lo largo del oviducto (Gannon, Warnes, Carati, & Verco, 2004), el desarrollo folicular, (McConnell et al., 2002), la formación del blastocisto (Watson, Natale, & Barcroft, 2004), la implantación del embrión en el útero (Richard et al., 2003) y la reabsorción del líquido amniótico (Huang et al., 2006). En un número elevado de casos, se ha determinado que la expresión de las AQPs en el tracto reproductor femenino está regulado por las gonadotropinas.

En el sistema reproductor masculino se ha demostrado que las AQP están presentes en la membrana plasmática de las células epiteliales secretoras de las vesículas seminales y la próstata (Huang et al., 2006). También son responsables

de la regulación del equilibrio osmótico y de la adaptación de los espermatozoides a los cambios de osmolaridad del entorno. Estos cambios son esenciales después de la deposición del semen en el tracto reproductor femenino, dado que éste tiene una osmolaridad inferior a la del aparato reproductor masculino (C. H. Yeung, Callies, Tüttelmann, Kliesch, & Cooper, 2010).

Aquaporin	Major tissue distribution
AQP0	Testis
AQP1	Vagina, ovary, oviduct, uterus, placenta, fetal membrane, embryo, testis, efferent ducts, epididymis, vas deferens, seminal vesicles and prostate
AQP2	Uterus, ovary, testis, efferent ducts, epididymis and vas deferens
AQP3	Uterus, cervix, ovary, placenta, fetal membrane, embryo, epididymis and prostate
AQP4	Uterus, cervix and ovary
AQP5	Ovary, uterus, cervix, oviduct, granulose cells, embryo and epididymis
AQP6	Embryo
AQP7	Ovary, embryo, testis, epididymis, spermatids, testicular and epididymal spermatozoa and ejaculated sperm
AQP8	Uterus, cervix, ovary, oviduct, placenta, fetal membranes, embryo, testis and epididymis
AQP9	Ovary, oviduct, uterus, granulose cells of follicles, placenta, fetal membrane, embryo, testis, efferent ducts, epididymis, vas deferens, prostate and coagulating gland
AQP10	Testis, efferent ducts and epididymis
AQP11	Testis
AQP12	N/A

N/A, data not available

Fig. 8 Localización de las acuaporinas en los tractos reproductores femenino y masculino (Huang et al., 2006; Sales et al., 2013)

5.3 Acuaporinas en espermatozoides de diferentes especies

Aunque una gran cantidad de estudios han descrito el posible papel de las AQPs en el tracto reproductivo masculino, la identificación, localización y función de estas proteínas en los espermatozoides ha sido menos estudiado, a pesar de su interés y sus posibles implicaciones. Así, mientras que AQP3, AQP7, AQP9 y AQP11 son las

más investigadas, las AQP2, AQP4, AQP5, AQP6, AQP10 y AQP12 no se han identificado en el espermatozoide de mamífero (resvisado por Yeste et al., 2017).

Entre esas investigaciones, las acuaporinas se han encontrado en espermatozoides de diversas especies, entre ellas: el hombre, el ratón, el jabalí, el toro, el perro e incluso en varias especies de peces (Domeniconi, Orsi, Justulin, Beu, & Felisbino, 2007; Moretti, Terzuoli, Mazzi, Iacoponi, & Collodel, 2012; Noelia Prieto-Martínez, Morató, Muiño, et al., 2017; Noelia Prieto-Martínez et al., 2016; C. H. Yeung & Cooper, 2010) (figura 9). Por otra parte, se ha observado que el tipo de AQP y su ubicación varían según la especie examinada; por esta razón, se puede afirmar que son proteínas cuya localización es especie-específica. Por ejemplo, en humanos y en ratón, se han identificado las AQP3, AQP7 y AQP11. Sin embargo, mientras que la AQP3 se encuentra en la cola espermática de ambas especies, la AQP7 se localiza en la cabeza y la cola del espermatozoide humano (C.-H. Yeung, Callies, Rojek, Nielsen, & Cooper, 2008) y la AQP 11 se halla en la cola del espermatozoide de ratón (C. H. Yeung & Cooper, 2010).

AQP	Localization	Species	Reference
AQP1	Not present in sperm, only reported in dogs	N/A	Curry et al. (1995); Ito et al. (2008); Liu et al. (1995)
AQP3	Sperm mid-piece	Human Mouse Pig Cattle	Chen et al. (2011); Laforenza et al. (2016); Prieto-Martínez et al. (2015); Prieto-Martínez, Morató et al. (2016)
AQP7	Elongated spermatids, testicular and epididymal sperm tail	Mouse	Yeung et al. (2009)
	Elongated spermatids, testicular and epididymal sperm tail	Rat	Calamita, Mazzone, Bizzoca et al. (2001); Ishibashi et al. (1997); Suzuki-Toyota et al. (1999)
	Spermatids, sperm head and tail	Human	Laforenza et al. (2016); Moretti et al. (2012); Saito et al. (2004); Yeung et al. (2010)
	Tail and cytoplasmic droplet of epididymal spermatozoa. Connecting, mid- and principal pieces of ejaculated spermatozoa	Pig	Prieto-Martínez, Vilagran et al. (2016); Vicente-Carrillo et al. (2016)
	Mid-piece	Cattle	Kasimanickam et al. (2017); Prieto-Martínez et al. (2015)
AQP8	Epididymal sperm tail	Mouse	Yeung et al. (2009)
	Primary spermatocytes and elongated spermatids	Rat	Calamita, Mazzone, Bizzoca et al. (2001)
	Mid-piece of ejaculated sperm	Human	Laforenza et al. (2016); Yeung et al. (2010)
AQP9	Caudal epididymal sperm and head of ejaculated sperm	Pig	Vicente-Carrillo et al. (2016)
	Not present	Human	Yeung et al. (2010)
AQP11	Elongated spermatids and ejaculated sperm tail	Rat Mouse	Yeung and Cooper (2010)
	Ejaculated sperm tail	Human Pig	Laforenza et al. (2016); Prieto-Martínez, Morató et al. (2016)

Fig. 9 Localización de las AQPs más abundantes en los espermatozoides de mamífero (tomado de Yeste et al., 2017)

En el cerdo (Prieto-Martínez, Morató, Vilagran, et al., 2017; Prieto-Martínez et al., 2016), se ha determinado que la AQP3 se encuentra en la pieza intermedia y el acrosoma, la AQP7 en la pieza de conexión y la AQP11 en la cabeza y en la cola del espermatozoide (Prieto-Martínez, Vilagran, Morató, et al., 2017) .

Asimismo, hay que destacar que la relación de dichas AQPs con la resistencia de los espermatozoides a la criopreservación difiere entre los espermatozoides porcinos y bovinos. Así, la AQP3 está relacionada con la criotolerancia del

espermatozoide porcino, la AQP7 es más abundante en los eyaculados de bovino buenos congeladores, y la AQP11 se asocia con la resistencia de los espermatozoides a la congelación en ambas especies (Prieto-Martínez, Vilagran, Morató, et al., 2017a; Prieto-Martínez, Morató, Vilagran, et al., 2017b; Prieto-Martínez et al., 2016).

OBJECTIVES

This thesis has three main objectives that seek to address the presence and function of AQP3, AQP7 and AQP11 in stallion spermatozoa. These objectives were:

1. To identify the presence of AQP3, AQP7 and AQP11 in stallion spermatozoa by immunoblotting and to investigate whether their relative content in good (GFE) and poor freezability ejaculates (PFE) differed before cryopreservation (Article 1).

2. To determine the localization of AQP3, AQP7 and AQP11 in stallion spermatozoa and to address whether changes in the localization of these three AQPs occurred in response to cryopreservation (Article 2).

3. To evaluate the relevance of each of these three AQPs during stallion sperm cryopreservation by using different concentrations three separate inhibitors (1, 2-propanediol, PDO; phloretin, PHL; and acetazolamide, AC). These inhibitors target either orthodox AQPs or aquaglyceroporins (GLPs) (Article 3).

Artículo 1

First evidence for the presence of aquaporins in stallion sperm.

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Reproduction in domestic animals

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1.

Figure. 1 Western blots showing the presence of AQP3 (a), AQP7 (b) and AQP11 (c) in stallion spermatozoa. α-Tubulin controls are shown below (d–f). Lane 1: Ladder. Lane 2: empty. Lanes 3, 5, and 7: GFE. Lanes 4 and 6: PFE

Figure. 2 Relative content of AQP3, AQP7 and AQP11 in good (GFE) and poor freezability ejaculates (PFE). (*) means significant ($p \leq .05$) differences between GFE and PFE

Table. 1 Sperm viability and total motility (mean ± SEM) in good (GFE) and poor freezability ejaculates (PFE) at 0 and 2 hr post-thaw

Artículo 2

Localization of AQP3, AQP7 and AQP11 in stallion spermatozoa and their changes in response to cryopreservation.

(Sebastian Bonilla-Correal).

Title

Localization of AQP3, AQP7 and AQP11 in stallion spermatozoa and their changes in response to cryopreservation.

Abstract

Background: Aquaporins (AQPs) are transmembrane proteins present in all cell types and species, and their involved in the transport of water and other solutes, such as glycerol and hydrogen peroxide. Aquaporins are divided into three groups: orthodox AQPs, aquaglyceroporins (GLPs) and superAQPs. Two GLPs, AQP3 and AQP7, and one superAQP, AQP11, have previously been found in the sperm of different species, such as horses, pigs, cattle, fish and humans. Cryopreservation damages the cell, affecting the cytoskeleton and plasma membrane, and other compartments, such as mitochondria. In addition, cryopreservation may also induce the translocation of some sperm proteins, which can alter the intracellular signalling pathways in which these proteins are integrated. Against this background, the aims of this work were: a) to evaluate the localization of AQP3, AQP7 and AQP11 in stallion spermatozoa; and b) to determine whether cryopreservation induces changes in the localization of these three AQPs.

Results: Localization of AQP3, AQP7 and AQP11 was evaluated by immunofluorescence in both fresh and frozen-thawed stallion spermatozoa. AQP3 was clearly present in the mid-piece of both fresh and frozen-thawed stallion spermatozoa. Whereas AQP7 was found in the mid-piece of fresh spermatozoa, freeze-thawing induced the relocalization of this protein to the main piece and post-acrosome region. Finally, while AQP11 was found in the main piece and post-acrosome region of fresh spermatozoa, the staining of the main piece was mainly lost following freeze-thawing and only a slight staining in the post-acrosome region was observed.

Conclusions: The present work has demonstrated for the first time that AQP3, AQP7 and AQP11 have a specific localization in stallion spermatozoa. In addition,

AQP7 and AQP11 were found to be translocated in response to cryopreservation. While this supports the relationship of these two AQPs with stallion sperm cryotolerance, more research is needed to address their particular role during cryopreservation.

Keywords: AQP3; AQP7; AQP11; cryopreservation; stallion; sperm

Introduction

Metabolite concentration strictly depends on plasma membrane permeability to water and solutes, which are crucial for cell function and survival [1]. Aquaporins (AQPs) are transmembrane proteins widely present in all cellular types and species, with a role as water channels and, some of them, also as facilitators of small solutes transport –such as glycerol and hydrogen peroxide [2]. To date, 13 different mammalian AQPs (AQP0-12) have been identified, which are classified into three different groups depending on their sequence similarity and solute permeability: orthodox AQPs, aquaglyceroporins (GLPs) and superaquaporins (superAQPs). AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 are orthodox AQPs, and are permeable to water. GLPs includes AQP3, AQP7, AQP9 and AQP10, which are permeable to water, glycerol, urea and other small electrolytes. Finally, the members of superAQPs group are specifically localised in the membrane of intracellular organelles, where they regulate organelle volume and intravesicular homeostasis through water and glycerol permeability and comprises AQP11 and AQP12 [2]. In spite of being ubiquitous proteins, their presence differs between species and cellular types. Concerning mammalian sperm cells, AQP3, AQP7 and AQP11 are present in stallion [3], boar [4,5], mouse [6–8], human [9,10] and cattle [11,12] sperm cells; AQP8 has been identified in mouse [7] and human [9,10] spermatozoa; and AQP9 in boar sperm cells [13]. AQPs in sperm cells have a key role in osmoregulation and volume regulation, which are crucial both in spermatogenesis and post-ejaculation events in the female tract.

Cryopreservation is the most efficient method for long-term storage of spermatozoa. Even so, it has collateral effects at both lethal and sub-lethal extents in stallion that cause a drastic impairment of sperm quality, and which are known as cryodamage. In fact, cryopreserved spermatozoa undergo a hyperosmotic insult during freezing and hypotonic stress upon thawing, and as a consequence these cells are subjected to drastic volume changes that injury cytoskeleton, mitochondria and cell membranes (reviewed in [14]). Among these structures, mitochondria are particularly sensitive to osmotic stress, and as a consequence of cryodamage they increase reactive oxygen species (ROS) production, thus becoming a major source of oxidative stress [15]. Concerning plasma membrane injuries, they alter membrane-associated and transmembrane proteins. As a consequence, intracellular signalling pathways to which these proteins are integrated become altered too, thus impairing sperm functions that may end up impairing fertility [16].

Although a previous study has demonstrated that AQP3, AQP7 and AQP11 are present in stallion spermatozoa, its localization has not been yet identified. Against this background, the present study aimed to evaluate the localization of these three AQPs in stallion spermatozoa. In addition, since relocalization of these AQPs has been found in response to the cryopreservation of boar and bull spermatozoa [11, 12, 24], the current study also investigated whether changes in the localization of AQP3, AQP7 and AQP11 also occur during stallion sperm cryopreservation.

Materials and Methods

Stallions and ejaculates

A total of 8 ejaculates from different stallions (n=8) were used in this study. Animals were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Spain), which is an EU-approved equine semen collection centre (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. Since all stallions used in this study were semen donors and were housed at the Equine Reproduction Service, the local ethics committee at our University indicated that no further ethics authorization was required.

Semen samples were collected using a Hannover-type artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line nylon mesh filter to separate the gel fraction. Gel-free semen was subsequently diluted 1:5 (v:v) in a Kenney extender [27], previously warmed at 37°C. Sperm concentration was assessed with a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), and each sample was split into two different fractions. The first one was used to assess the quality of fresh semen, whereas the other one was cryopreserved.

Sperm cryopreservation

The fraction intended for cryopreservation was centrifuged in a programmable refrigerated centrifuge (Medifriger BL-S; JP Selecta S.A., Barcelona) at 600 × *g* for 15 min at 20°C. After discarding the supernatants, pellets were resuspended in 2 mL of INRA-Freeze (INRA, Paris, France) commercial freezing extender prior to the evaluation of sperm concentration, motility and membrane integrity. After that, sperm concentration was adjusted to 2×10^6 viable spermatozoa per mL with INRA-Freeze medium. Samples were then packaged into 0.5-mL straws (Minitüb) and frozen using a controlled-rate freezer (Ice-Cube 14S-B; Minitüb), using the following cooling rates: i) -0.25°C/min from 20 to 5°C (60 min), ii) -4.75°C/min from 5°C to -90°C (20 min)

and iii) -11.11°C/min from -90°C to -120°C (2.7 min). Finally, straws were plunged into liquid nitrogen (-196°C) for storage.

Cryopreserved sperm quality was evaluated after thawing. With this purpose, two straws per ejaculate were immersed and agitated in a water bath at 37°C for 20 s. Thereafter, the content of these straws was pooled and diluted 1:3 (v:v) in pre-warmed Kenney medium. Diluted, frozen-thawed samples were incubated at 37°C for 1 h, and sperm quality was evaluated at two different time points: 10 min and 2 h.

Sperm motility

Sperm motility was evaluated in both fresh and frozen-thawed samples in all ejaculates through a computer-assisted sperm analysis (CASA) system, consisting of a phase contrast microscope (Olympus BX41; Olympus, Tokyo, Japan) equipped with a video camera and ISAS software (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). Whereas extended samples were directly examined, frozen-thawed samples were examined after 10 min or 2 h of incubation. For each sample and time point, three replicates of 1,000 spermatozoa per sample were evaluated in a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel), and observed under a negative phase-contrast field (Olympus 10× 0.30 PLAN objective, Olympus).

The following parameters were recorded for each motility assessment: total (TMOT, %) and progressive sperm motility (PMOT, %); curvilinear velocity (VCL, $\mu m \cdot s^{-1}$); straight line velocity (VSL, $\mu m \cdot s^{-1}$); average path velocity (VAP, $\mu m \cdot s^{-1}$); amplitude of lateral head displacement (ALH, μm); beat cross frequency (BCF, Hz); linearity (LIN, %), which was calculated assuming that LIN=VSL/VCL×100; straightness (STR, %), resulting from VSL/VAP×100; and motility parameter wobble (WOB, %), obtained from VAP/VCL×100. A sperm cell was considered to be motile when its VAP was higher than 15 μm/s, and progressively motile when its VAP was higher

than 70 µm/s. The corresponding mean ± standard error of the mean (SEM) was calculated for each parameter.

Sperm membrane integrity

Flow cytometry was used to assess eight different sperm quality parameters in both fresh and frozen-thawed semen samples: viability, membrane lipid disorder, early changes in sperm membrane permeability, mitochondrial membrane potential, intracellular calcium levels in sperm head and mid-piece, intracellular levels of superoxide (O_2^{-}•) radicals and intracellular levels of hydrogen peroxide (H_2O_2). All fluorochromes used were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). Before fluorochrome staining, samples were diluted to a final concentration of 1×10^6 sperm/mL with HEPES buffered saline solution (10 mmol/L HEPES, 150 mmol/L NaCl, 10 % BSA; pH=7.4), and after the addition of the corresponding fluorochromes they were incubated at 38°C in the dark. A total of three replicates per sample were assessed for each parameter.

All parameters were evaluated using a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, CA, USA). Samples were excited with an argon ion laser (488 nm) set at a power of 22 mW. Coulter principle for volume assessment allowed cell diameter/volume determination using the Cell Lab Quanta™ SC cytometer, which is based on measuring changes in electrical resistance produced in an electrolyte solution by suspended, non-conductive particles. In this system, forward scatter (FS) is replaced by electronic volume (EV). Furthermore, EV-channel calibration was performed using 10-µm Flow-Check fluorospheres (Beckman Coulter), by positioning this size of bead at channel 200 on the EV-scale.

A total of three optical filters were used: FL-1 (Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width: 505-545 nm), FL-2 (DRLP: 600 nm, BP filter: 575 nm, detection width: 560-590 nm) and FL-3 (LP filter: 670 nm/730 nm, detection width: 655-685 nm). FL-1 allowed detection of green fluorescence from SYBR-14, YO-PRO-1, JC-1 monomers ($JC\text{-}1_{mon}$), Fluo-3AM and 2',7'-dichlorofluorescein (DCF^+);

FL-2 was used to detect orange fluorescence from JC-1 aggregates ($JC\text{-}1_{agg}$); and FL-3 was used for red fluorescence detection from propidium iodide (PI), Merocyanine 540 (M540), Rhod-5N and ethidium (E^+). Signal was logarithmically amplified, and the adjustment of photomultiplier settings was performed according to particular staining methods.

Sheath flow rate was set at 4.17 µL/min and a minimum of 10,000 events were evaluated per replicate. For all particles, EV and side scatter (SS) were measured and linearly recorded. On the EV channel, the analyser threshold was adjusted to exclude subcellular debris (particle diameter <7 µm) and cell aggregates (particle diameter >12 µm). Therefore, on the basis of EV and SS distributions the sperm-specific events were positively gated, whereas the others were gated out.

Data obtained from flow cytometry were analysed using Flowing Software (Ver. 2.5.1; University of Turku, Finland) according to the recommendations of the International Society for Advancement of Cytometry (ISAC). Corresponding mean ± SEM was calculated for each parameter.

Sperm viability was evaluated through the assessment of plasma membrane integrity using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA), and the protocol of Garner and Johnson was followed [27]. Briefly, sperm were incubated with SYBR-14 (final concentration: 100 nmol/L) for 10 min, and PI was added (final concentration: 12 µmol/L) prior to an additional 5 min incubation. Three sperm populations were observed in flow cytometry dot plots: 1) viable, green-stained sperm ($SYBR\text{-}14^+/PI^-$); 2) non-viable, red-stained sperm ($SYBR\text{-}14^-/PI^+$); 3) non-viable, both red and green-stained sperm ($SYBR\text{-}14^+/PI^+$). The remaining dot plot population corresponded to unstained, non-sperm particles ($SYBR\text{-}14^-/PI^-$). Sperm viability assessment was assessed through green-stained, viable sperm, and SYBR-14 fluorescence spill over into FL-3 channel was compensated (2.45 %).

Immunofluorescence

Localization of AQP3, AQP7 and AQP11 in fresh and frozen-thawed spermatozoa was evaluated through immunofluorescence. Sperm samples were washed by dilution with phosphate buffered saline (PBS) 1× to a final concentration of $3×10^6$ cells per mL and centrifugation at 155 × *g* at room temperature for 5 min. Fixation of sperm cells was performed with paraformaldehyde (1.5 %; w:v) at room temperature for 30 min. After that, samples were centrifuged two times at 155 × *g* at room temperature for 5 min and were further resuspended with PBS 1×. Two drops per sample were placed in ethanol-rinsed slides, which were subsequently blocked and permeabilised with a blocking solution (TBS-Tween20 supplemented with 0.25 % [v:v] Triton X-100 and 3 % [w:v] BSA). After that, samples were incubated with anti-AQP3 (1:500; v:v), anti-AQP7 (1:500; v:v) and anti-AQP11 (1:100; v:v) rabbit polyclonal antibodies, which were diluted in blocking solution. This incubation was performed overnight in a humid chamber in the dark at 4°C. After that, five consecutive washes with PBS 1× for 5 min were performed. Slides were then incubated with an anti-rabbit secondary antibody conjugated with Alexa Fluor488 (Molecular Probes; 1:1,000) and diluted in blocking solution, at room temperature for 1 h. Slides were washed five times with PBS 1× for 5 min. A 10-µL drop of Vectashield mounting medium with 125 ng/mL of 4,6-diamidino-2-phenylindole (DAPI; Vectorlabs, Burlingame, CA, USA) was added prior to covering with a coverslip and sealing with nail varnish.

Samples were evaluated under a confocal laser-scanning microscope (CLSM, Nikon, A1R; Nikon Corp., Tokyo, Japan). Excitation at 405 nm was used to determine the localization of DAPI-stained nuclei, whereas AQP3, AQP7 and AQP11 were localised through excitation of samples at 496 nm. Negative controls were performed omitting incubations with primary antibodies.

The specificity of primary antibodies was confirmed through peptide competition assays with Aquaporin-3 (ref. ab195690; Abcam; Cambridge, UK), Aquaporin-7 (NBP1-30862PEP; Novus Biologicals, Littleton, CO, USA) and Aquaporin-11 (ref.

LS-E7981; LifeSpan BioSciences Inc., Seattle, WA, USA) immunising peptides, 20× in excess regarding their respective primary antibodies.

Results

Localization of AQP3, AQP7 and AQP11 was assessed by immunofluorescence in fresh and frozen-thawed stallion spermatozoa. The specificity of antibodies was confirmed through negative controls without primary antibody and peptide competition assays.

Regarding AQP3, it was clearly present in the mid-piece of both fresh and frozen-thawed stallion spermatozoa, whereas the principal piece was faintly stained. No changes in the localization of this AQP in response to cryopreservation (Fig. 1)

In fresh spermatozoa, AQP7-staining was clearly restricted to the mid-piece of fresh spermatozoa. However, this AQP relocalised following cryopreservation as AQP7-staining was mainly found in connecting and principal pieces, and in the post-acrosomal region.

Finally, AQP11 was localised in the principal piece and post-acrosomal region in fresh stallion spermatozoa. However, the staining in the principal piece disappeared in frozen-thawed spermatozoa and only a faint staining in the post-acrosomal region was observed. In addition, the intensity of AQP11-staining varied greatly between spermatozoa, both fresh and frozen-thawed.

Discussion

The results showed in this work confirm the presence of AQP3, AQP7 and AQP11 in stallion spermatozoa already identified in stallion spermatozoa (Bonilla-Correal et al., 2017). These proteins appear to crucial role for the regulation of sperm cell volume, which is vital during spermiogenesis and for the adaptation of spermatozoa to the female environment (Yeste et al., 2017). The most relevant results of this work, however, are the changes in the localization of AQP7 and AQP11 observed following cryopreservation. The main finding of this study are discussed into much detail in the following pages.

On the one hand, it is worth noting that, in fresh spermatozoa, all AQPs were found in the sperm tail, and only AQP (AQP11) was found in the post-acrosomal region. The localization of these three AQPs slightly differs from that reported in other species. As found herein for stallion spermatozoa, AQP3 is also present in the mid-piece of boar and bull spermatozoa (Prieto-Martínez et al., 2016a; 2016b). The localization of AQP7, which was found in the mid-piece, was similar to that of bull spermatozoa (Prieto-Martínez et al., 2016b), but differ from that of boar spermatozoa, in which this protein is restricted the connecting piece (Prieto-Martínez et al., 2015). Finally, AQP11 in stallion spermatozoa was found to be localised in the principal piece and post-acrosomal region, in a similar fashion that reported for boar and bull spermatozoa (Prieto-Martínez et al., 2015; Morató et al., 2017).

On the other hand, we found that cryopreservation induced changes in the localization of AQP7 and AQP11. Indeed, AQP7 relocalized from the mid-piece of fresh spermatozoa to connecting and principal pieces, and to the post-acrosomal region in frozen-thawed stallion spermatozoa. AQP11 also changed its localization in response to cryopreservation, as it was localised in the principal piece and post-acrosomal region in fresh stallion spermatozoa, and most of the staining of the principal piece disappeared in frozen-thawed spermatozoa and only a faint staining in the post-acrosomal region was observed. While relocalization of sperm proteins in response to freeze-thawing is not a rare event, the induced changes in the case

of AQPs differ across species. In effect, in pigs, relocalization of AQP7 from the connecting piece to the mid-piece and post-acrosomal region was identified in response to cryopreservation, whilst no differences between fresh and frozen-thawed sperm samples were observed for AQP3 and AQP11 (Prieto-Martínez et al., 2017). In contrast, we observed changes in the relocalization of AQP7 and AQP11, which does not occur in boar and bull spermatozoa (Prieto-Martínez *et al*., 2015; 2016a; 2016b). Based on our results, we cannot explain why such differences between species exist. However, relocalization of AQP7 and AQP11 could be explained by changes in plasma membrane domains due to the reduced membrane integrity of frozen-thawed stallion spermatozoa. In order for these results to be confirmed, furher research evaluated the localization of AQP3 and AQP11 in fresh and frozen-thawed spermatozoa should be conducted using immunogold and transmission electron microscopy. In any case, cryopreservation is the most useful technology to preserve mammalian spermatozoa for long-term (Yeste, 2016). The main inconvenience with this technique is the decreased fertilizing ability associated to the reduced post-thaw sperm survival, which is, in turn, caused by the high susceptibility of boar sperm to cold shock (Gilmore *et al*., 1996). The changes in the localization of AQP7 and AQP11 could be related to the function of these proteins during cryopreservation, in support to what has been previously reported in horse (Bonilla-Correal et al., 2017), pigs (Prieto-Martínez et al., 2017) and cattle (Prieto-Martínez et al., 2016; Morató et al., 2017). The fact that specific proteins are related to the cryotolerance of stallion spermatozoa is not new, as not only AQPs (Prieto-Martínez et al., 2016; Morató et al., 2017; Prieto-Martínez et al., 2017) but also heat-shock protein 90 (HSP90AA1), acrosin-binding protein (ACRBP), triosephosphate isomerase (TPI), and voltage-dependent anion channel 2 (VDAC2; Casas *et al*., 2009; Casas *et al*., 2010; Vilagran *et al*., 2013; 2014) have been identified as sperm freezability markers. In the case of AQPs, these proteins are involved in the regulation of transport of water, and in some case other solutes such as glycerol, across plasma membrane, and allow modification of sperm volume in response to osmotic changes (Edashige *et al.,* 2006; Kumar *et al*., 2015). Therefore, in addition to the fact that differences between GFE and PFE exist in terms of the relative

content of AQP3, AQP7 and AQP11, the data provided herein supports that the changes in the localization of AQP7 and AQP11 could also be involved in that response.

In conclusion, the present work has shown for the first time that AQP3, AQP7 and AQP11 have distinct localization in stallion spermatozoa, and change in response to cryopreservation. While AQP3 is present in the mid-piece of both fresh and frozen-thawed stallion spermatozoa and its localization does not change in response to cryopreservation, AQP7 is localized in the mid-piece of fresh spermatozoa, but translocates to the connecting and principal pieces, and to the post-acrosomal region following cryopreservation. Finally, AQP11 is found in the principal piece and post-acrosomal region in fresh stallion spermatozoa, but it is mostly lost after cryopreservation. While changes in the localization of AQP7 and AQP11 occur in response to cryopreservation, further research using immunogold and electron transmission microscope are needed to explain why the different relocalization differs from that observed in boar and bull spermatozoa.

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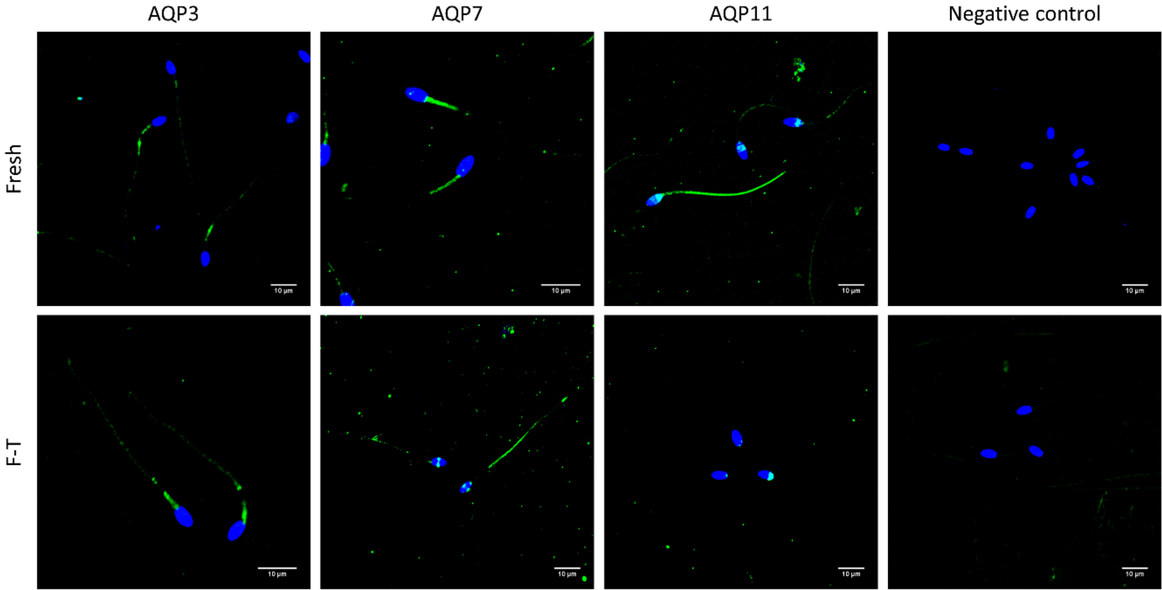
Figure legends

Figure 1 Localization of AQP3, AQP7 and AQP11 in fresh and frozen-thawed (F-T) spermatozoa.

Figure 1.



Artículo 3

Cryotolerance of stallion spermatozoa relies on aquaglyceroproteins rather than orthodox aquaporins.

(Sebastian Bonilla-Correal).

Title

Cryotolerance of stallion spermatozoa relies on aquaglyceroproteins rather than orthodox aquaporins

Abstract

Background: Aquaporins (AQPs), a family of ubiquitous water channels that are divided into orthodox AQPs, aquaglyceroporins (GLPs) and superAQPs. AQP3, AQP7 and AQP11, have a crucial role for sperm maturation and osmoregulation and are present in stallion spermatozoa. The exchange of water and solutes is crucial during cryopreservation, which is the most efficient method for long-term storage of stallion spermatozoa. However, freezing and thawing have a detrimental impact on sperm structure and function and this reduces their fertilising ability. Therefore, the aim of this study was to elucidate the functional relevance of each group of AQPs in stallion sperm cryopreservation through the use of three different inhibitors: acetazolamide (AC), phloretin (PHL) and propanediol (PDO).

Results: Different effects on stallion sperm cryotolerance were observed in response to the inhibition of each AQP group. When orthodox AQPs were inhibited with AC, alterations in different sperm quality and functionality parameters were observed ($P<0.05$), but not in a concentration- or time-depending manner. PHL decreased sperm motility, viability, acrosome integrity, and percentages of spermatozoa with low membrane lipid disorder, high mitochondrial membrane potential (MMP), and high intracellular levels of calcium and superoxides ($P<0.05$), but had no effect on the intracellular levels of peroxides. Finally, sperm motility, viability, acrosome integrity, percentages of spermatozoa with low membrane lipid disorder, high MMP and high intracellular calcium levels were higher ($P<0.05$) in PDO treatments than in the control.

Conclusions: The sperm response to AC, PHL and PDO indicates that GLPs rather than orthodox AQPs play a crucial role during stallion sperm cryopreservation. Furthermore, post-thaw sperm quality was higher in PDO treatments than in the

control, suggesting that this molecule could be added as a permeable cryoprotectant.

Keywords: Acetazolamide; Aquaporins; Phloretin; Propanediol; Sperm; Stallion

1. Introduction

The permeability of plasma membrane to water and solutes is crucial for proper cell function and homeostasis. Because of the amphipathic nature of the plasma membrane, simple diffusion of water molecules does not occur at high rates [1]. Aquaporins (AQPs) are a family of ubiquitous transmembrane proteins that allow the facilitated diffusion of water. Some AQPs are also permeable to small solutes [2]. To date, 13 AQPs (AQP0-AQP12) have been identified in mammalian cells and have been classified into three different groups (orthodox AQPs, aquaglyceroporins (GLPs) and superAQPs), which differ on their sequence and solute permeability. Regarding orthodox AQPs, this group includes AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8, which are exclusively permeable to water. GLPs are permeable to water, glycerol, urea and other small electrolytes; this group comprises AQP3, AQP7, AQP9 and AQP10. Finally, AQP11 and AQP12 are members of the superAQPs group, are localised in the membranes of intracellular organelles and are involved in the transport of water and glycerol. Even if ubiquitous, the presence of AQPs varies between cell types and species. In mammalian spermatozoa, AQP3, AQP7 and AQP11 have been identified in equine [3], porcine [4,5], murine [6–8], human [9,10] and bovine species [11,12]; AQP8 has been found in mouse [7] and human spermatozoa [9,10]; and AQP9 is present in boar spermatozoa [13]. In the male gamete, AQPs are involved in the regulation of cell volume and osmotic balance, which are crucial for spermatogenesis [14] and post-ejaculation events, including the activation of sperm motility upon ejaculation and the sperm adaptation to the female environment (reviewed in [2]).

Cryopreservation, which is the most efficient method for the long-term storage of stallion spermatozoa, causes a drastic impairment in sperm quality at both lethal and

sub-lethal levels. The hyperosmotic shock during freezing followed by a hypotonic stress during thawing induce a dramatic modification of the sperm cell volume that injuries cytoskeleton, mitochondria and plasma membrane (reviewed in [15]). Mitochondria are the most sensitive organelle to osmotic stress and, when damaged, they are a major source of oxidative stress through generation of reactive oxygen species (ROS) [16]. Moreover, alterations in the plasma membrane affect embedded and membrane-associated proteins. This, in turn, affects specific signalling pathways and may detrimentally impact sperm fertilising ability [17].

It is worth mentioning that there is a high variability between and within stallions in the ability of their ejaculates to withstand cryopreservation, i.e. cryotolerance or freezability. This inter- and intra-individual has also been described in other mammalian species [18–20]. Good (GFE) and poor freezability ejaculates (PFE) differ in their post-thaw sperm quality and function parameters, such as sperm membrane integrity, motility [21], ROS production and mitochondrial membrane potential (MMP) [22], which may ultimately affect the fertilising capacity [23].

During the last decade, the efforts to improve the efficiency of sperm cryopreservation protocols have been focused on the use of alternative cryoprotectant agents (CPA) and antioxidants to reduce osmotic and oxidative stresses, respectively (reviewed in [15]). In addition, the presence of damaged and non-viable sperm cells after thawing, which release factors that have deleterious effects on viable spermatozoa, has evidenced the importance of selecting stallion spermatozoa after cryopreservation [24]. In spite of these advances, increasing cryopreservation efficiency in stallion spermatozoa still needs further optimisation through the prediction of sperm cryotolerance (i.e. identification of GFE). In this context, AQPs are potential freezability biomarkers, since their permeability to water and small molecules are crucial for the sperm response to osmotic stress. In fact, the AQP involvement in sperm cryopreservation has previously been confirmed in bull [11,12], boar [25] and stallion spermatozoa ([3] Bonilla-Correal et al., 2017). In effect, AQP3 and AQP7 are related to the cryotolerance of boar spermatozoa [26],

AQP7 [4] and AQP11 [12] are associated with that of bull spermatozoa, and AQP3, AQP7 and AQP11 are related to stallion sperm freezability ([3] Bonilla-Correal et al., 2017). As one may assume that the inhibition of these AQPs may affect the sperm ability to withstand cryopreservation, the present study aimed to elucidate the functional relevance of orthodox AQPs and GLPs during stallion sperm cryopreservation by using three separate inhibitors (AC, PHL and PDO).

2. Methods

2.1. Stallions and ejaculates

A total of 12 ejaculates coming from different stallions (n=12) were used. Animals were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Spain), which is an EU-approved equine semen collection centre (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. Since all stallions used in this study were semen donors and were housed at the Equine Reproduction Service, the local ethics committee at our University indicated that no further ethics authorization was required.

Semen samples were collected using a Hannover-type artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line nylon mesh filter to separate the gel fraction. Gel-free semen was subsequently diluted 1:5 (v:v) in a Kenney extender [27], previously warmed at 37°C. Sperm concentration was assessed with a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), and each sample was split into two different fractions. The first one was used to assess the quality of fresh semen, whereas the other was divided into four different sub-fractions that were cryopreserved in the presence or absence of different concentrations of the three AQP-inhibitors.

2.2. AQP inhibitors

Prior to cryopreservation, three AQP inhibitors were added to semen samples: 1,3-propanediol (PDO, Sigma-Aldrich, St. Louis, MO, USA), acetazolamide (AC, Sigma-Aldrich), and phloretin (PHL, Sigma-Aldrich). PDO was diluted in the commercial freezing extender used for cryopreservation (see next section) to a working concentration of 100 mmol/L; AC was diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to a working concentration of 450 mmol/L, and PHL was diluted in methanol (Fisher Chemical, ThermoFisher Scientific; Waltham, Massachusetts, USA) to a working concentration of 365 mmol/L. Each inhibitor was assayed at the following concentrations: 0.1, 1 and 10 mmol/L for PDO; 250, 500 and 1000 µmol/L for AC; and 350 and 800 µmol/L for PHL.

2.3. Stallion sperm cryopreservation

Cryopreservation of stallion spermatozoa was performed in order to assess how each AQP affected sperm cryotolerance. The fraction of the ejaculate intended for cryopreservation was centrifuged in a programmable centrifuge (Medifriger BL-S; JP Selecta S.A., Barcelona) at 600 × *g* and 20°C for 15 min, and the supernatants were discarded. Pellets were subsequently resuspended in 2 mL of INRA-Freeze commercial extender (INRA, Paris, France), and sperm concentration, motility and membrane integrity were evaluated for subsequent adjustment to a final concentration of 2×10^6 viable spermatozoa per mL. After that, samples were packaged into 0.5-mL straws (Minitüb) prior to freezing in a controlled-rate freezer (Ice-Cube 14S-B; Minitüb), using the following cooling rates: i) -0.25°C/min from 20 to 5°C (60 min), ii) -4.75°C/min from 5°C to -90°C (20 min) and iii) -11.11°C/min from -90°C to -120°C (2.7 min). Finally, straws were plunged into liquid nitrogen (-196°C) for storage.

Frozen-thawed sperm quality was evaluated after thawing. Two straws per ejaculate were thawed at 37°C by immersion in a water bath for 20 s. The content of these straws was then diluted 1:3 (v:v) in pre-warmed Kenney medium. After that, samples were incubated at 37°C for 2 h, and sperm quality was assessed twice: at 10 min (0 h) and 2 h post-thaw.

2.4. Sperm motility

Sperm motility was evaluated before and after freeze-thawing through a computer-assisted sperm analysis (CASA) system, consisting of a phase-contrast microscope (Olympus BX41; Olympus, Tokyo, Japan) equipped with a video camera and ISAS software (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). The assessment of sperm motility in extended samples was performed after 15 min of incubation at 37ºC; frozen-thawed samples were evaluated after 10 min (0 h) and 2 h of thawing. Three replicates of 1,000 spermatozoa per sample and time point were evaluated using a pre-warmed (at 37°C) Makler counting chamber (Sefi-Medical

Instruments, Haifa, Israel), and observed under a negative phase-contrast field (Olympus 10× 0.30 PLAN objective, Olympus).

For each motility assessment, the evaluation of the following parameters was performed: total (TMOT, %) and progressive sperm motility (PMOT, %); curvilinear velocity (VCL, $\mu m \cdot s^{-1}$); straight line velocity (VSL, $\mu m \cdot s^{-1}$); average path velocity (VAP, $\mu m \cdot s^{-1}$); amplitude of lateral head displacement (ALH, μm); beat cross frequency (BCF, Hz); linearity (LIN, %), which was calculated assuming that LIN=VSL/VCL×100; straightness (STR, %), resulting from VSL/VAP×100; and motility parameter wobble (WOB, %), obtained from VAP/VCL×100. A sperm cell was considered to be motile when its VAP was higher than 10 μm/s, and progressively motile when its STR was higher than 75%. For each parameter, the corresponding mean ± standard error of the mean (SEM) was calculated.

2.5. Flow cytometry analyses

Flow cytometry analyses were performed in order to evaluate different sperm quality parameters in both fresh and frozen-thawed sperm samples: viability, acrosome integrity, membrane lipid disorder, MMP, intracellular calcium levels, and intracellular levels of superoxides ($O_2^{-}\bullet$) and peroxides (H_2O_2). Samples were diluted to a final concentration of 1×10^6 sperm/mL with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution (10 mmol/L HEPES, 150 mmol/L NaCl, 10 % BSA; pH=7.4) prior to staining with the corresponding fluorochromes (ThermoFisher Scientific, Waltham, MA, USA). After that, samples were incubated at 38°C in the dark, and a total of three replicates per sample and parameter were evaluated.

Flow cytometry analyses were performed using a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, CA, USA), and samples were excited with an argon ion laser (488 nm) set at a power of 22 mW. Cell diameter/volume was determined using the Cell Lab Quanta™ SC cytometer, through the Coulter principle for volume assessment, which is based on the changes in electrical resistance

produced in an electrolyte solution by suspended, non-conductive particles. In this system, forward scatter (FS) is replaced by electronic volume (EV). The EV-channel was calibrated using 10-µm Flow-Check fluorospheres (Beckman Coulter) and this size of beads was positioned at channel 200 on the EV-scale.

Three different optical filters were used: FL1 (Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width: 505-545 nm), FL2 (DRLP: 600 nm, BP filter: 575 nm, detection width: 560-590 nm) and FL3 (LP filter: 670 nm/730 nm, detection width: 655-685 nm). Whereas FL1 allowed the detection of green fluorescence from SYBR-14, *Arachis hypogaea* lectin -peanut agglutinin- conjugated with fluorescein isothiocyanate (PNA-FITC), YO-PRO-1, JC1 monomers ($JC1_{mon}$), Fluo3 and 2',7'-dichlorofluorescein (DCF); FL2 was used to detect orange fluorescence from JC1 aggregates ($JC1_{agg}$); and FL3 allowed the detection of red fluorescence from propidium iodide (PI), Merocyanine 540 (M540), Rhod5 and ethidium (E). Signals were logarithmically amplified, and the adjustment of photomultiplier settings was performed according to particular staining methods.

Sheath flow rate was set at 4.17 $\mu L \cdot min^{-1}$, and EV and side scatter (SS) were measured and linearly recorded for all particles. The analyser threshold on the EV channel was adjusted to exclude subcellular debris (particle diameter <7 µm) and cell aggregates (particle diameter >12 µm), and the sperm-specific events were positively gated on the basis of EV/SS distributions.
Data obtained from flow cytometry evaluations were analysed with Flowing Software (Ver. 2.5.1; University of Turku, Finland), and the recommendations of the International Society for Advancement of Cytometry (ISAC) were adhered. Following Petrunkina *et al.* [28], the events appearing in the lower-left quadrant of all protocols except SYBR14/PI were corrected using the percentage of non-sperm debris particles of the SYBR14-/PI- population. The percentages of all the other sperm populations were recalculated. Finally, for each parameter the corresponding mean ± SEM was calculated.

2.5.1. Plasma membrane and acrosome integrity

Plasma membrane and acrosome integrity were evaluated through two different tests: SYBR14/PI and PNA-FITC/PI.

On the one hand, the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) was used following the protocol of Garner and Johnson [29]. In brief, sperm were incubated with SYBR14 at a final concentration of 100 nmol/L for 10 min, and PI was added at a final concentration of 12 μmol/L prior to an additional incubation of 5 min. Three different sperm populations were observed: 1) viable, green-stained spermatozoa ($SYBR14^+/PI^-$); 2) non-viable, red-stained spermatozoa ($SYBR14^-/PI^+$); 3) non-viable spermatozoa stained in both green and red ($SYBR14^+/PI^+$). The remaining population in the dot-plots, which corresponded to unstained, non-sperm particles ($SYBR14^-/PI^-$), was not included in the calculation of the final percentages of each sperm population ($SYBR14^+/PI^-$, $SYBR14^-/PI^+$, and $SYBR14^+/PI^+$). SYBR14 fluorescence spill over into FL3 channel was compensated (2.45 %).

In the second case, spermatozoa were co-stained with PNA-FITC and PI, following the procedure described by Nagy *et al.* [30] with minor modifications. Briefly, spermatozoa were incubated with PNA-FITC (final concentration: 2.5 μg/mL) and with PI (12 μmol/L) at 38ºC in the dark for 10 min. Flow cytometry dot-plots allowed the identification of four different populations: 1) spermatozoa with an intact plasma membrane ($PNA\text{-}FITC^-/PI^-$); 2) spermatozoa with a damaged plasma membrane ($PNA\text{-}FITC^+/PI^-$); 3) spermatozoa with a damaged plasma membrane and a partially altered outer acrosome membrane ($PNA\text{-}FITC^+/PI^+$); and 4) spermatozoa a with damaged plasma membrane and lost outer acrosome membrane ($PNA\text{-}FITC^-/PI^+$). Thereafter, spermatozoa were classified into two different categories: a) spermatozoa with an intact plasma membrane and acrosome (population 1; $PNA\text{-}FITC^-/PI^-$); and b) spermatozoa with acrosome and/or plasma membrane damage, which included populations 2, 3 and 4. Events appearing in the $PNA\text{-}FITC^-/PI^-$ quadrant were corrected using the percentages of non-sperm debris particles found in the $SYBR14^-/PI^-$ quadrant; the percentages of the other three populations were

recalculated. PNA-FITC fluorescence spill over into FL-3 channel was compensated (2.45 %).

2.5.2. Sperm membrane lipid disorder

The evaluation of sperm membrane lipid disorder was performed according the protocol from Rathi *et al.* [31] with minor modifications [32], using M540 and YO-PRO-1. M540 detects the decrease in packing order of phospholipids in the outer monolayer of the plasma membrane(INRA, Paris, France) j. Samples were incubated with M540 (final concentration: 2.6 µmol/L) and YO-PRO-1 (final concentration: 25 nmol/L) at 38ºC in the dark for 10 min. Four populations were observed in flow cytometry dot-plots: 1) non-viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$); 2) non-viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$); 3) viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$); and 4) viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$). Percentages of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) were corrected using proportions of non-sperm debris particles found in the $SYBR\text{-}14^-/PI^-$ quadrant; the percentages of the other three populations were recalculated. Data were not compensated.

2.5.3. Mitochondrial membrane potential (MMP)

JC1-staining was used for the assessment of MMP, following the protocol of Ortega-Ferrusola *et al.* [33] with minor modifications. In brief, samples were incubated in the presence of JC1 at a final concentration of 0.3 µmol/L at 38ºC in the dark for 30 min. JC1 molecules form aggregates ($JC1_{agg}$) in the presence of high MMP, whereas remains as monomers in the presence of low MMP ($JC1_{mon}$). Three different populations were identified in flow cytometry dot-plots: 1) spermatozoa with low MMP ($JC1_{mon}$; $FL1^+/FL2^-$); 2) spermatozoa with high MMP ($JC1_{agg}$; $FL1^-/FL2^+$; and 3) spermatozoa with heterogeneous mitochondria ($JC1_{agg}$ and $JC1_{mon}$; $FL1^+/FL2^+$) in the same cell. Percentages of double-negative particles ($FL1^-/FL2^-$) were corrected using the proportions of $SYBR14^-/PI^-$; the percentages of the other populations were

recalculated. Spermatozoa with high MMP resulted from the sum of populations 2) and 3). FL1 spill-over into the FL2 channel was compensated (70.29%).

2.5.4. Intracellular calcium levels

For the evaluation of intracellular calcium levels, two different co-staining tests were performed: Fluo3-AM/PI and Rhod5-N/YO-PRO-1.

In the first test, intracellular calcium levels were evaluated with Fluo3-AM, which penetrates cell membranes and has more affinity for the calcium residing in the sperm mid-piece [34]. This test was performed according to the protocol of Harrison *et al.* [35] modified by Kadirvel *et al.* [36]. In brief, spermatozoa were incubated in the presence of 1 µmol/L of Fluo3-AM and 12 µmol/L of PI at 38ºC in the dark for 10 min. Four different sperm populations were identified in dot-plots: 1) non-viable spermatozoa with low intracellular calcium levels ($Fluo3^-/PI^+$); 2) non-viable spermatozoa with high intracellular calcium levels ($Fluo3^+/PI^+$); 3) viable spermatozoa with low intracellular calcium levels ($Fluo3^-/PI^-$); and 4) viable spermatozoa with high intracellular calcium levels ($Fluo3^+/PI^-$). Percentages of viable spermatozoa with low intracellular calcium levels ($Fluo3^-/PI^-$) were corrected using proportions of non-sperm debris particles found in the $SYBR14^-/PI^-$ quadrant; the percentages of the other three populations were recalculated. Spill over of PI into the FL1-channel and Fluo3 spill over the FL3-channel were compensated (28.72 % and 2.45 %, respectively).

Intracellular calcium levels were also assessed through Rhod5-N staining following the protocol described by Yeste *et al.* [34], in which Rhod5-N was found to have more affinity for the calcium residing in the sperm head. Briefly, samples were incubated with Rhod5-N at a final concentration of 5 µmol/L, and YO-PRO-1 at a final concentration of 25 nmol/L at 38ºC in the dark for 10 min. Four different populations were identified in flow cytometry dot-plots: 1) non-viable spermatozoa with low levels of intracellular calcium ($Rhod5^-$/YO-PRO-1^+); 2) non-viable spermatozoa with high levels of intracellular calcium ($Rhod5^+$/ YO-PRO-1^+); 3) viable spermatozoa with low levels of intracellular calcium ($Rhod5^-$/YO-PRO-1^-); and 4)

viable spermatozoa with high levels of intracellular calcium ($Rhod5^+$/ YO-PRO-1^-). Percentages of viable spermatozoa with low intracellular calcium levels ($Rhod5N^-$/YO-PRO-1^-) were corrected using the percentages of non-sperm debris particles found in the $SYBR14^-$/PI^- quadrant; the percentages of the other three populations were recalculated. Rhod5 spill over into the FL1-channel was compensated (3.16 %).

2.5.5. Intracellular superoxide levels ($O_2^{-\bullet}$)

Evaluation of intracellular superoxide ($O_2^{-\bullet}$) radical levels was performed following a modification of the protocol from Guthrie & Welch [37], through hydroethidine (HE) and YO-PRO-1 co-staining. HE is able to penetrate the sperm plasma membrane, and it is oxidised by $O_2^{-\bullet}$ to ethidium (E^+) and other products at the intracellular environment. In brief, samples were incubated with HE (final concentration: 4 µmol/L) and YO-PRO-1 (final concentration: 40 nmol/L) for 20 min. Four different sperm populations were identified in flow cytometry dot-plots: 1) non-viable spermatozoa with low superoxide levels (E^-/YO-PRO-1^+); 2) non-viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^+); 3) viable spermatozoa with low superoxide levels (E^-/YO-PRO-1^-); and 4) viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-). Percentages of viable spermatozoa with low superoxide levels (E^-/YO-PRO-1^-) were corrected using the percentages of non-sperm debris particles found in the $SYBR14^-$/PI^- quadrant; the percentages of the other three populations were recalculated. YO-PRO-1 spill over into the FL3-channel was compensated (5.06 %).

2.5.6. Intracellular hydrogen peroxide levels (H_2O_2)

Determination of intracellular levels of hydrogen peroxide (H_2O_2) through co-staining with 2’,7’-dichlorodihydrofluorescein diacetate (H_2DCFDA) and PI fluorochromes was performed following the protocol from Guthrie & Welch [37] with minor modifications. H_2DCFDA is a non-fluorescent probe that penetrates the sperm plasma membrane and is intracellularly de-esterified and converted into highly fluorescent, 2’,7’-dichlorofluorescein (DCF^+) upon oxidation. In brief, samples were

incubated with H_2DCFDA (final concentration: 200 μmol/L) and PI (final concentration: 12 μmol/L) for 30 min. Four different populations were identified in flow cytometry dot-plots: 1) viable spermatozoa with high peroxide levels (DCF^+/PI^-); 2) non-viable spermatozoa with high peroxide levels (DCF^+/PI^+); 3) viable spermatozoa with low peroxide levels (DCF^-/PI^-); and 4) non-viable spermatozoa with low peroxide levels (DCF^-/PI^+). Percentages of viable spermatozoa with low peroxide levels (DCF^-/PI^-) were corrected using the percentages of non-sperm debris particles found in the $SYBR14^-/PI^-$ quadrant; the percentages of the other three populations were recalculated. DCF-spill over into FL3-channel was compensated (2.45 %).

2.6. Statistical analyses

All data were analysed using a statistical package (IBM SPSS Statistics 25.0; Armonk, New York, USA). Data were first tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). Following this, the effects of each inhibitor and cryopreservation step (i.e. fresh, frozen-thawed at 0 h, frozen-thawed at 2 h) were tested through a mixed model followed by the post-hoc Sidak test for pair-wise comparisons. The intra-subjects factor (i.e. repeated measures) was the cryopreservation step (i.e. fresh, frozen-thawed at 0 h, frozen-thawed at 2 h) and the inter-subjects factor was the treatment (C, and different concentrations of PDO, AC or PHL). The level of significance was set at $P \leq 0.05$ and data are shown as mean ± SEM.

3. Results

As previously mentioned, sperm quality and functioy parameters were evaluated in both fresh and frozen-thawed samples in order to determine the effects of AQP inhibition during sperm cryopreservation. Regarding fresh samples, the absence of differences between controls and treated samples in any sperm parameter was due to the fact that inhibitors were added immediately before cryopreservation (P>0.05; Table 1).

3.1. Sperm motility

Total and progressive motilities of frozen-thawed spermatozoa are shown in Fig. 1. Percentages of total motile spermatozoa were significantly (P<0.05) higher in PDO treatments than in the control at both 0 h and 2 h post-thaw (Fig. 1A). Whereas the two PHL concentrations caused a significant (P<0.05) decrease in total motility at both post-thaw time points, the presence of AC did not cause any significant effect (P>0.05).

Progressive sperm motility (Fig. 1B) significantly (P<0.05) increased in the presence of both AC and PDO immediately after thawing, whereas PHL did not have any significant effect (P>0.05). Two hours after thawing, there were no significant differences between controls and samples treated with either AC or the lowest concentration of PDO (P>0.05). In contrast, progressive sperm motility in the treatments containing 1 and 10 mmol/L PDO was significantly (P<0.05) higher than in the control. Moreover, progressive sperm motility in the treatments containing PHL was significantly (P<0.05) lower than in the control.

3.2. Sperm viability: SYBR14/PI test

Percentages of viable spermatozoa ($SYBR14^{+}/PI^{-}$) did not differ between AC treatments and the control immediately after thawing (0 h), but the presence of this inhibitor induced a significant (P<0.05) decrease at 2 h post-thaw (Fig. 2A). Whereas the percentages of viable spermatozoa in PHL treatments were significantly (P<0.05) lower than in the control at 0 h and 2 h post-thaw, all PDO treatments

showed significantly ($P<0.05$) higher percentages of viable spermatozoa than the control.

3.3. *Acrosome integrity: PNA-FITC/PI test*

The PNA-FITC/PI test was carried out to determine the integrity of both acrosome and plasma membranes, and PNA-FITC$^-$/PI$^-$ spermatozoa were those having an intact acrosome and plasma membrane (Fig. 2B). Samples treated with AC did not show significant differences with the control at any concentration immediately after thawing ($P>0.05$), but there was a significant ($P<0.05$) decrease in the percentage of spermatozoa that presented an intact plasma and acrosome membrane in the samples treated with the lowest concentrations of AC at 2 h post-thaw. Percentages of spermatozoa with an intact plasma and acrosome membrane were significantly ($P<0.05$) lower in PHL treatments than in the control at both 0 h and 2 h post-thaw. In contrast, all treatments containing PDO showed significantly ($P<0.05$) higher percentages of spermatozoa with an intact plasma and acrosome membrane at both 0 h and 2 h post-thaw.

3.4. *Membrane lipid disorder: M540/YO-PRO-1 test*

The M540/YO-PRO-1 test allowed the evaluation of sperm membrane lipid disorder, the population of M540$^-$/YO-PRO-1$^-$ spermatozoa corresponding to those viable cells with low membrane lipid disorder (Fig. 3A). Whereas right after thawing, samples treated with 1000 µmol/L AC showed significantly ($P<0.05$) lower percentages of viable spermatozoa with low membrane lipid disorder than the control, the treatments containing 250 and 500 µmol/L AC also showed reduced ($P<0.05$) percentages of viable spermatozoa with low membrane lipid disorder than the control at 2 h post-thaw. Percentages of viable spermatozoa with low membrane lipid disorder at the highest PHL concentration were significantly ($P<0.05$) lower than the control immediately after thawing. At 2 h post-thaw, both 350 µmol/L and 800 µmol/L concentrations showed significantly ($P<0.05$) lower percentages of viable spermatozoa with low membrane lipid disorder than the control. Finally, percentages

of viable spermatozoa with low membrane lipid disorder were significantly (*P*<0.05) higher than the control in all PDO treatments, both at 0 h and 2 h post-thaw.

3.5. Mitochondrial membrane potential (MMP): JC1 test

Percentages of spermatozoa with high MMP were significantly (*P*<0.05) lower in the treatment containing AC at 1000 μmol/L than in the control immediately after thawing, and in the treatment containing AC at 250 μmol/L at 2 h post-thaw (Fig. 3B). When treated with PHL, samples showed a decrease in MMP at any time point after thaw (*P*<0.05). In contrast, percentages of spermatozoa with high MMP in the treatment containing 1 mmol/L PDO were significantly (*P*<0.05) higher than in the control both at 0 h and 2 h post-thaw.

3.6. Intracellular calcium of spermatozoa: Fluo3/PI test

Intracellular calcium levels were assessed through the Fluo3/PI test, and the population of Fluo3 $^{+}$/PI^{-} spermatozoa corresponded to viable cells with high intracellular calcium levels (Fig. 4A). Whereas, compared to the control, samples treated with any concentration of AC showed a significant (*P*<0.05) decrease in the percentage of viable spermatozoa with high levels of intracellular calcium immediately after thawing, no significant (*P*>0.05) differences between AC treatments and the control were found after 2 h of thawing. Treatments containing any concentration of PHL showed significantly (*P*<0.05) lower percentages of spermatozoa with high MMP at both 0 h and 2 h post-thaw. In contrast, percentages of viable spermatozoa with high levels of intracellular calcium were significantly (*P*<0.05) higher in all PDO concentrations than in the control right after thawing (0 h), and were significantly (*P*<0.05) higher than the control at the two highest PDO concentrations after 2 h of thawing.

3.7. Intracellular calcium of spermatozoa: Rhod5/YO-PRO-1 test

The Rhod5/YO-PRO-1 test was also performed to determine intracellular calcium levels, and the population of Rhod5^{+}/YO-PRO-1^{-} spermatozoa corresponded to viable cells with high intracellular calcium levels (Fig. 4B). When AC was present, no

significant (P>0.05) differences with regard to the control were observed either at 0 h or 2 h post-thaw. The treatments containing PHL showed no significant differences compared to the control at 0 h post-thaw, but those containing the two lowest PHL concentrations showed significantly (P>0.05) lower percentages of viable spermatozoa with high intracellular calcium levels after 2 h of thawing. In contrast, percentages of viable spermatozoa with high intracellular calcium levels were significantly (P<0.05) higher in PDO treatments than in the control at 0 h but not at 2 h post-thaw.

3.8. Intracellular superoxide levels ($O_2^{-\bullet}$): HE/YO-PRO-1 test

Intracellular $O_2^{-\bullet}$ levels were evaluated through the HE/YO-PRO-1 test, in which the population of E^+/YO-PRO-1^- spermatozoa corresponded to viable cells with high intracellular levels of $O_2^{-\bullet}$. Percentages of viable spermatozoa with high intracellular levels of $O_2^{-\bullet}$ did not differ (P>0.05) between AC treatments and the control either at 0 h or at 2 h post-thaw. In contrast, the treatment containing 800 µmol/L PHL showed significantly (P<0.05) lower percentages of viable spermatozoa with high intracellular levels of $O_2^{-\bullet}$ both at 0 h and 2 h post-thaw (P<0.05). Concerning samples treated with PDO, the single change that was observed was a significant (P<0.05) increase in the percentages of viable spermatozoa with high intracellular levels of $O_2^{-\bullet}$ at 1 mmol/L immediately after thawing.

3.9. Intracellular peroxide levels (H_2O_2): H_2DCFDA /PI test

Intracellular H_2O_2 levels were assessed through the H_2DCFDA /PI test, and the DCF^+/PI^- sperm population corresponded to viable spermatozoa with high intracellular levels of peroxides. Percentages of viable spermatozoa with high intracellular levels of peroxides were significantly (P<0.05) higher than the control in the treatment with 1000 µmol/L AC after 2 h of thawing. Whereas percentages of viable spermatozoa with high intracellular levels of peroxides were significantly (P<0.05) higher in the 800 µmol/L PHL treatment than in the control at 0 h post-thaw, those percentages in the treatment containing 350 µmol/L PHL were significantly (P<0.05) higher than in the control after 2 h of thawing. Finally, the treatment

containing 1 mmol/L PDO showed significantly (*P*<0.05) lower percentages of viable spermatozoa with high intracellular levels of peroxides at 0 h post-thaw.

4. Discussion

While AQPs have been identified in sperm cells from different mammalian species in the last decade (reviewed in [38]), their precise function and mechanism of action are yet to be fully addressed. In the case of horses, AQP3, AQP7 and AQP11 have been identified in stallion spermatozoa (Bonilla-Correal et al., 2017; [3]). Taking this into account, this study aimed to unveil the relevance of each group of AQPs during stallion sperm cryopreservation. With this purpose, three different inhibitors were added at different concentrations that were chosen according to preliminary experiments conducted in our laboratory and previous studies (REF): acetazolamide (AC; 250, 500 and 1000 μmol/L), phloretin (PHL; 350 and 500 μmol/L) and 1,3-propanediol (PDO; 0.1, 1 and 10 mmol/L). It is worth mentioning that AC is known to inhibit AQP1 and AQP4 [39,40], PHL inhibits both AQP3 and AQP7 [41–43], and PDO has been proven to inhibit orthodox AQPs (AQP1, AQP2, AQP5 and AQP4) with high efficiency, and GLPs (the family to which AQP3, AQP7 and AQP9 belong) with low intensity [44,45]. The effects of each AQP-inhibitor on sperm function and survival after cryopreservation were assessed on the basis of sperm motility, sperm viability, acrosome integrity, membrane lipid disorder, MMP, intracellular calcium levels and intracellular levels of ROS.

Upon thawing, samples treated with AC showed significantly higher percentages of PMOT and significantly lower percentages of viable spermatozoa with high $Fluo3^{+}$ levels than the control. In addition, the highest AC concentration significantly reduced the percentages of viable spermatozoa with low membrane lipid disorder and those with high MMP. At 2 h post-thaw, the treatments containing AC exhibited reduced sperm viability in all treatments. In addition, the lowest AC concentrations also showed a reduction in the percentages of spermatozoa with low membrane lipid disorder and with high MMP, and the highest one (1000 μmol/L) exhibited an increased percentage of viable spermatozoa with high H_2O_2 levels. The second inhibitor, PHL, showed significantly lower percentages of TMOT, PMOT, viable and acrosome-intact spermatozoa, viable spermatozoa with low membrane lipid disorder, spermatozoa with high MMP, viable spermatozoa with high levels of

intracellular calcium (mainly Fluo3$^+$-spermatozoa) and viable spermatozoa with high levels of intracellular superoxides. In contrast, treatments containing PDO showed higher percentages of TMOT, PMOT, viable and acrosome-intact spermatozoa, viable spermatozoa with low membrane lipid disorder, spermatozoa with high MMP, and viable spermatozoa with high intracellular calcium levels.

Differences between the effects of these three inhibitors appear to result from their specificity for separate AQPs and from the collateral effects on other proteins that are present in the sperm cell. In fact, AQP1 and AQP4 have not been previously identified in mammalian spermatozoa (Reviewed in [2]), which supports the absence of consistent effects immediately after thawing in the presence of AC. Nevertheless, the presence of AC did alter some sperm function parameters, although some of these changes did not appear to depend on the concentration of the inhibitor or on the post-thaw incubation time. In this context, it is worth mentioning that not only does AC inhibit some AQPs but also carbonic anhydrase, whose function is to convert CO_2 and H_2O to bicarbonate and protons [46]. Therefore, inhibition of carbonic anhydrase through AC could decrease the concentration of intracellular bicarbonate. Bicarbonate, together with Ca^{2+}, is crucial for the activation of the soluble adenylate cyclase (sAC) which, in turn, produces cAMP that triggers protein kinase A (PKA). Protein kinase A activates different complexes of the electron transport chain, and the inhibition of PKA has been reported to reduce the electron flow passing through Complex I [47]. This mechanism could explain the reduction of the mitochondrial membrane potential observed in the presence of some AC concentrations. In addition, the decrease in the percentages of spermatozoa with intracellular calcium levels observed in the presence of AC could be related to the lack of activation of the CatSper calcium channel. CatSper channel is responsible for the increase of intracellular calcium levels that mediate sperm capacitation (Reviewed in [48,49]), and lower activation of this channel would also be related to the decreased levels of bicarbonate via the AC-inhibition of carbonic anhydrase. Since sperm motility is also mediated by the increase of intracellular bicarbonate and calcium levels, our hypothesis would not match with the higher sperm progressive

motility observed right after thawing. Thus, further research is needed to understand why our sperm motility results would not match with the decreased MMP and intracellular calcium levels observed immediately after thawing. Finally, while neither sperm viability nor acrosome integrity were altered at 0 h, both parameters decreased at 2 h post-thaw. This suggests that the aforementioned collateral effects of AC induce sub-lethal alterations that are not apparent right after thawing but only at 2 h post-thaw.

The hydrophobic nature of PHL allows its penetration through the sperm plasma membrane [50] and its binding to an internal site of GLPs [51]. This internal binding would specifically inhibit GLPs, which in turn would disrupt the transport of water and small solutes, such as glycerol. As glycerol, which is the most used permeating CPA in stallion sperm cryopreservation, was present in the freezing medium used in this study, it is reasonable to suggest that the addition of PHL decreased the transport of this CPA, which had detrimental effects on sperm quality and function parameters. The reduced influx of glycerol together with a limited water efflux though PHL-inhibition of GLPs could cause extreme osmotic stress to spermatozoa, thus compromising membrane integrity (including membranes of intracellular organelles) and increasing membrane lipid disorder. Related with this, we observed reduced sperm motility and survival at 0 h and 2 h post-thaw, which could match with this hypothesis. In addition, an impairment in the sperm membrane integrity could also explain the drop in the intracellular calcium levels which, in turn, could be related to the compromised mitochondrial function that we observed through the decrease of spermatozoa with high MMP. Whereas the reduction in the percentages of viable spermatozoa with high superoxide and peroxide levels immediately after thawing could be related to the decrease of MMP, the observed increase in the percentage of viable spermatozoa with high peroxide levels at 2 h post-thaw could be due to the inhibition of H_2O_2 efflux through AQP3 and AQP9 [52,53]. Related with this, some studies have unravelled that H_2O_2 efflux through AQP3 and AQP9 plays a vital role for human sperm function [10].

Regarding PDO, it strongly inhibits orthodox AQPs remaining inside their pore [44,45]. PDO also inhibits GLPs, though less efficiently, since the pore diameter of GLPs is broader than that of orthodox AQPs. This broader diameter of GLPs allows PDO to pass through these channels [44] and, in fact, Cooper *et al.* [54] assessed the permeability of epididymal murine sperm to PDO. Therefore, PDO would be able to impair the cell permeability to both water and small solutes, including glycerol, so that the expected effects should be a combination of those observed in the presence of AC and PHL. However, the cryodamage-related effects observed in the presence of PHL because of the inhibition of GLPs were not observed in the case of PDO. In fact, our results showed that the addition of PDO to the freezing medium had a positive effect on sperm quality and function parameters (motility, viability, acrosome integrity and membrane lipid disorder). In this context, it could be hypothesised that PDO could function as a CPA itself, thereby mitigating the potential cryodamage that one would have expected to observe as a consequence of the lower intracellular concentration of glycerol. In fact, Widiasih *et al.* [55] observed that the use of PDO as a CPA yielded higher post-thaw motility and viability of human spermatozoa than glycerol. Moreover, PDO has also been used as a CPA for the cryopreservation of canine ovarian cortex [56] and human multipotent stromal cells [57]. Considering all the aforementioned, one could consider that the combination of two different CPAs at low concentrations would restrict the widely known collateral toxic effects of each CPA, including glycerol (reviewed in [49]). In fact, a less toxic and more efficient combination of CPAs would limit cryodamage and thus membrane alterations, which would yield higher post-thaw sperm viability and motility. In spite of this, it is also worth noting that when samples were treated with the intermediate concentration of PDO there was an increase in MMP that did not have an apparent cause. A potential reason would be an interaction of PDO with AQP11, which is present in the membrane of intracellular organelles, including those of mitochondria [2]. Nevertheless, since, to the best of our knowledge, the ability of PDO to inhibit superAQPs has not been assessed, further studies are needed to address the mechanism through which MMP is altered in the presence of certain PDO concentrations. The increase in superoxide levels might be a direct consequence of

the increased MMP, since mitochondria are the main source of ROS. Finally, the rise in the percentages of viable spermatozoa with intracellular calcium levels could be related to a better maintenance of sperm survival rather than to the specific effect of PDO on the calcium transport (Reviewed in [48,49]). In this context, while the increase in both total and progressive motilities and in MMP would be due to this calcium increase, the cause of this augmented calcium levels would remain unexplained, since sperm membranes were intact according to the results of separate tests (SYBR14/PI, PNA-FITC/PI and M540/YO-PRO-1). Considering all the aforementioned, the use of PDO as a CPA alone or in combination with other agents might lead to obtaining frozen-thawed stallion spermatozoa of better quality.

5. Conclusion

In conclusion, the effects of AQP inhibition are highly dependent on the specificity of each inhibitor and its collateral effects on other sperm proteins. The observed effects when samples were supplemented with AC, which mainly inhibits orthodox AQPs, suggest that these proteins are not involved in the response to osmolality changes produced during stallion sperm cryopreservation, since the observed changes seem to be caused by the side-effects on other sperm proteins. On the other hand, the dramatic impairment of post-thaw sperm quality observed in the presence of PHL suggests that GLPs play a crucial role in the response of stallion sperm to the osmolality changes that occur during cryopreservation. Finally, the improvement of the overall sperm quality and function parameters in the presence of PDO supports its role as a permeable CPA, whether alone or in combination with other cryoprotectants.

6. List of abbreviations

AQP: aquaporin; AC: acetazolamide; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; cAMP: cyclic AMP; CASA: computer-assisted sperm analysis; CPA: cryoprotective agent; DCF^+: 2’,7’-dichlorofluorescein ; DMSO: dimethyl sulfoxide; E^+: ethidium ; EV: electronic volume; FS: forward scatter; GFE: good freezability ejaculates; GLP: aquaglyceroporins; H_2DCFDA: 2’,7’-dichlorodihydrofluorescein diacetate; H_2O_2: hydrogen peroxide; HE: hydroethidine; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ISAC: International Society for Advancement of Cytometry; JC1: 5,5’,6,6’-tetrachloro-1,1’,3,3’tetraethyl-benzimidazolylcarbocyanine iodide; $JC1_{agg}$: JC1 aggregates; $JC1_{mon}$: JC1 monomers; LIN: linearity; M540: Merocyanine 540; MMP: mitochondrial membrane potential; O_2^{-}•: superoxide; PDO: 1,3-propanediol; PFE: poor freezability ejaculates; PHL: phloretin; PI: propidium iodide; PKA: cAMP dependent protein kinase; PMOT: progressive motility; PNA-FITC: *Arachis hypogaea* lectin -peanut agglutinin-conjugated with fluorescein isothiocyanate; ROS: reactive oxygen species; SEM: standard error of the mean; SS: side scatter; STR: straightness; TMOT: total motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity; WOB: motility parameter wobble.

7. Declarations

7.1. Ethics approval and consent to participate

Not applicable.

7.2. Consent for publication

Not applicable.

7.3. Availability of data and materials

The datasets and/or analysed during the current study are available from the corresponding author on reasonable request.

7.4. Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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7.6. Author's contributions

ADB performed the experiments, undertook sperm quality and statistical analyses and wrote the draft. FN and EGB contributed to performing CASA and flow cytometry evaluations. SBC, JC and MP collected the sperm samples and provided help evaluating sperm quality parameters. JM and MY conceived the study, help conduct data analysis and contributed to the critical revision of the Manuscript. All authors read and approved the final manuscript.

7.7. Acknowledgements

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Figure legends

Fig. 1 Sperm motility of samples after cryopreservation with standard freezing medium (control), or with freezing medium supplemented with acetazolamide (AC) at three different concentrations (250 μmol/L, 500 μmol/L and 1000 μmol/L), with phloretin (PHL) at two different concentrations (350 μmol/L and 800 μmol/L), or with 1,3-propanediol (PDO) at three concentrations (0.1 mmol/L, 1 mmol/L and 10 mmol/L). A) Percentages of total motile spermatozoa (TMOT). B) Percentages of progressively motile spermatozoa (PMOT). Data, shown as mean ± SEM, correspond to 0 h and 2 h post-thaw. Different letters (*a-d*) indicate significant differences ($P<0.05$) between treatments within a given time point.

Fig. 2 Sperm viability (SYBR14/PI) and acrosome integrity (PNA-FITC/PI) after cryopreservation with standard freezing medium (control), or with freezing medium supplemented with acetazolamide (AC) at three different concentrations (250 μmol/L, 500 μmol/L and 1000 μmol/L), with phloretin (PHL) at two different concentrations (350 μmol/L and 800 μmol/L), or with 1,3-propanediol (PDO) at three concentrations (0.1 mmol/L, 1 mmol/L and 10 mmol/L). A) Percentages of viable spermatozoa ($SYBR\text{-}14^{+}/PI^{-}$ spermatozoa). B) Percentage of viable spermatozoa with an intact acrosome ($PNA\text{-}FITC^{-}/PI^{-}$ spermatozoa). Data, shown as mean ± SEM, correspond to 0 h and 2 h post-thaw. Different letters (*a-d*) indicate significant differences ($P<0.05$) between treatments within a given time point.

Fig. 3 Sperm membrane lipid disorder (M540/YO-PRO-1) and mitochondrial membrane potential (JC1) after cryopreservation with standard freezing medium (control), or with freezing medium supplemented with acetazolamide (AC) at three different concentrations (250 μmol/L, 500 μmol/L and 1000 μmol/L), with phloretin (PHL) at two different concentrations (350 μmol/L and 800 μmol/L), or with 1,3-propanediol (PDO) at three concentrations (0.1 mmol/L, 1 mmol/L and 10 mmol/L). A) Percentages of viable spermatozoa with low membrane lipid disorder ($M540^{-}/YO\text{-}PRO\text{-}1^{-}$ spermatozoa). B) Percentages of spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$ spermatozoa). Data, shown as mean ± SEM, correspond to 0 h and 2 h post-thaw. Different letters (*a-e*) indicate significant differences ($P<0.05$) between treatments within a given time point.

Fig. 4 Intracellular calcium levels (Fluo3/PI and Rhod5/YO-PRO-1) after cryopreservation with standard freezing medium (control), or with freezing medium supplemented with acetazolamide (AC) at three different concentrations (250 μmol/L, 500 μmol/L and 1000 μmol/L), with phloretin (PHL) at two different

concentrations (350 μmol/L and 800 μmol/L), or with 1,3-propanediol (PDO) at three concentrations (0.1 mmol/L, 1 mmol/L and 10 mmol/L). A) Percentages of viable spermatozoa with high levels of intracellular calcium levels ($Fluo3^+/PI^-$ spermatozoa). B) Percentages of viable spermatozoa with high levels of intracellular calcium levels ($Rhod5^+$/YO-PRO-1^- spermatozoa). Data, shown as mean ± SEM, correspond to 0 h and 2 h post-thaw. Different letters (*a-d*) indicate significant differences (P<0.05) between treatments within a given time point.

Fig. 5 Intracellular levels of reactive oxygen species (ROS) after cryopreservation with standard freezing medium (control), or with freezing medium supplemented with acetazolamide (AC) at three different concentrations (250 μmol/L, 500 μmol/L and 1000 μmol/L), with phloretin (PHL) at two different concentrations (350 μmol/L and 800 μmol/L), or with 1,3-propanediol (PDO) at three concentrations (0.1 mmol/L, 1 mmol/L and 10 mmol/L). A) Percentages of viable spermatozoa with high levels of superoxide (E^+/YO-PRO-1^- spermatozoa). B) Percentages of viable spermatozoa with high levels of peroxide (DCF^+/PI^- spermatozoa). Data, shown as mean ± SEM, correspond to 0 h and 2 h post-thaw. Different letters (*a-d*) indicate significant differences (P<0.05) between treatments within a given time point.

Fig. 1

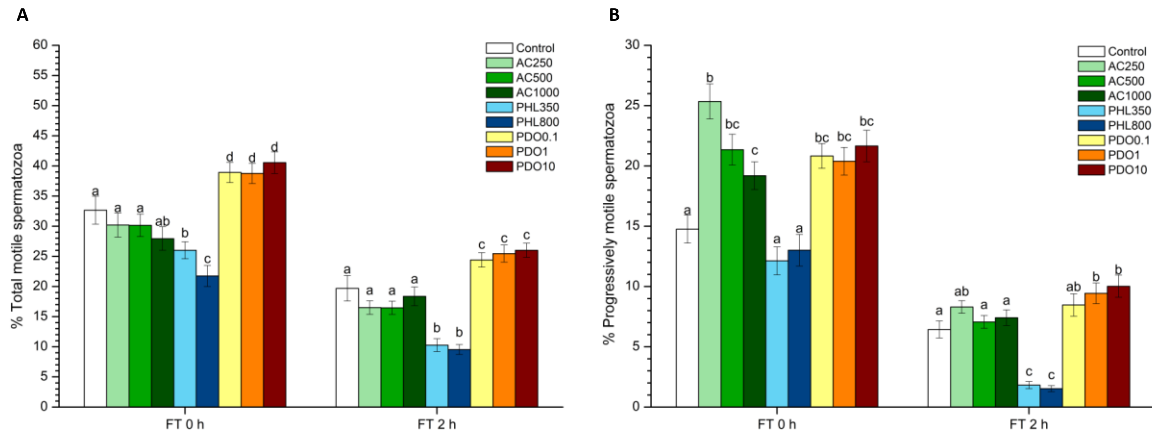


Fig. 2

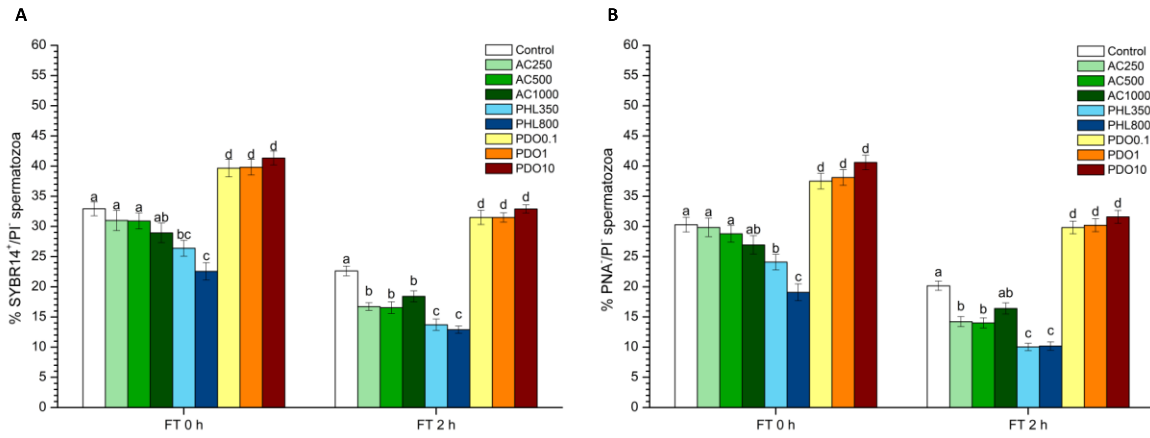


Fig. 3

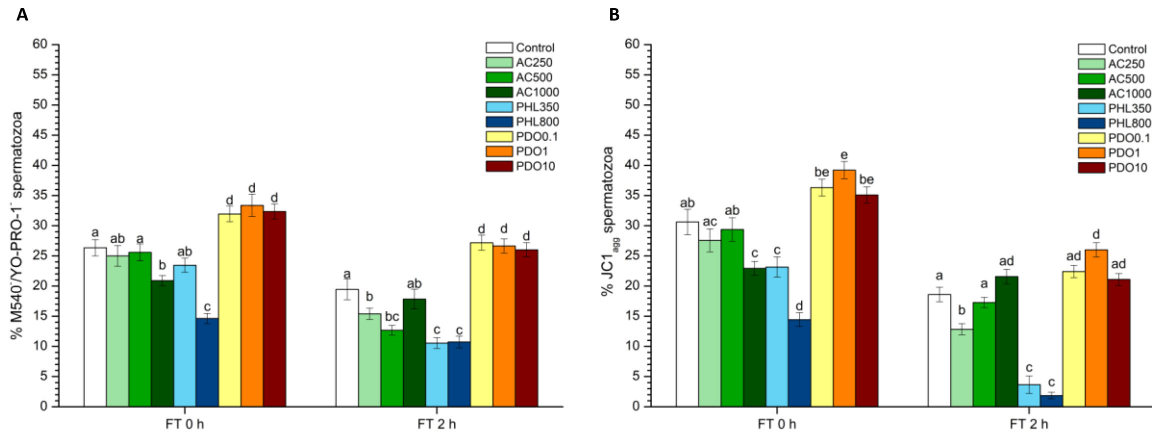


Fig. 4

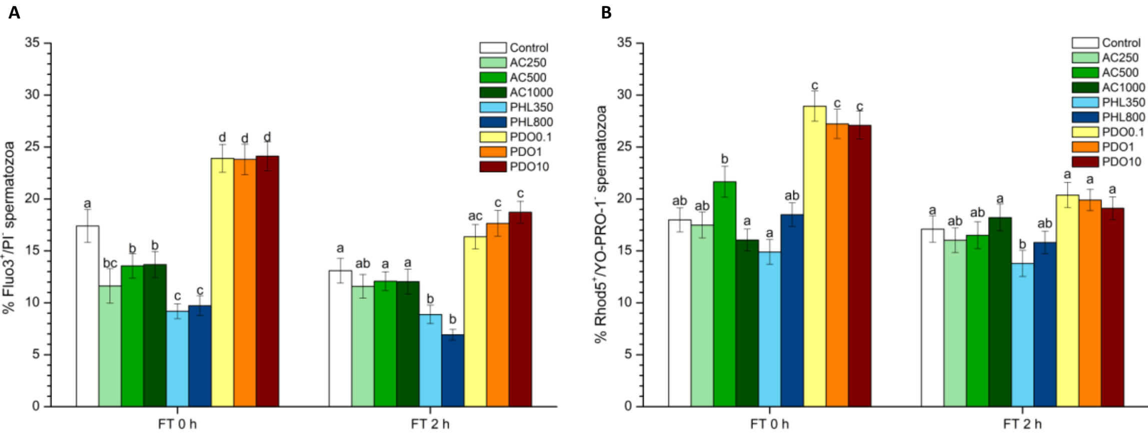
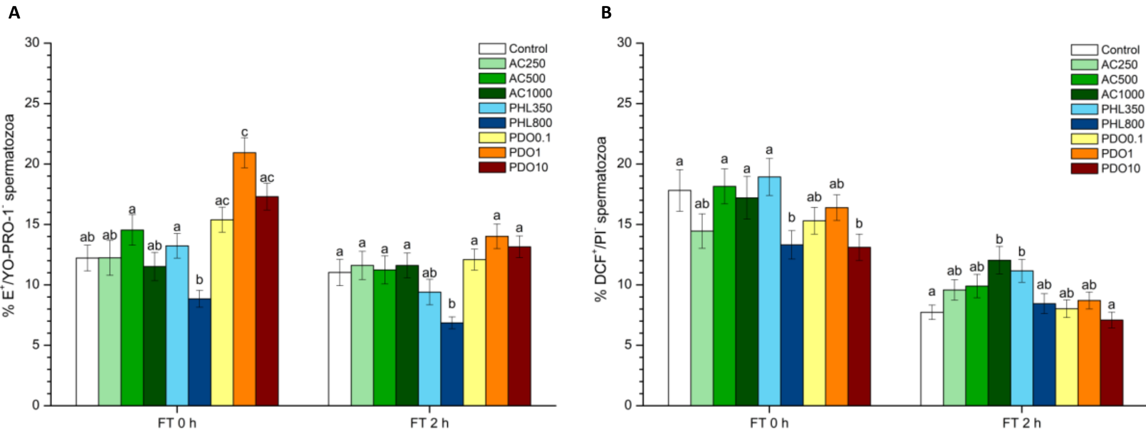


Fig. 5



DISCUSIÓN

El agua juega un papel fundamental en todos los procesos biológicos y su tráfico está involucrado en la respuesta de las células a los cambios en los gradientes osmóticos (Gravelle et al. 2013). Sin embargo, y debido a la naturaleza hidrofóbica de las membranas lipídicas, el agua penetra lentamente en esta estructura por difusión simple (revisado por Huang et al. 2006a y Matsuzaki et al. 2002), de modo que la mayor parte de su transporte se lleva a cabo mediante la difusión facilitada (Noiles et al. 1993; Huang et al. 2006a). La existencia de dicha difusión facilitada fue sugerida por primera vez por Sidel y Salomon en 1957, quienes hipotetizaron la existencia de poros de agua selectivos en las membranas biológicas (Sidel y Salomon, 1957). Sin embargo, no fue hasta principios de los noventa cuando Peter Agre y sus colegas descubrieron las acuaporinas (AQPs), una familia de proteínas de canal transmembrana específicas para el agua y otros solutos, incluidos los crioprotectores permeables como el glicerol (Agre et al., 2002).

Está bien establecido que las AQPs se distribuyen de forma ubicua en los tejidos de los mamíferos. En particular, su presencia en el tracto reproductivo de los seres humanos (revisado por Huang et al. 2006a) indica que juegan un papel importante en la fisiología reproductiva (Thoroddsen et al. 2011). Sin embargo, y en contraste con el profundo conocimiento y los numerosos estudios sobre las AQPs en las células somáticas, el conocimiento acerca de las AQPs en los espermatozoides es limitada. Concretamente, las AQPs se han estudiado en los espermatozoides de mamíferos de roedores (Yeung y Cooper, 2010; Chen y Duan, 2011) perros (Ito et al. 2008), humanos (Yeung et al. 2010; Moretti et al. 2012), cerdos y toros (Prieto-Martínez, Morató et al., 2016; Prieto-Martínez et al., 2017; Prieto-Martínez et al.2015). Sin embargo, la identificación, localización y función de estas proteínas en los espermatozoides de caballo no han sido estudiadas.

En el primer trabajo, se demostró por primera vez que las AQP3, AQP7 y AQP11 están presentes en el espermatozoide de caballo. En todos los casos, se utilizó la inmunotransferencia y se observaron dos bandas tanto a 30 como a 60 kDa, lo que sugiere la presencia de formas diméricas o agregados de estas proteínas después

de la extracción. Con respecto a este punto, vale la pena mencionar que la especificidad de estos anticuerpos se confirmó con ensayos utilizando péptidos de bloqueo. Por otro lado, y a pesar de su carácter preliminar, los resultados del primer trabajo sugieren que las AQP3 y AQP11 podrían estar involucrados en la capacidad del espermatozoide del caballo de a resistir a los procesos de congelación/descongelación. Dicha conclusión se alcanzó al observarse que los espermatozoides frescos de los eyaculados buenos congeladores (GFE) presentaban uno niveles relativos de AQP3 y AQP3 (banda de 60 kDa) significativamente superiores a los de los eyaculados más altos de AQP3 y AQP11) que el grupo de malos congeladores (PFE). Por lo tanto, estos datos sugieren que cuando las células espermáticas de caballo tienen unos niveles relativos más elevados de AQP3 y AQP11, su movilidad y supervivencia post-descongelación se mantienen mejor. Por el contrario, se determinó que el contenido relativo de la AQP7 en los espermatozoides frescos de caballo no está relacionado con su criotolerancia, dado que no se observaron diferencias entre GFE y PFE después de la descongelación. Estos datos están parcialmente de acuerdo con los resultados obtenidos en cerdo y toro, ya que la AQP3 se identificó como marcador de congelabilidad en espermatozoides de cerdo (Prieto-Martínez et al., 2017), y se determinó que la AQP11 era un marcador de congelabilidad del espermatozoide de toro (Morató et al., 2018) se identificó. Sin embargo, mientras que tanto en cerdo como en toro se determinó que los niveles relativos de AQP7 en el espermatozoide fresco estaban relacionados con su criotolerancia (Prieto-Martinez et al., 2017; Morató, et al., 2018), éste no parece ser el caso en el del caballo.

Por otro lado, en el segundo trabajo, se confirmó, mediante inmunofluorescencia, la presencia de las AQP3, AQP7 y AQP11 en los espermatozoides de caballo, ya identificadas en el primer trabajo (Bonilla-Correal et al., 2017). Estas proteínas parecen desempeñar un papel crucial en la regulación del volumen de las células espermáticas, que es de vital importancia durante la espermiogénesis y en la adaptación de los espermatozoides al tracto reproductor femenino (Yeste et al., 2017). Sin embargo, los resultados más relevantes de este trabajo fueron los cambios en la localización de la AQP7 y AQP11 observados después de la

criopreservación. Por un lado, vale la pena señalar que, en espermatozoides frescos, todas las AQPs se encontraron en la cola del espermatozoide, y solo se encontró la AQP11 en la región post-acrosómica. La localización de estas tres AQPs difiere ligeramente de la observada en otras especies. De modo parecido a lo observado en este trabajo, la AQP3 también está presente en la pieza intermedia de los espermatozoides de cerdo y toro (Prieto-Martínez et al., 2016a; 2016b). La localización de la AQP7, que se encontró en la pieza intermedia, fue similar a la de los espermatozoides de toro (Prieto-Martínez et al., 2016b), pero difirió de la de los espermatozoides de cerdo, en los que esta proteína está restringida a la pieza de conexión (Prieto-Martínez et al., 2015). Finalmente, se encontró que la AQP11 en espermatozoides de caballo se localizaba en la pieza principal y la región post-acrosómica, de una manera similar a lo observado en los espermatozoides de cerdo y toro (Prieto-Martínez et al., 2015; Morató et al., 2017).

Por otra parte, en este segundo trabajo se determinó que la criopreservación inducía cambios en la localización de la AQP7 y la AQP11. De hecho, la AQP7 se desplazó de la pieza intermedia a la cabeza, la región post-acrosómica y la cola del espermatozoide después de la congelación/descongelación. La AQP11 también cambió su localización en respuesta a la criopreservación, dado que en los espermatozoides frescos dicha proteína se localizaba en la pieza principal y la región post-acrosómica, pero la mayor parte del marcaje de la pieza principal de la misma desapareció en los espermatozoides descongelados y solo se observó una débil señal en la región post-acrosómica. Si bien la relocalización de las proteínas de los espermatozoides en respuesta a la criopreservación no es un evento raro, los cambios inducidos en el caso de las AQPs difieren entre las especies. En efecto, en el espermatozoide porcino, se identificó la relocalización de AQP7 de la pieza de conexión a la pieza intermedia y a la región post-acrosómica en respuesta a la criopreservación, mientras que no se observaron diferencias entre los espermatozoides frescos y congelados para la AQP3 y la AQP11 (Prieto- Martínez et al., 2017). Por el contrario, en este segundo trabajo se observaron cambios en la localización de AQP7 y AQP11, lo que no ocurre en los espermatozoides de cerdo y toro (Prieto-Martínez et al., 2015; 2016a; 2016b). Con los experimentos llevados

a cabo en este segundo trabajo no es posible explicar por qué existen tales diferencias entre especies. Sin embargo, la relocalización de la AQP7 y la AQP11 podrían explicarse por los cambios en los dominios de la membrana plasmática debido a la reducción de su integridad en los espermatozoides criopreservados de caballo. Para que estos resultados se confirmen, se debe realizar una investigación más exhaustiva evaluando la localización de AQP7 y AQP11 en espermatozoides frescos y criopreservados mediante técnicas de microscopía electrónica de transmisión e inmunogold. En cualquier caso, la criopreservación es la tecnología más útil para preservar los espermatozoides de mamíferos a largo plazo (Yeste, 2016). El principal inconveniente de esta técnica es la disminución de la capacidad fecundante asociada a la reducción de la supervivencia de los espermatozoides después de la descongelación, que a su vez es causada por la alta susceptibilidad de los mismos al choque térmico (Gilmore et al., 1996).

Los cambios en la localización de AQP7 y AQP11 podrían estar relacionados con la función de estas proteínas durante la criopreservación, de acuerdo con los estudios previos llevados cabo en caballos (Artículo 1; Bonilla-Correal et al., 2017), cerdos (Prieto-Martínez et al., 2017) y toros (Prieto-Martínez et al., 2016; Morató et al., 2017). El hecho de que determinadas proteínas estén relacionadas con la criotolerancia de los espermatozoides de caballo no es un hallazgo raro, ya que no solo las AQPs (Prieto-Martínez et al., 2016; Morató et al., 2017; Prieto-Martínez et al., 2017) sino también otras proteínas como la heat-shock protein 90 (HSP90AA1), la proteína de unión a acrosina (ACRBP), la triosefosfato isomerasa (TPI) y el canal aniónico dependiente de voltaje 2 (VDAC2); (Casas et al., 2009; Casas et al., 2010; Vilagran et al. , 2013; 2014) han sido identificadas como marcadores de congelación en los espermatozoides de mamífero. En el caso de las AQPs, estas proteínas están involucradas en la regulación del transporte de agua y, en algunos casos, de solutos como el glicerol a través de la membrana plasmática y permiten la modificación del volumen del espermatozoide en respuesta a los cambios osmóticos (Edashige et al., 2006; Kumar et al., 2015). Por lo tanto, además del hecho de que existen diferencias entre GFE y PFE en cuanto al contenido relativo de AQP3 y AQP11, los datos proporcionados en este segundo trabajo sugieren que los cambios en la

localización de la AQP7 y la AQP11 durante la criopreservación también podrían estar involucrados en esta respuesta.

En cuanto a la función y el mecanismo de acción de las AQPs, hay todavía muy pocos estudios que hayan abordado esta cuestión, a pesar de su relevancia, especialmente en el caso de la criopreservación espermática. Siguiendo la identificación y localización de las AQP3, AQP7 y AQP11 en los espermatozoides de caballo efectuadas en los Trabajos 1 y 2, se realizó un tercer estudio que tuvo como objetivo investigar la relevancia de cada grupo de AQPs durante la criopreservación de espermatozoides de caballo. Con este propósito, se agregaron tres inhibidores diferentes a diferentes concentraciones que se eligieron de acuerdo con experimentos preliminares realizados en nuestro laboratorio y estudios previos (Delgado-Bermúdez et al., 2019): acetazolamida (AC; 250, 500 y 1000 µmol / L), floretina (PHL; 350 y 500 µmol / L) y 1,3-propanodiol (PDO; 0.1, 1 y 10 mmol / L). Vale la pena mencionar que la AC inhibe las AQP1 y AQP4 (Gao et al., 2016; Tanimura et al., 2009), la PHL inhibe tanto la AQP3 como la AQP7 (Resk et al.,2002; Przybylo et al.,2014), y se ha demostrado que el PDO inhibe las AQPs ortodoxas (AQP1, AQP2, AQP5 y AQP4) con alta eficiencia, y la aquagliceroporinas (GLPs), familia a la que pertenecen las AQP3, AQP7 y AQP9, con menos intensidad (Yu et al., 2016a, Yu et al.,2016b). Los efectos de cada inhibidor de AQPs sobre la función y la supervivencia de los espermatozoides después de la criopreservación se evaluaron sobre la base de la motilidad, la viabilidad de los espermatozoides, la integridad del acrosoma, el desorden lipídico de la membrana, el potencial de membrana mitocondrial (MMP), los niveles de calcio intracelular y los niveles intracelulares de ROS.

Al descongelarse, las muestras tratadas con AC mostraron porcentajes significativamente más altos de motilidad progresiva (PMOT) y porcentajes significativamente más bajos de espermatozoides viables con niveles altos de Fluo3 $^{+}$ que el control. Además, la concentración más alta de AC redujo significativamente los porcentajes de espermatozoides viables con elevado desorden lipídico de membrana y aquéllos con un alto MMP. A las 2 h después de la descongelación, los

tratamientos que contenían AC presentaron una viabilidad espermática en todos los tratamientos. Además, las concentraciones más bajas de AC también mostraron una reducción en los porcentajes de espermatozoides con bajo desorden lipídico y alto MMP, y la más alta (1000 µmol/L) exhibió un mayor porcentaje de espermatozoides viables con niveles altos de H_2O_2. El segundo inhibidor, esto es, la PHL, mostró porcentajes significativamente más bajos de espermatozoides móviles totales (TMOT) y móviles progresivos (PMOT), espermatozoides viables y con el acrosoma intacto, espermatozoides viables con bajo desorden lipídico de membrana, espermatozoides con alto MMP, espermatozoides viables con altos niveles de calcio intracelular (principalmente espermatozoides Fluo3$^+$) y espermatozoides viables con altos niveles intracelulares de superóxidos. Por el contrario, los tratamientos que contenían PDO mostraron porcentajes más altos de TMOT, PMOT, espermatozoides viables y con el acrosoma intacto, espermatozoides viables con bajo desorden lipídico de membrana, espermatozoides con alto MMP y espermatozoides viables con altos niveles de calcio intracelular.

Las diferencias entre los efectos de estos tres inhibidores parecen ser el resultado de su especificidad para las distintas AQPs y de los efectos colaterales sobre otras proteínas que están presentes en los espermatozoides. De hecho, la AQP1 y la AQP4 no se han identificado previamente en espermatozoides de mamífero (revisado en Yeste et al., 2017), lo que respalda la ausencia de efectos consistentes inmediatamente después de la descongelación en presencia de la AC. Sin embargo, la presencia de la AC alteró algunos parámetros de la función espermática, aunque algunos de estos cambios no parecían depender de la concentración del inhibidor o del tiempo de incubación posterior a la descongelación. En este contexto, vale la pena mencionar que la AC no solo inhibe algunas AQPs sino también la anhidrasa carbónica, cuya función es convertir el CO_2 y el agua en bicarbonato y protones [46]. Por lo tanto, la inhibición de la anhidrasa carbónica a través de la AC podría disminuir la concentración de bicarbonato intracelular. El bicarbonato, junto con el Ca^{2+}, es crucial para la activación de la adenilato ciclasa soluble (sAC) que, a su vez, produce AMPc que activa la proteína quinasa A (PKA). La proteína quinasa A

activa diferentes complejos de la cadena de transporte de electrones, y se ha observado que la inhibición de la PKA reduce el flujo de electrones a través del Complejo I (Lark et al., 2015). Este mecanismo podría explicar la reducción del potencial de membrana mitocondrial observado en presencia de algunas concentraciones de AC. Además, la disminución en los porcentajes de espermatozoides con altos niveles intracelulares de calcio observados en presencia de la AC podría estar relacionada con la falta de activación del canal de calcio CatSper. El canal CatSper es responsable del aumento de los niveles de calcio intracelular que participan en la capacitación de los espermatozoides (revisado en Nishigaki et al., 2014; Yeste, 2016), y una menor activación de este canal también estaría relacionada con la disminución de los niveles de bicarbonato a través de la inhibición de la anhidrasa carbónica. Dado que la motilidad de los espermatozoides también está regulada por el aumento de los niveles intracelulares de bicarbonato y calcio, esta hipótesis no coincidiría con la mayor motilidad progresiva de los espermatozoides observada justo después de la descongelación. Por lo tanto, se requieren otros estudios para comprender por qué los resultados de motilidad espermática no coincidieron con la disminución del MMP y los niveles de calcio intracelular observados en los espermatozoides post-descongelados. Finalmente, aunque ni la viabilidad espermática ni la integridad del acrosoma se alteraron a las 0 h, ambos parámetros disminuyeron a las 2 h post-descongelación. Esto sugiere que los efectos colaterales de la AC mencionados anteriormente inducen alteraciones sub-letales que no son evidentes inmediatamente después de la descongelación, sino solo a las 2 h después de la descongelación.

La naturaleza hidrofóbica de la PHL permite su penetración a través de la membrana plasmática de los espermatozoides (Pohl et al., 1997) y su unión a un dominio intracelular de las GLPs (Wacker et al.,2013). Esta unión interna inhibiría específicamente los AGP, lo que a su vez interrumpiría el transporte de agua y pequeños solutos, como el glicerol. Dado que el glicerol, que es el CPA permeable más utilizado para la criopreservación de los espermatozoides, estaba presente en el medio de congelación utilizado en este estudio, es razonable sugerir que la adición de PHL disminuyó el transporte de este CPA, lo que tuvo efectos

perjudiciales sobre la calidad y funcionalidad de los espermatozoides. Una menor entrada de glicerol junto con un flujo de agua limitado podría causar un estrés osmótico extremo a los espermatozoides, comprometiendo así la integridad de la membrana (incluidas las membranas de los orgánulos intracelulares) y aumentando su desorden lipídico. En apoyo de esta hipótesis, se observó una reducción de la motilidad y supervivencia espermáticas a las 0 h y 2 h post-descongelación. Además, un deterioro de la integridad de la membrana de los espermatozoides también podría explicar la reducción en el porcentaje espermatozoides con elevados niveles de calcio intracelular, lo que, a su vez, podría estar relacionado con la disminución del porcentaje de espermatozoides con alto MMP. Mientras que la reducción en los porcentajes de espermatozoides viables con altos niveles de superóxidos y peróxidos inmediatamente después de la descongelación podría estar relacionada con la disminución del MMP, el aumento observado en el porcentaje de espermatozoides viables con altos niveles de peróxidos a las 2 h post-descongelación podría deberse a la inhibición del flujo de H_2O_2 a través de las AQP3 y AQP9 (Miller et al., 2010; Watanabe et al., 2016). Relacionado con este punto, estudios previos han demostrado que el flujo de H_2O_2 a través de las AQP3 y AQP9 juega un papel vital para la funcionalidad del espermatozoide humano (Laforenza et al., 2016).

Con respecto al PDO, éste inhibe las AQPs ortodoxas, situándose dentro de sus poros (Yu et al 2016a, Yu et al., 2016b). El PDO también inhibe las GLPs, aunque de manera menos eficiente, ya que el diámetro del poro de éstas es más ancho que el de las ortodoxas. Este diámetro más amplio lo que permite que el PDO pase a través de estos canales (Yul et al., 2016 a), tal y como demostraron Cooper y colaboradores (2008) en los espermatozodes epididimarios de ratón. Por lo tanto, el PDO podría afectar la permeabilidad celular tanto al agua como a los solutos pequeños, incluido el glicerol, de modo que los efectos esperados deberían ser una combinación de los observados en presencia de la AC y la PHL. Sin embargo, los dichos efectos esperados acerca del daño causado por la criopreservación observados en presencia de la PHL y debidos a la inhibición de las GLPs, no se observaron en el caso del PDO. De hecho, los resultados del tercer trabajo

sugirieron que la adición de PDO al medio de congelación tiene un efecto positivo sobre la calidad y funcionalidad de los espermatozoides de caballo descongelados (observado en la motilidad, viabilidad, integridad del acrosoma y desorden lipídico de la membrana). En este contexto, se podría plantear la hipótesis de que el PDO podría funcionar como CPA, mitigando así el posible daño criogénico que se hubiera podido esperar como consecuencia de la presencia de una concentración intracelular reducida de glicerol. En concordancia con estos hallazgos, Widiasih y colaboradores (Widiasih et al., 2009) observaron que el uso del PDO como CPA produjo una mayor motilidad y viabilidad de los espermatozoides humanos a la post-descongelación que el glicerol. Además, el PDO también se ha utilizado como CPA para la criopreservación de la corteza ovárica canina (Lopes et al., 2016) y las células madre multipotentes humanas (Pogozhykh et al., 2015). Teniendo en cuenta todo lo anterior, se podría considerar que la combinación de dos CPA diferentes a bajas concentraciones restringiría los efectos tóxicos que cada uno de estos CPAs, como el glicerol, induce de manera individual a altas concentraciones (revisado en Yeste, 2016). De hecho, una combinación menos tóxica y más eficiente de CPA limitaría el daño criogénico y, por lo tanto, las alteraciones de la membrana, lo que produciría una mayor viabilidad y motilidad de los espermatozoides después de la descongelación. A pesar de esto, también vale la pena señalar que cuando las muestras se trataron con una concentración intermedia de PDO, hubo un aumento del MMP, lo que no tuvo una causa clara. Una razón potencial sería una interacción del PDO con la AQP11, que está presente en la membrana de los orgánulos intracelulares, incluidas las de las mitocondrias (Yeste et al., 2017). Sin embargo, dado que no hay estudios que hayan evaluado la capacidad del PDO para inhibir las superAQP, se necesitan más estudios para abordar el mecanismo a través del cual se altera la MMP en presencia de ciertas concentraciones de PDO. Con todo, el aumento en los porcentajes de espermatozoides con niveles elevados de superóxidos podría ser una consecuencia directa del aumento del MMP, ya que las mitocondrias son la principal fuente de ROS. Finalmente, el aumento en los porcentajes de espermatozoides viables con altos niveles intracelulares de calcio podría estar relacionado con un mejor mantenimiento de la supervivencia

espermática a la post-descongelación, más que con un efecto específico del PDO sobre el transporte de calcio (revisado en Nishigaki et al., 2014; Yeste, 2016). En este contexto, si bien el incremento de las motilidades totales y progresivas y del MMP se debería a este aumento de calcio, la causa de dicho aumento permanecería sin explicación, ya que las membranas espermáticas estaban intactas de acuerdo con los resultados de distintos test (SYBR14 / PI, PNA-FITC / PI y M540 / YO-PRO-1). Teniendo en cuenta todo lo anterior, el uso del PDO como un CPA solo o en combinación con otros agentes podría incrementar el rendimiento de los protocolos de criopreservación de los espermatozoides de caballo.

CONCLUSIONES

1. Las AQP3, AQP7 y AQP11 están presentes en los espermatozoides de caballo y que existe una relación entre los niveles relativos de AQP3 y AQP11 (60 kDa) y la criotolerancia de los espermatozoides equinos.

2. AQP3, AQP7 y AQP11 tienen una localización específica en los espermatozoides del caballo. En el semen fresco, la AQP3 y AQP7 están localizadas en la pieza intermedia, mientras que la AQP11 se encuentra en la pieza principal y la región post-acrosomica de estas células. Esta localización se modifica en dos de ellas (AQP7 y AQP11) como respuesta a la criopreservación.

3. El efecto de inhibición sobre las AQPs depende en gran medida de la especificidad del inhibidor y sus efectos colaterales sobre otras proteínas espermáticas.

4. El efecto de la Acetazolamida como un inhibidor principal de AQPs ortodoxas sugiere que estas proteínas no están involucradas en la respuesta a cambios de osmolaridad.

5. El uso de la floretina como inhibidor de las GLPs demostró el papel crucial que juegan estas proteínas en la regulación de la osmolaridad durante la criopreservación.

6. La mejora de la calidad general de los espermatozoides descongelados después de la suplementación con el PDO indica que éste tiene un papel crioprotector, ya sea solo o en combinación con otros agentes como el glicerol.

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