UNIVERSIDAD DE EXTREMADURA

Tesis Doctoral

"PAPEL DE LA CADENA MITOCONDRIAL DE TRANSPORTE DE ELECTRONES EN EL DESENCADENAMIENTO DE LA SENESCENCIA EN EL ESPERMATOZOIDE EQUINO"

María Plaza Dávila

Facultad de Veterinaria

Departamento de Medicina Animal

Conformidad del Director: Fernando Juan Peña Vega

Cáceres, 2015

"Entonces Dios tomó un puñado de viento y sopló creando el caballo: Te llamarás árabe y la virtud inundará el pelo de tus crines y tu grupa. Serás mi preferido entre todos los animales porque te he hecho amo y amigo.....porque del viento vienes y viento debes ser en la carrera"

(Proverbio árabe)

Prof. Dr. Fernando J Peña
Diplomado Colegio Europeo de Reproducción Animal
Laboratorio de Reproducción Equina
Hospital Clínico Veterinario, Universidad de Extremadura

D. Fernando J Peña Vega, Profesor Titular de Universidad, en su calidad de Director de la Tesis Doctoral de Dª MARIA PLAZA DÁVILA:

INFORMA:

Que Dª MARIA PLAZA DÁVILA ha venido trabajando en la Tesis Doctoral titulada ***"PAPEL DE LA CADENA MITOCONDRIAL DE TRANSPORTE DE ELECTRONES EN EL DESENCADENAMIENTO DE LA SENESCENCIA EN EL ESPERMATOZOIDE EQUINO»*** desde el año 2011 bajo mi dirección. Durante este periodo ha realizado también una estancia de siete meses en la Universidad de Bolonia (Italia). La Tesis está formada por cuatro publicaciones en revistas internacionales recogidas en bases de datos del I.S.I. (tres publicadas en revistas dentro del primer cuartil, y una en revisión) y ha sido informada favorablemente por dos expertos Europeos de reconocido prestigio en el área de Reproducción. Por todo ello considero que la Tesis cumple todos los requisitos para ser juzgada y optar a la mención "Doctor Internacional".

En Cáceres a 10 de Noviembre de 2015.

Fdo. Fernando Juan Peña Vega

ESTE TRABAJO HA SIDO FINANCIADO POR:

- Ministerio de Economía y Competitividad (AGL2013-43211-R)
- Ministerio de Economía y Competitividad (AGL2010-20758)
- Gobierno de Extremadura-FEDER GR 10010, GR 15029 y PCE 1002
- Se agradece especialmente la colaboración al Servicio de Cría Caballar de las Fuerzas Armadas
- María Plaza Dávila ha disfrutado de una beca-contrato Pre-doctoral de La Fundación Fernando Valhondo Calaff y de una beca de investigación de Acción III de la Universidad de Extremadura

Agradecimientos

Sin duda, esta va a ser la parte más difícil de escribir por la emoción de agradecer a todas las personas que han querido recorrer, vivir y sobre todo apoyarme en esta aventura……por lo tanto, esta parte de la tesis se la dejo al corazón……..

En primer lugar, gracias a mi director, mi super jefe y cabeza pensante del equipo, Fernando. Tenemos caracteres muy diferentes pero hemos sido capaces de llegar a un equilibrio y me has sabido entender y apoyar en momentos difíciles…….gracias por creer en mí y por aguantar las "tonterías de niña pija" ……..

A la Fundación Fernando Valhondo Calaff por el apoyo económico que me ha ayudado tanto en esta aventura de enriquecimiento del conocimiento.

A mis padres, por luchar por mí desde el primer segundo de mi vida. A ti papá, por transmitirme siempre paz y tranquilidad. Por tantas horas a mi lado cuando tenía que estudiar, ya fuese en el colegio, como en el instituto o en la carrera. Has sido siempre mi inspiración y seguiré luchando y superándome para que sigas estando orgulloso de mí desde allá donde estés. Gracias por ser mi ángel de la guarda y cuidarme siempre. A ti mamá, la protección personificada. Por estar a mi lado cada día y apoyarme en todas las decisiones que tomo, aunque a veces te cueste……porque eres la persona más importante de mi vida y con la que sé que podré contar para lo que necesite y cuando lo necesite. Porque gracias a tí estoy AQUÍ. OS QUIERO.

A mi familia. A mis abuelos, mis tíos, mis primos y mis sobrinos-primos. Gracias por protegerme y quererme tanto, a pesar de ser "la rebelde" de la familia ;) gracias por estar orgullosos de mí. Silvia, Gabriela y Mateo, viendo en vuestros ojos el amor que sentís por mí me haceís la persona más feliz del mundo!! OS QUIERO MUCHISIMO A TODOS.

A los 5 pilares de mi vida: Laura, Ana María, Esther, Lorena (mi mandy constante) y mi prima Cristina. Gracias de todo corazón por estar cada día a mi lado, por aconsejarme siempre, aunque a veces no os haga mucho caso…….por quererme tal y como soy, por todos nuestros grandes y únicos momentos que me acompañarán el resto de mi vida, vaya donde vaya……sois mis luces, MIS HERMANAS. OS QUIERO.

A ti Vilma, mi pikipiki, mi princesita. Tú no eres consciente de lo importante que eres para mí…..incluso en los días más duros me recibes con esa alegría que hace que se vaya todo el mal que pueda tener. Mi compañera de viaje más fiel en esta aventura, y la que sin duda ha estado más horas a mi lado en el transcurso de esta Tesis. Eres mi vida.

A mis compañeros del departamento, porque el trabajo en equipo ha sido fundamental. A Cristina, por acogerme con los brazos abiertos desde el primer momento y enseñarme tanto sobre la reproducción equina. Sólo me quedo con nuestros momentos buenos, que han sido muchos!! A ti Patri, porque tú me haces ver la luz cuando estoy perdida en la oscuridad de mis bloqueos….gracias por trabajar codo con codo a mi lado desde tu llegada hasta hoy. A vosotros Antolín y Carolinaaaaa, por enseñarme todos los secretos del laboratorio y de la repro de pequeños. A vuestro lado he aprendido muchísimo!! A Guille, el nuevo!! Eres un gran veterinario y un compañero genial, me has ayudado en todo lo que he necesitado desde tu entrada en nuestro equipo. A Juan, nuestro técnico, por tus ratos tan divertidos entre experimento y experimento…..y por enseñarme los secretos de la citometría de flujo……"8 isoooooo". A Manuel, aunque ha sido poco tiempo, has estado siempre dispuesto a ayudarme tanto informáticamente como con los caballos. A mi caspasa Juanma, a mi hermano, por TODO. Siempre has estado en las buenas y en las malas. Las palabras se quedan cortas para decir lo enormemente agradecida que me siento por todos esos momentos……laboratorio, quirófano, patio de caballos, findes de guardia, biblioteca…..porque gracias a ti mi amor por los caballos es aún más grande. A TODOS por los buenos ratos que hemos pasado fuera del departamento, por nuestras risas que serán imborrables…….nos lo hemos pasado pipa chicos!! GRACIAS. A Mari Cruz por tu amabilidad siempre acompañada de una sonrisa y a Germán.

A mis internos (tanto de grandes como de pequeños animales), el motor y la luz del departamento: Joze (por la gran persona que eres y por estar siempre a mi lado), Julia (la mia bella bolognesa y especial confidente), Guada, MJ Montero (mi mini-pija), Yerga, Cris H y la gran lista de nombres que dejo sin escribir, pero que estáis AQUÍ….porque sin vosotros todo este trabajo no sería posible. Sois imprescindibles chicos!! Un placer haber trabajado y crecido a vuestro lado. Por todos esos momentos dentro y fuera del departamento…….SOIS GENIALES!!

A los compañeros de Fisio. Tapia e Inés, gracias por compartir vuestros conocimientos conmigo, por ayudarme siempre en el laboratorio, y por orientarme cuando me asaltan las dudas laborales!! Tapia, mil gracias por tu paciencia a la hora de mi pesadilla con la informática…… porque gracias a ti el diseño de la tesis ha salido perfecto!! A Patri, mi churri, un placer tenerte como compañera y amiga, eres una gran investigadora y mejor persona!! A Ana, Leticia, Natalia, Esther y Miriam por vuestra alegría, compañerismo, ayuda y ratos divertidos!! A Ginés, porque todo lo que he necesitado me lo ha concedido sin problemas. Un gran honor haber pertenecido al mismo grupo de investigación que una eminencia como usted. A Mere, por la alegría que contagias a todos los que te rodeamos, no cambies nunca!! A Marisa Campos, Catedrática de Bioquímica, por la ayuda y orientación prestadas en el inicio de la redacción de esta tesis doctoral.

A los compañeros de Anatomía Patológica (el que fue mi departamento en la época de estudiante). A chuchi, mi amigo y gran compañero, un placer haber compartido esta aventura

contigo, tanto fuera como dentro de la Facul. A Galapero y Edu, por vuestras risas y porque sois unos cracks!! A Luis Gómez y Vicente Roncero por enseñarme tanto del mundo de las necropsias, por confiar en mí durante los 3 años de internado en vuestro departamento, y seguir confiando en los años posteriores.

A los compañeros de Médica. A Javi Duque, mi paisano, un honor aprender cada día del mejor, gracias. A Patri, por cuidar tan bien de mi Vilmita. A Concha, a Rafa y a vuestros internos, sois un gran equipo y un gran departamento.

A Prado y Cari de Toxicología y a José Luis de Genética, por la ayuda prestada en todo momento, me hacéis sentir siempre muy querida y valorada. GRACIAS.

A los compañeros de Cirugía, Enfermedades Parasitarias y Enfermedades Infecciosas, siempre dispuestos a ayudar en todo lo que sea necesario. Y a ti Ana Cris, por esa maravillosa foto de Usagre, supiste reflejar toda su belleza, eres una artista!!

Al Personal del HCV: Marisa, Yolanda y Paqui, dispuestas a echarme una mano en cualquier momento y siempre acompañadas de una sonrisa. A Abel, Chiqui y Jesús Quintanilla, nuestros chicos de los caballos, porque bajar al patio de caballos y encontraros siempre listos para ayudarme o sacarme una sonrisa es una gran alegría!! A Elisa, Vero y Noelia, me ha encantado disfrutar a vuestro lado esos ratitos de pausa, "charletas" y risas….sois geniales.

A Manolo, Luisa y sobre todo a ti Pedro, porque siempre consigues sacarme una sonrisa aunque el día haya sido duro…….

A los subtenientes de la Yeguada Militar de Écija Jesús Ruíz y Paco Remesal, un honor trabajar a vuestro lado y aprender tanto de caballos.

A mis niñas de Mérida: Anais, Chelo y Raquel. Porque no importan los kilómetros que nos separen, siempre siento vuestro calor y apoyo. Ha sido genial crecer a vuestro lado y haber compartido tantos y tantos momentos……me quedo con los felices, que han sido muchos y sé que vendrán muchos más!! A ti Inma, porque sigues viva en mi corazón.

A mi peña S.A. de Torre de Santa María. Sois IMPRESCINDIBLES en mi vida (especialmente vosotras, mis chicas). Porque unas birras en el Tioky con vosotros son la mejor inyección de positividad y felicidad. Hemos crecido juntos y ahora estamos viendo cómo crece el grupo con nuestros niños, que son la alegría!! Por muchos y muchos años más juntos, gracias por creer siempre en mi valía y sobre todo gracias por esa ENERGÍA.

A mis niñas de la carrera: Leticia, Pili, Rosario y Estelia. Grandes compañeras y grandes amigas que siempre estarán presentes en todos los momentos de mi vida. Por tantos momentos imborrables, especiales, felices, divertidos y por todos los que aún nos quedan por compartir y VIVIR!!

A ti Emiliano porque eres especial y para mí como un hermano. A vosotros Diego y David, porque siempre estáis ahí para apoyarme, aconsejarme, echarme la bronca cuando me la merezco y por ayudarme siempre. GRACIAS A LOS 3.

A ti Lalito, por compartir conmigo todos tus conocimientos equinos, por conseguir que me enamore cada día más de los caballos, por estar a mi lado, por todo tu cariño, por ser como eres…….GRACIAS.

A mis chicas de "la resi" Chon, Chin y Bea. Por todos nuestros grandes momentos vividos en la época de estudiante y por todos los que nos quedan por vivir juntas!! Aunque nos reunamos pocas veces, esas veces son siempre una gran felicidad. A ti Yolanda por las horas juntas en la Facultad y sobre todo por tantos y tantos ratos de conversación acompañadas de una buena Paulaner y mejor música!!

A mis chicos de Mérida, porque sois increíbles!! Conseguís que en el día más nublado salga el sol. Por muchos años más de cervecitas y raciones en "el Boca" y lo que surja……

A mi grupito de Madrid. Sois geniales todos y cada uno de vosotros. Desde el primer momento me he sentido una más y por eso os echo tanto de menos!! Os considero el aire fresco que llena de alegría y energía positiva tantos momentos de mi vida. Mil gracias Juanma por el diseño de la portada porque es realmente precioso. Por muchos años más juntos chicos!!

A ti Alfonso, por darme tanto amor y tranquilidad durante esos años, por creer en mi valía y porque siempre me has conocido mejor que yo a mi misma. Que seas muy feliz porque te lo mereces.

A ti Javi, por apoyarme, ayudarme y sentirte siempre orgulloso de mí. GRACIAS

A nuestra "otra familia". Nati y Jesús, Alicia y Pedro, Rosa y Juanjo y Ayo y Ángel, siempre habéis formado parte de nuestras vidas y nos habéis ayudado muchísimo, por ello gracias de todo corazón.

Vorrei anche ringraziare la sezione di Fisiologia della Facoltà di Medicina Veterinaria dell'Università di Bologna. Dal primo giorno che sono arrivata mi sono sentita amata e protetta da tutti voi. Grazie alla Professoressa Marcella Spinaci, Il mio capo (la mia zia Marci): abbiamo lavorato lunghe ore, ma ne è valsa la pena perché ho imparato molto al tuo fianco, è stato un piacere essere la tua discepola, sei bravissima!! Grazie alla Professoressa Giovanna Galeati, sempre molto gentile ed affettuosa con me ed al Professor Carlo Tamanini, con l'umorismo ironico che lo caratterizza e tanto mi piace; è stato un onore imparare e lavorare con eminenze come voi. Il Dottore Diego Bucci e la Dottoressa Elisa Giaretta, colleghi e sopratutto amici, per il nostro divertimento nel laboratorio e fuori, il mio soggiorno all'Università di Bologna non sarebbe stato lo stesso senza di voi due. Cinzia Cappannari, la mia bellezza bionda, sei una

persona meravigliosa e ho sempre apprezzato i tuoi consigli e tutto il tuo amore. Bea è stato poco tempo ma mi è piaciuto molto imparare e lavorare al tuo fianco, sei una ragazza dolce e simpatica, non cambiare mai. Ringrazio anche Il Professore Gaetano Mari per tutto l'aiuto dato coi cavalli. A Danilo, Antonio, Carmen e gli altri componenti del gruppo di Fisiologia, Luigi e le sue ragazze, a tutti voi per avermi dato sempre un sorriso, perché così è un piacere andare a lavorare ogni giorno.

I miei amici italiani. Chiara, perché tu sei speciale per me, ci capiamo perfettamente e hai saputo ascoltarmi e consigliarmi nel modo migliore, sei e sarai sempre la mia churritina, la mia sorella. Ilaria, grazie mille per tutto l'aiuto dato dal mio primo giorno a Bologna, non siamo riuscite a vederci molto ma mi sono piaciuti i nostri momenti insieme. Giulia, mi nena e la migliore coinquillina, grazie per i mesi che mi hai accolto in casa tua, sono stati meravigliosi i nostri momenti di birra/vino con le sigarrette e le chiacchiere nel nostro salotto, per quei giorni a XM24, non li menticarò mai. A voi Pamela, Rafik, Silvio ed Alessio, i miei amici, tanti momenti divertenti, cene, feste, chiacchiere a casa ....a te Pamela, amica mia, a te Rafik, amico mio, grazie mille per essere stati al mio fianco in ogni momenti felici e meno felici del mio soggiorno a Bologna, per essere sempre lì, per amarmi ed accettarmi come sono.

Quando sei lontano dal tuo paese e dalla tua gente è difficile…..ma trovare persone come voi, che ti fanno sentire come in famiglia è una delle cose più belle che una persona può sentire. Avete un posto speciale nel mio cuore e ci rimarrete per sempre. Sono innamorata di Bologna grazie a voi. Grazie mille di tutto……..ci vediamo presto ragazzi!!

No puedo terminar de escribir estas líneas sin agradecer a los protagonistas de esta Tesis, los caballos.

Como dije al inicio, ha sido muy complicado expresar por escrito todo lo que siento…….si me he olvidado de alguien ha sido involuntariamente, espero me disculpéis.

A mis padres

Tesis doctoral por compendio de publicaciones

1. **Inhibition of mitochondrial complex I leads to decreased motility and membrane integrity related to increased hydrogen peroxide and reduced ATP production, while the inhibition of glycolysis has less impact on sperm motility. (2015)**Plaza Davila M, Martín Muñoz P, Tapia JA, Ortega Ferrusola C, Balao da Silva C, Peña FJ. Plos One. Vol: 10 (9) e0138777. PUBLIC LIBRARY SCIENCE. San Francisco (USA).

2. **Epigallocatechin-3-gallate (EGCG) reduces rotenone effect on stallion sperm-zona pellucida heterologous binding. (2015)** Plaza Davila M, Bucci D, Galeati G, Peña F, Mari G, Giaretta E, Tamanini C, Spinaci M.Reproduction in Domestic Animals. Vol: 50 (6): 1011-1016. WILEY-BLACKWELL. Hoboken (Holland).

3. **Mitochondrial ATP is required for maintenance of membrane integrity in the stallion spermatozoa, while motility requires both glycolysis and oxidative phosphorylation. (2015)** Plaza Davila M, Martin Muñoz P, Gallardo Bolaños JM, Stout TAE, Gadella BM, Tapia JA, Ortega Ferrusola C, Balao da Silva C, Peña FJ. (submitted)

4. **The impact of reproductive tecnologies on stallion mitochondrial function. (2015)** Peña FJ, Plaza Davila M, Ball BA, Squires EL, Martín P, Ortega Ferrusola C. Reproduction in Domestic Animals Vol: 50 (4) 529-537.WILEY-BLACKWELL. Hoboken (Holland).

Ignacio Caballero Posadas
Equipe VIRIM

Centre Val de Loire
Tèl. : 02 47 42 78 65
Email: icaballerop@tours.inra.fr

Unité ISP - Site 311
Route de Crotelles
37380 Nouzilly
www.inra.fr

To whom it may concern

20th October 2015

Dear Sir/Madam

I have studied Ms **María Plaza Dávila** doctoral thesis entitled "Papel de la cadena mitocondrial de transporte de electrones en el desencadenamiento de la senescencia en el espermatozoide equino".

The present work presented in the thesis aims to decipher the role of oxidative phosphorylation in energy production, oxidative stress and senescence in stallion sperm. Although ATP generation, oxidative stress and senescence have been studied for years in other species, little is known at present in stallion. Unlike, boar, human, mouse and bull sperm, which rely on glycolysis, the data presented in this thesis indicates that oxidative phosphorylation is the main source of energy in stallion, while glycolysis has only a supportive role. It is also very interesting the concept that reactive oxygen species may not be a simply toxic by-product but they may have an important function on stallion sperm function.

In my opinion this thesis represents a cohesive scientific work of interest in the field of animal reproduction and a valuable contribution to increase our understanding of stallion sperm mitochondria. The data produced in this work may help in the future to improve stallion sperm extenders and the functionality of spermatozoa subjected to reproductive biotechnologies. The quality of the work is corroborated by the fact that it has already produced 3 publication (and a fourth has been submitted) in respected peer reviewed international scientific journals, being Ms María Plaza Davila the first author in 3 of the publications.

I have no hesitation to recommend this thesis for a doctoral degree.

Yours sincerely,

Ignacio Caballero Posadas

Carmen Almiñana Brines, PhD
Institut National de la Recherche Agronomique (INRA)
Physiologie de la Reproduction et des Comportements
UMR 7247 Inra-Cnrs-Université de Tours-
37380 NOUZILLY
France
Teléfono: + 33 2 47 42 7508
Email: calminana@tours.inra.fr

Nouzilly, 21 st October 2015

To whom it may concern

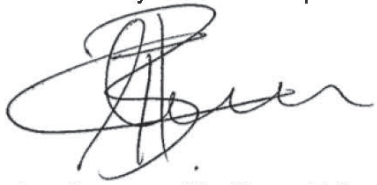
I have reviewed the work performed by **Miss María Plaza Dávila** for her PhD thesis entitled **"Papel de la cadena mitocondrial de transporte de electrones en el desencadenamiento de la senescencia en el espermatozoide equino".**

The thesis is of a coat type built upon four articles published in international ranked journals with peer-review system. Her work concerns the evaluation of the mitochondria in stallion sperm as key structures in sperm function and possible targets to optimize current sperm technologies in equine species. This task has been faced by different approaches: 1) Determining the importance of oxidative phosphorylation as the main source of ATP for stallion sperm motility; 2) Evaluating the mitochondrial electron transport chain in the origin of oxidative stress in stallion spermatozoa by using specific inhibitors of complex I (rotenone) and III (antimycin-A); and 3) Examining the response of equine sperm after inhibiting mitochondrial complex I by rotenone *during in vitro* capacitation conditions and evaluate the role of EGCG, a natural polyphenol component of green tea to counteract this effect.

The studies have been performed by well-designed experiments, which are presented and discussed in a clear way. The obtained results demonstrated that unlike other mammalian species, stallion spermatozoa rely on oxidative phosphorilation to generate energy for motility while glycolysis is only required to achieve high velocities. Moreover, the inhibition of mitochondrial complex I in stallion spermatozoa lead to decreased motility and the number of sperm bound to zona pellucida; Effect that could be counteracted by EGCG, suggesting a protective role of this green tea antioxidant.

This research work has led to the publication of three original papers as first author in respected peer reviewed international scientific journals. The obtained results represent an important step towards increasing our understanding of mitochondrial functional on stallion sperm quality that can be used in future developments of sperm conservation. In this regard, the thesis represents a significant contribution to the international efforts to optimize reproductive technologies such as cryopreservation and sex sorting, which caused sperm mitochondria damage.

On the basis of the above considerations, I fully support **Miss María Plaza Dávila** ´s candidacy to the European Doctorate.



Dr. Carmen Almiñana Brines

Índice

INDICE

Abreviaturas

ABREVIATURAS

2-DG: 2-Deoxiglucosa

2,4-DNP: 2,4-dinitrofenol

Acetil-CoA: Acetil Coenzima A

ADP: Adenosín Difosfato

ADN: Ácido Desoxirribonucleico

AMP_C: Adenosín Monofosfato cíclico

ATP: Adenosín 5'-trifosfato

BSA: Albúmina Sérica Bovina

CCCP: Carbonyl cyanide-m-chlorophenylhydrazone

CMTE: Cadena Mitocondrial de Transporte de Electrones

CRM: Cadena Respiratoria Mitocondrial

EGCG: Epigalocatequina-3-galato

EIM: Espacio Intermembrana

ERO_S: Especies Reactivas de Oxígeno

Eth.: Homodímero de Etidio

FAD: Flavín Adenín Dinucleótido

HEPES: Ácido N-2 (hidroxietil) piperazina N-2-etano sulfónico

IA: Inseminación Artificial

MM: Matriz Mitocondrial

MME: Membrana Mitocondrial Externa

MMI: Membrana Mitocondrial Interna

MT: Motilidad Total/Motiles Totales

MP: Motilidad Progresiva/Motiles Progresivos

NAD: Nicotinamida Adenina Dinucleótido

P_i: Fósforo inorgánico

PKA: Proteína Kinasa A

PUFA: Ácidos Grasos Poliinsaturados

TNF: Factor de Necrosis Tumoral

VAP: Velocidad Media

VCL: Velocidad Circular

VSL: Velocidad Rectilínea

ZP: Zona Pelúcida

Introducción

INTRODUCCIÓN

La inseminación artificial es una técnica que mejora la gestión de los eyaculados de sementales con alto valor genético, al permitir maximizar la distribución geográfica de las dosis seminales. Por lo tanto, es una herramienta imprescindible para el comercio de semen.

Los resultados de la inseminación artificial con semen fresco y refrigerado pueden superar a los obtenidos mediante monta natural, debido a un mejor control de todas las fases del proceso. Además, su uso ha promovido el comercio de dosis tanto nacional como internacional, evitando el traslado de animales.

El objetivo de la refrigeración del semen equino es prolongar la vida media de los espermatozoides y de este modo poder ser enviado para inseminar yeguas alojadas distantes del semental. Sin embargo, el semen equino en refrigeración dura viable pocos días, incluso horas dependiendo del individuo. El principal responsable de este descenso en la viabilidad se denomina shock por frío "cold shock" (Chantler et al., 2000). Además, experimenta daños como la peroxidación lipídica (White, 1993), depleción del ATP, o contaminación bacteriana (Ortega-Ferrusola et al., 2009a). Todo ello puede acelerar la muerte del espermatozoide durante la refrigeración.

Otra técnica que está facilitando el comercio de dosis seminales es la congelación, pero también acelera la muerte del espermatozoide. La criopreservación de los espermatozoides equinos permite almacenar el semen

durante períodos de tiempo mucho más largos. El inconveniente de esta técnica es que provoca la muerte, por término medio, de aproximadamente el 50% de los espermatozoides iniciales. Además de producir cambios sub-letales en la mayoría de los supervivientes (Gao et al., 1995; Vidament et al., 1997).

El principal problema que se presenta en el equino es la gran variabilidad existente entre sementales. Tanto la calidad de los eyaculados como la capacidad para la refrigeración se afectan por dicha variabilidad (Moran et al., 1992). Este hecho deriva del tipo de selección que se realiza en esta especie, basada en la búsqueda de determinados rasgos funcionales y morfológicos, sin prestar atención a la calidad seminal. Particularmente, la variabilidad influye en la crio-resistencia de los eyaculados. Se ha estimado que aproximadamente el 20% de los sementales son buenos congeladores, el 60% tiene una tolerancia aceptable al proceso y el 20% restantes son considerados malos congeladores (Gao et al., 1995; Vidament et al., 1997).

Por lo tanto, es posible que el semen fresco o refrigerado de sementales que presentan buena fertilidad, pueda tener, sin embargo, una pésima calidad tras la descongelación. La selección de los sementales en base a su calidad post-refrigeración y post-descongelación mejoraría el proceso, como ha ocurrido en el ganado bovino (Rath et al., 2009). Por otro lado, podría ser que estas diferencias no fueran sólo dependientes del individuo, por lo que es necesario realizar baterías de pruebas con el fin de clasificar al semental como buen o mal refrigerador o congelador, aunque todavía no existe una prueba de referencia que haya sido suficientemente estandarizada y de aplicación sencilla (Kuisma et

al., 2006). Los procesos de refrigeración y criopreservación provocan cambios moleculares en el espermatozoide que disminuyen su supervivencia. Principalmente el daño se asocia a cambios drásticos de temperatura (shock térmico), composición del medio extracelular (shock osmótico) y formación de especies reactivas de oxígeno (EROs) con origen en la mitocondria del espermatozoide (Ball, 2008).

1. EL ESPERMATOZOIDE EQUINO

El espermatozoide (del griego *esperma*, semilla, y *zoon*, animal) o gameto masculino, es una célula haploide, muy diferenciada y especializada, que tiene por función transportar el genoma masculino y fusionarse con el gameto femenino (el ovocito), para dar lugar a un nuevo individuo diploide y propagar así la especie.

Se origina en los túbulos seminíferos de los testículos en un proceso denominado espermatogénesis (Figura 1). Los túbulos seminíferos están tapizados por un gran número de células epiteliales germinales llamadas espermatogonias, que son células diploides que proliferan continuamente para mantener su número. Un porcentaje de estas células sufrirán una serie de modificaciones hasta transformarse en los gametos masculinos; el resto de espermatogonias actúan como células madre asegurando la producción continua de espermatozoides. Los espermatozoides, células haploides ya diferenciadas, carecen de capacidad fecundante, poseen escasa motilidad y

necesitan una última fase de maduración durante su tránsito por el epidídimo, para ser plenamente funcionales (Amann, 2008).

1.1 La espermatogénesis

Es el proceso de proliferación, maduración y diferenciación que tiene lugar en los túbulos seminíferos y mediante el cual las espermatogonias se transforman en espermatozoides. Consta de cuatro etapas consecutivas en las que las células primordiales cambian su morfología, pierden algunas de sus organelas y se transforman en células diferenciadas.

1.1.a La espermatocitogénesis

Poco antes de llegar a la pubertad, los gonocitos germinales se diferencian a espermatogonias. En esta primera etapa, las espermatogonias experimentan una serie de divisiones mitóticas para mantener su número (espermatogonias tipo A) en primer lugar, y para producir espermatogonias tipo B que se dividirán nuevamente, dando espermatocitos primarios.

1.1.b La espermatidogénesis

En esta segunda fase comienza la primera división meiótica, mediante la cual el espermatocito primario diploide origina dos espermatocitos secundarios haploides. Posteriormente, se producirá la segunda meiosis en estos espermatocitos secundarios originando las espermátidas, que son células de menor tamaño y más diferenciadas.

1.1.c La espermiogénesis

La espermátida esférica sufre una serie de cambios morfológicos hasta diferenciar la cabeza y la cola; sin alterarse ya la carga genética.

1.1.d La espermiación

En esta última fase se rompen las uniones de la espermátida madura y las células de Sertoli quedando liberada al lumen de los túbulos seminíferos. La espermátida pierde la mayor parte del citoplasma, su núcleo se condensa, y se produce la maduración del flagelo y del acrosoma.

La espermatogénesis tiene una duración de 57 días en el caballo. La participación de las células de Sertoli en la regulación y desarrollo de la espermatogénesis es de vital importancia, ya que proporcionan soporte estructural y nutricional. Además, las células de Sertoli intervienen en la formación de la barrera hemato-testicular, favorecen el desarrollo de las células germinales dentro del epitelio seminífero, participan en la descarga de espermátidas maduras en el proceso de espermiación, promueven la fagocitosis de las células degeneradas, segregan fluidos con proteínas para bañar las células germinales en desarrollo y facilitar el transporte de los espermatozoides a la rete testi, entre otras funciones (Amann, 2008; Johnson et al., 2008).

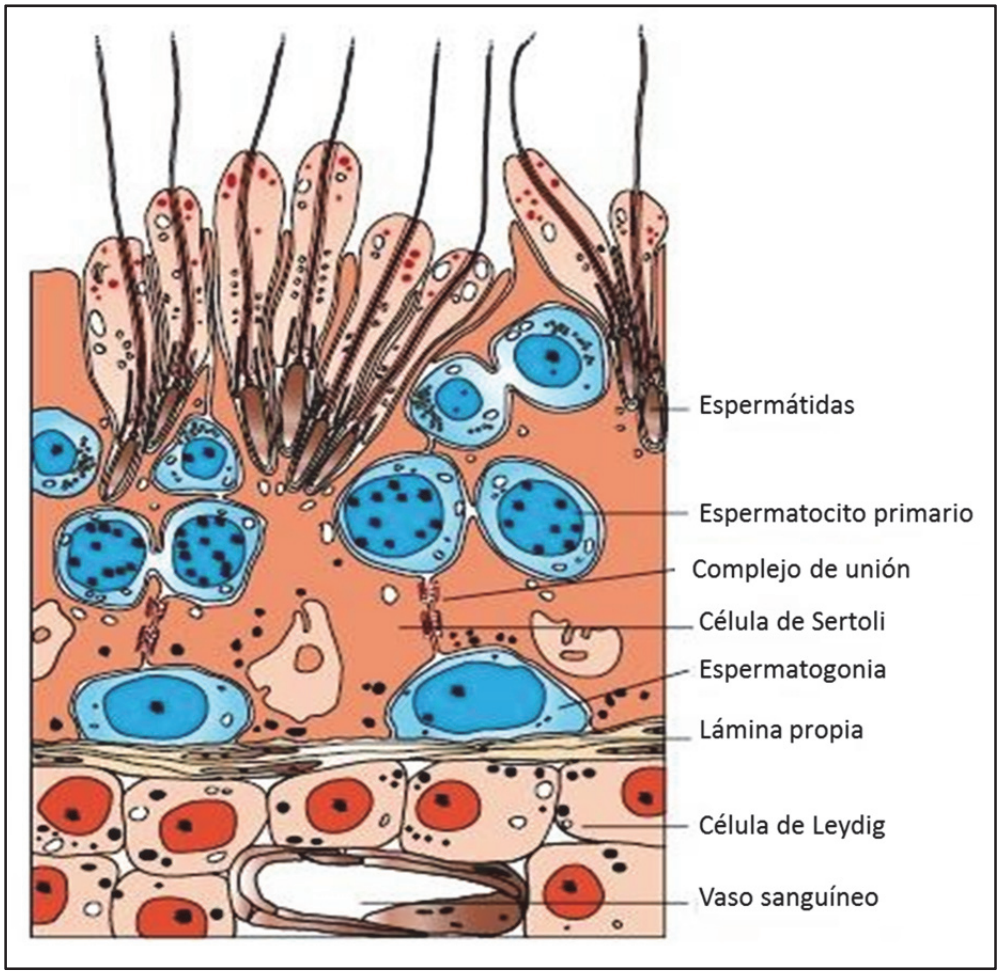


Figura 1. Organización celular del epitelio del túbulo seminífero. Modificada de (Samper, 2009).

1.2 Maduración espermática

El epidídimo es un conducto sinuoso (80 metros en caballos) que se divide en 3 segmentos: cabeza, cuerpo y cola. Está formado por un epitelio pseudo-estratificado compuesto por varios tipos celulares. El más importante es la *célula principal,* que constituye el 80% del epitelio. Este tipo celular es responsable de la producción de la mayoría de las proteínas que se secretan a la luz del epidídimo. Además, de forma similar a las células de Sertoli en los túbulos, las células principales crean una barrera inmunoprotectora necesaria para la correcta maduración de los espermatozoides (Cornwall, 2009).

El epidídimo no es solo un conducto de transporte para los espermatozoides, sino que aporta un medio adecuado en el que se concentran, maduran e inician el proceso de adquirir capacidad fecundante. Los espermatozoides procedentes de la *rete testi* migran a través de la cabeza y el cuerpo del epidídimo donde experimentan modificaciones morfológicas y funcionales moduladas por las secreciones procedentes de las células epiteliales que lo componen. Al conjunto de todas estas modificaciones se las denomina "maduración epididimaria". En la mayoría de los mamíferos, el espermatozoide tarda de 2 a 5 días en atravesarlo. Una vez que los espermatozoides completan el tránsito se almacenan en la cola del epidídimo hasta la eyaculación. Finalmente, los espermatozoides que alcanzan la cola del epidídimo forman un reservorio espermático conocido como "reservas extragonadales" (Toshimori, 1998; Tulsiani, 2006).

1.3 Regulación de la espermatogénesis

El mantenimiento de los túbulos seminíferos y por tanto de la espermatogénesis se consigue mediante el equilibrio en la proliferación y degeneración celular (Franca et al., 2005; Johnson et al., 2008). La apoptosis o muerte celular programada, tiene un papel importante en la regulación de la espermatogénesis de todos los mamíferos, incluido el caballo (Heninger et al., 2004). En el testículo, la apoptosis controla el número de espermatozoides que se producen, eliminando los anormales y en definitiva controlando la eficiencia final del proceso (Franca et al., 2005; Johnson et al., 2008). Este fenómeno puede afectar a las células germinales en todas sus fases siendo más frecuente en

espermatogonias y espermatocitos. Se estima que un 75% de las espermatogonias tipo A y un 25% de espermatocitos y espermátidas degeneran por apoptosis de forma fisiológica en los testículos de un animal adulto (Levy and Seifer-Aknin, 2001; Franca et al., 2005).

2. ESTRUCTURA DEL ESPERMATOZOIDE EQUINO

Desde el punto de vista funcional, es un transportador de la información genética. El espermatozoide de los mamíferos consta de cinco regiones: cabeza, cuello, pieza intermedia, pieza principal y pieza terminal (Figura 2). Destacan la presencia de un núcleo muy condensado, una membrana plasmática muy sensible a los cambios térmicos y osmóticos y las mitocondrias (Neill, 2006).

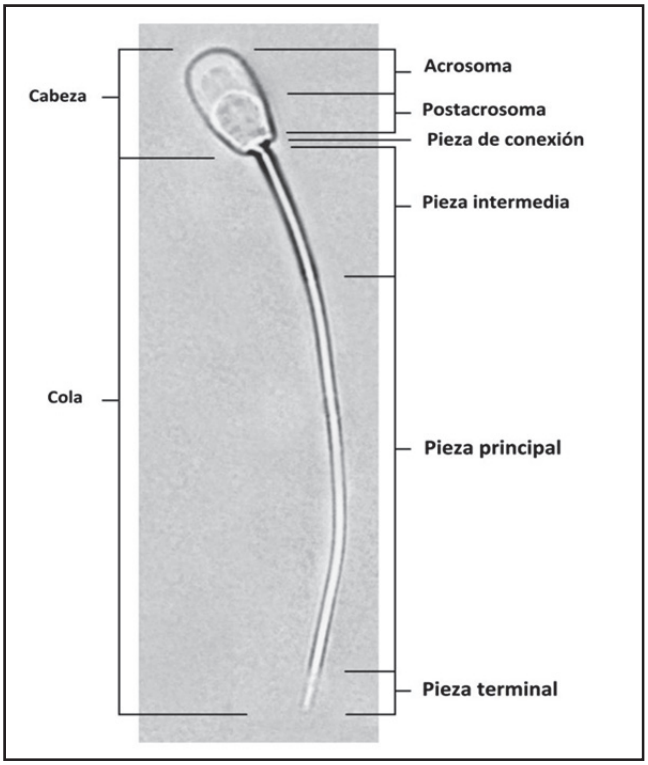


Figura 2. Estructura del espermatozoide.Modificada de (Tapia and Peña, 2009).

2.1 La membrana plasmática

Es una doble capa lipídica, que recubre al espermatozoide. En condiciones normales, los grupos hidrófilos (cabezas) de los fosfolípidos de la membrana se disponen formando las capas externa e interna de la bicapa lipídica, mientras que las colas hidrófobas se mantienen entre ambas capas. Los lípidos predominantes son los fosfolípidos y el colesterol. La composición de fosfolípidos de membrana y la relación colesterol/fosfolípidos difiere entre especies (en el caballo aproximadamente el 70% son fosfolípidos, el 25% lípidos neutros y un 5% son glicolípidos) (Garcia et al., 2011). La membrana plasmática consta además de una serie de proteínas integradas entre los lípidos. Estas proteínas actúan a modo de poros o canales, como receptores de unión a determinadas estructuras del aparato genital femenino (oviducto) (Parks and Lynch, 1992).

2.2 La cabeza

La cabeza contiene el acrosoma, el núcleo, estructuras del citoesqueleto y una pequeña cantidad de citoplasma. En función de la localización del acrosoma, en la cabeza se pueden distinguir varias regiones, siendo las principales la región apical, la región ecuatorial y la región post-acrosómica. La región apical está situada en la parte anterior de la cabeza y contiene el acrosoma, en el extremo contrario se encuentra la región post-acrosómica y entre estas dos regiones se encuentra la región ecuatorial (Boerke et al., 2008) (Figura 3).

El núcleo es la estructura que ocupa la mayor parte de la cabeza del espermatozoide y se encuentra rodeado por el acrosoma. Al contrario que ocurre en la células somáticas, las proteínas asociadas mayoritariamente al ADN son las protaminas, que se unen entre sí mediante puentes disulfuro dando lugar a una alta compactación de la cromatina (Miller et al., 2005; Neill, 2006)

El acrosoma es una estructura vesicular derivada del aparato de Golgi que se forma durante las últimas fases de la espermatogénesis. Está formado por una membrana acrosómica externa adyacente a la membrana plasmática que rodea a todo el espermatozoide, y una membrana acrosómica interna adyacente a la membrana nuclear. En su interior se encuentran diversas enzimas hidrolíticas que se liberarán al unirse el espermatozoide a la zona pelúcida del ovocito en la denominada "reacción acrosómica". Esto permite la degración de la zona pelúcida y la subsiguiente fusión del espermatozoide con el ovocito y, por tanto, la fecundación (Abou-haila and Tulsiani, 2009).

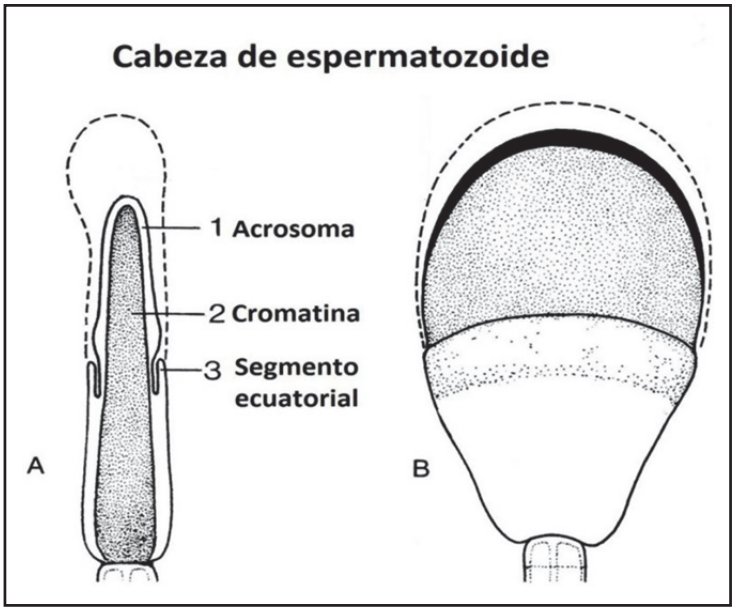


Figura 3. Cabeza del espermatozoide equino. Modificada de (Neill, 2006).

2.3 El flagelo

Es el responsable del movimiento del espermatozoide y lo forman cuatro regiones: la pieza conectora, la pieza intermedia, la pieza principal y la pieza terminal (Mortimer, 1997; Inaba, 2003) (Figura 4).

2.3.a La Pieza Conectora (PC)

El cuello o pieza conectora del espermatozoide es la unión entre la cabeza y la pieza intermedia. Está constituida por el *capitulum,* estructura fibrosa y densa que se ajusta a la fosa de implantación y es la parte principal de la pieza conectora, el *centriolo proximal* y una serie de *columnas laminadas* que proporcionan gran flexibilidad al espermatozoide para moverse lateralmente durante la batida flagelar (Neill, 2006) (Figura 5).

2.3.b La Pieza Intemedia (PI)

La forman, desde dentro hacia fuera, el axonema, las fibras densas, la vaina mitocondrial y la vaina fibrosa.

El axonema se organiza en 2 microtúbulos centrales rodeados por 9 pares de microtúbulos periféricos (denominados A y B en cada par), constituyendo el complejo "9+2". Los microtúbulos están formados por α-tubulina y β-tubulina, de 56 y 54 kDa respectivamente. Toda la red de microtúbulos, centrales y periféricos, se mantienen unidos entre sí por brazos de dineína (Gibbons and Grimstone, 1960; Hecht et al., 1984).

A continuación encontramos las fibras densas, que son nueve en total y rodean al axonema formando el complejo "9+(9+2)". Están compuestas por proteínas ricas en cisteína y por otros aminoácidos como la prolina, serina o treonina. Se distribuyen a lo largo de la pieza intermedia y tienen forma de lágrima, encontrándose su parte más gruesa en la sección proximal de la PI. Esta estructura confiere propiedades elásticas a la cola del espermatozoide, además de fuerza y resistencia durante la batida (Calvin and Bedford, 1971; Phillips, 1972; Bedford and Calvin, 1974; Fawcett, 1975).

La vaina mitocondrial está constituida por mitocondrias que envuelven helicoidalmente a las fibras densas. Se distribuyen a lo largo de la PI, pero el número de hélices y de giros varían entre especies. La longitud de la pieza intermedia está relacionada con la longitud del espermatozoide y en cada especie animal es diferente. En la mayoría de los mamíferos oscila entre 8 y 12 μm, sin embargo en la rata macho es de 64 μm y en el hámster chino de 100 μm. El número de giros de la vaina mitocondrial depende de la longitud de la PI, así por ejemplo, en el espermatozoide de toro encontramos 64 giros y en la rata macho 362 giros (Phillips, 1977). Las mitocondrias, además de ser las responsables del metabolismo energético y de la regulación de la muerte celular, se ha comprobado que son la mayor fuente de ERO_S (Koppers et al., 2010)

Por último, la vaina fibrosa está formada por dos columnas longitudinales, paralelas y contrapuestas que se distribuyen a lo largo de la PI en su parte más superficial. Ambas columnas están formadas por diferentes proteínas,

siendo A-kinase anchor protein 3 (AKAP3), A-kinase anchor protein 4 (AKAP4) y Testi-specific A-kinase-anchoringprotein 80 (TAKAP-80) las más importantes (Mandal et al., 1999; Vijayaraghavan et al., 1999). Además, se mantienen unidas a través de filamentos estrechamente fusionados que envuelven a la vaina mitocondrial y al resto de estructuras internas de la pieza intermedia. La función principal de la vaina fibrosa es modular el plano de la batida flagelar oponiendo resistencia al deslizamiento de los microtúbulos del axonema, además de limitar la flexión del flagelo y proteger todas las estructuras internas que componen la PI (Fawcett, 1975; Eddy et al., 2003).

2.3.c La Pieza Principal (PP)

Es la porción mayor de la cola. Está formada por el axonema, las nueve fibras densas y la vaina fibrosa, que se continúan desde la pieza intermedia. Las fibras van reduciendo su tamaño hasta desaparecer al final de la PP (Neill, 2006).

2.3.d La Pieza Terminal (PT)

Forma la pieza final de la cola, y está formada por el axonema, sin vaina fibrosa (Neill, 2006).

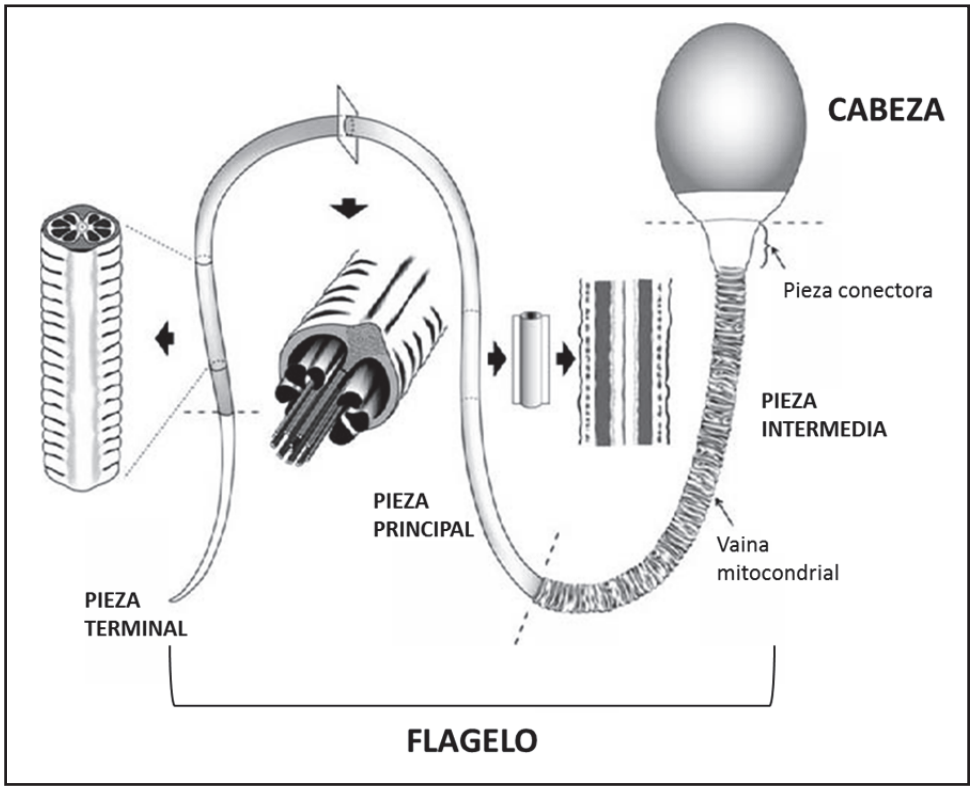


Figura 4. Estructura del flagelo del espermatozoide. Modificada de (Neill, 2006).

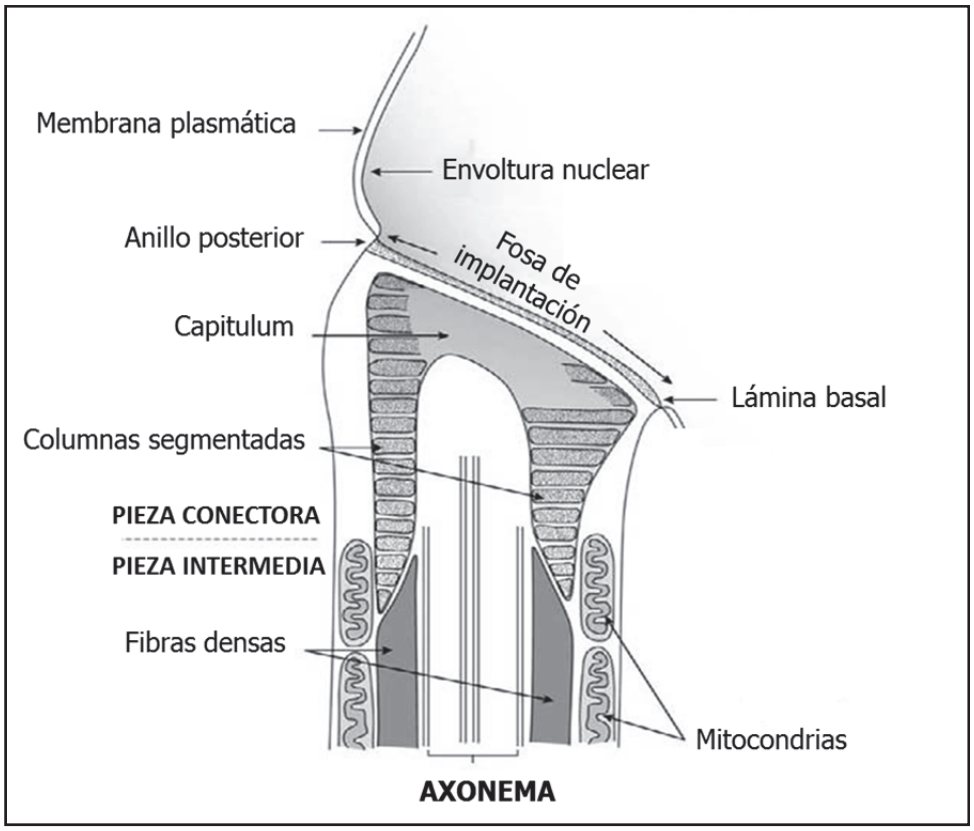


Figura 5. Estructura de la pieza conectora del espermatozoide. Modificada de (Neill, 2006).

3. FISIOLOGÍA ESPERMÁTICA

La función del espermatozoide es el transporte del material genético masculino, la penetración del ovocito y la fusión con su ADN. El espermatozoide de los mamíferos es incapaz de fecundar el ovocito tras la eyaculación. Durante su tránsito por el aparato reproductor femenino, experimenta una serie de transformaciones denominadas "capacitación", que consisten básicamente, en cambios en la fluidez y la permeabilidad iónica de la membrana, en un cambio en el patrón de motilidad y en la activación de vías de señalización intracelular dependientes de la AMPc/PKA (Gadella et al., 2001; de Lamirande and O'Flaherty, 2008). Estos espermatozoides capacitados son ya capaces de alcanzar el ovocito, unirse a la zona pelúcida y experimentar un nuevo cambio (la reacción acrosómica). Las proteasas liberadas por el acrosoma, mediante exocitosis, degradan las glicoproteínas que envuelven la membrana plasmática del ovocito, permitiendo que el espermatozoide fusione su núcleo con el del gameto femenino (Gadella et al., 1999; Gadella, 2008).

3.1 Motilidad espermática

La motilidad es una de las características funcionales básicas del espermatozoide. Existen dos tipos de movimiento espermático, movimiento activo e hiperactivo. El movimiento activo es propio de los espermatozoides eyaculados y cuya finalidad es avanzar por el tracto reproductor de la hembra (Turner, 2006). Este movimiento se caracteriza por ser simétrico, progresivo y con baja amplitud de onda. El movimiento hiperactivo, es el que experimentan

los espermatozoides capacitados. La batida flagelar es asimétrica y de mayor amplitud con objeto de penetrar en la membrana del ovocito (Yanagimachi, 1970).

Como anteriormente dijimos, la estructura que le confiere motilidad al espermatozoide es el axonema. Éste necesita ATP para poder realizar el movimiento de la cola. Las mitocondrias son las organelas encargadas de la producción de energía en el espermatozoide mediante fosforilación oxidativa. Sin embargo, su distribución se encuentra limitada al tracto intermedio. Turner y colaboradores en el 2006 (Turner, 2006), demostraron que el ATP producido en las mitocondrias era suficiente para permitir la motilidad y que el ATP tendría que difundirse hasta alcanzar la totalidad de la cola. Sin embargo, diversos estudios han comprobado que el metabolismo aeróbico no es el único que proporciona energía al espermatozoide (Escalier, 2006; Pena et al., 2009). El ATP puede provenir también de la glucólisis. La utilización de una vía u otra va a depender de la especie y de las condiciones del espermatozoide. Así por ejemplo, el espermatozoide humano mantiene un alto rango de glucólisis incluso durante la respiración aeróbica, y es capaz de mantener la motilidad en ausencia de oxígeno y/o en presencia de inhibidores mitocondriales, por lo que sólo depende de forma muy limitada de la respiración aeróbica (Williams and Ford, 2001). Por tanto, el espermatozoide es capaz de generar energía por ambas vías metabólicas, aeróbica y anaeróbica.

3.2 La maquinaria contráctil del axonema

El axonema es la estructura responsable de la motilidad del espermatozoide. Está compuesto por proteínas estructurales del citoesqueleto, proteínas motoras, chaperonas, elementos de regulación como las proteínas de unión a calcio y proteínas cinasas y fosfatasas (Inaba, 2007).

Las dineínas son el "motor molecular" del axonema (Figura 6). Cuando los brazos de dineína se fosforilan, las ATPasas de dineína se activan e hidrolizan ATP, provocando un cambio en su conformación que origina el movimiento (Tash, 1989). Para ello, los brazos de dineína interaccionan con sus dobletes de microtúbulos adyacentes y generan un movimiento que causa que los microtúbulos se deslicen uno sobre otro. Debido a que el axonema está anclado a la base de la cabeza, esta fuerza de deslizamiento global se transforma en un giro en el flagelo que, finalmente, genera el desplazamiento del espermatozoide (Satir, 1968; Brokaw, 1989).

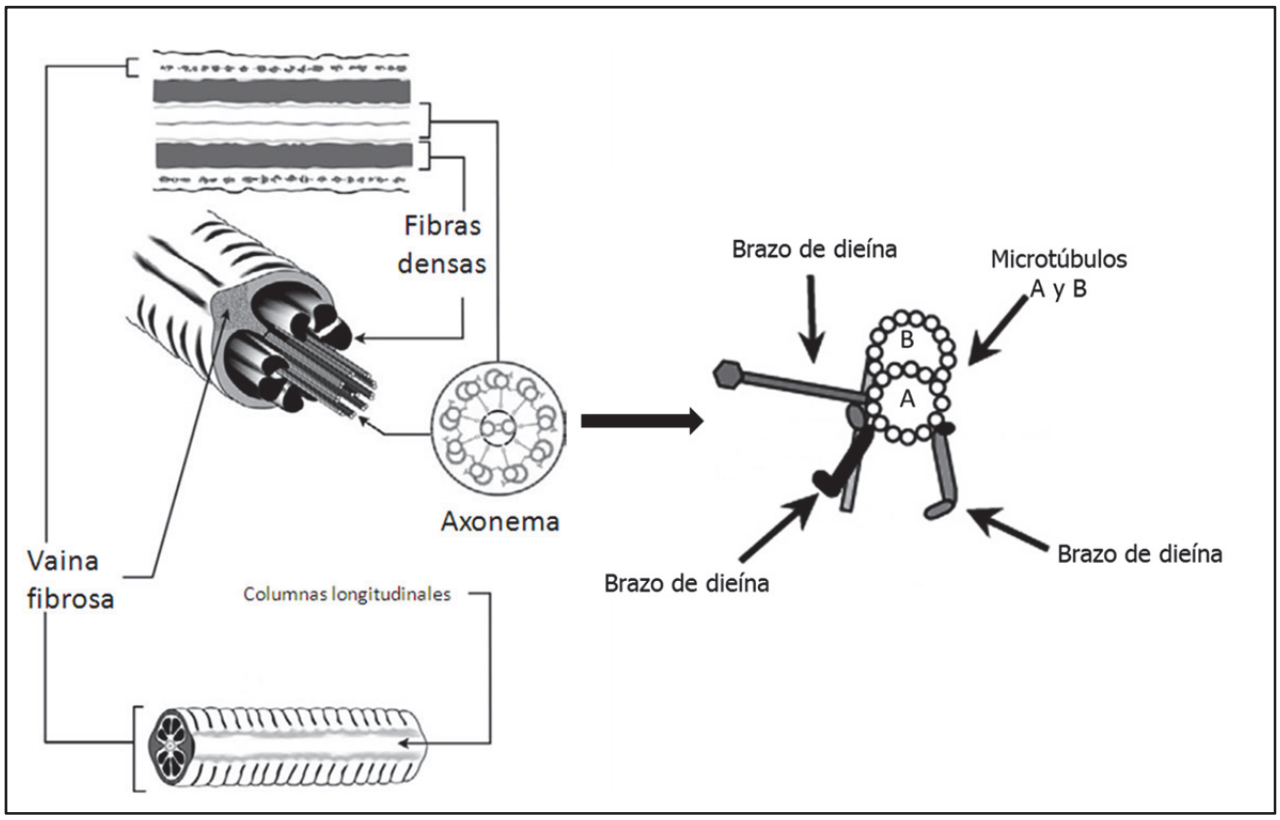


Figura 6. Componentes del citoesqueleto del flagelo (izda.) y detalle del axonema (dcha.) Modificada de (Barratt, 2006) y (Neill, 2006).

3.3 Capacitación

Durante la capacitación el espermatozoide experimenta cambios morfológicos y funcionales hasta adquirir capacidad de fecundar el ovocito (Aitken et al., 1997; de Lamirande et al., 1997). Este proceso ocurre "in vivo", durante el tránsito del espermatozoide por el aparato reproductor de la hembra. Entre otros cambios, tiene lugar la pérdida de proteínas decapacitantes que envuelven al espermatozoide y que provienen del plasma seminal.

Además, se produce un cambio en la distribución de los lípidos de las membranas, necesario para el reconocimiento y fusión con el ovocito. La albúmina y lipoproteínas de alta densidad presentes en medios de cultivo o en

el tracto reproductivo de la hembra, extraen el colesterol de la membrana del espermatozoide, y la hace más permeable a iones (Suzuki and Wakabayashi, 1988). Posteriormente se produce un aumento del calcio intracelular, de bicarbonato y de peróxido de hidrógeno, que activan la adenil-ciclasa para producir AMPc. El aumento de AMPc activa la PKA, que finalmente acaba por fosforilar las proteínas necesarias para la capacitación (de Lamirande et al., 1997).

3.4 Reacción acrosómica

La reacción acrosómica es un proceso de exocitósis mediante el que se liberan enzimas hidrolíticas presentes en el acrosoma. Estas enzimas degradan la zona pelúcida (ZP) del ovocito para permitir la fusión del ADN del espermatozoide con el gameto femenino (Neill, 2006). Esta reacción sólo se produce tras la capacitación espermática. La ZP del ovocito está constituida por 3 tipos de glicoproteínas (ZP1, ZP2, ZP3). La ZP3 es la que desencadena un aumento de pH y la activación de una serie de receptores desencadenando la reacción acrosómica (Bleil and Wassarman, 1980).

Durante la reacción acrosómica se produce una fusión de la membrana plasmática y la acrosomal externa. La fusión de las membranas provoca la formación de vesículas y la liberación progresiva del contenido acrosomal. La reacción es lenta y está regulada por cambios en la membrana y modificaciones en la distribución y composición de los lípidos de la membrana plasmática, que conducen a un aumento en la fluidez. Una de las transformaciones es la eliminación de colesterol, que permitirá la entrada de calcio extracelular. Todo

ello es necesario para la fusión de las membranas. Por último, la liberación de las enzimas hidrolíticas junto con la hiperactivación espermática hacen posible que el espermatozoide penetre en el ovocito y lo fecunde (Abou-haila and Tulsiani, 2009).

4. LA MITOCONDRIA

Las mitocondrias han captado el interés de los bioquímicos desde hace más de 50 años. Últimamente se han trasladado a áreas muy importantes del saber, como el estudio de la apoptosis, la biología molecular y la medicina evolutiva. Originalmente, fue la constatación de que juegan un papel central en el metabolismo energético de la célula lo que atrajo la atención de fisiólogos celulares y bioquímicos, y lo que condujo al Premio Nobel Peter Mitchell a desarrollar la Teoría Quimiosmótica (Mitchell, 1967).

Las mitocondrias generan la mayor parte del ATP que necesita la célula, también intervienen en la diferenciación y señalización celular, almacenamiento de Ca^{2+}, apoptosis y muerte celular, así como el control del ciclo y crecimiento celular (Voet, 2006). Muchos de estos procesos son desencadenados o mediados por el Ca^{2+}, por ERO_S o por ambos. El número de mitocondrias varía ampliamente dependiendo del tipo de organismo o tejido y de los requerimientos energéticos de sus células. En el hígado hay aproximadamente 1000-2000 mitocondrias por célula, sin embargo, en las células del miocardio, los túbulos contorneados distales del riñón, músculo esquelético y en otras

células que necesitan una mayor cantidad de energía, son mucho más abundantes (Rappaport et al., 1998; Zhao et al., 2003).

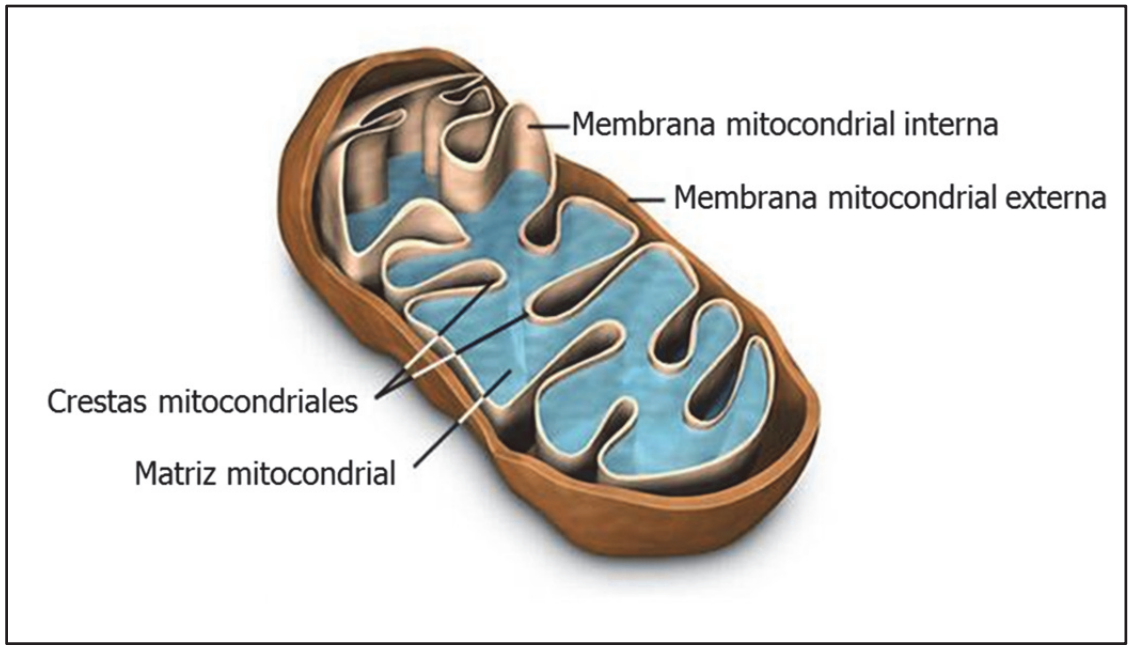


Figura 7. Esquema de la mitocondrial y sus diferentes compartimentos. Modificado de (Krauss, 2001).

4.1 Estructura

Son orgánulos celulares de aproximadamente 0.5-1 μm de diámetro y hasta 7 μm de longitud, rodeados de una doble membrana. La mitocondria está constituida por la membrana mitocondrial externa (MME), el espacio intermembrana (EIM), membrana mitocondrial interna (MMI), las crestas mitocondriales y la matriz mitocondrial (MM) (Rappaport et al., 1998) (Figura 7). La MME tiene una relación de proteínas/fosfolípidos similar a la de la membrana plasmática (50:50) y contiene un gran número de proteínas

integrales llamadas "porinas" que forman canales para permitir el paso de moléculas de más de 5000 Daltons (Rappaport et al., 1998; Krauss, 2001). Las proteínas más grandes también pueden entrar en la mitocondria si una secuencia de señalización en su extremo N-teminal se une a las traslocasas (complejo proteico de transporte de la MME). La alteración de la MME permite la fuga de las proteínas del EIM al citosol, dando lugar a la muerte celular (Chipuk et al., 2006). Debido a que la MME es completamente permeable a moléculas de pequeño tamaño, como iones y azúcares, sus concentraciones son similares en el EIM y en el citosol (Rappaport et al., 1998). Sin embargo, como las proteínas grandes deben tener una secuencia de señalización específica para poder ser transportadas a través de la MME, no todas consiguen entrar en la mitocondria, por ello la composición es diferente entre el EIM y el citosol. Una proteína que se localiza de ésta manera en el EIM es el citocromo c (Chipuk et al., 2006).

La MMI contiene proteínas que realizan varias funciones, como las reacciones de óxido-reducción o REDOX de la fosforilación oxidativa, regulan el transporte de metabolitos desde la matriz mitocondrial (porinas) y además en ella se encuentra la ATP-sintasa (Rappaport et al., 1998; McBride et al., 2006). La proporción de proteínas/fosfolípidos es de 80:20 y es rica en el fosfolípido cardiolipina. Dicho fosfolípido contiene cuatro ácidos grasos que le confieren impermeabilidad a la MMI (Krauss, 2001; Voet, 2006). A diferencia de la MME, la MMI no contiene porinas y es muy poco permeable, de modo que todos los iones y moléculas requieren transportadores de membrana para entrar o salir de la matriz mitocondrial (Rappaport et al., 1998).

4.2 Características de las mitocondrias

La morfología, la localización y el metabolismo energético de las mitocondrias cambian marcadamente durante la espermatogénesis. En el epitelio germinal se distinguen tres tipos diferentes de mitocondrias (Ramalho-Santos et al., 2009). Las mitocondrias "ortodoxas", ovoidales y con las crestas mitocondriales laminares, se encuentran en las espermatogonias y espermatocitos tempranos. Las mitocondrias "condensadas", con las crestas mitocondriales replegadas, son consideradas las más eficientes y están en espermatocitos tardíos y espermátidas (Figura 8). Por último, las mitocondrias "intermedias" las podemos encontrar tanto en espermatocitos primarios como tardíos, y como su propio nombre indica, es una estructura de evolución entre las ortodoxas y las condensadas (De Martino et al., 1979; Amaral et al., 2013).

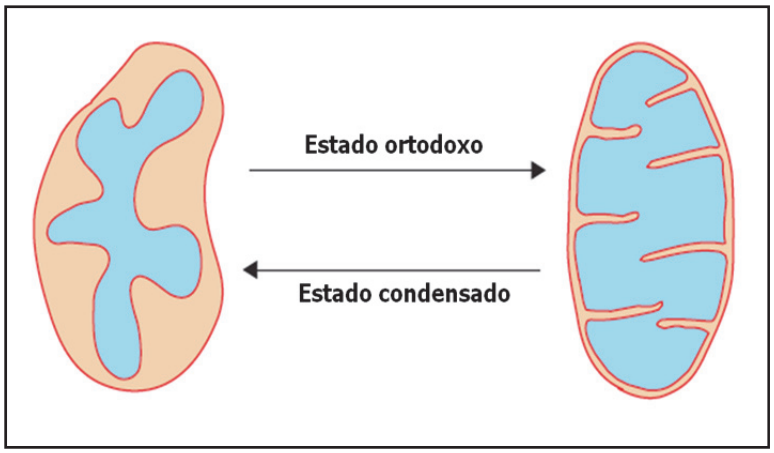


Figura 8. Cambio en la configuración de las mitocondrias. Modificada de (Paniagua R, 2007).

Durante la espermiogénesis, las mitocondrias se organizan en estructuras tubulares helicoidales ancladas alrededor de la porción anterior de nueve pares de fibras densas y del axonema formando la pieza intermedia. Los complejos filamentosos llamados retículo sub-mitocondrial sostienen el anclaje de la vaina mitocondrial (Olson and Winfrey, 1990; 1992). Una estructura de queratina denominada "cápsulas mitocondriales" cubre la MME de las mitocondrias espermáticas. Está formada por puentes disulfuro entre moléculas de cisteína y de prolina rica en selenoproteínas (Ursini et al., 1999). Todo esto puede conferir protección a las mitocondrias y al ADN mitocondrial (Amaral et al., 2013).

4.3 Función

Las mitocondrias son elementos muy importantes en la muerte celular programada, ya que regulan de forma activa la apoptosis tanto en levaduras, como en eucariotas inferiores y células humanas (Kroemer et al., 2007). Son capaces de emitir señales de peligro, como la producción de ERO_S, dentro de la célula, para alertar de perturbaciones en la homeóstasis mitocondrial (Krysko et al., 2011). Además, el incremento de la permeabilidad de la MME representa un punto de no retorno que conduce a la muerte de la célula (Tait and Green, 2010; Galluzzi et al., 2012d). Dicho incremento ocurre en una gran variedad de patologías incluyendo enfermedades infecciosas, condiciones isquémicas, alteraciones neurodegenerativas y cáncer (Kroemer et al., 2007; Tait and Green, 2010). En la mitocondria, el potencial de membrana del EIM lo produce la actividad de las enzimas de la cadena mitocondrial de transporte de

electrones (CMTE), también llamada cadena respiratoria mitocondrial (CRM) de la MMI y por el ciclo de Krebs de la matriz mitocondrial (también conocido como ciclo de los ácidos tricarboxílicos o ciclo del ácido cítrico) (Zhao et al., 2003; McBride et al., 2006). Cada molécula de piruvato producida en la glucólisis anaerobia es transportada desde el citosol a través de la MMI hacia la matriz mitocondrial donde sufre una descarboxilación oxidativa y se une a la coenzima A para formar CO_2, acetil-CoA y poder reductor en forma de NADH (Voet, 2006).

El acetil-CoA es el sustrato primario para entrar en el ciclo de Krebs. Las enzimas de dicho ciclo están situadas en la matriz mitocondrial, con la excepción de la succinato deshidrogenasa, que está unida a la MMI formando parte del complejo II de la CMTE. El ciclo de Krebs oxida la acetil-CoA a CO_2, y produce tres moléculas de NADH (mediante la isocitrato deshidrogenasa, cetoglutarato deshidrogenasa y malato deshidrogenasa) y una molécula de $FADH_2$ (mediante la succinato deshidrogenasa). Estas moléculas son una fuente de transporte de electrones a lo largo de los complejos respiratorios de la CRM. Además, el ciclo de los ácidos tricarboxílicos produce una molécula de GTP que se convierte fácilmente en ATP (Voet, 2006).

La CMTE está compuesta por cinco complejos (I-V). Las proteínas que forman estos complejos interactúan de forma específica para formar estructuras supramoleculares denominadas "supercomplejos respiratorios" o "respirasomas" (Dudkina et al., 2008). La NADH deshidrogenasa (complejo I), la succinato deshidrogenasa (complejo II), la citocromo c reductasa (complejo III) y

lacitocromo c oxidasa (complejo IV) realizan la transferencia de electrones y liberación gradual de la energía que se utiliza para la bomba de protones en el EIM (Voet, 2006) (Figura 9). A medida que la concentración de protones aumenta en el EIM, se establece un gradiente electroquímico a través de la MMI. Los protones pueden volver a la matriz mitocondrial mediante el complejo ATP-sintasa (complejo V), y su energía se utiliza para sintetizar ATP a partir de ADP y fosfato inorgánico (P_i) (Voet, 2006). Este proceso, llamado quimiosmosis, fue descrito por primera vez por Peter Mitchell (Mitchell, 1967) que fue galardonado por su trabajo con el Premio Nobel de Química en 1978, como hemos mencionado anteriormente.

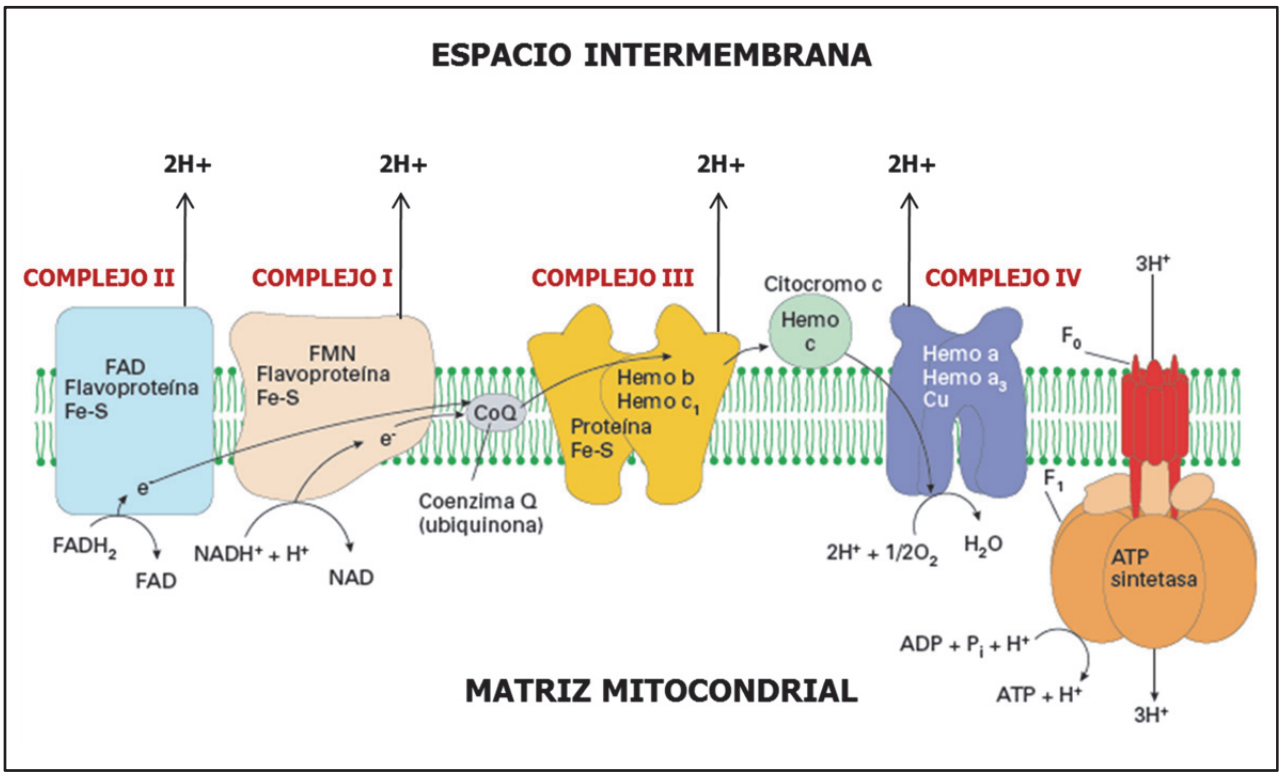


Figura 9. Cadena mitocondrial de transporte de electrones. Modificada de (Paniagua R, 2007).

5. LA RESPIRACIÓN CELULAR: CADENA DE TRANSPORTE DE ELECTRONES MITOCONDRIAL Y FOSFORILACIÓN OXIDATIVA

La mitocondria es el principal orgánulo encargado de la producción de energía en la célula. Dicho orgánulo produce ATP mediante la fosforilación oxidativa. Esta vía es más eficiente que la glucólisis; de una molécula de glucosa se obtienen 36 moléculas de ATP en la fosforilación oxidativa y 2 moléculas de ATP en la glucólisis (Johnstone et al., 2002).

La fosforilación oxidativa se realiza a través de la CMTE y la ATP-sintasa. Los componentes de dicha vía están embebidos en la MMI. Existen dos transportadores de electrones (coenzima Q y citocromo c) y cinco complejos enzimáticos (I-V), como hemos comentado anteriormente. El complejo I (NADH ubiquinona oxidorreductasa) está compuesto por la NADH deshidrogenasa, su grupo prostético flavin mononucleótido (FMN) y centros Fe-S. El complejo II (Succinato ubiquinona oxidorreductasa) está constituido por el citocromo b, tres centros Fe-S y el FAD (flavin adenin dinucleótido). El complejo III (ubiquinol citocromo c oxidorreductasa) está formado por un centro Fe-S y los citocromos b y c_1. El complejo IV (citocromo c oxidasa) está compuesto por CuA, CuB, citocromo a y citocromo a_3. El complejo V es la denominada ATP-sintasa o F_1-F_0asa (Johnstone et al., 2002).

El $NADH^+$ y el $FADH_2$ ceden sus dos electrones, que son conducidos por la CMTE, separándose de los protones. Al final de la cadena se reúnen de nuevo

los electrones con los protones y el O_2 para generar H_2O y ATP, a partir de ADP y P_i, a través de la ATP-sintasa (Johnstone et al., 2002).

5.1 COMPLEJO I (NADH deshidrogenasa o ubiquinona oxidorreductasa) y COMPLEJO II (Succinato deshidrogenasa o Succinato ubiquinona oxidorreductasa)

Los complejos I y II son las vías de entrada de los electrones a la cadena respiratoria mitocondrial. El complejo I es un complejo multienzimático donde la FMN cataliza la transferencia de electrones del NADH (procedente del ciclo de Krebs) a la coenzima Q (ubiquinona Q) en la cadena respiratoria. El NADH utiliza la coenzima Q como aceptor de dos electrones, y una vez transferidos, se bombean dos protones al EIM generando un gradiente electroquímico y fuerza motriz de protones por cada par de protones transferidos. El complejo II o succinato deshidrogenasa (que forma parte del ciclo de Krebs) reduce el FAD a $FADH_2$ introduce dos electrones en la CMTE o CRM y bombea dos protones al EIM.

Aunque el mecanismo real del bombeo de protones no es del todo conocido, sabemos que al final de la CMTE, la energía producida por el transporte se utilizará para el funcionamiento de la ATP-sintasa. En primer lugar dará paso a los protones concentrados en el EIM hacia la matriz mitocondrial para unirse al O_2 y formar H_2O, y en segundo lugar la fosforilación del ADP y posterior conversión en ATP ($ADP+P_i \rightarrow ATP+H_2O$), acumulándose en la matriz mitocondrial (Lambert and Brand, 2004b).

El complejo I es probablemente el lugar donde se producen más ERO_S de la mitocondria (Zoccarato et al., 2007). Se han propuesto como fuentes de producción de ERO_S varios sitios entre la FMN y la ubiquinona Q dentro del complejo I (Chance et al., 1979; Herrero and Barja, 2000; Kushnareva et al., 2002; Liu et al., 2002; Genova et al., 2003; Verkhovskaya et al., 2008). Uno de los lugares y fuente más probable podría ser la flavoproteína Fe-S (Herrero and Barja, 2000; Genova et al., 2001; Ohnishi et al., 2005). Aunque también la ubiquinona Q o la FMN *per se*, podrían ser lugares de generación de ERO_S (Herrero and Barja, 2000; Liu et al., 2002; Genova et al., 2003; Lambert and Brand, 2004a).

La rotenona es un inhibidor irreversible de la ubiquinona Q, dando lugar a la producción de radicales libres, sobre todo de anión superoxido ($O_2^{\bullet}$), que puede alterar la homeóstasis celular (Zoccarato et al., 2007; Moldzio et al., 2010). La rotenona es un isoflavonoide lipofílico que se puede encontrar en diferentes especies de plantas, como *Derris or lonchocarpus* (Singer and Ramsay, 1994; Moldzio et al., 2010). Se cree que la rotenona aumenta la liberación de $O_2^{\bullet}$ en la matriz mitocondrial sólo a través del complejo I (St-Pierre et al., 2002; Lambert and Brand, 2004a; Ohnishi et al., 2005).

5.2 COMPLEJO III (complejo b-c_1, ubiquinona/citocromo c reductasa) y COMPLEJO IV (complejo citocromo c oxidasa).

El complejo III se cree que está constituido por dos partes de una ubiquinona Q. La Q_1 que está orientada hacia la matriz mitocondrial y la Q_0 orientada hacia el EIM (Boveris and Cadenas, 1975; Boveris et al., 1976; Lenaz et al., 1978;

Rich and Bonner, 1978; Turrens et al., 1985; Crofts et al., 1999a; Crofts et al., 1999b; Starkov and Fiskum, 2001; St-Pierre et al., 2002; Turrens, 2003). Esto sugiere que existen al menos dos sitios diferentes de transferencia de electrones (Boveris et al., 1976).

En primer lugar la ubiquinona Q se reduce a QH_2 en la parte interna de la MMI y migra hacia la parte externa. Durante este proceso se transfieren 2 protones al EIM y un electrón al citocromo c_1 (parte del complejo III), que posteriormente pasará al complejo IV. El segundo electrón pasa del Q_0 al Q_1reduciéndose el citocromo b del complejo III y completándose de esta manera el ciclo. El citocromo c y la citocromo c oxidasa (ambos forman el complejo IV), sólo aceptan los electrones de uno en uno secuencialmente (Gille and Nohl, 2001).

La antimicina-A (antibiótico procedente del género Streptomyces) se une al Q_1 bloqueando el paso del segundo electrón al citocromo b. Por lo tanto la antimicina-A estimula la fuga de electrones inhibiendo la formación de QH_2 (Starkov and Fiskum, 2001). Todo ello indica que tanto Q_0 como Q_1 podrían ser lugares de generación de ERO_S, especialmente de $O_2^{\bullet}$ (Raha et al., 2000).

El cianuro tiene la capacidad de inhibir el complejo IV, bloqueando a la citocromo c oxidasa. Ello va a desencadenar la fuga de electrones, por lo que se pierde energía para la producción de ATP (Stowe and Camara, 2009).

5.3 ATP-sintasa (F_1-F_0asa).

La ATP-sintasa utiliza la fuerza motriz de los protones para convertir el ADP y P_i en ATP. Se trata de una proteína trans-membrana cuya estructura es la más compleja de toda la MMI.

Los protones bombeados al EIM por los complejos de la cadena y los electrones que se transportan a través de dichos complejos proporcionan la energía que la ATP-sintasa utiliza para devolver los protones a la matriz mitocondrial, donde junto con los electrones y el O_2 se transformarán en H_2O. Al mismo tiempo fosforila una molécula de ADP para obtener ATP (Figura 10). La subunidad F_0 se abre y permite el paso de los protones desde el EIM a la matriz, mientras que la F_1 es la que tiene función catalítica. Paul Boyer (Boyer, 1997) propuso que la ATP-sintasa pasa por tres estados: uno abierto (donde permite el paso de los protones), otro donde está unido el ADP y el Pi y por último uno donde se encuentra unido el ATP. Al final cada molécula de ATP será liberada a la matriz mitocondrial junto con las moléculas de H_2O (Krauss, 2001).

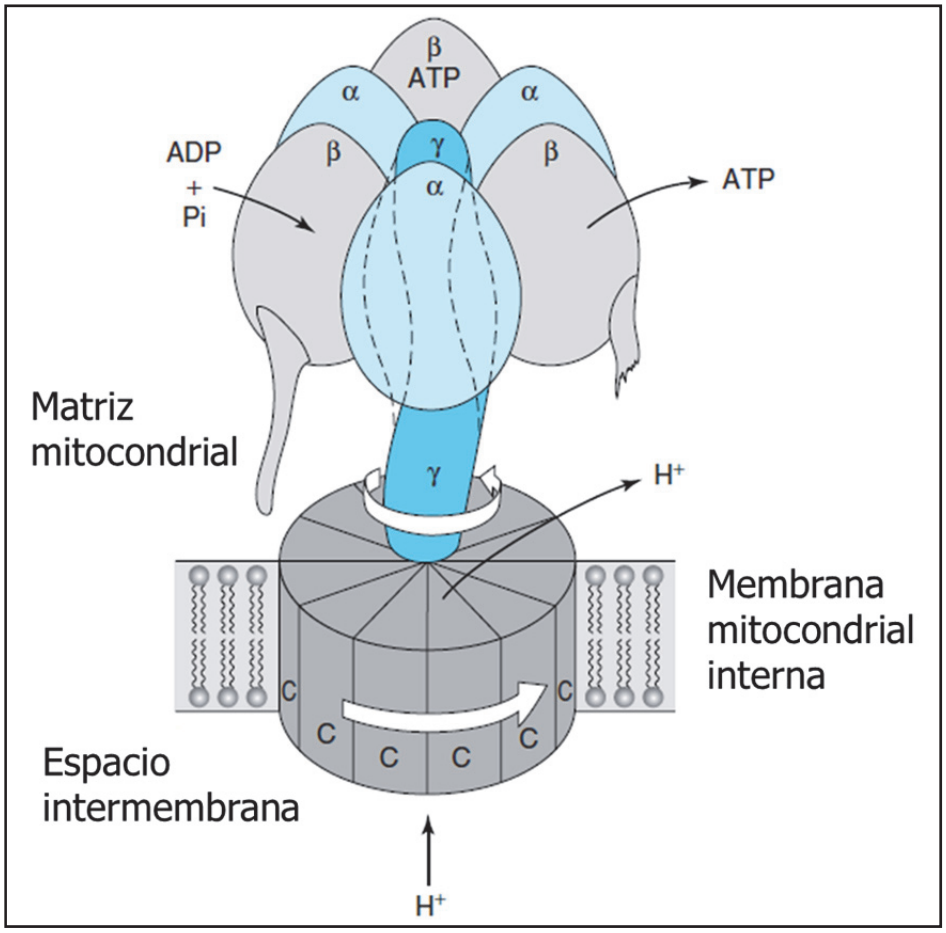


Figura 10. Mecanismo de producción de ATP por la ATP-sintasa. Modificada de (Murray, 2003).

La oligomicina-A es un inhibidor de la ATP-sintasa, concretamente bloquea la subunidad F_0 de tal manera que impide el paso de los protones desde el EIM a la matriz mitocondrial. Se trata de un macrólido producido por bacterias del género Streptomyces (Vernet et al., 2001; Erkkila et al., 2006).

Además de los inhibidores de la cadena de transporte de electrones mitocondrial anteriormente citados, también existen agentes desacoplantes de la mitocondria, como son el carbonylcyanide-m-chlorophenylhydrazone (CCCP) y el 2,4-dinitrofenol (2,4-DNP). Dichos agentes favorecen el paso de protones desde el EIM a la matriz antes de que sean utilizados por la ATP-sintasa.

Disipan el gradiente electroquímico y desacoplan el transporte de electrones lo que inhibe la formación de ATP y H_2O (Erkkila et al., 2006; Koppers et al., 2008; Dong et al., 2010). La razón es que ambos agentes son solubles en lípidos y por tanto, atraviesan con facilidad la MMI, devolviendo los protones a la matriz en lugar de que los devuelva la ATP-sintasa.

6. PRODUCCIÓN DE ESPECIES REACTIVAS DE OXÍGENO (EROs)

En 1943, el científico John McLeod demostró que los espermatozoides humanos producían especies reactivas de oxígeno, y que éstas tenían efectos deletéreos sobre ciertas funciones de estas células. Las EROs son moléculas muy reactivas debido a que contienen oxígeno con electrones desapareados en su último orbital. En el caso de los espermatozoides, la mitocondria es el principal orgánulo implicado en la producción de las EROs (Koppers et al., 2008), aunque también pueden provenir de leucocitos presentes en el eyaculado (Aitken and Baker, 1995), radiaciones electromagnéticas externas (De Iuliis et al., 2009), xenobióticos (Bennetts et al., 2008), como consecuencia del metabolismo de los propios espermatozoides (Baker and Aitken, 2004) o debido a cualquier factor que interfiera con la cadena mitocondrial de transporte de electrones.

El estrés oxidativo es un fenómeno causado por la existencia de un desequilibrio entre la producción y la detoxificación de las EROs. El entorno reductor de las células somáticas se mantiene gracias a la presencia de enzimas como la superóxido dismutasa, la glutatión peroxidasa y la catalasa que actúan

neutralizando radicales libres. Estas enzimas también están presentes en los espermatozoides (Nissen and Kreysel, 1983; Jeulin et al., 1989). Los desequilibrios que se produzcan en las reacciones de oxidación-reducción (estado REDOX) pueden causar efectos tóxicos a través de la producción de peróxidos y radicales libres que dañan componentes esenciales de las células, como el ADN (Tunc et al., 2009; Tunc and Tremellen, 2009), proteínas (Sikka et al., 1995) y lípidos (Koppers et al., 2010; Maia Mda et al., 2010).

Diversas investigaciones han demostrado que la producción de bajas cantidades de EROs es necesaria en el control de la hiperactivación (de Lamirande and Gagnon, 1993a), la reacción acrosómica (Griveau et al., 1994), y la fusión del espermatozoide con el ovocito (de Lamirande and Gagnon, 1993b). Por el contrario, la producción de grandes cantidades de EROs tiene efectos adversos en la viabilidad y la función de los espermatozoides, debiéndose mantener un equilibrio entre la producción y la detoxificación de las EROs (de Lamirande and Gagnon, 1995).

En el caso de los espermatozoides, durante la respiración celular en la mitocondria, el anión $O_2^{\bullet}$ puede dismutar espontánea o enzimáticamente (mediante la acción de la superóxido dismutasa) a peróxido de hidrógeno (H_2O_2). Aunque el $O_2^{\bullet}$ puede producir radicales libres más tóxicos que el H_2O_2, su vida media es muy corta (1ms) y su permeabilidad es baja (Iwasaki and Gagnon, 1992; Ball et al., 2001). Los espermatozoides tienen una capacidad limitada para almacenar enzimas antioxidantes y una membrana plasmática muy abundante en ácidos grasos poliinsaturados que los hacen muy

susceptibles a la acción de estas EROs (Baker and Aitken, 2005; Wathes et al., 2007).

El espermatozoide de mamíferos de interés ganadero es sometido con frecuencia a procesos biotecnológicos como la criopreservación; durante este proceso tiene lugar un incremento de la producción de EROs (Chatterjee and Gagnon, 2001) que está asociado, a su vez, a incrementos en la peroxidación de los lípidos de la membrana plasmática (Ortega Ferrusola et al., 2009), a un incremento en la fragmentación del ADN (Bucak et al., 2010) y a una disminución de la motilidad espermática (Awda et al., 2009) entre otros efectos. La adición de antioxidantes (Neagu et al., 2010) y enzimas reductoras (Gadea et al., 2011) han demostrado su eficacia a la hora de proteger y mejorar la calidad de los espermatozoides descongelados.

Todas estas EROs además de producir oxidaciones que dañan proteínas, lípidos y ADN, activan factores pro-apoptóticos desencadenando un fenómeno similar a la apoptosis (*apoptosis-like*), responsable tanto de la muerte de los espermatozoides como de los daños subletales que disminuyen la vida media y la capacidad fecundante de las células (Ortega-Ferrusola et al., 2008).

7. CONTROL DE LA MUERTE CELULAR POR LA MITOCONDRIA

En el pasado se pensaba que las mitocondrias eran meros orgánulos productores de energía. Actualmente sabemos que desarrollan muchas otras funciones, como hemos mencionado anteriormente. Varios autores han

descubierto que intervienen en la muerte celular producida por alteraciones en la homeóstasis. Ellas modulan la apoptosis de la célula mediante el desencadenamiento de una cascada de eventos que tendrá como fin la muerte de la célula (Zamzami et al., 1995; Kroemer et al., 2007; Tait and Green, 2010).

Existen dos vías principales implicadas en el proceso de apoptosis: la vía intrínseca o mitocondrial y la vía extrínseca o de los receptores de muerte celular. Ambas están interconectadas a distintos niveles, de hecho, la extrínseca puede utilizar parte de la vía mitocondrial para amplificar su señal. Dentro de la mitocondria existen proteínas que tienen un papel clave en el desencadenamiento de la apoptosis, como el citocromo c, que al liberarse al citoplasma, activa las denominadas caspasas (cisteinil aspartato proteasas) (Igney and Krammer, 2002; Galluzzi et al., 2012c; Galluzzi et al., 2012d).

Estas proteínas activadas tanto en la vía intrínseca como en la extrínseca, se sintetizan como proteínas precursoras (pro-caspasas) y que son finalmente activadas como caspasas por determinados factores como el TNF (Factor de Necrosis Tumoral), miembros de la familia Bcl-2 o las ERO_S. Existen dos tipos, las iniciadoras y las efectoras. Las iniciadoras (Caspasa 8 y 9 principalmente) son necesarias para comenzar la cascada apoptótica mediante la activación de las efectoras. Una vez activadas las efectoras (Caspasas 3 y 7 principalmente), son las encargadas de llevar a cabo la lisis que conlleva a la muerte de la célula (Thornberry and Lazebnik, 1998).

7.1. Vía extrínseca

Esta vía se inicia por estímulos extracelulares, mediante la activación de receptores de muerte celular de la familia del TNF que se encuentran anclados en la membrana plasmática (Gupta, 2003).

Se produce la activación de la caspasa 8, iniciadora de la vía extrínseca. La 8 activa a las caspasas efectoras 3 y 7 (Fuentes-Prior and Salvesen, 2004), que darán lugar a la proteólisis de sustratos y finalmente a la muerte celular.

7.2. Vía intrínseca

Se caracteriza por el papel fundamental que ejerce la mitocondria (Kroemer et al., 2007). En la membrana externa mitocondrial están presentes proteínas de la familia Bcl-2 que regulan procesos de permeabilización mitocondrial y constituyen un punto clave en la vía intrínseca de apoptosis. Algunos de los miembros de esta familia desempeñan funciones anti-apoptóticas (Bcl-2 o Bcl-XL) y otras funciones pro-apoptóticas (BAX, BAD, BAK) (Figura 11). Durante situaciones de estrés celular, estas proteínas con función anti-apoptótica pueden desestabilizarse. Si el equilibrio entre factores pro-apoptóticos y anti-apoptóticos se rompe a favor de los primeros se activará la vía intrínseca de la apoptosis. Este desequilibrio favorece la unión de las proteínas pro-apoptóticas Bax-Bak, dando lugar a oligómeros en la superficie de la membrana externa de la mitocondria, originando la aparición de canales mitocondriales y comprometiendo la integridad de dicha membrana (Pavlov et al., 2001). Como consecuencia, los factores pro-apoptóticos presentes en el EIM se liberan al

citoplasma. Entre ellos se encuentran el citocromo c, que activa directamente al factor proteasa Apaf-1, y que en presencia de ATP, promueve la formación de un complejo multimérico denominado apoptosoma. Este apoptosoma media en la activación de la caspasa iniciadora 9, que activará las caspasas efectoras 3, 6 y 7 (Fuentes-Prior and Salvesen, 2004).

No sólo los miembros de la familia Bcl-2 son capaces de desestabilizar la membrana mitocondrial y liberar los factores pro-apoptóticos. Otros factores como ciertas proteasas (Johnson, 2000; Suzuki et al., 2004), EROs o la presencia de Ca^{2+} pueden inducir también la apoptosis por esta vía (Kroemer et al., 2007).

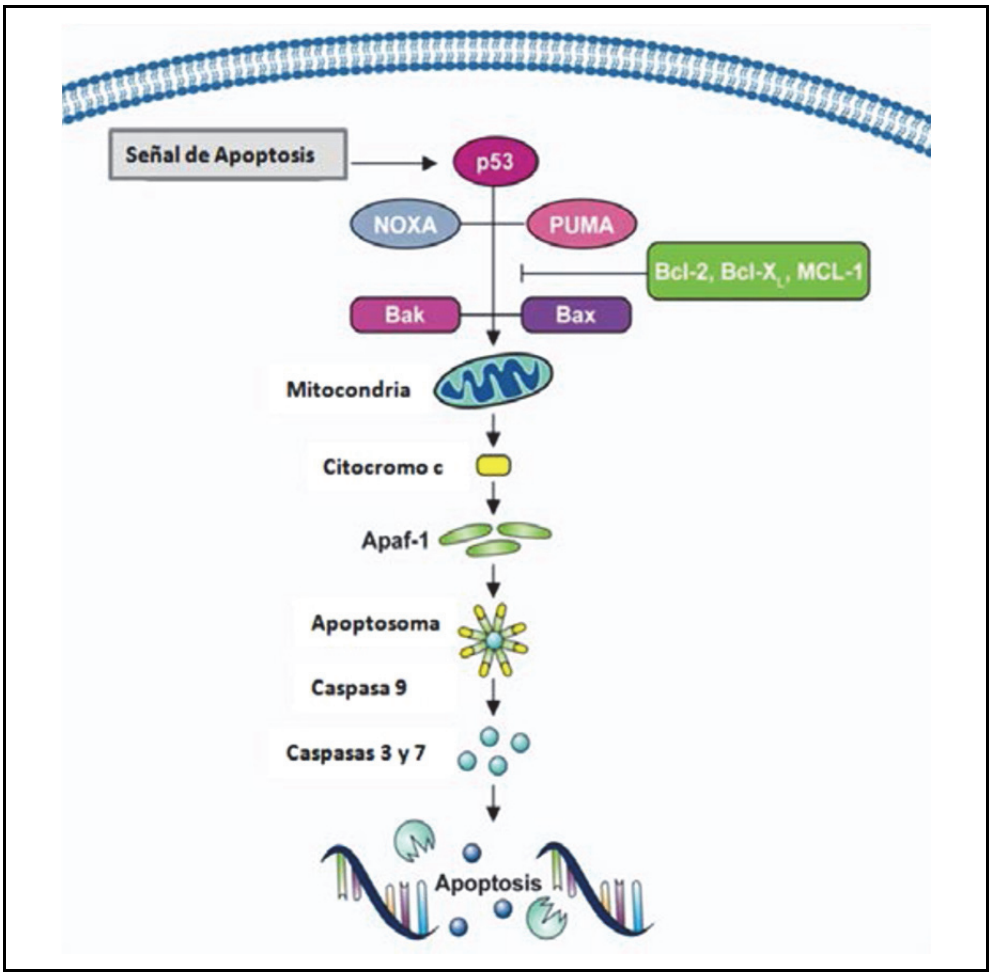


Figura 11. Vía intrínseca de la apoptosis. Modificada de http://www.e-allscience.com/blogs/news/12684405-apoptosis-muerte-celular-programada

Estudios previos en nuestro laboratorio (Ortega Ferrusola et al., 2010) evidencian la importancia de la vía intrínseca de la apoptosis en tecnologías reproductivas importantes en el caballo, como la congelación. Factores de la MMI y MME forman el denominado poro mitocondrial (Fiore et al., 1998; Rasola and Bernardi, 2007), que juega un papel muy importante en el proceso de congelación y descongelación del espermatozoide equino. Durante este proceso, la caída del potencial de membrana mitocondrial origina la apertura del poro, lo que provoca la liberación de factores pro-apoptóticos como el citocromo c, que desencadena la activación de caspasas, y posterior fragmentación del ADN y el aumento de la permeabilidad de membrana plasmática (Fiore et al., 1998; Anzar et al., 2002; Pena et al., 2003; Rasola and Bernardi, 2007). Todo ello se ha observado en espermatozoides humanos (Paasch et al., 2004) bovinos (Martin et al., 2004; Martin et al., 2007) y equinos (Brum et al., 2008; Ortega-Ferrusola et al., 2008).

Las mitocondrias se sitúan en el centro de un complejo sistema de sensores que detectan perturbaciones en la homeostasis intracelular, incluyendo el estrés oxidativo o alteraciones de la permeabilidad o de pH entre otras. Desde una posición tan central, responden a grados relativamente leves de estrés dando lugar a respuestas adaptativas dirigidas al restablecimiento de la homeostasis. Un ejemplo de ello es la repuesta ante una infección viral, donde desencadenan la autofagia mediante la activación de la proteína mitocondrial NLRX1 (nucleotide-binding oligomerization domain leucine rich repeat containing X1), el C1QBP (complement component 1q subcomponent-binding protein) y TOM (translocase of outer mitocondrial membrane 70 kDa subunit). A continuación,

dicha activación dará lugar a la expresión de moléculas antivirales, como el IFNβ (interferón β) y la IL-6 (Interleucina-6). A la inversa, cuando el daño está más allá de la recuperación, las mitocondrias desencadenan señales de peligro que darán lugar a la muerte celular (Galluzzi et al., 2012b).

8. ENVÍO DE SEÑALES DE PELIGRO POR LA MITOCONDRIA

Las mitocondrias no sólo decodifican los peligros para la célula en forma de activación de respuestas adaptativas o mediante el desencadenamiento de la muerte celular, sino también emiten señales de peligro que alertan de situaciones de estrés mitocondrial (Nunnari and Suomalainen, 2012). Mucho antes de causar alteraciones en la permeabilidad de la MME, la mitocondria activa la producción de ERO_S, que en condiciones fisiológicas se mantienen bajo control mediante moléculas antioxidantes (Hamanaka and Chandel, 2009; Green et al., 2011; Krysko et al., 2011; Tait and Green, 2012). Sin embargo, en respuesta a estímulos como hipoxia, infección viral y perturbaciones mitocondriales, como la pérdida del potencial de membrana mitocondrial, o aumento de los niveles de ERO_S libres, se traducen en señales de peligro para la célula (Al-Mehdi et al., 2012).

La disipación del potencial de membrana mitocondrial, como ocurre durante la alteración de la permeabilidad de la MME, es suficiente para inducir una crisis energética debido a la pérdida de protones resultante de dicha disipación. Todo

ello desencadena una situación citotóxica que da lugar a la muerte celular (Kroemer et al., 2009; Galluzzi et al., 2012d).

Otros factores mitocondriales que pueden actuar como señales de peligro son el ATP o la cardiolipina. El ATP estimula la respuesta inflamatoria mediante la activación del NLRP3 (nucleotide-binding oligomerization domain leucine rich repeat containing P3), un inflamasoma que promueve la maduración de citoquinas e induce la piroptosis celular (proceso de muerte celular programada diferente a la apoptosis) (Zitvogel et al., 2010). La cardiolipina puede ser liberada o expuesta en la superficie de la célula, posteriormente se une a los receptores CD1d de las células presentadoras de antígenos y todo ello desencadenará la activación de las células T o linfocitos T de la respuesta inflamatoria (Schlame, 2008; Dieude et al., 2011).

Así, múltiples componentes mitocondriales, una vez expuestos o liberados en el microambiente extracelular o en el torrente sanguíneo transmitirán señales de peligro que son decodificadas por el organismo para promover respuestas sistémicas innatas (Galluzzi et al., 2012a).

Por lo tanto, las mitocondrias, además de ser la principal fuente intracelular de ATP y tener un papel destacado en la regulación de la muerte celular, están profundamente involucradas en la señalización de peligro de alteración de la homeostasis. Por una parte, las mitocondrias son capaces de decodificar señales de peligro entrantes (por ejemplo en una infección viral) y traducirlas en respuestas adaptativas. Por otra parte, son capaces de emitir señales de peligro intracelulares cuando se producen alteraciones en la homeostasis, y si

dicha homeostasis no se puede restablecer y se produce la muerte celular, las señales emitidas por las mitocondrias serán interpretadas por el sistema inmune que desencadenará una respuesta inflamatoria estéril. Por estas razones, las mitocondrias se consideran como grandes reguladoras de la señalización de peligro celular (Galluzzi et al., 2012a).

Objetivos

OBJETIVOS

Basándonos en la importancia de la mitocondria en el espermatozoide y el efecto que en ellas tienen las tecnologías reproductivas actuales nos propusimos estudiar en la presente Tesis Doctoral la función mitocondrial en el espermatozoide equino. Para ello nos planteamos los siguientes objetivos específicos:

- Estudiar la importancia de la fosforilación oxidativa como fuente de energía para el espermatozoide equino.

- Estudiar la mitocondria como generadora de especies reactivas de oxígeno y determinar el papel de la misma como reguladora de la senescencia en el espermatozoide de caballo.

- Evaluar la respuesta de los espermatozoides equinos en condiciones de capacitación, a la inhibición del complejo I mitocondrial en un ensayo de unión heterólogo y comprobar si el antioxidante epigalocatequina-3-galato podría contrarrestar ese efecto.

Resultados

Artículo 1º

PLOS | ONE

RESEARCH ARTICLE

Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility

María Plaza Davila[1], Patricia Martin Muñoz[1], Jose A. Tapia[2], Cristina Ortega Ferrusola[1], Carolina Balao da Silva C[1], Fernando J. Peña[1]*

1 Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain, **2** Department of Physiology, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain

* fjuanpvega@unex.es

OPEN ACCESS

Citation: Plaza Davila M, Martin Muñoz P, Tapia JA, Ortega Ferrusola C, Balao da Silva C C, Peña FJ (2015) Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility. PLoS ONE 10(9): e0138777. doi:10.1371/journal.pone.0138777

Editor: Joël R Drevet, Clermont-Ferrand Univ., FRANCE

Received: April 16, 2015

Accepted: September 3, 2015

Published: September 25, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: The authors received financial support for this study from Ministerio de Economia y Competitividad-FEDER in Madrid, Spain, grants AGL2013-43211-R and Junta de Extremadura-FEDER (GR 10010 and PCE1002).

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Mitochondria have been proposed as the major source of reactive oxygen species in somatic cells and human spermatozoa. However, no data regarding the role of mitochondrial ROS production in stallion spermatozoa are available. To shed light on the role of the mitochondrial electron transport chain in the origin of oxidative stress in stallion spermatozoa, specific inhibitors of complex I (rotenone) and III (antimycin-A) were used. Ejaculates from seven Andalusian stallions were collected and incubated in BWW media at 37°C in the presence of rotenone, antimycin-A or control vehicle. Incubation in the presence of these inhibitors reduced sperm motility and velocity (CASA analysis) ($p<0.01$), but the effect was more evident in the presence of rotenone (a complex I inhibitor). These inhibitors also decreased ATP content. The inhibition of complexes I and III decreased the production of reactive oxygen species ($p<0.01$) as assessed by flow cytometry after staining with CellRox deep red. This observation suggests that the CellRox probe mainly identifies superoxide and that superoxide production may reflect intense mitochondrial activity rather than oxidative stress. The inhibition of complex I resulted in increased hydrogen peroxide production ($p<0.01$). The inhibition of glycolysis resulted in reduced sperm velocities ($p<0.01$) without an effect on the percentage of total motile sperm. Weak and moderate (but statistically significant) positive correlations were observed between sperm motility, velocity and membrane integrity and the production of reactive oxygen species. These results indicate that stallion sperm rely heavily on oxidative phosphorylation (OXPHOS) for the production of ATP for motility but also require glycolysis to maintain high velocities. These data also indicate that increased hydrogen peroxide originating in the mitochondria is a mechanism involved in stallion sperm senescence.

Introduction

The mitochondria of the spermatozoa control numerous functions and are considered to be hallmarks of sperm functionality [1, 2]. In addition to their role as an ATP source via oxidative phosphorylation (OXPHOS), other functions in regulating the lifespan of spermatozoa have attracted major research attention to these organelles [1] [3]. Important cellular functions in the spermatozoa are redox-regulated; the production of reactive oxygen species (ROS) is an early event during the series of the modifications that occur during capacitation[4]. However alteration in the redox homeostasis of the cell leads to sperm senescence and finally death[5]; in humans, it has been reported that the mitochondria of defective sperm are the major source of ROS originating from electron leakage in the electron transport chain (ETC) [6]. This has also been assumed to be true for horses, as reviewed in [7], and recent data support this hypothesis[8]. Moreover, sperm biotechnologies that are widely used in animal breeding are known to alter the redox status of these cells [9]. The preservation of spermatozoa for short periods in a liquid state or frozen for longer periods is at the core of the horse industry, so the investigation of mechanisms leading to sperm senescence, infertility and finally death, has increased in the recent years. Apoptotic-like mechanisms [10–13] are responsible for sperm deterioration during conservation. One mechanism explaining sperm senescence (and ultimately sperm death) during storage is lipid peroxidation [14]. This is related to the highly unsaturated nature of the lipids that comprise sperm membranes, which predisposes them to oxidative attack [15]. Interestingly the mitochondria are considered to be more sensitive to the changes induced by cryopreservation and cooling than other organelles in spermatozoa [16, 17]. The disruption of the mitochondrial electron transport chain is known to induce the production of mitochondrial ROS both in somatic cells [18] and human sperm [6]. To specifically assess the situation in stallion and test the hypothesis that the disruption of complexes I and III leads to sperm malfunction due to oxidative stress, split samples were incubated in presence of rotenone and antimycin-A. The production of reactive oxygen species was monitored using flow cytometry. Other effects on sperm parameters were investigated as well, moreover the role of glycolysis in sperm function was investigated using specific inhibitors and through the incubation of stallion spermatozoa in media free of glucose and pyruvate.

Materials and Methods

Reagents and media

Ethidium homodimer, 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyne iodine (JC-1), YO-PRO-1, CellRox Deep Red Reagent, Hoechst 33342, hydroethidine, dichlorodihydrofluoresceindiacetate, Mitosox, Sytox green and the ATP detection Kit were from Molecular Probes (Molecular Probes, Leiden The Netherlands). Rotenone, antimycin-A, 2-Deoxy-D-glucose and all other chemicals were from Sigma (St Louis MO)

Semen collection and processing

The use of animals for semen collection was approved by the ethics committee on animal experimentation of the University of Extremadura with authorization number 103/2013. Semen was obtained from seven Pure Spanish horses (PRE) (three ejaculates each from animals that were 6 to 14 years old and of proven fertility due to regular use as stud stallions in our center) that were individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, and the sperm samples were collected on a regular basis (two collections/week) during the 2013 and 2014 breeding seasons. Ejaculates were collected by staff

veterinarians, using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. The semen was immediately transported to the laboratory for evaluation and processing. The ejaculate was extended 1:2 in INRA-96 (IMV l'Aigle, France), centrifuged (600 x g for 10 min), and re-suspended in BWW media (91.06 mM NaCl, 4.78 mM KCl, 2.44 mM MgSO4, 1.17 mM KPO4, 21.0 mM HEPES, 5.5 mM glucose [anhydrous], 0.25 mM sodium pyruvate, 1.71 mM lactic acid hemicalcium salt, and 21.55 mM sodium lactate supplemented with 1% PVA at 300 mOsm/kg). The pH of the suspension was adjusted to 7.2. All media were filtered through a 0.45-mm filter. The final concentration for sperm incubation was 40 $x10^6$ spmtz/ml.

All experiments followed a split sample design, with every ejaculate divided to create control and treatment groups; for this, the sperm aliquots were incubated at 37° C in presence of the inhibitors or controls (vehicle) and aliquots were taken after 60, 120, and 180 min for CASA, flow cytometry and ATP measurements.

Sperm motility

Sperm motility and kinematics were assessed by analyzing undiluted aliquots taken at 60, 90 and 180 minutes of incubation with a CASA system (ISAS® Proiser, Valencia, Spain) and 20 μm deep Leja chambers (Leja Products B.V. Nieuw Vennep, The Netherlands) placed on a warmed (37°C) stage. A minimum of 500 spermatozoa per sample were analyzed in four different randomly selected fields. The analysis was based on the examination of 60 consecutive digital images in a lapse time of one second (60 Hz) obtained using a 10x negative phase contrast objective. The number of objects incorrectly identified as spermatozoa was minimized on the monitor using the playback function. With respect to the setting parameters for the program, spermatozoa with a VAP <15 μm/s were considered to be immotile, whereas spermatozoa with a velocity >15 μm/s were considered to be motile. Spermatozoa deviating <45% from a straight line were designated as linearly motile, and spermatozoa with a curvilinear velocity (VCL) > 45 μm/s were designated as rapid sperm. The absolute and re-calculated kinematic parameters measured by CASA included the following: curvilinear velocity (VCL; μm/s), which measures the sequential progression along the true trajectory; straight line velocity (VSL; μm/s), which measures the straight trajectory of the spermatozoa per unit time; and average path velocity (VAP; μm/s), which measures the mean trajectory of the spermatozoa per unit of time.

Flow cytometry

Flow cytometry analyses were conducted using a MACSQuant Analyser 10 (Miltenyi Biotech) flow cytometer equipped with three lasers emitting at 405 nm, 488 nm, and 635 nm and 10 photomultiplier tubes (PMTs) (V1 (excitation 405 nm, emission 450/50 nm), V2 (excitation 405 nm, emission 525/50 nm), B1 (excitation 488 nm, emission 525/50 nm), B2 (excitation 488 nm, emission 585/40 nm), B3 (excitation 488 nm, emission 655–730 nm (655LP + split 730), B4 (excitation 499 nm, emission 750 LP), R1 (excitation 635 nm, emission 655–730 nm (655LP+split 730) and R2 (excitation 635 nm, emission filter 750 LP). The system was controlled using MACSQuantify software. Sperm subpopulations were divided by quadrants to quantify the frequency of each subpopulation. Forward and sideways light scatter were recorded for a total of 50,000 events per sample. Non-sperm events were eliminated by gating the sperm population after Hoechst 33342 staining. The instrument was calibrated daily using specific calibration beads provided by the manufacturer, and compensation overlap was performed before each experiment using appropriate unstained and single-stained controls.

Simultaneous flow cytometric assessment of subtle membrane changes, viability and oxidative stress (reactive oxygen species ROS). Aliquots were taken at specific time intervals and diluted in PBS for staining to a final concentration 5 x 10^6 spermatozoa/mL. Aliquots were stained with CellRox (5μM) and Hoechst 33342 (0.5μM). After thorough mixing, the sperm suspension was incubated at RT in the dark for 25 min. The spermatozoa were then washed with PBS, then ethidium homodimer (0.35μM) and Yo-Pro-1 (25nM) were added, and the mixture was incubated for five minutes and read in a flow cytometer. This staining protocol was a modified version [19] of previously published protocols [20–22] and distinguishes four sperm subpopulations while simultaneously measuring oxidative stress. The first subpopulation, positive for only Hoechst 33342, was considered to be alive and without any membrane alterations. Another subpopulation, the Yo-Pro-1-positive cells emitting green fluorescence, represents cells whose membranes have become slightly permeable, enabling Yo-Pro-1 but not ethidium homodimer to cross the plasma membrane. Finally, two subpopulations of dead spermatozoa were easily detected; spermatozoa staining both with Yo-Pro-1 and ethidium homodimer (emitting both green and red fluorescence) and cells staining with only ethidium homodimer, emitting red fluorescence. Spermatozoa exhibiting oxidative stress emit fluorescence in the far red spectrum, whereas Hoechst 33342-positive sperm emit blue fluorescence. The positive controls for oxidative stress were samples supplemented with 800 μM SO_4Fe and 1.7 M of H_2O_2 (Sigma) to induce the Fenton reaction. Representative dot plots of this assay depicting positive controls are present in Fig 1.

Evaluation of mitochondrial membrane potential (ΔΨm). The probe 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyne iodine (JC-1), forms multimeric aggregates in mitochondria with high membrane potential; these aggregates emit in the high orange wavelength of 590 nm when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers that emit in the green wavelength (525 to 530 nm) when excited at 488 nm. Published staining protocols from our laboratory and others were followed [16, 23]. Briefly each sperm sample,was stained with JC-1 (1.5μM) were incubated at 37°C in the dark for 40 min before flow cytometry analysis.

Determination of anion superoxide (O2•-) and hydrogen peroxide (H2O2) production. Following protocols that have been previously validated for stallion sperm [24, 25], the samples were stained with hydroethidine (1.4μM) to detect superoxide anion (O_2•-) and dichlorodihydrofluorescein diacetate (H_2DCFDA 5 μM) to detect H_20_2. To restrict our analysis to spermatozoa Hoechst 33342 (0.5μM) was added. The samples were incubated for 30 min at 38°C before analyzing 100.000 events in the flow cytometer. HE and H_2DCFDA were excited at 488 nm, and fluorescence was recorded at 530 and 610 nm, respectively. Hoechst 33342 was excited at 405 nm, and fluorescence was recorded at 450 nm.

Mitosox red assay. The generation of mitochondrial superoxide anion was investigated using Mitosox Red (MSR, Molecular probes) as previously described [26, 27] with slight adaptations for stallion sperm in our laboratory[19]. Spermatozoa (5 × 10^6 /mL) were stained with 2 μM MitoSox Red, incubated for 15 min at 37° C, centrifuged for 5 min at 600 × *g*, and resuspended in BWW. SYTOX Green (0.05 μM) was then added for a final 15 min incubation. The MSR (red) and SYTOX Green (green) fluorescence were then measured using 530/30 band pass (green) and 585/42 band pass (red) filters. Non sperm-specific events were gated out after staining with Hoechst 33342 (0.5μM), and 10,000 cells were examined.

Determination of intracellular ATP

Intracellular ATP content in sperm lysates was measured using the ATP determination Kit (A22066) (Molecular Probes, Leiden Holland) according to the instructions provided by the

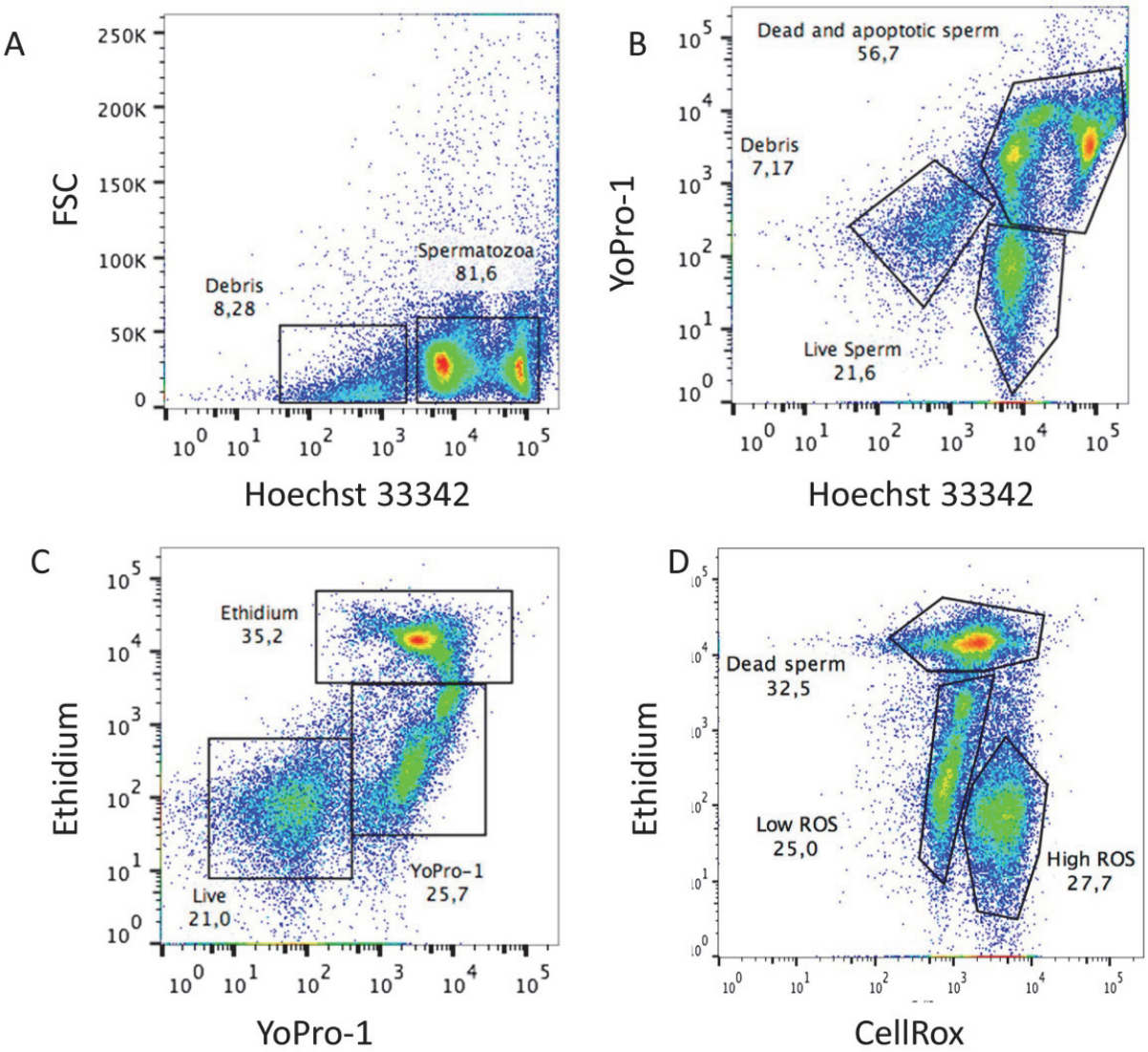


Fig 1. Representative cytograms for simultaneous flow cytometric assessment of subtle membrane changes, viability and reactive oxygen species (ROS). Samples were stained with Hoechst 33342, Yo-Pro-1, ethidium homodimer (Eth) and CellROX Deep Red, as described in the 'Materials and methods' section. Hierarchical gating was applied to exclude debris from the analysis and simultaneously measure viability, apoptosis, necrosis and ROS. (A) Hoechst 33342 fluorescence was detected using the V1 channel (Ex 405 bandpass filter 450/50 nm), and a gate was applied to positive (DNA-containing particles) events to gate out debris. The gated region was analysed. (B) Yo-Pro-1 was detected using the B1 channel (Ex 488 bandpass Filter 525/50 nm) and Hoechst 33342 fluorescence was detected using the V1 channel (Ex 405 bandpass filter 450/50 nm) (C,) Yo-Pro-1 was detected using the B1 channel (Ex 488 bandpass Filter 525/50 nm) and Eth was detected using the B3 channel (Ex 488 bandpass filter 655–730 nm) and (D) CellROX Deep Red was detected using the R1 channel (Ex 635 nm bandpass filter 655–730 nm). Positive controls for oxidation and compromised membranes are presented obtained as describe in material and methods.

doi:10.1371/journal.pone.0138777.g001

manufacturer and previously published protocols from our laboratory and others [8, 28]. Aliquots of spermatozoa (200 μL) were snap frozen in liquid nitrogen and stored at -80°C until analysis. On the day of analysis, the samples were thawed on ice and centrifuged at 20,000 x g for 15 min at 4° C, and the supernatant was used for the assay. The assay is based on the luciferase's requirement for ATP in producing light (emission maxima at approximately 560 nm at pH 7.8). The ATP content was normalized to pM/100 μg of protein quantified colorimetrically [29].

Statistical analysis

Three ejaculates from 7 individual stallions were collected. All experiments were repeated at least 21 times (three ejaculates from each of the seven stallions). The normality of the data was previously assessed using the Kolmogorov-Smirnoff test. In light of the normality of the distribution, results were analyzed by ANOVA, and Dunnett´s t-test was used to perform comparisons. The Pearson test was used to study the correlations, in control samples, between the production of reactive oxygen species and parameters of sperm functionality. Power analysis was set at 0.8 and $P < 0.05$ was regarded as significant, with * $p<0.05$ and ** $p<0.01$. The results are provided as the means ± SD. All analyses were conducted using SPSS 19.0 software for Mac.

Results

Inhibition of complex I (NADH-ubiquinone oxidoreductase) and complex III (co-enzyme Q-cytrochrome c reductase) reduces stallion sperm motility

Stallion sperm were washed and extended in BWW media supplemented with increasing concentrations of the mitochondrial complex I inhibitor rotenone (0 (vehicle) 100 and 500 nM and 5 and 10 μM). After 1 and 3 h of incubation at 37° C, motility was determined using a CASA system. After one hour of incubation, all the concentrations of the inhibitors tested reduced the percentage of motile spermatozoa, whereas after 3 hours of incubation, total motility was reduced in samples incubated in presence of concentrations of rotenone over 5 μM. Progressive motility dropped with all the concentrations tested after 1 and 3 hours of incubation at 37°C (Fig 2A and 2B).

Incubation of stallion spermatozoa in presence of the mitochondrial complex III inhibitor resulted in decreased total and progressive motilities after 1 and three hours of incubation at 37°C in BWW media at all the concentrations tested (Fig 2C and 2D).

Inhibition of complex I (NADH-ubiquinone oxidoreductase) and complex III (co-enzyme Q-cytrochrome c reductase) reduces stallion sperm velocities

To determine the effect of mitochondrial complex I inhibition on sperm velocities, split samples were incubated in presence of rotenone, and sperm velocities were determined after one and three hours of incubation using a CASA system. All the concentrations tested reduced circular, straight line, and average velocities after one hour of incubation. After three hours of incubation, VCL and VAP followed the same tendency, but VSL was reduced only in the presence of concentrations of rotenone greater than 5 μM (Fig 3). The mitochondrial complex III inhibitor antimycin- A reduced circular velocity at all concentrations tested after one hour of incubation and at concentrations of 100 nM and 500 nM after three hours of incubation.

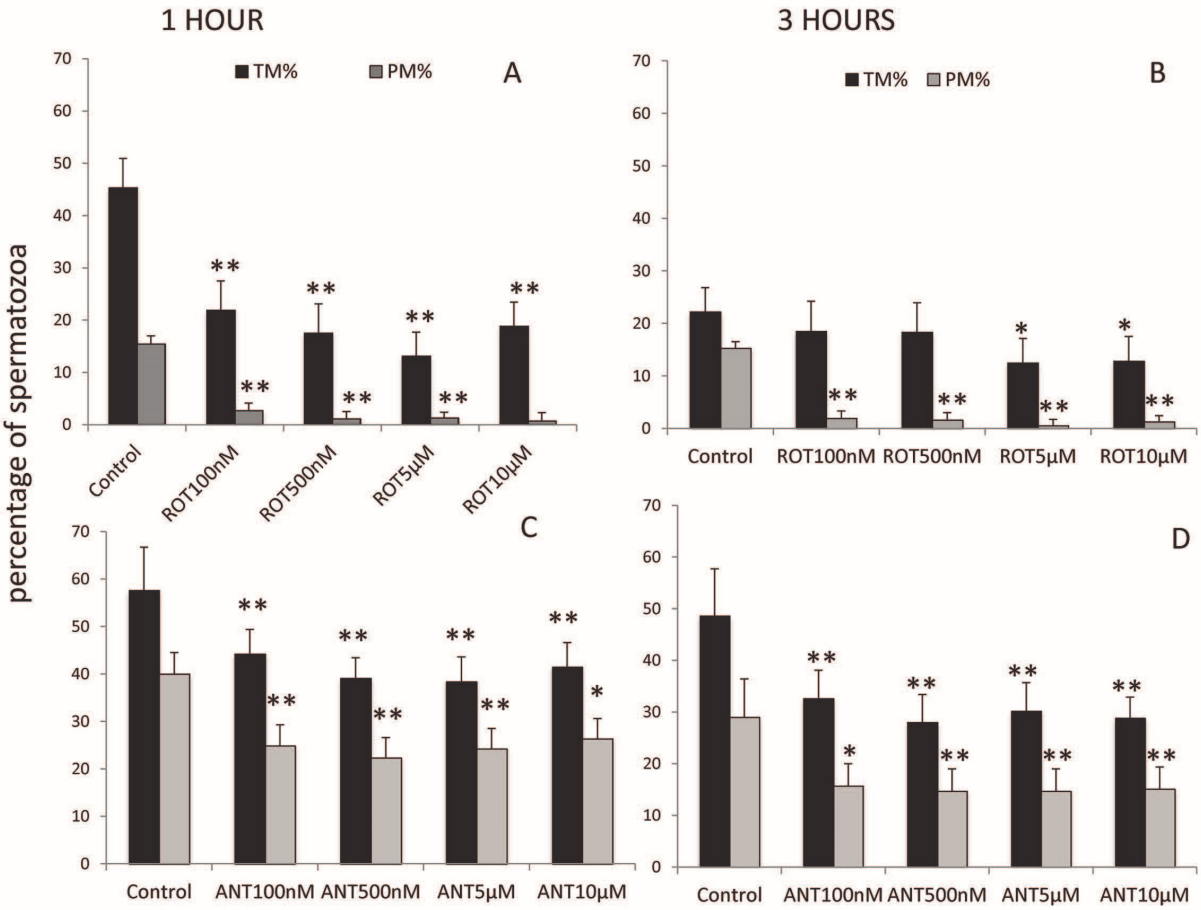


Fig 2. Effect of the inhibition of Complex I of the electron transport chain on stallion sperm motility (CASA analysis). Stallion spermatozoa were extended in BWW media and incubated in presence of rotenone (0 nm (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) and antimycin-A (0 vehicle DMSO, 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods section; motility was evaluated after 1 and three hours of incubation. TM%—percentage of total motile spermatozoa, PM %—percentage of progressive motile spermatozoa. Comparisons are made against controls. * P<0.05, ** P<0.01. The results are given as the means ± SD. The graphics represent two independent experiments (one for antimycin-A and one for rotenone) following a split sample design (the same ejaculate was split into the treatment and control groups).

doi:10.1371/journal.pone.0138777.g002

Straight-line and average path velocities were reduced by all the concentrations tested and after one and three hours of incubation (Fig 4).

Inhibition of complex I (NADH-ubiquinone oxidoreductase) and complex III (co-enzyme Q-cytochrome c reductase) reduces the percentage of spermatozoa with intact membranes

Rotenone had no effect on the percentage of intact sperm membranes after 1 hour of incubation, but after three hours of incubation, 10 µM rotenone reduced the percentage of intact sperm. On the other hand, the inhibitor of complex III reduced the percentages of intact sperm ($p<0.05$) after 1 and three hours of incubation at concentrations of 5 and 10 µM (Fig 5).

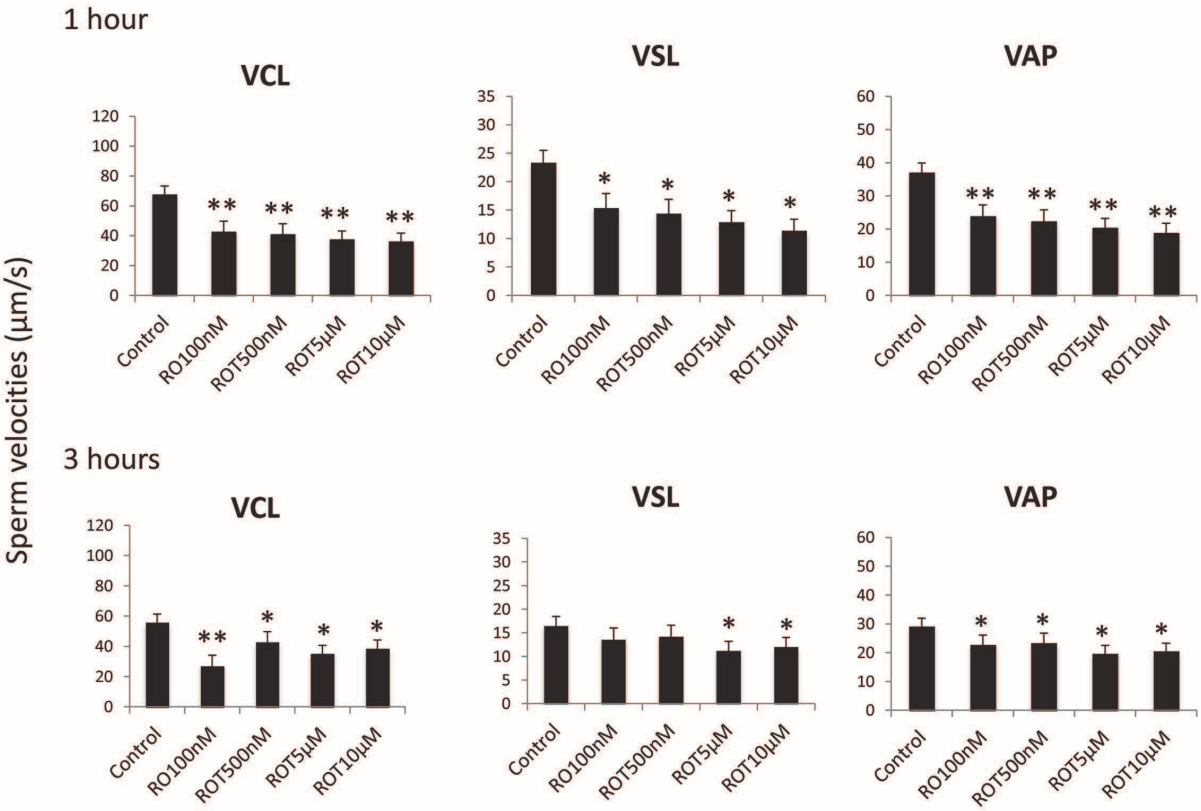


Fig 3. Effect of the inhibition of Complex I of the electron transport chain on stallion sperm velocities (CASA analysis). Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone (0 nm (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods section; velocities were evaluated after 1 and three hours of incubation, VCL—curvilinear velocity (µM/s), VSL straight line velocity (µM/), VAP average path velocity (µM/s). * P<0.05, ** P<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g003

Effect of complex I (NADH-ubiquinone oxidoreductase) and complex III (co-enzyme Q-cytochrome c reductase) on reactive oxygen species (ROS) production by stallion spermatozoa

The production of reactive oxygen species was initially monitored using CellRox using previously published protocols from our laboratory [19]. Both inhibitors significantly decreased ROS production at all incubation times (Fig 6). In light of these paradoxical results, reactive oxygen species production was also monitored using specific probes for superoxide anion, hydrogen peroxide and mitochondrial superoxide anion. Rotenone increased hydrogen peroxide production during incubation at 37°C, with no changes in non-mitochondrial superoxide anion (Fig 7). Following a similar pattern, rotenone induced mitochondrial superoxide production after 6 hours of incubation at 37°C (Fig 8). The inhibition of complex III with antimycin resulted in increased superoxide production only after one hour of incubation.

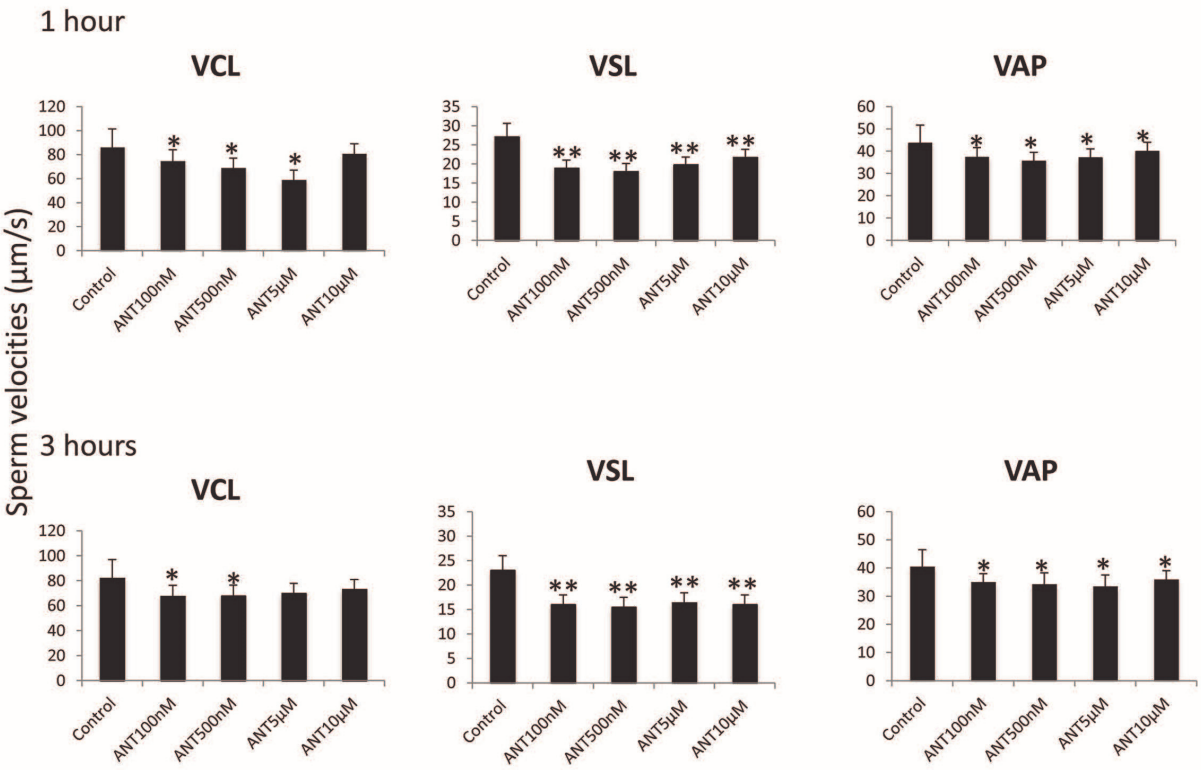


Fig 4. Effect of the inhibition of Complex III of the electron transport chain on stallion sperm velocities (CASA analysis). Stallion spermatozoa were extended in BWW media and incubated in the presence of antimycin-A (0 vehicle DMSO, 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods; velocities were evaluated after 1 and three hours of incubation, VCL—curvilinear velocity (µM/s), VSL—straight-line velocity (µM/s), VAP—average path velocity (µM/s). * P<0.05, ** P<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g004

Inhibition of complex I (NADH-ubiquinone oxidoreductase) and complex III (co-enzyme Q-cytochrome c reductase) induces mitochondrial uncoupling and reduces ATP content in stallion spermatozoa

One possible explanation for reduction of sperm motility and velocities is reduced ATP production due to mitochondrial uncoupling. To evaluate this possibility mitochondrial membrane potential was evaluated using JC-1 and ATP content using a luciferin-luciferase based assay. Inhibition of both complexes I and III lead to mitochondrial uncoupling when rotenone and antimycin were present at concentrations over 5 µM (Fig 9). The dynamics of ATP production was assessed in samples incubated in presence of both inhibitors up to 250 minutes. Both inhibitors reduced ATP content in stallion spermatozoa after 100 minutes of incubation (Fig 10).

Effect of inhibition of glycolysis on stallion sperm function

Because ATP production is not completely abolished and motility and membrane integrity are not completely suppressed, either membrane potential are not completely suppressed or other

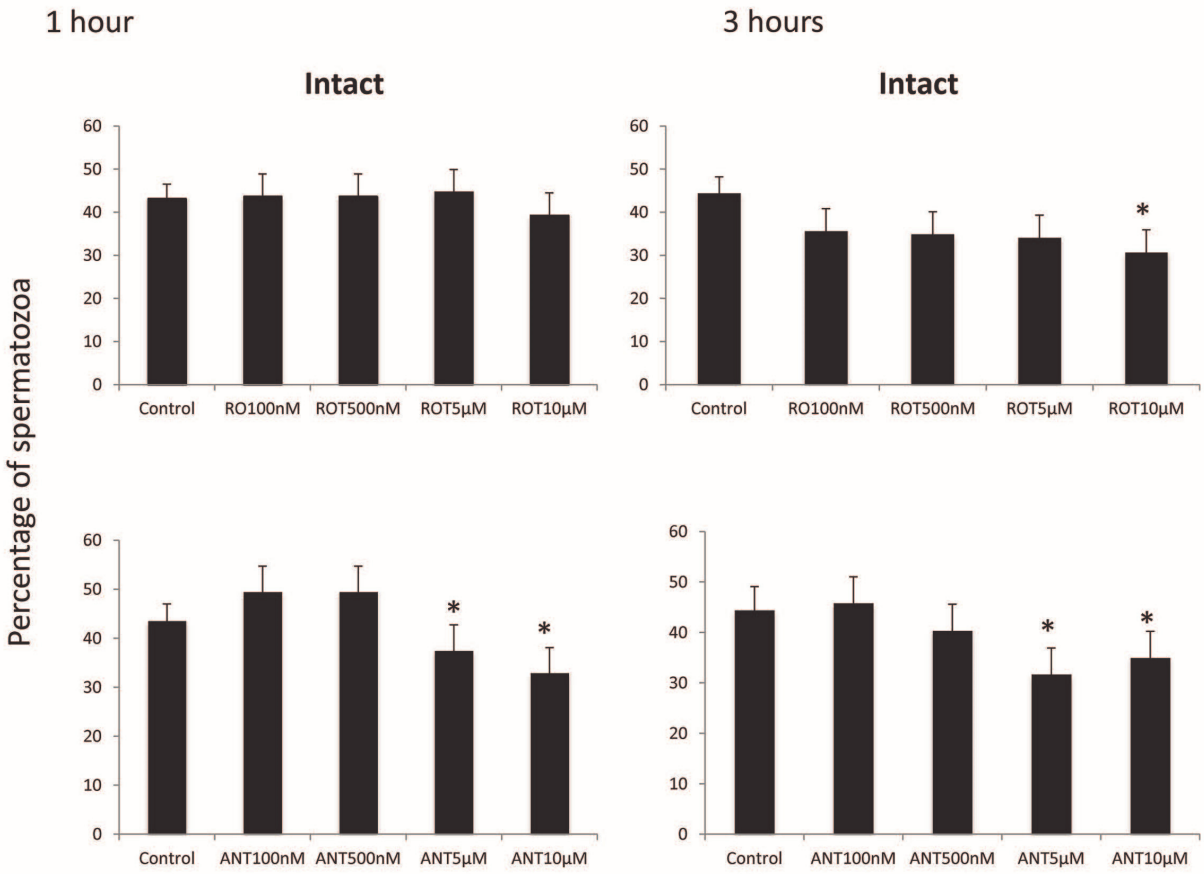


Fig 5. Effects of the inhibition of Complexes I and III of the electron transport chain on the percentage of stallion spermatozoa showing intact membranes. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone (0 nm (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) or antimycin-a (0 (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods; membrane integrity was determined using the H33342/YoPr/Ethidium assay as described in the materials and methods. ** P<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g005

sources of ATP may be present. To test this hypothesis, stallion sperm were incubated in presence of 0, 5, and 10 mM of 2-Deoxy-D-Glucose. This is a glucose analog that inhibits glycolysis via its actions on hexokinase, the rate limiting step of glycolysis. It is phosphorylated by hexokinase to 2-DG-P and cannot be further metabolized by phosphoglucose isomerase. This leads to the accumulation of 2-DG-P in the cell and the depletion in cellular ATP. The inhibitor 2-deoxyglucose induced a 17% reduction in total motility after 1 hour of incubation and 45.8% after 1 hour of incubation ($p<0.01$) (Fig 11) without any effect on the percentage of progressive motile sperm, mitochondria or membrane permeability and integrity, the percentage of spermatozoa with intact membranes after 1 h of incubation was 54.1 in controls, 56.0, 56.7 and 55.7 in samples incubated in the presence of 2, 5 and 10 mM of the inhibitor. After 3 h of incubation these percentages were 37 for controls, 31, 26 and 29 for samples supplemented with 2, 5 and 10 mM 2-DG. The percentages of spermatozoa with low mitochondrial membrane

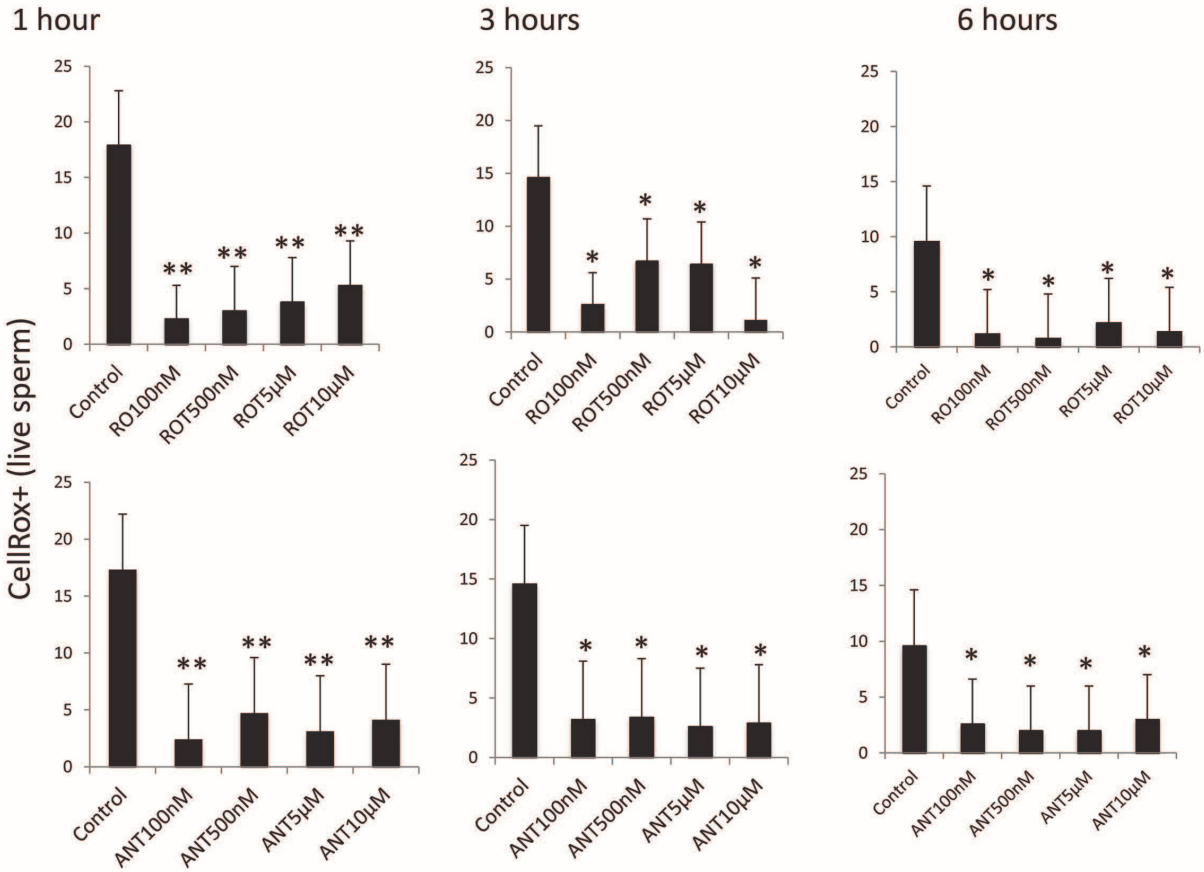


Fig 6. Effect of the inhibition of Complexes I and III of the electron transport chain on the percentage of stallion spermatozoa showing ROS production using CellRox deep red reagent. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone (0 (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) or antimycin (0 (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods. ROS were measured using flow cytometry after CellRox deep red staining as described in the materials and methods. * $p<0.05$, ** $p<0.01$. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g006

potential after 1 hour of incubation were 36, 36, 35 and 38 in controls and samples incubated in presence of 2, 5 and 10 mM of the analog. After 3 h of incubation these percentages were 47, 51, 51 and 54 in samples incubated in presence of 0, 2, 5, and 10 mM of the analog respectively. Additionally stallion sperm was incubated in a medium devoid of any kind of glucose, and in the absence of glucose and pyruvate. The only effect observed was a reduction in the percentage of total motile sperm after three hours of incubation in media devoid of pyruvate and glucose (Fig 12B and 12C, and a reduction in the VCL in media devoid of pyruvate and glucose compared to media devoid only of pyruvate (Fig 12E); this occurred only at three hours of incubation and this difference was not longer present after 6 hours. No changes were observed in ATP content (Fig 13).

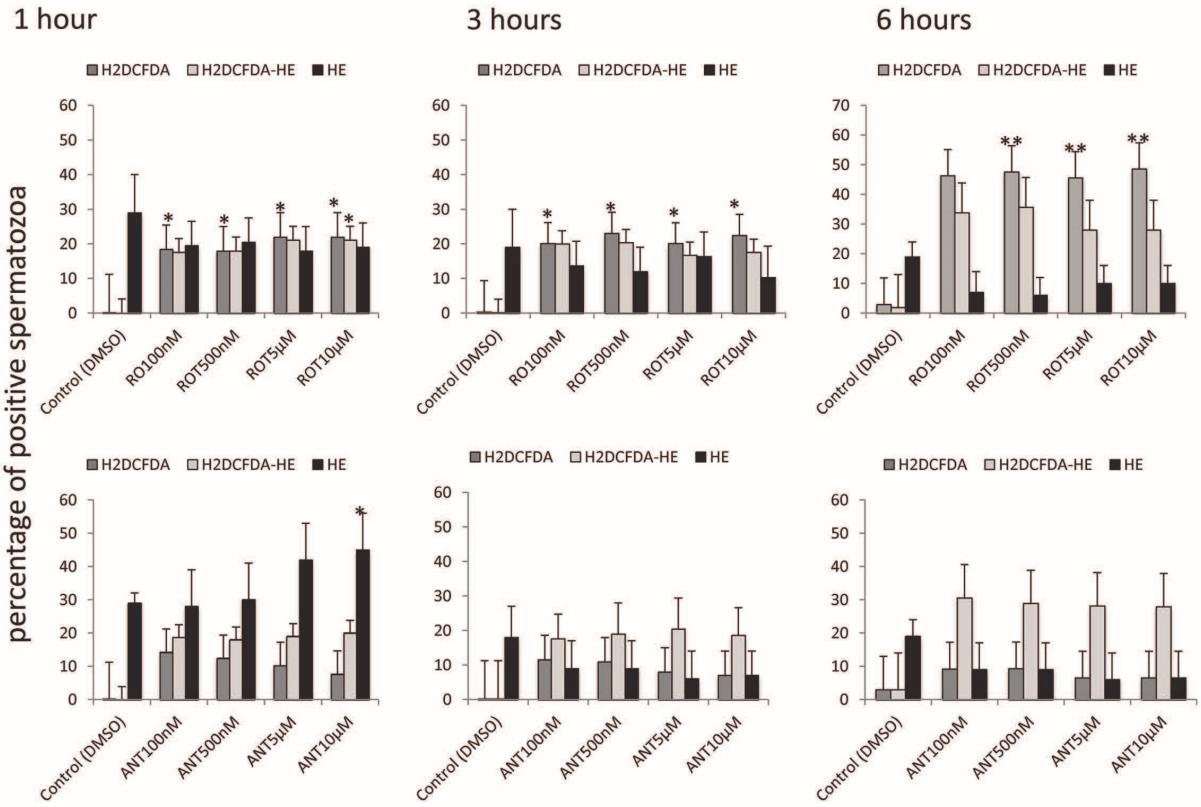


Fig 7. Effect of the inhibition of Complexes I and III of the electron transport chain on superoxide and hydrogen peroxide production by stallion spermatozoa. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone or antimycin as indicated in the materials and methods. Superoxide and hydrogen peroxide were measured using flow cytometry after HE (superoxide) and H_2DCFDA (hydrogen peroxide) staining as described in the materials and methods. * p<0.05, ** p<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g007

Relationship between reactive oxygen species and motility, membrane integrity, and mitochondrial membrane potential

Positive correlations were observed between different indicators of ROS production and sperm motility and velocity, with Mitosox-positive spermatozoa showing small but positive correlations with the percentage of total motile sperm (R^2 = 0.219, p<0.05) and sperm velocities (VCL, R = 0.314, p<0.01; VAP R = 0.229, p<0.01) (Table 1). Similarly significant correlations were found between CellRox-positive spermatozoa and the percentages of total motile spermatozoa (R = 0,180, p<0.05); progressive motile spermatozoa (R = 0.251, p<0.01); and VCL, VSL and VAP (Table 2). Interestingly the percentage of CellRox-positive sperm correlated with the percentage of intact sperm (R = 0.501, p<0.01) and the percentage of spermatozoa with high mitochondrial membrane potential (R = 0.363, p<0.01); other correlations found are summarized in Table 3.

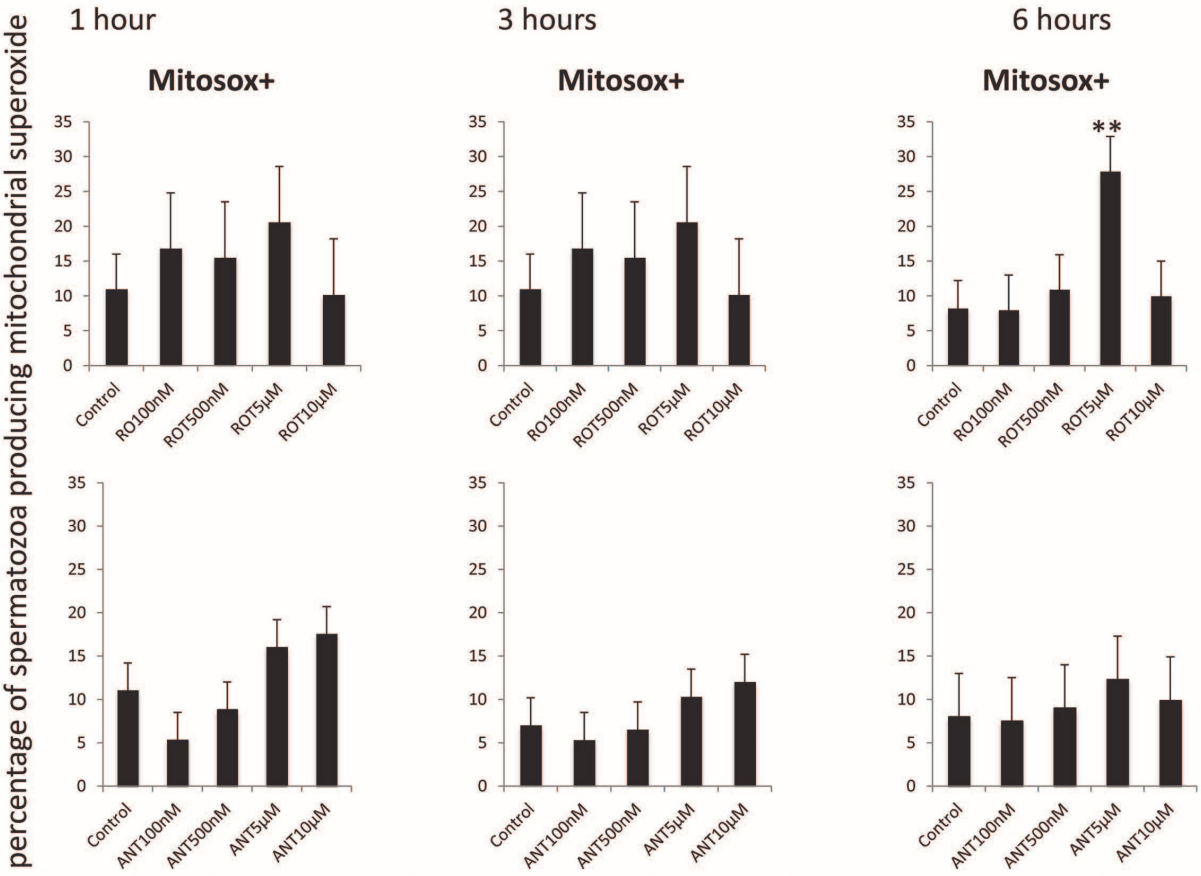


Fig 8. Effect of the inhibition of Complexes I and III of the electron transport chain on the production of mitochondrial superoxide in stallion spermatozoa. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone or antimycin as indicated in the materials and methods. Mitochondrial superoxide was measured using flow cytometry after Mitosox staining as described in the materials and methods. Data represent Mitosox positive sperm in the whole population. * $p<0.05$, ** $p<0.01$. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g008

Discussion

We studied the effects of interrupting the electron flux in the mitochondria of stallion sperm at two specific points, complex I (NADH-ubiquinone oxidoreductase), and complex III (co-enzyme Q-cytochrome c reductase). The major effects of these interventions were reductions in motility and velocities of the sperm. ATP content was also reduced, as were the mitochondrial membrane potential and membrane intactness and permeability. Intriguing effects were observed in terms of ROS production. The inhibition of complexes I and III reduced ROS production as assayed using the CellRox deep red reagent to measure oxidative stress. This is a paradoxical result because complexes I and III are recognized as major sources of ROS, both in somatic cells [18] and in spermatozoa [6]. This observation may be due to the fact that this probe is mainly sensitive to superoxide anion. According to the manufacturer the probe used in our experiment is sensitive to only the hydroxyl radical and the superoxide anion. Because significant amounts of divalent cations are unlikely in our model, the most likely explanation

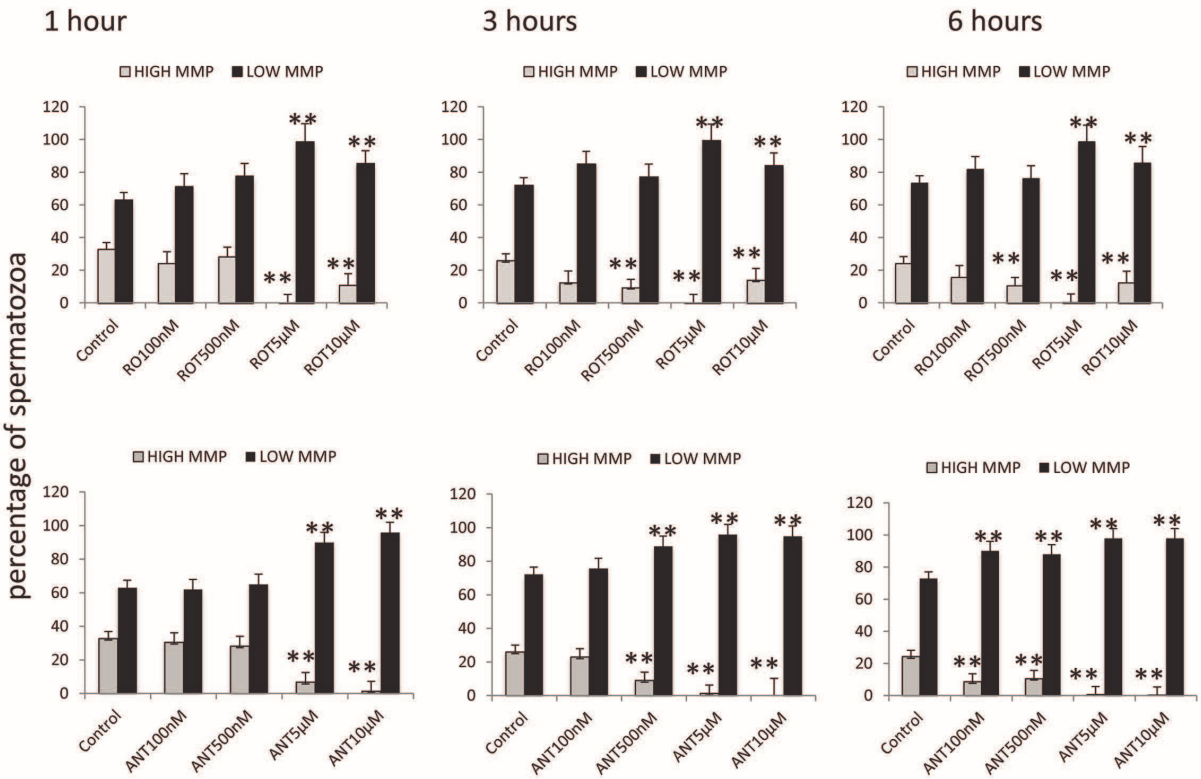


Fig 9. Effects of the inhibition of Complexes I and III of the electron transport chain on the mitochondrial membrane potential of stallion spermatozoa. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone (0 (DMSO), 100 nm, 500 nm, 5 μM and 10 μM) or antimycin-A (0 (DMSO), 100 nm, 500 nm, 5 μM and 10 μM) as indicated in the materials and methods. Mitochondrial membrane potential was assessed using flow cytometry after JC-1 staining. High MMP, spermatozoa showing high mitochondrial membrane potential, Low MMP, spermatozoa showing low mitochondrial membrane potential ** P<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g009

for our findings is that once the ETC is disrupted, the mitochondrial potential collapses and superoxide is no longer produced due to the interruption of electron transfer and subsequent electron leakage. Hydroethidine detected increased superoxide production in samples incubated in presence of antimycin 10 μM after 1 hour of incubation. However, the discrepancy between CellRox and hydrotheidine can be explained by the fact the CellRox was used to detect superoxide only in live cells, whereas hydroethidine was used in the whole population. The observed increase is therefore explained by superoxide produced by dead cells. This theory is also supported by the decrease in the percentage of intact sperm observed in the samples supplemented with antimycin and by the positive correlations found between CellRox-positivity and motility and membrane integrity

This hypothesis is also supported by the rapid collapse in mitochondrial membrane potential observed after the inhibition of both complexes, further suggesting that the interruption of the ETC reduces electron leakage. On the other hand, previous reports have demonstrated that mitochondrial inhibitors either increase or decrease ROS production [30]. In somatic cells, a small mitochondrial depolarization leads to increased ROS generation, whereas a more profound mitochondrial depolarization reduces ROS; this is consistent with the model where

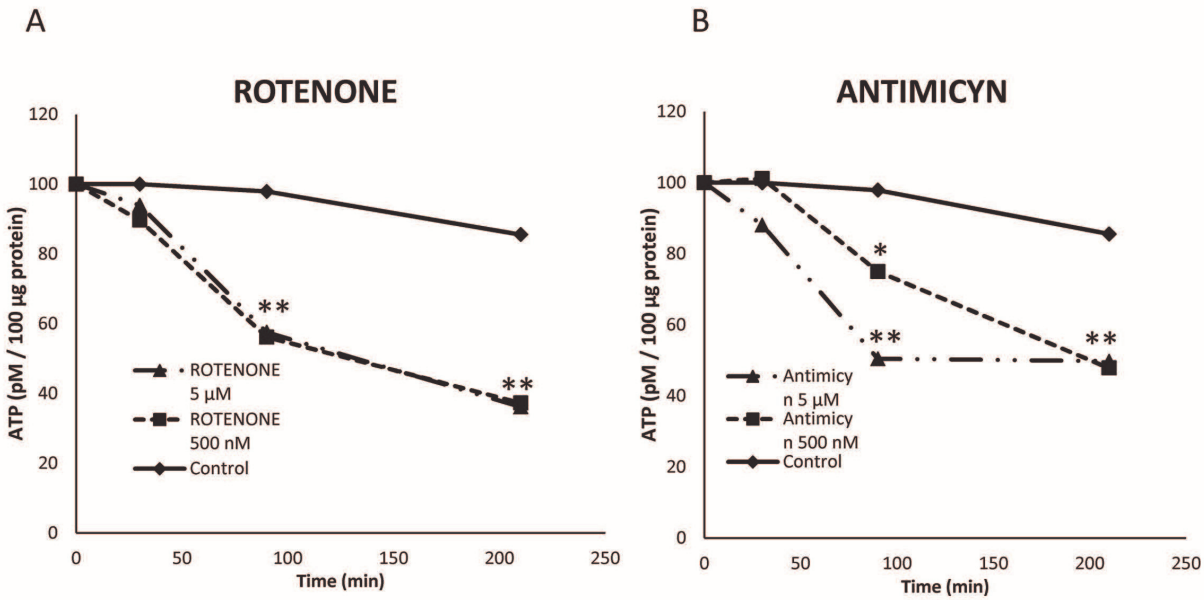


Fig 10. Effect of the inhibition of Complexes I and III of the electron transport chain on the ATP content of stallion spermatozoa. Stallion spermatozoa were extended in BWW media and incubated in the presence of rotenone (0 (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) or antimycin (0 (DMSO), 100 nm, 500 nm, 5 µM and 10 µM) as indicated in the materials and methods. ATP was determined as described in the materials and methods * p<0.05, ** p<0.01. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g010

under resting conditions, 1% to 2% or O2 used in the ETC is not completely reduced, leading to the generation of superoxide anion [31].

Superoxide anion may be an indicator of intensely metabolically active spermatozoa [8]. Further evidence in favor of this assumption can be found in the observations that both inhibitors reduced ATP and that positive (but weak) correlations were found between Mitosox-positive cells and sperm motility and velocity. To further investigate the mitochondrial production of reactive oxygen species, a probe specific for the mitochondrial superoxide production was used. With this reagent, only the inhibition of complex I led to increased mitochondrial superoxide production after six hours of incubation; this was also accompanied by parallel increases in hydrogen peroxide. However the increase in mitosox positivity may also be due to an artifact, due to ethidium contamination of the probe, and this is a clear limitation of this assay[32]. Rotenone induces hydrogen peroxide in somatic cell's mitochondria[18], and this was also evident in stallion spermatozoa.

Antimycin induced hydrogen peroxide production has been described in somatic cells [18]. Antimycin leads to the generation of the semiquinone radical [33], which then stabilizes by shedding its electrons to oxygen to create superoxide in the intramembranous space, dismutates to H_2O_2 under the influence of superoxide dismutase, and escapes to the outside of the cell. We were unable to detect increases in hydrogen peroxide, even after long incubation periods; this may be due to the presence of antioxidant enzymes in this space or reduced superoxide production.

Both inhibitors reduced ATP production in stallion spermatozoa accompanied by a significant reduction in sperm motility and velocity and by increases in membrane permeability. However, sperm motility and velocity were not abolished, To determine the potential role of

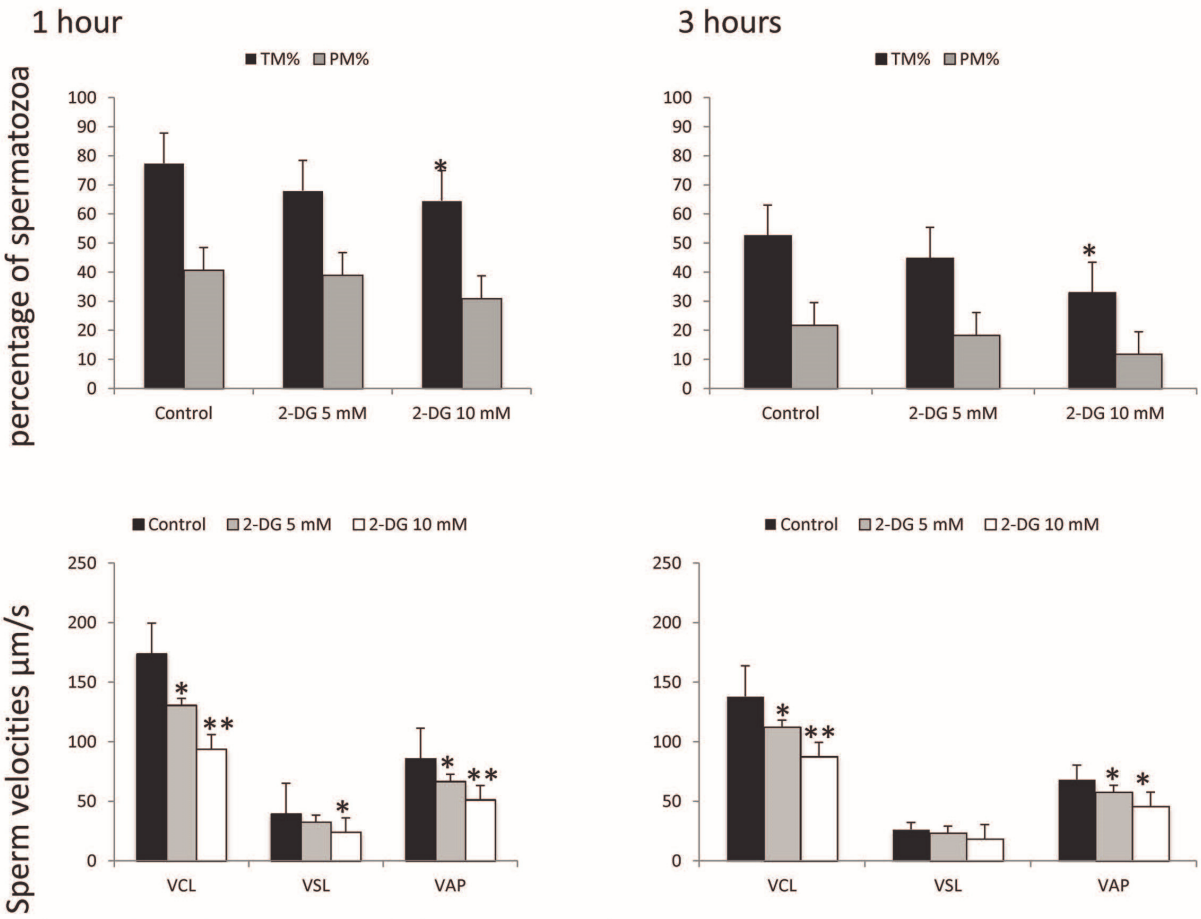


Fig 11. Effect of the inhibition of glycolysis on stallion sperm motility and kinematics. Stallion sperm were incubated in presence of 0, 5, and 10 mM of 2-deoxy-D-glucose as described in the materials and methods. The percentages of total motile sperm and progressively motile sperm and the sperm velocities were analyzed using a CASA system. *p<0.05, ** p<0.001. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g011

glycolysis as a source of energy for motility, glycolysis was inhibited using a specific inhibitor, and also stallion sperm was incubated in media devoid of any kind of glucose and devoid of glucose and pyruvate. The inhibition of glycolysis resulted in dramatically decreased sperm velocities, and also induced changes in the percentage of progressive motile sperm with the higher dosage. However this approach has some caveats, the futile phosphorylation of 2-DG by hexokinase will result in ATP depletion, for this reason is difficult to determine whether decreased ATP production is due to depletion or reduced rate of ATP production. Incubation of stallion spermatozoa in media devoid of glucose and pyruvate had no effect on ATP, although there was an effect on motility and sperm velocities after three hours of incubation. These results suggest that although OXPHOS is the main source for sperm motility, glycolysis is necessary to support the sperm movement after long incubation periods.

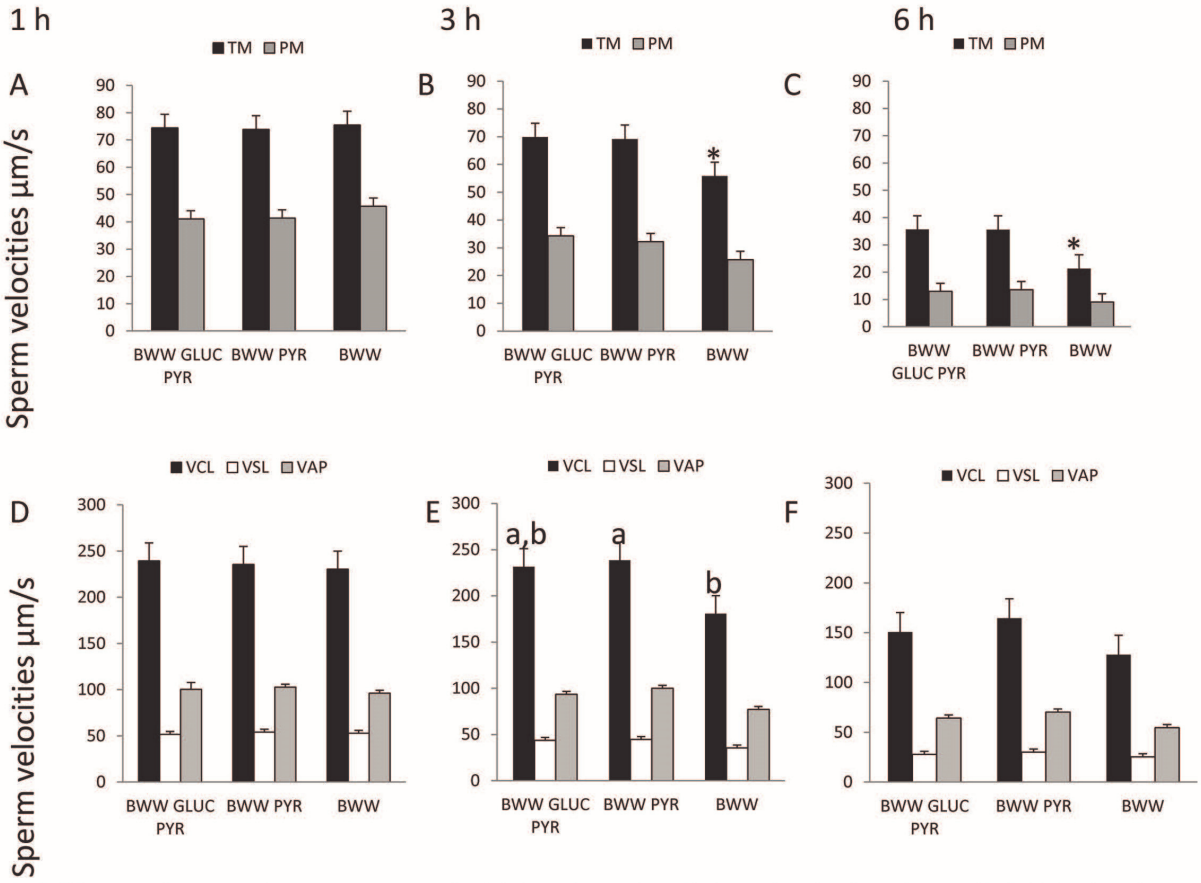


Fig 12. Effect of the incubation of stallion spermatozoa in BWW complete media (BWW GLUC PYR) devoid of glucose (BWW PYR) or devoid of glucose and pyruvate (BWW) on stallion sperm motility and kinematics. A, B and C, effects on the percentages of total motile (TM) and progressive motile (PM) after 1, 3 and 6 hours of incubation. D,E, F, effect on sperm velocities after 1, 3 and 6 hours of incubation. The percentages of total motile sperm and progressively motile sperm and the sperm velocities were analyzed using a CASA system. *p<0.05, in E bars with different superscripts represent significant differences (a, b p<0.05). The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g012

Although previous reports indicate that stallion spermatozoa are highly dependent on mitochondrial ATP [8, 34], the role of glycolysis have not been investigated to date. Our results indicate that on one hand, stallion spermatozoa are highly dependent on mitochondrial ATP for motility, confirming recent findings[8, 34]. However, this study also demonstrated for the first time that glycolysis has a role supporting sperm movement.

Stallion spermatozoa have high concentration of antioxidant enzymes [35, 36], probably representing an evolutionary adaptation to their intense mitochondrial activity. Supporting this assumption are the positive correlations found between markers of oxidative stress and sperm function in the present study. Recent findings indicate a relationship between oxidative stress and field fertility in stallions [8]. The correlations found in our study, although significant, were weak and moderate. Previous studies have demonstrated that cryopreservation

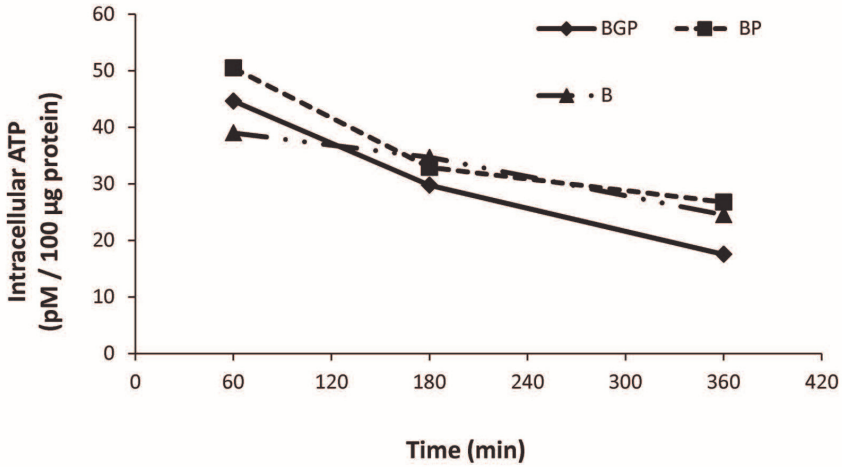


Fig 13. Effect of the incubation of stallion spermatozoa in BWW complete media (BWW GLUC PYR) devoid of glucose (BWW PYR) or devoid of glucose and pyruvate (BWW) on on the ATP content of stallion spermatozoa. ATP was determined as described in the materials and methods. The results are given as the means ± SD.

doi:10.1371/journal.pone.0138777.g013

induces lipid peroxidation [14] and that antioxidants controversially improve the outcome of cryopreservation [37–39]. In other species, antioxidant supplementation has successfully [9, 40] improved the outcome of cryopreservation. Our understanding of the function of mitochondrial ROS in somatic cells has changed in recent years [41, 42], and ROSs are no longer believed to be simply a toxic byproduct of oxidative metabolism. On the contrary, they are considered to be important regulators of many cellular functions; ROS can cause reversible post-translational protein modifications to regulate signaling pathways. This role may be of special importance in spermatozoa; spermatozoa are translationally silent cells that are unable to synthetize de novo proteins and can only rely on post-translational modifications to regulate their functions[43].

In short, it can be concluded that stallion sperm mostly rely on OXPHOS to produce ATP for motility. The inhibition of complex I of the ETC in the mitochondria leads to reduced motility due decreased ATP and to increased hydrogen peroxide production. These findings have implications for the meaning and significance of reactive oxygen production by stallion spermatozoa and may influence strategies for sperm preservation.

Table 1. Significant correlations between Mitosox-positivity and sperm motility and velocity.

	TM%	VCL µm/s	VAP µm/s	VSL µm/s
Mitosox +	R = 0.219*	R = 0.314**	R = 0.319**	R = 0.198*

Mitosox + spermatozoa showing mitochondrial production of superoxide radical, TM%—percentage of total motile sperm, VCL—curvilinear velocity, VAP—average path velocity, VSL—straight-line velocity.

*p<0.05

** p<0.01

doi:10.1371/journal.pone.0138777.t001

Table 2. Significant correlations between different indicators of the production of ROS and parameters of sperm motility and velocities.

	TM%	PM%	VCL μm/s	VAP μm/s	VSL μm/s
CellRox (live cells)	R = 0.180*	R = 0.251**	R = 0.313**	R = 0.229**	R = 0.285**

*p<0.05

** p<0.01

CellRox (Live Cells) percentage of live spermatozoa showing ROS production, TM%—percentage of total motile sperm, VCL—curvilinear velocity, VAP—average path velocity, VSL—straight-line velocity.

doi:10.1371/journal.pone.0138777.t002

Table 3. Significant correlations between different indicators of the production of ROS and membrane intactness and mitochondrial functionality.

	Intact sperm	YoPro+	JC-high
Mitosox+	R = 0.302**	R = 0.200**	R = 0.286**
H_2DCFDA	R = 0.269**	R = 0.218**	R = 0.302**
CellRox (live cells)	R = 0.501**	n.s	R = 0.363**
HE	R = -0.348**	n.s	n.s.

** p<0.01, n.s non-significant

Mitosox+ spermatozoa show mitochondrial production of superoxide radical, H_2DCFDA—percentage of spermatozoa showing hydrogen peroxide production, CellRox (Live Cells)—percentage of live spermatozoa showing ROS production, HE—percentage of spermatozoa showing superoxide production, Intact sperm—percentage of spermatozoa with completely intact membranes, YoPro+—percentage of spermatozoa with intact membranes but with increased permeability, JC- high percentage of spermatozoa with high mitochondrial membrane potential.

doi:10.1371/journal.pone.0138777.t003

Author Contributions

Conceived and designed the experiments: FJP. Performed the experiments: MPD PMM COF CBdSC. Analyzed the data: FJP. Contributed reagents/materials/analysis tools: JAT. Wrote the paper: FJP.

References

1. Amaral A, Lourenco B, Marques M, Ramalho-Santos J. Mitochondria functionality and sperm quality. Reproduction. 2013; 146(5):R163–74. doi: 10.1530/REP-13-0178 PMID: 23901129
2. Pena FJ, Plaza Davila M, Ball BA, Squires EL, Martin Munoz P, Ortega Ferrusola C, et al. The Impact of Reproductive Technologies on Stallion Mitochondrial Function. Reproduction in domestic animals = Zuchthygiene. 2015; 50(4):529–37. doi: 10.1111/rda.12551 PMID: 26031351
3. Pena FJ, Rodriguez Martinez H, Tapia JA, Ortega Ferrusola C, Gonzalez Fernandez L, Macias Garcia B. Mitochondria in mammalian sperm physiology and pathology: a review. Reproduction in domestic animals = Zuchthygiene. 2009; 44(2):345–9. doi: 10.1111/j.1439-0531.2008.01211.x PMID: 19144010
4. O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free radical biology & medicine. 2006; 41(4):528–40. doi: 10.1016/j.freeradbiomed.2006.04.027 PMID: 16863985
5. Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative stress and male reproductive health. Asian journal of andrology. 2014; 16(1):31–8. doi: 10.4103/1008-682X.122203 PMID: 24369131; PubMed Central PMCID: PMC3901879
6. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. The Journal of clinical endocrinology and metabolism. 2008; 93(8):3199–207. doi: 10.1210/jc.2007-2616 PMID: 18492763
7. Pena FJ, Garcia BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology. 2011; 76(7):1177–86. doi: 10.1016/j.theriogenology.2011.06.023 PMID: 21835453
8. Gibb Z, Lambourne SR, Aitken RJ. The Paradoxical Relationship Between Stallion Fertility and Oxidative Stress. Biology of reproduction. 2014. doi: 10.1095/biolreprod.114.118539 PMID: 25078685

9. Pena FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci. 2003; 78(1–2):85–98. PMID: 12753785

10. Gallardo Bolanos JM, Miro Moran A, Balao da Silva CM, Morillo Rodriguez A, Plaza Davila M, Aparicio IM, et al. Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. PLOS ONE. 2012; 7(1):e30688. doi: 10.1371/journal.pone.0030688 PMID: 22292020; PubMed Central PMCID: PMC3266901

11. Caselles AB, Miro-Moran A, Morillo Rodriguez A, Gallardo Bolanos JM, Ortega-Ferrusola C, Salido GM, et al. Identification of apoptotic bodies in equine semen. Reproduction in domestic animals=Zuchthygiene. 2014; 49(2):254–62. doi: 10.1111/rda.12264 PMID: 24467598

12. Ortega-Ferrusola C, Sotillo-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, Gonzalez-Fernandez L, et al. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl. 2008; 29(2):213–21. doi: 10.2164/jandrol.107.003640 PMID: 17978341

13. Ball BA. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Animal reproduction science. 2008; 107(3–4):257–67. doi: 10.1016/j.anireprosci.2008.04.014 PMID: 18524506

14. Ortega Ferrusola C, Gonzalez Fernandez L, Morrell JM, Salazar Sandoval C, Macias Garcia B, Rodriguez-Martinez H, et al. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction. 2009; 138(1):55–63. doi: 10.1530/REP-08-0484 PMID: 19380427

15. Garcia BM, Fernandez LG, Ferrusola CO, Salazar-Sandoval C, Rodriguez AM, Martinez HR, et al. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. Reproduction in domestic animals=Zuchthygiene. 2011; 46(1):141–8. doi: 10.1111/j.1439-0531.2010.01609.x PMID: 20456666

16. Garcia BM, Moran AM, Fernandez LG, Ferrusola CO, Rodriguez AM, Bolanos JM, et al. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. J Androl. 2012; 33(1):105–13. doi: 10.2164/jandrol.110.011957 PMID: 21436310

17. Ortega-Ferrusola C, Garcia BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, Tapia JA, et al. Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim Reprod Sci. 2009; 114(4):393–403. doi: 10.1016/j.anireprosci.2008.10.005 PMID: 19019584

18. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. The Journal of biological chemistry. 2003; 278(10):8516–25. doi: 10.1074/jbc.M210432200 PMID: 12496265

19. Gallardo Bolanos JM, Balao da Silva CM, Martin Munoz P, Morillo Rodriguez A, Plaza Davila M, Rodriguez-Martinez H, et al. Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction. 2014; 148(2):221–35. doi: 10.1530/REP-13-0191 PMID: 24850868

20. Nunez-Martinez I, Moran JM, Pena FJ. Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. International journal of andrology. 2007; 30(3):182–9. doi: 10.1111/j.1365-2605.2007.00743.x PMID: 17376220

21. Ortega Ferrusola C, Gonzalez Fernandez L, Macias Garcia B, Salazar-Sandoval C, Morillo Rodriguez A, Rodriguez Martinez H, et al. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol Reprod. 2009; 81(6):1106–11. Epub 2009/07/17. doi: 10.1095/biolreprod.109.078220 PMID: 19605785

22. Pena FJ, Saravia F, Johannisson A, Walgren M, Rodriguez-Martinez H. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. International journal of andrology. 2005; 28(2):107–14. doi: 10.1111/j.1365-2605.2005.00512.x PMID: 15811072

23. Gravance CG, Garner DL, Baumber J, Ball BA. Assessment of equine sperm mitochondrial function using JC-1. Theriogenology. 2000; 53(9):1691–703. PMID: 10968415

24. Macias-Garcia B, Gonzalez-Fernandez L, Gallardo-Bolanos JM, Pena FJ, Johannisson A, Morrell JM. Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS. Anim Reprod Sci. 2012; 132(1–2):74–82. Epub 2012/04/27. doi: 10.1016/j.anireprosci.2012.03.017 PMID: 22534021

25. Gallardo Bolanos JM, Balao da Silva C, Martin Munoz P, Plaza Davila M, Ezquerra J, Aparicio IM, et al. Caspase activation, hydrogen peroxide production and Akt dephosphorylation occur during stallion sperm senescence. Reproduction in domestic animals = Zuchthygiene. 2014; 49(4):657–64. doi: 10.1111/rda.12343 PMID: 24924976

26. Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage

in human spermatozoa. Biochem J. 2011; 436(3):687–98. Epub 2011/04/08. doi: 10.1042/BJ20110114 PMID: 21470189

27. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab. 2008; 93 (8):3199–207. Epub 2008/05/22. doi: 10.1210/jc.2007-2616 PMID: 18492763

28. Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Pena FJ, Mari G, et al. Effect of sex sorting on stallion spermatozoa: Heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci. 2013; 141(1–2):68–74. doi: 10.1016/j.anireprosci.2013.07.008 PMID: 23953198

29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976; 72:248–54. PMID: 942051

30. Xi Q, Cheranov SY, Jaggar JH. Mitochondria-derived reactive oxygen species dilate cerebral arteries by activating Ca2+ sparks. Circulation research. 2005; 97(4):354–62. doi: 10.1161/01.RES.0000177669.29525.78 PMID: 16020754; PubMed Central PMCID: PMC1352308

31. Turrens JF. Mitochondrial formation of reactive oxygen species. The Journal of physiology. 2003;552 (Pt 2):335–44. doi: 10.1113/jphysiol.2003.049478 PMID: 14561818; PubMed Central PMCID: PMC2343396

32. Zielonka J, Kalyanaraman B. Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free Radic Biol Med. 2010; 48(8):983–1001. doi: 10.1016/j.freeradbiomed.2010.01.028 PMID: 20116425; PubMed Central PMCID: PMC3587154

33. Sun J, Trumpower BL. Superoxide anion generation by the cytochrome bc1 complex. Archives of biochemistry and biophysics. 2003; 419(2):198–206. PMID: 14592463

34. Ortega Ferrusola C, Gonzalez Fernandez L, Salazar Sandoval C, Macias Garcia B, Rodriguez Martinez H, Tapia JA, et al. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. Theriogenology. 2010; 74(3):458–65. doi: 10.1016/j.theriogenology.2010.02.029 PMID: 20451990

35. Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine spermatozoa. American journal of veterinary research. 2001; 62(4):508–15. PMID: 11327456

36. Ball BA, Gravance CG, Medina V, Baumber J, Liu IK. Catalase activity in equine semen. American journal of veterinary research. 2000; 61(9):1026–30. PMID: 10976731

37. Morillo-Rodriguez A, Macias-Garcia B, Tapia JA, Ortega-Ferrusola C, Pena FJ. Consequences of butylated hydroxytoluene in the freezing extender on post-thaw characteristics of stallion spermatozoa in vitro. Andrologia. 2012; 44 Suppl 1:688–95. doi: 10.1111/j.1439-0272.2011.01251.x PMID: 22082046

38. Ball BA, Medina V, Gravance CG, Baumbe J. Effect of antioxidants on preservation of motility,viability and acrosomal integrity of equine spermatozoa during storage at 5 degrees C. Theriogenology. 2001; 56(4):577–89. PMID: 11572439

39. Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, et al. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. Journal of andrology. 2005; 26(1):15–24. PMID: 15611562

40. Neagu VR, Garcia BM, Sandoval CS, Rodriguez AM, Ferrusola CO, Fernandez LG, et al. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. Theriogenology. 2010; 73(5):645–50. doi: 10.1016/j.theriogenology.2009.10.021 PMID: 20018359

41. Stowe DF, Camara AK. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. Antioxidants & redox signaling. 2009; 11(6):1373–414. doi: 10.1089/ARS.2008.2331 PMID: 19187004; PubMed Central PMCID: PMC2842133

42. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Molecular cell. 2012; 48(2):158–67. doi: 10.1016/j.molcel.2012.09.025 PMID: 23102266; PubMed Central PMCID: PMC3484374

43. Gonzalez-Fernandez L, Ortega-Ferrusola C, Macias-Garcia B, Salido GM, Pena FJ, Tapia JA. Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. Biology of reproduction. 2009; 80 (6):1239–52. doi: 10.1095/biolreprod.108.073486 PMID: 19211810

Artículo 2º

Reproduction in Domestic Animals

Reprod Dom Anim **50**, 1011–1016 (2015); doi: 10.1111/rda.12628
ISSN 0936–6768

Epigallocatechin-3-Gallate (EGCG) Reduces Rotenone Effect on Stallion Sperm–Zona Pellucida Heterologous Binding

M Plaza Dávila[1], D Bucci[2], G Galeati[2], FJ Peña[1], G Mari[2,3], E Giaretta[2], C Tamanini[2] and M Spinaci[2]

[1]*Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain;* [2]*Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, Bologna, Italy;* [3]*AUB INFA, University of Bologna, Bologna, Italy*

Contents
Stallion spermatozoa are highly dependent on oxidative phosphorylation for ATP production to achieve normal sperm function and to fuel the motility. The aim of this study was to evaluate the response of equine sperm under capacitating conditions to the inhibition of mitochondrial complex I by rotenone and to test whether epigallocatechin-3-gallate (EGCG), a natural polyphenol component of green tea, could counteract this effect. After 2-h incubation of stallion spermatozoa in modified Tyrode's medium, rotenone (100 nM, 500 nM and 5 μM) and EGCG (10, 20 and 60 μM), alone or in combination, did not induce any significant difference on the percentage of viable cells, live sperm with active mitochondria and spermatozoa with intact acrosome. The inhibition of complex I of mitochondrial respiratory chain of stallion sperm with rotenone exerted a negative effect on heterologous ZP binding ability. EGCG at the concentrations of 10 and 20 μM (but not of 60 μM) induced a significant increase in the number of sperm bound to the ZP compared with that for control. Moreover, when stallion sperm were treated with rotenone 100 nM, the presence of EGCG at all the concentrations tested (10, 20 and 60 μM) significantly increased the number of sperm bound to the ZP up to control levels, suggesting that this green tea polyphenol is able to reduce the toxicity of rotenone.

Introduction

Spermatozoa require ATP to achieve normal sperm function and to fuel the motility. Mammalian sperm rely mainly on two metabolic pathways to produce ATP which are localized to different regions of the cell: oxidative phosphorylation (OXPHOS) occurs in mitochondria localized in the sperm mid-piece, while anaerobic glycolysis takes place mainly in the fibrous sheath of the flagellum where glycolytic enzymes are tightly anchored (Ferramosca and Zara 2014; Tourmente et al. 2015).

While human sperm rely mainly on glycolysis for ATP production, bull spermatozoa are characterized by both high respiration and glycolysis. On the other hand, stallion spermatozoa are highly dependent on OXPHOS for ATP production (Cummins 2009; Gibb et al. 2014). The great importance of sperm mitochondrial functionality in horse is confirmed by the observation that the most fertile stallion ejaculates exhibit the highest levels of OXPHOS activity (Gibb et al. 2014).

The inhibition of electron transport chain (ETC) along the respiratory complexes produces free radicals that damage the functionality of the mitochondria and decrease the intracellular ATP content resulting in a decrease in stallion sperm motility (Gibb et al. 2014), even in the presence of glucose (Plaza Dávila et al. 2015).

One of the most active inhibitors of mitochondrial respiratory chain (MRC) is rotenone, a lipophilic isoflavonoid that inhibits complex I (NADH reductase) (Singer and Ramsay 1994). Rotenone reduces ATP production by mitochondria, leading to increased formation of free radicals besides a deregulation of cell homoeostasis and ROS release into the mitochondrial matrix, where they can overwhelm the intramitochondrial antioxidant defence enzymes. This would account for the ability of rotenone to induce peroxidative damage in the mid-piece of the spermatozoa. The peroxidative damage, in turn, induces a progressive loss of motility in terms of the percentage of motile and progressive spermatozoa (Koppers et al. 2008). The presence of antioxidants, such as α-tocopherol, can prevent these negative effects of rotenone (Koppers et al. 2008).

Different natural antioxidants can help to reverse the negative effect of inhibitors of mitochondrial respiratory chain (MRC). Among Green tea catechins, the principal polyphenolic compound is epigallocatechin-3-gallate (EGCG) (Stewart et al. 2005), which can act as an eliminator of free radical by reaction with hydrogen, alkoxyl or peroxyl radicals (Wang et al. 2000) and as an iron chelator (Grinberg et al. 1997). In addition, its antioxidant capacity by removing free radicals can indirectly increase endogenous antioxidants activity (Guo et al. 1996; Skrzydlewska et al. 2002). Moreover, EGCG accumulates within the mitochondria and preserves catalase activity (Schroeder et al. 2008). Valenti et al. (2013) demonstrated that EGCG restores the overall rate of mitochondrial ATP synthesis of cells from subjects with Down's syndrome, in which the deficit of complex I and ATP synthase results in depressed energy production by mitochondrial OXPHOS.

Sperm mitochondria are organelles that greatly suffer due to damage induced by reproductive technologies, such as cryopreservation and sex sorting (Ortega Ferrusola et al. 2009; Balao da Silva et al. 2014; Peña et al. 2015). Attempts to protect mitochondria can be an

attractive strategy to improve the quality of stallion sperm that underwent such biotechnical procedures.

The aim of our study was to evaluate the response of equine sperm under capacitating conditions to the inhibition of mitochondrial complex I by rotenone and to test whether EGCG could counteract this effect.

Material and Methods

Experimental design

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Three ejaculates from each of three stallions of proven fertility were used. A control group (CTR) and fifteen different experimental groups for each ejaculate on the basis of the additions were considered: rotenone 100 nM (R100), rotenone 500 nM (R500), rotenone 5 μM (R5), EGCG 10 μM (E10), EGCG 20 μM (E20), EGCG 60 μM (E60), rotenone 100 nM + EGCG 10 μM (R100 + E10), rotenone 100 nM + EGCG 20 μM (R100 + E20), rotenone 100 nM + EGCG 60 μM (R100 + E60), rotenone 500 nM + EGCG 10 μM (R500 + E10), rotenone 500 nM + EGCG 20 μM (R500 + E20), rotenone 500 nM + EGCG 60 μM (R500 + E60), rotenone 5 μM + EGCG 10 μM (R5 + E10), rotenone 5 μM + EGCG 20 μM (R5 + E20) and rotenone 5 μM + EGCG 60 μM (R5 + E60).

The evaluation of viability, acrosome status and mitochondrial membrane potential was performed on fresh semen (CTR), and after 2 h of incubation in modified Tyrode's medium pH 7.4 (Rathi et al. 2001).

The heterologous binding assay was performed co-incubating for 1 h *in vitro*-matured porcine oocytes with semen previously pre-incubated for 1 h in the presence or absence of different concentrations of rotenone and EGCG.

Semen collection and preparation

The experiment was approved by the Ethic-scientific Committee of Alma Mater Studiorum, University of Bologna.

Semen was obtained from three different stallions of proven fertility (14, 15 and 18 years old) individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, from October to November 2013. Stallions jumped on a breeding phantom and ejaculates were collected with a Missouri artificial vagina equipped with a disposable liner and aniline filter (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately evaluated for volume and concentration (NucleoCounter SP-100; Chemometec, Allerød, Denmark), diluted 1 : 1 in Kenney's extender (Kenney et al. 1975) and sent to the laboratory within 1 h, maintained at 22°C.

Aliquots of the ejaculates were centrifuged twice for 2 min at 900 × ***g***. The supernatants were removed and the pellets resuspended in modified Tyrode's solution (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2{\cdot}2H_2O$, 0.4 mM $MgSO_4{\cdot}7H_2$, 0.3 mM $KHPO_4$, 20 mM HEPES, 5 mM glucose, 21.7 mM Na lactate, 1 mM Na pyruvate, 15 mM NaH_2CO_3, 7 mg/ml BSA, 50 μg/ml kanamycin) pH 7.4 (Rathi et al. 2001) to obtain 20 × 10^6 spermatozoa/ml.

For the evaluation of viability, acrosome status and mitochondrial membrane potential, 500 μl of semen suspensions was incubated for 2 h in Nunc 4-well multidish at 38°C in 95% humidity 5% CO2 in the presence or absence of different concentrations of rotenone and EGCG.

Viability assessment with SYBR-PI

Twenty-five microlitres of semen was incubated with 2 μl of a 300 μM solution of propidium iodide (PI) and 2 μl of a 10 μM solution of SYBR green-14, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA), for 5 min at 37°C in the dark. Aliquots of the stained suspensions were placed on clean microscope slides and carefully overlaid with coverslips, and at least 200 spermatozoa per sample were scored under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, the Netherlands). Spermatozoa stained with SYBR-14 and not stained with PI were considered as viable. Spermatozoa both SYBR-14+ and PI+ and those SYBR-14−/PI+ were considered with damaged membranes or dead.

Evaluation of mitochondrial membrane potential

For each sample, an aliquot (25 μl) of semen was incubated with 2 μl of a 300 μM propidium iodide (PI) stock solution, 2 μl of a 10 μM SYBR green-14 stock solution and 2 μl of a 150 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanineiodide (JC-1) solution for 20 min at 37°C in the dark. Ten microlitres of the sperm suspension was then placed on a slide, and at least 200 spermatozoa per samples were scored using the above-described microscope. JC-1 monomers emit a green fluorescence in mitochondria with low potential, while emitting a bright red-orange fluorescence in case of multimer formation (J-aggregates) in mitochondria with high membrane potential. Sperm cells SYBR+/PI− with an orange fluorescence in the mid-piece were considered as live spermatozoa with high mitochondrial membrane potential.

Evaluation of acrosome status

Acrosome integrity was evaluated using a FITC-conjugated lectin from Pisum sativum (FITC-PSA) which label acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended in ethanol 95% and fixed/permeabilized at 4°C for at least 30 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5 μg PSA-FITC/1 ml H2O) for 20 min in the dark. After staining, samples were washed in PBS and mounted with Vectashield mounting

medium with PI (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with a fluorescence microscope. The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absence of fluorescence was considered to indicate acrosome disruption or acrosome reaction.

In vitro maturation (IVM)

Porcine cumulus–oocyte complexes (COCs) were aspirated using an 18-gauge needle attached to a 10-mL disposable syringe from 4 to 6 mm follicles of ovaries collected at a local abattoir and transported to the laboratory within 1 h. Under a stereomicroscope, intact COCs were selected and transferred into a Petri dish (35 mm; Nunclon, Roskilde, Denmark) prefilled with 2 ml of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 (Petters and Wells 1993) supplemented with 5.0 mg/ml insulin, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 50 mm β-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 μl of the same medium per well and cultured at 39°C in a humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 h of *in vitro* maturation, the medium was supplemented with 1.0 mM db-cAMP, 10 IU/ml, eCG (Folligon; Intervet, Boxmeer, the Netherlands) and 10 IU/ml hCG (Chorulon; Intervet). For the last 22 h, COCs were transferred to fresh maturation medium (Funahashi et al. 1997). At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting in maturation medium containing 0.4% hyaluronidase.

Heterologous binding assay

For the binding assay, the semen was centrifuged twice for 2 min at 900 × *g* and resuspended in modified Tyrode's medium to obtain 1×10^6 spermatozoa/ml, and 500 μl of the sperm suspensions was pre-incubated for 1 h in the presence or absence of different concentrations of rotenone and EGCG. After oocyte maturation, 30–35 denuded oocytes were added in each well, and after 1 h of gamete co-incubation at 38°C in 95% humidity and 5% CO2 in air, the oocytes were washed four times in PBS/0.4% BSA with a wide bore glass pipette to remove the spermatozoa loosely attacked to zona pellucida. The oocytes were then fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated with 8.9 μM Hoechst 33342 for 10 min in PBS/0.4% BSA in the dark, washed twice in the same medium, and individually placed in droplets of Vectashield (Vector Laboratories) on a slide and covered with a coverslip. The number of spermatozoa attached to the zona pellucida of each oocyte was assessed using the above-described microscope and was expressed as standard deviation units (see Statistical analysis).

Statistical analysis

Statistical analysis was performed using R version 3.1.1 (R Core Team 2012).

Sperm analysis data are expressed as mean ± SD. Significance was set at $p < 0.05$. Data were checked for normality using the Shapiro–Wilk test; differences between the treatments were analysed using an ANOVA test.

As for heterologous binding assay, data were standardized by dividing the number of bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed as standard deviation units. Data were analysed using a linear mixed-effect model. Significance was set at $p < 0.05$.

Results

Evaluation of viability, mitochondrial membrane potential and acrosome status

Rotenone treatment of stallion semen at all the concentrations tested (100 nM, 500 nM and 5 μM) during a 2-h incubation in modified Tyrode's medium did not induce any significant difference on the percentage of viable cells, live sperm with active mitochondria and spermatozoa with intact acrosome (Fig. 1a–c). EGCG at all the concentrations tested (10, 20 and 60 μM) did not exert any significant effect on the parameter analysed when supplemented either alone or in the presence of rotenone (Fig. 1a–c).

Heterologous binding assay

To evaluate the effect of rotenone and EGCG on equine sperm capability to bind to swine ZP, denuded *in vitro*-matured porcine oocytes were co-incubated for 1 h with semen previously pre-incubated 1 h in the presence or absence of different concentrations of rotenone and EGCG (approximately 100 oocytes per treatment). The results are expressed as the number of sperm bound per oocyte normalized to the daily standard deviation (Fig. 2).

Rotenone at all the concentrations tested (100 nM, 500 nM and 5 μM) induced a significant decrease in the number of sperm bound to the ZP compared with that for control.

EGCG at the concentrations of 10 and 20 μM (but not of 60 μM) induced a significant increase in the number of sperm bound to the ZP compared with that for control.

When stallion sperm were treated with rotenone 100 nM, the presence of EGCG at all the concentrations tested (10, 20 and 60 μM) significantly increased the number of sperm bound to the ZP up to control levels. However, EGCG at the concentrations of 20 and 60 μM did not significantly increase the number of sperm bound to the ZP compared with R 100.

EGCG at all the concentrations tested (10, 20 and 60 μM) did not induce any increase in the number of spermatozoa bound when added in the presence of the higher concentration of rotenone (500 nM, 5 μM).

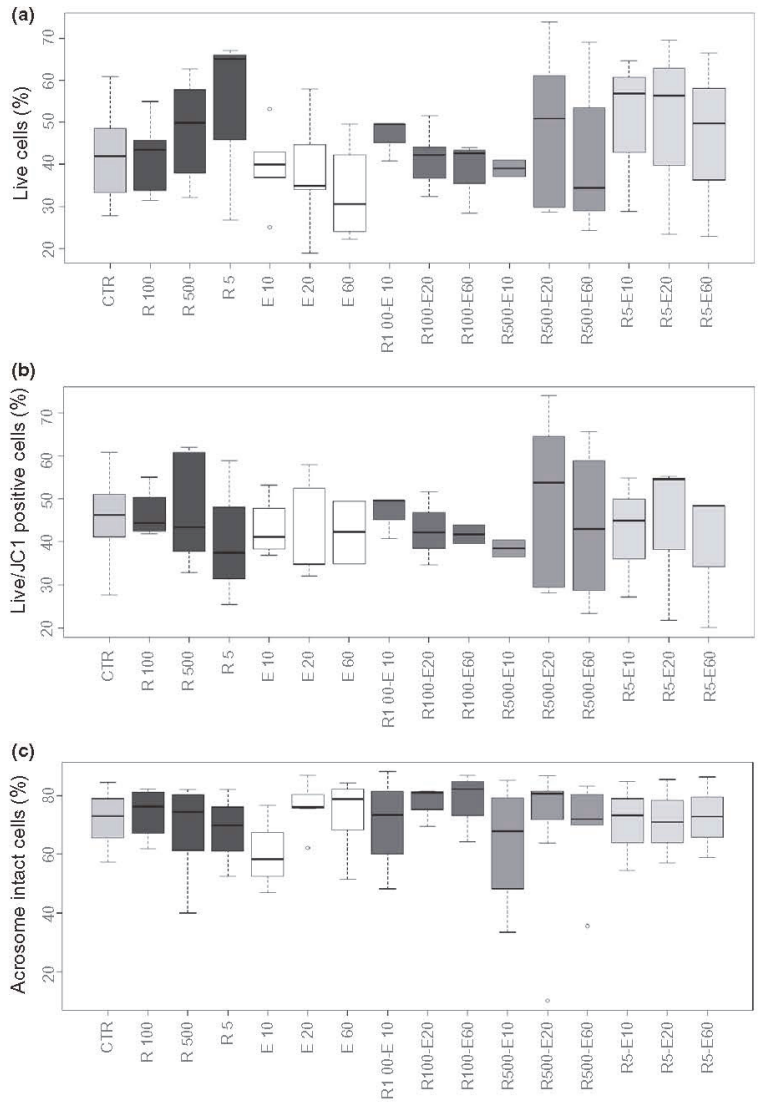


Fig. 1. Viability (a), mitochondrial membrane potential (b) and acrosome status (c) of stallion spermatozoa after 2-h incubation under capacitating condition in the presence of rotenone and/or EGCG. R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 μM; E10, EGCG 10 μM; E20, EGCG 20 μM; E60, EGCG 60 μM

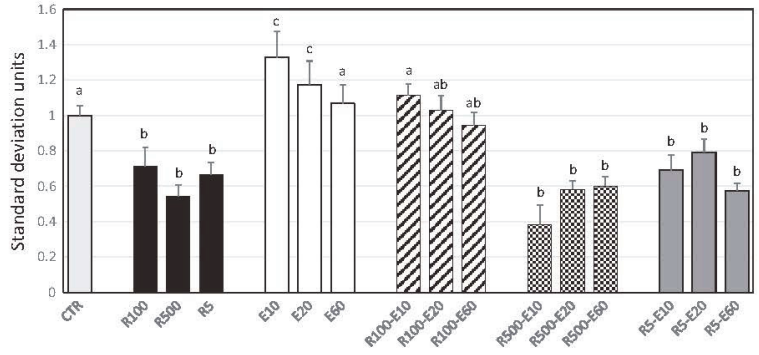


Fig. 2. Effect of rotenone and/or EGCG on heterologous zona pellucida binding ability of stallion sperm. Data were standardized by dividing the number of bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed as standard deviation units. R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 μM; E10, EGCG 10 μM; E20, EGCG 20 μM; E60, EGCG 60 μM. Different letters on the bars indicate a significant difference

Discussion

The aim of our study was to evaluate the response of equine sperm after inhibiting mitochondrial complex I by rotenone during *in vitro* capacitation for 2 h and to test whether EGCG, a natural polyphenol component of green tea, could counteract the effect of rotenone.

The evaluation of stallion sperm viability, acrosomal membrane integrity and mitochondrial activity did not evidence any significant effect of rotenone at all the concentrations tested (100 nM, 500 nM and 5 μM). The absence of significant differences on the percentage of viable stallion sperm agrees well with the data obtained by Gibb et al. (2014) and Plaza Dávila et al. (2015) who observed a sperm viability similar to that in control even using a higher rotenone concentration (10 μM) for 1 h; only after 3 h of incubation, rotenone at the concentration of 10 μM induced a significant reduction in the percentage of intact sperm (Plaza Dávila et al. 2015). In contrast to the results of those authors, we did not observe any significant decrease in the percentage of live spermatozoa with high mitochondrial membrane potential. This discrepancy could be due to different reasons: Gibb et al. (2014) and Plaza Dávila et al. (2015) evaluated JC-1 positivity by flow cytometry, while we used fluorescence microscopy possibly overestimating JC-1-positive cells classifying as JC-1 positive also those cells with only partial or spot-like JC-1-positive mitochondria. A further explanation could be the lower rotenone concentrations used in our work and the different conditions of the incubation with rotenone: capacitation in our study and non-capacitation in Gibb et al. (2014) and Plaza Dávila et al. (2015) studies.

To evaluate the effect of rotenone and EGCG on the *in vitro* function of equine spermatozoa, an heterologous binding assay was performed co-incubating denuded IVM porcine oocytes for 1 h with semen previously pre-incubated 1 h in the presence or absence of different concentrations of rotenone and EGCG. It has been demonstrated that sperm–oocyte binding assays offer a good reliability in the prediction of horse *in vivo* fertility (Fazeli et al. 1993, 1995; Meyers et al. 1996). Due to the low availability of equine oocytes, in our study a heterologous binding assay was performed as the efficiency/reliability of using bovine or swine oocytes has been demonstrated (Sinowatz et al. 2003; Clulow et al. 2010; Balao da Silva et al. 2013). As in the case of the homologous assay, the process of capacitation is needed for stallion spermatozoa to bind to heterologous oocytes (Clulow et al. 2010).

The results obtained in this study demonstrate for the first time that inhibition of complex I of MRC of stallion sperm with rotenone exerts a negative effect on ZP binding ability. In fact, rotenone at all the concentrations tested (100 nM, 500 nM and 5 μM) significantly decreased the number of sperm bound per oocyte in comparison with control group.

When stallion spermatozoa were treated under capacitating condition with 10 and 20 μM EGCG, stallion sperm–ZP binding activity was improved compared with control semen. A positive influence of EGCG addition on both fresh and frozen–thawed spermatozoa during IVF on ZP binding and oocyte penetration was already recorded in pig (Spinaci et al. 2008; Kaedei et al. 2012), suggesting a modulating action of this polyphenol on sperm capacitation. This effect could be exerted thanks to the antioxidant ability of EGCG that can act on the balance between excessive ROS production, which overwhelms the limited capacity of these cells to protect themselves from oxidative stress, and mild intracellular ROS generation, which stimulates intracellular cAMP generation, inhibits tyrosine phosphatase activity and enhances the formation of oxysterols, thus inducing a physiological capacitation (Aitken et al. 2015).

EGCG at 10 μM concentration significantly blunted the negative effect on stallion sperm–ZP binding activity of rotenone at the lower dose tested (100 nM). EGCG at the higher doses tested (20, 60 μM), even if it was not able to completely reverse the inhibitory effect of rotenone 100 nM, increased the number of sperm bound to ZP up to the levels of the control group. However, EGCG was not able to reduce the negative effect on heterologous binding induced by higher concentration of rotenone (500 nM and 5 μM).

Our results agree with the ability of epicatechin and EGCG (but not of other flavonoids such as genistein and baicalin) demonstrated by Kamalden et al. (2012) in protecting a transformed cell line (RGC-5 cells) from rotenone-induced toxicity. This positive effect, as suggested by the authors, could be mainly, but not exclusively, attributed to the antioxidant activity of these flavonoids. The ability of EGCG to counteract mitochondrial energy deficit due to impaired activities of complex I has been demonstrated by Valenti et al. (2013) in cultured fibroblasts and lymphoblasts from subjects with Down's syndrome. This effect was associated with EGCG-induced promotion of cAMP and PKA-dependent phosphorylation of complex I.

Rotenone inhibits oxidative glycolysis and ATP production in stallion spermatozoa inducing a reduction in sperm motility parameters (Plaza Dávila et al. 2015). It could be hypothesized that EGCG, counteracting rotenone-induced deficit in mitochondrial ATP synthesis, may ensure under capacitating conditions the adequate energy supply. In this way, the spermatozoa can sustain changes occurring during capacitation, such as hyperactivated motility and protein phosphorylation (Ferramosca and Zara 2014).

In conclusion, the inhibition of complex I by rotenone results in a decreased ZP binding ability of stallion spermatozoa, and the presence of EGCG is able to reduce the toxicity of rotenone at the lower dose (100 nM). Moreover, spermatozoa treated with EGCG attach better than non-treated ones, suggesting that they have a more advanced capacitation-like status.

Conflict of interest

None of the authors have conflict of interest to declare.

Author contributions

All the authors contributed to the research.

References

Aitken RJ, Baker MA, Nixon B, 2015: Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian J Androl **17**, 633–639.

Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Peña FJ, Mari G, Galeati G, 2013: Effect of sex sorting on stallion spermatozoa: heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci **141**, 68–74.

Balao da Silva C, Ortega Ferrusola C, Gallardo Bolanos J, Plaza Dávila M, Martin-Munoz P, Morrell J, Rodriguez Martinez H, Peña F, 2014: Effect of overnight staining on the quality of flow cytometric sorted stallion sperm: comparison with traditional protocols. Reprod Domest Anim **49**, 1021–1027.

Clulow JR, Evans G, Maxwell WM, Morris LH, 2010: Evaluation of the function of fresh and frozen–thawed sex-sorted and non-sorted stallion spermatozoa using a heterologous oocyte binding assay. Reprod Fertil Dev **22**, 710–717.

Cummins JM, 2009: Sperm motility and energetics. In: Birkhead TR, Hosken DJ, Pitnick S (eds), Sperm Biology, an Evolutionary Perspective. Academic Press, San Diego, CA, pp. 185–206.

Fazeli AR, Steenweg W, Bevers MM, de Loos FA, van den Broek J, Colenbrander B, 1993: Development of a sperm zona pellucida binding assay for bull semen. Vet Rec **132**, 14–16.

Fazeli AR, Steenweg W, Bevers MM, van den Broek J, Bracher V, Parlevliet J, Colenbrander B, 1995: Relation between stallion sperm binding to homologous hemizonae and fertility. Theriogenology **44**, 751–760.

Ferramosca A, Zara V, 2014: Bioenergetics of mammalian sperm capacitation. Biomed Res Int **2014**, 902953.

Funahashi H, Cantley TC, Day BN, 1997: Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod **57**, 49–53.

Gibb Z, Lambourne SR, Aitken RJ, 2014: The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod **91**, 1–10.

Grinberg LN, Newmark H, Kitrossky N, Rahamim E, Chevion M, Rachmilewitz EA, 1997: Protective effects of tea polyphenols against oxidative damage to red blood cells. Biochem Pharmacol **54**, 973–978.

Guo Q, Zhao BL, Li M, Zhen S, Xin W, 1996: Studies on protective mechanisms of four components of Green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta **1304**, 210–222.

Kaedei Y, Naito M, Naoi H, Sato Y, Taniguchi M, Tanihara F, Kikuchi K, Nagai T, Otoi T, 2012: Effects of (-)-epigallocatechin gallate on the motility and penetrability of frozen–thawed boar spermatozoa incubated in the fertilization medium. Reprod Domest Anim **47**, 880–886.

Kamalden TA, Ji D, Osborne NN, 2012: Rotenone-induced death of RGC-5 cells is caspase independent, involves the JNK and p38 pathways and is attenuated by specific green tea flavonoids. Neurochem Res **37**, 1091–1101.

Kenney RM, Bergman RV, Cooper WL, Morse GW, 1975: Minimal contamination techniques for breeding mares: techniques and preliminary findings. In: 21st Annual Convention of the American Association of Equine Practitioners, pp. 327.

Koppers AJ, De luliis GN, Finnie JM, McLaughlin EA, Aitken RJ, 2008: Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab **93**, 3199–3207.

Meyers SA, Liu IK, Overstreet JW, Vadas S, Drobnis EZ, 1996: Zona pellucida binding and zona-induced acrosome reactions in horse spermatozoa: comparisons between fertile and subfertile stallions. Theriogenology **46**, 1277–1288.

Ortega Ferrusola C, González Fernández L, Morrell JM, Salazar Sandoval C, MacíasGarcía B, Rodríguez-Martinez H, Tapia JA, Peña FJ, 2009: Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction **138**, 55–63.

Peña FJ, Plaza Dávila M, Ball BA, Squires EL, Martin Munoz P, Ortega Ferrusola C, Balao da Silva C, 2015: The impact of reproductive technologies on stallion mitochondrial function. Reprod Domest Anim **50**, 529–537.

Petters RM, Wells KD, 1993: Culture of pig embryos. J Reprod Fertil Suppl **48**, 61–73.

Plaza Dávila M, Martín Muñoz P, Tapia JA, Ortega Ferrusola C, Balao da Silva C, Peña FJ, 2015: Inhibition of mitochondrial complex I leads to decreased motility and membrane integrity related to increased hydrogen peroxide and reduced ATP production, while the inhibition of glycolysis has less impact on sperm motility. PLoS ONE **10**, e0138777.

R Core Team, 2012: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rathi R, Colenbrander B, Bevers MM, Gadella BM, 2001: Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod **65**, 462–470.

Schroeder EK, Kelsey NA, Doyle J, Breed E, Bouchard RJ, Loucks A, Harbison A, Linseman DA, 2008: Green tea epigallocatechin 3-gallate accumulates in mitochondria and displays a selective anti-apoptotic effect against inducers of mitochondrial oxidative stress in neurons. Antioxid Redox Signal **11**, 469–480.

Singer TP, Ramsay RR, 1994: The reaction sites of rotenone and ubiquinone with mitochondrial NADH dehydrogenase. Biochim Biophys Acta **1187**, 198–202.

Sinowatz F, Wessa E, Neumuller C, Palma G, 2003: On the species specificity of sperm binding and sperm penetration of the zona pellucida. Reprod Domest Anim **38**, 141–146.

Skrzydlewska E, Ostrowska J, Farbiszewski R, Michalak K, 2002: Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain. Phytomedicine **9**, 232–238.

Spinaci M, Volpe S, De Ambrogi M, Tamanini C, Galeati G, 2008: Effects of epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine oocytes. Theriogenology **69**, 877–885.

Stewart AJ, Mullen W, Crozier A, 2005: On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea. Mol Nutr Food Res **49**, 52–60.

Tourmente M, Villar-Moya P, Rial E, Roldan ER, 2015: Differences in ATP generation via glycolysis and oxidative phosphorylation, and relationships with sperm motility, in mouse species. J Biol Chem **290**, 20613–20626.

Valenti D, De Rasmo D, Signorile A, Rossi L, de Bari L, Scala I, Granese B, Papa S, Vacca RA, 2013: Epigallocatechin-3-gallate prevents oxidative phosphorylation deficit and promotes mitochondrial biogenesis in human cells from subjects with Down's syndrome. Biochim Biophys Acta **1832**, 542–552.

Wang LF, Kim DM, Lee CY, 2000: Effects of heat processing and storage on flavanols and sensory qualities of green tea beverage. J Agric Food Chem **48**, 4227–4232.

Submitted: 28 Jul 2015; Accepted: 13 Sep 2015

Author's address (for correspondence): Marcella Spinaci, Department of Veterinary Medical Sciences – DIMEVET, University of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia, 40064 Bologna, Italy. E-mail: marcella.spinaci@unibo.it

Artículo 3º

Mitochondrial ATP is required for maintenance of membrane integrity in the stallion spermatozoa, while motility requires both glycolysis and oxidative phosphorylation

Plaza Davila M[1], Martin Muñoz P[1] Gallardo Bolaños JM[1], Stout TAE.[2,3], Gadella BM[3,4], , Tapia JA[5], Ortega Ferrusola C[1], Balao da Silva C[1], Peña FJ[1]*

[1]Laboratory of Equine Reproduction and Equine Spermatology. Veterinary Teaching Hospital, University of Extremadura Cáceres, Spain: Departments of [2]Equine Sciences, [3]Farm Animal Health, and [4]Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, [5]Department of Physiology Faculty of Veterinary Medicine. University of Extremadura Cáceres, Spain.

*Correspondence to Dr. FJ Peña, Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine University of Extremadura Adv. de la Universidad s/n 10003 Cáceres Spain. E-mail fjuanpvega@unex.es
Phone + 34 927-257167
Fax +34 927257102

Acknowledgements
The authors received financial support for this study from Ministerio de Ciencia e Innovación-FEDER in Madrid, Spain grants AGL2013-43211-R and Junta de Extremadura-FEDER (GR 10010 and PCE1002).

ABSTRACT

To investigate the hypothesis that oxidative phosphorylation is a major source of ATP for stallion sperm motility, oxidative phosphorylation was suppressed using the mitochondrial uncouplers (CCCP and 2, 4,-dinitrophenol; DNP) and inhibiting mitochondrial respiration at complex IV with sodium cyanide or at the ATP-synthase step with oligomycin-A. Taking into account that mitochondrial dysfunction may also lead to oxidative stress, production of reactive oxygen species was monitored simultaneously. All inhibitors reduced ATP content, however oligomicyn-A induced the biggest reduction in ATP content. Oligomycin-A and CCCP significantly reduced mitochondrial membrane potential. Motility of the spermatozoa was nearly withdrawn by inhibition of mitochondrial respiration and reduced in presence of mitochondrial uncouplers as were sperm velocities. Inhibition of ATP synthase caused a collapse of sperm membranes and increased the production of reactive oxygen species in dead sperm. Inhibition of glycolysis by deoxyglucose led to reduced sperm velocities and also to reduced ATP content but not to a collapse of membrane integrity. Our results suggest that, in contrast to many other mammalian species, stallion spermatozoa rely mainly on oxidative phosphorylation to generate the energy required to maintain a functional Na^+/K^+ gradient which is dependent on an Na^+ K^+ antiporter ATPase: Under anaerobic conditions tested glycolysis is insufficient in maintenance of the Na+ K+ gradient and consequently the attraction of water causes sperm membrane collapse. However, under aerobic conditions glycolysis additionally provides energy for motility.

Key words: stallion, sperm, mitochondria, ATP, motility, CASA, flow cytometry

INTRODUCTION

The physiological and regulatory constraint of the equine breeding season implies that adequate fertility of stallions and mares is important. However, in contrast to other domestic species, stallions are primarily selected on the basis of pedigree, performance and conformation, with little consideration given to reproductive soundness (Varner et al, 2014). This has contributed to the marked variability in male fertility and semen quality among stallions and breeds, with male factor subfertility being a common problem in some horse breeds (Peña et al, 2012). Spermatozoa are highly specialized cells with the primary task to deliver a haploid set of male chromosomes to the oocyte during fertilization. During their generation in the testes, spermatozoa lose most of their cell organelles, with the notable exceptions of the acrosome (derived from the Golgi apparatus) and the mitochondria, where the latter become concentrated in the mid-piece. In recent years, the mitochondria of stallion spermatozoa have become the focus of research interest. Together with their role as a source of ATP, sperm mitochondria play other important roles during fertilization, and in the regulation of sperm lifespan by the activation of an apoptosis-like mechanism (Amaral et al., 2013; Ortega Ferrusola et al, 2008), probably related to their role as a source of reactive oxygen species (Koppers et al, 2008). Moreover, their sensitivity of cells to osmotic stress (Macías García et al, 2012, González Fernández et al, 2012) is linked to malfunctioning of the mitochondria (low ATP levels distort a functional Na^+ K^+ gradient allowing water attraction by the cell (ref). Dysfunction of mitochondria is also a potential source of sperm malfunction in the stallion (Peña et al, 2009, 2011), and it has been proposed that evaluation of mitochondrial activity is a robust tool for assessing sperm function and ability to withstand biotechnological procedures (Ortega Ferrusola et al; 2009ab). Despite all of these apparent properties, the role of mitochondria in the regulation of sperm motility remains a point of discussion. It has been proposed that equine spermatozoa are highly dependent on oxidative phosphorylation for motility, whereas, sperm from other species rely mostly on glycolysis to provide ATP for flagellar propulsion (Ferramosa and Zara, 2014; Mannowetz et al, 2012, Mukai and Okuno 2004, Nascimento et al, 2008, Odet et al, 2013). Previously, we studied the effect of inhibiting the adenine nucleotide translocator (ATN), a specific ATP/ADP transporter, on sperm function (Ortega Ferrusola et al, 2009c). The ATN catalyses the transmembrane exchange of ATP generated in the mitochondria by oxidative phosphorylation for cytosolic ADP (Klindemberg 2008). Our findings suggested that equine spermatozoa depend primarily on oxidative phosphorylation for the maintenance of activated motility. It is generally agreed that functional mitochondria are required for normal function of stallion sperm. To improve our understanding of the importance of oxidative phosphorylation as the main source of ATP for stallion sperm motility, oxidative phosphorylation was suppressed using mitochondrial uncouplers (carbonyl cyanide m-chlorophenyl hydrazine; CCCP and 2, 4, dinitrophenol; DNP) and inhibiting complex IV and ATP synthase. Moreover we studied the role of glycolysis using 2-deoxyglucose. Since mitochondrial dysfunction can also lead to oxidative stress, we simultaneously monitored the production of reactive oxygen species. Our results suggest that, in contrast to many other mammalian species, stallion spermatozoa rely mainly on oxidative phosphorylation to generate energy for motility and maintenance of membrane integrity, and that mitochondrial dysfunction may lead to a loss of motility via a decrease in ATP production in addition to an oxidative mechanism.

MATERIAL AND METHODS

Reagents and media

Ethidium homodimer; 5,5',6,6'–tetrachloro-1,1',3,3' tetra-ethylbenzymidazolyl carbocianyne iodine (JC-1); Yo-Pro-1; Cell Rox Deep Red Reagent; Hoechst 33342and the ATP detection Kit were all obtained from Molecular Probes (Molecular Probes Europe, Leiden, the Netherlands). Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), oligomycin, 2,4 dinitrophenol (2,4 DNP), sodium cyanide (NaCn), 2-deoxyglucose (2-DG) and all other chemicals were purchased from Sigma (St Louis, MO, USA)

Semen collection and processing

Semen was collected from 7 Purebred Spanish horses (PRE) (three ejaculates each) individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. Stallions were maintained according to institutional and European animal care regulations, and semen was collected on a regular basis (two collections/week) throughout the 2013 and 2014-breeding seasons. Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. After collection, the semen was immediately transported to the laboratory for evaluation and processing. The ejaculate was extended 1:2 in INRA-96 diluent (IMV L'Aigle, France), centrifuged at 600g for 10 min to remove the bulk of the seminal plasma and re-suspended at $40x10^6$ spermatozoa/ml in BWW medium supplemented with 1% polyvinyl alcohol. All of the experiments followed a split sample design with each ejaculate divided to yield control and treatment groups. For this, after centrifugation aliquots of stallion extended semen in BWW were supplemented with the different treatments and incubated in water bath at 37ºC up to 3 hours. Treatments consisted of CCCP (0 (vehicle) 100nM, 500nM 5μM and 10μM), 2,4 -DNP (0 (vehicle)10μM, 50μM, 200μM and 400μM), NaCn (0 (vehicle), 2 mM, 5mM and 10 mM), oligomycin (0 (vehicle), 30μM and 60 μM). In a particular set of experiments glucose was replaced by 2-DG. After 1 hours an three hours of incubation aliquots were taken for flow cytometry, computerized motility and ATP content analysis.

Sperm motility

Sperm motility and kinematics were assessed using a computer assisted sperm motility analysis (CASA) system (ISAS®: Proiser Valencia Spain) with samples loaded into 20μm deep Leja chambers (Leja Products B.V. Nieuw Vennep, the Netherlands) placed on a warmed (37ºC) stage. The analysis was based on the examination of 60 consecutive digitalized images in a lapsed time of one second (60 Hz) using a negative phase contrast objective at a magnification of x100. The number of objects incorrectly identified as spermatozoa was minimized retrospectively using the playback function. With respect to the motility parameters used, spermatozoa with a VAP <15 μm/s were considered to be immotile, whereas spermatozoa with a velocity >15μm/s were considered motile. Spermatozoa deviating <45 % from a straight line were considered to show linear motility and spermatozoa with a curvilinear velocity (VCL) > 45μm/s were designated as rapid. Absolute sperm motility parameters assessed by CASA

included: Curvilinear Velocity (VCL) μm/s, Linear Velocity (VSL) μm/s, and Mean Path Velocity (VAP) μm/s.

Flow cytometry

Flow cytometric analyses were conducted using a MACSQuant Analyser 10 (Miltenyi Biotech, Pozuelo de Alarcón, Spain) flow cytometer equipped with three lasers emitting at 405 nm, 488 nm, and 635 nm and 10 photomultiplier tubes (PMTs); (excitation 405 nm, emission 450-450 nm band pass), (excitation 405 nm, emission 525-550 nm band pass), (excitation 488 nm, emission 525-550 nm band pass), (excitation 488 nm, emission 585-640 nm band pass), (excitation 488 nm, emission 655-730 nm; 655nm long pass and split at 730nm), (excitation 499 nm, emission 750 nm long pass), (excitation 635 nm, emission 655-730 nm band pass; 655nm long pass and split at 730nm),) and (excitation 635 nm, emission filter 750 nm long pass). The system was controlled using MACSQuantify software (Miltenyi Biotech, Pozuelo de Alarcón, Spain). Sperm subpopulations on base of two fluorescent properties were visualized as two dimensional dot plots (on base of logarithmic increase of detected emission signals at a fixed PMT sensitivity setting). With the two dimensional intensity dot-plots a fixed four quadrants setting was used to quantify the frequency of each sperm subpopulation.

Confocal Laser Microscopy

Confocal microscopy was used to image sperm using a FV1000 spectral confocal microscope (Olympus, Hamburg). Sperm were stained with JC-1 and excited with a 488nm Argon laser. PMTs were utilized for fluorescence detection using a 510-535nm spectral interval for green fluorescence (low mitochondrial membrane potential) and a 580-620nm spectral interval for orange-red fluorescence (high mitochondrial membrane potential). Water immersion 60x objective was used. Plate temperature was set to 30ºC to decrease sperm motility. Acquisition time at 1600x1600 pixels resolution was 3.5 seconds.

Simultaneous flow cytometric assessment of early membrane changes, viability and oxidative stress (reactive oxygen species ROS)

The following stock solutions were prepared in DMSO: Yo-Pro-1 (25 μM), ethidium homodimer-1 (1.17 mM), and Cell Rox (5 mM). Hoechst 33342 (1.62 mM in water) was used to identify spermatozoa and allow debris to be gated out of the analysis. A sperm suspension (1 mL) containing 5 x 10^6 spermatozoa/mL was stained with 1 μL Yo-Pro-1, 1 μL CellRox, and 0.3 μL Hoechst 33342. After thorough mixing, the sperm suspension was incubated at RT in the dark for 25 minutes. The spermatozoa were then washed in PBS, and then incubated for 5 minutes with 0.3μL ethidium homodimer before analysis in the flow cytometer. This staining protocol was a modification (Gallardo Bolaños et al, 2014) of previous published protocols (Pena et al. 2005b, Nunez-Martinez et al. 2007, Ortega Ferrusola et al. 2009) and distinguishes four sperm subpopulations while simultaneously measuring oxidative stress. The first subpopulation, positive for only Hoechst 33342, was considered to be alive and without any membrane alterations. The Yo-Pro-1-positive cells emitting green fluorescence, were considered to show signs of early damage and a shift to a different physiological state, because membranes become slightly permeable during the first steps of damage, which enables Yo-Pro-1 but not ethidium homodimer to cross the plasma membrane.

Neither probe enters intact cells. Finally, two subpopulations of dead spermatozoa were also detected. These were either apoptotic (spermatozoa stained both with Yo-Pro-1 and ethidium homodimer, emitting both green and red fluorescence) or necrotic (cells stained with only ethidium homodimer and emitting red fluorescence). Spermatozoa exhibiting oxidative stress emit fluorescence in the far-red spectrum. The positive controls for oxidative stress were samples that were supplemented with 800 μM $FeSO_4$ and 200 μL of H_2O_2 (Sigma) to stimulate the Fenton reaction.

Evaluation of mitochondrial membrane potential (ΔΨm)

The lipophilic cationic compound 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyne iodine (JC-1) has the unique ability to differentially label mitochondria with low and those with high membrane potential and was used to monitor mitochondrial membrane potential as described previously (Ortega Ferrusola et al. 2009). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength (590 nm), when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers that emit in the green wavelength (525 to 530 nm) when excited at 488 nm. Mitochondrial staining was done by adding 0.5 μL of a 3mM stock solution of JC-1 (in dimethylsulfoxide DMSO) in 1 mL of a sperm suspension in PBS (5 $x10^6$/mL). The samples were incubated at 37 °C in the dark for 40 minutes before flow cytometric analysis, and the staining patterns were also monitored using confocal laser microscopy

Determination of intracellular ATP

Intracellular ATP content in sperm lysates was measured using the ATP determination Kit (A22066) following the instructions of the manufacturer, as described previously (Balao da Silva et al, 2013). The assay is based on the requirement of the luciferase for ATP to produce light (emission maxima ~ 560 nm at pH 7.8). The ATP content was normalized to pM/100 μg of protein. Measurements were made in a Tecan Infinite M200 microplate reader (Männedorf, Switzerland).

Statistical analysis

Three ejaculates from 7 individual stallions were collected. All experiments were repeated at least three times with independent samples (three independent ejaculates from each of the seven stallions). The normality of the data was assessed using the Kolmogorov-Smirnoff test. Since the data show equivalence of variance the results were analyzed by ANOVA followed by a Tukey post –hoc test to perform pair wise comparisons (SPSS 19.0 for Mac). Differences were considered significant when $P < 0.05$, and are indicated as; * $p<0.05$ and ** $p<0.01$. Results are displayed as means ± SEM

RESULTS

Effect of CCCP and DNP, uncouplers of mitochondrial proton gradient on stallion spermatozoa on mitochondrial membrane potential

In order to establish the effect of CCCP and DNP on sperm mitochondria, a dose and time effect experiment was conducted. Stallion sperm were incubated in increasing concentrations of CCCP and DNP for up to three hours, and mitochondrial membrane potential was evaluated flow cytometrically after JC-1 staining. This staining allowed differentiate sperm with high inner mitochondrial membrane potential (IMMP; A in fig 1), and low membrane potential (B in Fig 1). CCCP concentrations in excess of 500 nM significantly decreased mitochondrial membrane potential in a dose dependent manner ($p<0.05$; fig 2 A-B). At a concentration of 100nM, CCCP had no noticeable effect on mitochondrial membrane potential. However DNP was unable to induce changes in IMMP (Fig. 2 C-D)

Inhibition of mitochondrial respiration reduces mitochondrial membrane potential

Incubation of stallion spermatozoa in presence of F_0F_1-ATP synthase inhibitor oligomycin-A, resulted in a marked and rapid drop in IMMP (Fig 2 G-H). The percentage of spermatozoa showing low MMP increased from 28% in control to 68 and 63 % ($p<0.001$) in samples treated with 30 and 60 μM oligomycin-A respectively. When complex IV was inhibited with sodium cyanide there was a significant drop ($p<0.05$) after three hours of incubation and when was present at a final concentration of 10 mM (Fig. 2 E-F).

Uncoupling mitochondrial proton gradient reduces ATP content of stallion spermatozoa

To determine the effect of mitochondrial uncoupling on ATP production, stallion sperm were incubated in the presence of CCCP and DNP before assessment of ATP content. CCCP concentrations above 500 nM reduced the ATP content of stallion spermatozoa (Fig 3 A). Concentrations of DNP above 400 μM also reduced ATP content in stallion spermatozoa (Fig 3 B).

Inhibition of mitochondrial respiration reduces intracellular ATP in stallion spermatozoa

Inhibition of the F_0F_1-ATP synthase was done incubating stallion spermatozoa in presence of oligomycin-A, the inhibition resulted in a dramatic reduction of ATP content of stallion spermatozoa (Fig 3 D). Inhibition of complex IV with sodium cyanide also resulted in reduced ATP content (Fig 3 C).

Effect of uncoupling the mitochondrial proton gradient on sperm motility and kinematics

When stallion sperm was incubated in the presence of increasing doses of CCCP to uncouple mitochondria, most marked reduction in overall sperm motility was observed at > 500 nM levels after one or after three hours of incubation ($p<0.01$); and with a

concomitant reduction in the percentage of progressively motile sperm ($p<0.05$). At 100nM, CCCP only caused a decrease in the percentage of progressively motile sperm after three hours of incubation ($p<0.05$; Fig. 4). Uncoupling mitochondria also had a profound impact on sperm kinematics, with significant ($p<0.05$) reductions in sperm velocity at CCCP levels > 500 nM after both 1 and 3 hours of incubation (Fig. 5). The uncoupler 2,4-DNP had a less pronounced effect; incubation of stallion sperm in presence of DNP 400μM reduced the percentage of total motile sperm after 1 and 3 hours of incubation but no effect was seen on the percentage of progressive motile sperm (Fig 4). In the same way 2,4-DNP reduced all sperm velocities when present in the media at a final concentration of 400 μM (Fig. 5)

Inhibition of mitochondrial respiration dramatically reduces sperm motility and kinematics

Stallion sperm was incubated in presence of the inhibitor of complex IV, sodium cyanide NaCn and the F_0F_1-ATP synthase inhibitor oligomycin-A. Both inhibitors exerted a dramatic effect on the percentage of total motile and progressive motile sperm. In absence of complex IV inhibitor sodium cyanide 80% of the sperm showed overall signs of motility. In presence of 2 mM sodium cyanide only 40% or less of the sperm showed overall signs of motility ($p<0.01$; Figure 4 E-F). The percentage of progressive motile sperm was nearly abolished in presence of this inhibitor (Fig 4). The ATP synthase inhibitor oligomycin-A also induced a dramatic drop in the percentages of total and progressive motile spermatozoa (Fig 4 G-H): In negative controls 80% total and 46% progressive motility were noted while in presence of 60 μM oligomycin-A these values were reduced to 12% and 4%, respectively, after 1 hour of incubation at 37ºC. The motility was virtually completely abolished after 3 hours of incubation with 60 μM oligomycin-A.

The reducing effect on sperm velocity was more prominent when mitochondrial respiration was inhibited when compared to, uncoupling the proton gradient of the mitochondria (Fig 5). All the doses tested of both inhibitors (oligomycin-A and sodium cyanide) induced a significant reduction in sperm velocities ($p<0.01$).

Uncoupling the mitochondrial proton gradient had no effect in the percentage of intact sperm

The mitochondrial proton gradient was inhibited by incubation of stallion spermatozoa in presence of CCCP and 2,4-DNP. No effect was observed in the percentage of spermatozoa with intact membranes at any time of incubation considered (Fig 6 A-D).

Uncoupling the mitochondrial proton gradient in stallion sperm allows trans-membrane influx of YoPro-1

To examine the sensitivity of sperm membrane integrity to mitochondrial uncoupling, stallion spermatozoa were incubated in the presence of CCCP and DNP. Both uncouplers had no effect on membrane integrity during the incubation (Fig 6). However, YoPro-1 fluorescence increased when CCCP was present at concentrations greater than 5 μM (Fig 7 A-B) and when 2, 4-DNP was present at concentration of 400 μM after three hours of incubation (Fig 7 D).

Inhibition of mitochondrial respiration affects membrane intactness and permeability, with inhibition of ATP synthase collapsing sperm membrane integrity

When complex IV was inhibited (with the already noted sharper decrease in ATP content Fig 3), there was a significant drop in the percentage of spermatozoa with intact membranes both after 1 and 3 hours of incubation (Fig 6 E-F); this drop was accompanied by a increase in the percentage of spermatozoa with increased membrane permeability (Fig 7 E-F). When ATP synthase was inhibited with oligomycin-A (causing the largest depletion of cellular ATP content; Fig 3) the membrane integrity virtually collapsed after 1 hour of incubation ($p<0.001$) (Fig 6 G-H), this drop was due both to an increase in the permeability of the membrane (increased YoPro-1 influx), and to a collapse of the integrity of the plasma membrane (Fig 7 G-H).

Uncoupling the mitochondrial proton gradient in stallion sperm increases or decreases reactive oxygen species (ROS) generation in a dose specific fashion

Since some effects of mitochondrial dysfunction may be due to increased ROS production, the effect of CCCP and 2, 4-DNP on ROS production was assessed. CCCP increased ROS production after 1 hour of incubation at 100 nM but decreased ROS production at 10μM (Fig 8). After 3 hours of incubation no effect of CCCP on ROS production was apparent when compared to control treated sperm, with the exception of a increase in ROS in dead cells in presence of 5 μM CCCP (Fig 8). DNP only induced an increase in ROS production in membrane-deteriorated spermatozoa after three hours of incubation (Fig 8 D).

Effect of inhibition of mitochondrial respiration in production of ROS by stallion spermatozoa

Inhibition of complex IV resulted in a reduction of the percentage of live stallion spermatozoa showing increased ROS production both after one and three hours of incubation ($p<0.01$), however ROS production in dead sperm increased ($p<0.05$) (fig 8 D E). When ATP synthase was inhibited with oligomycin, there was a dramatic decrease in ROS production in the subpopulation of live sperm ($p<0.001$), while in the population of spermatozoa with altered membranes ROS production increased only after 1 hour on incubation ($p< 0.01$) (Fig 8 F-G).

Inhibition of glycolysis decreases stallion sperm ATP content, reduces sperm motility and velocity but has no effect of membrane integrity

Although ATP production is inhibited when mitochondrial aerobic functioning (oxidative phosphorylation) is inhibited, alternative glycolytic production of ATP production may rescue sperm motility and membrane integrity properties. In order to test the importance of glycolytic ATP production stallion spermatozoa were incubated in presence of 2-deoxyglucose instead of glucose to inhibit glycolysis. The presence of 2-deoxyglucose resulted in reduction of ATP content in stallion spermatozoa (Fig 9 A); moreover effect on motility velocities and membrane integrity were studied. There was a reduction in the percentage of total motile sperm, but not in the percentage of progressive motile spermatozoa (Fig 9 B-C). The sperm velocities were also reduced in presence of 2-DG (Fig 9 D-E). On the other hand, inhibition of glycolysis with 2-deoxyglucose had no effect on membrane intactness (Fig 9 F-G).

DISCUSSION

Mitochondrial oxidative phosphorylation takes place around the inner mitochondrial membrane and is comprised of the electron transport chain and the ATP synthase. The respiratory chain generates a proton concentration gradient (change in pH) and a trans-membrane potential ($\Delta\Psi m$) across the inner membrane, which together allow the production of ATP by ATP synthase (Erkkila et al., 2006). We evaluated the effect of uncoupling the mitochondrial electron chain and inhibiting mitochondrial respiration on major sperm functions. Exposing stallion spermatozoa to CCCP reduced sperm motility and increased sperm labeling with YoPro-1, without a decrease in membrane integrity. The use of 2,4-DNP also resulted in reduced motility but had no effect on membrane integrity, however 2,4, -DNP was not able to decrease mitochondrial membrane potential in contrast to CCCP. Inhibition of complex IV of the electron transport chain (ETC) resulted in a marked drop in sperm motility and specially velocities, however effects on membrane integrity were only evident when sodium cyanide was used at a final concentration of 10 mM. Inhibition of the F_0 part of H^+-ATP-synthase with oligomycin significantly reduced sperm motility and velocity. Moreover inhibition of ATP- synthase almost completely collapsed sperm membrane integrity. These results indicate that mitochondrial ATP is essential for stallion sperm function confirming previous reports (Ortega Ferrusola et al; 2009 Gibb et al, 2014). In addition we here describe for the first time that mitochondrial ATP synthase activity is crucial for the maintenance of intact sperm membranes.

At present, it is assumed that mammalian sperm rely predominantly the glycolytic ATP production for motility (Mukai and Okuno, 2004). This was based on CCCP treatment of mouse sperm which did not affect sperm motility in glucose cointaining incubation media. In contrast to this finding we showed in stallion spermatozoa that uncoupling by CCCP or 2,4-DNP decreased both percentages of motile sperm and the velocity of motion despite the presence of glucose in the media. We also showed this effect in stallion sperm when inhibiting complex IV or the ATP synthase. Uncoupling mitochondria or inhibition of mitochondrial respiration and the consequently reduction in sperm ATP levels showed that motility of stallion spermatozoa is highly dependent on aerobic ATP production (Gibb et al., 2014) in clear contrast to other mammalian species, reported to rely more non-aerobic glucose consumption (glycolysis; Storey BT 2008).

Although reduced levels were observed, ATP was not completely depleted and likewise motility and sperm velocities were not completely reduced. Although oligomycin-A was able to abolish sperm membrane integrity and also dramatically reduced sperm motility after three hours of incubation, sperm velocities were not so dramatically affected. One possible explanation is that other sources of ATP are present in stallion sperm. To test this hypothesis glycolysis was inhibited with 2 –deoxyglucose. Inhibition of glycolysis lead to reduced ATP content without effect on membrane intactness. Also 10 mM 2-deoxyglucose reduced the percentage of total motile sperm, and sperm velocities without effect in the percentage of progressive motile sperm. These findings suggest that ATP generated by glycolysis also have a role providing energy for motility.

Though disruption of mitochondrial function in stallion sperm leads to reduced

motility, this could be a function of either reduced ATP production (Gibb et al., 2014) or increased reactive oxygen species production (Koppers et al, 2008). To determine whether the effect was most likely a function of decreased ATP or increased ROS production, both parameters were measured simultaneously. Uncoupling mitochondria and inhibition of mitochondrial respiration reduced ATP content. The most prominent ATP reduction was observed in presence of oligomycin-A which coincided with the highest incidence of a collapse in the MMP, motility and membrane integrity of the spermatozoa. At the same time ROS increased in dead sperm, but significantly decreased in the live sperm subpopulation. Interestingly, sperm velocities, although reduced, were not so markedly affected in comparison with motility or membrane integrity. Inhibition of complex IV markedly reduced motility and velocities but had no effect in mitochondrial membrane potential, and only at a final concentration of 10 mM reduced membrane integrity. Moreover, production of ROS was reduced. These findings indicate that the changes observed in addition to reduced ATP production are related to oxidative stress and affect in case of oligomycin-A incubations also the membrane integrity.

We further investigated whether these effects were related to ATP depletion and/or increased leakage of reactive oxygen species. Oligomycin-A treatment induced a massive increase in ROS production in dead sperm, while reduced ROS in live sperm; sodium cyanide increased ROS production in dead spermatozoa. CCCP increased ROS in live and dead sperm, while 2, 4-DNP only resulted in increased ROS in dead sperm after three hours of incubation. The relationship between ROS production and sperm function is poorly understood, with conflicting reports on their role in or impact on sperm function (reviewed in Varner et al, 2014); although importance of dead sperm in the generation of reactive oxygen species has been recently emphasized (Aitken et al, 2015). Our results underline this fact, indicating that the production of ROS ought to be adequately interpreted in equines. High ROS is a by-product of intense aerobic activity in stallion sperm (Gibb et al., 2014). ROS does not appear to contribute to reduce sperm motility under uncoupling conditions, but ROS production becomes intense when ATP synthase is inhibited in dead spermatozoa. Mitochondrial inhibitors can both increase or decrease ROS production depending of the dose employed (Xi et al, 2005). In somatic cells a small mitochondrial depolarization can apparently lead to an increase in ROS generation while a more profound mitochondrial depolarization reduces ROS. This is consistent with the concept that, under resting conditions, 1% to 2% of O_2 used in the electron transport chain (ETC) is not completely reduced leading to the generation of O_2 •- (Turrens 2003). In accordance with this observation, in our study 100 nM CCCP increased ROS production after 1 hour of incubation, and reduced the percentage of progressively motile sperm after three hours. This latter effect could be attributed to ROS oxidizing sperm proteins involved in the regulation of motility. Many types of amino acid can be oxidatively modified, although their susceptibilities vary (Bourdon and Blanche, 2001). Direct oxidation is mostly mediated by HO• and NO•. Among the amino acids, those containing sulfur such as methionine and cysteine are preferred targets

We tested a wide range of mitochondrial inhibitors, CCCP, 2,4-DNP, sodium cyanide and oligomycin-A and stallion sperm behaved largely as described for somatic cells. At lower concentrations and after shorter (e.g.1 hour) incubations, CCCP resulted in increased ROS production; however, this was not accompanied by decreased motility or

by mitochondrial membrane depolarization. Higher concentrations tended to reduce ROS production, but the effect was only significant after 1 hour of incubation. These effects are somewhat difficult to integrate with the general assumption that ROS are deleterious to stallion sperm function. The fact that increased ROS production was not initially associated with impaired sperm function may indicate, as has been previously suggested, that increased ROS production is not necessarily detrimental, and may instead reflect an active sperm metabolism (Gibb et al, 2014). Only when ROS exposure is continued over a longer period of time, may the deleterious effects become apparent as a reduction in the percentage of progressively motile sperm, such as that seen two hours after the increase in ROS observed in our study. Recently, Macías Garcia et al. (2012) described increased ROS production in sperm selected by single layer centrifugation, a technique that should selects 'better' spermatozoa further suggesting that increased ROS production may simply reflect active oxidative phosphorylation in metabolically active spermatozoa. Alternatively, the effect reported by Macias Garcia et al, may relate to oxidative stress induced by colloidal centrifugation, since it has recently been reported that colloidal sperm preparation media may be a source of oxidative damage due to the presence of transition metals (Aitken et al 2014). Inhibition of APT synthase induced a significant increase on ROS production, but only in dead spermatozoa. Recently, it has been demonstrated that an L amino acid oxidase is present in equine spermatozoa and that this enzyme was located in the acrosome responsible to aromatic amino acids and particularly active in non-viable cells (Aitken et al, 2015). Our finding confirmed this previous report, and others indicating that dead sperm in the sample increase oxidative stress (Roca et al, 2013)

Interestingly, we found that uncoupling mitochondria with CCCP led to increased sperm staining with YoPro-1, and inhibiting ATP synthase with olygomycin-A virtually reduced to 0 the percentage of spermatozoa with intact membranes. Since the plasma membrane of the sperm remained intact, the drop in ATP induced by uncoupling oxidative phosphorylation may result in insufficient outward pumping of incoming YoPro-1 by multidrug resistant (MDR) receptors, which operate in a ATP dependent fashion. Indeed YoPro-1 seems to be more sensitive to ATP sensitive MDR transporter deficiency that other probes, and ATP leakage through specific channels may contribute to ATP depletion (Tsujimoto, 1997, Chekeni et al 2010, Gallardo Bolaños et al, 2014). Also the significant increase in ROS production may have contributed to reduce membrane integrity. All these findings stress the importance of mitochondrial function in stallion spermatozoa, opening new areas of research and challenging the paradigm of ROS negatively influencing sperm function.

In summary, this study provides new evidence that indicate that stallion sperm rely more on mitochondrial ATP production for their functioning than other mammalian sperm. The alternative regulation of glycolysis for ATP production is also present in stallion spermatozoa but is of lesser importance for maintenance of sperm functioning. These findings may be helpful for designing assays to evaluate sperm quality and better sperm handling methods for instance for (cryo)preservation.

REFERENCES

Aitken RJ, Finnie JM, Muscio L, Whiting S, Connaughton HS, Kuczera L, RothKirch TB, De Iuliis GN, 2014 Potential importance of transitions metals in the induction of DNA damage by sperm preparation media Hum Reprod (epub ahead of print)

Aitken JB, Naumovsky N, Curry B, Grupen CG, Gibb Z, Aitken RJ 2015 Characterization of an L-amino oxidase in equine spermatozoa DOI 10.1095/biolreprod.114.126052

Amaral A, Lourenco B, Marques M, Ramalho-Santos J 2013 Mitochondria functionality and sperm quality Reproduction 146: 163-174

Balao da Silva CM, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolanos JM, Plaza Davila M, Morrell JM, Rodriguez Martinez H, Tapia JA, Aparicio IM & Pena FJ 2013 Sex sorting increases the permeability of the membrane of stallion spermatozoa. Animal Reproduction Science 138 241-251.

Bourdon E and Blache D. The importance of proteins in defense against oxidation. Antioxid Redox Signal 3: 293–311, 2001.

Caiza de la Cueva FI, Rigau T, Pujol R, Piedrafita J, Rodriguez Gil JE 1997 Resistance to hyperosmotic stress in boar spermatozoa: the role of the ionic pumps and the relationship with cryosurvival Anim Reprod Sci 48. 301-315

Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, Isakson BE, Bayliss DA & Ravichandran KS 2010 Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* 467 863-867.

Erkkila K Kyttanen S, Wikstrom M, Taari K, Hikim APS, Swerdloff RS, Dunkel L 2006. Regulation of human male germ cell death by modulators of ATP production Am J Physiol Endocrinol Metab 290: E1145-E1154

Ferramosa A, Zara V 2014 Bioenergetics of mammalian sperm capacitation Biomed Res Int 2014; 902953

Gallardo Bolaños JM , Balao da Silva CM, Martín Muñoz P, Morillo Rodríguez A, M Plaza Dávila M, Rodriguez Martínez H, Aparicio IM, Tapia JA, Ortega Ferrusola C. Peña FJ (2014). Phosphorylated Akt preserves stallion sperm viability and motility by inhibiting caspase 3 and 7. Reproduction 148, 221-235

Gibb Z, Lambourne SR, Aitken RJ 2014 The paradoxical relationship between stallion fertility and oxidative stress Biology of Reproduction DOI: 10.1095/biolreprod.114.118539

González Fernández L, Morrell JM, Peña FJ, Macías García B (2012) Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria Theriogenology 78: 415-422.

Holt WV, Van Lock (2004) Concepts in sperm heterogenicity, sperm selection and sperm competition as biological foundations for laboratory tests of sperm quality Reproduction 127: 527-535.

Holt WV 2005 Is quality assurance in semen analysis still really necessary? A spermatologist's point of view Hum Reprod 20: 2983-2986

Klindemberg M (2008) The ADP and ATP transport in mitochondria and its carrier Biochemica et Biophysica Acta 1778: 1978-2021

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA & Aitken RJ 2008 Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. The Journal of Clinical Endocrinology and Metabolism 93 3199-3207.

Macías García B, Miró Morán A, González Fernández L Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolaños JM, Rodríguez Martínez H, Tapia JA, Peña FJ (2012) The mitochondria of the stallion spermatozoa are more sensitive than the plasmalemma to osmotic induced stress: role of c-Jun NHP_2 terminal kinases (JNKs) (SPKAs). Journal of Andrology 33: 105-113

Macías García B, Gonzalez Fernandez L, Gallardo Bolaños JM, Peña FJ, Johannisson A, Morrell JM 2012 Androcoll-E large selects a subset of live spermatozoa capable of producing ROS Anim Reprod Sci 132: 74-82

Mannowetz N, Wandernoth PM, Wennemuth G 2012 Glucose is a pH-dependent motor for sperm beat frequency during early activation PLos One 7:e41030

Mukai Ch, Okuno M (2004) Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement Biol Reprod 71: 540-547

Nascimento JM, Shi LZ, Tam J, Chandsawangbhuwana C, Durrant B, Botvinick EL, Berns MW 2008 Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers and real time automated traking and trapping J Cell Physiol 217: 745-751

Nunez-Martinez I, Moran JM & Pena FJ 2007b Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. Int J Androl 30 182-189.

Odet F, Gabel S, London RE, Goldberg E, Eddy EM 2013 Glycolisis and mitochondrial respiration in mouse LDHC-null sperm Biol Reprod 88: 95, 1-7

Ortega- Ferrusola C , Sotillo-Galán Y, Varela –Fernández E, Gallardo-Bolaños JM, González-Fernández L, Tapia JA, Rodríguez Martinez H, Peña FJ (2009) Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa Animal Reproduction Science 114: 393-403.

Ortega- Ferrusola C, Sotillo-Galán Y, Varela –Fernández E, Gallardo-Bolaños JM, González-Fernández L, Tapia JA, Peña FJ (2008) Detection of apoptosis like changes during the cryopreservation process in equine sperm Journal of Andrology 29:213-221.

Ortega Ferrusola C, González Fernández L, García Macías B, Salazar Sandoval C, Morillo Rodríguez A, Rodríguez Martinez H, Tapia JA, Peña FJ (2009). Effect of cryopreservation on nitric oxide production in stallion spermatozoa Biology of Reproduction 81: 1106-1111.

Ortega Ferrusola C, González Fernández L, García Macías B, Salazar Sandoval C, Morell JM, Rodríguez Martinez H, Tapia JA, Peña FJ. (2009) Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa is stallion dependent and relates to "apoptosis like" changes. Reproduction 138: 55-63.

Ortega Ferrusola C, González Fernández L, García Macías B, Salazar Sandoval C, Tapia JA, Peña FJ (2010) Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like changes" during cryopreservation of stallion spermatozoa. Theriogenology 74: 458-465

Peña FJ, Saravia F, Johanisson A, Wallgren M, Rodriguez Martinez H. (2005) A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa International Journal of Andrology 28: 107-114.

Peña FJ, Rodríguez Martínez H, Tapia JA, Ortega Ferrusola C, Fernández González L, Macias García B (2009) Mitochondria in mammalian sperm physiology and pathology: a review Reproduction in Domestic Animals 44: 345-349.

Peña FJ, Macías García B, Samper JC, Aparicio IM, Tapia JA, Ortega Ferrusola C. (2011) Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? Theriogenology 76: 1177-1186.

Pena FJ, Ortega-Ferrusola C, Tapia JA, Aparicio IM 2012 How stallion sperm age in vitro? Scenario for preservation technologies Journal of Equine Veterinary Science 32 451-454

Roca J, Martinez Alborcia MJ, Gil MA, Parrilla I, Martinez EA 2013 Dead spermatozoa in raw semen samples impair in vitro fertilization outcomes of frozen thawed spermatozoa Fertil Steril 100; 875-81

Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrin P, Freitas A, Paiva A, Almeida Santos T, Ramalho-Santos J 2011 Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential Plos One 6: e18112

Storey BT (2008) Mammalian sperm metabolism: oxygen and sugar friend and foe Int J Dev Biol 52: 5-6 427-437

Turrens FJ 2003 Mitochondrial formation of reactive oxygen species J Physiol 552; 355-344

Tsujimoto Y 1997 Apoptosis and necrosis: Intracellullar ATP level as determinant for cell death modes Cell Death and Differentiation 4: 429-434

Varner DD, Gibb Z, Aitken RJ 2014 Stallion fertility: A focus on the spermatozoa Equine Vet J. 2014 Jun 18. doi: 10.1111/evj.12308.

Xi Q, Cheranov SY, Jaggar JH 2005 Mitochondria derived reactive oxygen species dilate cerebral arteries by activating Ca2+ sparks Circ Res 97; 3454-362

FIGURE 1. Confocal laser microscopy images of the staining patterns of stallion spermatozoa after loading with JC-1. A represent spermatozoa with active mitochondria and high $\Delta\psi m$, B represent spermatozoa with inactive mitochondria and low $\Delta\psi m$ 100x objective

Figure 2.- Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on mitochondrial membrane potential, assessed using JC-1 staining. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors as described in material and methods for up to three hours, A-C-E-G are results after one hour of incubation, B-D-D-H are results after three ours of incubation at 37 ºC. Results are depicted as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$

Figure 3. Effect of uncoupling agents (CCCP (A) and DNP (B)) and inhibitors of mitochondrial respiration (Na Cn (C) and Oligomycin (D)) on ATP content of stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors . Results are portrayed as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$., ** $P<0.01$

Figure 4.- Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on the percentages of total (TM%) and progressively motile (PM%) spermatozoa measured by computer assisted sperm analysis (CASA). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors as described in material and methods, for up to three hours. Results are shown as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$

Figure 5. Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on stallion sperm velocities after CASA analysis; VCL = curvilinear velocity (µm/s), VSL = straight line velocity (µm/s), VAP = average path velocity (µm/s). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors as described in material and methods for up to three hours. Results are depictedn as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$

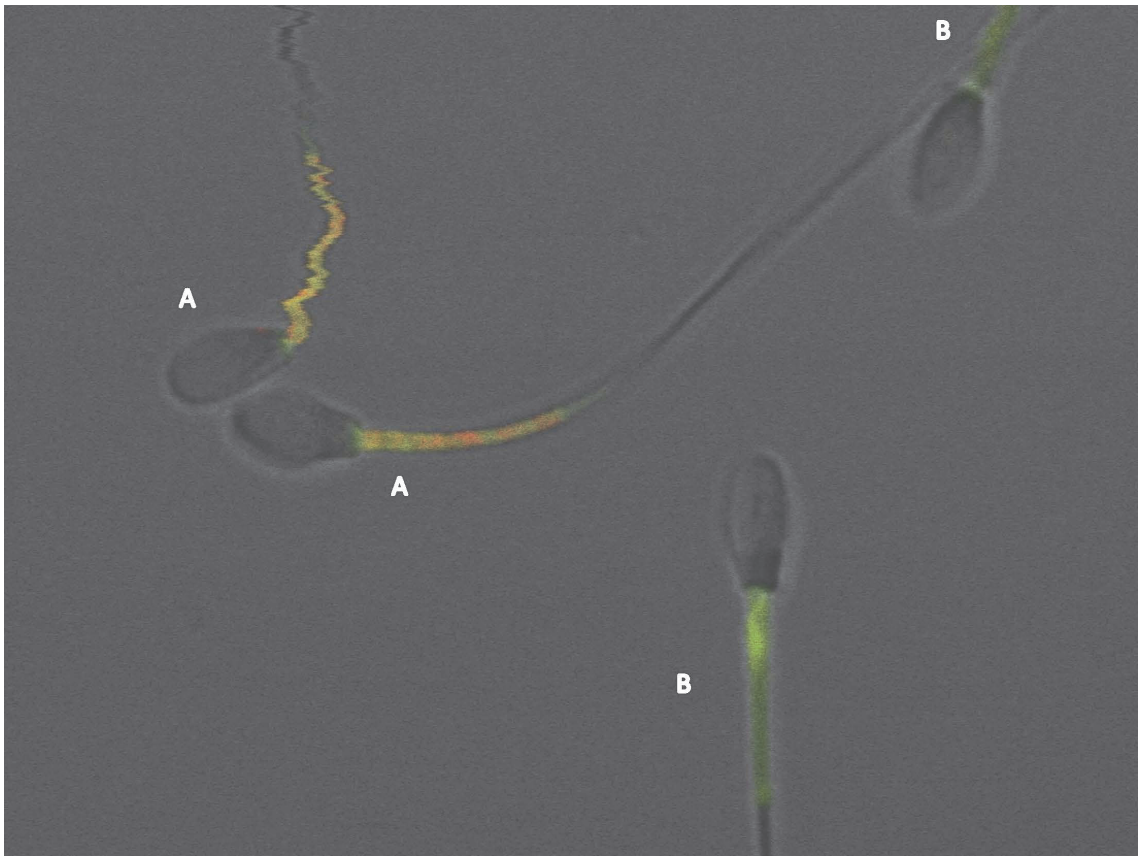
Figure 6. Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on the percentages of intact spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors as described in material and

methods for up to three hours. Results are depicted as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$

Figure 7. Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on the percentage of spermatozoa with signs of increased membrane permeability (YoPro+) or loss of membrane integrity (YoPro +Eth+). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors as described for material and methods for up to three hours. Results are shown as means ± SD. Comparisons were made between each sample and its control. n=7 stallions.

Figure 8.- Effect of uncoupling agents (CCCP (A-B) and DNP (C-D) an inhibitors of mitochondrial respiration (NaCn (E-F) and Oligmycin A (G-H)) on the production of reactive oxygen species (ROS) by stallion spermatozoa. ROS production was assessed flow cytometrically. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to three hours. Results are portrayed as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$

Figure 9.- Effect of inhibition of glycolysis sperm motility (B after 1 hour of incubation, C after three hours) and velocities (D 1 hour, E 3 hours), membrane intactness (F 1 hour, G three hours) and ATP content (A) in stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of 2- deoxyglucose 5 and 10 mM as described in material and methods up to three hours. Results are portrayed as means ± SD. Comparisons were made between each sample and its control. n=7 stallions * $p<0.05$, ** $p<0.01$


B
A
A
B

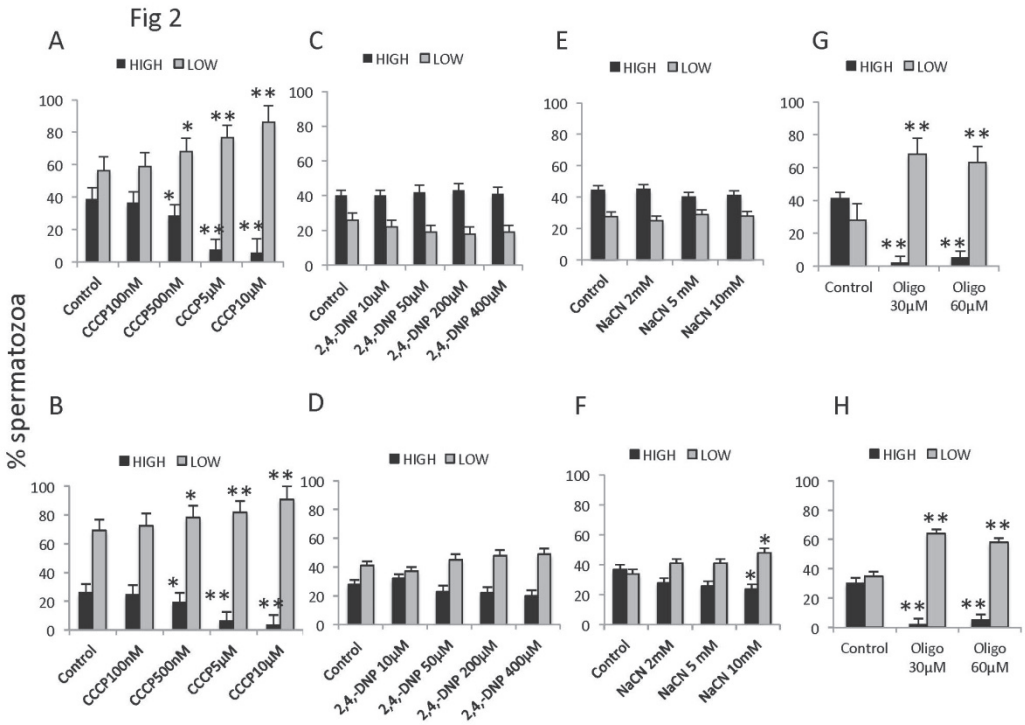


Fig 2
A
C
E
G
B
D
F
H
HIGH
LOW
% spermatozoa
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
NaCN 2mM
NaCN 5 mM
NaCN 10mM
Oligo 30µM
Oligo 60µM

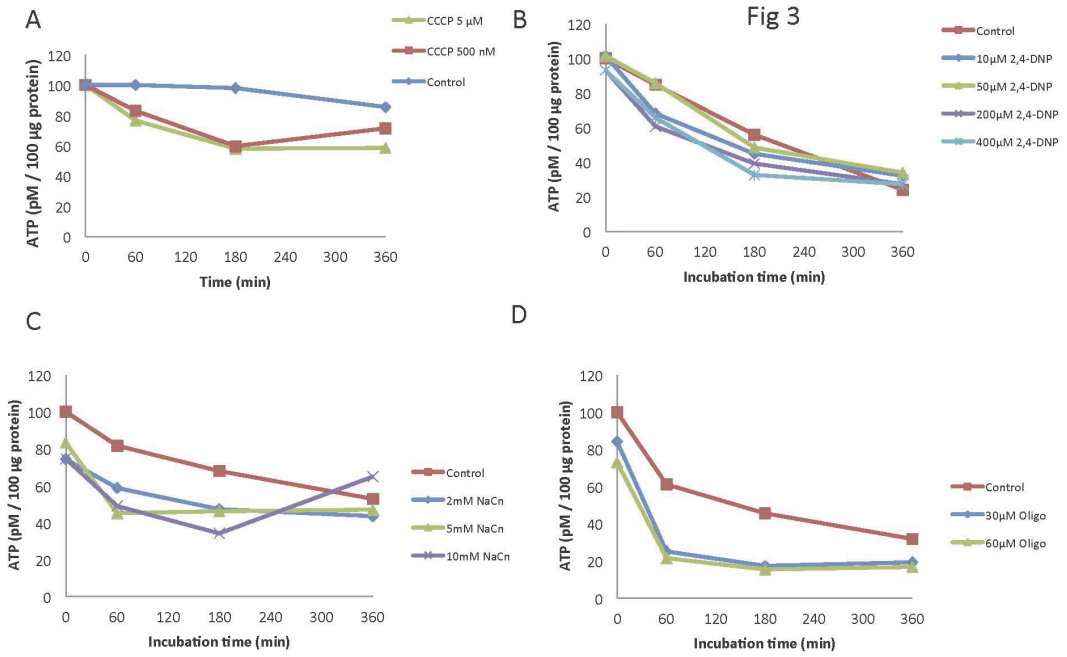


Fig 3
A
ATP (pM / 100 µg protein)
Time (min)
CCCP 5 µM
CCCP 500 nM
Control
B
ATP (pM / 100 µg protein)
Incubation time (min)
Control
10µM 2,4-DNP
50µM 2,4-DNP
200µM 2,4-DNP
400µM 2,4-DNP
C
ATP (pM / 100 µg protein)
Incubation time (min)
Control
2mM NaCn
5mM NaCn
10mM NaCn
D
ATP (pM / 100 µg protein)
Incubation time (min)
Control
30µM Oligo
60µM Oligo

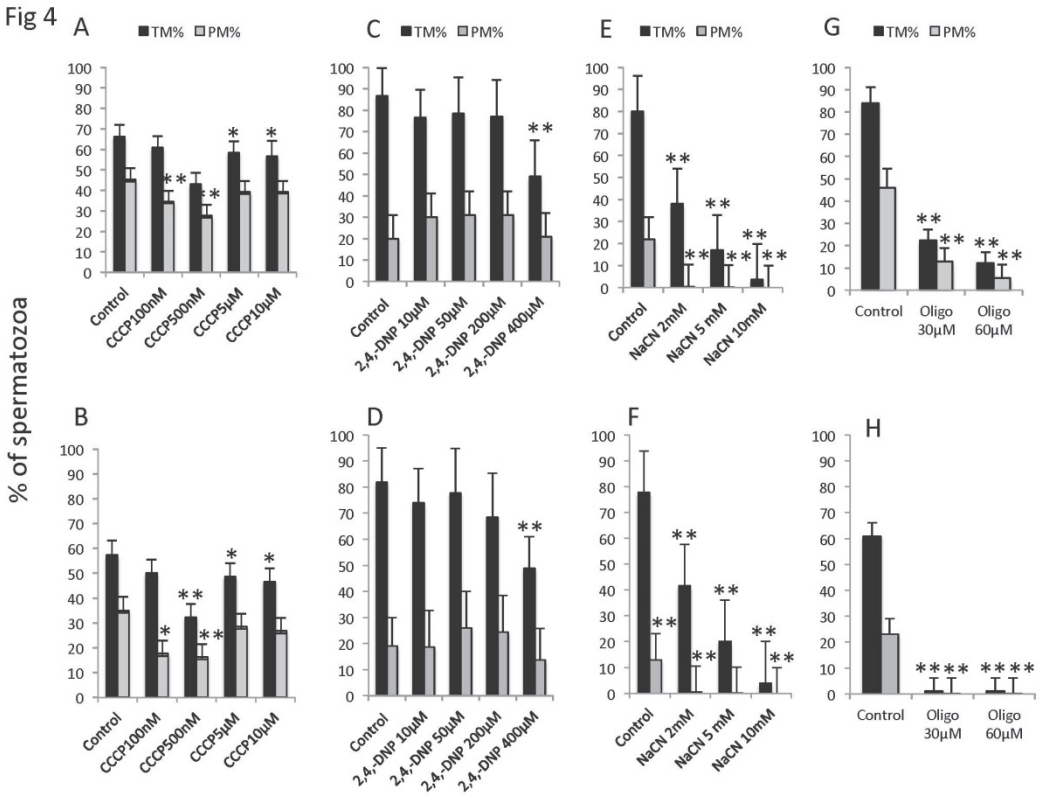


Fig 4
% of spermatozoa
A
TM% PM%
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
B
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
C
TM% PM%
Control
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
D
Control
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
E
TM% PM%
Control
NaCN 2mM
NaCN 5 mM
NaCN 10mM
F
Control
NaCN 2mM
NaCN 5 mM
NaCN 10mM
G
TM% PM%
Control
Oligo 30µM
Oligo 60µM
H
Control
Oligo 30µM
Oligo 60µM

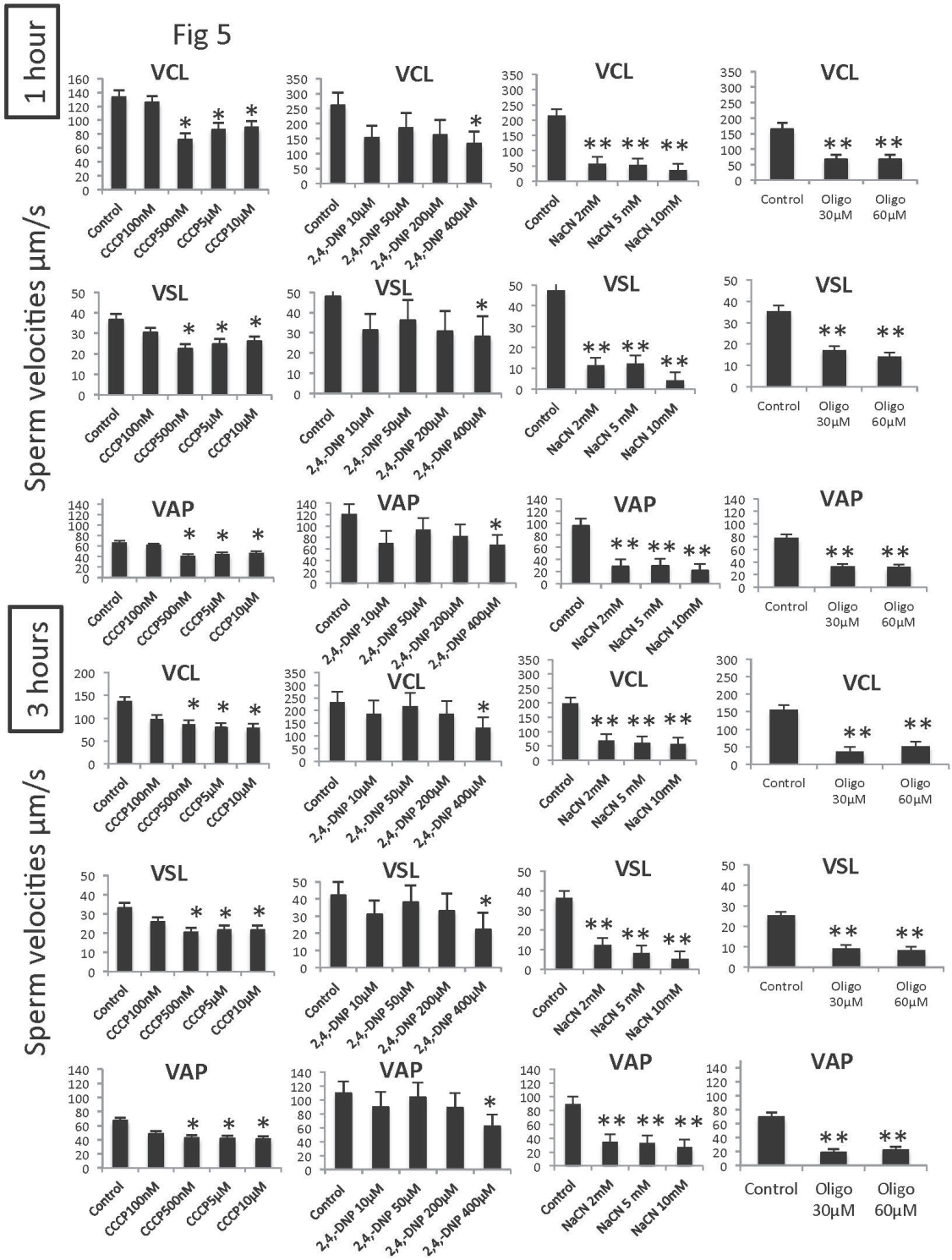


Fig 5
1 hour
3 hours
Sperm velocities µm/s
VCL
VSL
VAP
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
NaCN 2mM
NaCN 5 mM
NaCN 10mM
Oligo 30µM
Oligo 60µM

Fig 6

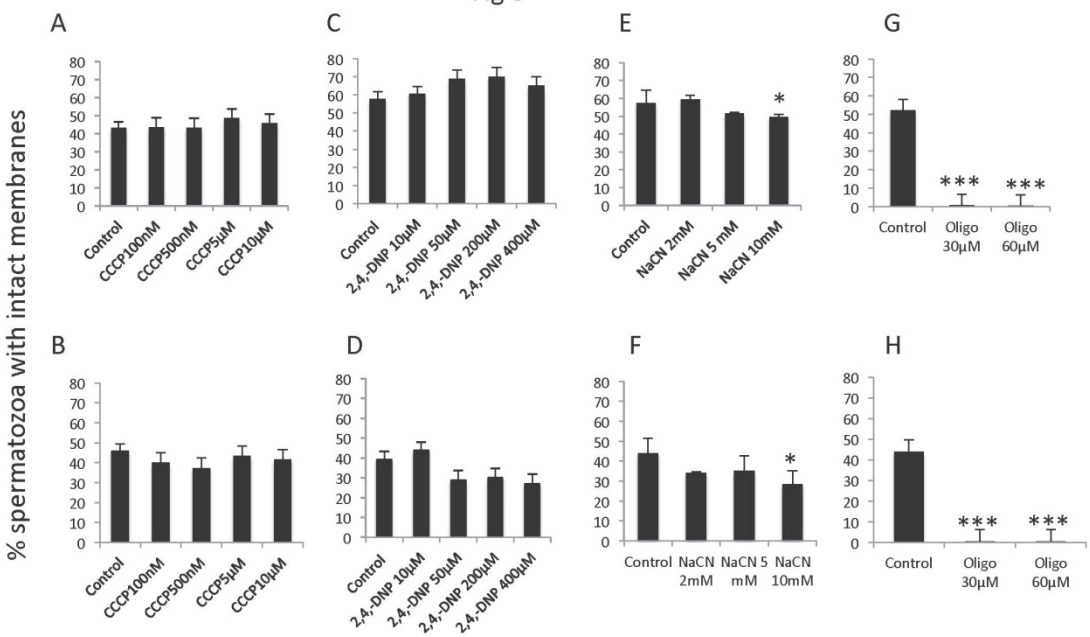
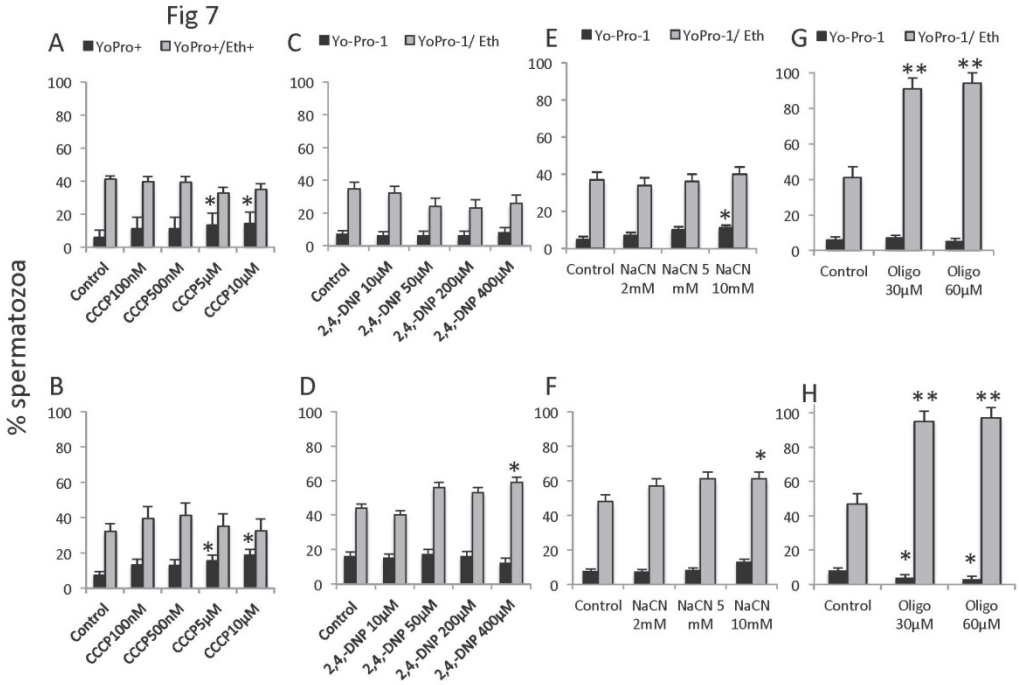

% spermatozoa with intact membranes
A
B
C
D
E
F
G
H
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
NaCN 2mM
NaCN 5 mM
NaCN 10mM
Oligo 30µM
Oligo 60µM

Fig 7


% spermatozoa
A YoPro+ YoPro+/Eth+
B
C Yo-Pro-1 YoPro-1/ Eth
D
E Yo-Pro-1 YoPro-1/ Eth
F
G Yo-Pro-1 YoPro-1/ Eth
H
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
NaCN 2mM
NaCN 5 mM
NaCN 10mM
Oligo 30µM
Oligo 60µM

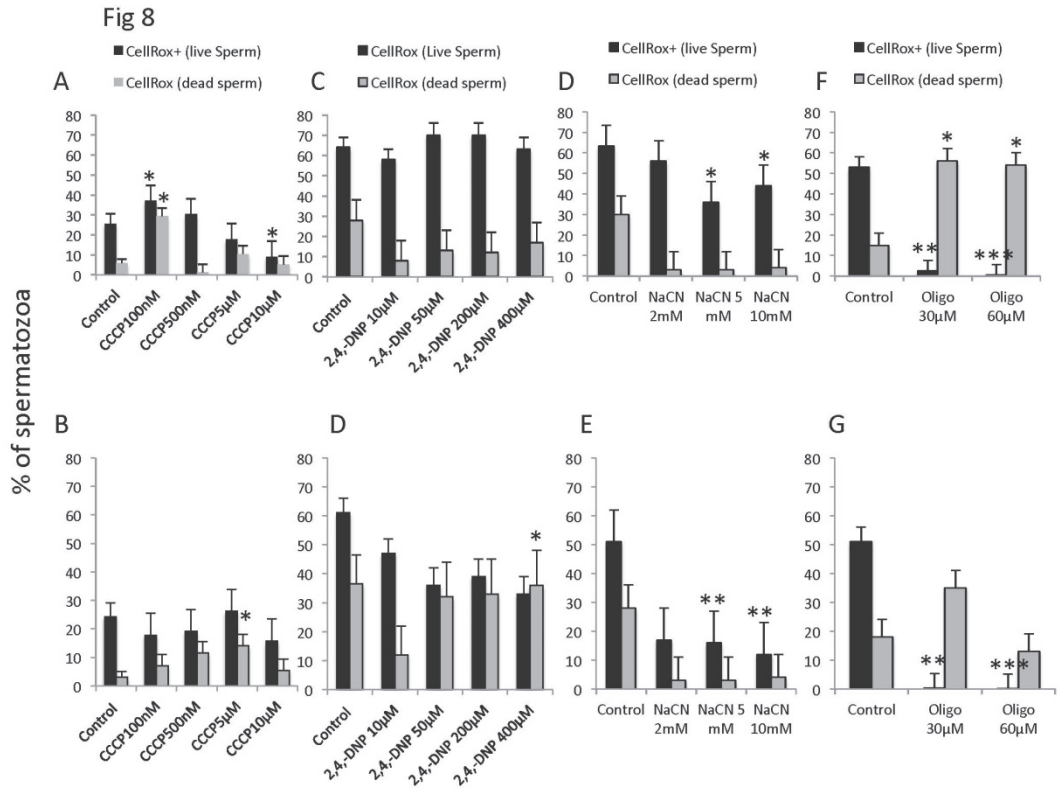


Fig 8
% of spermatozoa
A
CellRox+ (live Sperm)
CellRox (dead sperm)
Control
CCCP100nM
CCCP500nM
CCCP5µM
CCCP10µM
C
CellRox (Live Sperm)
CellRox (dead sperm)
Control
2,4,-DNP 10µM
2,4,-DNP 50µM
2,4,-DNP 200µM
2,4,-DNP 400µM
D
CellRox+ (live Sperm)
CellRox (dead sperm)
Control
NaCN 2mM
NaCN 5 mM
NaCN 10mM
F
CellRox+ (live Sperm)
CellRox (dead sperm)
Control
Oligo 30µM
Oligo 60µM
B
D
E
G

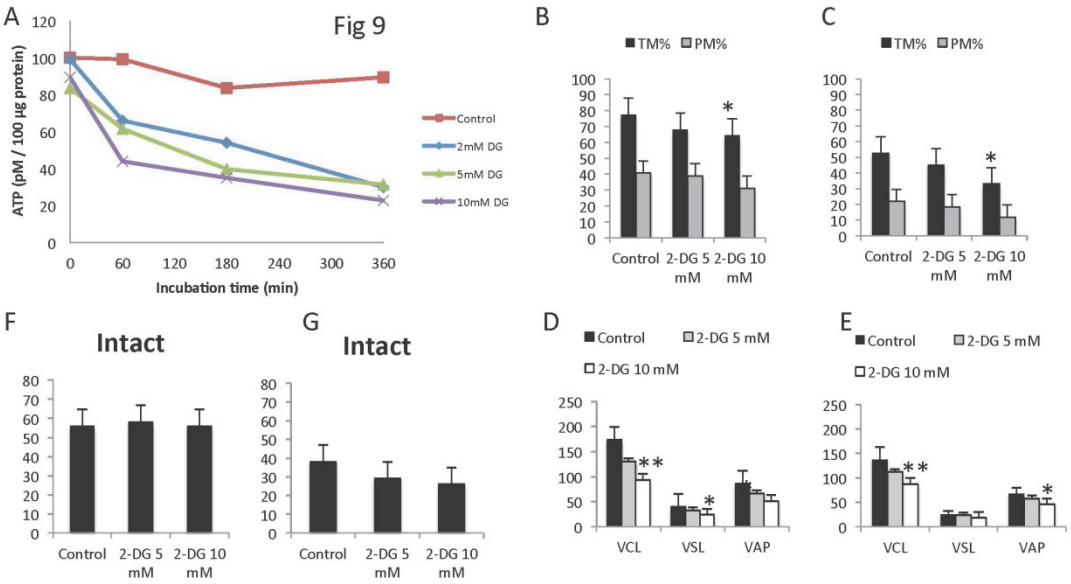


Fig 9
A
ATP (pM / 100 µg protein)
Incubation time (min)
Control
2mM DG
5mM DG
10mM DG
B
TM% PM%
Control
2-DG 5 mM
2-DG 10 mM
C
TM% PM%
Control
2-DG 5 mM
2-DG 10 mM
F
Intact
Control
2-DG 5 mM
2-DG 10 mM
G
Intact
Control
2-DG 5 mM
2-DG 10 mM
D
Control
2-DG 5 mM
2-DG 10 mM
VCL
VSL
VAP
E
Control
2-DG 5 mM
2-DG 10 mM
VCL
VSL
VAP

Artículo 4º

Reproduction in Domestic Animals

Reprod Dom Anim doi: 10.1111/rda.12551
ISSN 0936–6768

Review Article

The Impact of Reproductive Technologies on Stallion Mitochondrial Function

FJ Peña[1,2], M Plaza Davila[1], BA Ball[2], EL Squires[2], P Martin Muñoz[1], C Ortega Ferrusola[1] and C Balao da Silva[1]

[1]*Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain;* [2]*Gluck Equine Research Center, University of Kentucky, Lexington, KY, USA*

Contents
The traditional assessment of stallion sperm comprises evaluation of sperm motility and membrane integrity and identification of abnormal morphology of the spermatozoa. More recently, the progressive introduction of flow cytometry is increasing the number of tests available. However, compared with other sperm structures and functions, the evaluation of mitochondria has received less attention in stallion andrology. Recent research indicates that sperm mitochondria are key structures in sperm function suffering major changes during biotechnological procedures such as cryopreservation. In this paper, mitochondrial structure and function will be reviewed in the stallion, when possible specific stallion studies will be discussed, and general findings on mammalian mitochondrial function will be argued when relevant. Especial emphasis will be put on their role as source of reactive oxygen species and in their role regulating sperm lifespan, a possible target to investigate with the aim to improve the quality of frozen–thawed stallion sperm. Later on, the impact of current sperm technologies, principally cryopreservation, on mitochondrial function will be discussed pointing out novel areas of research interest with high potential to improve current sperm technologies.

Introduction

Due to the increased use of sperm technologies in equine species, the last decade has witnessed the development of intense research in sperm technologies in the stallion and thus its impact on sperm functionality. A central feature connecting both issues is the role of the mitochondrion. Among others, substantial increases of our current knowledge have occurred in areas such as oxidative stress (Aitken and Koppers 2011; da Silva et al. 2011; Gibb et al. 2014), apoptotic-like changes (Ortega-Ferrusola et al. 2008; Ortega Ferrusola et al. 2009b) and the pivotal effects of changes in osmolality during cryopreservation; interestingly, many of these studies have been performed using the stallion as a model. Technologies such as cryopreservation and sex sorting have experienced substantial advances in the last decade (Peña et al. 2011; Samper et al. 2012). In this review, we will discuss recent advances in the understanding of mitochondrial function, and the potential mechanisms explaining how mitochondrial dysfunction may impact fertility based in recent human studies. Moreover, evidence indicating that mitochondrial functionality may be used to identify the fertilizing subpopulation of spermatozoa, and the potential utility of separation of spermatozoa based on their mitochondrial status will be discussed.

Structure and Basic Mechanisms of Mitochondrial Function

Mitochondria are membrane-enclosed organelles that generate most of the ATP supply (Stowe and Camara 2009; Mizrahi and Breitbart 2014; Vadnais et al. 2014), although in some species glycolysis may be an important supply of ATP for sperm (Ferramosca et al. 2008). Mitochondrial compartments include the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), the cristae and matrix (Prince and Buttle 2004). In addition to ATP generation, mitochondria also have a role in cell signalling, Ca^{2+} buffering (Breitbart et al. 1996; Costello et al. 2009; Bravo et al. 2014), apoptosis (Koppers et al. 2010; Ortega Ferrusola et al. 2010; Mendoza et al. 2013) and cell death (Thompson et al. 2003). The OMM contains a large number of channels, formed by integral proteins termed 'porins', that allow molecules of <5000 Daltons to move freely across the OMM. Larger proteins can enter the mitochondrion if a signalling sequence at their n-terminus binds to translocases (large multisubunits proteins) that actively transport them across the OMM (Chipuk et al. 2006). Disruption of the OMM permits proteins in the IMS to leak into the cytoplasm leading to cell death. The OMM is permeable to small molecules and has a similar composition in ions and sugars as the cytoplasm. However, the protein composition is different from the cytosol. One protein localized only in the IMS is the cytochrome c. The IMM contains proteins involved in redox reactions of oxidative phosphorylation, ATP synthase and ATPase, transport and import proteins. The IMM is rich in the phospholipid cardiolipin, which contains four fatty acids that help to make the IMM impermeable (Schlame et al. 2005).

The membrane potential across the IMM is formed by the action of respiratory enzymes and the tricarboxylic acid (TCA) cycle. Each pyruvate molecule produced by glycolysis is actively transported across the IMM where is oxidized and combined with coenzyme A to form CO_2, acetyl-CoA and NADH (Becker and Hardin

2003). The acetyl-CoA is the primary substrate to enter the TCA cycle. The enzymes of this TCA cycle are located in the mitochondrial matrix with the exception of succinate dehydrogenase, which is bound to the IMM as part of complex II. The TCA cycle oxidizes the acetyl-CoA to CO_2, producing three molecules of NADH and two molecules of $FADH_2$, which are a source of electrons for transport along the respiratory complexes, and a molecule of GTP that is readily converted to ATP. The REDOX energy from NADPH and $FADH_2$ is transferred to O_2 in several steps via the electron transport chain (ETC.) along the respiratory complexes. The individual proteins of complexes I to IV interact to form supramolecular structures known as respiratory supercomplexes or respirasomes (Dudkina et al. 2008). Reducing equivalents from the cytoplasm can be imported by the malate aspartate shuttle system of antiporter proteins or fed into the electron transport system (ETS) using a glycerol phosphate buffer. NADH dehydrogenase, cytochrome c reductase and cytochrome c oxidase perform the transfer, and the incremental release of energy is used to pump protons into the IMS (Voet et al. 2006). As the proton concentration increases in the IMS, a strong electrochemical gradient is established across the IMM, the protons can return to the matrix through the ATP synthase complex, and their potential energy is used to synthesize ATP from ADP and inorganic phosphate (Mitchell 1967).

Characteristics of the sperm mitochondria

Depending of the cell type and functional status of the cell, mitochondria may present diverse morphologies and vary in numbers (Ramalho-Santos et al. 2009). Morphology, localization and energy metabolism change markedly during spermatogenesis. Different types of mitochondria are recognizable in the germinal epithelium during spermatogenesis (Ramalho-Santos et al. 2009). The mitochondria in A spermatogonia are ovoidal in shape and have lamellar cristae, characteristic of the 'orthodox' appearance. In B spermatogonia and resting and leptotene spermatocytes, the space delimited by the two membranes of the lamellar cristae increases in size. In zygotene and early pachytene, this process is observed in numerous mitochondria that are defined as 'intermediate'. In late pachytene spermatocytes, the inner space is flattened against the outer membrane by a considerable expansion of one or more intracristal spaces that communicate with the intermembrane space by a narrow aperture. These are considered the typical 'condensed' mitochondria of the germ cells (De Martino et al. 1979). While in spermatogonia and early spermatocytes, orthodox mitochondria are present, late spermatocytes and spermatids have more condensed mitochondria that are considered more efficient (Amaral et al. 2013). During spermiogenesis, mitochondria rearrange in tubular structures helically anchored around the anterior portion of the nine outer dense fibres and of the axoneme forming the mid-piece. Complex filaments called submitochondrial reticulum sustain the anchorage of the mitochondrial sheath (Olson and Winfrey 1990, 1992). A keratinous structure (mitochondrial capsule) covers the OMM of sperm mitochondria, formed by disulphide bounds between cysteine and proline-rich selenoproteins (Ursini et al. 1999). This may confer protection to sperm mitochondria and mitochondrial DNA (Amaral et al. 2013).

Fuel for sperm energy: glycolysis or OXPHOS?

There has been controversy regarding the main source of ATP for main sperm functions and particularly for motility (Ford 2006), however, mainly due to species differences. Sperm need a continuous supply of ATP for flagellar movement and signal transduction via protein phosphorylation (Gonzalez-Fernandez et al. 2009). There are species differences in energy generation. Human, mouse, boar and bull spermatozoa rely on glycolysis for ATP production; on the contrary, new evidences indicate that this is not the case for stallion spermatozoa (Ortega Ferrusola et al. 2010; Gibb et al. 2014), where mitochondrial oxidative phosphorylation (OXPHOS) is the main source of ATP. Thus, mitochondrial functionality is especially important in equids in terms of both sperm function and fertility due to their higher dependence of OXPHOS for ATP production, and moreover, highly fertile stallions exhibit higher levels of OXPHOS activity (Gibb et al. 2014). Recent experiments in our laboratory demonstrated that when the mitochondrial ETC was interrupted at both complexes I and II, a rapid decrease in intracellular ATP content was observed, accompanied by a rapid decrease in motility and velocities of the spermatozoa, even in the presence of glucose in the media. Previously, we studied the effect of the inhibition of the adenine nucleotide translocator (ATN), a specific ATP/ADP transporter in sperm function (Ortega Ferrusola et al. 2010). The ATP/ADP carrier catalyses the transmembrane exchange between ATP generated in the mitochondria by oxidative phosphorylation and cytosolic ADP (Klingenberg 2008). Our findings suggested that equine spermatozoa largely depend on oxidative phosphorylation for the maintenance of activated motility. More recently, these observations have been confirmed in other laboratories using CCCP, antimycin A and rotenone as mitochondrial inhibitors; incubation of stallion spermatozoa in the presence of these inhibitors resulted in a significant fall in motility and velocity (Gibb et al. 2014). The dependence of stallion spermatozoa on OXPHOS to obtain energy has important implications for stallion sperm evaluation, as intense mitochondrial activity may be reflected by increased superoxide production (Luo et al. 2013). The functional significance of this fact in terms of sperm functionality and fertility may break the so far assumed paradigm that detection of reactive oxygen species is always a sign of stallion sperm dysfunction, as highly active spermatozoa may produce more reactive oxygen species (ROS)

than defective spermatozoa (Macias-Garcia et al. 2012; Gibb et al. 2014).

Mitochondrial reactive oxygen species regulate sperm function

Reactive oxygen species are by-products of various metabolic processes and now are recognized as important regulators of many cellular functions (Stowe and Camara 2009). The mitochondrion is considered as the major source of reactive oxygen species in most cells. Superoxide ($O_2^{-}\bullet$) can be generated at different points within the electron transport chain, by univalent reduction of oxygen, and spontaneously or enzymatically dismutates to H_2O_2. Most superoxide is converted to H_2O_2 by superoxide dismutases inside and outside of the mitochondrial matrix, and superoxide in low and controlled amounts exerts important regulatory cellular functions. Excess of H_2O_2 can combine with Fe^{2+} to form reactive ferryl species. In the presence of nitric oxide (NO•), $O_2^{-}\bullet$ forms the reactant peroxynitrite (ONOO•), and ONOOH-induced nitrosylation of proteins, DNA and lipids can modify their structure and function (Stowe and Camara 2009). Numerous studies indicate that ROS are important regulators of sperm function (de Lamirande and Gagnon 1993, 2002, 2003; Zini et al. 1995; de Lamirande et al. 1997; de Lamirande and Lamothe 2009). An important aspect, sometimes neglected, is the type, origin and main role of the different ROS that can be formed during sperm metabolism. Superoxide is short-lived ($t_{1/2}$ 1 ms) and cell impermeant, while H_2O_2 is more stable and cell permeant. Nitric oxide (NO•) is synthesized through the conversion of l-arginine to l-citrulline by nitric oxide synthase (NOS). These enzymes are present in the stallion spermatozoa, possibly as sperm-specific isoforms (Ortega Ferrusola et al. 2009a). Moreover, recent evidence suggests that stallion sperm mitochondria produce significant amounts of NO (Ortega Ferrusola et al. 2009a). Nitric oxide has a relatively long half-life (1 s) and is more reactive than $O_2^{-}\bullet$. These compounds are considered, when produced in a controlled manner, as signalling molecules involved in a variety of sperm functions. Other molecules such as the hydroxyl radical (•OH), the peroxynitrite anion ($ONOO^{-}$) and lipid peroxides are considered more toxic to the spermatozoa and with less regulatory functions. Functions believed to be redox regulated in spermatozoa include activated and hyperactivated motility, chemotaxis, capacitation and the acrosome reaction. Controlled ROS production occurs during capacitation in spermatozoa (Agarwal et al. 2014); this controlled production triggers signalling pathways initiated by an increase in cyclic adenosine 3′-5′ monophosphate cAMP. Increased cAMP activates protein kinase A (PKA) and the subsequent phosphorylation of extracellular-regulated kinase-like proteins and finally tyrosine phosphorylation of proteins in the fibrous sheath of the spermatozoa, leading to sperm hyperactivation. Acrosome reaction and sperm oocyte fusion also depend on ROS-activated cellular pathways.

Do mitochondria control the lifespan of ejaculated spermatozoa?

Although sperm death after ejaculation is due to ATP depletion, other forms of sperm demise are described. Both 'apoptotic-like events' (Aitken et al. 2012a,c) (Gallardo Bolanos et al. 2014) and an 'autophagy-like' mechanism may also be involved in sperm death after ejaculation (Gallardo Bolanos et al. 2012; Bolanos et al. 2014). More interestingly, different subpopulations of spermatozoa are in more advanced stages of senescence and may die at different intervals after ejaculation (Auger et al. 1993; Barroso et al. 2006; Gallon et al. 2006). Many spermatozoa with apoptotic changes appear in the ejaculate, and apoptotic changes including phosphatidylserine (PS) translocation (Pena et al. 2003; Martin et al. 2004; Brum et al. 2008), increase in membrane permeability (Ortega Ferrusola et al. 2009a), low mitochondrial membrane potential (da Silva et al. 2011; Aitken et al. 2012a; Garcia et al. 2012), activated caspases (Caselles et al. 2014; Gallardo Bolanos et al. 2014) and DNA fragmentation (Gillan et al. 2005; Smith et al. 2013) have been characterized in the ejaculate. Although the significance of these changes is still under debate, some points of consensus are being achieved. Perhaps the most important aspect is that all spermatozoa are programmed to die and that only one sperm reaches immortality through fertilization (Aitken and Koppers 2011; Aitken et al. 2012a). Importantly, many sperm biotechnologies accelerate this pathway to sperm death (Ortega-Ferrusola et al. 2008; Peña et al. 2011; Balao da Silva et al. 2013a; Petyim et al. 2014). This form of sperm death appears to be dependent on the activation of an intrinsic apoptotic cascade originated in the mitochondria after unbalanced mitochondrial ROS generation (Koppers et al. 2008; Aitken and Curry 2011). This latter may occur after exhaustion of intracellular antioxidant defences, particularly intracellular glutathione. Although this mechanism has been primarily described in humans, evidences suggest that a similar scenario occurs in stallions, mainly during processes of conservation of sperm (Ortega-Ferrusola et al. 2008; Ortega Ferrusola et al. 2009b). The maintenance of sperm viability depends on the phosphorylation status of specific pro-survival proteins. One of this, Akt, is activated through phosphorylation at threonine 308 or serine 473 (Alessi et al. 1997; Yu et al. 2005). After phosphorylation, Akt functions through phosphorylation and inhibition of Bad (serine 136) or caspase-9 (Cardone et al. 1998). Bad is a pro-apoptotic member of the Bcl family that promotes cell death by dimerization with Bcl-2 or Bcl-X_L (Yang et al. 1995). Bad phosphorylation at four different serine residues (serine 112, 136, 155 or 170) has been characterized as inactivating Bad (Datta et al. 1997; Lizcano et al. 2000; Dramsi et al. 2002; Danial et al. 2003). Provided that

sperm Akt is phosphorylated at Ser473 and/or Thr308 sperm motility and integrity are maintained, with dephosphorylation of Akt, caspases are activated and motility is rapidly lost (Gallardo Bolanos et al. 2014), a similar mechanism that occurs in human sperm (Koppers et al. 2011). It appears that dephosphorylation of Akt depends of different factors such as ATP depletion, unbalanced ROS and removal of pro-survival factors (Pujianto et al. 2010). On the contrary, dead receptor-mediated sperm death may also occur. Some evidences indicate that the sperm death can be triggered through the activation of Toll-like receptors by bacteria (Das et al. 2011; Fujita et al. 2011), ROS released by death sperm (Roca et al. 2013), and TNF-α or other pro-death factors released by other spermatozoa-activating extrinsic apoptotic pathways (Macias Garcia et al. 2012; Mendoza et al. 2013).

The Impact of Sperm Technologies on Mitochondria

Cryopreservation

Sperm preservation as liquid or frozen semen is an important part of the equine industry. Cryopreservation facilitates international commerce of genetic material from highly valuable stallions. However, sperm conservation technologies, and particularly cryopreservation, cause sperm death and sublethal damage to the surviving population. This sperm mortality and reduced sperm lifespan compromises fertility. Reduced lifespan of the surviving population also increases the labour and costs related to the insemination procedure (Peña et al. 2011). To overcome the reduced lifespan of frozen–thawed sperm, inseminations have to be performed close to ovulation, thus requiring more demanding and costly mare management. Sperm death during cryopreservation is due primarily to osmotic stress (Willoughby et al. 1996; Mazur and Koshimoto 2002). The sublethal damages leading to reduced lifespan of sperm have been termed capacitation-like changes (Fuller and Whittingham 1997; Bravo et al. 2005) or, more recently, apoptotic-like changes (Ortega-Ferrusola et al. 2008). In any case, cryopreserved sperm experiences accelerated ageing and premature senescence. Recent studies point out that mitochondria are especially sensitive to changes induced by sperm cryopreservation and that cryopreservation has an important role in the accelerated senescence occurring in the population that survives (Ortega-Ferrusola et al. 2009). The effects of osmotic changes have been investigated (Pommer et al. 2002; Ball 2008). Mitochondria experienced changes in their dimensions when subjected to osmotic challenges, increasing or decreasing their volume when spermatozoa were incubated in 75 mOsm and 900 mOsm solutions, respectively. Spermatozoa subjected to the hypoosmotic solution (75 mOsm) showed mitochondrial vacuolation, which was irreversible, remaining even after the return to isosmolar conditions. Changes in mitochondrial arrangement and detachment of the mid-piece plasma membrane were found in spermatozoa previously subjected to 900 and 75 mOsm when returned to isosmolarity (Gonzalez-Fernandez et al. 2012). Functional studies also revealed deleterious changes in mitochondria subjected to osmotic challenges (Garcia et al. 2012), and more interestingly, changes in mitochondria preceded membrane changes, suggesting that mitochondria are more sensitive to osmotic damage. These effects were also evident when stallion spermatozoa were subjected to cryopreservation (Ortega Ferrusola et al. 2009b; Garcia et al. 2012). These studies provide evidences that mitochondria are especially sensitive to changes induced by cooling and cryopreservation. When mitochondria were assessed at three specific points of the cryopreservation procedure, a significant drop in mitochondrial membrane potential was evident after equilibration at 5°C and a further decrease after thawing; other parameters of sperm quality were only affected by freezing and thawing and not during refrigeration (Ortega-Ferrusola et al. 2008). The special sensitivity of mitochondria to cooling may have rapid practical applications. Mitochondrial activity was assessed before freezing and receiving operating system (ROC) curves were used to evaluate the value of this assay as predictors of potential freezability. The percentage of spermatozoa showing simultaneously orange and green fluorescence when stained with JC-1 was the mitochondrial parameter with the highest diagnostic value. For stallions with poor semen freezability (<25% of intact spermatozoa post-thaw), the significant area under the ROC curve was 0.985, with 100% sensitivity and 99.8% specificity for a cut-off value of 55.7 (Ortega-Ferrusola et al. 2009). Selection of ejaculates with high mitochondrial activity for freezing seems a reasonable strategy to improve the outcome of cryopreservation of equine semen. Independent studies support this finding, and Yeste et al. (2015) also reported that good freezability stallion ejaculates are characterized by higher mitochondrial membrane potential. In view of all the evidences so far reported, the molecular mechanism behind sublethal damage might be as follows (Fig. 1). Osmotic stress induces mitochondrial swelling (Gonzalez-Fernandez et al. 2012) resulting in the opening of the mitochondrial permeability transition pore (PT-pore) (Ortega Ferrusola et al. 2010), the release of pro-apoptotic factors, apoptosis-inducing factor (AIF) and cytochrome C (Mendoza et al. 2013) and increased superoxide leakage due to disruption of the ETC. The release of pro-apoptotic factors due to osmotic stress may be reinforced by adducts originated by lipid peroxidation (LPO) (Ortega Ferrusola et al. 2009b) of the highly unsaturated lipids of the sperm membranes (Garcia et al. 2011), with 4-hydroxynonenal (4-HNE) (Aitken et al. 2012b,c) as a major adduct participating in this process. Another adduct, 8-iso-prostaglandin F2α, has been detected in our laboratory. These compounds activate a self-perpetuating cascade that triggers further release of pro-apoptotic

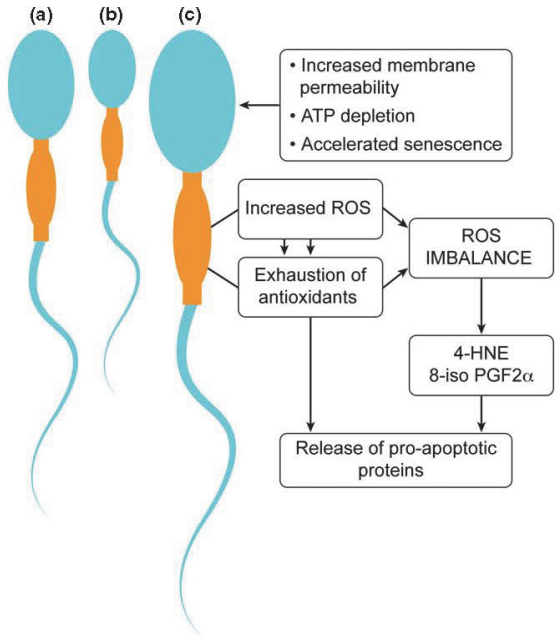


Fig. 1. Proposed mechanism explaining the mitochondrial origin of the short lifespan of frozen–thawed spermatozoa. (a) Fresh sperm; (b) frozen sperm; and (c) thawed sperm. Osmotic stress especially at thawing causes mechanical and biochemical damage to the mitochondria. Sperm experiences an hyperosmotic shock and shrinks at freezing (b) and a hypoosmotic shock and swells at thawing (c). As a consequence of these osmotic shocks, there is a leakage of cytochrome c from the mitochondria, activating an 'apoptotic-like' mechanism. Furthermore, mitochondrial dysfunction results in increased release of reactive oxygen species (ROS) that exhaust antioxidant defences leading to oxidative stress, and formation of toxic adducts from the phospholipids of the membranes. These adducts cause further mitochondrial dysfunction and increased release of ROS and pro-apoptotic proteins accelerating sperm senescence in a self-perpetuating positive feedback

factors from the mitochondria and inducing mitochondrial malfunction resulting in additional release of mitochondrial reactive oxygen species (Johnston et al. 2012) in a self-perpetuating cascade of events finally resulting in sperm demise. Depending on the intrinsic antioxidant defences of the spermatozoa and on the presence or absence of pro-survival factors, this chain of events can occur more or less rapidly. This latter fact opens new strategies to improve current cryopreservation protocols.

Sex sorting

Selecting the sex of offspring may offer advantages to the equine industry as certain breeds have particular preferences for a specific gender, as for example stallions being preferentially used for dressage, while fillies are preferred for polo competition (Gibb et al. 2011). To date, the only repeatable method to select spermatozoa for chromosomal sex is the Beltsville sorting technology using flow cytometry (FC) (Johnson et al. 1999; Johnson 2000). This technology has reached commercial status in the bovine industry (Frijters et al. 2009), and substantial advances have occurred recently in swine (Garcia et al. 2007; Vazquez et al. 2009) and ovine (de Graaf et al. 2006; Leahy et al. 2010). Although foals were born from sexed semen already in 2000 (Buchanan et al. 2000), the technology lags behind for equine compared to other species. For sex sorting, spermatozoa that are highly diluted are exposed to a toxic dye, assigned an electrical charge and resuspended in various media, and spermatozoa experience mechanical forces during transit through the FC (Leahy et al. 2011). While the effects of sex sorting have been studied in terms of sperm functionality in bulls, boars and rams (Bathgate 2008; Vazquez et al. 2009; Leahy and Gadella 2011), similar studies in horses are scarce. Recently, attempts to understand the molecular and proteomic changes that sex sorting induces on spermatozoa have been performed in rams (Leahy et al. 2011) and boars (Spinaci et al. 2006). Recent studies in stallions have disclosed new aspects of the changes induced by the procedure, and the possible mitochondrial modifications occurring. Although contradictory reports exist, mitochondrial damage and oxidative damage have been described as consequence of the sorting procedure (Balao da Silva et al. 2013b, 2014; Gibb et al. 2013). Probably, these effects are due to increased hydrostatic pressure experienced by the spermatozoa during the procedure, and the removal of the seminal plasma also occurring during sex sorting. It is well known that equine seminal plasma is rich in antioxidants (Ball et al. 2000).

Sperm selection

In contrast to other domestic species, stallions have been selected largely for phenotype and performance, and selection for sperm quality has been largely ignored. As a result, large variability in sperm quality among stallions is common. In recent years, methods to select the best spermatozoa from an ejaculate have been developed and, nowadays, are routinely used in stallion andrology practice (Macias Garcia et al. 2009a,b; Morrell et al. 2011; Edmond et al. 2012). Most reports indicate that colloidal centrifugation improves sperm characteristics, although less attention has been paid to improvements in mitochondrial function. Reports in our laboratory indicate that in frozen–thawed samples, colloidal centrifugation can select a population of spermatozoa with high mitochondrial membrane potential (MMP) (Macias Garcia et al. 2009b). However, this effect was not present when fresh sperm subjected to osmotic stress were selected through colloidal centrifugation, and the MMP assessed after JC-1 staining (Macias-Garcia et al. 2012) was not improved. Yet in this study, SLC selected a subpopulation of spermatozoa producing higher amount of superoxide that, in view of recent reports (Gibb et al. 2014), may be

indicating that this subpopulation has more metabolically active mitochondria. However, a recent human study suggests that colloidal centrifugation media may induce oxidative stress due to the presence of transition metals (Aitken et al. 2014).

Are Mitochondria Markers of the Fertilizing Sperm Subpopulation?

Although all sperm may look morphologically similar to a casual observer, the heterogeneous nature of the mammalian ejaculate is now widely accepted, and only a small percentage of the whole population is considered to have fertilizing capacity (Holt and Van Look 2004). Mitochondrial function is commonly monitored using cationic fluorescent probes which accumulate in mitochondria depending on the transmembrane electrical gradient (mitochondrial membrane potential – MMP). Examples include rhodamine 123 (Rh123) and various MitoTracker probes (Molecular Probes, Eugene, OR, USA), which accumulate in polarized mitochondria and fluoresce at different wavelengths, depending on their nature. Other probe widely used is 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). This probe is known by its unique property of discriminating between high and low MMP. Although the molecule fluoresces green in its monomeric form, high MMP leads to the formation of aggregates, and fluorescence change reversibly from green to orange (Amaral et al. 2013). Recent independent studies (Gallon et al. 2006; Sousa et al. 2011) based on fluorescence-activated cell sorting to separate spermatozoa with active mitochondria identified using the probe MitoTracker Green FM proposed that the fertilizing subpopulation is made up of those spermatozoa showing better mitochondrial function, as this particular subpopulation contained spermatozoa better able to decondense and participate in the early events of development after chemical induction and injection into mature bovine oocytes (Sousa et al. 2011). All these findings stress the importance of mitochondrial function in stallion spermatozoa, opening new areas of research and challenging the paradigm of ROS negatively influencing sperm function.

Concluding remarks

Recent advances in the knowledge of mitochondrial function stress the need to adapt these data to the assessment of stallion sperm quality and be used in future developments of sperm conservation. Current knowledge indicates that mitochondrial assessment should be included in the spermiogram, for assessment of both breeding soundness and quality control of frozen–thawed samples. Available evidences warrant further investigation on mitochondrial pathophysiology in the stallion and also the development of strategies to diminish mitochondrial stress during cryopreservation, based on recent knowledge of mitochondrial function.

Acknowledgements

The authors received financial support for their studies from Ministerio de Economía y Competitividad-FEDER in Madrid, Spain (grant AGL2013-43211-R); Ministerio de Educación PRX14000/95; and Junta de Extremadura-FEDER (GR 10010 and PCE1002).

Author contribution

All authors contributed to the literature review and commented and drafted the final version.

Conflict of interest

None of the authors have conflict of interest to declare.

References

Agarwal A, Virk G, Ong C, du Plessis SS, 2014: Effect of oxidative stress on male reproduction. World J Mens Health **32**, 1–17.

Aitken RJ, Curry BJ, 2011: Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal **14**, 367–381.

Aitken RJ, Koppers AJ, 2011: Apoptosis and DNA damage in human spermatozoa. Asian J Androl **13**, 36–42.

Aitken RJ, De Iuliis GN, Gibb Z, Baker MA, 2012a: The Simmet lecture: new horizons on an old landscape–oxidative stress, DNA damage and apoptosis in the male germ line. Reprod Domest Anim **47** (Suppl 4), 7–14.

Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN, 2012b: Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. Biol Reprod **87**, 110–118.

Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA, 2012c: Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. J Biol Chem **287**, 33048–33060.

Aitken RJ, Finnie JM, Muscio L, Whiting S, Connaughton HS, Kuczera L, Rothkirch TB, De Iuliis GN, 2014: Potential importance of transition metals in the induction of DNA damage by sperm preparation media. Hum Reprod **29**, 2136–2147.

Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, Bownes M, 1997: 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol **7**, 776–789.

Amaral A, Lourenco B, Marques M, Ramalho-Santos J, 2013: Mitochondria functionality and sperm quality. Reproduction **146**, R163–R174.

Auger J, Leonce S, Jouannet P, Ronot X, 1993: Flow cytometric sorting of living, highly motile human spermatozoa based on evaluation of their mitochondrial activity. J Histochem Cytochem **41**, 1247–1251.

Balao da Silva CM, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolanos JM, Plaza Davila M, Morrell JM, Rodriguez Martinez H, Tapia JA, Aparicio IM, Pena FJ, 2013a: Sex sorting increases the permeability of the membrane of stallion spermatozoa. Anim Reprod Sci, **138**, 241–251.

Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Pena FJ, Mari G, Galeati G, 2013b: Effect of sex sorting on stallion spermatozoa: Heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci **141**, 68–74.

Balao da Silva C, Ortega Ferrusola C, Gallardo Bolanos J, Plaza Davila M, Martin-Munoz P, Morrell J, Rodriguez Martinez H, Pena F, 2014: Effect of overnight staining on the quality of flow cytometric sorted stallion sperm: comparison with tradtitional protocols. Reprod Domest Anim **49**, 1021–1027.

Ball BA, 2008: Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim Reprod Sci **107**, 257–267.

Ball BA, Gravance CG, Medina V, Baumber J, Liu IK, 2000: Catalase activity in equine semen. Am J Vet Res **61**, 1026–1030.

Barroso G, Taylor S, Morshedi M, Manzur F, Gavino F, Oehninger S, 2006: Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. Fertil Steril **85**, 149–154.

Bathgate R, 2008: Functional integrity of sex-sorted, frozen-thawed boar sperm and its potential for artificial insemination. Theriogenology **70**, 1234–1241.

Becker WMKL, Hardin J, 2003: The world of the cell Benjamin Cummings San Francisco.

Bolanos JM, Moran AM, da Silva CM, Davila MP, Munoz PM, Aparicio IM, Tapia JA, Ferrusola CO, Pena FJ, 2014: During cooled storage the extender influences processed autophagy marker light chain 3 (LC3B) of stallion spermatozoa. Anim Reprod Sci **145**, 40–46.

Bravo MM, Aparicio IM, Garcia-Herreros M, Gil MC, Pena FJ, Garcia-Marin LJ, 2005: Changes in tyrosine phosphorylation associated with true capacitation and capacitation-like state in boar spermatozoa. Mol Reprod Dev **71**, 88–96.

Bravo A, Treulen F, Uribe P, Boguen R, Felmer R, Villegas JV, 2014: Effect of mitochondrial calcium uniporter blocking on human spermatozoa. Andrologia doi:10.1111/and.12314 [Epub ahead of print].

Breitbart H, Levinshal T, Cohen N, Friedmann H, Lubart R, 1996: Changes in calcium transport in mammalian sperm mitochondria and plasma membrane irradiated at 633 nm (HeNe laser). J Photochem Photobiol, B **34**, 117–121.

Brum AM, Sabeur K, Ball BA, 2008: Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology **69**, 1041–1055.

Buchanan BR, Seidel GE Jr, McCue PM, Schenk JL, Herickhoff LA, Squires EL, 2000: Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenology, **53**, 1333–1344.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC, 1998: Regulation of cell death protease caspase-9 by phosphorylation. Science **282**, 1318–1321.

Caselles AB, Miro-Moran A, Morillo Rodriguez A, Gallardo Bolanos JM, Ortega-Ferrusola C, Salido GM, Pena FJ, Tapia JA, Aparicio IM, 2014: Identification of apoptotic bodies in equine semen. Reprod Domest Anim, **49**, 254–262.

Chipuk JE, Bouchier-Hayes L, Green DR, 2006: Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ **13**, 1396–1402.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S, 2009: Ca2+-stores in sperm: their identities and functions. Reproduction **138**, 425–437.

Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, Datta SR, Greenberg ME, Licklider LJ, Lowell BB, Gygi SP, Korsmeyer SJ, 2003: BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature **424**, 952–956.

Das SC, Isobe N, Yoshimura Y, 2011: Expression of Toll-like receptors and avian beta-defensins and their changes in response to bacterial components in chicken sperm. Poult Sci **90**, 417–425.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME, 1997: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91**, 231–241.

De Martino C, Floridi A, Marcante ML, Malorni W, Scorza Barcellona P, Bellocci M, Silvestrini B, 1979: Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. Cell Tissue Res **196**, 1–22.

Dramsi S, Scheid MP, Maiti A, Hojabrpour P, Chen X, Schubert K, Goodlett DR, Aebersold R, Duronio V, 2002: Identification of a novel phosphorylation site, Ser-170, as a regulator of bad pro-apoptotic activity. J Biol Chem **277**, 6399–6405.

Dudkina NV, Sunderhaus S, Boekema EJ, Braun HP, 2008: The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. J Bioenerg Biomembr **40**, 419–424.

Edmond AJ, Brinsko SP, Love CC, Blanchard TL, Teague SR, Varner DD, 2012: Effect of centrifugal fractionation protocols on quality and recovery rate of equine sperm. Theriogenology **77**, 959–966.

Ferramosca A, Focarelli R, Piomboni P, Coppola L, Zara V, 2008: Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency. Int J Androl **31**, 337–345.

Ford WC, 2006: Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? Hum Reprod Update **12**, 269–274.

Frijters AC, Mullaart E, Roelofs RM, van Hoorne RP, Moreno JF, Moreno O, Merton JS, 2009: What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology **71**, 64–67.

Fujita Y, Mihara T, Okazaki T, Shitanaka M, Kushino R, Ikeda C, Negishi H, Liu Z, Richards JS, Shimada M, 2011: Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. Hum Reprod **26**, 2799–2806.

Fuller SJ, Whittingham DG, 1997: Capacitation-like changes occur in mouse spermatozoa cooled to low temperatures. Mol Reprod Dev **46**, 318–324.

Gallardo Bolanos JM, Miro Moran A, Balao da Silva CM, Morillo Rodriguez A, Plaza Davila M, Aparicio IM, Tapia JA, Ortega Ferrusola C, Pena FJ, 2012: Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. PLoS One, **7**, e30688.

Gallardo Bolanos JM, Balao da Silva CM, Martin Munoz P, Morillo Rodriguez A, Plaza Davila M, Rodriguez-Martinez H, Aparicio IM, Tapia JA, Ortega Ferrusola C, Pena FJ, 2014: Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction, **148**, 221–235.

Gallon F, Marchetti C, Jouy N, Marchetti P, 2006: The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil Steril **86**, 1526–1530.

Garcia EM, Vazquez JM, Parrilla I, Calvete JJ, Sanz L, Caballero I, Roca J, Vazquez JL, Martinez EA, 2007: Improving the fertilizing ability of sex sorted boar spermatozoa. Theriogenology **68**, 771–778.

Garcia BM, Fernandez LG, Ferrusola CO, Salazar-Sandoval C, Rodriguez AM, Martinez HR, Tapia JA, Morcuende D, Pena FJ, 2011: Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. Reprod Domest Anim **46**, 141–148.

Garcia BM, Moran AM, Fernandez LG, Ferrusola CO, Rodriguez AM, Bolanos JM, da Silva CM, Martinez HR, Tapia JA, Pena FJ, 2012: The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. J Androl **33**, 105–113.

Gibb Z, Morris LH, Maxwell WM, Grupen CG, 2011: Use of a defined diluent increases the sex-sorting efficiency of stallion sperm. Theriogenology **75**, 610–619.

Gibb Z, Butler TJ, Morris LH, Maxwell WM, Grupen CG, 2013: Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted

stallion sperm. Theriogenology **79**, 1001–1009.

Gibb Z, Lambourne SR, Aitken RJ, 2014: The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod **91**, 77–87.

Gillan L, Evans G, Maxwell WM, 2005: Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology **63**, 445–457.

Gonzalez-Fernandez L, Ortega-Ferrusola C, Macias-Garcia B, Salido GM, Pena FJ, Tapia JA, 2009: Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. Biol Reprod **80**, 1239–1252.

Gonzalez-Fernandez L, Morrell JM, Pena FJ, Macias-Garcia B, 2012: Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology **78**, 415–422.

de Graaf SP, Evans G, Maxwell WM, O'Brien JK, 2006: In vitro characteristics of fresh and frozen-thawed ram spermatozoa after sex sorting and re-freezing. Reprod Fertil Dev **18**, 867–874.

Holt WV, Van Look KJ, 2004: Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction **127**, 527–535.

Johnson LA, 2000: Sexing mammalian sperm for production of offspring: the state-of-the-art. Anim Reprod Sci **60–61**, 93–107.

Johnson LA, Welch GR, Rens W, 1999: The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci **77**(Suppl 2), 213–220.

Johnston SD, Satake N, Zee Y, Lopez-Fernandez C, Holt WV, Gosalvez J, 2012: Osmotic stress and cryoinjury of koala sperm: an integrative study of the plasma membrane, chromatin stability and mitochondrial function. Reproduction **143**, 787–797.

Klingenberg M, 2008: The ADP and ATP transport in mitochondria and its carrier. Biochim Biophys Acta **1778**, 1978–2021.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ, 2008: Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab **93**, 3199–3207.

Koppers AJ, Garg ML, Aitken RJ, 2010: Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med **48**, 112–119.

Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ, 2011: Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. Biochem J **436**, 687–698.

de Lamirande E, Gagnon C, 1993: Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. Fertil Steril **59**, 1291–1295.

de Lamirande E, Gagnon C, 2002: The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod **8**, 124–135.

de Lamirande E, Gagnon C, 2003: Redox control of changes in protein sulfhydryl levels during human sperm capacitation. Free Radic Biol Med **35**, 1271–1285.

de Lamirande E, Lamothe G, 2009: Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. Free Radic Biol Med **46**, 502–510.

de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C, 1997: Reactive oxygen species and sperm physiology. Rev Reprod **2**, 48–54.

Leahy T, Gadella BM, 2011: Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction **142**, 759–778.

Leahy T, Marti JI, Mendoza N, Perez-Pe R, Muino-Blanco T, Cebrian-Perez JA, Evans G, Maxwell WM, 2010: High pre-freezing dilution improves post-thaw function of ram spermatozoa. Anim Reprod Sci **119**, 137–146.

Leahy T, Marti JI, Crossett B, Evan G, Maxwell WM, 2011: Two-dimensional polyacrylamide gel electrophoresis of membrane proteins from flow cytometrically sorted ram sperm. Theriogenology **75**, 962–971.

Lizcano JM, Morrice N, Cohen P, 2000: Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. Biochem J **349**, 547–557.

Luo SM, Schatten H, Sun QY, 2013: Sperm mitochondria in reproduction: good or bad and where do they go? J Genet Genomics **40**, 549–556.

Macias Garcia B, Gonzalez Fernandez L, Morrell JM, Ortega Ferrusola C, Tapia JA, Rodriguez Martinez H, Pena FJ, 2009a: Single-layer centrifugation through colloid positively modifies the sperm subpopulation structure of frozen-thawed stallion spermatozoa. Reprod Domest Anim **44**, 523–526.

Macias Garcia B, Morrell JM, Ortega-Ferrusola C, Gonzalez-Fernandez L, Tapia JA, Rodriguez-Martinez H, Pena FJ, 2009b: Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim Reprod Sci **114**, 193–202.

Macias Garcia B, Ortega Ferrusola C, Aparicio IM, Miro-Moran A, Morillo Rodriguez A, Gallardo Bolanos JM, Gonzalez Fernandez L, Balao da Silva CM, Rodriguez Martinez H, Tapia JA, Pena FJ, 2012: Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology, **77**, 1280–1289.

Macias-Garcia B, Gonzalez-Fernandez L, Gallardo-Bolanos JM, Pena FJ, Johannisson A, Morrell JM, 2012: Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS. Anim Reprod Sci **132**, 74–82.

Martin G, Sabido O, Durand P, Levy R, 2004: Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod **71**, 28–37.

Mazur P, Koshimoto C, 2002: Is intracellular ice formation the cause of death of mouse sperm frozen at high cooling rates? Biol Reprod **66**, 1485–1490.

Mendoza N, Casao A, Perez-Pe R, Cebrian-Perez JA, Muino-Blanco T, 2013: New insights into the mechanisms of ram sperm protection by seminal plasma proteins. Biol Reprod **88**, 149–159.

Mitchell P, 1967: Proton current flow in mitochondrial systems. Nature **214**, 1327–1328.

Mizrahi R, Breitbart H, 2014: Mitochondrial PKA mediates sperm motility. Biochim Biophys Acta **1840**, 3402–3412.

Morrell JM, Garcia BM, Pena FJ, Johannisson A, 2011: Processing stored stallion semen doses by Single Layer Centrifugation. Theriogenology **76**, 1424–1432.

Olson GE, Winfrey VP, 1990: Mitochondria-cytoskeleton interactions in the sperm midpiece. J Struct Biol **103**, 13–22.

Olson GE, Winfrey VP, 1992: Structural organization of surface domains of sperm mitochondria. Mol Reprod Dev **33**, 89–98.

Ortega Ferrusola C, Gonzalez Fernandez L, Macias Garcia B, Salazar-Sandoval C, Morillo Rodriguez A, Rodriguez Martinez H, Tapia JA, Pena FJ, 2009a: Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol Reprod **81**, 1106–1111.

Ortega Ferrusola C, Gonzalez Fernandez L, Morrell JM, Salazar Sandoval C, Macias Garcia B, Rodriguez-Martinez H, Tapia JA, Pena FJ, 2009b: Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction **138**, 55–63.

Ortega Ferrusola C, Gonzalez Fernandez L, Salazar Sandoval C, Macias Garcia B, Rodriguez Martinez H, Tapia JA, Pena FJ, 2010: Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. Theriogenology **74**, 458–465.

Ortega-Ferrusola C, Sotillo-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, Gonzalez-Fernandez L, Tapia JA, Pena FJ, 2008: Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl **29**, 213–221.

Ortega-Ferrusola C, Garcia BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, Tapia JA, Pena

FJ, 2009: Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim Reprod Sci **114**, 393–403.

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

Peña FJ, Garcia BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO, 2011: Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology **76**, 1177–1186.

Petyim S, Neungton C, Thanaboonyawat I, Laokirkkiat P, Choavaratana R, 2014: Sperm preparation before freezing improves sperm motility and reduces apoptosis in post-freezing-thawing sperm compared with post-thawing sperm preparation. J Assist Reprod Genet **31**, 1673–1680.

Pommer AC, Rutllant J, Meyers SA, 2002: The role of osmotic resistance on equine spermatozoal function. Theriogenology **58**, 1373–1384.

Prince FP, Buttle KF, 2004: Mitochondrial structure in steroid-producing cells: three-dimensional reconstruction of human Leydig cell mitochondria by electron microscopic tomography. Anat Rec A Discov Mol Cell Evol Biol **278**, 454–461.

Pujianto DA, Curry BJ, Aitken RJ, 2010: Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. Endocrinology **151**, 1269–1279.

Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP, Amaral A, 2009: Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. Hum Reprod Update **15**, 553–572.

Roca J, Martinez-Alborcia MJ, Gil MA, Parrilla I, Martinez EA, 2013: Dead spermatozoa in raw semen samples impair in vitro fertilization outcomes of frozen-thawed spermatozoa. Fertil Steril **100**, 875–881.

Samper JC, Peña FJ, Plough TA, 2012: Commercial breeding with sexed stallion sperm: reality of fiction? J Equine Vet Sci **32**, 471–474.

Schlame M, Ren M, Xu Y, Greenberg ML, Haller I, 2005: Molecular symmetry in mitochondrial cardiolipins. Chem Phys Lipids **138**, 38–49.

da Silva CM, Macias-Garcia B, Miro-Moran A, Gonzalez-Fernandez L, Morillo-Rodriguez A, Ortega-Ferrusola C, Gallardo-Bolanos JM, Stilwell G, Tapia JA, Pena FJ, 2011: Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. J Pineal Res **51**, 172–179.

Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS, Aitken RJ, 2013: The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. J Cell Sci **126**, 1488–1497.

Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrin P, Freitas A, Paiva A, Almeida-Santos T, Ramalho-Santos J, 2011: Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. PLoS One **6**, e18112.

Spinaci M, Volpe S, Bernardini C, de Ambrogi M, Tamanini C, Seren E, Galeati G, 2006: Sperm sorting procedure induces a redistribution of Hsp70 but not Hsp60 and Hsp90 in boar spermatozoa. J Androl **27**, 899–907.

Stowe DF, Camara AK, 2009: Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. Antioxid Redox Signal **11**, 1373–1414.

Thompson WE, Ramalho-Santos J, Sutovsky P, 2003: Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. Biol Reprod **69**, 254–260.

Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, Flohe L, 1999: Dual function of the selenoprotein PHGPx during sperm maturation. Science **285**, 1393–1396.

Vadnais ML, Lin AM, Gerton GL, 2014: Mitochondrial fusion protein MFN2 interacts with the mitostatin-related protein MNS1 required for mouse sperm flagellar structure and function. Cilia **3**, 5–25.

Vazquez JM, Parrilla I, Roca J, Gil MA, Cuello C, Vazquez JL, Martinez EA, 2009: Sex-sorting sperm by flow cytometry in pigs: issues and perspectives. Theriogenology **71**, 80–88.

Voet DJG, Voet JG, Pratt CW, 2006: Fundamentals of Biochemistry, 2nd edn. John Wiley and Sons, New York.

Willoughby CE, Mazur P, Peter AT, Critser JK, 1996: Osmotic tolerance limits and properties of murine spermatozoa. Biol Reprod **55**, 715–727.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ, 1995: Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell **80**, 285–291.

Yeste M, Estrada E, Rocha LG, Marin H, Rodriguez-Gil JE, Miro J, 2015: Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. Andrology **3**, 395–407.

Yu F, Sugawara T, Maier CM, Hsieh LB, Chan PH, 2005: Akt/Bad signaling and motor neuron survival after spinal cord injury. Neurobiol Dis **20**, 491–499.

Zini A, De Lamirande E, Gagnon C, 1995: Low levels of nitric oxide promote human sperm capacitation in vitro. J Androl **16**, 424–431.

Submitted: 15 Feb 2015; Accepted: 9 May 2015

Author's address (for correspondence): Dr. FJ Peña, Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine, University of Extremadura Adv. de la Universidad s/n 10003 Cáceres, Spain.
E-mail: fjuanpvega@unex.es

Discusión

DISCUSIÓN

En nuestro primer trabajo ***"Inhibition of mitocondrial complex I leads to decreased motility and membrane integrity to increased hydrogen peroxide, and the inhibition of glycolysis reduces sperm velocities in stallion spermatozoa"*** estudiamos los efectos de interrumpir el flujo de electrones en dos puntos específicos (complejo I y complejo III). Su inhibición provocó la disminución de las motilidades (total y progresiva) y velocidades de los espermatozoides ligada a la reducción en la producción de ATP y del potencial de membrana mitocondrial.

Con respecto a la producción de ERO_S, se observaron efectos interesantes. En la inhibición de los complejos I y III se redujo dicha producción como se vio en el experimento usando CellRox® Deep Red para medir el estrés oxidativo. Este hallazgo parece una paradoja, ya que dichos complejos son reconocidos en la bibliografía como las principales fuentes de producción de ERO_S, tanto en células somáticas (Genova et al., 2003) como en espermatozoides (Koppers et al., 2008). Sin embargo, nuestro hallazgo se puede explicar por el hecho de que esta sonda es específicamente sensible al anión superóxido ($O_2^{\bullet}$). Según el fabricante de CellRox® Deep Red, la sonda reconoce sólo el anión $O_2^{\bullet}$ y el radical hidroxilo ($HO^{\bullet}$). Debido a que es poco probable la presencia significativa de cationes divalentes en nuestro modelo, la explicación de nuestros hallazgos, es que una vez se interrumpe la cadena de transporte de electrones, la

mitocondria colapsa y cesa la pérdida de electrones, y por lo tanto la producción del anión $O_2^{\bullet}$.

Al aplicar la sonda hidroetidina detectamos un aumento de la producción de $O_2^{\bullet}$ sólo en las muestras tratadas con antimicina-A 10 µM después de una hora de incubación, La discrepancia entre CellRox® Deep Red e hidroetidina puede explicarse por el hecho de que CellRox® Deep Red se utilizó para detectar ERO_S sólo en células vivas, mientras que la hidroetidina fue utilizada en toda la población. Por consiguiente, el aumento observado se explica porque, en este último caso, el $O_2^{\bullet}$ fue producido por las células muertas. Esta teoría también se apoya en el aumento en el número de células muertas tras el tratamiento con antimicina-A, y por la correlación positiva observada entre la motilidad de la muestra, la integridad de membrana y los espermatozoides CellRox® Deep Red -positivos. También apoya nuestra hipótesis el rápido colapso del potencial de membrana mitocondrial observado después de la inhibición de ambos complejos, lo que refuerza además la hipótesis de que la interrupción de la CMTE reduce la fuga de electrones.

Por otro lado, estudios anteriores han demostrado que los inhibidores mitocondriales pueden aumentar o disminuir la producción de ERO_S (Xi et al., 2005). En las células somáticas, una pequeña despolarización mitocondrial conduce a un aumento de la generación de ERO_S, mientras que una despolarización más intensa reduce dicha producción. Esto tiene sentido ya que sabemos que del 1 al 2 % del oxígeno utilizado por la CMTE no se reduce completamente, sino que da lugar al anión $O_2^{\bullet}$ (Turrens, 2003).

Nuestros resultados junto a trabajos recientes de otros grupos indican que la elevada producción de dicho anión puede ser un indicador de espermatozoides con una intensa actividad mitocondrial (Gibb et al., 2014), y no necesariamente de espermatozoides sufriendo un intenso estrés oxidativo. Una prueba más a favor de esta hipótesis es la reducción en la producción de ATP, que acompaña al descenso en los espermatozoides CellRox® Deep Red-positivos, inducida por ambos inhibidores, y las correlaciones positivas encontradas entre los espermatozoides MitoSOX-positivos (con mayor producción de $O_2^{\bullet}$ mitocondrial) y sus motilidades y velocidades. Para investigar más en profundidad la presencia de ERO_S mitocondrial, se usó una sonda específica en la producción de $O_2^{\bullet}$ (MitoSOX). La inhibición del complejo I dio lugar a un aumento en el $O_2^{\bullet}$ mitocondrial después de 6 horas de incubación, acompañado por incremento paralelo en H_2O_2. La rotenona induce generación de H_2O_2 mitocondrial en células somáticas (Genova et al., 2003), y en nuestro estudio también fue evidente en el espermatozoide de caballo.

La antimicina-A conduce a la generación del radical semiquinona (Sun and Trumpower, 2003), que luego se estabiliza cediendo sus electrones al oxígeno para formar $O_2^{\bullet}$ en el espacio intermembrana. Posteriormente el $O_2^{\bullet}$dismuta a H_2O_2 mediante la superoxido dismutasa y se escapa al exterior de la célula. No hemos podido detectar aumentos significativos de H_2O_2, incluso después de largos periodos de incubación. Esto podría ser debido a la reducida producción de $O_2^{\bullet}$ provocada por la acción de la antimicina-A.

Aunque estudios anteriores parecen indicar que los espermatozoides de caballo son muy dependientes del ATP generado en la mitocondria (Ortega Ferrusola et al., 2010; Gibb et al., 2014), el papel de la glucólisis aún no ha sido estudiado en profundidad. Por esta razón, quisimos determinar el papel de la glucólisis como fuente de energía para la motilidad. Para ello utilizamos la 2-deoxiglucosa (2-DG), un inhibidor específico de dicha vía metabólica. Al bloquear la glucólisis, las velocidades disminuyeron inmediatamente, pero no hubo cambios en el porcentaje de espermatozoides motiles progresivos. Además, para reforzar nuestros hallazgos, también incubamos los espermatozoides en dos medios diferentes; uno desprovisto completamente de glucosa y otro sin glucosa y sin piruvato. Observamos una reducción de la motilidad total y de la VCL después de la incubación durante 3 horas en el medio sin glucosa y sin piruvato. Con respecto a la cantidad de ATP no se observaron cambios. Nuestros resultados sugieren que la fosforilación oxidativa es la principal fuente de energía para la motilidad del espermatozoide equino como se había sugerido previamente (Ortega Ferrusola et al., 2010; Gibb et al., 2014). En resumen, podemos concluir diciendo que la fosforilación oxidativa es la principal fuente de energía en el espermatozoide equino, para mantener las motilidades, pero también necesitan la glucólisis para mantener las velocidades. La inhibición del complejo I de la CMTE conduce a la reducción de la motilidad debido principalmente a la disminución en la producción de ATP, y probablemente también a una mayor producción de H_2O_2 sobre todo tras largos periodos de incubación. Un aspecto aplicativo inmediato de nuestros resultados indican que la producción de ERO_S

debe ser valorada correctamente en el eyaculado equino, al no ser necesariamente un reflejo de estrés oxidativo

Como hemos comentado anteriormente, la producción de ERO_S influye en el proceso de capacitación, por lo tanto, nos planteamos que sería interesante investigar cómo respondería dicha célula en un ensayo de unión heterólogo.

En el artículo ***"Epigallocatechin-3-gallate (EGCG) reduces rotenone effect on stallion sperm zona pellucida heterologous binding"*** el objetivo de nuestro estudio fue evaluar la respuesta de los espermatozoides equinos después de la inhibición del complejo I de la CMTE y además probar si el EGCG, un componente polifenol natural del té verde, podría contrarrestar el efecto de la rotenona.

La evaluación de la viabilidad de los espermatozoides de caballo, la integridad del acrosoma y la actividad mitocondrial no evidenciaron diferencias significativas en todas las concentraciones de rotenona aplicadas (100 nM, 500 nM, 5 µM). La ausencia de diferencias significativas en el porcentaje de espermatozoides viables está de acuerdo con los datos obtenidos previamente (Gibb et al., 2014; Plaza Davila et al., 2015), donde se demuestra que la viabilidad de las muestras tratadas eran similares a los controles incluso utilizando la concentración más alta de rotenona (10 µM) durante una hora. Sólo después de 3 horas de incubación, la rotenona a una concentración 10 µM indujo diferencias significativas en el porcentaje de espermatozoides intactos (Plaza Davila et al., 2015); por el contrario nosotros no observamos una disminución significativa en el porcentaje de espermatozoides vivos con un alto

potencial de membrana mitocondrial, probablemente debido a que en este experimento usamos microscopía de fluorescencia en vez de citometría de flujo. Además, nos planteamos la posibilidad de añadir un antioxidante (EGCG) tanto a las muestras tratadas con las diferentes concentraciones de rotenona, como a las no tratadas.

Para evaluar el efecto de la rotenona y del EGCG sobre la función in vitro de los espermatozoides de caballo, realizamos un ensayo de unión heterólogo mediante co-incubación con ovocitos de cerda. Debido a la baja disponibilidad de ovocitos equinos, en nuestro estudio realizamos el ensayo de unión heterólogo ya que otros estudios han demostrado la fiabilidad de ésta prueba (Sinowatz et al., 2003; Clulow et al., 2010; Balao da Silva et al., 2013). Se ha demostrado que los ensayos de unión homólogo ofrecen una buena fiabilidad en la predicción de la fertilidad del caballo (Fazeli et al., 1993; Fazeli et al., 1995; Meyers et al., 1996).

Los resultados obtenidos en este trabajo demuestran por primera vez que la inhibición con rotenona del complejo I de la CMTE del espermatozoide equino ejerce un efecto negativo en la capacidad de unión al ovocito. De hecho, de todas las concentraciones ensayadas, 100 nM, 500 nM y 5 µM disminuyeron significativamente el número de espermatozoides unidos por ovocito con respecto al grupo control.

Cuando los espermatozoides se incubaron en condiciones de capacitación en presencia de EGCG (10 µM y 20 µM), la unión al ovocito aumentó con respecto al control. La influencia positiva de la adicción de EGCG tanto en

espermatozoides frescos como congelados, ya fue demostrada en otros estudios en cerdo (Spinaci et al., 2008; Kaedei et al., 2012) sugiriendo una acción moduladora. Este efecto podría deberse a la acción antioxidante del EGCG en la producción excesiva de ERO_S cuando la capacidad antioxidante de la célula está superada por el estrés oxidativo (Aitken et al., 2015b).

La concentración 10 µM de EGCG redujo el efecto negativo de 100 nM de rotenona sobre el binding. Las dosis más altas de EGCG (20 µM y 60 µM) no fueron capaces de revertir completamente el efecto inhibidor de la rotenona a 100 nM, pero sí aumentaron el número de espermatozoides unidos al ovocito con respecto al grupo control. Sin embargo, EGCG no fue capaz de reducir el efecto negativo de las concentraciones más altas de rotenona (500 nM y 5 µM) en el ensayo de unión heterólogo.

Se ha demostrado que la rotenona inhibe la fosforilación oxidativa y disminuye la producción de ATP en los espermatozoides equinos reduciendo la motilidad (Plaza Davila et al., 2015). Se podría plantear que el EGCG, contrarrestando la caída de ATP inducida por la rotenona, conseguiría garantizar el suministro de energía necesario para dicha función fisiológica del espermatozoide (Ferramosca and Zara, 2014).

Para avanzar en el estudio de la funcionalidad de la mitocondria nos planteamos en el tercer artículo ***"Mitochondrial ATP is required for maintenance of membrane integrity in the stallion spermatozoa, while motility requires both glycolysis and oxidative phosphorylation"***

evaluar el efecto del desacoplamiento de la CMTE y la inhibición respiración mitocondrial del espermatozoide equino.

La exposición de los espermatozoides al carbonylcyanide-m-chlorophenylhydrazone (CCCP) redujo su motilidad y aumentó los marcados con YOPRO-1, sin disminución de la integridad de membrana. El uso de 2,4-dinitrofenol (2,4-DNP) también dio lugar a una disminución de la motilidad, no tuvo efecto sobre la integridad de membrana, y a diferencia del CCCP, el 2,4-DNP no fue capaz de disminuir el potencial de membrana mitocondrial. La inhibición del complejo IV de la CMTE por parte de NaCN dio lugar a un marcado descenso de la motilidad y especialmente en las velocidades. Sin embargo, la integridad de membrana se vio afectada sólo a 10 mM. La inhibición de la subunidad F_0 de la ATP-sintasa por la oligomicina-A redujo significativamente tanto las motilidades como las velocidades de los espermatozoides. Además produjo un gran colapso en la integridad de la membrana mitocondrial. Estos resultados indican que el ATP mitocondrial es esencial para la funcionalidad espermática, como sugieren otros estudios (Ortega Ferrusola et al., 2010; Gibb et al., 2014). Sin embargo, se describe por primera vez la importancia de la actividad de la ATP-sintasa mitocondrial para el mantenimiento de las membranas de los espermatozoides.

Aunque la respiración mitocondrial es superior a la glucólisis con respecto a la eficiencia de síntesis de ATP, los espermatozoides de la mayoría de especies de mamíferos parecen basarse en la glucólisis para producir la energía necesaria para la motilidad y velocidad (Mukai and Okuno, 2004). Experimentos

realizados con espermatozoides de ratón mostraron que el tratamiento con CCCP no afectó a la motilidad cuando la glucosa estaba presente en los medios de incubación. Esto se contrapone con nuestros hallazgos en espermatozoides de caballo, donde el desacoplamiento de las mitocondrias por parte del CCCP y el 2,4-DNP, la inhibición del complejo IV por el NaCN y de la ATP-sintasa por la oligomicina-A disminuyeron las motilidades y velocidades a pesar de la presencia de glucosa.

Por lo tanto, el desacoplamiento de la CMTE o la inhibición de la respiración mitocondrial y la consecuente disminución en la producción de ATP muestran que, como habíamos subrayado anteriormente, la motilidad del espermatozoide de caballo es muy dependiente de la generación aeróbica de ATP (Gibb et al., 2014). Mientras que en otras especies se ha demostrado que es más dependiente de la glucólisis anaerobia (Storey, 2008).

En nuestros resultados, aunque la cantidad de ATP estaba reducida no desaparecía, con la posible excepción de la inhibición de la ATP-sintasa, cuando se produce una reducción del 70% en el contenido de ATP. Además, la oligomicina-A fue capaz de abolir la integridad de membrana y reducir drásticamente la motilidad de los espermatozoides después de 3 horas de incubación, no observándose un efecto tan dramático en las velocidades. Una posible explicación podría ser que existen otras fuentes complementarias de ATP en el espermatozoide de caballo. Para comprobar esta hipótesis se inhibió la glucólisis con 2-deoxiglucosa (2-DG). La inhibición de la vía glucolítica redujo el contenido de ATP, pero sin efecto en la integridad de membrana. También

10 mM de 2-DG redujo el porcentaje de espermatozoides motiles totales y las velocidades, sin tener efecto sobre los espermatozoides motiles progresivos. Estos hallazgos sugieren que el ATP generado en la glucólisis también tiene un papel en el suministro de energía para la motilidad. Por otro lado, la relación entre la producción de ERO_S y la función espermática es poco conocida en el caballo (Varner et al., 2015), por ello creímos importante investigarla. Aunque la interrupción de la función mitocondrial del espermatozoide equino conduce a la reducción de la motilidad, debido a la menor producción de ATP (Gibb et al., 2014); pero podría ser debido también al incremento en la producción de ERO_S (Koppers et al., 2008). Para determinar si el efecto era más probable por la disminución de ATP o por el aumento de ERO_S, se midieron diferentes parámetros simultáneamente. El desacoplamiento de la CMTE y la inhibición de la respiración mitocondrial redujeron el contenido de ATP, siendo el efecto particularmente marcado como consecuencia de la acción de la oligomicina-A,. Al mismo tiempo, la inhibición de la ATP-sintasa indujo un aumento muy significativo de la producción de ERO_S en los espermatozoides muertos. La importancia de la generación de dichas moléculas por espermatozoides muertos también fue observada recientemente (Aitken et al., 2015a). La inhibición del complejo IV redujo notablemente la motilidad, las velocidades y la producción de ERO_S, pero no tuvo ningún efecto sobre el potencial de membrana mitocondrial. Sólo la concentración de 10 mM redujo la integridad de membrana. Estos hallazgos indican que la pérdida de la homeostasis redox en el espermatozoide pueden tener que ver con el exceso de ERO_S producidos por los espermatozoides muertos.

Además, como hemos mencionado, los inhibidores mitocondriales pueden aumentar o disminuir la producción de ERO_S en función de la dosis empleada (Xi et al., 2005). De acuerdo con esa observación, en nuestro estudio, 100 nM de CCCP aumentó la concentración de ERO_S después de una hora de incubación, y redujo el porcentaje de espermatozoides motiles progresivos después de 3 horas de incubación. Este último efecto podría atribuirse a las proteínas oxidantes de las ERO_S implicadas en la regulación de la motilidad. Muchos aminoácidos pueden ser modificados por la oxidación, aunque sus sensibilidades varían (Bourdon and Blache, 2001). La oxidación directa está mediada principalmente por $O_2^{\bullet}$ y oxido nítrico (NO). Entre los aminoácidos, los que contienen azufre, como la metionina o la cisteína, son las principales dianas.

Muchos de los resultados de nuestra tesis son difíciles de integrar con la suposición general de que las ERO_S son perjudiciales para la función espermática del caballo. El hecho de que el aumento de la producción de ERO_S no se asoció inicialmente con una función espermática alterada puede indicar, como se ha sugerido anteriormente, que dicho aumento no es necesariamente perjudicial, y en su lugar puede reflejar la presencia de espermatozoides activos (Gibb et al., 2014). Sólo cuando la exposición a ERO_S continua durante un periodo largo de tiempo, son evidentes los efectos deletéreos, como la bajada significativa de la motilidad.

Recientemente, (Macias-Garcia et al., 2012) describieron el aumento de la producción de ERO_S en los espermatozoides seleccionados después de

someterlos a centrifugación en coloide. Esta técnica selecciona los espermatozoides mejores, sugiriendo además, que ese aumento en la producción de ERO_S podría reflejar que dichos espermatozoides están metabólicamente activos mediante la fosforilación oxidativa. Alternativamente, el efecto observado por (Macias-Garcia et al., 2012) puede hacer referencia al estrés oxidativo provocado por la centrifugación en coloide, ya que recientemente se ha descrito que los medios de centrifugación en coloide pueden ser una fuente de daño oxidativo debido a la presencia de metales de transición (Aitken et al., 2015a).

Por otro lado, nosotros observamos que desacoplar las mitocondrias con CCCP conducía a un aumento de espermatozoides YOPRO-1 positivos, y la inhibición de la ATP-sintasa con oligomicina-A reducía a 0 el porcentaje de espermatozoides con membrana intactas. En el primer caso (efecto del CCCP), se producía una caída de ATP inducida por el desacoplamiento de la CMTE resultando en un bombeo insuficiente de YOPRO-1, ya que este transporte depende del ATP. De hecho, parece ser que YOPRO-1 es más sensible a la caída en la producción de ATP que otras sondas (Tsujimoto, 1997; Chekeni et al., 2010; Gallardo Bolanos et al., 2014). También el aumento significativo en la producción de ERO_S puede haber contribuido a la reducción de la integridad de membrana. Todos estos hallazgos subrayan la importancia de la función mitocondrial en el espermatozoide equino, la apertura a nuevas áreas de investigación, y desafiar el paradigma de la influencia negativa de ERO_S sobre la función espermática. En resumen, este estudio proporciona nuevas pruebas que indican que los espermatozoides de caballo, en contraste con otros mamíferos,

dependen principalmente de la fosforilación oxidativa como fuente principal de ATP para el mantenimiento de la integridad de membrana, mientras que la energía para la motilidad, aunque principalmente es de origen mitocondrial, también se apoya en la glucólisis. Esta gestión de la producción de energía por parte del espermatozoide equino puede tener implicaciones importantes para la evaluación de la calidad del semen y del desarrollo de métodos para la conservación del esperma, como por ejemplo, la criopreservación.

La revisión final ***"The impact of reproductive technologies on stallion mitocondrial function"*** incluida en la presente Tesis Doctoral se creyó interesante debido a la variabilidad, complejidad y desconocimiento en ciertos aspectos de la bibliografía existente.

Conclusiones / Conclusions

CONCLUSIONES

- La fosforilación oxidativa es la principal fuente de energía para la motilidad y sobre todo para el mantenimiento de la integridad de membrana en espermatozoide equino, aunque la glucólisis es necesaria para la velocidad espermática.

- La inhibición del complejo I disminuye la capacidad de unión del espermatozoide equino a ovocitos heterólogos, pudiéndose contrarrestar dicho efecto con antioxidantes como el EGCG.

- El aumento en la generación de ERO_S (especialmente el $O_2^{\bullet}$) no es necesariamente un síntoma de daños en el espermatozoide equino, siendo en muchos casos un reflejo de la presencia de espermatozoides activos con una intensa actividad mitocondrial.

CONCLUSIONS

- Oxidative phosphorylation (OXPHOS) is the main energy source for motility and maintenance of membrane integrity in equine sperm, although glycolysis is also necessary to maintain sperm velocity.

- The inhibition of complex I result in a decreased ZP binding ability of stallion spermatozoa and the presence of EGCG is able to reduce this effect.

- The increased ROS production (especially $O_2^{\bullet}$) is not necessarily detrimental to equine sperm and may instead reflect an active sperm metabolism.

Resumen / Summary / Riassunto

RESUMEN

Las técnicas de reproducción asistida en medicina veterinaria están avanzando mucho en los últimos años. En la especie equina, estas técnicas tienen cada vez más importancia, ya que permiten almacenar y conservar el material genético de individuos muy valiosos, consiguiendo también maximizar su progenie. Sin embargo, estos individuos son a menudo considerados valiosos en base a criterios morfológicos o deportivos y no por su fertilidad o calidad reproductiva. Por todo ello, los gametos de este grupo de animales presentan una gran heterogeneidad en su respuesta frente a los diferentes procesos de manipulación y conservación habituales en las técnicas de reproducción asistida, como por ejemplo en la criopreservación.

La evaluación tradicional del semen equino comprende tanto el análisis de la motilidad y morfoanomalías espermáticas, como la integridad de membrana. La implantación progresiva de la citometría de flujo en el análisis seminal equino está determinando en la actualidad un aumento de las pruebas analíticas disponibles, permitiendo la evaluación de más parámetros seminales. Sin embargo, dentro de esta revolución, el estudio de la mitocondria en los espermatozoides equinos ha recibido una menor atención en comparación con otras estructuras y funciones en la andrología del semental.

Las mitocondrias son orgánulos celulares con múltiples funciones en el espermatozoide, entre las más importantes destacan el aporte de energía, el

mantenimiento de la integridad de membrana, la producción de especies reactivas de oxígeno (ERO_S) y la regulación de la muerte celular. Las mitocondrias han sido propuestas como la principal fuente de ERO_S en células somáticas y espermatozoides humanos. Sin embargo, no existen datos publicados sobre la producción de ERO_S mitocondrial en espermatozoides equinos.

En la presente Tesis Doctoral se estudió el papel de la cadena mitocondrial de transporte de electrones como origen del estrés oxidativo y de la senescencia del espermatozoide equino. Para ello utilizamos una amplia gama de inhibidores específicos. La rotenona como inhibidora del complejo I, la antimicina-A como inhibidora del complejo III, el NaCN como inhibidor del complejo IV y la oligomicina-A como inhibidora de la ATP-sintasa, además también fueron empleados dos desacopladores del gradiente de protones mitocondrial (CCCP y 2,4-Dinitrofenol).

Las consecuencias principales de la aplicación de los inhibidores y de los desacopladores fueron la disminución de las motilidades y velocidades de los espermatozoides, la reducción en la producción de ATP y la reducción del potencial de membrana (excepto el 2,4-DNP y NaCN). La inhibición de la ATP-sintasa produjo un colapso en la integridad de la membrana mitocondrial, lo que sugiere por primera vez su importancia para el mantenimiento de las membranas en los espermatozoides equinos.

La inhibición del complejo I también ejerció un efecto negativo en la capacidad de unión del espermatozoide a ovocitos heterólogos. Para comprobar si la

adicción de antioxidantes podría contrarrestar el efecto negativo de la inhibición del complejo I añadimos el flavonoide EGCG y comprobamos que contrarrestaba las dosis más bajas de rotenona y mejoraba los controles cuando se comparaba con muestras no tratadas con el inhibidor. Ello sugiere que el EGCG podría tener efectos positivos para la elaboración de medios de conservación del semen.

Los resultados obtenidos por la acción de todos los inhibidores y del CCCP utilizados en nuestro estudio produjeron un aumento en la producción de ERO_S en los espermatozoides muertos, y una disminución en la población de espermatozoides vivos.

La inhibición de los complejos I y III, produjo además un colapso en el potencial de membrana mitocondrial que dio lugar a disminución de ERO_S debido a que se redujo la fuga de electrones. La inhibición del complejo I indujo además la generación de H_2O_2 con el tiempo. Sin embargo, este fenómeno no se detectó con la inhibición del complejo III.

Para comprobar si la fosforilación oxidativa es la única vía metabólica de producción de energía para la motilidad del espermatozoide equino inhibimos la glucólisis con 2-deoxiglucosa. Además, para reforzar nuestros hallazgos, también incubamos los espermatozoides en dos medios diferentes; uno sin glucosa y otro sin glucosa y sin piruvato. Nuestros resultados demostraron que la fosoforilación oxidativa es la principal fuente de energía del espermatozoide equino, y también mostraron por primera vez, que la glucólisis es además necesaria para el mantenimiento de las velocidades espermáticas.

SUMMARY

Veterinary assisted reproduction techniques are developing very fast in recent years. In the equine industry these techniques are of utmost importance, because they allow breeders to store and preserve the genetic material of the most valuable individuals, and maximizing their offspring production at the same time. These individuals are commonly considered of great value based morphology and sportive performance criteria; those criteria are not related with their fertility or reproductive quality. The gametes of these groups of valuable selected animals show a high heterogeneity on their response to the different manipulation and conservation processes commonly used in assisted reproduction.

The traditional assessment of stallion sperm comprises the evaluation of sperm motility and membrane integrity and identification of abnormal morphology of the spermatozoa. More recently, the progressive introduction of flow cytometry in stallion sperm analysis is increasing the number of analytical tests available, allowing the study and assessment of new seminal parameters. However, inside this revolution, the evaluation of stallion sperm mitochondria has received much less attention in comparison with other sperm structures and functions in stallion andrology.

Mitochondria are cellular organelles with multiple roles in the spermatozoa, among the most important roles stands out the energy supply, the maintenance of membranes integrity, the production of reactive oxygen species (ROS) and

the regulation of cell death. Mitochondria have been proposed as the main source of ROS in human somatic cells and spermatozoa. However, there is not any published data on the production of mitochondrial ROS in equine spermatozoa.

In this Doctoral Thesis we studied the role of the mitochondria's electron transport chain (ETC) and oxidative stress as origin of senescence in equine sperm. In order to study that relation, a wide range of specific inhibitors of this organelle have been used: rotenone as complex I inhibitor, antimycin-A as complex III inhibitor, NaCN as complex IV inhibitor and oligomycin-A as ATP-synthase inhibitor; in addition two uncouplers of the mitochondrial proton gradient were also used (CCCP and 2,4-Dinitrophenol).

The main consequences of the application of the inhibitors and uncouplers were the reduction in sperm motility and velocity, the reduction in ATP production and the reduction of the membrane potential (except 2,4-DNP and NaCN). The inhibition of the ATP-synthase produced a collapse of mitochondrial membrane integrity, suggesting for the first time its importance for the maintenance of the equine sperm membranes.

Inhibition of complex I also leaded a negative effect on the spermatozoa-oocyte binding ability. To test if the addition of antioxidants could counteract the negative effect of the inhibition of complex I we added the flavonoid EGCG, and we found that it counteracted the lowest dose of rotenone and improved controls when compared with samples not treated with the inhibitor. This

finding suggests that EGCG may have positive effects on production of semen preservation media.

All inhibitors and CCCP used in our study led to an increase in the production of ROS in dead spermatozoa, and a decrease in the population of living spermatozoa. This decrease could be a good indication of the presence of active spermatozoa in the ejaculate.

In addition, the inhibition of complex I and III induced a collapse of the mitochondrial membrane potential resulting in a decrease of ROS because of a reduction in the electron flux. Inhibition of complex I was found to induce the generation of H_2O_2 over the time, however this effect was not found with inhibition of complex III.

To test if the oxidative phosphorylation (OXPHOS) was the only metabolic pathway of energy production for the motility of equine sperm we inhibited glycolysis with 2-deoxyglucose. Additionally, to reinforce our findings, stallion sperm was incubated in a medium devoid of any kind of glucose, and in the absence of glucose and pyruvate. Our results showed that OXPHOS is the main energy source of equine sperm, and showed for the first time that glycolysis is also necessary for maintaining sperm velocity.

RIASSUNTO

Le tecniche di riproduzione assistita in medicina veterinaria sono notevolmente evolute negli ultimi anni. Nella specie equina, queste tecniche sono diventate sempre più importanti, in quanto consentono di conservare e preservare il materiale genetico di individui di evelato valore, permettendo anche di massimizzare la loro produzione di prole. Tuttavia, questi individui sono spesso considerati di elevato valore sulla base di valutazioni morfologiche o sulla base delle performance sportive, criteri che non sono in relazione con la loro fertilità. Pertanto, i gameti di questianimali presentano risposte notevolmente eterogenee nei confronti dei diversi processi di manipolazione comunemente utilizzati nelle tecniche di riproduzione assistita come la crioconservazione.

La valutazione tradizionale del materiale seminale di stallone include la valutazione delle anomalie morfologiche,della motilità e dell'integrità delle membrane degli spermatozoi. La recente e progressiva introduzione della citometria a flusso per l'analisi dello sperma equino sta determinando un aumento dei test analitici disponibili, permettendo la valutazione di più parametri seminali. Tuttavia, all'interno di questa rivoluzione, lo studio dei mitocondri degli spermatozoi di stallone ha ricevuto meno attenzione rispetto ad altre strutture e funzioni.

I mitocondri sono organelli cellulari che giocano differenti ruoli negli spermatozoi: l'approvvigionamento di energia, il mantenimento dell'integrità della membrana, la produzione di specie reattive dell'ossigeno (SRO) e la regolazione della morte cellulare. I mitocondri sono stati proposti come la

principale fonte di SRO nelle cellule somatiche e negli spermatozoi umani. Tuttavia, non esistono dati pubblicati sulla produzione di SRO mitocondriale negli spermatozoi di stallone.

In questa Tesi di dottorato abbiamo studiato il ruolo della catena di trasporto degli elettroni mitocondriale edello stress ossidativo come origine della senescenza degli spermatozoi equini. A tal fine abbiamo utilizzato una vasta gamma di inibitori specifici che agiscono a livello di questo organello: il rotenone come inibitore del complesso I, l'antimicina-A come inibitore del complesso III, il NaCN come inibitore del complesso IV e l'oligomicina come inibitore della ATP-sintasi. Sono stati inoltre utilizzati due disaccoppianti del gradiente protonico mitocondriale (CCCP e 2,4-Dinitrophenol).

Le principali conseguenze deltrattamento con gli inibitori e i disaccoppiantisono state la diminuzione della motilità e della velocità degli spermatozoi, la riduzione della produzione di ATP e la riduzione del potenziale di membrana (tranne con l'utilizzo di 2,4-DNP e NaCN). L´inibizione della ATP-sintasi ha indotto una riduzione dell'integrità della membrana mitocondriale, suggerendo, per la prima volta, la sua importanza per il mantenimento delle membrane nello sperma equino.

L´inibizione del complesso I ha inoltre esercitato un effetto negativo sulla capacità di legame dello spermatozoo con l'oocita. Per verificare se l'aggiunta di antiossidanti potrebbe contrastare l'effetto negativo dell'inibizione del complesso I è stata addizionata l'EGCG, flavonoide del te verde,che è risultata in grado di neutralizzare gli effetti negativi indotti dalla dose minima di

rotenone. Ciò suggerisce che l'EGCG possa avere effetti positivi sulla capacitazione degli spermatozoi di stallonenonchè il suo possibile utilizzo come antiossidante nella produzione di extender per la conservazione dello sperma.

Nel nostro studio tutti gli inibitori e il CCCP hanno portato ad un aumento o diminuzione della produzione di SRO. Tale variazione dipendeva dal dosaggio e dal tempo dell'esposizione. Si è constatato che una piccola depolarizzazione mitocondriale porta ad un aumento della perdita di elettroni che comporta un aumento della produzione di SRO. Quindi possiamo dire che l'aumento della produzione di SRO è una buona indicazione della presenza di spermatozoi attivi nell'eiaculato.

L'inibizione dei complessi I e III ha condotto a un crollo del potenziale di membrana mitocondriale con la conseguente riduzione di SRO a causa delle riduzione del trasporto di elettroni. L'inibizione del complesso I ha indotto anche la produzione di H_2O_2 nel corso del tempo. Tuttavia, questo fenomeno non è stato rilevato con l'inibizione del complesso III.

Per verificare se la fosforilazione ossidativa è l'unica via metabolica per la produzione di energia per motilità degli spermatozoi equini abbiamo inibito la glicolisi con 2-deossiglucosio. Inoltre, per rafforzare i nostri risultati, abbiamo incubato gli spermatozoi in due medium diversi; senza glucosio e senza glucosio e piruvato. I nostri risultati hanno dimostrato che la fosoforilazione ossidativa è la fonte principale di energia per gli spermatozoi di stallone e, per la prima volta, che la glicolisi è necessaria per il mantenimento della velocità dei spermatozoi.

Bibliografía

BIBLIOGRAFÍA

Abou-haila, A., Tulsiani, D.R., 2009. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Arch Biochem Biophys 485, 72-81.

Aitken, R.J., Baker, H.W., 1995. Seminal leukocytes: passengers, terrorists or good samaritans? Hum Reprod 10, 1736-1739.

Aitken, R.J., Fisher, H.M., Fulton, N., Gomez, E., Knox, W., Lewis, B., Irvine, S., 1997. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. Mol Reprod Dev 47, 468-482.

Aitken, J.B., Naumovski, N., Curry, B., Grupen, C.G., Gibb, Z., Aitken, R.J., 2015a. Characterization of an L-amino acid oxidase in equine spermatozoa.Biol Reprod 92, 125.

Aitken, R.J., Baker, M.A., Nixon, B., 2015b. Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian J Androl 17, 633-639.

Al-Mehdi, A.B., Pastukh, V.M., Swiger, B.M., Reed, D.J., Patel, M.R., Bardwell, G.C., Pastukh, V.V., Alexeyev, M.F., Gillespie, M.N., 2012. Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. Sci Signal 5, ra47.

Amann, R.P., 2008. The cycle of the seminiferous epithelium in humans: a need to revisit? J Androl 29, 469-487.

Amaral, A., Lourenco, B., Marques, M., Ramalho-Santos, J., 2013. Mitochondria functionality and sperm quality.Reproduction 146, R163-174.

Anzar, M., He, L., Buhr, M.M., Kroetsch, T.G., Pauls, K.P., 2002.Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 66, 354-360.

Awda, B.J., Mackenzie-Bell, M., Buhr, M.M., 2009. Reactive oxygen species and boar sperm function. Biol Reprod 81, 553-561.

Baker, M.A., Aitken, R.J., 2004. The importance of redox regulated pathways in sperm cell biology. Mol Cell Endocrinol 216, 47-54.

Baker, M.A., Aitken, R.J., 2005. Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. Reprod Biol Endocrinol 3, 67.

Balao da Silva, C.M., Spinaci, M., Bucci, D., Giaretta, E., Pena, F.J., Mari, G., Galeati, G., 2013. Effect of sex sorting on stallion spermatozoa: Heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay.Anim Reprod Sci 141, 68-74.

Ball, B.A., Vo, A.T., Baumber, J., 2001. Generation of reactive oxygen species by equine spermatozoa.Am J Vet Res 62, 508-515.

Ball, B.A., 2008. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim Reprod Sci 107, 257-267.

Barratt, C.J.D.J.a.C.L., 2006. The sperm cell.Production, maturation, fertilization, regeneration.Cambridge University Press.

Bedford, J.M., Calvin, H.I., 1974. Changes in -S-S- linked structures of the sperm tail during epididymal maturation, with comparative observations in sub-mammalian species. J Exp Zool 187, 181-204.

Bennetts, L.E., De Iuliis, G.N., Nixon, B., Kime, M., Zelski, K., McVicar, C.M., Lewis, S.E., Aitken, R.J., 2008. Impact of estrogenic compounds on DNA integrity in human spermatozoa: evidence for cross-linking and redox cycling activities. Mutat Res 641, 1-11.

Bleil, J.D., Wassarman, P.M., 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell 20, 873-882.

Boerke, A., Tsai, P.S., Garcia-Gil, N., Brewis, I.A., Gadella, B.M., 2008. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology 70, 1188-1196.

Bourdon, E., Blache, D., 2001.The importance of proteins in defense against oxidation. Antioxid Redox Signal 3, 293-311.

Boveris, A., Cadenas, E., 1975. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Lett 54, 311-314.

Boveris, A., Cadenas, E., Stoppani, A.O., 1976. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J 156, 435-444.

Boyer, P.D., 1997.The ATP synthase--a splendid molecular machine. Annu Rev Biochem 66, 717-749.

Brokaw, C.J., 1989. Direct measurements of sliding between outer doublet microtubules in swimming sperm flagella. Science 243, 1593-1596.

Brum, A.M., Sabeur, K., Ball, B.A., 2008. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology 69, 1041-1055.

Bucak, M.N., Tuncer, P.B., Sariozkan, S., Baspinar, N., Taspinar, M., Coyan, K., Bilgili, A., Akalin, P.P., Buyukleblebici, S., Aydos, S., Ilgaz, S., Sunguroglu, A., Oztuna, D., 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. Cryobiology 61, 248-253.

Calvin, H.I., Bedford, J.M., 1971. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J Reprod Fertil Suppl 13, Suppl 13:65-75.

Clulow, J.R., Evans, G., Maxwell, W.M., Morris, L.H., 2010. Evaluation of the function of fresh and frozen-thawed sex-sorted and non-sorted stallion spermatozoa using a heterologous oocyte binding assay. Reprod Fertil Dev 22, 710-717.

Cornwall, G.A., 2009. New insights into epididymal biology and function. Hum Reprod Update 15, 213-227.

Crofts, A.R., Barquera, B., Gennis, R.B., Kuras, R., Guergova-Kuras, M., Berry, E.A., 1999a. Mechanism of ubiquinol oxidation by the bc(1) complex: different domains of the quinol binding pocket and their role in the mechanism and binding of inhibitors. Biochemistry 38, 15807-15826.

Crofts, A.R., Hong, S., Ugulava, N., Barquera, B., Gennis, R., Guergova-Kuras, M., Berry, E.A., 1999b.Pathways for proton release during ubihydroquinone oxidation by the bc(1) complex. Proc Natl Acad Sci U S A 96, 10021-10026.

Chance, B., Sies, H., Boveris, A., 1979.Hydroperoxide metabolism in mammalian organs. Physiol Rev 59, 527-605.

Chantler, E., Abraham-Peskir, J.V., Little, S., McCann, C., Medenwaldt, R., 2000. Effect of cooling on the motility and function of human spermatozoa.Cryobiology 41, 125-134.

Chatterjee, S., Gagnon, C., 2001.Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 59, 451-458.

Chekeni, F.B., Elliott, M.R., Sandilos, J.K., Walk, S.F., Kinchen, J.M., Lazarowski, E.R., Armstrong, A.J., Penuela, S., Laird, D.W., Salvesen, G.S., Isakson, B.E., Bayliss, D.A., Ravichandran, K.S., 2010. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature 467, 863-867.

Chipuk, J.E., Bouchier-Hayes, L., Green, D.R., 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ 13, 1396-1402.

De Iuliis, G.N., Newey, R.J., King, B.V., Aitken, R.J., 2009. Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One 4, e6446.

de Lamirande, E., Gagnon, C., 1993a. Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. Fertil Steril 59, 1291-1295.

de Lamirande, E., Gagnon, C., 1993b. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 16, 21-25.

de Lamirande, E., Gagnon, C., 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum Reprod 10 Suppl 1, 15-21.

de Lamirande, E., Leclerc, P., Gagnon, C., 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 3, 175-194.

de Lamirande, E., O'Flaherty, C., 2008. Sperm activation: role of reactive oxygen species and kinases. Biochim Biophys Acta 1784, 106-115.

De Martino, C., Floridi, A., Marcante, M.L., Malorni, W., Scorza Barcellona, P., Bellocci, M., Silvestrini, B., 1979. Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. Cell Tissue Res 196, 1-22.

Dieude, M., Striegl, H., Tyznik, A.J., Wang, J., Behar, S.M., Piccirillo, C.A., Levine, J.S., Zajonc, D.M., Rauch, J., 2011. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. J Immunol 186, 4771-4781.

Dong, Q., Tollner, T.L., Rodenburg, S.E., Hill, D.L., VandeVoort, C.A., 2010. Antioxidants, Oxyrase, and mitochondrial uncoupler 2,4-dinitrophenol improved postthaw survival of rhesus monkey sperm from ejaculates with low cryosurvival. Fertil Steril 94, 2359-2361.

Dudkina, N.V., Sunderhaus, S., Boekema, E.J., Braun, H.P., 2008. The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. J Bioenerg Biomembr 40, 419-424.

Eddy, E.M., Toshimori, K., O'Brien, D.A., 2003. Fibrous sheath of mammalian spermatozoa. Microsc Res Tech 61, 103-115.

Erkkila, K., Kyttanen, S., Wikstrom, M., Taari, K., Hikim, A.P., Swerdloff, R.S., Dunkel, L., 2006. Regulation of human male germ cell death by modulators of ATP production.Am J Physiol Endocrinol Metab 290, E1145-1154.

Escalier, D., 2006. Knockout mouse models of sperm flagellum anomalies. Hum Reprod Update 12, 449-461.

Fawcett, D.W., 1975. The mammalian spermatozoon. Dev Biol 44, 394-436.

Fazeli, A.R., Steenweg, W., Bevers, M.M., de Loos, F.A., van den Broek, J., Colenbrander, B., 1993. Development of a sperm zona pellucida binding assay for bull semen. Vet Rec 132, 14-16.

Fazeli, A.R., Steenweg, W., Bevers, M.M., van den Broek, J., Bracher, V., Parlevliet, J., Colenbrander, B., 1995. Relation between stallion sperm binding to homologous hemizonae and fertility. Theriogenology 44, 751-760.

Ferramosca, A., Zara, V., 2014. Bioenergetics of mammalian sperm capacitation.Biomed Res Int 2014, 902953.

Fiore, C., Trezeguet, V., Le Saux, A., Roux, P., Schwimmer, C., Dianoux, A.C., Noel, F., Lauquin, G.J., Brandolin, G., Vignais, P.V., 1998. The mitochondrial ADP/ATP carrier: structural, physiological and pathological aspects. Biochimie 80, 137-150.

Franca, L.R., Avelar, G.F., Almeida, F.F., 2005. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology 63, 300-318.

Fuentes-Prior, P., Salvesen, G.S., 2004.The protein structures that shape caspase activity, specificity, activation and inhibition.Biochem J 384, 201-232.

Gadea, J., Molla, M., Selles, E., Marco, M.A., Garcia-Vazquez, F.A., Gardon, J.C., 2011. Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology 62, 40-46.

Gadella, B.M., Flesch, F.M., van Golde, L.M., Colenbrander, B., 1999. Dynamics in the membrane organization of the mammalian sperm cell and functionality in fertilization. Vet Q 21, 142-146.

Gadella, B.M., Rathi, R., Brouwers, J.F., Stout, T.A., Colenbrander, B., 2001. Capacitation and the acrosome reaction in equine sperm. Anim Reprod Sci 68, 249-265.

Gadella, B.M., 2008. The assembly of a zona pellucida binding protein complex in sperm.Reprod Domest Anim 43 Suppl 5, 12-19.

Gallardo Bolanos, J.M., Balao da Silva, C.M., Martin Munoz, P., Morillo Rodriguez, A., Plaza Davila, M., Rodriguez-Martinez, H., Aparicio, I.M., Tapia, J.A., Ortega Ferrusola, C., Pena, F.J., 2014. Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction 148, 221-235.

Galluzzi, L., Kepp, O., Kroemer, G., 2012a. Mitochondria: master regulators of danger signalling. Nat Rev Mol Cell Biol 13, 780-788.

Galluzzi, L., Kepp, O., Trojel-Hansen, C., Kroemer, G., 2012b.Mitochondrial control of cellular life, stress, and death. Circ Res 111, 1198-1207.

Galluzzi, L., Kepp, O., Trojel-Hansen, C., Kroemer, G., 2012c. Non-apoptotic functions of apoptosis-regulatory proteins. EMBO Rep 13, 322-330.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., Gottlieb, E., Green, D.R., Hengartner, M.O., Kepp, O., Knight, R.A., Kumar, S., Lipton, S.A., Lu, X., Madeo, F., Malorni, W., Mehlen, P., Nunez, G., Peter, M.E., Piacentini, M., Rubinsztein, D.C., Shi, Y., Simon, H.U., Vandenabeele, P., White, E., Yuan, J., Zhivotovsky, B., Melino, G., Kroemer, G., 2012d. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19, 107-120.

Gao, D.Y., Liu, J., Liu, C., McGann, L.E., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, E.S., Critser, J.K., 1995. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol.Hum Reprod 10, 1109-1122.

Garcia, B.M., Fernandez, L.G., Ferrusola, C.O., Salazar-Sandoval, C., Rodriguez, A.M., Martinez, H.R., Tapia, J.A., Morcuende, D., Pena, F.J., 2011. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation.Reprod Domest Anim 46, 141-148.

Genova, M.L., Ventura, B., Giuliano, G., Bovina, C., Formiggini, G., Parenti Castelli, G., Lenaz, G., 2001. The site of production of superoxide radical in mitochondrial Complex I is not a bound ubisemiquinone but presumably iron-sulfur cluster N2. FEBS Lett 505, 364-368.

Genova, M.L., Pich, M.M., Biondi, A., Bernacchia, A., Falasca, A., Bovina, C., Formiggini, G., Parenti Castelli, G., Lenaz, G., 2003. Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant.Exp Biol Med (Maywood) 228, 506-513.

Gibb, Z., Lambourne, S.R., Aitken, R.J., 2014. The paradoxical relationship between stallion fertility and oxidative stress.Biol Reprod 91, 77.

Gibbons, I.R., Grimstone, A.V., 1960. On flagellar structure in certain flagellates. J Biophys Biochem Cytol 7, 697-716.

Gille, L., Nohl, H., 2001. The ubiquinol/bc1 redox couple regulates mitochondrial oxygen radical formation. Arch Biochem Biophys 388, 34-38.

Green, D.R., Galluzzi, L., Kroemer, G., 2011. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science 333, 1109-1112.

Griveau, J.F., Renard, P., Le Lannou, D., 1994. An in vitro promoting role for hydrogen peroxide in human sperm capacitation. Int J Androl 17, 300-307.

Gupta, S., 2003.Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). Int J Oncol 22, 15-20.

Hamanaka, R.B., Chandel, N.S., 2009.Mitochondrial reactive oxygen species regulate hypoxic signaling. Curr Opin Cell Biol 21, 894-899.

Hecht, N.B., Kleene, K.C., Distel, R.J., Silver, L.M., 1984. The differential expression of the actins and tubulins during spermatogenesis in the mouse. Exp Cell Res 153, 275-280.

Heninger, N.L., Staub, C., Blanchard, T.L., Johnson, L., Varner, D.D., Forrest, D.W., 2004. Germ cell apoptosis in the testes of normal stallions. Theriogenology 62, 283-297.

Herrero, A., Barja, G., 2000. Localization of the site of oxygen radical generation inside the complex I of heart and nonsynaptic brain mammalian mitochondria. J Bioenerg Biomembr 32, 609-615.

Igney, F.H., Krammer, P.H., 2002. Immune escape of tumors: apoptosis resistance and tumor counterattack. J Leukoc Biol 71, 907-920.

Inaba, K., 2003. Molecular architecture of the sperm flagella: molecules for motility and signaling. Zoolog Sci 20, 1043-1056.

Inaba, K., 2007. Molecular basis of sperm flagellar axonemes: structural and evolutionary aspects. Ann N Y Acad Sci 1101, 506-526.

Iwasaki, A., Gagnon, C., 1992. Formation of reactive oxygen species in spermatozoa of infertile patients. Fertil Steril 57, 409-416.

Jeulin, C., Soufir, J.C., Weber, P., Laval-Martin, D., Calvayrac, R., 1989.Catalase activity in human spermatozoa and seminal plasma. Gamete Res 24, 185-196.

Johnson, D.E., 2000. Noncaspase proteases in apoptosis. Leukemia 14, 1695-1703.

Johnson, L., Thompson, D.L., Jr., Varner, D.D., 2008. Role of Sertoli cell number and function on regulation of spermatogenesis. Anim Reprod Sci 105, 23-51.

Johnstone, R.W., Ruefli, A.A., Lowe, S.W., 2002. Apoptosis: a link between cancer genetics and chemotherapy. Cell 108, 153-164.

Kaedei, Y., Naito, M., Naoi, H., Sato, Y., Taniguchi, M., Tanihara, F., Kikuchi, K., Nagai, T., Otoi, T., 2012. Effects of (-)-epigallocatechin gallate on the motility and penetrability of frozen-thawed boar spermatozoa incubated in the fertilization medium. Reprod Domest Anim 47, 880-886.

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., Aitken, R.J., 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa.J Clin Endocrinol Metab 93, 3199-3207.

Koppers, A.J., Garg, M.L., Aitken, R.J., 2010. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med 48, 112-119.

Krauss, S., 2001. Mitochondria: Structure and Role in Respiration.Encyclopedia of Life Sciences, Nature Publishing Group.

Kroemer, G., Galluzzi, L., Brenner, C., 2007. Mitochondrial membrane permeabilization in cell death. Physiol Rev 87, 99-163.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Malorni, W., Nunez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G., 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16, 3-11.

Krysko, D.V., Agostinis, P., Krysko, O., Garg, A.D., Bachert, C., Lambrecht, B.N., Vandenabeele, P., 2011. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. Trends Immunol 32, 157-164.

Kuisma, P., Andersson, M., Koskinen, E., Katila, T., 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta Vet Scand 48, 14.

Kushnareva, Y., Murphy, A.N., Andreyev, A., 2002. Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. Biochem J 368, 545-553.

Lambert, A.J., Brand, M.D., 2004a. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Biol Chem 279, 39414-39420.

Lambert, A.J., Brand, M.D., 2004b. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem J 382, 511-517.

Lenaz, G., Landi, L., Cabrini, L., Pasquali, P., Sechi, A.M., Ozawa, T., 1978.On the sidedness of the ubiquinone redox cycle. Kinetic studies in mitochondrial membranes. Biochem Biophys Res Commun 85, 1047-1053.

Levy, R., Seifer-Aknin, I., 2001.Apoptosis during spermatogenesis and in ejaculated spermatozoa: importance for fertilization. Ann Biol Clin (Paris) 59, 531-545.

Liu, Y., Fiskum, G., Schubert, D., 2002. Generation of reactive oxygen species by the mitochondrial electron transport chain.J Neurochem 80, 780-787.

Macias-Garcia, B., Gonzalez-Fernandez, L., Gallardo-Bolanos, J.M., Pena, F.J., Johannisson, A., Morrell, J.M., 2012. Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS. Anim Reprod Sci 132, 74-82.

Maia Mda, S., Bicudo, S.D., Sicherle, C.C., Rodello, L., Gallego, I.C., 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. Anim Reprod Sci 122, 118-123.

Mandal, A., Naaby-Hansen, S., Wolkowicz, M.J., Klotz, K., Shetty, J., Retief, J.D., Coonrod, S.A., Kinter, M., Sherman, N., Cesar, F., Flickinger, C.J., Herr, J.C., 1999. FSP95, a testis-specific 95-kilodalton fibrous sheath antigen that undergoes tyrosine phosphorylation in capacitated human spermatozoa. Biol Reprod 61, 1184-1197.

Martin, G., Sabido, O., Durand, P., Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 71, 28-37.

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., Levy, R., 2007. Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum Reprod 22, 380-388.

McBride, H.M., Neuspiel, M., Wasiak, S., 2006. Mitochondria: more than just a powerhouse. Curr Biol 16, R551-560.

Meyers, S.A., Liu, I.K., Overstreet, J.W., Vadas, S., Drobnis, E.Z., 1996. Zona pellucida binding and zona-induced acrosome reactions in horse spermatozoa: comparisons between fertile and subfertile stallions. Theriogenology 46, 1277-1288.

Miller, D., Ostermeier, G.C., Krawetz, S.A., 2005. The controversy, potential and roles of spermatozoal RNA. Trends Mol Med 11, 156-163.

Mitchell, P., 1967. Proton current flow in mitochondrial systems. Nature 214, 1327-1328.

Moldzio, R., Radad, K., Krewenka, C., Kranner, B., Duvigneau, J.C., Wang, Y., Rausch, W.D., 2010. Effects of epigallocatechin gallate on rotenone-injured murine brain cultures. J Neural Transm 117, 5-12.

Moran, D.M., Jasko, D.J., Squires, E.L., Amann, R.P., 1992.Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa.Theriogenology 38, 999-1012.

Mortimer, S.T., 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update 3, 403-439.

Mukai, C., Okuno, M., 2004. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod 71, 540-547.

Murray, R.K., G.D., Mayes, P.A., Rodwell, V.W. 2003. Harper's Illustrated Biochemistry. McGraw-Hill.

Neagu, V.R., Garcia, B.M., Sandoval, C.S., Rodriguez, A.M., Ferrusola, C.O., Fernandez, L.G., Tapia, J.A., Pena, F.J., 2010. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. Theriogenology 73, 645-650.

Neill, J.D., 2006. Physiology of Reproduction.ELSEVIER.

Nissen, H.P., Kreysel, H.W., 1983. Superoxide dismutase in human semen. Klin Wochenschr 61, 63-65.

Nunnari, J., Suomalainen, A., 2012. Mitochondria: in sickness and in health. Cell 148, 1145-1159.

Ohnishi, S.T., Ohnishi, T., Muranaka, S., Fujita, H., Kimura, H., Uemura, K., Yoshida, K., Utsumi, K., 2005. A possible site of superoxide generation in the complex I segment of rat heart mitochondria. J Bioenerg Biomembr 37, 1-15.

Olson, G.E., Winfrey, V.P., 1990. Mitochondria-cytoskeleton interactions in the sperm midpiece. J Struct Biol 103, 13-22.

Olson, G.E., Winfrey, V.P., 1992. Structural organization of surface domains of sperm mitochondria.Mol Reprod Dev 33, 89-98.

Ortega-Ferrusola, C., Sotillo-Galan, Y., Varela-Fernandez, E., Gallardo-Bolanos, J.M., Muriel, A., Gonzalez-Fernandez, L., Tapia, J.A., Pena, F.J., 2008. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm.J Androl 29, 213-221.

Ortega-Ferrusola, C., Gonzalez-Fernandez, L., Muriel, A., Macias-Garcia, B., Rodriguez-Martinez, H., Tapia, J.A., Alonso, J.M., Pena, F.J., 2009a. Does the microbial flora in the ejaculate affect the freezeability of stallion sperm? Reprod Domest Anim 44, 518-522.

Ortega Ferrusola, C., Gonzalez Fernandez, L., Morrell, J.M., Salazar Sandoval, C., Macias Garcia, B., Rodriguez-Martinez, H., Tapia, J.A., Pena, F.J., 2009b. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction 138, 55-63.

Ortega Ferrusola, C., Gonzalez Fernandez, L., Salazar Sandoval, C., Macias Garcia, B., Rodriguez Martinez, H., Tapia, J.A., Pena, F.J., 2010. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. Theriogenology 74, 458-465.

Paasch, U., Grunewald, S., Dathe, S., Glander, H.J., 2004. Mitochondria of human spermatozoa are preferentially susceptible to apoptosis. Ann N Y Acad Sci 1030, 403-409.

Paniagua, R., N.M., Sesma, P., Álvarez-Uría, M., Fraile, B., Anadón, R., Sáez, F.J. 2007. Biología Celular.McGraw-Hill.

Parks, J.E., Lynch, D.V., 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29, 255-266.

Pavlov, E.V., Priault, M., Pietkiewicz, D., Cheng, E.H., Antonsson, B., Manon, S., Korsmeyer, S.J., Mannella, C.A., Kinnally, K.W., 2001. A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. J Cell Biol 155, 725-731.

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

Pena, F.J., Rodriguez Martinez, H., Tapia, J.A., Ortega Ferrusola, C., Gonzalez Fernandez, L., Macias Garcia, B., 2009. Mitochondria in mammalian sperm physiology and pathology: a review. Reprod Domest Anim 44, 345-349.

Phillips, D.M., 1972. Comparative analysis of mammalian sperm motility. J Cell Biol 53, 561-573.

Phillips, D.M., 1977. Mitochondrial disposition in mammalian spermatozoa.J Ultrastruct Res, 144-154.

Plaza Davila, M., Martin Munoz, P., Tapia, J.A., Ortega Ferrusola, C., Balao da Silva, C.C., Pena, F.J., 2015. Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility. PLoS One 10, e0138777.

Raha, S., McEachern, G.E., Myint, A.T., Robinson, B.H., 2000. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. Free Radic Biol Med 29, 170-180.

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P.C., Sousa, A.P., Amaral, A., 2009.Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. Hum Reprod Update 15, 553-572.

Rappaport, L., Oliviero, P., Samuel, J.L., 1998. Cytoskeleton and mitochondrial morphology and function. Mol Cell Biochem 184, 101-105.

Rasola, A., Bernardi, P., 2007. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12, 815-833.

Rath, D., Moench-Tegeder, G., Taylor, U., Johnson, L.A., 2009. Improved quality of sex-sorted sperm: a prerequisite for wider commercial application. Theriogenology 71, 22-29.

Rich, P.R., Bonner, W.D., 1978. The sites of superoxide anion generation in higher plant mitochondria. Arch Biochem Biophys 188, 206-213.

Samper J.C., 2009.Equine breeding management and artificial insemination. Saunders, ELSEVIER.

Satir, P., 1968. Studies on cilia 3. Further studies on the cilium tip and a "sliding filament" model of ciliary motility. J Cell Biol 39, 77-94.

Schlame, M., 2008.Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes. J Lipid Res 49, 1607-1620.

Sikka, S.C., Rajasekaran, M., Hellstrom, W.J., 1995.Role of oxidative stress and antioxidants in male infertility. J Androl 16, 464-468.

Singer, T.P., Ramsay, R.R., 1994. The reaction sites of rotenone and ubiquinone with mitochondrial NADH dehydrogenase. Biochim Biophys Acta 1187, 198-202.

Sinowatz, F., Wessa, E., Neumuller, C., Palma, G., 2003.On the species specificity of sperm binding and sperm penetration of the zona pellucida.Reprod Domest Anim 38, 141-146.

Spinaci, M., Volpe, S., De Ambrogi, M., Tamanini, C., Galeati, G., 2008.Effects of epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine oocytes. Theriogenology 69, 877-885.

St-Pierre, J., Buckingham, J.A., Roebuck, S.J., Brand, M.D., 2002.Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 277, 44784-44790.

Starkov, A.A., Fiskum, G., 2001. Myxothiazol induces H(2)O(2) production from mitochondrial respiratory chain. Biochem Biophys Res Commun 281, 645-650.

Storey, B.T., 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol 52, 427-437.

Stowe, D.F., Camara, A.K., 2009. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. Antioxid Redox Signal 11, 1373-1414.

Sun, J., Trumpower, B.L., 2003. Superoxide anion generation by the cytochrome bc1 complex. Arch Biochem Biophys 419, 198-206.

Suzuki, M., Wakabayashi, T., 1988.Packaging of DNA in cricket sperm.A compact mode of DNA packaging. J Mol Biol 204, 653-661.

Suzuki, Y., Takahashi-Niki, K., Akagi, T., Hashikawa, T., Takahashi, R., 2004. Mitochondrial protease Omi/HtrA2 enhances caspase activation through multiple pathways. Cell Death Differ 11, 208-216.

Tait, S.W., Green, D.R., 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11, 621-632.

Tait, S.W., Green, D.R., 2012. Mitochondria and cell signalling. J Cell Sci 125, 807-815.

Tapia J.A., Peña F.J., 2009. Apoptosis events in male germ cells and in mature mammalian spermatozoa.Springer.

Tash, J.S., 1989. Protein phosphorylation: the second messenger signal transducer of flagellar motility. Cell Motil Cytoskeleton 14, 332-339.

Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312-1316.

Toshimori, K., 1998. Maturation of mammalian spermatozoa: modifications of the acrosome and plasma membrane leading to fertilization. Cell Tissue Res 293, 177-187.

Tsujimoto, Y., 1997. Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. Cell Death Differ 4, 429-434.

Tulsiani, D.R., 2006. Glycan-modifying enzymes in luminal fluid of the mammalian epididymis: an overview of their potential role in sperm maturation. Mol Cell Endocrinol 250, 58-65.

Tunc, O., Thompson, J., Tremellen, K., 2009. Improvement in sperm DNA quality using an oral antioxidant therapy. Reprod Biomed Online 18, 761-768.

Tunc, O., Tremellen, K., 2009. Oxidative DNA damage impairs global sperm DNA methylation in infertile men. J Assist Reprod Genet 26, 537-544.

Turner, R.M., 2006. Moving to the beat: a review of mammalian sperm motility regulation. Reprod Fertil Dev 18, 25-38.

Turrens, J.F., Alexandre, A., Lehninger, A.L., 1985. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 237, 408-414.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. J Physiol 552, 335-344.

Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., Flohe, L., 1999.Dual function of the selenoprotein PHGPx during sperm maturation. Science 285, 1393-1396.

Varner, D.D., Gibb, Z., Aitken, R.J., 2015. Stallion fertility: a focus on the spermatozoon. Equine Vet J 47, 16-24.

Verkhovskaya, M.L., Belevich, N., Euro, L., Wikstrom, M., Verkhovsky, M.I., 2008. Real-time electron transfer in respiratory complex I. Proc Natl Acad Sci U S A 105, 3763-3767.

Vernet, P., Fulton, N., Wallace, C., Aitken, R.J., 2001. Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. Biol Reprod 65, 1102-1113.

Vidament, M., Dupere, A.M., Julienne, P., Evain, A., Noue, P., Palmer, E., 1997. Equine frozen semen: freezability and fertility field results. Theriogenology 48, 907-917.

Vijayaraghavan, S., Liberty, G.A., Mohan, J., Winfrey, V.P., Olson, G.E., Carr, D.W., 1999.Isolation and molecular characterization of AKAP110, a novel, sperm-specific protein kinase A-anchoring protein. Mol Endocrinol 13, 705-717.

Voet, D., Judith, G., Voet, J.G., Pratt, C.W., 2006. Fundamentals of biochemistry.John Wiley and Sons.

Wathes, D.C., Abayasekara, D.R., Aitken, R.J., 2007.Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 77, 190-201.

White, I.G., 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod Fertil Dev 5, 639-658.

Williams, A.C., Ford, W.C., 2001. The role of glucose in supporting motility and capacitation in human spermatozoa. J Androl 22, 680-695.

Xi, Q., Cheranov, S.Y., Jaggar, J.H., 2005. Mitochondria-derived reactive oxygen species dilate cerebral arteries by activating Ca2+ sparks. Circ Res 97, 354-362.

Yanagimachi, R., 1970. The movement of golden hamster spermatozoa before and after capacitation. J Reprod Fertil 23, 193-196.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X., Kroemer, G., 1995.Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 181, 1661-1672.

Zhao, Y., Wang, Z.B., Xu, J.X., 2003. Effect of cytochrome c on the generation and elimination of O2*- and H2O2 in mitochondria. J Biol Chem 278, 2356-2360.

Zitvogel, L., Kepp, O., Kroemer, G., 2010. Decoding cell death signals in inflammation and immunity. Cell 140, 798-804.

Zoccarato, F., Cavallini, L., Bortolami, S., Alexandre, A., 2007. Succinate modulation of H2O2 release at NADH:ubiquinone oxidoreductase (Complex I) in brain mitochondria. Biochem J 406, 125-129.