Universitat Autònoma de Barcelona

Photostimulation in boar sperm: mid-term effects, mechanism of action and “in-farm” applicability

Olga Blanco Prieto

Photostimulation in boar sperm: mid-term effects, mechanism of action and *"in-farm"* applicability.

PhD Thesis

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Olga Blanco Prieto

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Director and Tutor:

Juan Enrique Rodríguez Gil

Director:

María Montserrat Rivera del Álamo

UAB
Universitat Autònoma de Barcelona

FACULTAT DE VETERINÀRIA

Universitat Autònoma de Barcelona

El Dr. **Juan Enrique Rodríguez Gil**, Catedrático del Departamento de Medicina y Cirugía Animal.

La Dra. **María Montserrat Rivera del Álamo**, Profesora Asociada del Departamento de Medicina y Cirugía Animal.

CERTIFICAN:

Que la Tesis Doctoral titulada ***"Photostimulation in boar sperm: mid-term effects, mechanism of action and "in-farm" applicability"*** presentada por Olga Blanco Prieto para optar por el grado de Doctor en Medicina y Sanidad Animal por la Universidad Autónoma de Barcelona se ha realizado bajo su dirección y, considerándola terminada y cumpliendo los requisitos para poder optar a la Mención Internacional, autorizan su presentación para ser juzgada por la Comisión correspondiente.

Y para que conste a efectos oportunos, firman el presente documento en Bellaterra, a 11 de julio de 2020.

Juan Enrique Rodríguez Gil

María Montserrat Rivera del Álamo

UNIVERSIDAD AUSTRAL DE CHILE
INSTITUTO DE CIENCIA ANIMAL

FACULTAD DE CIENCIAS VETERINARIAS
CAMPUS ISLA TEJA
VALDIVIA – CHILE
FONO – FAX: 63 – 222 12 12
CASILLA 567
EMAIL.: ciencia.animal@uach.cl

CERTIFICADO

Valdivia 3 de enero del 2020.

Mediante el presente documento, se certifica que la Srta. **Olga Blanco Prieto con DNI 16636370-X** y estudiante del Doctorado en Medicina y Sanidad Animales de la Universitat Autónoma de Barcelona, realizó una estancia el laboratorio de Criobiología y Análisis de Funcionalidad Espermática de la Facultad de Ciencias Veterinarias de la Universidad Austral de Chile entre el 30 de septiembre de 2019 al 3 de enero de 2020.

Durante su estancia, la estudiante recibió entrenamiento en el trabajo rutinario del laboratorio, y participo en el montaje de técnicas analíticas avanzadas, relacionadas a con su formación y avance de objetivos planteados en su tesis doctoral. En lo específico, participo en el desarrollo de los siguientes trabajos experimentales, ambos vinculados al estudio de la fotoestimulación espermática con LED-630nm:

- Caracterización de los efectos de la fotoestimulación y descongelación del semen bovino en la calidad seminal, exposición de grupos SH y cuantificación del grado de descompactación de la cromatina inducida por tioglicolato.
- Evaluación de los efectos de 3 protocolos de fotoestimulación de semen equino refrigerado, en la calidad seminal y longevidad a largo plazo a 5° y 17°C.

Se extiende el presente certificado, a solicitud de la interesada, para los fines que estime convenientes

Alfredo Ramírez, PhD
Laboratorio de Criobiología y
Análisis de Funcionalidad Espermática
Instituto de Ciencia Animal
Facultad de Ciencias Veterinarias
Universidad Austral de Chile

A ti que lo estás leyendo y
sobre todo a mi Chipichichurri,
ya sabes tú por qué.

"Defiende tu derecho a pensar, porque incluso pensar de manera errónea es mejor que no pensar".
Hipatia

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ABSTRACT

Exposition of sperm from separate species of mammalian and non-mammalian sperm such as rabbit, turkey, ram, human, tilapia, dog and bull to specific light patterns improves motion parameters as well as increases mitochondria cytochrome C oxidase activity. In boar sperm, the red light LED exposure in discontinuous irradiation with a specific protocol of 10 minutes light followed by 10 minutes in darkness and then again, a 10 minutes irradiation period (10'-10'-10' procedure) increases both *in vitro* semen quality parameters and *in vivo* fertility rates, although several doubts raise regarding the practical *in-farm* application of red light stimulation of boar sperm. These doubts are mainly linked to a poor knowledge regarding very important questions such as the mechanism/s of action of red light and how the utilized irradiation protocols can be optimized. In this sense, this PhD dissertation has been focused on the following questions:

- Did the effects of red-light LED on *in vitro* semen quality parameters of boar sperm last over time?
- Is mitochondrial activity related with the observed effects of red-light LED irradiation of boar sperm?
- Is red light LED irradiation a practical and feasible tool to improve fertility rates in pig farming?

Results shown in this dissertation indicate that:

1. Red-light LED stimulation of boar sperm increases their resilience to withstand thermal stress over the first 48 h of storage at 17ºC.
2. Red-light LED stimulation contributes to the maintenance of an optimal nucleoprotein structure of boar sperm stored at 17ºC for 96h.
3. Effects of red-light LED irradiation on boar sperm stored at 17ºC for 96h depend on the specific utilized irradiation pattern.
4. Effects induced by red LED light stimulation on boar sperm are related to the action on the activity of mitochondria photosensitive components such as cytochrome C

oxidase. This action will cause, as a consequence, significant changes in the activity of mitochondrial electron chain.

5. The impact of red-light LED on boar sperm mitochondrial activity depends on the precise, previous sperm functional status and on the time of exposure.
6. The action of red LED light on the boar sperm electron chain does not exclude other concomitant mechanisms of action.
7. Red-light LED stimulation of boar semen prior to AI in pig farms can have a positive effect on the overall reproductive performance of these farms. Otherwise, the overall final effectiveness of this method would depend on other factors such as a proper farm management.
8. Stimulation with red-light LED could be an economical and profitable way to improve fertility and prolificacy in sow farms, provided the appropriate management of farms.

RESUMEN

La exposición a patrones de luz específicos de los espermatozoides de diferentes especies animales, ya sean mamíferos o no mamíferos, como conejo, pavo, pequeños rumiantes, humanos, tilapia, perro y toro; mejora los parámetros de movimiento y aumenta la actividad mitocondrial del citocromo C oxidasa. En los espermatozoides de cerdo, la exposición a luz roja LED de forma discontinua con un protocolo especifico de 10 minutos de luz, 10 minutos de oscuridad y otros 10 minutos de irradiación (proceso 10'-10'-10') aumenta tanto los parámetros de calidad del semen *in vitro* como las parametros de fertilidad *in vivo*, aunque surgen varias dudas respecto a la aplicación práctica en la granja de la estimulación con luz roja del esperma de cerdo. Estas dudas se deben a un escaso conocimiento sobre cuestiones muy importantes como, por ejemplo, los mecanismos de acción de la luz roja y cómo se pueden optimizar los protocolos de irradiación utilizados. Por ello, esta tesis doctoral se centra en las siguientes preguntas:

- ¿Los efectos de la luz roja LED sobre los parámetros de calidad del semen de cerdo *in vitro* perdurarán en el tiempo?
- ¿La actividad mitocondrial está relacionada con los efectos observados de la irradiación con luz roja LED de los espermatozoides de cerdo?
- ¿La irradiación con luz roja LED es una herramienta práctica y factible para mejorar las tasas de fertilidad en la cría de cerdos?

Los resultados presentados en esta disertación muestran que:

1. La estimulación con luz roja LED de los espermatozoides de cerdo aumenta su resistencia frente el estrés térmico durante las primeras 48 h de almacenamiento a 17ºC.
2. La estimulación con luz roja LED contribuye al mantenimiento de una estructura óptima de nucleoproteína del esperma de cerdo almacenado a 17ºC durante 96 h.

3. Los efectos de la irradiación con luz roja LED sobre los espermatozoides de cerdo almacenados a 17ºC durante 96 h dependen del patrón de irradiación específico utilizado.
4. Los efectos inducidos por la estimulación de la luz LED roja en los espermatozoides de cerdo están relacionados con la acción sobre la actividad de los componentes fotosensibles de la mitocondria, como la citocromo C oxidasa. Esta acción causará como consecuencia cambios significativos en la actividad de la cadena de electrones mitocondriales.
5. El impacto de la luz roja en la actividad mitocondrial de los espermatozoides de cerdo depende del estado funcional previo y preciso del esperma y del tiempo de exposición.
6. La acción de la luz LED roja en la cadena de electrones de esperma de cerdo no excluye otros mecanismos de acción concomitantes.
7. La estimulación con luz roja LED del semen de cerdo antes de la IA en granjas porcinas puede tener un efecto positivo en el rendimiento reproductivo general de estas granjas. Por otro lado, la efectividad final general de este método dependería de otros factores, como el manejo adecuado de la granja.
8. La estimulación con LED de luz roja podría ser una forma económica y rentable de mejorar la fertilidad y la prolificidad en las granjas de cerdas, siempre que el manejo de las granjas sea adecuado.

RESUM

L'exposició a patrons de llum específics dels espermatozoides de diferents espècies animals, ja siguin mamífers o no mamífers, com conill, gall dindi, petits remugants, humans, tilàpia, gos i toro, millora els paràmetres de motilitat i augmenta l'activitat mitocondrial de la citocrom C oxidasa. En els espermatozoides de porc, l'exposició a llum vermella LED de forma discontínua amb un protocol específic de 10 minuts de llum, 10 minuts de foscor i 10 minuts d'irradiació una altra vegada (procés 10'-10'-10 ') augmenta tant els paràmetres de qualitat de l'esperma *in vitro* com les taxes de fertilitat *in vivo*, encara que sorgeixen diversos dubtes respecte a l'aplicació pràctica a la granja de l'estimulació amb llum vermella de l'esperma de porc. Aquests dubtes es deuen a un escàs coneixement sobre qüestions molt importants com, per exemple, els mecanismes d'acció de la llum vermella i com poder optimitzar els protocols d'irradiació utilitzats. Per això, aquesta tesi doctoral se centra en les següents preguntes:

- Els efectes de la llum vermella LED sobre els paràmetres de qualitat de l'esperma de porc i*n vitro* perduressin en el temps?
- L'activitat mitocondrial està relacionada amb els efectes observats de la irradiació amb llum vermella LED dels espermatozoides de porc?
- La irradiació amb llum vermella LED és una eina pràctica i factible per millorar les taxes de fertilitat en la cria de porcs?

Els resultats presentats en aquesta dissertació mostren que:

1. L'estimulació amb llum vermella LED dels espermatozoides de porc augmenta la seva resistència davant l'estrès tèrmic durant les primeres 48 h d'emmagatzematge a 17ºC.
2. L'estimulació amb llum vermella LED contribueix a el manteniment d'una estructura òptima de nucleoproteïnes de l'esperma de porc emmagatzemat a 17ºC durant 96 h.
3. Els efectes de la irradiació amb llum vermella sobre els espermatozoides de porc emmagatzemats a 17ºC durant 96 h depenen de el patró d'irradiació específic utilitzat.

4. Els efectes induïts per l'estimulació de la llum LED vermella en els espermatozoides de porc estan relacionats amb l'acció sobre l'activitat dels components fotosensibles del mitocondri, com la citocrom C oxidasa. Aquesta acció causarà com a conseqüència canvis significatius en l'activitat de la cadena d'electrons mitocondrials.
5. L'impacte de la llum vermella LED en l'activitat mitocondrial dels espermatozoides de porc depèn de l'estat funcional previ i precís de l'esperma i del temps d'exposició.
6. L'acció de la llum LED vermella a la cadena d'electrons d'esperma de porc no exclou altres mecanismes d'acció concomitants.
7. L'estimulació amb llum vermella LED de semen de porc abans de la IA en granges porcines pot tenir un efecte positiu en el rendiment reproductiu general d'aquestes granges. D'altra banda, l'efectivitat final general d'aquest mètode dependria d'altres factors, com el maneig adequat de la granja.
8. L'estimulació amb LED de llum vermella podria ser una forma econòmica i rendible de millorar la fertilitat i la prolificitat en les granges de truges, sempre que el maneig de les granges sigui adequat.

RIASSUNTO

L’esposizione a diversi tipi specifici di emanazioni luminose migliora i parametri di movimento e aumenta l'attività mitocondriale del citocromo C ossidasi degli spermatozoi. Tali cambi favorevoli si sono osservati in svariate specie animali, sia mammiferi, come il coniglio, i piccoli ruminanti, l’uomo, il cane o il toro, sia altri tipi di vertebrati, come il tacchino o la tilapia. Nel seme di suino l'esposizione discontinua alla luce LED rossa con un protocollo specifico di 10 minuti di luce, 10 minuti di oscurità ed ulteriori 10 minuti di irradiazione (processo 10'-10'-10 ') aumenta entrambi i parametri di qualità del seme *in vitro* ed i parametri di fertilità *in vivo*. Tuttavia sorgono numerosi dubbi sull'applicazione di questa tecnica a livello pratico, in quanto esiste una mancanza di informazioni su questioni molto importanti, come i meccanismi di azione della luce LED rossa o come l’ottimizzazione dei protocolli di irradiazione utilizzati. Perciò, questa tesi di dottorato vuol rispondere ad i seguenti quesiti:

- Quanto durano nel tempo gli effetti della luce LED rossa sui parametri di qualità del seme di verro *in vitro*?
- L'attività mitocondriale è correlata in maniera diretta agli effetti osservati nel seme suino irradiato con luce LED rossa?
- L'irradiazione con luce LED rossa è una tecnica pratica ed applicabile in allevamento per migliorare i parametri di fertilità dei suini?

I risultati presentati in questa tesi dimostrano che:

1. La stimolazione per mezzo de luce LED rossa del seme di suino aumenta la loro resistenza allo stress termico nelle prime 48 ore di conservazione a 17°C.
2. La stimolazione con luce LED rossa contribuisce al mantenimento di una struttura nucleoproteica ottimale del seme di maiale conservato a 17°C per 96 ore.
3. Gli effetti dell'irradiazione con luce LED rossa sugli spermatozoi di suino conservato a 17°C per 96 ore dipendono dal protocollo specifico di irradiazione utilizzato.
4. Gli effetti indotti dalla stimolazione per mezzo della luce LED rossa del seme di verro sono correlati all'azione sull'attività dei componenti fotosensibili dei mitocondri,

come la citocromo-C ossidasi. Questa azione causa cambiamenti significativi nell'attività della catena di elettroni mitocondriali.

5. L'impatto della luce LED rossa sull'attività mitocondriale del seme suino dipende dallo stato funzionale precedente del seme e dal tempo di esposizione.
6. L'azione della luce LED rossa sulla catena di elettroni del seme di maiale non esclude altri meccanismi d'azione concomitanti.
7. La stimolazione con luce LED rossa del seme di maiale prima dell'inseminazione artificiale può avere un effetto positivo sul rendimento riproduttivo generale dell'allevamento suino, ma solo nel caso in cui la gestione dell'allevamento sia adeguata.
8. La stimolazione con luce LED rossa potrebbe essere un metodo economico e redditizio per migliorare la fertilità e la prolificità negli allevamenti di scrofe, a condizione che la gestione dell'allevamento in questione sia appropriata.

ABBREVIATONS AND SYMBOLS

’	Minute
ºC	Celsius degrees
AI	Artificial Insemination
AKT II	Protein Kinase B II
ALH	Mean Lateral Head Displacement
AMPK	AMP-Activated Protein Kinase
AQN-1	Carbohydrate-Binding Protein 1
AR	Acrosome Reaction
ATP	Adenosine Triphosphate
BCF	Mean Frequency of Head Displacement
Ca^{+2} C	Ionic Calcium
cAMP	Cyclic Adenosine Monophosphate
CCO	Cytochrome C Oxidase
CIA	Intracervical Artificial Insemination
CLCs	Cholesterol-Loaded Cyclodextrins
Cm	Centimetre
CoA	Coenzyme A
DNA	Deoxyribonucleic Acid
DUAI	Deep Intrauterine Artificial Insemination
E. Coli	Escherichia Coli
FAD/FADH2	Flavin Adenine Dinucleotide
FCCP	Carbonyl Cyanide-4(Trifluoromethoxy)Phenylhydrazone
Fe	Ferro
GLUTs	Glucotransporters By Facilized Diffusion
GSK3α	Glycogen Synthase Kinase 3α A
H	Hydrogen
H^{+}	Hydrogen Ion
He	Helium
hsp 90	Heat-Shock Protein 90
IP3	Inositol Triphosphate

IVC	In Vitro Capacitation
J	Julius
JC-1	1H-Benzimidazolium,5,6-Dichloro-2-[3-(5,6-Dichloro1,3diethyl1,3-Dihydro-2H-Benzimidazol-2-Ylidene)-1propenyl]-1,3-Diethyl-, Iodide
LDH	Lactate Dehydrogenase
LDH-X	Sperm LDH
LED	Light-Emitting Diode
LIN	Linearity Coefficient
LNAase	L-Leucyl-B-Naphtylamidase
MAPK	Mitogen-Activated Protein Kinase
Min	Minute
mL	Millilitre
MMP	Mitochondrial Membrane Potential
mW	Milliwatts
NAD/NADH	Nicotinamide Adenine Dinucleotide
Ne	Neon
Nm	Nanometre
NO	Nitric Oxide
NSF	N-Ethylmaleimide-Sensitive Factor
O2	Oxygen
ºC	Grades Centigrade
OH-	Hydroxyl
Oligo A	Oligomycin A
p32	Protein Mitochondrial Matrix Of 32 KDa
PCIA	Post-Cervical Artificial Insemination
pH	Hydrogenions Potential
PhD	Doctorate
PI3K	Phosphoinositide 3-Kinase
PK	Pyruvate Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PSI	Spermadesin I

PSII	Spermadesin II
ROS	Reactive Oxygen Species
S	Sulphur
SGLTs	Sodium/Glucose Transporters
SNAREs	Attachment Protein Receptor
STR	Straightness Coefficient
TRPs	Transient Receptor Potential
TRPM8	Transient Receptor Potential M8
TRPV4	Transient Receptor Potential V4
UV	Ultraviolet
VAMP	Vesicle-Associated Membrane Protein
VAP	Mean Velocity
VCL	Curvilinear Velocity
VDAC2	Voltage-Dependent Anion Channel 2
VSL	Linear Velocity
WOB	Wobble Coefficient
ZP	Zona Pellucida

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bars), and samples irradiated with red LED light for 1 min (light grey bars), 5 min (dark grey bars) or 10 min (black bars), and subsequently stored at 17 ºC for 96 h.

Figure 5. Effects of light-stimulation on free cysteine radicals of sperm head proteins determined through dibromobimane.

Figure 6. Effects of light-stimulation on free cysteine radicals of sperm head proteins determined through dibromobimane in non-irradiated samples (white bars), and samples irradiated with red LED light for 1 min (light grey bars), 5 min (dark grey bars) or 10 min (black bars), and subsequently stored at 17 ºC for 96 h.

Manuscript II

Figure 1. Effects of light stimulation on percentages of membrane intact SYBR14+/PI-;

Table 1. Effects of photostimulation of boar spermatozoa on the mean motion parameters in the presence or absence of either oligomycin A or FCCP.

Figure 2. Effects of light-stimulation on the motile sperm subpopulation structure in the presence/absence of either oligomycin A or FCCP.

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Figure 8. Effects of light-stimulation on cytochrome C oxidase activity in the presence/absence of either oligomycin A or FCCP

Supplementary Figure 1. Representative plots corresponding to the determination of the O_2 consumption rate of boar sperm incubated in the presence/absence of either oligomycin A or FCCP.

Supplementary Figure 2. Effects of light-stimulation on percentages of spermatozoa with a non-intact acrosome (PNA-FITC$^-$) in the presence/absence of either oligomycin A or FCCP.

Manuscript III

Table 1. Comparison of farrowing rates and litter sizes mean values of all analysed farms between control and photo-stimulated groups.

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INTRODUCTION

1.- THE MAMMALIAN EJACULATE

The mammalian ejaculate is defined as the fluid comprised by sperm cells and secretions from the accessory sexual glands during ejaculation through the male reproductive tract. The whole ejaculate appears as a viscous, creamy, slightly yellowish or greyish fluid (Bonet et al., 2013). Under a biological point of view, the mammalian ejaculate is the foremost component of semen. In turn, semen is the result of mixing ejaculate with female secretions after mating and subsequent ejaculation inside the female genital tract. Taking all of these concepts into account, the behaviour and function of sperm in the *ex vivo* collected ejaculate will differ from that of cells placed inside the female genital tract after ejaculation. Thus, information collected from ejaculated sperm would be taken only as an approximation to the real sperm function inside the female. Focusing on boar, first ejaculation usually occurs around 5-6 months of life and boar reaches the full adultness status at about 1 year old (Martín, 1982; Hugues and Varley, 1984; Sancho, 2002; Cordova-Izquierdo et al., 2004). The average volume of boar ejaculate is between 200-300 mL, with a sperm total count in the range of $10*10^9$ sperm and $10*10^{10}$ sperm (Foote, 2002; Casas et al., 2010), whereas pH is in the range of 6.85-7.9 (Martín, 1982).

1.1.- Fractions of the mammalian ejaculate

Following the mechanistic of ejaculation, the mammalian ejaculate is divided in 3 fractions:

- Pre-sperm fraction: contains fluid from the prostate gland, seminal vesicles and bulbourethral gland. In boars, the volume of this fraction is about 10–15 mL. The pre-sperm fraction does not contain spermatozoa and usually has a transparent or clear appearance, with a distinct, species-specific smelling caused by the presence of pheromones. It serves to flush urine and bacteria out of the urethra Thibault et al.; 1993;(Illera-Marin et al., 1994; Setchell and Brooks, 1994; Bonet et al., 2013).

- Sperm fraction: The volume of this fraction in boars' ejaculate is approximately 70–100 mL and has a whitish colour, varying in other species from whitish to yellowish (Thibault et al.; 1993; Illera-Marin et al., 1994; Setchell and Brooks, 1994). Boar sperm fraction has a milky-white appearance. This fraction contains the 80-90% of sperm. Additionally, the sperm fraction also contains secretions produced by both the prostate and seminal vesicles. In pig farming, this fraction is the only one that is collected to produce seminal doses for artificial insemination (AI; see Bonet et al., 2013).

- Post-sperm fraction: The volume of this fraction in the boar is in the range of 150 mL-200 mL. Like in other mammalian species,sperm concentration of this fraction is usually below 10^6 spermatozoa/mL (Thibault et al., 1993; Illera-Marin et al., 1994; Setchell and Brooks, 1994;). In addition, boar post-sperm fraction contains secretions of gelatinous consistency coming from the prostate and bulbourethral glands (Bonet et al., 2013).

As a whole, mammalian ejaculate is composed by spermatozoa and by fluids secreted by vas deferens fluid and the accessory glands secretion, the contribution varying of each gland and section among species. The mixture of these secretions is known as seminal plasma. The following points are centred in a succinct description of these two main ejaculate components.

1.2.- Accessory glands

As indicated above, seminal plasma is the final result of the mixture of secretions from *vas deferens* and all accessory glands. The most important accessory glands in mammals are the following:

- Prostate: this gland is present in the majority of species with some exceptions such as the hedgehog (Thibault, 1993). Prostate secretion usually contains transglutaminase, which will induce the coagulation of ejaculate. This compound is absent in species in which ejaculate does not coagulates such as dog (Thibault, 1993). Furthermore, prostatic secretion in all species contains high levels of ions like zinc, calcium, magnesium potassium as well as high phosphatase activity (Thibault, 1993). Focusing

on boar, prostate secretion contains granules that are rich in neutral and carboxylated acid mucosubstances as well as immunomodulatory spermadhesins, such as PSI and PSII. These spermadhesins act by lowering the immune response of uterus against the ejaculate, whereas the agglutination induced by the granules increases the protection of sperm against the aggressive environment in which they are placed. Furthermore, prostatic acid phosphatases provide stability to the sperm plasma membrane, being also involved in the modulation of cellular metabolism by promoting sperm motility (Ekhlasi-Hundrieser et al., 2002; Manaskova et al., 2002; Garcia et al., 2006; 2008; Manaskova and Jonakova, 2008).

- Seminal vesicles: This gland is especially important in species such as guinea pig, horse, rat and human. Among the main components of its secretion are fructose and prostaglandins (Thibault, 1993). Additionally, the secretion of seminal vesicles is very rich in potassium ions, showing concentrations ten times higher than that of blood plasma (Cosentino and Condckett, 1986). Secretions from seminal vesicles in boar are also very rich in ions such as sodium, potassium, calcium, magnesium, manganese, as well as energy substrates like glucose, fructose and inositol (Baronos, 1971; Mann, 1975). Furthermore, seminal vesicles secretions contain approximately 80-90% of total seminal plasma proteins (Lavon and Boursnell, 1971). All of these components are important in the modulation of sperm functions like motility and the achievement of sperm capacitation status (Saiz-Cidoncha et al., 1991; Badia, 2003). Furthermore, secretion from seminal vesicles also contains spermadhesins, which are key in the promotion of sperm-oocyte interaction as well as in the protection of sperm against processes such as head-to-head agglutination (Harayama et al., 1994; 1999; 2000) and cell membrane peroxidation (Kordan et al., 1998). Finally, another very important component of this secretion is acid phosphatase, which is involved in sperm metabolism by maintaining plasmalemma integrity through the synthesis of antioxidative metabolites (Dostalova et al., 1994; Töpfer-Petersen et al., 1995; Calvete et al., 1996).

- Bulbourethral gland: This is especially prominent in boars. Bulbourethral gland secretion has a high content in sialoproteins (Thibault, 1993), which renders a highly viscous secretion that is mainly released at the end of the ejaculation creating a gel plug that prevents backflow of semen (Badia, 2003; 2006).

- Urethral gland: in men, its secretion occurs during sexual arousal (Thibault, 1993). The gland discharges its secretion into pre-sperm fraction of the human ejaculate (Cossu et al., 1988). These secretions also contain significant levels of L-leucyl-β-naphtylamidase (LNAase) activity, which acts as lubricant on the urethral walls (Cossu et al., 1988).

- Preputial glands: these glands are present in practically all mammals. The secretion from these glands contains pheromones that attract the female, thus favouring mating (Thibault, 1993).

2.- THE EJACULATED SPERMATOZOON

Mammalian spermatozoa are designed following a species-specific evolutionary strategy. Therefore, mature spermatozoa are very complex and specific cells, with very few common functional features among species. In this way, boar sperm is specifically designed to optimize fertilization following the whole reproductive strategy of this species. This strict specificity makes very difficult to draw an optimal profile for explaining the overall mammalian sperm function in a simple description such as that of this Introduction. Taking this into consideration, this point has been centred on boar sperm as the main target of this dissertation.

2.1.- Morphology of the boar spermatozoon

The mature boar spermatozoon is an elongated cell of about 43–45 μm in length with a specifically designed morphology to carry out the function which is intended for (Briz, 1994). The cell has two major distinguishable regions, the head and the tail, which are separated by a short linking segment called the connecting piece or neck. The head is flattened and contains the acrosome and a haploid nucleus, whereas the tail has a composed structure with three sections, the apical is the midpiece, the central is named the principal piece and the distal is the terminal piece. A flagellum crosses the three parts, becoming the central axis of the whole tail. Furthermore, the flagellum is covered in its midpiece and principal piece areas by a complex fibrous structure responsible to maintain

optimal motility patterns of sperm in each point of their time life (Bonet et al, 2013; See Fig. 1).

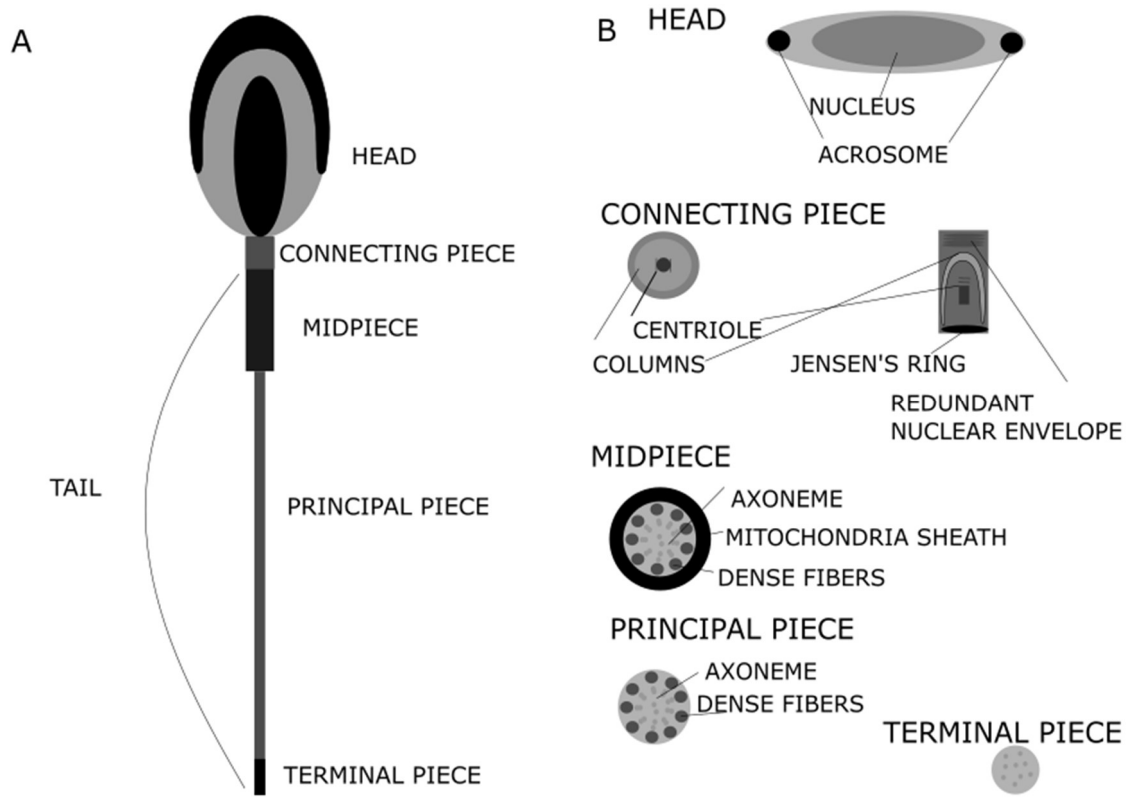


Figure 1. Schematic representation of a mammalian sperm. A: longitudinal representation of the whole spermatozoon. B: cross section at head, connecting piece, midpiece, principal piece and terminal piece showing the most important features of each section. Figures are not at the same scale in order to show details more easily (Adapted from Bonet et al., 2013).

Taking into account this overall description, the most important features of each segment of the whole sperm structure are the following:

- Head: The most important organelle of the sperm head is the nucleus. Sperm nucleus is flattened and ovoid-shaped. Sperm nucleus structure is very rigid. This rigidity is attributable to its extremely condensed and electrodense chromatin fibers. Accompanying the nucleus is the acrosome, a membrane-bound vesicle that forms a cap over the anterior part of the nucleus covering, in boar sperm, approximately 80 % of its length. The acrosomal content is mainly formed by a homogeneously packed set of hydrolytic enzymes, such as acrosin (Suter and Habenicht, 1998). The head area comprising the acrosome is called the acrosomal region. Three acrosomal segments

are clearly distinguishable under electron microscope, according to their electrodensity: the apical segment, the principal segment and the equatorial segment. The equatorial segment shows the highest electrodensity. The postacrosomal region is the head area that contains the postacrosomal dense lamina. The cell membrane enclosing the whole sperm head region is firmly adhered to the postacrosomal dense lamina but detaches very easily from the outer acrosomal membrane. The subacrosomal, or perinuclear space is the limit separating the nucleus from the inner acrosomal membrane or the innermost face of the postacrosomal dense lamina (Bonet et al, 2013).

- Neck or connecting piece: this is a small linking segment between the base of the sperm head and the midpiece. It is trapezoid in shape. The major components are a basal plate, the basal body, redundant nuclear envelope and the centriole at an angle of 45° relative to the axoneme (Bonet et al., 2013). The complexity of this structure can be explained by the main functions of the neck. The basal body is responsible of the transmission of movement to the tail and to the head (Wan and Goldstein, 2016). The redundant nuclear envelope also plays a role as an intracellular calcium store derived from calreticulin-containing vesicles present in germinal cells before spermiogenesis (Publicover et al., 2007). The centriole is involved in the formation of the mitotic spindle during the first mitosis of the zygote (Thibault et al., 1993). As well, in the neck is where the columns and the fibrous structure that continue in the tail are originated.

- Tail: as mentioned above, three regions are distinguishable in the tail, the midpiece, the principal piece and the terminal piece. The axoneme that starts at the conneting piece is stretched and thus developed into the flagellum along the three pieces of the spermatozoon tail. The midpiece contains the mitochondrial sheath. This sheath consists of about 200 elongated mitochondria lying end to end in a helical arrangement around the underlying axoneme. The principal piece is the longest segment of the spermatozoon tail, extending from the annulus or Jensen's ring to the proximal end of the terminal piece. The Jensen's ring consists of an electrodense ring-shaped structure underlying the plasmalemma, marking the junction of the midpiece and the principal piece. The mitochondrial sheath is replaced by the fibrous sheath in the principal piece. It is formed by two continuous longitudinal axes or columns

(dorsal and ventral) that are coplanar placed with respect to the central microtubule pair. These two columns are joined by a series of circumferential ribs regularly distributed around the principal piece. The thickness is maximal in the first third and decreases throughout the principal piece. The terminal piece is the last and shorter segment of the spermatozoon tail. At this point, the microtubular structure that forms the flagellum becomes disorganized, losing thus the axoneme its main characteristics (Bonet and Briz, 1991; Briz et al., 1993; 1995; Briz, 1994; Bonet et al., 1994; 2000; 2006; Bonet et al., 2013).

It is noteworthy that the maintenance of a complete and intact structure of the whole cell is essential for maintaining sperm full fertilizing ability. In this sense, if a significant percentage of sperm in an ejaculate shows malformations in any structure, the ejaculate will be likely subfertile or, in the worst of cases, infertile.

2.2.- Functionality of ejaculated boar sperm. Sperm capacitation

The ultimate goal of sperm is to fertilize an oocyte. However, in mammals this goal is only reached by a very low number of sperm in an ejaculate. This aspect is related with the fact. That, immediately after ejaculation, mammalian sperm must undergo a full series of function changes that allow reaching the fertilization site for a selected pool of sperm cells. For this purpose, ejaculated sperm have a very active and dynamic functionality, which allows them to fulfil the ultimate target of fertilization. In this sense, it must be reminded that the freshly ejaculated sperm is not able at this precise moment to penetrate an oocyte. Sperm must undergo a whole series of physiological and biochemical changes during their journey inside the female genital tract before being competent for fertilization. This whole series of changes, which were firstly described in 1951 in rats and rabbits, are known as sperm capacitation (Austin, 1951; Chang, 1951; 1959; Suarez, 2007; Signorelli, et al., 2012).

It is noteworthy that the time that sperm needs to achieve the full capacitation status varies depending on the species. This is due to the time spanned between the ejaculation inside the female genital tract and the subsequent ovulation varies from hours to days, depending on the species (Fraser, 2010; Bonet et al., 2013). In pigs, this time lapse is about 2h (Botto et al., 2010). This implies that *in vivo* capacitation of boar sperm is achieved in a minimal time of 2 hours. The complete sequence of steps to achieve the capacitation status is not

full known yet (Visconti, 2009; Fraser, 2010). One of the aspects that makes difficult the complete understanding of the capacitation process lies in the difficulty of extrapolating *in vitro* results to *in vivo* without breaking the homeostasis of the female genital tract (Rodríguez-Martínez, 2007). This explains the pre-eminence of *in vitro* studies regarding capacitation mechanisms studies (Bonet et al., 2013). However, information yielded by these *in vitro* studies can be biased because they do not take into account all of the components, from substrates to environmental conditions, present in *in vivo* conditions. Considering these limitations, it is known that capacitation is launched through the activation of sperm cAMP-dependent protein kinase A (PKA). The PKA activation seems to be induced through two concomitant ways. The first way is the presence in seminal plasma of agents that directly activates PKA, such as prostaglandins (Jiménez et al., 2003; Wu et al., 2006). Concomitantly, the start of the process is simultaneously induced by the reorganization of the whole cell membrane structure due to the loss of cell membrane cholesterol. This loss, in turn, makes cell membrane more permeable to the uptake of extracellular bicarbonate and calcium. The uptake of these products together with the stimulating action of seminal plasma factors such as the Fertilization Promoting Peptide (FPP) and adenosine will in turn increase intracellular cAMP, activating thus PKA (Baldi et al., 1996; Visconti et al., 1999; Funahashi et al., 2000a; b; Gadella et al., 2008; Visconti, 2009). Following the PKA activation, sperm will undergo a sequential series of function changes, from which the most important are the following:

- Loss of cell membrane cholesterol, with the subsequent increase in membrane fluidity (Harrison et al., 1996; Gadella and Harrison, 2002; Harrison and Gadella, 2005).

- Following the up-regulation of key cellular signalling pathways linked to the cAMP rise, there will be the activation of other intracellular molecular cascades. One of the most important is the activation of calcium-responsive pathways. These are in fact related with the activation of the cAMP-dependent pathways, being of great importance in the activation of calcium fluxes that will subsequently increase intracellular calcium levels (Adeoya-Osiguwa and Fraser, 2003)
- Changes in flagellar activity which will concomitantly modify sperm kinematic parameters (Cancel et al. 2000; García-Herreros et al., 2005; Aparicio et al., 2007). These changes conform a characteristic motion pattern that is known as hyperactivated motility. The hyperactivated motility is characterized by rapid changes

of linearity patterns together with high velocity and strength (Yanagimachi, 1994). In boar sperm, hyperactivated motility is characterized by an increase of motion parameters values like those of curvilinear velocity (VCL), linear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR) and wobble coefficient (WOB; see Rivera et al., 2008). The mechanism/s by which hyperactivated motility is launched are complex and linked to the subsequent activation of several protein kinases due to the previous activation of the PKA (Lackey and Gray, 2015). Thus, activation of protein kinases such as glycogen synthase kinase 3α A (GSK3αA), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase bII (Akt II) and AMP-activated protein kinase (AMPK) are involved in the achievement of hyperactivated motility (Aparicio et al., 2007; Lackey and Gray, 2015; Hurtado de Llera et al., 2016).

- Exposure of carbohydrate-binding protein 1 (AQN-1) from the acrosomal surface. The AQN-1 is a protein included in the spermadhesin family that have a high affinity for binding to glycoconjugates of the zona pellucida (ZP; see Calvete et al., 1997). Upon anchoring sperm to the oocyte through the ZP, the signalling cascade leading to acrosomal exocytosis of sperm is activated allowing sperm to pass through the zona pellucida (Töpfer-Petersen et al., 2008).

- Overall increase in the phosphorylation status of many sperm proteins linked to the above-mentioned PKA-launched activation of a whole array of separate sperm protein kinases (Kalab et al., 1998; Petrunkina et al., 2004). At this respect, as indicated above, the achievement of boar sperm capacitation is concomitant with an increase of p32 tyrosine phosphorylation levels (Tardif et al., 2001). This protein has been subsequently identified as pro-acrosin (Polakoski, 1977; Phi-Van et al., 1983; Fini et al., 1996). Indirect immunolocalization showed that phosphorylated p32 was also redistributed through the acrosome during capacitation, being thus the combination of an increase of tyrosine phosphorylation and relocation the mechanisms implied in the activation of pro-acrosin to acrosin (Tardif et al., 2001; Puigmulé et al., 2011).
- The outer acrosome membranes become fusogenic (Flesch and Gadella, 2000). This phenomenon is linked to the relocation of several proteins included in the proteins from the Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor (SNAREs) family such as syntaxin1, syntaxin 2 and vesicle-associated

membrane protein (VAMP) from the apical ridge of sperm head to a more homogeneous distribution exactly matching the site of sperm zona binding and subsequent induced acrosomal exocytosis (Tsai et al., 2007; 2010). On the other hand, the presence of AQN-1 allows the recognition of the zona pellucida of the oocyte (Calvete et al., 1997).

2.3.- Sperm energy management during the achievement of capacitation

The above described capacitation-linked molecular events require a strict modulation of energy metabolism during the entire process. Thus, mammalian sperm, including those of boar, can use lactate, pyruvate, glycerol and citrate to obtain energy (Jones et al., 1992; Jones, 1997; Albarracin et al., 2004; Medrano et al., 2006a; Storey, 2008). From these metabolites, whereas citrate is metabolised through glycolysis, others are further processed through mitochondrial oxidative phosphorylation power (Baker and Aitken, 2004). However, there is general agreement in considering monosaccharides as the main energy source for mammalian ejaculated sperm. In fact, non-monosaccharides metabolites are less efficient to yield ATP than monosaccharides, although they are important in synthesizing NADH as the most important reductive power of the cell (Baker and Aitken, 2004; Medrano et al., 2006b).

Energy production from monosaccharides of mammalian sperm can be carried out through two main pathways, namely glycolysis and mitochondrial oxidative phosphorylation as a subsequent extension of the glycolytic pathway (Eddy et al., 2003; Storey, 2008; Matsuura et al., 2017). The importance of each of these pathways for ATP production depends on the animal species (Mann et al., 1975; Medrano et., al 2006a; Storey, 2008). The most important pathway found in boar sperms is glycolysis (Marin et al., 2003). Glycolysis is essential for the movement of sperm (Mukai and Okuno, 2004), especially to regulate the concentration of ATP in the distal part of the tail (Takei et al., 2014). At this respect, there are four factors that maximize ATP production through a tail sperm-focused system (Mukai and Travis, 2012):

- The overall ratio surface/volume in the tail area is large.

- The cytoplasmic space between the plasma membrane and fibrous sheath is almost virtual.

- There is a large amount of glycolytic enzymes placed through the whole tail.

- There is a high potential usage of ATP by the tail dynein ATPases, drawing the reactions in the forward direction through the entire tail structure.

Boar sperm requires a strict modulation system of glycolytic rate to maintain their function. The glycolytic pathway has several important checkpoints that allow cells for this strict control. The first of these points is the regulation of monosaccharides uptake. In eukaryotic cells, this uptake is performed through specific hexoses transporters that can belong to two separate protein families, the sodium/glucose transporters family (SGLTs, see Turk and Wright, 1997) and the GLUTs family (Bell et al., 1990). Mammalian sperm contain transporters from both SGLT and GLUT families (Scheepers et al., 2004; Bucci el al., 2011), although the most relevant studies so far are those pertaining to the GLUT family. From these, GLUT 1, GLUT 2, GLUT 3 and GLUT 5 have been found in sperm from separate mammalian species such as dog, horse and donkey sperm (Angulo et al., 1998; Bucci et al., 2010a; b). Regarding boar, GLUT 1, 2, 3 and 5 have been described and localized (Bucci et al., 2010a). It must be stressed that each GLUT has separate affinities for specific monosaccharides, presenting separate locations in the spermatozoon. Thus, whereas both GLUT 1 and GLUT 2 have high affinity for glucose, although they can also transport other monosaccharides, GLUT 3 is almost exclusively devoted to glucose transport and GLUT 5 is fructose-specific (Elsas and Longo, 1992; Scheepers et al., 2004). In this sense, it is noteworthy that in boar sperm, whereas GLUT 1 was localized along the whole sperm tail and in the acrosomal membrane with a spotted pattern (Bucci et al 2010a), GLUT 2 and GLUT 3 were localized in the acrosome, with further GLUT 3 location at the head subequatorial band and a faintly reactivity at the tail. On the contrary, GLUT 5 was mainly located at the tail, with a fainter reactivity at the acrosome (Bucci et al., 2010a). This would imply that whereas glucose would be uptaken in the whole sperm in a similar rhythm, fructose does not; having a more intense fructose uptake at the tail. Furthermore, achievement of *in vitro* capacitation (IVC) and subsequent acrosome reaction (AR) do not affect their disposition. We can only speculate about the biological significance of this GLUTs location, although

the specificity in the location of each GLUT combined with their monosaccharide specificity seems to indicate that there would be separate glucydic metabolic pathways depending on each GLUT location in sperm (Medrano et al., 2006b; Bonet et al., 2013).

The second glycolysis checkpoint is the phosphorylation rate of the uptaken monosaccharides through specific hexokinases. It is important to stress that boar sperm have different efficacy rates of ATP production through glycolysis depending upon the specific metabolized hexose. The most efficient of them is glucose, which induces a fast and intense activation of glycolysis (Jones and Conor, 2000; Medrano et al., 2006b). This effect is related with its much greater sensitivity to hexokinase than that of other monosaccharides as mannose, fructose or sorbitol (Medrano et al., 2006b). Otherwise, the absence of fructokinase in boar sperm could explain the difference between glucose and fructose (Medrano et al., 2006b).

Glycolytic rate is also modulated by the activity of the control enzymes of its final stages. From these, pyruvate kinase (PK) is the most important checkpoint. This is due to that a saturation of PK activity will limit the overall glycolysis rate. In this way, PK kinetics is key for the overall glycolysis rate (Odet et al., 2011; Margaryan et al., 2015; Liu et al., 2016).

Finally, the last key modulatory point on monosaccharides metabolism that will be mentioned here is that linked to lactate dehydrogenase (LDH) activity. The regulation of LDH is very important, since this enzyme regulates the entry of monosaccharide substrate derivatives into the mitochondrial oxidative phosphorylation through transformation of pyruvate in lactate and conversely (Kohsaka et al., 1992). In this way, LDH activity will modulate the fate of the final glycolytic derivative products, which will be derived either to be subsequent secreted to the extracellular environment or to be converted to acetyl CoA to be subsequently processed through the mitochondrial oxidative phosphorylation (Medrano et al., 2006b). Sperm LDH is an isoenzyme that has been described in several species (Coronel et al., 1983; Pan et al., 1983; Pawlowski and Brinkmann, 1992; Jones, 1997). In the specific case of boar sperm, it has been shown that the sperm-specific LDH isoform (LDH-X) is the most important form found (Medrano et al., 2006a). LDH is located in the head, midpiece and tail of spermatozoa (Kohsaka et al., 1992). This enzyme is also very important to regulate the reductive potential NAD^+ in sperm (Jones, 1997).

Thus, the combination of the LDH-X regulatory functions on both oxidative phosphorylation rate and $NADH^+$ synthesis will allow boar sperm for yielding enough energy and reductive power to keep all the key sperm functions along their journey inside the female genital tract (Medrano et al., 2006a).

2.4.- Main roles of mammalian sperm mitochondria

As indicated above, pyruvate obtained from the metabolism of monosaccharides through the glycolytic pathway can be transformed in acetyl CoA in order to be subsequent metabolized into the mitochondrial oxidative phosphorylation pathway. This pathway is composed by three consecutive reaction chains, namely, the Krebs cycle, the electronic chain and the chemiosmosis.

- Krebs cycle: The Krebs cycle, also known as tricarboxylic acid or citric acid cycle, is the gateway to the aerobic metabolism of any molecule that can be transformed into an acetyl group or dicarboxylic acid (Krebs and Johnson, 1980; Berg et al., 2002). The cycle includes a series of oxidation-reduction reactions that result in the oxidation of an acetyl group to two molecules of carbon dioxide. The cycle removes electrons from acetyl CoA and then derives these electrons to form NADH and $FADH_2$ (Krebs and Johnson, 1980).

- Electron transport chain: The electron transport chain is composed by four complexes and a coenzyme (Ahmad and Kahwaji, 2018). In this chain, electrons released during deoxidation of NADH and $FADH_2$ in the Krebs cycle are flown through a series of membrane proteins and organic molecules to generate a proton gradient across the membrane.

    - Ubiquinone oxidoreductase (Complex I): In this complex, NADH is oxidized and the electrons are transferred from NADH to coenzyme Q while translocating a proton across the membrane (Ahmad and Kahwaji, 2018).

    - Succinate dehydrogenase (Complex II): In this complex, $FADH_2$ is oxidized and passes one electron per each oxidized molecule down to Coenzyme Q.

This complex does not translocate protons across the membrane and less ATP is produced with this pathway (Ahmad and Kahwaji, 2018).

- Coenzyme Q (ubiquinone): It is as an electron carrier and transfers 2 electrons to complex III. It is a redox reaction of coenzyme Q that allows the transfer of electrons (Ahmad and Kahwaji, 2018).

- Cytochrome oxidoreductase (Complex III): It is formed by cytochrome B and cytochrome C proteins. Cytochrome C accepts 1 electron from ubiquinone and passes it to complex IV while pumping protons across the membrane (Ahmad and Kahwaji, 2018).

- Complex IV: It is formed by the cytochrome C, the cytochrome A and the cytochrome A3. In this complex, oxygen is reduced and through the capture of 2 protons per each oxygen molecule (Ahmad and Kahwaji, 2018). Protons that were captured by oxygen molecules are subsequently flown through to generate a proton-motive force that facilitates the intra-membranous proton transport (Berg et al., 2002). These protons will be finally needed to activate the following, final step of oxidative phosphorylation, namely chemiosmosis.

- Chemiosmosis: this step constituted the generation of ATP thanks to the formation of a protons flux through the internal membrane of mitochondria (Mitchell and Moyle, 1967; Berg et al., 2002). The energy that the electron chain uses to pass protons from the matrix of the mitochondria to the intermembrane space creates an electrochemical gradient of protons that is used to produce ATP through the activation of the electrochemical-dependent mitochondria ATP synthase. The synthesis of ATP also renders a proton for each synthetized ATP. This proton finally returns to the mitochondrial matrix (Berg et al., 2002), whereas the final acceptor of electrons of the chain is oxygen (Berg et al., 2002; See Fig. 2).

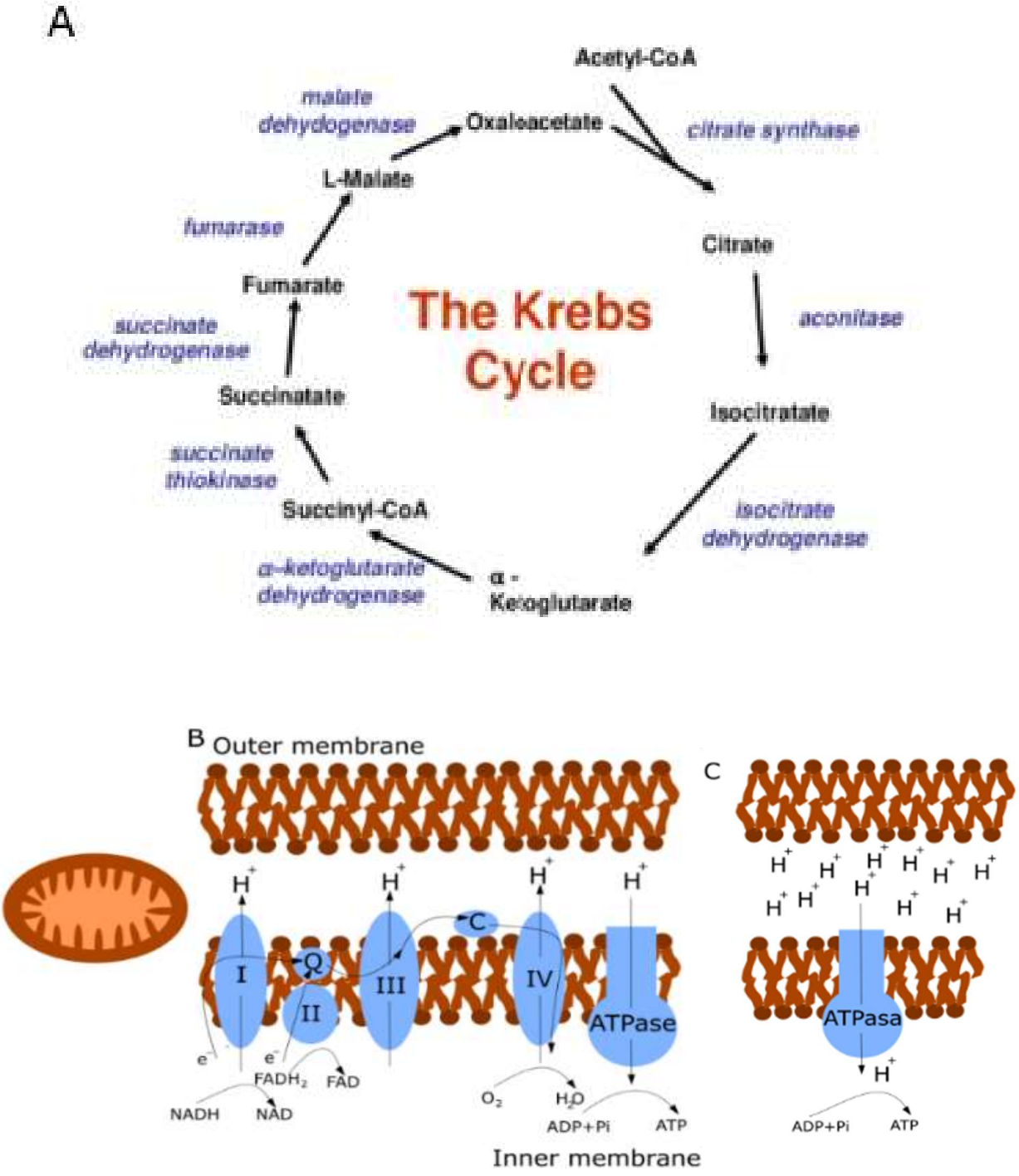


Figure 2. schematic representation of the mitochondria oxidative phosphorylation. A: scheme of the Krebs cycle. B: scheme of the electro chain. C: scheme of chemiosmosis. All schemes are drawn to only shown the most important features of each step (From Ahmad and Kahwaji, 2018).

2.4.1- Oxidative phosphorylation in spermatozoa

Mitochondria are found only in the midpiece, thus oxidative phosphorylation occurs only at this level (Bonet and Briz, 1991a; Briz et al., 1993; 1995; Briz, 1994; Bonet et al., 1994; 2000; 2006). In boar sperm, the pyruvate needed to fuel the Krebs cycle can be obtained from glycolysis or through the uptake of extracellular lactate. In both cases, lactate will be transformed to acetyl CoA by means of LDH-X activity (Medrano et al., 2006a). The maintenance of a correct energy production ratio between glycolysis and oxidative phosphorylation depends on several factors such as O_2 pressure, intracellular levels of ATP or intracellular concentrations of metabolic modulators such as nitric oxide (NO; see Peña et al., 2009). In any case, the amount of ATP yielded by boar sperm mitochondria is not enough to support motility (Turner, 2003). Thus, boar sperm require glycolysis-synthesized ATP to maintain the overall sperm function (Marin et al., 2003).

2.4.2- Regulation of sperm death/sperm capacitation

Although energy production is a very relevant role of sperm mitochondria, there are several features that suggest the existence of other mitochondrial roles that can be equally or even more relevant than ATP synthesis for maintaining overall sperm function. Thus, it is noteworthy that all mammalian sperm studied so far have a similar number of mitochondria, although the relative importance of oxidative phosphorylation contribution to the final sperm yielding is very variable among species (Martin et al., 2003; Baker and Aitken, 2004; Mukai and Okuno, 2004). In fact, there are several mitochondrial roles that are of the greatest importance for maintaining mammalian sperm function. Thus, the maintenance of both the appropriate redox balance and the reductive strength by modulating the $NADH/NAD^+$ balance as well as the reactive oxygen species (ROS) production rate, and the buffering of intracellular pH, would be very relevant (Rodriguez-Gil, 2006). Centering on ROS buffering, mitochondria generate these ROS through the activity of complexes I and III of the respiratory chain (Krahenbuhl et al., 1991; Gonzalvez and Gottlieb, 2007). The spermatozoon has different antioxidants to protect from excessive intracellular ROS levels (Sies, 1993; Saleh and Agarwal, 2002). Protection against excessive ROS is a very important issue, since spermatozoa are particularly susceptible to damage induced by oxidative stress because their plasma

membranes contain a high proportion of polyunsaturated fatty acids (Alvarez and Storey, 1995; Saleh and Agarwal, 2002). Concomitantly, sperm cytoplasm contains relatively low concentrations of scavenging enzymes (Jones et al, 1979; Aitken and Fisher, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996; Saleh and Agarwal, 2002). In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by the seminal plasma antioxidant (Iwasaki and Gagnon 1992; Zini et al, 1993; Saleh and Agarwal, 2002).

Another very important role of sperm mitochondria is the modulation of intracellular calcium levels through the decrease of the mitochondria membrane potential (Publicover et al., 2007; Peña et al., 2009). This is important, taking into account the key role of the release of intramitochondrial calcium together with the increase of extracellular calcium uptake in the achievement of the capacitation status (Marquez and Suarez, 2004).

A very interesting question that arises from all of the above described mitochondrial roles is that all of them are related with the control of apoptosis in somatic cells (Erkkila et al., 2006; Lesnefsky and Hoppel, 2006; Ott et al., 2007; Peña et al., 2009). There is some controversy regarding the existence and role of apoptosis in mammalian sperm. From all of this controversy, it seems to surge the image that there is a close relationship between molecular mechanisms linked to apoptosis and those implied in the rendering of sperm capacitation status. In this way, the mitochondria-generated ROS is very important to release cytochrome C, another pro-apoptotic protein, outside of mitochondria. This release, in turn, will activate the intrinsic apoptosis pathway via caspase 3 activation (Mishra and Shana, 2005; Chan 2006; Tsujimoto and Shimizu, 2007). On the other hand, anions such as peroxides, superoxides and peroxynitrites act on cysteine residues of tyrosine phosphatase, inducing thus its inactivation (Aitken and Nixon, 2013). This is important in the maintenance of the overall protein phosphorylation levels as a key factor to maintain sperm function. Superoxide also participates in the direct activation of soluble adenylate cyclase, increasing thus intracellular levels of cAMP (Aitken and Nixon, 2013). Furthermore, the oxidative stress produced by the generation of ROS potentiates the decrease of cholesterol in the membrane, making it more permeable to calcium (Morales et al., 2012). Combining thus all of these actions, a key role for the mitochondrial modulation of intracellular ROS levels, as well as its modulation of apoptotic pathways,

will imply the existence of a key role for the overall mitochondria activity in the regulation of the correct achievement of sperm capacitation status (Fig. 3).

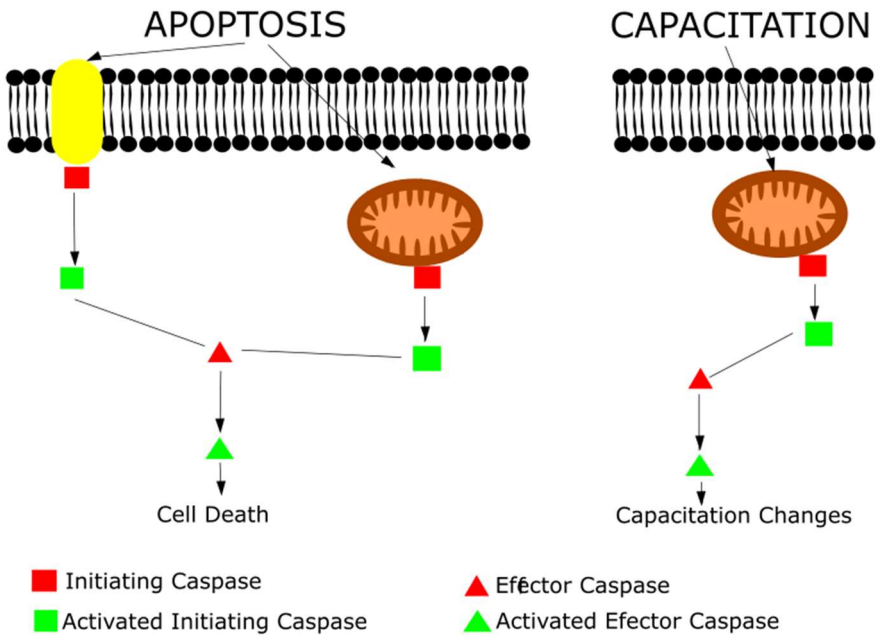


Figure 3. Schematic representation of the main molecular pathways that can be considered as common for the launching of both apoptosis and sperm capacitation. There are represented only the most relevant common points between both ways.

2.5.- Sperm subpopulations

Mammalian ejaculate, including that of boar, presents a hierarchical structure in its composition when sperm are evaluated following parameters like motility (Holt, 1996; Quintero-Moreno et al., 2003; 2004; 2007; Satake et al., 2006; Dorado et al., 2011a), morphology (Rubio-Guillé et al., 2007) or mitochondrial activity (Ramió-Lluch et al., 2011). Although the biological significance of this hierarchical, subpopulational structure is not well understood, several publications suggest a role of subpopulations in the optimization of the fertilizing ability of ejaculates. Thus, during the achievement of IVC of boar sperm is linked with an increase on the percentage of motile sperm subpopulation with the highest values of velocity and the lowest values of linearity as well as a concomitant increase of other subpopulation with high values of both velocity and linearity (Ramió-Lluch et al., 2008). Furthermore, IVC also induces an increase in the percentage of sperm included in the subpopulation with the highest mitochondria activity when determined by the JC-1 technique (Ramió-Lluch et al., 2011). Likewise,

cryopreservation also modifies the subpopulational structure in different species such as bull (Rubio-Guillén et al., 2007; Muiño et al., 2008; Ferraz et al., 2014), dog (Peña et al., 2012), boar (Flores et al., 2008b; 2009) and donkey (Flores et al., 2008b). Modifications on subpopulational structure have been also observed to be induced by centrifugation (Dorado et al., 2011b), being these changes related with the specific resistance to cryopreservation of a specific ejaculate (Quintero-Moreno et al., 2004; Flores et al., 2008a; b; 2009).

3.- ARTIFICIAL INSEMINATION IN PIG FARMING

The AI is a key technology for the development of intensive production farming (Bonet et al., 2013). In pig farming, AI started to be routinely utilized in farms at the eighth decade of the 20th century, since it was during that decade that all the previous work finally reached the moment for the profitable application of AI in farm conditions. Currently, commercial AI in pig farming is based on the utilization of seminal doses that are stored in refrigeration at about 15ºC-17ºC. Concomitantly, the utilized commercial semen extenders have as common features a high glucose content as well as strong buffering substances that maintain the pH of the extender in the physiological range. In these conditions, boar semen can be stored without a significant loss of their fertilizing ability for as long as fifteen days (Pursel and Johnson, 1975; Gottardi et al., 1980; Martín-Rillo, 1984; Johnson et al., 1988; 2000; Gadea, 2003; Szymanowicz et al., 2019). Currently, more than 90% of the farms devoted to intensive production worldwide uses AI (Gerrits et al., 2005; Vyt, 2007; Bonet et al., 2013; Knox, 2016). At the beginning of the implantation of AI, it was standardized and all farms used Intracervical Artificial Insemination (CAI). In this way, more that ninety percent of European and North American farms used it in the 2000's years. The broad implantation of AI in pig farming is due to a series of factors. Among these, there are a significant decrease in the cost per pregnant sow, an easier and faster access to new improving genetic lines in order to optimize production and an easier implantation of sanitary procedures in sows' farms (Reed, 1985; Crabo, 1990; Johnson et al., 2000; Maes et al., 2008; 2011). In the last years, the porcine industry has changed quickly. Now, every day Post-Cervical Artificial Insemination (PCAI) is increasingly used in Europe, gradually replacing traditional CAI. The increasing use of PCAI is based in factors such asits simplicity or the reduced sperm

cells number utilized in each AI. Furthermore, PCAI can be easily combined with other reproductive biotechnologies, such as the use of frozen-thawed sperm, fixed-time AI or sperm-mediated gene transfer, facilitating thus the application of these technologies in pig farming (Watson and Behan, 2002; Bortolozzo et al., 2015; García-Vazquez et al., 2019).

The main goal of optimizing AI is based on two concomitant concepts. The first concept is the minimization of the time elapsed between the moment of insemination and that of ovulation. The second one is the deposition of semen doses as near as possible to the oviduct. Regarding the minimization of the time between AI and ovulation, it is noteworthy that this synchronization is already observed in *in vivo* conditions. The *in vivo* synchronization is based in three concomitant mechanisms: (Bonet et al., 2013).

- Olfactory, auditory and mechanical stimuli induced by the boar during mating. These stimuli will induce an increase of the rhythm and intensity of uterine contractions in the sow, which will facilitate the arrival of sperm to the oviduct. Additionally, a short, daily exposure of sows to boar has beneficial effects in the improvement of follicle development, ovulation induction and stimulation of myometral contractions, allowing thus for optimizing the AI timing (Ulguim et al., 2018).

- Mechanical dilation of cervix by the penis inside the cervix during mating as well as uterine dilation induced by the placement of AI doses into uterus will induce neuro-endocrine signals in a similar manner to that described for the utero-hypophyseal reflex induced by the foetus during the first steps of the expulsive phase of partum (Pawlinski et al., 2017).

- Ovulatory-inducing compounds present in the ejaculate. The seminal plasma contains substances like oestrogens and prostaglandins that potentiate the uterine mechanical reflex induced by penis and ejaculate (Pawlinski et al., 2017). Furthermore, these substances also yield a direct signal on ovarian follicles accelerating their rupture (Langendijk et al., 2000).

Otherwise, the currently utilized AI protocols, namely the CAI protocol and the PCAI one, are designed to place semen as near as possible to the oviduct. Centring on the CAI, its first feature is the stimulation of ovulatory signals in the sow by passing the boar in

front of the sow to launch the olfactory and auditory stimulus above described. This boar-induced stimulus is complemented by a mechanical stimulation exerted by the farmer on the sow, which will also show the presence of a strong sow's standing reflex as a sure signal for oestrus. Then, the vulva is thoroughly cleaned before the introduction of the intracervical catheter, named spirette. The spirette must be introduced slowly, since this is the best manner to induce an optimal mechanical stimulation in the cervix. Then, a mechanical flank pressure is applied by the farmer at the same time that the seminal dose is slowly introduced into the female through the spirette. Semen deposition must be carried out slowly, because a too fast deposition will cause a backflow out through the vulva. Moreover, the spirette catheter must be left in place for several minutes, either standing static or while applying a rotational movement of the *s*pirette inside the cervix by hand in order to imitate the mechanic stimulation induced by the penis *in vivo*. This process can be repeated a maximum of three times each oestrus (Bonet et al., 2013).

The PCAI system is very similar to that of the CAI. The main difference, however, is centred in the spirette. Thus, the PCAI spirette is formed by two elements. The first element is a catheter similar to that utilized in the CAI system, whereas the second one is a narrow, flexible catheter, which is placed inside the other catheter. The role of the narrow element is to pass through the cervix and reach the uterine body, depositing thus the semen in a much more cranial position in the genital tract than that performed by the CAI system (Roberts and Bilkei, 2005; Roca et al., 2006; Garcia-Vazquez et al., 2019). In this way, the near-oviduct placement of semen doses causes that the boar-induced stimulatory effects on sow were less important in the PCAI system when compared with the CAI one, becoming thus expendable. On the other hand, this placement also implies the utilization of much less sperm, extended in a much smaller volume, with the consequent advantages from the economical point of view. This implies that the implementation of PCAI is increasing in the last years on farms around the world (Bortolozzo et al., 2015).

Finally, the Deep Intrauterine Insemination (DUAI) could be another possibility to inseminate sows using a catheter longer and less flexible than PCAI and less volume of ejaculate, because semen is placed even closer to oviduct than in PCAI (Martinez et al., 2001). However, this is not practical in a farm because this is a more difficult technique, which needs the utilization of an endoscope, timing is long (more than 4 minutes per sow)

and fertility rates are not better than those yielded by conventional AI (Martinez et al., 2001). Furthermore, the incidence of unilateral fertilizations is much greater after applying DUAI when compared with both CAI and PCAI, decreasing thus prolificacy (Martinez et al., 2006).

4.- LABORATORY ANALYSIS OF SEMEN QUALITY

AI-utilized semen has to be previously tested for evaluating their fertilizing ability. For this purpose, a whole array of laboratory parameters have been developed and integrated in the semen quality analysis. The classic semen quality analysis consists in the determination of parameters such as sperm concentration, total and progressive motility and different stains to measure viability, sperm membrane integrity and morphology. However, results obtained from these parameters are not strongly correlated with the *in vivo* fertilizing ability of the analysed sample (Hirai et al., 2001; Matsuura et al., 2017). In spite of the low predictive ability of the classical semen analysis, it is important to carry out it because it can be a feasible marker for semen alterations such as an excessive rhythm of extraction, epididymal problems or teratozoospermia, which will lead to a decrease in the *in vivo* fertilizing ability of samples. At this moment, the obtaining of adequate information regarding the fertilizing ability of a boar semen dose requires the launching of a whole arrays of analysis. From these, the most important would be the evaluation of plasma membrane integrity and fluidity, sperm osmotic tolerance, the achievement of the capacitation and/or apoptosis status, nuclear integrity, mitochondria function, levels of ROS, acrosome integrity and the activity of different metabolites and enzymes. However, the application of all of these techniques is very expensive, being thus fairly impractical for the majority of boar farms, which will carry out quality semen analysis based upon only some, but not all, of these parameters (Bonet et al., 2013).

5.- POSSIBLE WAYS OF IMPROVEMENT OF ARTIFICIAL INSEMINATION

5.1.- Succinct description of improving techniques for AI

The currently applied pig farming AI techniques are very efficient, reaching very high rates of both fertility and prolificacy. This implies that any future improvement of these techniques will not be easy to reach, since the applied putative improvement/s might have more efficiency with, at least, the same economical cost. At this moment, there are three main technologies that are in study to improve farm AI results or, at least, to complement farming AI in some specific situations. These technologies are the use of frozen semen, the utilization of sex-sorted sperm and the application of photostimulation of refrigerated samples before AI.

- AI with frozen sperm: Currently, the *in-farm* application of AI with frozen semen is very limited in pigs. This is due to that AI with frozen semen has lower values of *in vivo* fertility and prolificacy than that obtained with refrigerated samples. Furthermore, the economic cost of the frozen doses is much higher than those of refrigerated samples (Bailey et al., 2008; Yeste et al., 2017). Despite these problems, AI with frozen semen is still interesting to the pig industry–breeding farms in specific situations. For instance, AI with frozen semen allows breeders of gene-selection devoted farms to optimize their genetic selection programs much faster than AI with refrigerated samples, since the availability for separate gene lines is much greater if utilizing frozen doses. Moreover, frozen doses can be transported worldwide easily and securely, facilitating thus the availability of separate genetic lines for selection purposes (Martínez et al., 2001; Roca et al., 2003; Casas et al., 2010).

  The main cause by which frozen semen has a lower *in vivo* fertilizing ability than refrigerated one is the wide number of functional and structural alterations that sperm undergo during the cryopreservation process. Thus, freezing affects the integrity of the sperm plasma membrane, acrosome, and nucleus, as well as mitochondrial function, sperm motility, inducing thus an overall decline of sperm vitality (Johnson et al., 2000; Vadnais and Althouse, 2011; Bryła and Trzcińska, 2015; Pezo et al., 2019). These alterations can be minimized if ejaculates with good freezability characteristics are utilized in combination with optimized freezing extenders and protocols and appropriate insemination techniques (Yeste et al., 2017). Sperm cryotolerance is directly related to the cholesterol-to-phospholipid ratio of the plasma membrane, namely, the relative abundance of saturated fatty acids in the phospholipid fraction (Parks and Lynch, 1992). Additionally, other factors such as the relative

abundance of sperm proteins like heat-shock protein 90 (hsp 90), acrosin, acrosin-binding protein, triosephosphate isomerase, and voltage-dependent anion channel 2 (VDAC2) are also linked with the ability of boar sperm to withstand cryoinjuries (Casas et al., 2010; Vilagran et al., 2013, 2014; Pinart et al., 2015; Estrada et al., 2017). To improve freezability, freezing extenders have been complemented with different additives such as several antioxidants, seminal plasma fractions and cholesterol-loaded cyclodextrins, which have shown to have beneficial effects (Yeste et al., 2017). In this way, some of the antioxidants that have shown this improving effects so far are reduced glutathione, L-cysteine, α-tocopherol, butylated hydroxytoluene, Trolox, and ascorbic acid (Roca et al., 2004; Chanapiwat et al., 2009; Jeong et al., 2009; Kaeoket et al., 2010; Satorre et al., 2012; Varo-Ghiuru et al., 2015; Estrada et al., 2014; 2017; Giaretta et al., 2015). The inclusion of seminal plasma to cryopreservation has yielded contradictory results. On the contrary, the addition of specific seminal plasma proteins like fibronectin 1 or N-acetyl-β-hexosaminidase (Vilagran et al., 2015; Wysocki et al., 2015) increases motility, membrane integrity, and *in vitro* fertilization outcomes of frozen-thawed sperm from poor-freezability ejaculates (Hernández et al., 2007). Supplementing thawing media with cholesterol-loaded cyclodextrins (CLCs) increases the integrity of the plasma membrane and acrosome of frozen-thawed sperm, but decreases sperm motility (Blanch et al., 2012; Tomás et al., 2013; 2014). In this way, the combination of all of these compounds to the freezing medium can improve cryosurvival. However, the success of AI with frozen semen also depends on the specific applied AI method. At this respect, the placement of thawed samples as close as possible to the oviduct is paramount for the success of the technique. This implies that techniques such as the CPAI and the DUAI are those of election for frozen samples in pig farming (Yeste et al., 2017).

- Sex-sorted sperm: Sex-sorted semen in pig farming can be applied in order to create a disbalance in the males/females ratio at each parturition. The implantation of sex-sorted semen in farms can accelerate genetic progress by allowing the production of preselected female livestock (Rath and Johnson, 2008). In this way, insemination with X chromosome-bearing sperm can be an alternative to the castration of male piglets to prevent the distasteful *boar taint* (Johnson et al., 2005). At this moment, there are separate techniques to carry out sperm sex-sorting. The most utilized currently is flow cytometry by adding the fluorescent nuclear dye Hoechst 33342 (Johnson et al., 1987a, 1987b, 1989; Rath et al., 2015). This staining facilitates the differentiation

between X chromosome-bearing sperm and Y chromosome-bearing ones based on the differences in the quantity of DNA between both types of cells (Johnson, 1997; Rens et al., 1998; Johnson and Welch, 1999; Rath et al., 2009). More recently, other techniques are being developed. Among them, the development of an oligonucleotide probe conjugated with gold particles which recognizes and hybridizes to Y chromosome sequences forming a Hoogsteen triple helix stands out. These triple helices are biochemically inert without affecting gametes and the subsequent fertilization (Rehbock et al., 2014; Taylor et al., 2014; Tiedemann et al., 2014). At this moment, the main limitations of all the currently applied sorting procedures are a relatively low efficiency in the number of sorted sperm as well as a significant loss of the sperm fertilizing ability after sorting and subsequent storage in cryopreservation conditions before AI. Besides these limitations, the sorting technique by itself also induces important structural alterations in sorted sperm. Thus, cytometer-based sorting requires a correct orientation of sperm heads in front of the laser, lasting the whole process about 8-to-10 hours. Sperm orientation is performed by applying electrostatic forces, which affects sperm mitochondria function as well as other cell structures such as cell membrane. In this way, the sensitivity of sorted sperm against processes like high dilution or cryopreservation is greatly altered (Bucci et al., 2012; Rath et al., 2015). On the other hand, the gold particles-based technique implies the pass of nanoparticles through cell membrane, altering thus the whole cell membrane structure during the process (Taylor et al., 2014). All of these drawbacks limit thus at this moment the routine use of sex-sorted boar semen to specific situations such as elite breeding units or nucleus herds (Spinaci et al., 2016), being necessary further progress for a wider application of the technique to the whole pig farming.

- Photostimulation: This is the technique on which this PhD Thesis is centred. Semen photostimulation is a particular case of the utilization of light as a therapeutically tool for several processes. Thus, phototherapy, as the therapeutic use of light is named, is currently applied to different disciplines, including dermatology, surgery and rheumatology (Goldman et al., 1980; Abergel et al., 1984; Wheeland and Walker, 1986; Trelles and Mayayo, 1987; Desan et al., 2007; Saltmarche, 2008; Avci et al., 2013). The light is an electromagnetic radiation in which colour is the final result of the interaction of energy and matter (Azeemi and Raza, 2005). The molecular basis by which light exerts their therapeutically effects are currently under discussion. At

this respect, it is well known that light effects will depend on factors such as the specific wavelength and the applied doses. Each wavelength has specific characteristics regarding their emitting energy and capacity of penetration, which implies separate therapeutically uses of light depending on wavelength (Azeemi and Raza, 2005; Hochman, 2018). In any case, light irradiation has been shown to increase mitochondrial respiration and ATP production in *E. Coli*, yeast, HeLa cells and hepatocytes (Karu, 1989; Gao and Xing, 2009). Furthermore, light concomitantly affects ROS production in cells such as neurones, inducing thus an oxidative effect or an antioxidative one, depending on the utilized specific wavelength and dose. In this sense, low doses increase ROS production, whereas high ones induce cytotoxic effects (Huang et al., 2011).

5.2.- Effects of photostimulation on sperm

In the last years, several groups have centred their work on determining the effect of light on sperm function. Thus, centring in no-mammalian species, light stimulation of turkey semen stored at 5 °C with a He-Ne laser at a wavelength of 632.8 nm (power=6 mW; 3.24 to 5.4 J cm−2) increases the sperm motility for 48h (Iaffaldano et al., 2005). Furthermore, stimulation with a He-Ne laser (λ=632.8 nm; power=6 mW; energy density=3.96 J cm^{-2}) increased motility and the overall mitochondria cytochrome C oxidase (CCO) activity in frozen-thawed turkey sperm but not in cells from chickens and pheasants (Iaffaldano et al., 2013). Zan-Bar et al. (2005) compared the effects of the UV (294 nm; energy density: 0.1 $mWcm^{-2}$) blue, (360 nm; energy density: 1.5$mWcm^{-2}$) red (660 nm; energy density: 10$mWcm^{-2}$) and white light (the whole 400 nm–to-800 nm range; energy density: 40$mWcm^{-2}$) in tilapia and ram. Results indicated that, whereas ram sperm only showed an increase of motility and *in vitro* fertilizing ability after irradiation with red light, tilapia cells shown a similar response against both red and white light irradiation. On the other hand, both UV and blue light had negative effects, as they both decreased motility, fertility and ROS production in both species. These results suggest the existence of species-specific differences in the response of sperm function to light.

Works exclusively focused in mammalian sperm are more numerous. Thus, the application of a Krypton Laser red light at a wavelength of 647 nm and with an energy density of 32 J cm^{-2} in human sperm increased the percentage of motile spermatozoa as

well as progressive motility (Sato et al.,1984; Lenzi et al., 1989). Also in human sperm, Firestone et al. (2012) observed and increase of motility after applying a λ=905 nm; energy density=50mWcm−2; 1.5 J cm−2 laser at a wavelength of 905 nm and an energy density of 1.5 J cm^{-2}. On the contrary, photostimulation with a 655-nm diode laser with an output power of 21.7 mW and a spot: 0.56 cm^2 did not modify dog sperm total motility, although mean motion parameters such as VAP, LIN and BCF were significantly increased (Corral-Baqués et al., 2005). Further studies on dog sperm also indicated that this effect of light on dog sperm was accompanied with specific changes in the motile sperm subpopulational structure of ejaculates (Corral-Baqués et al., 2009). Likewise, bull sperm irradiated with a He-Ne laser of 633 nm wavelength at output powers of 5mW (energy density=0.510mWcm^{-2}), 7.5mW (energy density=0.765mWcm^{-2}) and 10mW (energy density=1.020mWcm^{-2}) showed a significant increase in mean motions parameters such as VCL, VAP, ALH, as well as a concomitant increase in the percentage of sperm with intermediate mitochondrial membrane potential (Siqueira et al., 2016). Similarly, irradiation with a diode pumped solid state laser at a wavelength of 532 nm, and output power of 1 mW and a power density of 0.31 J cm^{-2} increased total and progressive motility and also mean values of VSL, VAP, VCL and ALH in buffalo sperm after 4 minutes of exposure (Abdel-Salam et al., 2011). Likewise, frozen ram sperm irradiated with an He-Ne laser (λ=632.8 nm; power=6 mW; spot size=1 cm^2, power density=6.12 J cm^{-2}) increased the percentage of total and progressive motility as well as the ability to maintain cell integrity against an environmental stress such as thermal stress of freezing/thawing (Iaffaldano et al., 2016). Similarly, red light irradiation at a wavelength of 780 nm and an energy between 0.2 and 6.0 J increased the percentage of spermatozoa with high mitochondrial membrane potential of bull sperm (Lubart et al., 1997). Red light stimulation (630-nm He-Ne laser, power source=13 mW) also increased the *in vitro* fertilizing ability of mouse sperm in the presence of calcium in a defined fertilization medium (Cohen et al., 1998). Finally, centring on boar semen, a discontinuous irradiation with red LED light using an specific protocol of 10 minutes light followed by 10 minutes in the darkness and then again a 10 minutes irradiation period (10'-10'-10' procedure) increased mean values of VSL, VCL and VAP (Yeste et al., 2016). Aside from motility, this light irradiation pattern also increased the efficacy of *in vitro* capacitation (Ocaña-Quero et al. 1997; Yeste et al., 2016). Likewise, the above described red LED light 10'-10'-10' procedure increased *in vivo* fertility and prolificacy

in pig farming conditions (Yeste et al., 2016), indicating thus a positive effect of light irradiation on the fertilizing ability of this species.

The effect of light on the fertilizing ability of mammalian sperm is accompanied with changes in several intracellular modulatory pathways. Thus, the 10'-10'-10' procedure increased mitochondria activity of boar sperm (Yeste et al., 2016). This light-induced increase in mitochondrial activity seems to be related with concomitant changes in several mitochondria-modulated mechanisms. In this way, photostimulation with a He-Ne laser at an energy density of 6.12 J cm^{-2} increases ATP content and also the CCO activity in frozen ram sperm (Iaffaldano et al., 2016), as well as the intramitochondrial calcium content in boar sperm subjected to the 10'-10'-10' procedure (Yeste et al., 2016). It is interesting to remark that the effects of light on intramitochondrial calcium content seems to depend on the irradiation dose, since low power irradiation procedures increased this content (Bonet et al., 2013; Correia et al., 2015), whereas high irradiation doses induced the contrary effect (Breitbart et al., 1996). Following with the mitochondria-related effects of photostimulation, it has been described that light stimulation at a wavelength range of 400–800 nm and a power density range of 40–80mWcm^{-2} also increased the production of cell hydroxyl radicals (OH^-). This effect is related with the observed increase in both overall ROS and NO levels of bull sperm irradiated in these conditions and then subjected to *in vitro* capacitation and subsequent acrosome reaction (Cohen et al., 1998; Lavi et al. 2010).

5.3.- Mechanism/s of action of light on the overall sperm function

The above described effects clearly indicate the existence of a strong effect of light on the overall sperm function. However, the exact mechanism/s by which light is exerting these effects are not well known. At these moments, there are several hypotheses about this/these mechanism/s, in the understanding that these hypothetic mechanisms would not be mutually exclusive, but, probably, would act in a cooperative manner.

- Intracellular photosensitive molecules: any eukaryotic cell, including sperm, contains molecules that can absorb light, inducing thus changes in their energy status and, hence, in their activity. Among these molecules, porphyrins, cytochromes, pyridine cofactors, NADH, NADPH, Fe-S clusters and flavin stand out (Kessel, 1982;

Cunningham et al., 1985; Pottier and Truscott, 1986; Kim and Jung, 1992; Fraikin et al., 1996; Klebanov et al., 1998; Edwards and Silva, 2001; Aba and Wolfgang, 2011). Photosensitive molecules reabsorb the electromagnetic radiation of the light and transfer the ionized energy to adjacent molecules (Lubart et al., 1992). Following this mechanism, light irradiation would induce an increase in the mitochondrial electrochemical potential, increasing thus mitochondria ATP production. The increase of the mitochondrial electrochemical potential induced by light fill follow three steps. The first step would be the formation of singlet oxygen by endogenous porphyrins, increasing thus ROS synthesis rate. This first step will be concomitant with the second one, namely, an increase in the activity of the whole mitochondrial electron chain, concomitantly increasing mitochondria membrane potential. Finally, the third step would be the oxidation of NAD^+ groups as a consequence of the two previous steps. The oxidation of NAD^+ molecules would ultimately modify the whole redox status in both mitochondria and the whole cell (Karu, 1988; Friedmann et al., 1991; Tamura, 1993; Breitbart et al., 1996). The final consequences of the light-induced changes in the redox state would affect the active transport of H^+ and also of Ca^{2+}. Centring on Ca^{2+} ions, the changes on redox status would increase their uptake from the extracellular medium as well as the release of mitochondrial Ca^{2+} to cytosol (Breitbart et al., 1996). These changes in intracellular calcium distribution could explain the described increase in the achievement of sperm *in vitro* capacitation status (Baker et al., 2004). Other photosensitive molecules could be also in the basis of light action on sperm. From these, the hemo and flavin groups that form part of the NADPH oxidase system would be also involved in the light-induced increase of ROS production (Jones et al., 2000).

- Nitric oxide: the NO is synthesized in the mitochondria. Its role is binding to mitochondria CCO, avoiding thus the union of the enzyme with oxygen molecules. This binding, in turn, would inhibit the whole activity of the electron transport chain (Karu, 2008; Gao and Xing, 2009; Huang et al., 2011). Light irradiation induces NO dissociation from their binding sites on the heme, iron and copper centres of CCO, thereby resuming the activity of the mitochondrial electron chain (Karu, 2008; Gao and Xing, 2009; Huang et al., 2011).

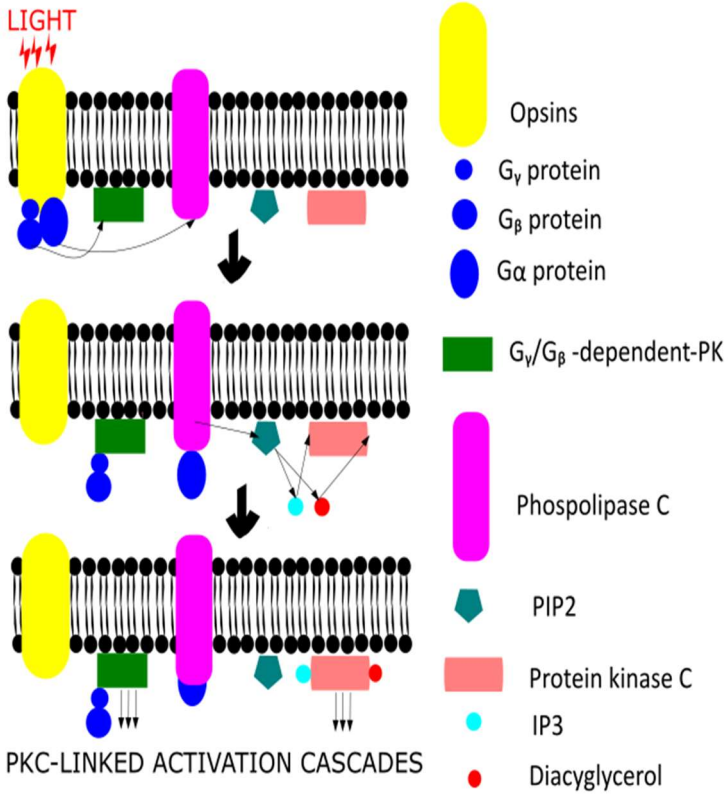
- Opsins: they are G proteins-coupled molecules that, in sperm, seems to play a role as thermosensors via canonical phospholipase C and cycling nucleotides pathways (Pérez-Cerezales et al., 2015). Interestingly, opsins are activated by light when associated to photosensitive molecules like retina, flavins or bilins. These complexes transform the photon energy in an electrochemical intracellular signal, which, in turn, activates sub-cellular cascades modulated by separate mechanisms. Human and mice sperm contain rhodopsin, an RGR opsin activated by red light through retinal-dependent mechanisms (Terakita, 2005). This is not the only opsin present in sperm, since other such as melanopsin, neuropsin and encephalopsin are also described here (Foster et al., 1991; Buczyłko et al., 1996; Newman et al., 2003; Terakita, 2005; Hichida and Matsuyama, 2009; Larusso et al., 2008; Buhr et al., 2015; Pérez-Cerezales et al., 2015). The exact role/s of these proteins in sperm, as well as the specific wavelengths to which they are sensitive, are presently under investigation. In sperm, opsins have specific locations, suggesting in this way a specific role linked with their location. In fact, *in vitro* studies suggest that photo/thermostimulation induces the release of intramitochondrial calcium through calmodulin-related mechanisms (Foster et al., 1991; Buczyłko et al., 1996; Newman et al., 2003; Terakita, 2005; Hichida and Matsuyama, 2009; Larusso et al., 2008; Buhr et al., 2015; Pérez-Cerezales et al., 2015). In this way, light activation of rhodopsin would be a mechanism involved in the light-induced launching of sperm capacitation through calcium-modulated mechanisms (see Fig. 4).

- Transient Receptor Potential proteins family (TRPs): the TRPs receptors are transmembrane proteins involved in thermotactical response of all eukaryotic cells (Wu et al., 2010; Islam, 2011; Zheng, 2013). Centring on mammalian sperm, it has been described that TRPV4 and TRPM8 receptors, both included in the TRP family, are the main temperature-sensitive ion channels in these cells (Mundt et al., 2018). Interestingly, it is known that at least TRPV4 is related with the initial membrane depolarization observed in sperm incubated in capacitating conditions, acting thus in the early phenomena linked with the launching of capacitation (Mundt et al., 2018). It is noteworthy that both TRPV4 and TRPM8 act through Ca^{2+}-calmodulin mediated mechanisms (De Blas et al., 2009; Martínez-López et al., 2011; Mundt et al., 2018). This is important, since this signal transduction cascade is the same that modulates sperm rhodopsin action (Foster et al., 1991; Buczyłko et al., 1996;

Newman et al., 2003; Terakita, 2005; Hichida and Matsuyama, 2009; Larusso et al., 2008; Buhr et al., 2015; Pérez-Cerezales et al., 2015). This concomitancy would indicate the existence of a cooperative mechanism of action between TRPs and opsins as thermotaxis-regulatory elements. Concomitantly, although probably light has not a direct action on both TRPV4 and TRPM8, the existence of these common mechanisms of action between TRPs and opsins would imply that light would also modify the molecular signal exerted by TRPs through modification of the Ca^{2+}-calmodulin-related transduction cascade (See Fig. 4). In this way, TRPs would be also implied in the overall effect of light in sperm function, albeit in an indirect manner through Ca^{2+}-calmodulin-PKC related mechanisms.

Figure 4. Scheme representing the putative opsins and/or TRPs-mediated mechanism of action of light on the overall sperm function.

- Other proteins: low irradiation doses at a wavelength of 780 nm did modify the 3D conformation of non-chromophoric proteins. These conformational changes will modify the specific activity of the affected proteins (Lubart et al., 1997). In this way, it has been hypothesized that light-induced changes in the conformational status of



proteins linked to calcium transport would be in the basis of the observed decrease of mitochondria calcium uptake, being thus another modulatory mechanism related with

the action of light in the achievement of the sperm capacitation status (Breitbart et al., 1990).

As a conclusion, data yielded until now by separate research teams seem to indicate that light would exert its effects on the overall sperm function through the simultaneous activation of several, independent mechanisms liked to photosensitive molecules as well as to the activation of the Ca^{2+}-calmodulin-PKC intracellular transduction cascade (see Fig. 5).

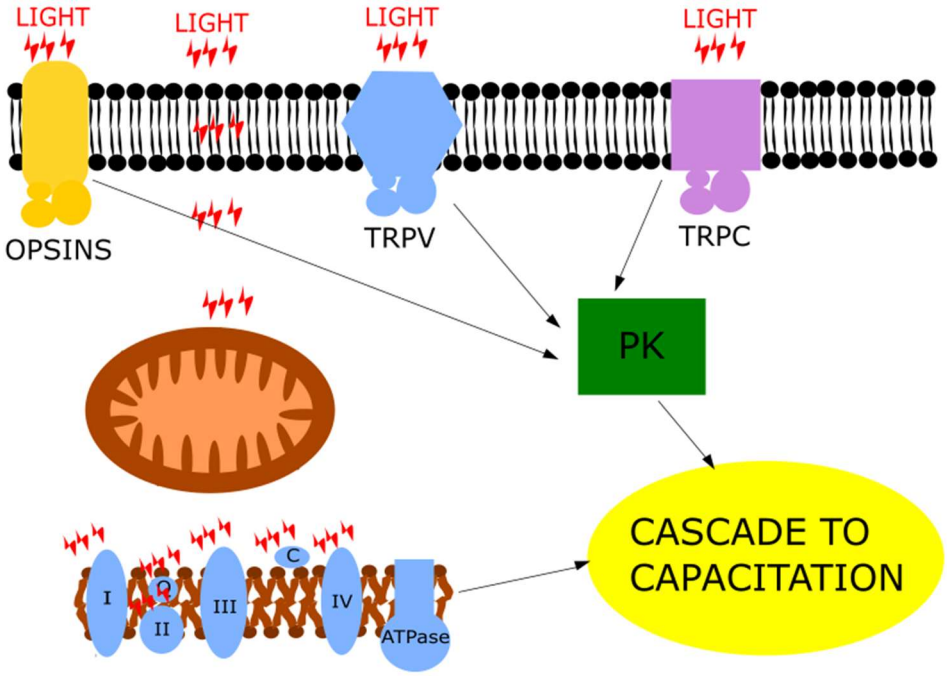


Figure 5. Scheme representing the main pathways through which light is exerting its action on the overall sperm function.

OBJECTIVES

Taking into account all of the above described information, the main objectives of this PhD Thesis dissertation are:

1- Evaluating the duration over the time of light effects *in vitro* on diluted boar sperm stored at 16-17ºC during 96 hours.

2- Deeping further in the knowledge of the light action on the modulation of the mitochondrial electron transport chain of boar sperm in order to a better understanding of the mechanism of action involved in light effect on the overall sperm function.

3- Evaluating the effects of the 10'-10'-10' photostimulation pattern in the main *in vivo* reproductive parameters of commercial pig farms distributed worldwide.

With this purpose, the following three experimental designs were carried out:

1- Boar seminal doses were exposed to 4 different light protocols: Without light, 1 minute of red LED, 5 minutes of red LED and 10 minutes of red LED. Immediately after irradiation and after 24 h, 48 h, 72 h and 96 h of storage at 17ºC, the sperm resilience to withstand thermal stress was tested through incubation at 37ºC for 120 min. Before starting the assay (0 min) and after incubation at 37 ºC for 120 min, sperm viability, acrosome integrity, motility and kinematics were determined, and only at 0 minutes free cysteine radicals of sperm head proteins were determined.

2- Seventeen boar seminal doses were exposed to 4 different light protocols: Without light, 1 mint of red LED, 5 minutes of red LED and 10 minutes of red LED. Each light treatment protocol in turn was subdivided in three separate treatment groups. The first group were formed by cells that were light-irradiated without previous incubation with any effector. The second group were sperm previously incubated with oligomycin A, a specific inhibitor of the mitochondria ATP synthase (Chappell and Greville, 1961) before photostimulation. The third group was composed by sperm previously incubated with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), an specific uncoupler of the mitochondria electron chain (Choen et al., 1998), before photostimulation. Motility characteristics and motile sperm subpopulations structure, as well as plasma membrane integrity, acrosome integrity, mitochondrial membrane potential (MMP), intracellular levels of superoxides,

peroxides and calcium, intracellular levels of ATP, O_2 consumption rate and CCO activity were compared among groups.

3- In 30 farms around the world, animals were divided into two homogeneous groups. The first group was inseminated following the usual routine of the farm (Control Group), whereas the other group was inseminated with semen doses previously light-stimulated with the MaXipig® system (IUL, S.A.; Photo Group), a commercial device designed for photostimulation of refrigerated doses immediately before applying AI in farms. Both fertility and prolificacy were compared between the two groups.

In the following chapters of this PhD Thesis dissertation, these experimental designs will be explained following a 3-manuscript display.

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MANUSCRIPT I

Irradiation of pig sperm with red LED light maintains the integrity of nucleoprotein structure over liquid-storage and increases their resilience to withstand thermal stress

Olga Blanco-Prieto, Jaime Catalán, Lina Trujillo Rojas, Ariadna Delgado-Bermúdez, Marc Llavanera, Teresa Rigau, Sergi Bonet, Marc Yeste, Maria Montserrat Rivera del Álamo, Joan E. Rodríguez-Gil

Theriogenology, In Revision

Title

Irradiation of pig sperm with red LED light maintains the integrity of nucleoprotein structure over liquid-storage and increases their resilience to withstand thermal stress

Authors

Olga Blanco-Prieto[1,#], Jaime Catalán[1,#], Lina Trujillo Rojas[1], Ariadna Delgado-Bermúdez[2,3], Marc Llavanera[2,3]; Teresa Rigau[1], Sergi Bonet[2,3], Marc Yeste[2,3], Maria Montserrat Rivera del Álamo[1], Joan E. Rodríguez-Gil[1,*]

Affiliations

[1]Unit of Animal Reproduction, Department of Animal Medicine and Surgery, School of Veterinary Medicine. Autonomous Uiversity of Barcelona. E-08193 Bellaterra (Cerdanyola del Vallès), Spain

[2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, E-17003 Girona, Spain

[3]Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, E-17003 Girona, Spain

[#]Joint first authors

[*]Corresponding author: Phone: +34-935811045; e-mail: juanenrique.rodriguez@uab.cat

Abstract

This study sought to determine the effects of irradiating pig seminal doses with red LED light irradiation on their quality and longevity over liquid-storage at 17 ºC. For this purpose, boar ejaculates were diluted in a commercial extender at a final concentration of 3×10^7 sperm/mL and stored at 17 ºC for 96 h. Upon arrival to our laboratory (5-6 h within collection), samples were subjected to irradiation with a temperature-controlled red light-

emitting diode (LED) for 1 min, 5 min or 10 min. Controls consisted of non-irradiated spermatozoa. Samples were stored at 17 ºC for 96 h, and plasma membrane and acrosome integrity, motility and free cysteine radicals of sperm head proteins were evaluated every 24 h. In addition, the sperm resilience to withstand thermal stress following irradiation was evaluated at 24 h, 48 h, 72 h and 96 h by incubating stored seminal doses at 37 ºC for 120 min. Light-stimulation for 5 min and 10 min counteracted the decrease in thermal stress observed in non-irradiated samples during the first 48 h of storage. Moreover, all irradiation protocols counteracted the decrease in percentages of spermatozoa with altered acrosomes observed in non-irradiated samples after 72 h of storage. The improving effects of light-stimulation upon sperm motility parameters were more transient, and not detected after 48 h of storage. While liquid-storage also led to an increase in the free cysteine levels of sperm head proteins, this increment was partially mitigated through light-stimulation for 5 min and 10 min. Our results suggest that, although irradiation with red LED light may contribute to better maintain the quality of liquid-stored pig semen, these effects are only maintained for the first 48 h. In addition, not only does the persistence of these effects appear to vary between sperm parameters but also between irradiation patterns.

Keywords: pigs; semen preservation; light-stimulation, liquid-storage

1. Introduction

Artificial Insemination (AI) with liquid-stored semen is the most common technique for pig breeding in farms across Western countries [1]. This pre-eminence has led to seek for new procedures aimed at improving sperm quality and reproductive performance. In the last years, stimulation of pig semen with red-light using an emitting diode (LED) has been described as a relatively easy technique that allows for a significant increment in farrowing rates and prolificacy, despite this increase not having been observed across all farms [2,3].

Stimulation with low-energy laser red-light increases sperm motility and ATP generation in humans, mice, rabbits, dogs, cattle and sheep [4–8]. In addition, Iaffaldano et al. demonstrated that irradiating turkey sperm with a He-Ne laser at 632.8 nm (6 mW and 3.96 J/cm^2) increases their longevity when stored at 5 ºC for 48 h [9]. A later study from the same group showed that stimulating rabbit sperm with a low-energy He-Ne laser also augments their motility, viability and acrosome integrity, these effects being

maintained after 48 h of storage at 15 ºC [10]. Moreover, irradiation of bull sperm with a Al-Ga-Id-P laser diode at 660 nm (30 mW) increments their cryotolerance [11]. All this previous research supports that sperm irradiation improves their longevity in liquid and frozen storage.

With regard to pig sperm, irradiation using red LED light with 10 min of light followed by 10 min of darkness and 10 min of further light increases their reproductive performance in a significant number of worldwide farms [2,3]. In spite of this, previous studies devoted to the effects of red LED light on sperm quality are inconsistent. In effect, while Luther et al. [12] did not observe differences on the quality of thermically stressed pig sperm after stimulation with red-light, Yeste et al. [3] and Pezo et al. [13] found an improvement in sperm quality, resilience to withstand thermal stress and the ability to elicit in vitro capacitation and trigger progesterone-induced acrosome exocytosis. Whilst the exact mechanisms through red-light exerts these effects have not been fully elucidated, new insights into how light-stimulation works would be very important, as it would contribute to optimize the practical use of this technique. Related with this, three different hypotheses have been raised. The first one contemplates that red-light affects mitochondrial cytochromes, which would affect mitochondrial function and subsequently sperm motility and ATP production [7]. The other two hypotheses envisage an impact on thermotaxis, either through Transient Receptor Potential Cation V-Member channels (TRPV) or opsins, especially rhodopsin [14]. All these hypotheses are not, however, mutually exclusive.

From a practical point of view, elucidating whether red-light stimulation may improve the longevity of pig semen doses intended to AI along with the potential endurance of its beneficial effects on reproductive performance would help develop the use of sperm irradiation on farm conditions. Taking this into account, the current work sought to evaluate the span during which the positive effects of exposing sperm to red LED light are maintained over storage at 17 ºC. With this purpose, pig semen doses diluted in standard conditions in a commercial extender were stored at 17 ºC for 96 h without any previous treatment (control samples) or exposed to red LED light for 1 min, 5 min or 10 min (irradiated samples). Sperm viability, acrosome integrity, sperm motility and kinematics, and the disruption of disulfide bonds of the nucleoprotein structure of the sperm head were determined every 24 h. Samples were also incubated at 37 ºC for 120 min in order to analyze the sperm resilience to withstand thermal stress.

2. Material and methods

2.1. Animals and samples

Seven healthy boars from a commercial farm, aged from two to three years, were used in the study. All boars were from Piétrain breed and had proven fertility after AI using liquid-stored semen. Semen collection was performed twice a week through the gloved-hand method and analyzed to ensure the quality and homogeneity of all ejaculates. Immediately after collection, sperm-rich fractions were diluted to a final concentration of 3×10^7 spermatozoa/mL in a commercial extender (MR-A, Kubus SA, Majadahonda, Spain). Extended semen samples were cooled and maintained at 17 ºC during shipment to our laboratory within 5-6 h post-collection.

Boar ejaculates utilized in this study were not collected for the unique purpose of investigation, but for their commercial use. Thus, the farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain) provided us with some doses, whereas the remaining ones were used for AI. Therefore, and because no animal was manipulated by the authors, there was no need for any specific ethical approval to perform this work.

2.2. Irradiation of spermatozoa

Sperm irradiation was performed with a red LED light PhastBlue® device (IUL, S.A.; Barcelona, Spain). Prior to light-stimulation, semen samples were split into 1.5-mL tubes specifically designed for being used with the PhastBlue® device, as follows: 1) control (no exposure to red-light; non-irradiated samples); 2) irradiation for 1 min; 3) irradiation for 5 min; and 4) irradiation for 10 min. Light-stimulation was performed at 20 ºC, and non-irradiated samples were kept at the same temperature for 10 min. Upon irradiation (0 h) and after 24 h, 48 h, 72 h and 96 h of storage at 17 ºC, sperm viability, acrosome integrity, motility and kinematics, and free cysteine radicals of sperm head proteins were evaluated.

2.3. Thermal stress assay

Immediately after irradiation and after 24 h, 48 h, 72 h and 96 h of storage at 17ºC, the sperm resilience to withstand thermal stress was tested through incubation at 37ºC for

120 min. Before starting the assay (0 min) and after incubation at 37 ºC for 120 min, sperm viability, acrosome integrity, motility and kinematics were determined. The thermal resilience in each of these sperm parameters was determined by subtracting the value obtained at 0 min from that observed at 120 min.

2.4. Evaluation of sperm viability and acrosome integrity through flow cytometry

Sperm viability and acrosome integrity were determined through flow cytometry following the recommendations set by Lee et al. [15]. In all cases, sperm concentration was previously adjusted to 1×10^6 spermatozoa/mL in a final volume of 500 µL, and three technical replicates were assessed. Samples were examined using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA), and the flow rate was set at 4.17 µL/min. Electronic volume (EV) and side scatter (SSC) were recorded in a log-linear mode (EV/SSC dot plots) and 10,000 events per replicate were evaluated. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 µm) and cell aggregates (particle diameter > 12 µm). Furthermore, compensation was utilized to minimize fluorescence spill over into a different channel. All data were collected in List-mode Data files (EV, SSC, FL1, FL2 and FL3), which were subsequently analyzed with the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). All fluorochromes were purchased from Molecular Probes® (Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) and diluted with dimethyl sulfoxide (DMSO).

Percentages of viable spermatozoa, defined as membrane-intact spermatozoa, were analyzed through the LIVE/DEAD® Sperm Viability Kit (SYBR14/PI; [16]). Following staining, three separate sperm populations were identified. The first population was composed of all membrane-intact spermatozoa ($SYBR14^+/PI^-$), whereas the other two subpopulations ($SYBR14^-/PI^+$ and $SYBR14^+/PI^+$) included sperm with different degrees of plasma membrane alterations. Debris and non-sperm particles were detected as $SYBR14^-/PI^-$ and were subtracted from the percentages of total spermatozoa. Percentages of the three sperm populations were recalculated. Single-stained samples were used to set EV gain, FL1 and FL3 PMT-voltages and for compensation of FL1 spill over into the FL3-channel (2.45%).

Acrosome integrity was analyzed through staining with *Arachis hypogaea* lectin conjugated with fluorescein isothiocyanate (PNA-FITC) and ethidium homodimer (EthD-1; used as a vital stain) as described by Cooper and Yeung [17] and adapted to pig spermatozoa by Rocco et al. [18]. Following this procedure, four sperm populations were identified. The first population included all viable spermatozoa with intact acrosome membranes (PNA-FITC$^+$/EthD-1$^-$). The second population was composed of viable spermatozoa with altered acrosome membranes (PNA-FITC$^-$/EthD-1$^-$). The third population contained non-viable spermatozoa with intact acrosome membranes (PNA-FITC$^+$/EthD-1$^+$). Finally, the fourth population comprised all non-viable spermatozoa with altered acrosomes (PNA-FITC$^-$/EthD-1$^+$). Following the recommendations set by Petrunkina et al. [19], percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were used to correct the percentages of viable spermatozoa with altered acrosome membranes (PNA-FITC$^-$/EthD-1$^-$); the other percentages were recalculated. Percentages of spermatozoa with altered acrosomes resulted from summing up PNA-FITC$^-$/EthD-1$^-$ and PNA-FITC$^-$/EthD-1$^+$ populations. FL1 spill over into the FL3 channel (2.70%) was compensated.

2.5. Evaluation of sperm motility and kinematics through computer-assisted sperm analysis (CASA)

Computer-assisted analysis of sperm motility (CASA) analysis was performed using a commercial system (Integrated Sperm Analysis System V1.0, Proiser SL, Valencia, Spain). Prior to analysis, samples were warmed at 38 ºC for 5 min in a water bath, and 5 µL of each sample was placed onto a pre-warmed (38 ºC) Makler chamber (Sefi-medical Instruments, Haifa, Israel). The following parameters were assessed: percentages of total motile spermatozoa; percentages of progressively motile spermatozoa; curvilinear velocity (VCL, µm/s); straight-line velocity (VSL, µm/s); average path velocity (VAP, µm/s); linearity coefficient (LIN, %); straightness coefficient (STR, %); wobble coefficient (WOB, %); mean amplitude of lateral head displacement (ALH, µm); and frequency of head displacement (BCF, Hz). A spermatozoon was considered as motile when VAP ≥ 10 µm/s, and progressively motile when STR ≥ 45% [20]. A total of 1,000 spermatozoa were evaluated per assessment, and three technical replicates were counted.

2.6. Evaluation of free cysteine radicals of sperm head proteins

To determine free cysteine radicals of sperm head proteins, 1.5-mL aliquots from control and irradiated samples were centrifuged at 2,000×g and 17 ºC for 30 s. Supernatants were discarded, and pellets were resuspended with 250 µL of Cysteine Buffer, composed of 50 mM Tris/HCl, 150 mM NaCl, 1% (v:v) Nonidet P-40, 0.5% (w:v) sodium deoxycolate 0.5%, 1 mM Na_2VO_4, 10 µmol/mL leupeptin, 1 mM benzamidin and 0.5 mM phenylmethyl sulfonil fluoride (PMSF); the pH was adjusted to 7.4. Samples were homogenized at 4 ºC through sonication, subjecting samples to 11 consecutive ultra-sound pulses at maximal intensity; this step also led to detach sperm heads from tails.

After preparation of samples, two different techniques were used. The first one was based on the method described by Brocklehurst et al. [21] and adapted to pig sperm by Flores et al. [22]. Briefly, homogenized samples were centrifuged at 850×g and 4 ºC for 20 min and the resulting supernatants along with the upper layer of the pellet were discarded. Thereafter, pellets were resuspended in 1 mL of cysteine buffer, and 20 µL of this suspension was mixed with 1 mL of 0.2 mM 2,2'-dipyrydil disulfide. Samples were incubated at 37 ºC for 1 h, prior to determining free cysteine levels in isolated sperm head proteins through spectrophotometric analysis at 343 nm wavelength. Data were normalized through a parallel determination of the total protein content of samples by utilizing a commercial kit based on the Bradford method [23](BioRad; Hercules, CA, USA).

The second technique was based on the use of dibromobimane, as described by Ijiri et al. [24] and adapted to pig sperm as follows. Forty µL of each semen sample were addedwith 40 mM dibromobimane in 99.9% (v:v) DMSO until a final dibromobimane concentration of 40 µM. Samples were subsequently incubated at 20 ºC for 40 min in the dark. Afterwards, samples were centrifuged at 850×*g* at 4 °C for 20 min. Supernatants were recovered and diluted to a final concentration of 2 µg protein/µL with Cysteine Buffer. These diluted samples were mixed at a 1:4 (v:v) ratio with an electrophoresis loading buffer (pH 6.8) containing 250 mM TRIS, 50 mM ditiothreitol (DTT), 10% (w:v) sodium dodecyl sulphate (SDS), 0.5% (v:v) bromophenol blue and 50% (v:v) glycerol. Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE); the percentage of polyacrylamide in the separating gel was 10% (w:v). Since protein concentration was high, the power was set at 23 V. Gels were washed for 30 min in TBST

solution and then examined under a UV transilluminator after 38 s of exposure. Protein bands that had free cysteine radicals were fluorescent, due to the specific binding of dibromobimane to these bands, and images were taken and saved as TIFF files. Subsequently, gels were thoroughly washed with TBST and then stained with Coomassie Blue using a commercial kit (InstantBlue™ Ultrafast Protein Stain; Sigma; St. Louis, USA). Thereafter, gels stained with Coomassie blue were scanned and saved as TIFF files. Relative intensities of both dibromobimane-stained and Coomassie Blue-stained bands were analyzed with the Fiji© image processing package (National Institute of Health; Bethesda, USA). The relative amount of free cysteine radicals in each lane was calculated in two steps. First, the intensity of dibromobimane stained bands was divided into their corresponding Coomasie blue-stained ones. The resulting values were used to calculate the ratio between the 20 kDa dibromobimane band which corresponded to free nucleoproteins [25], and the 20 kDa Coomasie Blue one. A similar dibromobimane/Coomasie Blue ratio was calculated with the 60 kDa band, which would coincide with nucleoprotein/DNA aggregates [26]. Finally, the dibromobimane/Coomasie Blue intensity ratio of the entire lane was calculated. In all cases, the relative intensity observed at 0 h was adjusted to 100 and was taken as the baseline for the other experimental points.

2.7. Statistical analyses

Statistical analyses were conducted using a statistical package (SPSS® Ver. 25.0 for Windows; IBM corp., Armonk, NY, USA). Data were first tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests, respectively. When required, data were transformed through arcsine $\sqrt{x}$. The effects of irradiation on sperm viability (SYBR-14/PI), acrosome integrity (PNA-FITC/EthD-1), sperm motility and kinematic parameters, and free cysteine radicals of sperm head proteins evaluated through two separate techniques were analyzed through a linear mixed model (inter-subjects factor: irradiation protocols; intra-subjects factor: time of storage) followed by post-hoc Sidak's test for pair-wise comparisons.

Motile sperm subpopulations were determined as described in Luna et al. [27]. Briefly, individual motion parameters taken for each individual spermatozoon, i.e, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, DANCE and MDA were utilized as

independent variables in a Principal Component Analysis (PCA). The obtained matrix was rotated with the Varimax method and Kaiser normalization. The resulting regression scores were used to run a two-step cluster analysis based on the log-likelihood distance and the Schwarz's Bayesian Criterion. This cluster analysis led each spermatozoon to be classified in one of the three resulting subpopulations. Following this, percentages of spermatozoa belonging to each of the three motile subpopulations were calculated per sample (i.e. irradiation protocol, storage time and semen dose) and compared through a linear mixed model (inter-subjects factor: irradiation protocols; intra-subjects factor: time of storage) followed by post-hoc Sidak's test for pair-wise comparisons.

In all analyses, the level of significance was set at $P \leq 0.05$. Data are shown as mean ± standard error of the mean (SEM).

3. Results

3.1. Effects of irradiation on sperm viability and acrosome integrity over liquid-storage at 17 ºC for 96 h

At the beginning of the experiment, the percentage of viable spermatozoa ($SYBR14^+/PI^-$) was 85.6% ± 3.4% (mean ± SEM). This percentage decreased progressively and reached a figure of 80.7% ± 3.0% after 96 h of storage (Fig. 1A). Although irradiation protocols (1 min, 5 min and 10 min) had not significant effects on sperm viability, irradiation for 1 min showed slight, although significantly (P<0.05), higher percentages of viable spermatozoa ($SYBR14^+/PI^-$) than non-irradiated samples after 72 h of storage (1 min: 86.0% ± 3.7% *vs.* non-irradiated control: 82.7% ± 2.9%; Fig. 1A). With regard to the thermal stress test, there was a significant (P<0.05) decrease in the percentages of viable spermatozoa after incubation of all samples at 37 ºC for 120 min, since the beginning of the experiment (Figs. 1C and 1E). While the extent of that decrease was larger after 48 h, 72 h and 96 h of storage at 17 ºC (P<0.05), no significant differences between the control (non-irradiated) and irradiated samples were observed (Figs. 1C and 1E).

As far as acrosome integrity is concerned, there was an increase in the percentages of spermatozoa with altered acrosomes over storage, which went from 11.6% ± 0.8% at 0 h to 18.6% ± 1.1% at 96 h (Fig. 1B). However, previous exposure to red-light mitigated that increase, which was much more apparent after 72 h of storage (Fig. 1B). This counteracting effect was also observed when the sperm resilience to withstand thermal stress was evaluated. In effect, while incubating sperm at 37 ºC for 120 min increased the percentages of altered acrosomes and that this increment was higher as storage extended further than 72 h, there was a counteracting effect in irradiated samples (Fig. 1E).

3.2. Effects of irradiation on total sperm motility over liquid-storage at 17 ºC for 96 h

Percentages of total motile spermatozoa significantly (P<0.05) decreased in non-irradiated samples over storage (0 h: 68.0% ± 2.9% *vs.* 96 h: 58.1% ± 2.7% after 96 h of storage; Fig. 2A). Irradiation protocols induced separate effects depending on the time of exposure. Thus, whereas light-stimulation for 10 min maintained the percentages of total motile spermatozoa during the first 48 h of storage to values equal or significantly (P<0.05) higher than those of non-irradiated (control) samples, not only was total sperm

motility at 0 h significantly (P<0.05) lower in both 1 min and 5 min patterns than in non-irradiated samples, but this parameter showed lower values over storage in samples irradiated for 1 min (Fig. 2A).

In spite of the aforementioned, percentages of total motile spermatozoa following thermal stress at 37 ºC for 120 min showed a completely different picture (Fig. 2C). In effect, samples that were irradiated (especially for 1 min or 5 min), stored for 24 h and 48 h and then incubated at 37 ºC for 120 min showed significantly (P<0.05) higher percentages of total motile spermatozoa than non-irradiated samples. However, these differences were much less evident or even disappeared after 72 h of storage (Fig. 2D).

3.3. Effects of irradiation on progressive sperm motility over liquid-storage at 17 ºC for 96 h

Regarding percentages of progressively motile spermatozoa, although they were significantly (P<0.05) higher after 24 h of storage than at 0 h in non-irradiated samples, they dropped after 72 h and 96 h of storage (24 h: 47.0% ± 1.9% *vs.* 96 h: 21.3% ± 1.1%, P<0.05; Fig. 2B). While percentages of progressively motile spermatozoa were significantly (P<0.05) higher in non-irradiated samples than in their irradiated counterparts, this tendency reversed and, after 96 h of storage, they were significantly (P<0.05) higher in samples irradiated for 1 min and 5 min than in the non-irradiated ones (Fig. 2B).

The effects of light-stimulation on progressive sperm motility changed when samples were assessed after incubation at 37 ºC for 120 min. As shown in Fig. 2E, whereas the percentages of progressively motile spermatozoa after incubation at 37 ºC for 120 min dropped throughout storage time, the extent of that decrease was significantly (P<0.05) smaller when samples were irradiated for 5 min and 10 min. (Fig. 2E). This increase in the resilience to withstand thermal stress was observed at 0 h, and after 24 h and 48 h of storage at 17 ºC. In contrast, this counteracting effect was not observed after 96 h of storage at 17 ºC (Fig. 2E).

3.4. Effects of irradiation on motile subpopulations over liquid-storage at 17 ºC for 96 h

A total of three motile subpopulations (SP) were identified. Whereas SP1 showed the lowest VCL, VSL and VAP values, those of SP2 were than in SP1, and those of SP3 were higher than in SP2 (Table 1).

At 0 h, most spermatozoa belonged to SP1 in non-irradiated samples (61.2%), followed by SP2 (27.6%) and SP3 (11.2%; Fig. 3A). This subpopulation structure was roughly maintained during the first 48h of storage. At longer times of storage, however, proportions of spermatozoa belonging to SP1 significantly (P<0.05) increased, whereas those of SP2 and SP3 decreased (SP1: 70.7%, SP2: 25.5% and SP3: 3.8% at 96 h; Fig. 3A). No irradiation protocol modified the structure of motile sperm subpopulations found in non-irradiated samples over storage (Figs. 3C, 3E and 3G).

When non-irradiated samples were thermally stressed at 37 ºC for 120 min, proportions of spermatozoa belonging to SP1 increased (94.5%), whereas those of SP2 (4.6%) and SP3 (0.9%) decreased (Fig. 3B) with regard to that observed before incubation (0 min). Storage at 17 ºC for 48 h and further exposure to thermal stress decreased the percentage of SP1-spermatozoa (70.2%) and increased those of SP2 (24.5%) and SP3 (5.3%; Fig. 3B). Remarkably, light-stimulation for 1 min and 10 min and further incubation at 37 ºC for 120 min significantly (P<0.05) increased the percentages of spermatozoa belonging to SP2 observed in non-irradiated samples at 0 h (non-irradiated samples: 4.6% *vs.* 1 min: 13.0% and 10 min: 14.3%; Figs. 3D and 3H). This effect was maintained during the first 24 h of storage, but not later (Figs. 3D, 3F and 3H).

3.5. Effects of irradiation on free cysteine radicals of sperm head proteins over liquid-storage at 17 ºC for 96 h

As shown in Fig. 4, free cysteine radicals of sperm head proteins, evaluated through 2,2'-dipyrydil disulfide, in non-irradiated samples significantly (P<0.05) increased over storage and reached maximal values at the end of the experiment (96 h: 8.9 nmol/µg protein ± 1.4 nmol/µg protein *vs.* 0 h: 4.1 nmol/µg protein ± 0.6 nmol/µg protein). Free cysteine radicals of sperm head proteins in samples irradiated for 5 min and 10 min were significantly (P<0.05) lower than in their non-irradiated counterparts, not only at 0 h but also after storage at 17 ºC (Fig. 4).

When free cysteine radicals of sperm head proteins were determined with dibromobimane, there was an increase in non-irradiated samples in the 60 kDa-band at 0 h, and in the 20 kDa-band after 96 h of storage (Figs. 5,6A,6B). The effects of irradiation were variable and depended upon the light-stimulation pattern (i.e. 1 min, 5 min or 10 min) and the specific electrophoretic band (i.e. 20 kDa or 60 kDa).

Free cysteine radicals of sperm head proteins in the 20 kDa-band were significantly (P<0.05) lower in samples irradiated for 10 min than in non-irradiated samples after 24 h of storage. In addition, free cysteine radicals of sperm head proteins in the whole lane were significantly (P<0.05) lower in samples irradiated for 1 min than in non-irradiated samples after 24 h and until the end of storage (Figs. 6A, 6B and 6C). Moreover, and compared to non-irradiated samples, light-stimulation for 5 min and 10 min significantly (P<0.05) decreased free cysteine radicals of sperm head proteins after 72 h of storage in both 20 kDa- and 60 kDa-bands, and in the whole electrophoresis lane (Figs. 6A, 6B and 6C).

4. Discussion

Results showed herein suggest that the effects of stimulating pig spermatozoa with red-light are maintained over the first two days of storage, in a similar fashion to that observed in turkey [9] and rabbit sperm [10]. Furthermore, our data indicate that the maintenance of these irradiation effects rely on the applied protocol as well as on the sperm parameter. With regard to sperm viability and acrosome integrity, it is worth mentioning that while light-stimulation had no impact on plasma membrane integrity occurring over liquid-storage in rabbit sperm, all irradiation patterns partially counteracted the increase in the percentages of spermatozoa with altered acrosomes observed after 48 h of storage, which is in agreement with previous studies [10].

The most interesting results are those observed in the sperm resilience to withstand thermal stress (i.e. following incubation at 37 ºC for 120 min). In effect, previous irradiation of sperm for 5 min and 10 min partially mitigated the decrease in sperm viability observed in non-irradiated samples stored at 17 ºC for 24 h and exposed to thermal stress. In addition, the three light-stimulation protocols also counteracted the increase in the percentages of spermatozoa with altered acrosomes observed in non-irradiated samples stored at 17 ºC for 72 h and exposed to thermal stress. The increased sperm resilience to withstand thermal stress in response to light-stimulation was also observed in total and sperm progressive motility; this effect was maintained over the first 48 h of storage and also relied upon the irradiation pattern. These data, evidencing that light-stimulation increases the resilience of pig sperm to withstand thermal stress, are in line with previous studies in which not only were light-stimulation protocols different but they were also conducted in other species (turkey and sheep [9,10]). While the mechanisms through which irradiation can increase that resilience remain unknown, they are likely to be related with the presence, in the sperm plasma membrane, of receptors that are sensitive to both light and temperature. In effect, sperm plasma membrane is known to contain both TRVP and opsins receptors, which have been suggested to be involved in the modulation of thermotaxis [14]. Sperm thermotaxis has been related with the regulation of lifespan and capacitation of mammalian sperm, since is one of the mechanisms that ultimately coordinates oocyte fertilization [14]. Therefore, one may suggest that any factor that affects the response of TRPV and/or opsins to thermotaxis can modulate the sperm resilience to withstand environmental stress.

Another finding in this study was that the effects of light-stimulation depend on the specific irradiation pattern. This suggests that both the intensity and rhythm at which energy is applied are crucial for the sperm response to red-light. Light would thus act on several, highly sensitive points, such as phosphodiester bonds that link phosphate molecules in the ATP structure [28], thereby increasing the energy stored in these bonds. Light could also act on other sensitive points, such as the mitochondrial electron chain, thereby changing its rhythm [29,30]. Irrespective of the exact mechanism, light appears to cause positive or negative effects on these points, depending on the irradiation pattern [29]. Furthermore, it is worth noting that mammalian sperm are extremely sensitive to thermotaxis, since they respond to temperature variations as low as 0.0006ºC [31]. Since the device used in the current study to irradiate pig sperm is not able to regulate the environmental temperature at such a low range (0.0006ºC), we suggest that there could be a mixed effect of direct energy stimulation and thermotactic response linked to slight, non-controllable temperature increase during irradiation. Therefore, the different impact of each irradiation pattern could result from both the specific energy intake and the thermotaxis-modulating effect.

While determining free cysteine radicals of sperm head proteins, we observed that, as expected, storage at 17 ºC induced a time storage-dependent increase in the disruption of nucleoprotein disulfide bonds, evaluated through 2,2'-dipyrydil disulfide. Nevertheless, it is worth mentioning that these results are apparently contradictory with those observed following dibromobimane staining. These apparent discrepancies can be due to the different sensitivity of both techniques. In effect, any spectrophotometer-based technique, such as that of 2,2'-dipyrydil disulfide, is much more sensitive than a one based on semi-quantitative analysis of electrophoretic band intensities and on their ratios (calculated between dibromobimane- and Coomassie Blue-stained bands). Thus, it is reasonable to suggest that whereas the 2,2'-dipyrydil disulfide technique is able to detect small differences, the dibromobimane one is not able to. In spite of this, dibromobimane can detect changes in specific nucleoproteins and nucleoprotein-DNA aggregates [32], whereas the 2,2'-dipyrydil disulfide technique evaluates the whole sperm head extract. Therefore, these two techniques are complementary and allow us to consider through which specific manner disulfide bonds are gradually breaking. While free cysteine radicals of the 60 kDa band significantly increased at 0 h, they only did so after 96 h of storage in the case of the 20 kDa band. In this context, one should bear in mind that

whereas the 20 kDa band corresponds to free nucleoproteins, the 60 kDa one coincides with specific nucleoproteins-DNA fragments that result from sperm head sonication [33]. Whereas the lack of increase in the free cysteine radicals of the 20 kDa band at 0 h could be explained if we assume that free nucleoproteins have the same number of cysteine radicals, the observed increase after 96 h of storage would most probably be caused by an increase in the total amount of free nucleoproteins. On the contrary, since the 60 kDa band corresponds to nucleoprotein/DNA aggregates, with a variable number of disulfide bonds, the gradual increment observed as soon as after 24 h of storage could result from breaking of disulfide bonds between proteins and DNA. In any case, our data indicate that irradiation for 5 min and 10 min counteracted, at least partially, this gradual increase. We could hypothesize that light increases the energy of disulfide bonds since, as aforementioned, energy accumulates in covalent bonds, such as the phosphodiester ones [28]. The increase in the accumulated energy of these chemical bonds makes them more difficult to be broken, although their breaking would release much more energy than in standard conditions [26]. Hence, our data suggest that when light-stimulation reaches specific energy levels, it helps maintain the disulfide bonds-based structures that, otherwise, would be progressively deteriorating over storage.

5. Conclusions

In conclusion, our results indicate that not only does irradiation of pig sperm increase specific quality parameters, but also their resilience to withstand thermal stress over the first 48 h of storage at 17 ºC. Apart from the fact the light-stimulation contributes to the maintenance of nucleoprotein structure over storage, it is noticeable that the effects of irradiation along with their durability depend on the irradiation pattern.

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Author contribution

Conceptualization, M.M.R.A. and J.E.R.G.; Data curation, O.B.P. and J.E.R.G.; Formal analysis: J.E.R.G.; Funding acquisition: T.R., S.B., M.Y. and J.E.R.G.; Investigation: O.B.P., J.C., A.D.M., M.L., L.T.R., M.M.R.A. and J.E.R.G.; Methodology, O.B.P., J.C., A.D.M., M.L., M.M.R.A., L.T.R. and J.E.R.G.; Project administration, M.M.R.A. and J.E.R.G.; Resources, T.R., S.B., M.Y. and J.E.R.G.; Supervision, M.M.R.A. and J.E.R.G.; Writing - original draft: O.B.P. and J.E.R.G.; Writing - review & editing: M.Y. and M.M.R.A.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported herein.

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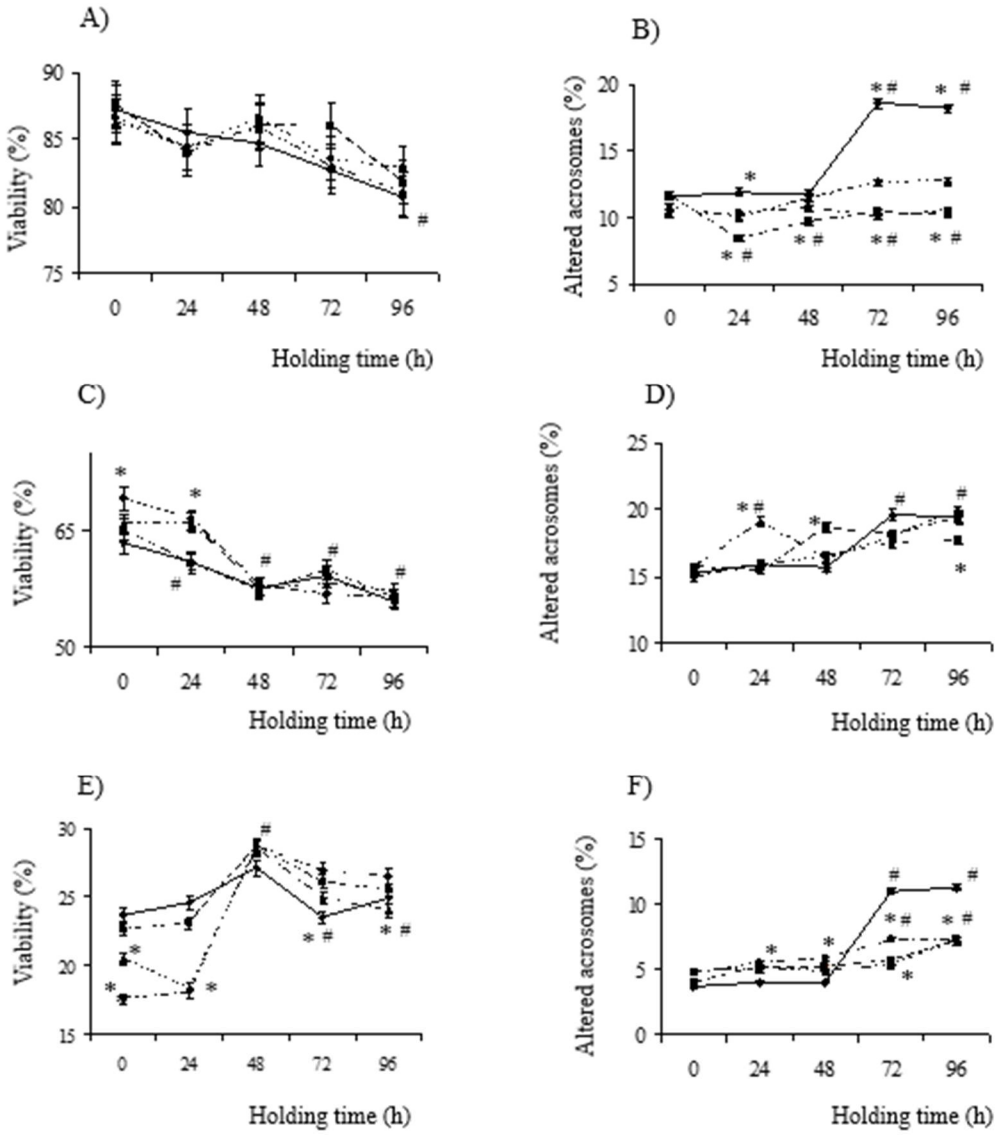

Figure 1. Effects of light-stimulation on the percentages of viability (plasma membrane integrity; **A, C, E**) and altered acrosomes (**B, D, F**). Samples were non-irradiated (♦) or irradiated with red LED light for 1 min (■), 5 min (▲) or 10 min (●), and subsequently stored at 17 ºC for 96 h. Figures 1A and 1B represent results obtained with no previous incubation at 37 ºC (0 min). Figures 1C and 1D show data obtained after incubation of samples at 37 ºC for 120 min (thermal stress test). Figures 1E and 1F represent differences between thermally-stressed (incubated at 37 ºC for 120 min) and non-incubated samples (0 min). (*) means significant (P<0.05) differences between irradiated samples and their respective non-irradiated control within a given time point. (#) means significant (P<0.05) differences between storage times at 17 ºC (24 h, 48 h, 72 h and 96 h) and the results obtained immediately after irradiation (0 h) within a given light-stimulation protocol. Results are expressed as means±SEM for seven separate experiments.

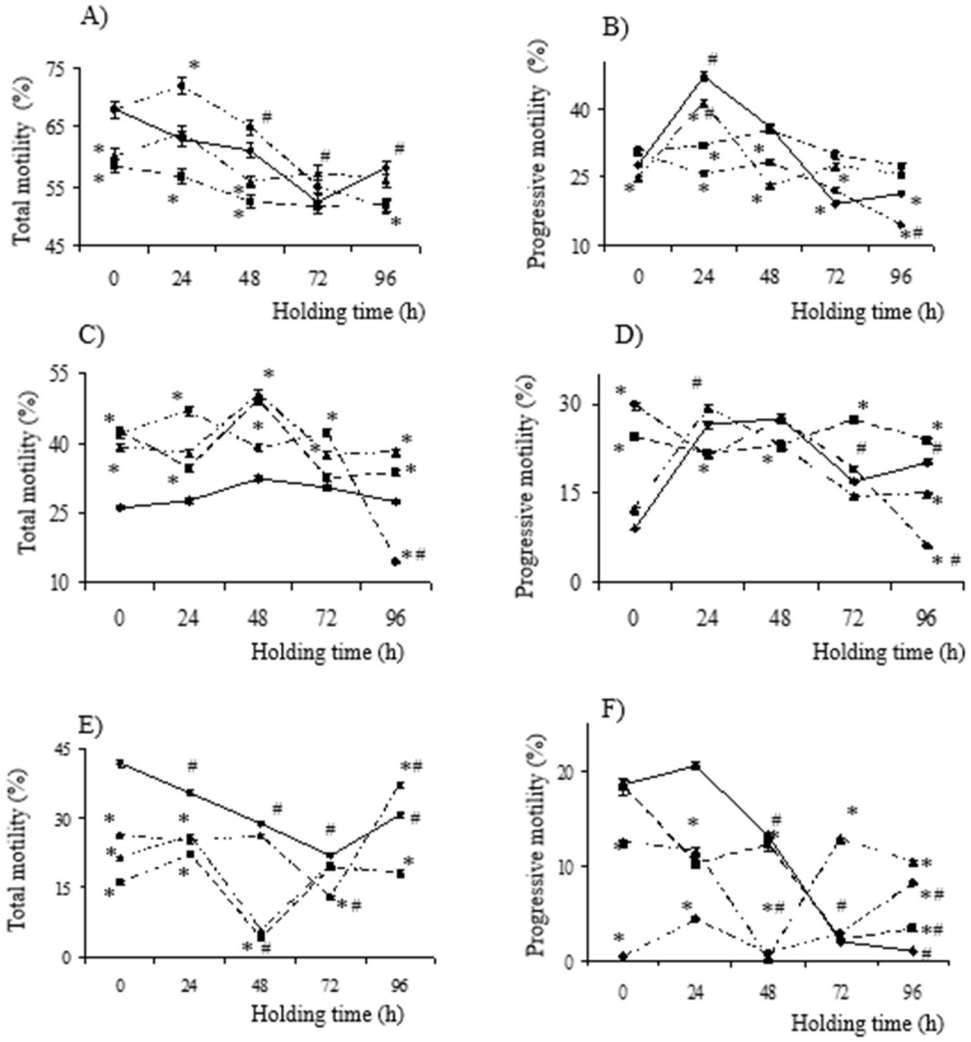

Figure 2. Effects of light-stimulation on percentages of total motile spermatozoa (**A, C, E**) and progressively motile spermatozoa (**B, D, F**). Samples were non-irradiated (♦) or irradiated with red LED light for 1 min (■), 5 min (▲) or 10 min (●), and subsequently stored at 17 ºC for 96 h. Figures 2A and 2B represent results obtained with no previous incubation at 37 ºC (0 min). Figures 2C and 2D show data obtained after incubation of samples at 37 ºC for 120 min (thermal stress test). Figures 2E and 2F represent differences between thermally-stressed (incubated at 37 ºC for 120 min) and non-incubated samples (0 min). (*) means significant (P<0.05) differences between irradiated samples and their respective non-irradiated control within a given time point. (#) means significant (P<0.05) differences between storage times at 17 ºC (24 h, 48 h, 72 h and 96 h) and the results obtained immediately after irradiation (0 h) within a given light-stimulation protocol. Results are expressed as means±SEM for seven separate experiments.

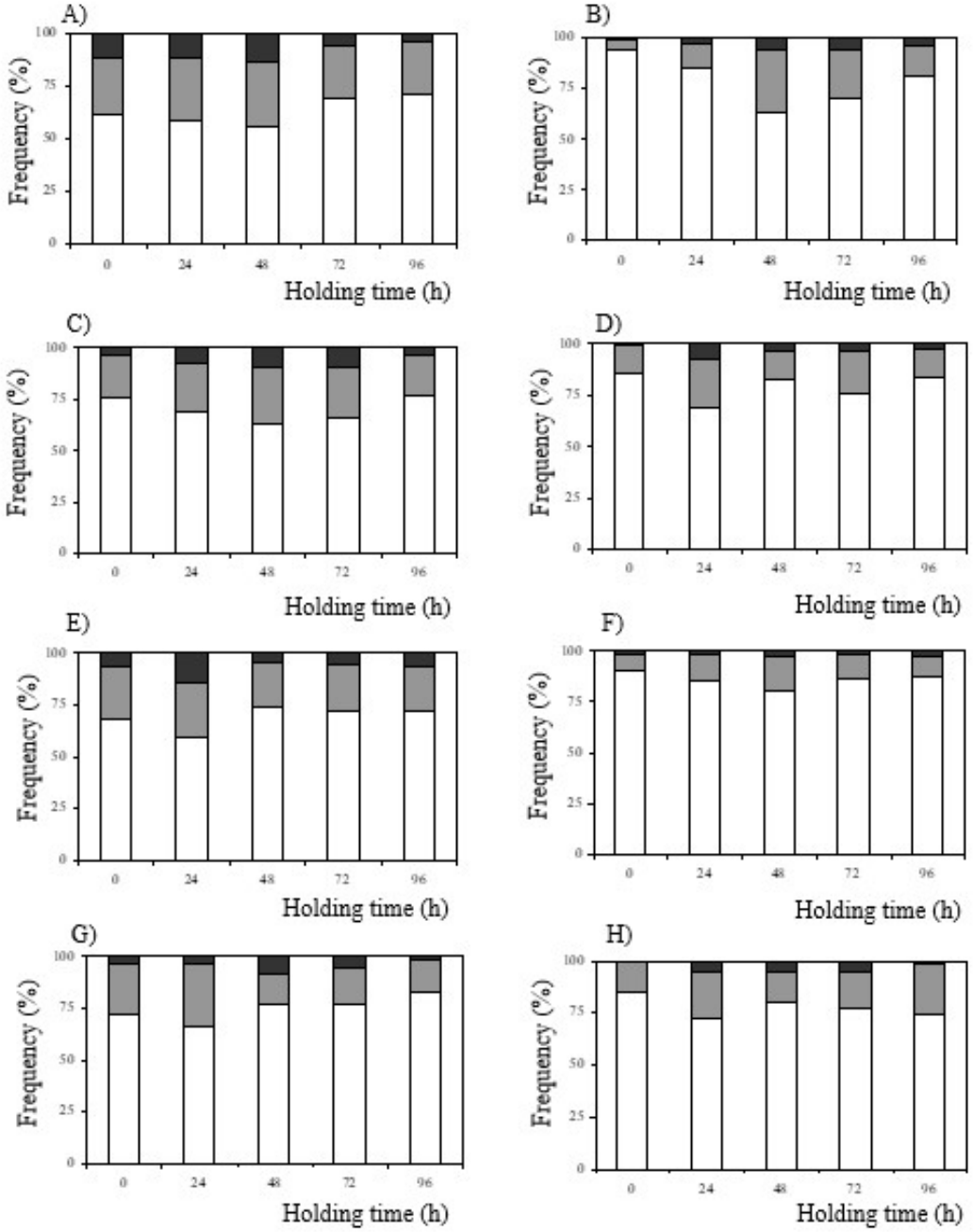

Figure 3. Effects of light-stimulation on motile sperm subpopulations. Data are shown as proportions of sperm belonging to Subpopulation 1 (white areas), Subpopulation 2 (grey areas) and Subpopulation 3 (black areas) in non-irradiated samples (**A, B**), and samples irradiated with red LED light for 1 min (**C, D**), 5 min (**E, F**) or 10 min (**G, H**), and subsequently stored at 17 ºC for 96 h. Figures 3A, 3C, 3E and 3G represent results obtained with no previous incubation at 37 ºC (0 min). Figures 3B, 3D, 3F and 3H show data obtained after incubation of samples at 37 ºC for 120 min (thermal stress test). Results are expressed as mean ± SEM for seven separate experiments.

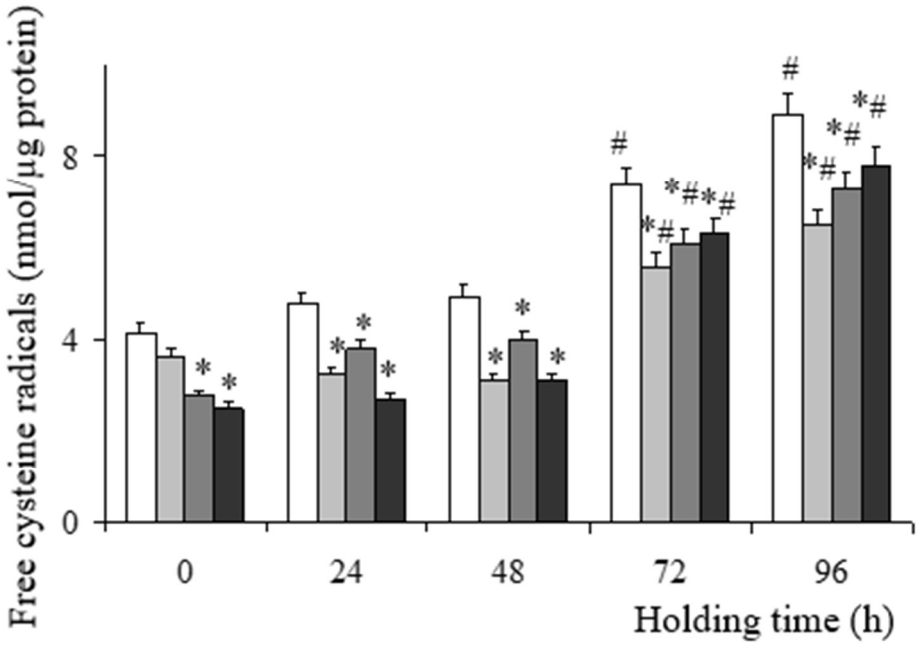

Figure 4. Effects of light-stimulation on free cysteine radicals of sperm head proteins determined through the 2,2-dipyrydil disulfide technique in non-irradiated samples (white bars), and samples irradiated with red LED light for 1 min (light grey bars), 5 min (dark grey bars) or 10 min (black bars), and subsequently stored at 17 ºC for 96 h. (*) means significant (P<0.05) differences between irradiated samples and their respective non-irradiated control within a given time point. (#) means significant (P<0.05) differences between storage times at 17 ºC (24 h, 48 h, 72 h and 96 h) and the results obtained immediately after irradiation (0 h) within a given light-stimulation protocol. Results are expressed as means±SEM for seven separate experiments.

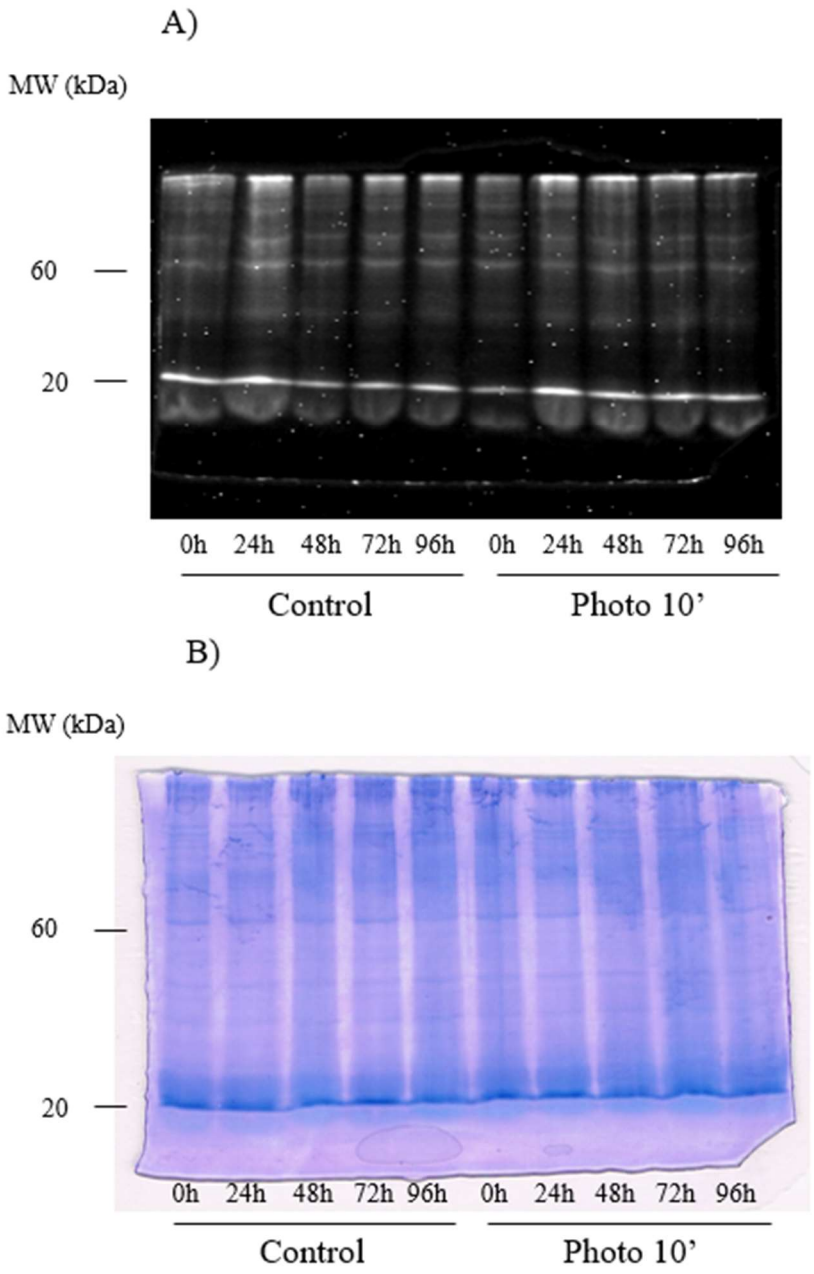

Figure 5. Effects of light-stimulation on free cysteine radicals of sperm head proteins determined through dibromobimane. (**A**) Representative dibromobimane-stained electrophoresis gel (after revealing with UV light) in non-irradiated samples (control) and samples irradiated with red LED light for 10 min (Photo 10') and stored at 17 ºC for 96 h. (**B**) The same electrophoresis gel after washing and subsequent staining with Coomassie Blue; the 20 kDa-band corresponds to free nucleoproteins, and the 60 kDa-band corresponds to nucleoprotein/DNA aggregates. Similar electrophoresis were conducted for samples irradiated for 1 min and 5 min. In all cases, non-irradiated samples for the same experiment were loaded onto the same gel so as to standardize the obtained intensities.

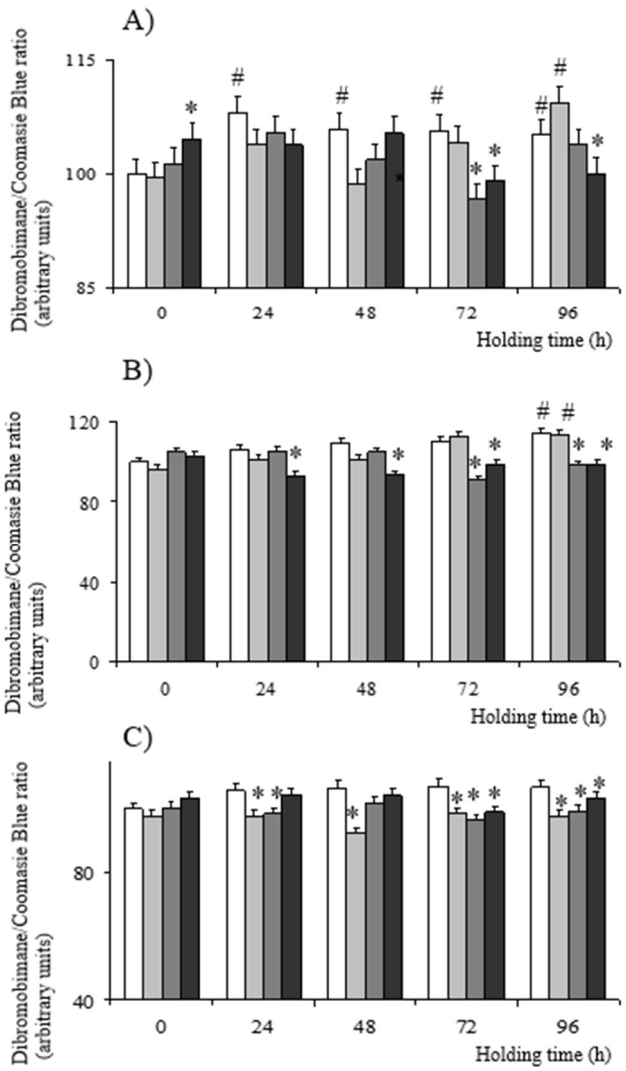

Figure 6. Effects of light-stimulation on free cysteine radicals of sperm head proteins determined through dibromobimane in non-irradiated samples (white bars), and samples irradiated with red LED light for 1 min (light grey bars), 5 min (dark grey bars) or 10 min (black bars), and subsequently stored at 17 ºC for 96 h. (**A**) Dibromobimane/Coomassie Blue ratios for the specific 60 kDa band. (**B**) Dibromobimane/Coomassie Blue ratios for the specific 20 kDa band. (**C**) Dibromobimane/Coomassie Blue ratios for the entire electrophoresis lane. (*) means significant (P<0.05) differences between irradiated samples and their respective non-irradiated control within a given time point. (#) means significant (P<0.05) differences between storage times at 17 ºC (24 h, 48 h, 72 h and 96 h) and the results obtained immediately after irradiation (0 h) within a given light-stimulation protocol. Results are expressed as means±SEM for seven separate experiments.

MANUSCRIPT II

Red LED light acts on the mitochondrial electron chain of mammalian sperm via light-time exposure-dependent mechanisms

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Cells, In Revision

Title

Red LED light acts on the mitochondrial electron chain of mammalian sperm via light-time exposure-dependent mechanisms

Authors

Olga Blanco-Prieto[1,#], Jaime Catalán[1,#], Lina Trujillo[1], Alejandro Peña[1], Maria Montserrat Rivera del Álamo[1], Marc Llavanera[2,3,], Sergi Bonet[2,3], Josep Maria Fernández-Novell[4], Marc Yeste[2,3,‡,*], Joan E. Rodríguez-Gil[1,‡,*]

Affiliations

[1]Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra (Cerdanyola del Vallès), Spain

[2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, E-17003 Girona, Spain

[3]Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, E-17003 Girona, Spain

[4]Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, E-08028 Barcelona, Spain.

[#]**Joint first authors**

[‡]**Joint senior authors**

[*]**Corresponding authors**

Marc Yeste, Unit of Cell Biology, Department of Biology, University of Girona, Faculty of Sciences, C/Maria Aurèlia Campany, 69, Campus Montilivi, E-17003 Girona, Spain. Electronic address: marc.yeste@udg.edu. Phone: +34 972 419514. Fax: +34 972 418150; Joan E. Rodríguez Gil: Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra (Cerdanyola del Vallès), Spain. e-mail: juanenrique.rodriguez@uab.cat. Phone: +34 93 5811045. Fax: +34 93 5812006.

Abstract

This work analyzes the effect of red LED light on boar sperm mitochondrial function. Thus, liquid-stored boar semen was stimulated with red-light for 1 min, 5 min and 10 min in the presence or absence of oligomycin A, a specific inhibitor of mitochondrial ATP synthase, or FCCP, a specific disruptor of mitochondrial electron chain. Whereas exposure for 1 min and 5 min significantly (P<0.05) decreased total motility and ATP levels, 10 min irradiation induced the opposite effect. Oligomycin A abolished light-effects on ATP levels, O_2 consumption and mitochondrial membrane potential, whereas FCCP significantly (P<0.05) increased O_2 consumption. Both oligomycin A and FCCP significantly (P<0.05) decreased total motility. Red light increased cytochrome C oxidase activity with a maximal effect at 5 min stimulation, which was abolished by both oligomycin A and FCCP. In conclusion, red-light modulates boar sperm mitochondria activity through affecting electron chain activity in an exposition time-dependent manner.

Keywords: Sperm; photobiology; mitochondrial function; pig; oligomycin A; FCCP

Introduction

Since about 95% of swine farms in Western countries use Artificial Insemination (AI) with liquid-stored boar semen, yielding an estimated mean fertility of 90%, its optimization is crucial in that sector (Rodríguez-Gil and Estrada, 2013; Knox, 2016; Yeste, 2017). Therefore, any technique aimed at increasing reproductive performance needs to be contemplated, that is why several approaches have been undertaken in the last years. One of these approaches is red-light irradiation, which increases both farrowing rates and litter sizes showing, however, significant variations across farms worldwide (Yeste et al., 2016; Blanco Prieto et al., 2019). In addition, the literature remains inconsistent on how light-irradiation affects sperm function and their ability to elicit *in vitro* capacitation (Yeste et al., 2016; Luther et al., 2018; Pezo et al., 2019). In this context, additional research on which mechanism/s underlie the boar sperm response to red-light is much warranted.

At present, three potential mechanisms have been suggested to be related to the sperm response to red-light (reviewed in Rodríguez-Gil, 2019). The first one hypothesizes that light interacts with plasma membrane receptors, such as those belonging to the Transient Receptor Potential (TRP) family, linked to thermotaxis. The TRP family is a highly heterogeneous group and only proteins included in vanilloid TRP (TRPV), ankyrin TRP (TRPA) and melastanin TRP (TRPM) subfamilies are involved in the control of thermotaxis (Vriens et al., 2014). As part of the first subfamily, TRPV4 has been demonstrated to be the most important temperature-sensitive ion channel found in mammalian sperm (Dusenbery, 2009). The second mechanism is related to the ability of light to interact with specific receptors belonging to the opsins family. At this respect, not only has rhodopsin, a retinal-dependent opsin sensitive to red-light (Islam, 2011), been identified in mouse and human spermatozoa (Zheng, 2013), but also other opsins, such as the blue light-sensitive melanopsin, the ultraviolet-responsive neuropsin and PNN3/encephalopsin (Gunthorpe et al., 2002; Rosenbaum et al., 2004; Harteneck, 2005; Guimaraes & Jordt, 2007; Wu et al., 2010; Nilius & Owsianik, 2011; Masuyama et al., 2012; Zheng, 2013; Pérez-Cerezales et al., 2015). While the exact function of these opsins in mammalian sperm still remains unknown, mounting evidence suggests that these proteins, especially rhodopsin, are involved, together with TRP proteins, in the modulation of sperm thermotaxis (Zheng, 2013; Pérez-Cerezales et al., 2015). Finally, the third mechanism contemplates the direct effect on intracellular, light-sensitive proteins, notably the impact on photosensitizers, such as mitochondria cytochromes,

which are crucial for the regulation of the overall mitochondrial function. Cytochromes are an essential component of the mitochondria electron chain and play a vital role in the control of oxidative phosphorylation, generation of reactive oxygen (ROS) and apoptosis (Ortega-Ferrusola et al., 2009). A common characteristic of cytochromes is the presence of the heme group, which accepts and donates electrons across the electron chain (Kessel, 1982; Pottier & Truscott, 1986). Since the heme group is light-sensitive, cytochromes are known to react against specific light wavelengths depending on their structure (Lynch & Copeland, 1992). Thus, whereas cytochrome P450 has its higher absorption peak at 450 nm, other cytochrome complexes, such as cytochrome C, has two peaks at 610-630 nm and 660-680 nm (Lynch & Copeland, 1992).

Against this background, this study aimed to examine how irradiation of boar sperm with red-light affects mitochondrial function, since this is one of the hypothesized mechanisms of action. With this purpose, boar semen stored at 17ºC was irradiated with red-light for 1 min, 5 min and 10 min, in the presence/absence of oligomycin A, a well-known specific inhibitor of mitochondrial ATP synthase; or FCCP, a potent disruptor of electron chain function. Our hypothesis is that the use of these two agents should disrupt electron chain function and abolish the stimulating effects of red-light upon mitochondrial function when they block the crucial, involved part of the electron chain.

Materials and Methods

Semen samples

Semen samples were provided by a commercial farm (Servicios Genéticos Porcinos, S.L. Roda de Ter, Spain) and came from 17 separate boars. Animals were post-pubertal (2-3 years old), healthy and belonged to the Piétrain breed. Studs were housed in strictly climate-controlled conditions, fed with a standard diet and provided with water *ad libitum*. Semen was collected manually through the gloved hand method, and the obtained rich-sperm fractions were immediately diluted at 17ºC to a final concentration of 2×10^7 sperm/mL in a commercial extender (Duragen®, Magapor, S.L.; Ejea de los Caballeros, Zaragoza, Spain). Diluted semen was split into 90-mL commercial AI doses, and two doses of each ejaculate were transported at 17ºC to our laboratory for 30-60 min.

Ethics

Boar ejaculates utilized in this study were not collected for the unique purpose of investigation, but for their commercial use. Thus, the farms provided us with some doses,

and the remaining ones were used for AI. Therefore, there was no need for any specific ethical approval to perform this work, as no animal was manipulated by the authors.

Experimental design

Upon arrival, samples were confirmed to fulfill quality standards, which were ≥85% membrane-intact spermatozoa ($SYBR14^+/PI^-$), ≥85% morphologically normal spermatozoa, and ≥80% total motile spermatozoa. Following this, each semen sample was split into separate aliquots of 1.5-mL that were subjected to three red-light irradiation protocols (LED, 620-630 nm; PhastBlue®, IUL, S.L.; Barcelona, Spain). These three protocols consisted of exposing sperm to red-light for 1 min (1'), 5 min (5') or 10 min (10'). In all cases, the temperature within the PhastBlue® system was maintained at 20ºC. The control consisted of 1.5-mL Eppendorf tubes kept at 20ºC in the dark for 10 min. In addition to the aforementioned, semen samples were also exposed to the same three protocols (i.e. 1', 5' and 10') or non-exposed (control) in the presence of either 5 µM oligomycin A or 5 µM FCCP. Both inhibitors were added to semen samples 10 min before exposure to red-light.

Following exposure to red-light, samples were used to evaluate sperm motility through a computer-assisted sperm analysis system (CASA); sperm membrane and acrosome integrity, mitochondrial membrane potential and intracellular ROS and calcium levels by flow cytometry; O_2 consumption; intracellular ATP levels; and total cytochrome C oxidase (CCO) activity. In the case of intracellular ATP levels and total cytochrome C oxidase (CCO) activity, samples were first centrifuged at 2,000×g and 20ºC for 30 s and the resulting pellet was frozen by plunging into liquid N_2. Samples were stored at -80ºC until use.

Sperm motility

Sperm motility was determined through a CASA system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain), based on the settings and parameters described in Ramió et al. (2008). The following kinematic parameters were evaluated: curvilinear velocity (VCL), straight line velocity (VSL), average pathway velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR), wobble coefficient (WOB), amplitude of lateral head displacement (mALH), beat cross frequency (BCF), dance (DNC, VCL×ALH), absolute mean angular displacement (absMAD) and algebraic mean angular displacement (algMAD). Briefly, samples were warmed at 38ºC for 5 min

in a water bath prior to placing 6 µL into a 20-micron Leja® Standard Count Chamber Slide (Leja Products B.V.; Nieuw Vennep, The Netherlands). Three replicates of 1,000 sperm each were evaluated before calculating the corresponding mean ± standard error of the mean (SEM). Total motility was defined as the percentage of sperm with VAP ≥10 µm/s, whereas progressive motility was considered to be the percentage of motile sperm with a STR ≥45%. Individual kinematic parameters (VSL, VCL, VAP, LIN, STR and BCF) were used to determine motile sperm subpopulations.

Flow cytometry

Flow cytometry was used to determine plasma membrane and acrosome integrity, mitochondrial membrane potential (MMP), and intracellular levels of superoxides, peroxides and calcium, following the recommendations set by Lee et al. (2008). In all analyses, sperm concentration was previously adjusted to 1×10^6 spermatozoa/mL in a final volume of 500 µL, and three technical replicates were evaluated. Samples were examined using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA), and the sheath flow rate was set at 4.17 µL/min. Electronic volume (EV) and side scatter (SS) were recorded in a log-linear mode (EV/SS dot plots) for 10,000 events per replicate. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter>12 µm), and compensation was used to minimize fluorescence spill-over into a different channel. Information on all events was collected in List-mode Data files (EV, SS, FL1, FL2 and FL3) and processed using the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). In all assessments data were corrected using the procedure described by Petrunkina et al. (2010), based on the percentage of debris particles ($SYBR14^-/PI^-$) determined through SYBR14/PI staining. Fluorochromes were purchased from Molecular Probes® (Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) and diluted with dimethyl sulfoxide (DMSO).

Plasma membrane integrity

Percentages of membrane-intact spermatozoa were determined using the LIVE/DEAD® Sperm Viability Kit (SYBR14/PI; Garner & Johnson, 1995). Three separate sperm populations were identified. One of these populations corresponded to membrane-intact spermatozoa ($SYBR14^+/PI^-$), whereas the other two subpopulations ($SYBR14^-/PI^+$ and $SYBR14^+/PI^+$) corresponded to spermatozoa with different degrees of

plasma membrane alterations. Debris and non-sperm particles were detected as SYBR14$^-$/PI$^-$, and were not taken into account to calculate the percentages of the three sperm populations. Single-stained samples were used to set EV gain, FL1 and FL3 PMT-voltages and for compensation of FL1 spill over into the FL3-channel (2.45%).

Acrosome integrity

Acrosome integrity was determined through staining with peanut agglutinin from *Arachis hypogaea* conjugated with fluorescein isothiocyanate (FITC-PNA) and ethidium homodimer (EthD-1), following the protocol set by Cooper & Yeung (1998) and adapted to boar spermatozoa by Rocco et al. (2018). Following this procedure, four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1$^-$); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1$^-$); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1$^+$); and (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1$^+$). Total percentages of spermatozoa with a non-intact acrosome membrane resulted from summing PNA-FITC$^-$/EthD-1$^-$ and PNA-FITC$^-$/EthD-1$^+$ populations. FL1 spill over into the FL3 channel (2.70%) was compensated.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was evaluated by incubation with 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide (JC1; final concentration: 0.3 µM), as described by Gillan et al. (2005). Three subpopulations were distinguished: (i) spermatozoa with high MMP, characterized by the formation of JC1 aggregates, which showed orange staining; (ii) spermatozoa with intermediate/medium MMP, characterized by mitochondria that were stained in both orange and green; and (iii) spermatozoa with low MMP, characterized by the presence of JC1 monomers, which emitted green fluorescence.

Intracellular levels of superoxides and peroxides

Intracellular levels of superoxide (O_2^-) radicals were analyzed through staining with hydroethidine and YO-PRO-1 (HE/YO-PRO-1) fluorochromes, whereas peroxide (H_2O_2) radicals were determined through staining with 2'-7'-dichlorodihydrofluorescein diacetate and propidium iodide (H_2DCFDA/PI). Both tests were performed as described in Guthrie & Welch, (2006), and results are shown as percentages of viable spermatozoa

with high superoxide levels (E^+/YO-PRO-1^-) and of viable spermatozoa with high peroxide levels (DCF^+/PI^-). In the case of HE/YO-PRO-1, FL3 spill-over into the FL1-channel was compensated (5.06%), and in that of H_2DCFDA/PI, FL1 spill-over into the FL3-channel was also compensated (2.45%).

Intracellular calcium levels

Previous studies found that Fluo3 mainly stains mitochondrial calcium in boar sperm (Yeste et al., 2015). For this reason, we combined this fluorochrome with propidium iodide (Fluo3/PI), as described by Kadirvel et al. (2009), and the following four populations were identified: (i) viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^-); (ii) viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^-); (iii) non-viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^+); and (iv) non-viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^+). FL1 spill-over into the FL3-channel (2.45%) and FL3 spill-over into the FL1-channel (28.72%) were compensated.

Determination of JC1-staining with laser confocal laser microscopy

Spermatozoa stained with JC1 were also analyzed by confocal laser scanning microscopy to detect the specific localization of mitochondria with high MMP (stained in orange) in the mid-piece. With this purpose, sperm were incubated at the same conditions than those used for flow cytometry, i.e. 38ºC for 30 min (final concentration of JC1: 0.3 µM). In order to avoid an excessive motility constraint that could alter JC1-staining along mitochondrial piece, 20 µL of each sample were placed onto the bottom of a well (4-well plates). Samples were observed under a Leica TCS 4D confocal laser scanning microscope (Leica lasetechnik; Wertrieb, Germany) adapted to an inverted Leitz DMIRBE microscope at 63× (NA=1.4 oil) Leitz Plan-Apo Lens (Leitz; Stuttgarts, Germany). The light source was an argon/krypton laser (74 mW) and fluorescence detection was performed through an excitation wavelength of 488 nm. Depending on how mitochondria were stained, two emission wavelengths were detected. The first emission wavelength corresponded to low MMP mitochondria (JC1 monomers), which emitted at 530 nm (green), whereas the second one corresponded to high MMP mitochondria (JC1 aggregates), which emitted at 590 nm (orange). Since JC1-stained sperm maintained their motility, a sequential track of images per sample was taken at 38ºC for 20 s (rate: one capture/s).

Determination of intracellular ATP levels.

Intracellular ATP levels were determined following the protocol set by Chida et al. (2012). Briefly, 1-mL semen aliquots were spun at 17ºC for 30 s after light-stimulation, and pellets were immediately plunged into liquid N_2. Frozen pellets were subsequently stored at -80ºC for three weeks. Thereafter, pellets were resuspended in 300 µL ice-cold 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer containing 250 mM sucrose (pH was adjusted to 7.4). Resuspended pellets were sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070; Bandelin Electronic GmbH and Co., Berlin, Germany) and tubes were kept on ice to avoid specimen heating. Samples were subsequently centrifuged at 1,000×g and 4ºC for 10 min and supernatants were kept. Twenty µL was used to determine total protein content, and the remaining volume was mixed with 300 µL ice-cold 10% (v:v) trichloroacetic acid and kept at 4ºC for 20 s. Samples were subsequently centrifuged at 1,000×*g* and 4ºC for 30 s, and supernatants were carefully separated from the pellet and again centrifuged at 1,000×*g* and 4ºC for 10 min. Resulting supernatants were mixed with two volumes of 1 M Tris-acetate buffer (pH=7.75), and ATP content was determined in these final suspensions using the Invitrogen® ATP Determination Kit (ThermoFisher Bioscientific; Waltham, USA; catalogue number: A22066), following the manufacturer's instructions. Determinations of ATP content were carried out through an Infinite F200 fluorimeter (TECAN®), using 96-wells microplates for fluorescence-based assays (Invitrogen®). To normalize data, total protein of samples was determined through the Bradford method (Bradford, 1976), by using a commercial kit (Bio-Rad laboratories; Hercules, CA, USA).

Determination of O_2 consumption rate

Determination of O_2 consumption rate was performed using the SensorDish® Reader (SDR) system (PreSens Gmbh; Regensburg, Germany). One-mL semen aliquots, previously exposed to red-light, were transferred onto an Oxodish® OD24 plate (24 wells/plate) specifically designed for this device. Plates were sealed with Parafilm®, introduced in the SDR system, and incubated at 37ºC (controlled atmosphere) for 2 h. During that time, O_2 concentration was recorded in each well at a rate of one reading per min. Data were exported to an Excel file (see Suppl. Fig. 1 for representative curves) and final O_2 consumption rate was normalized against the total number of viable spermatozoa per sample, which was determined through flow cytometry ($SYBR14^+/PI^-$ spermatozoa) using another aliquot.

Determination of cytochrome C oxidase activity

Activity of cytochrome C oxidase (CCO) was determined in mitochondria-enriched sperm fractions, as described in McLean et al. (1993). Briefly, 1-mL sperm aliquots, previously irradiated with red-light, were centrifuged at 1,000×*g* and 17ºC for 30 s. The resulting pellets were immediately plunged into liquid N_2 and stored for three weeks. Pellets were resuspended in 500 µL ice-cold PBS and sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070). Thereafter, 500 µL Percoll® at a concentration of 1.055 mg/mL in PBS at 4ºC was placed onto each sperm homogenate. Samples were centrifuged at 3,000×*g* and 10ºC for 45 min and the mitochondria-enriched fraction was carefully harvested with a micropipette and transferred into a new 1.5-mL tube. Samples were again centrifuged at 12,000×*g* and 20ºC for 2 min and the resulting pellets were resuspended in 100 µL PBS at 20ºC. These mitochondria-enriched suspensions were split into two separate aliquots. The first one was used to determine CCO activity using a commercial kit (Cytochrome C Oxidase Assay Kit; Sigma-Aldrich; catalogue number CYTOCOX1). The other aliquot (10 µL) was used to determine total protein content through a commercial kit based on the Bradford method (Bradford, 1976; KIT). Enzyme activity was normalized against the total protein content.

Statistical analyses

Statistical analyses were conducted using a statistical package (SPSS® Ver. 25.0 for Windows; IBM corp., Armonk, NY, USA). Data were first tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests, respectively. When required, data were transformed through arcsin √x. The effects of red-light stimulation patterns on motility parameters; percentages of spermatozoa with an intact plasma membrane ($SYBR14^+/PI^-$), spermatozoa with an intact acrosome ($PNA\text{-}FITC^+$), spermatozoa with high MMP ($JC1_{agg}^+$), viable spermatozoa with high intracellular calcium levels ($Fluo3^+/PI^-$), viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-), and viable spermatozoa with high peroxide levels (DCF^+/PI^-); geometric mean fluorescence intensity (GMFI) of $JC1_{agg}^+$, $Fluo3^+$, E^+ and DCF^+; intracellular levels of ATP; O_2 consumption rate; and cytochrome C oxidase activity were evaluated through one-way analysis of variance (ANOVA) followed by post-hoc Sidak's test.

Motile sperm subpopulations were determined through the protocol described in Luna et al. (2017). In brief, individual kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) recorded for each sperm cell were used as independent variables

in a Principal Component Analysis (PCA). Kinematic parameters were sorted into PCA components and the obtained matrix was subsequently rotated using the Varimax method with Kaiser normalization. As a result, each spermatozoon was assigned regression scores for the new PCA components and these values were subsequently used to run a two-step cluster analysis based on the log-likelihood distance and the Schwarz's Bayesian Criterion. Three sperm subpopulations were identified and each individual spermatozoon was assigned to one of these subpopulations (SP1, SP2 or SP3). Following this, percentages of spermatozoa belonging to each subpopulation were calculated per sample, and were further used to evaluate the effects of red-light stimulation on the distribution of motile sperm subpopulations through one-way ANOVA and Sidak's post-hoc test.

In all analyses, the level of significance were set at $P \leq 0.05$. Data are shown as mean ± standard error of the mean (SEM).

Results

Effects of red-light stimulation on plasma membrane and acrosome integrity in the presence or absence of either oligomycin A or FCCP

No red-light stimulation protocol had any effect on the percentages of membrane-intact spermatozoa ($SYBR14^+/PI^-$) when compared to the control (80.0% ± 2.0%; Fig. 1A). Similarly, no protocol induced any change on the percentage of acrosome-intact spermatozoa when compared to the control (control: 18.7% ± 1.9%; Suppl. Fig. 2).

Effects of red-light stimulation on sperm motility in the presence or absence of either oligomycin A or FCCP

Percentages of total motile spermatozoa in Oligomycin A (26.1% ± 0.4%) and FCCP (37.1% ± 0.8%) were significantly (P<0.05) lower than those of control (70.8% ±2.5%; Fig. 1B). These differences were also observed in the percentage of progressively motile spermatozoa (Fig. 1C). Light-stimulation altered total and progressive sperm motility, but the impact differed between the three protocols. Thus, whereas light irradiation for 1 and 5 min significantly (P<0.05) decreased total sperm motility compared to the control, it did not make such an impact when samples were irradiated for 10 min (Fig. 1B). Moreover, percentages of progressively motile spermatozoa were significantly (P<0.05) lower in 1 and 10 min protocols than in the control, but they did not differ between the 5 min protocol and the control (Fig. 1C). It is worth noting that the presence of oligomycin A and FCCP during light-stimulation led to a significant reduction in both total and progressive sperm motility (Figs. 1B and 1C).

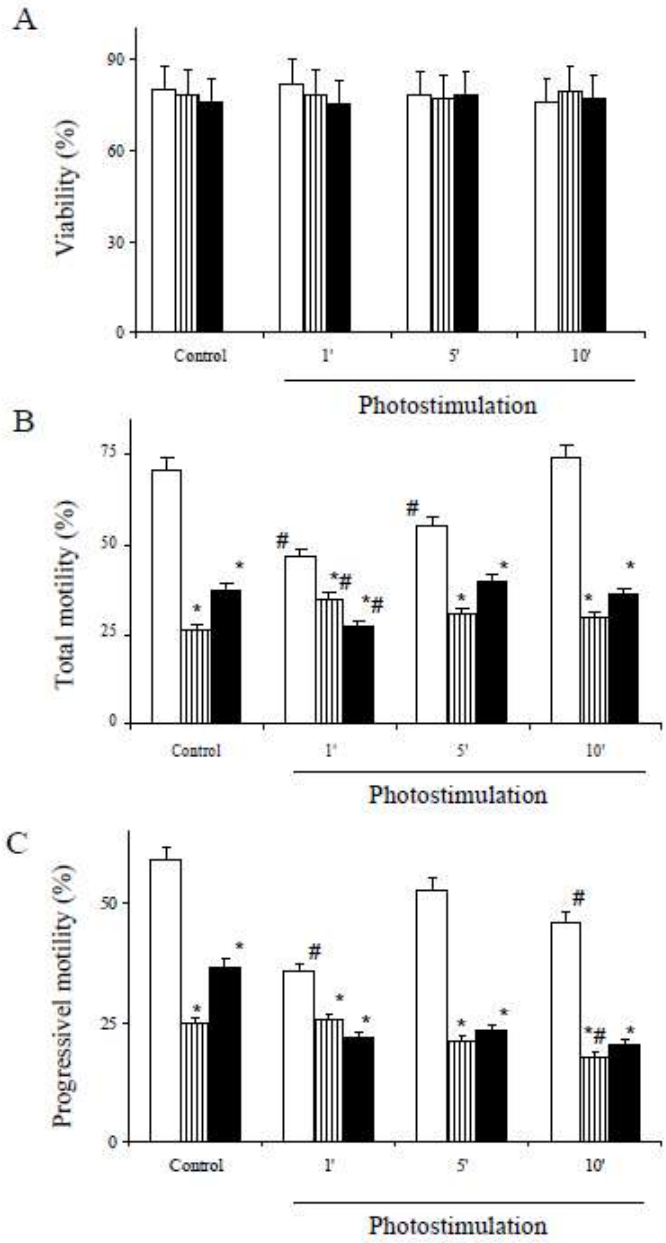

Figure 1 Effects of light stimulation on percentages of membrane intact SYBR14^{+}/PI^{-}; **A**), total (**B**) and progressive (**C**) sperm motility in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 µM oligomycin A. Black bars: sperm incubated with 5 µM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant (P<0.05) differences with respect to non-irradiated sperm (control). (*) indicates significant (P<0.05) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

With regard to the effects of light-stimulation on sperm kinematic parameters, irradiating samples for 1 min led to a significant (P<0.05) decrease on VCL, VAP and VSL; in contrast, the other two light-stimulation protocols (5 min and 10 min) had a much less impact, if any (Table 1). On the other hand, the presence of either oligomycin A or FCCP also exerted a negative impact on sperm kinematic parameters, regardless of whether or not sperm were exposed to red-light (Table 1). This was particularly apparent in the case of VCL and DNC; however, whereas the presence of oligomycin A or FCCP had less effect following light-stimulation for 1 min, the extent of that reduction was higher when sperm were irradiated for 5 and 10 min (Table 1).

Finally, the percentages of spermatozoa belonging to each motile subpopulation changed following light-stimulation, and in response to the presence of oligomycin A or FCCP. Although three motile sperm subpopulations were identified in all treatments, irradiation of sperm with red-light for 1 min significantly (P<0.05) decreased the percentages of Subpopulation 3, which was the one that exhibited the highest kinematic parameters .The presence of either oligomycin A or FCCP did not alter the structure of sperm subpopulations observed after irradiating spermatozoa for 1 min. Moreover, percentages of spermatozoa belonging to each subpopulation following light-irradiation for 5 min and 10 min did not differ from the control (Fig. 2). Interestingly, the presence of oligomycin A or FCCP significantly (P<0.05) decreased the percentages of spermatozoa belonging to Subpopulation 3 in non-irradiated samples (Fig. 2), and in the other two light-stimulation protocols (5 min and 10 min).

Table 1. Effects of photostimulation of boar spermatozoa on the mean motion parameters in the presence or absence of either oligomycin A or FCCP.

	No Photostimulation			1' Photostimulation			5' Photostimulation			10' Photostimulation		
	Control	Olig. A	FCCP	Control	Olig. A	FCCP	Control	Olig. A	FCCP	Control	Olig. A	FCCP
VCL (μm/s)	70.6±6.5^{a}	48.1±3.8^{b}	54.0±4.3^{b}	52.0±4.0^{b}	50.7±4.3^{b}	49.2±4.6^{b}	65.6±5.2^{a}	45.8±3.9^{b}	52.0±4.4^{b}	63.1±5.0^{a}	49.1±4.6^{b}	53.3±4.7^{b}
VSL (μm/s)	28.9±2.6^{a}	19.5±1.7^{b}	20.3±1.8^{b}	22.6±2.0^{b}	21.4±1.9^{b}	20.2±1.9^{b}	26.6±2.0^{a}	18.1±1.4^{b}	18.4±1.5^{b}	26.6±1.9^{a}	17.9±1.3^{b}	17.8±1.4^{b}
VAP (μm/s)	51.2±5.5^{a}	29.7±2.6^{b}	31.1±2.9^{b}	36.3±3.0^{b}	32.4±3.0^{b}	30.8±2.6^{b}	42.0±4.4^{c}	27.0±2.6^{b}	29.7±3.1^{b}	42.9±4.8^{c}	27.5±2.6^{b}	29.8±3.0^{b}
LIN (%)	47.0±3.0^{a}	46.6±3.1^{ab}	42.2±2.7^{b}	50.2±3.5^{a}	49.1±3.0^{a}	45.2±2.6^{ab}	47.8±3.2^{a}	46.6±3.0^{ab}	38.6±2.3^{bc}	49.2±3.9^{a}	43.5±3.0^{ab}	35.6±2.1^{c}
STR (%)	63.0±3.4^{a}	70.1±3.8^{ab}	66.7±3.0^{a}	66.7±3.2^{a}	70.0±3.9^{ab}	68.9±3.6^{a}	61.2±3.5^{a}	72.4±4.0^{b}	63.9±2.9^{a}	67.3±3.5^{a}	69.6±3.9^{ab}	61.0±3.1^{a}
WOB (%)	72.9±4.9^{a}	65.0±4.1^{b}	60.6±4.4^{b}	70.5±4.9^{a}	67.4±4.3^{ab}	63.4±3.8^{b}	77.5±5.2^{a}	62.4±4.1^{b}	58.9±4.0^{b}	70.6±4.8^{a}	60.3±4.2^{b}	57.1±4.9^{b}
mALH (μm)	2.78±0.11^{a}	2.22±0.09^{b}	2.49±0.12^{ab}	2.15±0.08^{b}	2.26±0.10^{b}	2.21±0.10^{b}	2.44±0.12^{ab}	2.14±0.09^{b}	2.40±0.11^{ab}	2.53±0.12^{a}	2.28±0.12^{ab}	2.47±0.14^{ab}
BCF (Hz)	7.7±0.8^{ab}	6.7±0.4^{a}	7.7±0.7^{ab}	7.3±1.0^{a}	7.5±0.8^{a}	7.5±0.7^{a}	7.4±0.6^{a}	7.2±0.8^{a}	7.2±0.9^{a}	8.5±0.8^{b}	7.5±0.4^{a}	7.7±0.8^{ab}
DNC ($\mu m^2/s$)	247.7±10.9^{a}	142.9±5.0^{b}	168.8±7.2^{c}	157.5±5.5^{bc}	154.3±4.9^{bc}	134.1±4.6^{b}	252.3±11.4^{a}	137.5±4.4^{b}	156.6±5.8^{bc}	207.4±8.6^{d}	152.5±4.8^{bc}	159.1±5.0^{bc}
|MAD| (º)	95.8±6.1^{ab}	106.9±6.9^{a}	107.0±7.0^{a}	97.6±5.9^{ab}	105.2±6.9^{a}	107.2±6.9^{a}	81.1±5.4^{b}	115.3±7.9^{a}	114.7±7.6^{a}	99.7±6.3^{a}	113.7±7.3^{a}	118.2±8.6^{a}
algMAD (º)	0.03±0.01^{a}	-1.83±0.13^{b}	-0.35±0.10^{c}	0.20±0.16^{ad}	0.30±0.18^{d}	0.20±0.17^{ad}	0.41±0.17^{d}	-0.04±0.10^{a}	0.24±0.15^{ad}	0.27±0.18^{ad}	1.05±0,24^{e}	-0.56±0.23^{f}

Results are expressed as mean±S.E.M. for 7 separate experiments. The analysis was conducted on a total number of 20,966 motile spermatozoa. Different superscripts in a row indicate significant (P<0.05) difference when compared with the corresponding Control value.

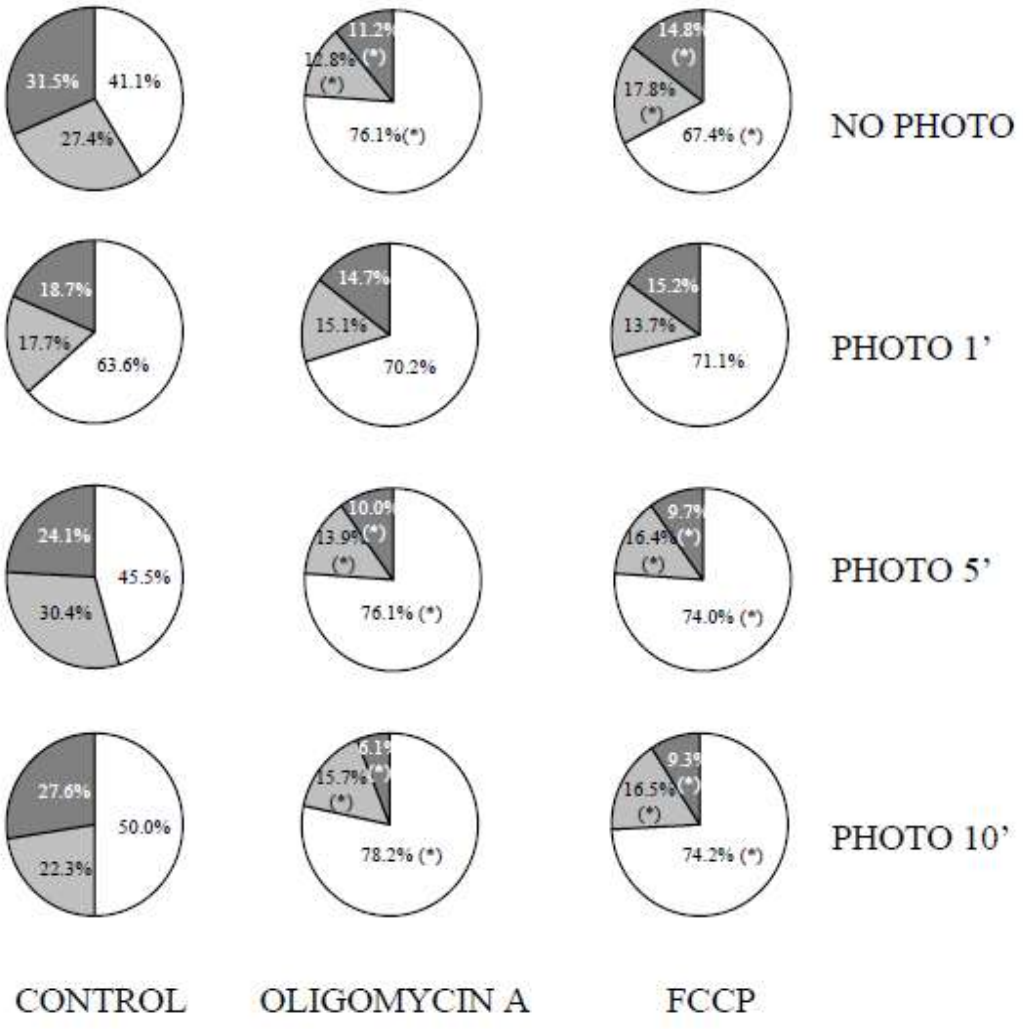

Figure 2. Effects of light-stimulation on the motile sperm subpopulation structure in the presence/absence of either oligomycin A or FCCP. Results are shown in three columns, as follows: CONTROL, sperm incubated without either oligomycin A or FCCP; OLIGOMYCIN A, sperm incubated with 5 µM oligomycin A; FCCP, sperm incubated with 5 µM FCCP. Data are also split into four rows as follows: NO PHOTO: non-irradiated sperm; PHOTO 1': sperm irradiated for 1 min; PHOTO 5': sperm irradiated for 5 min; PHOTO 10': sperm irradiated for 10 min. White areas: % motile spermatozoa belonging to Subpopulation 1. Light grey areas: % motile spermatozoa belonging to Subpopulation 2. Dark grey areas: % motile spermatozoa belonging to Subpopulation 3. Results are shown as mean ± SEM for 7 separate experiments. (*) indicates significant (P<0.05) differences between the control and olygomycin A or FCCP within a given light-stimulation treatment (i.e., control, 1 min, 5 min or 10 min).

Effects of red-light stimulation on mitochondrial membrane potential in the presence or absence of either oligomycin A or FCCP

As expected, percentages of spermatozoa high MMP and the FL2:FL1 in the sperm population with high MMP were low (0.59% ± 0.02%; 1.19 arbitrary units ± 0.08

arbitrary units; Figs. 3B and 3D) in control, non-irradiated samples. While the addition of oligomycin A did not modify these parameters, that of FCCP induced a significant (P<0.05) increase in both the percentage of spermatozoa with high MMP cells and the FL2:FL1 ratio in the sperm population with high MMP (Figs. 3B and 3D).

On the other hand, light-stimulation for 5 min induced a significant (P<0.05) increase in the percentage of spermatozoa with high MMP, and all light-stimulation protocols significantly (P<0.05) increased the FL1:FL2 ratio in the sperm population with high MMP (e.g. 1 min: 1.58 arbitrary units ± 0.17 arbitrary units *vs.* control: 1.19 arbitrary units ± 0.08 arbitrary units; Fig. 3D). While both oligomycin A and FCCP significantly (P<0.05) increased the FL2:FL1 ratio in the sperm population with high MMP following light-stimulation for 1 and 5 min, this effect was not observed when samples were irradiated for 10 min (Fig. 3D).

In all treatments, percentages of spermatozoa with intermediate MMP was much higher than those of spermatozoa with high MMP; the FL2:FL1 ratio in this intermediate MMP population was lower than that of high-MMP one (Figs. 3A and 3C). While percentages of spermatozoa exhibiting intermediate MMP were not affected by light-stimulation protocols and presence of oligomycin A and FCCP, the FL2:FL1 ratio in this sperm population was significantly (P<0.05) increased in light-stimulated spermatozoa, especially after irradiating sperm for 5 min (Fig. 3C). Moreover, although the presence of oligomycin A did not alter the hike observed in the FL2:FL1 ratio after light-stimulation, FCCP counteracted that increase in the three irradiation protocols (Fig. 3C).

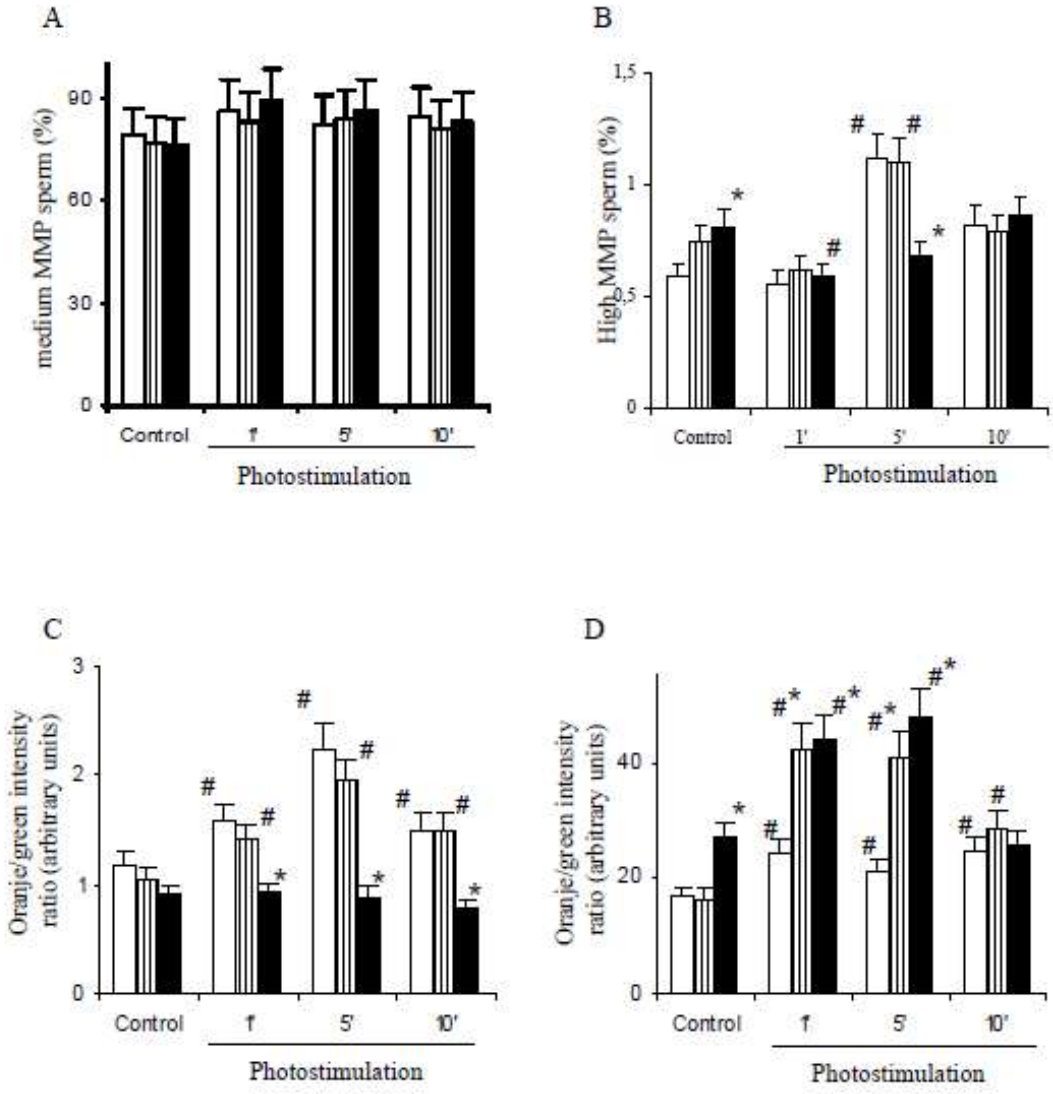

Figure 3. Effects of light-stimulation on mitochondrial membrane potential in the presence/absence of either oligomycin A or FCCP. Results are shown as percentages of spermatozoa with intermediate (medium) MMP (**A**) and high MMP (**B**), and as FL2:FL1 (orange/green) ratio in medium-MMP (**C**) and high-MMP sperm populations (**D**). Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 μM oligomycin A. Black bars: sperm incubated with 5 μM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant ($P<0.05$) differences with respect to non-irradiated sperm (control). (*) indicates significant ($P<0.05$) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

It is worth mentioning that the specific localization of orange-stained mitochondria (corresponding to spermatozoa with high MMP) differed between control and oligomycin A- and FCCP-treated samples. Thus, whereas orange-stained mitochondria in the absence of these two inhibitors were found to be confined to the two mid-piece poles (Fig. 4A), sperm treated with oligomycin A showed a distinct pattern, with orange-stained mitochondria being distributed along the entire mid-piece (Fig. 4B). Sperm treated with FCCP showed faint but uniform orange-staining pattern along the mid-piece (Fig. 4C). No light-stimulation pattern changed the distribution of orange-stained mitochondria along the mid-piece (data not shown).

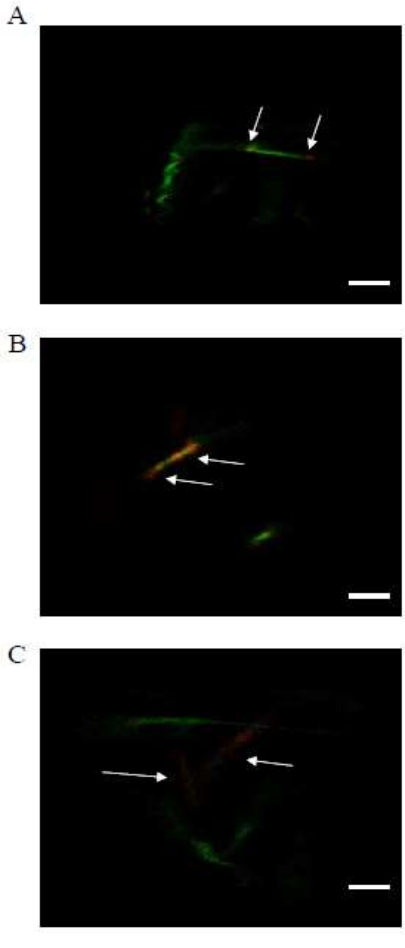

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Figure 4. Specific localization of mitochondria with high mitochondrial membrane potential (JC1 staining) in boar sperm incubated in the presence/absence of either oligomycin A or FCCP. Representative images of laser confocal microscope for control (**A**), oligomycin A (**B**) and FCCP (**C**). Arrows indicate the localization of mitochondria with high MMP (orange staining). The appearance of sashed mid-pieces is due to the frame rate of the laser confocal microscope, which was lower than sperm velocity. Images are representative for 7 separate experiments. Bar size: 10 μm.

Effects of red-light stimulation on superoxide and peroxide levels in the presence or absence of either oligomycin A or FCCP

With regard to the control, percentages of spermatozoa with high intracellular superoxide levels were significantly (P<0.05) lower only when samples were irradiated for 1 min (Fig. 5A). Although neither oligomycin A nor FCCP affected the impact of light-stimulation on the percentages of viable spermatozoa with high intracellular superoxide levels, those percentages in the control, non-irradiated spermatozoa were significantly higher than in non-irradiated spermatozoa exposed to FCCP (control: 22.5% ± 1.5% *vs.* FCCP: 17.1% ± 1.0%).

On the other hand, light-stimulation, especially for 1 min and 10 min, significantly (P<0.05) decreased the proportion of spermatozoa with high peroxide levels (Fig. 5B). While this decrease was not altered by the presence of either oligomycin A or FCCP following irradiation for 1 min and 5 min, both inhibitors counteracted the decrease observed in the 10 min protocol, reaching values similar to those found in non-irradiated spermatozoa (Fig. 5B). Finally, non-irradiated spermatozoa did not show significant differences between the control and oligomycin A- and FCCP-treated samples.

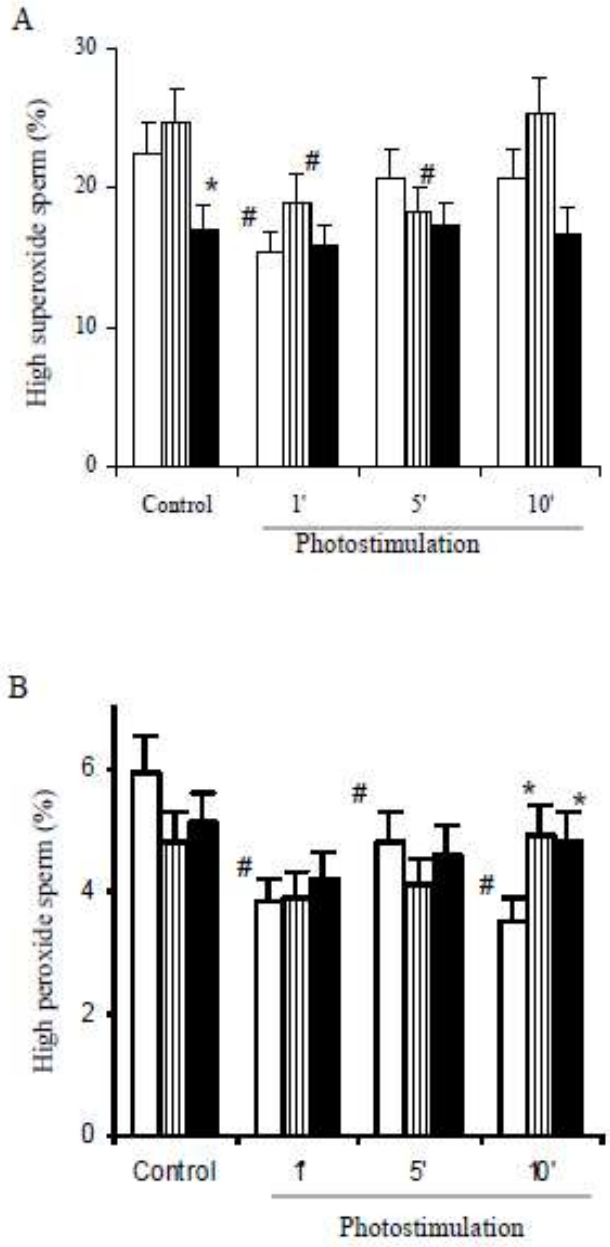

Figure 5. Effects of light-stimulation on intracellular levels of superoxides (**A**) and peroxides (**B**) in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 μM oligomycin A. Black bars: sperm incubated with 5 μM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant (P<0.05) differences with respect to non-irradiated sperm (control). (*) indicates significant (P<0.05) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

Effects of red-light stimulation on intracellular calcium levels in the presence or absence of either oligomycin A or FCCP

Percentages of viable spermatozoa with high intracellular calcium level did not differ amongst non-irradiated samples (i.e. control *vs.* oligomycin A and FCCP; Fig. 6A). However, light-stimulation for 5 min significantly (P<0.05) augmented the geometric mean of fluorescence intensity of $Fluo3^+$ in viable sperm. This increase was counteracted by both oligomycin A and FCCP (Fig. 6B).

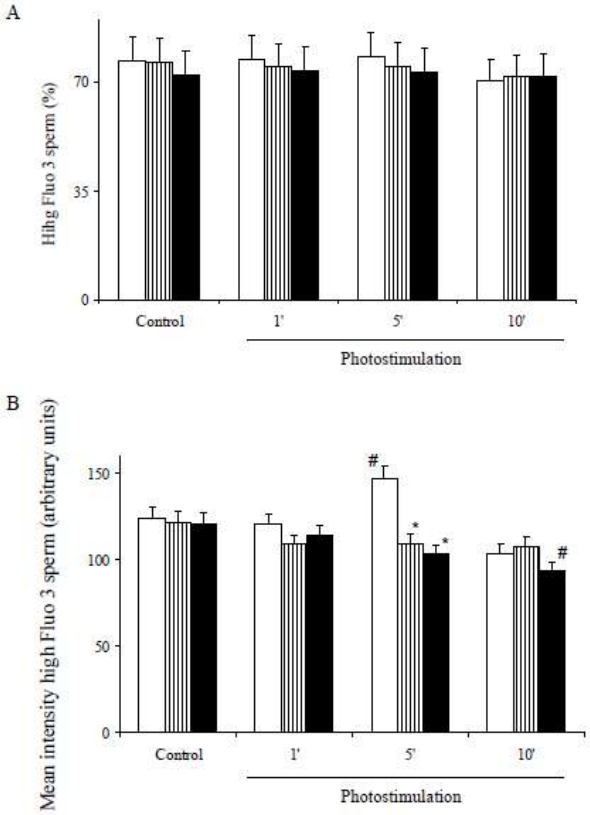

Figure 6. Effects of light-stimulation on percentages of spermatozoa with high intracellular calcium levels ($Fluo3^+$, **A**) and geometric mean intensity of $Fluo3^+$ (**B**) in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 µM oligomycin A. Black bars: sperm incubated with 5 µM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant (P<0.05) differences with respect to non-irradiated sperm (control). (*) indicates significant (P<0.05) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

Effects of red-light stimulation on intracellular levels of ATP and oxygen consumption in the presence or absence of either oligomycin A or FCCP

Control, non-irradiated spermatozoa showed low intracellular ATP levels (0.79 nmol/mg protein ± 0.03 nmol/mg protein; Fig. 7A). These levels were significantly (P<0.05) increased in the presence of oligomycin A, and significantly (P<0.05) decreased in the presence of FCCP (Fig. 7A). Light-stimulation for 1 min and 5 min significantly (P<0.05) decreased intracellular ATP levels, but such decreases were completely counteracted completely or partially by oligomycin A, when spermatozoa were irradiated for 1 min and 5 min, respectively (Fig. 7A). Strikingly, oligomycin A strongly (P<0.05) decreased intracellular ATP levels when spermatozoa were exposed to red-light (non-irradiated sperm + oligomycin A: 0.31 nmol/mg protein ± 0.01 nmol/mg protein *vs.* irradiation for 10 min + oligomycin A: 0.86 nmol/mg protein ± 0.04 nmol/mg protein; Fig. 7A). On the other hand, while the presence of FCCP did not affect the sperm response to light in terms of intracellular ATP levels after exposure for 1 and 5 min, it significantly (P<0.05) decreased those levels when sperm were light-stimulated for 10 min (non-irradiated + FCCP: 0.29 nmol/mg protein ± 0.01 nmol/mg protein *vs.* 10 min + FCCP: 0.86 nmol/mg protein ± 0.04 nmol/mg protein; Fig. 7A). In non-irradiated sperm, O_2 consumption rate was significantly (P<0.05) decreased by oligomycin A, and increased by FCCP (Fig. 7B). Light-stimulation significantly (P<0.05) increased O_2 consumption rate in the three protocols (i.e. 1 min, 5 min and 10 min; Fig. 7B). It is worth noting that while the presence of oligomycin A in irradiated spermatozoa dramatically (P<0.05) decreased O_2 consumption rates, completely abolishing the effects of light, combining red-light exposure and FCCP led to higher O_2 consumption rates than those observed in the absence of FCCP (Fig. 7B)

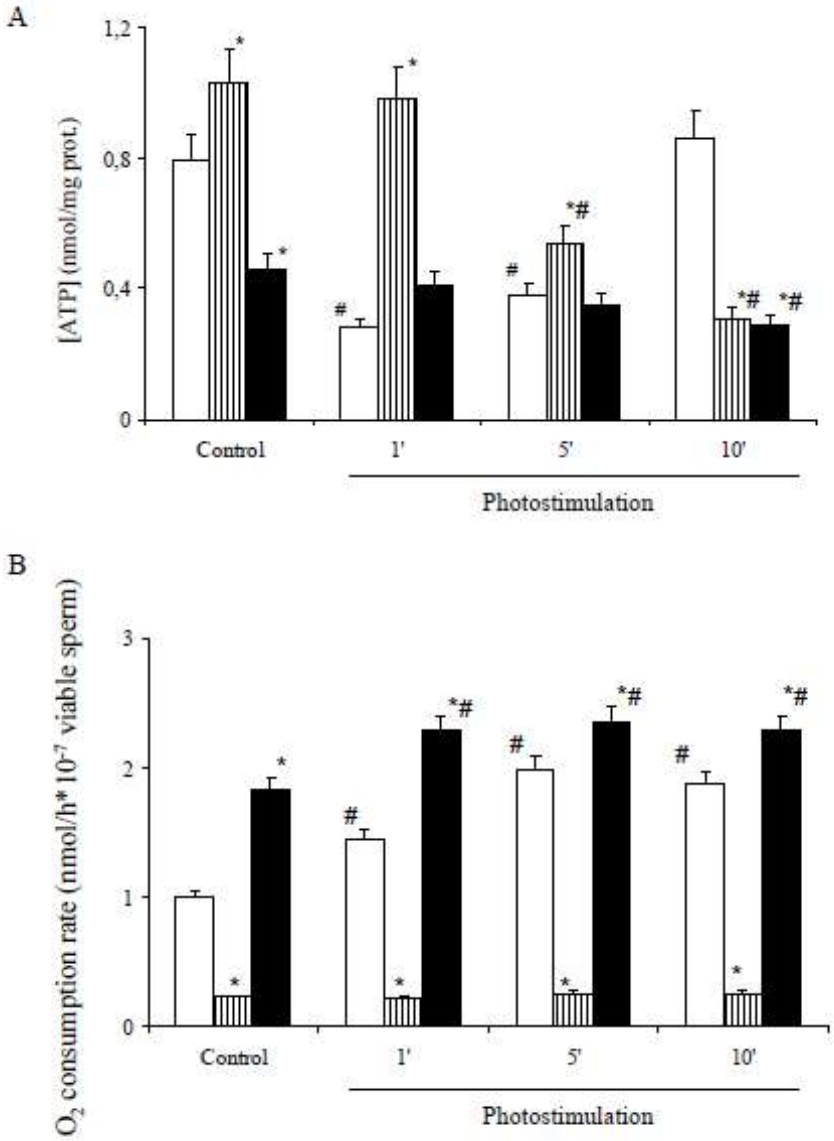

Figure 7. Effects of light-stimulation on intracellular ATP levels (**A**) and O_2 consumption rate (**B**) in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 μM oligomycin A. Black bars: sperm incubated with 5 μM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant ($P<0.05$) differences with respect to non-irradiated sperm (control). (*) indicates significant ($P<0.05$) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

Effects of red-light stimulation on cytochrome C oxidase activity in the presence or absence of either oligomycin A or FCCP

Non-irradiated spermatozoa showed low CCO activity (43.8 mIU/mg protein ± 3.2 mIU/mg protein; Fig. 8), regardless of the presence of oligomycin A or FCCP. Light-stimulation induced a significant (P<0.05) increase in CCO activity, which reached a peak after sperm were irradiated for 5 min (253.8 mIU/mg protein ±9.7 mIU/mg protein; Fig.8). While neither oligomycin A nor FCCP affected the aforementioned increase when sperm irradiated for 1 min and 10 min, they both significantly (P<0.05) counteracted the increase in CCO activity induced by the exposure of sperm to light for 5 min (Fig. 8).

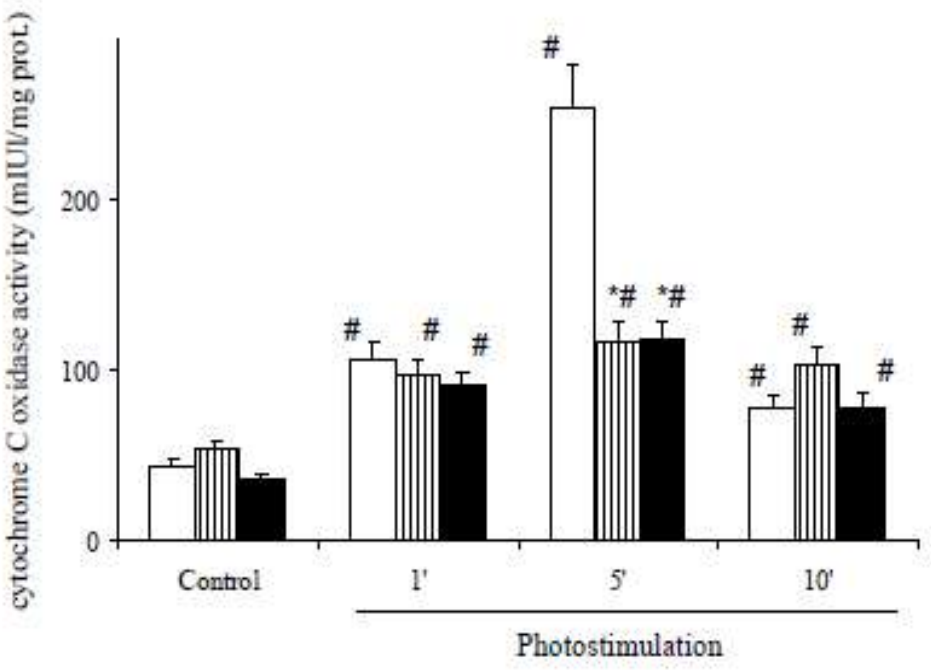

Figure 8. Effects of light-stimulation on cytochrome C oxidase activity in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 µM oligomycin A. Black bars: sperm incubated with 5 µM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant (P<0.05) differences with respect to non-irradiated sperm (control). (*) indicates significant (P<0.05) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

Discussion

The present study clearly indicates that the effects of red LED light on boar sperm function are related with changes in the mitochondrial electronic chain rate. Moreover, the impact of red-light appears to rely on the time of exposure, as indicated by the differences observed between protocols. Regarding the effects of red-light on the mitochondrial electronic chain, this could be explained through different hypothesis. The main one would be underpinned by the direct action on the photosensitizers of the mitochondrial electronic chain. In effect, crucial components of the electronic chain, such as cytochrome C, are sensitive to light at a wavelength range from 630 nm to 660 nm (Lynch & Copeland, 1992). This sensitivity would be on the basis of results on our CCO activity. In fact, the effects of red-light on the mitochondrial electron chain can have a significant impact on mammalian sperm. Indeed, while electron chain activity is usually related with energy production (McLean et al., 1993), it is also involved in the modulation of the overall eukaryotic cell function. At this respect, electron chain plays a pivotal role in the generation of reactive oxygen species (ROS), since mitochondria are the most important generating source of ROS in eukaryotic cells (Zhao et al., 2019). In addition, cytochrome complexes are also involved in the intrinsic apoptotic pathway (Cai et al., 1998), and both ROS generation and modulation of apoptotic-like changes are crucial in eliciting and controlling sperm capacitation (Ortega-Ferrusola et al., 2009). Therefore, red light-induced changes in cytochrome C complex activity could ultimately affect how sperm capacitate and which their survival is.

Our results indicate that the presence of oligomycin A in non-irradiated sperm decreased O_2 consumption, although this reduction was not concomitant with a complete inhibition of motility or a decrease in intracellular ATP levels. These results differ from other previously published in which oligomycin A did abolish motility without modifying either O_2 consumption rate or ATP content (Ramió-Lluch et al., 2013). These differences could be explained by the separate experimental conditions. In effect, whereas the study by Ramió-Lluch et al. (2013) was performed under *in vitro* capacitation conditions, the current work was centered on semen stored in a commercial extender, specifically designed to maintain sperm activity as low as possible. At this respect, similar results of both oligomycin A and FCCP on sperm boar motility and overall mitochondrial function have been recently published in cells resuspended in commercial extender (Nesci et al., 2020). The different environmental conditions founbd by boar sperm in capacitating medium and in basal commercial extender would lead cells to be kept in very different

functional status, which, in turn, would support separate reactivity against agents such as oligomycin A. At this respect, it is worth bearing in mind that oligomycin A inhibits the activity of mitochondrial ATP synthase via binding its regulatory F0 subunit complex (Shchepina et al., 2002). The F0 subunit complex modulates the proton flux that ultimately activates ATP synthase (Neupane et al., 2019). This implies that the activity of the F0 subunit complex will depend on the precise proton flux that reaches it. Thus, the inhibitory activity of the oligomycin A would be different depending on the proton flux. This would imply that in conditions in which mitochondrial activity was very low or basal, as in this work, the effect of oligomycin A will be much less pronounced than in conditions with a stimulated mitochondrial activity like in Ramió-Lluch et al., (2013). Regarding the results observed in both ATP content and motility, Marin et al. (2003) showed that when boar sperm are maintained in conditions similar to those of the current work, not only are ATP levels much lower than those of Ramió-Lluch et al. (2013), but ATP is mainly produced via glycolysis (Marin et al., 2003). Thus, the combination of different ATP levels with separate glycolysis/mitochondria oxidative phosphorylation balance would be on the basis of the observed differences in both oligomycin A-sensitivity and specific motility parameters of sperm incubated in basal and capacitating conditions.

Results observed for JC1-staining and motile sperm subpopulations suggest that the effects of red LED light on boar semen are not homogeneous, but highly rely upon the specific functional status of each individual spermatozoon. Thus, the observed percentages of spermatozoa with high and intermediate MMP, and their FL2:FL1 ratios, suggests that the exposure time to red-light has a different impact on mitochondrial membrane potential and that not all spermatozoa exhibit the same response. Microscope confocal images supports that hypothesis. In addition, the different response of each spermatozoon to red-light agrees with the presence of separate sperm subpopulations based on JC1-staining (Ramió-Lluch et al., 2011) and also concur with our motile sperm subpopulations data. In fact, it would be logical to assume that the different response to red LED light of each spermatozoon would also induce variations on the motile subpopulation structure similar to those observed when sperm are cryopreserved or subjected to *in vitro* capacitation (Ramió et al., 2008; Flores et al., 2009; Estrada et al., 2017). Another interesting result is that the effects of red LED light depend on the time of exposure. Related with this, our data are in agreement with previous studies about the sperm response to different red LED light patterns in terms of thermal resistance and *in*

vitro capacitation (Yeste et al., 2016). While the mechanisms underlying this different time-response remain to be elucidated, it is reasonable to suggest that the energy supplied to the mitochondrial electron chain by red-light is proportional to the time of exposure and intensity. It is worth mentioning that each specific photon has different energy potential levels depending on the wavelength. Since energy goes from high levels at the ultraviolet range to low levels at the infrared one), photons in the red range utilized herein have relatively low energy levels (http://www.photon.t.u-tokyo.ac.jp/~maruyama/nanoheat/Energy.pdf). Because the intensity of the device was kept at the same in all protocols and wavelength ranged from 620 to 630 nm, the different pattern effects observed herein are likely to be due to the different time of exposure, which fuel separate energy levels to mitochondrial photosensitizers. The energy supplied to photosensitizers modulate their rate of e^-/H^+ flux (Iaffaldano et al., 2016). As a consequence, it is reasonable to hypothesize that energy-linked, light-induced changes on electron chain activity affect the overall mitochondria function, which leads to variations in capacitation rate, sperm motility and intracellular ion fluxes (Iaffaldano et al., 2016). The final consequence is that different exposure times have different impact on the overall boar sperm function, depending on the exact energy level and the rate at which this energy is fueled into mitochondria. A corollary of this hypothesis is that any condition affecting the number of photons that reach cytochromes have different impact on the sperm cell. In this context, it is worth mentioning that the sperm response to red-light has been suggested to be reliant upon other factors, such as the recipient in which are sperm contained (Luther et al., 2018). However, explaining these effects should also take into consideration other potential sources of variation. The utilization of the same device and the same light regime for sperm stored in similar recipients regarding volume and color have been reported to render different effects on the overall sperm function (Luther et al., 2018; Pezo et al., 2019). In effect, differences in the storage time of sperm before light-stimulation between Luther et al. (2018) and Pezo et al. (2019) could explain these apparently contradictory results. Whereas Luther et al., (2018) stored sperm for 14 h prior to irradiation, that time lasted about 1 h in Pezo et al. (2019). This is important, since the functional status of boar sperm is different in both conditions. In support of this hypothesis, one should bear in mind that cryotolerance of boar sperm when previously stored at 15-17ºC for 24 h is higher than when cryopreserved immediately after collection (Yeste et al., 2014; Torres et al., 2019). These differences have been linked with proteins related to the cell resilience to withstand environmental changes, such as heat shock protein 70 (HSPA1A), which

shows much higher serine-phosphorylation levels after 24 h of storage at 15-17ºC (Yeste et al., 2014). Therefore, the functional sperm status is likely to lead to different sensitiveness to light-provided energy.

In spite of the aforementioned, there could be other factors that modulate the effects of red-light. This includes the presence of light-sensitive receptors, such as opsins (Perez-Cerezales et al., 2015) and transient receptor potential proteins (TRP; see De Blas et al., 2009; Bahat & Eisenbach, 2010; Gibbs et al., 2011), in mammalian spermatozoa. While the exact role of these receptors in sperm is not completely known, the regulation of thermotaxis has been suggested to be one of their most probable functions (Wu et al., 2010; Islam, 2011; Perez-Cerezales et al., 2015). Thermotaxis could be, in fact, an important modulator of light-stimulation, since mammalian sperm are sensitive to environmental temperature changes as low as 0.0006ºC (Bahat et al., 2012). Given that these small changes cannot be controlled by devices such as the one used to irradiate sperm in this study, it is reasonable to suggest that red-light stimulation could also act through inducing temperature changes higher than 0.0006ºC.

In conclusion, our results indicate that the effects induced by red LED light stimulation on boar sperm are related to mitochondrial photosensitizers, such as CCO, modifying mitochondrial electron chain activity. In addition, this study has also shown that the impact of red-light depends on the precise, previous sperm functional status and on the time of exposure. These findings do not exclude, however, that this mitochondrial mechanism acts together with thermotaxis, via other receptors.

Materials and Methods

Semen samples

Semen samples were provided by a commercial farm (Servicios Genéticos Porcinos, S.L. Roda de Ter, Spain) and came from 17 separate boars. Animals were post-pubertal (2-3 years old), healthy and belonged to the Piétrain breed. Studs were housed in strictly climate-controlled conditions, fed with a standard diet and provided with water *ad libitum*. Semen was collected manually through the gloved hand method, and the obtained rich-sperm fractions were immediately diluted at 17ºC to a final concentration of 2×10^7 sperm/mL in a commercial extender (Duragen®, Magapor, S.L.; Ejea de los Caballeros, Zaragoza, Spain). Diluted semen was split into 90-mL commercial AI doses, and two doses of each ejaculate were transported at 17ºC to our laboratory for 30-60 min.

Ethics

Boar ejaculates utilized in this study were not collected for the unique purpose of investigation, but for their commercial use. Thus, the farms provided us with some doses, and the remaining ones were used for AI. Therefore, there was no need for any specific ethical approval to perform this work, as no animal was manipulated by the authors.

Experimental design

Upon arrival, samples were confirmed to fulfill quality standards, which were ≥85% membrane-intact spermatozoa ($SYBR14^+/PI^-$), ≥85% morphologically normal spermatozoa, and ≥80% total motile spermatozoa. Following this, each semen sample was split into separate aliquots of 1.5-mL that were subjected to three red-light irradiation protocols (LED, 620-630 nm; PhastBlue®, IUL, S.L.; Barcelona, Spain). These three protocols consisted of exposing sperm to red-light for 1 min (1'), 5 min (5') or 10 min (10'). In all cases, the temperature within the PhastBlue® system was maintained at 20ºC. The control consisted of 1.5-mL Eppendorf tubes kept at 20ºC in the dark for 10 min. In addition to the aforementioned, semen samples were also exposed to the same three protocols (i.e. 1', 5' and 10') or non-exposed (control) in the presence of either 5 µM oligomycin A or 5 µM FCCP. Both inhibitors were added to semen samples 10 min before exposure to red-light.

Following exposure to red-light, samples were used to evaluate sperm motility through a computer-assisted sperm analysis system (CASA); sperm membrane and acrosome integrity, mitochondrial membrane potential and intracellular ROS and calcium levels by flow cytometry; O_2 consumption; intracellular ATP levels; and total cytochrome C oxidase (CCO) activity. In the case of intracellular ATP levels and total cytochrome C oxidase (CCO) activity, samples were first centrifuged at 2,000×g and 20ºC for 30 s and the resulting pellet was frozen by plunging into liquid N_2. Samples were stored at -80ºC until use.

Sperm motility

Sperm motility was determined through a CASA system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain), based on the settings and parameters described in Ramió et al. (2008). The following kinematic parameters were evaluated: curvilinear velocity (VCL), straight line velocity (VSL), average pathway velocity

(VAP), linearity coefficient (LIN), straightness coefficient (STR), wobble coefficient (WOB), amplitude of lateral head displacement (mALH), beat cross frequency (BCF), dance (DNC, VCL×ALH), absolute mean angular displacement (absMAD) and algebraic mean angular displacement (algMAD). Briefly, samples were warmed at 38ºC for 5 min in a water bath prior to placing 6 µL into a 20-micron Leja® Standard Count Chamber Slide (Leja Products B.V.; Nieuw Vennep, The Netherlands). Three replicates of 1,000 sperm each were evaluated before calculating the corresponding mean ± standard error of the mean (SEM). Total motility was defined as the percentage of sperm with VAP ≥10 µm/s, whereas progressive motility was considered to be the percentage of motile sperm with a STR ≥45%. Individual kinematic parameters (VSL, VCL, VAP, LIN, STR and BCF) were used to determine motile sperm subpopulations.

Flow cytometry

Flow cytometry was used to determine plasma membrane and acrosome integrity, mitochondrial membrane potential (MMP), and intracellular levels of superoxides, peroxides and calcium, following the recommendations set by Lee et al. (2008). In all analyses, sperm concentration was previously adjusted to 1×10^6 spermatozoa/mL in a final volume of 500 µL, and three technical replicates were evaluated. Samples were examined using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA), and the sheath flow rate was set at 4.17 µL/min. Electronic volume (EV) and side scatter (SS) were recorded in a log-linear mode (EV/SS dot plots) for 10,000 events per replicate. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter>12 µm), and compensation was used to minimize fluorescence spill-over into a different channel. Information on all events was collected in List-mode Data files (EV, SS, FL1, FL2 and FL3) and processed using the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). In all assessments data were corrected using the procedure described by Petrunkina et al. (2010), based on the percentage of debris particles ($SYBR14^-/PI^-$) determined through SYBR14/PI staining. Fluorochromes were purchased from Molecular Probes® (Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) and diluted with dimethyl sulfoxide (DMSO).

Plasma membrane integrity

Percentages of membrane-intact spermatozoa were determined using the LIVE/DEAD® Sperm Viability Kit (SYBR14/PI; Garner & Johnson, 1995). Three separate sperm populations were identified. One of these populations corresponded to membrane-intact spermatozoa ($SYBR14^+/PI^-$), whereas the other two subpopulations ($SYBR14^-/PI^+$ and $SYBR14^+/PI^+$) corresponded to spermatozoa with different degrees of plasma membrane alterations. Debris and non-sperm particles were detected as $SYBR14^-/PI^-$, and were not taken into account to calculate the percentages of the three sperm populations. Single-stained samples were used to set EV gain, FL1 and FL3 PMT-voltages and for compensation of FL1 spill over into the FL3-channel (2.45%).

Acrosome integrity

Acrosome integrity was determined through staining with peanut agglutinin from *Arachis hypogaea* conjugated with fluorescein isothiocyanate (FITC-PNA) and ethidium homodimer (EthD-1), following the protocol set by Cooper & Yeung (1998) and adapted to boar spermatozoa by Rocco et al. (2018). Following this procedure, four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane ($PNA\text{-}FITC^+/EthD\text{-}1^-$); (ii) viable spermatozoa with a non-intact acrosome membrane ($PNA\text{-}FITC^-/EthD\text{-}1^-$); (iii) non-viable spermatozoa with an intact acrosome membrane ($PNA\text{-}FITC^+/EthD\text{-}1^+$); and (iv) non-viable spermatozoa with a non-intact acrosome membrane ($PNA\text{-}FITC^-/EthD\text{-}1^+$). Total percentages of spermatozoa with a non-intact acrosome membrane resulted from summing $PNA\text{-}FITC^-/EthD\text{-}1^-$ and $PNA\text{-}FITC^-/EthD\text{-}1^+$ populations. FL1 spill over into the FL3 channel (2.70%) was compensated.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was evaluated by incubation with 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide (JC1; final concentration: 0.3 µM), as described by Gillan et al. (2005). Three subpopulations were distinguished: (i) spermatozoa with high MMP, characterized by the formation of JC1 aggregates, which showed orange staining; (ii) spermatozoa with intermediate/medium MMP, characterized by mitochondria that were stained in both orange and green; and (iii) spermatozoa with low MMP, characterized by the presence of JC1 monomers, which emitted green fluorescence.

Intracellular levels of superoxides and peroxides

Intracellular levels of superoxide (O_2^-) radicals were analyzed through staining with hydroethidine and YO-PRO-1 (HE/YO-PRO-1) fluorochromes, whereas peroxide (H_2O_2) radicals were determined through staining with 2'-7'-dichlorodihydrofluorescein diacetate and propidium iodide (H_2DCFDA/PI). Both tests were performed as described in Guthrie & Welch, (2006), and results are shown as percentages of viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-) and of viable spermatozoa with high peroxide levels (DCF^+/PI^-). In the case of HE/YO-PRO-1, FL3 spill-over into the FL1-channel was compensated (5.06%), and in that of H_2DCFDA/PI, FL1 spill-over into the FL3-channel was also compensated (2.45%).

Intracellular calcium levels

Previous studies found that Fluo3 mainly stains mitochondrial calcium in boar sperm (Yeste et al., 2015). For this reason, we combined this fluorochrome with propidium iodide (Fluo3/PI), as described by Kadirvel et al. (2009), and the following four populations were identified: (i) viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^-); (ii) viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^-); (iii) non-viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^+); and (iv) non-viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^+). FL1 spill-over into the FL3-channel (2.45%) and FL3 spill-over into the FL1-channel (28.72%) were compensated.

Determination of JC1-staining with laser confocal laser microscopy

Spermatozoa stained with JC1 were also analyzed by confocal laser scanning microscopy to detect the specific localization of mitochondria with high MMP (stained in orange) in the mid-piece. With this purpose, sperm were incubated at the same conditions than those used for flow cytometry, i.e. 38ºC for 30 min (final concentration of JC1: 0.3 µM). In order to avoid an excessive motility constraint that could alter JC1-staining along mitochondrial piece, 20 µL of each sample were placed onto the bottom of a well (4-well plates). Samples were observed under a Leica TCS 4D confocal laser scanning microscope (Leica lasetechnik; Wertrieb, Germany) adapted to an inverted Leitz DMIRBE microscope at 63× (NA=1.4 oil) Leitz Plan-Apo Lens (Leitz; Stuttgarts, Germany). The light source was an argon/krypton laser (74 mW) and fluorescence detection was performed through an excitation wavelength of 488 nm. Depending on how

mitochondria were stained, two emission wavelengths were detected. The first emission wavelength corresponded to low MMP mitochondria (JC1 monomers), which emitted at 530 nm (green), whereas the second one corresponded to high MMP mitochondria (JC1 aggregates), which emitted at 590 nm (orange). Since JC1-stained sperm maintained their motility, a sequential track of images per sample was taken at 38ºC for 20 s (rate: one capture/s).

Determination of intracellular ATP levels.

Intracellular ATP levels were determined following the protocol set by Chida et al. (2012). Briefly, 1-mL semen aliquots were spun at 17ºC for 30 s after light-stimulation, and pellets were immediately plunged into liquid N_2. Frozen pellets were subsequently stored at -80ºC for three weeks. Thereafter, pellets were resuspended in 300 µL ice-cold 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer containing 250 mM sucrose (pH was adjusted to 7.4). Resuspended pellets were sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070; Bandelin Electronic GmbH and Co., Berlin, Germany) and tubes were kept on ice to avoid specimen heating. Samples were subsequently centrifuged at 1,000×g and 4ºC for 10 min and supernatants were kept. Twenty µL was used to determine total protein content, and the remaining volume was mixed with 300 µL ice-cold 10% (v:v) trichloroacetic acid and kept at 4ºC for 20 s. Samples were subsequently centrifuged at 1,000×*g* and 4ºC for 30 s, and supernatants were carefully separated from the pellet and again centrifuged at 1,000×*g* and 4ºC for 10 min. Resulting supernatants were mixed with two volumes of 1 M Tris-acetate buffer (pH=7.75), and ATP content was determined in these final suspensions using the Invitrogen® ATP Determination Kit (ThermoFisher Bioscientific; Waltham, USA; catalogue number: A22066), following the manufacturer's instructions. Determinations of ATP content were carried out through an Infinite F200 fluorimeter (TECAN®), using 96-wells microplates for fluorescence-based assays (Invitrogen®). To normalize data, total protein of samples was determined through the Bradford method (Bradford, 1976), by using a commercial kit (Bio-Rad laboratories; Hercules, CA, USA).

Determination of O_2 consumption rate

Determination of O_2 consumption rate was performed using the SensorDish® Reader (SDR) system (PreSens Gmbh; Regensburg, Germany). One-mL semen aliquots, previously exposed to red-light, were transferred onto an Oxodish® OD24 plate (24

wells/plate) specifically designed for this device. Plates were sealed with Parafilm®, introduced in the SDR system, and incubated at 37ºC (controlled atmosphere) for 2 h. During that time, O_2 concentration was recorded in each well at a rate of one reading per min. Data were exported to an Excel file (see Suppl. Fig. 1 for representative curves) and final O_2 consumption rate was normalized against the total number of viable spermatozoa per sample, which was determined through flow cytometry ($SYBR14^+/PI^-$ spermatozoa) using another aliquot.

Determination of cytochrome C oxidase activity

Activity of cytochrome C oxidase (CCO) was determined in mitochondria-enriched sperm fractions, as described in McLean et al. (1993). Briefly, 1-mL sperm aliquots, previously irradiated with red-light, were centrifuged at 1,000×*g* and 17ºC for 30 s. The resulting pellets were immediately plunged into liquid N_2 and stored for three weeks. Pellets were resuspended in 500 µL ice-cold PBS and sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070). Thereafter, 500 µL Percoll® at a concentration of 1.055 mg/mL in PBS at 4ºC was placed onto each sperm homogenate. Samples were centrifuged at 3,000×*g* and 10ºC for 45 min and the mitochondria-enriched fraction was carefully harvested with a micropipette and transferred into a new 1.5-mL tube. Samples were again centrifuged at 12,000×*g* and 20ºC for 2 min and the resulting pellets were resuspended in 100 µL PBS at 20ºC. These mitochondria-enriched suspensions were split into two separate aliquots. The first one was used to determine CCO activity using a commercial kit (Cytochrome C Oxidase Assay Kit; Sigma-Aldrich; catalogue number CYTOCOX1). The other aliquot (10 µL) was used to determine total protein content through a commercial kit based on the Bradford method (Bradford, 1976; KIT). Enzyme activity was normalized against the total protein content.

Statistical analyses

Statistical analyses were conducted using a statistical package (SPSS® Ver. 25.0 for Windows; IBM corp., Armonk, NY, USA). Data were first tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests, respectively. When required, data were transformed through arcsin √x. The effects of red-light stimulation patterns on motility parameters; percentages of spermatozoa with an intact plasma membrane ($SYBR14^+/PI^-$), spermatozoa with an intact acrosome ($PNA\text{-}FITC^+$), spermatozoa with high MMP ($JC1_{agg}^+$), viable spermatozoa with high intracellular

calcium levels ($Fluo3^+/PI^-$), viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-), and viable spermatozoa with high peroxide levels (DCF^+/PI^-); geometric mean fluorescence intensity (GMFI) of $JC1_{agg}{}^+$, $Fluo3^+$, E^+ and DCF^+; intracellular levels of ATP; O_2 consumption rate; and cytochrome C oxidase activity were evaluated through one-way analysis of variance (ANOVA) followed by post-hoc Sidak's test.

Motile sperm subpopulations were determined through the protocol described in Luna et al. (2017). In brief, individual kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) recorded for each sperm cell were used as independent variables in a Principal Component Analysis (PCA). Kinematic parameters were sorted into PCA components and the obtained matrix was subsequently rotated using the Varimax method with Kaiser normalization. As a result, each spermatozoon was assigned regression scores for the new PCA components and these values were subsequently used to run a two-step cluster analysis based on the log-likelihood distance and the Schwarz's Bayesian Criterion. Three sperm subpopulations were identified and each individual spermatozoon was assigned to one of these subpopulations (SP1, SP2 or SP3). Following this, percentages of spermatozoa belonging to each subpopulation were calculated per sample, and were further used to evaluate the effects of red-light stimulation on the distribution of motile sperm subpopulations through one-way ANOVA and Sidak's post-hoc test.

In all analyses, the level of significance were set at $P \leq 0.05$. Data are shown as mean ± standard error of the mean (SEM).

Acknowledgements

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Competing interests

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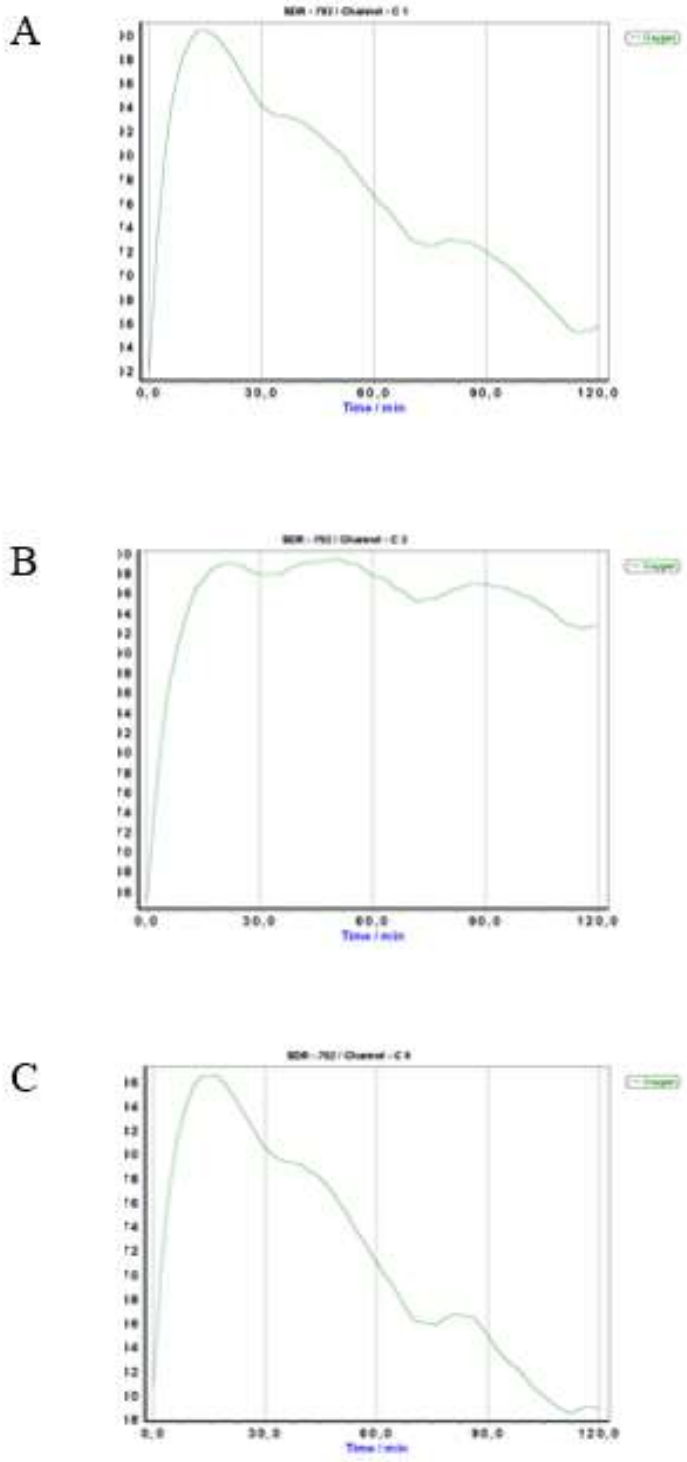
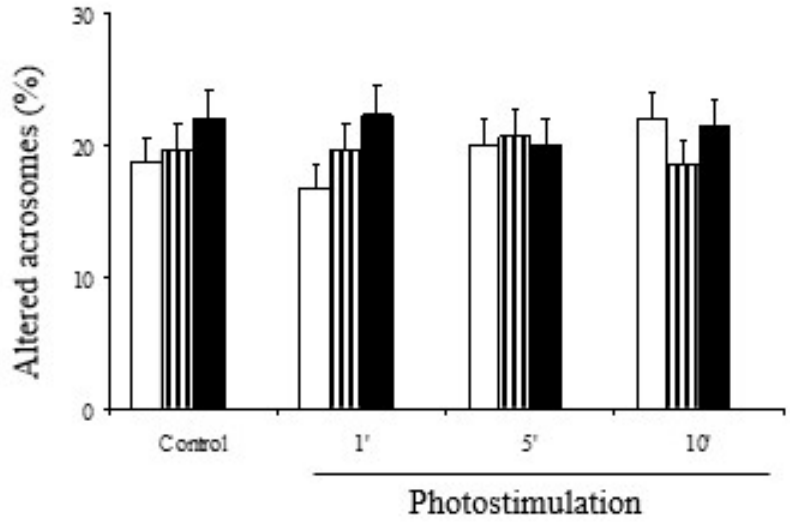
Supplementary Figures



Supplementary Figure 1. Representative plots corresponding to the determination of the O_2 consumption rate of boar sperm incubated in the presence/absence of either oligomycin A or FCCP. **A**: Control. **B**: Sperm incubated with 5 µM oligomycin A. **C**: Sperm incubated with 5 µM FCCP.



Supplementary Figure 2. Effects of light-stimulation on percentages of spermatozoa with a non-intact acrosome (PNA-FITC$^-$) in the presence/absence of either oligomycin A or FCCP. Sperm were irradiated for 1 min (1'), 5 min (5') and 10 min (10'). Control: non-irradiated spermatozoa. White bars: sperm not incubated with either oligomycin A or FCCP. Stripped bars: sperm incubated with 5 μM oligomycin A. Black bars: sperm incubated with 5 μM FCCP. Results are expressed as mean ± SEM for 7 separate experiments. (#) indicates significant (P<0.05) differences with respect to non-irradiated sperm (control). (*) indicates significant (P<0.05) differences between olygomicin A or FCCP with respect to the treatment without the two inhibitors (white bars) for a given light-stimulation protocol (i.e., control, 1 min, 5 min or 10 min).

MANUSCRIPT III

Red LED light acts on the mitochondrial electron chain of mammalian sperm via light-time exposure-dependent mechanisms

Olga Blanco Prieto, Jaime Catalán, Marcel Lleonart, Sergi Bonet, Marc Yeste, Joan E. Rodríguez-Gil. (2019). Red-light stimulation of boar semen prior to artificial insemination improves field fertility in farms: A worldwide survey. Reprod Domest Anim, 54:1145-1148. doi: 10.1111/rda.13470.

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SHORT COMMUNICATION

Reproduction in Domestic Animals WILEY

Red-light stimulation of boar semen prior to artificial insemination improves field fertility in farms: A worldwide survey

Olga Blanco Prieto[1] | Jaime Catalán[1] | Marcel Lleonart[2] | Sergi Bonet[3] | Marc Yeste[3] | Joan E. Rodríguez-Gil[1]

[1]Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Barcelona, Spain
[2]IUL, Barcelona, Spain
[3]Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain

Correspondence
Joan E. Rodríguez-Gil, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra (Cerdanyola del Vallès), Barcelona, Spain.
Email: juanenrique.rodriguez@uab.cat

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Abstract

A survey of in vivo fertility data from 31 pig farms distributed worldwide was conducted to determine whether stimulating boar semen with LED-based red light increases its reproductive performance following artificial insemination (AI). Red-light stimulation with MaXipig® was found to increase farrowing rates (mean ± *SEM*, control: 87.2% ± 0.4% vs. light stimulation 90.3% ± 0.5%) and the number of both total and live newborn piglets. Red-light stimulation increased farrowing rates in 27 farms, with an increase ranging from 0.2% to 9.1%. Similar results were observed in litter sizes. Suboptimal management after AI was suggested in those farms with no response to red-light stimulation. Our results indicate that a routine use of red-light stimulation of boar semen can have a positive effect on the reproductive performance. However, the effectiveness of this system appears to highly rely upon proper management of pig farms.

KEYWORDS
artificial insemination, boar semen, fertility, prolificacy, red-light stimulation

1 | INTRODUCTION

Artificial insemination (AI) with liquid-stored semen is the most utilized method for pig breeding worldwide (Knox, 2016; Yeste, 2017). Thus, optimization of AI at profitable costs can be a powerful tool to improve the reproductive performance of pig farms.

Motility and in vitro fertilizing ability of mammalian spermatozoa can be improved by red-light stimulation with different light sources (reviewed in Yeste, Castillo-Martín, Bonet, and Rodríguez-Gil (2018)). In the case of boar semen, a previous study found that LED-based red-light stimulation increased its in vivo fertilizing ability (Yeste et al., 2016). While this study was performed in one farm, further reports showed inconsistent effects of red-light stimulation on sperm quality parameters evaluated in vitro (Luther, Thi, Schäfer, Schulze, & Waberski, 2018; Pezo et al., 2019).

Against this background, this work sought to determine whether red-light stimulation with MaXipig® immediately before AI improves the reproductive performance of boar semen. With this purpose, a worldwide survey involving 31 farms was conducted and the collected data are shown herein.

2 | MATERIALS AND METHODS

2.1 | AI trials

Farrowing rates and litter sizes (total and live born piglets) were collected from 31 farms distributed in three continents as follows: Europe: Portugal (1), Belgium (2), Finland (2), France (3), UK (3), and Spain (13); America: Canada (3); and Asia: China (1), South Korea (1), and Japan (2). AI trials were performed following the routine

TABLE 1 Comparison of farrowing rates and litter sizes mean values of all analysed farms between control and photo-stimulated groups

Group	*n*	Farrowing rates (%)	Total piglets/birth	Live piglets/birth
Control	20,279	87.2 ± 0.4[a]	14.9 ± 0.1[a]	13.9 ± 0.1[a]
Photo	13,363	90.3 ± 0.5[b]	15.3 ± 0.2[b]	14.2 ± 0.1[a]

Note: *n*: total number of inseminated sows. Different superscripts indicate significant differences ($p < 0.05$) between groups.

TABLE 2 Farm-by-farm comparison of reproductive traits between control and photo-stimulated groups

Country/ Farm	AI type	*n* control	*n* photo	Fertility control (%)	Fertility photo (%)	ΔFertility	Total piglets/birth control	Total piglets/birth photo	ΔTotal piglets	Live piglets/ birth control	Live piglets/ birth photo	ΔLive piglets
Belgium 1	T	103	103	94.2	99.0	+5.8	15.5	16.4	+0.9	14.3	15.1	+0.8
Belgium 2	T	345	335	96.7	98.5	+1.2	15.2	15.6	+0.4	14.8	15.5	+0.7
Canada 1	T	244	240	78.0	82.0	+4.0	16.1	16.4	+0.3	15.5	15.8	+0.3
Canada 2	T	680	700	91.0	89.7	−1.3	14.9	15.1	+0.2	14.2	15.0	+0.8
Canada 3	T	59	52	89.8	98.1	+8.3	16.7	16.9	+0.2	15.8	16.0	+0.2
China 1	T	179	121	93.3	94.2	+0.9	16.9	17.3	+0.4	16.0	16.8	+0.8
Finland 1	T	1,078	887	80.7	83.1	+2.4	16.9	17.2	+0.3	15.5	15.7	+0.2
Finland 2	T	982	621	84.3	84.5	+0.2	17.0	17.3	+0.3	15.5	15.7	+0.2
France 1	T	88	85	86.4	90.4	+4.0	14.9	15.0	+0.1	14.2	14.8	+0.6
France 2	T	236	248	96.2	97.6	+1.4	15.8	15.8	0.0	14.8	14.9	+0.1
France 3	T	303	301	76.9	84.7	+7.8	14.8	15.5	+0.7	14.0	14.0	0.0
Japan 1	T	56	65	82.1	90.8	+8.7	12.1	12.3	+0.3	11.7	11.7	0.0
Japan 2	T	136	133	72.1	81.2	+9.1	10.6	13.1	+2.5	8.2	9.1	+0.9
Portugal 1	P	997	207	92.0	95.8	+3.8	15.2	15.8	+0.6	13.5	14.4	+0.9
South Korea 1	T	270	49	91.0	93.5	+2.5	13.9	14.7	+0.8	13.0	13.7	+0.7
Spain 1	T	500	531	78.4	85.7	+7.3	10.4	10.5	+0.1	8.7	9.3	+0.6
Spain 2	T	288	367	90.3	95.1	+4.8	14.0	15	+1.0	13.7	14.5	+0.8
Spain 3	T	505	367	87.1	90.5	+3.4	14.3	14.5	+0.2	13.3	13.5	+0.2
Spain 4	P	81	129	88.9	85.3	−3.6	15.9	16.3	+0.4	15.0	15.7	+0.3
Spain 5	P	173	150	83.8	90.0	+6.2	16.4	16.9	+0.5	16.0	16.5	+0.5
Spain 6	P	346	292	92.5	91.8	−0.7	13.1	13.0	−0.1	11.3	11.1	−0.2
Spain 7	P	939	300	90.5	90.7	+0.2	10.9	10.7	−0.2	8.9	8.9	0.0
Spain 8	P	1,217	1,152	86.8	87.8	+1.0	16.0	16.1	+0.1	14.4	14.6	+0.2
Spain 9	P	144	320	94.8	96.4	+1.6	18.6	18.6	0.0	17.4	16.8	−0.6
Spain 10	P	210	210	95.7	96.2	+0.5	17.8	17.9	+0.1	16.5	16.7	+0.2
Spain 11	P	911	514	92.3	98.6	+6.3	12.1	12.5	+0.4	11.4	12.0	+0.6
Spain 12	P	520	234	89.6	94.8	+5.2	14.3	14.8	+0.5	13.5	13.9	+0.4
Spain 13	M	6,673	3,191	86.7	89.4	+2.7	17.9	17.7	−0.2	17.0	17.2	+0.2
UK 1	M	660	228	79.5	81.0	+1.5	14.8	14.7	−0.1	14.2	14.0	−0.2
UK 2	M	576	499	83.5	83.5	0.0	14.2	14.9	+0.7	13.3	13.8	+0.5
UK 3	M	780	732	76.4	79.1	+2.7	15.4	14.4	−1.0	15.0	14.1	−0.9

Note: AI, AI technique; Country/Farm, country in which farms were located; M, mixed AI techniques; *n*, total number of sows inseminated in each farm either with and standard procedure (Control) or after red-light stimulation of boar semen (Photo); P, post-cervical AI; T, standard trans-cervical AI. ΔFertility, ΔTotal Piglets, ΔLive Piglets: differences in values of farrowing rates, total piglets/birth and live piglets/birth.

procedure of each farm. These trials were performed in all seasons during years 2017 and 2018 without following any uniform temporal distribution during seasons. In each farm, animals were divided into two groups. The first group was inseminated following the usual routine of the farm (Control Group), whereas the other group was inseminated with semen doses previously light-stimulated with

MaXipig® (IUL, S.A.; Photo Group). Both experimental groups had homogeneous distributions regarding breeds and age of sows. The age of the utilized sows ranged from first to fifth parturition, and sows were randomly assigned to each group. Positive gestations were confirmed through ultrasonography 42 days after AI, and farrowing rates were calculated at parturition. Numbers of total piglets per parturition (TP) and live piglets per parturition (LP) were counted during the first 3 hr after delivery. A total number of 33,642 sows were involved in AI trials: 20,279 were included in Control Group and 13,363 were included in Photo one. Finally, a more detailed description of the procedure has been included as a Supporting information.

2.2 | Statistical analysis

Since data were not enough to run a logistic model, differences of results between Groups were tested through the Student–Newman–Keuls test after analysing normality and homogeneity of variances of data through Shapiro–Wilk and Levene tests, respectively. When required, data were previously normalized through logit transformation. Significance level was set at $p \leq 0.05$.

3 | RESULTS

3.1 | Effects of stimulating boar semen with red light upon reproductive performance

Red-light stimulation of semen prior to AI induced a significant ($p < 0.05$) increase in farrowing rates (Table 1). Likewise, the number of both total and live piglets born per birth was also significantly ($p < 0.05$) increased in the light-stimulated group (Table 1).

3.2 | Farm-by-farm analysis of data after red-light stimulation

Red-light stimulation of boar semen prior to AI increased farrowing rates in 26 out of the 31 tested farms (Table 2). This increase ranged from 0.2% to 9.1%. The other three farms showed a decrease in farrowing rates (Table 2). Regarding prolificacy, whereas 24 farms showed an increase in the number of total piglets born per parturition after light stimulation, a decrease in this number was observed in five farms (Table 2). Similar results were observed when the number of live newborn piglets per parturition was analysed (Table 2). A close data analysis from farms in which light stimulation had negative effects showed a high percentage of non-cyclic, non-returning sows, which was concomitant with a high variation in the number of both total and live piglets born per parturition (Table S1).

4 | DISCUSSION

This worldwide survey indicates that routine application of red-light stimulation of boar semen can increase reproductive performance in pig farms. However, a careful analysis might be necessary to establish the profitability of this procedure in each farm. Related to this, one should bear in mind that the statistical relevance of boar semen quality in explaining fertility outcomes following AI is as much as approximately 30% (Quintero-Moreno, Rigau, & Rodríguez-Gil, 2004). This implies that factors other than sperm quality, such as seasonality, are underlying the fertility outcome following AI, regardless of the impact of sperm photo-stimulation (Amann, Saacke, Barbato, & Waberski, 2018; Auvigne, Leneveu, Jehannin, Peltoniemi, & Sallé, 2010; De Rensis, Ziecik, & Kirkwood, 2017). Yet, proper management of the farm is required for optimal results, as data from those farms with negative results suggest. In those farms, returning animals showing no-cycling signs were higher than in the ones with a positive response to red-light stimulation of boar semen. Yet, dispersion indexes in prolificacy data after parturition were also higher in farms with a negative photo-stimulation response (Table S1). All these data suggest suboptimal management of sows after AI. For this reason, a relationship between the suboptimal management of animals after AI and the lack of effects of red-light stimulation should not be dismissed.

In conclusion, the data showed herein suggest that red-light stimulation of boar semen prior to AI can have a positive effect on the reproductive performance of commercial pig farms. Yet, the effectiveness of this method may depend on other factors, such as the proper farm management.

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CONFLICT OF INTEREST

We declare that no author have any disclosure or conflict of interests with the work and data shown in this manuscript.

AUTHOR CONTRIBUTIONS

O. Blanco Prieto co-performed the main statistical analyses of data. M. Lleonart co-performed the main responsible for collecting data from all farms. M. Yeste designed and co-performed the statistical analysis of data. Moreover, he collaborated in the writing of the manuscript. J.E. Rodríguez-Gil designed the procedure, collaborated in the collection of data and organized them, and wrote the manuscript and was the maximal responsible for all of the work.

ORCID

Marc Yeste https://orcid.org/0000-0002-2209-340X

Joan E. Rodríguez-Gil https://orcid.org/0000-0002-1112-9884

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SUPPORTING INFORMATION

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

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DISCUSSION

Results yielded in this PhD Thesis have several, important implications in the knowledge of the utilization of red LED light as an improving tool of reproductive performance in pig farming. Thus, results indicate that the effects of red LED light on *in vitro* semen quality parameters last for as long as 48 hours after irradiation (see MANUSCRIPT 1), suggesting a medium-to-long term mechanisms of action. Regarding this/ese mechanism/s of action, results also showed that red LED light acts on the activity of the mitochondria electron chain, modifying thus the whole sperm function and being this action dependent on the time of exposure to light (MANUSCRIPT 2). Finally, from a practical point of view, results also indicate that the in-farm application of red LED light irradiation can increase *in vivo* reproductive performance of pigs after following a worldwide survey in farm conditions, depending on the light effect on the precise conditions by which AI is performed (MANUSCRIPT 3). Taking these evidences into account, when considering the medium-term effects of red LED light, a similar result has been observed in turkey (Iaffaldano et al., 2005) and rabbit sperm (Iaffaldano et al., 2010). The maintenance of these irradiation effects relies on the applied protocol as well as on the analyzed sperm parameter, regardless of the fact that several important aspects of the overall sperm function were not analyzed and, in this way, we do not know the endurance of the light effect on these no analyzed points. Meanwhile, regarding sperm viability and acrosome integrity, it is worth mentioning that while light-stimulation had no impact on plasma membrane integrity, all irradiation patterns partially counteracted the increase in the percentages of spermatozoa with altered acrosomes observed after 48 h of storage, which is in agreement with previous studies conducted by Iaffaldano et al. (2010). This implies a separate sensitivity to light irradiation of acrosome membrane when compared with that of the whole sperm cell. Otherwise, the most interesting results are those observed in the sperm resilience to withstand thermal stress (i.e. following incubation at 37 ºC for 120 min) in which light irradiation counteracted the increase in the percentages of spermatozoa with altered acrosomes exposed to thermal stress. This effect was maintained over the first 48 h of storage and also relied upon the irradiation pattern. The mechanisms through which irradiation can increase that resilience remain unknown. At this respect, we can hypothesize about the putative role that the specific plasma membrane receptors which are sensitive to both light and temperature could play in these

mechanisms. Focusing on that, sperm plasma membrane contains both TRPs and opsins, which have been suggested to be involved in the modulation of thermotaxis (Nilius et al., 2011). Both types of receptors are sensitive to light (Foster et al., 1991; Terakita, 2005; Rodriguez-Gil, 2019). Furthermore, rhodopsin, an opsin present in sperm (Pérez-Cerezales et al., 2015), is specifically sensitive to red light (Foster et al., 1991). In this way, red LED light could exert these medium-term effects through the stimulation of thermotactic signals involving receptors like rhodopsin.

The medium-term effects of light-stimulation depend on the specific irradiation pattern. In this way, both intensity and rhythm at which light, and hence energy, are applied are crucial for the sperm response to light. Depending thus on the specific energy that irradiation has, light would act on several, highly sensitive points, such as covalent bonds that link phosphate molecules like those of ATP, similar that photosynthesis (Arnon, 1971), thereby increasing the energy stored in these bonds. Furthermore, as results yielded in MANUSCRIPT 2 show, light can also act on other sensitive points, such as the mitochondrial electron chain, thereby changing its rhythm (Hamblin et al., 2011). Irrespective of the exact mechanism, light appears to cause positive or negative effects on these points, depending on the irradiation pattern (Hamblin et al., 2011). We could hypothesize that light increases the energy of disulphide bonds since, as aforementioned, energy accumulates in covalent bonds, such as the disulphide and the phosphate ones (Arnon, 1971). The increase in the accumulated energy of these chemical bonds makes them more difficult to be broken, although their breaking would release much more energy than in standard conditions (Nakai et al., 2019). Hence, our data suggest that when light-stimulation reaches specific energy levels, it helps to maintain the disulphide bonds-based structures that, otherwise, would be progressively deteriorate over storage.

One of the most interesting results yielded by the study conducted in MANUSCRIPT 2 are those regarding to changes in the head sperm free cysteine radicals levels, which were determined by using two parallel techniques, the spectrophotometric analysis based upon the 2,2'-dipyrydil disulfide reaction and the electrophoretical analysis based upon the dibromobimane staining. At this respect, storage at 17 ºC induced a time storage-dependent increase in the disruption of nucleoprotein disulfide bonds, evaluated through 2,2'-dipyrydil disulfide. Nevertheless, it is worth mentioning that these results are apparently contradictory with those observed following dibromobimane staining. These

apparent discrepancies can be due to the different sensitivity of both techniques. It must be stressed that the 2,2'-dipyrydil disulfide spectrophotometer-based technique is much more sensitive than that of dibromobimane staining, which is based on a semi-quantitative analysis of electrophoretic band intensities. This implies that whereas the 2,2'-dipyrydil disulfide technique is able to detect small differences, the dibromobimane one is not able to. In spite of this, the fact that the dibromobimane stain is able to detect changes in specific nucleoproteins and nucleoprotein-DNA aggregates, shown in the electrophoresis process as separate bands (Flores et al., 2008), implies that dibromobimane is more able to detect changes in some of these specific aggregates. Therefore, both techniques are complementary, allowing thus to a better understanding of the dynamics by which head sperm disulfide bonds gradually break. At this respect, while free cysteine radicals of the 60 kDa band significantly increased at 0 h, they only did so after 96 h of storage in the case of the 20 kDa band. It is important to consider that whereas the 20 kDa band corresponds to free nucleoproteins, the 60 kDa one coincides with specific nucleoproteins-DNA fragments that result from sperm head sonication (Cree et al., 2011). Taking this into account, the observed increase of disulfide bonds breaking after 96 h of storage would be related with an increase in the total amount of free nucleoproteins. On the contrary, since the 60 kDa band corresponds to nucleoprotein/DNA aggregates, the gradual increase observed after 24 h of storage could result from the breaking of disulfide bonds between proteins and DNA. In any case, our data indicate that irradiation for 5 min and 10 min counteracted, at least partially, this gradual increase. Thus, we could hypothesize that light increases the energy of disulfide bonds. The increase in the accumulated energy of these chemical bonds makes them more difficult to be broken, although their breaking would release much more energy than in standard conditions (Nakai et al., 2019). Hence, our data suggest that when light-stimulation reaches specific energy levels, it helps maintaining the disulfide bonds-based structures that, otherwise, would be progressively deteriorating over storage. As indicated above, results yielded in MANUSCRIPT 2 indicate that one of the mechanisms of action of the red-light stimulation is related with the modification of the sperm mitochondria activity and, more specifically, with changes in the mitochondrial electronic chain rate. At this respect, it is noteworthy that several checkpoints of the electron chain, such as the cytochrome C complex, are sensitive to light at a wavelength range from 630 nm to 660 nm (Lynch & Copeland, 1992), which is in the range of the applied light in our study. Mitochondria electron chain activity is related with energy production (McLean et al., 1993), being also

involved in the modulation of the overall eukaryotic cell function. At this respect, electron chain plays a pivotal role in the generation of reactive oxygen species (ROS), since mitochondria are the most important generating source of ROS in eukaryotic cells (Zhao et al., 2019) and modulation of apoptotic-like changes are crucial in eliciting and controlling sperm capacitation (Ortega-Ferrusola et al., 2009). Under these bases, it is obvious that the action of red light on the mitochondrial electron chain activity will have a significant impact on mammalian sperm.

Notwithstanding, the comparison of the results obtained in this study with other previously conducted strongly suggests that the action of red light on sperm mitochondria function will be dependent on the precise functional status in which mitochondria are at the moment of irradiation. At this respect, results shown here indicate that the ATPase inhibition by oligomycin A in the basal condition in which the study was performed, i.e., in sperm suspended in a commercial extender at 16ºC, decreased the sperm O_2 consumption rate, although this reduction was not concomitant with a complete inhibition of motility or a decrease in intracellular ATP levels. These results are similar to those reported by Nesci et al. (2020). Strikingly, when sperm were incubated in capacitating conditions and, hence, mitochondrial activity was increased immediately after suspension of sperm in the capacitation medium, the inhibition of the ATPase activity by oligomycin A abolishes motility without modifying both O_2 consumption rate and intracellular ATP content (Ramió-Lluch et al., 2014). It is noteworthy that oligomycin A inhibits the activity of mitochondrial ATP synthase via binding its regulatory F0 subunit complex (Shchepina et al., 2002). The F0 subunit complex modulates the proton flux that ultimately activates ATP synthase (Neupane et al., 2019). This implies that the activity of the F0 subunit complex will depend on the precise proton flux that reaches it. Thus, the inhibitory activity of the oligomycin A would be different depending on the proton flux. This would imply that in conditions in which mitochondrial activity was very low or basal, as in this work, the effect of oligomycin A will be much less pronounced than in conditions with a stimulated mitochondrial activity like in Ramió-Lluch et al. (2014).

Results obtained with light stimulation in the presence of either oligomycin A or FCCP also indicate that the effects of red light are not homogeneous, but highly rely upon the specific functional status of each individual spermatozoon. In this sense, the exposure time to red light has a different impact on mitochondrial membrane potential depending

on the individual function status of each sperm. Thus, not all spermatozoa will exhibit the same response to irradiation. It would be logical to assume that the different response to red LED light of each spermatozoon would also induce variations on the motile subpopulation structure similar to those observed when sperm are cryopreserved or subjected to *in vitro* capacitation (Ramió et al., 2008; Flores et al., 2009; Estrada et al., 2017).

Another interesting result is that the effects of red LED light depend on the time of exposure. Related with this, our data are in agreement with previous studies centred on the sperm response to different red LED light patterns in terms of thermal resistance and *in vitro* capacitation (Yeste et al., 2016). While the mechanisms underlying this different time-response remain to be elucidated, it is reasonable to suggest that the energy supplied to the mitochondrial electron chain by red light is proportional to the time of exposure and intensity. It is reasonable to hypothesize that energy-linked, light-induced changes on electron chain activity affect the overall mitochondrial function, which leads to variations in capacitation rate, sperm motility and intracellular ion fluxes (Iaffaldano et al., 2016). The final consequence is that different exposure times have different impact on the overall boar sperm function, depending on the exact energy level and the rate at which this energy is fuelled into mitochondria. Explaining these effects should also take into consideration other potential sources of variation. The utilization of the same device and the same light regime for sperm stored in similar recipients regarding volume and color have been reported to render different effects on the overall sperm function (Luther et al., 2018; Pezo et al., 2019). This is important, since the functional status of boar sperm is different in both conditions. In support of this hypothesis, one should bear in mind that cryotolerance of boar sperm when previously stored at 15-17ºC for 24 h is higher than when cryopreserved immediately after collection (Yeste et al., 2014; Torres et al., 2019). These differences have been linked with proteins related to the cell resilience to withstand environmental changes, such as heat shock protein 70 (HSPA1A), which shows much higher serine-phosphorylation levels after 24 h of storage at 15-17ºC (Yeste et al., 2014). Therefore, the functional sperm status is likely to lead to different sensitiveness to light-provided energy. On the other hand, thermotaxis could be, in fact, an important modulator of light-stimulation, since mammalian sperm are sensitive to environmental temperature changes as low as 0.0006ºC (Bahat et al., 2012).

Finally, the analysis of the use of photostimulation as an improving tool of reproductive performance in pig farms worldwide raises several interesting points. The worldwide study clearly indicates that, taking results as a whole, the routine application of a 10'-10'-10' protocol to AI in farms can induce a profitable increase of farms reproductive efficiency. However, the variability of obtained results, including farms in which photostimulation had no effect, also indicate the existence of factors external to the photostimulation procedure that are influencing in the final results. This is logical, since it is well known that factors other than sperm quality, such as seasonality or the technical skills of the staff who carry out the AI, are underlying the fertility outcome of pig farms, regardless of the intrinsic quality and fertilizing ability of sperm (Amann et al.., 2018; Auvigne et al., 2010; De Rensis et al., 2018). For this reason, the optimal application of a routine photostimulation procedure to increase reproductive performance of sows farms will need for a concomitant optimization of farming conditions and management, including a proper training of the staff performing AI.

CONCLUSIONS

1. Red light LED stimulation of boar semen increases their resilience to withstand thermal stress over the first 48 h of storage at 17 ºC.

2. Red-light LED stimulation contributes to the maintenance of nucleoprotein structure over storage.

3. The effects of irradiation along with their durability depend on the irradiation pattern.

4. The effects induced by red LED light stimulation on boar sperm are related to mitochondrial photosensitizers, such as cytochrome C oxidase, which modifies the activity of mitochondrial electron chain.

5. The impact of red-light LED depends on the precise, previous sperm functional status and on the time of exposure.

6. The action of red LED light in the electronic transport chain does not exclude other mechanisms of action.

7. Red LED light stimulation of boar semen prior to AI can have a positive effect on the reproductive performance of commercial pig farms. Yet, the effectiveness of this method may depend on other factors, such as the proper farm management.

8. Stimulation with red-light LED could be an economical and profitable way to improve fertility and prolificacy in sow farms, provided the appropriate management of farms.

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