Universitat Autònoma de Barcelona

# EFECTOS DE LA FOTOESTIMULACIÓN EN EL SEMEN DE BURRO Y CABALLO

**Jaime Catalán Bahamondes**

# EFECTOS DE LA FOTOESTIMULACIÓN EN EL SEMEN DE BURRO Y CABALLO

TESIS DOCTORAL


Presentada por:

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Bajo la dirección de:

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Para optar al título de Doctor con Mención Internacional en el programa de Doctorado en Medicina y Sanidad Animal


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**Jordi Miró Roig**, Profesor Titular del Departamento de Medicina y Cirugía Animal de la Universidad Autónoma de Barcelona, y **Marc Yeste Oliveras**, Profesor Agregado del Departamento de Biología de la Universidad de Girona,

**CERTIFICAN**

Que la Tesis titulada "EFECTOS DE LA FOTOESTIMULACIÓN EN EL SEMEN DE BURRO Y CABALLO" presentada por **Jaime Catalán Bahamondes** para optar al grado de Doctor en Medicina y Sanidad Animales 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.

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# CERTIFICADO

Valdivia, 3 de enero del 2020.

Mediante el presente documento, se certifica que el Sr. Jaime Catalán Bahamondes con NIE Y4930029-Q 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 participó en el montaje de técnicas analíticas avanzadas, relacionadas a con su formación y avance de objetivos planteados en su tesis doctoral. En lo específico, participó en el desarrollo de los siguientes trabajos experimentales, ambos vinculados al estudio de la fotoestimulación espermática con LED-630nm:

- 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.
- 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.

Se extiende el presente certificado a solicitud del interesado, para los fines académicos que estime conveniente.

Alfredo Ramírez, PhD
Laboratorio de Criobiología y
Análisis de Funcionalidad Espermática
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*Compos sui*

*A mis padres*

,

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# ÍNDICE DE CONTENIDOS

# ABREVIATURAS Y/O ACRÓNIMOS

| | |
|---|---|
| **ADN** | Ácido desoxirribonucleico |
| **ADP** | Adenosín Difosfato |
| **ALH** | Amplitud media del desplazamiento lateral de la cabeza del espermatozoide |
| **AMP** | Adenosín monofosfato |
| **ATP** | Adenosín trifosfato |
| **BCF** | Frecuencia de batido cruzado del espermatozoide, expresada como el número de veces que la trayectoria curvilínea cruza la lineal |
| **$Ca^{2+}$** | Ion Calcio |
| **cAMP** | Monofosfato de adenosina cíclico |
| **CASA** | Sistema de análisis espermático asistido por ordenador |
| **CAT** | Catalasa |
| **cGMP** | Monofosfato de guanosina cíclico |
| **EMP** | Espermatozoides con motilidad progresiva |
| **g** | Unidad de fuerza gravitacional |
| **GPX** | Glutatión peroxidasa |
| **GSR** | Glutatión reductasa |
| **He-Ne** | Helio-Neón |
| **$H_2O_2$** | Peróxido de hidrogeno |
| **IA** | Inseminación artificial |
| **LED** | Diodo emisor de luz |
| **M540** | Merocianina 540 |
| **min** | Minuto |
| **mL** | Mililitro |
| **NAD** | Nicotinamida Adenina Dinucleótido |
| **nm** | Nanómetro |
| **$O_2$** | Oxígeno molecular |

| | |
|---|---|
| **pH** | Potencial de hidrogeno |
| **PLC** | Fosfolipasa C |
| **PS** | Plasma seminal |
| **ROS** | Especies reactivas de oxígeno |
| **SOD** | Superóxido dismutasa |
| **TRP** | Receptor de potencial transitorio |
| **TRPA** | Receptor de potencial transitorio anquirina |
| **TRPM** | Receptor de potencial transitorio melastanina |
| **TRPV** | Receptor de potencial transitorio vainilloide |
| **TRPV4** | Receptor de potencial transitorio vainilloide 4 |
| **µm** | Micrómetro |
| **WBFSH** | Federación Mundial para la Cría de Caballos de Deporte (World Breeding Federation for Sport Horses) |

**RESUMEN**

**RESUM**

**ABSTRACT**

# RESUMEN

Estudios previos realizados en otras especies, incluyendo humanos, perros, cerdos, toros, conejos y carneros, han demostrado que la estimulación con luz roja aumenta la motilidad espermática. Además, otros estudios han sugerido que la capacidad fecundante de los espermatozoides tanto *in vitro* como *in vivo* puede incrementarse después de la irradiación. Por otra parte, se ha observado que la fotoestimulación con luz roja podría mejorar la longevidad del semen refrigerado y la criotolerancia de los espermatozoides. Aunque los mecanismos a través de los cuales la luz ejerce sus efectos sobre los espermatozoides se desconocen en gran medida, la evidencia creciente otorga a los fotosensibilizadores celulares endógenos, especialmente los presentes en las mitocondrias, un papel crucial.

Por todo ello, el objetivo de esta Tesis Doctoral fue evaluar los efectos de diferentes protocolos de fotoestimulación con un luz LED roja a una longitud de onda de 620-630 nm sobre los parámetros de calidad espermática, en semen de burro y caballo; fresco, refrigerado y congelado/descongelado, además de evaluar la influencia del medio/diluyente y del color de la pajuela utilizada en los efectos observados, así como estudiar si los efectos producidos por la estimulación con luz roja en los espermatozoides están provocados por cambios en la función mitocondrial inducidos por la acción de la luz sobre los citocromos mitocondriales como el citocromo C.

Con este propósito, se diseñaron cinco estudios: en el primero se evaluaron los efectos de diferentes patrones de estimulación con luz LED roja sobre los parámetros de motilidad espermática, la integridad de la membrana plasmática, el potencial de membrana mitocondrial y los niveles intracelulares de ROS en semen de burro fresco y refrigerado. En el segundo estudio, se analizaron los efectos de diferentes patrones de irradiación con luz roja sobre la motilidad espermática, las subpoblaciones de espermatozoides móviles, el potencial de membrana mitocondrial, la tasa de consumo de $O_2$, los niveles intracelulares de ATP, la integridad de la membrana plasmática y la integridad del ADN espermático en semen fresco de caballo. En el tercer estudio se propuso determinar si la irradiación con luz roja del semen de caballo congelado-descongelado podría mejorar la calidad espermática y aumentar su capacidad de resistir al estrés térmico a 38°C durante 120 minutos. En el cuarto estudio se examinó si el color de la pajuela y/o la turbidez del diluyente utilizado para la refrigeración del semen afectaba a la respuesta inducida por la estimulación con luz roja en semen de caballo refrigerado. Finalmente, en el quinto trabajo se estudió si los efectos producidos por la fotoestimulación estaban

relacionados con cambios en la función mitocondrial inducidos sobre los citocromos mitocondriales, como el citocromo C. Para ello, se analizaron los efectos de la irradiación espermática en presencia y ausencia de Oligomicina A (un inhibidor específico de la ATP sintasa) y de Carbonilcianuro-p-trifluorometoxifenilhidrazona (FCCP; un disruptor de la cadena de electrones mitocondriales), sobre la motilidad, las subpoblaciones de espermatozoides móviles, la integridad de la membrana plasmática y del acrosoma, el potencial de membrana mitocondrial, la tasa de consumo de $O_2$, los niveles intracelulares de ATP, la actividad de la citocromo C oxidasa y los niveles intracelulares de ROS en semen de burro fresco.

Los resultados presentados en esta Tesis Doctoral indican que la irradiación de los espermatozoides de burro y caballo con luz LED roja modifica algunos parámetros de motilidad espermática, la estructura de las subpoblaciones de espermatozoides móviles, la tasa de consumo de $O_2$, la actividad de la citocromo C oxidasa y los niveles intracelulares de ROS, sin afectar a la integridad de la membrana plasmática, el acrosoma o el ADN espermático, mejorando además su resiliencia al estrés térmico. Estos efectos varían según el tiempo y patrón de exposición, la especie, el tipo de semen (fresco, refrigerado y congelado), el color de la pajuela y la turbidez del diluyente utilizado.

En conclusión, esta Tesis Doctoral muestra que la irradiación de los espermatozoides de burro y caballo con luz LED roja (620-630 nm) es un método seguro que produce una mejora sobre algunos parámetros de calidad espermática en el semen de burro y caballo, efectos que están relacionados con la acción de la luz sobre los fotosensibilizadores mitocondriales, como el citocromo C que modifica la actividad de la cadena de electrones mitocondrial. Así, la fotoestimulación podría tener un impacto positivo en el rendimiento reproductivo y en las tecnologías de reproducción asistida en estas especies, así como también en la conservación de semen. No obstante, se requieren más estudios para confirmar estos efectos beneficiosos.

## RESUM

Estudis previs realitzats en altres espècies, incloent humans, gossos, porcs, toros, conills i marrans, han demostrat que la fotoestimulació amb llum vermella augmenta la motilitat espermàtica. A més, altres estudis indiquen que la capacitat fecundant dels espermatozoides tant *in vitro* com *in vivo* pot incrementar després de la irradiació, així com també s'ha observat que l'estimulació amb llum vermella podria millorar la longevitat del semen refrigerat, la criotolerància i la qualitat del semen congelat/descongelat. Tot i que els mecanismes a través dels quals la llum exerceix els seus efectes sobre els espermatozoides es desconeixen en gran mesura, l'evidència creixent atorga als fotosensibilitzadors cel·lulars endògens, especialment els presents en les mitocòndries, un paper fonamental.

Per tot això, l'objectiu d'aquesta tesi doctoral va ser avaluar els efectes de diferents protocols de fotoestimulació amb un llum LED vermella a una longitud d'ona de 620-630 nm sobre els paràmetres de qualitat espermàtica, en semen de ruc i cavall; fresc, refrigerat i congelat/descongelat, a més d'avaluar la influència del medi i del color de la palleta utilitzada en els efectes observats, i determinar si els efectes produïts per l'estimulació amb llum vermella en els espermatozoides estan provocats per canvis en la funció mitocondrial induïts per l'acció de la llum sobre els citocroms mitocondrials com el citocrom C.

Amb aquest propòsit es van dissenyar cinc estudis: en el primer es van avaluar els efectes de diferents patrons d'estimulació amb llum LED vermella sobre els paràmetres de motilitat espermàtica, la integritat de la membrana, el potencial de membrana mitocondrial i els nivells intracel·lulars de ROS en semen de ruc fresc i refrigerat. En el segon estudi es van analitzar els efectes de diferents patrons d'irradiació amb llum vermella sobre la motilitat espermàtica, les subpoblacions d'espermatozoides mòbils, el potencial de membrana mitocondrial, la taxa de consum d'$O_2$, els nivells intracel·lulars d'ATP, la integritat de la membrana i la integritat de l'ADN espermàtic en semen fresc de cavall. El tercer estudi tenia com objectiu determinar si la irradiació amb llum vermella de l'esperma de cavall congelada-descongelada podria millorar la qualitat espermàtica i augmentar la seva capacitat de resistir la incubació a 38 °C per 120 minuts. En el quart estudi, es va examinar si el color de la palleta i / o la terbolesa del medi utilitzat per a la refrigeració del semen afectaven a la resposta induïda per l'estimulació amb llum vermella en el semen de cavall refrigerat. Finalment, en el cinquè treball es va estudiar si els efectes produïts per la fotoestimulació estaven relacionats amb canvis en la funció mitocondrial induïts sobre els citocroms mitocondrials, com el citocrom C. Amb aquest propòsit, es van analitzar els efectes

de la irradiació dels espermatozoides en presència i absència d'oligomicina A (un inhibidor específic de l'ATP sintasa) i de carbonilcianuro-p-trifluorometoxifenilhidrazona (FCCP; un disruptor de la cadena d'electrons mitocondrials) sobre la motilitat espermàtica, les subpoblacions d'espermatozoides mòbils, la integritat del membrana plasmàtica i de l'acrosoma , el potencial de membrana mitocondrial, la taxa de consum d'$O_2$, els nivells intracel·lulars d'ATP, l'activitat de la citocrom C oxidasa i els nivells intracel·lulars de ROS en semen de ruc fresc.

Els resultats obtinguts en aquesta Tesi Doctoral indiquen que la irradiació dels espermatozoides de ruc i cavall amb llum LED vermella modifica alguns paràmetres de motilitat espermàtica, l'estructura de les subpoblacions d'espermatozoides mòbils, la taxa de consum d'$O_2$, l'activitat de la citocrom C oxidasa i els nivells intracel·lulars de ROS, sense afectar la integritat de la membrana plasmàtica, l'acrosoma o l'ADN espermàtic, millorant a més la seva resiliència a l'estrès tèrmic. Aquests efectes varien segons el temps i el patró d'exposició, l'espècie, el tipus de semen (fresc, refrigerat i congelat), el color de la palleta i la terbolesa del medi utilitzat.

En conclusió, aquesta Tesi Doctoral mostra que la irradiació dels espermatozoides de ruc i cavall amb llum LED vermella (620-630 nm) és un mètode segur que produeix una millora sobre alguns paràmetres de qualitat espermàtica en el semen de burro i cavall, efectes que estan relacionats amb l'acció de la llum sobre els fotosensibilitzadors mitocondrials, com el citocrom C que modifica l'activitat de la cadena d'electrons mitocondrial. Així, la fotoestimualació podria tenir un impacte positiu en el rendiment reproductiu i en les tecnologies de reproducció assistida en aquestes espècies, així com també en la conservació del semen. No obstant això, són necessaris més estudis que confirmin aquest efecte positiu.

## ABSTRACT

Previous studies carried out in other species including humans, dogs, pigs, cattle, rabbits, and sheep, and using different light sources, showed that stimulating sperm with red light increases their motility. In addition, other studies indicate that in vitro and in vivo sperm fertilizing ability can be increased after irradiation, and suggest that stimulation with red light could improve the longevity of liquid-stored semen, sperm cryotolerance and the quality of irradiated frozen-thawed semen. While the mechanisms through which light exerts its effects on sperm are largely unknown, mounting evidence gives endogenous cellular photosensitizers, especially those present in mitochondria, a crucial role.

Therefore, the objective of this Dissertation was to evaluate the effects of different irradiation protocols using a red LED light at a wavelength of 620-630 nm on freshly ejaculated, liquid-stored and frozen-thawed sperm from stallions and jackasses. The potential influence of the medium/extender and the colour of the straw was also investigated, as well as whether the effects produced by red light resulted from changes in mitochondrial function via modulation of cytochromes, such as cytochrome C.

For this purpose, five studies were designed. In the first one, the effects of different red LED light patterns on sperm motility, integrity of plasma membrane, mitochondrial membrane potential, and intracellular ROS levels were investigated in fresh and stored donkey semen. The second study analysed the effects of separate red light irradiation patterns on motility, membrane integrity, motile sperm subpopulations, mitochondrial membrane potential, $O_2$ consumption rate, intracellular ATP levels and the integrity of DNA of fresh horse sperm. The third study sought to determine whether red light irradiation of frozen-thawed horse semen could improve sperm quality and increase their ability to withstand incubation at 38 °C for 120 minutes. In the fourth study, whether the colour of the straw and / or the turbidity of the medium affected the response induced by red light stimulation was examined in liquid-stored horse semen. Finally, the fifth work studied whether the effects produced by red light on sperm were related to changes in mitochondrial function induced by mitochondrial cytochromes, such as cytochrome C. With this aim, the effects of sperm irradiation were tested in the presence/absence of Oligomycin A (a specific ATP synthase inhibitor) and Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (a mitochondrial electron chain disruptor). Different sperm variables, including total motility, motile sperm subpopulations, acrosome and plasma membrane integrity, mitochondrial membrane potential, $O_2$ consumption rate, intracellular ATP

levels, cytochrome C oxidase activity, and intracellular ROS levels, were evaluated in fresh donkey semen.

The results shown in this Dissertation indicate that irradiation of donkey and horse sperm with red LED light modifies their sperm motility, the structure of motile sperm subpopulations, the rate of $O_2$ consumption, the activity of cytochrome C oxidase and intracellular levels of ROS, without affecting the integrity of plasma membrane and acrosome or sperm DNA. In addition, stimulation with red light also improves the sperm resilience to thermal stress. These effects vary according to the time and pattern of exposure, species, type of semen (fresh, stored and frozen), colour of the straw and the turbidity of the medium.

In conclusion, this Dissertation supports that irradiation of donkey and horse sperm with red LED light (620-630 nm) is a safe method that may improve some sperm quality parameters in donkey and horse semen. These effects are related to the action of light on mitochondrial photosensitizers, such as cytochrome C, which modifies the activity of the mitochondrial electron chain. Therefore, irradiation of sperm with red light could have a positive impact on reproductive performance and assisted reproductive technologies in these species, as well as on semen preservation. However, more studies are needed to confirm this beneficial effect.

# CAPÍTULO I

## INTRODUCCIÓN

# 1. EL BURRO Y EL CABALLO

## *1.1. Taxonomía y evolución*

Los burros (*Equus asinus*) y los caballos (*Equus caballus*) son mamíferos del orden Perissodactyla, caracterizados por presentar un número impar de dedos terminados en pezuñas (ungulados). Dentro de este orden, pertenecen a la familia *Equidae*, compuesta por diferentes especies del género *Equus* (Houghton Brown et al., 2003; Steiner and Ryder, 2011). De acuerdo con los mapas de división del ADN mitocondrial y los registros fósiles (revisado de Forstén, 1992), el género *Equus* incluye seis especies: tres cebras (*E. grevyi, E. zebra, E. burchelli*), dos burros (*E. asinus, E. hemionus*) y el caballo verdadero (*E. caballus*, que incluye el caballo doméstico y el de Przewalski). Aunque estas especies compartieron un ancestro común hace millones de años (Orlando et al., 2013), difieren tanto morfológica como cromosómicamente (Wilborn and Pugh, 2011), así como en su fisiología y comportamiento (Merkies et al., 2020). En el caso particular de burros y caballos, se ha demostrado que presentan importantes diferencias reproductivas, no solo por el hecho de que sus espermatozoides son distintos en motilidad y morfología, sino también por la manera en que éstos interactúan con el endometrio (Miró and Papas, 2018). A pesar de estas diferencias, casi todos los individuos de las especies del género *Equus* son capaces de reproducirse entre sí dando lugar a híbridos interespecíficos (Wilborn and Pugh, 2011).

## *1.2. Domesticación*

Los burros y los caballos fueron domesticados hace más de 5000 años (Merkies et al., 2020). En el caso de los caballos, se estima que el proceso de domesticación se inició hace aproximadamente 5500 años, mientras que el de los burros se inició hace alrededor de 6000 años (Clutton-Brock, 1992). Desde entonces, los humanos les han asignado diversas funciones que van desde el trabajo y el transporte hasta el deporte y el ocio, centrando la selección de los animales reproductores en rasgos específicos como el color, la conformación, el comportamiento, la aptitud y el carácter. Con el tiempo, esta presión de selección ha dado lugar a una amplia variedad de razas con características físicas muy diversas; desde voluminosos caballos de tiro hasta los caballos árabes de huesos finos, los caballos en miniatura o diferentes tipos de burros y mulas (Merkies et al., 2020).

## *1.3. Importancia y principales usos*

Los burros se han utilizado durante siglos como animales de trabajo y transporte, así como para

producir mulas. Aunque la mecanización de la agricultura y la industrialización durante el último siglo redujo la importancia y el número de los burros y sus híbridos, principalmente en Europa occidental, éstos todavía se utilizan como animales de carga y trabajo en países menos desarrollados (Camillo et al., 2018; Canisso et al., 2019; Panzani et al., 2020). Además de esto, y dado que la industrialización llevó a la casi extinción de muchas razas europeas, se han puesto en marcha varios programas de conservación en los últimos años (Canisso et al., 2019; Panzani et al., 2020), lo que sumado a una creciente demanda de nuevos usos del burro (producción de leche, cosmética, silvicultura, turismo rural, ocio…), ha aumentado el interés en desarrollar estudios para comprender y mejorar el desempeño reproductivo en esta especie (Camillo et al., 2018; Canisso et al., 2019; Miró et al., 2020; Papas et al., 2020a).

Al igual que en el caso del burro, el caballo ha estado ligado desde un comienzo a la vida del hombre (Morel, 1999), siendo un importante impulsor de la civilización humana (Schubert et al., 2014; Librado et al., 2016; Orlando, 2020; Schrimpf et al., 2020). En un principio, el caballo se usó como instrumento de guerra, transporte y trabajo, mientras que en la actualidad su uso es principalmente de tipo recreativo y deportivo (Morel, 1999). El impacto económico anual de la industria equina, generado principalmente por los caballos de deporte, es muy significativo. Como ejemplo, esta industria generó en el año 2019 unos 300 mil millones de dólares y 1,6 millones de empleos a tiempo completo (Littiere et al., 2020). Por otro lado, en los países menos desarrollados el caballo sigue siendo hasta hoy un activo esencial para la agricultura y el transporte (Swann, 2006; Librado et al., 2016).

El gran interés de las personas principalmente por el caballo ha motivado su producción y el conocimiento de sus características reproductivas. En este sentido, es importante considerar que la temporada de reproducción del caballo está limitada por factores fisiológicos y en algunas ocasiones regulatorios, por lo que una fertilidad adecuada de los sementales y las yeguas es fundamental para el éxito reproductivo (Davila et al., 2016). Sin embargo, a diferencia de otras especies domésticas, los sementales se seleccionan como reproductores principalmente sobre la base de su pedigrí, su conformación y su rendimiento deportivo o de trabajo, dejando poca consideración por la solidez reproductiva o la fertilidad (Colenbrander et al., 2003; Varner et al., 2015; Davila et al., 2016). Esto ha contribuido a la marcada variabilidad en la fertilidad y la calidad del semen observada entre sementales, por lo que la subfertilidad es un problema común en algunas razas de caballos (Colenbrander et al., 2003; Peña et al., 2011; Davila et al., 2016). Esta problemática ha llevado a la búsqueda de nuevas estrategias y biotecnologías para favorecer el

desarrollo de la especie. Dentro de las biotecnologías utilizadas, la gran relevancia de la inseminación artificial (IA) en las últimas décadas tanto en burros y caballos como en otras especies (Morel, 1999; Canisso et al., 2008), ha promovido el interés por estudios centrados en el semen, con la finalidad de maximizar su viabilidad y poder fecundante (Loomis, 2006).

## 2. SEMEN EQUINO

### *2.1. Definición y composición*

El semen es un conglomerado de fluidos donde se encuentran suspendidos los espermatozoides, vesículas celulares (epididimosomas y prostasomas) y otras células (llamadas clásicamente células redondas; ya sean células que recubren los conductos de extrusión, el epidídimo o glándulas accesorias, leucocitos migratorios e incluso células espermatogénicas) (Beyler and Zaneveld, 1982; Rodríguez-Martínez et al., 2011). Su composición varía entre especies e incluso entre individuos dentro de la misma especie. Entre otras, dichas variaciones pueden observarse en el número de espermatozoides, volumen, pH y contenido total de proteínas (Mann et al., 1981). En el semen, por definición, se distinguen dos partes: una, formada por los espermatozoides producidos por los testículos y la otra, formada por el plasma seminal, el cual se constituye mediante la contribución combinada de los fluidos de la cola del epidídimo y las glándulas sexuales accesorias durante la eyaculación (Beyler and Zaneveld, 1982; Meyers, 2009; Rodríguez-Martínez et al., 2011; Carretero et al., 2016; Papas et al., 2019a).

Tanto los burros como los caballos poseen el conjunto completo de glándulas accesorias, que incluyen ámpulas, próstata, vesículas seminales y glándulas bulbouretrales. La morfología y capacidad secretora del aparato accesorio masculino se basa en atributos individuales y vinculados a la especie (Mann et al., 1981), generando diferencias que en el caso del burro y el caballo pueden verse reflejadas en el volumen total del eyaculado (Mann et al., 1981). El eyaculado en estas especies está constituido por tres fracciones, con diferentes aportes de fluidos por parte de las glándulas sexuales accesorias en cada una de ellas: la fracción pre-espermática, que tiene un aspecto transparente y se produce en las glándulas bulbouretrales y la próstata; la fracción rica en espermatozoides, que se deriva del epidídimo y ámpula; y la fracción post-espermática, que está formada principalmente por los espermatozoides que quedan en el canal uretral y las secreciones liberadas por las vesículas seminales (Varner et al., 1987; Amann and Graham, 1993; Lindeberg et al., 1999; Kareskoski et al., 2010; 2011; Oliveira et al., 2020).

### *2.2. Plasma seminal*

El plasma seminal (PS) se define como una secreción compleja compuesta por iones inorgánicos, azúcares, sales orgánicas, lípidos, enzimas, prostaglandinas, proteínas y varios factores procedentes de los testículos, epidídimos y glándulas sexuales accesorias masculinas (Maxwell et al., 2007; Carretero et al., 2016), que se mezclan con los espermatozoides durante la eyaculación (Papas et al., 2019a; Szczykutowicz et al., 2019).

Durante mucho tiempo, el PS fue considerado como un medio pasivo que acompañaba a los espermatozoides después de la eyaculación (Szczykutowicz et al., 2019). Sin embargo, con el tiempo se ha visto que más que un diluyente natural y un vehículo de transporte, el PS juega un papel importante para la fertilidad de los mamíferos, tanto en los procesos relacionados con los espermatozoides como dentro del tracto reproductivo femenino (Carretero et al., 2016; Papas et al., 2019a; Szczykutowicz et al., 2019).

En este sentido, se ha visto que el PS no solo ayuda a regular la osmolaridad del semen a través de componentes inorgánicos, sino que también es fuente de energía por los azúcares que contiene, brinda protección amortiguadora (*buffer*) contra los cambios de pH, protege a los espermatozoides de los efectos deletéreos de las especies reactivas de oxígeno (ROS) a través de antioxidantes (enzimáticos y no enzimáticos) y regula la respuesta inmune del tracto reproductivo de la hembra generada por el semen (Troedsson et al., 2005; Jonakova et al., 2010; Carretero et al., 2016; Szczykutowicz et al., 2019).

Además, las proteínas presentes en el PS se han asociado a eventos relacionados con la fecundación tales como la maduración espermática (Dacheux and Paquignon, 1980; Dacheux et al., 1998; Carretero et al., 2016; Szczykutowicz et al., 2019), la capacitación espermática (Chang, 1957; Triphan et al., 2007; Carretero et al., 2016; Szczykutowicz et al., 2019), la interacción con el oviducto mediante la formación de reservorios espermáticos (Gwathmey et al., 2006; Carretero et al., 2016; Szczykutowicz et al., 2019) e incluso en la interacción con el oocito (Töpfer-Petersen et al., 2009; Alghamdi et al., 2009; Carretero et al., 2016; Szczykutowicz et al., 2019).

En cuanto a la actividad enzimática de algunas proteínas halladas en el PS, en estudios recientes se observó una mayor actividad de algunas enzimas antioxidantes (superóxido dismutasa, SOD; catalasa, CAT; glutatión peroxidasa, GPX y glutatión reductasa, GSR) en el semen de burros que en el de caballos (Papas et al., 2019a). Sumado a esto, en el burro se ha visto que la actividad de estas enzimas antioxidantes está asociada con la motilidad y varía entre

temporadas (Papas et al., 2019a). Además, en burro y caballo la actividad total y específica de la SOD está relacionada con la criotolerancia de los espermatozoides (Papas et al., 2019b; 2020a).

Sin embargo, también se ha visto que el PS puede tener efectos perjudiciales tanto para la conservación del semen refrigerado en burro y caballo (Miró et al., 2009; Papas et al., 2019a; 2020b) como para la criopreservación de semen equino (Varner et al., 1987; Brinsko et al., 2000; Sieme et al., 2004; Moore et al., 2005; Kareskoski and Katila, 2008; Kareskoski et al., 2010; Papas et al., 2019a).

## *2.3. El espermatozoide*

El espermatozoide o gameto masculino es una célula haploide altamente especializada que tiene como función llevar el genoma masculino hasta su unión con el femenino, con la finalidad de dar origen a un nuevo individuo y propagar la especie. Para este objetivo, se deposita una gran cantidad de espermatozoides en el tracto genital femenino (Flesch and Gadella, 2000; Rigby et al., 2000), miles de millones en el caso de los équidos. Sin embargo, solo el 0,0007% de los espermatozoides depositados en el útero de la yegua alcanzan el lumen del oviducto – donde ocurre la fecundación – (Rigby et al., 2000; Varner et al., 2015), y solamente uno logra fecundar con éxito el óvulo (Flesch and Gadella, 2000). En este viaje asombroso, el espermatozoide tiene que ser capaz de responder y adaptarse a entornos que cambian dramáticamente (Peña, 2020). Para esto, los espermatozoides presentan estructuras específicas que están preparadas para reaccionar adecuadamente con el tracto genital femenino y el oocito (Flesch and Gadella, 2000).

### 2.3.1. Anatomía y estructura espermática

El espermatozoide generalmente se divide anatómicamente en cabeza, cuello y flagelo (o cola) (Fig. 1). El flagelo se puede subdividir a su vez en pieza intermedia, pieza principal y pieza final (Pesch and Bergmann, 2006; Brito, 2007), aunque otros autores lo dividen en cabeza y cola, incluyendo la pieza de conexión o cuello como parte del flagelo (Varner and Johnson, 2007; Varner et al., 2015; Toshimori and Eddy, 2015). En su totalidad, el espermatozoide se encuentra rodeado por la membrana plasmática o plasmalema (Amann and Graham, 1993; Brito, 2007; Varner and Johnson, 2007; Varner et al., 2015; Toshimori and Eddy, 2015). En el caso del espermatozoide de caballo, la longitud es de aproximadamente 60 μm (Pesch and Bergmann, 2006; Brito, 2007; Meyers, 2009), y las características de cada una de las partes son:

- **Cabeza**: la cabeza del espermatozoide de caballo es elíptica, aplanada dorsoventralmente y

más gruesa en la porción posterior (Johnson et al., 1978; Johnson, 1991; Varner et al., 2000; Brito, 2007; Varner and Johnson, 2007; Varner et al., 2015). Sus dimensiones aproximadas incluyen una longitud de 5 a 7 μm y un ancho que varía según los autores: 2,5 a 3 μm (Gravance et al., 1996; Casey et al., 1997; Hidalgo et al., 2005) o 3,5 a 4,0 μm (Amann and Graham, 1993; Meyers, 2009). La cabeza contiene el acrosoma, la lámina post-acrosomal y el núcleo, además de un complemento reducido de elementos citosólicos (Brito, 2007; Varner and Johnson, 2007; Varner et al., 2015; Toshimori and Eddy, 2015). Los dos tercios anteriores del núcleo están cubiertos por el acrosoma, una vesícula especializada que contiene enzimas hidrolíticas esenciales para la penetración del oocito (Brito, 2007; Meyers, 2009). La lámina post-acrosomal está compuesta por laminillas estrechas y cubre la porción caudal del núcleo y el anillo posterior. El núcleo comprende la mayor parte de la cabeza del espermatozoide, contiene el material genético en forma de ADN altamente condensado y está separado del citoplasma circundante por la envoltura nuclear de doble capa (Brito, 2007; Varner and Johnson, 2007; Meyers, 2009; Varner et al., 2015). La base del núcleo termina en la fosa de implantación, donde la capa externa de la envoltura nuclear se engrosa para formar la placa basal (Brito, 2007; Varner and Johnson, 2007), que proporciona la unión de la cabeza al capítulo del cuello (Pesch and Bergmann, 2006; Brito, 2007). El borde entre la cabeza y el cuello está claramente definido por un anillo posterior y corresponde al lugar de unión del plasmalema con la envoltura nuclear en la base de la cabeza (Brito, 2007).

- **Cuello:** el cuello o pieza de conexión del espermatozoide es un segmento de enlace de aproximadamente 1 μm (Pesch and Bergmann, 2006), que sirve como pieza articular de conexión entre la cabeza del espermatozoide y la pieza intermedia del flagelo (Pesch and Bergmann, 2006; Brito, 2007; Varner and Johnson, 2007; Varner et al., 2015) y para estabilizar algunos componentes estructurales del flagelo (Varner and Johnson, 2007; Varner et al., 2015; Toshimori and Eddy, 2015). El cuello está unido anteriormente a la placa basal y posteriormente a las fibras densas de la cola (Pesch and Bergmann, 2006; Brito, 2007). El cuello está compuesto por las columnas segmentadas y el capítulo (Pesch and Bergmann, 2006; Varner and Johnson, 2007; Meyers, 2009; Varner et al., 2015; Toshimori and Eddy, 2005). El capítulo se articula con la cabeza al nivel de la fosa de implantación, uniéndose mediante finos filamentos que conectan el capítulo a la placa basal. Las columnas segmentadas anclan las fibras densas del flagelo (Varner and Johnson, 2007; Varner et al., 2015).

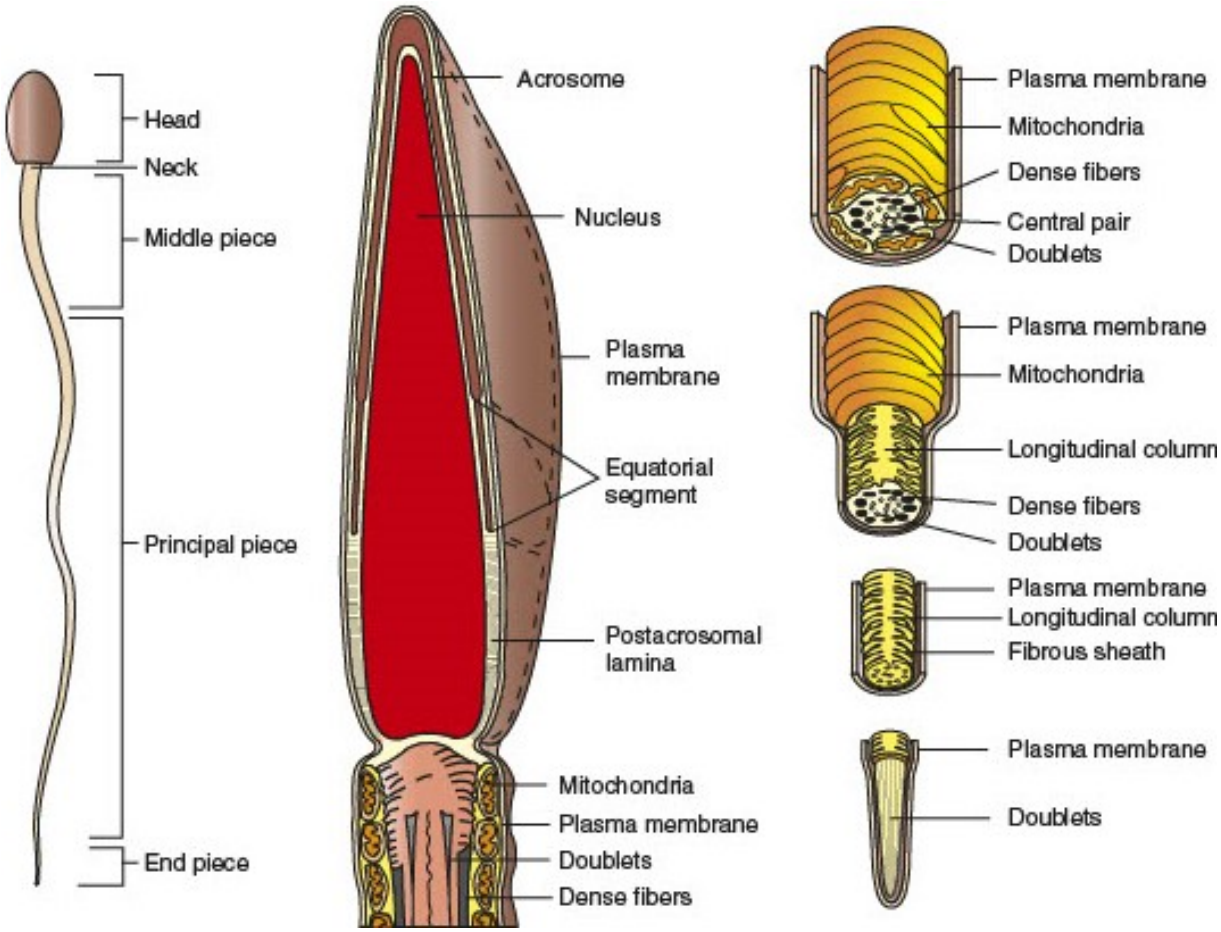


**Figura 1.** Diagrama de un espermatozoide de caballo, que está constituido por cabeza, cuello y cola, la cual se divide a su vez en pieza intermedia, principal y final. Todo el espermatozoide se encuentra cubierto por la membrana plasmática. La cabeza incluye el núcleo (que contiene la información genética en forma de ADN altamente condensado), el acrosoma en forma de capucha (que contiene las enzimas necesarias para la fecundación), una porción especializada del acrosoma denominada segmento ecuatorial y la lámina post-acrosomal. El cuello es el punto de unión de la cola a la cabeza, mediante una disposición de articulación esferoidal. El par central y los nueve pares de microtúbulos que constituyen el axonema están rodeados por las fibras densas. Todos estos elementos se extienden desde la región del cuello, a través de la pieza central y la pieza principal, hasta la pieza final, donde terminan en sitios ligeramente diferentes. Debido a que estas fibras densas son afiladas, la cola se vuelve progresivamente más delgada. Los dobletes de microtúbulos son los elementos contráctiles, que se contraen diferencialmente para inducir un movimiento de deslizamiento y flexionan la cola en un patrón helicoidal. Esto impulsa al espermatozoide. Las mitocondrias son estructuras membranosas donde se produce la mayor parte de la energía necesaria para el movimiento de los espermatozoides. Las columnas longitudinales y la vaina fibrosa de la pieza principal y las fibras densas proporcionan la rigidez necesaria para el movimiento normal de la cola (Meyers, 2009). Modificado de Amann and Pickett (1987).

- **Flagelo o cola**: el flagelo del espermatozoide consta de tres segmentos distintos: pieza intermedia o mitocondrial, pieza principal y pieza final o terminal (Pesch and Bergmann,

2006; Brito, 2007; Meyers, 2009). Los elementos estructurales primarios del flagelo incluyen el axonema, las fibras densas externas, las mitocondrias (vaina mitocondrial) y la vaina fibrosa (Pesch and Bergmann, 2006; Varner and Johnson, 2007; Varner et al., 2015; Toshimori and Eddy, 2015). El flagelo proporciona la fuerza motriz, que es esencial para impulsar a los espermatozoides a través del tracto genital femenino para llegar al lugar de fecundación en la unión ámpulo-ístmica del oviducto de la burra o yegua (Meyers, 2009). En la mayoría de los espermatozoides de burro y caballo, la fosa de implantación y la placa basal tienen una posición excéntrica (descentrada) con respecto al ancho de la célula, por lo que los espermatozoides con cola abaxial se consideran normales (Brito, 2007; Meyers, 2009). Los espermatozoides con tal implantación de la cola tienden a nadar en amplios movimientos circulares, lo cual es considerado anormal o, al menos, no progresivo en una especie no équida (Meyers, 2009). El lugar donde dos mitocondrias perpendiculares comienzan a girar en espiral hacia la hélice mitocondrial denota el comienzo de la pieza intermedia (Brito, 2007).

- **Pieza intermedia**: la pieza intermedia está formada por el axonema rodeado por las fibras densas y la vaina mitocondrial. Se extiende desde el extremo caudal del cuello hasta el anillo (anillo de Jensen) (Pesch and Bergmann, 2006; Brito, 2007; Varner and Johnson, 2007; Toshimori and Eddy, 2015), punto donde la vaina mitocondrial se reemplaza por una vaina fibrosa (Pesch and Bergmann, 2006; Varner and Johnson, 2007; Toshimori and Eddy, 2015). En el caso de los espermatozoides de caballo, la pieza intermedia tiene una longitud aproximada de 8 μm a 10,5 μm (Amann and Graham, 1993; Brito, 2007; Meyers, 2009) y un diámetro aproximado de 0,6 μm (Brito, 2007). El axonema está formado por nueve pares de microtúbulos que forman un haz cilíndrico dispuesto uniformemente alrededor de un par central de microtúbulos, que son los elementos que se contraen para producir el movimiento de la cola del espermatozoide. Esta disposición de microtúbulos se conoce como patrón 9x2 + 2 (Brito, 2007; Varner and Johnson, 2007; Meyers, 2009; Inaba, 2011; Varner et al., 2015). Los microtúbulos del axonema se extienden desde la región del cuello a través de la pieza intermedia y la pieza principal hasta la pieza terminal, donde terminan en sitios ligeramente diferentes (Brito, 2007; Meyers, 2009). Cada par de microtúbulos del axonema está rodeado por una de las nueve fibras densas externas longitudinales que proporcionan rigidez y elasticidad al flagelo, que se extienden desde su origen en aposición a las columnas segmentadas del cuello a

lo largo de la pieza intermedia y la mayor parte de la pieza principal, mientras que no están presentes en la pieza final (Brito, 2007; Varner and Johnson, 2007; Meyers, 2009). Las fibras densas externas a su vez están rodeadas por mitocondrias dispuestas de punta a punta en una doble espiral continua (Brito, 2007; Meyers, 2009). Se observan aproximadamente entre 50 a 60 espirales mitocondriales en el espermatozoide de caballo, que forman la vaina mitocondrial (Amann and Graham, 1993; Brito, 2007; Meyers, 2009). Las mitocondrias son los orgánulos membranosos que producen la mayor parte de la energía necesaria para la motilidad de los espermatozoides (Brito, 2007; Meyers, 2009), la cual almacenan en forma de adenosin trifosfato (ATP), que es utilizado por el axonema para el desarrollo del movimiento flagelar coordinado que se origina en la región del cuello (Meyers, 2009). En el extremo caudal de la vaina mitocondrial se encuentra el anillo (o anillo de Jensen), que consta de subunidades filamentosas muy compactas orientadas circunferencialmente, y finaliza en el extremo anterior de la vaina fibrosa de la pieza principal, demarcando el extremo caudal de la pieza intermedia (Brito, 2007).

- **Pieza principal**: la pieza principal es el segmento más largo de la cola. En el espermatozoide de caballo mide aproximadamente entre 30 μm y 44 μm (Amann and Graham, 1993; Brito, 2007; Meyers, 2009). El axonema y las fibras densas de la pieza intermedia continúan a través de la pieza principal, pero las fibras densas se vuelven más estrechas y terminan en diferentes niveles en la pieza principal en su porción distal (Brito, 2007; Varner and Johnson, 2007; Meyers, 2009). La singularidad de la pieza principal es la presencia de la vaina fibrosa, que rodea las fibras densas externas (Pesch and Bergmann, 2006; Brito, 2007; Meyers, 2009), y se compone de dos columnas fibrosas dispuestas longitudinalmente que están puenteadas por una serie de nervaduras fibrosas dispuestas circunferencialmente (Pesch and Bergmann, 2006; Brito, 2007). Las fibras densas y la vaina fibrosa no se contraen, pero proporcionan el soporte estructural y la flexibilidad esenciales para la traducción efectiva del movimiento deslizante de los dobletes del axonema en movimientos de la cola de flexión y amplitud definitiva (Amann and Pickett, 1987; Pesch and Bergmann, 2006; Brito, 2007). La vaina fibrosa termina abruptamente a unos micrómetros de la punta de la cola donde la pieza principal se fusiona con la pieza final (Brito, 2007).
- **Pieza final:** la pieza final carece de vaina fibrosa o componentes de fibra densa externa y contiene solo el axonema (Brito, 2007; Toshimori and Eddy, 2015). En la pieza final,

la disposición microtubular 9x2 + 2 continúa aproximadamente hasta la mitad y luego se pierde gradualmente en el fragmento restante (Pesch and Bergmann, 2006; Meyers, 2009). En los caballos, la pieza final tiene una longitud aproximada de 4 a 5 μm (Amann and Graham, 1993; Meyers, 2009).

# 3. ANÁLISIS SEMINAL

## *3.1. Definición*

La evaluación del semen *in vitro*, complementaria a la exploración clínica en el examen de la capacidad reproductiva de un semental, tiene un alto valor diagnóstico para evaluar la calidad seminal, función testicular y epididimaria, y/o el tracto genital del semental, permitiendo la detección de casos claros de infertilidad o incluso de subfertilidad potencial (Rodríguez-Martínez, 2003).

## *3.2. Usos*

En este sentido, el análisis seminal es también un componente fundamental en la aplicación de las diferentes biotecnologías reproductivas utilizadas en el burro y caballo (Rodríguez et al., 2008), ya que es necesario establecer la calidad de un eyaculado para su posterior utilización de forma fresca, refrigerada o congelada. Las pruebas de laboratorio que comprende el análisis seminal permiten correlacionar la calidad del semen con la fertilidad, evitando así el desperdicio de tiempo, recursos y esfuerzos (Holt et al., 2007). Además, el análisis seminal es necesario para determinar tanto el número de dosis para IA como el número de pajuelas que se pueden obtener de un eyaculado (Foxcroft et al., 2008). Por otro lado, también permite hacer una estimación precisa del daño sufrido por la célula espermática después de los procesos de refrigeración o criopreservación, contribuyendo con esto al desarrollo de nuevos procedimientos con la finalidad de mantener la integridad y la función del espermatozoide (Hernández-Avilés et al., 2019).

## *3.3. Procedimiento*

El proceso de la evaluación seminal comienza después de la recolección, por lo que el semen crudo se debe manipular con cuidado para evitar que cualquier cambio de temperatura o manejo inapropiado pueda afectar y conducir a una interpretación incorrecta de la calidad del eyaculado (Estrada and Samper, 2007). De este modo, una vez realizada la colecta, el semen debe ser transportado al laboratorio inmediatamente. La fracción gel y otros residuos deben eliminarse

utilizando un filtro en línea durante la recolección o después de esta. Posteriormente, tras registrar el volumen total y tomar una muestra para evaluar la concentración de espermatozoides, el semen se diluye con un diluyente previamente calentado a 37ºC (Samper, 2007).

Actualmente, para realizar el análisis espermático del burro o caballo se dispone de pruebas de diagnóstico de rutina y de otras más avanzadas, cuyo uso se determina en función del tiempo, la disponibilidad de equipo especializado y las limitaciones económicas del propietario (Brinsko et al., 2011; Restrepo-Betancur et al., 2013). Las pruebas de rutina incluyen la evaluación de las características macroscópicas del semen, como el olor, el color, el volumen y el pH, y la evaluación de características microscópicas, como la concentración, motilidad, viabilidad y morfología de los espermatozoides (Dowsett, 1988; Estrada and Samper, 2007). Las pruebas complejas o avanzadas, realizadas en aquellos sementales seleccionados, pueden incluir el análisis del plasma seminal, el estudio con microscopia electrónica de la ultraestructura del espermatozoide, el análisis de la estructura de la cromatina y varias pruebas de la funcionalidad espermática (por ejemplo, integridad de la membrana plasmática y del acrosoma, potencial de la membrana mitocondrial y los niveles intracelulares de calcio, entre otras pruebas a través de citometría de flujo) (Brinsko et al., 2011).

Para asegurar la confiabilidad de los resultados, el análisis seminal debe realizarse de manera minuciosa y metódica por una persona con experiencia en un laboratorio adecuadamente equipado. Asimismo, las técnicas utilizadas deben ser objetivas, estandarizadas y lo suficientemente sensibles como para reconocer desviaciones de los rangos normales (Mortimer, 1994; Brinsko et al., 2011).

### *3.4. Aplicación*

El enfoque convencional para evaluar la calidad espermática en burros y caballos basado fundamentalmente en las pruebas de diagnóstico de rutina se remonta a varias décadas, y se realiza tanto inmediatamente después de la colecta como después de su almacenamiento *in vitro* (Katila, 2001; Varner, 2008; Atroshchenko et al., 2019). Si bien este análisis posee un valor predictivo, el cual en el último tiempo se ha mejorado conla incorporación de técnicas más desarrolladas (Varner, 2008; Ball, 2008a; Barrier Battut et al., 2016), es importante tener en cuenta que la fiabilidad de la evaluación seminal como indicador de la fertilidad de un semental no es absoluta. En muchos casos, dicha evaluación no es suficiente para determinar las posibles causas de la disminución de la fecundidad (Estrada and Samper, 2007; Ball, 2008a; Atroshchenko et al.,

2019), estando ésta influenciada además por factores independientes de la fertilidad del semental, tales como la fertilidad de la hembra y el manejo reproductivo (Estrada and Samper, 2007).

En la última década, con la incorporación de la lipidómica, epigenómica, proteómica y metabolómica, así como los avances en el análisis de imágenes (por ejemplo, microscopia y sistema de análisis espermático asistido por ordenador (CASA)) y la citometría de flujo (Peña, 2020; Hernández-Avilés et al., 2019), se han producido enormes progresos en la comprensión de la biología del espermatozoide. Estos hallazgos han proporcionado nuevas pistas que pueden ayudar a comprender de mejor manera la infertilidad y el impacto de las tecnologías reproductivas en la función espermática (Peña, 2020), y han mejorado la capacidad del clínico y del investigador para explicar las diferencias en el potencial reproductivo entre los sementales, y evaluar críticamente el efecto de varios métodos de preservación del espermatozoide equino (Hernández-Avilés et al., 2019).

# 4. INSEMINACIÓN ARTIFICIAL

## *4.1. Definición*

La IA es la técnica mediante la cual se deposita una cantidad adecuada de espermatozoides viables en el útero de una hembra en el momento óptimo para lograr tasas óptimas de preñez (Samper, 2009). En la actualidad, es una herramienta ampliamente utilizada en la reproducción equina (Aurich, 2012; Kowalczyk et al., 2019), así como también en animales de granja, animales de compañía y especies silvestres (Aurich, 2012).

## *4.2. Origen y desarrollo*

Cuenta la leyenda que el caballo fue el primer animal en el cual la IA se practicó con éxito. Se dice que en 1322 un jefe árabe que codiciaba el semental de un rival envió a su campamento un espía que extrajo parte del semen de este semental desde la vagina de una yegua recién apareada, lo diluyó con leche de camello en una bolsa de piel de cabra, lo transfirió a otra yegua y al año siguiente, nació un potro lindo y sano. Sin embargo, no se relata cómo se realizó el procedimiento de inseminación (Bowen, 1969; Pickett and Voss, 1999; Pickett et al., 2000; Allen, 2005). Pese a lo interesante de esta historia, no fue hasta 1784 cuando el italiano Lázzaro Spallanzani comunicó la primera IA realizada con éxito en mamíferos, concretamente en perro (Mendiola et al., 2005; Aurich, 2012; Soriano-Úbeda et al., 2013; Ombelet and Van Robays, 2015). En el caso de los équidos, si bien Hunter en Estados Unidos en 1799, Repiquet en Francia en 1885 y Heape en

1898 en Gran Bretaña (Morel, 1999) utilizaron la IA en yeguas, no fue hasta principios del siglo XX cuando el ruso Ivanoff realizó experimentos controlados de IA en caballos (Allen, 2005). En este sentido, se afirma que en 1909 fueron inseminadas las primeras yeguas en Rusia y que en el año 1938 el Instituto Central de Inseminación Artificial de Moscú ya registraba 138.000 yeguas inseminadas artificialmente (Morel, 1999). Asimismo, tanto rusos como chinos ampliaron el uso diario de la IA con semen fresco en caballos entre 1930 y 1960, hasta el punto de que en 1959 se inseminaron 600.000 yeguas en China (Pickett and Voss, 1999; Allen, 2005).

Sin embargo, otros autores consideran que no fue realmente hasta finales de la década de 1960 cuando el profesor Pickett y su grupo del Laboratorio de Reproducción Animal en Fort Collins (Colorado) comenzaron una amplia gama de estudios fisiológicos básicos sobre los factores que influyen en la espermatogénesis en el semental, los métodos de recolección, dilución, enfriamiento y congelación del semen e inseminación, lo que desencadenó que la IA equina despegara bajo un amplio escrutinio científico (Allen, 2005). Otro hito importante en la historia de la IA en équidos ocurrió algunos años antes cuando, en 1957, Barker y Gandier reportaron el nacimiento del primer potrillo a través de IA con semen congelado (Loomis, 2001).

Si bien las ventajas que ofrece la IA son tan vastas que, tal vez, sea la biotecnología con mayor impacto en la producción equina cuando es empleada adecuadamente en sus diferentes modalidades (Canisso et al., 2008), no fue hasta comienzos de este siglo cuando la IA devino una técnica de gran proyección para la producción equina (Squires, 2009). Esto se debe a que durante muchos años el desarrollo y utilización de la IA en équidos, principalmente con semen congelado, estuvo limitado por imposiciones de muchas asociaciones de criadores que no permitían su uso (Samper, 2007; Canisso et al., 2008; Squires, 2009). Sin embargo, las regulaciones de las asociaciones de criadores de diversos países del mundo se han ido flexibilizando en los últimos años (a excepción de la raza Pura Sangre Inglés), permitiendo el registro de productos oriundos de la IA y otras tecnologías de reproducción asistida, ejerciendo con esto un gran impacto en la industria ecuestre (Loomis, 2006; Canisso et al., 2008; Squires, 2019).

### *4.3. Ventajas de la IA*

La IA ofrece numerosas ventajas (Samper, 2009), entre las cuales cabe destacar las siguientes:

a) Acelera la mejora genética por la mayor difusión de sementales de alto valor.
b) Evita el desplazamiento de las yeguas con los problemas sanitarios que ello puede acarrear.

c) Reduce el riesgo de contracción de enfermedades de transmisión venérea.
d) Evita la sobreutilización de un semental.
e) Permite la utilización de un semental ubicado a miles de kilómetros de la yegua.
f) Además, y en el caso del semen criopreservado, permite el uso del semen procedente de sementales que ya han fallecido.

La IA es una técnica viable y utilizada también en burros, donde el material estándar para caballos puede utilizarse para la recolección, evaluación, transporte e inseminación (Chamba-Ochoa et al., 2017). Sin embargo, en comparación con el caballo, los estudios y conocimiento sobre la utilización de esta técnica en burros es aún limitada (Chamba-Ochoa et al., 2017; Fanelli et al., 2019).

### *4.4. Tipos de IA*

En los équidos, se utilizan tres métodos de IA (Samper, 2009; Sanchez et al., 2009):

a. IA con semen fresco: tras su recolección, el semen se usa inmediatamente en su estado crudo o diluido con un diluyente apropiado. Este tipo de IA presenta limitaciones, dado que debe llevarse a cabo dentro de los 30 minutos posteriores a la recolección en el caso del semen sin diluir, por lo que la yegua y el semental deben encontrarse en el mismo lugar, o durante las 3 horas siguientes cuando el semen es diluido.
b. IA con semen refrigerado: en este caso, el semen se colecta del mismo modo que en el caso anterior, pero, tras su dilución con un medio apropiado y su análisis, se refrigera a 5-8 ºC. La IA con este tipo de semen puede llevarse a cabo hasta 48 horas después de su recolección.
c. IA con semen congelado: aquí, el semen es colectado, diluido, analizado y finalmente congelado (preferiblemente con un biocongelador, o congelador con tasa de enfriamiento controlada) a -196 ºC. Las pajuelas resultantes, habitualmente de 0,5 mL, se almacenan en tanques de nitrógeno líquido para ser utilizadas en una IA varios días, meses o años después de la recolección.

### *4.5. Factores implicados en el éxito de la IA*

Hay numerosos factores que influyen en la tasa de preñez alcanzada con el uso de la IA (Sieme et al., 2003), incluyendo la fertilidad inherente al semental y a la hembra; el tipo de semen utilizado para la inseminación (fresco, refrigerado-transportado o congelado-descongelado), así

como su calidad y resistencia a los procesos de conservación; el número de espermatozoides de la dosis de inseminación; la concentración del semen diluido; el tiempo que se almacena el semen refrigerado antes de la IA; el manejo de la hembra y la destreza del veterinario con las distintas técnicas y estrategias empleadas para maximizar la fertilidad (Sieme et al., 2003; Brinsko, 2006; Alonso et al., 2016).

En yeguas inseminadas con semen fresco, las dosis recomendadas están en el rango de 250-500×$10^6$ espermatozoides con motilidad progresiva (EMP) depositados en el cuerpo del útero, siendo la concentración de 500×$10^6$ de espermatozoides la más frecuente. Para semen refrigerado, se adopta la dosis de IA recomendada para semen fresco, es decir 500×$10^6$ EMP, dosis que se podría reducir en al menos 100×$10^6$ EMP en el caso de sementales y yeguas con fertilidad probada acompañado de un buen manejo (Brinsko, 2006). En el caso del semen congelado/descongelado, no existe una dosis estándar que haya sido críticamente evaluada en el caballo, además de que cada centro tiene su propio protocolo y que, mientras que algunos estudios se refieren al número de espermatozoides mótiles por dosis, otros lo hacen al número de espermatozoides totales. Sin embargo, la dosis recomendada para alcanzar tasas de preñez óptimas para la inseminación en el cuerpo del útero es de un mínimo de entre 200 y 250×$10^6$ EMP (Pickett et al., 2000; Lyle and Ferrer, 2005; Metcalf, 2007; Cazales Penino et al., 2020). En un esfuerzo por aumentar la uniformidad en cuanto al número total de espermatozoides por dosis, la Federación Mundial para la Cría de Caballos de Deporte (WBFSH) ha establecido como valor estándar para la inseminación con semen congelado un mínimo de 250×$10^6$ EMP depositados en el cuerpo uterino, con un porcentaje de motilidad progresiva después de la descongelación de al menos 35% (Alonso et al., 2016).

Es importante mencionar que en el caso del semen congelado/descongelado, se utiliza la técnica de IA profunda con el objetivo de reducir el número de espermatozoides requeridos y aumentar los porcentajes de preñez (Brinsko et al., 2003; Brinsko, 2006; Hayden et al., 2012; Cazales Penino et al., 2020). Esta técnica consiste en depositar un pequeño volumen de semen en el cuerno uterino (cerca de la unión útero-tubárica) ipsilateral al folículo ovulatorio, a través de histeroscopia o mediante un catéter guiado por vía transrectal (Brinsko, 2006; Cazales Penino et al., 2020). La dosis que se utiliza y las tasas de preñez logradas varían ampliamente en los diferentes estudios, independiente del método utilizado (Brinsko, 2006), donde existe una tendencia a emplear el mínimo de dosis necesaria para lograr la preñez. Mientras que diversos autores señalan que la histeroscopia debe utilizarse cuando las dosis de inseminación contiene

menos de $20\times10^6$ EMP espermatozoides (Squires, 2005), otros indican que es posible obtener resultados aceptables con dosis bajas de entre 50 y $100\times10^6$ de EMP usando la inseminación con catéter guiado por vía transrectal (Petersen et al., 2002; Alonso et al., 2016). En este sentido, algunos centros en Europa abogan por la IA profunda en el cuerno uterino utilizando como mínimo por dosis de inseminación una pajuela de 0,5 mL, en la que generalmente se envasan entre 100 y $200\times10^6$ espermatozoides/mL (Brinsko, 2006). En el caso de las burras, debido a que la concentración media de espermatozoides en el semen de burro es superior a la del caballo, al parecer las dosis inseminantes deben ser también superiores (De Oliveira et al., 2016).

### *4.6. Uso de la IA*

En un trabajo de revisión llevado a cabo por Squires (2009) se indicó que, del total de las yeguas en las que se utilizaba IA, el 50-60% se inseminaban con semen fresco, el 30-40% con semen refrigerado y el 5-10% con semen congelado/descongelado. De acuerdo con este autor, las tasas de preñez obtenidas por ciclo varían entre los diferentes estudios publicados, principalmente cuando se trataba de IA con semen congelado/descongelado. Así, las tasas de preñez con semen fresco se encontraban en el rango del 65% al 70% por ciclo; con semen refrigerado del 50% al 60% y con semen congelado del 40% al 50%. Actualmente, se estima que estos porcentajes han aumentado respecto a los datos recogidos por Squires (2009), debido al mayor conocimiento de las técnicas, su uso generalizado y las mejoras en el transporte, los contenedores, los diluyentes y los crioproctectores del semen. En este sentido, en una revisión recientemente publicada por Kowalczyk et al. (2019), se menciona que en Europa la IA se usa en alrededor del 50% de las yeguas destinadas a la reproducción. Este porcentaje va en aumento principalmente por el uso de la IA en las yeguas de deporte, a pesar de su disminución en las de cría y en el resto de las yeguas. Además, en Europa, la IA con semen refrigerado ha ido ganando popularidad en las yeguas de deporte y en el resto de yeguas, mientras se ha aumentado el uso de la IA con semen congelado para las yeguas de cría (Kowalczyk et al., 2019).

En caballos, los índices de preñez obtenidos actualmente mediante los diferentes métodos de IA (semen fresco, refrigerado o congelado) se consideran suficientes como para dar márgenes de confiabilidad comercialmente aceptables (Losinno and Aguilar, 2002). No obstante, la tasa de preñez es menor (<50%) cuando se insemina con semen congelado/descongelado en comparación al semen fresco o refrigerado (Blottner et al., 2001; Vidament, 2005; Brinsko, 2006; Nikitkina et al., 2020). Esta diferencia es aún más patente en burros, ya que, a pesar de presentar

una viabilidad y motilidad aceptables, penetrar ovocitos *in vitro* y fecundar yeguas, los resultados obtenidos con semen congelado/descongelado después de inseminar burras son muy deficientes (Miró and Papas, 2018), dónde las tasas de preñez varían desde el 0% al 36% (De Oliveira et al., 2016). De entre los factores implicados en estos resultados se apunta a la intensa reacción endometrial posterior a la IA que se genera en esta especie (Miró and Papas, 2018).

El uso generalizado de la IA en los últimos años ha incrementado el interés de investigadores y clínicos por las técnicas de procesamiento y conservación del semen, lo que ha llevado al desarrollo de métodos, protocolos o procedimientos que tienen como objetivo minimizar el daño espermático y maximizar su viabilidad, supervivencia y capacidad fecundante (Loomis 2006; Kowalczyk et al., 2019). Sumado a lo anterior, los propietarios a menudo solicitan a los veterinarios de équidos que mejoren el rendimiento reproductivo de sus sementales, en donde, desafortunadamente, la calidad seminal muchas veces se desvía de las expectativas y conduce a tasas de gestación insatisfactorias (Kowalczyk et al., 2019). Todo ello pone de manifiesto la necesidad de desarrollar métodos de conservación del semen y técnicas de mejora de la calidad espermática.

# 5. CONSERVACIÓN Y MEJORA DE LA CALIDAD ESPERMÁTICA

## *5.1. Métodos de conservación*

La implementación de la IA y otras técnicas de reproducción asistida ha llevado a que, en la actualidad, los métodos de conservación del semen (refrigeración y criopreservación) sean de gran importancia (Varela et al., 2018).

El semen equino es muy sensible a la manipulación y a los procesos de conservación, debido principalmente a que, durante el procesamiento y almacenamiento, los espermatozoides sufren estrés oxidativo, estrés osmótico (Ball, 2008b) y otros cambios propios de la apoptosis (Brum et al., 2008), tales como la activación de caspasas o el desorden en los lípidos de la membrana plasmática (Thomas et al., 2006). Todos estos eventos llevan a la disminución de la calidad del semen (Ball, 2008b), lo que ha motivado al estudio y desarrollo de diluyentes y crioprotectores del semen (Squires et al., 2004; Squires, 2005; Restrepo-Betancur et al., 2014; Nikitkina et al., 2020).

## *5.2. Semen refrigerado*

El semen diluido refrigerado es el método más utilizado para su transporte en la industria equina,

permitiendo una amplia distribución de las dosis con una calidad espermática aceptable (Varela et al., 2018). Esto se debe a que, en el caso de los équidos, el semen fresco diluido con los medios actualmente disponibles, no se puede conservar por más de 12 horas entre 15ºC y 20ºC, mientras que el semen diluido refrigerado a 4-6ºC se puede almacenar hasta, según Samper (2007), 72 horas, con una gran variabilidad entre sementales.

Los espermatozoides son sensibles a muchos factores ambientales, incluida la temperatura, la luz, el daño físico y una gran variedad de sustancias químicas. En consecuencia, el proceso de conservación del semen comienza desde la recolección, y por ello, una vez realizada la colecta, el semen debe ser transportado al laboratorio inmediatamente sin agitarlo, evitando su exposición a la luz y a cambios bruscos de temperatura (Brinsko et al., 2011). Después de extraer la fracción gel, registrado el volumen total y tomada una muestra para evaluar la concentración de los espermatozoides, el semen se diluye con un diluyente previamente precalentado (37ºC). Los diluyentes del semen protegen a los espermatozoides del daño por frío y les proporcionan una solución amortiguadora de pH y nutrientes (Samper, 2007). Los diluyentes de semen equino comúnmente utilizados para su uso en semen fresco y refrigerado varían en su composición, aunque la mayoría contienen leche, antibióticos (Samper, 2007; Brinsko et al., 2011; Nikitkina et al., 2020) o yema de huevo (Samper, 2007; Nikitkina et al., 2020). Los antibióticos más utilizados son una combinación de penicilina, amikacina y/o gentamicina (Samper 2007; Brinsko et al., 2011). Tras realizar la dilución, el semen de caballo y burro debe ser enfriado desde 37°C (la temperatura del diluyente) a 4-6°C (la temperatura de refrigeración). En este punto, es importante considerar que dichos espermatozoides requieren una velocidad de enfriamiento lenta, si bien el descenso desde 37°C a 20°C se puede llevar a cabo rápidamente. Entre 20°C y 4-6°C, la velocidad de enfriamiento debe ser de −0,5°C a −0,1°C por minuto para maximizar la viabilidad espermática. Para esto, además, el envasado del semen se debe realizar en condiciones anaeróbicas (Samper, 2007).

Algunos caballos tienen una reducción significativa de la calidad de su semen cuando se enfría y almacena durante más de 12 horas (Samper, 2007). En este sentido, se ha descrito un efecto perjudicial del plasma seminal en la motilidad espermática del semen equino durante su almacenamiento a 4-6ºC durante 24 y 48 horas (Jasko et al., 1991). Otros estudios también han demostrado que la eliminación del 90% del plasma seminal contribuye a la estabilización de la membrana plasmática de los espermatozoides (Brinsko et al., 2000; Barrier-Battut et al., 2013). Además, la eliminación del plasma seminal se ha correlacionado con un mayor mantenimiento

de la viabilidad espermática después de 48 horas de almacenamiento a 4-6ºC en burros (Serres et al., 2002; Miró et al., 2009). No obstante, en caballos, algunos estudios muestran que la presencia de una pequeña proporción, del 1% al 20%, de plasma seminal mejora la motilidad espermática (Jasko et al., 1992). Esta proporción de plasma seminal en el volumen final de semen refrigerado se logra mediante su dilución con un diluyente de refrigeración. Si bien la centrifugación del eyaculado y la posterior eliminación del sobrenadante pueden ser una alternativa, se han observado diferencias entre individuos y especies con respecto a la sensibilidad de los espermatozoides a la centrifugación, siendo las velocidades iguales o inferiores a 600 g aquéllas en las que no se han detectado daños significativos en los espermatozoides de mamíferos (Mann et al., 1981).

Finalmente, debido a la alta variabilidad entre individuos en la tolerancia de sus espermatozoides al almacenamiento a bajas temperaturas y en sus tasas de preñez después de la IA con semen refrigerado (Brinsko et al., 2000; Hartwig et al., 2014; Varela et al., 2018), los burros y caballos pueden clasificarse como buenos o malos refrigeradores dependiendo de cómo se mantenga la motilidad progresiva y la viabilidad de sus eyaculados durante el almacenamiento a 4ºC-6ºC (Hartwig et al., 2014).

A pesar del esfuerzo de muchos investigadores por mejorar las técnicas de refrigeración de semen en burros y caballos, la pérdida de viabilidad en el espermatozoide después de la refrigeración continúa siendo un factor limitante para el amplio uso de la técnica (Varela et al., 2018). Esto se debe principalmente a los diluyentes utilizados, por lo que existe una oportunidad para el desarrollo de otros métodos o mejoras en los diluyentes de semen para que este mantenga la viabilidad por más de 48 horas (Squires, 2009).

### *5.3. Semen congelado*

El primer artículo sobre congelación de semen equino fue publicado por Smith y Polge en 1950 obteniendo una supervivencia del 25% de espermatozoides congelados a -79°C en una solución de glucosa y glicerol. Algunos años más tarde, en 1957, se comunicó la primera gestación de una yegua inseminada con semen congelado-descongelado, aunque no fue hasta fines de 1980 cuando se generalizó el uso del semen congelado en equinos (Loomis, 2001).

En general, los procedimientos utilizados para la criopreservación de semen de burro y caballo implican el examen andrológico del macho, la recolección del semen, la evaluación seminal, la dilución, centrifugación y descarte del sobrenadante, la resuspensión del pellet con el

diluyente de congelación, la refrigeración y/o estabilización del semen, el envasado y, finalmente, la congelación y la evaluación seminal después de la descongelación (Samper and Morris, 1998; Canisso et al., 2019). Sin embargo, hasta el momento no existe un procedimiento estandarizado para cada una de estas etapas; de allí que exista una alta variación entre los resultados experimentales y los procedimientos rutinarios de campo (Squires et al., 1999; Vidament, 2005; Aurich and Aurich, 2006).

El uso de semen criopreservado en équidos ha adquirido una gran importancia en las últimas décadas debido principalmente a los beneficios que ofrece (Loomis, 2001; Mesa and Henao, 2012), ya que permite su almacenamiento a largo plazo, el transporte del semen a cualquier destino, la conservación del semen de animales genéticamente superiores y el desarrollo de un programa de mejora genética (Pugliesi et al., 2014; Peña et al., 2011), así como también el control o la erradicación de algunas enfermedades venéreas (Loomis and Graham, 2008; Pérez et al., 2017). Desde el punto de vista práctico, una de las principales ventajas del uso de semen congelado es que permite inseminar una yegua en el momento óptimo de reproducción sin depender de la disponibilidad del semental (Loomis and Squires, 2005). También la crioconservación es un enfoque seguro de almacenamiento de gametos que, además, permite la conservación de la biodiversidad y protege las especies en peligro de extinción (Rodrigues et al., 2015).

Sin embargo, diversos autores señalan que el éxito de la congelación de semen equino es menor que en otras especies domésticas y la tasa de preñez disminuye cuando se insemina con semen congelado-descongelado en comparación con la utilización de semen fresco o refrigerado (Blottner et al., 2001; Vidament, 2005; Brinsko, 2006; Nikitkina et al., 2020), lo cual, como se explicó anteriormente (véase apartado 4.6.), se acentúa aún más en el caso de semen de burro (Miró and Papas, 2018).

Uno de los factores más importantes implicados en la criotolerancia espermática en equinos es la variabilidad individual existente entre los sementales (Dowsett and Knott, 1996; Sieme et al., 2008; Loomis and Graham, 2008; Mari et al., 2011; Cabrera et al., 2014) e incluso entre los eyaculados de un mismo animal (Dowsett and Knott, 1996; Cabrera et al., 2014). Esta variabilidad se debe en gran medida al tipo de selección a la que han sido sometidos, basada principalmente en la genealogía, el rendimiento atlético o la morfología, más que en la eficiencia reproductiva (Varner et al., 2008; Mari et al., 2011).

Se considera que un semental es "apto para congelación" cuando presenta valores de

motilidad progresiva ≥50-60% en semen fresco y ≥30-35% en la post-descongelación (Loomis and Graham, 2008). Otros autores mencionan que antes de iniciar el proceso de congelación, el semen fresco debe tener como mínimo un 40% de espermatozoides morfológicamente normales, una motilidad progresiva superior al 60% y una concentración mínima de $100x10^6$ de espermatozoides por mL (Restrepo-Betancur et al., 2014), aunque se han desarrollado protocolos especiales para procesar eyaculados de bajo volumen y baja concentración (Barrier-Battut et al., 2013).

Algunos estudios muestran que solo el 20-30% de los sementales producen un semen con buena capacidad de congelación, llamados "buenos congeladores"; otro 40-60% aproximadamente tiene una capacidad aceptable (aunque se ve afectada negativamente por la criopreservación) y el 20-30% restante son sementales que producen un semen que no es congelable por lo que, aunque estos animales pueden tener buenos índices de fertilidad en monta natural, reciben el nombre de "malos congeladores" (Brinsko et al., 2000; Loomis and Graham, 2008).

Otro factor que limita el uso del semen congelado es la baja tolerancia de los espermatozoides equinos a los procesos de congelación y descongelación (Fagundes et al., 2011; Candeias et al., 2012). Los daños causados por la criopreservación a los espermatozoides se han descrito ampliamente en la literatura (Blach et al., 1989; Heitland et al., 1996; Ortega-Ferrusola et al., 2007; Yeste et al., 2015). La reducción de la tasa de fertilidad observada después de los procesos de congelación y descongelación se relaciona principalmente con los daños causados en el funcionamiento y en la estructura de la membrana espermática (Parks and Graham, 1992; Candeias et al., 2012). En equinos, se ha demostrado que la criopreservación induce la fragmentación del ADN espermático (Baumber et al., 2003; Cabrera et al., 2014; Yeste et al., 2015), disminuye el potencial de membrana mitocondrial (Brum et al., 2008; Cabrera et al., 2014; Yeste et al., 2015), daña el acrosoma (Cabrera et al., 2014; Yeste et al., 2015) y genera ROS (Burnaugh et al., 2007; Cabrera et al., 2014; Yeste et al., 2015). Por este motivo, se necesitan estrategias que permitan mejorar los factores fisicoquímicos que afectan la supervivencia de los espermatozoides durante los procesos de congelación y descongelación para lograr una mayor viabilidad y fertilidad (Watson, 1995; Pugliesi et al., 2014).

Hasta ahora, los grandes esfuerzos han ido principalmente enfocados a la búsqueda de las tasas optimas de dilución, enfriamiento y congelación del semen, y en los métodos de criopreservación (Salamon and Maxwell, 1995), así como también en el descubrimiento y

desarrollo de sustancias que protegen a las células del daño producido por la criopreservación, como aditivos, diluyentes y crioprotectores (Salamon and Maxwell, 1995; Squires et al., 2004; Restrepo-Betancur et al., 2014; Nikitkina et al., 2020).

### *5.4. Técnicas de mejora de calidad espermática*

Debido a la necesidad de aumentar el rendimiento reproductivo de burros y caballos, entre otras especies, actualmente se están empleando y desarrollando otros enfoques que buscan mejorar la calidad del semen mediante varias técnicas y además que esta mejora en la calidad se mantenga durante el almacenamiento (Loomis, 2006; Johannisson et al., 2009; Kowalczyk et al., 2019; Blanco-Prieto et al., 2020a). Algunas de las técnicas empleadas con este fin son: la separación del plasma seminal (Brinsko et al., 2000; Loomis, 2006; Aurich, 2008), la centrifugación amortiguada (colchones de centrifugación) (Ecot et al., 2005; Waite et al., 2008) y las técnicas de selección de espermatozoides (por ejemplo: migración espermática, filtración y centrifugación coloidal o con gradiente de densidad). El resultado de estas técnicas es un número menor de espermatozoides que la muestra inicial pero con una mayor calidad; por ejemplo, una mayor motilidad y una mayor proporción de espermatozoides morfológicamente normales, y en el caso de la centrifugación coloidal, también una mejora en su integridad de membrana y de la cromatina (Ayuso-Hernando et al., 2011; Morrell et al., 2016; Papas et al., 2020b).

En este contexto, la fotoestimulación espermática emerge como un nuevo enfoque en la búsqueda de la mejora de la calidad, la capacidad fecundante y la criotolerancia de los espermatozoides (Blanco-Prieto et al., 2020b).

### *5.5. Fotoestimulación espermática*

#### 5.5.1. Definición

En la década de 1980, la irradiación de células animales con luz visible (luz de bajo nivel), también conocida como fotobiomodulación, se comenzó a utilizar para el tratamiento médico (fototerapia) en diferentes disciplinas. Además, dado que la estimulación de la luz aumenta la producción de ATP en la célula, y esto es crucial para la motilidad de los espermatozoides y la maduración de los ovocitos, se iniciaron una serie de estudios con la finalidad de investigar los efectos de la luz sobre los gametos, demostrándose que la irradiación ejercía efectos positivos en particular sobre los espermatozoides (Lubart et al., 1992; Abdel-Salam and Harith, 2015; Yeste et al., 2018; Zupin et al., 2020). Así pues, en este campo de investigación, la fotoestimulación

emerge como una herramienta muy útil para el estudio del efecto de la luz sobre la fertilidad, motilidad y criotolerancia de los espermatozoides (Iaffaldano et al., 2013; Blanco-Prieto et al., 2020b; Zupin et al., 2020).

La fotoestimulación espermática consiste en irradiar muestras de semen a una longitud de onda fija o variable durante un corto período de tiempo. La fuente de luz puede ser un láser, que proporciona luz en longitud de onda fija (discreta), o un diodo emisor de luz (LED), que proporciona luz en longitud de onda variable (espectro). En algunos casos, el proceso alterna fases de luz y oscuridad (Yeste et al., 2016).

### 5.1.2. Efectos

Si bien se ha podido observar que el primer efecto de la fotoestimulación es la formación de ROS mediante fotosensibilizadores celulares endógenos (Lubart et al., 2000; Zan-Bar et al., 2005), se ha demostrado, en experimentos *in vitro* e *in vivo*, que la fotoestimulación aumenta la motilidad espermática, la generación de ATP y los niveles de calcio intracelular en los espermatozoides de diferentes especies, así como también su viabilidad y capacidad fecundante (Abdel-Salam and Harith, 2015; Gabel et al., 2018; Yeste et al., 2018; Zupin et al., 2020; Blanco-Prieto et al., 2020a; 2020b). Estos estudios emplearon diferentes longitudes de onda (Zupin et al., 2020) y observaron que, si bien había una variación en los resultados obtenidos, la estimulación con luz roja (620-750 nm) era la más beneficiosa para mejorar la motilidad espermática y la fertilidad (Zan-Bar et al., 2005).

La fotoestimulación con luz roja, ya sea con luz láser o con LED, aumenta la motilidad de los espermatozoides de humano, perro, toro, carnero, cerdo, pollo, conejo, pavo, faisán y tilapia (Sato et al., 1984; Ban Frangez et al., 2015; Salama and El-Sawy, 2015; Preece et al., 2017; Gabel et al., 2018; Corral-Baqués et al., 2005; 2009; Siqueira et al., 2016; Yeste et al., 2016; Pezo et al., 2019; Iaffaldano et al., 2005; 2010; 2013; 2016; Zan-Bar et al., 2005), la capacidad de inducir la capacitación espermática *in vitro* en cerdo (Yeste et al., 2016), y su poder fecundante en cerdo (Yeste et al., 2016; Pezo et al., 2019; Blanco-Prieto et al., 2020a), ratón (Cohen et al., 1998), carnero y tilapia (Zan-Bar et al., 2005). Asimismo, la irradiación con luz roja mejora la conservación de semen refrigerado de pavo, conejo y cerdo (Iaffaldano et al., 2005; 2010; Yeste et al., 2016; Blanco-Prieto et al., 2020a), la resistencia térmica del semen refrigerado de cerdo (Yeste et al., 2016), la congelación de semen de toro (Fernandes et al., 2015) y la viabilidad y calidad del semen congelado/descongelado de carnero (Iaffaldano et al., 2016).

Estudios previos han analizado el efecto de la fotoestimulación con luz roja sobre la fertilidad *in vitro*. Cohen et al. (1998) estudiaron los efectos de la irradiación en espermatozoides de ratón con un láser Helio-Neón (He-Ne) de 630 nm (luz roja) y con un láser de diodo a una longitud de onda de 780 nm (luz infrarroja). La irradiación a 630 nm pero no la de 780 nm mejoró los porcentajes de ovocitos fecundados *in vitro*, lo que se observó juntamente con un aumento en los niveles de calcio intracelular. De acuerdo con estos resultados, Zan-Bar et al. (2005) también encontraron un aumento en el porcentaje de ovocitos fecundados *in vitro* después de irradiar semen de carnero y tilapia con varias fuentes de luz (400-800 nm luz blanca, luz roja de 660 nm, luz azul de 360 nm y ultravioleta de 294 nm); en concreto, los espermatozoides de tilapia mostraron mayor motilidad y poder fecundante después de la irradiación con luz roja y blanca. En el caso de los espermatozoides de carnero, la motilidad y la fertilidad solo aumentaron con la irradiación con luz roja, mientras que se obtuvo un efecto negativo sobre la motilidad y la fertilidad de los espermatozoides de ambas especies tras la irradiación con luz ultravioleta y azul, lo que se atribuyó a un aumento excesivo de los niveles de ROS en las muestras irradiadas (Zan-Bar et al., 2005).

Con respecto a los efectos de la estimulación lumínica en el rendimiento reproductivo evaluado después de la IA, Yeste et al. (2016) encontraron que la irradiación de semen de cerdo refrigerado con un dispositivo LED con luz roja (620-630 nm) a través de un patrón discontinuo (10 min de luz, 10 min de oscuridad, 10 min de luz) aumentó las tasas de partos y el tamaño de la camada, resultados que fueron confirmados más tarde por Blanco Prieto et al. (2019) y Pezo et al. (2019), si bien dicho aumento no se observó en todas las explotaciones (Yeste et al., 2016; Blanco-Prieto et al., 2019; 2020a; 2020b).

### 5.1.3. Mecanismos de acción

Dado que los mecanismos de acción de la fotoestimulación en el espermatozoide no están del todo claros, se han sugerido tres posibles hipótesis para explicarlos (Yeste et al. 2018; Blanco-Prieto et al., 2020b). La primera plantea que la luz interactúa con **receptores** específicos de la **membrana plasmática** que pertenecen a la familia de receptores de potencial transitorio (TRP) y que están vinculados a la termotaxis. La familia TRP es un grupo muy heterogéneo y solo las proteínas incluidas en las subfamilias TRP vainilloide (TRPV), TRP anquirina (TRPA) y TRP melastanina (TRPM) están implicadas en el control de la termotaxis (Vriens et al., 2014). Como parte de la primera subfamilia, se ha visto que TRPV4 es el canal iónico sensible a la temperatura

más importante en los espermatozoides de mamífero (Dusenbery, 2009; Blanco-Prieto et al., 2020b), dado que juega un papel crucial durante la capacitación de los espermatozoides (Lishko et al., 2012; Singh and Rajender, 2014; Sun et al., 2017; Mundt et al., 2018) y se ha relacionado con la respuesta termotáctica tanto en ratones (Hamano et al., 2016) como en espermatozoides humanos (Mundt et al., 2018).

La segunda hipótesis está relacionada con la capacidad de la luz para interactuar con receptores específicos que pertenecen a la familia de las **opsinas**, acopladas a una proteína G (transducina) y localizadas en la membrana plasmática de los espermatozoides de mamíferos (Pérez-Cerezales et al., 2015). Los diferentes miembros de esta familia de proteínas (melanopsina, encefalopsina, rodopsina y neuropsina) parecen funcionar como termosensibilizadores, más que como proteínas intracelulares fotosensibles (fotosensibilizadores), a través de la fosfolipasa C canónica (PLC) y las vías de los nucleótidos cíclicos (cAMP / cGMP) (Pérez-Cerezales et al., 2015). Si bien aún se desconoce la función exacta de estas opsinas en los espermatozoides de mamífero, la evidencia creciente sugiere que estas proteínas, especialmente la melanopsina y la rodopsina, están involucradas, junto con las proteínas TRPV antes mencionadas, en la respuesta del espermatozoide a la termotaxis (Zheng, 2013; Pérez-Cerezales et al., 2015; Roy et al., 2020). En este contexto, es relevante indicar que la termotaxis podría ser un modulador importante de la estimulación lumínica, ya que los espermatozoides de mamífero son sensibles a cambios de temperatura tan pequeños como 0,0006ºC (Bahat et al., 2012; Pérez-Cerezales et al., 2015; Blanco-Prieto et al., 2020b). Por tanto, es razonable sugerir que la estimulación con luz roja podría actuar también a través de esta vía.

Finalmente, el tercer mecanismo hipotético contempla el efecto directo de la irradiación de luz sobre proteínas intracelulares fotosensibles (**fotosensibilizadores**), como son los citocromos mitocondriales, cruciales para la regulación de la función mitocondrial global (Yeste et al., 2018; Blanco-Prieto et al., 2020b). Los citocromos son componentes esenciales de la cadena de electrones de la mitocondria y juegan un papel vital en el control de la fosforilación oxidativa, la generación de ROS y la activación de la vía apoptótica intrínseca (Ortega-Ferrusola et al., 2009; Blanco-Prieto et al., 2020b). Los citocromos se caracterizan por poseer un grupo hemo, que acepta y cede electrones (Kessel, 1982; Pottier and Truscott, 1986) y es sensible a la luz. Sin embargo, es importante considerar que no todos los citocromos reaccionan a las mismas longitudes de onda, ya que su respuesta a longitudes de onda de luz específicas depende de su estructura (Lynch and Copeland, 1992). Así, mientras que el citocromo P450 tiene su pico de

absorción más alto a 450 nm, el citocromo C, que forma el complejo IV de la cadena de electrones mitocondriales, tiene dos picos a 610-630 nm y 660-680 nm (Lynch and Copeland, 1992).Los fotosensibilizadores, entre los que se encuentran los citocromos mitocondriales, ionizan y transfieren la energía a moléculas adyacentes al absorber la luz de la radiación electromagnética (Lubart et al., 1992). Por tanto, la estimulación lumínica produce un potencial electroquímico extra en las mitocondrias, lo que aumenta la síntesis de ATP. Este potencial electroquímico adicional generado por la estimulación de la luz (luz visible y roja lejana) se convierte en energía de la fuerza motriz del protón y aumenta la síntesis de ATP. Las teorías para explicar la formación de ATP a partir del potencial electroquímico generado por la luz fueron propuestas por primera vez por Lubart et al. (1992) como sigue: i.) Formación de oxígeno singlete por porfirinas endógenas, que posteriormente activarían la cadena respiratoria mitocondrial; y ii.) excitación de cromóforos mitocondriales (fotosensibilizadores/fotoaceptores endógenos, como porfirinas o citocromos), que luego desencadenarían reacciones redox en la cadena respiratoria (Karu, 1988; Friedmann et al., 1991; Tamura, 1993; Breitbart et al., 1996; Yeste et al., 2018). Así, el primer paso después de la irradiación con luz visible es la formación de ROS por fotosensibilizadores endógenos excitados electrónicamente. Esto da como resultado un aumento de la actividad de la cadena respiratoria y del potencial de membrana a través de la membrana mitocondrial interna, que oxida la reserva de NAD y altera el estado redox tanto del citosol como de las mitocondrias. Estos cambios son los que provocan un aumento en la producción de ATP (Yeste et al., 2018). Además, el aumento en el potencial de la membrana mitocondrial altera los niveles de $Ca^{2+}$ citosólico ya que, por un lado, aumenta la actividad de las bombas dependientes de ATP (ATPasa) que captan $Ca^{2+}$ del espacio extracelular y, por otro lado, aumenta la liberación mitocondrial de $Ca^{2+}$ (Fig. 2) (Yeste et al., 2018).

De las tres hipótesis planteadas hasta ahora, la evidencia indica que la irradiación de luz acelera la respiración mitocondrial y la producción de ATP (Karu, 1989; Gao and Xing, 2009), dando a la tercera hipótesis de los citocromos mitocondriales (especialmente el citocromo C) una importancia vital en la respuesta de la célula espermática a la luz roja (Zan-Bar et al., 2005; Yeste et al., 2018; Blanco-Prieto et al., 2020b), ya que en estudios realizados en diferentes especies se ha demostrado una relación directa entre la motilidad y actividad mitocondrial (Iaffaldano et al., 2010; Abdel-Salam and Harith, 2015; Iaffaldano et al., 2016; Siqueira et al., 2016; Yeste et al., 2016). Se ha visto que la irradiación con un láser de He-Ne (632,8 nm) de espermatozoides congelados/descongelados de carnero aumentó la motilidad, el contenido de

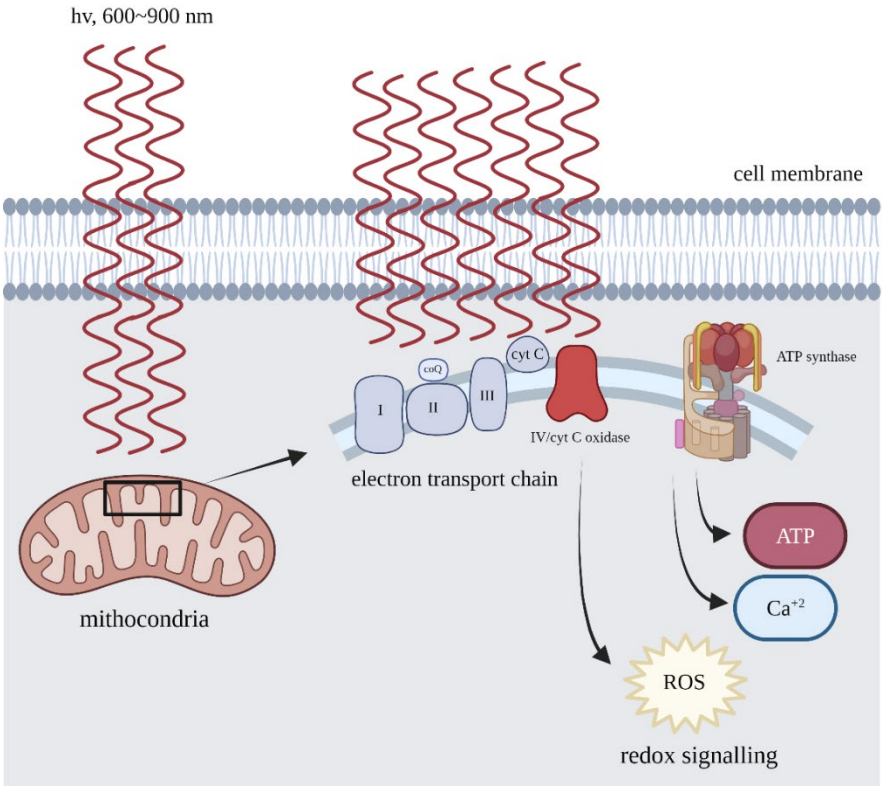


**Figura 2.** Diagrama que ilustra las vías de señalización que se activarían mediante la irradiación de luz de los espermatozoides. Los fotones son absorbidos por cromóforos fotosensibilizadores en las mitocondrias. Esto conduce a un aumento de la fosforilación oxidativa , producción de ATP, $Ca^{2+}$ y ROS. Adaptado de Huang et al. (2011).

ATP y la actividad de la citocromo C oxidasa (Iaffaldano et al., 2016). Efectos similares se observaron en espermatozoides refrigerados de conejo, pues la irradiación con luz roja aumentó la motilidad, la actividad de la citocromo C oxidasa y la carga de energía celular, que correspondía a la suma de las fracciones de ATP, ADP y AMP (Iaffaldano et al., 2010). De acuerdo con esto, también se han investigado los efectos de la irradiación con láser de He-Ne sobre la motilidad y la integridad funcional de espermatozoides congelados-descongelados de algunas especies de aves (Iaffaldano et al., 2013). Aunque en este trabajo se observó un aumento en la actividad de la citocromo C oxidasa, dicho incremento no se pudo correlacionar con un aumento en la motilidad. En dichos estudios, la actividad de la citocromo C oxidasa se determinó como una medida del efecto de la irradiación en la mitocondria a nivel bioenergético. En este sentido, es sabido que la energía química requerida para la motilidad espermática es suministrada por la producción de ATP en la célula espermática que deriva, dependiendo de la especie, de la glucólisis y/o de la fosforilación oxidativa (Iaffaldano et al., 2013). Este último proceso

bioquímico se produce como resultado de la actividad de la citocromo C oxidasa (Arnold, 2012). Dado que los espermatozoides deben mantener la motilidad para fecundar el ovocito, los procesos metabólicos implicados en la producción de ATP son esenciales para apoyar la motilidad de los espermatozoides. Recientemente, se han observado efectos similares en espermatozoides porcinos refrigerados, pues, de acuerdo con Blanco-Prieto et al. (2020b), la fotoestimulación con luz LED roja (620-630 nm) aumenta el ATP, la actividad mitocondrial, y la actividad de la citocromo C en las muestras irradiadas.

Sin embargo, y a pesar de los resultados que apoyan la relevancia de las mitocondrias en los efectos de la luz sobre la célula espermática, no se debe descartar que más de uno de los mecanismos hipotéticos anteriormente mencionados pueda estar involucrado en la respuesta del espermatozoide a la luz roja, así como que la influencia de la luz en la conformación de otras proteínas también pueda estar también implicada en dicha respuesta (Yeste et al., 2018).

### 5.1.4. Aplicaciones

Los datos hasta ahora publicados indican que los efectos de la estimulación lumínica dependen del estado de la muestra, la irradiación del haz de luz utilizado (Gabel et al., 2018), el tiempo o el patrón de exposición (Yeste et al., 2016; Blanco-Prieto et al., 2020a; 2020b) y la especie (Zan-Bar et al., 2005). En este sentido, ningún estudio ha observado un efecto perjudicial de la luz visible sobre la viabilidad espermática (Yeste et al., 2016; Pezo et al., 2019; Blanco-Prieto et al., 2020a; 2020b), y la integridad del ADN (Preece et al., 2017; Gabel et al., 2018; Yeste et al., 2018) y del acrosoma (Iaffaldano et al., 2016; Blanco-Prieto et al., 2020a; 2020b), por lo que se postula que la fotoestimulación puede tener un efecto positivo sobre la motilidad espermática y su capacidad de fecundar los ovocitos tanto *in vivo* como *in vitro* (Gabel et al., 2018).

Finalmente, desde un punto de vista práctico, se debe tener en cuenta que la aplicación de un láser requiere material específico y a menudo costoso que las granjas comerciales no se pueden permitir en condiciones ordinarias. De este modo, la posibilidad de utilizar otros sistemas más económicos como sistemas de luz estimuladores que puedan tener una eficiencia similar a los dispositivos láser merece una mayor atención. En este contexto, un sistema basado en LED es más barato y fácil de mantener y utilizar que los láseres, y también tiene una elevada eficiencia fotónica. Además, como se ha mencionado anteriormente, se ha visto que la fotoestimulación con luz LED roja (620-630 nm) del semen antes de la IA proporciona buenos resultados con un

incremento significativo del rendimiento reproductivo en granjas comerciales de cerdo (Yeste et al., 2016).

### 5.1.5. Justificación del estudio

A pesar de todo lo dicho anteriormente, los efectos de la fotoestimulación sobre el espermatozoide de burro y caballo todavía no se han estudiado. En la presente Tesis no solo se evaluaron los efectos de diferentes protocolos de fotoestimulación con luz roja sobre los parámetros de calidad espermática, sino también sobre su capacidad para soportar el estrés térmico y mejorar su longevidad. Además, se investigaron los efectos del diluyente y del color del recipiente (pajuela), así como también los posibles mecanismos por los cuales la luz actúa sobre la célula espermática y su efecto en el metabolismo. Para ello, se utilizaron dos dispositivos con triple LED rojo (Fig. 3) a una longitud de onda de 620-630 nm, y los recipientes que contenían el semen y se irradiaron fueron pajuelas 0,5 mL o tubos Eppendorf de 1,5 mL.

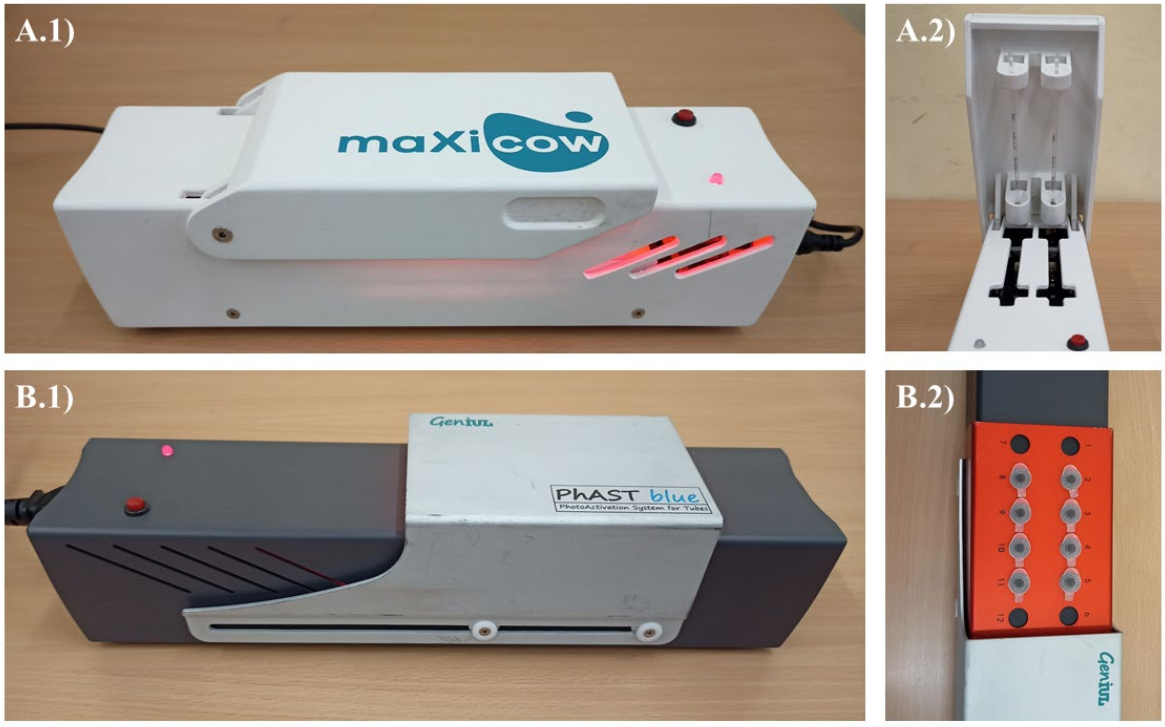


**Figura 3.** Equipos utilizados para la fotoestimulación utilizados en este estudio, ambos formados por un dispositivo con triple LED rojo (620-630 nm). A) Máquina para fotoestimular pajuelas de 0,25 o 0,5 mL; B) Máquina para fotoestimular tubos Eppendorf 1,5 mL. 1) Detalle del perfil de la máquina; 2) Detalle de la máquina cargada.

CAPÍTULO II

# HIPÓTESIS Y OBJETIVOS

# 1. HIPÓTESIS

La hipótesis de esta Tesis Doctoral es que la irradiación con luz LED roja del semen de burro y caballo, fresco, refrigerado y congelado produce una mejora en los parámetros de calidad espermática y su capacidad para soportar el estrés térmico. Estos efectos, que estarían impulsados por cambios en la función mitocondrial producidos por la luz, podrían variar según la especie (burro o caballo), el patrón de irradiación, la turbidez del diluyente y el color de la pajuela utilizados para realizar el procedimiento de fotoestimulación.

# 2. OBJETIVOS

Para comprobar las hipótesis planteadas en esta Tesis Doctoral, se establecieron los siguientes objetivos:

1- Evaluar cómo afectan diferentes patrones de estimulación con luz LED roja (620-630 nm) a la motilidad, la integridad de la membrana, el potencial de membrana mitocondrial y los niveles intracelulares de ROS intracelulares en semen de burro fresco y refrigerado.

2- Analizar cómo diferentes patrones de estimulación con luz LED roja (620-630 nm) modulan la motilidad, la integridad de la membrana, las subpoblaciones de espermatozoides móviles, el potencial de membrana mitocondrial, la tasa de consumo de $O_2$, los niveles intracelulares de ATP y la integridad del ADN espermático en semen fresco de caballo.

3- Verificar si la estimulación del semen de caballo congelado-descongelado con luz LED roja (620-630 nm) podría mejorar su calidad y aumentar su resistencia térmica a 38°C.

4- Determinar si el color de la pajuela y la turbidez del diluyente utilizado para la refrigeración del semen afectan a la respuesta inducida por la estimulación con luz LED roja (620–630 nm) en semen de caballo refrigerado.

5- Estudiar si los efectos producidos por la estimulación con luz LED roja (620-630 nm) están provocados por cambios en la función mitocondrial inducidos por la acción de la luz sobre los citocromos mitocondriales, como el citocromo C.

CAPÍTULO III

# COMPENDIO DE ARTÍCULOS

# 1. Effects of red-light irradiation on the function and survival of fresh and liquid-stored donkey semen

Jaime Catalán; Marion Papas; Sabrina Gacem; Federico Noto; Ariadna Delgado-Bermúdez; Joan E. Rodríguez-Gil; Jordi Miró; Marc Yeste

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# Effects of red-light irradiation on the function and survival of fresh and liquid-stored donkey semen

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ABSTRACT

This study sought to determine whether sperm irradiation using a light emission diode (LED) at 620–630 nm affects the motility, membrane integrity (viability), mitochondrial activity and intracellular levels of reactive oxygen species (ROS) in fresh diluted and liquid-stored donkey semen. With this purpose, sixteen ejaculates (eight fresh diluted and eight cooled-stored) were collected from eight adult jackasses. Fresh semen samples were diluted in Kenney extender and stimulated with red-light after collection, whereas cooled semen was stored at 4 °C for 24 h after dilution and then irradiated. In all cases, semen samples were packed into 0.5-mL transparent straws, which were then randomly divided into control and 19 treatments: six consisted of single red-light exposure, and the other 13 involved irradiation at light-dark-light intervals. Upon irradiation, sperm motility, membrane integrity mitochondrial membrane potential (MMP) and intracellular levels of superoxide anion ($\cdot O_2^-$) and hydrogen peroxide ($H_2O_2$) were evaluated. While specific light-patterns increased both sperm motility and mitochondrial activity, they did not affect sperm membrane integrity and had no clear impact on intracellular ROS levels. The effects of irradiation patterns differed between fresh and cooled semen since, whereas 1 and 4 min patterns induced the greatest increments in the total and progressive motility of fresh semen, 4 min, 4-1-4 and 4-4-4 were the most suitable for cooled-stored samples. In both fresh diluted and cooled-stored semen, the motility increase observed after light-stimulation for 4 min was concomitant with changes in the percentages of spermatozoa with high mitochondrial membrane potential. In summary, this study shows, for the first time, that specific irradiation patterns increase sperm motility and mitochondrial activity in the donkey. Furthermore, the precise effect of red-light appears to depend on the specific functional status of cells, with separate effects on fresh and cooled samples.



## 1. Introduction

The great development of artificial insemination (AI) in the last years has increased the interest of professionals and researchers in semen processing techniques, highlighting the need of developing methods, protocols or procedures that minimize sperm damage while maximizing their survival and fertilizing capacity [1]. In this sense, although several studies have described and characterized the semen of domestic horses, studies on donkey sperm are scarce. At this respect, it is worth noting that while both horses and donkeys are phylogenetically close species, they have important reproductive differences, including sperm characteristics such as motility patterns, morphology and the sperm interaction with the female endometrium [2].

In this context, studies carried out in other species have shown that reproductive performance can be partially increased through stimulation of semen with red-light before AI [3]. Other studies have also shown that light-stimulation increases sperm motility, which, in species such as equids, may have a direct impact upon reproductive performance [4]. Therefore, red-light irradiation has

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been suggested to exhibit a positive effect on sperm motility and fertilizing ability, both *in vivo* and *in vitro* [5].

Although the mechanisms underlying light-effects on mammalian spermatozoa remain largely unknown, mounting evidence gives cell photosensitizers a crucial role. Accordingly, when these cell photosensitizers are stimulated, ATP production and $Ca^{2+}$ influx increase. In addition, the fact that opsins are present in mammalian spermatozoa suggests that they could be involved in the sperm response to red-light (Reviewed in Ref. [6]). Matching with these hypotheses, previous studies conducted with low level laser therapy devices (LLLT) and light-emitting diodes (LED) have reported an increase in ATP production through the mitochondrial respiratory chain [5,7], without neither damaging the irradiated tissue [8], nor affecting the integrity of sperm DNA at very high doses (810 nm at 850 nm wavelength). However, it has been reported that the first step after irradiation with visible light is the formation of ROS by endogenous cellular photosensitizers, which could affect sperm motility and fertilizing ability [9]. In this context, one must bear in mind that the effects of light-stimulation rely upon sample source (i.e. either fresh or frozen-thawed sperm [5]), time and pattern of exposure [3], and species [9]. From a practical point of view, the application of lasers requires specific and often expensive material. Therefore, a system based on LED is much cheaper and easier than lasers with high photonic efficiency. In addition, stimulation with red-light (620–630 nm) using LED has been found to improve *in vitro* capacitation and fertilizing ability of boar spermatozoa, without affecting their viability [3].

In spite of all the aforementioned, the effects of red-light stimulation on equid sperm have not yet been studied. Therefore, the present work aimed to evaluate, for the first time, how different light-stimulation patterns with a red-light emission diode (620–630 nm) affect the motility, membrane integrity, mitochondrial activity and intracellular ROS levels (hydrogen peroxide, $H_2O_2$, and superoxide anion, $\cdot O_2^-$) of fresh diluted and cooled-stored donkey semen.

## 2. Materials and methods

### 2.1. Suppliers

All reagents were of analytical grade and were purchased from Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Saint Louis, MO, USA). Plastic materials were provided by Nunc (Roskilde, Dinamarca). As far as fluorochromes are concerned, and unless otherwise stated, all were purchased from Molecular Probes (Invitrogen; Eugene, Oregon, USA) and were previously resuspended in dimethyl sulfoxide (Sigma-Aldrich).

### 2.2. Animals and ejaculates

This study involved sixteen ejaculates coming from eight healthy, adult jackasses of proven fertility. Animals were allocated at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), and collected through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line nylon mesh filter to separate the gel fraction. Immediately after collection, gel-free semen was diluted 1:5 (v:v) with Kenney extender [10], previously pre-warmed at 37 °C. Sperm concentration was determined in all samples through a haemocytometer (Neubauer chamber, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

### 2.3. Experimental design

Prior to light-stimulation, a conventional seminal analysis was performed and sperm concentration was adjusted to $25 \times 10^6$ spermatozoa/mL with a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Eight ejaculates were packed into transparent 0.5-mL straws (fresh diluted semen; Minitüb GmbH, Tiefenbach, Germany), whereas the others were stored in 50-mL tubes at 4 °C for 24 h (cooled-stored semen) and then packed into 0.5-mL straws.

### 2.4. Light-stimulation procedures

Light-stimulation of samples was carried out using the aforementioned transparent 0.5-mL straws, containing either fresh diluted or cooled-stored semen. After packaging, straws were placed into a programmable photo-activation system (MaxiCow; IUL, S.A., Barcelona, Spain). In this device, each straw is in contact with a triple LED configuration system that emits red-light (wavelength window: 620 nm–630 nm). The device is equipped with a supporting software (IUL, S.A.) that allows the regulation of the intensity and exposure time. In all cases, intensity was set at 100%.

A total of 19 light-stimulation protocols were evaluated. Six out of these 19 protocols consisted of single light-emission periods (1, 2, 3, 4, 5 and 10 min), whereas the other 13 treatments consisted of light-dark-light intervals: 1-1-1, 2-1-2, 2-2-2, 3-1-3, 3-3-3, 4-1-4, 4-4-4, 5-1-5, 5-5-5, 10-5-10, 10-10-10, 15-5-15 and 15-15-15. Controls consisted of aliquots from the same samples without light-stimulation. Upon irradiation, sperm samples were transferred into 1.5-mL tubes, and incubated in a water bath at 37 °C for 5 min prior to analysis of sperm motility, membrane integrity, mitochondrial membrane potential and intracellular levels of peroxides and superoxides.

### 2.5. Analysis of sperm motility

Sperm motility was evaluated using a computer-assisted sperm-analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). Five μL of each sperm sample was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel) previously warmed at 37 °C. Samples were analyzed with a 10 × negative phase-contrast objective and an Olympus B×41 microscope (Olympus), and at least 1000 sperm cells were counted per analysis. In each evaluation, percentages of total (%TMOT) and progressively motile spermatozoa (%PMOT) were recorded along with the following kinetic parameters: curvilinear velocity (VCL, μm/s), which is the mean path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, μm/s), which is the mean path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, μm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of linearity (LIN, %), which is the quotient between VSL and VCL multiplied by 100; percentage of straightness (STR, %), which is the quotient between VSL and VAP multiplied by 100; percentage of oscillation (WOB, %), which is the quotient between VAP and VCL multiplied by 100; mean amplitude of lateral head displacement (ALH, μm), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle; and frequency of head displacement (BCF, Hz), which is the frequency at which the actual sperm trajectory crosses the average path trajectory (Hz).

Settings of the CASA system were those recommended by the manufacturer: frames/s: 25 images captured per second; particle area >4 and <75 $\mu m^2$; connectivity: 6; minimum number of images

to calculate the ALH: 10. Cut-off values were VAP ≥10 μm/s for a sperm cell to be considered as motile, and STR ≥75% to be graded as progressively motile.

### 2.6. Flow cytometry analyses

#### 2.6.1. General information about the flow cytometric analyses performed in this work

All flow cytometry assessments were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter; Fullerton, CA, USA). Information regarding flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry [11]. Sperm concentration was previously adjusted to $1 \times 10^6$ total spermatozoa per mL (in a final volume of 0.5 mL) with HEPES buffered saline solution (10 mM HEPES, 150 mM NaCl, 10% BSA; pH = 7.4), and stained with the corresponding fluorochromes.

Samples were excited with an argon ion laser emitting at 488 nm (power: 22 mW). The sheath flow rate was set at 4.17 μL min. Spermatozoa were selected on the basis of electronic volume (EV) and side scatter (SS), and non-sperm specific events were gated out. The EV channel was periodically calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in Channel 200 on the EV scale. A total of 10,000 events were evaluated using the following three optical filters: (1) FL1 (green fluorescence), dichroic/splitter, dichroic long pass (DRLP): 550 nm and band pass (BP) filter: 525 nm; (2) FL2 (orange fluorescence), DRLP 600 nm and BP filter 575 nm; and (3) FL3 (red fluorescence), long pass (LP) filter: 670/730 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to each staining method. FL1 was used to detect green fluorescence (SYBR14; YO-PRO-1; JC1 monomers; and 2′,7′-dichlorodihydrofluorescein diacetate, $H_2$DCFDA), FL2 was used to detect orange fluorescence (JC1 aggregates), and FL3 was used to detect red fluorescence (hydroethidine, HE; and propidium iodide, PI). Three technical replicates were evaluated.

Information on each event (i.e. EV, SS, FL1, FL2 and FL3) was collected as list-mode data (.LMD). These data were then analyzed using Cell Laboratory Quanta SC MPL Analysis Software version 1.0 (Beckman Coulter) to quantify each sperm population. Data for JC1, HE/YO-PRO-1 and $H_2$DCFDA/PI were corrected using the percentage of debris particles found in SYBR14/PI dot plots. This avoided overestimation of sperm cells due to the presence of non-sperm, alien particles [13].

#### 2.6.2. Evaluation of sperm plasma membrane integrity

Sperm plasma membrane integrity was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR-14 and PI; Live/Dead Sperm Viability kit; Molecular Probes), according to the protocol described by Garner and Johnson [14] adapted to donkey spermatozoa. Briefly, sperm samples were incubated at 38.5 °C for 10 min with SYBR14 at a final concentration of 100 nM, and then with PI at a final concentration of 12 μM for 5 min at the same temperature. All incubations were performed in the dark. FL1 was used to measure the green fluorescence from SYBR14, and FL3 was used to detect the red fluorescence from PI. Three sperm populations were identified: (1) spermatozoa with an intact plasma membrane, stained in green ($SYBR14^+/PI^-$; viable spermatozoa); (2) spermatozoa with a damage plasma membrane, stained in red ($SYBR14^-/PI^+$); and (3) spermatozoa with a damage plasma membrane, stained in orange ($SYBR14^+/PI^+$). Non-sperm debris particles were identified as those that were not stained with either SYBR14 or PI ($SYBR14^-/PI^-$). SYBR14 spill-over into the PI channel was compensated for (2.45%).

#### 2.6.3. Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined through staining with JC1 following a modified protocol based upon Ortega-Ferrusola et al. [12]. Briefly, sperm samples were incubated with 0.5 μM JC1 at 38.5 °C for 30 min in the dark. Green fluorescence from JC1 monomers ($JC1_{mon}$) was collected through FL1 ($JC1_{mon}$), and orange fluorescence from JC1 aggregates ($JC1_{agg}$) was collected through FL2. Two sperm populations were distinguished: (i) spermatozoa with low MMP, in which all mitochondria were stained in green ($FL1^+/FL2^-$); and (ii) spermatozoa with high MMP, which contained spermatozoa with heterogeneous mitochondria stained both in green and orange in the same cell ($FL1^+/FL2^+$) and spermatozoa that had all their mitochondria stained in orange. The ratio between JC1 aggregates ($JC1_{agg}$; $FL2^+$) and JC1 monomers ($JC1_{mon}$; $FL1^+$) in the sperm population with high MMP was also calculated, as another marker of mitochondrial activity. FL1 spill-over into the FL2 channel was compensated (70.29%).

#### 2.6.4. Evaluation of intracellular ROS levels: $H_2O_2$ and $\cdot O_2^-$

Hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) were used to evaluate intracellular levels of ($\cdot O_2^-$) and $H_2O_2$ respectively, following protocols modified from Guthrie and Welch [15] and Morrell et al. [16].

For $\cdot O_2^-$, sperm samples were mixed with HE (final concentration: 4 μM) and YO-PRO-1 (final concentration: 25 nM) and then incubated at 25 °C for 30 min in the dark. Oxidation of HE to ethidium ($E^+$) was detected through FL3 (red fluorescence), and green fluorescence from YO-PRO-1 was collected through FL1. Four sperm populations were identified: (1) non-viable spermatozoa with low $\cdot O_2^-$ levels ($E^-/YO\text{-}PRO\text{-}1^+$); (2) non-viable spermatozoa with high $O_2^-$ levels ($E^+/YO\text{-}PRO\text{-}1^+$); (3) viable spermatozoa with low $\cdot O_2^-$ levels ($E^-/YO\text{-}PRO\text{-}1^-$); and (4) viable spermatozoa with high $O_2^-$ levels ($E^+/YO\text{-}PRO\text{-}1^-$). Geometric mean fluorescence intensity of $E^+$ (FL-3) in all sperm populations was also recorded, and YO-PRO-1 spill-over into the FL3-channel was compensated (5.06%).

For $H_2O_2$, sperm samples were stained with final concentrations of 140 μM $H_2$DCFDA and 12 μM PI and incubated at 25 °C for 30 min in the dark. Oxidation of $H_2$DCFDA to dichlorofluorescein ($DCF^+$) was detected through FL1 (green fluorescence), and red fluorescence from PI was collected through FL3. Four sperm populations were identified: (1) viable spermatozoa with high peroxide levels ($DCF^+/PI^-$); (2) non-viable spermatozoa with high peroxide levels ($DCF^+/PI^+$); (3) viable spermatozoa with low peroxide levels ($DCF^-/PI^-$); and (4) non-viable spermatozoa with low peroxide levels ($DCF^-/PI^+$). Geometric mean of fluorescence intensity of $DCF^+$ (FL1) in all sperm populations was also recorded, and $DCF^+$ spill-over into the FL3 channel was compensated (2.45%).

### 2.7. Statistical analyses

Statistical analyses were performed using a statistical software package (IBM® SPSS® 25.0 for Windows; IBM corp., Armonk, NY, USA). Data were tested for normality (Shapiro-Wilk) and homogeneity of variances (Levene) prior to running a two-way ANOVA (factors: light-stimulation pattern, and semen source – fresh diluted or cooled-stored –) followed by a post-hoc Sidak's test for pair-wise comparisons. The level of statistical significance was set at $P \leq 0.05$, and data are shown as mean ± standard error of the mean (SEM).

## 3. Results

### 3.1. Effects of red-light irradiation on fresh diluted donkey semen

#### 3.1.1. Sperm motility

As shown in Fig. 1a, percentages of total motile spermatozoa in fresh semen increased ($P < 0.05$) when exposed to red-light for 1 min and 4 min (1 min: 81.7% ± 2.3%; 4 min: 79.2% ± 2.0% *vs.* control: 70.6% ± 1.7%). Furthermore, progressive motility (Fig. 1b) significantly increased when semen was irradiated for 1 min and 4 min (1 min: 54.7% ± 2.5%; 4 min: 56.4% ± 2.6% *vs.* control: 44.1% ± 2.0%).

On the other hand, red-light also increased significantly some sperm kinematic parameters (Table 1) compared to the control; as in the case of VCL (1 min: 137.7 μm/s ± 4.0 μm/s *vs.* control 1.9 μm/s ± 3.1 μm/s), VSL (1 min: 87.5 μm/s ± 2.7 μm/s; 3 min: 87.8 μm/s ± 2.7 μm/s; 1-1-1: 90.7 μm/s ± 2.7 μm/s; 2-1-2 min: 87.9 μm/s ± 2.7 μm/s; 2-2-2: 88.6 μm/s ± 2.7 μm/s; and 4-1-4: 88.5 μm/s ± 2.9 μm/s *vs.* control: 76.0 μm/s ± 2.1 μm/s), and VAP (1 min: 114.2 μm/s ± 3.3 μm/s; 3 min: 114.7 μm/s ± 3.3 μm/s; 1-1-1: 112.7 μm/s ± 3.3 μm/s; 2-1-2: and 111.0 μm/s ± 3.3 μm/s *vs.* control: 99.1 μm/s ± 2.6 μm/s). Percentages of LIN (2-2-2: 73.8% ± 2.3% *vs.* control: 61.7 ± 1.8) and WOB (10 min: 88.0% ± 1.2%; 2-2-2: 88.1% ± 1.2% *vs.* control: 79.6% ± 0.9%) were also found to be increased ($P < 0.05$) following exposure to red-light. In contrast, no significant differences were observed between the control and irradiation patterns in terms of STR ($P > 0.05$). Finally, ALH decreased significantly with 5-1-5 pattern (3.0 μm ± 0.1 μm *vs.* control: 3.7 μm ± 0.1 μm), and BCF was lower ($P < 0.05$) than the control after stimulation with red-light for 10 min and 5-1-5 pattern (10 min: 8.1 Hz ± 0.4 Hz; 5-1-5: 8.1 Hz ± 0.4 Hz *vs.* control: 9.9 Hz ± 0.3 Hz).

#### 3.1.2. Sperm membrane integrity

Percentages of membrane-intact spermatozoa ($SYBR14^+/PI^-$) following light-stimulation in fresh diluted semen did not differ from the control ($P > 0.05$; Supplementary Fig. 1).

#### 3.1.3. Mitochondrial membrane potential

As Fig. 2a shows, percentages of spermatozoa with high MMP were higher ($P < 0.05$) than the control (69.5% ± 2.8%) in the following light-stimulation patterns: 4 min (83.9% ± 3.0%), 3-3-3 (81.9% ± 2.8%), 4-1-4 (85.4% ± 2.9%), 4-4-4 (81.8% ± 2.5%), 5-5-5 (85.9% ± 2.9%), 10-5-10 (84.5% ± 2.9%), 10-10-10 (84.9% ± 2.8%) and 15-15-15 (80.2% ± 3.1%).

Fig. 2b exhibits the ratio between JC1 aggregates ($JC1_{agg}$; $FL2^+$) and JC1 monomers ($JC1_{mon}$; $FL1^+$) in the sperm population with high MMP. In nine patterns, 3 min (0.93 ± 0.04), 4 (1.03 ± 0.04), 3-3-3 (1.02 ± 0.04), 4-1-4 (1.02 ± 0.04), 4-4-4 (1.06 ± 0.04), 5-5-5 (1.01 ± 0.04), 10-5-10 (1.06 ± 0.04), 10-10-10 (1.03 ± 0.04) and 15-15-15 (1.05 ± 0.02), this ratio was significantly higher than in the control (0.79 ± 0.04).

#### 3.1.4. Intracellular ROS levels: $H_2O_2$ and $\cdot O_2^-$

Fig. 3a shows percentages of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$). Some light-stimulation protocols significantly increased those percentages compared to the control; these patterns were: 1 min, 2 min, 3-3-3 min, 10-5-10 and 10-10-10. In contrast, other patterns, such as 5 min, 10 min, 1-1-1, 5-5-5 and 15-5-15, showed lower percentages ($P < 0.05$) of viable spermatozoa with high levels of peroxides than the control. In addition, and as indicated by Fig. 3b, the GMFI of $DCF^+$ (FL1 channel) in the population of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$) was significantly lower in patterns 1 min, 3 min, 5 min, 10 min, 2-1-2, 2-2-2, 3-1-3, 3-3-3, 4-1-4, 4-4-4, 5-5-5, 10-5-10, 10-10-10 and 15-15-15 than in the control.

Percentages of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$; Fig. 3c) were significantly higher in patterns 4-1-4, 4-4-4, 5-1-5, 5-5-5, 10-5-10, 10-10-10, 15-5-15 and 15-15-15 than in the control. In contrast, those percentages were lower ($P < 0.05$) in patterns 2 min, 3 min, 4 min, 1-1-1, 2-1-2, 2-2-2 and 3-1-3 than in the control. With regard to the GMFI of $E^+$ (FL3 channel) in the population of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$), six irradiation patterns (1 min, 5 min, 10 min, 4-1-4, 4-4-4 and 5-1-5) significantly increased those values with regard to the control.

### 3.2. Effects of red-light irradiation on cooled-stored donkey semen

#### 3.2.1. Sperm motility

As shown in Fig. 4a, percentages of total motile spermatozoa were higher ($P < 0.05$) in 4 min (55.6% ± 3.0%), 4-1-4 (58.4% ± 3.2%) and 4-4-4 patterns (56.2% ± 3.1%) than in the control (45.2% ± 2.3%). Percentages of progressively motile spermatozoa (Fig. 4b) were

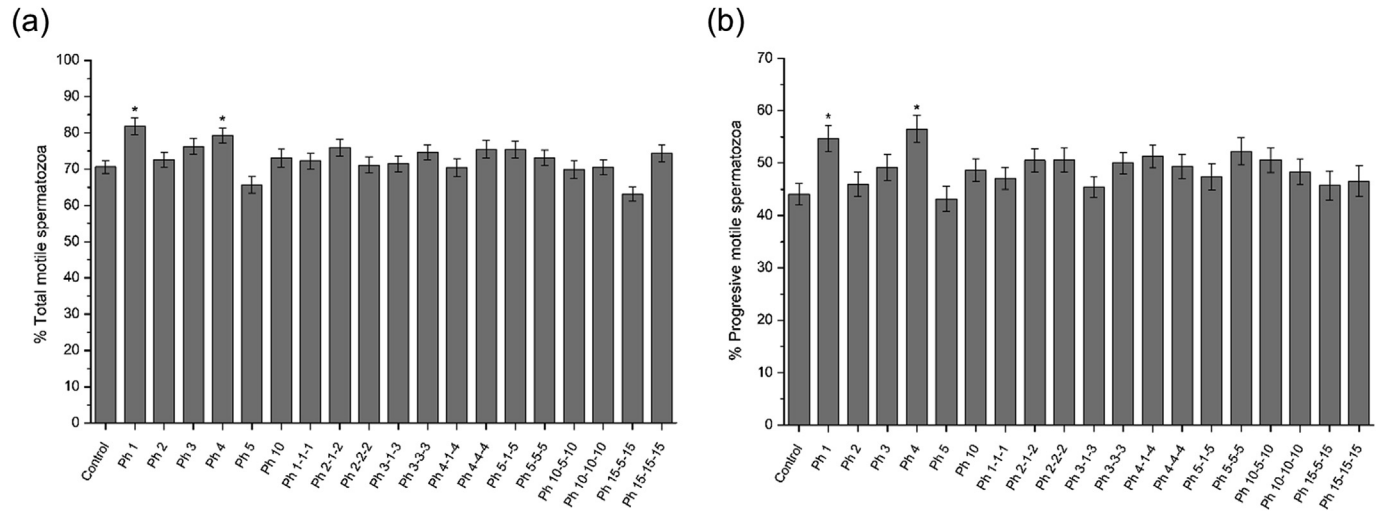


**Fig. 1.** Percentages of total (TMOT; a) and progressively motile spermatozoa (PMOT; b) in the control and different irradiation patterns (fresh diluted semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

**Table 1**
Kinetic parameters in the control and different irradiation patterns (fresh diluted semen). Different superscript letters (*a-b*) indicate significant differences ($P \leq 0.05$) between red-light stimulation patterns and the control. Data are shown as mean ± SEM for eight experiments.

| Patterns | Kinetic parameters (mean ± SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) | LIN (%) | STR (%) | WOB (%) | ALH (μm) | BCF (Hz) |
| Control | 121.9 ± 3.1[a] | 76.0 ± 2.1[a] | 99.1 ± 2.6[a] | 61.7 ± 1.8[a] | 77.2 ± 1.6[a] | 79.6 ± 0.9[a] | 3.7 ± 0.1[a] | 9.9 ± 0.3[a] |
| Photo 1 | 137.7 ± 4.0[a] | 87.5 ± 2.7[b] | 114.2 ± 3.3[b] | 62.4 ± 2.3[a] | 75.7 ± 2.1[a] | 82.0 ± 1.2[a] | 3.6 ± 0.1[a] | 9.5 ± 0.4[a] |
| Photo 2 | 126.6 ± 4.0[a] | 83.5 ± 2.7[a] | 106.2 ± 3.3[a] | 65.0 ± 2.3[a] | 77.8 ± 2.1[a] | 83.3 ± 1.2[a] | 3.6 ± 0.1[a] | 8.8 ± 0.4[a] |
| Photo 3 | 134.7 ± 4.0[a] | 87.8 ± 2.7[b] | 114.7 ± 3.3[a] | 64.9 ± 2.3[a] | 76.2 ± 2.1[a] | 84.9 ± 1.2[a] | 3.5 ± 0.1[a] | 9.0 ± 0.4[a] |
| Photo 4 | 122.2 ± 4.2[a] | 81.8 ± 2.9[a] | 101.0 ± 3.5[a] | 66.6 ± 2.4[a] | 80.6 ± 2.2[a] | 82.3 ± 1.3[a] | 3.3 ± 0.1[a] | 10.2 ± 0.4[a] |
| Photo 5 | 123.3 ± 4.0[a] | 78.0 ± 2.7[a] | 101.2 ± 3.3[a] | 64.2 ± 2.3[a] | 77.6 ± 2.1[a] | 82.1 ± 1.2[a] | 3.6 ± 0.1[a] | 9.2 ± 0.4[a] |
| Photo 10 | 120.0 ± 4.0[a] | 85.4 ± 2.7[a] | 105.5 ± 3.3[a] | 71.6 ± 2.3[a] | 81.0 ± 2.1[a] | 88.0 ± 1.2[a] | 3.2 ± 0.1[a] | 8.1 ± 0.4[a] |
| Photo 1-1-1 | 131.4 ± 4.0[a] | 90.7 ± 2.7[b] | 112.7 ± 3.3[b] | 68.9 ± 2.3[a] | 80.3 ± 2.1[a] | 85.5 ± 1.2[b] | 3.5 ± 0.1[a] | 9.4 ± 0.4[a] |
| Photo 2-1-2 | 131.1 ± 4.0[a] | 87.9 ± 2.7[b] | 111.0 ± 3.3[a] | 66.5 ± 2.3[a] | 78.2 ± 2.1[a] | 84.5 ± 1.2[a] | 3.5 ± 0.1[a] | 9.2 ± 0.4[a] |
| Photo 2-2-2 | 120.1 ± 4.0[a] | 88.6 ± 2.7[b] | 105.8 ± 3.3[a] | 73.8 ± 2.3[b] | 83.7 ± 2.1[a] | 88.1 ± 1.2[b] | 3.2 ± 0.1[a] | 8.7 ± 0.4[a] |
| Photo 3-1-3 | 124.5 ± 3.8[a] | 84.5 ± 2.6[a] | 106.6 ± 3.2[a] | 68.3 ± 2.2[a] | 79.5 ± 2.0[a] | 85.8 ± 1.1[b] | 3.5 ± 0.1[a] | 8.5 ± 0.4[a] |
| Photo 3-3-3 | 121.8 ± 4.0[a] | 84.5 ± 2.7[a] | 104.3 ± 3.3[a] | 69.5 ± 2.3[a] | 80.8 ± 2.1[a] | 85.7 ± 1.2[b] | 3.4 ± 0.1[a] | 8.8 ± 0.4[a] |
| Photo 4-1-4 | 124.2 ± 4.2[a] | 88.5 ± 2.9[b] | 105.3 ± 3.5[a] | 71.1 ± 2.4[a] | 83.9 ± 2.2[a] | 84.4 ± 1.3[a] | 3.2 ± 0.1[a] | 10.3 ± 0.4[a] |
| Photo 4-4-4 | 128.0 ± 4.2[a] | 84.6 ± 2.9[a] | 107.3 ± 3.5[a] | 66.3 ± 2.4[a] | 79.0 ± 2.2[a] | 83.6 ± 1.3[a] | 3.3 ± 0.1[a] | 10.3 ± 0.4[a] |
| Photo 5-1-5 | 115.8 ± 4.0[a] | 80.5 ± 2.7[a] | 101.4 ± 3.3[a] | 67.9 ± 2.3[a] | 78.1 ± 2.1[a] | 86.2 ± 1.2[b] | 3.0 ± 0.1[b] | 8.1 ± 0.4[a] |
| Photo 5-5-5 | 122.2 ± 4.2[a] | 84.8 ± 2.9[a] | 103.2 ± 3.5[a] | 69.6 ± 2.4[a] | 82.2 ± 2.2[a] | 84.5 ± 1.3[a] | 3.3 ± 0.1[a] | 9.9 ± 0.4[a] |
| Photo 10-5-10 | 117.8 ± 4.2[a] | 81.4 ± 2.9[a] | 99.9 ± 3.5[a] | 69.6 ± 2.4[a] | 81.5 ± 2.2[a] | 85.2 ± 1.3[a] | 3.1 ± 0.1[a] | 9.6 ± 0.4[a] |
| Photo 10-10-10 | 111.1 ± 4.2[a] | 77.3 ± 2.9[a] | 92.5 ± 3.5[a] | 70.7 ± 2.4[a] | 84.1 ± 2.2[a] | 83.8 ± 1.3[a] | 3.1 ± 0.1[a] | 9.7 ± 0.4[a] |
| Photo 15-5-15 | 113.1 ± 4.2[a] | 75.8 ± 2.9[a] | 92.2 ± 3.5[a] | 68.3 ± 2.4[a] | 83.5 ± 2.2[a] | 81.6 ± 1.3[a] | 3.2 ± 0.1[a] | 10.6 ± 0.4[a] |
| Photo 15-15-15 | 123.1 ± 6.0[a] | 76.6 ± 4.1[a] | 100.9 ± 5.0[a] | 63.6 ± 3.5[a] | 77.2 ± 3.2[a] | 82.4 ± 1.8[a] | 3.6 ± 0.1[a] | 8.8 ± 0.6[a] |

significantly higher in 4 min (39.2% ± 2.0%), 4-1-4 (43.3% ± 2.4%), 4-4-4 (38.6% ± 2.0%) and 10-10-10 patterns (37.2% ± 1.9%) than in the control (29.1% ± 1.6%).

On the other hand, red-light also increased some kinematic parameters (mean ± SEM; Table 2). In effect, VSL augmented ($P < 0.05$) when compared to the control (73.9 μm/s ± 3.1 μm/s), with the following light-stimulation patterns: 10 min (94.0 μm/s ± 3.6 μm/s), 2-2-2 (95.1 μm/s ± 3.9 μm/s) and 3-3-3 (100.9 μm/s ± 3.9 μm/s). Average path velocity (VAP) significantly increased after irradiation with the following patterns: 10 min (109.5 μm/s± 4.0 μm/s), 2-2-2 (110.0 μm/s ± 4.3 μm/s) and 3-3-3 (113.8 μm/s ± 4.3 μm/s *vs.* control: 88.2 μm/s ± 3.4 μm/s).

Furthermore, LIN increased ($P < 0.05$) with the following patterns: 2 min (75.2% ± 1.4%), 10 min (75.6% ± 1.4%), 1-1-1 (76.5% ± 1.4%), 2-2-2 (75.7% ± 1.6%) and 3-3-3 (78.7% ± 1.6% *vs.* control: 66.8% ± 1.2%). Finally, WOB values were significantly higher in the following patterns: 10 min (88.4% ± 1.4%), 1-1-1 (88.3% ± 1.4%) and 3-3-3 (88.3% ± 1.5%) than in the control (80.6% ± 1.2%).

### 3.2.2. Sperm membrane integrity

Percentages of membrane-intact spermatozoa ($SYBR14^+/PI^-$) following light-stimulation of cooled-stored spermatozoa did not differ between the control and light-stimulation patterns (Supplementary Fig. 1).

### 3.2.3. Mitochondrial membrane potential

Fig. 5a shows percentages of spermatozoa with high MMP in cooled-stored semen samples. Light-stimulation patterns of 4 min (55.7% ± 1.2%), 10 min (60.1% ± 1.1%), 1-1-1 (58.1% ± 1.1%), 2-2-2 (55.9% ± 1.4%), and 3-3-3 (59.6% ± 1.0%) showed higher ($P < 0.05$) percentages of spermatozoa with high MMP than the control (43.5% ± 1.0%). In contrast, three light-stimulation patterns: 4-1-4 (33.0% ± 0.7%), 4-4-4 (31.4% ± 0.8%), and 5-1-5 (31.9% ± 0.8%) exhibited lower ($P < 0.05$) percentages of spermatozoa with high MMP than the control.

With regard to the ratio between JC1 aggregates ($JC1_{agg}$; $FL2^+$) and JC1 monomers ($JC1_{mon}$; $FL1^+$) in the sperm population with high MMP (Fig. 5b), the following patterns: 4 min (0.95 ± 0.09), 10 min (1.13 ± 0.11), 1-1-1 (0.95 ± 0.04), 2-2-2 (0.93 ± 0.03), 4-1-4 (0.94 ± 0.04), 4-4-4 (0.95 ± 0.03), 5-1-5 (0.95 ± 0.04), 5-5-5 (0.94 ± 0.04), 10-5-10 (0.93 ± 0.03), 10-10-10 (0.92 ± 0.03) and 15-15-15 (0.93 ± 0.02), exhibited significantly higher $JC1_{agg}$: $JC1_{mon}$ ratios than the control (0.80 ± 0.03).

### 3.2.4. Intracellular ROS levels: $H_2O_2$ and $\cdot O_2^-$

Fig. 6a shows percentages of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$). Irradiation with 2 min, 3 min, 4 min, 5 min, 2-2-2, 5-1-5, 15-5-15 and 15-15-15 patterns decreased ($P < 0.05$) these percentages. In addition, and as shown in Fig. 6b, the GMFI of $DCF^+$ (FL1 channel) in the population of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$) was significantly higher in 2 min, 3 min, 4 min, 5 min, 2-1-2, 2-2-2 and 4-1-4 patterns than in the control.

Percentages of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$; Fig. 6c) were lower ($P < 0.05$) in 1 min, 2 min, 3 min, 10 min and 15-15-15 patterns than in the control. In contrast, no significant differences ($P > 0.05$) between the control and light-stimulation patterns were observed for the GMFI of $E^+$ (FL-3 channel) in the population of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$; Fig. 6d).

## 4. Discussion

Our results indicate that stimulation with red LED-based light at 620–630 nm wavelength affects the motility, mitochondrial membrane potential and intracellular levels of ROS ($\cdot O_2^-$) and ($H_2O_2$) of donkey spermatozoa, without affecting their plasma membrane integrity. However, the extent and the patterns required to trigger these effects differ between fresh diluted and cooled-stored semen.

The results obtained in this study clearly show that the effects of exposing donkey sperm to red-light also depend on the light/dark intervals of each light-stimulation pattern. These findings match with previous studies using laser- and LED-based light-systems and conducted in other mammalian species, such as dogs, buffalos and humans [17–19]. While red-light is known to be the most suitable to improve sperm motility and other functional parameters in animal and human sperm (see Refs. [6] for review), those light-effects have been suggested to rely on the precise rhythm and rates of

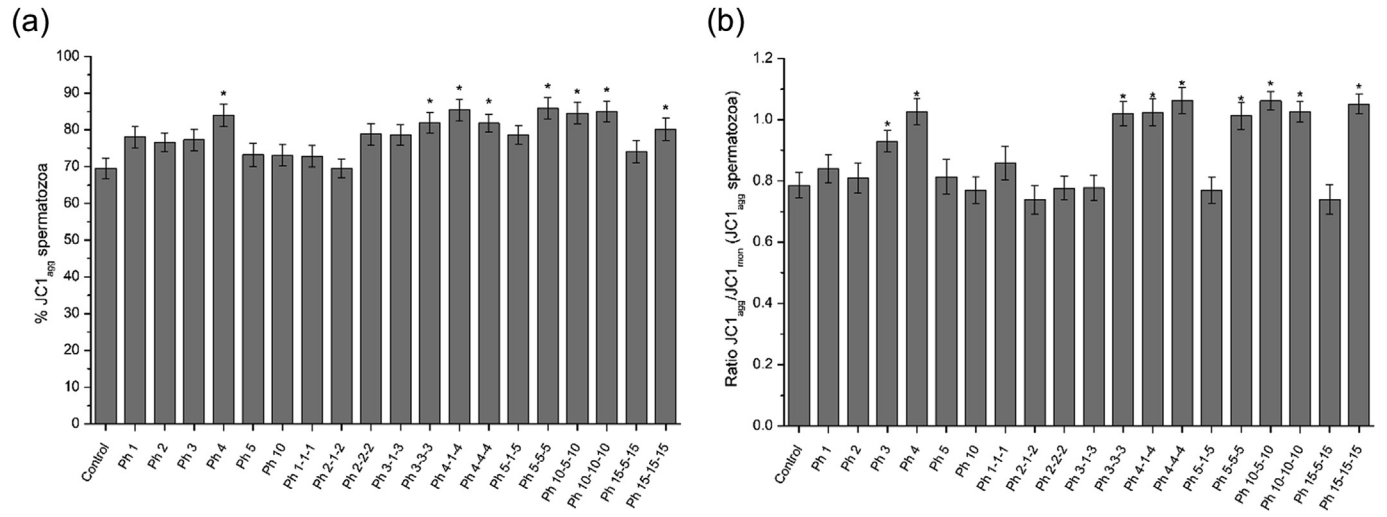


**Fig. 2.** (a) Percentages of spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$; a); and (b) ratios between JC1 aggregates ($JC1_{agg}$; FL2) and JC1 monomers ($JC1_{mon}$; FL1) for the sperm population with high mitochondrial membrane potential in the control and different irradiation patterns (fresh diluted semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

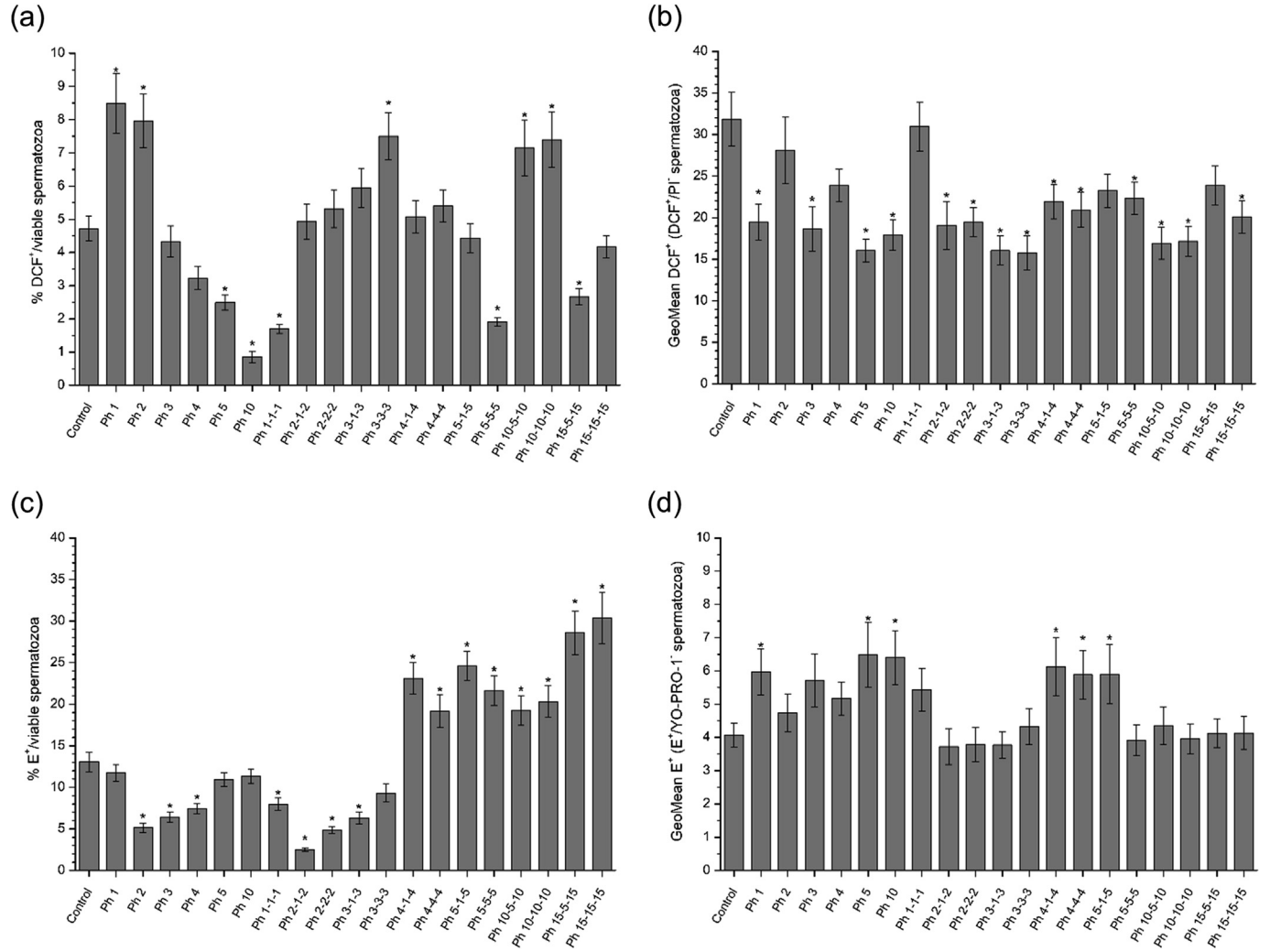


**Fig. 3.** (a) Percentages of viable spermatozoa with high intracellular peroxide ($H_2O_2$) levels ($DCF^+/PI^-$); (b) geometric mean of fluorescence intensity (GMFI; arbitrary units) of $DCF^+$ in the population of viable spermatozoa with high intracellular peroxide levels ($DCF^+/PI^-$); (c) percentages of viable spermatozoa with high intracellular superoxide ($\cdot O_2^-$) levels ($E^+$/YO-PRO-$1^-$); and (d) GFMI of $E^+$ in viable spermatozoa with high intracellular superoxide levels ($E^+$/YO-PRO-$1^-$) in the control and different irradiation patterns (fresh diluted semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

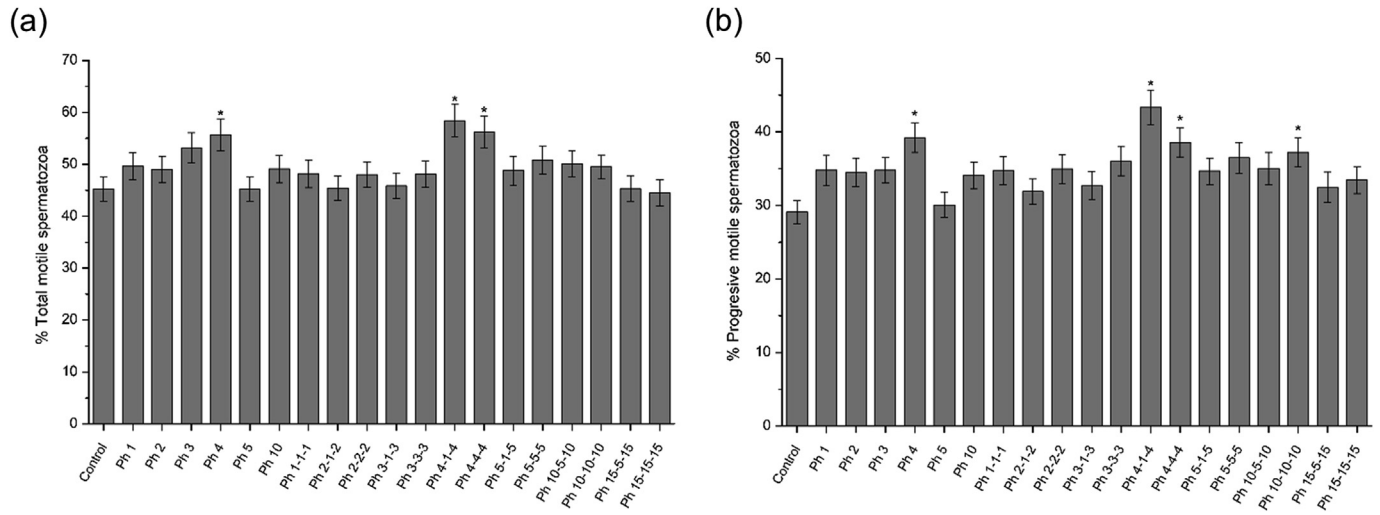


**Fig. 4.** Percentages of total (TMOT; a) and progressively motile spermatozoa (PMOT; b) in the control and different irradiation patterns (cooled-stored semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

application, regardless of the light source. In fact, different wavelengths have different effects, and despite the optimum wavelength varying between species, mounting evidence indicates that blue or green light are detrimental for mammalian spermatozoa [5,9,20].

One of the most interesting findings of our study is that the effects of light-stimulation highly rely upon the sample source (i.e. fresh diluted *vs.* cooled-stored semen). Therefore, we could identify which protocols were more suitable for either fresh diluted or cooled-stored donkey semen. These results agree with Gabel et al. [5], who observed that fresh and frozen-thawed human sperm differed on the most suitable light-stimulation regimens for each semen source. However, these authors did not really compare the same protocols in both semen sources, but rather shorter protocols were used for frozen-thawed human sperm.

With regard to the impact of light-stimulation upon sperm motility, we observed that single exposure of fresh diluted semen to red-light for 1 min and 4 min increased total and progressive sperm motility. In contrast, whereas the pattern of 1 min failed to affect total and progressive motilities of cooled-stored spermatozoa and that of 4 min augmented both motility parameters, other protocols that did not affect fresh diluted semen increased the motility of cooled-stored spermatozoa (4-1-4 and 4-4-4). As far as kinematic parameters are concerned, fresh diluted and cooled-stored semen again differed in the protocols that exerted a significant impact. In effect, fresh semen needed shorter combined patterns (1-1-1 and 2-2-2), which significantly increased VSL, VAP, LIN and WOB, whereas cooled-stored semen needed longer exposure (single exposure: 10 min; combined: 3-3-3). While it was surprising that the patterns increasing progressive and total motility differed from those increasing the average kinematic parameters (e.g. VSL, VAP), the stimulating effects of light, from LED and laser sources, on these kinematic parameters have previously been reported in humans [19,21–25], dogs [17,26] bulls [27], buffalos [18], rams [28], and pigs [3,29]. Besides, we observed that the 5-1-5 pattern significantly decreased the ALH of fresh semen. Similarly, Corral-Baqués et al. [17] also found that exposing dog spermatozoa to laser light at 655 nm decreased ALH. Therefore, not only does light-stimulation increase sperm velocity, but also the way through which sperm move.

At present, there is no clear explanation of how exposure of mammalian spermatozoa to red-light increases their motility. However, the main sperm compartment targeted by red-light has been suggested to be the mitochondrion (see Ref. [6] for review). Our mitochondrial membrane potential data agree with this possibility, as percentages of spermatozoa with high MMP were increased when fresh diluted semen was light-stimulated with 4 min, 3-3-3, 4-1-4, 4-4-4, 5-5-5, 10-5-10, 10-10-10 and 15-15-15 patterns. In the case of cooled-stored semen, less patterns were able to increase the percentages of spermatozoa with high MMP (4 min, 10 min, 1-1-1, 2-2-2, and 3-3-3), and some protocols even decreased those percentages (4-1-4, 4-4-4 and 5-1-5). The increase in the mitochondrial membrane potential observed in this study matches with previous works conducted in other species, in which mitochondrial membrane potential was also evaluated by JC1. In effect, Siqueira et al. [27] found that the percentage of sperm with intermediate mitochondrial membrane potential increased following irradiation with a He–Ne laser at 633 nm. Furthermore, Yeste et al. [3], using LED at 620–630 nm, reported an increase in the percentage of boar spermatozoa with high mitochondrial membrane potential.

In addition to the evaluation of the percentages of spermatozoa with high MMP, we determined the ratios between $JC1_{agg}$ (FL2; orange) and $JC1_{mon}$ (FL1; green) in the sperm cells belonging to the high MMP population. We observed that $JC1_{agg}$:$JC1_{mon}$ ratio in that population increased following nine light-stimulation patterns in fresh diluted semen (3 min, 4 min, 3-3-3, 4-1-4, 4-4-4, 5-5-5, 10-5-10, 10-10-10, and 15-15-15), and 11 patterns in cooled-stored semen (4 min, 10 min, 1-1-1, 2-2-2, 4-1-4, 4-4-4, 5-1-5, 5-5-5, 10-5-10, 10-10-10, 15-15-15). This suggests that not only does irradiation rise the percentages of spermatozoa with high MMP, but it also increases the intensity of that mitochondrial activity. These findings support the hypothesis that red-light exerts its effects via activation of mitochondrial photosensitizers, such as the cytochrome C/cytochrome C oxidase complex [30]. This photonic absorption leads to an increase in ATP production and in mitochondrial $Ca^{2+}$ uptake, and has been suggested to increase sperm motility and fertilizing ability [31].

We also analyzed intracellular levels of ROS ($H_2O_2$) and ($\cdot O_2^-$), since they are mainly synthesized in the mitochondria as a product of the electronic chain [32], and light-stimulation has been suggested to increase their production [9][33]. However, our results were very variable since, while some patterns increased the percentages of viable spermatozoa with high levels of $H_2O_2$ and $\cdot O_2^-$, others had no effect or decreased those percentages. In spite of this,

**Table 2**
Kinetic parameters in the control and different irradiation patterns (cooled-stored semen). Different superscript letters (*a-b*) indicate significant differences ($P \leq 0.05$) between red-light stimulation patterns and the control. Data are shown as mean ± SEM for eight experiments.

| Patterns | Kinetic parameters (mean ± SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) | LIN (%) | STR (%) | WOB (%) | ALH (μm) | BCF (Hz) |
| Control | 108.8 ± 3.5[a] | 73.9 ± 3.1[a] | 88.2 ± 3.4[a] | 66.8 ± 1.2[a] | 82.9 ± 1.0[a] | 80.6 ± 1.2[a] | 3.4 ± 0.1[a] | 9.7 ± 0.4[a] |
| Photo 1 | 104.6 ± 4.2[a] | 75.7 ± 3.7[a] | 88.9 ± 4.1[a] | 72.2 ± 1.5[a] | 84.9 ± 1.3[a] | 84.8 ± 1.5[a] | 3.1 ± 0.1[a] | 8.5 ± 0.5[a] |
| Photo 2 | 116.0 ± 4.0[a] | 87.9 ± 3.6[a] | 101.7 ± 4.0[a] | 75.2 ± 1.4[b] | 85.8 ± 1.2[a] | 86.7 ± 1.4[a] | 3.2 ± 0.1[a] | 8.6 ± 0.5[a] |
| Photo 3 | 108.9 ± 4.8[a] | 76.6 ± 4.2[a] | 94.0 ± 4.7[a] | 69.7 ± 1.7[a] | 81.0 ± 1.5[a] | 84.6 ± 1.7[a] | 3.3 ± 0.1[a] | 7.9 ± 0.6[a] |
| Photo 4 | 110.9 ± 4.8[a] | 79.6 ± 4.2[a] | 94.1 ± 4.7[a] | 71.4 ± 1.7[a] | 84.6 ± 1.5[a] | 84.1 ± 1.7[a] | 3.4 ± 0.1[a] | 8.8 ± 0.6[a] |
| Photo 5 | 112.7 ± 4.0[a] | 82.6 ± 3.6[a] | 95.5 ± 4.0[a] | 72.5 ± 1.4[a] | 82.6 ± 1.2[a] | 83.8 ± 1.4[a] | 3.3 ± 0.1[a] | 9.8 ± 0.5[a] |
| Photo 10 | 122.4 ± 4.0[a] | 94.0 ± 3.6[b] | 109.5 ± 4.0[b] | 75.6 ± 1.4[b] | 85.2 ± 1.2[a] | 88.4 ± 1.4[b] | 3.1 ± 0.1[a] | 8.7 ± 0.5[a] |
| Photo 1-1-1 | 118.4 ± 4.0[a] | 90.1 ± 3.6[a] | 104.8 ± 4.0[a] | 76.5 ± 1.4[b] | 86.2 ± 1.2[a] | 88.3 ± 1.4[b] | 3.1 ± 0.1[a] | 8.8 ± 0.5[a] |
| Photo 2-1-2 | 116.7 ± 4.4[a] | 88.3 ± 3.9[a] | 102.6 ± 4.3[a] | 73.6 ± 1.6[a] | 84.7 ± 1.3[a] | 86.6 ± 1.5[a] | 3.3 ± 0.1[a] | 8.6 ± 0.5[a] |
| Photo 2-2-2 | 124.2 ± 4.4[a] | 95.1 ± 3.9[b] | 110.0 ± 4.3[b] | 75.7 ± 1.6[b] | 86.9 ± 1.3[a] | 86.9 ± 1.5[a] | 3.0 ± 0.1[a] | 9.6 ± 0.5[a] |
| Photo 3-1-3 | 118.7 ± 4.4[a] | 88.0 ± 3.9[a] | 102.4 ± 4.3[a] | 72.8 ± 1.6[a] | 85.4 ± 1.3[a] | 85.0 ± 1.5[a] | 3.3 ± 0.1[a] | 8.8 ± 0.5[a] |
| Photo 3-3-3 | 128.1 ± 4.4[a] | 100.9 ± 3.9[b] | 113.8 ± 4.3[b] | 78.7 ± 1.6[b] | 88.5 ± 1.3[a] | 88.3 ± 1.5[b] | 3.2 ± 0.1[a] | 9.9 ± 0.5[a] |
| Photo 4-1-4 | 111.3 ± 4.8[a] | 81.4 ± 4.2[a] | 96.2 ± 4.7[a] | 72.3 ± 1.7[a] | 83.6 ± 1.5[a] | 85.8 ± 1.7[a] | 3.3 ± 0.1[a] | 8.6 ± 0.6[a] |
| Photo 4-4-4 | 118.7 ± 4.8[a] | 84.5 ± 4.2[a] | 102.5 ± 4.7[a] | 70.5 ± 1.7[a] | 82.3 ± 1.5[a] | 85.2 ± 1.7[a] | 3.5 ± 0.1[a] | 9.0 ± 0.6[a] |
| Photo 5-1-5 | 121.8 ± 4.8[a] | 90.5 ± 4.2[a] | 106.4 ± 4.7[a] | 75.0 ± 1.7[a] | 85.6 ± 1.5[a] | 86.9 ± 1.7[a] | 3.3 ± 0.1[a] | 8.8 ± 0.6[a] |
| Photo 5-5-5 | 109.0 ± 4.8[a] | 80.3 ± 4.2[a] | 94.7 ± 4.7[a] | 72.9 ± 1.7[a] | 83.4 ± 1.5[a] | 86.2 ± 1.7[a] | 3.3 ± 0.1[a] | 8.1 ± 0.6[a] |
| Photo 10-5-10 | 116.7 ± 4.8[a] | 84.4 ± 4.2[a] | 101.6 ± 4.7[a] | 72.7 ± 1.7[a] | 83.3 ± 1.5[a] | 86.7 ± 1.7[a] | 3.5 ± 0.1[a] | 8.3 ± 0.6[a] |
| Photo 10-10-10 | 114.0 ± 4.8[a] | 84.5 ± 4.2[a] | 98.8 ± 4.7[a] | 74.3 ± 1.7[a] | 85.6 ± 1.5[a] | 86.4 ± 1.7[a] | 3.5 ± 0.1[a] | 8.6 ± 0.6[a] |
| Photo 15-5-15 | 109.7 ± 4.8[a] | 77.6 ± 4.2[a] | 93.3 ± 4.7[a] | 69.8 ± 1.7[a] | 82.0 ± 1.5[a] | 84.4 ± 1.7[a] | 3.1 ± 0.1[a] | 8.4 ± 0.6[a] |
| Photo 15-15-15 | 108.2 ± 5.3[a] | 79.7 ± 4.7[a] | 93.2 ± 5.3[a] | 71.9 ± 1.9[a] | 84.6 ± 1.6[a] | 84.8 ± 1.9[a] | 3.1 ± 0.1[a] | 8.4 ± 0.7[a] |

it is worth noting that a bi-phasic response between exposure time and $O_2^-$ generation was observed in fresh diluted semen since, with regard to the control, shorter protocols were found to decrease the percentages of viable spermatozoa with high $\cdot O_2^-$ levels, and longer protocols were observed to increase those percentages. This increase in the percentage of viable spermatozoa with high $\cdot O_2^-$ levels matched with mitochondrial membrane potential, especially with regard to the ratio between JC1 aggregates ($JC1_{agg}$; $FL2^+$) and JC1 monomers ($JC1_{mon}$; $FL1^+$) in the sperm population with high MMP, which could be explained by the involvement of electronic chain in ROS production. In the case of cooled-stored semen, however, the control showed higher percentages of viable spermatozoa with high levels of $H_2O_2$ and $\cdot O_2^-$ than some irradiation protocols. Moreover, and in contrast to what observed in fresh diluted semen, the GMFI of $DCF^+$ in $DCF^+/PI^-$ population (i.e. high $H_2O_2$) was higher in some light-stimulation patterns than in the control. Therefore, while our data do not show a clear relationship of irradiation with generation of $H_2O_2$ and $\cdot O_2^-$, it is clear that this response differed between fresh diluted and cooled-stored semen. Nevertheless, those changes in ROS production had no effect on sperm viability.

The results obtained in this study and those observed in other species (see Ref. [6] for review) indicate that irradiation has several potential uses in assisted reproduction technology in the donkey. However, two crucial points must be taken into account: a) the source of the semen, as we have observed differences between fresh diluted and cooled-stored semen, and; b) the specificity of the species, since the most appropriate protocols found in this study differ from those that show the best results in pigs [3]. Additional research on sperm irradiation should address the impact on the fertilizing ability of donkey sperm of 1 min and 4 min (fresh diluted semen), and 4-1-4 and 4-4-4 patterns (cooled-stored semen). The most interesting protocol is 4 min, since the observed motility increase was concomitant with changes in the percentages of

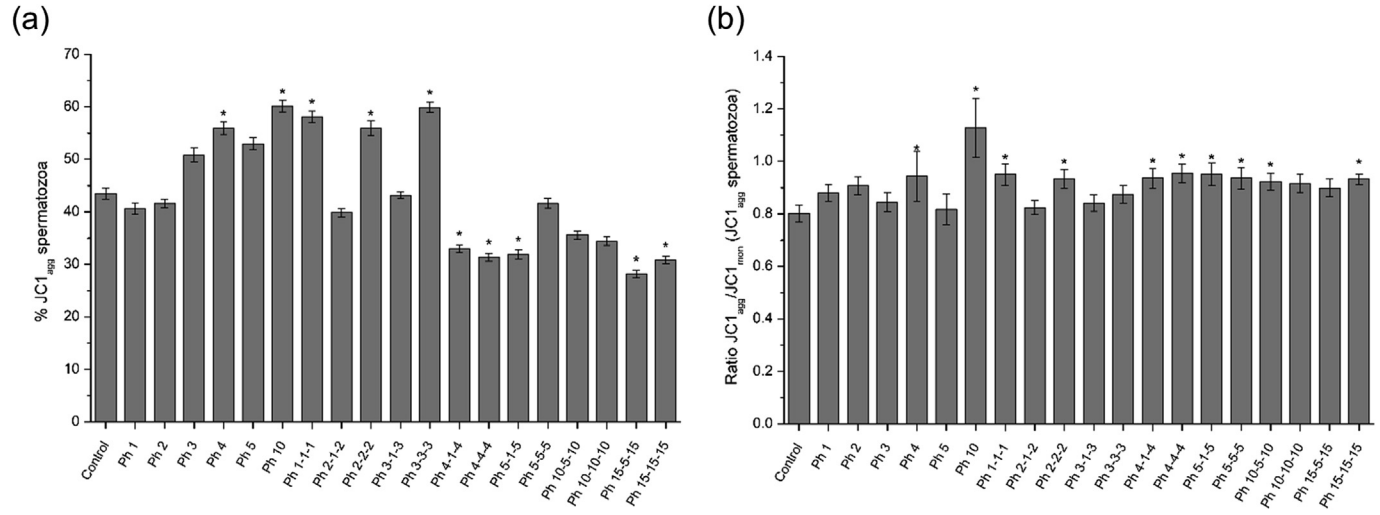


**Fig. 5.** (a) Percentages of spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$); and (b) ratios between JC1 aggregates ($JC1_{agg}$; FL2) and JC1 monomers ($JC1_{mon}$; FL1) for the sperm population with high mitochondrial membrane potential in the control and different irradiation patterns (cooled-stored semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

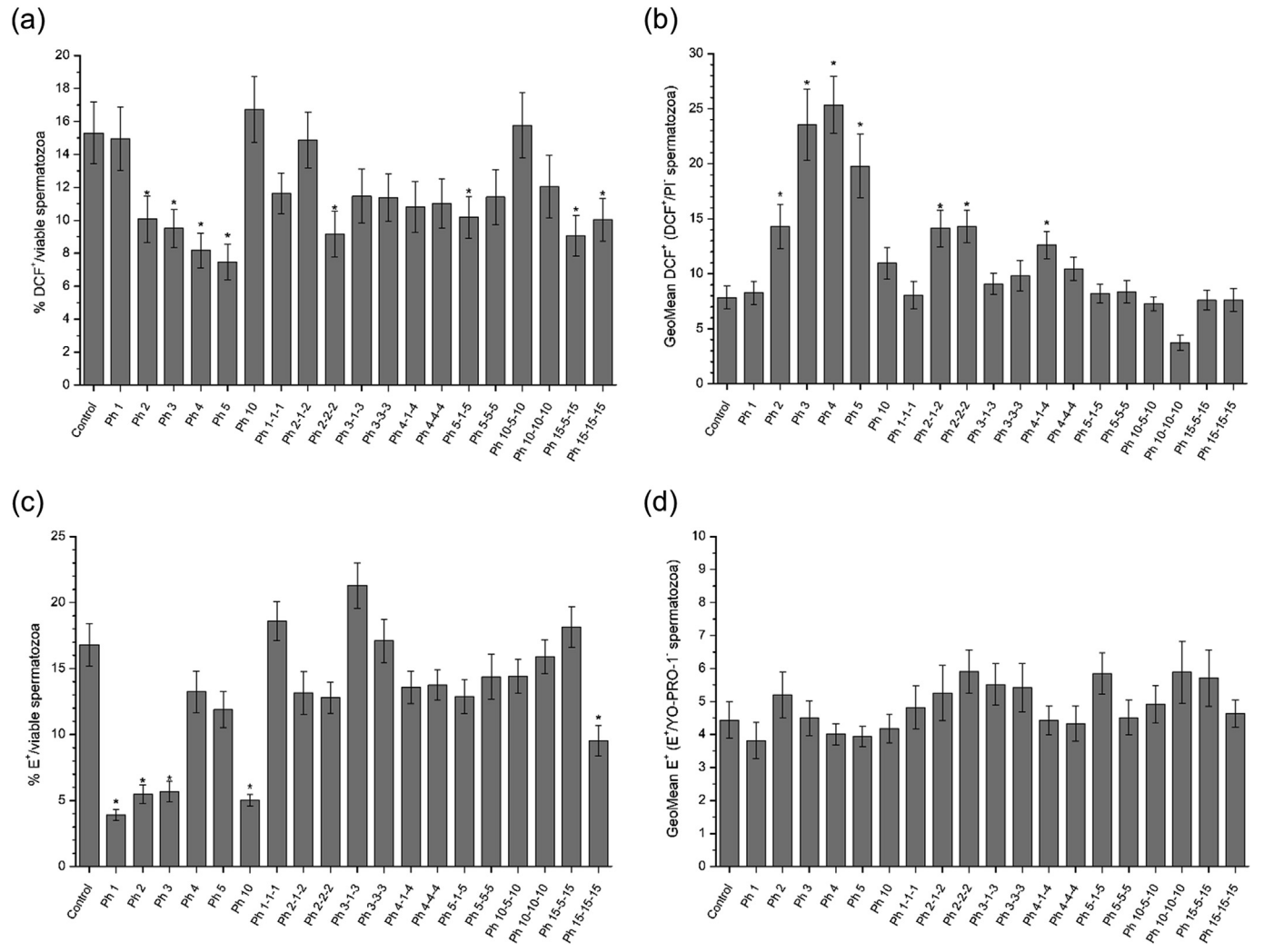


**Fig. 6.** (a) Percentages of viable spermatozoa with high intracellular peroxide ($H_2O_2$) levels ($DCF^+/PI^-$); (b) geometric mean of fluorescence intensity (GMFI; arbitrary units) of $DCF^+$ in the population of viable spermatozoa with high intracellular peroxide levels ($DCF^+/PI^-$); (c) percentages of viable spermatozoa with high intracellular superoxide ($\cdot O_2^-$) levels ($E^+$/YO-PRO-1$^-$); and (d) GFMI of $E^+$ in viable spermatozoa with high intracellular superoxide levels ($E^+$/YO-PRO-1$^-$) in the control and different irradiation patterns (cooled-stored semen). Asterisks (*) indicate significant differences ($P \leq 0.05$) between red-light stimulation protocols and the control. Data are shown as mean ± SEM for eight experiments.

spermatozoa with high MMP. Finally, previous works in other species have shown that exposure to red-light maintains better the sperm quality during liquid storage for 24 and 48 h [32], and also increases the longevity of frozen-thawed semen [28]. Therefore, it is reasonable to suggest that further studies should interrogate on whether light-stimulation could improve the sperm preservation technology, and extend the insemination interval in the donkey.

### 4.1. Conclusions

In conclusion, this work has demonstrated, for the first time, that red-light stimulation increases the motility and mitochondrial activity of fresh diluted and cooled-stored donkey spermatozoa, without affecting their viability. However, a clear relationship between the different irradiation patterns and ROS generation was not found. Morever, the effects of red-light stimulation differed between fresh diluted and cooled-stored semen, with only one pattern (4 min) being suitable for both semen sources. While the observed increase in sperm motility and mitochondrial activity could have a beneficial impact upon their fertility capacity and longevity, further research interrogating on whether such a positive effect exists is warranted. Furthermore, future experiments should also address which mechanism(s) of action underlie this sperm response to red-light.

## Declaration of competing interest

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

## CRediT authorship contribution statement

**Jaime Catalán:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Marion Papas:** Investigation, Methodology. **Sabrina Gacem:** Investigation. **Federico Noto:** Investigation. **Ariadna Delgado-Bermúdez:** Investigation. **Joan E. Rodríguez-Gil:** Conceptualization, Project administration, Supervision, Writing - review & editing. **Jordi Miró:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. **Marc Yeste:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.03.024.

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# 2. Red-light irradiation of horse spermatozoa increases mitochondrial activity and motility through changes in the motile sperm subpopulation structure

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# Red-Light Irradiation of Horse Spermatozoa Increases Mitochondrial Activity and Motility through Changes in the Motile Sperm Subpopulation Structure

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**Abstract:** Previous studies in other mammalian species have shown that stimulation of semen with red-light increases sperm motility, mitochondrial activity, and fertilizing capacity. This study sought to determine whether red-light stimulation using a light emitting diode (LED) at 620–630 nm affects sperm motility and structure of motile subpopulations, sperm viability, mitochondrial activity, intracellular ATP levels, rate of $O_2$ consumption and DNA integrity of horse spermatozoa. For this purpose, nine ejaculates were collected from nine different adult stallions. Upon collection, semen was diluted in Kenney extender, analyzed, its concentration was adjusted, and finally it was stimulated with red-light. In all cases, semen was packaged in 0.5-mL transparent straws, which were randomly divided into controls and 19 light-stimulation treatments; 6 consisted of a single exposure to red-light, and the other 13 involved irradiation with intervals of irradiation and darkness (light-dark-light). After irradiation, sperm motility was assessed using a Computerized Semen Analysis System (CASA). Flow cytometry was used to evaluate sperm viability, mitochondrial membrane potential and DNA fragmentation. Intracellular levels of ATP and $O_2$ consumption rate were also determined. Specific red-light patterns were found to modify kinetics parameters (patterns: 4, 2-2-2, 3-3-3, 4-4-4, 5-1-5, and 5-5-5 min), the structure of motile sperm subpopulations (patterns: 2, 2-2-2, 3-3-3, and 4-1-4 min), mitochondrial membrane potential (patterns: 4, 3-3-3, 4-4-4, 5-1-5, 5-5-5, 15-5-15, and 15-15-15 min), intracellular ATP levels and the rate of $O_2$ consumption (pattern: 4 min), without affecting sperm viability or DNA integrity. Since the increase in some kinematic parameters was concomitant with that of mitochondrial activity, intracellular ATP levels and $O_2$ consumption rate, we suggest that the positive effect of light-irradiation on sperm motility is related to its impact upon mitochondrial activity. In conclusion, this study shows that red LED light stimulates motility and mitochondrial activity of horse sperm. Additional research is needed to address the impact of red-light irradiation on fertilizing ability and the mechanisms through which light exerts its effects.

**Keywords:** photobiology; red-light; horse; semen; motility; mitochondrial activity

## 1. Introduction

Artificial insemination (AI) is an effective technique to improve the use of stallions in breeding programs [1], and it is used worldwide not only for the reproduction of horses, but also for that of farm animals, companion animals, and wild species [2]. Its advantages are so vast that it is considered as a biotechnology with great impact on equine reproduction; in effect, when used correctly, a stallion can leave hundreds of descendants throughout its reproductive life [3]. While pregnancy rates following AI with any semen source have increased enough to provide commercially acceptable margins of reliability in the horse [4], equine veterinarians often find owners seeking methods that can improve the reproductive performance of their stallions. In this context, different strategies have been developed to improve and handle sperm, thus maximizing their survival and fertilizing ability [5,6].

Studies conducted with pig semen have reported that reproductive performance can be increased through light-stimulation of semen prior to AI [7,8]. In effect, previous works using different light sources, such as lasers and light-emitting diodes (LED), have suggested that light-stimulation has a positive effect on the motility and ability of sperm to fertilize the oocyte in pigs [7–9], mice [10], and sheep [11]. Remarkably, no study has reported any detrimental effect of visible light on DNA integrity [12–14]. Light-stimulation of semen consists of irradiating sperm samples at a fixed (laser) or variable (LED) wavelength for a short period of time [15]. Data from previous studies indicate that the effects of light-stimulation depend on the state of the sample, the irradiation of the light beam used [13], the time or pattern of exposure [7], and the species [11].

While the mechanisms through which light exerts its effects on sperm are largely unknown, mounting evidence gives endogenous cellular photosensitizers, especially those present in mitochondria, a crucial role. Consequently, when these molecules are stimulated with light, both the production of ATP and the entry of $Ca^{2+}$ into the sperm cell raise [14]. This raise in energy supply underlies the subsequent increase of sperm motility [14,16–18]. In addition to this, opsins, which are present in sperm, and the potential influence of light on the conformation of other proteins like those belonging to the Transient Receptor Potential (TRP) family may also be involved in the sperm response to light [14,19].

The evidence accumulated so far in studies conducted with both low-level laser therapy devices and LED agree with these hypotheses, since they indicate that light irradiation accelerates mitochondrial respiration and ATP production in sperm [14,20,21]. Furthermore, it has become apparent that the sperm response to light-stimulation is biphasic, as low doses produce stimulating effects, moderate doses have no effect, and high doses exert cytotoxic effects [14].

As far as light devices are concerned, the positive results of low-level lasers and LED suggest that any method can be used safely [13]; however, practically speaking, not only are LED-based systems much cheaper and easier to maintain than lasers, but they also show high photonic efficiency [7]. Among all visible light spectra tested, red-light has been shown to be the one that improves sperm motility the most [15]. In addition to this, it has been reported that red-light stimulation (620–630 nm) by LED increases the fertilizing ability of pig sperm [7–9], without affecting their viability [7,9].

In spite of all the aforementioned, the effects of red-light stimulation on freshly ejaculated horse sperm are yet to be investigated. Therefore, the present work aimed at evaluating, for the first time, whether stimulation with red LED light (620–630 nm) affects the viability, motility, mitochondrial activity, intracellular levels of ATP, $O_2$ consumption rate, DNA integrity, and motile sperm subpopulations of fresh horse spermatozoa.

## 2. Materials and Methods

### 2.1. Suppliers

All reagents used were of analytical grade and were purchased from Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany), and Sigma-Aldrich (Saint Louis, MO, USA). As far as fluorochromes are concerned, and unless otherwise stated, all were purchased from Molecular

Probes (Thermo Fisher Scientific; Waltham, MA, USA) and were previously resuspended with dimethyl sulfoxide (Sigma-Aldrich). Plastic materials were provided by Nunc (Roskilde, Denmark).

### 2.2. Animals and Ejaculates

This study included nine ejaculates from nine different adult stallions, with proven fertility, housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All animals were semen donors and were collected under the health conditions set by the EU (free of equine arteritis, infectious anemia, and contagious metritis). Since this service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation to animals other than semen collection was carried out, the ethics committee of our institution indicated that no further ethical approval was required.

Ejaculates were collected through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) and an in-line nylon mesh filter was used to separate the gel fraction from the semen. Upon collection, gel-free semen was diluted 1:5 (v:v) with Kenney extender [22], previously prewarmed to 38 °C. Sperm concentration was determined in all samples through a hemocytometer (Neubauer Chamber, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

### 2.3. Experimental Design

Prior to light-stimulation, sperm concentration of samples was adjusted to $25 \times 10^6$ spermatozoa/mL. Following this, samples were packed into 0.5-mL transparent straws (Minitüb GmbH; Tiefenbach, Germany). After semen packaging, straws were placed in a programmable photo-activation system (Maxicow, IUL, S.A.; Barcelona, Spain). In this system, each straw is in contact with a triple LED configuration system that emits red-light (wavelength window: 620–630 nm). The system is equipped with a supporting software (IUL, S.A.) that allows the regulation of intensity and exposure time. In all cases, intensity was set at 100%.

A total of 19 irradiation protocols (red light) were evaluated. This aimed at determining which light exposure protocols had greater effects, which ones had no effect and which ones caused deleterious effects on sperm motility, viability, mitochondrial activity, intracellular levels of ATP, $O_2$ consumption rate, or DNA integrity. Six of these protocols consisted of single periods of light emission (1, 2, 3, 4, 5, and 10 min), whereas the other 13 treatments consisted of light-dark-light intervals (1-1-1, 2-1-2, 2-2-2, 3-1-3, 3-3-3, 4-1-4, 4-4-4, 5-1-5, 5-5-5, 10-5-10, 10-10-10, 15-5-15, and 15-15-15 min). Control samples were not irradiated. In order to determine the impact of each irradiation pattern, sperm viability, motility and motile subpopulations, mitochondrial activity, intracellular levels of ATP, $O_2$ consumption rate, and DNA fragmentation were assessed. Prior to evaluation, irradiated and control samples, previously transferred into 1.5-mL tubes, were incubated in a water bath at 38 °C for 5 min.

### 2.4. Analysis of Sperm Motility

Sperm motility was evaluated using a computer-assisted sperm-analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain) following incubation at 38 °C for 5 min. Five μL of each sperm sample was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel) previously warmed at 38 °C. Samples were analyzed with a 10 × negative phase-contrast objective and an Olympus BX41 microscope (Olympus), and at least 1000 sperm cells per analysis were counted. In each evaluation, percentages of total (%TMOT) and progressively motile spermatozoa (%PMOT) were recorded together with the following kinetic parameters: curvilinear velocity (VCL, μm/s), which is the mean path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, μm/s), which is the mean path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, μm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of linearity (LIN, %), which is the quotient between VSL and VCL multiplied by 100; percentage of straightness (STR, %), which is the quotient between VSL and VAP multiplied by 100; percentage of oscillation (WOB, %), which is the quotient between VAP and VCL multiplied by

100; mean amplitude of lateral head displacement (ALH, μm), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle; and frequency of head displacement (BCF, Hz), which is the frequency at which the actual sperm trajectory crosses the average path trajectory (Hz). In addition, individual kinetic parameters for each spermatozoon were also recorded and used to investigate the effects of light-stimulation upon sperm motile subpopulations.

Settings of the CASA system were those recommended by the manufacturer, i.e., frames/s: 25 images captured per second; particle area >4 and <75 $\mu m^2$; connectivity: 6; minimum number of images to calculate the ALH: 10. Cut-off values were VAP ≥ 10 μm/s for a sperm cell to be considered as motile, and STR ≥ 75% for being considered as progressively motile.

### *2.5. Flow Cytometry Analyses*

#### 2.5.1. General Information about Flow Cytometry Analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry [23]. Prior to staining, sperm concentration (except for SCSA test) was adjusted to $1 \times 10^6$ total spermatozoa per mL in a final volume of 0.5 mL with HEPES buffered saline solution (10 mM HEPES, 150 mM NaCl, 10% BSA; pH = 7.4). In addition, a correction procedure that consisted of differentiating into DNA-containing and non-DNA-containing particles was made for JC1 test, since the presence of alien particles could overestimate the percentages of intact spermatozoa [24,25].

All flow cytometry assessments were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter; Fullerton, CA, USA), and samples were excited with an argon ion laser (488 nm) set at a power of 22 mW. For each event, the cytometer provided the electronic volume (EV, equivalent to forward scatter, FS, in other equipment) and the side scatter (SS). Calibration of this device was made periodically through 10-μm Flow-Check fluorospheres (beads; Beckman Coulter); the bead size was positioned at channel 200 on the volume scale.

A total of three optical filters (FL1, FL2 and FL3), with the following particular characteristics, were used: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm; and FL3 (red fluorescence): LP filter: 670/730 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL1 was used to detect green fluorescence (SYBR14, JC1 monomers and SCSA), FL2 was used to detect orange fluorescence (JC1 aggregates, $JC1_{agg}$), and FL3 was used to detect red fluorescence (PI and SCSA). When required, and as stated below, compensation was used to minimize spill over between channels.

Sheath flow rate was set at 4.17 μL/min in all analyses, and EV and SS were recorded in a log-linear mode (in EV vs. SS dot plots) for a minimum of 10,000 events per assessment. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris and cell aggregates, and the sperm-specific events were positively gated on the basis of EV/SS distributions. Information on the events was collected in list-mode data files (*.LMD), and files were subsequently analyzed through the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). Three replicates using independent tubes were evaluated, and the corresponding mean ± standard error of the mean (SEM) was subsequently calculated.

#### 2.5.2. Plasma Membrane Integrity

Sperm membrane integrity was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR14/PI; Molecular Probes, ThermoFisher Scientific, Waltham, Massachusetts, MA, USA), according to the protocol described by Garner and Johnson [26] adapted to horse spermatozoa. Briefly, sperm samples were incubated at 37.5 °C for 10 min with SYBR14 at a final concentration of 100 nM, and then with PI at a final concentration of 12 μM for 5 min at the same temperature. All incubations were performed in the dark. FL1 was used to measure the green fluorescence from SYBR14, and FL3 was used to detect the red fluorescence from PI. Three sperm populations were identified: (i) spermatozoa with an intact plasma

membrane, stained in green ($SYBR14^+/PI^-$); (ii) spermatozoa with a damaged plasma membrane, stained in red ($SYBR14^-/PI^+$); and (iii) spermatozoa with a damaged plasma membrane, stained in orange ($SYBR14^+/PI^+$). Non-sperm particles (debris) were found in the $SYBR14^-/PI^-$ quadrant, and were used to correct JC1-data. Single-stained samples were used for setting the EV-gain, FL1 and FL3 photomultiplier (PMT)-voltages, and for compensation of SYBR14 spill over into the PI channel (2.45%).

#### 2.5.3. Evaluation of Mitochondrial Membrane Potential (ΔΨm, JC1)

Mitochondrial membrane potential of horse spermatozoa was determined after modification of the protocol described in [27]. Sperm samples were incubated with JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide) at a final concentration of 0.5 µM at 37.5 °C for 30 min in the dark. Green fluorescence from JC1-monomers was collected through FL1, and orange fluorescence from JC1 aggregates ($JC1_{agg}$) was collected through FL2. Two populations were distinguished: (i) spermatozoa with low MMP, in which all mitochondria were stained in green ($FL1^+/FL2^-$); and (ii) spermatozoa with high MMP ($JC1_{agg}$), which contained spermatozoa with heterogeneous mitochondria stained both in green and orange in the same cell ($FL1^+/FL2^+$) and spermatozoa that had all their mitochondria stained in orange ($FL1^-/FL2^+$). Spillover of FL1 into FL2-channel was compensated (68.5%). Following the protocol of Petrunkina et al. [24], the percentages of non-sperm, debris particles found in the SYBR14/PI test ($SYBR14^-/PI^-$) were used to correct the percentages of non-stained events in the sperm population with low MMP; the percentages of the sperm population with high MMP were recalculated.

#### 2.5.4. DNA Integrity (SCSA Test)

DNA fragmentation of horse spermatozoa in control and irradiated samples was evaluated through SCSA test [28,29], as modified by Morrell et al. [30]. Briefly, sperm samples were diluted in a buffer solution (TNE; 0.15 M NaCL, 0.01 M Tris-HCl, 1 mM EDTA, pH = 7.4) to a final concentration of $2 \times 10^6$ spermatozoa/mL. Next, 200 µL of this solution containing $2 \times 10^6$ spermatozoa/mL were mixed with 400 µL of an acid-detergent solution (80 mM HCl, 150 mM NaCl, and 0.1% Triton X-100; pH = 1.2) in ice. After 30 s, 1.2 mL of an acridine orange (AO) solution (6 µg/mL in 37 mM citric acid, 126 mM $Na_2HPO_4$, 1.1 mM EDTA, 150 mM NaCl, pH = 6.0) was added, and samples were kept in ice for further 3 min. Immediately after this time, samples were evaluated and green and red fluorescence were collected through FL1 and FL3 filters, respectively. Percentages of DNA fragmentation (DNA fragmentation index, %DFI), which was the ratio between red (ssDNA) fluorescence and red (ssDNA) + green (dsDNA) fluorescence, mean fluorescence intensity of single stranded DNA (ssDNA, mean DFI) and percentages of high DNA stainability (HDS) were determined.

### *2.6. Determination of Intracellular ATP Levels*

Intracellular ATP levels were determined following the protocol set by Chida et al. [31]. Briefly, after irradiated, 1-mL semen aliquots were centrifuged at 17 °C for 30 s and pellets were immediately plunged into liquid $N_2$. Frozen pellets were subsequently stored at −80 °C 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), while being kept on ice to avoid specimen heating. Samples were subsequently centrifuged at 1000× g and 4 °C for 10 min and supernatants were collected. 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 4 °C for 20 s. Samples were subsequently centrifuged at 1000× *g* and 4 °C for 30 s, and supernatants were carefully separated from the pellet and again centrifuged at 1000× *g* and 4 °C 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, MA, 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 [32] using a commercial kit (Bio-Rad laboratories; Hercules, CA, USA).

### *2.7. Determination of $O_2$ Consumption Rate*

For the determination of the $O_2$ consumption rate, the unirradiated control sample and five light stimulation patterns of short and long exposure to light were used, which had obtained significant differences in some kinetic parameters of sperm motility and mitochondrial activity, these parameters were; 2, 4, 3-3-3, 5-5-5, and 15-5-15 min. 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 Oxodish® OD24 plates (24 wells/plate) specifically designed for this device. Plates were sealed with Parafilm®, introduced in the SDR system, and incubated at 37 °C (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 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. In addition, $O_2$ consumption rates in irradiated samples were standardized against the control ($O_2$ consumption rate $_{\text{irradiation pattern}}/O_2$ consumption rate $_{\text{control}} \times 100$).

### *2.8. Statistical Analyses*

Statistical analyses were conducted using a statistical package (IBM® SPSS® 25.0 for Windows; IBM corp., Armonk, NY, USA). Data were first tested for normality and homogeneity of variances with Shapiro–Wilk and Levene tests, respectively. When required, data were transformed through arcsin $\sqrt{x}$. The effects of red-light stimulation patterns on total and progressive motility, kinematic parameters, percentages of viable spermatozoa, percentage of spermatozoa with high MMP, intracellular ATP levels, $O_2$ consumption rates, and DNA fragmentation were evaluated through one-way analysis of variance (ANOVA) followed by post-hoc Sidak's test.

Sperm motile subpopulations were set according to the procedure described in [33]. Briefly, individual kinematic parameters obtained for each spermatozoon (VSL, VCL, VAP, LIN, STR, WOB, ALH, and BCF) were used as independent variables in a principal component analysis (PCA). These kinematic parameters were sorted into PCA components and the obtained matrix was subsequently rotated using the Varimax procedure with the Kaiser normalization. For each spermatozoon, regression scores of the resulting PCA components were worked out.

Based on the regression scores of each individual spermatozoon, a two-step cluster analysis based on the log-likelihood distance and the Schwarz's Bayesian Criterion was run. Following identification of four motile sperm subpopulations, percentages of spermatozoa belonging to each subpopulation (SP1, SP2, SP3, or SP4) were calculated in each treatment and replicate. These percentages were subsequently used to evaluate the effects of red-light stimulation on sperm subpopulations through one-way ANOVA and Sidak's post-hoc test.

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

## 3. Results

### *3.1. Effects of Red-Light Irradiation on Sperm Viability*

Figure 1 shows representative histograms and dot-plots for the control (Figure 1a,b) and one light-stimulation pattern (15-15-15; Figure 1c,d). Figure 2a shows, as mean ± SEM, the percentages of viable spermatozoa following light-stimulation. No significant differences between control and irradiation patterns were observed ($p > 0.05$).

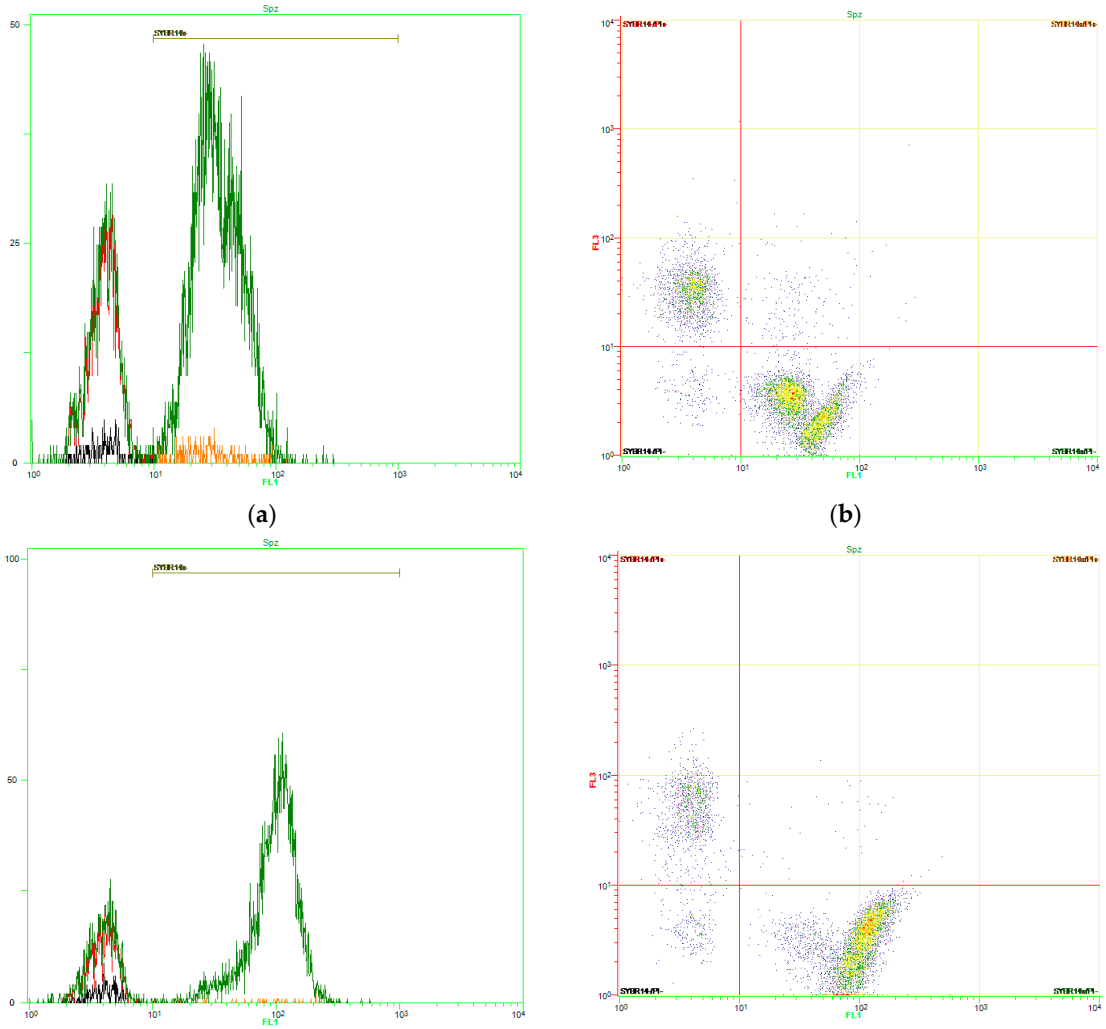



**Figure 1.** Sperm viability (SYBR14/PI). Representative SYBR14 (FL1) histograms for the control (**a**) and 15-15-15 light-stimulation pattern (**c**). Representative FL1/FL3 dot-plots for the control (**b**) and 15-15-15 light-stimulation pattern (**d**). Viable spermatozoa (SYBR14$^+$/PI$^-$) appear in the lower right quadrant. No significant differences between the control and treatments were observed ($p > 0.05$).

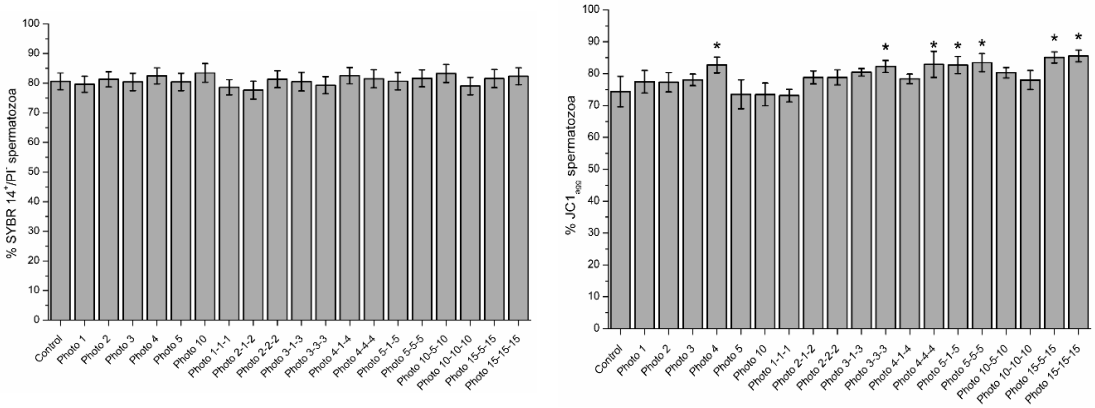



**Figure 2.** (**a**) Percentages of spermatozoa with an intact plasma membrane (SYBR14$^+$/PI$^-$; viable spermatozoa) in the control and the different light-stimulation patterns. No significant differences

between the control and light-stimulation patterns were observed ($p > 0.05$). (**b**) Percentages of spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$) in the control and different irradiation patterns. The superscript (*) means significant differences ($p \leq 0.05$) between the control and the different light-stimulation patterns. Data are shown as mean ± SEM of nine independent experiments.

### 3.2. *Effects of Red-Light Irradiation on Mitochondrial Membrane Potential (ΔΨm)*

As shown in Figure 2b, percentages of spermatozoa with high $JC1_{agg}$ were significantly ($p < 0.05$) higher, compared with the control samples (74.4% ± 4.8%), in the following light-stimulation patterns: 4 (82.7% ± 2.5%), 3-3-3 (82.3% ± 1.9%), 4-4-4 (82.9% ± 4.1%), 5-1-5 (82.7% ± 2.7%), 5-5-5 (83.4% ± 2.8%), 15-5-15 (85.1% ± 1.7%), and 15-15-15 (85.5% ± 1.8%). Figure 3 shows representative histograms and dot-plots for the control (Figure 3a,b) and 15-15-15 pattern (Figure 3c,d).

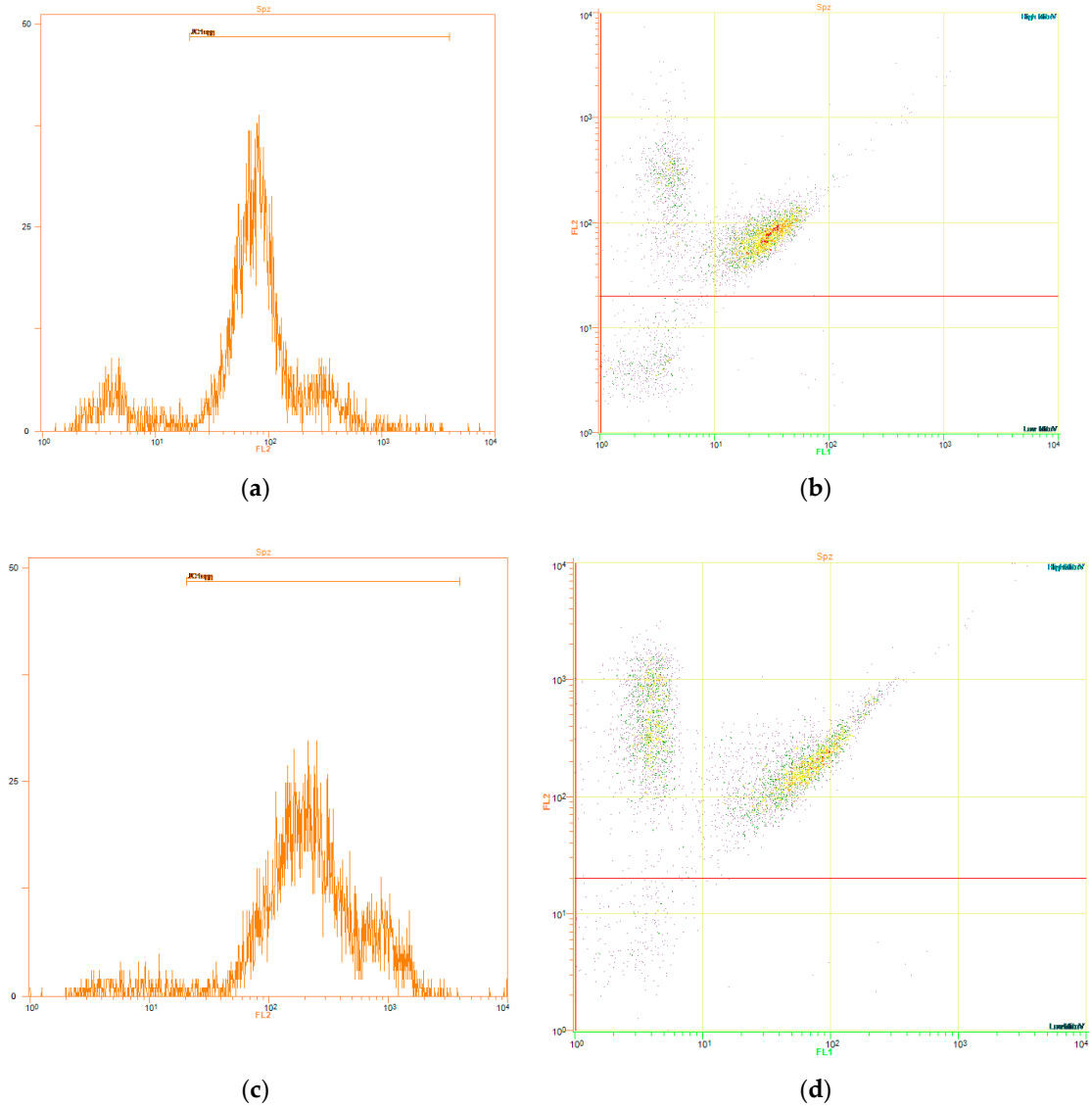
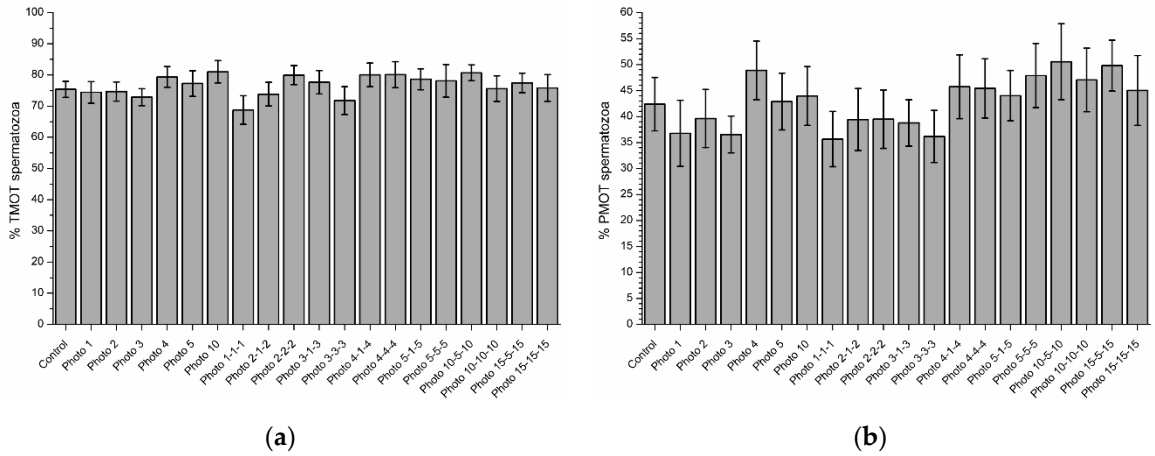



**Figure 3.** Mitochondrial membrane potential (ΔΨm, JC1). Representative $JC1_{agg}$ (FL2 channel) histograms for the control (**a**) and 15-15-15 light-stimulation pattern (**c**). Representative FL1/FL2 dot-plots for the control (**b**) and 15-15-15 light-stimulation pattern (**d**). Spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$) appear in the upper half of the Figure. Percentages of spermatozoa with high mitochondrial membrane potential ($JC1_{agg}$) were significantly ($p < 0.05$) higher in the 15-15-15 light-stimulation pattern than in the control.

### 3.3. Effects of Red-Light Irradiation on Sperm Motility

Total and progressive sperm motility did not differ between control and irradiation patterns ($p > 0.05$; Figure 4). However, red-light stimulation affected several sperm kinematic parameters when compared to the control in a fashion that depended on the specific light regime applied to cells. In effect, VSL increased after the application of irradiation patterns 3-3-3 (65.7 µm/s ± 4.8 µm/s) and 5-5-5 (66.7 µm/s ± 2.0 µm/s) when compared to control samples (56.9 µm/s ± 2.5 µm/s; see Table 1). Furthermore, VAP significantly ($p < 0.05$) increased with patterns 4 (94.4 µm/s ± 4.6 µm/s), 2-2-2 (96.0 µm/s ± 4.7 µm/s), 3-3-3 (96.4 µm/s ± 5.6 µm/s), and 4-4-4 (94.8 µm/s ± 3.9 µm/s) with regard to the control (81.9 µm/s ± 4.7 µm/s; see Table 1). Finally, BCF significantly ($p < 0.05$) decreased after the application of patterns 3-3-3 min (8.4 Hz ± 0.6 Hz) and 5-1-5 (8.4 Hz ± 0.6 Hz) when compared to the control (9.7 Hz ± 0.6 Hz; Table 1).




(**a**) (**b**)

**Figure 4.** Percentages of total (**a**; TMOT) and progressively motile spermatozoa (**b**; PMOT) in the control and different irradiation patterns. No significant differences between the control and light-stimulation patterns were observed. Data are shown as mean ± SEM of nine independent experiments.

**Table 1.** Kinetic parameters (mean ± SEM) of horse spermatozoa in the control and different irradiation patterns.

| Patterns | VCL (µm/s) | VSL (µm/s) | VAP (µm/s) | LIN (%) | STR (%) | WOB (%) | ALH (µm) | BCF (Hz) |
|---|---|---|---|---|---|---|---|---|
| Control | 107.2 ± 5.9 $^{a}$ | 56.9 ± 2.5 $^{a}$ | 81.9 ± 4.7 $^{a}$ | 55.6 ± 4.7 $^{a}$ | 71.0 ± 4.6 $^{a}$ | 77.6 ± 2.1 $^{a}$ | 2.7 ± 0.1 $^{a}$ | 9.7 ± 0.6 $^{a}$ |
| Ph 1 | 116.0 ± 8.3 $^{a}$ | 58.9 ± 3.9 $^{a}$ | 88.5 ± 4.7 $^{a}$ | 52.2 ± 6.2 $^{a}$ | 66.3 ± 5.7 $^{a}$ | 77.2 ± 2.8 $^{a}$ | 2.9 ± 0.2 $^{a}$ | 9.3 ± 0.4 $^{a}$ |
| Ph 2 | 117.2 ± 4.8 $^{a}$ | 62.3 ± 5.3 $^{a}$ | 92.0 ± 4.9 $^{a}$ | 53.6 ± 4.8 $^{a}$ | 68.0 ± 4.2 $^{a}$ | 78.1 ± 2.6 $^{a}$ | 2.9 ± 0.1 $^{a}$ | 9.1 ± 0.3 $^{a}$ |
| Ph 3 | 113.2 ± 7.0 $^{a}$ | 60.7 ± 4.0 $^{a}$ | 90.4 ± 6.3 $^{a}$ | 54.5 ± 4.2 $^{a}$ | 68.2 ± 4.5 $^{a}$ | 79.6 ± 2.1 $^{a}$ | 2.9 ± 0.2 $^{a}$ | 9.1 ± 0.2 $^{a}$ |
| Ph 4 | 117.7 ± 5.2 $^{a}$ | 62.5 ± 4.5 $^{a}$ | 94.4 ± 4.6 $^{b}$ | 53.2 ± 4.6 $^{a}$ | 66.2 ± 4.8 $^{a}$ | 80.2 ± 2.2 $^{a}$ | 3.0 ± 0.3 $^{a}$ | 9.2 ± 0.8 $^{a}$ |
| Ph 5 | 111.3 ± 6.6 $^{a}$ | 58.9 ± 2.6 $^{a}$ | 87.7 ± 4.3 $^{a}$ | 54.8 ± 5.0 $^{a}$ | 68.6 ± 5.0 $^{a}$ | 79.2 ± 2.0 $^{a}$ | 2.6 ± 0.1 $^{a}$ | 9.4 ± 0.5 $^{a}$ |
| Ph 10 | 115.4 ± 6.9 $^{a}$ | 62.6 ± 3.3 $^{a}$ | 91.8 ± 6.3 $^{a}$ | 55.4 ± 4.2 $^{a}$ | 69.4 ± 4.3 $^{a}$ | 79.5 ± 2.0 $^{a}$ | 2.9 ± 0.1 $^{a}$ | 9.3 ± 0.2 $^{a}$ |
| Ph 1-1-1 | 112.4 ± 8.5 $^{a}$ | 60.7 ± 2.5 $^{a}$ | 84.6 ± 5.7 $^{a}$ | 55.0 ± 5.6 $^{a}$ | 70.6 ± 5.3 $^{a}$ | 77.0 ± 2.3 $^{a}$ | 3.2 ± 0.3 $^{a}$ | 9.1 ± 0.7 $^{a}$ |
| Ph 2-1-2 | 116.3 ± 7.4 $^{a}$ | 62.9 ± 3.3 $^{a}$ | 90.6 ± 4.5 $^{a}$ | 56.1 ± 5.5 $^{a}$ | 70.6 ± 5.0 $^{a}$ | 78.6 ± 2.4 $^{a}$ | 3.2 ± 0.3 $^{a}$ | 8.8 ± 0.6 $^{a}$ |
| Ph 2-2-2 | 121.6 ± 7.3 $^{a}$ | 62.4 ± 3.4 $^{a}$ | 96.0 ± 4.7 $^{b}$ | 53.5 ± 5.0 $^{a}$ | 65.9 ± 4.6 $^{a}$ | 79.6 ± 2.5 $^{a}$ | 2.9 ± 0.3 $^{a}$ | 8.6 ± 0.8 $^{a}$ |
| Ph 3-1-3 | 115.6 ± 6.8 $^{a}$ | 61.1 ± 3.5 $^{a}$ | 91.4 ± 6.2 $^{a}$ | 53.7 ± 3.5 $^{a}$ | 68.2 ± 4.1 $^{a}$ | 78.6 ± 1.8 $^{a}$ | 3.3 ± 0.3 $^{a}$ | 8.6 ± 0.6 $^{a}$ |
| Ph 3-3-3 | 119.5 ± 6.9 $^{a}$ | 65.7 ± 4.8 $^{b}$ | 96.4 ± 5.6 $^{b}$ | 55.6 ± 4.8 $^{a}$ | 68.0 ± 4.7 $^{a}$ | 79.7 ± 2.5 $^{a}$ | 3.1 ± 0.3 $^{a}$ | 8.4 ± 0.6 $^{b}$ |
| Ph 4-1-4 | 112.3 ± 7.7 $^{a}$ | 63.9 ± 2.8 $^{a}$ | 93.3 ± 5.7 $^{a}$ | 59.6 ± 4.5 $^{a}$ | 72.6 ± 5.0 $^{a}$ | 81.9 ± 1.4 $^{a}$ | 2.8 ± 0.1 $^{a}$ | 9.6 ± 0.3 $^{a}$ |
| Ph 4-4-4 | 112.0 ± 7.6 $^{a}$ | 64.4 ± 3.2 $^{a}$ | 94.8 ± 3.9 $^{b}$ | 59.0 ± 4.3 $^{a}$ | 71.6 ± 4.7 $^{a}$ | 82.2 ± 1.7 $^{a}$ | 2.6 ± 0.1 $^{a}$ | 9.0 ± 0.5 $^{a}$ |
| Ph 5-1-5 | 113.0 ± 6.8 $^{a}$ | 64.0 ± 2.0 $^{a}$ | 91.0 ± 4.1 $^{a}$ | 58.1 ± 4.0 $^{a}$ | 71.2 ± 3.8 $^{a}$ | 81.1 ± 1.9 $^{a}$ | 3.1 ± 0.3 $^{a}$ | 8.4 ± 0.6 $^{b}$ |
| Ph 5-5-5 | 112.4 ± 7.4 $^{a}$ | 66.7 ± 2.0 $^{b}$ | 92.7 ± 4.3 $^{a}$ | 61.3 ± 4.4 $^{a}$ | 74.4 ± 4.4 $^{a}$ | 82.4 ± 1.5 $^{a}$ | 2.9 ± 0.1 $^{a}$ | 8.9 ± 0.3 $^{a}$ |
| Ph 10-5-10 | 105.8 ± 8.3 $^{a}$ | 61.3 ± 2.5 $^{a}$ | 86.0 ± 5.4 $^{a}$ | 60.8 ± 5.4 $^{a}$ | 73.7 ± 5.8 $^{a}$ | 81.9 ± 1.7 $^{a}$ | 2.5 ± 0.1 $^{a}$ | 9.1 ± 0.3 $^{a}$ |
| Ph 10-10-10 | 109.4 ± 12.3 $^{a}$ | 58.1 ± 5.1 $^{a}$ | 84.0 ± 8.8 $^{a}$ | 57.3 ± 6.5 $^{a}$ | 73.0 ± 6.9 $^{a}$ | 77.4 ± 3.4 $^{a}$ | 2.8 ± 0.3 $^{a}$ | 9.7 ± 0.6 $^{a}$ |
| Ph 15-5-15 | 109.2 ± 8.9 $^{a}$ | 60.5 ± 3.4 $^{a}$ | 83.6 ± 4.9 $^{a}$ | 58.6 ± 6.2 $^{a}$ | 74.3 ± 6.1 $^{a}$ | 77.4 ± 2.8 $^{a}$ | 3.0 ± 0.2 $^{a}$ | 9.8 ± 0.4 $^{a}$ |
| Ph 15-15-15 | 112.2 ± 9.4 $^{a}$ | 59.6 ± 6.1 $^{a}$ | 85.7 ± 5.1 $^{a}$ | 56.7 ± 7.4 $^{a}$ | 70.9 ± 7.5 $^{a}$ | 77.7 ± 3.7 $^{a}$ | 2.8 ± 0.2 $^{a}$ | 9.7 ± 0.3 $^{a}$ |

Different letters (*a*, *b*) indicate significant differences ($p \leq 0.05$) between the control and irradiation patterns.

### 3.4. Sperm Subpopulations

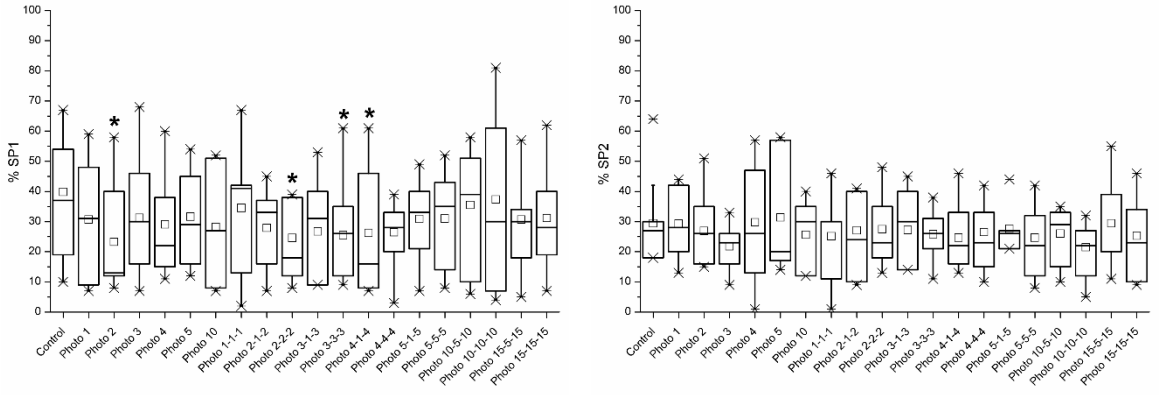
Four different sperm subpopulations were identified following cluster analyses based on the analyzed individual kinematic parameters. Table 2 shows the kinematic parameters for these sperm subpopulations, which were identified as SP1, SP2, SP3, and SP4. SP1 was the slowest sperm subpopulation, since it showed low VCL, VSL, VAP, and ALH. SP2 presented intermediate values (higher than SP1 but lower than SP3) in most sperm kinetic parameters (VCL, VSL, VAP, LIN, STR, and WOB) and exhibited higher ALH than SP1 and SP3. In addition, SP2 also showed the highest BCF. SP3 was the most linear subpopulation, as displayed the highest values in most kinetic parameters (i.e., VSL, VAP, LIN, STR, and WOB). Finally, SP4 was the subpopulation that, despite showing the highest values of VCL and ALH, its VSL was similar to that of SP1, which was the slowest subpopulation (SP1), and its LIN, STR, and WOB was lower than in the other three subpopulations (SP1, SP2, and SP3).

**Table 2.** Descriptive parameters (mean ± SEM; range) of the four sperm subpopulations (SP1, SP2, SP3, and SP4) identified in stallion fresh semen.

| | SP1 | | SP2 | | SP3 | | SP4 | |
|---|---|---|---|---|---|---|---|---|
| **N** | **11,893** | | **10,098** | | **11,210** | | **8142** | |
| **Parameter** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** |
| VCL (μm/s) | 72.7 ± 0.1 | 0.0–120.0 | 109.1 ± 0.1 | 71.6–181.9 | 120.1 ± 0.2 | 87.2–213.9 | 158.3 ± 0.3 | 83.1–372.2 |
| VSL (μm/s) | 45.9 ± 0.1 | 0.0–84.6 | 64.7 ± 0.2 | 0.9-105.6 | 94.5 ± 0.2 | 43.4–199.0 | 47.2 ± 0.3 | 0.6–221.2 |
| VAP (μm/s) | 59.9 ± 0.1 | 0.0–112.9 | 85.1 ± 0.1 | 34.4–129.3 | 112.7 ± 0.2 | 83.9–224.8 | 109.5 ± 0.2 | 24.1-282.5 |
| LIN (%) | 63.2 ± 0.2 | 0.0–100.0 | 60.7 ± 0.2 | 0.8–97.7 | 79.4 ± 0.1 | 33.8–99.3 | 30.0 ± 0.2 | 0.4–84.4 |
| STR (%) | 72.1 ± 0.2 | 0.0–100.0 | 76.6 ± 0.2 | 1.4–99.7 | 84.3 ± 0.1 | 35.4–100.0 | 43.7 ± 0.2 | 0.5–98.3 |
| WOB (%) | 80.6 ± 0.1 | 0.0–100.0 | 78.6 ± 0.1 | 28.1–100.0 | 93.8 ± 0.1 | 71.2–100.0 | 69.7 ± 0.2 | 17.6–100.0 |
| ALH (μm) | 2.2 ± 0.1 | 0.0–5.9 | 3.5 ± 0.1 | 0.9–6.6 | 2.5 ± 0.1 | 0.4–5.30 | 5.7 ± 0.1 | 2.0–16.9 |
| BCF (Hz) | 7.7 ± 0.1 | 0.0–21.0 | 11.0 ± 0.1 | 3.6–22.0 | 7.9 ± 0.1 | 0.0–20.0 | 8.4 ± 0.1 | 0.0–22.0 |

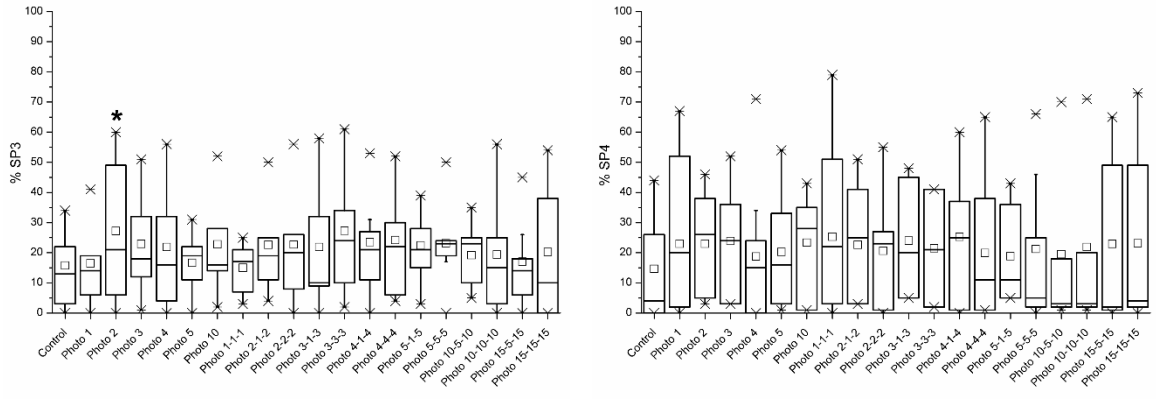
Data were obtained after classifying sperm cells into motile subpopulations through principal component and cluster analyses.

Figure 5a shows the percentages of spermatozoa belonging to SP1. These percentages were significantly ($p < 0.05$) higher in the control (39.8% ± 5.8%) than in the following irradiation patterns: 2 (23.3% ± 5.1%), 2-2-2 (24.6% ± 4.4%), 3-3-3 (25.5% ± 5.1%), and 4-1-4 (26.3% ± 5.8%). Percentages of spermatozoa belonging to SP3 significantly ($p < 0.05$) increased following light-stimulation for 2 min (27.2% ± 6.9% vs. 15.8% ± 3.4% in the control; Figure 5c). In contrast, no significant differences ($p > 0.05$) between the control and irradiation patterns were observed in SP2 and SP4 (Figure 5b,d).




(**a**) (**b**)

**Figure 5.** *Cont.*




(**c**) (**d**)

**Figure 5.** Box plots showing the percentages of sperm belonging to (**a**) Subpopulation 1 (SP1, which was the slowest subpopulation based on VCL, VSL, and VAP); (**b**) Subpopulation 2 (SP2, which presented intermediate values, higher than SP1 but lower than SP3); (**c**) Subpopulation 3 (SP3, which was the one with the highest VSL, VAP, and LIN); and (**d**) Subpopulation 4 (SP4, which was the one with the highest VCL and ALH but with the lowest LIN, STR, and WOB) in the control and different irradiation patterns. The superscript (*) means significant differences ($p \leq 0.05$) between the control and irradiation patterns. Data are shown as mean ± SEM of nine independent experiments.

### 3.5. Effects of Red-Light Stimulation on Intracellular ATP Levels

Figure 6 shows intracellular ATP levels in irradiated and control samples. Samples irradiated for 4 min showed significantly ($p < 0.05$) higher intracellular ATP levels (3.8 nmol/mg protein ± 0.3 nmol/mg protein) than the control (2.5 nmol/mg protein ± 0.2 nmol/mg protein). No significant differences between the control and the other light-stimulation patterns were observed.

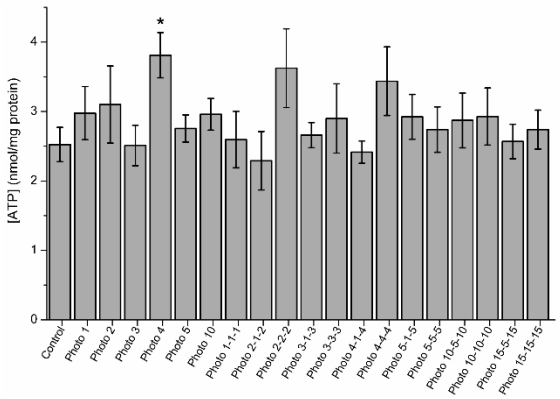
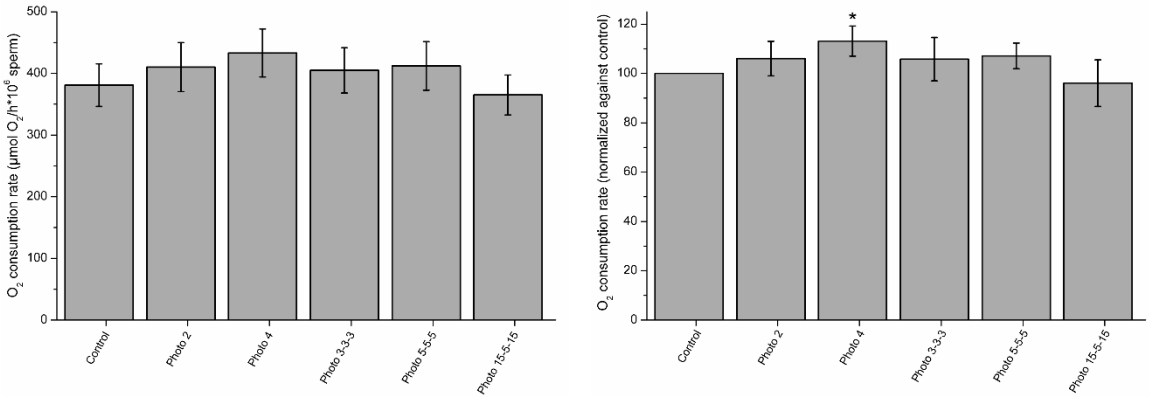



**Figure 6.** Intracellular ATP levels in control and irradiated samples. The superscript (*) means significant differences ($p \leq 0.05$) between the control and different light-stimulation patterns. Data are shown as mean ± SEM of nine independent experiments.

### 3.6. Effects of Red-Light Stimulation on Oxygen Consumption

Figure 7a shows $O_2$ consumption rates, which did not differ ($p > 0.05$) between irradiated and control samples. However, when, in order to remove individual variability, $O_2$ consumption rates in irradiated samples were normalized against their corresponding controls (Figure 7b), those standardized rates in samples irradiated for 4 min were significantly ($p < 0.05$) higher than in the control.




(**a**) (**b**)

**Figure 7.** $O_2$ consumption rates in control and irradiated samples (**a**), and rates in irradiated samples normalized against the corresponding control (**b**). The superscript (*) means significant differences ($p \leq 0.05$) between the control and irradiation patterns. Data are shown as mean ± SEM of nine independent experiments.

### *3.7. Effects of Red-Light Stimulation on DNA Fragmentation*

As shown in Table 3, percentages of DNA fragmentation (% DFI), mean fluorescence intensity of single-stranded DNA (mean DFI), and percentages of high DNA stainability (% HDS) did not differ ($p > 0.05$) between the control and irradiated samples.

**Table 3.** DNA fragmentation parameters of horse sperm in control and irradiated samples. Data (mean ± SEM) are given as percentages of sperm with damaged chromatin (% DFI), mean fluorescence intensity of ssDNA (mean DFI), and percentages of high stainability (% HDS).

| Patterns | % DFI | Mean DFI | % HDS |
|---|---|---|---|
| Control | 15.4 ± 3.0 | 322.9 ± 30.1 | 21.1 ± 5.1 |
| Photo 1 | 15.4 ± 0.8 | 335.5 ± 33.3 | 22.8 ± 8.9 |
| Photo 2 | 15.4 ± 1.2 | 332.1 ± 19.5 | 22.8 ± 7.6 |
| Photo 3 | 15.9 ± 2.9 | 327.5 ± 25.4 | 22.2 ± 5.1 |
| Photo 4 | 14.2 ± 1.4 | 319.0 ± 21.3 | 18.1 ± 5.6 |
| Photo 5 | 15.8 ± 1.4 | 326.9 ± 29.9 | 19.7 ± 8.9 |
| Photo 10 | 14.6 ± 0.9 | 319.7 ± 25.4 | 19.3 ± 8.5 |
| Photo 1-1-1 | 17.0 ± 1.4 | 326.0 ± 34.8 | 25.3 ± 8.1 |
| Photo 2-1-2 | 15.6 ± 0.7 | 319.7 ± 29.0 | 21.1 ± 5.8 |
| Photo 2-2-2 | 17.1 ± 1.7 | 332.6 ± 30.1 | 25.9 ± 6.1 |
| Photo 3-1-3 | 16.4 ± 2.9 | 351.7 ± 46.2 | 22.7 ± 8.9 |
| Photo 3-3-3 | 15.8 ± 2.9 | 330.4 ± 30.2 | 21.8 ± 3.1 |
| Photo 4-1-4 | 14.3 ± 1.0 | 332.5 ± 26.4 | 18.7 ± 8.3 |
| Photo 4-4-4 | 16.8 ± 2.9 | 357.7 ± 44.1 | 32.3 ± 7.2 |
| Photo 5-1-5 | 17.5 ± 3.9 | 323.7 ± 28.1 | 28.2 ± 12.2 |
| Photo 5-5-5 | 17.5 ± 2.7 | 350.0 ± 37.5 | 31.6 ± 6.0 |
| Photo 10-5-10 | 18.4 ± 3.8 | 341.4 ± 27.0 | 31.9 ± 7.0 |
| Photo 10-10-10 | 18.4 ± 3.3 | 324.6 ± 27.4 | 31.4 ± 10.4 |
| Photo 15-5-15 | 19.3 ± 4.5 | 313.7 ± 24.3 | 29.7 ± 9.2 |
| Photo 15-15-15 | 18.3 ± 4.3 | 314.5 ± 19.3 | 25.1 ± 9.6 |

No significant differences between control and irradiated samples were observed ($p > 0.05$). DFI: DNA fragmentation index. HDS: High DNA stainability.

## 4. Discussion

Our results clearly show that irradiation with LED-based red-light modifies some kinetic parameters and the structure of motile sperm subpopulations. These changes occurred together with an increase in mitochondrial activity of horse spermatozoa, without affecting the integrity of plasma membrane or DNA. Interestingly, our data suggest that the effects of exposing horse spermatozoa to red-light heavily rely upon the specific utilized light-dark interval. These findings are similar to those observed in previous studies based on the use of both laser and LED-based light sources in species such as dogs [17], buffalos [34], and humans [35].

In addition, our data are consistent with those reported by other authors, who have suggested that irradiation effects depend on the precise rhythm and intervals of light-darkness regardless of the light source (LED or laser) used [7,36,37]. In fact, distinct wavelengths have different effects and, although the optimal wavelength varies between species, mounting evidence indicates that blue- or green-light is detrimental for mammalian spermatozoa [11,13,38]. Related with this, it is important to emphasize that, in previous studies carried out in other mammalian species, red-light has been demonstrated to be the one that mostly improves motility and other functional parameters in both human and animal spermatozoa (see [14] for review).

The results shown herein suggest that stallion sperm exposed to short patterns of red-light stimulation, both single or combining light with dark regimens, exhibit better response on motility parameters than the long ones. This is very apparent when looking at the changes in kinetic parameters (patterns 4, 2-2-2, 3-3-3, 4-4-4, 5-1-5, and 5-5-5), and in the sperm subpopulation structure (patterns 2, 2-2-2, 3-3-3, and 4-1-4). In this context, it is worth mentioning that, in a study conducted using liquid-stored boar semen irradiated with LED red-light, the shortest irradiation patterns were the ones that exerted the most intense effects [7]. A similar result was also observed in a study conducted with human sperm, in which irradiation with a single exposition to red LED light for 50 s, 100 s, and 200 s increased sperm motility, whereas the exposure for 400 s had inhibitory effects [13]. Therefore, all these results, including those of our study, would be consistent with the fact that the two-phase response to light-doses follows the Arndt–Schultz curve [38,39]. In fact, these data also suggest that low levels of irradiation have greater but variable effect on tissues than higher levels of irradiation. This variable response, including the impact upon sperm motility, has already been described elsewhere [12,13,35,37,40,41] although one study with human sperm suggested a linear relationship between the dose of red-light irradiation and sperm motility [42]. However, taking into account the considerable difference in the response to light between species, a more in-depth study is needed to establish better the mechanisms underlying the observed variability.

Focusing on sperm motility, our findings in some kinetic patterns were similar to those observed in other species. These parameters were VSL (patterns: 3-3-3 min and 5-5-5 min), with similar effects to those found in humans [41], dogs [17], buffalos [34], and pigs [7]; and VAP (patterns: 4 min, 2-2-2 min, 3-3-3 min, and 4-4-4 min), with a similar impact to that reported in dogs [16,17], bulls [43], buffalos [34], and pigs [7]. In addition, we observed a significant decrease in BCF (patterns 3-3-3 min and 5-1-5 min) compared to the control, which matched with previous reports in humans [42], but differed from what found in dogs [16,17] and pigs [7].

Another important point to highlight is the lack of differences in total and progressive motility between control and light-stimulated samples. While these results are in agreement with those previously reported in dogs [16,17] and cattle [43], irradiation of sperm with red-light has been found to increase total and progressive motility in humans [35,37,41,42,44], buffalos [34], sheep [45], and pigs [7]. Therefore, the aforementioned differences also support that the effects of light-dose on sperm function rely upon species [11].

When evaluating the presence of sperm motile subpopulations in horse ejaculates, we identified four separate subpopulations. These results are similar to those reported previously in horses [46], donkeys [47], cattle [48], and goats [49,50]. Remarkably, we observed that percentages of spermatozoa belonging to SP1, which was the slowest subpopulation based on VCL, VSL, and VAP, were significantly

higher in the control than after the following light-stimulation patterns: 2 min, 2-2-2 min, 3-3-3 min, and 4-1-4 min. In addition, percentages of sperm belonging to SP3, which showed the highest values in most kinetic parameters, including VSL, VAP, and LIN, were significantly lower in the control than following light-stimulation for 2 min. Therefore, our data suggest that irradiation of horse sperm with red-light modifies the structure of motile sperm subpopulations by decreasing the percentage of the slowest sperm subpopulation and increasing the percentage of the most linear and fastest one. It is worth mentioning that these results are in agreement with a previous study conducted with dog semen, in which stimulation with laser red-light significantly increased the proportions of the fastest sperm subpopulation [17]. These changes in the characteristics of motile sperm subpopulations, in addition to those observed in sperm kinetic parameters, indicate that not only does irradiation with red-light increase sperm velocity, but it also modifies the way through which sperm move. At this moment, there is no clear explanation of how these effects occur, since the exact mechanism(s) through which red-light stimulates sperm remain(s) unclear. However, it has been hypothesized that a mechanism related to the activation of sperm mitochondria could be essential to explain those effects [14]. In fact, our results from the analysis of mitochondrial membrane potential agree with this possibility, since irradiation with patterns 4, 3-3-3, 4-4-4, 5-1-5, 5-5-5, 15-5-15, and 15-15-15 increased the percentages of spermatozoa with high $\Delta\Psi m$. A similar increase on $\Delta\Psi m$ has been described in pig sperm [7,43]. Thus, these data suggest that stimulation with red-light could increase mitochondrial activity through photosensitizers that are present in the electronic chain, such as cytochrome C [7,51], which would result in higher sperm motility and greater fertilization potential [52].

Recent studies have concurred that oxygen consumption is an alternative way to measure mitochondrial activity, which could be better than the use of markers of mitochondrial membrane potential, such as JC1 [53,54]. In addition to this, the rate of oxygen consumption also provides an indirect measure of ATP produced by oxidative phosphorylation in sperm [54]. The results obtained in this study agree with this possibility, since they showed an increase in intracellular ATP levels and $O_2$ consumption rate ($O_2$ consumption rate normalized against the corresponding control) in samples irradiated for 4 min, compared with the non-irradiated control. Furthermore, these results agree with those obtained in the evaluation of the percentages of viable sperm with high MMP with this stimulation pattern. In this context, it is important to take into account that the increase in the potential of the mitochondrial membrane is associated with changes in the consumption of ATP and the activity of the enzymes of the respiratory chain [55,56]. Related with this, Iaffaldano et al. [45] observed that light stimulation of frozen-thawed ram sperm with a He-Ne laser increased ATP content and the activity and affinity of cytochrome C oxidase (CCO) for their substrate (cytochrome C). Interestingly, these authors found that CCO activity and ATP content were positively correlated with each other and with sperm motility, supporting the hypothesis that the effects of red light upon sperm are mediated by mitochondria.

## 5. Conclusions

In conclusion, this work has shown, for this first time, that irradiation of horse sperm with red-light modifies the structure of motile sperm subpopulations, increases some kinetic parameters, intracellular ATP levels, the rate of $O_2$ consumption, and mitochondrial membrane potential, without affecting the integrities of DNA and plasma membrane. Therefore, we suggest that the effects of light on sperm are related to mitochondrial function. These effects, however, rely upon the specific light-stimulation pattern. While these changes could have a beneficial impact upon the fertility ability of horse spermatozoa, further research investigating whether such a positive effect exists is much warranted. In addition to contemplating in vivo or in vitro fertility assays, future studies should also address which mechanism(s) underlie(s) this sperm response to red light.

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J.E.R.-G., J.M. and M.Y.; Supervision, J.E.R.-G., J.M. and M.Y.; Project administration, J.E.R.-G., J.M. and M.Y.; Funding acquisition, J.E.R.-G., J.M. and M.Y. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** J.E.R.-G. and M.Y. are inventors of a patent entitled 'Method and apparatus for improving the quality of mammalian sperm' (European Patent Office, no. 16199093.2; EP-3-323-289-A1), which is owned by Instruments Utils de Laboratori Geniul, SL (Barcelona, Spain).

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# 3. Irradiating frozen-thawed stallion sperm with red-light increases their resilience to withstand post-thaw incubation at 38ºC

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# Irradiating frozen-thawed stallion sperm with red-light increases their resilience to withstand post-thaw incubation at 38 °C

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ABSTRACT

The aim of this study was to evaluate whether red-light stimulation increases the longevity and resilience of cryopreserved stallion sperm to withstand post-thaw incubation for 120 min. Sixteen frozen straws of 0.5 mL from eight stallions were used. Samples were cryopreserved, thawed through incubation at 38 °C for 30 s and divided into the control and samples exposed to red-light using a triple LED photo-activation system (wavelength: 620–630 nm). Three irradiation protocols consisting of different light-dark-light intervals (1-1-1, 2-2-2 and 3-3-3 min) were tested. Sperm quality parameters were analyzed immediately after light-stimulation (0 min) and after 120 min of incubation at 38 °C. Sperm motility was evaluated using a Computerized Semen Analysis System (CASA), and flow cytometry and different fluorochromes were used to evaluate the integrity and lipid disorder of plasma membrane, mitochondrial membrane potential and intracellular levels of peroxides and superoxides. Irradiation significantly increased the percentages of spermatozoa with high mitochondrial membrane potential (1-1-1 pattern) and the intracellular levels of peroxides (2-2-2 pattern) at 0 min. In addition, sperm kinematic parameters (2-2-2 and 3-3-3 patterns) and percentages of viable spermatozoa with low membrane lipid disorder (3-3-3 pattern) were significantly higher in irradiated samples than in the control at 120 min. Our results indicate that red-light stimulation could help increase the resilience of frozen-thawed stallion sperm to withstand post-thaw incubation at 38 °C for 120 min and that these effects rely on the irradiation pattern. Further research should evaluate whether light-stimulation could also have a positive on fertility rates after artificial insemination.



## 1. Introduction

Cryopreservation is the best method for long-term preservation of stallion spermatozoa and facilitates their international trade [1,2]. While the development of slow-freezing in Equidae has occurred together with that of artificial insemination (AI) [3,4], pregnancy rates of frozen-thawed sperm following AI are lower than those of fresh or cooled-stored semen [5–7].

One of the most important factors underlying the aforementioned reduction in reproductive performance is related to the decreased sperm function and survival after freezing and thawing [8,9]. Indeed, cryodamage has been extensively described in the literature [2,10–13], and includes a drop in the integrity of plasma membrane and acrosome, sperm motility and mitochondrial membrane potential [2,14–18], along with a concomitant increase in both DNA fragmentation and intracellular levels of reactive oxygen species (ROS) [2,14,19,20]. These injuries lead to a decreased sperm lifespan, which may involve a diminished ability to survive within the female reproductive tract [21,22]. Several efforts have been primarily focused on trying to improve cryopreservation procedures by finding suitable freezing media and/or evaluating the use of different additives to protect sperm during freezing and thawing procedures [4,22–24]. All these attempts have aimed at better preserving sperm membrane integrity,

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motility and fertilizing ability through controlling the physico-chemical factors (osmotic and thermal stress, ice nucleation …) that may induce cryodamage [17,25,26].

Irradiation with laser red-light has been described to improve the cryotolerance and longevity of frozen-thawed ram and bull spermatozoa [22,27]. Red-light stimulation, either with low-level lasers or light emitting diodes (LED), has also been reported to increase the motility, ability to elicit *in vitro* capacitation, fertilizing ability and lifespan of fresh and liquid-stored semen in fish [28], birds [29], humans [30–35], pigs [13,36,37], sheep [38], dogs [39,40], buffalos [41] and donkeys [42], without causing any damage on sperm DNA [22,32,43]. While, at present, the mechanisms through which light exerts its effects upon sperm are unclear, different hypothesis have been raised. One of these hypothesis contemplates the involvement of endogenous cellular photosensitizers, especially those located within mitochondria, such as porphyrins, cytochromes, pyridine cofactors, nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), Fe–S clusters and flavins [28,44]. These photosensitizers absorb light from electromagnetic radiation, and then ionize and transfer the absorbed energy into adjacent molecules [45]. This increased energy boosts electrochemical potential in mitochondria, which could ultimately augment ATP synthesis [44]. Another hypothesis takes into consideration the potential role of opsins, which are present in mammalian sperm cells, may absorb light from different spectra [44] and have been proven to underlie the sperm response to thermotaxis [46]. Finally, a third hypothesis envisages the interaction of red-light with transient receptor potential proteins (TRP [47–49]), which reside in the mammalian sperm membrane and have also been suggested to be involved in the modulation of thermotaxis [50,51].

Therefore, irradiation of frozen-thawed stallion sperm with red-light using low-level lasers and LED could better maintain their survival and extend their lifespan. Because application of lasers requires specific and often expensive material, a LED-based system, which is much cheaper, easier to maintain and has high photonic efficiency [13], was used as a light-source in this study. Briefly, cryopreserved sperm straws were thawed and subsequently irradiated using a red-light emitting diode (LED, 620–630 nm) with three different patterns (light, dark, light (min); 1-1-1, 2-2-2 and 3-3-3). Immediately after irradiation (0 min) and after 120 min of incubation at 38 °C, sperm motility, integrity and lipid disorder of plasma membrane, mitochondrial activity, and intracellular levels of hydrogen peroxides ($H_2O_2$) and superoxide anions ($\cdot O_2^-$) were evaluated.

## 2. Materials and methods

### 2.1. Suppliers

All reagents were of analytical grade and were purchased from Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Saint-Louis, MO, USA). Fluorochromes were purchased from Molecular Probes (Thermo Fisher Scientific; Waltham, Massachusetts, USA) and resuspended with dimethyl sulfoxide (DMSO; Sigma-Aldrich). The photo-activation LED system (PhastBlue®) emitting at 620–630 nm and the required tubes (1.5 mL) specifically designed for that device were provided by IUL S.L (Barcelona, Spain).

### 2.2. Stallions and ejaculates

Eight ejaculates, each coming from a separate stallion, were collected and cryopreserved. All stallions were adult (age: 5–12 years old) and of proven fertility, and were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), which is a European Union (EU) approved collection center for equine semen (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. Since all stallions used in this study were semen donors and were allocated at the Equine Reproduction Service, the local committee at our university indicated that no further ethics authorization was required.

Ejaculates were collected using a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) containing an in-line nylon mesh filter to remove the gel fraction. Upon collection, gel-free semen was diluted 1:5 (v:v) in Kenney extender [52] previously warmed to 37 °C and transferred into 50-mL corning tubes. Each sample was evaluated on basis of total volume, sperm concentration (Neubauer chamber, Paul Marienfeld GmbH and Co. KG; Lauda-Königshofen, Germany), motility (Computer Assisted Semen Analysis, CASA; see section 2.7 for details), morphology (eosin-nigrosin staining) and plasma membrane integrity (SYBR14/PI). All samples were confirmed to be above the standard thresholds (≥60% $SYBR14^+/PI^-$ sperm and ≥70% morphologically normal sperm) prior to freezing.

### 2.3. Sperm cryopreservation

Before sperm cryopreservation and in order to remove seminal plasma, diluted semen samples were centrifuged at 600×*g* and 20 °C for 15 min (Medifriger BL-S, JP Selecta S.A., Barcelona, Spain). Supernatants were discarded and pellets were resuspended in a commercial freezing medium (Botucrio®, Botupharma Animal Biotechnology; Botucatu, Brazil), which contains 1% glycerol and 4% methylformamide as permeable cryoprotectants. Thereafter, sperm concentration, motility and plasma membrane integrity were re-evaluated, and freezing medium (Botucrio®) was added to obtain a final concentration of $200 \times 10^6$ viable sperm per mL (standardized in all cases). Samples were packaged into 0.5-mL straws and cryopreserved using a controlled-rate freezer (Ice-Cube 14S; Minitüb). Cooling/freezing was performed in three stages as follows: (i) a first cooling step from 20 °C to 5 °C for 60 min, at a rate of −0.25 °C/min; (ii) a second freezing step from 5 °C to −90 °C for 20 min, at a rate of −4.75 °C/min; and (iii) a third freezing step from −90 °C to −120 °C for 2.7 min, at a rate −11.11 °C/min. Straws were subsequently plunged into liquid nitrogen and stored in appropriate tanks for one month until thawed and irradiated.

### 2.4. Sperm thawing

Sixteen 0.5-mL straws (two per ejaculate) were thawed through incubation at 38 °C for 30 s in a water bath with gently shaking. The content of the two straws coming from the same ejaculate was poured into a 15-mL tube and added with three mL of Kenney medium, previously warmed at 38 °C (total volume: 4 mL).

### 2.5. Experimental design

After thawing and dilution, each frozen-thawed sperm sample coming from the same stallion (n = 8) and resulting from pooling the content of two straws was split into four different 1-mL aliquots using transparent plastic 1.7-mL tubes (IUL S.L.), specifically designed for the PhastBlue® device. One aliquot was used as a control, and the other three were placed into the PhastBlue® system and irradiated. In all cases, the temperature within the PhastBlue® equipment was maintained at 20 °C, as this device has an air ventilation system that avoids the thermal effect associated with light-stimulation. The device, which was equipped with a triple LED system that emitted red-light at 620–630 nm, was

connected to a computer that had a built-in software allowing the adjustment of intensity and exposure time. Irradiation protocols consisted of two periods of light exposure separated by a period of darkness, as described in Yeste et al. [13]. Specifically, three light-stimulation protocols, consisting of different intervals of light and darkness (1-1-1: 1 min light, 1 min darkness and 1 min light; 2-2-2: 2 min light, 2 min darkness and 2 min light; and 3-3-3: 3 min light, 3 min darkness and 3 min light) were tested. Light intensity was maintained at 100% in all cases. As mentioned earlier, control samples were not irradiated and were kept at 20 °C for 9 min.

Upon light-stimulation (0 min) and after 120 min of incubation at 38 °C (120 min), sperm motility was evaluated with a CASA system, and the integrity and lipid disorder of plasma membrane, mitochondrial membrane potential and the intracellular levels of ROS (peroxides and superoxides) were determined through flow cytometry.

### 2.6. Flow cytometry

The integrity (SYBR14/PI) and lipid disorder (M540/YO-PRO-1) of sperm plasma membrane, mitochondrial membrane potential (JC1) and intracellular levels of peroxides ($H_2$DCFDA/PI) and superoxides (HE/YO-PRO-1) were determined through flow cytometry. Samples were stained and evaluated following the protocol described by Prieto-Martínez et al. [53] adjusted to stallion spermatozoa.

Management of the flow cytometer and analysis of samples were carried out in accordance with the recommendations of the International Society of Cytometry [54]. The flow cytometer used in this study was a Cell Lab Quanta SC™ (Beckman Coulter, Fullerton, CA, USA) and particles were excited with an argon laser (488 nm) at a power of 22 mW. Prior to staining, sperm concentration was adjusted to $1 \times 10^6$ sperm/mL. Every day, the electronic volume (EV) channel was calibrated with 10-μm diameter fluorescent beads (Beckman Coulter) following the manufacturer's instructions. The flow rate was set at 4.17 μL/min, and the analyzer threshold was established to exclude cell aggregates (particles with a diameter > 12 μm) and cell debris (particles with a diameter < 7 μm). Sperm cells were gated on the basis of EV and side scatter (SS) distributions. Three different optical filters were used (FL1 for the analysis of SYBR14, $H_2$DCFDA and JC1 monomers; detection width: 505–545 nm; FL2 for the analysis of JC1 aggregates; detection width: 560–590 nm; and FL3 for the analysis of PI, M540 and HE; detection width: 655–685 nm).

Dot plots were examined using Cell Lab Quanta SC™ MPL Analysis Software (version 1.0; Beckman Coulter), and data from M540/YO-PRO-1, JC1, $H_2$DCFDA/PI and superoxides (HE/YO-PRO-1) were corrected using the percentage of non-stained, debris particles found in SYBR14/PI staining, as recommended by Petrunkina et al. [55].

#### 2.6.1. Analysis of plasma membrane integrity

Sperm viability (plasma membrane integrity) was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR14/PI; Molecular Probes, Thermo Fisher Scientific), according to the protocol described by Garner and Johnson [56] and adapted to stallion spermatozoa. In brief, samples were first incubated with SYBR14 (final concentration: 100 nM) at 38 °C for 10 min, and then with PI (final concentration: 12 μM) at 38 °C for 5 min. Three sperm populations were distinguished: (i) viable spermatozoa emitting green fluorescence ($SYBR14^+/PI^-$), which appeared on the right side of the lower half of FL1/FL3 dot plots; (ii) non-viable spermatozoa emitting red fluorescence ($SYBR14^-/PI^+$), which appeared on the left side of the upper half of the FL1/FL3 dot plots; and (iii) non-viable spermatozoa emitting both green and red fluorescence ($SYBR14^+/PI^+$), which appeared on the right side of the upper half of FL1/FL3 dot plots. Non-stained particles ($SYBR14^-/PI^-$), which appeared on the left side of the lower half of the FL1/FL3 dot plots, showed EV/SS distributions similar to spermatozoa and were considered as non-DNA, debris particles. Percentages of non-stained particles were used to correct the percentages of double-negative sperm populations in the other assessments. Spill-over of FL1-into FL3-channel was compensated (2.45%).

#### 2.6.2. Analysis of membrane lipid disorder

Membrane lipid disorder was determined through staining with M540 and YO-PRO-1 fluorochromes. Sperm were incubated with M540 (final concentration: 2.6 μM) and YO-PRO-1 (final concentration: 25 nM) at 38 °C for 10 min in the dark [57]. Four sperm populations were detected: (i) viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$); (ii) viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$); (iii) non-viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$); and (iv) non-viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$), and percentages of all sperm populations were recalculated.

#### 2.6.3. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined through incubation with JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3'tetraethyl-benzimidazolylcarbocyanine iodide; final concentration: 0.3 μM) at 38 °C for 30 min in the dark. When MMP is low, JC1 forms monomers emitting green fluorescence ($JC1_{mon}$), which is collected through FL1. When mitochondrial membrane potential is high, JC1 forms aggregates emitting orange fluorescence ($JC1_{agg}$), which is detected through FL2. Three sperm populations were distinguished: (i) spermatozoa with green-stained mitochondria (low MMP); (ii) spermatozoa with orange-stained mitochondria (high MMP); and (iii) spermatozoa with heterogeneous mitochondria stained both green and orange in the same cell (intermediate MMP). Ratios between FL2 ($JC1_{agg}$) and FL1 fluorescence ($JC1_{mon}$) for each of these sperm populations were also evaluated. Spill-over of FL1-into FL2-channel was compensated (68.5%). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of spermatozoa with low MMP, and percentages of all sperm populations were recalculated.

#### 2.6.4. Analysis of intracellular ROS levels: $H_2O_2$ and $\cdot O_2^-$

Intracellular ROS levels were determined through two oxidation sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) and hydroethidine (HE), which detect hydrogen peroxide ($H_2O_2$) and superoxide anion ($\cdot O_2^-$), respectively [58]. Following a modified procedure from Guthrie and Welch [59], a simultaneous differentiation of viable and non-viable sperm was performed using PI ($H_2$DCFDA) or YO-PRO-1 (HE).

In the case of peroxides, spermatozoa were incubated with $H_2$DCFDA (final concentration: 200 μM) and PI (final concentration: 12 μM) at room temperature for 30 min in the dark. $H_2$DCFDA is a stable, cell-permeable, non-fluorescent probe that deesterifies intracellularly and converts into 2′,7′-dichlorofluorescein (DCF) in the presence of $H_2O_2$ [59]. Fluorescence of DCF was measured through FL1 filter and that of PI was detected through FL3. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of peroxides ($DCF^-/PI^-$); (ii) viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$); (iii) non-viable spermatozoa with low levels of peroxides ($DCF^-/PI^+$); and (iv) non-viable spermatozoa with high levels of peroxides ($DCF^+/PI^+$). Percentages of

debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from those of viable spermatozoa with low levels of peroxides (DCF$^-$/PI$^-$), and percentages of all sperm populations were recalculated. Spill-over of FL1 into the FL3 channel was compensated (2.45%). Data are shown as corrected percentages of viable spermatozoa with high levels of peroxides (DCF$^+$/PI$^-$), and geometric mean of DCF$^+$-fluorescence intensity in the DCF$^+$/PI$^-$ sperm population.

Regarding superoxide anions, samples were incubated with HE (final concentration: 4 μM) and YO-PRO-1 (final concentration: 25 nM) at room temperature for 30 min in the dark [59]. Hydroethidine diffuses freely through plasma membrane and converts into ethidium (E$^+$) in the presence of superoxide anions ($\cdot O_2^-$) [60]. Fluorescence of ethidium (E$^+$) was detected through FL3 and that of YO-PRO-1 was collected through FL1. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of superoxides (E$^-$/YO-PRO-1$^-$); (ii) viable spermatozoa with high levels of superoxides (E$^+$/YO-PRO-1$^-$); (iii) non-viable spermatozoa with low levels of superoxides (E$^-$/YO-PRO-1$^+$); and (iv) non-viable spermatozoa with high levels of superoxides (E$^+$/YO-PRO-1$^+$). Percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from those of viable spermatozoa with low levels of superoxides (E$^-$/YO-PRO-1$^-$), and percentages of all sperm populations were recalculated. Spill-over of FL3 into the FL1 channel was compensated (5.06%). Data are shown as corrected percentages of viable spermatozoa with high levels of superoxides (E$^+$/YO-PRO-1$^-$), and geometric mean of E$^+$-fluorescence intensity in the E$^+$/YO-PRO-1$^-$ sperm population.

### 2.7. Analysis of sperm motility

Sperm motility was evaluated by using a computer-assisted sperm-analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). Samples were incubated at 38 °C in a water bath for 5 min, and 5 μL of each sperm sample was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel) previously warmed at 38 °C. Samples were then analyzed under a 10 × negative phase-contrast objective (Olympus B×41 microscope; Olympus, Tokyo, Japan). A minimum of 1000 sperm cells were counted per analysis. In each evaluation, percentages of total motility (TMOT, %) and progressively motile spermatozoa (PMOT, %) were recorded together with the following kinetic parameters: curvilinear velocity (VCL, μm/s), which is the mean path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, μm/s), which is the mean path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, μm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of linearity (LIN, %), which is the quotient between VSL and VCL multiplied by 100; percentage of straightness (STR, %), which is the quotient between VSL and VAP multiplied by 100; percentage of oscillation (WOB, %), which is the quotient between VAP and VCL multiplied by 100; mean amplitude of lateral head displacement (ALH, μm), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle; and frequency of head displacement (BCF, Hz), which is the frequency at which the actual sperm trajectory crosses the average path trajectory.

CASA settings were those recommended by the manufacturer, i.e. frames/s: 25 images captured per second; particle area >4 and < 75 μm$^2$; connectivity: 6; minimum number of images to calculate the ALH: 10. Cut-off value for motile spermatozoa was VAP ≥10 μm/s, and for progressively motile spermatozoa was STR ≥75%.

### 2.8. Statistical analyses

Results were analyzed with a statistical package (IBM® SPSS® 25.0 for Windows; Armonk, NY, USA). Prior to performing any test, normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) were confirmed. When required, data were linearly transformed through arcsine $\sqrt{x}$.

Effects of irradiation treatment (control, 1-1-1, 2-2-2 and 3-3-3) and post-thaw incubation time after irradiation (0 min and 120 min) were checked through a linear mixed model, followed by post-hoc Sidak test for pair-wise comparisons. Post-thaw incubation time after irradiation was the within-subjects factor and the irradiation protocol was the fixed-effects factor. All sperm parameters were considered as dependent variables. Data that, despite being linearly transformed, did not match with parametric assumptions were analyzed through Friedman and Wilcoxon tests.

The significance level was set at $P \leq 0.05$ and in all cases they are shown as mean ± standard error of the mean (SEM).

## 3. Results

### 3.1. Effects of red-light stimulation on plasma membrane integrity

Percentages of membrane-intact spermatozoa did not significantly differ between control samples and any of the performed irradiation protocols either immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C (Fig. 1a).

### 3.2. Effects of red-light stimulation on membrane lipid disorder

Fig. 1b shows the percentages of viable spermatozoa with low plasma membrane lipid disorder. No significant differences between the control and light-stimulation protocols were observed at 0 min. On the contrary, percentages of viable spermatozoa with low membrane lipid disorder were higher ($P < 0.05$) in samples irradiated with the 3-3-3 pattern than in the control at 120 min (Fig. 1b). No other significant differences between the control and the separate irradiation patterns were observed.

### 3.3. Effects of red-light stimulation on mitochondrial membrane potential

No significant differences in the percentages of spermatozoa with both high (Fig. 2a) and intermediate MMP (Fig. 2b) were observed between the control and light-stimulation patterns, either upon irradiation (0 min) or after 120 min of incubation at 38 °C. Meanwhile, incubation at 38 °C for 120 min induced a decrease ($P < 0.05$) in the percentages of both high MMP and intermediate MMP in control samples (Fig. 2a and b). A similar decrease was observed in sperm subjected to the 1-1-1 irradiation pattern. However, both 2-2-2 and 3-3-3 irradiation patterns abolished these decreases after 120 min of incubation (Fig. 2a and b).

Fig. 2c shows the geometric mean of $JC1_{agg}$-intensity in the sperm population with high MMP. An increase ($P < 0.05$) was found immediately after irradiation of samples with the 1-1-1 pattern (i.e. 0 min). In contrast, no differences ($P > 0.05$) between the control and light-stimulation patterns were observed after 120 min of incubation at 38 °C. Moreover, geometric means of $JC1_{agg}$-intensity in the sperm population with intermediate MMP did not differ between the control and irradiation protocols at 0 min and 120 min (Fig. 2d). Additionally, whereas this intensity did decrease ($P < 0.05$) from 0 min to 120 min in the control and 1-1-1 pattern, that reduction was not significant in 2-2-2 and 3-3-3 treatments (Fig. 2d).

Finally, we also evaluated $JC1_{agg}/JC1_{mon}$ ratios (GMFI) in the

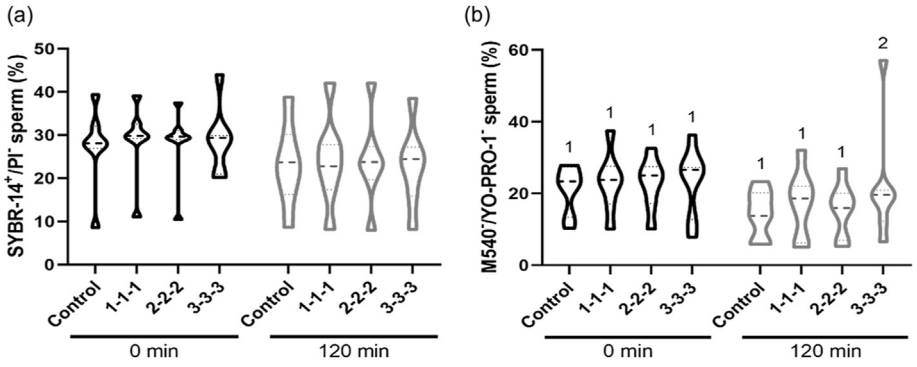


**Fig. 1.** Percentages of spermatozoa with an intact plasma membrane (SYBR14$^+$/PI$^-$, viable spermatozoa; **a**) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C and (**b**) percentages of viable spermatozoa with low plasma membrane lipid disorder (M540$^-$/YO-PRO-1$^-$) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. Different numbers (*1*, *2*) indicate significant differences ($P \leq 0.05$) between the control and irradiation patterns after 0 min or 120 min of light-stimulation and incubation at 38 °C. Data are shown as mean ± SEM of eight separate experiments.

sperm populations with high (Fig. 2e) and intermediate MMP (Fig. 2f). In the case of the sperm population with high MMP, whereas higher values ($P < 0.05$) were obtained with the 1-1-1 pattern compared to the control at 0 min, no significant differences between the control and any of the tested irradiation protocols at 120 min were observed. With regard to the sperm population with intermediate MMP, $JC1_{agg}/JC1_{mon}$ ratios did not differ ($P > 0.05$) between the control and irradiation patterns either immediately after light-stimulation (0 min) or after incubation at 38 °C for 120 min. However, while those ratios decreased ($P < 0.05$) over incubation time in the control and 1-1-1 pattern, such a reduction was not observed in 2-2-2 and 3-3-3 protocols (Fig. 2f).

### 3.4. Effects of red-light stimulation on intracellular ROS levels ($H_2O_2$ and $\cdot O_2^-$)

No significant differences in the percentages of viable sperm with high peroxide levels between the control and irradiation patterns were observed either immediately after light-stimulation (0 min) or after incubation at 38 °C for 120 min (Fig. 3a). In contrast, the GMFI of DCF$^+$ in the population of viable sperm with high levels of peroxides (DCF$^+$/PI$^-$) was higher ($P < 0.05$) in samples irradiated with the 2-2-2 pattern than in the control at 0 min (Fig. 3b). No significant differences between the control and irradiation patterns were observed after 120 min of incubation.

As shown in Fig. 4a, no significant differences between the control and any of the tested light-stimulation patterns were found in the percentages of viable spermatozoa with high levels of superoxides either upon irradiation or after incubation at 38 °C for 120 min. With regard to the GMFI of E$^+$ in the population of viable sperm with high levels of superoxides (E$^+$/YO-PRO-1$^-$), no significant differences between the control and irradiation patterns were observed at either 0 min or 120 min (Fig. 4b).

### 3.5. Effects of red-light stimulation on sperm motility

Percentages of TMOT (Fig. 5a) and PMOT (Fig. 5b) did not differ between the control and irradiation protocols either immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. However, while PMOT decreased ($P < 0.05$) from 0 min to 120 min in the control, that reduction was not significant when samples were irradiated with each of the three protocols.

On the other hand, stimulation with red-light increased ($P < 0.05$) VAP (2-2-2 and 3-3-3 patterns), and STR (3-3-3 pattern) after 120 min of incubation at 38 °C (Table 1). In addition, while incubation for 120 min led to a decrease ($P < 0.05$) in VSL, VAP, STR and BCF in the control, that reduction was not statistically significant in 2-2-2 and 3-3-3 patterns (Table 1). Moreover, ALH decreased ($P < 0.05$) following incubation at 38 °C for 120 min in the control and samples irradiated with 1-1-1 and 2-2-2 patterns, but not in those exposed to the 3-3-3 protocol (Table 1).

## 4. Discussion

This study suggests that stimulation with red LED light at a wavelength of 620–630 nm could help better maintain the sperm quality of frozen-thawed stallion spermatozoa. In effect, we found that irradiating frozen-thawed stallion sperm with red-light increased some kinematic parameters and the intensity of mitochondrial membrane potential, especially when these parameters were evaluated after 120 min of incubation at 38 °C. Moreover, and despite the observed increment in the intensity of intracellular peroxide levels, light-stimulation did not affect the integrity of sperm plasma membrane and mitigated the increase in membrane lipid disorder observed in the control after 120 min of incubation.

One interesting finding of this work was that the effects of red-light stimulation on each sperm parameter depended on the irradiation pattern and time of exposure. These findings coincide with previous studies using laser- and LED-based red-light conducted in other mammalian species, such as dogs, buffalo, humans and donkeys [33,40–42]. In effect, the impact of red-light on mammalian spermatozoa has been revealed to rely heavily upon the precise simulation pattern, time and intensity [13] and on the functional status of the cell [42]. Although the mechanisms underlying this different time response have not yet been elucidated, based on the hypothesis that light acts on mitochondrial endogenous cellular photosensitizers, it is reasonable to suggest that the energy supplied to the mitochondrial electron chain by red-light is proportional to exposure time and intensity. The final consequence of this phenomenon would be that separate exposure times have a different impact on the overall function of sperm, depending on the exact level of energy and the rate at which this energy is fed into mitochondria.

Focusing on sperm motility, the effects of red-light stimulation on kinetic parameters observed herein were similar to those observed in other species such as humans [32], dogs [40], buffalos [41] and pigs [13]. In effect, we found that percentages of both TMOT and PMOT did not differ between the control and light-stimulated samples. While these results are in agreement with those previously reported in dogs [39,40] and bulls [61], sperm

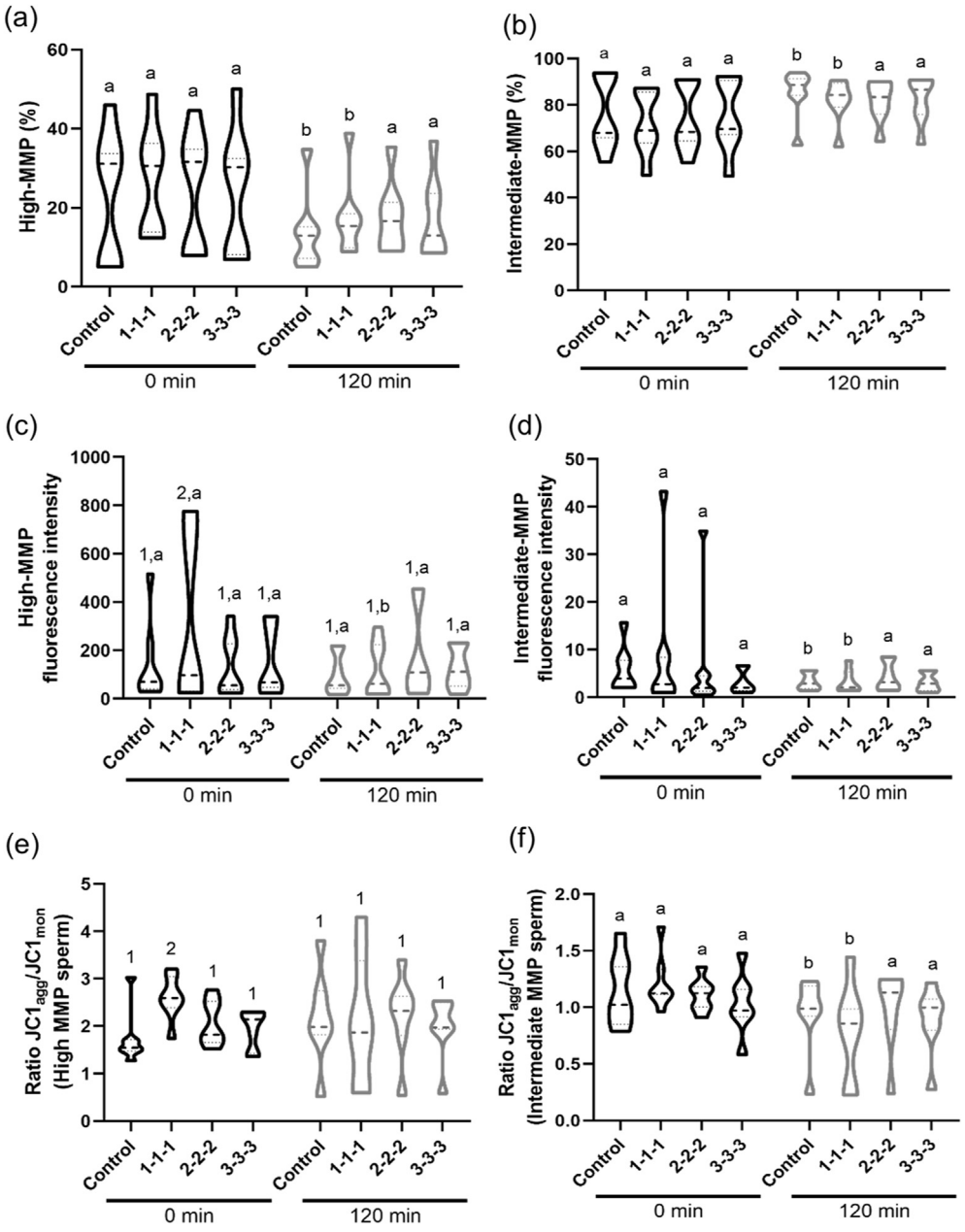


**Fig. 2.** Percentages of spermatozoa with high mitochondrial membrane potential (MMP; $JC1^{++}_{agg}$, **a**) and with intermediate mitochondrial membrane potential (MMP; $JC1^{+}_{agg}$; **b**); geometric mean of $JC1_{agg}$-fluorescence intensity (GMFI, FL2) in the sperm populations with high (**c**) and intermediate MMP (**d**); and $JC1_{agg}/JC1_{mon}$ ratios (GMFI FL2/GMFI FL1) in the sperm populations with high (**e**) and intermediate MMP (**f**) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. Different numbers (*1, 2*) indicate significant differences ($P \leq 0.05$) between the control and the different light-stimulation patterns after 0 min or 120 min of incubation at 38 °C, and different letters (*a, b*) indicate significant differences ($P \leq 0.05$) between 0 min or 120 min of incubation at 38 °C within the control or irradiation patterns. Data are shown as mean ± SEM of eight separate experiments.

irradiation has been found to increase total and progressive motility in humans [30,32–34,62], buffalos [41], sheep [22], pigs [13] and donkeys [42]. Therefore, these differences indicate that the effects of light-dose on sperm function differ between species [28]. On the other hand, it is worth noting that, in spite of the fact that no significant differences in most motility indicators were found between the control and irradiation protocols at 0 min or 120 min, the significant decrease observed in these parameters in the control was not seen in irradiated samples. The fact that irradiated spermatozoa showed higher resilience to withstand post-thaw incubation than the control could be related the extended sperm longevity observed by Iaffaldano et al. [29]. Furthermore, the increases in kinematic parameters observed after irradiation in 2-2-2 and 3-3-3 patterns would indicate that not only could light-stimulation help maintain post-thaw motility of stallion spermatozoa better, but could also modify their patterns of movement, as observed in frozen-thawed human sperm and in fresh and cooled-stored donkey semen [42,43].

At present, there is no a consensus on how red-light modifies some sperm function parameters. Although one must consider the relationship between light exposure and thermotaxis [46], the involvement of mitochondria photosensitizers also needs to be contemplated [44]. Herein, we observed that immediately after irradiating sperm with the 1-1-1 pattern (i.e. 0 min), there was a

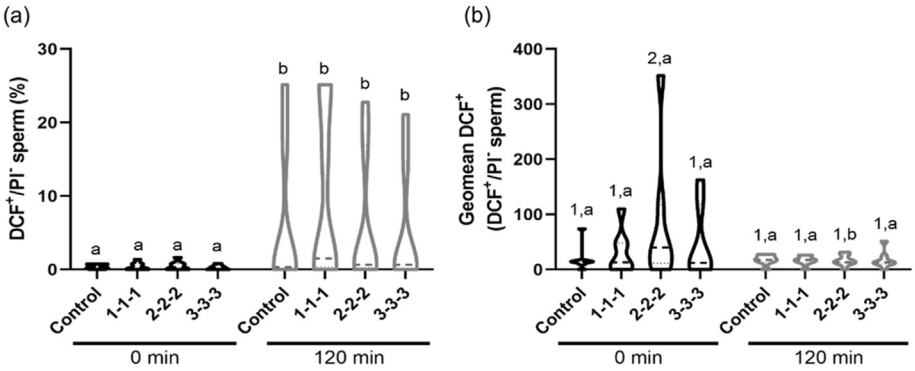


**Fig. 3.** Percentages of viable spermatozoa with high peroxide levels ($DCF^+/PI^-$; **a**) and geometric mean of $DCF^+$-intensity (GMFI, FL1 channel) in the population of viable spermatozoa with high peroxide levels (**b**) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. Different numbers (*1, 2*) indicate significant differences ($P \leq 0.05$) between the control and the different light-stimulation patterns after 0 min or 120 min of incubation at 38 °C, and different letters (*a, b*) indicate significant differences ($P \leq 0.05$) between 0 min and 120 min of incubation at 38 °C within the control or irradiation patterns. Data are shown as mean ± SEM of eight separate experiments.

transient increase in the $JC1_{agg}/JC1_{mon}$ ratio of the sperm population with high MMP. However, there was no clear relationship between this increase in the intensity of MMP and the effects of red-light stimulation on sperm motility. These results differ from those observed by Siqueira et al. [61], who found that irradiation of frozen-thawed bull sperm with a He–Ne laser (wavelength: 633 nm) increased sperm motility and mitochondrial membrane potential immediately after exposure to laser-based red-light for 10 min. Irradiation of liquid-stored boar semen with red LED light at a wavelength of 620–630 nm (10-10-10 pattern) has also been reported to increase sperm motility and the percentages of high MMP sperm, immediately after light-stimulation (0 min) and after 90 min of incubation at 37 °C [13]. While mitochondrial activation does not seem to be the only mechanism involved in the sperm response to red-light [44], the effects observed in the present and other aforementioned studies could be due to its impact on mitochondrial photosensitizers. This hypothesis would be in agreement with the results published by other authors [13,22,38,42], in which the cytochrome C/cytochrome C oxidase complex was demonstrated to be stimulated with red and infrared light [63]. It has been suggested that the photonic absorption by cytochrome C oxidase augments ATP production, which could underlie the increase in sperm motility and fertilizing ability, as observed in this and other studies [64].

Since ROS are mainly generated in the mitochondria as a by-product of the electronic chain, it has been purported that the light-mediated increase in mitochondrial activity could stimulate ROS production, which could, in turn, be detrimental to sperm cells [38]. In this regard, while sperm irradiation did not affect superoxide generation, it did increase peroxides in viable spermatozoa immediately after light-stimulation. This effect, which was observed in a specific irradiation pattern (2-2-2), agrees with Zan-Bar et al. [28] and Cohen et al. [65], who suggested that the first step of irradiation is the formation of ROS through endogenous cellular photosensitizers. Low ROS levels have been reported to be beneficial for sperm motility and fertilizing ability [28]. In fact, $H_2O_2$ has been suggested as the active molecule involved in the light-mediated changes on sperm fertilizing ability [65]. This is consistent with what described by Zan-Bar et al. [28] and de Lamirande et al. [66], who found that low concentrations of ROS participate in signaling transduction pathways related to sperm capacitation and acrosome reaction. In agreement with this possibility, both the increase in intracellular ROS levels and mitochondrial activity were observed immediately after light-stimulation. However, the extent of that increase was not enough to detrimentally affect sperm membrane integrity, which matched with our previous findings in fresh and cooled-stored jackass semen [42]. Moreover, our results warrant further research on the potential link between ROS generation and the increment in intracellular $Ca^{2+}$ levels, since superoxide anion is rapidly switched by endogenous superoxide

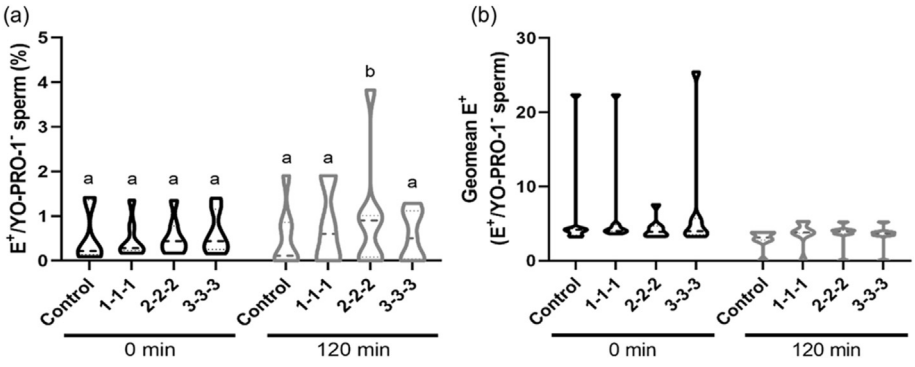


**Fig. 4.** Percentages of viable spermatozoa with high superoxide levels ($E^+$/YO-PRO-$1^-$; **a**) and geometric mean of $E^+$-intensity (GMFI, FL3 channel) in the population of viable spermatozoa with high superoxide levels (**b**) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. Different letters (*a, b*) indicate significant differences ($P \leq 0.05$) between 0 min and 120 min of incubation at 38 °C within the control or irradiation patterns. No significant differences between the control and light-stimulation patterns were observed either at 0 min or at 120 min. Data are shown as mean ± SEM of eight separate experiments.

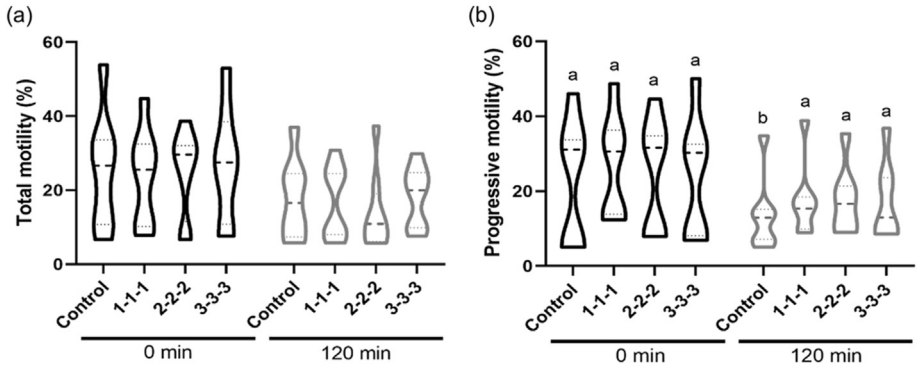


**Fig. 5.** Percentages of total motile spermatozoa (TMOT; **a**) and progressively motile spermatozoa (PMOT; **b**) in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C. Different letters (*a*, *b*) indicate significant differences ($P \leq 0.05$) within the control and irradiation patterns between 0 min and 120 min of light-stimulation and incubation at 38 °C. No significant differences between the control and light-stimulation patterns were observed either at 0 min or at 120 min. Data are shown as mean ± SEM of eight separate experiments.

dismutase (SOD) into $H_2O_2$, and the resulting transient $H_2O_2$ elevation leads to a fast increase in intracellular $Ca^{2+}$ levels [65,67–69].

Through M540/YO-PRO-1 staining, we found that the percentages of viable spermatozoa with low membrane lipid disorder decreased after 120 min of incubation at 38 °C. However, when frozen-thawed stallion sperm were previously irradiated with the 3-3-3 pattern, percentages of viable spermatozoa with low membrane lipid disorder at 120 min were significantly higher than in the control. This is a positive result since it indicates that the sperm resilience to withstand post-thaw incubation at 38 °C could be increased when irradiated with the 3-3-3 pattern, which agrees with a previous study conducted with cryopreserved ram sperm irradiated with a He–Ne laser [22]. While M540 evaluates the early changes in membrane lipid disorder and the increment in membrane lipid disorder is one of the changes that occur during sperm capacitation, observing such an increase at post-thaw suggests that the sperm plasma membrane is losing their functional integrity [70–73]. It is worth mentioning, however, that this lower increase in membrane lipid disorder observed in irradiated sperm was not reflected on sperm membrane integrity, which was evaluated through $SYBR14^+/PI^-$. In effect, no significant differences in the percentages of spermatozoa with an intact plasma membrane (sperm viability) were observed between the control and irradiated samples. Not only could these results indicate that the extent of early changes in the membrane detected by M540/YO-PRO-1 is not enough to be identified by the SYBR14/PI test, but they would also demonstrate that light-stimulation does not negatively affect sperm viability. These findings agree with previous studies conducted in fresh and cooled-stored semen in pigs and donkeys [13,36,42], but do not match with those reported by Iaffaldano et al. [22] in cryopreserved ram sperm. These differences could be explained by the fact that not only were our study and that of Iaffaldano et al. [22] conducted in separate species (horse *vs.* sheep), but also because two different light-sources were used (red LED light *vs.* He–Ne laser).

The results obtained in this study would suggest that light-stimulation could better maintain the quality of frozen-thawed stallion sperm. Since the improvement in sperm motility and the reduced membrane lipid disorder could be observed after 120 min of thawing and irradiation, which agrees with previous studies carried out with frozen-thawed turkey and ram sperm [22,29,38],

**Table 1**
Kinetic parameters of frozen-thawed stallion sperm in the control and irradiation patterns (light-darkness-light (min): 1-1-1, 2-2-2 and 3-3-3) immediately after light-stimulation (0 min) or after 120 min of incubation at 38 °C.

| Treatments | | Control | | 1-1-1 | | 2-2-2 | | 3-3-3 | |
|---|---|---|---|---|---|---|---|---|---|
| N | | 8 | | 8 | | 8 | | 8 | |
| Parameters | Time (min) | mean ± SEM | Range | mean ± SEM | Range | mean ± SEM | Range | mean ± SEM | Range |
| **VCL (μm/s)** | 0 min | $71.1\pm4.6^{1,a}$ | 13.4–130.4 | $73.8\pm5.5^{1,a}$ | 15.3–132.2 | $72.5\pm5.8^{1,a}$ | 16.0–133.0 | $72.9\pm5.1^{1,a}$ | 14.4–131.4 |
| | 120 min | $48.7\pm5.9^{1,a}$ | 13.4–107.2 | $51.1\pm5.0^{1,a}$ | 13.9–109.5 | $55.6\pm5.5^{1,a}$ | 17.4–113.1 | $56.6\pm3.9^{1,a}$ | 19.8–113.6 |
| **VSL (μm/s)** | 0 min | $40.8\pm5.6^{1,a}$ | 29.3–52.1 | $43.8\pm6.3^{1,a}$ | 32.4–55.2 | $41.4\pm5.5^{1,a}$ | 29.9–52.8 | $42.4\pm6.8^{1,a}$ | 31.0–53.9 |
| | 120 min | $24.8\pm5.6^{1,b}$ | 14.3–37.2 | $29.7\pm4.6^{2,a}$ | 15.5–38.4 | $33.2\pm6.3^{2,a}$ | 19.7–42.5 | $33.1\pm4.6^{2,a}$ | 19.5–42.4 |
| **VAP (μm/s)** | 0 min | $49.3\pm5.9^{1,a}$ | 37.7–60.9 | $51.1\pm6.6^{1,a}$ | 40.5–63.7 | $49.1\pm5.8^{1,a}$ | 37.4–60.7 | $51.6\pm7.1^{1,a}$ | 40.0–63.3 |
| | 120 min | $34.2\pm5.1^{1,b}$ | 22.6–45.8 | $37.8\pm4.8^{1,b}$ | 23.2–46.4 | $39.5\pm6.2^{1,a}$ | 27.9–51.1 | $39.0\pm4.5^{1,a}$ | 27.4–50.6 |
| **LIN (%)** | 0 min | $55.5\pm5.2^{1,a}$ | 43.8–67.2 | $57.6\pm5.9^{1,a}$ | 45.9–69.3 | $57.4\pm5.1^{1,a}$ | 45.7–69.1 | $57.0\pm5.8^{1,a}$ | 45.3–68.7 |
| | 120 min | $50.0\pm6.8^{1,a}$ | 38.3–61.7 | $51.6\pm5.6^{1,a}$ | 39.9–63.2 | $57.7\pm6.6^{1,a}$ | 42.0–65.4 | $58.0\pm5.7^{1,a}$ | 43.2–66.6 |
| **STR (%)** | 0 min | $81.4\pm2.3^{1,a}$ | 73.7–89.1 | $81.9\pm2.6^{1,a}$ | 74.1–89.6 | $83.9\pm1.4^{1,a}$ | 76.2–91.6 | $82.6\pm2.5^{1,a}$ | 72.9–88.4 |
| | 120 min | $68.9\pm6.4^{1,b}$ | 62.1–77.6 | $75.9\pm4.8^{1,a}$ | 68.1–83.6 | $76.7\pm4.6^{1,a}$ | 68.0–83.4 | $79.8\pm3.7^{2,a}$ | 70.1–85.6 |
| **WOB (%)** | 0 min | $67.5\pm4.7^{1,a}$ | 57.9–77.0 | $70.2\pm4.9^{1,a}$ | 60.7–79.7 | $67.9\pm5.0^{1,a}$ | 58.3–77.4 | $69.0\pm5.7^{1,a}$ | 59.5–78.5 |
| | 120 min | $70.1\pm4.1^{1,a}$ | 60.5–79.7 | $67.1\pm4.0^{1,a}$ | 57.6–76.7 | $69.6\pm4.9^{1,a}$ | 60.0–79.4 | $69.8\pm4.3^{1,a}$ | 60.2–79.3 |
| **ALH (μm)** | 0 min | $2.6\pm0.2^{1,a}$ | 2.1–3.1 | $2.6\pm0.2^{1,a}$ | 2.1–3.1 | $2.6\pm0.2^{1,a}$ | 2.0–3.1 | $2.5\pm0.2^{1,a}$ | 2.0–3.0 |
| | 120 min | $1.5\pm0.3^{1,b}$ | 1.0–2.0 | $1.8\pm0.3^{1,b}$ | 1.2–2.3 | $1.6\pm0.3^{1,b}$ | 1.1–2.1 | $1.8\pm0.2^{1,a}$ | 1.5–2.5 |
| **BCF (Hz)** | 0 min | $9.1\pm0.8^{1,a}$ | 7.3–10.9 | $9.4\pm0.7^{1,a}$ | 7.6–11.1 | $9.7\pm0.8^{1,a}$ | 7.8–11.5 | $9.8\pm0.8^{1,a}$ | 8.0–11.6 |
| | 120 min | $5.8\pm1.2^{1,b}$ | 4.0–7.6 | $6.9\pm1.1^{1,a}$ | 5.1–8.7 | $6.4\pm1.0^{1,a}$ | 4.6–8.2 | $7.8\pm0.7^{1,a}$ | 6.0–11.6 |

Different numbers (*1, 2*) indicate significant differences ($P \leq 0.05$) between the control and the different light-stimulation patterns after 0 min or 120 min of incubation at 38 °C, and different letters (*a, b*) indicate significant differences ($P \leq 0.05$) between 0 min or 120 min of incubation at 38 °C within the control or irradiation patterns. Data are shown as mean ± SEM of eight separate experiments.

we suggest that light-stimulation could increase the longevity of frozen-thawed sperm, which could help extend the AI-interval for frozen-thawed stallion semen. This could have a practical relevance, since cryopreservation is known to reduce sperm life span [74] and fertilizing ability [75], and thus AI with frozen-thawed sperm needs to be performed near to the time of ovulation. Remarkably, mares that are to be inseminated with frozen-thawed semen are monitored through ultrasonography, and an insemination-to-ovulation interval of 6 h is known to yield the best pregnancy outcomes [75]. While this studys open up the possibility of considering light-stimulation as a way to extend the longevity of frozen-thawed stallion sperm, its main limitation is the reduced number of animals and ejaculates, which should be increased the confirm this surmise. In addition, further research should also evaluate, through AI trials, the impact of light-irradiation on the fertilizing ability of frozen-thawed sperm, as this would represent the best approach to determine to which extent that better resilience against post-thaw incubation translates into field fertility. Finally, since some permeable cryoprotectants, such as glycerol, have been associated with higher post-thaw sperm motility, and despite the fact that, herein, all samples contained the same concentration of cryoprotectants, further studies should also confirm that the effects observed were due to light-stimulation rather than any other factor, such as media components.

## 5. Conclusions

We can conclude that light-stimulation, especially with 2-2-2 and 3-3-3 patterns, could help increase the resilience of frozen-thawed stallion sperm to withstand post-thaw incubation at 38 °C, which was more apparent from sperm motility and membrane lipid disorder evaluated at 120 min. This, together with previous studies conducted with liquid-stored and frozen-thawed turkey and ram sperm, suggests that irradiation could be used to increase the longevity of frozen-thawed stallion sperm prior to AI. However, additional experiments are required to evaluate whether this beneficial effect is translated into higher sperm fertilizing ability, and to confirm that the irradiation patterns tested herein have no detrimental impact on other sperm parameters, such as DNA integrity. Finally, our results warrant further research on the mechanisms involved in the response of frozen-thawed stallion sperm to red LED light.

## CRediT authorship contribution statement

**Jaime Catalán:** Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Marc Llavanera:** Data curation, Investigation, Methodology. **Sebastián Bonilla-Correal:** Methodology. **Marion Papas:** Investigation, Methodology. **Sabrina Gacem:** Investigation, Methodology. **Joan E. Rodríguez-Gil:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. **Marc Yeste:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - review & editing, Supervision. **Jordi Miró:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

## Declaration of competing interest

J.E.R.G. and M.Y. are inventors of a patent entitled 'Method and apparatus for improving the quality of mammalian sperm' (European Patent Office, No. 16199093.2; EP-3-323-289-A1), which is owned by Instruments Utils de Laboratori Geniul, SL (Barcelona, Spain).

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# 4. The effects of light on mammalian sperm rely upon the color of the straw and the medium used

Jaime Catalán; Iván Yánez-Ortiz; Sabrina Gacem; Marion Papas; Sergi Bonet; Joan E. Rodríguez-Gil; Marc Yeste; Jordi Miró

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# The Effects of Red Light on Mammalian Sperm Rely upon the Color of the Straw and the Medium Used

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**Simple Summary:** Several studies have shown that the exposure of semen to red light improves sperm quality and fertilizing ability, which could improve the efficiency of assisted reproductive techniques with irradiated semen. However, despite being considered as possible sources of variation, the effects of the color of the container (straws) or the medium have not yet been evaluated. In this study, 13 ejaculates from different stallions were split into equal fractions, diluted either with Kenney or Equiplus extender, and subsequently packed into straws of five different colors. After storage at 4 °C for 24 h, the sperm were irradiated and different variables, including sperm motility, plasma membrane integrity, and mitochondrial membrane potential, were evaluated. Our results confirm that irradiation increases some motion characteristics and mitochondrial membrane potential without affecting sperm viability and demonstrate that the effects depend on the color of the straw and the extender used.

**Abstract:** Previous research has determined that irradiation of mammalian sperm with red light increases motility, mitochondrial activity, and fertilization capacity. In spite of this, no study has considered the potential influence of the color of the straw and the extender used. Therefore, this study tests the hypothesis that the response of mammalian sperm to red light is influenced by the color of the straw and the turbidity/composition of the extender. Using the horse as a model, 13 ejaculates from 13 stallions were split into two equal fractions, diluted with Kenney or Equiplus extender, and stored at 4 °C for 24 h. Thereafter, each diluted fraction was split into five equal aliquots and subsequently packed into 0.5-mL straws of red, blue, yellow, white, or transparent color. Straws were either nonirradiated (control) or irradiated with a light–dark–light pattern of 3–3–3 (i.e., light: 3 min, dark: 3 min; light: 3 min) prior to evaluating sperm motility, acrosome and plasma membrane integrity, mitochondrial membrane potential, and intracellular ROS and calcium levels. Our results showed that irradiation increased some motion variables, mitochondrial membrane potential, and intracellular ROS without affecting the integrities of the plasma membrane and acrosome. Remarkably, the extent of those changes varied with the color of the straw and the extender used; the effects of irradiation were more apparent when sperm were diluted with Equiplus extender and packed into red-colored straws or when samples were diluted with Kenney extender and packed into transparent straws. As the increase in sperm motility and intracellular ROS levels was parallel to that of mitochondrial activity, we suggest that the impact of red light on sperm function relies upon the specific rates of energy provided to the mitochondria, which, in turn, vary with the color of the straw and the turbidity/composition of the extender.



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## 1. Introduction

Artificial insemination (AI) is a tool widely used today for horse breeding, especially when looking for genetic improvement [1]. The increasing use of this technology, both in the horse and other species, has augmented the interest for semen processing techniques and their optimization, aimed at maximizing their survival and fertilization capacity [2,3]. Unfortunately, semen quality often deviates from expectations and leads to unsatisfactory pregnancy rates [1]. In this context, any protocol or procedure that optimizes its use and helps increase reproductive performance should be considered; for this reason, several approaches have been undertaken in recent years [3,4]. One of these approaches is sperm irradiation; in effect, previous research has demonstrated that red light stimulation, either with low-level lasers or light-emitting diodes (LEDs), increases the motility, ability to elicit in vitro capacitation, fertilizing ability, and lifespan of fresh, liquid-stored, and frozen-thawed sperm in fish [5], birds [6], humans [7–12], pigs [13–15], sheep [16], dogs [17,18], buffalos [19] donkeys [3], and horses [20,21]. In addition to this, recent studies have shown that the increase in sperm motility in response to LED-based red light is concomitant with that of mitochondrial activity in pigs, donkeys, and horses [3,13,20,22].

The mechanisms through which light exerts its effects are not entirely clear. Three potential mechanisms have been surmised to explain the response of mammalian sperm to red light (reviewed in Yeste et al. [23]). The first of these hypotheses is related to the possible influence of light on transient receptor proteins (TRPs) [24–26], which reside in sperm plasmalemma and have been purported to participate in the modulation of thermotaxis [27,28]. The second hypothesis is related to the presence of opsins in mammalian spermatozoa, which absorb light from different spectra [23]; despite being mainly related to the response to thermotaxis [28], they could also be involved in the sperm response to light. Finally, mounting evidence supports the third hypothesis, which confers a crucial role on endogenous cellular photosensitizers, especially those present in the mitochondria [22,23,29]. These photosensitizers absorb light from electromagnetic radiation and then ionize and transfer the absorbed energy into adjacent molecules [30]. This increased energy induces a rise in electrochemical mitochondria potential, which may result in an augmentation of ATP and $Ca^{2+}$ levels [23]. In spite of this, it cannot be ruled out that more than one of the mechanisms proposed by these hypotheses are involved in the sperm response to red light [22,29].

Previous studies carried out with low-level laser therapy devices and light-emitting diodes (LEDs) have reported an increase in ATP production via the mitochondrial electron chain [31,32] without damaging the irradiated cells [32,33] or the integrity of their DNA [32,34]. Therefore, it has been suggested that light stimulation can have a safe and positive effect on sperm motility and fertilizing ability both in vivo and in vitro [32]. Nevertheless, the sperm response to irradiation has been reported to depend on different factors, including the type (i.e., fresh, cooled-stored or frozen-thawed) and state of the sample [3], the irradiation of the light beam used [32], the time or pattern of exposure [13], and the species [5]. Given the properties of light emission/absorption, other factors such as the color of the straw and choice of extender could also affect the sperm response to red light. However, to the best of our knowledge, no previous study has examined this possibility despite the wide variety of extenders and colors of commercial straws.

Taking the results obtained in the aforementioned studies (especially those conducted with fresh, cooled-stored, and frozen-thawed horse sperm) [20,21] into account, this study aims at determining whether the color of the straw and the extender used affect the response of cooled-stored sperm to LED-based red light (620–630 nm). Our hypothesis is that the effects of red light on horse sperm depend on the color of the straw and the extender.

## 2. Materials and Methods

### *2.1. Suppliers*

All reagents used were of analytical grade and were purchased from Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany), and Sigma-Aldrich (Saint Louis, MO, USA). As far as fluorochromes are concerned, unless otherwise stated, all were purchased from Molecular Probes (Thermo Fisher Scientific; Waltham, MA, USA) and were previously prepared with dimethyl sulfoxide (Sigma-Aldrich). Plastic materials were provided by Nunc (Roskilde, Denmark), and empty straws of different colors (transparent, red, white, blue, and yellow) were purchased from Minitüb GmbH (Tiefenbach, Germany).

### *2.2. Animals and Ejaculates*

This study included 13 ejaculates from 13 different adult stallions (age: 5–8 years old) with proven fertility. Animals were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), which is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All animals were semen donors and were collected under CEE health conditions (free of equine arteritis, infectious anemia, and contagious metritis). As indicated in Catalán et al. [21], this Service runs under the rules of the Regional Government of Catalonia, Spain, and no manipulation of the animals other than semen collection was carried out. The study was approved by the Ethics Committee, Autonomous University of Barcelona (Code: CEEAH 1424).

Ejaculates were collected through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany), and an in-line nylon mesh filter was used to remove the gel fraction. Upon collection, gel-free semen was split into two fractions of equal volume and immediately diluted 1:5 (*v:v*) either in Kenney [35] or Equiplus extender (Minitüb GmbH; Tiefenbach, Germany), which were selected for their different turbidity. The absorbance of these two extenders was evaluated at 625 nm with a spectrophotometer (Biochrom WPA, Lightwave II; Cambridge, UK) and sterilized; ultrafiltered Milli-Q water was used as blank. Absolute absorbance values of the Equiplus and Kenney extenders were 0.090 and >2.5, respectively. Both extenders were preheated to 37 °C, and sperm concentration was adjusted in all cases to $30 \times 10^6$ sperm/mL with a Neubauer chamber (Paul Marienfeld GmbH and Co. KG; Lauda-Königshofen, Germany). Following this, sperm motility (Computer Assisted Semen Analysis, CASA), morphology (eosin–nigrosin staining), and plasma membrane integrity (SYBR14/PI) of each sample were evaluated. All samples were confirmed to fulfill the standard thresholds: $\geq$60% SYBR14$^+$/PI$^-$ spermatozoa and $\geq$70% morphologically normal spermatozoa. Thereafter, semen samples were stored in a refrigerator at 4 °C for 24 h.

### *2.3. Experimental Design*

After 24 h of storage, samples extended in either Kenney or Equiplus were split and packed into 0.5-mL straws (Minitüb GmbH) of five different colors (blue, red, yellow, white, and transparent); the sperm concentration was maintained at $30 \times 10^6$ sperm/mL at all experimental points. Straws were placed within a programmable photoactivation system (MaxiCow; IUL, SA, Barcelona, Spain). In this device, each straw is in contact with a triple-LED configuration system that emits red light (wavelength window: 620 to 630 nm). The apparatus is equipped with software (IUL, SA) that allows the regulation of intensity and time of exposure. In all cases, the intensity was set at 100%.

Straws of different colors containing sperm diluted by both extenders were irradiated with a light–dark–light interval pattern of 3–3–3 min. Nonirradiated samples (control) were also packed into 0.5-mL straws and left for 9 min in the dark, which was the same time used to irradiate the samples. Upon light stimulation, irradiated and nonirradiated samples were transferred into 1.5-mL tubes. Sperm motility was evaluated with a computer-assisted sperm analysis (CASA) system, and plasma membrane and acrosome integrity, mitochon-

drial membrane potential, intracellular ROS (peroxides and superoxides), and calcium levels were determined through flow cytometry.

### *2.4. Analysis of Sperm Motility*

Sperm motility was evaluated using a computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). In brief, samples were incubated at 38 °C in a water bath for 5 min, and 5 µL of each sperm sample was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel), previously warmed at 38 °C. Samples were then analyzed under a 10× negative phase-contrast objective (Olympus BX41 microscope; Olympus, Tokyo, Japan). A minimum of 1000 sperm cells was counted per analysis. In each evaluation, percentages of total motility (TMOT, %) and progressively motile spermatozoa (PMOT, %) were recorded together with the following kinetic measures: curvilinear velocity (VCL, µm/s), which is the mean path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, µm/s), which is the mean path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, µm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of linearity (LIN, %), which is the quotient between VSL and VCL multiplied by 100; percentage of straightness (STR, %), which is the quotient between VSL and VAP multiplied by 100; percentage of oscillation (WOB, %), which is the quotient between VAP and VCL multiplied by 100; mean amplitude of lateral head displacement (ALH, µm), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle; frequency of head displacement (BCF, Hz), which is the frequency at which the actual sperm trajectory crosses the average path trajectory.

CASA settings were those recommended by the manufacturer, i.e., frames: 25 images captured per second; particle area >4 and <75 $\mu m^2$; connectivity = 6; minimum number of images to calculate ALH: 10. The cut-off value for motile spermatozoa was VAP $\geq$ 10 µm/s; for progressively motile spermatozoa, the cut-off value was STR $\geq$ 75%.

### *2.5. Flow Cytometry*

The integrity of sperm plasma membrane (SYBR14/PI), acrosome integrity (PNA-FITC/PI), mitochondrial membrane potential (JC1), and intracellular levels of peroxides ($H_2$DCFDA/PI), superoxides (HE/YO-PRO-1), and calcium (Fluo3/PI) were determined through flow cytometry. Samples were stained and evaluated following the protocol described by Prieto-Martínez et al. [36] and adjusted to horse spermatozoa.

Management of the flow cytometer and analysis of the samples were carried out in accordance with the recommendations of the International Society of Cytometry [37]. The flow cytometer used in this study was a Cell Lab Quanta SC™ (Beckman Coulter, Fullerton, CA, USA), and particles were excited with an argon laser (488 nm) at a power of 22 mW. Prior to staining, sperm concentration was adjusted to $1 \times 10^6$ sperm/mL. Every day, the electronic volume (EV) channel was calibrated with 10-µm diameter fluorescent beads (Beckman Coulter), following the manufacturer's instructions. The flow rate was set at 4.17 µL/min, and the analyzer threshold was established to exclude cell aggregates (particles with a diameter >12 µm) and debris (particles with a diameter < 7 µm). Sperm cells were gated on the basis of EV and side scatter (SS) distributions. Three different optical filters were used (FL1 for the analysis of SYBR14, PNA, $H_2$DCFDA, Fluo3, and JC1 monomers, detection width: 505–545 nm; FL2 for the analysis of JC1 aggregates, detection width: 560–590 nm; FL3 for the analysis of PI and HE, detection width: 655–685 nm).

Dot plots were examined using Cell Lab Quanta SC™ MPL Analysis Software (version 1.0; Beckman Coulter) and data from PNA/PI, JC1, $H_2$DCFDA/PI, HE/YO-PRO-1; Fluo3/PI were corrected using the percentage of nonstained debris particles found in SYBR14/PI staining, as recommended by Petrunkina et al. [38].

#### 2.5.1. Analysis of Plasma Membrane Integrity

Sperm viability (plasma membrane integrity) was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR14/PI; Molecular Probes, Thermo Fisher Scientific; Waltham, MA, USA), according to the protocol described by Garner and Johnson [39], and adapted to horse spermatozoa. In brief, samples were first incubated with SYBR14 (final concentration: 100 nM) at 38 °C for 10 min, and then with PI (final concentration: 12 µM) at 38 °C for 5 min. Three sperm populations were distinguished: (i) viable spermatozoa emitting green fluorescence ($SYBR14^+/PI^-$), which appeared on the right side of the lower half of the FL1/FL3 dot plots; (ii) nonviable spermatozoa emitting red fluorescence ($SYBR14^-/PI^+$), which appeared on the left side of the upper half of the FL1/FL3 dot plots; (iii) nonviable spermatozoa emitting both green and red fluorescence ($SYBR14^+/PI^+$), which appeared on the right side of the upper half of the FL1/FL3 dot plots. Nonstained particles ($SYBR14^-/PI^-$), which appeared on the left side of the lower half of the FL1/FL3 dot plots, showed EV/SS distributions similar to spermatozoa and were considered non-DNA debris particles. Percentages of nonstained particles were used to correct the percentages of double-negative sperm populations in the other assessments. Spill-over of FL1 into the FL3 channel was compensated (2.45%).

#### 2.5.2. Analysis of Acrosome Integrity

Plasma membrane integrity was evaluated through PNA/PI costaining, following the procedure described for horse spermatozoa by Rathi et al. [40]. With this purpose, spermatozoa were stained with PNA conjugated with FITC (final concentration: 5 µg/mL) and PI (final concentration: 12 µm) and incubated at 38 °C for 10 min in the dark. Green fluorescence from PNA was collected through FL1, whereas red fluorescence from PI was collected through FL3. As spermatozoa were not previously permeabilized, they were identified and placed in one of the four following populations: (i) spermatozoa with intact plasma membranes ($PNA^-/PI^-$); (ii) spermatozoa with damaged plasma membranes that presented an acrosome membrane that could not be fully intact ($PNA^+/PI^+$); (iii) spermatozoa with damaged plasma membranes and lost outer acrosome membranes ($PNA^-/PI^+$); (iv) spermatozoa with damaged plasma membranes ($PNA^+/PI^-$). Therefore, after PNA/PI staining, two main categories were detected: (i) spermatozoa with an intact plasma membrane ($PNA^-/PI^-$) and (ii) spermatozoa that had damaged their plasma membrane and/or their acrosome membrane (these were represented by the other three categories: $PNA^+/PI^-$, $PNA^+/PI^+$, $PNA^-/PI^+$). Unstained and single-stained samples were used for setting the EV gain, FL1 and FL3 PMT voltages, and for compensation of PNA spill over into the PI channel (2.45%).

#### 2.5.3. Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was determined through incubation with JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide; final concentration: 0.3 µM) at 38 °C for 30 min in the dark. When MMP is low, JC1 forms monomers emitting green fluorescence ($JC1_{mon}$), which are collected through FL1. When mitochondrial membrane potential is high, JC1 forms aggregates emitting orange fluorescence ($JC1_{agg}$), which are detected through FL2. Three sperm populations were distinguished: (i) spermatozoa with green-stained mitochondria (low MMP), (ii) spermatozoa with orange-stained mitochondria (high MMP), and (iii) spermatozoa with heterogeneous mitochondria, stained both green and orange in the same cell (intermediate MMP). Ratios between FL2 ($JC1_{agg}$) and FL1 fluorescence ($JC1_{mon}$) for each of these sperm populations were also evaluated. Spill-over of FL1 into the FL2 channel was compensated (68.5%). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of spermatozoa with low MMP, and the percentages of all sperm populations were recalculated.

2.5.4. Analysis of Intracellular ROS Levels: $H_2O_2$ and $O_2^-$

Intracellular ROS levels were determined through two oxidation sensitive fluorescent probes, 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) and hydroethidine (HE), which detect hydrogen peroxides ($H_2O_2$) and superoxide anions ($\cdot O_2^-$), respectively [41]. Following a modified procedure from Guthrie and Welch [42], a simultaneous differentiation of viable and nonviable sperm was performed using PI ($H_2$DCFDA) or YO-PRO-1 (HE).

In the case of peroxides, spermatozoa were incubated with $H_2$DCFDA (final concentration: 200 µM) and PI (final concentration: 12 µM) at room temperature for 30 min in the dark. $H_2$DCFDA is a stable, cell-permeable, nonfluorescent probe that is converted into 2′,7′-dichlorofluorescein (DCF) in the presence of $H_2O_2$ [42]. Fluorescence of $DCF^+$ was measured through FL1 and that of PI was detected through FL3. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of peroxides ($DCF^-/PI^-$), (ii) viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$), (iii) nonviable spermatozoa with low levels of peroxides ($DCF^-/PI^+$), and (iv) nonviable spermatozoa with high levels of peroxides ($DCF^+/PI^+$). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of viable spermatozoa with low levels of peroxides ($DCF^-/PI^-$) and the percentages of all sperm populations were recalculated. Spill-over of FL1 into the FL3 channel was compensated (2.45%). Data are shown as corrected percentages of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$) and the geometric mean of $DCF^+$-fluorescence intensity in the $DCF^+/PI^-$ sperm population.

Regarding superoxide anions, samples were incubated with HE (final concentration: 4 µM) and YO-PRO-1 (final concentration: 25 nM) at room temperature for 30 min in the dark [42]. Hydroethidine diffuses freely through the plasma membrane and converts into ethidium ($E^+$) in the presence of superoxide anions ($O_2^-$) [43]. Fluorescence of ethidium ($E^+$) was detected through FL3 and that of YO-PRO-1 was detected through FL1. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^-$), (ii) viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$), (iii) nonviable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^+$), and (iv) nonviable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^+$). Percentages of debris particles found in the SYBR14/PI test ($SYBR14^-/PI^-$) were subtracted from those of viable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^-$) and the percentages of all sperm populations were recalculated. Spill-over of FL3 into the FL1 channel was compensated (5.06%). Data are shown as corrected percentages of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$) and the geometric mean of $E^+$-fluorescence intensity in the $E^+$/YO-PRO-$1^-$ sperm population.

2.5.5. Intracellular Calcium Levels

Previous studies found that Fluo3 mainly stains mitochondrial calcium in mammalian sperm [44]. For this reason, we combined this fluorochrome with propidium iodide (Fluo3/PI), as described by Kadirvel et al. [45]; 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) nonviable spermatozoa with low levels of intracellular calcium ($Fluo3^-/PI^+$), and (iv) nonviable 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.

*2.6. 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 normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test), and, if required, they were transformed with arcsin $\sqrt{x}$. The effects of the color of the straw and the extender on the response of horse sperm to red light were tested with a two-way analysis of variance (ANOVA), followed by a post hoc Sidak test. Sperm motility measures; percentages of spermatozoa with an intact plasma membrane ($SYBR14^+/PI^-$), acrosome-intact spermato-

zoa (PNA-FITC$^-$/PI$^-$), spermatozoa with high and intermediate mitochondrial membrane potential, 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$^-$); and geometric mean fluorescence intensities (GMFI) of JC1$_{agg}$, Fluo3$^+$, E$^+$, and DCF$^+$ were analyzed.

Motile sperm subpopulations were determined through the protocol described in Luna et al. [46]. In brief, individual kinematic variables (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) recorded for each spermatozoon were used as independent variables in a principal component analysis (PCA). Kinematic measures were sorted into PCA components, and the obtained matrix was subsequently rotated using the Varimax method with Kaiser normalization. As a result, each sperm cell was assigned a regression score for each of the new PCA components, and these values were subsequently used to run a two-step cluster analysis based on the log-likelihood distance and Schwarz's Bayesian criterion. Four sperm subpopulations were identified, and each individual spermatozoon was assigned to one of these subpopulations (SP1, SP2, SP3, or SP4). Following this, percentages of spermatozoa belonging to each subpopulation were calculated per sample and used to determine the effects of the color of the straw and the extender on the response of horse sperm to red light through two-way ANOVA and Sidak's post hoc test.

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

As expected, no differences in variables were observed between the straws of different colors in the nonirradiated group. For this reason, and in order to simplify the presentation of data, all these results have been grouped and identified as "control" (nonirradiated samples).

### *3.1. Plasma Membrane Integrity*

Percentages of membrane-intact spermatozoa (Figure S1a) did not differ between nonirradiated and irradiated samples. In addition, neither the color of the straw nor the type of extender had any effect on the percentages of membrane-intact spermatozoa in irradiated and nonirradiated samples (e.g., nonirradiated sperm in Equiplus extender: 46.2% ± 3.9% vs. sperm diluted in Equiplus, packed into blue straws, and irradiated: 47.9% ± 3.6% vs. sperm diluted in Kenney, packed into blue straws, and irradiated: 38.6% ± 3.0%).

### *3.2. Acrosomal Integrity*

In a similar fashion to that observed for plasma membrane integrity, percentages of acrosomal-intact spermatozoa (Figure S1b) did not differ between nonirradiated and irradiated samples, regardless of the color of the straw or the extender (e.g., nonirradiated sperm in Kenney extender: 39.2% ± 3.1% vs. sperm diluted in Kenney, packed into red straws, and irradiated: 39.7% ± 3.2% vs. sperm diluted in Equiplus, packed into red straws, and irradiated: 49.8% ± 3.6%).

### *3.3. Sperm Motility*

No significant differences were observed in the percentages of sperm with total (Figure S2a) and progressive motility (Figure S2b) between nonirradiated and irradiated samples when compared within each extender. In addition, neither the color of the straw nor the type of extender had any effect on the percentages of sperm with total and progressive motility when the two extenders were compared within each of the colored straws (e.g., total motility: nonirradiated sperm in Equiplus: 56.0% ± 4.5% vs. sperm diluted in Equiplus, packed into yellow straws, and irradiated: 56.5% ± 3.8% vs. sperm diluted in Kenney, packed into yellow straws, and irradiated: 56.3% ± 3.4%; progressive motility: nonirradiated sperm diluted in Kenney: 27.1% ± 2.4% vs. sperm diluted in Kenney,

packed into transparent straws, and irradiated: 31.9% ± 2.9% vs. sperm diluted in Equiplus, packed into transparent straws, and irradiated: 28.4% ± 2.6%).

Regarding sperm kinetic variables (Table 1), VCL, VSL, and VAP were significantly ($p < 0.05$) higher in samples diluted in Equiplus and packed into red straws than in their respective control (nonirradiated samples). In addition, VCL and VAP were significantly ($p < 0.05$) higher in sperm diluted in Kenney extender and packed into transparent straws than in the nonirradiated control. In addition to this, STR in samples packed into blue straws and irradiated was significantly higher ($p < 0.05$) in sperm diluted in Kenney than in those diluted in Equiplus extender.

As shown in Table 2, four different motile sperm subpopulations were identified (SP1, SP2, SP3, and SP4); SP1 was characterized as the fastest subpopulation since it exhibited the highest values in VCL, VSL, and VAP. SP2 was the slowest sperm subpopulation. SP3, although characterized by intermediate speed values (but lower than SP1 and SP4) and LIN and STR values similar to SP1, was the one that showed the highest BCF. Finally, SP4 was characterized by intermediate speed values, which were higher than in SP3, but it was the least linear.

Figure 1a shows the percentages of sperm belonging to SP1. Compared to their respective controls, these percentages were significantly ($p < 0.05$) higher in irradiated samples diluted in Equiplus extender and packed into blue, yellow, or red straws and in irradiated samples diluted in Kenney extender and packed into transparent straws. Percentages of sperm belonging to SP2 were significantly ($p < 0.05$) higher in the control than in irradiated samples diluted in Equiplus extender and packed into yellow, red, or transparent straws (Figure 1b). On the contrary, no significant differences ($p > 0.05$) between nonirradiated and irradiated samples were observed for SP3 and SP4 (Figure 1c,d).

### *3.4. Mitochondrial Membrane Potential*

As shown in Figure 2a and Figure S3, percentages of sperm with high MMP were significantly ($p < 0.05$) higher in samples diluted in Equiplus and packed into yellow, red, and transparent straws and in those diluted in Kenney and packed into transparent straws than in their respective controls. In contrast, no significant differences between extenders were observed when nonirradiated and irradiated samples packed into straws of different color were compared. With regard to the percentages of sperm with intermediate MMP, no significant differences between nonirradiated and irradiated samples were observed, regardless of the color of the straw and the extender (Figure 2b).

No significant differences in the geometric mean of $JC1_{agg}$ intensity (orange, FL2) of sperm populations with high (Figure 2c) and intermediate MMP (Figure 2d) were observed between nonirradiated and irradiated samples, regardless of the color of the straw and the extender used. However, the geometric mean of $JC1_{agg}$ intensity (orange, FL2) of the sperm population with a high MMP (Figure 2c) was significantly ($p < 0.05$) higher in samples diluted in Kenney extender and nonirradiated (control) or packed into blue, yellow, red, white, or transparent straws than in their counterparts diluted in Equiplus extender. In addition, the geometric mean of $JC1_{agg}$ intensity (orange, FL2) of the sperm population, with an intermediate MMP in nonirradiated samples (control), was significantly ($p < 0.05$) higher when they were diluted in Kenney than when they were diluted in Equiplus extenders (Figure 2d).

Finally, we also evaluated $JC1_{agg}/JC1_{mon}$ ratios of sperm populations with high (Figure 2e) and intermediate MMP (Figure 2f). No significant differences ($p > 0.05$) were observed when comparing nonirradiated and irradiated samples, regardless of the color of the straw and extender used, either within the same diluent or when comparing the two extenders.

**Table 1.** Effects of the color of the straw (blue, yellow, red, white, and transparent), extender, and light stimulation on sperm kinetic variables in nonirradiated (control) and irradiated samples.

| Extender | Treatment (Straw Color) | Kinetic Variables (Mean ± SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) | LIN (%) | STR (%) | WOB (%) | ALH (μm) | BCF (Hz) |
| **Equiplus** | Control | 91.2 ± 3.1 [1] | 54.0 ± 2.9 | 74.2 ± 3.2 [1] | 59.3 ± 1.4 | 73.5 ± 1.9 | 80.8 ± 1.1 | 3.0 ± 0.2 | 8.9 ± 0.2 |
| | Blue | 98.5 ± 4.4 | 60.4 ± 3.6 | 82.6 ± 4.3 | 61.2 ± 214 | 72.9 ± 1.3 [a] | 83.0 ± 1.4 | 3.0 ± 0.2 | 8.9 ± 0.4 |
| | Yellow | 102.0 ± 4.7 | 62.1 ± 4.1 | 82.8 ± 3.4 | 61.0 ± 1.7 | 75.6 ± 1.8 | 80.7 ± 1.4 | 3.3 ± 0.2 | 9.2 ± 0.1 |
| | Red | 106.5 ± 3.2 [2] | 68.8 ± 2.6 [2] | 89.3 ± 3.0 [2] | 64.7 ± 2.1 | 77.2 ± 2.9 | 83.8 ± 0.9 | 3.3 ± 0.2 | 8.8 ± 0.2 |
| | White | 96.7 ± 4.0 | 61.3 ± 3.9 | 81.2 ± 4.3 | 63.1 ± 1.4 | 75.5 ± 1.6 | 83.5 ± 0.8 | 2.9 ± 0.2 | 8.7 ± 0.2 |
| | Transparent | 102.9 ± 4.9 | 62.9 ± 3.1 | 83.6 ± 4.2 | 62.8 ± 1.8 | 77.8 ± 2.1 | 82.7 ± 0.6 | 3.2 ± 0.2 | 8.9 ± 0.2 |
| **Kenney** | Control | 93.8 ± 2.8 [1] | 56.7 ± 3.6 | 72.3 ± 3.1 [1] | 59.9 ± 2.6 | 78.7 ± 2.2 | 76.1 ± 2.7 | 2.9 ± 0.1 | 10.1 ± 0.4 |
| | Blue | 97.5 ± 4.3 | 63.3 ± 2.8 | 77.7 ± 4.5 | 63.8 ± 2.2 | 82.4 ± 1.6 [b] | 81.0 ± 2.2 | 2.8 ± 0.1 | 9.2 ± 0.4 |
| | Yellow | 100.9 ± 4.1 | 63.4 ± 2.5 | 80.4 ± 3.9 | 63.3 ± 2.0 | 81.2 ± 2.5 | 78.6 ± 2.3 | 3.0 ± 0.1 | 9.9 ± 0.4 |
| | Red | 96.1 ± 3.6 | 62.6 ± 2.5 | 77.7 ± 4.3 | 63.7 ± 1.8 | 81.8 ± 1.8 | 78.6 ± 2.3 | 3.0 ± 0.1 | 9.8 ± 0.5 |
| | White | 92.1 ± 3.2 | 56.0 ± 2.5 | 70.8 ± 3.7 | 60.7 ± 2.1 | 80.1 ± 2.0 | 76.2 ± 2.5 | 3.0 ± 0.1 | 10.4 ± 0.4 |
| | Transparent | 107.4 ± 3.0 [2] | 66.3 ± 2.8 | 86.0 ± 3.0 [2] | 62.0 ± 2.2 | 78.8 ± 2.4 | 79.1 ± 2.5 | 3.1 ± 0.1 | 9.8 ± 0.5 |

SEM: standard error of the mean; VCL (μm/s): curvilinear velocity; VSL (μm/s): straight line velocity; VAP (μm/s): average path velocity; LIN (%): linearity; STR (%): straightness; WOB (%): wobble; ALH (μm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency. Different numbers ([1, 2]) indicate significant differences ($p < 0.05$) between nonirradiated and irradiated samples packed into straws of different color within the same extender (i.e., Kenney and Equiplus). Different letters ([a, b]) indicate significant differences ($p < 0.05$) between extenders within nonirradiated or samples packed into straws of different color. The absence of numbers indicates the lack of statistical differences ($p > 0.05$) between irradiated and nonirradiated samples within the same diluent. The absence of letters means the lack of significant differences between irradiated/nonirradiated sperm between the two extenders. Data are shown as mean ± SEM of 13 separate experiments.

**Table 2.** Descriptive parameters (mean ± SEM; range) of the four sperm subpopulations (SP1, SP2, SP3, and SP4) identified in nonirradiated and irradiated samples diluted in the two extenders and packed into straws of different color.

| | **SP1** | | **SP2** | | **SP3** | | **SP4** | |
|---|---|---|---|---|---|---|---|---|
| **N** | **10,647** | | **12,622** | | **14,674** | | **8748** | |
| **Parameter** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** | **Mean ± SEM** | **Range** |
| VCL (μm/s) | 146.7 ± 0.4 | 108.2–247.3 | 61.9 ± 0.2 | 10.0–126.6 | 94.5 ± 0.1 | 54.9–149.2 | 126.3 ± 0.3 | 71.9–248.3 |
| VSL (μm/s) | 108.5 ± 0.2 | 63.4–198.3 | 33.4 ± 0.1 | 4.0–54.3 | 71.4 ± 0.1 | 42.6–123.6 | 40.0 ± 0.2 | 4.2–112.6 |
| VAP (μm/s) | 126.6 ± 0.2 | 10.2–121.0 | 45.4 ± 0.1 | 7.6–80.4 | 82.5 ± 0.1 | 48.9–120.0 | 97.4 ± 0.3 | 25.1–201.5 |
| LIN (%) | 75.0 ± 0.1 | 10.0–99.5 | 56.4 ± 0.2 | 8.3–96.6 | 76.6 ± 0.1 | 42.0–99.3 | 32.0 ± 0.2 | 2.7–59.8 |
| STR (%) | 85.9 ± 0.1 | 5.3–96.9 | 74.6 ± 0.2 | 4.0–95.2 | 87.1 ± 0.08 | 52.1–99.7 | 42.4 ± 0.2 | 1.5–97.0 |
| WOB (%) | 87.2 ± 0.1 | 37.8–100.0 | 75.1 ± 0.2 | 23.6–98.6 | 88.0 ± 0.09 | 45.9–100.0 | 78.0 ± 0.2 | 19.3–99.2 |
| ALH (μm) | 3.9 ± 0.02 | 0.5–11.6 | 2.2 ± 0.01 | 0.2–6.8 | 2.6 ± 0.01 | 0.3–7.4 | 3.9 ± 0.02 | 1.0–10.3 |
| BCF (Hz) | 8.2 ± 0.03 | 0.0–21.0 | 8.7 ± 0.03 | 0.0–22.0 | 9.1 ± 0.03 | 0.0–22.0 | 7.7 ± 0.05 | 0.0–21.6 |

SEM: standard error of the mean; VCL (μm/s): curvilinear velocity; VSL (μm/s): straight line velocity; VAP (μm/s): average path velocity; LIN (%): linearity; STR (%): straightness; WOB (%): wobble; ALH (μm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency.

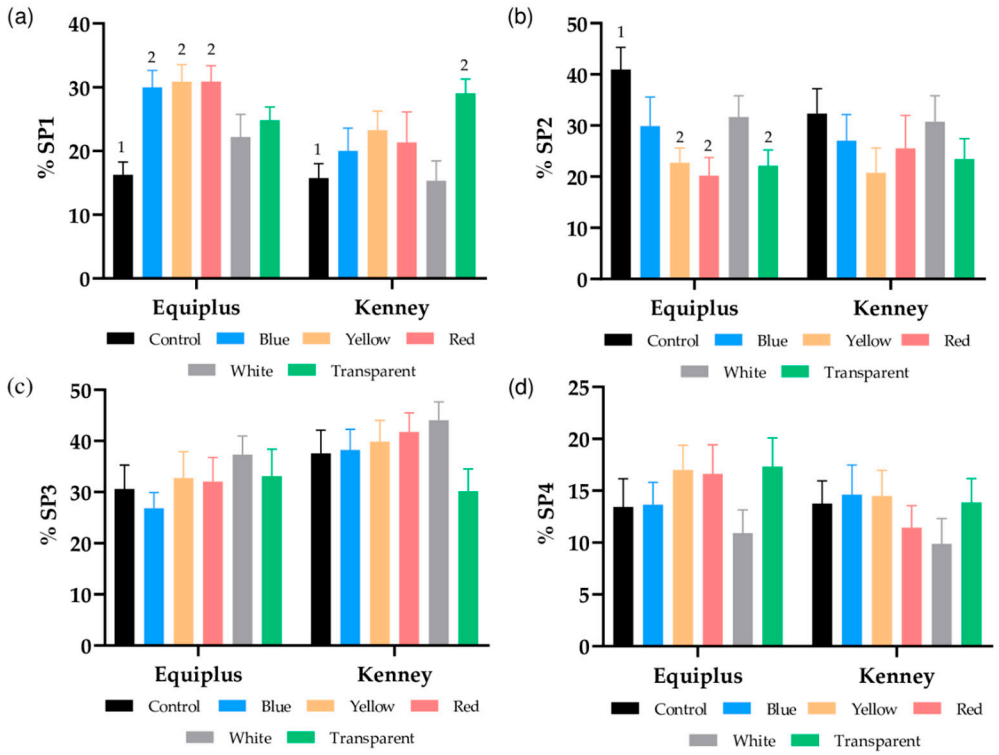


**Figure 1.** Effects of the color of the straw, extender, and light stimulation on the structure of motile sperm subpopulations in control (nonirradiated) and irradiated samples packed into straws of different color and extended either with Equiplus or Kenney extender. (**a**) Subpopulation 1 (SP1, which was the fastest subpopulation for VCL, VSL and VAP); (**b**) Subpopulation 2 (SP2, the slowest); (**c**) Subpopulation 3 (SP3); (**d**) Subpopulation 4 (SP4). The different numbers (1, 2) indicate significant differences ($p < 0.05$) between irradiated and nonirradiated samples packed into different colored straws within the same diluent. The absence of numbers indicates the lack of statistical differences between irradiated and nonirradiated samples packed into different colored straws within the same diluent. On the other hand, no significant differences between nonirradiated and irradiated samples were observed when the two extenders were compared within the same treatment. Data are shown as mean ±SEM of 13 separate experiments.

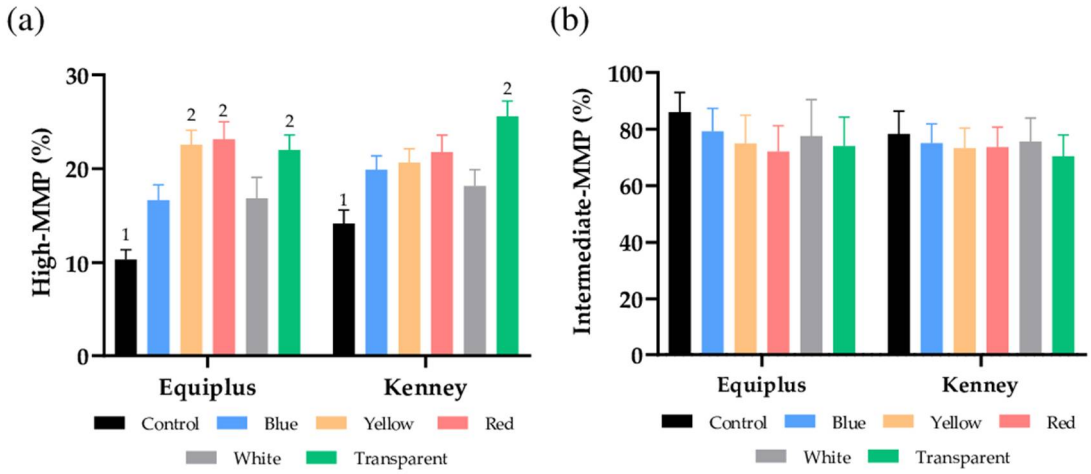


**Figure 2.** *Cont.*

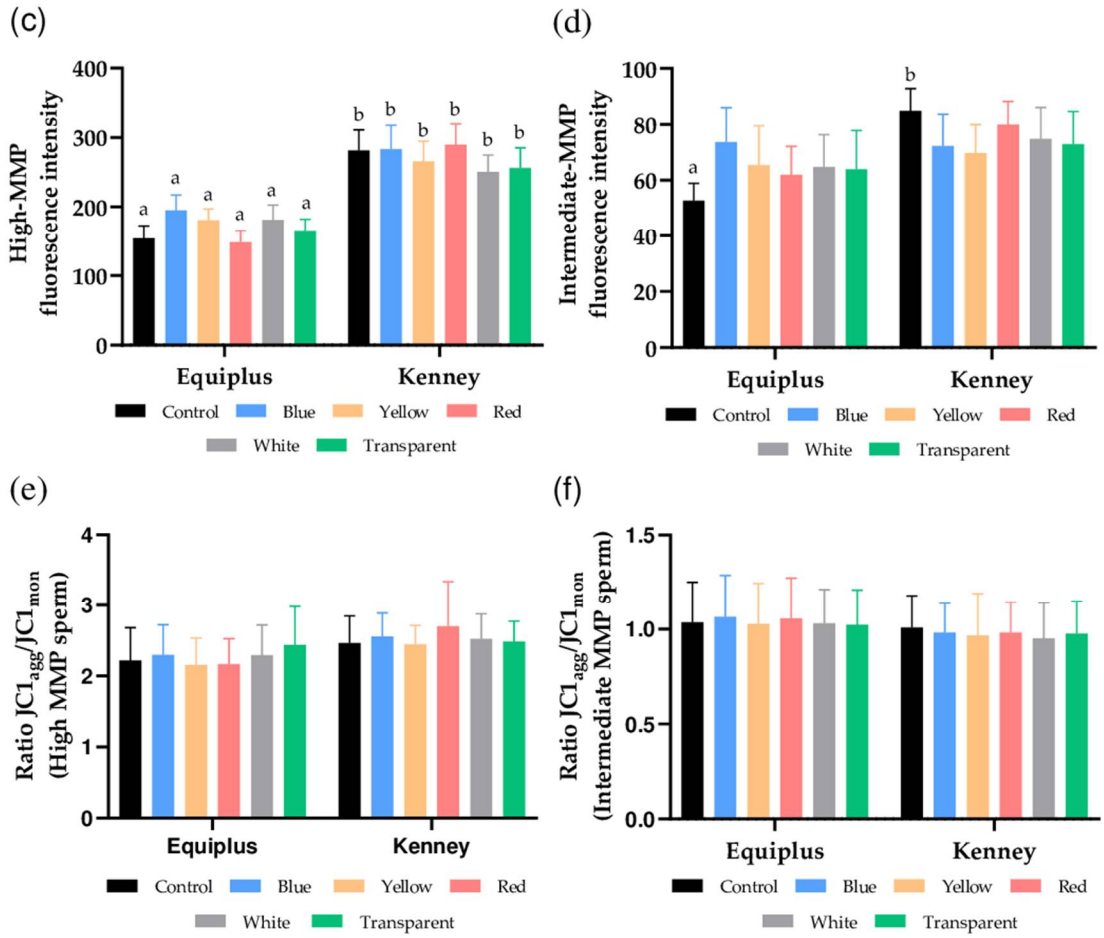


**Figure 2.** Effects of the color of the straw, extender, and light stimulation on mitochondrial membrane potential in control (nonirradiated) and irradiated samples packed into straws of different color and extended either with Equiplus or Kenney extender. The results are presented as percentages of sperm with high mitochondrial membrane potential (MMP; **a**) and with intermediate mitochondrial membrane potential (MMP; **b**), geometric mean of fluorescence intensity of $JC1_{agg}$ (GMFI, FL2) in sperm populations with high (**c**) and intermediate MMP (**d**), and $JC1_{agg}/JC1_{mon}$ ratios (GMFI FL2/GMFI FL1) in sperm populations with high (**e**) and intermediate MMP (**f**) in nonirradiated (control) and irradiated samples. Different numbers (1, 2) indicate significant differences ($p < 0.05$) between nonirradiated and irradiated samples packed into straws of different color within the same diluent. Different letters (a, b) indicate significant differences ($p < 0.05$) between the two extenders within nonirradiated or samples irradiated and packed into straws of different color. The absence of numbers indicates the lack of statistical differences between irradiated and nonirradiated samples within the same diluent, and the absence of letters indicates the lack of differences when comparing a given treatment between both diluents. Data are shown as mean ± SEM of 13 separate experiments.

### *3.5. Intracellular Peroxide and Superoxide Levels*

Figure 3a shows the percentage of viable sperm with high peroxide levels. No significant differences between nonirradiated and irradiated samples were observed, regardless of the color of the straw or the extender used. However, as Figure 3b shows, GMFI of $DCF^+$ in the population of viable sperm with high levels of peroxides ($DCF^+/PI^-$) was significantly higher ($p < 0.05$) in transparent, irradiated straws diluted in Equiplus extender than in their respective control (i.e., nonirradiated samples diluted in Equiplus) and transparent, irradiated straws diluted in Kenney extender.

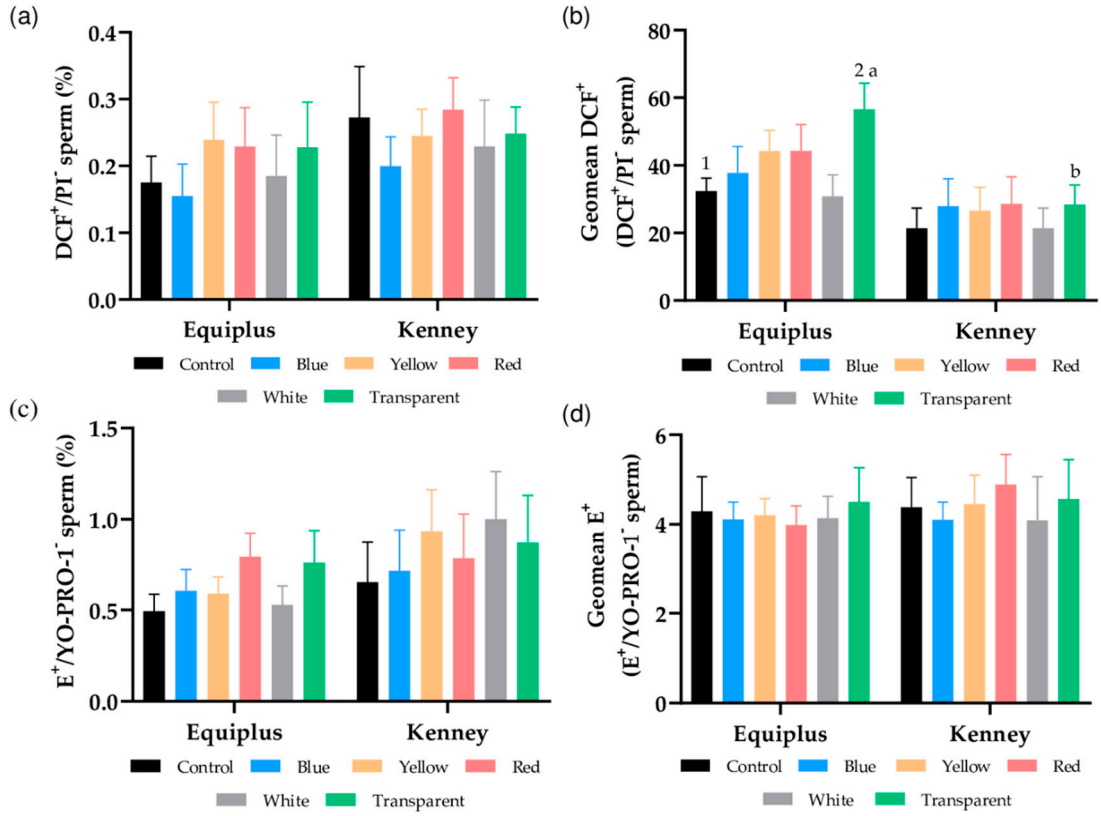


**Figure 3.** Effects of the color of the straw, extender, and light stimulation on intracellular ROS levels in control (nonirradiated) and irradiated samples packed into straws of different color and extended either with Equiplus or Kenney extender. Data are shown as (**a**) percentages of viable spermatozoa with high peroxide levels ($DCF^+/PI^-$); (**b**) geometric mean of $DCF^+$-intensity (GMFI, FL1 channel) in the population of viable spermatozoa with high peroxide levels ($DCF^+/PI^-$); (**c**) percentages of viable spermatozoa with high superoxide levels ($E^+$/YO-PRO-$1^-$); (**d**) geometric mean of $E^+$-intensity (GMFI, FL3 channel) in the population of viable spermatozoa with high superoxide levels ($E^+$/YO-PRO-$1^-$). Different numbers (1, 2) indicate significant differences ($p < 0.05$) between nonirradiated and irradiated samples packed into straws of different color within the same diluent. Different letters (a, b) indicate significant differences ($p < 0.05$) between the two extenders within nonirradiated or samples irradiated packed into straws of different color. The absence of numbers or letters indicates the lack of statistical difference between irradiated and nonirradiated samples within the same diluent or when comparing a given treatment between Kenny and Equiplus extenders. Data are shown as mean ± SEM of 13 separate experiments.

As shown in Figure 3c, percentages of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$) and GMFI of $E^+$ in the population of viable spermatozoa with high levels of superoxide (Figure 3d) did not differ ($p > 0.05$) between irradiated and nonirradiated samples, regardless of the color of the straw and the extender used.

### *3.6. Intracellular Calcium Levels*

Percentages of viable sperm with high intracellular calcium levels ($Fluo3^+/PI^-$; Figure S4a) did not differ between nonirradiated and irradiated samples, regardless of the color of the straw and the extender used (e.g., nonirradiated samples diluted in Equiplus: 0.5% ± 0.1% vs. sperm diluted in Equiplus, packed into red straws, and irradiated: 0.8% ± 0.1% vs. samples diluted in Kenney, packed into red straws, and irradiated: 0.8% ± 0.2%). Similar results were observed for the GMFI of $Fluo3^+$ in the viable sperm population with high intracellular calcium levels (Figure S4b; e.g., nonirradiated sperm diluted in Kenney: 4.4 ± 0.2 vs. sperm diluted in Kenney, packed into white straws, and irradiated: 4.1 ± 0.2 vs. sperm diluted in Equiplus, packed into white straws, and irradiated: 4.1 ± 0.1).

## 4. Discussion

The results of this study agree with previous research, as irradiation with LED-based red light was found to modify some sperm motion variables and increase mitochondrial membrane potential and intracellular ROS of horse sperm without affecting the integrity of the plasma membrane and acrosome. The most remarkable and novel finding, however, was that these effects varied with the color of the straw used to pack sperm before irradiation and with the turbidity of the extender.

Regarding the effects on sperm motility, red light stimulation did not affect TMOT or PMOT, regardless of the color of the straw or the type of diluent used, which agrees with the data reported for dogs [17,18], bulls [47], and horses [20,21]. However, other studies found that irradiation of sperm with red light increases total and progressive motility in humans [7–11], buffaloes [19], sheep [4], pigs [13], and donkeys [3]. In evaluating the presence of motile subpopulations of sperm in horse ejaculates, we identified four separate subpopulations. These results are similar to those previously reported for this species [21,48]. In addition to this, we observed that the percentages of sperm belonging to SP1, which was the fastest subpopulation according to VCL, VSL, and VAP, were significantly higher in irradiated samples that were either diluted with Equiplus extender and packed into blue, yellow, and red straws or diluted with Kenney extender and packed into transparent straws. Furthermore, samples diluted in Equiplus extender and packed into red, yellow, and transparent straws showed significantly lower percentages of sperm belonging to SP2 (the slowest subpopulation) than the control. Therefore, our data confirm the results obtained in previous studies, where irradiation with red light was found to modify the structure of motile sperm subpopulations by decreasing the percentages of the slowest sperm subpopulation [21] and increasing those of the fastest one [18,21,22]. Moreover, we observed that light-stimulation increased some kinetic measures, which agrees with the data reported for other species such as humans [9,34], dogs [17,18], cattle [47], buffaloes [19], pigs [13], donkeys [3,22], and horses [20,21]. These observed differences reinforce the hypothesis that the effects of red light on spermatozoa depend on the specific irradiation pattern [3,13,22,29] and also differ between species [3,5,20–22]. At this point, the increase of VCL, VSL, and VAP observed in sperm diluted in Equiplus extender, packed into red straws, and irradiated and the increase of VCL and VAP found in semen diluted in Kenney extender, packed into transparent straws, and irradiated should be emphasized. Moreover, STR also increased in sperm diluted in Kenney extender, packed into blue straws, and irradiated. All these data suggest that the effects of red light on sperm depend on the color of the straw and the medium used. Based on these results, it is reasonable to surmise that the color of the straw and the turbidity of the extender modify the amount of light/energy that reaches the sperm cells.

At present, there is no clear explanation of how irradiation affects these sperm motion measures as the exact mechanism(s) through which red light stimulates sperm still remains unknown. However, one of the established hypotheses postulates that red light may boost mitochondrial activity, which could be relevant to explain the effects observed in sperm kinetics. Related to this, our data on the analysis of mitochondrial membrane potential (JC1) would agree with this possibility because there was an increase in the percentages of sperm with high mitochondrial membrane potential in samples packed into transparent and red straws and irradiated, regardless of the extender used (Equiplus or Kenney). This matches with Siqueira et al. [47], who found that irradiation of bovine sperm with a He-Ne laser at a wavelength of 633 nm increases the percentage of sperm cells with high mitochondrial membrane potential, and with Yeste et al. [13], who observed that irradiation with red LED light at a wavelength between 620–630 nm augments the percentages of pig sperm with high mitochondrial membrane potential. All these data suggest that red light stimulation could increase mitochondrial activity through photosensitizers present in the electronic chain, such as cytochrome C [13,22,29,49], which would underlie the increase observed in sperm motility.

In addition to the aforementioned, because ROS are mainly generated in the mitochondria as a byproduct of the electronic chain and following the previously established hypothesis, which points out that one of the first effects of light on sperm is the production of ROS [5,50], the generation of intracellular peroxide and superoxide levels was also evaluated. While sperm irradiation did not affect superoxide generation, we found an increase in the levels of peroxides in those irradiated after dilution in Equiplus and packing into transparent straws. This rise in intracellular ROS levels agrees with Zan-Bar et al. [5], Catalán et al. [20], and Cohen et al. [50], who suggested that ROS formation would be mediated through specific endogenous cellular photosensitizers such as mitochondrial cytochromes. In this sense, it has been reported that although an excess of ROS production produced by irradiation with light could be detrimental to sperm cells [5,50], low ROS levels are beneficial for sperm motility and fertilizing ability [5]. Whilst more studies are needed to set a relationship between fertilization ability and high mitochondrial membrane potential, intracellular ROS, and sperm motility, $H_2O_2$ has been suggested to be the active molecule involved in the light-mediated changes of sperm fertilizing capacity [50], which is consistent with Zan-Bar et al. [5] and de Lamirande et al. [51], who indicated that low concentrations of ROS participate in the signaling transduction pathways related to sperm capacitation and acrosomal reaction. Therefore, ROS can have both harmful and beneficial effects on sperm, and the delicate balance between the amounts of ROS produced and ROS scavengers at any time point determines whether a particular sperm function parameter is compromised or boosted [50]. In this sense, the extent of increase in intracellular ROS levels observed in this study was not enough to negatively affect sperm motility and viability, which is similar to that reported by Catalán et al. [3] in a study conducted with fresh and cooled-stored donkey semen. This increase in intracellular ROS (peroxides), observed herein after irradiation, together with the variation seen due to the color of the straw and the extender used, was concomitant with a rise in mitochondrial activity. These findings reinforce the conjecture that ROS formation caused by light would be mediated by specific endogenous cellular photosensitizers such as mitochondrial cytochromes. Furthermore, cytochrome complexes are also known to be implicated in the intrinsic apoptotic pathway [52], and both ROS generation and modulation of apoptotic-like changes are crucial to cause and control sperm capacitation [53]. Therefore, red light-induced changes in cytochrome C complex activity could ultimately affect sperm capacitation and survival. Surprisingly, however, our results did not show an increase in intracellular calcium levels, which is a crucial secondary messenger involved in the modulation of sperm motility and capacitation [54,55]. Related to this, it is worth noting that our data differ from those reported in previous studies, where light-stimulation was found to increase intracellular levels of calcium [30,50]. This could be explained by different conditions of time and intensity of radiation between the current study and the others, as previous research indicates that sperm irradiation can have stimulatory or inhibitory effects on calcium transport, depending on the intensity of the light used [56].

Regarding the effects of irradiation on the integrity of the plasma membrane, no significant differences were found between irradiated and nonirradiated samples. These results were similar to those reported by Yeste et al. [13] and Pezo et al. [14] in pigs and by Catalán et al. in horses and donkeys [3,20,22]. Similarly, no negative impact of irradiation on acrosomal integrity was observed, which concurs with previous studies in rabbits [16], pigs [13,57], and donkeys [22]. This supports the idea that under the conditions tested herein, stimulation of sperm with red light is safe and can have a positive effect on sperm motility and mitochondrial membrane potential, in agreement with Gabel et al. [32].

Finally, the differences observed in this study between straw colors and extenders with regard to mitochondrial activity, intracellular levels of peroxides, and motility suggest that these two factors also influence the sperm response to light. In fact, the impact of red light on mammalian sperm has been previously reported to rely on the precise rhythm and intensity of light [13] and the functional status of the cell [3]. In agreement with this and with the hypothesis that light acts on endogenous cellular photosensitizers of

mitochondria, it is reasonable to suggest that the energy supplied to the mitochondrial electron chain by red light is proportional to the exposure time and the intensity of the light used. The final consequence of this phenomenon would be that the color of the straw and the opacity/turbidity of the medium influence the intensity of the light that feeds mitochondria, which would generate a different effect on sperm cells.

## 5. Conclusions

Our results confirm that LED-based red light irradiation increases some sperm motion variables, mitochondrial membrane potential, and intracellular ROS without affecting the integrity of the sperm membrane and acrosome. However, these effects vary with the color of the straw and the extender/medium used. Given that increased motility and intracellular ROS levels are concomitant with a rise in mitochondrial activity, we suggest that the impact of irradiation on sperm depends on the precise rates of energy provided by the light that feeds the mitochondria. Remarkably, such an energy rate, sensed by mitochondrial photosensitizers, varies with the color of the straw and the extender/medium used, so that these two aspects have to be taken into consideration when sperm are irradiated. In effect, as could be observed in this study, the greatest effects were obtained in samples diluted in Equiplus extender, packed into red straws, and irradiated and samples diluted in Kenney extender, packed into transparent straws, and irradiated.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2076-2615/11/1/122/s1: Figures S1–S3.

**Author Contributions:** Conceptualization, S.B., J.E.R.-G., J.M., and M.Y.; methodology, J.C., I.Y.-O., S.G., and M.P.; validation, J.E.R.-G., J.M., and M.Y.; formal analysis, J.C.; investigation, J.C., S.G., M.P., J.E.R.-G., M.Y., and J.M.; resources, S.B., J.E.R.-G., J.M., and M.Y.; data curation, J.C.; writing—original draft preparation, J.C.; writing—review and editing, S.B., J.E.R.-G., J.M., and M.Y.; supervision, S.B., J.E.R.-G., J.M., and M.Y.; project administration, S.B., J.E.R.-G., J.M., and M.Y.; funding acquisition, S.B., J.E.R.-G., J.M., and M.Y. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee, Autonomous University of Barcelona (Code: CEEAH 1424).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

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**Conflicts of Interest:** J.E.R.-G. and M.Y. are inventors of a patent entitled "Method and apparatus for improving the quality of mammalian sperm" (European Patent Office, No. 16199093.2; EP-3-323-289-A1), which is owned by Instruments Útils de Laboratori Geniul, SL (Barcelona, Spain).

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# 5. Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light

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# Red LED Light Acts on the Mitochondrial Electron Chain of Donkey Sperm and Its Effects Depend on the Time of Exposure to Light

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This work aimed to investigate how stimulation of donkey sperm with red LED light affects mitochondrial function. For this purpose, freshly diluted donkey semen was stimulated with red light for 1, 5, and 10 min, in the presence or absence of oligomycin A (Omy A), a specific inhibitor of mitochondrial ATP synthase, or FCCP, a specific disruptor of mitochondrial electron chain. The results obtained in the present study indicated that the effects of red LED light on fresh donkey sperm function are related to changes in mitochondria function. In effect, irradiation of donkey sperm resulted in an increase in mitochondrial membrane potential (MMP), the activity of cytochrome C oxidase and the rate of oxygen consumption. In addition, in the absence of oligomycin A and FCCP, light-stimulation augmented the average path velocity (VAP) and modified the structure of motile sperm subpopulations, increasing the fastest and most linear subpopulation. In contrast, the presence of either Omy A or FCCP abolished the aforementioned effects. Interestingly, our results also showed that the effects of red light depend on the exposure time applied, as indicated by the observed differences between irradiation protocols. In conclusion, our results suggest that exposing fresh donkey sperm to red light modulates the function of their mitochondria through affecting the activity of the electron chain. However, the extent of this effect depends on the irradiation pattern and does not exclude the existence of other mechanisms, such as those related to thermotaxis.

**Keywords: sperm, red light stimulation, mitochondrial function, donkey, oligomycin A, FCCP**

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## INTRODUCTION

In recent years, the use and development of artificial insemination (AI) in equine species has grown considerably (Canisso et al., 2008; Crowe et al., 2008; Squires, 2009), and is currently being considered as the basis of modern equine reproduction worldwide (Pagl et al., 2006; Kowalczyk et al., 2019). However, while a significant number of previous studies have described

and characterized the semen of domestic horses, studies on donkey sperm are scarce (Miró and Papas, 2018; Catalán et al., 2020a). In addition to this, at present, the most of European donkey breeds are in danger of extinction, which added to a mounting world demand for new donkey uses (milk, cosmetics or skin production, oncotherapy, silviculture, rural tourism. . .). For these reasons, the interest in developing studies to improve the reproductive performance in this species has increased (Miró et al., 2020). At this respect, it is worth noting that while both horses and donkeys are phylogenetically close species, they show important reproductive differences, and not only do their sperm vary in motility and morphology but also on how they interact with the female endometrium (Miró and Papas, 2018). Be that as it may, the fact that AI has been largely developed in the horse over the last years has fostered the application of those techniques in the donkey, which necessarily entails the need of developing methods, protocols or procedures aimed at optimizing sperm survival and fertilizing capacity, as in the horse (Loomis, 2006; Varner, 2016).

Irradiation of sperm with red light (laser and LED) has been demonstrated to jointly increase sperm motility and mitochondrial activity in sheep, cattle, pigs, donkeys and horses (Iaffaldano et al., 2016; Siqueira et al., 2016; Yeste et al., 2016; Catalán et al., 2020a,b,c). These effects seem, however, to rely upon the light-stimulation pattern, species and other factors, since while it has also been found to increase the *in vivo* fertilizing ability in pigs (Yeste et al., 2016; Blanco-Prieto et al., 2019), its use in separate farms around the world brings significant differences (Blanco-Prieto et al., 2019). In addition, the literature remains inconsistent on how irradiation affects sperm function and, at present, the mechanisms underlying that impact on mammalian spermatozoa remain largely unknown (reviewed in Yeste et al., 2018). Hence, further studies aimed at addressing the machinery that potentially guides the response of sperm to red light in separate species are warranted. In this context, the donkey could be an interesting model since previous studies have demonstrated that irradiation affects some sperm parameters in this species (Catalán et al., 2020a).

At present, three mechanisms have been suggested as being related to the response of sperm to red light (Yeste et al., 2018). The first hypothesizes that irradiation is linked to thermotaxis, via its interaction with specific members of the Transient Receptor Potential (TRP) family. While the TRP family is a highly heterogeneous class of plasma membrane receptors, only those belonging to vanilloid TRP (TRPV), ankyrin TRP (TRPA), and melastanin TRP (TRPM) subfamilies have been reported to be involved in the control of thermotaxis (Vriens et al., 2014). Remarkably, ion channels are crucial during sperm capacitation (Lishko et al., 2012; Singh and Rajender, 2014; Sun et al., 2017; Mundt et al., 2018), and TRPV4 has been linked to the thermotactic response both in mouse (Hamano et al., 2016) and human sperm (Mundt et al., 2018). A second potential mechanism envisages the participation of opsins, which are coupled to a G-protein (transducin) and also reside in the plasma membrane of mammalian sperm (Pérez-Cerezales et al., 2015). The different members of this family of proteins (melanopsin, encephalopsin, rhodopsin, and neuropsin) appear to work as thermosensitizers rather than light-sensitive intracellular proteins (photosensitizers), via canonical phospholipase C (PLC) and cyclic nucleotide pathways (cAMP/cGMP; Pérez-Cerezales et al., 2015). In effect, opsins, especially melanopsin and rhodopsin, are involved, along with the aforementioned TRPV proteins, in the sperm response to thermotaxis (Zheng, 2013; Pérez-Cerezales et al., 2015; Roy et al., 2020). Interestingly, a recent study has demonstrated that rhodopsin and melanopsin trigger a different signaling pathway but, unlike vision, both types of opsin coexist in the same sperm cells (Roy et al., 2020). The third hypothesis considers the role of endogenous photosensitizers, particularly cytochromes residing in the mitochondria (Yeste et al., 2018). It is widely known that cytochromes are an essential component of the mitochondrial electron chain and control oxidative phosphorylation, generation of reactive oxygen species and apoptosis (Ortega-Ferrusola et al., 2009). Cytochromes have a heme group, which not only accepts and donates electrons (Kessel, 1982; Pottier and Truscott, 1986), but is sensitive to light. However, not all cytochromes react against the same wavelengths (Lynch and Copeland, 1992). Thus, whereas cytochrome P450, which is also present in the endoplasmic reticulum of somatic cells, has its highest absorption peak at 450 nm, cytochrome C, which forms the complex IV of the mitochondrial electron chain, has two peaks at 610–630 nm and 660–680 nm (Lynch and Copeland, 1992). However, and despite unpublished data supporting the relevance of mitochondria in pig sperm (Blanco-Prieto et al., personal communication), one should not rule out that more than one of these hypothetical mechanisms could be involved in the sperm response to red light, as well as that the potential influence of light on the conformation of other proteins might also be implied in that response (Yeste et al., 2018).

Against this background, the objective of this study was to determine if the previously reported effects of light stimulation in donkey sperm (Catalán et al., 2020a) are related to changes caused by the action of light on mitochondrial function, since this is one of the hypothetical mechanisms of action. Our hypothesis is that the effects of irradiating donkey sperm with red-light are driven by changes in mitochondrial function. For this reason, freshly diluted donkey semen was irradiated with red light for 1, 5, and 10 min, in the presence/absence of Omy A, a specific inhibitor of the mitochondrial ATP synthase activity, or FCCP, which acts as a disruptor of the electron chain function. If mitochondria are involved in the sperm response to irradiation, utilizing these two effectors should affect the impact from red light. In addition, the use of both oligomycin A and FCCP should allow elucidating the exact part of the mitochondrial electron chain that is involved in the sperm response to light, and should also contribute to shed light onto the different effects between species.

# MATERIALS AND METHODS

## Animals, Semen Samples, and Ethics

Eight ejaculates, each coming from a separate jackass, were used. Animals were allocated to individual paddocks at the Equine Reproduction Service, Autonomous University of Barcelona

(Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. Jackasses were adult, healthy and of proven fertility, and were fed grain forage, straw and hay, with water being provided *ad libitum*.

Ejaculates were collected through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) and an in-line nylon mesh filter to remove the gel fraction. Upon collection, gel-free semen was diluted 1:5 (v:v) with Kenney extender (Kenney et al., 1975), previously preheated to 37°C. Thereafter, a conventional spermiogram, based on total ejaculate volume, sperm concentration (Neubauer chamber, Paul Marienfeld GmbH, and Co. KG; Lauda-Königshofen, Germany), motility (Computer Assisted Semen Analysis, CASA), morphology (eosin-nigrosin staining) and plasma membrane integrity (SYBR14/PI), was performed. All samples were confirmed to be above the standard thresholds ($\geq$70% of total motility and $SYBR14^+/PI^-$ sperm and $\geq$ 70% morphologically normal sperm).

All jackasses used in this study were semen donors and no manipulation to animals, apart from semen collection in the authorized center, was made. The animal study was reviewed and approved by the Ethics Committee for Animal and Human Experimentation (CEEAH) of our institution (Autonomous University of Barcelona; authorization code: CEEAH 1424).

## Experimental Design

Prior to light-stimulation, sperm concentration was adjusted to $30 \times 10^6$ spermatozoa/mL with Kenney extender using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Königshofen, Germany). Following this, each semen sample was split into separate aliquots of 1.5 mL that were subjected to three red light irradiation protocols with an air-refrigerated red LED light system (PhastBlue®, IUL, S.L.; Barcelona, Spain; wavelength range: 620–630 nm; intensity per sample: 35.05 $W/m^2$). These three protocols consisted of exposing sperm to red light for 1 min (P1), 5 min (P5) or 10 min (P10). In all cases, the temperature within the PhastBlue® system was maintained at 20°C ± 0.1°C. The control consisted of 1.5 mL tubes kept at 20°C in the dark for 10 min. In addition to the aforementioned, semen samples were also exposed to the same three protocols (i.e., P1, P5, and P10) or non-exposed (control), in the presence of either 5 μM oligomycin A or 5 μM FCCP. In order to achieve a maximal effect of both Omy A and FCCP, these two molecules were added to semen samples 10 min prior to exposure to red light. Following exposure to red light, sperm motility was evaluated through a computer-assisted sperm analysis system (CASA); sperm membrane integrity, mitochondrial membrane potential (MMP), and intracellular ROS and calcium levels were determined through flow cytometry; $O_2$ consumption rate; intracellular ATP levels and total cytochrome C oxidase (CCO) activity were also assessed. In the case of intracellular ATP levels and total CCO activity, samples were first centrifuged at 2,000 × *g* and 20°C for 30 s; the resulting pellets were immediately frozen by plunging them into liquid $N_2$. Samples were stored at −80°C until use.

## Sperm Motility

As indicated above, sperm motility was evaluated using a CASA system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). For this purpose, samples were incubated in a water bath at 38°C for 5 min prior to analysis. Five microliter of each sperm sample was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel) previously warmed at 38°C. Samples were observed under a 10 × negative phase-contrast objective (Olympus BX41 microscope; Olympus Corporation, Tokyo, Japan), and at least 1,000 sperm cells were counted per analysis. In each evaluation, percentages of total (TMOT) and progressively motile spermatozoa (PMOT) were recorded along with the following kinetic parameters: curvilinear velocity (VCL, μm/s), which is the mean path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, μm/s), which is the mean path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, μm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of linearity (LIN,%), which is the quotient between VSL and VCL multiplied by 100; percentage of straightness (STR,%), which is the quotient between VSL and VAP multiplied by 100; percentage of oscillation (WOB,%), which is the quotient between VAP and VCL multiplied by 100; mean amplitude of lateral head displacement (ALH, μm), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle; and frequency of head displacement (BCF, Hz), which is the frequency at which the actual sperm trajectory crosses the average path trajectory (Hz).

Settings of the CASA system were those recommended by the manufacturer: frames/s: 25 images captured per second; particle area > 4 and < 75 $\mu m^2$; connectivity: 6; minimum number of images to calculate the ALH: 10. Cut-off values were VAP ≥ 10 μm/s for TMOT, and STR ≥ 75% for PMOT. In addition, individual kinematic parameters (VSL, VCL, VAP, LIN, STR, and BCF) were used to determine motile sperm subpopulations. Three replicates were evaluated before calculating the corresponding mean ± SD.

### Flow Cytometry

Flow cytometry was used to determine plasma membrane integrity, MMP, and intracellular levels of superoxides, peroxides and calcium, following the recommendations set by the International Society for Advancement of Cytometry (ISAC; Lee et al., 2008). In all analyses, sperm concentration was previously adjusted to $1 \times 10^6$ 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, United States), 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). When required, 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, United States) and diluted with dimethyl sulfoxide (DMSO).

### Plasma Membrane Integrity

Sperm membrane integrity was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR14/PI; Molecular Probes, Thermo Fisher Scientific), according to the protocol described by Garner and Johnson (1995) and adapted to donkey spermatozoa. In brief, samples were first incubated with SYBR14 (final concentration: 100 nM) at 38°C for 10 min, and then with PI (final concentration: 12 μM) at 38°C for 5 min. Three sperm populations were distinguished: (i) viable spermatozoa emitting green fluorescence ($SYBR14^+/PI^-$), which appeared on the right side of the lower half of FL1/FL3 dot plots; (ii) non-viable spermatozoa emitting red fluorescence ($SYBR14^-/PI^+$), which appeared on the left side of the upper half of FL1/FL3 dot plots; and (iii) non-viable spermatozoa emitting both green and red fluorescence ($SYBR14^+/PI^+$), which appeared on the right side of the upper half of FL1/FL3 dot plots. Non-stained particles ($SYBR14^-/PI^-$), which appeared on the left side of the lower half of FL1/FL3 dot plots, had similar EV/SS distributions than spermatozoa and were considered as non-DNA, debris particles. Percentages of non-stained particles were used to correct the percentages of double-negative sperm populations in the other assessments. Spill-over of FL1- into FL3-channel was compensated (2.45%).

### Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined through incubation with JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl−benzimidazolylcarbocyanine iodide; final concentration: 0.5 μM) at 38°C for 30 min in the dark. At low MMP, JC1 remains as a monomer ($JC1_{mon}$) emitting green fluorescence, which is detected through FL1. At high MMP, JC1 forms aggregates ($JC1_{agg}$) emitting orange fluorescence, which is collected through FL2. Three sperm populations were distinguished: (i) spermatozoa with green-stained mitochondria (low MMP); (ii) spermatozoa with heterogeneous mitochondria stained both green and orange in the same cell (intermediate MMP); and (iii) spermatozoa with orange-stained mitochondria (high MMP). Ratios between $JC1_{agg}$ (FL2) and $JC1_{mon}$ (FL1) fluorescence for each of these sperm populations were also evaluated. Spill-over of FL1- into FL2-channel was compensated (68.50%). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of spermatozoa with low MMP, and percentages of all sperm populations were recalculated.

### Analysis of Intracellular ROS Levels: $H_2O_2$ and $\cdot O_2^-$

Intracellular ROS levels were determined through two oxidation sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) and hydroethidine (HE), which detect hydrogen peroxide ($H_2O_2$) and superoxide anion ($\cdot O_2^-$), respectively (Murillo et al., 2007). Sperm were counterstained with PI ($H_2$DCFDA) or YO-PRO-1 (HE), following a protocol modified from Guthrie and Welch (2006).

With regard to peroxides, spermatozoa were incubated with $H_2$DCFDA (final concentration: 200 μM) and PI (final concentration: 12 μM) at room temperature for 30 min in the dark. $H_2$DCFDA is a stable, cell-permeable, non-fluorescent probe that, in the presence of $H_2O_2$, is deesterified and converted into 2′,7′-dichlorofluorescein (DCF). Fluorescence of DCF and PI were measured through FL1 and FL3 detectors, respectively. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of peroxides ($DCF^-/PI^-$); (ii) viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$); (iii) non-viable spermatozoa with low levels of peroxides ($DCF^-/PI^+$); and (iv) non-viable spermatozoa with high levels of peroxides ($DCF^+/PI^+$). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of viable spermatozoa with low levels of peroxides ($DCF^-/PI^-$), and percentages of all sperm populations were recalculated. Spill-over of FL1- into the FL3-channel was compensated (2.45%). Data are shown as corrected percentages of viable spermatozoa with high levels of peroxides ($DCF^+/PI^-$), and geometric mean of $DCF^+$-fluorescence intensity in the $DCF^+/PI^-$ sperm population.

As far as superoxides are concerned, samples were incubated with HE (final concentration: 4 μM) and YO-PRO-1 (final concentration: 25 nM) at room temperature for 30 min in the dark (Guthrie and Welch, 2006). Hydroethidine diffuses freely through plasma membrane and converts into ethidium ($E^+$) in the presence of superoxide anions ($\cdot O_2^-$) (Zhao et al., 2003). Fluorescence of ethidium (E) was detected through FL3 and that of YO-PRO-1 was detected through FL1. Four sperm populations were distinguished: (i) viable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^-$); (ii) viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$); (iii) non-viable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^+$); and (iv) non-viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^+$). Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from those of viable spermatozoa with low levels of superoxides ($E^-$/YO-PRO-$1^-$), and percentages of all sperm populations were recalculated. Spill-over of FL3-into the FL1-channel was compensated (5.06%). Data are shown as corrected percentages of viable spermatozoa with high levels of superoxides ($E^+$/YO-PRO-$1^-$), and geometric mean of $E^+$-fluorescence intensity in the $E^+$/YO-PRO-$1^-$ sperm population.

### Intracellular Calcium Levels

Previous studies found that Fluo3 mainly stains mitochondrial calcium in pig sperm (Yeste et al., 2015). For this reason, we combined this fluorochrome with PI (Fluo3/PI), as described by Kadirvel et al. (2009). Four sperm 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 ($Fluo3^+/PI^+$). FL3 spill-over into the FL1-channel (28.72%) and FL1 spill-over into the FL3-channel (2.45%) were compensated.

### Determination of Intracellular ATP Levels

Intracellular ATP levels were determined following the protocol set by Chida et al. (2012). Immediately after light-stimulation, 1-mL semen aliquots were centrifuged at 17°C for 30 s; pellets were plunged into liquid $N_2$, and frozen pellets were subsequently stored at −80°C for a maximum of 3 weeks. Pellets were then 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), and subsequently sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070; Bandelin Electronic GmbH and Co., Berlin, Germany). During this process, tubes were kept on ice to avoid specimen heating. Next, samples were centrifuged at 1,000× g and 4°C for 10 min, supernatants were harvested for further use and pellets were discarded. Twenty μL of supernatant was used to determine total protein content, and the remaining volume was mixed with 300 μL ice-cold 10% (v:v) trichloroacetic acid and held at 4°C for 20 s. Samples were subsequently centrifuged at 1,000 × *g* and 4°C for 30 s, and supernatants were carefully separated from the pellet and again centrifuged at 1,000 × *g* and 4°C for 10 min. Supernatants were mixed with two volumes of 1 M Tris-acetate buffer (pH = 7.75), and the resulting suspension was used to determine the ATP content using the Invitrogen® ATP Determination Kit (Thermo Fisher Scientific, Waltham, United States; catalog number: A22066). Determinations of ATP content were carried out with an Infinite F200 fluorimeter (TECAN®), using 96-wells microplates for fluorescence-based assays (Invitrogen®). Data were normalized against the total protein content determined with the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad laboratories; Hercules, CA, United States).

### Determination of $O_2$ Consumption Rate

Determination of $O_2$ consumption rate was performed through the SensorDish® Reader (SDR) system (PreSens Gmbh; Regensburg, Germany). After light-stimulation, 1 mL of each sperm sample was transferred onto an Oxodish® OD24 plate (24 wells/plate). Plates were sealed with Parafilm®, placed within the SDR system, and incubated at 37°C (controlled atmosphere) for 2 h. During that time, $O_2$ concentration was recorded in each well at a rate of one reading/min. Data were exported to an Excel file 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) as described above.

### Determination of Cytochrome C Oxidase Activity

The CCO activity 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 17°C for 30 s. The resulting pellets were immediately plunged into liquid $N_2$ and stored for 3 weeks. Pellets were resuspended in 500 μL ice-cold PBS and sonicated (10 kHz, 20 pulses; Bandelin Sonopuls HD 2070). Thereafter, 500 μL Percoll® (concentration: 1.055 mg/mL in PBS) at 4°C was placed onto each sperm homogenate. Samples were centrifuged at 3,000 × *g* and 10°C 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 20°C for 2 min and the resulting pellets were resuspended in 100 μL PBS at 20°C. 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; catalog number CYTOCOX1). Enzyme activity was normalized against the total protein content. Therefore, the other aliquot (10 μL) was used to determine total protein content through a commercial kit (Bio-Rad laboratories) based on the Bradford method (Bradford, 1976).

## Statistical Analysis

Statistical analyses were conducted using a statistical package (SPSS® Ver. 25.0 for Windows; IBM Corp., Armonk, NY, United States). Data were first tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) and, if required, they were transformed with arcsin $\sqrt{x}$. The effects of irradiating donkey with red light and the presence of oligomycin A/FCCP were tested on sperm motility parameters, percentages of spermatozoa with an intact plasma membrane ($SYBR14^+/PI^-$), spermatozoa with high and intermediate MMP, 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 intensities (GMFI) of $JC1_{agg}$, $Fluo3^+$, $E^+$ and $DCF^+$; $JC1_{agg}/JC1_{mon}$ GMFI-ratios; intracellular levels of ATP; $O_2$ consumption rate; and cytochrome C oxidase activity were evaluated through two-way analysis of variance (ANOVA; factor 1: irradiation protocol; factor 2: presence/absence of Omy A or FCCP) followed by *post hoc* Sidak test for pair-wise comparisons.

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 a regression score for each of 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 Bayesian Criterion. Four sperm subpopulations were identified and each individual spermatozoon was assigned to one of these subpopulations (SP1, SP2, SP3, or SP4). Following this, percentages of spermatozoa belonging to each subpopulation were calculated per sample and used to determine

the effects of irradiation and Omy A/FCCP on the distribution of motile sperm subpopulations through two-way ANOVA followed by Sidak test.

In all analyses, the level of significance was set at $P \leq 0.05$. Data are shown as mean ± standard deviation (SD), median and interquartile range (i.e., Q1 and Q3).

## RESULTS

### Effects of Red Light Stimulation on Plasma Membrane Integrity in the Presence or Absence of Either Omy A or FCCP

Irradiating donkey sperm with red light did not affect sperm membrane integrity, as no significant differences between the control and light-stimulation protocols were observed with regard to the percentages of membrane-intact spermatozoa ($SYBR14^+/PI^-$; **Figure 1**), neither did the presence of Omy A or FCCP alter that parameter.

### Effects of Red Light Stimulation on Sperm Motility in the Presence or Absence of Either Omy A or FCCP

As shown in **Figure 2**, percentages of TMOT (**Figure 2A**) and PMOT (**Figure 2B**) did not differ between non-irradiated and irradiated sperm samples, in the presence/absence of either Omy A or FCCP. Moreover, the presence of Omy A or FCCP, irrespective of irradiation/non-irradiation, led to a significant ($P < 0.05$) decrease in total (**Figure 2A**) and progressive (**Figure 2B**) sperm motility.

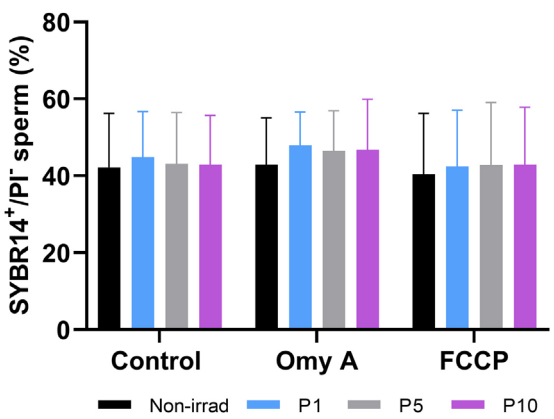


**FIGURE 1 |** Effects of light-stimulation on the percentages of membrane-intact sperm ($SYBR14^+/PI^-$) in the presence/absence of Omy A or FCCP. No significant differences between non-irradiated and irradiated sperm in the presence/absence of oligomycin A or FCCP were observed, nor between samples in the presence of Omy A or FCCP and the treatment without the two disruptors for a given light-stimulation protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

Regarding the effects of light-stimulation on sperm kinetic parameters (**Table 1**), irradiation for 5 min and 10 min significantly ($P < 0.05$) increased VAP, when Omy A and FCCP were absent. In contrast, the presence of Omy A or FCCP in non-irradiated samples led to a significant ($P < 0.05$) decrease in VCL, VSL, VAP, LIN, and WOB (**Tables 1A,B**). Moreover, treatments with Omy A and FCCP showed significantly ($P < 0.05$) higher values of BCF (**Table 1C**) and STR in non-irradiated and irradiated samples.

Finally, as shown in **Table 2**, four different motile sperm subpopulations were identified in all treatments (SP1, SP2, SP3, and SP4). Interestingly, irradiation was found to affect the proportions of sperm belonging to each motile subpopulation. Light-stimulation of spermatozoa with red light for 1 min significantly increased ($P < 0.05$) the proportions of sperm belonging to SP1 (**Figure 3A**), which was the subpopulation that exhibited the highest kinematic parameters (**Table 2**). Regardless of whether samples were or not irradiated, the presence of Omy A or FCCP significantly ($P < 0.05$) decreased the proportions of sperm belonging to SP1, SP2, and SP4 (**Figures 3A,B,D**) and increased those of sperm belonging to SP3 (**Figure 3C**), which was the motile subpopulation that exhibited the lowest values in most kinematic parameters (**Table 2**).

### Effects of Red Light Stimulation on Mitochondrial Membrane Potential in the Presence or Absence of Either Omy A or FCCP

As shown in **Figure 4A**, irradiation for 1, 5, and 10 min significantly ($P < 0.05$) increased the percentages of spermatozoa with high MMP in samples without Omy A or FCCP. In treatments containing Omy A, irradiation for 1 min or 5 min significantly ($P < 0.05$) augmented the percentages of spermatozoa with high MMP compared to non-irradiated samples. Furthermore, percentages of spermatozoa with high MMP in samples containing FCCP were significantly ($P < 0.05$) higher in samples irradiated for 10 min than in their non-irradiated counterparts. In addition to this, percentages of spermatozoa with high MMP in non-irradiated samples and samples irradiated for 1, 5, or 10 min were significantly lower ($P < 0.05$) when FCCP was present than in the absence of this disruptor (**Supplementary Figure 1**).

As shown in **Figure 4B**, no significant differences in the percentages of sperm with intermediate MMP were observed between non-irradiated and irradiated samples, either in the presence or absence of Omy A and FCCP. In addition, no significant differences in the geometric mean of $JC1_{agg}$ intensity (orange, FL2) of sperm populations with high (**Figure 4C**) and intermediate MMP (**Figure 4D**) were observed between irradiated and non-irradiated samples, either in the presence or absence of Omy A or FCCP. However, geometric mean of the intensity of $JC1_{agg}$ (orange, FL2) in the sperm population with high MMP was significantly ($P < 0.05$) lower in the non-irradiated sample containing FCCP than in that without any inhibitor/disruptor (**Figure 4C**).

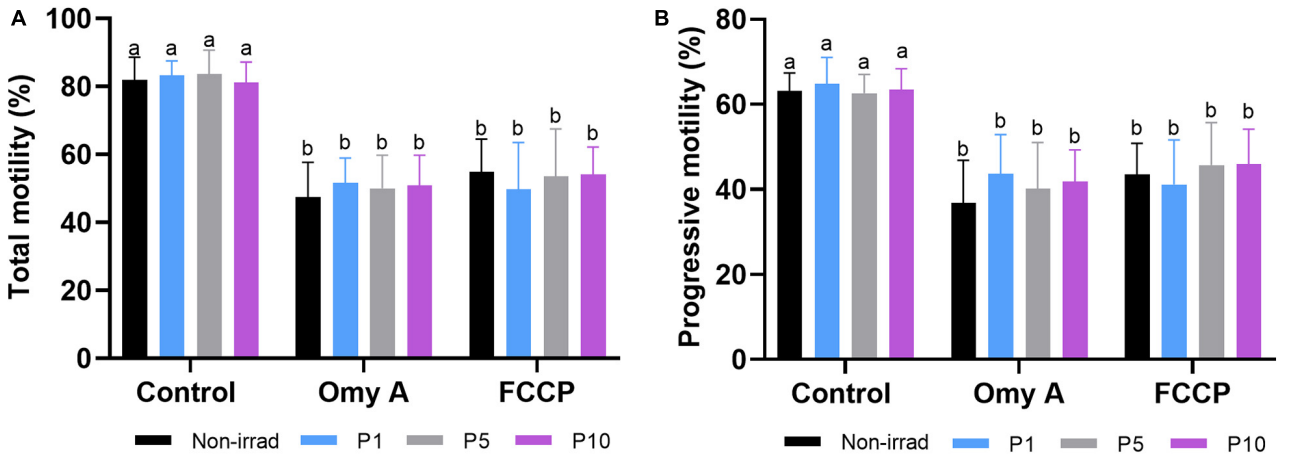


**FIGURE 2 |** Effects of light-stimulation on the percentages of total **(A)** and progressive **(B)** sperm motility in the presence/absence of oligomycin A (Omy A) or FCCP. Different letters (*a b*) indicate significant differences ($P < 0.05$) between oligomycin A or FCCP with respect to the treatment without the two for a given protocol (i.e., control, P1, P5, or P10). No significant differences were found between the samples exposed to the different irradiation times with respect to non-irradiated sperm. Data are shown as mean ± SD from eight separate experiments.

**TABLE 1A |** Kinetic parameters (VSL, VCL, and VAP) of donkey sperm in the control and the different light-stimulation patterns in the presence or absence of oligomycin A (Omy A) and FCCP.

| Treatments | | Kinetic parameters | | | | | |
|---|---|---|---|---|---|---|---|
| | | VCL (μm/s) | | VSL (μm/s) | | VAP (μm/s) | |
| | | Mean ± SD | Q1, median, Q3 | Mean ± SD | Q1, median, Q3 | Mean ± SD | Q1, median, Q3 |
| Control | Control | $130.7 \pm 13.5^{Aa}$ | 110.0, 133.0, 147.5 | $102.1 \pm 10.9^{Aa}$ | 89.0, 105.3, 111.6 | $117.6 \pm 6, 9^{Aa}$ | 101.3, 121.0, 131.1 |
| | Omy A | $66.0 \pm 8.7^{Ab}$ | 55.6, 64.7, 77.1 | $42.4 \pm 8.7^{Ab}$ | 30.8, 44.2, 47.6 | $46.5 \pm 6.0^{Ab}$ | 36.0, 49.1, 53.7 |
| | FCCP | $75.8 \pm 9.8^{Ab}$ | 69.5, 75.9, 87.8 | $44.0 \pm 9.5^{Ab}$ | 35.1, 46.4, 49.9 | $49.2 \pm 6.9^{Ab}$ | 40.7, 52.2, 57.4 |
| P1 | Control | $135.2 \pm 15.6^{Aa}$ | 110.6, 137.4, 156.4 | $105.1 \pm 13.3^{Aa}$ | 88.6, 107.3, 120.5 | $126.3 \pm 9.9^{Aa}$ | 103.2, 129.0, 138.8 |
| | Omy A | $68.4 \pm 8.1^{Ab}$ | 59.4, 66.6, 77.1 | $43.2 \pm 7.4^{Ab}$ | 35.2, 46.9, 50.7 | $47.7 \pm 5.5^{Ab}$ | 39.2, 50.4, 55.0 |
| | FCCP | $73.4 \pm 11.8^{Ab}$ | 63.5, 70.1, 91.5 | $41.2 \pm 10.6^{Ab}$ | 31.9, 45.7, 51.8 | $46.5 \pm 8.9^{Ab}$ | 34.1, 50.8, 58.3 |
| P5 | Control | $139.6 \pm 11.6^{Aa}$ | 123.7, 141.5, 153.4 | $109.2 \pm 8.9^{Aa}$ | 94.9, 108.2, 117.4 | $130.7 \pm 6.2^{Ba}$ | 115.3, 131.8, 142.5 |
| | Omy A | $69.2 \pm 10.8^{Ab}$ | 55.8, 65.9, 82.6 | $44.5 \pm 10.6^{Ab}$ | 35.1, 46.0, 54.4 | $49.5 \pm 5.5^{Ab}$ | 38.9, 51.1, 60.7 |
| | FCCP | $80.4 \pm 12.3^{Ab}$ | 69.2, 81.8, 92.2 | $48.6 \pm 11.5^{Ab}$ | 39.4, 49.6, 54.2 | $53.0 \pm 6.4^{Ab}$ | 43.7, 54.8, 59.2 |
| P10 | Control | $141.2 \pm 10.7^{Aa}$ | 132.6, 142.2, 158.7 | $110.0 \pm 11.6^{Aa}$ | 99.5.0, 111.2, 119.5 | $132.1 \pm 6.4^{Ba}$ | 122.4, 133.9, 142.3 |
| | Omy A | $66.9 \pm 8.1^{Ab}$ | 57.4, 66.2, 76.8 | $41.9 \pm 7.5^{Ab}$ | 34.5, 42.8, 49.8 | $46.1 \pm 5.5^{Ab}$ | 38.4, 47.3, 54.0 |
| | FCCP | $78.2 \pm 11.7^{Ab}$ | 68.4, 76.9, 90.5 | $47.1 \pm 10.6^{Ab}$ | 37.1, 49.1, 53.7 | $54.2 \pm 6.2^{Ab}$ | 41.4, 53.7, 59.3 |

*Different letters (A,B) indicate significant differences ($P < 0.05$) between the control and the different light stimulation patterns used in the presence or absence of oligomycin A (Omy A) or FCCP. Different letters (a,b) indicate significant differences ($P < 0.05$) between Omy A or FCCP with respect to treatment without the two inhibitors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD, median and interquartile range from eight separate experiments.*

Finally, we also evaluated $JC1_{agg}/JC1_{mon}$ ratios of sperm populations with high (**Figure 4E**) and intermediate MMP (**Figure 4F**). No significant differences between non-irradiated and irradiated samples were observed, either in the presence/absence of Omy A/FCCP, or within the same irradiation pattern comparing samples with and without disruptors.

## Effects of Red Light Stimulation on Intracellular ROS Levels in the Presence or Absence of Either Omy A or FCCP

**Figures 5A,B** show the proportions of viable spermatozoa with high intracellular levels of peroxides (% $DCF^+/PI^-$ spermatozoa) and the GMFI of $DCF^+$ in the population of viable sperm with high levels of peroxides ($DCF^+/PI^-$). No significant differences between irradiated and non-irradiated samples were observed, either in the presence or absence of Omy A or FCCP. In addition, no significant differences were found within each irradiation pattern when samples with and without disruptors (Omy A and FCCP) were compared.

Percentages of viable sperm with high levels of superoxides (% $E^+/YO\text{-}PRO\text{-}1^-$ spermatozoa; **Figure 5C**) and GMFI of $E^+$ in the viable sperm population with high levels of superoxides ($E^+/YO\text{-}PRO\text{-}1^-$; **Figure 5D**) did not significantly differ between irradiated and non-irradiated samples, or between treatments with and without disruptors (Omy A and FCCP) within each irradiation pattern.

**TABLE 1B |** Kinetic parameters (LIN, STR and WOB) of donkey sperm in the control and the different light-stimulation patterns in the presence or absence of oligomycin A (Omy A) and FCCP.

| Treatments | | Kinetic parameters | | | | | |
|---|---|---|---|---|---|---|---|
| | | LIN (%) | | STR (%) | | WOB (%) | |
| | | Mean ± SD | Q1, median, Q3 | Mean ± SD | Q1, median, Q3 | Mean ± SD | Q1, median, Q3 |
| Control | Control | $77.8 \pm 3.4^{Aa}$ | 69.6, 79.7, 84.8 | $85.2 \pm 2.1^{Aa}$ | 78.5.6, 87.1, 90.7 | $91.3 \pm 3.6^{Aa}$ | 88.7, 91.1, 96.2 |
| | Omy A | $63.9 \pm 7.7^{Ab}$ | 55.0, 64.6, 72.0 | $90.3 \pm 2.4^{Ab}$ | 87.3, 91.7, 92.8 | $70.6 \pm 8.0^{Ab}$ | 63.1, 70.4, 79.6 |
| | FCCP | $56.2 \pm 7.5^{Ab}$ | 49.3, 59.2, 63.1 | $88.0 \pm 2.9^{Aa}$ | 87.8, 90.4, 90.8 | $62.9 \pm 7.9^{Ab}$ | 56.1, 65.2, 69.6 |
| P1 | Control | $78.3 \pm 2.6^{Aa}$ | 72.9, 77.8, 84.2 | $84.9 \pm 1.8^{Aa}$ | 79.7, 85.2, 90.3 | $92.1 \pm 2.8^{Aa}$ | 91.2, 91.7, 93.3 |
| | Omy A | $63.2 \pm 6.9^{Ab}$ | 56.9, 62.0, 69.1 | $90.4 \pm 1.4^{Ab}$ | 89.2, 90.5, 91.3 | $69.8 \pm 7.9^{Ab}$ | 63.5, 68.9, 76.3 |
| | FCCP | $56.0 \pm 7.8^{Ab}$ | 49.4, 54.7, 61.6 | $88.3 \pm 2.2^{Aa}$ | 87.3, 88.5, 90.1 | $63.3 \pm 8.3^{Ab}$ | 56.7, 61.2, 69.8 |
| P5 | Control | $78.1 \pm 3.2^{Aa}$ | 67.7, 81.1, 86.2 | $84.2 \pm 2.2^{Aa}$ | 78.9, 86.8, 90.7 | $93.0 \pm 3.6^{Aa}$ | 89.1, 91.4, 95.8 |
| | Omy A | $64.3 \pm 6.2^{Ab}$ | 54.6, 67.5, 72.1 | $89.7 \pm 2.4^{Ab}$ | 86.6, 89.8, 92.5 | $71.5 \pm 8.6^{Ab}$ | 63.0, 74.1, 78.5 |
| | FCCP | $59.5 \pm 9.7^{Ab}$ | 50.1, 56.9, 69.9 | $90.3 \pm 1.4^{Ab}$ | 88.6, 91.1, 91.6 | $65.7 \pm 10.3^{Ab}$ | 55.9, 63.0, 75.5 |
| P10 | Control | $78.3 \pm 3.5^{Aa}$ | 71.0, 77.9, 86.4 | $83.8 \pm 2.7^{Aa}$ | 80.3, 84.8, 90.6 | $93.1 \pm 4.8^{Aa}$ | 88.4, 91.9, 96.0 |
| | Omy A | $62.6 \pm 8.3^{Ab}$ | 55.3, 60.1, 70.8 | $90.8 \pm 1.6^{Ab}$ | 89.2, 90.9, 92.5 | $68.8 \pm 8.7^{Ab}$ | 61.9, 65.3, 77.7 |
| | FCCP | $60.2 \pm 10.0^{Ab}$ | 50.9, 58.7, 68.5 | $90.4 \pm 1.1^{Ab}$ | 89.6, 90.3, 90.9 | $66.5 \pm 10.4^{Ab}$ | 57.0, 65.1, 74.3 |

*Different letters (A,B) indicate significant differences ($P < 0.05$) between the control and the different light stimulation patterns used in the presence or absence of oligomycin A (Omy A) or FCCP. Different letters (a,b) indicate significant differences ($P < 0.05$) between Omy A or FCCP with respect to treatment without the two inhibitors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD, median and interquartile range from eight separate experiments.*

## Effects of Red Light Stimulation on Intracellular Calcium Levels in the Presence or Absence of Either Omy A or FCCP

Percentages of viable sperm with high intracellular calcium levels did not differ between irradiated and non-irradiated samples in the presence/absence of Omy A and FCCP, nor between the presence/absence of disruptors within each irradiation pattern (**Figure 6A**). Similar results were obtained in the case for the GMFI of $Fluo3^+$ in the viable sperm population with high intracellular calcium levels ($Fluo3^+/PI^-$; **Figure 6B**).

**TABLE 1C |** Kinetic parameters (ALH and BCF) of donkey sperm in the control and the different light-stimulation patterns in the presence or absence of oligomycin A (Omy A) and FCCP.

| Treatments | | Kinetic parameters | | | |
|---|---|---|---|---|---|
| | | ALH (μm) | | BCF (Hz) | |
| | | Mean ± SD | Q1, median, Q3 | Mean ± SD | Q1, median, Q3 |
| Control | Control | $2.5 \pm 0.7^{Aa}$ | 1.8, 2.6, 3.3 | $9.2 \pm 1.0^{Aa}$ | 8.1, 9.0, 10.3 |
| | Omy A | $2.4 \pm 0.3^{Aa}$ | 2.1, 2.4, 2.7 | $12.9 \pm 1.0^{Ab}$ | 10.4, 12.6, 13.5 |
| | FCCP | $2.8 \pm 0.3^{Aa}$ | 2.5, 2.9, 3.0 | $13.3 \pm 0.7^{Ab}$ | 12.3, 13.3, 13.8 |
| P1 | Control | $2.6 \pm 0.4^{Aa}$ | 1.9, 2.5, 3.2 | $9.1 \pm 1.1^{Aa}$ | 7.7, 8.9, 11.0 |
| | Omy A | $2.5 \pm 0.2^{Aa}$ | 2.3, 2.5, 2.7 | $12.7 \pm 0.8^{Ab}$ | 11.0, 12.5, 13.1 |
| | FCCP | $2.9 \pm 0.4^{Aa}$ | 2.5, 2.8, 3.2 | $12.9 \pm 0.9^{Ab}$ | 12.2, 12.7, 13.7 |
| P5 | Control | $2.6 \pm 0.6^{Aa}$ | 2.0, 2.5, 3.4 | $8.9 \pm 1.0^{Aa}$ | 7.7, 8.8, 10.4 |
| | Omy A | $2.5 \pm 0.4^{Aa}$ | 2.2, 2.4, 2.8 | $11.8 \pm 0.9^{Ab}$ | 10.6, 12.0, 12.9 |
| | FCCP | $2.9 \pm 0.3^{Aa}$ | 2.6, 3.0, 3.1 | $13.8 \pm 1.0^{Ab}$ | 11.8, 13.6, 14.2 |
| P10 | Control | $2.7 \pm 0.6^{Aa}$ | 2.0, 2.8, 3.5 | $9.4 \pm 0.9^{Aa}$ | 8.2, 9.4, 10.6 |
| | Omy A | $2.5 \pm 0.3^{Aa}$ | 2.2, 2.4, 2.7 | $12.3 \pm 0.9^{Ab}$ | 11.0, 12.4, 13.3 |
| | FCCP | $2.8 \pm 0.4^{Aa}$ | 2.4, 2.8, 3.1 | $13.8 \pm 0.8^{Ab}$ | 12.1, 13.7, 14.0 |

*Different letters (A,B) indicate significant differences ($P < 0.05$) between the control and the different light stimulation patterns used in the presence or absence of oligomycin A (Omy A) or FCCP. Different letters (a,b) indicate significant differences ($P < 0.05$) between oligomycin A or FCCP with respect to treatment without the two inhibitors for a given protocol (i.e., P1, P5, or P10). Data are shown as mean ± SD, median and interquartile range from eight separate experiments.*

## Effects of Red Light Stimulation on Intracellular Levels of ATP and Oxygen Consumption in the Presence or Absence of Either Omy A or FCCP

**Figure 7A** shows the intracellular levels of ATP observed in non-irradiated and irradiated samples in the presence or absence of Omy A or FCCP. No significant differences were observed between non-irradiated and irradiated samples were observed, either in the presence or absence of oligomycin A. In the presence of FCCP, intracellular ATP levels were significantly ($P < 0.05$) higher in sperm irradiated for 1 min than in those non-irradiated. In addition, samples irradiated for 1 min showed significantly ($P < 0.05$) higher intracellular levels of ATP in the presence than in the absence of FCCP.

**Figure 7B** shows that light stimulation ($P < 0.05$) significantly increased the $O_2$ consumption rate in all three protocols (i.e., P1, P5, and P10) compared to the non-irradiated control. In samples containing Omy A, no significant differences between non-irradiated and irradiated samples were observed. However, $O_2$ consumption rates in non-irradiated and irradiated samples containing Omy A were significantly ($P < 0.05$) lower than in the ones that did not contain this inhibitor. On the other hand, no significant differences between irradiated and non-irradiated samples were observed in samples containing FCCP. However, samples containing FCCP showed significantly ($P < 0.05$) higher $O_2$ consumption rates

**TABLE 2 |** Descriptive parameters (mean ± SD, median and interquartile range) of the four sperm subpopulations (SP1, SP2, SP3, and SP4) identified in donkey fresh semen, in the presence or absence of oligomycin A (Omy A) or FCCP.

| | SP1 | | SP2 | | SP3 | | SP4 | |
|---|---|---|---|---|---|---|---|---|
| **N** | **7,611** | | **9,455** | | **16,551** | | **6,694** | |
| **Parameter** | **Mean ± SD** | **Q1, median, Q3** | **Mean ± SD** | **Q1, median, Q3** | **Mean ± SD** | **Q1, median, Q3** | **Mean ± SD** | **Q1, median, Q3** |
| VCL μm/s) | 168.4 ± 13.6 | 153.1, 168.1, 182.0 | 114.2 ± 16.1 | 98.0, 116.4, 132.8 | 70.2 ± 14.9 | 54.5, 68.7, 86.3 | 151.4 ± 19.5 | 132.3, 155.7, 173.9 |
| VSL μm/s) | 132.9 ± 18.0 | 113.5, 132.9, 150.9 | 101.7 ± 15.6 | 84.6, 102.3, 119.7 | 41.8 ± 9.3 | 30.5, 43.1, 53.8 | 54.5 ± 17.8 | 35.7, 56.0, 74.2 |
| VAP μm/s) | 152.4 ± 14.9 | 137.0, 151.8, 166.8 | 109.2 ± 16.1 | 92.1, 111.0, 128.2 | 47.4 ± 7.6 | 36.2, 47.8, 58.4 | 115.0 ± 19.1 | 98.9, 117.8, 134.2 |
| LIN (%) | 78.9 ± 9.2 | 69.8, 80.7, 88.7 | 89.1 ± 5.7 | 85.4, 91.3, 94.7 | 59.4 ± 13.2 | 49.8, 61.3, 71.2 | 35.9 ± 12.1 | 24.3, 37.4, 48.2 |
| STR (%) | 87.1 ± 7.2 | 81.8, 88.7, 94.4 | 93.3 ± 4.2 | 90.5, 95.5, 97.9 | 86.5 ± 12.2 | 84.9, 92.6, 95.5 | 47.3 ± 16.5 | 33.3, 49.2, 61.8 |
| WOB (%) | 90.3 ± 4.3 | 84.6, 91.1, 95.0 | 95.1 ± 3.6 | 92.4, 96.4, 98.8 | 68.1 ± 10.5 | 59.5, 68.5, 73.3 | 75.9 ± 9.1 | 69.3, 77.9, 84.2 |
| ALH (μm) | 3.7 ± 0.6 | 3.1, 3.6, 4.2 | 2.1 ± 0.5 | 1.6, 2.0, 2.5 | 2.6 ± 0.5 | 2.1, 2.5, 3.0 | 4.6 ± 1.1 | 3.7, 4.6, 5.5 |
| BCF (Hz) | 10.9 ± 2.5 | 9.0, 11.0, 13.0 | 8.2 ± 2.0 | 6.8, 8.0, 10.0 | 11.6 ± 3.1 | 9.0, 12.0, 14.0 | 8.0 ± 2.4 | 6.0, 8.0, 10.0 |

*These data were obtained after classifying sperm cells into motile subpopulations through principal component and cluster analyses.*

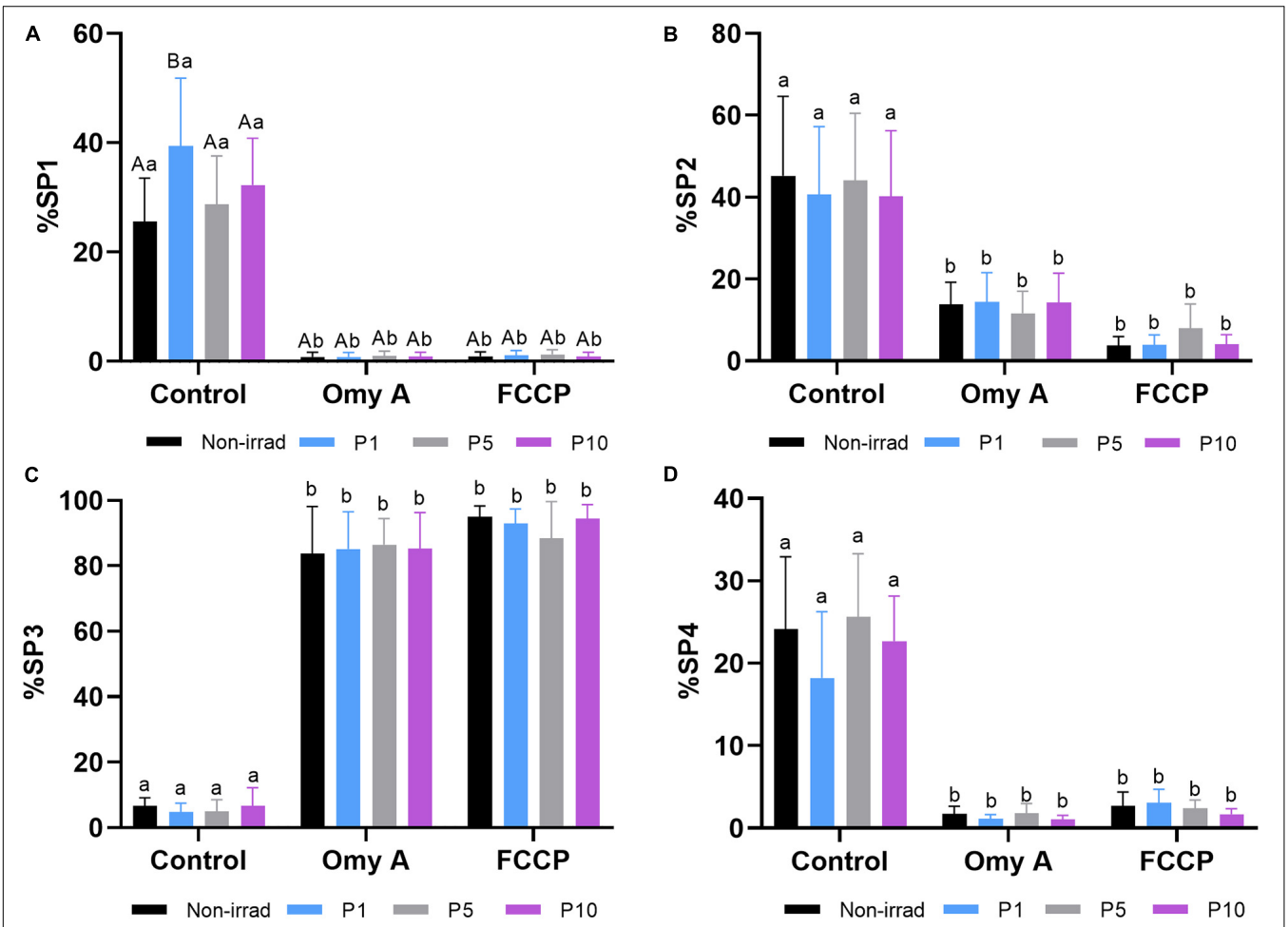


**FIGURE 3 |** Effects of light stimulation on the structure of the motile sperm subpopulation in the presence/absence of oligomycin A (Omy A) or FCCP. **(A)** Subpopulation 1 (SP1, the fastest and most linear); **(B)** Subpopulation 2 (SP2 presented intermediate values, lower than SP1 and SP4, but more linear than SP4; **(C)** Subpopulation 3 (SP3, the slowest, but more linear than SP4); and **(D)** Subpopulation 4 (SP4 presented intermediate values, higher than SP2, but was the least linear). Different letters (*A,B*) indicate significant differences ($P < 0.05$) between non-irradiated samples and the different light stimulation patterns used in the presence or absence of oligomycin A (Omy A) or FCCP. Different letters (*a,b*) indicate significant differences ($P < 0.05$) between oligomycin A (Omy A) or FCCP with respect to the treatment without the two disruptors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

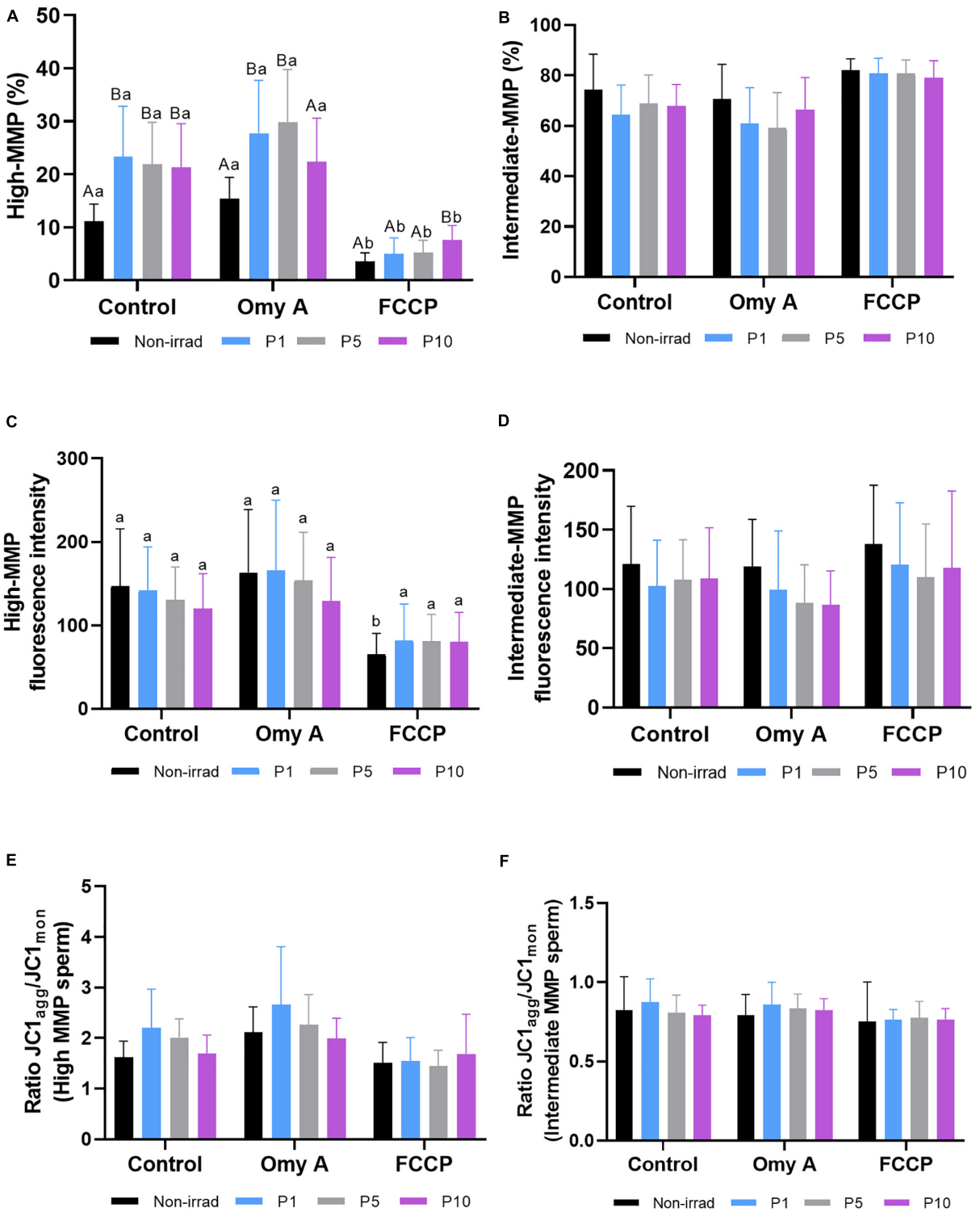


**FIGURE 4 |** Effects of light stimulation on the mitochondrial membrane potential in the presence/absence of oligomycin A (Omy A) or FCCP. The results are presented as percentages of sperm with high mitochondrial membrane potential (MMP; $JC1_{agg}^{++}$; **(A)** and with intermediate mitochondrial membrane potential (MMP; $JC1_{agg}^{+}$; **(B)**; geometric mean of the $JC1_{agg}$ fluorescence intensity (GMFI, FL2) in the sperm populations with high **(C)** and intermediate **(D)** MMP; and the $JC1_{agg}/JC1_{mon}$ (GMFI FL2/GMFI FL1) ratios in sperm populations with high **(E)** and intermediate **(F)** MMP in control and irradiation patterns (P1, P5, or P10), in the presence/absence of Omy A. Different letters (*A, B*) indicate significant differences ($P < 0.05$) between non-irradiated samples and the different light-stimulation patterns used in the presence or absence of Omy A or FCCP. Different letters (*a, b*) indicate significant differences ($P < 0.05$) between Omy A or FCCP with respect to the treatment without the two disruptors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

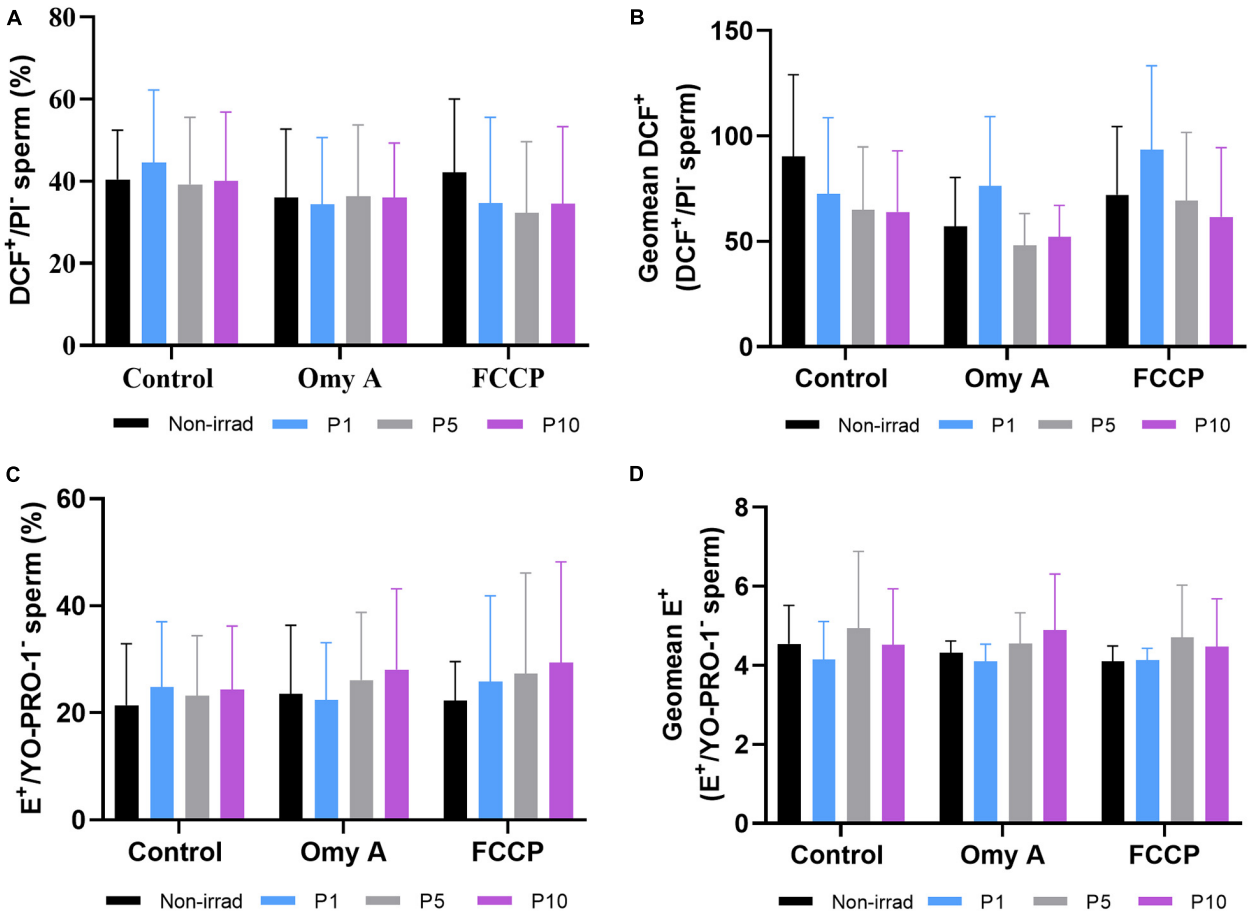


**FIGURE 5 |** Effects of light-stimulation on the percentages of viable spermatozoa with high peroxide levels ($DCF^+/PI^-$; **(A)** geometric mean of $DCF^+$ -intensity (GMFI, FL1 channel) in the population of viable spermatozoa with high peroxide levels **(B)** and percentages of viable spermatozoa with high superoxide levels ($E^+/YO\text{-}PRO\text{-}1^-$; **(C)** and geometric mean of $E^+$ -intensity (GMFI, FL3 channel) in the population of viable spermatozoa with high superoxide levels **(D)** in the control and irradiated samples in the presence or absence of oligomycin A (Omy A) or FCCP. No significant differences were observed between non-irradiated sperm and the different light-stimulation patterns used in the presence/absence of oligomycin A or FCCP, nor between the results of the samples in the presence of Omy A or FCCP with respect to the treatment without the two disruptors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

than those without this disruptor, regardless of whether they were irradiated.

## Effects of Red Light Stimulation on Cytochrome C Oxidase Activity in the Presence or Absence of Either Omy A or FCCP

As shown in **Figure 8**, irradiation for 1, 5, or 10 min induced a significant increase ($P < 0.05$) in CCO activity compared to non-irradiated samples. Although these effects were observed in both the presence and absence of Omy A or FCCP, the highest CCO activity was observed in sperm irradiated for 5 min in the absence of Omy A/FCCP.

While neither Omy A nor FCCP affected the significant ($P < 0.05$) increase in CCO activity observed in irradiated sperm compared to non-irradiated sperm, samples containing Omy A or FCCP showed significantly ($P < 0.05$) lower CCO activity than when these two molecules were absent from non-irradiated sperm and sperm irradiated for 5 min and 10 min. In contrast, CCO activity in sperm irradiated for 1 min was significantly ($P < 0.05$) lower when Omy A was present than when this inhibitor was absent.

## DISCUSSION

The results obtained in the present study with regard to the potential of mitochondrial membrane, sperm kinetic parameters, the structure of motile sperm subpopulations, CCO activity and oxygen consumption rate support that irradiation with red LED light affects mitochondrial activity of donkey sperm. Furthermore, the impact of red light has been found to rely upon the time of exposure (i.e., 1, 5, or 10 min), regardless of the presence of Omy A and FCCP, which is in agreement with

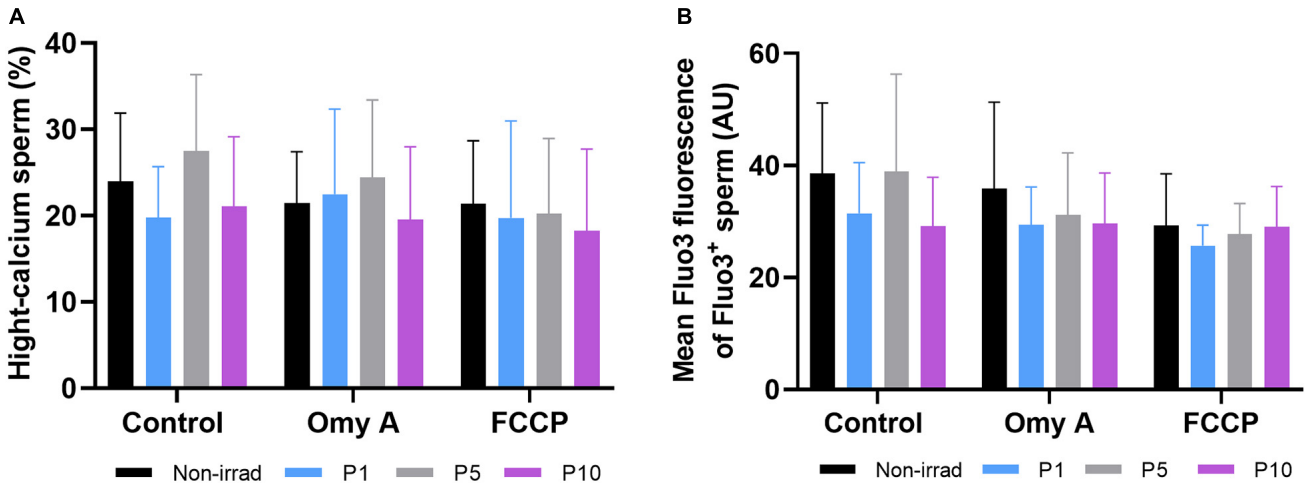


**FIGURE 6 |** Effects of light-stimulation on percentages of spermatozoa with high intracellular calcium levels ($Fluo3^+$); **(A)** and geometric mean intensity of $Fluo3^+$ **(B)** in the presence/absence of either oligomycin A or FCCP. No significant differences were observed between non-irradiated sperm and the different light stimulation patterns used in the presence or absence of oligomycin A (Omy A) or FCCP, nor between the results of the samples in the presence of Omy A or FCCP with respect to the treatment without the two inhibitors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

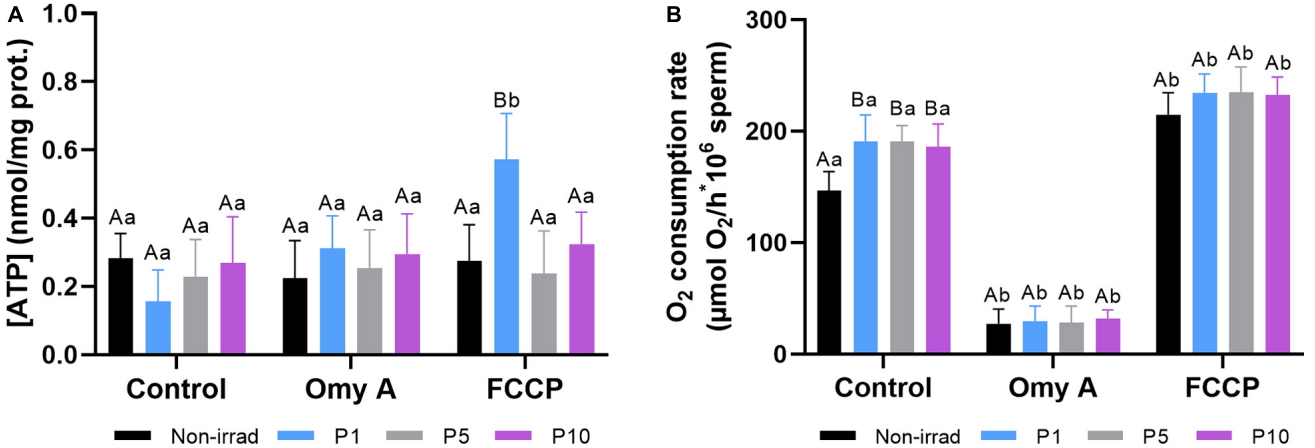


**FIGURE 7 |** Effects of light-stimulation on intracellular ATP levels **(A)** and $O_2$ consumption rate **(B)** in the presence/absence of either oligomycin A (Omy A) or FCCP. Different letters (*A, B*) indicate significant differences ($P < 0.05$) between non-irradiated sperm and the different light stimulation patterns used in the presence or absence of Omy A or FCCP. Different letters (*a, b*) indicate significant differences ($P < 0.05$) between Omy A or FCCP with respect to the treatment without the two disruptors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

previous studies conducted in other mammalian species, such as pigs, dogs, buffalos, humans, donkeys and horses (Corral-Baqués et al., 2009; Abdel-Salam et al., 2011; Salman Yazdi et al., 2014; Yeste et al., 2016; Catalán et al., 2020a,b,c). In this context, it is worth emphasizing that a high individual variation was observed, which indicates that apart from the relevance of the stimulation pattern, time and intensity (Yeste et al., 2016), the sperm response to red light also depend on the functional status of the cell (Catalán et al., 2020a). Although the mechanisms underlying this different response have yet to be elucidated, this work suggests that, in donkey sperm, red light acts on mitochondrial photosensitizers and that the energy supplied to the mitochondrial electron chain following irradiation is related to the time of exposure and intensity. Therefore, the exact level of energy provided to sperm through red light stimulation and the overall sperm function status appear to be on the basis of the different impact observed between treatments and species.

With regard to the effects of red light on the mitochondrial electron chain observed in this work, it is reasonable to surmise that this could be explained by the direct action of red light on mitochondrial photosensitizers (reviewed in Yeste et al., 2018). Indeed, we observed that red light affected the activity of CCO, which is a crucial component of the mitochondrial electron chain and is sensitive to light at a wavelength ranging from 630 to 660 nm (Lynch and Copeland, 1992). These observed

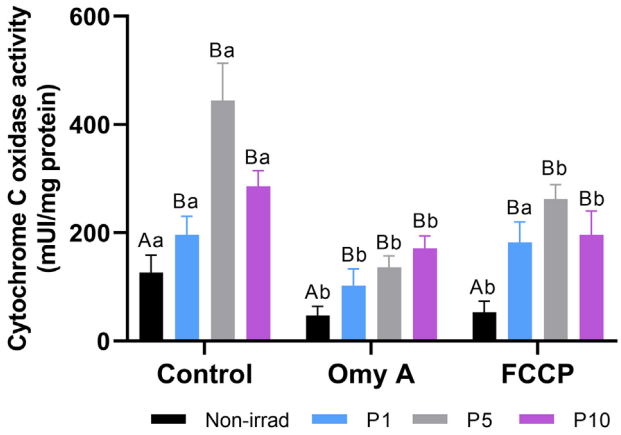


**FIGURE 8 |** Effects of light-stimulation on cytochrome C oxidase activity in the presence/absence of either oligomycin A (Omy A) or FCCP. Different letters (*A, B*) indicate significant differences ($P < 0.05$) between non-irradiated sperm and the different light stimulation patterns used in the presence or absence of Omy A or FCCP. Different letters (*a, b*) indicate significant differences ($P < 0.05$) between Omy A or FCCP with respect to the treatment without the two inhibitors for a given protocol (i.e., control, P1, P5, or P10). Data are shown as mean ± SD from eight separate experiments.

increases in CCO activity are similar to those reported in ram semen irradiated with He-Ne laser (632.8 nm; Iaffaldano et al., 2016) and in liquid-stored pig semen irradiated with LED (Blanco-Prieto et al., personal communication), which would also support the hypothesis that mitochondrial photosensitizers play a relevant role in the effects of red light upon sperm cells. On the other hand, our results showed that neither irradiation nor the presence of Omy A or FCCP had any detrimental effect on sperm viability, which was evaluated through the $SYBR14^{+}/PI^{-}$ test. These results are in agreement with Yeste et al. (2016) and Pezo et al. (2019), who conducted their studies in liquid-stored pig semen; Catalán et al. (2020a), who evaluated the effects of red light irradiation on fresh and cooled-stored donkey semen; and Catalán et al. (2020b,c) that used frozen-thawed and fresh horse sperm.

As far as the effects of red light on sperm motility are concerned, it is worth mentioning that while no significant differences were observed in the percentages of TMOT and PMOT between non-irradiated and samples irradiated with red light, light-stimulation for 5 min or 10 min in the absence of Omy A/FCCP significantly increased VAP, which is in agreement with previous studies conducted in dogs (Corral-Baqués et al., 2005, 2009), cattle (Siqueira et al., 2016), buffalos (Abdel-Salam et al., 2011), pigs (Yeste et al., 2016), donkeys (Catalán et al., 2020a), and horses (Catalán et al., 2020b,c). We also evaluated the effects of red light on motile sperm subpopulations and we identified four different subpopulations, which is in agreement with that previously reported in the donkey (Miró et al., 2005). Remarkably, we observed that irradiation for 1 min increased the percentages of sperm belonging to SP1, which was the one that included the fastest and most linear motile sperm. Thus, our data suggest that irradiation of fresh donkey sperm with red light modifies the structure of motile sperm subpopulations by increasing the proportions of faster and more linear sperm cells. In addition, our results concur with a previous study conducted on dog semen, in which stimulation with red light (laser) significantly increased the proportions of the fastest sperm subpopulation (Corral-Baqués et al., 2009) and another one carried out with fresh horse sperm where, in a similar fashion to this work, an increase in the most rapid and linear subpopulation was observed (Catalán et al., 2020c). These changes in the characteristics of the motile sperm subpopulations, together with those observed in sperm kinetic parameters, indicate that not only does irradiation with red light increase the speed but also alters the motility pattern of donkey sperm. While there is not, at present, a clear explanation about how red light stimulation affects sperm motility, the aforementioned impact on mitochondrial function could provide some clues. Related to this, it is worth mentioning that the increase observed in VAP and in the proportions of spermatozoa belonging to SP1 was concomitant with a rise in the percentages of sperm with high MMP, determined through JC1. These findings agree with Siqueira et al. (2016), who found that irradiation with a He-Ne laser at a wavelength of 633 nm augmented the percentage of sperm with intermediate MMP. Similar results were found in pig, donkey and horse sperm, since irradiation with red LED light at a wavelength between 620 and 630 nm increased the percentages of sperm with high MMP (Yeste et al., 2016; Catalán et al., 2020a,b,c). Therefore, the current study suggests that stimulation with red light increases mitochondrial activity through endogenous photosensitizers, such as cytochrome C (Begum et al., 2013; Iaffaldano et al., 2016; Yeste et al., 2016; Catalán et al., 2020a,b,c), which could, in turn, lead to greater motility of sperm and higher fertilization potential (Breitbart et al., 1996).

Recent studies indicate that oxygen consumption represents an alternative measure of mitochondrial activity, which could be better than the use MMP markers, such as JC1 (Moscatelli et al., 2017; Meyers et al., 2019). Oxygen consumption rate would also provide an indirect measure of ATP produced by oxidative phosphorylation in mammalian sperm (Meyers et al., 2019). The results obtained in this study showed an increase in oxygen consumption on all irradiated samples (namely, light-stimulated for 1, 5, or 10 min) in the absence of Omy A or FCCP compared to the non-irradiated control. While these results are in agreement with those obtained from the evaluation of some motility parameters, the structure of motile subpopulations, the percentages of viable sperm with high MMP and the activity of cytochrome C oxidase, it is surprising that no link to intracellular levels of ATP was observed, since no differences in this parameter were found when non-irradiated and irradiated samples without oligomycin A/FCCP were compared. Moreover, no relationship between intracellular ATP levels and other mitochondrial parameters was observed in samples irradiated for 1 min in the presence of FCCP. The increase in the potential of the mitochondrial membrane is associated with changes in the consumption of ATP and in the activity of respiratory chain enzymes

(Alberts et al., 2002; Zorova et al., 2018). Related with this, Iaffaldano et al. (2016) observed that light stimulation of frozen-thawed ram sperm with a He-Ne laser increased ATP content and the activity and affinity of cytochrome C oxidase for its substrate (cytochrome C). Interestingly, these authors found that CCO activity and ATP content were positively correlated with each other and with sperm motility, supporting the hypothesis that the effects of red light upon sperm are mediated by mitochondria. However, and as aforementioned, we did not observe that correspondence in the case of donkey sperm. An explanation about why irradiation did not result in a clear increase of intracellular ATP levels could be that ATP in sperm is mainly synthesized through two routes, glycolysis and mitochondrial oxidative phosphorylation, without dismissing other energy sources available to sperm such as β-oxidation (Storey and Keyhani, 1974; Amaral et al., 2013). The precise balance of ATP production from both sources is very dynamic and multifactorial, undergoing rapid changes not only between species, but also within the same cell under different physiological and environmental conditions. The intracellular changes that are related to the complex relationship between mitochondrial oxidative phosphorylation and glycolysis, and that could explain these effects will be later discussed in more detail.

In addition to the aforementioned, the electron chain is known to play a fundamental role in the generation of reactive oxygen species (ROS), since mitochondria are the most important source of ROS generation in eukaryotic cells (Zhao et al., 2019). A previously established hypothesis indicates that light-stimulation increases ROS production by sperm (Cohen et al., 1998; Zan-Bar et al., 2005). Furthermore, cytochrome complexes are also involved in the intrinsic apoptotic pathway (Cai et al., 1998), and it has been surmised that both generation of ROS and modulation of apoptotic-like changes could be crucial to elicit and modulate the achievement of capacitated status by sperm (Ortega-Ferrusola et al., 2009; Aitken et al., 2015). Against this background, one could reasonably suggest that the red light-induced changes in CCO activity observed herein could ultimately affect the lifespan and capacitation status of mammalian sperm. However, our results showed that light-stimulation of fresh donkey sperm did not increase intracellular levels of ROS or calcium, which is a crucial secondary messenger involved in early and late capacitation events (Yeste, 2013; Correia et al., 2015). Thus, our data differ from those observed in donkeys and other species in which irradiation was found to increase intracellular ROS (Cohen et al., 1998; Zan-Bar et al., 2005; Catalán et al., 2020a) and intracellular calcium levels (Lubart et al., 1992; Cohen et al., 1998). These differences could be due to the high individual variability in the sperm response to red light, as well as to differences in the light source, intensity and irradiation pattern, as previous reports found for intracellular levels of ROS (Catalán et al., 2020a) and calcium (Breitbart et al., 1996). Moreover, the existence of a complex homeostasis system aimed at maintaining ROS levels within a physiological range, which would include systems such as the glutathione peroxidase-glutathione reductase complex (GPX/GSR) and enzymes such as intracellular peroxidases (see Peña et al., 2019 for review), would allow sperm to maintain ROS levels in the event of a temporarily induced stress like the one caused by light-stimulation. Nevertheless, further research should be conducted to confirm this hypothesis.

Regarding our results obtained in samples containing Omy A or FCCP, one should note that irradiation in the presence of these two molecules increased the percentages of sperm with high MMP, compared to the non-irradiated control in the presence of these molecules. Furthermore, non-irradiated samples in the presence of FCCP exhibited lower intensity of $JC1_{agg}$ fluorescence in the sperm population with high MMP, which did not occur in irradiated samples. These findings also support that light-stimulation of sperm exerts its effects via MMP. At this point, it is worth mentioning that some studies have recently questioned the use of MMP probes to assess the potential of the mitochondrial membrane due to its non-specific nature (Meyers et al., 2019). These reports point up that MMP probes are cationic and their accumulation rate inside the mitochondria is inversely proportional to the potential of the internal mitochondrial membrane; hence, MMP probes like JC1, which was the one used in this study, may not be reliable as a quantitative measure of MMP without adequate controls. Therefore, these previous studies suggest that high/low MMP controls, such as ATP synthase inhibitors like Omy A and MMP uncouplers like FCCP or DNP, are needed (Meyers et al., 2019). Remarkably, the results obtained in this work with Omy A/FCCP showed this controlling effect, since percentages of sperm with high MMP in samples without disruptors were lower than those containing Omy A, and significantly higher than those containing FCCP. Regarding sperm motility parameters in the presence of these two molecules, they were similar to those found by Ramió-Lluch et al. (2014) and Nesci et al. (2020) in previous studies conducted with pig semen. According to Nesci et al. (2020), the reduced motility observed in treatments containing Omy A or FCCP could result from the decrease in ATP content caused by these two molecules. However, our results indicate that the presence of Omy A and FCCP in irradiated and non-irradiated sperm reduces sperm motility without causing a decrease on overall sperm ATP levels. These findings observed in the presence of Omy A are similar to those seen by Ramió-Lluch et al. (2014), who reported that the control exerted by Omy A-sensitive ATP synthase over pig sperm motility does not seem to be related to its inhibiting effect upon ATP levels. In this context, it is worth mentioning that a previous study reported that mitochondrial respiration of pig sperm incubated in the presence of glucose only contributes to 5% of the total energy produced by the cell, the other 95% being obtained from glycolysis (Marín et al., 2003). Therefore, it is surprising that the relatively low levels of energy produced through ATP synthase affected sperm motility as much as they did in the study of Ramió-Lluch et al. (2014), especially if one bears in mind that these authors did not observe an alteration of overall sperm energy levels. Related with this, in a recent study, Nesci et al. (2020) observed that, despite pig sperm being regarded to rely upon glycolysis, the motility of these cells is highly dependent on the ATP produced through mitochondrial oxidative phosphorylation. In other studies, such as those carried

out by Iaffaldano et al. (2016) on ram sperm and Odet et al. (2013) on mouse sperm, an association between overall ATP content and sperm motility has also been observed. In the case of mice, one should keep in mind that their sperm have been reported to maintain their function through the ATP originated from glycolysis or mitochondrial respiration indistinctly (Pasupuleti, 2007). In addition to this, a recent study published by Balbach et al. (2020) has demonstrated a functional link between these two pathways during mouse sperm capacitation. All these data strongly point to the existence of species-specific differences on the mechanisms by which mammalian spermatozoa produce ATP via glycolysis or oxidative phosphorylation (Rodríguez-Gil and Bonet, 2016). Our results suggest that changes in intracellular ATP levels cannot be taken as a direct indicator of changes affecting mitochondrial function, since glycolysis and probably other metabolic pathways present in sperm also produce ATP; thus, further metabolomics studies in donkey sperm are needed to understand the precise glycolysis/oxidative phosphorylation balance in these cells.

While the evidence reported in this and another study in pigs (Blanco-Prieto et al., personal communication) indicates that the effects of red light on sperm would be related to the direct effect on intracellular light-sensitive proteins, especially the impact on endogenous photosensitizers, such as mitochondrial cytochromes, other pathways or factors that might modulate the effects of red light on mammalian sperm could also be involved (reviewed in Yeste et al., 2018). These pathways or factors include light-sensitive receptors, such as opsins (Pérez-Cerezales et al., 2015) and transient receptor potential proteins (TRP) (De Blas et al., 2009; Bahat and Eisenbach, 2010; Gibbs et al., 2011), whose one of their most probable functions in mammalian sperm is the regulation of thermotaxis (Wu et al., 2010; Pérez-Cerezales et al., 2015). Thermotaxis could be, in fact, an important modulator of light stimulation, since mammalian sperm are sensitive to temperature changes as small as 0.0006°C (Bahat et al., 2012; Pérez-Cerezales et al., 2015). Therefore, it is reasonable to suggest that red light stimulation could also act through this pathway, especially if one takes into consideration that, in this study, the effects of irradiation on MMP and ATP content, which rely on glycolysis/oxidative phosphorylation balance, differed. In addition, one should not discard that signaling transduction pathways triggered when opsins and TRP receptors are activated could change ATP production via glycolysis as well as the balance between glycolysis and oxidative phosphorylation.

## CONCLUSION

In conclusion, our results indicate that the effects induced by the stimulation of fresh donkey sperm with red LED light are related to mitochondrial photosensitizers, such as CCO, which modify the activity of the mitochondrial electron chain; the effect of red light on these photosensitizers depends on the time of exposure, among other factors. However, these findings do not exclude that this mitochondrial mechanism could work in conjunction with other pathways, such as thermotaxis, via plasma membrane receptors.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

All jackasses used in this study were semen donors and no manipulation to animals, apart from semen collection in the authorized center, was made. The animal study was reviewed and approved by the Ethics Committee for Animal and Human Experimentation (CEEAH) of our institution (Autonomous University of Barcelona; authorization code: CEEAH 1424.

## AUTHOR CONTRIBUTIONS

MY, JR-G, and JM: conceptualization. JC, MP, LT-R, OB-P, JM, MY, and JR-G: methodology and investigation. JM, MY, and JR-G: validation, project administration, and supervision. JR-G: formal analysis and data curation. JC: writing—original draft preparation. JM and MY: writing—review and editing, funding acquisition, and resources. All authors have read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.588621/full#supplementary-material

**Supplementary Figure 1 |** Representative flow-cytometry dot-plots observed in non-irradiated samples and samples irradiated for 5 min, in the presence/absence of FCCP and Omy A.

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**Conflict of Interest:** JR-G and MY were inventors of a patent entitled 'Method and apparatus for improving the quality of mammalian sperm' (European Patent Office, No. 16199093.2; EP-3-323-289-A1), which is owned by Instruments Utils de Laboratori Geniul, SL (Barcelona, Spain).

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

CAPÍTULO IV

# DISCUSIÓN

Los resultados obtenidos en esta Tesis Doctoral aportan varios avances importantes respecto a la aplicación de luz LED roja sobre los espermatozoides de burro y caballo, tanto en relación a su posible uso como herramienta de mejora de la calidad espermática y del rendimiento reproductivo, como en cuanto a la comprensión de cómo la luz roja ejerce sus efectos sobre los gametos masculinos.

Considerados en conjunto, los datos recogidos indican que la estimulación con luz LED roja a una longitud de onda de 620-630 nm modifica los **parámetros de motilidad espermática**, **la estructura de las subpoblaciones de espermatozoides móviles**, **el potencial de membrana mitocondrial**, **el consumo de $O_2$**, **la actividad de la citocromo C oxidasa** y **los niveles intracelulares de ROS de los espermatozoides**, **sin afectar** a la **integridad de la membrana plasmática**, el **acrosoma** o el **ADN espermático**. Sin embargo, el **tiempo** y los **patrones de exposición** necesarios para desencadenar estos efectos dependen del tipo de muestra (semen fresco, refrigerado o congelado), de la especie (burro o caballo), de la turbidez del diluyente y del color de pajuela utilizado.

Comenzando con los efectos sobre **los parámetros de motilidad espermática**, en el caso del burro la estimulación con luz roja aumentó la motilidad total y progresiva, coincidiendo con lo observado en humanos (Sato et al., 1984; Lenzi et al., 1989; Firestone et al., 2012; Salman Yazdi et al., 2014; Salama and El-Sawy, 2015; Ban Frangez et al., 2015), búfalos (Abdel-Salam et al., 2011), carneros (Iaffaldano et al., 2016) y cerdos (Yeste et al., 2016). Por el contrario, en espermatozoides de caballo, la fotoestimulación no afectó a los porcentajes de motilidad total o progresiva, lo que concuerda con los datos reportados en perros (Corral-Baqués et al., 2005; 2009) y toros (Siqueira et al., 2016). Esto demuestra que los efectos de la irradiación con luz roja varían entre las especies (Zan-Bar et al., 2005). Con respecto a los efectos de la fotoestimulación sobre los parámetros cineticos que definen la motilidad espermática, se observó un aumento en la velocidad, rectitud y linealidad en los espermatozoides de burro y caballo, de igual modo que lo publicado previamente en humanos (Lenzi et al., 1989; Firestone et al., 2012; Preece et al., 2017), perros (Corral-Baqués et al., 2005; 2009), toros (Siqueira et al., 2016), búfalos (Abdel-Salam et al., 2011) y cerdos (Yeste et al., 2016; Pezo et al., 2019). Además, en los espermatozoides de burro se pudo detectar una disminución de la amplitud media del desplazamiento lateral de la cabeza (ALH), similar a lo observado en perros (Corral-Baqués et al., 2009) y, en el caso del caballo, una disminución de la frecuencia de batido (BCF), que coincidió con investigaciones

previas en humanos (Lenzi et al., 1989) pero difirió de lo hallado anteriormente en perros (Corral-Baqués et al., 2005; 2009) y cerdos (Yeste et al., 2016).

Al evaluar la presencia de **subpoblaciones de espermatozoides móviles** en los eyaculados de burro y caballo, se identificaron cuatro subpoblaciones separadas en ambas especies, correspondiendo con lo anteriormente publicado tanto en burros (Miró et al., 2005) como en caballos (Quintero-Moreno et al., 2003). Sorprendentemente, se observó que la irradiación con luz roja aumentó los porcentajes de espermatozoides pertenecientes a la subpoblación más rápida en el semen de burro y caballo, lo que concuerda con lo determinado anteriormente por Corral-Baqués et al. (2009) en un estudio realizado con luz roja (láser) en espermatozoides de perro. Además, en el caballo se pudo observar una disminución del porcentaje de espermatozoides en la subpoblación más lenta.

Estos cambios en las características de las subpoblaciones de espermatozoides móviles, junto con los observados en los parametros de la motilidad espermática global de la muestra, indican que la irradiación con luz roja no solo aumenta la velocidad, sino que también altera el patrón de motilidad de los espermatozoides de burro y caballo. En este momento, no existe una explicación clara de cómo ocurren estos efectos, ya que los mecanismos que subyacen a la estimulación de los espermatozoides por parte de la luz roja no se conocen con exactitud. Sin embargo, se hipotetiza que un mecanismo relacionado con la activación de las mitocondrias de los espermatozoides podría ser esencial para explicarlos (Yeste et al., 2018). De hecho, los resultados obtenidos en esta Tesis Doctoral apuntan hacia esta posibilidad, ya que en los estudios realizados tanto en burro como en caballo se pudo observar una relación entre el aumento de la actividad mitocondrial y la motilidad en las muestras irradiadas, lo que también se determinó en estudios realizados en conejos (Iaffaldano et al., 2010), toros (Abdel-Salam and Harith, 2015; Siqueira et al., 2016), carneros (Iaffaldano et al., 2016) y cerdos (Yeste et al., 2016). Cabe destacar que este aumento de la actividad mitocondrial provocado por la fotoestimulación se detectó a través del **potencial de membrana mitocondrial** evaluado mediante el fluorocromo JC-1[1] así como por los **niveles de consumo de oxígeno**, lo que concuerda con lo hallado por Blanco-Prieto et al. (2020b) en cerdos. Además, en los resultados presentados en esta Tesis Doctoral en el estudio realizado con espermatozoides de burro irradiados en presencia y ausencia de

[1] JC-1: sonda para el análisis del potencial de membrana mitocondrial (5,5 6,6-tetracloro-1,1,3,3-yoduro de tetraetilbenzimidazolicarbocianina)

Oligomicina A (un inhibidor específico de la ATP sintasa) y FCCP (un disruptor de la cadena de electrones mitocondriales), se pudo observar que, en efecto, la irradiación en presencia de estas dos moléculas inhibió los efectos sobre los parámetros de motilidad y las subpoblaciones de espermatozoides móviles y se produjo un incremento del porcentaje de espermatozoides con alto potencial de membrana mitocondrial. Estos hallazgos también apoyan la hipótesis de que los efectos producidos por la fotoestimulación de los espermatozoides con luz roja se deben a un aumento de la actividad mitocondrial. En efecto, de acuerdo con los resultados obtenidos, es razonable suponer que el aumento de la actividad mitocondrial se debe a la acción directa de la luz roja sobre las moléculas fotosensibilizadoras mitocondriales endógenas, como el citocromo C (Begum et al., 2013; Iaffaldano et al., 2016; Yeste et al., 2016; 2018; Blanco-Prieto et al., 2020b). De hecho, en espermatozoides de burro se vio que la irradiación con luz roja aumentaba **la actividad de la citocromo C oxidasa** –lo que también se pudo observar en las muestras irradiadas en presencia de Oligomicina A y FCCP–, un componente crucial de la cadena de electrones mitocondrial sensible a la luz en una longitud de onda que varía entre 630 y 660 nm (Lynch and Copeland, 1992; Blanco-Prieto et al., 2020b). Esta hipótesis estaría de acuerdo con los resultados publicados por otros autores en ovino y porcino (Iaffaldano et al., 2010; 2016; Blanco-Prieto et al., 2020b), que demostraron que el complejo citocromo C/citocromo C oxidasa se estimula con luz roja e infrarroja (Begum et al., 2013). En base a estas observaciones, se ha sugerido que la absorción fotónica por la citocromo C oxidasa aumenta la producción de ATP, lo que podría explicar el aumento de la motilidad y la capacidad fecundante de los espermatozoides irradiados (Breitbart et al., 1996). En esta línea, Iaffaldano et al. (2016) observaron que la fotoestimulación de espermatozoides de carnero congelados-descongelados con un láser He-Ne aumentaba el contenido de ATP y la actividad y afinidad de la citocromo C oxidasa por su sustrato (citocromo C). Curiosamente, estos autores encontraron una correlación positiva entre la actividad de la citocromo C oxidasa, el contenido de ATP y la motilidad de los espermatozoides, lo que, de nuevo, respalda la hipótesis de que los efectos de la luz roja sobre los espermatozoides están mediados por las mitocondrias.

Por otra parte, y de acuerdo con lo anterior, en los estudios incluidos en esta Tesis Doctoral y llevados a cabo con semen fresco de caballo se observó un aumento del ATP intracelular en las muestras irradiadas junto con un incremento de la actividad mitocondrial, el consumo de oxígeno y algunos parámetros cinéticos de los espermatozoides. Sin embargo, en el caso de los espermatozoides de burro, si bien se obtuvo una correlación entre la actividad

mitocondrial, el consumo de oxígeno, algunos parámetros cinéticos y la actividad de la citocromo C oxidasa, no se observó un aumento de los niveles intracelulares de ATP en las muestras irradiadas, lo que concuerda con los resultados publicados por Blanco-Prieto et al. (2020b) en porcino. Una posible explicación de estas diferencias debería contemplar que el ATP en los espermatozoides se sintetiza principalmente a través de dos rutas, la glucólisis y la fosforilación oxidativa mitocondrial, sin descartar otras fuentes de energía disponibles para los espermatozoides como la *β*-oxidación (Storey and Keyhani, 1974; Amaral et al., 2013). El equilibrio preciso de la producción de ATP entre ambas fuentes es muy dinámico y depende de muchos factores, experimentando cambios rápidos no solo entre especies, sino también dentro de la misma célula bajo diferentes condiciones fisiológicas y ambientales. Por lo tanto, los cambios intracelulares que están vinculados a la compleja relación entre la fosforilación oxidativa mitocondrial y la glucólisis podrían explicar los efectos observados en el caso del burro.

Es bien conocido que las mitocondrias son la fuente más importante de generación de ROS en células eucariotas y que, por tanto, la cadena de electrones juega un papel fundamental en la generación de ROS (Zhao et al., 2019); por ello, se ha sugerido que el aumento de la actividad mitocondrial mediado por la luz estimularía la producción de ROS por los espermatozoides (Iaffaldano et al., 2010). En los resultados aquí presentados, y a pesar de que hubo variación en el caso del semen de burro, se pudo observar un **aumento de los niveles intracelulares de ROS** (peróxidos y superóxidos) en las muestras irradiadas. Este aumento concuerda con lo publicado por Zan-Bar et al. (2005) y Cohen et al. (1998), quienes plantearon que la formación de ROS estaría mediada por fotosensibilizadores celulares endógenos específicos como los citocromos mitocondriales. En este sentido se considera que, aunque un exceso de producción de ROS generado por la irradiación con luz podría ser perjudicial para los espermatozoides (Zan-Bar et al., 2005; Cohen et al., 1998), los niveles bajos de estas especies moleculares son beneficiosos para la motilidad de los espermatozoides y su poder fecundante (Zan-Bar et al., 2005). Si bien se requieren más estudios para establecer una relación entre la capacidad fecundante, el alto potencial de membrana mitocondrial, los niveles de ROS intracelulares y la motilidad de espermática, se ha propuesto que el $H_2O_2$ es la molécula activa involucrada en los cambios de la funcionalidad espermática mediados por la luz (Cohen et al., 1998). En definitiva, las ROS pueden tener efectos tanto perjudiciales como beneficiosos sobre los espermatozoides, y el delicado equilibrio entre las cantidades de ROS producidas y los captadores de ROS determina si un parámetro particular de la función espermática se ve

comprometido o potenciado (Cohen et al., 1998). En este sentido, el grado de aumento de los niveles de ROS intracelulares observados en los estudios de esta Tesis Doctoral no fueron suficientes para afectar negativamente la motilidad y la viabilidad de los espermatozoides. Aquí es importante considerar la existencia de un sistema de homeostasis complejo en los espermatozoides destinado a mantener los niveles de ROS dentro de un rango fisiológico. Se sugiere que dicho sistema, que incluiría el complejo glutatión peroxidasa-glutatión reductasa (GPX/GSR) y enzimas como las peroxidasas intracelulares (ver Peña et al. (2019) para revisión), permitiría a los espermatozoides mantener los niveles de ROS cuando se induce un estrés transitorio como el causado por la estimulación de la luz. Sin embargo, se deben realizar más investigaciones para confirmar esta hipótesis.

El citocromo C también está involucrado en la vía apoptótica intrínseca (Cai et al., 1998) y se ha sugerido que tanto la generación de ROS como la modulación de los cambios de tipo apoptótico podrían ser cruciales para desencadenar y regular la capacitación espermática (Ortega-Ferrusola et al., 2009; Aitken et al., 2015). Por ello, los cambios inducidos por la luz roja en la actividad mitocondrial y principalmente en el complejo citocromo C oxidasa podrían afectar en última instancia a la capacitación y supervivencia de los espermatozoides. Sin embargo, los resultados obtenidos en esta Tesis Doctoral no mostraron un aumento de los niveles de calcio intracelular en las muestras irradiadas de burro y caballo, que es un mensajero secundario crucial involucrado en el control de la motilidad y la capacitación de los espermatozoides (Yeste, 2013; Correia et al., 2015). En relación con esto, es importante señalar que los datos aquí presentados difieren de los reportados en estudios previos donde se encontró que la estimulación con luz roja aumentaba los niveles intracelulares de calcio (Lubart et al., 1992; Cohen et al., 1998). Esto podría explicarse por las diferentes condiciones de tiempo e intensidad de radiación utilizados en los distintos estudios donde se evaluaron los niveles de calcio intracelular, ya que investigaciones previas indican que la irradiación de los espermatozoides puede tener tanto efectos estimulantes como inhibidores sobre el transporte de calcio, lo cual depende de la intensidad de la luz utilizada (Breitbart et al., 1996).

Si bien los resultados incluidos en esta Tesis Doctoral, así como también lo observado recientemente por Blanco-Prieto et al. (2020b), indican, como ya se ha mencionado previamente, que los efectos de la luz roja sobre los espermatozoides estarían relacionados con el impacto directo sobre las proteínas intracelulares sensibles a la luz, especialmente los fotosensibilizadores endógenos como los citocromos mitocondriales, también podrían estar involucradas otras vías

o factores que podrían modular los efectos de la luz roja en los espermatozoides de mamífero (Yeste et al., 2018). Estas vías o factores incluyen receptores fotosensibles, como las opsinas (Pérez-Cerezales et al., 2015) y los receptores de potencial transitorio (TRP) (De Blas et al., 2009; Bahat and Eisenbach, 2010; Gibbs et al., 2011), cuya función más probable en los espermatozoides de mamíferos es la regulación de la termotaxis (Wu et al., 2010; Pérez-Cerezales et al., 2015). La termotaxis podría ser, de hecho, una respuesta importante a la estimulación lumínica, ya que ésta, sobretodo en el caso de la luz roja e infrarroja, calienta la muestra y los espermatozoides de mamífero son sensibles a cambios de temperatura tan pequeños como 0,0006 °C (Bahat et al., 2012; Pérez-Cerezales et al., 2015).

Otro de los hallazgos interesantes de esta Tesis Doctoral fue que los efectos de la luz en cada parámetro espermático evaluado dependían del **patrón de exposición** utilizado –que fueron diferentes según la especie (burros y caballos) – y el tipo de semen utilizado (fresco, refrigerado o congelado). Estos hallazgos coinciden con estudios previos que utilizaron luz roja basada en láser y LED y se llevaron a cabo en otras especies de mamíferos, como perros, humanos y cerdos (Corral-Baqués et al., 2009; Salman Yazdi et al., 2014; Blanco-Prieto et al., 2020a). En efecto, se ha revelado que el impacto de la luz roja en los espermatozoides de mamífero depende de la especie (Zan-Bar et al., 2005) y del ritmo preciso de aplicación (Yeste et al., 2016), así como también del estado de la célula y de la intensidad de luz utilizada (Gabel et al., 2018). De acuerdo con esto y con la hipótesis de que la luz actúa sobre los fotosensibilizadores celulares endógenos mitocondriales, es razonable sugerir que la energía suministrada a la cadena de electrones mitocondriales por la luz roja es proporcional al tiempo de exposición y a la intensidad de la luz. Esto explicaría los resultados obtenidos en el estudio sobre el efecto del color de la pajuela y el diluyente en la respuesta a la fotoestimulación espermática, ya que el color de aquélla y la opacidad/turbidez de éste influirían en la intensidad de la luz que alimenta a las mitocondrias, generando un impacto diferente en los espermatozoides. En este sentido, es importante destacar que los mejores resultados se obtuvieron al utilizar pajuelas transparentes y un diluyente con turbidez alta; en cambio, cuando se utilizó un diluyente transparente, los resultados más óptimos se observaron con una pajuela de color rojo.

Al analizar los efectos de la irradiación sobre los **espermatozoides congelados-descongelados**, se pudo observar una disminución del porcentaje de espermatozoides viables que presentaban trastorno lipídico de la membrana tras 120 minutos de incubación a 38ºC

después de la descongelación e irradiación. Si bien el marcaje utilizado (M540[2]/YO-PRO-1[3]) para este parámetro evalúa los cambios tempranos en el desorden de lípidos de membrana y el incremento de éste es uno de los cambios que ocurren durante la capacitación espermática, observar un aumento de este tipo después de la descongelación indica, sin embargo, que la membrana plasmática del espermatozoide está perdiendo su integridad funcional (Bonet, 2006; Martínez-Pastor et al., 2010; Hossain et al., 2011). La disminución de la presencia de espermatozoides con esta alteración tras la irradiación post-descongelación es un resultado muy positivo ya que, además, fue concomitante con una mejora en algunos parámetros de cinética espermática. Todo ello indicaría que la resiliencia de los espermatozoides a la incubación post-descongelación a 38ºC podría incrementarse después de ser irradiados. Estas mejoras en la calidad espermática en el semen irradiado post-descongelación concuerdan con un estudio previo realizado con espermatozoides de carnero criopreservados e irradiados con un láser He-Ne (Iaffaldano et al., 2016). Sin embargo, cabe mencionar que, en esta Tesis, los efectos positivos de la irradiación post-descongelación no se reflejaron en la integridad de la membrana espermática. En efecto, no se observaron diferencias significativas en los porcentajes de espermatozoides con una membrana plasmática intacta (viabilidad de los espermatozoides) entre las muestras de control y las irradiadas. En este sentido, dichos resultados difieren de lo observado por Iaffaldano et al. (2016) en espermatozoides criopreservados de carnero. Estas diferencias podrían explicarse no solo por el hecho de que ambos estudios fueron realizados en especies diferentes (caballo *vs.* carnero), sino también porque se utilizaron patrones, intensidades y fuentes de luz distintas (luz LED roja *vs.* láser He-Ne).

Como se ha mencionado anteriormente, la irradiación con luz roja **no afectó a la integridad de la membrana** plasmática de los espermatozoides (viabilidad), lo que concuerda con lo publicado en porcino (Yeste et al., 2016; Pezo et al., 2019; Blanco-Prieto et al., 2020b). Del mismo modo, no se observó ningún impacto negativo de la irradiación sobre la integridad acrosómica, coincidiendo con estudios previos en conejos (Iaffaldano et al., 2010) y cerdos (Yeste et al., 2016; Blanco-Prieto et al., 2020a), así como **tampoco se hallaron daños en la integridad del ADN**, tal y como se publicó en humanos por Preece et al. (2017) y Gabel et al.

[2] M540: sonda para el análisis del desorden lipídico de la membrana plasmática
[3] YO-PRO-1: nombre comercial de una sonda para la evaluación de los cambios tempranos en la integridad de la membrana plasmática (C24H29I2N3O). Se utiliza como marcador de viabilidad espermática.

(2018). Por todo ello, se sugiere que la fotoestimulación puede tener un efecto positivo y seguro sobre la calidad espermática y la capacidad fecundante de los espermatozoides tanto *in vivo* como *in vitro* (Gabel et al., 2018).

En definitiva, los resultados obtenidos en esta Tesis Doctoral y los observados en otras especies (ver Yeste et al. (2018) para revisión) indican que la irradiación tiene varios usos potenciales en la tecnologías de reproducción asistida en el burro y caballo. Sin embargo, se deben tener en cuenta algunos puntos cruciales tales como el tipo de semen (fresco, refrigerado o congelado), la especie, el diluyente y el color de la pajuela o recipiente utilizado. Asimismo, las investigaciones ulteriores en el campo de la irradiación de los espermatozoides deben abordar el impacto sobre el poder fecundante de éstos en burros y caballos, así como el tipo de semen y los protocolos a utilizar en cada especie. Además de esto, trabajos previos en otras especies han demostrado que la exposición a la luz roja mantiene mejor la calidad y longevidad espermáticas durante la refrigeración (Iaffaldano et al., 2010; Blanco-Prieto et al., 2020a), y también mejora los resultados de la criopreservación espermática en semen de toro (Fernandes et al., 2015) y la calidad del semen de carnero irradiado post-descongelación (Iaffaldano et al., 2016), lo que concuerda con lo observado en esta Tesis Doctoral con el semen de caballo congelado/descongelado. Por lo tanto, es razonable sugerir que otros estudios deberían evaluar si la estimulación con luz roja podría mejorar la tecnología de preservación de semen y ayudar a extender el intervalo de inseminación en el burro y caballo.

CAPÍTULO V

# CONCLUSIONES

1. La irradiación de los espermatozoides de burro y caballo con luz roja modifica los parámetros de motilidad espermática, la estructura de las subpoblaciones de espermatozoides móviles, el potencial de membrana mitocondrial, el consumo de $O_2$, la actividad de la citocromo C oxidasa y los niveles intracelulares de ROS.
2. Los efectos de la luz roja sobre los espermatozoides dependen del tiempo y patrón de exposición y la especie.
3. El impacto de los patrones de exposición a la luz LED roja utilizados sobre los espermatozoides varía según el tipo de semen utilizado (fresco, refrigerado y congelado), la turbidez del diluyente y el color de la pajuela.
4. Los efectos inducidos por la estimulación con luz LED roja en los espermatozoides de burro y caballo están relacionados con los fotosensibilizadores mitocondriales, como el citocromo C y con la modificación de la actividad de la cadena de electrones mitocondrial. Sin embargo, esta acción de la luz LED roja en la cadena de transporte de electrones no excluye otros mecanismos de acción.
5. La irradiación con luz roja no afecta a la viabilidad, la integridad del acrosoma ni la integridad del ADN de los espermatozoides, por lo que puede tener un efecto positivo y seguro sobre la calidad espermática y el poder fecundante.
6. La estimulación con luz LED roja del semen de caballo post-descongelación aumenta la resiliencia al estrés térmico de los espermatzoides incubados a 38° C durante 120 min.
7. La estimulación con luz LED roja del semen de burro y caballo podría ser útil para aumentar la longevidad del semen congelado-descongelado antes de la IA, lo que podría suponer un aumento en el intervalo de inseminación-ovulación. Asimismo, la estimulación con luz LED roja del semen de burro y caballo podría tener un impacto positivo en el rendimiento reproductivo y las tecnologías de reproducción asistida en dichas especies. Sin embargo, se requieren más estudios, incluídos los relacionados con el mecanismo subyacente, para confirmar dichos efectos beneficiosos.

CAPÍTULO VI

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