**UAB**

Universitat Autònoma de Barcelona

# RELEVANCIA DE LOS ANTIOXIDANTES EN LA REGULACIÓN DE LAS ESPECIES REACTIVAS DE OXÍGENO (ROS) EN EL SEMEN DE BURRO Y CABALLO

## TESIS DOCTORAL

**Iván Patricio Yánez Ortiz**

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

Departamento de Medicina y Cirugía Animal
Facultad de Veterinaria
Universidad Autónoma de Barcelona

**Dr. Jordi Miró Roig**
Co-director y Tutor

**Dr. Marc Yeste Oliveras**
Co-director

Bellaterra (Cerdanyola del Vallès)
2022

*"La suerte es lo que sucede cuando la preparación se encuentra con la oportunidad."*

*"La sort és el que passa quan la preparació es troba amb l'oportunitat."*

*"Luck is what happens when preparation meets opportunity."*

**Séneca (Córdoba, 4 a.C. – Roma, 65 a.C.)**

**UAB**

**Universitat Autònoma
de Barcelona**

**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 Doctoral titulada *"Relevancia de los antioxidantes en la regulación de las especies reactivas de oxígeno (ROS) en el semen de burro y caballo"* presentada por **Iván Patricio Yánez Ortiz** para optar al grado de Doctor en Medicina y Sanidad Animal por la Universidad Autónoma de Barcelona se ha realizado bajo su dirección y, considerándola terminada y cumpliendo los requisitos para poder optar a la Mención Internacional, autorizan su presentación para ser juzgada por la Comisión correspondiente.

Y para que conste a los efectos oportunos, firman el presente documento en Bellaterra (Cerdanyola del Vallès), a 19 de mayo de 2022.

Dr. Jordi Miró Roig                    Dr. Marc Yeste Oliveras

# CERTIFICADO

Quito, 04 de febrero de 2022

Mediante el presente documento, se certifica que el Sr. **Iván Patricio Yánez Ortiz**, con pasaporte 1721229480 y estudiante del Programa de Doctorado en Medicina y Sanidad Animal del Departamento de Medicina y Cirugía Animal de la Universidad Autónoma de Barcelona, realizó una estancia en el instituto de investigaciones en Biomedicina – iBioMed de la Universidad San Francisco de Quito – USFQ entre el 01 de noviembre de 2021 y el 03 de febrero de 2022.

Durante su estancia, el estudiante, junto con un equipo interdisciplinario de nuestro Instituto, participó en la toma y procesamiento de biopsias de endometrio de yeguas en celo adaptadas al Ecuador. Este trabajo está dentro del marco del proyecto "Análisis de la estacionalidad en yeguas de tres diferentes latitudes mediante evaluación hormonal y biopsias endometriales", proyecto HUBI# 17374. Dichas biopsias, junto con muestras de suero sanguíneo de las mismas yeguas, serán enviadas a la Universidad Autónoma de Barcelona para su análisis histológico y determinación del grado de endometritis, análisis inmunohistoquímico y expresión génica de citoquinas antiinflamatorias y citoquinas que favorecen la endometritis, así como para realizar la medición de la concentración de varias hormonas de la reproducción como: estrógenos, progesterona, LH y kisspeptina, entre otras.

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

**Dr. Ramiro Díaz B., DMVZ. MSc. PhD.**

**Director del Instituto de Investigaciones en Biomedicina "Una Salud"**
**Profesor/Investigador**
**Tel.: + 593 2 2971700 ext. 2071**
**@: rdiaz@usfq.edu.ec**
**USFQ**

Universidad San Francisco de Quito
Diego de Robles y Francisco de Orellana - Cumbayá
T.: 297 1700 Ext. 2071
Email: rdiaz@usfq.edu.ec

*A mi familia*
*A la meva família*
*To my family*

# ÍNDICE DE CONTENIDOS

## AGRADECIMIENTOS

Esta Tesis Doctoral se realizó en el Servicio de Reproducción Equina, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Autónoma Barcelona, Bellaterra (Cerdanyola del Vallès), España, con la colaboración del Grupo de Biotecnología de la Reproducción Animal y Humana (TechnoSperm), Instituto de Tecnología Agroalimentaria, Departamento de Biología, Facultad de Ciencias, Universidad de Girona, Girona, España.

Parece que fue ayer cuando decidí empezar esta aventura llamada "Doctorado", sabiendo que tenía que dejar parte de mi vida en mi querido país y sobre todo alejarme de mi familia y amigos por un largo tiempo... De esto, ya han pasado ya casi tres años. Durante este camino no puedo evitar acordarme de todas las personas que, de una u otra manera, han contribuido en mi crecimiento personal y profesional, y sobre todo han permitido que esta Tesis se haga realidad. No soy una persona tan expresiva, de hecho soy alguien de pocas palabras y siempre trato de cuidar mis emociones, pero espero que estas líneas sirvan para transmitir mi sincero agradecimiento y cariño a cada una de las personas que han estado ahí conmigo.

A mis directores, **Dr. Jordi Miró Roig** y **Dr. Marc Yeste Oliveras**. Jordi, gracias por ser una luz al final del túnel y cruzarse en mi camino en el momento en el que quizá más lo necesitaba. Mi mundo era o es otro, pero usted me acogió a su lado sin ningún complejo y sobre todo sin tener absolutamente nada de experiencia en el mundo de los caballos. Ahora puedo decir que usted me ha enseñado todo lo que sé de reproducción equina y también puedo decir orgullosamente que he estado con el mejor, no solo en su área, sino también con una persona increíble que siempre ha estado pendiente de todo y de todos. Sé que la apuesta que hice por el "caballo ganador" la gané en todos los aspectos. Marc, gracias por aceptarme en su equipo, pese a las circunstancias en las que me encontraba, y contribuir significativamente en la realización de esta Tesis. Sé que no ha habido la oportunidad de compartir muchas cosas con usted como me hubiese gustado, pero no hay duda que los momentos en los que hemos coincidido me han servido para darme cuenta que es una gran persona y sobre todo un magnífico investigador. Jordi y Marc gracias por creer en mí.

A mis amigos y compañeros del Servicio de Reproducción Equina de la Universidad Autónoma Barcelona, **Jaime**, **Marta** y **Miguel**. Jaime, gracias por ser ese amigo incondicional y echarme la mano cuando más lo necesité. Sé que tuviste mucho que ver para que Jordi y Marc me acogieran como Doctorando, y realmente te la jugaste por mí. Es más fácil cuando trabajas con un amigo que entiende lo que implica estar lejos de casa. Marta y Miguel, gracias por su compañerismo en todo momento y por ese apoyo durante este tiempo. Sé que son excelentes profesionales, ya consolidados, y estoy seguro que ustedes también muy pronto estarán escribiendo sus agradecimientos de la Tesis.

A los profesores de Reproducción Animal de la Facultad de Veterinaria de la Universidad Autónoma Barcelona, **Xus**, **Joan Enric**, **Montse**, **Teresa Rigau** y **Teresa Mogas**. Gracias a todos por hacerme sentir en casa y por sus palabras de aliento durante estos años. Sé que son grandiosos profesionales, catedráticos e investigadores, y con un componente humano admirable, que me han enseñado lo que implica la enseñanza y la investigación en la academia, además de la historia y cultura de la hermosa Catalunya.

Al técnico de laboratorio, **Álex**. Gracias por tu disposición para siempre colaborar con nosotros en todo lo que hemos necesitado, pero sobre todo por tus consejos, anécdotas y amistad.

A mis compañeras de despacho, **Iris**, **Tania**, **Sabrina**, **Linda**, **Judith**, **Ana**, **Magdalena** y **Ariana**. Gracias a todas por ser parte del día a día durante este tiempo. Ustedes saben muy bien todo lo que implica realizar una Tesis, obviamente con muchas más cosas buenas que malas, pero que al final se traducen en satisfacción. Como dije anteriormente, estoy seguro que ustedes, Iris, Judith, Ana y Ariana, también muy pronto estarán escribiendo sus agradecimientos de la Tesis.

A los chicos de TechnoSperm de la Universidad de Girona, **Yentel**, **Ariadna**, **Sandra**, **Carolina**, **Isabel**, **Estela**, **Marc**, **Jordi** y **Albert**. Gracias a todos por estar siempre dispuestos a colaborar con sus consejos y enseñanzas las veces que fui al laboratorio. Sé que no hubo la oportunidad de compartir tantos momentos fuera del trabajo, pero de lo poco que los pude conocer son excelentes personas y unos investigadores de élite.

A mi familia, **Inés**, **Wilson** y **Mauricio**. Inés, madre mía, gracias por darme la vida y ser el motor que impulsa mi corazón para siempre seguir adelante. Tu amor infinito y tus bendiciones depositadas sobre mí han hecho posible que cada día tenga esa fuerza necesaria para seguir adelante con este sueño. Wilson y Mauricio, queridos hermanos, gracias por su amor, apoyo y confianza, pero sobre todo por estar siempre ahí en todo momento. Familia, ustedes son pilares fundamentales de este logro... Los quiero mucho.

*Iván Patricio Yánez Ortiz*

# ABREVIATURAS

| | |
|---|---|
| -SH | Grupo sulfhidrilo |
| ºC | Grados centígrados |
| AA | Ácido ascórbico |
| ADN | Ácido desoxirribonucleico |
| ARN | Ácido ribonucleico |
| ART | Técnicas de reproducción asistida |
| ATP | Adenosín trifosfato |
| CAT | Catalasa |
| COX2 | Ciclooxigenasa 2 |
| $Cu^+$ | Ion cuproso |
| $Cu^{2+}$ | Ion cúprico |
| CUPRAC | Capacidad antioxidante reductora cúprica |
| $Fe^{2+}$ | Ion ferroso |
| $Fe^{3+}$ | Ion férrico |
| FRAP | Capacidad de reducción férrica del plasma |
| $g$ | Fuerza centrífuga relativa |
| GPX | Glutatión peroxidasa |
| GSH | Glutatión reducido |
| GSR | Glutatión reductasa |
| GSSG | Disulfuro de glutatión |
| H | Hidrógeno |
| $H_2O$ | Agua |
| $H_2O_2$ | Peróxido de hidrógeno |
| HDL | Lipoproteínas de alta densidad |
| HNO | Nitroxilo |
| HOCl | Ácido hipocloroso |
| IA | Inseminación artificial |
| IFN-$\gamma$ | Interferón gamma |
| IL-10 | Interleuquina 10 |
| IL-6 | Interleuquina 6 |
| IL-8 | Interleuquina 8 |
| IL1RA | Antagonista del receptor de interleuquina 1 |

| | |
|---|---|
| IL1β | Interleuquina 1 beta |
| LAAO | L-aminoácido oxidasa |
| LDL | Lipoproteínas de baja densidad |
| LOO$^-$ | Peroxilo lipídico |
| LOOH | Peróxido lipídico |
| mL | Mililitro |
| mM | Milimolar |
| $N_2O$ | Óxido nitroso |
| NADPH | Dinucleótido de nicotinamida y adenina fosfato reducido |
| NETs | Trampas extracelulares de neutrófilos |
| NO | Óxido nítrico |
| $NO_2$ | Dióxido de nitrógeno |
| $NO_3^-$ | Peroxinitrito |
| $O_2$ | Oxígeno |
| $O_2^-$ | Anión superóxido |
| $O_3$ | Ozono |
| OH$^-$ | Anión hidroxilo |
| OSI | Índice de estrés oxidativo |
| OXPHOS | Fosforilación oxidativa |
| pH | Potencial de hidrógeno |
| PMN | Neutrófilos polimorfonucleares |
| PON1 | Paraoxonasa tipo 1 |
| PUFA | Ácidos grasos poliinsaturados de cadena larga |
| RNS | Especies reactivas de nitrógeno |
| RO$^-$ | Alcoxilo |
| $RO_2$ | Peroxilo |
| ROS | Especies reactivas de oxígeno |
| SOD | Superóxido dismutasa |
| TAC | Capacidad antioxidante total |
| TAS | Estado antioxidante total |
| TEAC | Capacidad antioxidante equivalente de Trolox |
| TOS | Estado oxidativo total |
| v/v | Volumen/volumen |
| vs. | Versus |
| μm | Micrómetro |

# RESUMEN
# RESUM
# ABSTRACT

# RESUMEN

El semen constituye un complejo sistema oxidante/antioxidante debido a que involucra una acción prooxidante por parte de los propios espermatozoides, mediante la producción fisiológica de especies reactivas de oxígeno (ROS) derivada del metabolismo espermático y una acción antioxidante ejercida principalmente por el plasma seminal, mediante sus componentes enzimáticos y no enzimáticos. No obstante, cuando la producción de las ROS excede la capacidad de defensa antioxidante, se genera un desequilibrio oxidativo/antioxidante que conduce a una situación de estrés oxidativo, provocando graves alteraciones estructurales y funcionales en el espermatozoide. Si bien las ROS tienen un papel importante en el desarrollo de varios procesos fisiológicos relacionados con la fecundación, la producción exacerbada de éstas después de la entrada del semen al útero, debido a la NETosis de los neutrófilos polimorfonucleares (PMN) que actúan para combatir la reacción endometrial, influyen en el éxito reproductivo de los équidos. Esta situación aún se hace más evidente con el uso de semen criopreservado, ya que los espermatozoides pierden el soporte antioxidante del plasma seminal antes de la congelación, aumentando su susceptibilidad al ataque de las ROS. Por lo tanto, el objetivo principal de esta Tesis Doctoral fue determinar la relevancia de los antioxidantes en la regulación de las ROS en el semen de burro y caballo.

En el primer estudio, se determinó la cantidad de ROS extracelular producida por los espermatozoides de burro sometidos a estrés reductivo, a estrés oxidativo y a NETosis. Se evidenció que la exposición de los espermatozoides a concentraciones altas (iguales o superiores a 8 mM) de glutatión reducido (GSH) es capaz de generar más cantidad de ROS (medidas en niveles de peróxido de hidrógeno, $H_2O_2$) extracelular que la exposición a concentraciones altas (hasta 10 mM) de $H_2O_2$, lo cual resalta la gran resistencia de los espermatozoides de burro a la toxicidad que producen los altos niveles de GSH, así como su gran capacidad de defensa antioxidante en comparación con otras especies. Por otro lado, se observó que la NETosis de los PMN genera una gran cantidad de ROS extracelular, y que ésta aumenta cuando los PMN entran en contacto con los espermatozoides y en ausencia de plasma seminal.

En el segundo estudio, se evaluó la suplementación de distintas concentraciones de GSH al medio de congelación y su efecto sobre la criotolerancia de los espermatozoides de burro. Se observó que las concentraciones de GSH (entre 2 mM y 10 mM) no afectaron la viabilidad, la integridad de la membrana plasmática y del acrosoma, y el potencial de membrana mitocondrial de los espermatozoides después de la descongelación, mientras que la motilidad disminuyó únicamente en dosis iguales o superiores a 8 mM. Esto demostró, una vez más, la sorprendente resistencia de los espermatozoides de burro a dosis altas de GSH, incluso después de ser sometidos a la criopreservación. Por otro lado, los niveles intracelulares de ROS de los espermatozoides viables después de la descongelación se redujeron significativamente con la suplementación del medio de congelación con concentraciones altas de GSH (8 mM y 10 mM).

En el tercer y cuarto estudio, se investigó la relación entre la criotolerancia de los espermatozoides de burro y caballo y los antioxidantes enzimáticos y no enzimáticos, y el índice de estrés oxidativo (OSI) del plasma seminal. Se observó que los niveles de actividad de los antioxidantes enzimáticos, como la paraoxonasa tipo 1 (PON1) y la superóxido dismutasa (SOD), y de los antioxidantes no enzimáticos (medidos principalmente en términos de capacidad antioxidante equivalente de Trolox, TEAC) fueron significativamente superiores en los eyaculados clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad". Además, se encontraron correlaciones positivas con algunos parámetros de motilidad y funcionalidad espermática después de la descongelación. Por su parte, el OSI del plasma seminal fue más bajo en los eyaculados clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad", siendo mayor en el caballo que en el burro.

En conclusión, esta Tesis Doctoral sugiere que la regulación de las ROS por parte del sistema de defensa antioxidante, fundamentalmente del plasma seminal, constituye un mecanismo muy complejo. En efecto, tanto las características estructurales y funcionales de los espermatozoides, como los niveles de actividad de los antioxidantes enzimáticos y no enzimáticos presentes en el plasma seminal, hacen que el control del estrés oxidativo sea particularmente específico en el burro y en el caballo, lo cual está relacionado con la estrategia reproductiva de cada especie.

## RESUM

El semen constitueix un complex sistema oxidant/antioxidant perquè involucra una acció prooxidant per part dels propis espermatozoides, mitjançant la producció fisiològica d'espècies reactives d'oxigen (ROS) derivada del metabolisme espermàtic, i una acció antioxidant exercida principalment pel plasma seminal, mitjançant els seus components enzimàtics i no enzimàtics. No obstant això, quan la producció de les ROS excedeix la capacitat de defensa antioxidant, es genera un desequilibri oxidatiu/antioxidant que condueix a una situació d'estrès oxidatiu i provoca greus alteracions estructurals i funcionals a l'espermatozoide. Si bé les ROS tenen un paper important en el desenvolupament de diversos processos fisiològics relacionats amb la fecundació, la producció exacerbada després de l'entrada del semen a l'úter, a causa de la NETosi dels neutròfils polimorfonuclears (PMN) que actuen per combatre la reacció endometrial, influeixen en l'èxit reproductiu dels èquids. Aquesta situació encara es fa més evident amb l'ús del semen criopreservat, ja que els espermatozoides perden el suport antioxidant del plasma seminal abans de la congelació, la qual cosa augmenta la seva susceptibilitat a l'atac de les ROS. Per tant, l'objectiu principal d'aquesta Tesi Doctoral va ser determinar la rellevància dels antioxidants en la regulació de les ROS al semen de ruc i cavall.

Al primer estudi, es va determinar la quantitat de ROS extracel·lular produït pels espermatozoides de ruc sotmesos a estrès reductiu, a estrès oxidatiu i a la NETosi. Es va evidenciar que l'exposició dels espermatozoides a concentracions altes (iguals o superiors a 8 mM) de glutatió reduït (GSH) és capaç de generar més quantitat de ROS (mesurada en nivells de peròxid d'hidrogen, $H_2O_2$) extracel·lular que l'exposició a concentracions altes (fins a 10 mM) d'$H_2O_2$, cosa que ressalta la gran resistència dels espermatozoides de ruc a la toxicitat que produeixen els alts nivells de GSH, així com la gran capacitat de defensa antioxidant en comparació amb altres espècies. D'altra banda, es va observar que la NETosi dels PMN genera una gran quantitat de ROS extracel·lular, la qual augmenta quan els PMN entren en contacte amb els espermatozoides i en absència de plasma seminal.

Al segon estudi, es va l'efecte de la suplementació del medi de congelació amb diferents concentracions de GSH en la criotolerància dels espermatozoides de

ruc. Es va observar que les concentracions de GSH (entre 2 mM i 10 mM) no van afectar la viabilitat, la integritat de la membrana plasmàtica i de l'acrosoma, i el potencial de membrana mitocondrial dels espermatozoides després de la descongelació, mentre que la motilitat va disminuir únicament en dosis iguals o superiors a 8 mM. Això destaca, una vegada més, la resistència sorprenent dels espermatozoides de ruc a dosis altes de GSH, àdhuc quan es sotmeten a la criopreservació. D'altra banda, els nivells intracel·lulars de ROS dels espermatozoides viables després de la descongelació es van reduir significativament amb l'addició de concentracions altes de GSH (8 mM i 10 mM) al medi de congelació.

Al tercer i quart estudi, es va determinar la relació entre la criotolerància dels espermatozoides de ruc i cavall i els antioxidants enzimàtics i no enzimàtics, i l'índex d'estrès oxidatiu (OSI) del plasma seminal. Es va observar que els nivells d'activitat dels antioxidants enzimàtics, com la paraoxonasa tipus 1 (PON1) i la superòxid dismutasa (SOD), i dels antioxidants no enzimàtics (mesurats principalment en termes de capacitat antioxidant equivalent de Trolox, TEAC) eren significativament superiors en les ejaculacions classificades com de "bona congelabilitat" que en aquelles classificades com de "mala congelabilitat". A més, es van trobar correlacions positives amb alguns paràmetres de motilitat i funcionalitat espermàtica després de la descongelació. Per la seva banda, l'OSI del plasma seminal va ser més baix en les ejaculacions classificades com de "bona congelabilitat" que en les classificades com de "dolenta congelabilitat"; aquest índex era més gran en el cavall que en el ruc.

En conclusió, aquesta Tesi Doctoral suggereix que la regulació de les ROS per part del sistema de defensa antioxidant, fonamentalment del plasma seminal, constitueix un mecanisme molt complex. En efecte, tant les característiques estructurals i funcionals dels espermatozoides com els nivells d'activitat dels antioxidants enzimàtics i no enzimàtics presents al plasma seminal, fa que el control de l'estrès oxidatiu sigui particularment específic al ruc i al cavall, la qual cosa està relacionada amb l'estratègia reproductiva de cada espècie.

# ABSTRACT

In semen, there is a complex oxidant/antioxidant system, as the prooxidant activity from sperm via the physiological production of reactive oxygen species (ROS) derived from their metabolism is combined with the antioxidant action exerted mainly by the seminal plasma through its enzymatic and non-enzymatic components. When ROS production exceeds the antioxidant defense capacity, however, an oxidative/antioxidant imbalance is generated, thereby leading to oxidative stress, which causes serious structural and functional alterations on sperm. While ROS play an important role in several physiological processes related to fertilization, their exacerbated production after semen enters the uterus, which results from the NETosis of polymorphonuclear neutrophils (PMN) triggered to neutralize the endometrial reaction, influences the reproductive success in equids. This situation even becomes more apparent with the use of cryopreserved semen, because sperm lose the antioxidant support of the seminal plasma before freezing, increasing their susceptibility to ROS attack. The main objective of this Dissertation, therefore, was to determine the relevance of antioxidants in the regulation of ROS in donkey and horse semen.

In the first study, the amount of extracellular ROS produced by donkey sperm subjected to reductive stress, oxidative stress and NETosis was determined. The exposure of sperm to high concentrations (equal to or greater than 8 mM) of reduced glutathione (GSH) was found to be capable of generating more extracellular ROS (measured in levels of hydrogen peroxide, $H_2O_2$) than the incubation with high concentrations (up to 10 mM) of $H_2O_2$, which highlights the great resilience of donkey sperm to the toxicity produced by high levels of GSH and the larger antioxidant defense capacity compared to other species. On the other hand, NETosis of PMN was observed to generate a great amount of extracellular ROS, which increased when PMN came into contact with sperm and in the absence of seminal plasma.

In the second study, how the addition of different GSH concentrations to the freezing medium affects donkey sperm cryotolerance was evaluated. Whereas GSH concentrations (between 2 mM and 10 mM) did not affect viability, plasma membrane and acrosome integrity, and mitochondrial membrane potential of

sperm after thawing, motility only decreased in doses equal to or greater than 8 mM. This highlights, once again, the surprising resilience of donkey sperm to high GSH doses, even after cryopreservation. On the other hand, intracellular ROS levels of viable sperm after thawing significantly decreased with the addition of high GSH concentrations (8 mM and 10 mM) to the freezing medium.

In the third and fourth studies, the relationship of donkey and horse sperm cryotolerance with enzymatic and non-enzymatic antioxidants, and the oxidative stress index (OSI) of seminal plasma was determined. Activity levels of enzymatic antioxidants, such as paraoxonase type 1 (PON1) and superoxide dismutase (SOD), and of non-enzymatic antioxidants (measured mainly in terms of Trolox equivalent antioxidant capacity, TEAC) were found to be significantly higher in ejaculates classified as with "good freezability" than in those classified as with "poor freezability". In addition, positive correlations were seen with some motility and functionality parameters after thawing. Furthermore, the OSI of seminal plasma was lower in "good freezability" than in "poor freezability" ejaculates, and was higher in the horse than in the donkey.

In conclusion, this Dissertation shows that the regulation of ROS by the antioxidant defense system, largely from the seminal plasma, is a very complex mechanism. Indeed, both structural and functional characteristics of sperm and activity levels of the enzymatic and non-enzymatic antioxidants present in seminal plasma make the control of oxidative stress particularly specific in donkeys and horses, which is likely to be related to the reproductive strategy of each species.

# CAPÍTULO 1:

# INTRODUCCIÓN

## 1. Semen

El semen es un fluido corporal viscoso y gelatinoso resultante de la combinación de un componente celular (los espermatozoides) producido en el epitelio germinal de los testículos y de un componente líquido (plasma seminal) generado por la cola del epidídimo y las glándulas sexuales accesorias (Meyers, 2009; Rodríguez-Martínez et al., 2011; Ezzati et al., 2020; Barbagallo et al., 2021; Gibb et al., 2021). El semen se libera durante la eyaculación, que es un proceso complejo donde los dos componentes (celular y líquido) se mezclan en los conductos eyaculatorios comunes de las ámpulas de los conductos deferentes y las vesículas seminales, resultando en una suspensión celular heterogénea (Björndahl et al., 2010). Tanto las características como los componentes del semen (por ejemplo: número de espermatozoides, volumen, pH y composición del plasma seminal) son característicos de la especie y pueden incluso variar entre individuos de la misma (Mann et al., 1963; Mann y Lutwak-Mann, 1981). Del mismo modo, factores como la edad, la estación del año, el tamaño testicular, el tiempo de abstinencia y las condiciones ambientales (por ejemplo: nutrición, administración de fármacos y estrés social) hacen que el semen varíe en sus características y composición (Amann, 2011).

La eyaculación en burros y caballos se produce por una serie de contracciones fuertes y pulsátiles de los músculos uretral y bulboesponjoso, e involucra tres procesos secuenciales: erección, emisión y eyaculación propiamente dicha. La erección es el alargamiento y endurecimiento del pene, resultado del aumento del flujo sanguíneo sobre la musculatura cavernosa y esponjosa. La emisión es el movimiento y depósito de espermatozoides y plasma seminal en la uretra pélvica. La eyaculación propiamente dicha es la expulsión del semen a través de la uretra (Mann y Lutwak-Mann, 1981; Amann, 2011). Los diversos fluidos que componen el eyaculado proceden, en una pequeña parte, del propio testículo, de las ámpulas de los conductos deferentes y de las diferentes glándulas sexuales accesorias, y se liberan en un orden concreto. Como consecuencia, el eyaculado de los mamíferos se divide en tres fracciones: fracción pre-espermática, fracción espermática o rica en espermatozoides, y fracción post-espermática. La fracción pre-espermática presenta un aspecto acuoso y proviene de la próstata y de las glándulas bulbouretrales. La fracción rica en espermatozoides tiene un aspecto más

viscoso, pero sin llegar a serlo, y se origina en las ámpulas de los conductos deferentes y en el epidídimo. La fracción post-espermática tiene un aspecto viscoso y gelatinoso que se deriva de las secreciones de las vesículas seminales (Kareskoski et al., 2010; Amann, 2011; Kareskoski et al., 2011; Carvalho et al., 2016; Oliveira et al., 2020). Generalmente, los eyaculados de los équidos están formados por seis a nueve flujos consecutivos de semen (Magistrini et al., 2000; Tirpák et al., 2021), con aproximadamente el 70% de los espermatozoides en los primeros tres flujos (Kareskoski y Katila, 2008).

## 1.1. El espermatozoide

El espermatozoide es una célula reproductiva haploide producida por el epitelio germinal de los testículos durante la espermatogénesis (Meyers, 2009). Este gameto contiene la información genética masculina, la cual se combinará con la femenina durante el proceso de fecundación. Por lo tanto, su función es fecundar al oocito y así dar origen a un nuevo individuo (Varner et al., 2015). Para cumplir con este objetivo, es necesario que un gran número de espermatozoides sean liberados en el tracto reproductivo femenino, dado que durante su trayecto hasta alcanzar el oocito, éstos sufren una serie de cambios estructurales y funcionales que hacen que la inmensa mayoría acaben muriendo (Rigby et al., 2000; Flesch y Gadella, 2000). No obstante, cuando la ovulación es inminente, los espermatozoides que han logrado avanzar en su trayecto hasta poder unirse al epitelio del oviducto se capacitarán (apenas el 0,0007% de los espermatozoides depositados en el tracto reproductivo femenino en el caso de los équidos), adquiriendo así la capacidad de fecundar al oocito (Rigby et al., 2000; Maitan et al., 2021).

El espermatozoide fue descrito por primera vez por Anton van Leeuwenhoek en 1677 (Varner y Johnson, 2011), distinguiéndose básicamente dos partes: cabeza y cola o flagelo (Mann y Lutwak-Mann, 1981). No obstante, de forma general hoy en día se considera al cuello o pieza de conexión como una parte independiente de la cola (Meyers, 2009). De este modo, las tres partes principales, todas ellas cubiertas con la membrana plasmática, que se distinguen en el espermatozoide equino son: la cabeza, el cuello y la cola o flagelo (Figura 1), lo que resulta en una longitud promedio del gameto de 60 µm (Brito, 2007).
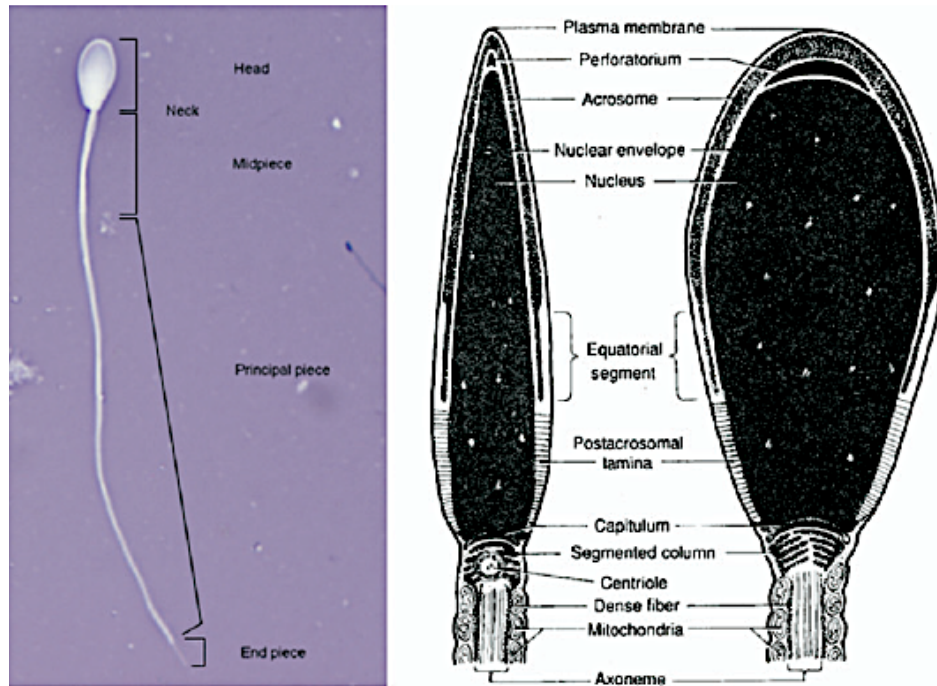
**Figura 1.** Partes del espermatozoide equino (Brito, 2007).

La cabeza es una estructura de forma ovalada y alargada, elíptica vista desde un plano longitudinal y aplanada vista desde un plano dorsoventral, ligeramente más gruesa en la porción posterior que en la anterior (Brito, 2007; Varner et al., 2015). Sus dimensiones aproximadas son: 5-7 μm de largo, 2,5-4 μm de ancho, 1,45 μm de ancho en la base, 14-16 μm de perímetro y 11-16 μm$^2$ de área (Brito, 2007; Meyers, 2009). Anatómicamente, la cabeza se subdivide en una región acrosómica, un segmento ecuatorial, una región post-acrosómica y un anillo posterior que delimita la unión entre la cabeza y la cola (Varner et al., 2015). En su interior, se encuentra el núcleo, el acrosoma, la lámina post-acrosómica (Brito, 2007) y un compartimento pequeño de citoplasma (Varner et al., 2015). El núcleo ocupa la mayor parte del espacio dentro de la cabeza y contiene el material genético en forma de ácido desoxirribonucleico (ADN) altamente condensado compuesto por el cromosoma X o Y, y un número haploide de cromosomas somáticos. El contenido del núcleo está envuelto por una doble membrana (cada una formada por una bicapa lipídica y proteínas) que lo separa del citoplasma circundante (Brito, 2007; Meyers, 2009; Varner et al., 2015). El acrosoma es un orgánulo específico del espermatozoide que tiene su origen en el aparato de Golgi de las espermátidas durante la espermiogénesis y contiene numerosas moléculas activas, entre las que se encuentran enzimas hidrolíticas y glucolíticas necesarias

para la fecundación (Brito, 2007; Meyers, 2009; Varner et al., 2015). El compartimento de citoplasma contiene proteínas citosólicas que son importantes para la función del espermatozoide (Varner et al., 2015).

El cuello es una estructura articular de conexión entre el borde posterior de la cabeza y la pieza intermedia de la cola (Meyers, 2009). Mide aproximadamente 1 µm de largo (Pesch y Bergmann, 2006) e incluye las columnas segmentadas, el capítulo, los centriolos proximal y distal, y varias mitocondrias pequeñas (Brito, 2007; Varner et al., 2015). Las columnas segmentadas anclan las fibras densas externas de la cola (Varner et al., 2015). El capítulo es una densa estructura fibrosa que sirve como unión con la placa basal de la cabeza mediante finos filamentos a nivel de la fosa de implantación (Brito, 2007; Varner et al., 2015). En los espermatozoides de burro y caballo, la fosa de implantación tiene una posición excéntrica con respecto al ancho de la célula, por lo que los espermatozoides con cola abaxial se consideran normales (Brito, 2007; Meyers, 2009). El centriolo proximal se encuentra unido a la fosa de implantación, mientras que el centriolo distal da lugar al axonema durante el desarrollo de la cola en la espermiogénesis (Brito, 2007; Varner et al., 2015), estando ausente en los espermatozoides maduros (Pesch y Bergmann, 2006). Las mitocondrias del cuello tienen una disposición perpendicular y comienzan a girar en espiral hacia la hélice mitocondrial, lo cual indica el comienzo de la pieza intermedia (Brito, 2007).

La cola es una estructura alargada que es esencial para el impulso motriz de los espermatozoides a través del tracto reproductor femenino durante la fecundación (Meyers, 2009). Comprende tres regiones bien definidas: una pieza intermedia, una pieza principal y una pieza final o terminal (Pesch y Bergmann, 2006; Brito, 2007).

- **Pieza intermedia:** Está formada por el axonema, una matriz de fibras densas externas y una vaina mitocondrial (Varner et al., 2015). Mide aproximadamente entre 8-10,5 µm de largo y 0,6 µm de diámetro, y se extiende desde el extremo caudal del cuello hasta el anillo de Jensen (Brito, 2007; Meyers, 2009), que es el sitio de unión con la pieza principal y el punto donde la vaina mitocondrial se reemplaza por una vaina fibrosa (Varner et al., 2015). El axonema es una matriz cilíndrica formada por nueve

pares de microtúbulos conectados por puentes espaciados que rodean un par central de microtúbulos conectados entre sí por un puente cruzado corto; estos microtúbulos centrales son los encargados de contraerse para producir el movimiento (Pesch y Bergmann, 2006; Brito, 2007; Varner et al., 2015). Las fibras densas externas rodean cada par de microtúbulos que conforman el axonema y también se encuentran en número de nueve, lo que da un patrón de configuración 9 × 2 + 2. Se extienden a lo largo de toda la longitud de la pieza intermedia, desde las columnas segmentadas del cuello hasta el final de la pieza principal y son las responsables de dar rigidez y elasticidad a la cola (Brito, 2007; Meyers, 2009; Varner et al., 2015). La vaina mitocondrial consiste en una superposición de mitocondrias dispuestas helicoidalmente alrededor de las fibras densas externas de la pieza intermedia (Varner et al., 2015). En el espermatozoide de los équidos, la disposición en espiral de un extremo a otro contiene aproximadamente entre 40-60 vueltas helicoidales de mitocondrias (Brito, 2007; Meyers, 2009; Varner et al., 2015). Las mitocondrias son los orgánulos responsables de producir la mayor parte de la energía en forma de adenosín trifosfato (ATP) necesaria para el movimiento de los espermatozoides (Brito, 2007; Meyers, 2009).

- **Pieza principal:** Representa la región más larga de la cola, midiendo aproximadamente entre 30-44 µm (Brito, 2007; Meyers, 2009). Contiene el axonema y las fibras densas externas, y se extiende a lo largo de toda la longitud de la pieza principal, desde el anillo de Jensen hasta la pieza final. Está rodeada por una vaina fibrosa compuesta de dos columnas longitudinales (dorsal y ventral) entre las cuales se entrecruzan una serie de nervaduras orientadas circunferencialmente a la mitad de la cola, pero que están interconectadas entre sí (Pesch y Bergmann, 2006; Brito, 2007; Varner et al., 2015). La vaina fibrosa y las fibras densas externas, si bien no se contraen, proporcionan el soporte estructural rígido y elástico a la cola, lo cual es esencial para que el movimiento deslizante de los pares de microtúbulos del axonema se traduzca en un movimiento efectivo de flexión y bateo de la cola (Pesch y Bergmann, 2006; Brito, 2007; Varner et al., 2015). El extremo distal de la vaina fibrosa termina abruptamente aproximadamente a unos 9-10 µm de la punta de la cola, donde la pieza

principal se fusiona con la pieza final (Pesch y Bergmann, 2006; Brito, 2007).

- **Pieza final:** Es la región más corta de la cola con una longitud de aproximadamente 4-5 µm (Meyers, 2009). Carece de vaina fibrosa y contiene únicamente el axonema (Brito, 2007). El patrón de configuración 9 × 2 + 2 se mantiene hasta más o menos la mitad de la pieza final, pero en el segmento restante, los pares de microtúbulos se separan, quedando dieciocho microtúbulos individuales, los mismos que se reducirán gradualmente conforme se acercan a la parte más distal de la cola (Pesch y Bergmann, 2006; Meyers, 2009).

La membrana plasmática de los espermatozoides de burro y caballo es una típica bicapa lipídica compuesta por una mezcla heterogénea de fosfolípidos, glucolípidos, esteroles, carbohidratos complejos y proteínas (Ladha, 1998; Meyers, 2009). En estas especies, como en otras de mamíferos, la membrana plasmática se caracteriza fundamentalmente por la presencia de ácidos grasos poliinsaturados de cadena larga (PUFA) (Wathes et al., 2007; Macías García et al., 2011; Agarwal et al., 2014; Aitken et al., 2018; Aurich et al., 2018; Papas et al., 2019b; Papas et al., 2020a). Estos PUFA son los responsables de conferir impermeabilidad y flexibilidad a la membrana plasmática, debido a que constituyen la parte hidrofóbica de los fosfolípidos (Flesch y Gadella, 2000). Así mismo, contribuyen a la fluidez requerida para los eventos de fusión de la membrana plasmática asociados con la fecundación (Aurich et al., 2018). Se ha descrito que el ácido docosapentaenoico es el PUFA más predominantes en la membrana plasmática de los espermatozoides de caballo, aunque con variaciones significativas entre individuos (Macías García et al., 2011).

## 1.2. Plasma seminal

El plasma seminal es una mezcla compleja de secreciones producidas por múltiples glándulas del tracto reproductor masculino (Meyers, 2009) y constituye el medio en el que se encuentran inmersos los espermatozoides durante la eyaculación (Rodríguez-Martínez et al., 2011). Se origina en el líquido intratubular del testículo y en la cola del epidídimo, y se enriquece con las secreciones de las

ámpulas de los conductos deferentes y las glándulas sexuales accesorias: próstata, glándulas bulbouretrales y vesículas seminales (Muiño-Blanco et al., 2008; Castiglione Morelli et al., 2021; Tirpák et al., 2021). Es un líquido isotónico y neutro que representa hasta el 98% del volumen total del eyaculado (Tirpák et al., 2021), y está compuesto de proteínas y sustancias no proteicas, como iones, sustancias orgánicas de bajo peso molecular, que incluyen aminoácidos libres, monosacáridos, lípidos, poliaminas, prostaglandinas y hormonas esteroideas (Al-Essawe et al., 2018b), y otras moléculas como ADN libre de células, ácido ribonucleico (ARN) y microARN (Castiglione Morelli et al., 2021).

Tradicionalmente, se consideró que el plasma seminal, visto en conjunto, cumplía con un única función, que es la de servir como vehículo pasivo para que los espermatozoides eyaculados puedan transportarse hacia y dentro del tracto reproductor femenino (Töpfer-Petersen et al., 2005). Sin embargo, hoy en día se considera que los componentes del plasma seminal desempeñan un papel importante en la fisiología de los espermatozoides (Al-Essawe et al., 2018b), lo que está relacionado fundamentalmente con el mantenimiento del metabolismo, el pH y la osmolalidad espermática (Muiño-Blanco et al., 2008), y la regulación del estrés oxidativo generado por las ROS a través de la acción de antioxidantes enzimáticos y no enzimáticos (Peña et al., 2019). Además, el plasma seminal tiene la capacidad de modular la respuesta inflamatoria en el ambiente uterino (Alghamdi et al., 2004), protegiendo a los espermatozoides de la endometritis inducida por la reproducción de ser fagocitados y destruidos (Troedsson et al., 2001), esto por su influencia en la formación de trampas extracelulares de neutrófilos (NETs) asociadas a la inmunidad uterina contra agentes infecciosos o espermatozoides (Mateo-Otero et al., 2021), así como con los procesos asociados a la fecundación (Töpfer-Petersen et al., 1998; Alghamdi et al., 2009; Szczykutowicz et al., 2019; Cecchini Gualandi et al., 2021).

## 2. Metabolismo espermático

El espermatozoide es una célula altamente especializada y su actividad metabólica requiere de una cantidad suficiente de energía libre para que se produzca el movimiento necesario en todas las etapas de su vida, incluso hasta llegar a su objetivo final que es la fecundación del oocito (Flores y Vilanova, 2015).

En este sentido, es sabido que la energía química requerida para la motilidad espermática es suministrada por la producción de ATP en condiciones aeróbicas (Kamp et al., 2007), principalmente por fosforilación oxidativa (OXPHOS) a nivel de las mitocondrias que se encuentran en la pieza intermedia del flagelo (Ortega Ferrusola et al., 2010; Gibb et al., 2014). Sin embargo, durante la capacitación el gasto de energía se incrementa considerablemente para alcanzar la hiperactividad del movimiento flagelar, motivo por el cual, se produce la activación de la maquinaria enzimática a nivel de la pieza principal del flagelo para generar ATP a partir de la glucólisis (Turner, 2005). Pese a que la OXPHOS muestra una mayor eficiencia en la producción de energía metabólica, generando más cantidad de ATP por molécula de glucosa, existen diferencias en cuanto a la dependencia en el metabolismo mitocondrial de los espermatozoides entre especies (Iaffaldano et al., 2013). Se considera que los espermatozoides de los équidos utilizan predominantemente la OXPHOS para la generación de energía en forma de ATP (Peña et al., 2019).

Dentro del proceso de producción de ATP vital para la motilidad espermática se pueden generar ROS por dos vías (Vernet et al., 2001). La primera surge como consecuencia de la fuga de electrones de los complejos I y II de la cadena de transportadora de electrones mitocondrial, siendo ésta considerada como principal causa en el daño oxidativo de los espermatozoides (Sabeur y Ball, 2006). La segunda surge a partir de un sistema enzimático ubicado en la membrana plasmática que utiliza el dinucleótido de nicotinamida y adenina fosfato reducido (NADPH) como sustrato a través de una oxidasa dependiente de NADPH que interviene en la activación de las vías de señalización interna. Estas vías regulan la fosforilación de las proteínas flagelares, que está relacionada con el movimiento de los espermatozoides (Olivera et al., 2016). Esta NADPH oxidasa ha demostrado generar ROS en caballos debido a que está involucrada en reacciones de óxido-reducción, además de que el NADPH es requerido por otras enzimas oxidantes para la generación de superóxidos (Sabeur y Ball, 2006; Pintus y Ros-Santaella, 2021).

## 3. Endometritis inducida por el semen

La estrategia reproductiva de los équidos marca que las hembras (burra y yegua) presenten una inflamación fisiológica normal del endometrio como

respuesta a la presencia del semen que ingresa directamente al útero tras la monta natural o la inseminación artificial (IA). Esta respuesta inflamatoria permite al sistema inmunológico fagocitar y remover del útero elementos contaminantes como bacterias, espermatozoides no viables, plasma seminal o diluyentes contenidos en el eyaculado o en las dosis seminales (Troedsson et al., 2001; Troedsson y Woodward, 2016; Miró y Papas, 2018a; Moya et al., 2022). Con la resolución exitosa de esta inflamación dentro de las 48 horas después del apareamiento, el endometrio es capaz de proporcionar un ambiente óptimo para la entrada del embrión en la luz uterina unos cinco días después y el posterior desarrollo de la gestación (Morris et al., 2020). No obstante, si esta inflamación no se resuelve más allá de las 48 horas, se producen una serie de cambios en el útero que son incompatibles con el establecimiento de la gestación. Ello puede comportar una condición patológica de endometritis inducida por la presencia de espermatozoides en la reproducción (Christoffersen y Troedsson, 2017; Canisso et al., 2020), condición que incluso puede progresar hasta una endometritis infecciosa o al desarrollo de una fibrosis endometrial o endometrosis (Morris et al., 2020), lo cual representa una de las principales causas de subfertilidad e infertilidad en estas especies (Gutjahr et al., 2000; LeBlanc, 2010; Canisso et al., 2020).

Aunque durante mucho tiempo se pensó que la inflamación del endometrio era debida a los microorganismos, hoy en día se sabe que son los propios espermatozoides, más que los agentes infecciosos, los responsables de esta reacción inflamatoria (Vilés et al., 2013; Canisso et al., 2020; Morris et al., 2020), más aún cuando se presentan situaciones de montas o inseminaciones continuas para asegurar una gestación, lo que genera una respuesta inmune adquirida hacia los espermatozoides que terminará en una endometritis persistente (Katila, 2012). No obstante, existen también condiciones previas que favorecen la endometritis, estrechamente relacionadas con defectos en la anatomía del tracto reproductor femenino que hacen que estas hembras sean propensas a aspirar aire o acumular líquido u orina en la vagina y en el útero (Canisso et al., 2016). Entre las causas principales se pueden mencionar una conformación vulvar y perineal deficiente, un posible desgarro del esfínter vestíbulo-vaginal, una posición pendular del útero, el deterioro de la contractibilidad uterina, una incompetencia en el cierre del cuello uterino o la atrofia de los pliegues endometriales (Trotter y McKinnon, 1988). Por lo tanto, solo las burras y yeguas con una respuesta inmune competente y una

anatomía funcional del tracto reproductivo pueden eliminar las infecciones de forma espontánea, adquiriendo una resistencia a la endometritis, mientras que las hembras con una respuesta inmune deficiente y una mala conformación anatómica pueden ser incapaces de combatir el desarrollo de una infección o pueden tener infecciones persistentes (Fumuso et al., 2007; Christoffersen et al., 2015). Cabe destacar que existen diferencias anatómicas importantes en el tracto reproductor femenino entre la burra y la yegua. La burra presenta un cuello uterino más largo y con una mayor sinuosidad que protruye hacia la vagina en comparación a la yegua, lo cual representa un factor de riesgo para el desarrollo de distocias (Vendramini et al., 1998; Renner-Martin et al., 2009). Además, durante el periodo de estro o celo, la burra tiende a acumular menos líquido en el útero, ocasionando que la presencia de edema sea menos característico de lo que es en la yegua (Climent et al., 2012).

## 3.1. Reacción endometrial

Como se mencionó anteriormente, después de la exposición del endometrio al semen producto de la monta natural o la IA, las burras y yeguas suelen mostrar un cierto grado de inflamación fisiológica que se resuelve dentro de las 48 horas posteriores. La resolución de esta inflamación se sustenta en la coordinación eficaz de varias vías de respuesta, donde la vía principal se basa en la respuesta inmune innata del endometrio para eliminar el exceso de espermatozoides y bacterias. No obstante, esto se complementa con una vía mecánica que involucra contracciones del miometrio para evacuar físicamente los desechos inflamatorios y en mucha menor medida, una vía de respuesta inmunológica clásica que contempla la unión antígeno-anticuerpo (Christoffersen y Troedsson, 2017; Morris et al., 2020).

Inmediatamente después de la introducción de los espermatozoides en el tracto reproductivo femenino se produce el reconocimiento de éstos como partículas extrañas y se produce la señalización por las células epiteliales de la mucosa en el endometrio para el reclutamiento y posterior movilización las células de defensa del sistema inmunológico del organismo de la hembra hacia los sitios de infección a través de la activación de varias citoquinas (Canisso et al., 2020; Morris et al., 2020). Las células encargadas de llevar a cabo la neutralización de los agentes extraños incluyen células fagocíticas como neutrófilos, macrófagos y

células dendríticas (Christoffersen y Troedsson, 2017), aunque también recientemente se ha identificado la presencia de eosinófilos en el endometrio de la burra (Miró et al., 2020a). De todas estas células de defensa inmunitaria, los granulocitos neutrófilos polimorfonucleares (PMN) son los que destacan por su rápida activación durante el estro o celo, pero no es hasta que se lleva a cabo la monta o la IA, cuando éstos se desplazan a través de la circulación sanguínea para llegar hasta el tejido endometrial, con el objetivo claro de contrarrestar un inminente proceso inflamatorio generado por la presencia de los espermatozoides en la luz uterina (Kotilainen et al., 1994; Katila, 1995; Troedsson et al., 2001; Rodriguez-Martinez et al., 2010; Vilés et al., 2013; Skarzynski et al., 2020). Este influjo de PMN en el endometrio es más elevado en la burra que en la yegua cuando se lleva a cabo la IA con semen congelado-descongelado, lo que implica una menor probabilidad de fecundación debido a una reacción inflamatoria exacerbada (Vidament et al., 2009; Vilés et al., 2013; Miró y Papas, 2018a; Miró et al., 2020b).

Durante las primeras 24 horas posteriores al apareamiento natural o artificial, además de producirse la quimiotaxis de los PMN para unirse y engullir bacterias y espermatozoides, también se evidencia una rápida activación de citoquinas proinflamatorias como IL1β, IL-8 e IFN-γ (Christoffersen et al., 2012). No obstante, en respuesta a la inflamación, se liberan adicionalmente citoquinas antiinflamatorias como IL1RA, IL6 y IL10 para mediar la respuesta, principalmente vía ciclooxigenasa 2 (COX2) (Kotilainen et al., 1994; Katila, 1995), ya que es necesario que se establezca un equilibrio entre los factores proinflamatorios y antiinflamatorios entre las 6 y 12 horas después del apareamiento para que el desenlace de la inflamación sea favorable para la reproducción (Woodward y Troedsson, 2015; Marth et al., 2018; Morris et al., 2020). Por el contrario, si la expresión endometrial de citoquinas proinflamatorias es inadecuada y prolongada, la modulación de la respuesta inflamatoria se ve alterada y las citoquinas antiinflamatorias reducen su actividad, lo que conduce a que un mayor número de PMN se trasladen al útero entre las 2 y 12 horas luego del darse la presencia de los espermatozoides (Christoffersen et al., 2012; Woodward et al., 2013; Christoffersen y Troedsson, 2017).

## 3.2. NETosis

La infiltración de PMN ocurre en el útero aproximadamente desde la media hora después de efectuarse el apareamiento y se mantiene hasta las 4-8 horas (Katila, 1995). Una vez activados, los PMN llevan a cabo su acción mediante dos mecanismos distintos, según el tamaño del patógeno. Mientras que en uno, los PMN liberan enzimas líticas que provocan la fagocitosis del agente infeccioso, en el otro, los PMN tienen la capacidad de degranular/extruir su ADN y moléculas como histonas y enzimas, incluidas la catepsina, elastasa y mieloperoxidasa, para la formación de NETs que se encuentran en el espacio extracelular y que tienen efectos inmunomoduladores y bactericidas (Branzk et al., 2014; Rebordão et al., 2014; Rebordão et al., 2018; Skarzynski et al., 2020; Moya et al., 2022). Así, la diseminación de los agentes infecciosos desde el sitio de la infección se ve afectada por la captura de las NETs (O'Brien et al., 2017), creando así un tipo único de muerte celular llamada NETosis (Mesa y Vasquez, 2013; Zambrano et al., 2016). Dentro del contexto reproductivo, la presencia de NETs se considera parte del desarrollo evolutivo del reino animal, reconociéndose a los espermatozoides como uno de los varios inductores de la formación NETs (Brinkmann, 2018). Es así que, recientemente, se ha observado en el burro que la unión de los espermatozoides a PMN podría inhibir la primera vía de acción de los PMN; es decir, la fagocitosis, lo cual conduciría a la activación de la segunda vía de acción de los PMN, esto es, la NETosis (Miró et al., 2020b).

Es importante destacar que existe una particularidad importante y clave dentro de la estrategia reproductiva de los équidos, la cual involucra únicamente la captura de los espermatozoides por parte de las NETs, pero sin comprometer su viabilidad (Zambrano et al., 2016). Relacionado con ello, hay que mencionar que el plasma seminal tiene un rol fundamental debido a que su presencia tiene efectos positivos sobre la motilidad de los espermatozoides y la supervivencia de éstos en el útero. Concretamente, el plasma seminal ayuda a evitar el daño oxidativo (Papas et al., 2019a; Papas et al., 2019b; Papas et al., 2020a) y disminuye la unión de los espermatozoides a los PMN y a otras células fagocíticas, gracias a la presencia de una ADNsa que facilita que algunos espermatozoides logren escapar de la NETs y puedan continuar, así, su trayecto hasta el oviducto, donde fecundarán al oocito (Alghamdi et al., 2004; Alghamdi y Foster, 2005).

No obstante de lo anterior, se ha observado que la NETosis de los PMN se induce por la presencia del plasma seminal (Miró et al., 2020b; Mateo-Otero et al., 2021), con lo cual, si bien existe una regulación fisiológica para permitir la liberación de los espermatozoides de las NETs, aquellos que quedan unidos a los PMN constituyen una fuente importante de generación de ROS hacia el medio extracelular (Foster y Cunningham, 1997; Moore et al., 1997). Relacionado con ello, hay que destacar que la producción de peróxido de hidrógeno ($H_2O_2$) es mucho mayor (aproximadamente entre 300 a 400 veces más) a la generada por el propio metabolismo espermático, lo que tiene un efecto deletéreo sobre la motilidad, tal y como se ha observado en caballos (Baumber et al., 2002). Sin embargo, en el burro los valores de producción de ROS derivada de la NETosis de los PMN aún son desconocidos.

## 4. Criopreservación espermática

La criopreservación de semen permite la conservación, por un tiempo indefinido, de los espermatozoides vivos y con sus estructuras intactas mediante el enfriamiento a temperaturas bajo cero hasta llegar a la congelación (Pegg, 2007). Es uno de los procedimientos más importantes en el desarrollo de las técnicas de reproducción asistida (ART) aplicadas a las especies de interés productivo (Yeste, 2017; Ugur et al., 2019; Riesco et al., 2021), los animales de compañía (Aurich et al., 2020; Sicherle et al., 2020) y las especies en peligro de extinción (Fickel et al., 2007).

En el caso de los équidos, y a pesar que en gran medida la selección de machos reproductores se basa principalmente en la genealogía, el rendimiento deportivo, el trabajo o la morfología (Poyato-Bonilla et al., 2021), más que en la eficiencia reproductiva (El-Badry et al., 2016; Al-Essawe et al., 2018a; Šichtař et al., 2019), la criopreservación de semen ha adquirido gran importancia en las últimas décadas. Ello es debido a los beneficios que ofrece su utilización (Loomis, 2001; Alvarenga et al., 2016), relacionados fundamentalmente con el almacenamiento a largo plazo, el transporte a largas distancias (Neuhauser et al., 2019), la selección de animales genéticamente superiores (Palmer y Chavatte-Palmer, 2020) y el establecimiento de bancos de germoplasma (Pugliesi et al., 2014), así como con la facilidad de inseminar a una hembra en el momento óptimo de reproducción, sin

depender de la disponibilidad del macho reproductor (Samper, 2007; Loomis y Graham, 2008; Aurich et al., 2020), la inseminación de varias hembras con un solo eyaculado (Peña et al., 2011), e incluso con el control de la diseminación de enfermedades venéreas (Pérez et al., 2017). Sin embargo, la congelación-descongelación implica una afectación negativa en la motilidad, morfología, funcionalidad y supervivencia de los espermatozoides (Holt, 2000; Blottner et al., 2001), así como en su capacidad de fecundación (Yeste et al., 2015), lo cual se ve reflejado en una menor tasa de preñez en comparación con el semen fresco o refrigerado (Vidament et al., 2009; Canisso et al., 2011; Oliveira et al., 2012). Esta reducción del poder fecundante del semen congelado-descongelado es especialmente importante en el caso del burro (Rota et al., 2012; Miró y Papas, 2018a; Álvarez et al., 2019).

Para contrarrestar la menor eficiencia de la IA del semen congelado-descongelado respecto al semen fresco o refrigerado, se han establecido parámetros mínimos que deben cumplir los eyaculados de un macho reproductor antes de ser sometidos a la criopreservación, como por ejemplo, tener un porcentaje de espermatozoides progresivamente móviles superior al 50%, un porcentaje de espermatozoides morfológicamente normales de al menos el 40% y una concentración mínima de $100 \times 10^6$ de espermatozoides/mL (Loomis y Graham, 2008; Hoffmann et al., 2011; Restrepo Betancur et al., 2014). Hay que mencionar además que, incluso en el caso del semen de los machos reproductores considerados "aptos para congelación" por cumplir con los parámetros antes mencionados, existe una variabilidad relacionada con la sensibilidad de los espermatozoides para resistir a los procedimientos de congelación y descongelación. Estas diferencias en la criotolerancia espermática se han observado, tanto entre animales pertenecientes a la misma especie (Loomis y Graham, 2008; Al-Essawe et al., 2018a), como entre razas (Alvarenga et al., 2003) e, incluso, entre eyaculados de un mismo animal (Yeste et al., 2015).

Las diferencias en términos de criotolerancia espermática hace posible que, mediante el uso de la estadística (Casas et al., 2009; Yeste et al., 2013a; Yeste et al., 2014; Prieto-Martínez et al., 2017; Morató et al., 2018), se puedan clasificar a los eyaculados de burro (Papas et al., 2020a) y caballo (Papas et al., 2019b) como de "buena congelabilidad" o "mala congelabilidad". En este sentido, se ha

observado que solo el 20-30% de los machos reproductores producen un semen con buena capacidad de congelabilidad, mientras que los animales que producen un semen que no es congelable por su baja criotolerancia representan el 20-30%, aunque se trate de animales con buenos índices de fertilidad en monta natural. El 40-60% restante de machos reproductores se consideran en una categoría intermedia; es decir, con una capacidad de congelabilidad aceptable, aunque su calidad espermática se vea afectada negativamente por la criopreservación (Vidament et al., 1997; Brinsko et al., 2000; Loomis y Graham, 2008; Hoffmann et al., 2011; Neuhauser et al., 2019; Šichtař et al., 2019).

### 4.1. Protocolo de criopreservación

Desde la publicación del primer artículo sobre congelación del semen de caballo en un medio con glucosa y glicerol por Smith y Polge en 1950 y el posterior nacimiento del primer potro con semen congelado en 1957 (Barker y Gandier, 1957), se han hecho notables esfuerzos para mejorar esta tecnología. Entre otras cuestiones, las investigaciones se han enfocado a la mejor comprensión de la gran variabilidad en cuanto a la calidad del semen congelado-descongelado disponible en el mercado y a los resultados obtenidos con su uso (Samper y Morris, 1998; Sieme et al., 2008), lo cual posiblemente tiene su origen en la falta de un protocolo estandarizado de criopreservación de semen que unifique los criterios experimentales y los procedimientos rutinarios de campo (Vidament, 2005; Aurich y Aurich, 2006). No obstante, hoy en día los protocolos de congelación de semen de burros y caballos requieren de una serie de procedimientos secuenciales que van desde el examen andrológico del macho reproductor hasta la evaluación seminal después de la descongelación, pasando por otras etapas como la recolección de semen y su evaluación, la dilución en un medio para centrifugación, el lavado o centrifugación en gradiente de densidad, la eliminación del plasma seminal, la resuspensión del pellet de espermatozoides con el medio de congelación, la adición del medio de congelación, la refrigeración y/o estabilización, el envasado y sellado de pajillas, la congelación y el almacenamiento en nitrógeno líquido (Samper y Morris, 1998; Alvarenga et al., 2016; Canisso et al., 2019).

Si bien todos los pasos mencionados anteriormente son importantes para la obtención de una calidad seminal y poder fecundante aceptables después de la

descongelación, hay dos pasos clave en todo protocolo de criopreservación de semen de burro (Papas et al., 2020c) y caballo (Catalán et al., 2020a). Dichas etapas se corresponden con la dilución elevada del eyaculado en un medio para la centrifugación en gradiente de densidad (Miró y Papas, 2018b) y a la eliminación del plasma seminal, dado que la presencia de éste afecta negativamente a la calidad de los espermatozoides durante su almacenamiento (Alvarenga et al., 2016), ya sea en estado refrigerado (Miró et al., 2009) o congelado (Kareskoski et al., 2010). Sin embargo, la eliminación del plasma seminal supone una mayor exposición de los espermatozoides al estrés oxidativo, ya que los antioxidantes enzimáticos y no enzimáticos que contiene este fluido también se eliminan. Por lo tanto, los espermatozoides pueden padecer alteraciones funcionales y estructurales graves que son producto de la oxidación y conducen a la muerte celular (Yeste et al., 2013a; Dutta et al., 2019;). Por lo tanto, se recomienda dejar una fracción de plasma seminal (entre el 5 y 20%) después de la centrifugación junto con los espermatozoides para la regulación del ambiente oxidante generado por la presencia de ROS procedente del metabolismo espermático, el cual es esencial, tanto para la supervivencia a la congelación (Jasko et al., 1991; Loomis, 2006; de Andrade et al., 2011) como para la modulación de la respuesta inflamatoria que se produce en la hembra después de la monta natural o la IA (Troedsson et al., 2001; Mari et al., 2011; Cecchini Gualandi et al., 2021).

Es importante considerar que la centrifugación puede inducir la peroxidación lipídica de la membrana plasmática de los espermatozoides por un incremento de la producción de ROS (Canisso et al., 2008; Marzano et al., 2020). Por ello, se recomiendan velocidades de centrifugación bajas que oscilan entre 400 y 900× $g$ por un tiempo que puede variar entre 10 y 20 minutos (Len et al., 2010; Alvarenga et al., 2016). En el caso específico del burro (Papas et al., 2020a) y el caballo (Catalán et al., 2020a), se recomienda realizar una dilución 1:5 (v/v) del eyaculado en un medio para la centrifugación en gradiente de densidad y centrifugarlo a una velocidad de 660× $g$ durante 15 minutos a una temperatura de 20 °C. Ello permite que se mantenga una mayor cantidad de espermatozoides vivos y morfológicamente normales antes de la congelación, lo que tiene un impacto positivo en la calidad seminal después de la descongelación.

## 4.2. Alteraciones en el espermatozoide

Independientemente del protocolo de criopreservación aplicado, la calidad del semen congelado-descongelado depende de la capacidad de los espermatozoides de resistir a cada uno de los procedimientos sin perder sus funciones principales (Sieme et al., 2008), ya que la pérdida de una sola de estas funciones puede reducir o suprimir totalmente su poder fecundante (Amann y Pickett, 1987; Yeste et al., 2015). Los daños que sufren los espermatozoides durante el proceso de criopreservación se han descrito ampliamente en la literatura (Bailey et al., 2000; Hezavehei et al., 2018; Yeste, 2016; Ezzati et al., 2020). Si bien, todas las alteraciones están interrelacionadas entre sí, es posible clasificarlas según su origen en daños debidos a los cambios de temperatura (estrés térmico), de osmolalidad (estrés osmótico) o a la acción de agentes oxidantes (estrés oxidativo) (Alvarenga et al., 2016). El estrés térmico es el resultado de la exposición de los espermatozoides a variaciones de temperatura, lo cual conduce a la formación de cristales de hielo dentro de la célula y en el entorno circundante (Morris et al., 2012). El estrés osmótico incluye, por un lado, la formación de un medio extracelular hipertónico durante la congelación, al que la célula responde perdiendo agua y disminuyendo su volumen con el objetivo de equilibrar los ambientes extra e intracelular (Yeste, 2016) y, por otro, la exposición a un medio extracelular hipotónico durante la descongelación, lo que permite que el agua ingrese a la célula por difusión pasiva con el consiguiente aumento de su volumen (Pommer et al., 2002). Finalmente, el estrés oxidativo se asocia a la producción de ROS (Yeste et al., 2015), lo cual se describe más adelante.

La estructura principal irreversiblemente afectada por la criopreservación es la membrana plasmática (Loomis y Graham, 2008), debido a las alteraciones de los complejos lipoproteicos durante los procesos de congelación y descongelación (Mazur et al., 1972). Con el descenso de temperatura, la configuración de los fosfolípidos se modifica a medida que se mueven lateralmente, lo que permite la adhesión de proteínas (Watson, 2000). Esto provoca que la membrana plasmática del espermatozoide se vuelva más rígida y frágil, debido a que pasa del estado líquido al gelificado (De Leeuw et al., 1990), lo que resulta en un aumento de su permeabilidad y a una disminución de la actividad metabólica (Hammerstedt y Graham, 1992). Aparte del daño observado en la membrana plasmática después

de la congelación-descongelación, en los équidos también se ha demostrado que la criopreservación induce la fragmentación del ADN, la disminución del potencial de membrana mitocondrial y la pérdida de integridad del acrosoma (Cabrera et al., 2014; Yeste et al., 2015).

## 5. Estrés oxidativo

El estrés oxidativo es una condición asociada con una mayor tasa de daño celular inducido por el oxígeno ($O_2$) y los oxidantes derivados del $O_2$, comúnmente conocidos como ROS (Sikka et al., 1995). Bajo ciertas condiciones, el aumento de oxidantes y la disminución de antioxidantes no se pueden prevenir, y al existir una producción no controlada de ROS que excede la capacidad antioxidante de la propia célula, el equilibrio oxidativo/antioxidante cambia hacia el estado oxidativo (Erel, 2004; Desai et al., 2010). Por lo tanto, el estrés oxidativo es una consecuencia del desequilibrio entre el sistema de defensa antioxidante y la producción de ROS (Celi, 2011; Agarwal et al., 2014; Sies, 2015; Papas et al., 2019a).

En el caso de los espermatozoides, que fueron las primeras células conocidas con capacidad de generar ROS como subproducto de la respiración celular (Tosic y Walton, 1946), esta situación de estrés oxidativo viene dada en gran medida por la manipulación del semen durante el proceso de criopreservación y por la aplicación de biotecnologías reproductivas como la IA (Bansal y Bilaspuri, 2011), donde las condiciones aeróbicas hacen que la producción de ROS sea prácticamente inevitable (Peña et al., 2019). Todo esto conlleva a que el espermatozoide sufra una serie de cambios drásticos de temperatura, pH, osmolalidad y presión de oxígeno que pueden exacerbar la producción de ROS, y en consecuencia el nivel de estrés oxidativo, lo cual comprometerá gravemente la calidad espermática y repercutirá negativamente en la fertilidad (Aitken y Baker, 2006; Guthrie y Welch, 2012; Sotgia et al., 2020).

### 5.1. Especies reactivas de oxígeno (ROS)

Las ROS son compuestos químicos altamente reactivos e inestables con un electrón desapareado (Agarwal et al., 2014; Peña et al., 2019) que se forman como subproductos del metabolismo del $O_2$ durante las reacciones enzimáticas normales

de señalización intracelular (Bansal y Bilaspuri, 2011) en los procesos metabólicos y fisiológicos, y pueden producirse reacciones oxidativas dañinas en organismos que las eliminan a través de mecanismos antioxidantes enzimáticos y no enzimáticos (Erel, 2004). Cuando este electrón desapareado pasa a formar parte de estructuras celulares cercanas, producto del emparejamiento con los electrones de otros átomos o moléculas en un intento por alcanzar un estado electrónicamente estable, se produce la oxidación de los lípidos de la membrana celular, los aminoácidos de las proteínas o los ácidos nucleicos (Agarwal et al., 2014; Wagner et al., 2018), lo que puede afectar a la célula, originando desde respuestas adaptativas hasta apoptosis o necrosis (Lushchak, 2014). La amplia gama de ROS incluyen radicales libres de oxígeno como el anión hidroxilo ($OH^-$), el anión superóxido ($O_2^-$), el peroxilo ($RO_2$), el alcoxilo ($RO^-$), el peroxilo lipídico ($LOO^-$), y moléculas no radicales como el $H_2O_2$, el ácido hipocloroso ($HOCl$), el peróxido lipídico ($LOOH$) y el ozono ($O_3$). Además, están las especies reactivas de nitrógeno (RNS) como el óxido nítrico ($NO$), el óxido nitroso ($N_2O$), el dióxido de nitrógeno ($NO_2$), el peroxinitrito ($NO_3^-$) y el nitroxilo ($HNO$), que son radicales libres de nitrógeno y se consideran una subclase de las ROS (Bansal y Bilaspuri, 2011; Agarwal et al., 2014; Peña et al., 2019; Juárez-Rojas et al., 2022).

## 5.2. Fuentes de ROS

La reproducción es una de las funciones biológicas que más energía demanda en su actividad, generando una elevada tasa metabólica que incurre en una mayor producción de ROS (Blount et al., 2016). Particularmente en los équidos, mientras en el macho, la generación de energía en forma de ATP (Peña et al., 2019) dependiente de la OXPHOS (Ortega Ferrusola et al., 2010; Gibb et al., 2014) se asocia con una elevada actividad mitocondrial en los espermatozoides, lo cual genera una gran cantidad de ROS que puede, incluso, alcanzar niveles tóxicos (Darr et al., 2016; Davila Plaza et al., 2016; Aitken et al., 2022), en la hembra, la inflamación endometrial que ocurre después de la monta natural o la IA por la presencia de espermatozoides es la principal responsable de la elevada producción de ROS (Vilés et al., 2013). Por lo tanto, los espermatozoides (Ball et al., 2001; Yeste et al., 2015) y los PMN (Foster y Cunningham, 1997; Moore et al., 1997) constituyen las dos fuentes principales de ROS dentro del contexto reproductivo de burros y caballos. No obstante, otra fuente importante de generación de ROS son los

espermatozoides inmaduros, los no viables, e incluso, los morfológicamente anormales (Morrell y Rodriguez-Martinez, 2009; Roca et al., 2016), los cuales favorecen la desaminación de los aminoácidos aromáticos por acción de la enzima L-aminoácido oxidasa (LAAO) presente en el acrosoma de los espermatozoides (Aitken et al., 2015).

En todos los escenarios vistos anteriormente, los espermatozoides intervienen activamente en la producción de ROS, debido fundamentalmente a la fuga de electrones de la cadena transportadora de electrones de la mitocondria, siendo esta considerada actualmente como la principal fuente de ROS en el espermatozoide (Koppers et al., 2008; Peña et al., 2019; Aitken et al., 2022). En condiciones aeróbicas, más del 90% del $O_2$ consumido se reduce directamente a agua ($H_2O$) gracias a la acción de la citocromo C oxidasa de la cadena transportadora de electrones y sin que se libere ROS. Sin embargo, menos del 10% restante del $O_2$ consumido genera ROS, primero por la reducción a $O_2^-$ y luego por la formación de $H_2O_2$ (Agarwal et al., 2014). De forma general, se estima que en una célula sana aproximadamente el 2% del $O_2$ reducido en las mitocondrias durante la OXPHOS se convierte en $O_2^-$ (Sotgia et al., 2020; Pintus y Ros-Santaella, 2021). En esta circunstancia, los niveles bajos de ROS son necesarios para que el espermatozoide pueda cumplir con funciones vitales relacionadas con la fertilidad, como la capacitación, la hiperactivación y la posterior reacción acrosómica (Baumber et al., 2003; Leahy y Gadella, 2011; Macías-García et al., 2015), así como para la penetración de la zona pelúcida del oocito (Agarwal et al., 2014; Gosalvez et al., 2017) e incluso para la formación de las capas germinales en la última etapa del desarrollo embrionario (Kodama et al., 1996).

### 5.3. Efectos adversos de las ROS

Los efectos adversos de las ROS se generan cuando la sobreproducción de estas moléculas supera los mecanismos reguladores antioxidantes de la célula (Peña et al., 2019; Gibb et al., 2021). Las moléculas comunes que reciben el electrón desapareado son los lípidos en las membranas y los carbohidratos en los ácidos nucleicos (Wagner et al., 2018). Por lo tanto, las principales alteraciones en los espermatozoides de los équidos se producen a nivel de la membrana plasmática, el ADN y algunos parámetros espermáticos como la motilidad,

influenciando directamente en los procesos fisiológicos y afectando la calidad seminal (Baumber et al., 2000; Baumber et al., 2002; Baumber et al., 2003; Bansal y Bilaspuri, 2011). Estos efectos del estrés oxidativo son particularmente importantes durante la conservación de los espermatozoides, ya sea por refrigeración o congelación, y este daño aumenta aún más en situaciones en las que gran parte del plasma seminal se extrae de una muestra de semen debido a que gran parte de la capacidad antioxidante del semen reside justamente en el plasma seminal (Ball, 2008).

### 5.3.1. Peroxidación lipídica de la membrana plasmática

Los fosfolípidos de la membrana plasmática de los espermatozoides de burro y caballo se caracterizan por contener una gran cantidad de PUFA (Wathes et al., 2007; Macías García et al., 2011; Agarwal et al., 2014; Aitken et al., 2018; Aurich et al., 2018; Papas et al., 2019b; Papas et al., 2020a). Esta situación, al mismo tiempo que le confiere a la membrana la fluidez y la flexibilidad necesarias para facilitar la fusión de las membranas durante la capacitación (membranas plasmática y acrosómica externa) y la fecundación (membranas plasmática del espermatozoide y del oocito) (Aitken et al., 2016b), también la convierte en un blanco para el ataque de los radicales libres de oxígeno debido a la presencia de dobles enlaces no conjugados en los ácidos grasos (Bansal y Bilaspuri, 2011). El daño oxidativo se produce por la gran susceptibilidad del hidrógeno (H) del enlace metil carbono-hidrógeno que se encuentra entre los ácidos grasos, lo cual en primera instancia forma $LOO^-$ que luego reaccionan con el $O_2$ para generar $RO_2$. Posteriormente estos $RO_2$ provocan una reacción en cadena autocatalítica para finalmente reaccionar con el H y formar LOOH (Saalu, 2010; Agarwal et al., 2014). El resultado es ello es la peroxidación lipídica de la membrana plasmática, condición que modifica la configuración estructural de la misma, provocando un deterioro de las funciones celulares, una reducción de la motilidad e incluso la inducción de la apoptosis de los espermatozoides (Bucak et al., 2010).

### 5.3.2. Fragmentación del ácido desoxirribonucleico (ADN)

El ADN espermático se caracteriza por la gran condensación y organización estructural de la cromatina presente en el núcleo, producto del reemplazo de las

histonas por proteínas de transición, y posteriormente, por protaminas durante la espermiogénesis (Agarwal et al., 2014). Esta organización del ADN no solo ayuda a proteger la cromatina del daño oxidativo, haciéndolo particularmente resistente a la acción de los radicales libres de oxígeno (Schulte et al., 2010), sino que es fundamental para para la transmisión del ADN paterno y de la información epigenética paterna al oocito durante la fecundación (Bao y Bedford, 2016). Sin embargo, cuando este proceso de condensación falla debido a la protaminación incompleta de la cromatina de los espermatozoides, el ADN se convierte en uno de los principales blancos de la oxidación (Agarwal et al., 2014); en concreto, los grupos nucleofílicos de la desoxirribosa y de las bases nitrogenadas quedan expuestos al ataque electrofílico de las ROS (Zorrilla García et al., 2004). En consecuencia, se modifican las bases nitrogenadas y se produce daño en el ADN (Zribi et al., 2011), caracterizado por la presencia de sitios sin bases nitrogenadas, deleciones, mutaciones, enlaces cruzados de ADN y reordenamientos cromosómicos (Agarwal et al., 2014). Asimismo, la oxidación de los nucleótidos puede causar pares abásicos en el ADN, aumentando el riesgo de que ocurran errores de replicación (Peña et al., 2019). Como resultado de la fragmentación del ADN, se producen alteraciones en los sistemas reguladores y de señalización de la muerte celular, con lo cual la ruptura de las cadenas de ADN inducida por las ROS puede desencadenar cambios similares a la apoptosis en los espermatozoides (Dutta et al., 2021).

## 6. Antioxidantes

Para mantener los mecanismos oxidativos fisiológicos mientras se minimiza el riesgo de daño celular, es necesario que exista un equilibrio entre la producción de ROS y los antioxidantes (Wagner et al., 2018). Los antioxidantes son agentes reductores que reaccionan con las ROS e interrumpen la secuencia de la cadena oxidativa (Bansal y Bilaspuri, 2011). Su función es dar electrones a otras moléculas que tienen uno o más electrones desapareados por la presencia de las ROS, y así evitar que se produzcan alteraciones en la estructura molecular de lípidos, proteínas y ADN espermático (Tremellen, 2008). Por lo tanto, los antioxidantes tienen la capacidad de neutralizar las ROS y posteriormente eliminarlas, considerándose una de las principales estrategias de defensa contra el estrés oxidativo (Sies, 1993).

A pesar de que el tracto reproductor masculino es capaz de proporcionar a los espermatozoides una protección antioxidante enzimática altamente especializada durante la maduración en el epidídimo (Wathes et al., 2007) y de que el propio espermatozoide contiene antioxidantes intracelulares proporcionados por las mitocondrias y el citosol (Ortega-Ferrusola et al., 2019), dichos mecanismos no son suficientes para asegurar la supervivencia de los espermatozoides debido a que éstos pierden la mayor parte de su citoplasma durante la espermiogénesis y la maduración en el epidídimo (Wathes et al., 2007), dejando un volumen muy pequeño que limita su capacidad de defensa antioxidante (Bollwein et al., 2008; Ball, 2009; Amidi et al., 2016; Dutta et al., 2019), Además, la producción de ROS se multiplica cuando los espermatozoides son eyaculados, así como durante la activación de la motilidad (Ball et al., 2001) y la posterior interacción con el tracto reproductor femenino (Miró et al., 2020b). Por lo tanto, al encontrarse limitada la presencia de antioxidantes intracelulares, los espermatozoides necesitan de moléculas extracelulares con propiedades antioxidantes que contrarresten el estrés oxidativo y que puedan prevenir el daño celular. En este escenario, el plasma seminal constituye la principal defensa antioxidante de los espermatozoides contra el daño producido por el estrés oxidativo, mediante la acción de factores enzimáticos y no enzimáticos que intervienen en la modulación y eliminación del exceso de ROS (Muiño-Blanco et al., 2008; Agarwal et al., 2014; Wagner et al., 2018; Papas et al., 2019a; Peña et al., 2019; Papas et al., 2020a).

## 6.1. Antioxidantes enzimáticos

Las enzimas antioxidantes encargadas de combatir el estrés oxidativo que han sido descritas en el plasma seminal de burro y caballo incluyen la superóxido dismutasa (SOD), la catalasa (CAT) y el sistema glutatión peroxidasa-glutatión reductasa (GPX/GSR) (Baumber y Ball, 2005; Papas et al., 2019a). El mecanismo enzimático de defensa antioxidante del semen consiste en una serie de reacciones químicas, donde cada enzima cumple con una función específica, y en la cual, los productos de una reacción química sirven de sustrato para la siguiente (Figura 2). En orden de intervención, la secuencia de reacciones que catalizan las enzimas antioxidantes empieza con la dismutación del $O_2^-$ por parte de la SOD para formar $H_2O_2$ y $O_2$ (Kowalowka et al., 2008; Walczak-Jedrzejowska et al., 2013). Posteriormente, el $H_2O_2$ entra en el sistema de equilibrio oxidación-reducción

utilizando el glutatión para mantener una homeostasis entre las ROS necesarias para las funciones fisiológicas del espermatozoide y los efectos adversos que podría causar su exceso (Luberda, 2005; Adeoye et al., 2018). Dentro de este equilibrio, el $H_2O_2$ puede ser convertido a $H_2O$ por la acción de la GPX, utilizando el glutatión reducido (GSH) y haciendo que el GSH se oxide a disulfuro de glutatión (GSSG), mientras que la GSR cataliza la reducción de GSSG para nuevamente obtener GSH y $H_2O_2$ (Baumber y Ball, 2005; Meseguer et al., 2007; Agarwal et al., 2012; Žaja et al., 2016; Papas et al., 2020b; Silvestre et al., 2021). Por otro lado, el exceso de $H_2O_2$ es tomado directamente por la CAT para reducirlo a $H_2O$ y $O_2$ (Ighodaro y Akinloye, 2018), y así terminar con el proceso de reducción del principal agente oxidativo y responsable del efecto citotóxico en los espermatozoide como es el $H_2O_2$ (Baumber et al., 2000; Ball, 2008).
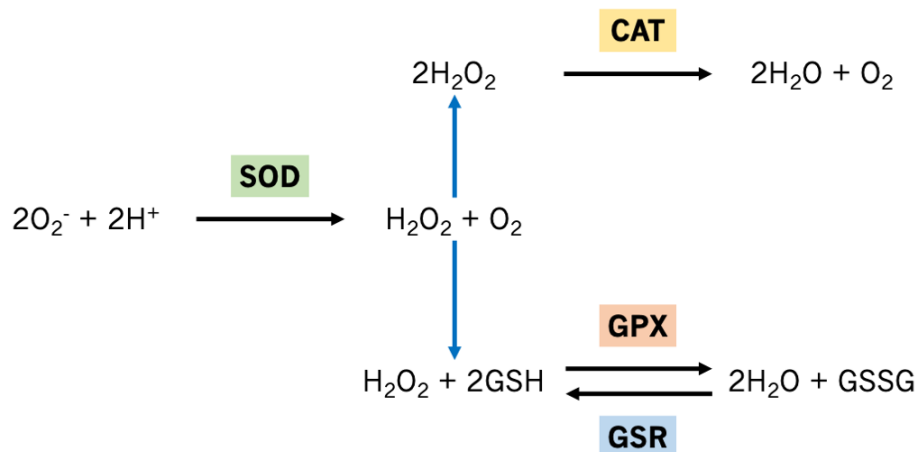


**Figura 2.** Reacciones químicas del sistema de defensa antioxidante enzimático del semen. Adaptado de Baumber y Ball (2005), y Papas et al. (2020b).

Recientemente, se han podido determinar los niveles de actividad de estas enzimas antioxidantes en el plasma seminal de los équidos, observándose que son más altos en el burro que en el caballo (Papas et al., 2019a). En el caso concreto del sistema enzimático antioxidante del plasma seminal del burro, se ha observado que los niveles de actividad de SOD y GPX presentan variaciones entre las estaciones del año, específicamente en el verano, donde son más altas en comparación con el resto de estaciones. Del mismo modo, en esta especie se ha visto que los niveles de actividad de SOD y CAT están asociados con el porcentaje total de espermatozoides móviles y con algunos de los parámetros cinemáticos de

movimiento, respectivamente. Por otro lado, en estudios llevados a cabo de forma individual en cada una de las especies de équidos, se ha establecido que los niveles de actividad total y específica de la SOD presente en el plasma seminal guardan relación con la criotolerancia de los espermatozoides, tanto de burro (Papas et al., 2020a) como de caballo (Papas et al., 2019b).

Finalmente, vale la pena señalar que, en estudios previos desarrollados en humanos (Verit et al., 2009; Marsillach et al., 2011) y cerdo (Barranco et al., 2015a), se ha descrito la presencia de otra enzima con actividad antioxidante en el plasma seminal, como es la paraoxonasa tipo 1 (PON1). Se trata de una enzima extracelular funcionalmente ligada al colesterol con propiedades antioxidantes y antiinflamatorias, cuya función es prevenir la oxidación del colesterol unido a las lipoproteínas de alta (HDL) y baja densidad (LDL) de la membrana plasmática, con lo cual, tiene la capacidad de proteger al espermatozoide del estrés oxidativo (Camps et al., 2009). Asimismo, en el cerdo se ha visto que los niveles de actividad de la PON1 se correlacionan con varios parámetros de calidad y funcionalidad de los espermatozoides refrigerados, se asocian positivamente con la fertilidad *in vivo* (Barranco et al., 2015b) y están relacionados con la criotolerancia de los espermatozoides, minimizando el estrés oxidativo debido a la manipulación criogénica (Li et al., 2018).

## 6.2. Antioxidantes no enzimáticos

Además de las enzimas antioxidantes que neutralizan la sobreproducción de ROS, existen antioxidantes no enzimáticos de bajo peso molecular que ayudan a la actividad de las enzimas del sistema de defensa antioxidante (Walczak-Jedrzejowska et al., 2013). Los antioxidantes no enzimáticos estudiados en el semen incluyen aminoácidos y péptidos pequeños como el glutatión, cisteína, hipotaurina, taurina, carnitina, glutamina y prolina; vitaminas como la C o ácido ascórbico (AA), la E o α-tocoferol y las del complejo B como la B5 o ácido pantoténico; carotenoides como el licopeno; polifenoles como el resveratrol, quercetina, procianidina, hidroxitirosol y 3,4-dihidroxifenilglicol; hormonas como la melatonina; minerales como el zinc, el selenio, el cobre y el cromo; y otras sustancias como la ubiquinona o la coenzima Q10 y el análogo sintético de la

vitamina E soluble en agua, denominado Trolox (Walczak-Jedrzejowska et al., 2013; Silvestre et al., 2021).

De todos los antioxidantes no enzimáticos mencionados anteriormente, el GSH ha sido uno de los más estudiados en el proceso de criopreservación de espermatozoides de équidos, fundamentalmente como un suplemento para los medios de refrigeración (Oliveira et al., 2018) y congelación (Oliveira et al., 2013), debido a que los protocolos actuales de criopreservación de semen, tanto de burro (Papas et al., 2020c) como de caballo (Catalán et al., 2020a), comprenden la eliminación del plasma seminal por centrifugación. Ello implica que los espermatozoides pierden el soporte antioxidante brindado por las enzimas (SOD, CAT, GPX y GSR) presentes en el plasma seminal, lo que provoca que sean presa fácil del ataque de las ROS y entren en una situación de estrés oxidativo generado por el propio metabolismo espermático. En consecuencia, los espermatozoides se hacen más susceptibles de sufrir daños funcionales, con efectos letales debido a la activación de las vías apoptóticas (Dutta et al., 2019), y daños estructurales graves, principalmente a nivel del ADN, lo que tiene un impacto significativo en la fertilidad individual (Yeste et al., 2013a).

El GSH es una molécula de bajo peso molecular elaborado formada por los aminoácidos cisteína, glutamato y glicina. Estos aminoácidos integran el grupo de los tioles, los cuales se caracterizan por contener un grupo sulfhidrilo (-SH) que es el grupo químico funcional esencial para las acción antioxidante del GSH (Silvestre et al., 2021). La protección ejercida por el GSH en los espermatozoides durante la criopreservación está dada por la capacidad de blindar los enlaces de disulfuro dispuestos entre las fibras de la cromatina del ADN del daño oxidativo provocado por las ROS, manteniendo así la integridad de la estructura nuclear espermática (Irvine, 1996; Luberda, 2005; Yeste et al., 2013b). El efecto reductor del GSH sobre las ROS se genera a través de la interacción con las enzimas del sistema de equilibrio oxidación-reducción (GPX/GSR) que usa el glutatión como coenzima (Luberda, 2005; Walczak-Jedrzejowska et al., 2013).

Pese al efecto protector del GSH en el control del estrés oxidativo durante el proceso de criopreservación, un exceso de éste puede ocasionar una situación contraria a la oxidación, la cual sería un estado de estrés reductivo, caracterizado

por una menor producción de ROS o por el cambio en el sistema de equilibrio oxidación-reducción a favor de los antioxidantes (Castagné et al., 1999). Se ha visto que este nuevo ambiente también provoca modificaciones estructurales y funcionales en los espermatozoides de caballo, observándose una baja tolerancia respecto a una alta concentración de antioxidantes (Oliveira et al., 2013). Es importante destacar que un estudio realizado con espermatozoides de burro congelado-descongelado en el cual se suplementó el medio de congelación con GSH (Kumar et al., 2019) sugirió que los espermatozoides de esta especie tienen una mayor tolerancia al estrés reductivo que los de caballo.

### 6.3. Capacidad antioxidante del plasma seminal

Como se mencionó anteriormente, los protocolos actuales de criopreservación de semen de los équidos comprenden la eliminación del plasma seminal por centrifugación. Esto ha llevado, en los últimos años, a que se intensifique el uso de antioxidantes en el medio de congelación con el objetivo claro de prevenir los efectos deletéreos de las ROS durante el proceso de congelación-descongelación de espermatozoides de burro (Kumar et al., 2019) y caballo (Contreras et al., 2020). Sin embargo, la diversidad de antioxidantes enzimáticos y no enzimáticos que componen el plasma seminal todavía sigue siendo incierta. En este sentido, el enfoque de la investigación actual se ha centrado en determinar la capacidad antioxidante del plasma seminal en estas especies, con la medición de los niveles de actividad de las enzimas antioxidantes presentes en el plasma seminal (Papas et al., 2019a) y su posterior relación con la criotolerancia espermática (Papas et al., 2019b; Papas et al., 2020a).

Debido a que la medición de diferentes moléculas antioxidantes por separado no es práctica y sus efectos antioxidantes contra el daño oxidativo son la expresión de acciones acumulativas (Erel, 2004; Cecchini Gualandi et al., 2021), estudios realizados en otras especies han considerado la determinación de un parámetro integrado que represente la actividad de todos los antioxidantes de una muestra biológica (por ejemplo: suero y plasma sanguíneo, saliva, semen, plasma seminal, leche, tejidos) en un único valor, en lugar de la simple suma de las sustancias individuales medibles, denominado estado antioxidante total (TAS) o capacidad antioxidante total (TAC) (Verit et al., 2006; Verit et al., 2009; Tvrdá et al.,

2016; Kapusta et al., 2018; Kargin et al., 2018; Attia et al., 2019; Rubio et al., 2019; Cecchini y Fazio, 2020; Grešáková et al., 2021; Gupta et al., 2021). Específicamente, en el plasma seminal, la medición de la TAC ha demostrado tener relación con la criotolerancia de los espermatozoides de cerdo (Barranco et al., 2015c). De igual forma, un estudio reciente llevado a cabo con semen fresco de burro evidenció la importancia de la TAC del plasma seminal sobre la funcionalidad espermática (Cecchini Gualandi et al., 2021).

La TAC mide la cantidad de antioxidantes totales presentes en el plasma seminal, principalmente antioxidantes no enzimáticos, capaces de neutralizar los componentes prooxidantes utilizando diferentes enfoques metodológicos, como los niveles totales de tioles (Cecchini Gualandi et al., 2021), la capacidad antioxidante reductora cúprica (CUPRAC) (Li et al., 2018), la capacidad de reducción férrica del plasma (FRAP) (Hitit et al., 2020) y la capacidad antioxidante equivalente de Trolox (TEAC) (Sotgia et al., 2020). Estos métodos generalmente se basan en el contenido de radicales libres eliminados por una solución de prueba, o en la capacidad para reducir una sustancia química oxidada, midiendo de esta forma la actividad antioxidante no enzimática de la muestra contra el radical (Erel, 2004; Cecchini y Fazio, 2020). Los ensayos empleados para obtener los valores que integran la TAC guardan relación con cambios de coloración de compuestos químicos que reaccionan con moléculas oxidantes en el caso de los niveles totales de tioles y de la TEAC, así como con la reducción del ion cúprico ($Cu^{2+}$) al ion cuproso ($Cu^{+}$) en el caso de la CUPRAC, y del ion férrico ($Fe^{3+}$) al ion ferroso ($Fe^{2+}$) en el caso de la FRAP (Benzie y Strain, 1996; Erel, 2004; Da Costa et al., 2006; Campos et al., 2009; Li et al., 2018; Hitit et al., 2020; Cecchini Gualandi et al., 2021; González-Arostegui et al., 2022). Mientras que la FRAP refleja el efecto de los antioxidantes no enzimáticos de bajo peso molecular, midiendo principalmente los niveles de ácido úrico, α-tocoferol y AA, la CUPRAC y la TEAC, a más de los anteriores, miden el efecto antioxidante de los factores no enzimáticas que contienen -SH en su estructura como los tioles (entre ellos el GSH) y la albúmina, respectivamente (Rubio et al., 2016; Rubio et al., 2017; Cecchini Gualandi et al., 2021). El α-tocoferol y el AA se insertan en la estructura de la membrana plasmática de los espermatozoides y son particularmente eficaces en la reducción de las ROS que se adhieren superficialmente a las membranas y causan la peroxidación lipídica de las mismas (Li et al., 2018). Es importante mencionar que el ensayo de la TEAC ha

sido utilizado como marcador para determinar la TAC del plasma seminal (Verit et al., 2009; Benedetti et al., 2012; Barranco et al., 2015c; Barranco et al., 2021).

Por otro lado, con este nuevo conocimiento de la TAC del plasma seminal (medida utilizando el ensayo de la TEAC), junto con la medición del estado oxidativo total (TOS) del plasma seminal (Erel, 2005; Verit et al., 2009; Cecchini Gualandi et al., 2021; Robert et al., 2021), ha sido posible también la determinación de un parámetro denominado índice de estrés oxidativo (OSI) (Wu et al., 2017). La determinación del OSI es una técnica rápida, fácil y económica para mostrar con precisión la relación oxidante/antioxidante en una muestra biológica, con lo cual, un aumento en esta relación indicaría riesgo de estrés oxidativo debido al aumento en la producción de ROS o al consumo de antioxidantes defensivos (Abuelo et al., 2013). Este nuevo concepto ha sido aplicado en el plasma seminal de otras especies como el humano (Verit et al., 2009) y el cerdo (Barranco et al., 2021), siendo esta la forma más objetiva para determinar el estrés oxidativo en muestras de semen, ya que muestra la relación oxidantes/antioxidantes presentes en el plasma seminal. De este modo, se ha reportado que el OSI del plasma seminal tiene relación con el almacenamiento del semen de cerdo refrigerado (Barranco et al., 2021).

# CAPÍTULO 2:

# HIPÓTESIS Y OBJETIVOS

## 1. Hipótesis

Nuestra hipótesis radica en que los niveles de las ROS producidas extracelularmente por la NETosis de los PMN son mucho mayores a las producidas por los espermatozoides de burro, debiendo resistir a un alto estrés oxidativo durante su permanencia en el tracto reproductivo femenino. Por otro lado, el entorno oxidativo en el que tienen que transportarse los espermatozoides de burro congelados-descongelados, en ausencia del plasma seminal, hasta alcanzar el oocito de la burra después de la IA, podría representar la necesidad de suplementar el medio de congelación con antioxidantes como el GSH, y así combatir el estrés oxidativo producido por las ROS. Del mismo modo, la medición de los niveles de actividad de los antioxidantes enzimáticos y no enzimáticos, así como del OSI del plasma seminal de burro y caballo podría tener relación con la resistencia de los espermatozoides a los procesos de congelación y descongelación.

## 2. Objetivos

Para comprobar la hipótesis planteada en esta Tesis Doctoral, se establecieron los siguientes objetivos:

1. Determinar la cantidad de ROS extracelular producida por los espermatozoides de burro expuestos a estrés reductivo y a estrés oxidativo (Artículo 1).

2. Determinar la cantidad de ROS extracelular producida por la NETosis de los PMN (Artículo 1).

3. Evaluar el efecto de la suplementación de distintas concentraciones de GSH al medio de congelación y su efecto sobre la criotolerancia de los espermatozoides de burro (Artículo 2).

4. Determinar la importancia de los antioxidantes enzimáticos y no enzimáticos, así como del OSI del plasma seminal en la criotolerancia espermática en el semen de burro (Artículo 3) y caballo (Artículo 4).

# CAPÍTULO 3:

# COMPENDIO DE ARTÍCULOS CIENTÍFICOS

La Tesis Doctoral *"Relevancia de los antioxidantes en la regulación de las especies reactivas de oxígeno (ROS) en el semen de burro y caballo"* es un compendio de las siguientes publicaciones científicas:

1. Extracellular reactive oxygen species (ROS) production in fresh donkey sperm exposed to reductive stress, oxidative stress and NETosis

2. Addition of reduced glutathione (GSH) to the freezing medium reduces intracellular ROS levels in donkey sperm

3. Impact of seminal plasma antioxidants on the freezability of donkey sperm

4. Seminal plasma antioxidants are related to sperm cryotolerance in the horse

# 1. Extracellular reactive oxygen species (ROS) production in fresh donkey sperm exposed to reductive stress, oxidative stress and NETosis

Iván Yánez-Ortiz, Jaime Catalán, Yente Mateo-Otero, Marta Dordas-Perpinyà, Sabrina Gacem, Natalia Yeste, Anna Bassols, Marc Yeste, Jordi Miró

*antioxidants*

*Article*

# Extracellular Reactive Oxygen Species (ROS) Production in Fresh Donkey Sperm Exposed to Reductive Stress, Oxidative Stress and NETosis

Iván Yánez-Ortiz [1,2,3,†], Jaime Catalán [1,2,3,†], Yentel Mateo-Otero [2,3], Marta Dordas-Perpinyà [1], Sabrina Gacem [1], Natalia Yeste [4], Anna Bassols [4], Marc Yeste [2,3,*,‡] and Jordi Miró [1,*,‡]

1   Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, Autonomous University of Barcelona, E-08193 Bellaterra, Cerdanyola del Vallès, Spain; ivan.yanez22@gmail.com (I.Y.-O.); dr.jcatalan@gmail.com (J.C.); martadordas@gmail.com (M.D.-P.); swp.sabrina.gacem@gmail.com (S.G.)
2   Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, E-17003 Girona, Spain; yentel.mateo@udg.edu
3   Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, E-17003 Girona, Spain
4   Department of Biochemistry and Molecular Biology, Faculty of Veterinary Sciences, Autonomous University of Barcelona, E-08193 Bellaterra, Cerdanyola del Vallès, Spain; nati31194@gmail.com (N.Y.); anna.bassols@uab.cat (A.B.)
*   Correspondence: marc.yeste@udg.edu (M.Y.); jordi.miro@uab.cat (J.M.); Tel.: +34-9-7241-9514 (M.Y.); +34-9-3581-4293 (J.M.)
†   These authors contributed equally to this work.
‡   These authors share senior authorship.

check for **updates**

**Abstract:** Jenny shows a large endometrial reaction after semen influx to the uterus with a large amount of polymorphonuclear neutrophils (PMN) migrating into the uterine lumen. PMN act as a sperm selection mechanism through phagocytosis and NETosis (DNA extrudes and, together with proteins, trap spermatozoa). While a reduced percentage of spermatozoa are phagocytosed by PMN, most are found to be attached to neutrophil extracellular traps (NETs). This selection process together with sperm metabolism produces a large amount of reactive oxygen species (ROS) that influence the reproductive success. The present study aimed to determine the extracellular ROS production in both sperm and PMN. With this purpose, (1) donkey sperm were exposed to reductive and oxidative stresses, through adding different concentrations of reduced glutathione (GSH) and hydrogen peroxide ($H_2O_2$), respectively; and (2) PMN were subjected to NETosis in the presence of the whole semen, sperm, seminal plasma (SP) or other activators such as formyl-methionyl-leucyl-phenylalanine (FMLP). Extracellular ROS production (measured as $H_2O_2$ levels) was determined with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit. Donkey sperm showed more resilience to oxidative stress than to the reductive one, and GSH treatments led to greater $H_2O_2$ extracellular production. Moreover, not only did SP appear to be the main inducer of NETosis in PMN, but it was also able to maintain the extracellular $H_2O_2$ levels produced by sperm and NETosis.

**Keywords:** reactive oxygen species (ROS); reduced glutathione (GSH); hydrogen peroxide ($H_2O_2$); polymorphonuclear neutrophils (PMN); NETosis; seminal plasma (SP); donkey

## 1. Introduction

Donkey reproductive strategy shows a large spermatogenic efficiency in the male [1] with very high sperm concentration, motility and viability values [2], and a high inflammatory response in the endometrium after Artificial Insemination (AI) in the female [3]. In fact, it has been established that sperm are the main factors responsible for the endometrial physiological inflammation that occurs in the donkey after mating (natural breeding or AI) [4], which has also been observed in other species [5–8]. This triggers the activation of the defense cells of the immune system, specifically polymorphonuclear neutrophils

(PMN), which move rapidly toward the endometrium to counteract the inflammatory process generated by the presence of sperm, which is necessary for pregnancy to be established [9]. This influx of PMN is particularly higher and even faster in jennies than in mares, reaching its maximum concentration between 6–12 h post-AI, which may underlie a lower probability of fertilization [4,10].

According to Branzk et al. [11], as a result of infection, the action of PMN can occur through two ways depending on the size of the pathogen. Indeed, not only can PMN eliminate infectious agents by phagocytosis, but they also have the ability to degranulate/extrude their DNA and bactericidal molecules (histones and enzymes) and form neutrophil extracellular traps (NETs), which are found in the extracellular space and create a unique type of cell death called NETosis [12,13]. Kotilainen et al. [14] reported that infusing spermatozoa rather than bacteria into the mare uterus provokes the influx of PMN. Within this context, Miró et al. [10] recently observed in donkeys that sperm:PMN binding could inhibit the first action of PMN, i.e., phagocytosis, which would lead to the activation of the second action of PMN, i.e., NETosis.

In the horse, it has been previously established that although the production of reactive oxygen species (ROS) is a consequence of the sperm metabolism itself [15,16], the generation of energy in the form of mitochondrial adenosine triphosphate (ATP) depending on oxidative phosphorylation (OXPHOS) [17,18], the action of PMN is also an important source of ROS generation in the extracellular medium [19,20]. In the horse, Baumber et al. [21] observed a significant increase (300–400 times more) in the production of hydrogen peroxide ($H_2O_2$), one of ROS molecules, by sperm incubated with PMN, compared with the amount of $H_2O_2$ produced by sperm only. This large amount of $H_2O_2$ produced by PMN causes a significant decrease (35–40%) in sperm motility [21]. However, these values in the donkey are still unknown.

Physiologically, the enzymatic and non-enzymatic antioxidants found in the seminal plasma (SP) are responsible for controlling the ROS produced by sperm [22], thus establishing a physiological homeostasis in the redox balance between ROS and antioxidant systems in sperm [23]. The imbalance can give rise to two situations, both with detrimental effects on sperm motility and viability [24]. The first one is a reductive stress state, due to lower ROS production or the change in the redox balance in favor of antioxidants. The second is an oxidative stress state, caused by an excessive ROS production or a decrease in the activity of antioxidants [25].

Papas et al. [26] demonstrated that the capacity of the enzymatic antioxidants present in the SP is higher in the donkey than in the horse. It is known that jenny PMN exposed to donkey sperm undergo NETosis [27], and that NETosis produces ROS [28]. On the other hand, SP modulates sperm-PMN binding [29] and is able to induce NETosis in the donkey [30]. Our hypothesis is that the amount of extracellular ROS (measured as $H_2O_2$ levels) produced by PMN during NETosis in the presence of sperm/SP is significantly higher than that produced in the presence of sperm without SP and that, for this reason, donkey sperm have higher capacity to withstand oxidative stress. Therefore, the objectives of this study were (1) to determine the amount of extracellular ROS produced by donkey sperm subjected to reductive and oxidative stresses, and (2) to assess the amount of extracellular $H_2O_2$ produced by NETosis.

## 2. Materials and Methods

### 2.1. Animals and Statement of Ethics

The study was performed with sperm samples obtained from four separate Catalonian jackasses between 3–6 years old, and with blood samples obtained from three Catalonian jennies aged 3–8 years old. All animals were clinically healthy, with a good body condition and of proven fertility (males with good fertility rates and females with at least one foaling). All animals were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an equine sperm collection center approved by the European Union (EU), with authorization number ES09RS01E. All

animal handling protocols in the center include strict animal health and welfare controls. Jackasses were maintained in individual paddocks and jennies were grouped in a big paddock (maximum 10 animals per paddock). Their diet was based on grain, straw and hay fodder, in addition to water ad libitum. The sperm collection was carried out under the sanitary guidelines established by the Council of the European Communities in Directive 82/894/CEE of 21 December 1982, which includes that housed animals are free of equine viral arteritis, equine infectious anemia and equine contagious metritis. The study was approved by the Ethics Committee of the Autonomous University of Barcelona (authorization code: CEEAH 1424).

*2.2. Experimental Design*

Two separate experiments were performed: (1) sperm exposure to reductive and oxidative stresses, and (2) determination of extracellular $H_2O_2$ production by NETosis.

*2.3. Experiment 1. Sperm Exposure to Reductive Stress and Oxidative Stress*
2.3.1. Sperm Collection

The jackasses were collected on a regular schedule in the morning. Sperm were collected through an artificial vagina (Hannover model; Minitüb GmbH, Tiefenbach, Germany) preheated to a temperature between 48 °C–50 °C and coupled with an in-line nylon filter to remove the gel fraction. Immediately after collection, each ejaculate was split into two fractions: one was intended to recovering the SP and the other was diluted 1:5 (*v:v*) in a cooling extender based on skimmed milk [31], previously tempered to 37 °C. Sperm concentration of diluted semen was evaluated by a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and adjusted to $100 \times 10^6$ sperm/mL.

2.3.2. Seminal Plasma (SP) Collection

The SP fraction of each ejaculate was obtained by successive centrifugations at $3000 \times g$ and 4 °C for 10 min (Medifriger BL-S; JP Selecta S.A., Barcelona, Spain). The process was repeated under the same conditions as many times as necessary (~5 depending on the ejaculate) until sperm were completely eliminated. Verification was carried out using a phase contrast microscope (Olympus Europa SE & Co. KG, Hamburg, Germany) at $200\times$ magnification. For all experiments, SP samples were maintained in a water bath at 37 °C until used.

2.3.3. Treatments

Ten treatments were prepared with the sperm sample obtained from each jackass and with the SP obtained from each jackass in a final volume of 1.5 mL as follows: T1 = Sperm and SP (control); T2 = Sperm and SP + 2 mM (final concentration) reduced glutathione (GSH; G4251; Sigma-Aldrich, St. Louis, MO, USA); T3 = Sperm and SP + 4 mM GSH; T4 = Sperm and SP + 6 mM GSH; T5 = Sperm and SP + 8 mM GSH; T6 = Sperm and SP + 10 mM GSH; T7 = Sperm and SP + 0.5 mM hydrogen peroxide ($H_2O_2$; 30% (*w:w*); 7722-84-1; Sigma-Aldrich, St. Louis, MO, USA); T8 = Sperm and SP + 1 mM $H_2O_2$; T9 = Sperm and SP + 5 mM $H_2O_2$; and T10 = Sperm and SP + 10 mM $H_2O_2$.

All treatments were incubated in a water bath at 37 °C and analyzed after 0 min, 30 min, 60 min and 120 min of incubation. All experiments were carried out by the same technician to avoid errors and biases due to the human factor. Sperm motility evaluation was performed using a computer-assisted sperm analysis (CASA) system (Proiser R + D, Valencia, Spain), and sperm viability was assessed through eosin-nigrosin staining. Extracellular ROS production (measured as $H_2O_2$ levels) was determined by the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188; ThermoFisher Scientific, Waltham, MA, USA).

### 2.3.4. Evaluation of Sperm Motility

Objective evaluation of sperm motility was performed using the motility module of the CASA ISAS® V 1.2 system (CASA-Mot; Proiser R + D, Valencia, Spain) combined with a 10× negative phase contrast microscope (UOP200i; Proiser R + D, Valencia, Spain). A high-resolution digital camera (MQ003MG-CM; Proiser R + D, Valencia, Spain), capable of capturing up to 100 frames per second (fps), was used. Briefly, 2 μL of each sperm sample was placed into a reusable counting chamber of 10 μm in depth (Spermtrack®10; Proiser R + D, Valencia, Spain), previously heated to 37 °C. A minimum of 500 spermatozoa was counted per analysis. In each evaluation, total (TM, %) and progressive motility (PM, %) were recorded, as well as the following kinematic parameters: curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity coefficient (LIN = [VSL/VCL] × 100, %), straightness coefficient (STR = [VSL/VAP] × 100, %), wobble coefficient (WOB = [VAP/VCL] × 100, %), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz). The CASA-Mot settings were those recommended by the manufacturer: particle area > 4 and <75 μm$^2$, connectivity = 6, minimum images number to calculate ALH = 10. The cut-off values for total and progressively motile sperm were VAP $\geq$ 10 μm/s and STR $\geq$ 75%, respectively. Three technical replicates were evaluated.

### 2.3.5. Evaluation of Sperm Viability

Sperm viability was determined through eosin-nigrosin staining [32]. Briefly, 10 μL of each sperm sample was placed on a slide, previously warmed to 37 °C, and 10 μL of the eosin-nigrosin stain was added. Subsequently, a smear of the mixture was prepared on the slide using a glass rod. Samples were air dried at room temperature. A minimum of 200 sperm/sample were evaluated under a bright field optical microscope (Carl Zeiss, Göttingen, Germany) at 1000× magnification using immersion oil. Three technical replicates were evaluated, and the percentage of viable spermatozoa (eosin negative) was recorded.

### 2.3.6. Determination of Extracellular H$_2$O$_2$ Production

The generation of extracellular H$_2$O$_2$ was determined through the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188; ThermoFisher Scientific, Waltham, MA, USA), a highly sensitive and stable probe for measuring H$_2$O$_2$ production.

The detection system consisted of the dye, 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red), horseradish peroxidase (HRP) and 50 mM sodium phosphate (pH = 7.4). In this system, 100 μM Amplex Red reagent in the presence of 0.2 U/mL HRP reacts with H$_2$O$_2$ in a 1:1 (*v:v*) stoichiometry to produce a highly red-fluorescent oxidation product, resorufin, that can be measured by fluorescence or absorbance.

The Amplex® Red assay was performed on each sample according to the manufacturer's instructions. Treatments and assay standards (final H$_2$O$_2$ concentrations: 0 μM, 0.5 μM, 1 μM, 2 μM, 2.5 μM, 4 μM, 5 μM, 8 μM, 10 μM and 20 μM) were pipetted into a 96-well microplate with duplicates for each sample. Absorbance was measured using spectrophotometry at 560 nm and monitored at multiple time points, every 30 min for 2 h, to follow the kinetics of the reaction at 37 °C. Results are expressed as a single blank-corrected concentration.

### 2.4. Experiment 2. Determination of Extracellular H$_2$O$_2$ Production by NETosis

#### 2.4.1. Samples

Sperm were obtained as explained in Section 2.3.1. For this experiment, sperm concentration was adjusted to 1.5 × 10$^6$ sperm/mL. SP was harvested as described in Section 2.3.2.

#### 2.4.2. PMN Isolation

For isolating PMN from the peripheral blood, the protocol set by Siemsen et al. [33] and Yildiz et al. [34] was followed. Briefly, five blood samples were collected through jugular venipuncture in 10 mL BD Vacutainer® tubes containing anticoagulant (18.0 mg

of ethylenediaminetetraacetic acid; EDTA; BD, Plymouth, UK). Immediately afterward, blood samples were incubated in a water bath at 37 °C for 30 min to separate red blood cells (RBC) and the plasma fraction rich in leukocytes. The fraction of 5 mL above the red blood cell (RBC) pellet was recovered and mixed with an equal volume of 0.02% EDTA solution (E8008; Sigma-Aldrich, St. Louis, MO, USA). In turn, this mixture was layered in sterile tubes with an equal volume of Ficoll® Paque Plus (GE17-1440-02; Merck KGaA, Darmstadt, Germany) and centrifuged at $500 \times g$ and 20 °C for 30 min (Medifriger BL-S; JP Selecta S.A., Barcelona, Spain) to extract blood plasma, lymphocytes and monocytes. The pellet containing RBC and PMN was mixed with 25 mL of sterile PBS $1\times$ and centrifuged at $500 \times g$ and 20 °C for 10 min. The fresh pellet obtained from this second centrifugation was resuspended in RBC $1\times$ lysis buffer (00-4333-57; ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Subsequently, samples were gently shaken for 15 min, and 25 mL of sterile PBS $1\times$ was added. The resulting mixture was centrifuged again ($500 \times g$ and 20 °C for 10 min), and the pellet was resuspended in 1 mL RPMI-1640 medium (R0883; Sigma-Aldrich, St. Louis, MO, USA) supplemented with Penicillin-Streptomycin at 1% (P4333; Sigma-Aldrich, St. Louis, MO, USA). Finally, samples were analyzed using a hematological flow cytometer (Sysmex XN-1000™; Sysmex Corporation, Kobe, Japan) to evaluate the purity and efficiency of the isolation method, and only those having >80% PMN were used to test the treatments. Concentration of PMN was adjusted to $3 \times 10^5$ PMN/mL.

### 2.4.3. Treatments

Six treatments were prepared in a final volume of 150 μL as follows: T1 = PMN (control): 75 μL of PMN + 75 μL of RPMI-1640 medium [30]; T2 = PMN + Whole semen (sperm + SP): 75 μL of PMN + 75 μL of Whole semen [10]; T3 = PMN + Sperm: 75 μL of PMN + 75 μL of Sperm [30]; T4 = PMN + SP: 75 μL of PMN + 37.5 μL of SP (25% of SP with respect to the total volume) + 37.5 μL of RPMI-1640 medium [30]; T5 = PMN + formyl-methionyl-leucyl-phenylalanine (FMLP): 75 μL of PMN + 15 μL 0.1 mM FMLP (F3506; Sigma-Aldrich, St. Louis, MO, USA) + 60 μL of RPMI-1640 medium [10,21,30]; and T6 = PMN + Kenney: 75 μL of PMN + 75 μL of Kenney [30].

All treatments were incubated at 37 °C for 2 h. After that, PMN were stained with SYTOX™ Orange Nucleic Acid Stain (S11368; ThermoFisher Scientific, Waltham, MA, USA) and the percentage of reacted PMN (NETosis) was evaluated using a confocal microscope. Extracellular ROS production (measured as $H_2O_2$ levels) was determined through the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188; ThermoFisher Scientific, Waltham, MA, USA). The evaluations were carried out by the same technician to avoid errors and biases due to the human factor.

### 2.4.4. Evaluation of NETosis

NETosis was evaluated following the protocol established by Mateo-Otero et al. [30]. In brief, treatments were incubated at 37 °C for 2 h in sterile Millicell® EZ SLIDES 8-well plates (Merck KGaA, Darmstadt, Germany). Thereafter, 200 μL of 4% Paraformaldehyde (158127; Sigma-Aldrich, St. Louis, MO, USA) was added to each well to fix the cells, and three washes were performed with 200 μL of sterile PBS $1\times$. Subsequently, 150 μL of SYTOX™ Orange Nucleic Acid Stain (S11368; ThermoFisher Scientific, Waltham, MA, USA) diluted 1:2500 (*v:v*) was placed in each well to stain the cells for 30 min in darkness. Finally, cells were washed with sterile PBS $1\times$ before mounting them on slides with DPX mounting medium (06522; Sigma-Aldrich, St. Louis, MO, USA). A minimum of 200 cells/treatment were evaluated with a confocal laser scanning microscope (Olympus FluoView™ FV1000; Olympus Corporation, Tokyo, Japan) at $200\times$ magnification. The percentage of reacted PMN (exhibiting expanded or elongated nuclei) was recorded, and two technical replicates were counted.

2.4.5. Determination of Extracellular $H_2O_2$ Production

Evaluation of extracellular $H_2O_2$ production was carried out as explained in Section 2.3.6.

*2.5. Statistical Analysis*

Data obtained from all experiments are included in the section of Supplementary Materials and were analyzed with the statistical package R (V 4.0.3, R Core Team; Vienna, Austria) and plotted using GraphPad Prism (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA). At first, normality of data was verified through the Shapiro–Wilk test and homoscedasticity using the Levene test. When necessary, data were linearly transformed with arcsin $\sqrt{x}$. When, even transformed, data did not present normal distribution and/or variances were not homogenous, a non-parametric analysis was performed using the Friedman test followed by the Wilcoxon test for pairwise comparison.

In experiment 1, sperm motility and viability were compared with a generalized linear model mixed (GLMM; repeated measures). The within-subjects factor was the incubation time (0 min, 30 min, 60 min and 120 min), the fixed-effects factor was the concentration of GSH (control, 2 mM, 4 mM, 6 mM, 8 mM and 10 mM) or $H_2O_2$ (control, 0.5 mM, 1 mM, 5 mM and 10 mM) and the random-effects factor was the jackass. The extracellular $H_2O_2$ production by sperm and SP in the different GSH and $H_2O_2$ concentrations was compared by a two-way ANOVA. In experiment 2, the percentage of reacted PMN in the different treatments (PMN [control], PMN + Whole semen, PMN + Sperm, PMN + SP, PMN + FMLP and PMN + Kenney) was compared by one-way ANOVA. The extracellular $H_2O_2$ production by NETosis in the different treatments was analyzed with a GLMM, including the time of incubation (0 min, 30 min, 60 min, 90 min and 120 min) as a within-subject factor and considering the jenny as a random factor. The Bonferroni post hoc test was used for pair-wise comparisons. The differences were considered to be statistically significant when $p \leq 0.05$. Results are expressed as means $\pm$ standard error of the mean (SEM).

**3. Results**

*3.1. Experiment 1: Exposure of Sperm to Reductive Stress*

3.1.1. Sperm Motility

No significant differences in TM and PM were observed when sperm were exposed to a GSH concentration of 8 mM, at any of the incubation times. However, 10 mM GSH was seen to reduce TM and PM after 120 min of incubation ($p \leq 0.05$). On the other hand, the impact of GSH on TM and PM was concentration-dependent; the higher the concentration, the earlier the reduction in these two motility parameters (30 min for 10 mM GSH; 60 min for 4 mM, 6 mM and 8 mM GSH; 120 min for the control and 2 mM GSH; Figure 1a,b).
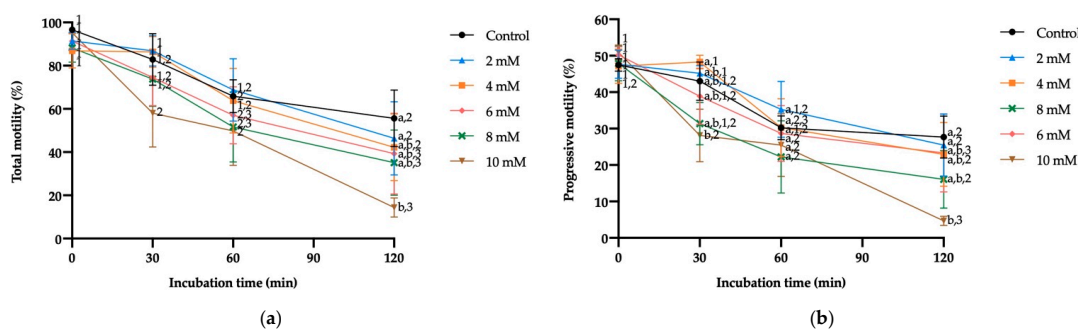


**Figure 1.** Mean $\pm$ SEM of total (**a**) and progressive (**b**) motility of donkey sperm exposed to reductive stress with different reduced glutathione (GSH) concentrations measured at distinct incubation times. (a,b) Different letters indicate significant differences ($p \leq 0.05$) between GSH concentrations within a given incubation time. (1–3) Different numbers indicate significant differences ($p \leq 0.05$) between incubation times within a given GSH concentration.

Regarding kinematic parameters (Table 1), the different GSH concentrations had no significant effect on VCL, VSL, VAP, STR or ALH at any of the incubation times. However, the treatment containing 8 mM GSH showed significantly lower LIN after 30 and 120 min of incubation ($p \leq 0.05$). Similarly, WOB was significantly lower in the treatments containing 6 mM and 8 mM GSH after 30 min and 120 min of incubation, respectively. Finally, the presence of 8 mM reduced BCF after 30 min, 60 min and 120 min of incubation ($p \leq 0.05$).

**Table 1.** Mean ± SEM of the kinematic parameters of donkey sperm exposed to reductive stress with different reduced glutathione (GSH) concentrations measured at different incubation times.

| Parameter | Incubation Time | GSH Concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | 2 mM | 4 mM | 6 mM | 8 mM | 10 mM |
| VCL (µm/s) | 0 min | 217.05 ± 15.87 [a,1] | 215.20 ± 15.02 [a,1] | 216.39 ± 21.43 [a,1] | 215.58 ± 14.79 [a,1] | 213.17 ± 21.01 [a,1] | 238.79 ± 14.66 [a,1] |
| | 30 min | 200.15 ± 22.28 [a,1,2] | 195.60 ± 11.89 [a,1] | 206.31 ± 15.46 [a,1] | 196.55 ± 22.39 [a,1] | 182.97 ± 25.66 [a,1,2] | 170.67 ± 24.31 [a,2] |
| | 60 min | 156.64 ± 12.80 [a,2] | 180.38 ± 20.14 [a,1] | 170.12 ± 21.63 [a,1] | 183.45 ± 16.87 [a,1] | 181.28 ± 21.96 [a,1,2] | 195.84 ± 24.81 [a,1,2] |
| | 120 min | 155.60 ± 18.77 [a,2] | 186.70 ± 14.20 [a,1] | 173.22 ± 9.72 [a,1] | 189.96 ± 28.20 [a,1] | 156.98 ± 13.22 [a,2] | 156.29 ± 15.83 [a,2] |
| VSL (µm/s) | 0 min | 80.68 ± 2.37 [a,1] | 82.30 ± 5.91 [a,1] | 84.67 ± 5.17 [a,1] | 83.38 ± 2.61 [a,1] | 80.77 ± 5.80 [a,1] | 88.24 ± 5.33 [a,1] |
| | 30 min | 78.37 ± 4.47 [a,1] | 78.99 ± 2.04 [a,1] | 82.48 ± 1.65 [a,1,2] | 71.68 ± 8.34 [a,1] | 58.79 ± 7.20 [a,1,2] | 63.45 ± 8.09 [a,2] |
| | 60 min | 60.96 ± 5.56 [a,1] | 68.60 ± 6.05 [a,1] | 60.90 ± 9.28 [a,2] | 64.44 ± 5.76 [a,1] | 58.77 ± 7.45 [a,1,2] | 68.01 ± 8.01 [a,1,2] |
| | 120 min | 65.40 ± 7.04 [a,1] | 71.73 ± 3.22 [a,1] | 63.63 ± 7.24 [a,1,2] | 66.79 ± 15.92 [a,1] | 51.90 ± 6.35 [a,2] | 48.37 ± 5.43 [a,2] |
| VAP (µm/s) | 0 min | 113.58 ± 6.16 [a,1] | 112.78 ± 9.07 [a,1] | 114.13 ± 10.00 [a,1] | 111.58 ± 5.76 [a,1] | 107.90 ± 9.10 [a,1] | 120.83 ± 6.54 [a,1] |
| | 30 min | 107.14 ± 9.62 [a,1,2] | 105.92 ± 2.58 [a,1] | 106.83 ± 4.72 [a,1,2] | 96.37 ± 12.06 [a,1] | 84.47 ± 12.31 [a,1,2] | 83.08 ± 10.96 [a,2] |
| | 60 min | 81.07 ± 5.28 [a,2] | 92.39 ± 9.73 [a,1] | 82.99 ± 12.00 [a,2] | 85.29 ± 8.37 [a,1] | 85.48 ± 9.67 [a,1,2] | 90.76 ± 11.20 [a,1,2] |
| | 120 min | 83.48 ± 8.21 [a,2] | 89.85 ± 5.75 [a,1] | 83.80 ± 6.50 [a,2] | 87.68 ± 15.96 [a,1] | 72.10 ± 6.39 [a,2] | 68.19 ± 8.61 [a,2] |
| LIN (%) | 0 min | 34.97 ± 1.52 [a,1] | 35.28 ± 1.46 [a,1] | 36.36 ± 0.60 [a,1] | 35.71 ± 1.04 [a,1] | 34.52 ± 0.80 [a,1] | 34.72 ± 0.26 [a,1] |
| | 30 min | 36.48 ± 1.57 [a,1] | 37.25 ± 2.66 [a,1] | 36.68 ± 1.88 [a,1] | 34.13 ± 1.44 [ab,1] | 28.57 ± 0.99 [b,1] | 33.57 ± 3.44 [a,1,2] |
| | 60 min | 35.03 ± 2.70 [a,1] | 35.10 ± 1.06 [a,1] | 32.21 ± 1.67 [a,1] | 31.64 ± 1.30 [a,1] | 29.24 ± 1.48 [a,1] | 32.15 ± 0.67 [a,1,2] |
| | 120 min | 37.76 ± 2.92 [a,1] | 34.68 ± 1.02 [ab,1] | 33.56 ± 2.16 [ab,1] | 31.19 ± 4.82 [ab,1] | 29.02 ± 2.69 [b,1] | 27.64 ± 1.47 [b,2] |
| STR (%) | 0 min | 65.16 ± 1.31 [a,1] | 66.17 ± 0.87 [a,1] | 67.88 ± 0.54 [a,1] | 67.87 ± 0.56 [a,1] | 66.95 ± 1.24 [a,1] | 67.31 ± 0.83 [a,1] |
| | 30 min | 66.55 ± 1.30 [a,1] | 67.52 ± 2.44 [a,1] | 69.53 ± 1.69 [a,1] | 67.52 ± 2.35 [a,1] | 61.26 ± 0.82 [a,1] | 67.23 ± 3.30 [a,1] |
| | 60 min | 65.18 ± 3.11 [a,1] | 66.64 ± 0.44 [a,1] | 64.29 ± 1.93 [a,1] | 66.75 ± 1.32 [a,1] | 61.41 ± 3.81 [a,1] | 67.98 ± 1.41 [a,1] |
| | 120 min | 68.78 ± 3.23 [a,1] | 70.73 ± 1.80 [a,1] | 67.47 ± 4.22 [a,1] | 65.57 ± 8.39 [a,1] | 61.69 ± 3.97 [a,1] | 63.32 ± 4.37 [a,1] |
| WOB (%) | 0 min | 51.44 ± 1.43 [a,1] | 51.33 ± 1.71 [a,1,2] | 51.45 ± 1.17 [a,1] | 50.60 ± 1.49 [a,1] | 49.33 ± 0.68 [a,1] | 49.78 ± 0.38 [a,1] |
| | 30 min | 52.36 ± 1.38 [a,1] | 52.79 ± 1.96 [a,1] | 50.50 ± 1.43 [a,1] | 48.62 ± 1.67 [ab,1,2] | 44.94 ± 1.30 [b,1] | 47.75 ± 2.43 [ab,1,2] |
| | 60 min | 50.78 ± 1.74 [a,1] | 50.31 ± 0.97 [a,1,2] | 47.92 ± 1.08 [a,1] | 46.07 ± 0.83 [a,1,2] | 47.40 ± 1.09 [a,1] | 46.98 ± 0.84 [a,1,2] |
| | 120 min | 52.23 ± 1.88 [a,1] | 47.48 ± 2.23 [ab,2] | 48.76 ± 0.59 [ab,1] | 45.70 ± 1.78 [b,2] | 47.24 ± 0.81 [ab,1] | 43.59 ± 1.96 [b,2] |
| ALH (µm) | 0 min | 2.56 ± 0.14 [a,1] | 2.58 ± 0.16 [a,1] | 2.63 ± 0.22 [a,1] | 2.73 ± 0.19 [a,1] | 2.77 ± 0.24 [a,1] | 2.96 ± 0.15 [a,1] |
| | 30 min | 2.34 ± 0.24 [a,1,2] | 2.40 ± 0.17 [a,1] | 2.63 ± 0.17 [a,1] | 2.59 ± 0.24 [a,1] | 2.44 ± 0.26 [a,1,2] | 2.25 ± 0.29 [a,1,2] |
| | 60 min | 2.00 ± 0.16 [a,1,2] | 2.29 ± 0.20 [a,1] | 2.22 ± 0.21 [a,1] | 2.45 ± 0.17 [a,1] | 2.40 ± 0.24 [a,1,2] | 2.58 ± 0.29 [a,1,2] |
| | 120 min | 1.93 ± 0.22 [a,2] | 2.45 ± 0.17 [a,1] | 2.24 ± 0.07 [a,1] | 2.51 ± 0.32 [a,1] | 2.12 ± 0.15 [a,2] | 2.20 ± 0.20 [a,2] |
| BCF (Hz) | 0 min | 34.39 ± 1.42 [a,1] | 33.21 ± 3.26 [a,1] | 33.27 ± 2.16 [a,1] | 31.41 ± 2.26 [a,1] | 28.45 ± 1.49 [a,1] | 32.58 ± 1.06 [a,1] |
| | 30 min | 35.00 ± 2.38 [a,1] | 34.77 ± 1.55 [a,1] | 31.43 ± 0.60 [ab,1] | 28.21 ± 3.29 [abc,1,2] | 22.44 ± 2.19 [c,1,2] | 24.09 ± 2.25 [bc,2] |
| | 60 min | 28.13 ± 1.49 [ab,1] | 29.75 ± 2.70 [a,1] | 26.33 ± 3.35 [ab,1] | 22.67 ± 2.22 [ab,2] | 20.62 ± 2.14 [b,2] | 21.67 ± 1.69 [ab,2] |
| | 120 min | 31.29 ± 1.86 [a,1] | 28.78 ± 2.04 [ab,1] | 27.64 ± 1.60 [abc,1] | 27.12 ± 2.19 [abc,1,2] | 23.04 ± 1.28 [bc,1,2] | 20.55 ± 1.99 [c,2] |

VCL (µm/s): curvilinear velocity; VSL (µm/s): straight line velocity; VAP (µm/s): average path velocity; LIN (%): linearity coefficient; STR (%): straightness coefficient; WOB (%): wobble coefficient; ALH (µm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency. ([a–c]) Different letters indicate significant differences ($p \leq 0.05$) between GSH concentrations within a given incubation time. ([1,2]) Different numbers indicate significant differences ($p \leq 0.05$) between incubation times within a given GSH concentration.

As far as the effects of incubation time on motility are concerned, kinematic parameters did not vary significantly in treatments containing 2 mM and 6 mM GSH, except for WOB and BCF ($p \leq 0.05$). At the other concentrations, the incubation time caused a significant decrease in VCL, VSL and VAP after 30 min (10 mM GSH) and 60 min (4 mM GSH) of incubation, and in LIN after 120 min of incubation (10 mM GSH). Likewise, the incubation time reduced WOB, ALH and BCF at GSH concentrations equal to or higher than 6 mM ($p \leq 0.05$).

3.1.2. Sperm Viability

Sperm viability was not significantly affected by exposure to GSH at any incubation time or concentration. On the contrary, the incubation time within each treatment produced a significant decrease from 30 min in 6 mM, 8 mM and 10 mM GSH; and from 60 min in the control, 2 mM and 4 mM GSH (Figure 2).
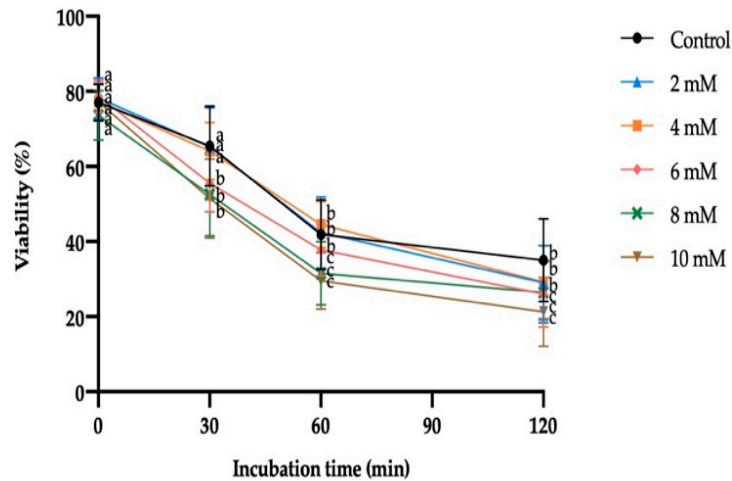
**Figure 2.** Mean $\pm$ SEM of viability of donkey sperm exposed to reductive stress with different reduced glutathione (GSH) concentrations measured at different incubation times. No significant differences were found between GSH concentrations within a given incubation time. (a–c) Different letters indicate significant differences ($p \leq 0.05$) between incubation times within a given GSH concentration.

3.1.3. Extracellular Hydrogen Peroxide ($H_2O_2$) Production

The extracellular $H_2O_2$ production by sperm exposed to reductive stress increased significantly at GSH concentrations equal to or higher than 8 mM. Moreover, the extracellular $H_2O_2$ production by SP was not significantly affected by the different GSH concentrations. Significant differences in the extracellular $H_2O_2$ produced by sperm vs. that produced by SP were only observed in the treatments containing 8 mM or 10 mM GSH (8 mM GSH: 8.11 $\mu$M $\pm$ 0.93 $\mu$M vs. 5.83 $\mu$M $\pm$ 0.66 $\mu$M; 10 mM GSH: 10.09 $\mu$M $\pm$ 1.51 $\mu$M vs. 5.47 $\mu$M $\pm$ 0.50 $\mu$M; Figure 3).



**Figure 3.** Mean $\pm$ SEM of extracellular hydrogen peroxide ($H_2O_2$) produced by donkey sperm and seminal plasma (SP) exposed to reductive stress with increasing reduced glutathione (GSH) concentrations at 37 °C for 30 min. (a–c) Different letters indicate significant differences ($p \leq 0.05$) between GSH concentrations in sperm or SP. (1,2) Different numbers indicate significant differences ($p \leq 0.05$) between sperm or SP within a given GSH concentration.

### 3.2. Experiment 1: Exposure of Sperm to Oxidative Stress

3.2.1. Sperm Motility

No significant differences in TM and PM were found when sperm were exposed to different $H_2O_2$ concentrations, at any of the incubation times. While incubating sperm with 0.5 mM $H_2O_2$ did not affect TM, higher concentrations of $H_2O_2$ decreased that sperm variable (from 30 min in the treatment containing 10 mM $H_2O_2$, and from 120 min in the treatments containing 1 mM and 5 mM $H_2O_2$). Although the incubation time led to a decrease in PM in all treatments, this reduction was only statistically significant ($p \leq 0.05$) in the control from 60 min of incubation (Figure 4a,b).



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

**Figure 4.** Mean $\pm$ SEM of total (**a**) and progressive (**b**) motility of donkey sperm exposed to oxidative stress with different hydrogen peroxide ($H_2O_2$) concentrations measured at different incubation times. No significant differences were found between $H_2O_2$ concentrations within a given incubation time. (**a**,**b**) Different letters indicate significant differences ($p \leq 0.05$) between incubation times within a given $H_2O_2$ concentration.

Regarding kinematic parameters (Table 2), the presence of 10 mM $H_2O_2$ led to a reduction in sperm velocity (VCL, VSL and VAP) after 30 min of incubation ($p \leq 0.05$). While LIN, WOB and BCF were not significantly affected by treatment at any of the incubation times, STR increased in 10 mM $H_2O_2$ after 60 min and 120 min of incubation ($p \leq 0.05$), respectively, and ALH significantly decreased in the treatment containing 5 mM $H_2O_2$ after 30 min of incubation.

**Table 2.** Mean $\pm$ SEM of the kinematic parameters of donkey sperm exposed to oxidative stress with different hydrogen peroxide ($H_2O_2$) concentrations measured at different incubation times.

| Parameter | Incubation Time | $H_2O_2$ Concentration | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.5 mM | 1 mM | 5 mM | 10 mM |
| VCL (µm/s) | 0 min | 217.05 ± 15.87 [a,1] | 207.18 ± 12.71 [a,1] | 198.34 ± 15.79 [a,1] | 200.12 ± 19.14 [a,1] | 200.68 ± 13.72 [a,1] |
| | 30 min | 200.15 ± 22.28 [a,1] | 173.46 ± 15.47 [ab,1,2] | 170.15 ± 4.75 [ab,1,2] | 154.00 ± 10.13 [b,2] | 152.15 ± 11.91 [b,2] |
| | 60 min | 156.64 ± 12.80 [a,2] | 158.57 ± 12.00 [a,2] | 146.71 ± 8.83 [a,2] | 152.04 ± 13.70 [a,2] | 164.64 ± 15.75 [a,1,2] |
| | 120 min | 155.60 ± 18.77 [a,2] | 154.92 ± 15.31 [a,2] | 167.50 ± 12.09 [a,1,2] | 160.25 ± 16.59 [a,2] | 169.35 ± 11.85 [a,1,2] |
| VSL (µm/s) | 0 min | 80.68 ± 2.37 [a,1] | 78.03 ± 2.08 [a,1] | 76.12 ± 3.37 [a,1] | 76.52 ± 5.10 [a,1] | 72.05 ± 2.40 [a,1,2] |
| | 30 min | 78.37 ± 4.47 [a,1] | 69.56 ± 3.33 [ab,1] | 74.73 ± 4.44 [ab,1] | 66.62 ± 4.12 [ab,1] | 56.47 ± 4.02 [b,2] |
| | 60 min | 60.96 ± 5.56 [a,2] | 67.32 ± 3.36 [a,1] | 63.29 ± 2.09 [a,1] | 68.86 ± 7.00 [a,1] | 72.61 ± 6.91 [a,1,2] |
| | 120 min | 65.40 ± 7.04 [a,1,2] | 62.58 ± 6.28 [a,1] | 74.44 ± 7.67 [a,1] | 68.07 ± 8.25 [a,1] | 78.77 ± 5.25 [a,1] |
| VAP (µm/s) | 0 min | 113.58 ± 6.16 [a,1] | 108.23 ± 5.43 [a,1] | 104.02 ± 6.23 [a,1] | 104.42 ± 8.38 [a,1] | 103.43 ± 5.96 [a,1] |
| | 30 min | 107.14 ± 9.62 [a,1] | 92.98 ± 5.55 [ab,1,2] | 94.95 ± 3.21 [ab,1,2] | 86.27 ± 4.41 [ab,1] | 79.06 ± 5.80 [b,2] |
| | 60 min | 81.07 ± 5.28 [a,2] | 86.72 ± 4.68 [a,1,2] | 81.46 ± 3.70 [a,2] | 85.99 ± 8.26 [a,1] | 90.97 ± 8.25 [a,1,2] |
| | 120 min | 83.48 ± 5.28 [a,2] | 81.37 ± 4.68 [a,2] | 92.09 ± 3.70 [a,1,2] | 86.20 ± 8.26 [a,1] | 94.32 ± 8.25 [a,1,2] |
| LIN (%) | 0 min | 34.97 ± 1.52 [a,1] | 34.94 ± 1.24 [a,1] | 35.72 ± 1.65 [a,1] | 34.97 ± 0.82 [a,2] | 33.46 ± 1.65 [a,2] |
| | 30 min | 36.48 ± 1.57 [a,1] | 36.71 ± 1.58 [a,1] | 40.58 ± 2.68 [a,1] | 39.94 ± 2.14 [a,1,2] | 34.51 ± 2.65 [a,2] |
| | 60 min | 35.03 ± 2.70 [a,1] | 40.24 ± 1.24 [a,1] | 40.98 ± 0.84 [a,1] | 42.22 ± 1.99 [a,1] | 42.12 ± 3.12 [a,1] |
| | 120 min | 37.76 ± 2.92 [a,1] | 36.87 ± 3.50 [a,1] | 41.35 ± 3.45 [a,1] | 39.34 ± 4.26 [a,1,2] | 43.57 ± 3.93 [a,1] |
| STR (%) | 0 min | 65.16 ± 1.31 [a,1] | 65.42 ± 1.00 [a,1] | 66.39 ± 1.52 [a,1] | 65.97 ± 0.26 [a,1] | 63.27 ± 1.40 [a,2] |
| | 30 min | 66.55 ± 1.30 [a,1] | 66.80 ± 1.10 [a,1] | 71.07 ± 2.81 [a,1] | 68.81 ± 1.94 [ab,1] | 63.96 ± 1.42 [a,2] |
| | 60 min | 65.18 ± 3.11 [b,1] | 70.34 ± 0.97 [ab,1] | 70.19 ± 1.79 [ab,1] | 71.65 ± 1.93 [ab,1] | 73.34 ± 3.38 [a,1] |
| | 120 min | 68.78 ± 3.23 [ab,1] | 67.96 ± 3.64 [b,1] | 73.13 ± 3.40 [ab,1] | 70.40 ± 4.01 [ab,1] | 75.85 ± 3.37 [a,1] |

**Table 2.** *Cont.*

| Parameter | Incubation Time | H$_2$O$_2$ Concentration | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.5 mM | 1 mM | 5 mM | 10 mM |
| WOB (%) | 0 min | 51.44 ± 1.43 [a,1] | 51.23 ± 1.21 [a,1] | 51.58 ± 1.52 [a,1] | 50.96 ± 1.31 [a,1] | 50.53 ± 1.71 [a,1] |
| | 30 min | 52.36 ± 1.38 [a,1] | 52.48 ± 1.60 [a,1] | 54.73 ± 1.68 [a,1] | 55.55 ± 1.91 [a,1] | 51.80 ± 2.69 [a,1] |
| | 60 min | 50.78 ± 1.74 [a,1] | 54.86 ± 1.26 [a,1] | 56.07 ± 0.70 [a,1] | 56.43 ± 1.19 [a,1] | 55.49 ± 2.11 [a,1] |
| | 120 min | 52.23 ± 1.88 [a,1] | 51.69 ± 2.34 [a,1] | 54.27 ± 2.86 [a,1] | 53.03 ± 2.99 [a,1] | 55.20 ± 3.08 [a,1] |
| ALH (μm) | 0 min | 2.56 ± 0.14 [a,1] | 2.51 ± 0.14 [a,1] | 2.44 ± 0.18 [a,1] | 2.45 ± 0.23 [a,1] | 2.43 ± 0.15 [a,1] |
| | 30 min | 2.34 ± 0.24 [a,1,2] | 2.15 ± 0.20 [ab,1,2] | 2.06 ± 0.05 [ab,1,2] | 1.86 ± 0.12 [b,2] | 1.87 ± 0.14 [b,2] |
| | 60 min | 2.00 ± 0.16 [a,2,3] | 1.95 ± 0.14 [a,2] | 1.79 ± 0.09 [a,2] | 1.81 ± 0.12 [a,2] | 1.98 ± 0.16 [a,2] |
| | 120 min | 1.93 ± 0.22 [a,3] | 1.92 ± 0.16 [a,2] | 2.02 ± 0.10 [a,2] | 1.96 ± 0.16 [a,2] | 1.98 ± 0.13 [a,2] |
| BCF (Hz) | 0 min | 34.39 ± 1.42 [a,1] | 32.23 ± 2.03 [a,1] | 32.46 ± 1.94 [a,1] | 31.76 ± 2.47 [a,1] | 32.01 ± 1.72 [a,1,2] |
| | 30 min | 35.00 ± 2.38 [a,1] | 32.29 ± 1.10 [a,1] | 34.61 ± 1.42 [a,1] | 32.33 ± 1.29 [a,1] | 27.94 ± 3.34 [a,2] |
| | 60 min | 28.13 ± 1.68 [a,1] | 32.00 ± 0.81 [a,1] | 32.33 ± 0.81 [a,1] | 34.66 ± 2.09 [a,1] | 34.01 ± 1.68 [a,1,2] |
| | 120 min | 31.29 ± 1.86 [a,1] | 31.27 ± 2.46 [a,1] | 36.27 ± 3.54 [a,1] | 33.28 ± 3.36 [a,1] | 38.17 ± 2.71 [a,1] |

VCL (μm/s): curvilinear velocity; VSL (μm/s): straight line velocity; VAP (μm/s): average path velocity; LIN (%): linearity coefficient; STR (%): straightness coefficient; WOB (%): wobble coefficient; ALH (μm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency. ([a,b]) Different letters indicate significant differences ($p \leq 0.05$) between H$_2$O$_2$ concentrations within a given incubation time. ([1–3]) Different numbers indicate significant differences ($p \leq 0.05$) between incubation times within a given H$_2$O$_2$ concentration.

The incubation time significantly decreased sperm velocity (VCL and VAP) and ALH; the higher the concentration of H$_2$O$_2$, the larger the extent of that reduction (from 30 min in 5 mM and 10 mM H$_2$O$_2$, and from 60 min in 0.5 mM and 1 mM H$_2$O$_2$). While LIN and STR increased along the incubation time in the treatments containing 5 mM and 10 mM H$_2$O$_2$ ($p \leq 0.05$), WOB was not affected. Finally, BCF significantly decreased after 30 min of incubation in the treatment containing 10 mM H$_2$O$_2$.

3.2.2. Sperm Viability

Exposure to different H$_2$O$_2$ concentrations did not significantly affect sperm viability at any of the incubation times. However, the incubation time caused a significant decrease in sperm viability regardless of H$_2$O$_2$ concentration (from 60 min in the control, 0.5 mM, 1 mM and 10 mM H$_2$O$_2$, and from 30 min in 5 mM H$_2$O$_2$; Figure 5).
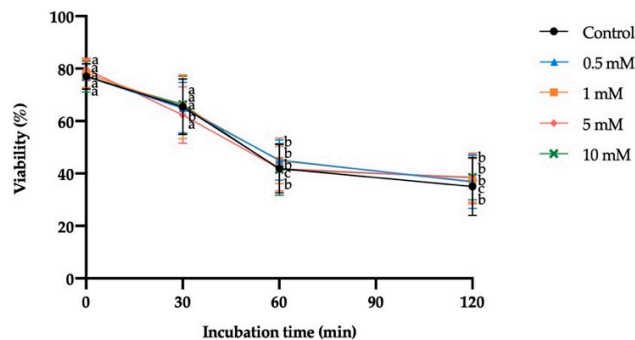


**Figure 5.** Mean ± SEM of viability of donkey sperm exposed to oxidative stress with different hydrogen peroxide (H$_2$O$_2$) concentrations measured at different incubation times. No significant differences were found between H$_2$O$_2$ concentrations within a given incubation time. (a,b) Different letters indicate significant differences ($p \leq 0.05$) between incubation times within a given H$_2$O$_2$ concentration.

3.2.3. Extracellular Hydrogen Peroxide (H$_2$O$_2$) Production

No significant differences in the extracellular H$_2$O$_2$ production were found between the control and treatments in which oxidative stress was induced with H$_2$O$_2$, either in sperm or in SP. In the same way, the extracellular H$_2$O$_2$ production in a given treatment did not significantly differ between sperm and SP.

*3.3. Experiment 2. Extracellular $H_2O_2$ Production by NETosis*

3.3.1. Reacted Polymorphonuclear Neutrophils (PMN)

There was a significant effect of the treatment on the percentage of reacted PMN (NETosis; $p \leq 0.001$; Figure 6a–c). The highest percentage of reacted PMN was found when 25% of SP was added (PMN + SP; 89.19% $\pm$ 1.02%), being significantly different from the other treatments. Removing SP from sperm (PMN + Sperm) induced less NETosis than semen containing SP (PMN + Whole semen; 33.03% $\pm$ 1.19% vs. 70.65% $\pm$ 1.17%; $p \leq 0.001$). Activation of PMN and, therefore, induction of NETosis was greater in the presence of FMLP (PMN + FMLP) than in both the control (58.07% $\pm$ 1.67% vs. 20.91% $\pm$ 2.27%; $p \leq 0.001$) and the treatment containing sperm but not SP (PMN + Sperm; 58.07% $\pm$ 1.67% vs. 33.03% $\pm$ 1.19%; $p \leq 0.001$). By contrast, NETosis in the treatments containing SP, either alone (PMN + SP; 89.19% $\pm$ 1.02%) or together with sperm (PMN + Sperm; 70.65% $\pm$ 1.17%), was significantly higher than in the treatment with FMLP (PMN + FMLP; 58.07% $\pm$ 1.67%; $p \leq 0.001$). Finally, the treatment that included the Kenney extender (PMN + Kenney) did not differ significantly from the control (18.73% $\pm$ 1.29% vs. 20.91% $\pm$ 2.27%).



**Figure 6.** Activation of PMN and NETosis. (**a**) Mean $\pm$ SEM of percentages of reacted PMN in each treatment. (a–e) Different letters indicate significant differences ($p \leq 0.05$) between treatments. (**b**) NETosis of PMN with degranulation/extrusion of their molecular components. (**c**) Representative images resulting from the PMN exposure to the different treatments. Scale bar = 15 μm.

3.3.2. Extracellular Hydrogen Peroxide ($H_2O_2$) Production

Treatments containing the whole semen (i.e., sperm and SP) generated a significantly higher amount of $H_2O_2$ after 0 min and 30 min of incubation compared with PMN (control), PMN + SP or PMN + FMLP (Figure 7). However, after 30 min of incubation, the treatment containing PMN + SP was found to produce more $H_2O_2$ than the control and the treatment with PMN + FMLP ($p \leq 0.05$). After 60 min of incubation, PMN (control), PMN + Whole semen and PMN + Sperm presented significantly higher $H_2O_2$ production than the other treatments. Only at 90 min of incubation did the whole semen (PMN + Whole semen) generate less $H_2O_2$ than the control, whereas PMN + Sperm produced more $H_2O_2$ than the control (PMN) after 120 min of incubation ($p \leq 0.05$).



**Figure 7.** Mean $\pm$ SEM of extracellular $H_2O_2$ production of jenny polymorphonuclear neutrophils (PMN) exposed to different treatments. (a–c) Different letters indicate significant differences ($p \leq 0.05$) between treatments within a given incubation time. (1–3) Different numbers indicate significant differences ($p \leq 0.05$) between incubation times within a given treatment.

Regarding the incubation time, the presence of the whole semen and SP significantly increased $H_2O_2$ levels after 30 min of incubation. After 60 min, the control, PMN + Sperm and PMN + FMLP treatments showed significantly higher production of $H_2O_2$ than at the beginning of the experiment ($p \leq 0.05$). By contrast, production of $H_2O_2$ did not differ along incubation in the treatment containing the Kenney extender (PMN + Kenney).

**4. Discussion**

Intracellular ROS production by sperm mainly results from electron loss in the mitochondrial transport chain during energy generation in the form of ATP [35]. This effect has been associated with greater mitochondrial activity in horse spermatozoa, where the electron loss favors an increase in ROS production even to toxic levels and alters the redox balance [36,37]. Within this context, it is important to mention that the chemical energy required for sperm motility, in the case of the horse, is supplied by the production of mitochondrial ATP derived from OXPHOS [17,18]. As donkey sperm present a significantly higher speed and progressivity compared with horse sperm [38], due to the fact that the former present a larger intermediate piece and a higher mitochondrial membrane potential [39,40] than the latter [41], one could suggest that ROS production by donkey sperm is even higher. However, our results indicate that exposure of donkey sperm to high doses (up to 10 mM) of GSH (reductive stress) and $H_2O_2$ (oxidative stress) does not affect TM, their values being maintained after 60 min and 120 min of incubation. In fact, only a significant reduction in PM was observed at 10 mM GSH. In human sperm, Panner Selvam et al. [23] observed a significant decrease in TM and PM under conditions

of reductive and oxidative stress from 30 min of incubation. This would indicate that donkey sperm have great resilience to the toxicity produced by high GSH levels and that their antioxidant capacity is very high. This capacity, together with SP, allows maintaining the redox balance following exposure to high levels of ROS.

When sperm are deposited in the female genital tract, they quickly lose the antioxidant support of SP. From then onward, sperm rely on their own antioxidant defense mechanisms [42], which, in the case of donkeys, are not enough to counteract the powerful oxidative environment of the endometrium due the infiltration of PMN as early as 6–10 h post-AI [3,43]. This leads to the activation of pathways that ultimately result in sperm death (apoptotic-like changes and necrosis after severe stress) [44]. Our results show that as the time of incubation increases, sperm viability is significantly reduced as early as 30 min after the contact occurs. This differs from what is found in humans; in effect, whereas human sperm reduce their viability significantly following exposure to oxidative conditions [23], this is not observed in donkeys because of the resistance of their sperm to high $H_2O_2$ concentrations.

The mechanisms through which donkey sperm are able to tolerate ROS better than antioxidants are still unknown. Surprisingly, we observed that high GSH concentrations caused higher $H_2O_2$ production, contrary to what might be expected once the antioxidant defense system was exogenously enhanced. The link found here could be related to two aspects. The first one would have to do with the presence of the enzyme L-amino acid oxidase (LAAO), which is found in the acrosome of sperm in several species, including humans [45], and is particularly active in the horse [46]. LAAO is responsible for the generation of $H_2O_2$ through deamination of L-amino or aromatic amino acids, such as phenylalanine, tyrosine and tryptophan [47]. In this circumstance, one could reasonably suggest that there could be a relationship between GSH and LAAO, which would support the suggestion that more ROS are produced under reductive conditions. The second aspect that could be envisaged to explain the greater ROS production in the presence of GSH would be the increase in the number of non-viable spermatozoa and even in that of morphologically abnormal ones, which results from incubation. These sperm cells have been shown to contain high levels of $H_2O_2$ synthesized in the mitochondria or in the cytoplasm of the sperm tail, so that they may be a potential source of ROS [48,49]. Furthermore, as non-viable sperm appear to be more sensitive to the presence of L-amino acids than viable cells, ROS production would likely be increased in the presence of non-viable sperm. Taking this aspect into account, we were able to quantify the amount of $H_2O_2$ produced by the non-viable donkey sperm released into the extracellular space, which ranged between 3.94 and 5.20 μM (average = 4.71 μM). The higher extracellular production of $H_2O_2$ began to occur when sperm were exposed to high antioxidant levels (i.e., 8 mM and 10 mM GSH). This makes the initially induced reductive environment become a spontaneous access route for lipid peroxidation in sperm that remain viable due to increased exposure to ROS [49]. Related to this, it has been described that TM and PM decrease significantly in horse sperm as the amount of ROS-producing non-viable sperm increases [50]. However, our CASA observations showed a reduction in sperm motility due to concentrations of GSH ≥ 8 mM after 60 min of incubation, but without this being associated with the presence of non-viable sperm. Furthermore, our findings also suggest that donkey sperm are more resilient to high concentrations of GSH than their horse counterpart, for which 2.5 mM GSH is already harmful [51,52].

The remarkable tolerance to oxidative stress found in our study is possibly an indication of the reproductive strategy developed by the donkey. Contrary to what was thought about the sperm susceptibility to oxidative stress, given by their inadequate cell repair systems and their low capacity for antioxidant defense due to their low cytoplasmic content [53], our data point out to a better antioxidant system in the donkey compared with other species. While the role of the antioxidant enzymes present in donkey SP has been widely described and their activities have been found to be higher than in the horse [26,54], non-enzymatic sperm antioxidants, such as GSH or ascorbic acid (AA), could increase

their biological effects in response to the stimulus given by oxidative stress, as previously reported in humans [55]. Paradoxically, we could define the behavior of donkey sperm found in our study as a "type of sperm survival" to oxidative stress. One could speculate that this tolerance would be similar to that observed in cancer cells, which have the ability to detoxify ROS by reconfiguring their metabolic activity, thus favoring their survival in an oxidizing environment and avoiding the activation of known cell death pathways [56]. This generates a broad research field in this animal species that could even serve to take it as a model to better understand what happens in other species. Likewise, the presence of several metabolites that could have a role in the maintenance of ROS balance has recently been described [57] and could become a potential research field in the donkey.

One of the hypotheses put forward in this study was that NETosis is capable of producing much more extracellular ROS (measured in $H_2O_2$ levels) than sperm themselves. While the amount of intracellular $H_2O_2$ produced by donkey sperm can be evaluated through the use of a fluorescent probe sensitive to oxidation (2′,7′-dichlorodihydrofluorescein diacetate; $H_2DCFDA$) [39,40], the amount of ROS produced specifically by NETosis is yet to be determined. In this study, NETosis in donkeys was seen to be induced by the presence of SP (either alone or in whole semen), in a similar fashion to that recently reported by Mateo-Otero et al. [30]. In addition, the current work also found that, in the donkey, SP controls extracellular ROS generation and maintains redox balance, as the extracellular $H_2O_2$ production is greater when SP is absent, thus complementing what was recently found by Papas et al. [58], who also demonstrated that the presence of SP improves the relative levels of intracellular superoxides in donkey sperm exposed to high $H_2O_2$ concentrations. This may be due to the fact that even without the existence of a pathogen, PMN are capable of activating one of their spontaneous apoptosis pathways (the intrinsic pathway) in response to a stimulus given by the presence of ROS [59]. This could explain the extracellular $H_2O_2$ levels generated when PMN come into contact with sperm and in the absence of SP, which contains antioxidants. In this scenario, the ROS generated by sperm as a by-product of their metabolism would trigger the intrinsic apoptosis pathway of PMN. In addition to the aforementioned, while PMN were observed to produce little extracellular $H_2O_2$ at the beginning of the experiment, they demonstrated to be capable of generating 5–6 times more extracellular $H_2O_2$ than their initial value along incubation. This effect could be attributed to a spontaneous or constitutive apoptosis process that PMN undergo as a mechanism to maintain the homeostasis of the immune system [60]. Interestingly, the Kenney extender seemed to prevent PMN from reaction, keeping the production of extracellular $H_2O_2$ constant even after 2 h of incubation. In this regard, we posit that one of the components of this extender, glucose, may account for preventing the activation of PMN and thus the release of extracellular ROS. In this sense, the data obtained in humans by Manosudprasit et al. [61] indicate that high concentrations of glucose lead to a significant delay in triggering the spontaneous apoptosis of PMN.

It is worth noting that while exposure to a chemotactic peptide (FMLP) was found to induce a quick activation of donkey PMN, the extent of that induction was larger than that of sperm but lesser than that of SP. Although the role of FMLP as an activating agent for PMN was previously reported by Baumber et al. [21] in horses and by Miró et al. [10] in donkeys, the current study was the first to combine PMN and FMLP without any other cellular component (e.g., sperm). In this scenario, the extracellular $H_2O_2$ production seems to be slower and with levels similar to those produced in the presence of SP. Thus, it could be established that FMLP has an effect similar to that of SP, but only with respect to its interaction with PMN. The comparison of the results obtained in our treatment of PMN + Sperm with those observed in horses [21] indicate that extracellular $H_2O_2$ production was four times higher in the donkey than in the horse after 30 min of incubation. Here, one must point out that the extracellular ROS amount produced by sperm:PMN binding relies upon the PMN concentration [21]. It seems that more PMN lead to more NETosis, resulting in higher extracellular ROS production.

According to the results obtained, it should be considered that PMN could undergo spontaneous apoptosis after 60 min of incubation, with the consequent production of high amounts of extracellular ROS. This would suggest that the activation of any of the cell death pathways, both in sperm and PMN, induced by an oxidative environment, would largely explain these high extracellular $H_2O_2$ levels ($4.69\ \mu M \pm 0.71\ \mu M$ in sperm and $5.95\ \mu M \pm 0.85\ \mu M$ in PMN). However, when sperm and PMN were put together, extracellular $H_2O_2$ levels did not increase after 60 min of incubation (PMN + Sperm; $5.98\ \mu M \pm 0.29\ \mu M$), which would rule out a cumulative effect on extracellular $H_2O_2$ production. Hence, the key factor would appear to be the presence of SP, which could be largely responsible for maintaining the redox balance in any environment (reductive or oxidative) and even in NETosis, keeping extracellular $H_2O_2$ levels almost constant at the same incubation time (60 min; $5.60\ \mu M \pm 0.26\ \mu M$, $4.81\ \mu M \pm 0.24\ \mu M$ and $4.46\ \mu M \pm 0.18\ \mu M$, respectively). Furthermore, it was also clear that the absence of SP led to higher amounts of extracellular $H_2O_2$ along the incubation time.

Be that as it may, NETosis is capable of producing an immense amount of ROS, which, in the long run, and if occurs repeatedly and/or excessively, can presumably contribute to chronic inflammation of the donkey endometrium and even end in fibrosis [62]. The action mechanism through which ROS production occurs by NETosis still raises several questions. However, it is known that $H_2O_2$ is produced by a redox reaction (dismutation) of the superoxide anion ($O_2^-$) mediated by the NADPH oxidase (NOX2) complex, generated by the NETs formation, which causes an electron loss of molecular oxygen ($O_2$). Finally, this $H_2O_2$ can be converted into hydroxyl anion ($OH^-$) through the Harber–Weiss or Fenton reaction [49,63–65]. Additionally, for NETosis of PMN to occur as a defense mechanism against pathogens, ROS must be generated through NOX2 [65–67]. This substrate is likely provided by both sperm themselves and the uterine environment. However, when NETs are formed, PMN have the ability to capture sperm with the clear objective of eliminating them by NETosis [13,27], possibly this programmed cell death process being the trigger for ROS generation in the extracellular space. Taking into account the fact that NETs formation in donkeys [10] and NETosis itself [30] are dependent on sperm concentration, some spermatozoa could have the ability to avoid their capture in the PMN vicinity, possibly because NETs would already be saturated with spermatozoa, or due to the action of the DNase present in the SP, which would block NETosis [68]. This would, in turn, give a greater number of motile and viable sperm the chance to reach the oviduct and fertilize the oocyte.

## 5. Conclusions

In conclusion, this study shows: (1) that donkey sperm have greater tolerance to oxidative than to reductive stress, as extracellular $H_2O_2$ production is larger at high GSH concentrations; and (2) that while SP is the inducer of NETosis of PMN, it also plays a fundamental role in the redox balance through its antioxidant contribution (enzymatic and non-enzymatic), as it controls the extracellular $H_2O_2$ production from both sperm and PMN. According to the results obtained, and from a practical point of view, the addition of antioxidants such as GSH could be necessary to improve the poor AI outcomes obtained in jennies when using frozen-thawed donkey sperm.

**References**

1. Canisso, I.F.; Panzani, D.; Miró, J.; Ellerbrock, R.E. Key aspects of donkey and mule reproduction. *Vet. Clin. N. Am. Equine Pract.* **2019**, *35*, 607–642. [CrossRef] [PubMed]
2. Miró, J.; Lobo, V.; Quintero-Moreno, A.; Medrano, A.; Peña, A.; Rigau, T. Sperm motility patterns and metabolism in Catalonian donkey semen. *Theriogenology* **2005**, *63*, 1706–1716. [CrossRef] [PubMed]
3. Miró, J.; Papas, M. Post–Artificial Insemination endometrial inflammation and its control in donkeys. *J. Equine Vet. Sci.* **2018**, *65*, 38–43. [CrossRef]
4. Vilés, K.; Rabanal, R.; Rodríguez-Prado, M.; Miró, J. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology* **2013**, *79*, 1019–1026. [CrossRef] [PubMed]
5. Troedsson, M.H.T.; Loset, K.; Alghamdi, A.M.; Dahms, B.; Crabo, B.G. Interaction between equine semen and the endometrium: The inflammatory response to semen. *Anim. Reprod. Sci.* **2001**, *68*, 273–278. [CrossRef]
6. O'Leary, S.; Jasper, M.J.; Warnes, G.M.; Armstrong, D.T.; Robertson, S.A. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* **2004**, *128*, 237–247. [CrossRef] [PubMed]
7. Scott, J.L.; Ketheesan, N.; Summers, P.M. Leucocyte population changes in the reproductive tract of the ewe in response to insemination. *Reprod. Fertil. Dev.* **2006**, *18*, 627. [CrossRef]
8. Chastant, S.; Saint-Dizier, M. Inflammation: Friend or foe of bovine reproduction? *Anim. Reprod.* **2019**, *16*, 539–547. [CrossRef]
9. Rodriguez-Martinez, H.; Saravia, F.; Wallgren, M.; Martinez, E.A.; Sanz, L.; Roca, J.; Vazquez, J.M.; Calvete, J.J. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. *J. Reprod. Immunol.* **2010**, *84*, 57–65. [CrossRef]
10. Miró, J.; Marín, H.; Catalán, J.; Papas, M.; Gacem, S.; Yeste, M. Seminal plasma, sperm concentration, and sperm-PMN interaction in the donkey: An in vitro model to study endometrial inflammation at post-insemination. *Int. J. Mol. Sci.* **2020**, *21*, 3478. [CrossRef]
11. Branzk, N.; Lubojemska, A.; Hardison, S.E.; Wang, Q.; Gutierrez, M.G.; Brown, G.D.; Papayannopoulos, V. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* **2014**, *15*, 1017–1025. [CrossRef] [PubMed]
12. Mesa, M.A.; Vasquez, G. NETosis. *Autoimmune Dis.* **2013**, *2013*, 651497. [CrossRef] [PubMed]
13. Zambrano, F.; Carrau, T.; Gärtner, U.; Seipp, A.; Taubert, A.; Felmer, R.; Sanchez, R.; Hermosilla, C. Leukocytes coincubated with human sperm trigger classic neutrophil extracellular traps formation, reducing sperm motility. *Fertil. Steril.* **2016**, *106*, 1053–1060. [CrossRef] [PubMed]
14. Kotilainen, T.; Huhtinen, M.; Katila, T. Sperm-induced leukocytosis in the equine uterus. *Theriogenology* **1994**, *41*, 629–636. [CrossRef]
15. Ball, B.A.; Vo, A.T.; Baumber, J. Generation of reactive oxygen species by equine spermatozoa. *Am. J. Vet. Res.* **2001**, *62*, 508–515. [CrossRef]
16. Yeste, M.; Estrada, E.; Rocha, L.G.; Marín, H.; Rodríguez-Gil, J.E.; Miró, J. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology* **2015**, *3*, 395–407. [CrossRef]
17. Ortega Ferrusola, C.; González Fernández, L.; Salazar Sandoval, C.; Macías García, B.; Rodríguez Martínez, H.; Tapia, J.A.; Peña, F.J. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. *Theriogenology* **2010**, *74*, 458–465. [CrossRef] [PubMed]
18. Gibb, Z.; Lambourne, S.R.; Aitken, R.J. The paradoxical relationship between stallion fertility and oxidative stress. *Biol. Reprod.* **2014**, *91*, 77–78. [CrossRef] [PubMed]

19. Foster, A.P.; Cunningham, P.M. Differential superoxide anion generation by equine eosinophils and neutrophils. *Vet. Immunol. Immunopathol.* **1997**, *59*, 225–237. [CrossRef]
20. Moore, T.; Wilcke, J.; Chilcoat, C.; Eyre, P.; Crisman, M. Functional characterization of equine neutrophils in response to calcium ionophore A23187 and phorbol myristate acetate ex vivo. *Vet. Immunol. Immunopathol.* **1997**, *56*, 233–246. [CrossRef]
21. Baumber, J.; Vo, A.; Sabeur, K.; Ball, B.A. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology* **2002**, *57*, 1025–1033. [CrossRef]
22. Muiño-Blanco, T.; Pérez-Pé, R.; Cebrián-Pérez, J.A. Seminal plasma proteins and sperm resistance to stress. *Reprod. Domest. Anim.* **2008**, *43*, 18–31. [CrossRef] [PubMed]
23. Panner Selvam, M.K.; Agarwal, A.; Henkel, R.; Finelli, R.; Robert, K.A.; Iovine, C.; Baskaran, S. The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa. *Free Radic. Biol. Med.* **2020**, *152*, 375–385. [CrossRef] [PubMed]
24. Baumber, J.; Ball, B.A.; Gravance, C.G.; Medina, V.; Davies-Morel, M.C.G. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *J. Androl.* **2000**, *21*, 895–902. [CrossRef]
25. Castagné, V.; Lefèvre, K.; Natero, R.; Becker, D.A.; Clarke, P.G.H. An optimal redox status for the survival of axotomized ganglion cells in the developing retina. *Neuroscience* **1999**, *93*, 313–320. [CrossRef]
26. Papas, M.; Arroyo, L.; Bassols, A.; Catalán, J.; Bonilla-Correal, S.; Gacem, S.; Yeste, M.; Miró, J. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology* **2019**, *140*, 180–187. [CrossRef]
27. Mateo-Otero, Y.; Zambrano, F.; Gacem, S.; Yeste, M.; Miró, J.; Fernandez-Fuertes, B. Sperm induce NETosis in jenny polymorphonuclear cells in a concentration and time dependent manner. *J. Equine Vet. Sci.* **2020**, *89*, 103037. [CrossRef]
28. Mantovani, A.; Cassatella, M.A.; Costantini, C.; Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* **2011**, *11*, 519–531. [CrossRef]
29. Miró, J.; Vilés, K.; García, W.; Jordana, J.; Yeste, M. Effect of donkey seminal plasma on sperm movement and sperm-polymorphonuclear neutrophils attachment in vitro. *Anim. Reprod. Sci.* **2013**, *140*, 164–172. [CrossRef] [PubMed]
30. Mateo-Otero, Y.; Zambrano, F.; Catalán, J.; Sánchez, R.; Yeste, M.; Miro, J.; Fernandez-Fuertes, B. Seminal plasma, and not sperm, induces time and concentration-dependent neutrophil extracellular trap release in donkeys. *Equine Vet. J.* **2021**, *00*, 1–12. [CrossRef]
31. Kenney, M.R. Minimal contamination techniques for breeding mares: Techniques and priliminary findings. *Proc. Am. Assoc. Equine Pract.* **1975**, 327–336.
32. Bamba, K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology* **1988**, *29*, 1245–1251. [CrossRef]
33. Siemsen, D.W.; Malachowa, N.; Schepetkin, I.A.; Whitney, A.R.; Kirpotina, L.N.; Lei, B.; Deleo, F.R.; Quinn, M.T. Neutrophil isolation from nonhuman species. *Methods Mol. Biol.* **2014**, *1124*, 19–37. [CrossRef]
34. Yildiz, K.; Gokpinar, S.; Sursal, N.; Babur, C.; Ozen, D.; Azkur, A.K. Extracellular trap formation by donkey polymorphonuclear neutrophils against *Toxoplasma gondii*. *J. Equine Vet. Sci.* **2019**, *73*, 1–9. [CrossRef]
35. Peña, F.J.; O'Flaherty, C.; Ortiz Rodríguez, J.M.; Martín Cano, F.E.; Gaitskell-Phillips, G.L.; Gil, M.C.; Ortega Ferrusola, C. Redox regulation and oxidative stress: The particular case of the stallion spermatozoa. *Antioxidants* **2019**, *8*, 567. [CrossRef]
36. Darr, C.R.; Varner, D.D.; Teague, S.; Cortopassi, G.A.; Datta, S.; Meyers, S.A. Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility, and ROS production. *Biol. Reprod.* **2016**, *95*, 34. [CrossRef] [PubMed]
37. Davila Plaza, M.; Muñoz Martin, P.; Bolaños Gallardo, J.M.; Stout, T.A.E.; Gadella, B.M.; Tapia, J.A.; Balao da Silva, C.; Ortega Ferrusola, C.; Peña, F.J. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. *Reproduction* **2016**, *152*, 683–694. [CrossRef] [PubMed]
38. Gacem, S.; Valverde, A.; Catalán, J.; Yánez-Ortiz, I.; Soler, C.; Miró, J. A new approach of sperm motility subpopulation structure in donkey and horse. *Front. Vet. Sci.* **2021**, *8*, 424. [CrossRef] [PubMed]
39. Catalán, J.; Papas, M.; Gacem, S.; Noto, F.; Delgado-Bermúdez, A.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Effects of red-light irradiation on the function and survival of fresh and liquid-stored donkey semen. *Theriogenology* **2020**, *149*, 88–97. [CrossRef]
40. Catalán, J.; Papas, M.; Trujillo-Rojas, L.; Blanco-Prieto, O.; Bonilla-Correal, S.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light. *Front. Cell Dev. Biol.* **2020**, *8*, 588621. [CrossRef] [PubMed]
41. Catalán, J.; Papas, M.; Gacem, S.; Mateo-Otero, Y.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Red-light irradiation of horse spermatozoa increases mitochondrial activity and motility through changes in the motile sperm subpopulation structure. *Biology* **2020**, *9*, 254. [CrossRef] [PubMed]
42. Moawad, A.R.; Fernandez, M.C.; Scarlata, E.; Dodia, C.; Feinstein, S.I.; Fisher, A.B.; O'Flaherty, C. Deficiency of peroxiredoxin 6 or inhibition of its phospholipase $A_2$ activity impair the *in vitro* sperm fertilizing competence in mice. *Sci. Rep.* **2017**, *7*, 1–13. [CrossRef] [PubMed]
43. Rota, A.; Panzani, D.; Sabatini, C.; Camillo, F. Donkey jack (*Equus asinus*) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. *Theriogenology* **2012**, *78*, 1846–1854. [CrossRef] [PubMed]

44. Guthrie, H.D.; Welch, G.R. Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. *Reprod. Fertil. Dev.* **2005**, *17*, 467. [CrossRef] [PubMed]
45. Houston, B.; Curry, B.; Aitken, R.J. Human spermatozoa possess an IL4I1 L-amino acid oxidase with a potential role in sperm function. *Reproduction* **2015**, *149*, 587–596. [CrossRef] [PubMed]
46. Aitken, J.B.; Naumovski, N.; Curry, B.; Grupen, C.G.; Gibb, Z.; Aitken, R.J. Characterization of an L-amino acid oxidase in equine spermatozoa. *Biol. Reprod.* **2015**, *92*, 1–13. [CrossRef]
47. Upreti, G.C.; Jensen, K.; Munday, R.; Duganzich, D.M.; Vishwanath, R.; Smith, J.F. Studies on aromatic amino acid oxidase activity in ram spermatozoa: Role of pyruvate as an antioxidant. *Anim. Reprod. Sci.* **1998**, *51*, 275–287. [CrossRef]
48. Aitken, R.; Koppers, A.J. Apoptosis and DNA damage in human spermatozoa. *Asian J. Androl.* **2011**, *13*, 36–42. [CrossRef]
49. Roca, J.; Parrilla, I.; Gil, M.A.; Cuello, C.; Martinez, E.A.; Rodriguez-Martinez, H. Non-viable sperm in the ejaculate: Lethal escorts for contemporary viable sperm. *Anim. Reprod. Sci.* **2016**, *169*, 24–31. [CrossRef]
50. Morrell, J.M.; Winblad, C.; Georgakas, A.; Stuhtmann, G.; Humblot, P.; Johannisson, A. Reactive oxygen species in stallion semen can be affected by season and colloid centrifugation. *Anim. Reprod. Sci.* **2013**, *140*, 62–69. [CrossRef] [PubMed]
51. Oliveira, R.A.; Wolf, C.A.; Viu, M.A.O.; Gambarini, M.L. Addition of glutathione to an extender for frozen equine semen. *J. Equine Vet. Sci.* **2013**, *33*, 1148–1152. [CrossRef]
52. Oliveira, R.A.; Piersanti, R.L.; Wolf, C.A.; Viu, M.A.O.; Gambarini, M.L. Glutathione for the freezing of cooled equine semen, using different protocols. *Anim. Reprod.* **2018**, *11*, 104–109.
53. Dutta, S.; Majzoub, A.; Agarwal, A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J. Urol.* **2019**, *17*, 87–97. [CrossRef] [PubMed]
54. Papas, M.; Catalan, J.; Barranco, I.; Arroyo, L.; Bassols, A.; Yeste, M.; Miró, J. Total and specific activities of superoxide dismutase (SOD) in seminal plasma are related with the cryotolerance of jackass spermatozoa. *Cryobiology* **2020**, *92*, 109–116. [CrossRef]
55. Moretti, E.; Collodel, G.; Fiaschi, A.I.; Micheli, L.; Iacoponi, F.; Cerretani, D. Nitric oxide, malondialdheyde and non-enzymatic antioxidants assessed in viable spermatozoa from selected infertile men. *Reprod. Biol.* **2017**, *17*, 370–375. [CrossRef] [PubMed]
56. Fiaschi, T.; Chiarugi, P. Oxidative stress, tumor microenvironment, and metabolic reprogramming: A diabolic liaison. *Int. J. Cell Biol.* **2012**, *762825*. [CrossRef] [PubMed]
57. Castiglione Morelli, M.A.; Ostuni, A.; Giangaspero, B.; Cecchini, S.; Carluccio, A.; Boni, R. Relationships between seminal plasma metabolites, semen characteristics and sperm kinetics in donkey (*Equus asinus*). *Animals* **2021**, *11*, 201. [CrossRef]
58. Papas, M.; Catalan, J.; Bonilla-Correal, S.; Gacem, S.; Miró, J.; Yeste, M. Seminal plasma has limited counteracting effects following induction of oxidative stress in donkey spermatozoa. *Reprod. Fertil. Dev.* **2020**, *32*, 619–628. [CrossRef]
59. Kennedy, A.D.; Deleo, F.R. Neutrophil apoptosis and the resolution of infection. *Immunol. Res.* **2009**, *43*, 25–61. [CrossRef]
60. Savill, J.S.; Wyllie, A.H.; Henson, J.E.; Walport, M.J.; Henson, P.M.; Haslett, C. Macrophage phagocytosis of aging neutrophils in inflammation: Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Investig.* **1989**, *83*, 865–875. [CrossRef]
61. Manosudprasit, A.; Kantarci, A.; Hasturk, H.; Stephens, D.; Van Dyke, T.E. Spontaneous PMN apoptosis in type 2 diabetes and the impact of periodontitis. *J. Leukoc. Biol.* **2017**, *102*, 1431–1440. [CrossRef] [PubMed]
62. Miró, J.; Gutiérrez-Reinoso, M.; Aguiar da Silva, J.; Fernandes, C.; Rebordão, M.R.; Alexandre-Pires, G.; Catalán, J.; Ferreira-Dias, G. Collagen and eosinophils in jenny's endometrium: Do they differ with endometrial classification? *Front. Vet. Sci.* **2020**, *7*, 631. [CrossRef] [PubMed]
63. Remijsen, Q.; Berghe, T.V.; Wirawan, E.; Asselbergh, B.; Parthoens, E.; De Rycke, R.; Noppen, S.; Delforge, M.; Willems, J.; Vandenabeele, P. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* **2011**, *21*, 290–304. [CrossRef]
64. Arażna, M.; Pruchniak, M.P.; Demkow, U. Reactive oxygen species, granulocytes, and NETosis. *Adv. Exp. Med. Biol.* **2015**, *836*, 1–7. [CrossRef]
65. Stoiber, W.; Obermayer, A.; Steinbacher, P.; Krautgartner, W.D. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. *Biomolecules* **2015**, *5*, 702–723. [CrossRef] [PubMed]
66. Fuchs, T.A.; Abed, U.; Goosmann, C.; Hurwitz, R.; Schulze, I.; Wahn, V.; Weinrauch, Y.; Brinkmann, V.; Zychlinsky, A. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **2007**, *176*, 231–241. [CrossRef] [PubMed]
67. Behnen, M.; Möller, S.; Brozek, A.; Klinger, M.; Laskay, T. Extracellular acidification inhibits the ROS-dependent formation of neutrophil extracellular traps. *Front. Immunol.* **2017**, *8*, 184. [CrossRef] [PubMed]
68. Alghamdi, A.S.; Foster, D.N. Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. *Biol. Reprod.* **2005**, *73*, 1174–1181. [CrossRef]

# 2. Addition of reduced glutathione (GSH) to the freezing medium reduces intracellular ROS levels in donkey sperm

Iván Yánez-Ortiz, Jaime Catalán, Ariadna Delgado-Bermúdez, Augusto Carluccio, Jordi Miró, Marc Yeste

*Article*

# Addition of Reduced Glutathione (GSH) to Freezing Medium Reduces Intracellular ROS Levels in Donkey Sperm

Iván Yánez-Ortiz [1,2,3,†] , Jaime Catalán [1,2,3,4,†] , Ariadna Delgado-Bermúdez [2,3] , Augusto Carluccio [4] , Jordi Miró [1,*,‡] and Marc Yeste [2,3,*,‡]

1   Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, Autonomous University of Barcelona, ES-08193 Barcelona, Cerdanyola del Vallès, Spain; ivan.yanez22@gmail.com (I.Y.-O.); dr.jcatalan@gmail.com (J.C.)
2   Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; ariadna.delgado@udg.edu
3   Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain
4   Faculty of Veterinary Medicine, University of Teramo, Loc. Piano d'Accio, IT-64100 Teramo, Italy; acarluccio@unite.it
*   Correspondence: jordi.miro@uab.cat (J.M.); marc.yeste@udg.edu (M.Y.); Tel.: +34-93-5814293 (J.M.); +34-972-419514 (M.Y.)
†   These authors contributed equally to this work.
‡   These authors share senior authorship.

**Abstract:** In donkeys, the use of frozen-thawed sperm for artificial insemination (AI) leads to low fertility rates. Furthermore, donkey sperm produce a large amount of reactive oxygen species (ROS), and post-AI inflammation induces the formation of neutrophil extracellular traps (NETosis), which further generates many more ROS. These high ROS levels may induce lipid peroxidation in the sperm plasma membrane, thus affecting its integrity. Enzymatic and non-enzymatic antioxidants, mainly found in the seminal plasma (SP), are responsible for maintaining the redox balance. However, this fluid is removed prior to cryopreservation, thereby exposing sperm cells to further oxidative stress. The exogenous addition of antioxidants to the freezing medium can reduce the detrimental effects caused by ROS generation. Therefore, the aim of this study was to evaluate how the addition of different reduced glutathione (GSH) concentrations (control, 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM) to fresh sperm affect their cryotolerance. Total and progressive motility, kinematic parameters and motile sperm subpopulations were significantly ($p < 0.05$) different from the control in treatments containing 8 mM and 10 mM GSH, but not at lower concentrations. Plasma and acrosome membrane integrity, mitochondrial membrane potential (MMP) and intracellular superoxide levels ($O_2^-$) were not affected ($p > 0.05$) by any GSH concentration. Interestingly, however, the addition of 8 mM or 10 mM GSH reduced ($p < 0.05$) the percentages of viable sperm with high overall ROS levels compared to the control. In conclusion, frozen-thawed donkey sperm are able to tolerate high GSH concentrations, which differs from what has been observed in other species. This antioxidant capacity suggests that ROS could be important during post-AI and that the impact of using exogenous antioxidants like GSH to improve the sperm resilience to freeze-thawing is limited in this species.

**Keywords:** sperm cryopreservation; reactive oxygen species (ROS); superoxide anion ($O_2^-$); reduced glutathione (GSH); antioxidants; donkey

## 1. Introduction

The cryopreservation of genetic material is a milestone for reproductive biotechnology, not only in humans but also in other vertebrates [1], since it may be applied together with other assisted reproductive technologies (ART) for the dissemination of genes of genetically superior animals [2], as well as for the conservation of biodiversity and the protection of endangered species [3]. This last aspect is very relevant in the donkey because the size

of the population has suffered a significant decrease in recent years [4], which directly affects the genetic variability of the existing breeds [5]. Research in this species has focused its efforts on improving sperm cryopreservation protocols, precisely to try to safeguard endangered donkey breeds [6–18]. However, the use of cryopreserved donkey sperm has been limited by the low conception rates obtained when jennies are inseminated [7,8,19]. This, which contrasts with the reproductive performance of mares, is largely due to the physiological endometritis that occurs in the jenny after artificial insemination (AI) [20,21].

In addition to the above, cryopreservation is known to induce freezing injuries in sperm due to their exposure to thermal and osmotic stresses and their high sensitivity to an oxidative environment, which leads to subsequent lipid peroxidation in the plasma membrane [22]. In the particular case of donkey sperm, and in a similar fashion to other species, this occurs because the plasma membrane contains a large amount of polyunsaturated fatty acids esterified into phospholipids [23]. Oxidative stress can be defined as a consequence of the imbalance between the cellular antioxidant defense system and the production of reactive oxygen species (ROS). Physiologically, sperm release ROS as a by-product of their metabolism; these ROS are scavenged by enzymatic and non-enzymatic antioxidants which play an instrumental role in maintaining the redox balance [24]. Both types of antioxidants, whose function is to prevent oxidative damage, are present in sperm or in seminal plasma (SP) [25]. In this context, it is worth mentioning that Papas et al. [26] showed that the antioxidant capacity of the enzymes present in the SP is significantly higher in donkeys than in horses. In spite of this, the sperm cryopreservation protocols currently applied in donkeys include the elimination of SP by centrifugation [27]. This makes sperm more susceptible to functional damage with lethal consequences [28] and lead them to undergo structural injuries, mainly at the chromatin level, which has a significant impact on male fertility [29]. In addition, it must be considered that, in equids, the presence of sperm during AI causes a powerful endometrial reaction with a large influx of polymorphonuclear neutrophils (PMN) into the uterine lumen [20,30–32]. These polymorphonuclear neutrophils act mainly via degranulating/extruding their DNA and bactericidal molecules (histones and enzymes), thereby forming neutrophil extracellular traps (NETs) that capture sperm without killing them [33]. Therefore, sperm:PMN binding in donkey activates one of the defense mechanisms of polymorphonuclear neutrophils, NETosis [34], which releases a large amount of ROS. These ROS levels are much greater than those generated by sperm [35] and even by the polymorphonuclear neutrophils themselves [36].

An alternative to counteract the negative effect that ROS generates on sperm from different mammalian species, including the human [37], is the addition of reduced glutathione (GSH) to the preservation medium in both cooling [38–41] and freezing [42–46]. Reduced glutathione is one of the most relevant non-enzymatic antioxidants in mammalian cells, and most of its scavenging effect results from the interaction with glutathione reductase (GSR) and glutathione peroxidase (GPX) [47]. Reduced glutathione contains a sulfhydryl group (-SH) that protects the disulfide bonds between chromatin fibers from the oxidative damage caused by ROS, thus maintaining the sperm nuclear structure during cryopreservation [47–49].

Despite the antioxidant properties of GSH to maintain the redox balance during cryopreservation, GSH concentrations greater than 2.5 mM have been found to be toxic to horse sperm when added to the freezing extender, as there is a significant reduction in the motility, viability and plasma membrane integrity after thawing [43]. The same effect has been observed in pigs, as the addition of 1 mM GSH to the freezing medium results in greater protection of sperm function than higher concentrations [41]. In donkeys, there is only one study that investigated the effects of supplementing the freezing medium with GSH; however, only one concentration was tested [16]. Testing the resilience of donkey sperm to oxidative stress using different GSH concentrations could shed some light into the reproductive strategy of this species [50–52]. In addition, supplementing freezing media with GSH could improve donkey sperm function and survival after thawing, which would help sperm to fight against the oxidative environment they encounter when reaching the

jenny uterus after AI [20]. Therefore, the objective of this study was to evaluate how different GSH concentrations added to the freezing medium affect the cryotolerance of donkey sperm.

## 2. Materials and Methods

### 2.1. Donkeys and Sperm Samples

A total of 12 ejaculates from 4 adult Catalonian jackasses (age: between 3 and 6 years old) were used. Animals were clinically healthy and of proven fertility (good fertility rates), and were maintained in individual paddocks at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is a center for the collection of semen from equids that holds an EU approval (authorization number: ES09RS01E) and operates under rigorous protocols which include the control of the health and welfare of animals. Thus, it was not necessary to receive a specific approval from the Ethics Committee of the Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain) to handle animals, since the four jackasses were semen donors. Complying with the sanitary guidelines established by the Council of the European Communities in Directive 82/894/EEC of 21 December 1982, animals were free of equine viral arteritis, equine infectious anemia and equine contagious metritis.

Sperm collection was performed on a regular schedule in the morning through an artificial vagina (Hannover model; Minitüb GmbH, Tiefenbach, Germany) with the use of an in-line nylon filter to remove the gel fraction. Once the ejaculate was obtained, it was immediately diluted 1:5 (*v:v*) in a skim milk-based extender [53], prewarmed to 37 °C. The quality of fresh sperm was checked through the analysis of concentration (Neubauer chamber; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), motility (CASA system; see Section 2.5 for details), and viability and morphology (eosin-nigrosin staining; see Section 2.6 for details). All samples were confirmed to be above the standard thresholds before freezing: viable sperm $\geq$85%, morphologically normal sperm $\geq$70%, and total motile sperm $\geq$80%.

### 2.2. Experimental Design

Before cryopreservation, each diluted sperm sample was centrifuged at $600 \times g$ and 20 °C for 15 min (Medifriger BL-S; JP Selecta SA, Barcelona, Spain); the supernatant was discarded and the pellet was resuspended in a commercial freezing medium containing glycerol and methylformamide as permeable cryoprotectants (BotuCRIO; Botupharma Animal Biotechnology, Botucatu, Brazil). Subsequently, sperm concentration and viability were again analyzed, and the same freezing medium (BotuCRIO) was added to obtain a final concentration of $200 \times 10^6$ viable sperm/mL (normalized in all cases). The final volume was divided into 6 aliquots of 3 mL each, and a different concentration of reduced glutathione (GSH; G4251; Saint Louis, MO, USA; final concentrations: 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM) was added; the control was not supplemented with GSH. A total of 6 separate 0.5 mL plastic straws were filled with the content of each aliquot (sperm sample with its respective treatment). Each straw was manually labeled with the freezing date, jackass name and treatment as follows: T1 = sperm (control); T2 = sperm + 2 mM GSH; T3 = sperm + 4 mM GSH; T4 = sperm + 6 mM GSH; T5 = sperm + 8 mM GSH; and T6 = sperm + 10 mM GSH.

### 2.3. Sperm Cryopreservation

All straws were cryopreserved in parallel using an automatic controlled-rate freezer (Ice-Cube 14S; Minitüb GmbH, Tiefenbach, Germany). Cooling/freezing was carried out in 3 stages: (1) cooling from 20 °C to 5 °C for 60 min at a rate of $-0.25$ °C/min, (2) freezing from 5 °C to $-90$ °C for 20 min at a rate of $-4.75$ °C/min, and (3) freezing from $-90$ °C to $-120$ °C for 2.7 min at a rate of $-11$ °C/min. Finally, straws were plunged into liquid nitrogen at $-196$ °C and stored in appropriate tanks for their conservation until analysis.

### 2.4. Sperm Thawing

Frozen sperm straws were thawed by incubation at 37 °C for 30 s in a hot water bath. The content of each straw was poured into a 10-mL conical tube for post-thaw sperm analysis. For each treatment and sperm sample, one straw was used for the assessment of motility (CASA system; see Section 2.5 for more details) and viability (eosin-nigrosin staining; see Section 2.6 for more details), and the other two were utilized for the evaluation of sperm parameters through flow cytometry (see Section 2.7 for details).

### 2.5. Sperm Motility Analysis

Sperm motility was evaluated by means of a CASA-Mot module of the ISAS v1.2 system (Proiser R + D, Valencia, Spain) equipped with a high-resolution digital camera model MQ003MG-CM (Proiser R + D, Valencia, Spain) capable of capturing up to 100 frames per second (fps). Before doing so, the content of each straw was diluted 1:2 (*v:v*) in a cooling extender based on skim milk prewarmed to 38 °C [53,54]. Briefly, 2 µL of each sperm sample was placed into a reusable Spermtrack10 chamber (Spk 10; Proiser R + D, Valencia, Spain), prewarmed to 38 °C. A minimum of 500 spermatozoa were counted per analysis under a 10× negative phase contrast microscope model UOP200i (Proiser R + D, Valencia, Spain). Total (TM, %) and progressive sperm motility (PM, %) were evaluated together regarding the following kinematic parameters: curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity coefficient (LIN = [VSL/VCL] × 100, %), straightness coefficient (STR = [VSL/VAP] × 100, %), wobble coefficient (WOB = [VAP/VCL] × 100, %), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz). The CASA-Mot settings were those recommended by the manufacturer: particle area > 4 and <75 µm$^2$, connectivity: 6, minimum number of images to calculate ALH: 10. VAP $\geq$ 10 µm/s and STR $\geq$ 75% were used as cut-off values for motile sperm and progressively motile sperm, respectively. Three technical replicates were examined.

### 2.6. Sperm Viability Analysis

Sperm viability was analyzed by eosin-nigrosin staining [55]. Briefly, 10 µL of the content of each sperm sample was placed on a slide which was previously preheated to 37 °C. Immediately afterwards, 10 µL of the eosin-nigrosin stain was placed onto the sperm sample and mixed; the mixture was smeared. A bright field light microscope (Carl Zeiss, Göttingen, Germany) at 1000× magnification using immersion oil objective was used to evaluate a minimum of 200 sperm/sample. The percentage of viable sperm (eosin negative) was recorded and three technical replicates were evaluated.

### 2.7. Evaluation of Sperm through Flow Cytometry

The sperm functional parameters evaluated through flow cytometry were plasma membrane integrity (SYBR-14/PI), acrosome membrane integrity (PNA-FITC/PI), mitochondrial membrane potential (MMP, JC-1), intracellular ROS levels (H$_2$DCFDA/PI), and intracellular superoxides levels (HE/YO-PRO-1). Fluorochromes were purchased from Molecular Probes (Thermo Fisher Scientific, Waltham, MA, USA). Sperm concentration was adjusted to $1 \times 10^6$ sperm/mL before staining. For each sample, a total of 10,000 events were analyzed, and 3 technical replicates were evaluated.

A CytoFLEX flow cytometer (Beckman Coulter Fullerton, CA, USA) was used with a sheath flow rate set at 10 µL/min. Samples were excited with an argon ion laser (488 nm) at a power of 50 mW. Cell diameter/volume was assessed through the Coulter principle. To exclude cell aggregates and debris, distributions of two different dot plots were used. Cell aggregates were excluded on the basis of forward scatter height (FSC-H) and altitude (FSC-A) dot plot distribution, whereas subcellular debris were excluded based on the distribution of FSC-A and side scatter altitude (SSC-A) dot plots.

A total of 3 different optical filters were used: FITC was used for the analysis of SYBR-14, PNA-FITC, JC-1 monomers (JC-1$_{mon}$), DCF$^+$ and YO-PRO-1 (band pass: 525–540 nm); PE for

the analysis of JC-1 aggregates (JC-1$_{agg}$) and E$^+$ (band pass: 585–542 nm), and PC5.5 for PI analysis (band pass: 690–650 nm). Information about each event (FSC-A, FSC-H, SSC-A, FITC, PE, and PC5.5) was collected in xit files and quantification from each sperm population was obtained using the CytExpert analysis software (Beckman Coulter Fullerton, CA, USA). The analyses were performed following the recommendations of the International Society for the Advancement of Cytometry (ISAC) [56]. For each parameter, the corresponding mean and standard error of the mean (SEM) were calculated.

### 2.7.1. Plasma Membrane Integrity Analysis (SYBR-14/PI)

The analysis of plasma membrane integrity was carried out following the protocol described by Garner and Johnson [57], adapted to donkey sperm, using the LIVE/DEAD sperm viability kit (SYBR-14/PI). Briefly, sperm samples were incubated in the dark at 38 °C with SYBR-14 (final concentration: 31.8 nM) for 10 min and PI (final concentration: 7.6 μM) for a further 5 min. Three sperm populations were identified: (1) sperm with an intact plasma membrane, green-stained (SYBR-14$^+$/PI$^-$), (2) sperm with a damaged plasma membrane, red-stained (SYBR-14$^-$/PI$^+$), and (3) sperm with a damaged plasma membrane, both green- and red-stained (SYBR-14$^+$/PI$^+$). The unstained sperm (SYBR-14$^-$/PI$^-$) were considered as non-sperm particles (debris) and were used to correct the data in the other evaluations. The spill over of SYBR-14 in the PC5.5 channel (8.34%) was compensated.

### 2.7.2. Acrosome Membrane Integrity Analysis (PNA-FITC/PI)

The integrity of the acrosome membrane was evaluated following the protocol described by Rathi et al. [58] adapted to donkey sperm using PNA-FITC and PI. Briefly, sperm samples were incubated in the dark at 38 °C with FITC-conjugated PNA (final concentration: 1.17 μg/mL) and PI (final concentration: 5.6 μm) for 10 min. Four sperm populations were identified: (1) sperm with an intact plasma membrane (PNA-FITC$^-$/PI$^-$), (2) sperm with a damaged plasma membrane that presented an acrosomal membrane that could not be completely intact (PNA-FITC$^+$/PI$^+$), (3) sperm with a damaged plasma membrane and missing outer acrosomal membrane (PNA-FITC$^-$/PI$^+$), and (4) sperm with a damaged plasma membrane (PNA-FITC$^+$/PI$^-$). Data were not compensated.

### 2.7.3. Mitochondrial Membrane Potential (MMP) Analysis (JC-1)

The MMP analysis was performed following the protocol described by Ortega-Ferrusola et al. [59] adapted to donkey sperm using JC-1 iodide (5.5′, 6,6′-tetrachloro-1,1′, 3,3′ tetraethylbenzimidazolylcarbocyanine). In brief, sperm samples were incubated in the dark at 38 °C for 30 min with JC-1 (final concentration: 750 nM). In the presence of low MMP, JC-1 molecules remain as green-fluorescent monomers (JC-1$_{mon}$), whereas in the presence of high MMP, they form orange-fluorescent aggregates (JC-1$_{agg}$). Two sperm populations were distinguished: (1) low MMP sperm (cells presenting a JC-1$_{mon}$ fluorescence intensity higher than a JC-1$_{agg}$ fluorescence intensity) and (2) high MMP sperm (cells presenting a JC-1$_{agg}$ fluorescence intensity higher than a JC-1$_{mon}$ fluorescence intensity). Data were not compensated. The fluorescence intensity of JC-1$_{mon}$ and JC-1$_{agg}$ was recorded in all sperm populations, and the ratio between JC-1$_{agg}$ and JC-1$_{mon}$ was calculated.

### 2.7.4. Intracellular Reactive Oxygen Species (ROS) Levels Analysis: H$_2$O$_2$ (H$_2$DCFDA/PI) and O$_2^-$ (HE/YO-PRO-1)

The analysis of the intracellular levels of ROS was carried out following the protocol described by Morrell et al. [60] and Guthrie and Welch [61], adapted to donkey sperm. The combination of 2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and PI was used to measure overall intracellular ROS levels, and the combination of hydroetidine (HE) and YO-PRO-1 was used to measure intracellular levels of superoxide anion (O$_2^-$).

For ROS evaluation, sperm samples were incubated in the dark at 38 °C with H$_2$DCFDA (final concentration: 50 μM) for 20 min; thereafter, PI (final concentration: 6 μM) was added and samples were incubated for 5 min. In the presence of ROS, H$_2$DCFDA is de-esterified

and oxidized into the highly fluorescent molecule $DCF^+$. Therefore, four sperm populations were identified: (1) viable sperm with high ROS levels ($DCF^+/PI^-$), (2) non-viable sperm with high ROS levels ($DCF^+/PI^+$), (3) viable sperm with low ROS levels ($DCF^-/PI^-$), and (4) non-viable sperm with low ROS levels ($DCF^-/PI^+$). Data were not compensated. The fluorescence intensity of $DCF^+$ was recorded in all sperm populations.

For the measurement of the intracellular levels of $O_2^-$, sperm samples were incubated in the dark at 38 °C with HE (final concentration: 5 μM) and with YO-PRO-1 (final concentration: 31.25 nM) for 30 min. In the presence of $O_2^-$, HE is oxidized into fluorescent ethidium ($E^+$). This allowed the observation of four sperm populations: (1) viable sperm with high $O_2^-$ levels ($E^+/YO$-PRO-$1^-$), (2) non-viable sperm with high $O_2^-$ levels ($E^+/YO$-PRO-$1^+$), (3) viable sperm with low·$O_2^-$ levels ($E^-/YO$-PRO-$1^-$), and (4) non-viable sperm with low $O_2^-$ levels ($E^-/YO$-PRO-$1^+$). The spill over of $E^+$ into the FITC channel (3.62%) was compensated. The fluorescence intensity of $E^+$ was recorded in all sperm populations.

*2.8. Statistical Analyses*

Statistical analyses were performed with the R statistical package (V 4.0.3, R Core Team; Vienna, Austria) and graphs were plotted with GraphPad Prism (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA). The Shapiro–Wilk test was run to verify the normal distribution of data and the Levene test checked the homoscedasticity. When necessary, data were transformed with arcsin $\sqrt{x}$ to match with parametric assumptions. When these assumptions were not met, a non-parametric analysis was performed using the Kruskal–Wallis test followed by the Wilcoxon test ($p \leq 0.05$) for pairwise comparisons. The effects of the different GSH concentrations (control, 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM) on motility, viability (eosin-nigrosin), plasma membrane integrity ($SYBR14^+/PI^-$), acrosome membrane integrity ($PNA$-$FITC^-/PI^-$), MPP ($JC$-$1_{agg}$), intracellular $H_2O_2$ levels ($DCF^+/PI^-$), and intracellular $O_2^-$ levels ($E^+/YO$-PRO-$1^-$), as well as the geometric mean of fluorescence intensity (GMFI) of $JC$-$1_{agg}$, $DCF^+$ and $E^+$ after thawing, were analyzed using a one-way ANOVA, followed by the Bonferroni post-hoc test ($p \leq 0.05$) for pairwise comparisons.

Motile sperm subpopulations were obtained by applying the procedure described by Martí et al. [62]. Briefly, a principal component analysis (PCA) was performed from the kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) of each sperm cell after thawing. The matrix obtained was rotated using the Varimax method with Kaiser normalization, where each spermatozoon was assigned a regression score. These values were used to perform a non-hierarchical, multivariate cluster analysis using the *k*-means model based on Euclidean distances. Finally, the proportion of spermatozoa in each subpopulation was calculated to analyze the effects of the different GSH concentrations through a one-way ANOVA, followed by the Bonferroni post-hoc test ($p \leq 0.05$) for pairwise comparisons.

In all analyses, the results are expressed as means ± SEM.

## 3. Results

*3.1. Sperm Motility*

The percentage of total motile sperm at post-thaw (Figure 1A) was significantly lower in the treatments containing 8 mM GSH (29.36% ± 6.88%) and 10 mM GSH (7.85% ± 3.10%) than in the control (62.56% ± 4.81%) and those containing 2 mM GSH (59.18% ± 5.42%) and 4 mM GSH (57.20% ± 5.50%). The concentration of 6 mM GSH (47.81% ± 6.36%) only differed from that of 10 mM ($p < 0.05$). In addition, the percentage of progressively motile sperm (Figure 1B) decreased significantly when GSH concentrations of 8 mM (9.76% ± 3.95%) and 10 mM (1.65% ± 0.96%) were added, compared to the control (34.05% ± 4.61%), 2 mM GSH (37.20% ± 5.04%) and 4 mM GSH (31.41% ± 5.30%). The concentration of 6 mM (27.14% ± 5.70%) was only different from that of 10 mM ($p < 0.05$).
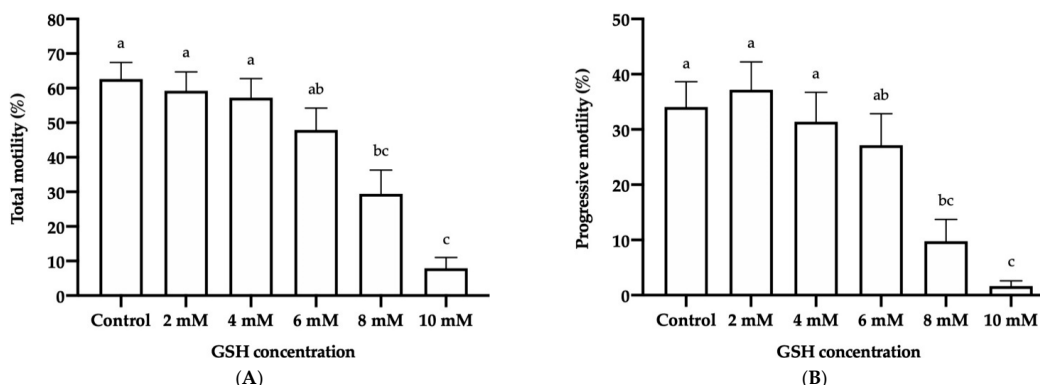
**Figure 1.** Mean $\pm$ SEM of the total (**A**) and progressive (**B**) motility of frozen-thawed donkey sperm following cryopreservation with different reduced glutathione (GSH) concentrations. (a–c) Different letters indicate significant differences ($p \leq 0.05$) between the control and GSH treatments.

On the other hand, the kinematic parameters of frozen-thawed donkey sperm remained without significant changes at concentrations $\leq 6$ mM GSH compared to the control (Table 1). Indeed, VCL, VAP, WOB and ALH were significantly lower in the treatment containing 10 mM than in the control ($p < 0.05$), whereas VSL, LIN, STR, and BCF were lower than the control in those having 8 mM and 10 mM GSH ($p < 0.05$).

**Table 1.** Mean $\pm$ SEM of the kinematic parameters of frozen-thawed donkey sperm following cryopreservation with different reduced glutathione (GSH) concentrations.

| Parameter | GSH Concentration | | | | | |
|---|---|---|---|---|---|---|
| | Control | 2 mM | 4 mM | 6 mM | 8 mM | 10 mM |
| VCL (μm/s) | 134.66 ± 8.53 [a] | 152.04 ± 6.74 [a] | 139.16 ± 7.43 [a] | 138.69 ± 9.47 [a] | 114.97 ± 13.76 [a] | 57.03 ± 18.80 [b] |
| VSL (μm/s) | 53.16 ± 4.55 [a] | 60.51 ± 4.09 [a] | 52.71 ± 4.11 [a] | 50.47 ± 5.20 [a] | 30.14 ± 4.76 [b] | 13.53 ± 5.08 [b] |
| VAP (μm/s) | 69.42 ± 4.75 [a] | 76.22 ± 3.75 [a] | 70.92 ± 2.98 [a] | 68.26 ± 4.51 [a] | 53.05 ± 5.95 [a] | 27.23 ± 8.54 [b] |
| LIN (%) | 37.02 ± 1.20 [a] | 37.96 ± 1.26 [a] | 35.59 ± 1.29 [a] | 33.96 ± 1.67 [a] | 23.06 ± 2.61 [b] | 10.98 ± 3.75 [c] |
| STR (%) | 70.07 ± 1.91 [a] | 74.07 ± 2.09 [a] | 68.06 ± 3.21 [a] | 67.37 ± 3.21 [a] | 47.72 ± 5.40 [b] | 23.19 ± 7.92 [c] |
| WOB (%) | 51.61 ± 0.39 [a] | 50.65 ± 0.65 [a] | 51.96 ± 1.50 [a] | 49.95 ± 0.88 [a] | 44.14 ± 4.51 [a] | 24.70 ± 7.55 [b] |
| ALH (μm) | 1.81 ± 0.08 [a] | 2.06 ± 0.08 [a] | 1.92 ± 0.10 [a] | 1.93 ± 0.11 [a] | 1.62 ± 0.18 [a] | 0.83 ± 0.27 [b] |
| BCF (Hz) | 28.23 ± 2.28 [a] | 29.27 ± 2.19 [a] | 25.84 ± 1.46 [a] | 23.57 ± 1.91 [ab] | 15.19 ± 1.90 [bc] | 8.05 ± 2.52 [c] |

VCL (μm/s): curvilinear velocity; VSL (μm/s): straight line velocity; VAP (μm/s): average path velocity; LIN (%): linearity coefficient; STR (%): straightness coefficient; WOB (%): wobble coefficient; ALH (μm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency. ([a–c]) Different letters indicate significant differences ($p \leq 0.05$) between the control and GSH treatments.

### 3.2. Motile Sperm Subpopulations

Four motile sperm subpopulations were identified in frozen-thawed donkey sperm (Table 2). Subpopulation 1 (SP1) was characterized by being the fastest (higher values of VCL, VSL, and VAP) and most progressive (high values of LIN, STR, ALH and BCF, although WOB was intermediate). Subpopulation 2 (SP2) was the slowest and least progressive, with the lowest ALH and BCF values. Subpopulation 3 (SP3) presented intermediate velocity and progressivity values similar to those of SP1, with an intermediate ALH and a BCF similar to SP1. Finally, subpopulation 4 (SP4) was also characterized by presenting intermediate speed, progressivity values, ALH and BCF. Although these parameters were lower than in SP1 and SP3, SP4 exhibited the highest WOB.

The proportions of motile spermatozoa of SP2 identified in frozen-thawed donkey sperm (Figure 2) were significantly higher in the presence of 8 mM (53.65% $\pm$ 7.10%) and 10 mM GSH (79.04% $\pm$ 9.01%) than in that of 2 mM GSH (24.56% $\pm$ 3.37%). On the contrary, SP3 reduced its proportion in the presence of 8 mM (12.88% $\pm$ 3.34%) and 10 mM

GSH (7.24% ± 4.66%) compared to 2 mM GSH (29.87% ± 2.77%; $p < 0.05$). Similarly, the proportion of sperm belonging to SP1 was significantly lower in the treatment containing 10 mM GSH than in that with 2 mM GSH (0.72% ± 0.58% vs. 11.52% ± 2.28%), and the proportion of SP4-sperm was lower in the presence of 10 mM GSH (13.01% ± 4.92%) than in the control (34.05% ± 1.83%) and treatments with 2 mM (34.11% ± 1.42%), 4 mM (33.23% ± 1.94%), and 6 mM GSH (29.67% ± 3.83%).

**Table 2.** Structure of the four motile sperm subpopulations identified in frozen-thawed donkey sperm following cryopreservation with different reduced glutathione (GSH) concentrations.

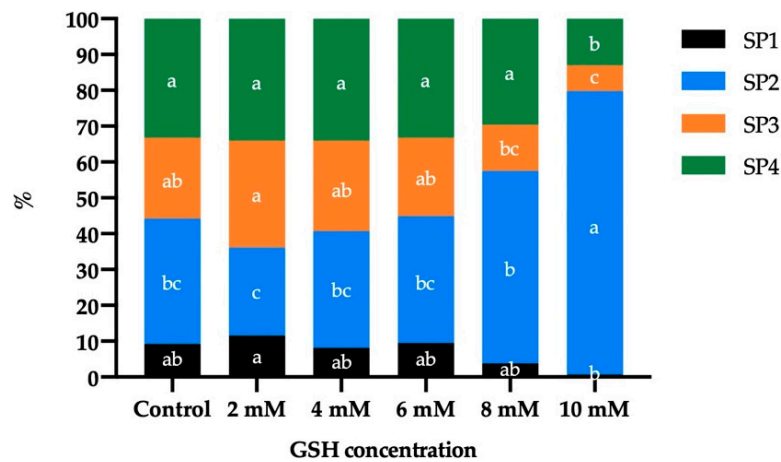| Parameter | SP1 | | SP2 | | SP3 | | SP4 | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± SEM | Range | Mean ± SEM | Range | Mean ± SEM | Range | Mean ± SEM | Range |
| VCL (µm/s) | 265.51 ± 0.81 | 197.90–429.30 | 69.32 ± 0.35 | 0.00–147.30 | 198.68 ± 0.35 | 127.50–276.00 | 134.67 ± 0.32 | 66.50–218.70 |
| VSL (µm/s) | 115.39 ± 0.54 | 10.80–191.30 | 15.79 ± 0.13 | 0.00–54.70 | 83.56 ± 0.30 | 3.70–152.10 | 49.42 ± 0.22 | 1.80–105.00 |
| VAP (µm/s) | 131.57 ± 0.40 | 78.60–216.50 | 33.31 ± 0.19 | 0.00–91.90 | 99.91 ± 0.22 | 44.30–165.90 | 69.05 ± 0.17 | 25.10–123.90 |
| LIN (%) | 44.13 ± 0.23 | 3.90–75.30 | 24.06 ± 0.19 | 0.00–92.30 | 42.75 ± 0.17 | 1.50–95.60 | 38.03 ± 0.19 | 1.20–100.00 |
| STR (%) | 87.42 ± 0.27 | 7.70–100.00 | 48.01 ± 0.29 | 0.00–99.20 | 83.59 ± 0.23 | 3.70–100.00 | 71.71 ± 0.25 | 2.30–100.00 |
| WOB (%) | 50.09 ± 0.18 | 27.00–85.30 | 48.88 ± 0.17 | 0.00–100.00 | 50.80 ± 0.13 | 19.20–100.00 | 52.32 ± 0.14 | 17.40–100.00 |
| ALH (µm) | 3.36 ± 0.02 | 1.50–6.40 | 1.10 ± 0.00 | 0.00–2.80 | 2.61 ± 0.01 | 0.80–4.00 | 1.83 ± 0.01 | 0.40–3.50 |
| BCF (Hz) | 36.67 ± 0.31 | 0.00–73.50 | 14.39 ± 0.10 | 0.00–52.40 | 35.29 ± 0.19 | 0.00–72.10 | 28.39 ± 0.14 | 0.00–70.80 |
| **n (%)** | 1776 (9.97%) | | 5609 (31.50%) | | 4339 (24.36%) | | 6085 (34.17) | |



**Figure 2.** Distribution of the four motile sperm subpopulations (SP) identified in frozen-thawed donkey sperm following cryopreservation with different concentrations of reduced glutathione (GSH). (a–c) Different letters indicate significant differences ($p \leq 0.05$) between the control and GSH treatments.

*3.3. Sperm Viability*

No significant differences in the viability of frozen-thawed donkey sperm (evaluated with eosin-nigrosin staining) between the different GSH concentrations and the control were found (Figure 3).
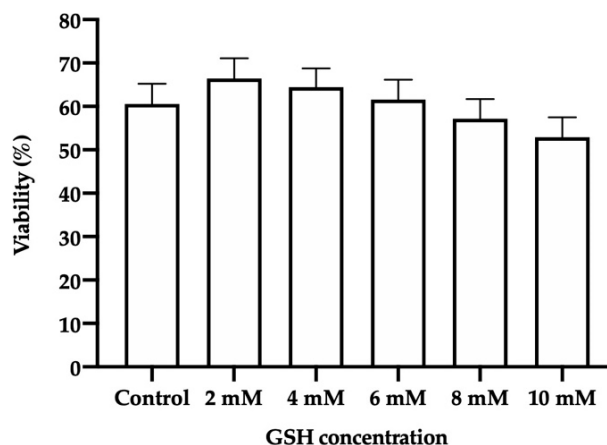
**Figure 3.** Mean $\pm$ SEM of the viability of frozen-thawed donkey sperm following cryopreservation with different reduced glutathione (GSH) concentrations. No significant differences ($p \leq 0.05$) between the control and GSH treatments were found.

### 3.4. Sperm Quality

3.4.1. Sperm Membrane Integrity

No significant differences in the percentage of frozen-thawed donkey sperm with intact plasma membrane (SYBR14$^+$/PI$^-$) were found between the control and the different GSH concentrations (Figure 4A).



**Figure 4.** Mean $\pm$ SEM of the percentages of sperm with an intact plasma membrane (SYBR14$^+$/PI$^-$; (**A**)) and with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$; (**B**)) observed in frozen-thawed donkey sperm after cryopreservation with different GSH concentrations. No significant differences ($p \leq 0.05$) between the control and GSH treatments were found.

3.4.2. Acrosome Membrane Integrity

Similarly, no significant differences in the percentage of frozen-thawed donkey sperm with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$) were observed between the different GSH concentrations and the control (Figure 4B).

3.4.3. Mitochondrial Membrane Potential (MPP)

No significant differences in the percentage of frozen-thawed donkey sperm with high-MMP (JC-1$_{agg}$) were seen between the different GSH concentrations and the control (Figure 5A). Similarly, the GMFI of JC-1$_{agg}$ in the high-MMP sperm population did not differ from the control when GSH was added to the freezing medium (Figure 5B).
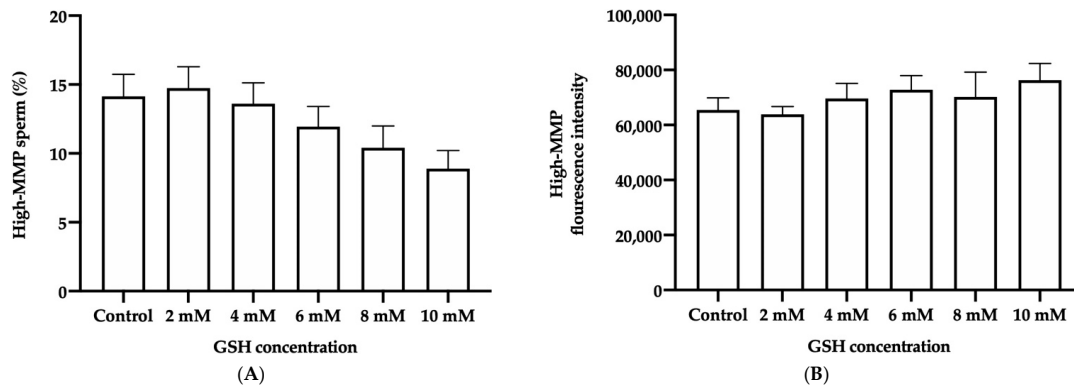
**Figure 5.** Mean ± SEM of the percentage of spermatozoa with high mitochondrial membrane potential (high-MMP; higher proportion of JC-1$_{agg}$ than JC-1$_{mon}$) (**A**) and geometric mean fluorescence intensity of JC-1$_{agg}$ (GMFI, PE channel) in the sperm population with high-MMP (**B**) observed in frozen-thawed donkey sperm following cryopreservation with different GSH concentrations. No significant differences ($p \leq 0.05$) between the control and GSH treatments were found.

### 3.4.4. Intracellular Reactive Oxygen Species (ROS) Levels: $H_2O_2$ and $O_2^-$

The GSH concentrations of 8 mM and 10 mM significantly ($p < 0.05$) decreased the percentage of frozen-thawed donkey sperm with high ROS levels (DCF$^+$/PI$^-$) compared to the control ($9.33\% \pm 1.91\%$ and $9.36\% \pm 1.81\%$ vs. $19.72\% \pm 3.13\%$, respectively; Figure 6A). Exposure to any GSH concentration also reduced ($p < 0.05$) the GMFI of DCF$^+$ in the DCF$^+$/PI$^-$ sperm population ($147{,}217.30 \pm 12{,}735.15$ in 2 mM GSH, $132{,}642.2 \pm 5966.27$ in 4 mM GSH, $145{,}301.20 \pm 9617.71$ in 6 mM GSH, $130{,}855.70 \pm 8405.67$ in 8 mM GSH, and $133{,}857.40 \pm 9184.45$ in 10 mM GSH) with respect to the control ($259{,}537.70 \pm 49{,}296.13$; Figure 6B).

On the other hand, no significant differences in either the percentage of frozen-thawed donkey sperm with high·$O_2^-$ levels (E$^+$/YO-PRO-1$^-$) or the GMFI of E$^+$ in the E$^+$/YO-PRO-1$^-$ sperm population were observed between the different GSH concentrations and the control (Figure 6C,D).
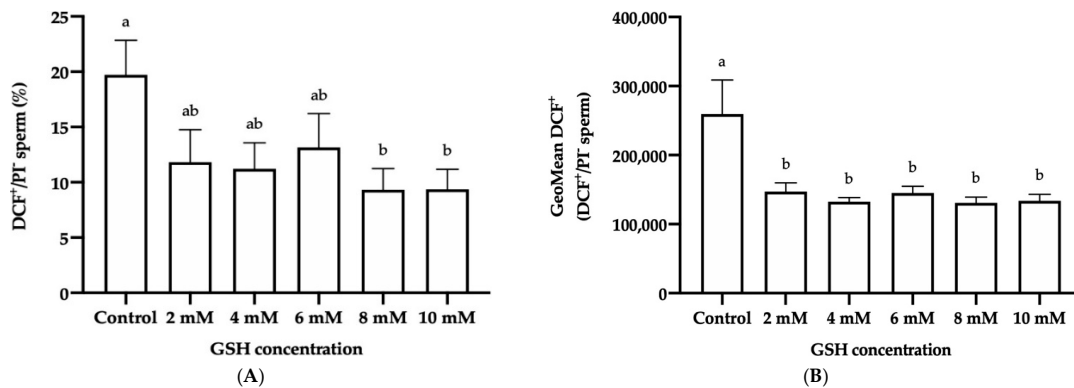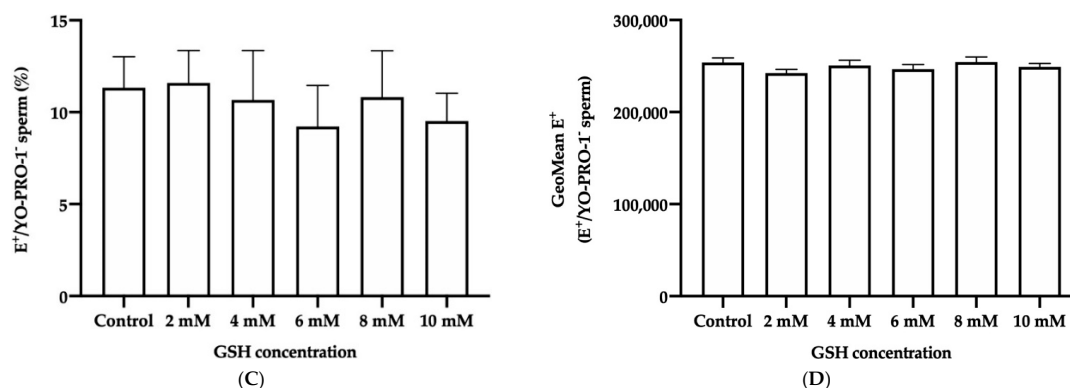


**Figure 6.** *Cont.*

**Figure 6.** Mean ± SEM of the percentage of viable sperm with high overall ROS levels (DCF$^+$/PI$^-$, (**A**)), geometric mean fluorescence intensity of DCF$^+$ (GMFI, FITC channel) in the DCF$^+$/PI$^-$ sperm population (**B**), percentage of viable spermatozoa with high·O$_2^-$ levels (E$^+$/YO-PRO-1$^-$, (**C**)), and geometric mean fluorescence intensity of E$^+$ (GMFI, channel PE) in the E$^+$/YO-PRO-1$^-$ sperm population (**D**) observed in frozen-thawed donkey sperm following cryopreservation with different reduced glutathione (GSH) concentrations. (a,b) Different letters indicate significant differences ($p \leq 0.05$) between the control and GSH concentrations in (**A**,**B**). No significant differences ($p \leq 0.05$) between the control and GSH treatments in (**C**,**D**) were found.

## 4. Discussion

The combination of the cellular component (sperm) produced by the germinal epithelium of the testes and the liquid component (SP) generated by the epididymis and the accessory sex glands [63,64] causes semen to become a complex redox system due to the antioxidant action exerted mostly by SP and, to a lesser extent, by sperm, as well as by the pro-oxidant action of sperm through ROS production [65]. This last action is more important during sperm cryopreservation in equids, because sperm cells lose the antioxidant support of SP before freezing. While intracellular GSH levels in horse sperm have been quantified [66], it is not clear whether this produced amount is high enough for sperm to have the ability to scavenge the oxidative stress generated by ROS and thus maintain redox balance without the antioxidant support of SP. In the case of donkeys, it has recently been shown that sperm are capable of tolerating high ROS concentrations (H$_2$O$_2$) and that in the presence of PMN, SP plays a decisive role in the regulation of the ROS produced during NETosis [36]. In this scenario, the addition of exogenous GSH could compensate in a certain way for the antioxidant component that sperm lose when cryopreserved.

The first hypothesis of this study envisaged that adding GSH to the freezing medium would increase the ability of donkey sperm to withstand cryopreservation. However, our results indicate that supplementation of the freezing medium with GSH at a concentration as high as 10 mM does not affect the viability, plasma and acrosome membrane integrity, and MMP of post-thaw sperm. However, sperm motility was reduced almost to zero. This surprising finding in the donkey provides us with valuable information to understand the reproductive strategy in this species, since the tolerance to GSH observed herein was much higher than that reported in horses [43], pigs [41], sheep [42], cattle [45], and dogs [44]. In effect, in those species, adding high concentrations of GSH to the freezing medium results in a significant decrease in all the aforementioned parameters of post-thaw sperm; in fact, in some cases, these high concentrations have even been reported to be cytotoxic [43].

Regarding the motility of frozen-thawed donkey sperm, we observed a significant effect of high GSH concentrations on TM and PM, which decreased in the presence of 8 mM (29.36% ± 6.88% and 9.76% ± 3.95%, respectively) and 10 mM GSH (7.85% ± 3.10% and 1.65% ± 0.96%, respectively) with respect to the control (62.56% ± 4.81% and 34.05% ± 4.61%, respectively). Interestingly, frozen-thawed donkey sperm were found to be more tolerant to exogenous GSH supplementation than their horse counterparts, as TM and PM in the latter

are known to be reduced in the presence of 5 mM and 2.5 mM GSH, respectively. Similarly, VSL, LIN, STR, and BCF values were significantly reduced at 8 mM and 10 mM GSH, whereas the other kinematic parameters (VCL, VAP, WOB, and ALH) were affected in the presence of 10 mM GSH. In addition, in horse sperm, LIN, STR, and BCF are decreased in the presence of a GSH concentration $\geq$2.5 mM; furthermore, VSL and ALH are decreased at $\geq$5 mM GSH, and VCL and VAP are decreased at $\geq$7.5 mM GSH [43]. Moreover, four separate subpopulations were herein identified in frozen-thawed donkey sperm, which is in agreement with previous research in this species [27,50,67]. We found that the treatment containing 10 mM GSH significantly increased the proportion of sperm belonging to SP2 (the slowest and least progressive subpopulation), reduced those of sperm belonging to SP3 and SP4 (the subpopulations with intermediate kinematic parameters), and did not affect that of SP1 (the fastest and most progressive subpopulation) with respect to the control. Remarkably, 2 mM, 4 mM, and 6 mM GSH were found not to modify the structure of the frozen-thawed motile sperm subpopulations compared to the control. The analysis of sperm subpopulations once again highlighted the cryotolerance of frozen-thawed donkey sperm as, while the presence of 2 mM GSH is known to modify the structure of those subpopulations in pigs, increasing the fastest subpopulation and decreasing the slowest one [68], no changes were observed herein for concentrations $\leq$6 mM GSH.

While sperm viability and integrities of plasma and acrosome membranes were not affected by the presence of high GSH concentrations, no treatment, even the ones containing low concentrations of this antioxidant, improved these sperm parameters post-thaw. This is contrary to what was found by Kumar et al. [16] in frozen-thawed sperm from exotic donkeys (Poitou breed, Martina Franca), where there was a significant increase in motility, viability and plasma membrane integrity when the freezing medium was supplemented with 2.5 mM. Our results, however, agree with the aforementioned study in the percentage of sperm with an intact acrosome membrane, since no differences in this parameter were observed in our study or in that of Kumar et al. [16], even when using two different techniques (flow cytometry vs. Giemsa stain + microscopy). Moreover, comparing our results with those found in other species may provide some clues, despite the fact that inconsistent data are seen in the literature. In cattle, Gangwar et al. [45] observed that adding 0.5 mM GSH to the freezing medium increased the viability, plasma and acrosome membrane integrity, and MMP of frozen-thawed sperm. In horses, Oliveira et al. [39] reported that a higher concentration of GSH (2.5 mM) was required to observe an improvement in the percentage of post-thaw sperm with an intact plasma membrane, despite the fact that acrosome integrity was similar to the control. In pigs, Estrada et al. [69] supplemented the freezing medium with 2 mM GSH and observed an increase in the percentages of viable and acrosome-intact sperm, and in fertilizing ability. On the contrary, Silva et al. [42] did not find any improvement in the quality (plasma and acrosome membrane integrity, and MMP) of frozen-thawed sheep sperm with 2 mM GSH in relation to the control, which was in agreement with our observations in the current study.

As previously mentioned, whereas TM and PM were found to decrease when 8 mM and 10 mM GSH were added to the freezing medium, viability, plasma and acrosome membrane integrity, and MMP did not differ from the control. Moreover, we observed that the percentage of viable sperm with high intracellular ROS levels was significantly lower in the treatments containing 8 mM and 10 mM GSH than in the control. These observations are in relative or slight agreement with our second hypothesis, which suggested that motility, viability, plasma and acrosome membrane integrity, MMP, and intracellular ROS levels of post-thaw sperm would be affected by the addition of GSH to the freezing medium. ROS production is known to alter the function and structure of sperm membranes [70], causing a detrimental effect on sperm motility and membrane integrity [22]. Our results indicate that the detrimental effects of ROS could be counteracted by the presence of GSH. However, the other parameters of sperm functionality did not improve with the presence of GSH. In fact, the results showed that GSH is effective in scavenging ROS up to 8 mM, and could have an effect on sperm motility. Further studies are needed to address whether fertility is

also compromised or if other antioxidants are able to improve the functionality and fertility of donkey sperm. As the post-translational modification of protein thiols is the most likely mechanism to maintain redox balance [41,71], one could speculate that supplementing the freezing medium with exogenous GSH could reverse the oxidative stress generated during the freezing and thawing of donkey sperm. This could maintain the integrity of plasma and acrosome membranes.

On the other hand, it is important to highlight that GSH supplementation did not affect the production of intracellular $O_2^-$ levels during cryopreservation. Physiologically, $O_2^-$ is scavenged by superoxide dismutase (SOD) [72], another antioxidant enzyme present in donkey SP, rather than by the glutathione complex enzymes (GSR and GPX). Freeze-thawing reduces SOD activity in response to an increase in $O_2^-$ production [65]; this enzyme converts $O_2^-$ into $H_2O_2$ by dismutation [73]. This leads one to posit that if there was high $O_2^-$ production in the long run, there would be a greater amount of $H_2O_2$. This excess of $H_2O_2$ could be scavenged by exogenous GSH and thus reduce the percentage of sperm with high $H_2O_2$ levels, as reported herein.

Another important implication of our results is related to what happens in the jenny's endometrium when AI is performed with frozen-thawed semen. In this scenario, donkey sperm are exposed to an oxidative environment in the uterus, which results from the production of ROS by PMN during the NETosis triggered by the presence of sperm [21]. Based on our results, GSH concentrations were not able to scavenge intracellular sperm ROS levels. Therefore, it seems reasonable to suggest that exogenous GSH supplementation between 2 mM and 6 mM could be a strategy against oxidative stress in this species after cryopreservation. This could be especially relevant after freeze-thawing if one bears in mind that SP, which is the main antioxidant source in semen, is removed before cryopreservation. However, one aspect that must be taken into account is that the degree of reduction in intracellular ROS levels observed at high GSH concentrations (8 mM and 10 mM) was not sufficient to positively affect sperm motility; in fact, it reduced sperm motility. This would not be in agreement with most observations in other mammalian species that indicate that sperm sensitivity to oxidative stress and cryogenic damage results in a reduction in motility after freeze-thawing [45,74,75]. In effect, we observed that, although high concentrations of GSH regulated the intracellular ROS levels, motility decreased. This opposite relationship advises that the impact of ROS on the other functional parameters in donkey sperm differs from other species.

## 5. Conclusions

According to the results obtained, frozen-thawed donkey sperm were found, on the one hand, to have a surprising capacity to tolerate high GSH concentrations compared to other species, maintaining viability, plasma membrane and acrosome integrity, and MMP. On the other hand, supplementation of the freezing medium with GSH appears to be necessary to control intracellular ROS levels, mainly $H_2O_2$, produced during freeze-thawing as well as post-AI, and may improve reproductive performance in the jenny. However, the highest GSH concentrations (8 mM and 10 mM) affected sperm motility, which suggests that how donkey sperm handle ROS generation differs from other species. Therefore, conducting in vivo studies is much warranted to complement the results obtained in this work.

**Institutional Review Board Statement:** The Ethics Committee of the Autonomous University of Barcelona (CEEAH) considered that the study is exempted of clearance.

**Informed Consent Statement:** The informed consent was obtained from owner of Catalonian jackasses in this study.

**Data Availability Statement:** All data is contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sherman, J.K. Low temperature research on spermatozoa and eggs. *Cryobiology* **1964**, *1*, 103–129. [CrossRef]
2. Loomis, P.R. Advanced methods for handling and preparation of stallion semen. *Vet. Clin. N. Am. Equine Pract.* **2006**, *22*, 663–676. [CrossRef] [PubMed]
3. Fickel, J.; Wagener, A.; Ludwig, A. Semen cryopreservation and the conservation of endangered species. *Eur. J. Wildl. Res.* **2007**, *53*, 81–89. [CrossRef]
4. Camillo, F.; Rota, A.; Biagini, L.; Tesi, M.; Fanelli, D.; Panzani, D. The current situation and trend of donkey industry in Europe. *J. Equine Vet. Sci.* **2018**, *65*, 44–49. [CrossRef]
5. Aranguren-Méndez, J.; Jordana, J.; Gomez, M. Genetic diversity in Spanish donkey breeds using microsatellite DNA markers. *Genet. Sel. Evol.* **2001**, *33*, 1–10. [CrossRef]
6. Trimeche, A.; Renard, P.; Tainturier, D. A procedure for Poitou jackass sperm cryopreservation. *Theriogenology* **1998**, *50*, 793–806. [CrossRef]
7. Vidament, M.; Vincent, P.; Martin, F.X.; Magistrini, M.; Blesbois, E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. *Anim. Reprod. Sci.* **2009**, *112*, 22–35. [CrossRef] [PubMed]
8. Oliveira, J.V.; Papa, F.O.; Melo-Oña, C.M.; Monteiro, G.A.; Puoli-Filho, J.N.P.; Alvarenga, M.A. New procedures to freeze donkey semen and its influence on mares and jennies fertility. *J. Equine Vet. Sci.* **2012**, *32*, 503–504. [CrossRef]
9. Rota, A.; Panzani, D.; Sabatini, C.; Camillo, F. Donkey jack (*Equus asinus*) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. *Theriogenology* **2012**, *78*, 1846–1854. [CrossRef]
10. Pukazhenthi, B.S.; Johnson, A.; Guthrie, H.D.; Songsasen, N.; Padilla, L.R.; Wolfe, B.A.; Coutinho da Silva, M.; Alvarenga, M.A.; Wildt, D.E. Improved sperm cryosurvival in diluents containing amides versus glycerol in the Przewalski's horse (*Equus ferus przewalskii*). *Cryobiology* **2014**, *68*, 205–214. [CrossRef]
11. Ortiz, I.; Dorado, J.; Morrell, J.M.; Crespo, F.; Gosálvez, J.; Gálvez, M.J.; Acha, D.; Hidalgo, M. Effect of single-layer centrifugation or washing on frozen–thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample? *Theriogenology* **2015**, *84*, 294–300. [CrossRef]
12. Acha, D.; Hidalgo, M.; Ortiz, I.; Gálvez, M.J.; Carrasco, J.J.; Gómez-Arrones, V.; Dorado, J. Freezability of Andalusian donkey (*Equus asinus*) spermatozoa: Effect of extenders and permeating cryoprotectants. *Reprod. Fertil. Dev.* **2016**, *28*, 1990–1998. [CrossRef] [PubMed]
13. Oliveira, J.V.; Oliveira, P.V.L.F.; Melo-Oña, C.M.; Guasti, P.N.; Monteiro, G.A.; Sancler da Silva, Y.F.R.; Papa, P.M.; Alvarenga, M.A.; Dell'Aqua, J.A.; Papa, F.O. Strategies to improve the fertility of fresh and frozen donkey semen. *Theriogenology* **2016**, *85*, 1267–1273. [CrossRef] [PubMed]
14. Bottrel, M.; Acha, D.; Ortiz, I.; Hidalgo, M.; Gósalvez, J.; Camisão, J.; Dorado, J. Cryoprotective effect of glutamine, taurine, and proline on post-thaw semen quality and DNA integrity of donkey spermatozoa. *Anim. Reprod. Sci.* **2018**, *189*, 128–135. [CrossRef]
15. Zhang, H.; Ye, H.; Shao, Y.; Wu, S.; Yu, J.; Ji, C.; Wang, S.; Zeng, S. The effects of egg yolk concentration and particle size on donkey semen preservation. *J. Equine Vet. Sci.* **2018**, *65*, 19–24. [CrossRef]
16. Kumar, P.; Kumar, R.; Mehta, J.S.; Chaudhary, A.K.; Ravi, S.K.; Chandra Mehta, S.; Ansari, M.M.; Legha, R.A.; Tripathi, B.N.; Talluri, T.R. Ameliorative effect of ascorbic acid and glutathione in combating the cryoinjuries during cryopreservation of exotic jack semen. *J. Equine Vet. Sci.* **2019**, *81*, 102796. [CrossRef]
17. Yu, X.; He, S.; Wang, L.; Kang, M.; Zhu, Y.; Wang, S.; Sun, X. Effects of Vitamin C and Vitamin E on cryopreservation of Guanzhong donkey semen. *Pak. J. Zool.* **2019**, *51*, 1777–1781. [CrossRef]
18. Li, N.; Yu, J.; Yang, F.; Shao, Y.; Wu, S.; Liu, B.; Li, M.; Wang, T.; Li, J.; Zeng, S. L-proline: An effective agent for frozen and post-thawed donkey semen storage. *J. Equine Vet. Sci.* **2021**, *101*, 103393. [CrossRef] [PubMed]

19. Canisso, I.F.; Carvalho, G.R.; Davis Morel, M.; Ker, P.G.; Rodrigues, A.L.; Silva, E.C.; Coutinho Da Silva, M.A. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Vet. J.* **2011**, *43*, 179–183. [CrossRef]

20. Vilés, K.; Rabanal, R.; Rodríguez-Prado, M.; Miró, J. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology* **2013**, *79*, 1019–1026. [CrossRef] [PubMed]

21. Miró, J.; Papas, M. Post–Artificial Insemination endometrial inflammation and its control in donkeys. *J. Equine Vet. Sci.* **2018**, *65*, 38–43. [CrossRef]

22. Yeste, M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology* **2016**, *85*, 47–64. [CrossRef] [PubMed]

23. Aitken, R.J.; De Iuliis, G.N.; Drevet, J.R. Role of oxidative stress in the etiology of male infertility and the potential therapeutic value of antioxidants. In *Oxidants, Antioxidants, and Impact of the Oxidative Status in Male Reproduction*; Elsevier: London, UK, 2018; pp. 91–100.

24. Peña, F.J.; O'Flaherty, C.; Ortiz Rodríguez, J.M.; Martín Cano, F.E.; Gaitskell-Phillips, G.L.; Gil, M.C.; Ortega Ferrusola, C. Redox regulation and oxidative stress: The particular case of the stallion spermatozoa. *Antioxidants* **2019**, *8*, 567. [CrossRef]

25. Kim, J.G.; Parthasarathy, S. Oxidation and the spermatozoa. *Semin. Reprod. Endocrinol.* **1998**, *16*, 235–239. [CrossRef]

26. Papas, M.; Arroyo, L.; Bassols, A.; Catalán, J.; Bonilla-Correal, S.; Gacem, S.; Yeste, M.; Miró, J. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology* **2019**, *140*, 180–187. [CrossRef] [PubMed]

27. Papas, M.; Catalán, J.; Recuero, S.; Morrell, J.M.; Yeste, M.; Miró, J. Single layer centrifugation improves the quality of fresh donkey semen and modifies the sperm ability to interact with polymorphonuclear neutrophils. *Animals* **2020**, *10*, 2128. [CrossRef] [PubMed]

28. Dutta, S.; Majzoub, A.; Agarwal, A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J. Urol.* **2019**, *17*, 87–97. [CrossRef]

29. Yeste, M.; Estrada, E.; Casas, I.; Bonet, S.; Rodríguez-Gil, J.E. Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. *Theriogenology* **2013**, *79*, 929–939. [CrossRef] [PubMed]

30. Kotilainen, T.; Huhtinen, M.; Katila, T. Sperm-induced leukocytosis in the equine uterus. *Theriogenology* **1994**, *41*, 629–636. [CrossRef]

31. Troedsson, M.H.T.; Loset, K.; Alghamdi, A.M.; Dahms, B.; Crabo, B.G. Interaction between equine semen and the endometrium: The inflammatory response to semen. *Anim. Reprod. Sci.* **2001**, *68*, 273–278. [CrossRef]

32. Rodriguez-Martinez, H.; Saravia, F.; Wallgren, M.; Martinez, E.A.; Sanz, L.; Roca, J.; Vazquez, J.M.; Calvete, J.J. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. *J. Reprod. Immunol.* **2010**, *84*, 57–65. [CrossRef]

33. Zambrano, F.; Carrau, T.; Gärtner, U.; Seipp, A.; Taubert, A.; Felmer, R.; Sanchez, R.; Hermosilla, C. Leukocytes coincubated with human sperm trigger classic neutrophil extracellular traps formation, reducing sperm motility. *Fertil. Steril.* **2016**, *106*, 1053–1060. [CrossRef] [PubMed]

34. Miró, J.; Marín, H.; Catalán, J.; Papas, M.; Gacem, S.; Yeste, M. Seminal plasma, sperm concentration, and sperm-PMN interaction in the donkey: An in vitro model to study endometrial inflammation at post-insemination. *Int. J. Mol. Sci.* **2020**, *21*, 3478. [CrossRef]

35. Baumber, J.; Vo, A.; Sabeur, K.; Ball, B.A. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology* **2002**, *57*, 1025–1033. [CrossRef]

36. Yánez-Ortiz, I.; Catalán, J.; Mateo-Otero, Y.; Dordas-Perpinyà, M.; Gacem, S.; Yeste, N.; Bassols, A.; Yeste, M.; Miró, J. Extracellular reactive oxygen species (ROS) production in fresh donkey sperm exposed to reductive stress, oxidative stress and NETosis. *Antioxidants* **2021**, *10*, 1367. [CrossRef]

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

38. Câmara, D.R.; Mello-Pinto, M.M.C.; Pinto, L.C.; Brasil, O.O.; Nunes, J.F.; Guerra, M.M.P. Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. *Small Rumin. Res.* **2011**, *100*, 44–49. [CrossRef]

39. Ansari, M.S.; Rakha, B.A.; Andrabi, S.M.H.; Ullah, N.; Iqbal, R.; Holt, W.V.; Akhter, S. Glutathione-supplemented tris-citric acid extender improves the post-thaw quality and in vivo fertility of buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Biol.* **2012**, *12*, 271–276. [CrossRef]

40. Oliveira, R.A.; Piersanti, R.L.; Wolf, C.A.; Viu, M.A.O.; Gambarini, M.L. Glutathione for the freezing of cooled equine semen, using different protocols. *Anim. Reprod.* **2018**, *11*, 104–109.

41. Ahmad, E.; Naseer, Z.; Aksoy, M. Glutathione supplementation in semen extender improves rabbit sperm attributes during refrigeration. *World Rabbit Sci.* **2021**, *29*, 81. [CrossRef]

42. Gadea, J.; García-Vazquez, F.; Matás, C.; Gardón, J.C.; Cánovas, S.; Gumbao, D. Cooling and freezing of boar spermatozoa: Supplementation of the freezing media with reduced glutathione preserves sperm function. *J. Androl.* **2005**, *26*, 396–404. [CrossRef] [PubMed]

43. Silva, S.V.; Soares, A.T.; Batista, A.M.; Almeida, F.C.; Nunes, J.F.; Peixoto, C.A.; Guerra, M.M.P. In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. *Reprod. Domest. Anim.* **2011**, *46*, 874–881. [CrossRef]

44. Oliveira, R.A.; Wolf, C.A.; Viu, M.A.O.; Gambarini, M.L. Addition of glutathione to an extender for frozen equine semen. *J. Equine Vet. Sci.* **2013**, *33*, 1148–1152. [CrossRef]

45. Ogata, K.; Sasaki, A.; Kato, Y.; Takeda, A.; Wakabayashi, M.; Sarentonglaga, B.; Yamaguchi, M.; Hara, A.; Fukumori, R.; Nagao, Y. Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. *J. Reprod. Dev.* **2015**, *61*, 116–122. [CrossRef] [PubMed]

46. Gangwar, C.; Saxena, A.; Patel, A.; Singh, S.P.; Yadav, S.; Kumar, R.; Singh, V. Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. *Anim. Reprod. Sci.* **2018**, *192*, 171–178. [CrossRef]

47. Luberda, Z. The role of glutathione in mammalian gametes. *Reprod. Biol.* **2005**, *5*, 5–17.

48. Irvine, D.S. Glutathione as a treatment for male infertility. *Rev. Reprod.* **1996**, *1*, 6–12. [CrossRef] [PubMed]

49. Yeste, M.; Flores, E.; Estrada, E.; Bonet, S.; Rigau, T.; Rodríguez-Gil, J.E. Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-thawing by stabilising disulfide bonds. *Reprod. Fertil. Dev.* **2013**, *25*, 1036–1050. [CrossRef]

50. Miró, J.; Lobo, V.; Quintero-Moreno, A.; Medrano, A.; Peña, A.; Rigau, T. Sperm motility patterns and metabolism in Catalonian donkey semen. *Theriogenology* **2005**, *63*, 1706–1716. [CrossRef]

51. Canisso, I.F.; Panzani, D.; Miró, J.; Ellerbrock, R.E. Key aspects of donkey and mule reproduction. *Vet. Clin. N. Am. Equine Pract.* **2019**, *35*, 607–642. [CrossRef]

52. Gacem, S.; Papas, M.; Catalan, J.; Miró, J. Examination of jackass (*Equus asinus*) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production. *Reprod. Domest. Anim.* **2020**, *55*, 181–188. [CrossRef]

53. Kenney, M.R. Minimal contamination techniques for breeding mares: Techniques and priliminary findings. *Proc. Am. Assoc. Equine Pract.* **1975**, 327–336.

54. Gacem, S.; Catalán, J.; Valverde, A.; Soler, C.; Miró, J. Optimization of CASA-mot analysis of donkey sperm: Optimum frame rate and values of kinematic variables for different counting chamber and fields. *Animals* **2020**, *10*, 1993. [CrossRef]

55. Bamba, K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology* **1988**, *29*, 1245–1251. [CrossRef]

56. Lee, J.A.; Spidlen, J.; Boyce, K.; Cai, J.; Crosbie, N.; Dalphin, M.; Furlong, J.; Gasparetto, M.; Goldberg, M.; Goralczyk, E.M.; et al. MIFlowCyt: The minimum information about a flow cytometry experiment. *Cytometry* **2008**, *73*, 926–930. [CrossRef] [PubMed]

57. Garner, D.L.; Johnson, L.A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **1995**, *53*, 276–284. [CrossRef] [PubMed]

58. Rathi, R.; Colenbrander, B.; Bevers, M.M.; Gadella, B.M. Evaluation of in vitro capacitation of stallion spermatozoa. *Biol. Reprod.* **2001**, *65*, 462–470. [CrossRef] [PubMed]

59. Ortega-Ferrusola, C.; Sotillo-Galán, Y.; Varela-Fernández, E.; Gallardo-Bolaños, J.M.; Muriel, A.; González-Fernández, L.; Tapia, J.A.; Peña, F.J. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. *J. Androl.* **2008**, *29*, 213–221. [CrossRef]

60. Morrell, J.M.; Winblad, C.; Georgakas, A.; Stuhtmann, G.; Humblot, P.; Johannisson, A. Reactive oxygen species in stallion semen can be affected by season and colloid centrifugation. *Anim. Reprod. Sci.* **2013**, *140*, 62–69. [CrossRef] [PubMed]

61. Guthrie, H.D.; Welch, G.R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J. Anim. Sci.* **2006**, *84*, 2089–2100. [CrossRef] [PubMed]

62. Martí, J.I.; Aparicio, I.M.; García-Herreros, M. Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. *Theriogenology* **2011**, *76*, 97–109. [CrossRef]

63. Meyers, S.A. Sperm physiology. In *Equine Breeding Management and Artificial Insemination*; Samper, J.C., Ed.; Saunders Elsevier: Philadelphia, PA, USA, 2009; pp. 47–55.

64. Rodríguez-Martínez, H.; Kvist, U.; Ernerudh, J.; Sanz, L.; Calvete, J.J. Seminal plasma proteins: What role do they play? *Am. J. Reprod. Immunol.* **2011**, *66*, 11–22. [CrossRef] [PubMed]

65. Gadea, J.; Sellés, E.; Marco, M.A.; Coy, P.; Matás, C.; Romar, R.; Ruiz, S. Decrease in glutathione content in boar sperm after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology* **2004**, *62*, 690–701. [CrossRef] [PubMed]

66. Ortega-Ferrusola, C.; Martin Muñoz, P.; Ortiz-Rodriguez, J.M.; Anel-López, L.; Balao da Silva, C.; Álvarez, M.; de Paz, P.; Tapia, J.A.; Anel, L.; Silva- Rodríguez, A.; et al. Depletion of thiols leads to redox deregulation, production of 4-hydroxinonenal and sperm senescence: A possible role for GSH regulation in spermatozoa. *Biol. Reprod.* **2019**, *100*, 1090–1107. [CrossRef]

67. Catalán, J.; Papas, M.; Trujillo-Rojas, L.; Blanco-Prieto, O.; Bonilla-Correal, S.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light. *Front. Cell Dev. Biol.* **2020**, *8*, 588621. [CrossRef] [PubMed]

68. Estrada, E.; Rivera del Álamo, M.M.; Rodríguez-Gil, J.E.; Yeste, M. The addition of reduced glutathione to cryopreservation media induces changes in the structure of motile subpopulations of frozen-thawed boar sperm. *Cryobiology* **2017**, *78*, 56–64. [CrossRef]

69. Estrada, E.; Rodríguez-Gil, J.E.; Rocha, L.G.; Balasch, S.; Bonet, S.; Yeste, M. Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. *Andrology* **2014**, *2*, 88–99. [CrossRef]
70. Ball, B.A.; Vo, A.T.; Baumber, J. Generation of reactive oxygen species by equine spermatozoa. *Am. J. Vet. Res.* **2001**, *62*, 508–515. [CrossRef] [PubMed]
71. Bilodeau, J.F.; Blanchette, S.; Gagnon, C.; Sirard, M.A. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* **2001**, *56*, 275–286. [CrossRef]
72. Fridovich, I. Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* **1995**, *64*, 97–112. [CrossRef] [PubMed]
73. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alex. J. Med.* **2018**, *54*, 287–293. [CrossRef]
74. Hammerstedt, R.H. Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: A review of the effect on design of storage preservation systems. *Reprod. Fertil. Dev.* **1993**, *5*, 675–690. [CrossRef] [PubMed]
75. Holt, W.V. Mechanisms of sperm storage in the female reproductive tract: An interspecies comparison. *Reprod. Domest. Anim.* **2011**, *46*, 68–74. [CrossRef] [PubMed]

# 3. Impact of seminal plasma antioxidants on the freezability of donkey sperm

Jaime Catalán, Iván Yánez-Ortiz, Asta Tvarijonaviciute, Luis Guillermo González-Arostegui, Camila P. Rubio, Marc Yeste, Jordi Miró, Isabel Barranco

*antioxidants*

MDPI

*Article*

# Impact of Seminal Plasma Antioxidants on Donkey Sperm Cryotolerance

Jaime Catalán [1,2,3,4,†], Iván Yánez-Ortiz [1,2,3,†], Asta Tvarijonaviciute [5,6],
Luis Guillermo González-Arostegui [5,6], Camila P. Rubio [6,7], Marc Yeste [2,3], Jordi Miró [1,*,‡]
and Isabel Barranco [8,*,‡]

[1] Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, Autonomous University of Barcelona, ES-08193 Cerdanyola del Vallès, Spain; dr.jcatalan@gmail.com (J.C.); ivan.yanez22@gmail.com (I.Y.-O.)
[2] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; marc.yeste@udg.edu
[3] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain
[4] Faculty of Veterinary Medicine, University of Teramo, Loc. Piano d'Accio, IT-64100 Teramo, Italy
[5] Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Murcia, ES-30100 Murcia, Spain; asta@um.es (A.T.); luisgarostegui@gmail.com (L.G.G.-A.)
[6] Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, Faculty of Veterinary Medicine, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, ES-30100 Murcia, Spain; camila.peres@uab.cat
[7] Department of Animal and Food Science, Faculty of Veterinary Sciences, Autonomous University of Barcelona, ES-08193 Cerdanyola del Vallès, Spain
[8] Department of Veterinary Medical Sciences, University of Bologna, IT-40064 Ozzano dell'Emilia, Italy
* Correspondence: jordi.miro@uab.cat (J.M.); isabel.barranco@unibo.it (I.B.);
Tel.: +34-93-5814293 (J.M.); +34-639923954 (I.B.)
† These authors contributed equally to this work.
‡ These authors share senior authorship.

check for updates

**Abstract:** This study investigated whether the activities of the antioxidant components of donkey seminal plasma (SP)—both enzymatic (superoxide dismutase (SOD), catalase-like (CAT), glutathione peroxidase-like (GPX), and paraoxonase type 1 (PON1)) and non-enzymatic (measured in terms of total thiol, copper-reducing antioxidant capacity (CUPRAC), ferric-reducing ability of plasma (FRAP), and Trolox equivalent antioxidant capacity (TEAC))—and oxidative stress index (OSI) are related to sperm cryotolerance. For this purpose, 15 ejaculates from jackasses (one per individual) were collected and split into two aliquots. The first one was used for measuring the activities levels of enzymatic and non-enzymatic antioxidants and OSI in SP, whereas the other aliquot was cryopreserved. Before cryopreservation, sperm quality parameters (concentration, motility, and viability) were evaluated. After thawing, sperm motility, plasma membrane integrity, lipid disorder, mitochondrial membrane potential, reactive oxygen species (ROS), and calcium intracellular levels were also determined. Based on the percentages of total motility (TM) and of sperm with an intact plasma membrane (SYBR14$^+$/PI$^-$) after thawing, samples were classified as good-freezability (GFE) or poor-freezability (PFE) ejaculates through cluster analyses. The SP activity levels of enzymatic (SOD and PON1) and non-enzymatic antioxidants (CUPRAC, FRAP, and TEAC) were higher ($p < 0.05$) in GFE than in PFE, whereas SP-OSI was higher ($p < 0.05$) in PFE than in GFE. In addition, the activity levels of SOD, PON1, GPX, CUPRAC, FRAP, and TEAC were positively ($p < 0.05$) related to post-thaw sperm motility and plasma membrane integrity and negatively to intracellular ROS levels. The SP-OSI was negatively correlated ($p < 0.05$) to post-thaw sperm quality parameters and positively to intracellular ROS levels. It can thus be concluded that donkey SP antioxidants are related to sperm cryotolerance and that measurements of antioxidants PON1, SOD, CUPRAC, FRAP, and TEAC, as well as SP-OSI, could be used as markers of sperm cryotolerance. Further research addressing the relationship of these antioxidants and SP-OSI with sperm cryotolerance and their potential use as freezing markers is warranted.

## 1. Introduction

In the last few decades, the donkey (*Equus asinus*) has been rediscovered in an attempt to protect biodiversity, endangered breeds, and develop marginal agricultural areas [1–3]. This fact, together with a growing demand for new uses (milk production, cosmetics, forestry, rural tourism, leisure...) has promoted a growing research interest in studying the reproductive characteristics of this species [3–5]. In this regard, the improvement of breeding management in donkeys with the use of assisted reproductive technologies, such as artificial insemination (AI) and semen cryopreservation, is crucial for their genetic selection and conservation [6].

Despite advances in cryopreservation, the use of frozen-thawed semen has been limited in donkeys due to the low conception rates obtained when jennies (0 to 36%) are inseminated compared to mares (33% to 56%) [7–10], which is attributed to the intense endometrial inflammatory response that occurs when the jenny is inseminated with frozen-thawed sperm [11–13]. In vivo and in vitro experiences have shown that SP, which is removed during the semen-freezing process because of its harmful effects on equids' sperm [14], may play a key role in modulating this response [12].

Cryopreservation reduces the survival and fertilizing capacity of mammalian sperm [14]. This can be explained by the impact of freeze-thawing on plasma membrane integrity, impairing sperm motility, viability, and morphology [15,16]. Exposure of sperm to a stressful environment during cryopreservation is associated with an overproduction of reactive oxygen species (ROS) [14]. This situation could cause an imbalance in relation to the antioxidant defense, leading to oxidative stress (OS) and subsequent lipid peroxidation (LPO) of the plasma membrane [17–20]. In the case of donkeys, although it also occurs in other species, their sperm are especially sensitive to these events due to the large amount of polyunsaturated fatty acids in their plasma membrane [6,17,19–21]. In addition, the low amount of antioxidant enzymes within the sperm cell, together with the large number of mitochondria, makes the antioxidants (enzymatic and non-enzymatic) present in SP the main defense against the OS-induced damage [6,20,22,23]. The removal of SP before freezing, therefore, may increase sperm susceptibility to OS [6]. In recent years, many efforts have been focused on improving cryopreservation procedures in different species, analyzing the supplementation of freezing/thawing media with different antioxidants and obtaining inconsistent results [24–28].

In donkeys, as in other mammalian species, a high inter- and intra-male variability in the ability of sperm to withstand cryopreservation has been reported [29–34], leading to the classification of males/ejaculates as of "good" (GFE) or "poor" (PFE) freezability [17,31,35,36]. Differences in the composition of SP surrounding sperm among individuals and ejaculates could explain such differences [37,38]. In this regard, a recent study from our research group investigated whether the activities of four enzymes with antioxidant properties present in donkey SP (superoxide dismutase (SOD), catalase-like (CAT), glutathione peroxidase-like (GPX), and glutathione reductase (GSR)) were related to the sperm resilience to freezing and thawing. The results of this study show that the activity of CAT and GSR and the total and specific activity of SOD in SP were associated with donkey sperm cryotolerance [6]. As far as we are aware, however, it has not yet been reported whether other components of SP with antioxidant properties, beyond these enzymes, are related to sperm freezability in this species, nor has the activity of the previously mentioned enzymes been linked to sperm quality parameters measured by flow cytometry. Related to this, a recent investigation performed in pig SP revealed that the activity of some enzymes, such as paraoxonase (PON1), and the total non-enzymatic antioxidant capacity were positively involved in sperm cryotolerance, minimizing the OS as result of freezing and thawing [39].

Against this background, this study aimed to determine whether antioxidant components of donkey SP, including enzymes (SOD, CAT, GPX, and PON1), non-enzymatic antioxidant capacity (measured in terms of total thiol, copper-reducing antioxidant capacity (CUPRAC), ferric-reducing ability of plasma (FRAP), and Trolox equivalent antioxidant capacity (TEAC), which are assays that evaluate the total non-enzymatic antioxidant capacity of SP), and the oxidative stress index (OSI), are related to donkey sperm cryotolerance.

## 2. Materials and Methods

### 2.1. Animals and Ejaculates

This study included 15 ejaculates from Catalonian jackasses (aged 4–11 years old) of proven fertility. All animals used were in good health condition and were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), a European Union-approved equine semen collection center (authorization number: ES09RS01E). This center works under strict protocols of health control and animal welfare. Because this Service already works under the approval of the Generalitat de Catalunya (Spain) and given that no manipulation on the animals beyond the collection of semen was conducted, the Ethics Committee of our institution indicated that no further ethical approval was required to carry out this study.

All animals were kept in individual paddocks and were fed a standard diet composed of mixed hay and basic concentrate. No antioxidant supplementation was used, and water was available ad libitum. A Hannover artificial vagina was used to collect the ejaculates (Minitüb GmbH, Tie-fenbach, Germany), with a nylon mesh filter connected in-line to remove the gel fraction. After removing the gel fraction, the total semen volume was evaluated, and an aliquot was used for sperm concentration assessment (Neubauer chamber, Paul Marienfeld GmbH and Co. KG; Lau-da-Königshofen, Germany). Then, the total volume of each ejaculate was divided into two equal aliquots. One was used to obtain the SP (see Section 2.2), and the other one was diluted in previously warmed (38 °C) Kenney extender (1:5v:v) [40]. This latter aliquot was utilized to evaluate sperm motility (by computer-assisted semen analysis, CASA; see Section 2.5), morphology, and viability (assessed by eosin-nigrosine staining [41]) immediately after ejaculate collection. All ejaculates fulfilled standard semen quality thresholds (>60% live sperm and >70% morphologically normal sperm).

### 2.2. Isolation of Seminal Plasma (SP)

After collection, semen was immediately centrifuged five times at $1500 \times g$ for 10 min (JP Selecta S.A., Barcelona, Spain) at 4 °C. Once each centrifugation cycle finished, the supernatant was examined to ensure the absence of spermatozoa under a phase-contrast microscope (Olympus Europa, Hamburg, Germany). Five mL-aliquots of SP were subsequently stored at −80 °C. Before antioxidants measurements, SP samples were thawed on ice.

### 2.3. Measurement of Antioxidants Activity Levels and OSI in SP

The total non-enzymatic antioxidant capacity of SP was assessed in terms of CUPRAC, FRAP, and TEAC, following the protocols described by Li et al. [39], and total thiol following the procedure described by Jocelyn [42] and da Costa et al. [43]. All these procedures were previously adapted to donkey SP. The TEAC method is based on a color change by 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate [44], the FRAP method is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ [45], and the CUPRAC method is based on the reduction of $Cu^{2+}$ to $Cu^{1+}$ [46]. The analysis of total thiol is based on the fact that thiols interact with 5, 5′-dithiobis- (2-nitrobenzoic acid) (DTNB), forming a highly colored anion with a maximum peak at 412 nm ($e412 = 13{,}600$ $M^{-1}$ $cm^{-1}$) [43]. All these assays were performed using an Olympus AU400 automatic chemistry analyzer (Olympus Europe GmbH, Hamburg, Germany). The results of TEAC, FRAP, and CUPRAC are expressed in mmol/L, and total thiol results are expressed in μmol/L.

Enzymatic SP antioxidants were measured as the activity of CAT, GPX, SOD, and PON1. Activities of CAT, GPX, and SOD were measured using commercially available assays following the manufacturer's instructions (CAT: Sigma-Aldrich, St. Louis, MO, USA; GPX and SOD: Randox, Crumlin, UK). Activity of PON1 was assessed by measuring the hydrolysis of p-nitrophenyl acetate into p-nitrophenol, based on the protocol of Barranco et al. [47] adapted to donkey SP. All measurements, except for the CAT activity, were performed using an Olympus AU400 automatic chemistry analyzer. Activity of CAT was measured using a micro-plate reader (PowerWave XS; Bio-Tek Instruments). Activities of SOD and CAT were expressed as IU/mL, whereas those of PON1 and GPX were expressed as IU/L.

To calculate SP-OSI, the total oxidative status (TOS) of SP was previously measured using the method described by Erel [48] and adapted to donkey SP. This assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. This test was also performed using an Olympus AU400 automatic chemistry analyzer. The results of TOS are expressed in µmol $H_2O_2$ equiv./L. Thereafter, SP-OSI value was calculated utilizing the following formula, as described by Wu et al. [49]: OSI (arbitrary unit) = TOS (µmol $H_2O_2$ equiv./L)/TEAC (mmol Trolox equiv./L).

Measurements of all analytes were performed in duplicate for each SP sample. In all the analytes, each test showed intra- and inter-assay coefficient variations below 10%.

### 2.4. Sperm Cryopreservation

Sperm cryopreservation was performed as previously described by Flores et al. [50]. For this purpose, before sperm cryopreservation, diluted semen was centrifuged at $600\times g$ for 15 min at 20 °C (Medifriger BL-S, JP Selecta S.A., Barcelona, Spain). After the supernatant removal, the pellets were resuspended in a commercial freezing extender (Botucrio®, Botupharma Animal Biotechnology; Botucatu, Brazil), which contains permeable cryoprotectants (4% methylformamide and 1% glycerol). Subsequently, sperm concentration, motility, and plasma membrane integrity were reevaluated, and freezing medium (Botucrio®) was added until reaching a final concentration of $200 \times 10^6$ viable sperm per mL (standardized in all cases). Prior to their cryopreservation using a controlled-rate freezer (Ice-Cube 14S; Minitüb), samples were packaged into 0.5-mL straws. Three stages of cooling/freezing were performed: (i) from 20 °C to 5 °C for 60 min, at a rate of −0.25 °C/min; (ii) from 5 °C to −90 °C for 20 min, at a rate of −4.75 °C/min; and (iii) from −90 °C to −120 °C for 2.7 min, at a rate of −11.11 °C/min. Once the last freezing step finished, straws were immediately submerged into liquid nitrogen and kept in tanks until their thawing and analysis. The thawing protocol consisted of incubating the straws for 30 s in a water bath at 38 °C followed by dilution with three volumes of Kenney extender, also prewarmed at 38 °C (final concentration: $50 \times 10^6$ sperm/mL)

### 2.5. Assessment of Sperm Motility

Before and after cryopreservation, sperm motility was evaluated using a CASA system (Proiser S.L.; Valencia, Spain). For that, 5 µL of each semen sample (diluted in Kenney extender at a concentration of $50 \times 10^6$ spermatozoa/mL) was placed onto a previously heated (38 °C) Makler chamber (Sefi Medical Instruments; Haifa, Israel). Then, samples were assessed under a 10× negative phase-contrast objective using an Olympus B × 41 microscope (Olympus, Tokyo, Japan) that also had a plate heated to 38 °C. A minimum of 1000 sperm cells per analysis were counted. Percentages of total (TM, %) and progressive motility (PM, %) were recorded in each evaluation together with kinetic parameters as follows: 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; curvilinear velocity (VCL, µm/s), which is the mean path velocity of the sperm head along its actual trajectory; average path velocity (VAP, µm/s), which is the mean velocity of the sperm head along its average trajectory; percentage of straightness (STR, %), which is the quotient between VSL and VAP

multiplied by 100; percentage of linearity (LIN, %), which is the quotient between VSL and VCL multiplied by 100; percentage of oscillation (WOB, %), which is the quotient between VAP and VCL multiplied by 100; frequency of head displacement (BCF, Hz), which is the frequency at which the head crosses the actual sperm track; and 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. The settings of CASA used were those recommended by the provider, i.e., frames/s: 25 images captured per second; connectivity: 6; particle area >4 and <75 μm$^2$; minimum number of images to calculate the ALH: 10. Cut-off value for motile spermatozoa was VAP $\geq$ 10 μm/s and for progressively motile spermatozoa was STR $\geq$ 75%.

*2.6. Flow Cytometry Analyses*

Flow cytometry was used to determine the integrity and lipid disorder of sperm plasma membrane, mitochondrial membrane potential (MMP), and intracellular levels of overall ROS, superoxides, and calcium in post-thawed semen samples, following the recommendations set by the International Society for Advancement of Cytometry [51].

In this study, the Cell Lab Quanta SC™ flow cytometer was used (Beckman Coulter, Fullerton, CA, USA), and particles were excited with an argon laser (488 nm) at a power of 22 mW. Firstly, sperm concentration was adjusted to $1 \times 10^6$ sperm/mL. Before starting to use the cytometer, the electronic volume (EV) channel was calibrated with 10-μm-diameter fluorescent beads (Beckman Coulter) following the provider's instructions. The analyzer threshold was established to exclude cell aggregates (particles with a diameter >12 μm) and cell debris (particles with a diameter <7 μm), and the flow rate was set at 4.17 μL/min. Based on EV and side scatter (SS) distributions, sperm cells were gated. Three different optical filters were used (FL1 for the analysis of H$_2$DCFDA, SYBR14, YO-PRO-1, JC1 monomers, and Fluo3, detection width: 505–545 nm; FL2 for the analysis of JC1 aggregates, detection width: 560–590 nm; FL3 for the analysis of PI, HE, and M540, detection width: 655–685 nm). Compensation was used when required to minimize fluorescence spill-over into a different channel. All events information was collected in List-mode Data files (EV, SS, FL1, FL2, and FL3) and processed using the Cell Lab Quanta SC MPL Analysis Software (version 1.0; Beckman Coulter). Data were corrected in all assessments based on the percentage of debris particles (SYBR14$^-$/propidium iodide (PI)$^-$) determined through SYBR14/PI staining, as previously described by Petrunkina et al. [52],. All fluorochromes used were purchased from Molecular Probes$^®$ (Invitrogen$^®$, Thermo Fisher Scientific, Waltham, MA, USA) and diluted in dimethyl sulfoxide (DMSO).

2.6.1. Evaluation of Sperm Plasma Membrane Integrity

Sperm plasma membrane integrity was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR14, viable spermatozoa, and PI, non-viable spermatozoa), following a protocol modified for donkey sperm, adapted from Garner and Johnson [53]. Briefly, sperm samples were incubated first with SYBR14 (final concentration: 100 nM) for 10 min at 38 °C and later with PI (final concentration: 12 mM) for 5 min at 38 °C. All incubations were performed protecting samples from the light. To measure the green fluorescence from SYBR14, FL1 was used, and to detect the red fluorescence from PI, FL3 was used. Three sperm populations were identified: (1) sperm with an intact plasma membrane (SYBR14$^+$/PI$^-$; viable sperm); (2) sperm with a damaged plasma membrane (SYBR14$^-$/PI$^+$); and (3) sperm with a damaged plasma membrane (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 (2.45%).

2.6.2. Evaluation of Membrane Lipid Disorder

Membrane lipid disorder was determined through staining with merocyanine 540 (M540) and YO-PRO-1 fluorochromes. Sperm were incubated with M540 (final concentration: 2.6 mM) and YO-PRO-1 (used as a vital stain; final concentration: 25 nM) at 38 °C for

10 min in the dark [54]. Four sperm populations were detected: (1) viable sperm with low membrane lipid disorder ($M540^-$/YO-PRO-1$^-$); (2) viable sperm with high membrane lipid disorder ($M540^+$/YO-PRO-1$^-$); (3) non-viable sperm with low membrane lipid disorder ($M540^-$/YO-PRO-1$^+$); and (4) non-viable sperm with high membrane lipid disorder ($M540^+$/YO-PRO-1$^+$). Percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from those of viable sperm with low membrane lipid disorder ($M540^-$/YO-PRO-1$^-$); thereafter, the percentages of all sperm populations were recalculated.

### 2.6.3. Evaluation of Mitochondrial Membrane Potential

The MMP was determined using JC-1 (5,50,6,60-tetrachloro-1,10,3,3′tetraethyl-benzimidazolylcarbocyanine iodide). Under dark conditions, sperm samples were stained with JC-1 (final concentration: 0.5 mM) at 38 °C for 30 min. When mitochondrial membrane potential is low, JC-1 monomers emit green fluorescence (JC-1$_{mon}$), which is collected through FL1. If mitochondrial membrane potential is high, JC-1 forms aggregates emitting orange fluorescence (JC-1$_{agg}$), which is detected through FL2. Three sperm populations were distinguished: (1) green-stained sperm with low mitochondrial membrane potential (low MMP); (2) orange-stained sperm (high MMP); and (3) sperm with heterogeneous mitochondria stained both green and orange in the same cell (intermediate MMP). Spillover 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 sperm with low MMP, and percentages of all sperm populations were recalculated.

### 2.6.4. Evaluation of Intracellular ROS Levels

Intracellular ROS levels were determined through two oxidation-sensitive fluorescent probes: hydroethidine (HE) and 2,7-dichlorodihydrofluorescein and diacetate (H$_2$DCFDA), which detect superoxide anion ($\cdot O_2^-$) and overall ROS, respectively [55]. Following a modified procedure from Guthrie and Welch [56], a simultaneous differentiation of viable and non-viable sperm was performed using YO-PRO-1 (HE) or PI (H$_2$DCFDA).

For $\cdot O_2^-$, sperm samples were mixed with Hydroethidine (HE, final concentration: 4 mM) 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 sperm with low $\cdot O_2^-$ levels (E$^-$/YO-PRO-1$^+$); (2) non-viable sperm with high $\cdot O_2^-$ levels (E$^+$/YO-PRO-1$^+$); (3) viable sperm with low $\cdot O_2^-$ levels (E$^-$/YO-PRO-1$^-$); and (4) viable sperm with high $\cdot O_2^-$ levels (E$^+$/YO-PRO-1$^-$). YO-PRO-1 spill-over into the FL3 channel was compensated (5.06%).

For overall ROS, sperm samples were stained with H$_2$DCFDA (final concentration: 140 mM) and PI (final concentration: 12 mM) 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 sperm with high ROS levels (DCF$^+$/PI$^-$); (2) non-viable sperm with high ROS levels (DCF$^+$/PI$^+$); (3) viable sperm with low ROS levels (DCF$^-$/PI$^-$); and (4) non-viable sperm with low ROS levels (DCF$^-$/PI$^+$). DCF$^+$ spill-over into the FL3 channel was compensated (2.45%).

### 2.6.5. Evaluation of Intracellular Calcium Levels

Previous studies in pig sperm found that Fluo3-acetomethoxyester fluorochrome (Fluo3) mainly stains mitochondrial calcium [57]. For this reason, we combined this fluorochrome with PI (Fluo3/PI), following the method described by Kadirvel et al. [58]. Four sperm populations were identified: (1) viable sperm with low levels of intracellular calcium (Fluo3$^-$/PI$^-$); (2) viable sperm with high levels of intracellular calcium (Fluo3$^+$/PI$^-$); (3) non-viable sperm with low levels of intracellular calcium (Fluo3$^-$/PI$^+$); and (4) non-viable sperm with high levels of intracellular calcium (Fluo3$^+$/PI$^+$). The FL3 overflow in the FL1 channel (28.72%) and the FL1 overflow in the FL3 channel (2.45%) were compensated.

*2.7. Experimental Design*

A total of 15 ejaculates from 15 different Catalan donkeys were used. After collection, each ejaculate was divided into two aliquots. The first aliquot was centrifuged (see Section 2.2) to obtain SP samples for the measurement of the activity levels of enzymatic and non-enzymatic antioxidants and OSI. The second aliquot was cryopreserved following the previously described procedure (see Section 2.4). Before cryopreservation, sperm concentration, motility, morphology, and viability were evaluated. Ten minutes after thawing, sperm motility, plasma membrane integrity and lipid disorder, MMP, and intracellular levels of overall ROS, superoxides and calcium were evaluated in each semen sample. Based on the percentage of total motile sperm (TM) and the percentage of viable sperm (SYBR14$^+$/PI$^-$) after thawing, donkey ejaculates were classified as GFE and PFE by hierarchical clustering.

*2.8. Statistical Analysis*

Data analyses were performed using the R statistical package (V 4.0.3, R Core Team; Vienna, Austria), and graphs were prepared with the GraphPad Prism software (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA). First, Shapiro–Wilk and Levene tests were run to verify normal distribution and homogeneity of variances, respectively. If necessary, data were transformed using the square root of arcsin (arcsin $\sqrt{x}$) to keep parametric assumptions. The minimum level of statistical significance for all analyzes was set at $p \leq 0.05$. Results are shown as mean $\pm$ standard error of the mean (SEM).

2.8.1. Hierarchical Clusters and Comparison between GFE and PFE

Classification of the 15 ejaculates into groups of GFE and PFE was carried out by means of a hierarchical cluster analysis of complete linkage using the Euclidean distances from the percentages of TM and viable sperm (SYBR14$^+$/PI$^-$) obtained in each sample after thawing, as described in Morató et al. [59].

The SP activity levels of enzymatic (PON1, CAT, GPX, and SOD) and non-enzymatic antioxidants (total thiol, CUPRAC, FRAP, and TEAC), as well as SP-OSI in donkey ejaculates were compared between GFE and PFE by means of a *t*-test for independent samples. When, even after being linearly transformed, these data did not match with parametric assumptions, the Mann–Whitney test was used as an alternative.

2.8.2. Correlation Matrix

Pearson's coefficients were calculated to develop the correlation matrix between the SP activity levels of enzymatic (PON1, CAT, GPX, and SOD) and non-enzymatic antioxidants (total thiol, CUPRAC, FRAP, and TEAC), as well as SP-OSI values in donkey ejaculates, and TM, PM, kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF), plasma membrane integrity (SYBR14$^+$/PI$^-$), MMP (JC-1$_{agg}$), intracellular ROS levels (DCF$^+$/PI$^-$), intracellular superoxides levels (E$^+$/YO-PRO-1$^-$), intracellular calcium levels (Fluo3$^+$/PI$^-$), and plasma membrane lipid disorder (M540$^+$/YO-PRO-1$^-$), obtained in each sample after thawing.

## 3. Results

*3.1. Classification of Donkey Ejaculates into GFE and PFE Groups According to Their Post-Thaw Sperm Quality and Functionality Parameters*

According to TM and sperm viability (SYBR14$^+$/PI$^-$) obtained after thawing, the 15 ejaculates were classified (by hierarchical clustering, $p < 0.01$) into GFE ($n = 8$) and PFE ($n = 7$). The percentages of TM and viable (SYBR14$^+$/PI$^-$) sperm after thawing were significantly higher ($p < 0.01$ and $p < 0.001$, respectively; Figure 1) in GFE (48.76 $\pm$ 1.58 and 48.85 $\pm$ 1.23%, respectively) than in PFE (32.29 $\pm$ 3.17 and 30.43 $\pm$ 2.64%, respectively).
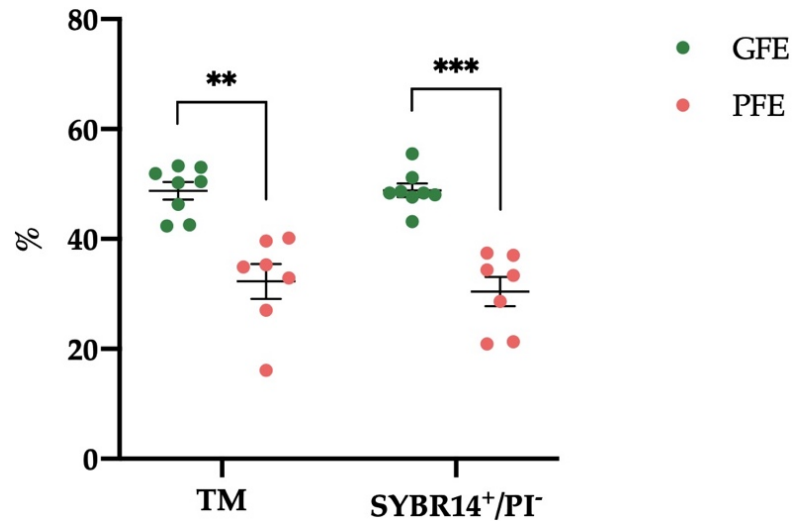
**Figure 1.** Mean $\pm$ SEM of the percentages of total sperm motility (TM) and of sperm with an intact plasma membrane (SYBR14$^+$/PI$^-$, viable sperm) after thawing in donkey ejaculates ($n$ = 15) classified as good-freezability (GFE) or poor-freezability ejaculates (PFE). The dots represent the TM and SYBR14$^+$/PI$^-$ individual values in GFE and PFE. ** $p \leq 0.01$; *** $p \leq 0.001$.

The ejaculates classified as GFE presented a range of TM between 42.35 and 53.27% and a range of SYBR14$^+$/PI$^-$ between 43.16 and 55.48%, whereas the PFE were in ranges of TM and SYBR14$^+$/PI$^-$ between 16.09 and 40.14% and between 20.89 and 37.40%, respectively (Tables S1 and S2).

### 3.2. Activity Levels of Enzymatic Antioxidants in Donkey SP

The activity levels of PON1 and SOD in SP were significantly higher in GFE ($p < 0.01$ and $p < 0.05$, respectively; $0.35 \pm 0.06$ and $2527 \pm 216$ IU/mL, respectively; Figure 2a,b) than in PFE ($0.14 \pm 0.02$ and $1760 \pm 347$ IU/mL, respectively). On the contrary, the SP of PFE showed higher activity levels of CAT than that of GFE ($0.35 \pm 0.04$ vs. $0.18 \pm 0.04$ IU/mL, respectively; $p < 0.05$; Figure 2c). Moreover, the activity levels of GPX in SP did not differ significantly between GFE and PFE (Figure 2d). Table S3 shows the mean $\pm$ SEM and range of each enzymatic antioxidant's activity levels in SP samples of all donkey ejaculates included in the study.

### 3.3. Activity Levels of Non-Enzymatic Antioxidants in Donkey SP

No differences were found in the levels of total thiol between GFE and PFE (Figure 3a). The activity levels of CUPRAC, FRAP, and TEAC in SP, however, were higher ($p < 0.05$) in donkey ejaculates classified as GFE than in those classified as PFE (Figure 3b–d). The CUPRAC values were $1.93 \pm 0.15$ and $1.37 \pm 0.19$ mmol/L, respectively. The FRAP values were $2.09 \pm 0.18$ and $1.27 \pm 0.25$ mmol/L, respectively. Meanwhile, TEAC values were $2.59 \pm 0.12$ and $1.97 \pm 0.26$ mmol/L, respectively. Table S3 shows the mean $\pm$ SEM and range of each non-enzymatic antioxidant's activity levels in SP samples of all donkey ejaculates included in the study.
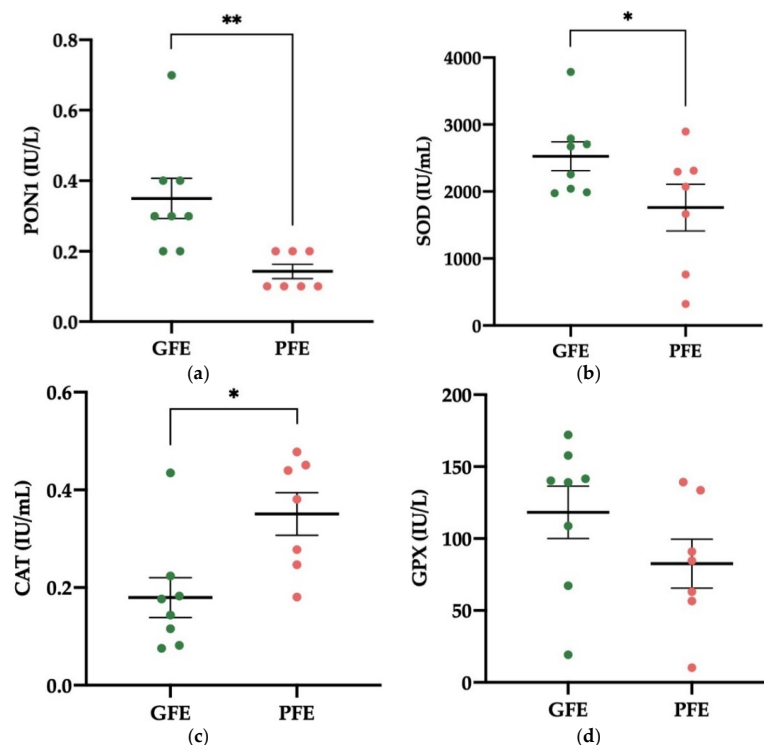
**Figure 2.** Mean $\pm$ SEM of the activity levels of paraoxonase type 1 (PON1, **a**), superoxide dismutase (SOD, **b**), catalase-like (CAT, **c**), and glutathione peroxidase-like (GPX, **d**) in seminal plasma (SP) in donkey ejaculates classified as having good (GFE, $n = 8$) or poor freezability (PFE, $n = 7$). * $p \leq 0.05$; ** $p \leq 0.01$.

### 3.4. Levels of OSI in Donkey SP

The levels of OSI in SP from donkey ejaculates were significantly lower ($p < 0.01$) in GFE than in PFE (Figure 4). The values were $2.65 \pm 0.36$ and $4.68 \pm 0.87$, respectively. TOS levels were $6.85 \pm 0.96$ and $8.04 \pm 0.34$ μmol/L in GFE and PFE, respectively. Table S3 shows the mean $\pm$ SEM and range of OSI and TOS in SP of all donkey ejaculates included in the study.

### 3.5. Correlations of the Activity Levels of Enzymatic and Non-Enzymatic Antioxidants and OSI in Donkey SP with Post-Thaw Sperm Motility Parameters

Figure 5 shows the correlation matrix between sperm motility and SP antioxidant parameters. Significant correlations between the activity levels of enzymatic and non-enzymatic antioxidants and OSI in SP and post-thaw sperm motility parameters were observed. For example, the activity levels of SOD and GPX were positively correlated with TM ($r = 0.64$, $p < 0.05$ and $r = 0.59$, $p < 0.05$, respectively) and with PM ($r = 0.59$, $p < 0.05$ and $r = 0.52$, $p < 0.05$, respectively). Furthermore, the activity levels of SOD were positively correlated with kinematic parameters ($r = 0.66$, $p < 0.01$ for VCL; $r = 0.55$, $p < 0.05$ for VSL; and $r = 0.57$, $p < 0.05$ for VAP). Similarly, the activity levels of non-enzymatic antioxidants (measured in terms of CUPRAC, FRAC, and TEAC) were positively correlated with TM ($r = 0.60$, $p < 0.05$; $r = 0.64$, $p < 0.05$; and $r = 0.65$, $p < 0.01$, respectively) and with PM ($r = 0.58$, $p < 0.05$; $r = 0.63$, $p < 0.05$; and $r = 0.66$, $p < 0.01$, respectively).
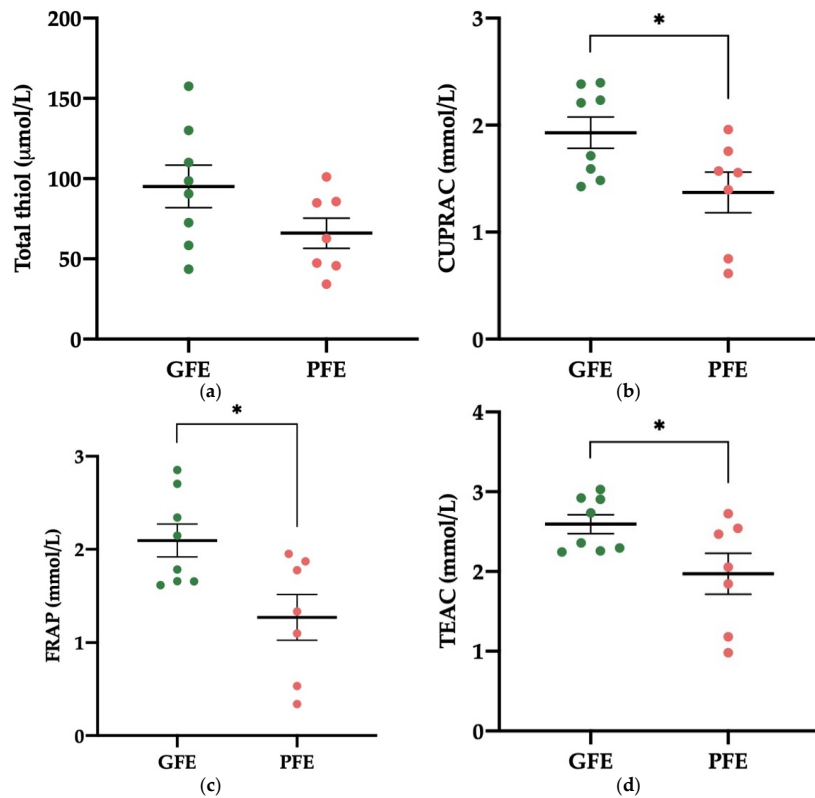
**Figure 3.** Mean $\pm$ SEM of the activity levels of non-enzymatic antioxidants (measured in terms of total thiol (**a**), cupric reducing antioxidant capacity (CUPRAC, **b**), plasma iron-reducing capacity (FRAP, **c**), and Trolox equivalent antioxidant capacity (TEAC, **d**)) in seminal plasma (SP) of donkey ejaculates classified as having good (GFE, $n = 8$) or poor freezability (PFE, $n = 7$). * $p \leq 0.05$.
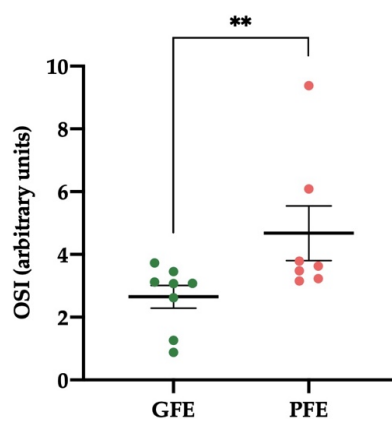


**Figure 4.** Mean $\pm$ SEM of the levels of seminal oxidative stress index (OSI) of donkey ejaculates classified as having good (GFE, $n = 8$) or poor freezability (PFE, $n = 7$). ** $p \leq 0.01$.
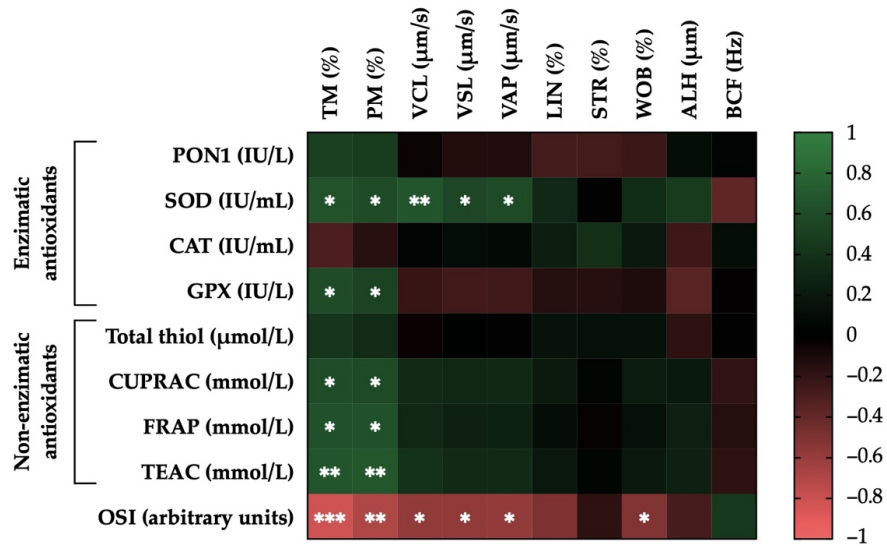
**Figure 5.** Correlations of the activity levels of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase-like, CAT; and glutathione peroxidase-like, GPX) and non-enzymatic antioxidants (measured in terms of total thiol; cupric-reducing antioxidant capacity, CUPRAC; plasma iron-reducing capacity, FRAP; and Trolox equivalent antioxidant capacity, TEAC) and oxidative stress index (OSI) in donkey seminal plasma (SP; *n* = 15) with post-thaw sperm motility parameters (percentage of total motility, TM; percentage of progressively motile spermatozoa, PM; curvilinear velocity, VCL; straight-line velocity, VSL; average path velocity, VAP; linearity coefficient, LIN; straightness coefficient, STR; wobble coefficient, WOB; amplitude of lateral head displacement, ALH; beat-cross frequency, BCF). The scale colors (1 to −1) indicate whether the correlation is positive (green) or negative (red). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

On the contrary, the levels of SP-OSI were negatively correlated with TM ($r = -0.82$, $p < 0.001$), PM ($r = -0.70$, $p < 0.01$), and the following kinematic parameters: VCL ($r = -0.58$, $p < 0.05$), VSL ($r = -0.57$, $p < 0.05$), VAP ($r = -0.59$, $p < 0.05$), and WOB ($r = -0.52$, $p < 0.05$). Table S1 shows the mean ± SEM and range of each sperm motility parameter after thawing in GFE and PFE.

*3.6. Correlations of the Activity Levels of Enzymatic and Non-Enzymatic Antioxidants and OSI in Donkey SP with Post-Thaw Sperm Functionality Parameters*

Figure 6 shows the correlation matrix between sperm functionality and SP antioxidant parameters. Significant correlations between the activity levels of enzymatic or non-enzymatic antioxidants and OSI in donkey SP and post-thaw sperm functionality parameters were observed. For example, activity levels of PON1 and SOD were positively correlated with plasma membrane integrity (SYBR14$^+$/PI$^-$; $r = 0.61$, $p < 0.05$; and $r = 0.67$, $p < 0.01$, respectively). On the contrary, activity levels of SOD and GPX were negatively correlated with intracellular ROS levels (DCF$^+$/PI$^-$; $r = -0.53$, $p < 0.05$; and $r = -0.70$, $p < 0.01$, respectively) and with intracellular superoxide levels (HE$^+$/YO-PRO-1$^-$; $r = -0.66$, $p < 0.01$; and $r = -0.58$, $p < 0.05$, respectively), whereas activity levels of PON1 were negatively correlated only with the latter (HE$^+$/YO-PRO-1$^-$; $r = -0.57$, $p < 0.05$). In addition, activity levels of SOD were negatively correlated with the proportion of sperm with high MMP ($r = -0.54$, $p < 0.05$). Activity levels of CAT were positively correlated with the proportion of sperm with intermediate MMP ($r = 0.69$, $p < 0.01$) and negatively with plasma membrane lipid disorder (M540$^+$/YO-PRO-1$^-$; $r = -0.57$, $p < 0.05$).
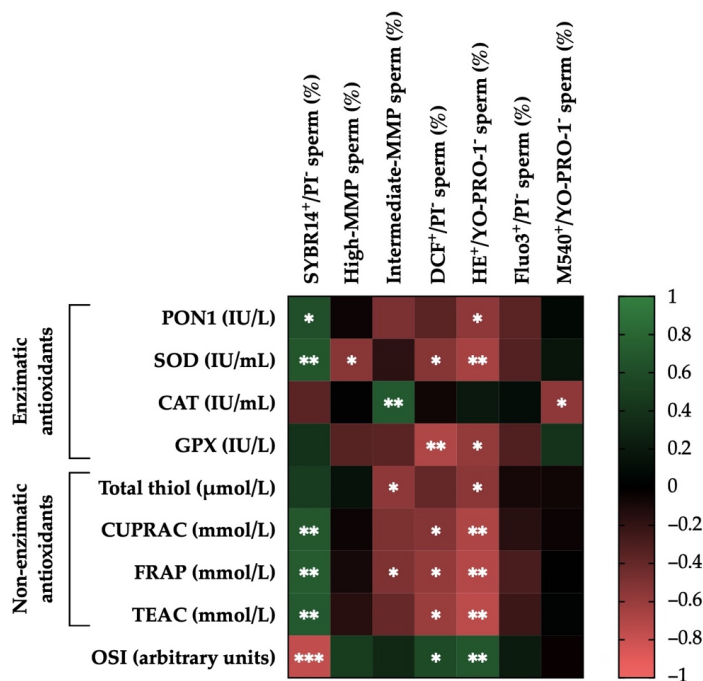
**Figure 6.** Correlations of the activity levels of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase-like, CAT; and glutathione peroxidase-like, GPX) and non-enzymatic antioxidants (measured in terms of total thiol; cupric reducing antioxidant capacity, CUPRAC; plasma iron-reducing capacity, FRAP; and Trolox equivalent antioxidant capacity, TEAC) and oxidative stress index (OSI) in donkey seminal plasma (SP; $n = 15$) with post-thaw sperm functionality parameters (plasma membrane integrity, SYBR14$^+$/PI$^-$; mitochondrial membrane potential, MMP; intracellular ROS levels, DCF$^+$/PI$^-$; intracellular superoxide levels, E$^+$/YO-PRO-1$^-$; intracellular calcium levels, Fluo3$^+$/PI$^-$; plasma membrane lipid disorder, M540$^+$/YO-PRO-1$^-$). The scale colors (1 to $-1$) indicate whether the correlation is positive (green) or negative (red). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Regarding the activity levels of non-enzymatic antioxidants, CUPRAC, FRAP, and TEAC showed strong positive correlations with the integrity of plasma membrane (SYBR14$^+$/PI$^-$; r = 0.69, $p < 0.01$; r = 0.71, $p < 0.01$; and r = 0.69, $p < 0.01$, respectively). In contrast, they were negatively correlated with intracellular ROS levels (DCF$^+$/PI$^-$; r = $-0.53$, $p < 0.05$; r = $-0.58$, $p < 0.05$; and r = $-0.61$, $p < 0.05$, respectively) and strongly with intracellular superoxide levels (HE$^+$/YO-PRO-1$^-$; r = $-0.69$, $p < 0.01$; r = $-0.71$, $p < 0.01$; and r = $-0.75$, $p < 0.01$, respectively). Activity levels of total thiol were also negatively correlated with HE$^+$/YO-PRO-1$^-$ (r = $-0.55$, $p < 0.05$). Furthermore, the activity levels of total thiol and FRAC were also negatively correlated with the proportion of sperm with intermediate MMP (r = $-0.57$, $p < 0.05$; and r = $-0.50$, $p < 0.05$, respectively).

Finally, the SP-OSI showed a strong negative correlation with the integrity of plasma membrane (SYBR14$^+$/PI$^-$; r = $-0.77$, $p < 0.001$) and was positively correlated with intracellular ROS levels (DCF+/PI$^-$; r = 0.57, $p < 0.05$) and intracellular superoxide levels (HE$^+$/YO-PRO-1$^-$; r = 0.67, $p < 0.01$). Table S2 shows the mean $\pm$ SEM and range of each sperm function parameter after thawing in GFE and PFE.

*3.7. Correlations between the Activity Levels of Enzymatic and Non-Enzymatic Antioxidants and OSI in Donkey SP*

Figure 7 shows the correlation matrix and the relationship between activity levels for all enzymatic and non-enzymatic antioxidants and OSI in donkey SP. Positive correlations were observed for GPX (r = 0.54, $p < 0.05$) and the four assays used for evaluating non-enzymatic antioxidants (total thiol: r = 0.50, $p < 0.05$; CUPRAC: r = 0.56, $p < 0.05$; FRAP: r = 0.73, $p < 0.01$; and TEAC: r = 0.61, $p < 0.05$) with PON1. The activity levels of four non-enzymatic antioxidants showed strong positive correlations with each other: CUPRAC (r = 0.81, $p < 0.001$), FRAP (r = 0.75, $p < 0.01$), and TEAC (r = 0.68, $p < 0.01$) with total thiol; FRAP (r = 0.95, $p < 0.001$) and TEAC (r = 0.96, $p < 0.001$) with CUPRAC; and TEAC (r = 0.97, $p < 0.001$) with FRAP. Furthermore, the activity levels of CUPRAC, FRAP, and TEAC were positively correlated with SOD (r = 0.56, $p < 0.05$; r = 0.58, $p < 0.05$; and r = 0.68, $p < 0.01$, respectively). On the contrary, negative correlations between CAT and PON1 (r = −0.58, $p < 0.05$) were observed. Likewise, levels of SP-OSI showed strong negative correlations with SOD (r = −0.87, $p < 0.001$), CUPRAC (r = −0.75, $p < 0.01$), FRAP (r = −0.72, $p < 0.01$) and TEAC (r = −0.80, $p < 0.001$). Table S3 shows the mean $\pm$ SEM and range of each enzymatic and non-enzymatic antioxidant's activity levels, as well as OSI in SP samples of all donkey ejaculates included in the study.
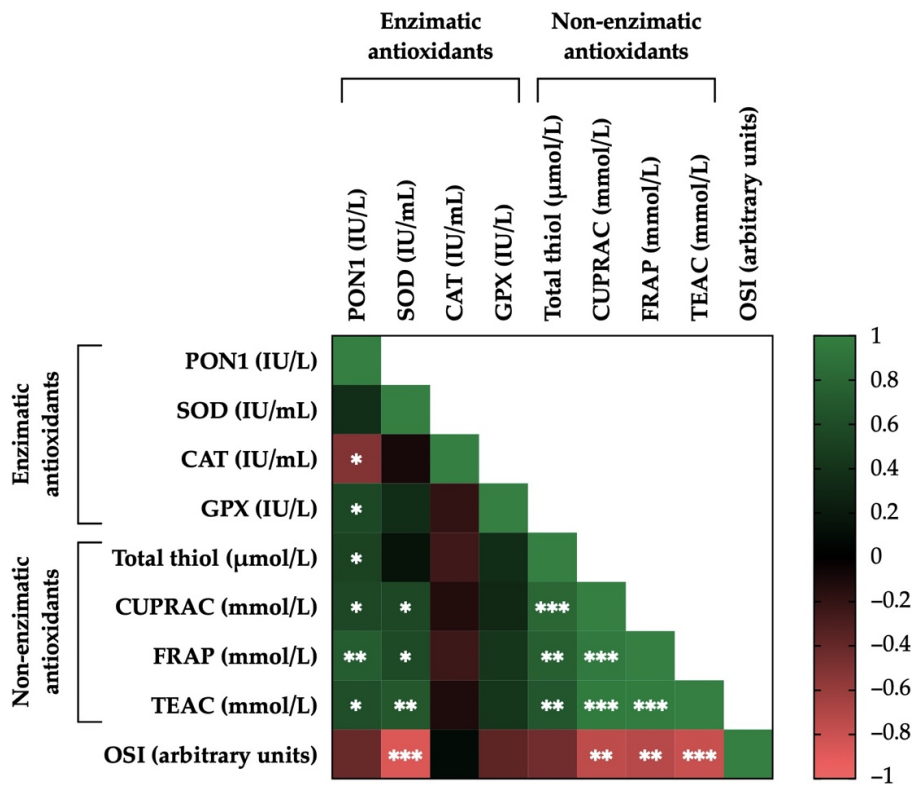


**Figure 7.** Correlations between the activity levels of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase-like, CAT; and glutathione peroxidase-like, GPX) and non-enzymatic antioxidants (measured in terms of total thiol; cupric reducing antioxidant capacity, CUPRAC; plasma iron-reducing capacity, FRAP; and Trolox equivalent antioxidant capacity, TEAC) and oxidative stress index (OSI) in donkey seminal plasma (SP; *n* = 15). The scale colors (1 to −1) indicate whether the correlation is positive (green) or negative (red). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

## 4. Discussion

The present study demonstrated that the activity levels of some SP components with antioxidant properties, both enzymatic and non-enzymatic, as well as the SP-OSI were related to the quality and functionality parameters (motility, plasma membrane integrity, and intracellular levels of ROS) of frozen-thawed donkey sperm. The main finding, however, was that the activity levels of enzymatic antioxidants SOD and PON1, those of non-enzymatic antioxidants (measured in terms of CUPRAC, FRAP, and TEAC), and the OSI assessed in donkey SP were directly related to the sperm resilience to cryopreservation in this species.

For a long time, SP was regarded as a passive medium that accompanies sperm during and after ejaculation [60]. More than a natural diluent and a transport vehicle, nevertheless, SP has recently been considered as an important player for fertility in mammals, both regarding sperm function and female-reproductive-tract-related processes [22,60,61]. In effect, this fluid also regulates the immune response of the female reproductive tract generated by the presence of semen [60–63]. It is well-known that SP represents the most important source of enzymatic and non-enzymatic antioxidants for sperm, being capable of eliminating the excess of ROS that induces OS in semen [6,17,22,64,65].. It is worth mentioning that the composition of SP as well as the capacity of sperm to withstand freeze-thawing differ not only between species, but also between and within individuals [6,17,63,66]. In addition, SP components, mainly those with antioxidant properties, may be related to the cryotolerance of sperm to freeze-thawing processes [6,17]. This study, therefore, aimed to evaluate whether differences between donkey ejaculates/individuals in the resilience of their sperm to cryopreservation could be explained by differences in their SP-antioxidant composition.

As far as we are aware, this is the first report addressing the relationship of the levels of antioxidants, enzymatic and non-enzymatic, and OSI in SP with the quality and functionality parameters of donkey sperm after freeze-thawing. When enzymatic antioxidants PON1 and SOD and non-enzymatic antioxidants (measured in terms of CUPRAC, FRAP, and TEAC) of SP were evaluated, their levels were higher in ejaculates categorized as GFE than in those graded as PFE. Activity levels of enzymatic antioxidant CAT and SP-OSI, however, were higher in ejaculates classified as PFE compared to those classified as GFE. These results are in agreement with those previously observed in donkey semen in the case of SOD [6] and in pig semen in the case of SOD, PON1, CAT, FRAP, and TEAC [39]. However, our results differ from those previously reported in donkey semen in the case of CAT. The lower CAT activity levels we found in donkey GFE ejaculates could be related to the high activity levels of PON1 detected in this group. PON1, therefore, might be playing a more relevant role in the control of ROS in donkey semen than CAT. This hypothesis could explain the negative correlation observed between CAT and PON1 activity levels. We also found differences in CUPRAC levels compared to pig SP, where these levels were lower in the fraction of semen with better freezability [39]. This could rely on the distinct SP composition and the different effect of antioxidants depending on species [17], reproductive season [22,67], and age [67]. Based on these considerations, it is reasonable to suggest that the resilience of donkey sperm to cryopreservation may be determined by the high activity levels of enzymatic antioxidants PON1 and SOD and total non-enzymatic antioxidants (measured in terms of CUPRAC, FRAP, and TEAC) in SP. The brief contact of sperm with SP before its removal could be sufficient to exert the beneficial effect of antioxidants on donkey sperm cryotolerance, which is similar to findings previously reported in donkey and horses [6,17].

Regarding the enzymatic antioxidants of SP analysed in this study, our results support previous studies that highlight the relevance of SOD for sperm cryotolerance in donkeys, horses, pigs, humans, and buffaloes [6,17,39,67,68], where SOD was positively related to post-thaw motility [39,67,68] and viability [6,17,39] and negatively to intracellular ROS levels [39]. This neutralization of ROS is produced through a cascade of reactions initiated by SOD, an essential antioxidant that avoids OS [39,69]. SOD protects the cell against $O_2^-$, as it catalyses the dismutation of this anion into $H_2O_2$ [70]. Furthermore, this reaction

prevents the formation of the highly reactive hydroxyl radical ($OH^-$) that occurs when $O_2^-$ and $H_2O_2$ react with the ferric ion [70,71]. In our study, the positive and negative correlation of SOD activity levels with post-thaw sperm quality and with ROS levels, respectively, together with the higher SOD activity levels found in GFE in comparison to PFE, would confirm that, in donkey semen, SOD is the main antioxidant SP enzyme involved in the detoxification of ROS [6]. Hence, the measurement of SOD activity levels in donkey SP could be used as a marker of sperm cryotolerance in this species. However, although the protocol used in this work and similar protocols to measure intracellular $O_2^-$ levels have been validated and tested in different studies and species [56,72,73], they could be evaluating $O_2^-$ imprecisely, as reported by Robinson et al. [74] and more recently by Gallo et al. [75]. When oxidized, hydroethidine generates two red fluorescent products, 2-hydroxyethidium (2-OH-$E^+$) and ethidium ($E^+$). The former selectively detects $O_2^-$, while the latter binds to DNA [74–76]. Therefore, the protocols used to assess intracellular $O_2^-$ levels should maximize the detection of specific product 2-OH-$E^+$. This issue should be considered in future studies.

In order for SOD to fulfil its function as an antioxidant, it is combined with additional enzymes able to metabolize the $H_2O_2$ generated [77], because an excess of $H_2O_2$ is toxic to tissues and cells [78]. In this sense, the activity of SOD is completed with that of enzymes such as GPX and CAT [70,78]. Regarding GPX, which is an enzyme whose function is to reduce $H_2O_2$ to water [17,70], its levels did not differ between GFE and PFE, which is in agreement with the results reported in previous studies using frozen-thawed donkey and horse sperm [6,17]. Curiously, our results show a positive relationship between the activity levels of GPX in SP and both TM and PM, differing from previous studies conducted in fresh and frozen-thawed donkey semen [6,22]. We also observed a negative correlation between GPX activity in SP and intracellular ROS levels, a parameter that was not evaluated in the aforementioned studies carried out in donkeys [6,22]. At this point, it is important to mention that previous reports also found differences in the functional role of GPX between species; in fresh, liquid-stored, or frozen semen; as well as between studies conducted in the same species [6,17]. In effect, while kinetic sperm parameters have been reported to be negatively correlated to GPX activity in sperm and SP from rams [79], this activity has been found to be positively related to the quality parameters and fertilizing capacity of fresh sperm from Arabian stallions. Other studies carried out in horses, however, reported a negative relationship between GPX activity in SP and motility of fresh and frozen-thawed sperm [17,22]. Taking into consideration our results and the previous studies in frozen-thawed donkey sperm [6], one could posit that the lack of differences in GPX activity between GFE and PFE would indicate that this enzyme cannot be used as a marker of sperm cryotolerance in donkeys.

The importance of CAT, an enzyme whose function is to break down $H_2O_2$ into water and molecular oxygen ($O_2$) and thus functions as a ROS scavenger, has been previously described in mammalian sperm [6,70,80]. In fact, CAT has been shown to counteract the deleterious effects of OS on human [81,82], mouse [83], horse [84], and pig sperm [56], which is mainly observed as an improvement in sperm motility parameters [70,85–87]. Our results, however, did not show a positive relationship between CAT activity, intracellular ROS, and motility parameters, which would not concur with our previous studies in fresh donkey semen [22] and frozen-thawed donkey and horse sperm [6,17]. Furthermore, in this study, CAT was found to be positively correlated with the proportions of sperm showing an intermediate MMP and negatively with those of sperm exhibiting high membrane lipid disorder, indicating a positive effect of this enzyme on one of the structural sperm parameters. However, these results, together with the aforementioned lower activity of CAT in GFE compared to PFE, would indicate that the activity of CAT in donkey semen could be less relevant as a modulator of $H_2O_2$ than other antioxidant enzymes, such as PON1 or GPX.

As mentioned above, in addition to SOD, CAT, and GPX, which are considered as fundamental participants in the first line of the cellular antioxidant defense [78], the activity

levels of PON1 in donkey SP were also evaluated in this study and were found to be higher than those previously reported in pigs [39,88] and lower than those observed in humans [89]. To the best of our knowledge, this is the first report identifying and quantifying this enzyme in donkey SP. It is well-known that PON1 is an extracellular enzyme associated with high-density lipoproteins (HDL) that has antioxidant and anti-inflammatory properties. It prevents the oxidation of lipoproteins of low (LDL) and high density [88,90], since it binds the cholesterol of the membrane to prevent its oxidation, probably hydrolyzing specific lipid peroxides, such as cholesterol esters and oxidized phospholipids [47,91,92]. In our study, a positive correlation between PON1 activity in SP and sperm with intact plasma membrane (viable) after thawing was found; in addition, intracellular levels of ROS after thawing were negatively correlated with PON1 activity. These data are in agreement with those reported in frozen-thawed pig sperm [39]. In addition, in liquid-stored pig semen, a high PON1 activity in SP was related to an improvement in sperm motility and fertility and a decrease in the generation of intracellular ROS [88]. In humans, while low levels of PON1 activity have been related to an increase in OS and infertility [89,93], high levels of this enzyme have been positively associated with some sperm quality parameters such as concentration, motility, and morphology [89]. Thanks to its antioxidant properties, PON1 protects sperm cells against OS [88,89,91] and exerts a positive influence on sperm quality parameters, as has been observed both in this study and in other species such as pigs [39,88] and humans [89]. This fact, added to the higher activity levels of PON1 in donkey GFE compared to PFE observed in this study and the higher activity of PON1 found in donkey SP when compared to its pig counterpart [39,88], suggests that this enzyme could play a key role in the resilience of donkey sperm to cryopreservation. The measurement of PON1 in donkey SP could thus be a potential freezability biomarker.

Our results show that the activity levels of non-enzymatic antioxidants (measured in terms of CUPRAC, FRAP, and TEAC) were positively correlated with the percentages of motile and viable sperm after thawing. As expected, their levels, together with total thiol amounts, were negatively correlated with intracellular ROS levels. Results obtained in a recent study in fresh donkey semen were similar to those presented herein, as a positive correlation was not observed between sperm motility and FRAP activity levels nor between sperm motility and total thiols [94]. In addition, our data are in agreement with those reported by Li et al. [39], who observed that the motility of frozen-thawed pig sperm was positively related to higher activity levels of FRAP and TEAC and their viability with higher levels of TEAC and FRAP. It is worth noting that in the case of CUPRAC, our findings differ from those reported by Li et al. [39] in pig semen. Indeed, herein, higher activity levels of CUPRAC, FRAP, and TEAC in GFE compared to PFE were observed, highlighting the involvement of non-enzymatic SP antioxidants in donkey sperm cryotolerance. While FRAP reflects the effect of low-molecular-weight antioxidants, mainly measuring the levels of uric acid, α-tocopherol, and ascorbic acid (AA), CUPRAC and TEAC evaluate the effect of antioxidants that contain sulfhydryl groups in their structure such as thiols (such as reduced glutathione; GSH) and albumin [94,95]. Ascorbic acid and α-tocopherol insert into the membrane structure and are particularly efficient at reducing ROS that interact superficially with membrane components. Specifically, AA reduces nitroxide radicals, and α-tocopherol reduces lipid peroxyl radicals [39]. Furthermore, both are individually sufficient to minimize plasma membrane LPO when present at adequate concentrations [96]. Uric acid is another powerful ROS scavenger that binds to plasma membrane but is not effective in minimizing LPO [39,96]. Reduced glutathione is probably the most important non-enzymatic ROS scavenger and acts mainly via neutralizing $\cdot OH^-$, which is among the most dangerous ROS generated from inactivated $H_2O_2$ [97]. As far as albumin is concerned, in vitro studies have shown that it is an effective antioxidant during LPO, capable of avoiding the damage produced by $\cdot OH^-$, a product of the reaction of $H_2O_2$ and iron [98]. Furthermore, it is known that, among the cationic ligands, copper and iron are very powerful for generating ROS after reaction with oxygen. Free ions of Cu (II) and Fe (II) can interact with $H_2O_2$, leading to the formation of $\cdot OH^-$ through the

Fenton reaction [99]. Bound to proteins such as albumin, copper and iron are generally less susceptible to participate in the Fenton reaction, and the $\cdot OH^-$ released by that reaction is mainly directed to proteins such as albumin, avoiding more important targets [99].

Because maintaining an adequate balance between ROS and antioxidant levels is essential for optimal sperm function [100], the analysis of SP-OSI, which is considered as a quick, easy, and inexpensive technique to accurately show the oxidant/antioxidant relationship in biological samples [101], was also conducted in this study. To our knowledge, this is the first report in which OSI has been measured in donkey SP in order to evaluate its presumed influence on sperm cryotolerance in mammals. In pig semen, SP-OSI has been related to the ability of sperm to tolerate cooling, showing a positive relationship with the loss of sperm motility and a negative relationship with in vivo fertility of liquid-stored semen [102]. This study found that SP-OSI values were lower in GFE than in PFE. Furthermore, SP-OSI levels were negatively correlated with sperm motility and viability after thawing. These results are in agreement with a previous study carried out in porcine, in which SP-OSI levels were found to be related with the sperm resilience to liquid storage at 17 °C [102]. In addition, SP-OSI levels were negatively correlated with the activity levels of SOD, CUPRAC, FRAP, and TEAC. These findings, together with the positive correlation found between OSI and intracellular levels of ROS, mainly superoxides, would indicate the great relevance of the balance between the cellular antioxidant defense system and the oxidative agents present in sperm, and they would also indicate the importance that the determination of SP-OSI could have to predicting donkey sperm cryotolerance.

Finally, individual variability in the capacity of sperm to withstand cryopreservation has already been described in donkeys [103]. In addition, it has been reported that antioxidant enzymes in SP, such as GPX, CAT, and especially SOD, play a vital function in sperm resilience to freeze-thawing in this species [6]. Our study, in addition to ratifying these previous findings, demonstrated the importance of other SP components with antioxidant properties for donkey sperm cryotolerance, including enzymatic antioxidants, such as PON1, and non-enzymatic components, measured in terms of CUPRAC, TEAC, FRAP and total thiols. The current study also investigated the complex balance between antioxidants and oxidative agents in SP and its influence on donkey sperm freezability, through the assessment of OSI. In relation to this, several efforts have been made to identify markers of frostbite in the whole ejaculate and SP in mammalian species, including donkeys. In this regard and as mentioned above, our study confirms that the activity of SOD in donkey SP could be used as a marker of sperm cryotolerance. In addition, our results also indicate, for the first time in this species, that other enzymatic antioxidants, such as PON1, and non-enzymatic antioxidants, such as CUPRAC, FRAP, and TEAC, as well as OSI in SP could be used as putative cryotolerance biomarkers. Further research addressing whether these SP components play such a role in other species is warranted.

## 5. Conclusions

The present study has shown, for the first time in donkeys, that SP components with antioxidant properties, both enzymatic and non-enzymatic, as well as SP-OSI are related to the sperm resilience to cryopreservation. Specifically, the results indicate that the activity levels of enzymatic (SOD and PON1) and non-enzymatic antioxidants (measured in terms of CUPRAC, FRAP, and TEAC) were higher in the SP of GFE than in that of PFE. An opposite pattern, however, was observed for SP-OSI levels, which were lower in the SP of ejaculates categorized as GFE than in those classified as PFE. These results, therefore, suggest that PON1, SOD, CUPRAC, FRAP, TEAC, and OSI in donkey SP could be used as sperm cryotolerance markers in this species. The measurement of these SP antioxidants and SP-OSI could help us identify those donkey ejaculates that will exhibit worse sperm quality after thawing, as well as those that would require antioxidant supplements to rescue sperm function and survival during conservation. Further research addressing the relationship of these antioxidants and SP-OSI with sperm cryotolerance in other species is warranted.

## References

1. Camillo, F.; Rota, A.; Biagini, L.; Tesi, M.; Fanelli, D.; Panzani, D. The current situation and trend of donkey industry in Europe. *J. Equine Vet. Sci.* **2018**, *65*, 44–49. [CrossRef]
2. Canisso, I.F.; Panzani, D.; Miró, J.; Ellerbrock, R.E. Key aspects of donkey and mule reproduction. *Vet. Clin. N. Am. Equine Pract.* **2019**, *35*, 607–642. [CrossRef] [PubMed]
3. Di Palma, T.; Cecchini, S.; MacChia, G.; Pasolini, M.P.; Cocchia, N.; Boni, R. Kinematic, bioenergetic and oxidative evaluations of donkey sperm preserved at +4 °C. *Zygote* **2020**, *28*, 300–307. [CrossRef]
4. Martini, M.; Altomonte, I.; Licitra, R.; Salari, F. Nutritional and nutraceutical quality of donkey milk. *J. Equine Vet. Sci.* **2018**, *65*, 33–37. [CrossRef]
5. Miró, J.; Marín, H.; Catalán, J.; Papas, M.; Gacem, S.; Yeste, M. Seminal plasma, sperm concentration, and sperm-pmn interaction in the donkey: An in vitro model to study endometrial inflammation at post-insemination. *Int. J. Mol. Sci.* **2020**, *21*, 3478. [CrossRef]
6. Papas, M.; Catalan, J.; Barranco, I.; Arroyo, L.; Bassols, A.; Yeste, M.; Miró, J. Total and specific activities of superoxide dismutase (SOD) in seminal plasma are related with the cryotolerance of jackass spermatozoa. *Cryobiology* **2020**, *92*, 109–116. [CrossRef] [PubMed]
7. Álvarez, C.; González, N.; Luño, V.; Martínez, F.; Gil, L. Alternatives in Donkey semen cryopreservation: Mare vs. Jenny Colostrum. *Reprod. Domest. Anim.* **2019**, *54*, 94–97. [CrossRef]
8. Vidament, M.; Vincent, P.; Martin, F.X.; Magistrini, M.; Blesbois, E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. *Anim. Reprod. Sci.* **2009**, *112*, 22–35. [CrossRef]
9. Canisso, I.F.; Carvalho, G.R.; Morel, M.D.; Ker, P.G.; Rodrigues, A.L.; Silva, E.C.; Da Silva, M.A.C. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Vet. J.* **2011**, *43*, 179–183. [CrossRef]
10. Oliveira, J.V.; Papa, F.O.; Melo-Oña, C.M.; Monteiro, G.A.; Puoli-Filho, J.N.P.; Alvarenga, M.A. New procedures to freeze donkey semen and its influence on mares and jennies fertility. *J. Equine Vet. Sci.* **2012**, *32*, 503–504. [CrossRef]
11. Vilés, K.; Rabanal, R.; Rodríguez-Prado, M.; Miró, J. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology* **2013**, *79*, 1019–1026. [CrossRef] [PubMed]

12. Miró, J.; Papas, M. Post–artificial insemination endometrial inflammation and its control in donkeys. *J. Equine Vet. Sci.* **2018**, *65*, 38–43. [CrossRef]

13. Mateo-Otero, Y.; Zambrano, F.; Catalán, J.; Sánchez, R.; Yeste, M.; Miro, J.; Fernandez-Fuertes, B. Seminal plasma, and not sperm, induces time and concentration-dependent neutrophil extracellular trap release in donkeys. *Equine Vet. J.* **2021**, *54*, 415–426. [CrossRef]

14. Yeste, M.; Estrada, E.; Rocha, L.G.; Marín, H.; Rodríguez-Gil, J.E.; Miró, J. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology* **2015**, *3*, 395–407. [CrossRef] [PubMed]

15. Holt, W.V. Basic aspects of frozen storage of semen. *Anim. Reprod. Sci.* **2000**, *62*, 3–22. [CrossRef]

16. Watson, P.F. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* **2000**, *60*, 481–492. [CrossRef]

17. Papas, M.; Catalán, J.; Fernandez-Fuertes, B.; Arroyo, L.; Bassols, A.; Miró, J.; Yeste, M. Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance. *Antioxidants* **2019**, *8*, 539. [CrossRef]

18. Yeste, M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology* **2016**, *85*, 47–64. [CrossRef]

19. Sieme, H.; Oldenhof, H.; Wolkers, W. Sperm membrane behaviour during cooling and cryopreservation. *Reprod. Domest. Anim.* **2015**, *50*, 20–26. [CrossRef]

20. Amidi, F.; Pazhohan, A.; Shabani Nashtaei, M.; Khodarahmian, M.; Nekoonam, S. The role of antioxidants in sperm freezing: A review. *Cell Tissue Bank.* **2016**, *17*, 745–756. [CrossRef]

21. Aitken, R.J.; De Iuliis, G.N.; Drevet, J.R. Role of Oxidative Stress in the Etiology of Male Infertility and the Potential Therapeutic Value of Antioxidants. In *Oxidants, Antioxidants, and Impact of the Oxidative Status in Male Reproduction*; Elsevier: London, UK, 2018; pp. 91–100. [CrossRef]

22. Papas, M.; Arroyo, L.; Bassols, A.; Catalán, J.; Bonilla-Correal, S.; Gacem, S.; Yeste, M.; Miró, J. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology* **2019**, *140*, 180–187. [CrossRef] [PubMed]

23. Muiño-Blanco, T.; Pérez-Pé, R.; Cebrián-Pérez, J. Seminal plasma proteins and sperm resistance to stress. *Reprod. Domest. Anim.* **2008**, *43*, 18–31. [CrossRef] [PubMed]

24. Oliveira, R.A.; Wolf, C.A.; Viu, M.A.O.; Gambarini, M.L. Addition of glutathione to an extender for frozen equine semen. *J. Equine Vet. Sci.* **2013**, *33*, 1148–1152. [CrossRef]

25. Gadea, J.; García-Vazquez, F.; Matás, C.; Gardón, J.C.; Cánovas, S.; Gumbao, D. Cooling and freezing of boar spermatozoa: Supplementation of the freezing media with reduced glutathione preserves sperm function. *J. Androl.* **2005**, *26*, 396–404. [CrossRef] [PubMed]

26. Silva, S.V.; Soares, A.T.; Batista, A.M.; Almeida, F.C.; Nunes, J.F.; Peixoto, C.A.; Guerra, M.M.P. In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and wupplemented with superoxide dismutase and reduced glutathione. *Reprod. Domest. Anim.* **2011**, *46*, 874–881. [CrossRef]

27. Gangwar, C.; Saxena, A.; Patel, A.; Singh, S.P.; Yadav, S.; Kumar, R.; Singh, V. Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. *Anim. Reprod. Sci.* **2018**, *192*, 171–178. [CrossRef] [PubMed]

28. Ogata, K.; Sasaki, A.; Kato, Y.; Takeda, A.; Wakabayashi, M.; Sarentonglaga, B.; Yamaguchi, M.; Hara, A.; Fukumori, R.; Nagao, Y. Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. *J. Reprod. Dev.* **2015**, *61*, 116–122. [CrossRef] [PubMed]

29. Dowsett, K.F.; Knott, L.M. The influence of age and breed on stallion semen. *Theriogenology* **1996**, *46*, 397–412. [CrossRef]

30. Sieme, H.; Harrison, R.A.P.; Petrunkina, A.M. Cryobiological determinants of frozen semen quality, with special reference to stallion. *Anim. Reprod. Sci.* **2008**, *107*, 276–292. [CrossRef]

31. Loomis, P.R.; Graham, J.K. Commercial semen freezing: Individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. *Anim. Reprod. Sci.* **2008**, *105*, 119–128. [CrossRef]

32. Mari, G.; Castagnetti, C.; Rizzato, G.; Mislei, B.; Iacono, E.; Merlo, B. Density gradient centrifugation of sperm from a subfertile stallion and effect of seminal plasma addition on fertility. *Anim. Reprod. Sci.* **2011**, *126*, 96–100. [CrossRef]

33. Cabrera, P.; Sánchez, R.; Risopatrón, J. Selección espermática en semen congelado/descongelado de equino: Evaluación de las membranas plasmática, acrosomal y potencial de membrana mitocondrial. *Int. J. Morphol.* **2014**, *32*, 725–731. [CrossRef]

34. Neuhauser, S.; Gösele, P.; Handler, J. Postthaw addition of autologous seminal plasma improves sperm motion characteristics in fair and poor freezer stallions. *J. Equine Vet. Sci.* **2019**, *72*, 117–123. [CrossRef]

35. Aurich, J.E.; Kühne, A.; Hoppe, H.; Aurich, C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenology* **1996**, *46*, 791–797. [CrossRef]

36. Brinsko, S.P.; Crockett, E.C.; Squires, E.L. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenology* **2000**, *54*, 129–136. [CrossRef]

37. Bromfield, J.J. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* **2016**, *152*, R223–R232. [CrossRef]

38. Mogielnicka-Brzozowska, M.; Kordan, W. Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. *Pol. J. Vet. Sci.* **2011**, *14*, 489–499. [CrossRef]

39. Li, J.; Barranco, I.; Tvarijonaviciute, A.; Molina, M.F.; Martinez, E.A.; Rodriguez-Martinez, H.; Parrilla, I.; Roca, J. Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. *Theriogenology* **2018**, *107*, 27–35. [CrossRef]
40. Kenney, R.M.; Bergman, R.V.; Cooper, W.L.; Morse, G.W. Minimal contamination techniques for breeding mares: Technique and preliminary findings. *Proc. Am. Assoc. Equine Pract.* **1975**, *21*, 327–336.
41. Bamba, K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology* **1988**, *29*, 1245–1251. [CrossRef]
42. Jocelyn, P.C. Spectrophotometric assay of thiols. *Methods Enzymol.* **1987**, *143*, 44–67. [CrossRef]
43. Da Costa, C.M.; Dos Santos, R.C.C.; Lima, E.S. A simple automated procedure for thiol measurement in human serum samples. *J. Bras. Patol. Med. Lab.* **2006**, *42*, 345–350. [CrossRef]
44. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **2004**, *37*, 277–285. [CrossRef]
45. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef]
46. Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, Á. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: The CUPRAC–BCS assay. *Anal. Biochem.* **2009**, *392*, 37–44. [CrossRef]
47. Barranco, I.; Roca, J.; Tvarijonaviciute, A.; Rubér, M.; Vicente-Carrillo, A.; Atikuzzaman, M.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H. Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Mol. Reprod. Dev.* **2015**, *82*, 58–65. [CrossRef]
48. Erel, O. A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* **2005**, *38*, 1103–1111. [CrossRef]
49. Wu, R.; Feng, J.; Yang, Y.; Dai, C.; Lu, A.; Li, J.; Liao, Y.; Xiang, M.; Huang, Q.; Wang, D.; et al. Significance of serum total oxidant/antioxidant status in patients with colorectal cancer. *PLoS ONE* **2017**, *12*, e0170003. [CrossRef]
50. Flores, E.; Taberner, E.; Rivera, M.M.; Peña, A.; Rigau, T.; Miró, J.; Rodríguez-Gil, J.E. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. *Theriogenology* **2008**, *70*, 936–945. [CrossRef]
51. Lee, J.A.; Spidlen, J.; Boyce, K.; Cai, J.; Crosbie, N.; Dalphin, M.; Furlong, J.; Gasparetto, M.; Goldberg, M.; Goralczyk, E.M.; et al. MIFlowCyt: The minimum information about a flow cytometry experiment. *Cytom. Part A* **2008**, *73A*, 926–930. [CrossRef]
52. Petrunkina, A.M.; Waberski, D.; Bollwein, H.; Sieme, H. Identifying non-sperm particles during flow cytometric physiological assessment: A simple approach. *Theriogenology* **2010**, *73*, 995–1000. [CrossRef]
53. Garner, D.L.; Johnson, L.A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **1995**, *53*, 276–284. [CrossRef] [PubMed]
54. Harrison, R.; Ashworth, P.; Miller, N. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **1996**, *45*, 378–391. [CrossRef]
55. Catalán, J.; Papas, M.; Trujillo-Rojas, L.; Blanco-Prieto, O.; Bonilla-Correal, S.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light. *Front. Cell Dev. Biol.* **2020**, *8*, 588621. [CrossRef]
56. Guthrie, H.D.; Welch, G.R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry1. *J. Anim. Sci.* **2006**, *84*, 2089–2100. [CrossRef]
57. Yeste, M.; Fernández-Novell, J.M.; Ramió-Lluch, L.; Estrada, E.; Rocha, L.G.; Cebrián-Pérez, J.A.; Muiño-Blanco, T.; Concha, I.I.; Ramírez, A.; Rodríguez-Gil, J.E. Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. *Andrology* **2015**, *3*, 729–747. [CrossRef] [PubMed]
58. Kadirvel, G.; Kumar, S.; Kumaresan, A.; Kathiravan, P. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Anim. Reprod. Sci.* **2009**, *116*, 244–253. [CrossRef]
59. Morató, R.; Prieto-Martínez, N.; Muiño, R.; Hidalgo, C.O.; Rodríguez-Gil, J.E.; Bonet, S.; Yeste, M. Aquaporin 11 is related to cryotolerance and fertilising ability of frozen–thawed bull spermatozoa. *Reprod. Fertil. Dev.* **2018**, *30*, 1099–1108. [CrossRef]
60. Szczykutowicz, J.; Kałuza, A.; Kaźmierowska-Niemczuk, M.; Ferens-Sieczkowska, M. The potential role of seminal plasma in the fertilization outcomes. *BioMed Res. Int.* **2019**, *2019*, 5397804. [CrossRef]
61. Carretero, M.I.; Fumuso, F.G.; Giuliano, M.S. Effect of seminal plasma in the conservation of semen in zootechnical species. *Spermova* **2016**, *6*, 93–98. [CrossRef]
62. Troedsson, M.H.T.; Desvousges, A.; Alghamdi, A.S.; Dahms, B.; Dow, C.A.; Hayna, J.; Valesco, R.; Collahan, P.T.; Macpherson, M.L.; Pozor, M.; et al. Components in seminal plasma regulating sperm transport and elimination. *Anim. Reprod. Sci.* **2005**, *89*, 171–186. [CrossRef]
63. Jonakova, V.; Jonak, J.; Ticha, M. Proteomics of Male Seminal Plasma. In *Reproductive Genomics in Domestic Animals*; Wiley-Blackwell: Oxford, UK, 2010; pp. 339–366.
64. Barranco, I.; Padilla, L.; Tvarijonaviciute, A.; Parrilla, I.; Martínez, E.A.; Rodriguez-Martinez, H.; Yeste, M.; Roca, J. Levels of activity of superoxide dismutase in seminal plasma do not predict fertility of pig AI-semen doses. *Theriogenology* **2019**, *140*, 18–24. [CrossRef]

65. Yánez-Ortiz, I.; Catalán, J.; Mateo-Otero, Y.; Dordas-Perpinyà, M.; Gacem, S.; Yeste, N.; Bassols, A.; Yeste, M.; Miró, J. Extracellular reactive oxygen species (ROS) production in fresh donkey sperm exposed to reductive stress, oxidative stress and NETosis. *Antioxidants* **2021**, *10*, 1367. [CrossRef] [PubMed]

66. Baumber, J.; Ball, B.A. Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. *Am. J. Vet. Res.* **2005**, *66*, 1415–1419. [CrossRef] [PubMed]

67. Waheed, M.M.; Gouda, E.M.; Khalifa, T.A.A. Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. *Anim. Reprod. Sci.* **2013**, *142*, 126–130. [CrossRef] [PubMed]

68. Buffone, M.G.; Calamera, J.C.; Brugo-Olmedo, S.; Vincentiis, S.; Calamera, M.M.; Storey, B.T.; Doncel, G.F.; Alvarez, J.G. Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa. *Fertil. Steril.* **2012**, *97*, 293–298. [CrossRef] [PubMed]

69. Kowalowka, M.; Wysocki, P.; Fraser, L.; Strzezek, J. Extracellular superoxide dismutase of boar seminal plasma. *Reprod. Domest. Anim.* **2008**, *43*, 490–496. [CrossRef]

70. Silvestre, M.A.; Yániz, J.L.; Peña, F.J.; Santolaria, P.; Castelló-Ruiz, M. Role of antioxidants in cooled liquid storage of mammal spermatozoa. *Antioxidants* **2021**, *10*, 1096. [CrossRef]

71. Kehrer, J.P. The Haber–Weiss reaction and mechanisms of toxicity. *Toxicology* **2000**, *149*, 43–50. [CrossRef]

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

73. Ghaleno, L.R.; Valojerdi, M.R.; Janzamin, E.; Chehrazi, M.; Sharbatoghli, M.; Yazdi, R.S. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: A flow cytometric study. *Arch. Gynecol. Obstet.* **2014**, *289*, 173–180. [CrossRef] [PubMed]

74. Robinson, K.M.; Janes, M.S.; Pehar, M.; Monette, J.S.; Ross, M.F.; Hagen, T.M.; Murphy, M.P.; Beckman, J.S. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 15038. [CrossRef] [PubMed]

75. Gallo, A.; Boni, R.; Buia, M.C.; Monfrecola, V.; Esposito, M.C.; Tosti, E. Ocean acidification impact on ascidian Ciona robusta spermatozoa: New evidence for stress resilience. *Sci. Total Environ.* **2019**, *697*, 134100. [CrossRef] [PubMed]

76. De Iuliis, G.N.; Wingate, J.K.; Koppers, A.J.; McLaughlin, E.A.; Aitken, R.J. Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 1968–1975. [CrossRef] [PubMed]

77. Aitken, R.J.; Iuliis, G.N. De Role of Oxidative Stress in the Etiology of Sperm DNA Damage. In *Sperm Chromatin*; Springer: New York, NY, USA, 2011; pp. 277–293. [CrossRef]

78. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alex. J. Med.* **2019**, *54*, 287–293. [CrossRef]

79. Kasimanickam, R.; Pelzer, K.D.; Kasimanickam, V.; Swecker, W.S.; Thatcher, C.D. Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. *Theriogenology* **2006**, *65*, 1407–1421. [CrossRef]

80. Rubio-Riquelme, N.; Huerta-Retamal, N.; Gómez-Torres, M.J.; Martínez-Espinosa, R.M. Catalase as a molecular target for male infertility diagnosis and monitoring: An overview. *Antioxidants* **2020**, *9*, 78. [CrossRef]

81. Aitken, R.J.; Buckingham, D.; Harkiss, D. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Reproduction* **1993**, *97*, 441–450. [CrossRef]

82. Aitken, R.J.; Drevet, J.R. The importance of oxidative stress in determining the functionality of mammalian spermatozoa: A two-edged sword. *Antioxidants* **2020**, *9*, 111. [CrossRef]

83. Baiardi, G.; Ruiz, R.D.; Cuneo, M.F.; Ponce, A.A.; Lacuara, J.L.; Vincent, L. Differential effects of pharmacologically generated reactive oxygen species upon functional activity of epididymal mouse spermatozoa. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 173–178. [CrossRef]

84. Baumber, J.; Ball, B.A.; Gravance, C.G.; Medina, V.; Davies-Morel, M.C.G. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *J. Androl.* **2000**, *21*, 895–902. [PubMed]

85. Maxwell, W.; Stojanov, T. Liquid storage of ram semen in the absence or presence of some antioxidants. *Reprod. Fertil. Dev.* **1996**, *8*, 1013–1020. [CrossRef] [PubMed]

86. Peruma, P.; Chamuah, J.K.; Rajkhowa, C. Effect of catalase on the liquid storage of mithun (Bos frontalis) semen. *Asian Pac. J. Reprod.* **2013**, *2*, 209–214. [CrossRef]

87. Michael, A.J.; Alexopoulos, C.; Pontiki, E.A.; Hadjipavlou-Litina, D.J.; Saratsis, P.; Ververidis, H.N.; Boscos, C.M. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim. Reprod. Sci.* **2009**, *112*, 119–135. [CrossRef] [PubMed]

88. Barranco, I.; Tvarijonaviciute, A.; Perez-Patiño, C.; Alkmin, D.V.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality, and in vivo fertility. *Andrology* **2015**, *3*, 315–320. [CrossRef]

89. Verit, F.F.; Verit, A.; Ciftci, H.; Erel, O.; Celik, H. Paraoxonase-1 activity in subfertile men and relationship to sperm parameters. *J. Androl.* **2009**, *30*, 183–189. [CrossRef]

90. Camps, J.; Marsillach, J.; Joven, J. The paraoxonases: Role in human diseases and methodological difficulties in measurement. *Crit. Rev. Clin. Lab. Sci.* **2009**, *46*, 83–106. [CrossRef]

91. Mackness, M.I.; Arrol, S.; Abbott, C.; Durrington, P.N. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* **1993**, *104*, 129–135. [CrossRef]
92. Aviram, M.; Rosenblat, M.; Billecke, S.; Erogul, J.; Sorenson, R.; Bisgaier, C.L.; Newton, R.S.; La Du, B. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic. Biol. Med.* **1999**, *26*, 892–904. [CrossRef]
93. Lazaros, L.A.; Xita, N.V.; Hatzi, E.G.; Kaponis, A.I.; Stefos, T.J.; Plachouras, N.I.; Makrydimas, G.V.; Sofikitis, N.V.; Zikopoulos, K.A.; Georgiou, I.A. Association of paraoxonase gene polymorphisms with sperm parameters. *J. Androl.* **2011**, *32*, 394–401. [CrossRef]
94. Cecchini, G.S.; Giangaspero, B.; Di Palma, T.; Macchia, G.; Carluccio, A.; Boni, R. Oxidative profile and protease regulator potential to predict sperm functionality in donkey (*Equus asinus*). *Sci. Rep.* **2021**, *11*, 20551. [CrossRef]
95. Rubio, C.P.; Martínez-Subiela, S.; Hernández-Ruiz, J.; Tvarijonaviciute, A.; Cerón, J.J.; Allenspach, K. Serum biomarkers of oxidative stress in dogs with idiopathic inflammatory bowel disease. *Vet. J.* **2017**, *221*, 56–61. [CrossRef] [PubMed]
96. Niki, E. Assessment of antioxidant capacity of natural products. *Curr. Pharm. Biotechnol.* **2010**, *11*, 801–809. [CrossRef] [PubMed]
97. Bathgate, R. Antioxidant mechanisms and their benefit on post-thaw boar sperm quality. *Reprod. Domest. Anim.* **2011**, *46*, 23–25. [CrossRef]
98. López-Rodriguez, G.; Suárez-Dieguez, T. Albumin and transferrin are antioxidants that prevent lipoperoxidation in vitro. *Rev. Latinoam. Quím.* **2010**, *38*, 159–167.
99. Roche, M.; Rondeau, P.; Singh, N.R.; Tarnus, E.; Bourdon, E. The antioxidant properties of serum albumin. *FEBS Lett.* **2008**, *582*, 1783–1787. [CrossRef] [PubMed]
100. Bansal, A.K.; Bilaspuri, G.S. Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med. Int.* **2011**, *2011*, 686137. [CrossRef] [PubMed]
101. Abuelo, A.; Hernández, J.; Benedito, J.L.; Castillo, C. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal* **2013**, *7*, 1374–1378. [CrossRef] [PubMed]
102. Barranco, I.; Rubio, C.P.; Tvarijonaviciute, A.; Rodriguez-Martinez, H.; Roca, J. Measurement of oxidative stress index in seminal plasma can predict in vivo fertility of liquid-stored porcine artificial insemination semen doses. *Antioxidants* **2021**, *10*, 1203. [CrossRef]
103. Contri, A.; Gloria, A.; Robbe, D.; Sfirro, M.P.; Carluccio, A. Effect of sperm concentration on characteristics of frozen-thawed semen in donkeys. *Anim. Reprod. Sci.* **2012**, *136*, 74–80. [CrossRef] [PubMed]

# 4. Seminal plasma antioxidants are related to sperm cryotolerance in the horse

Jaime Catalán, Iván Yánez-Ortiz, Asta Tvarijonaviciute, Isabel Barranco,
Marc Yeste, Jordi Miró

*antioxidants*

*Article* 1

# Seminal Plasma Antioxidants are Related to Sperm Cryotolerance in the Horse

**Jaime Catalán[1,2,3,4,†], Iván Yánez-Ortiz[1,2,3,†], Asta Tvarijonaviciute[5,6], Luis Guillermo González-Aróstegui[5,6*], Camila P. Rubio[6,7], Isabel Barranco[8], Marc Yeste[2,3,9,*,‡] and Jordi Miró[1,*,‡]**

1. Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, Autonomous University of Barcelona, ES-08193 Bellaterra (Cerdanyola del Vallès), Spain; dr.jcatalan@gmail.com (J.C.); ivan.yanez22@gmail.com (I.Y.-O.); jordi.miro@uab.cat (J.M.)
2. Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; marc.yeste@udg.edu (M.Y.)
3. Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain
4. Faculty of Veterinary Medicine, University of Teramo, Loc. Piano d'Accio, IT-64100 Teramo, Italy
5. Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, ES-30100 Murcia, Spain; asta@um.es (A.T.); luisgarostegui@gmail.com (L.G.G.-A.)
6. Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, Faculty of Veterinary Medicine, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, ES-30100 Murcia, Spain; camila.peres@uab.cat (C.P.R)
7. Department of Animal and Food Science, Faculty of Veterinary Sciences, Autonomous University of Barcelona, ES-08193 Cerdanyola del Vallès, Spain
8. Department of Veterinary Medical Sciences, University of Bologna, IT-40064 Ozzano dell'Emilia, Bologna, Italy; isabel.barranco@unibo.it (I.B.)
9. Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain.
* Correspondence: jordi.miro@uab.cat (J.M.); marc.yeste@udg.edu (M.Y.); Tel.: +34-93-5814293 (J.M.); +34-972-419514 (M.Y.)
† These authors contributed equally to this work.
‡ These authors share senior authorship.

**Abstract:** The objective of this study was to determine the relationship of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX) and non-enzymatic antioxidants (measured in terms of: cupric-reducing antioxidant capacity, CUPRAC; ferric-reducing ability of plasma, FRAP; and Trolox equivalent antioxidant capacity, TEAC), as well as oxidative stress index (OSI) in seminal plasma (SP) with the resilience of horse sperm to freeze-thawing. Twenty-one ejaculates (one per individual) were collected and split into two aliquots: the first was used to harvest the SP and assess the activity levels of antioxidants and the OSI, and the second one was cryopreserved. The following post-thaw sperm quality parameters were evaluated: sperm motility, plasma membrane and acrosome integrity, mitochondrial membrane potential, intracellular levels of reactive oxygen species (ROS), and plasma membrane lipid disorder. Based on post-thaw total motility (TM) and plasma membrane integrity (SYBR14+/PI-), ejaculates were hierarchically ($p < 0.001$) clustered into two groups of good (GFE) and poor freezability (PFE). The SP activity levels of PON1, SOD, and TEAC were higher ($p < 0.05$) in GFE than in PFE, showing a positive relationship ($p < 0.05$) with some sperm motility parameters and with plasma membrane (PON1 and TEAC) and acrosome (SOD and TEAC) integrity. In contrast, OSI was higher ($p < 0.05$) in the SP of PFE than in that of GFE, and was negatively correlated ($p < 0.05$) to some sperm motility parameters and to plasma membrane and acrosome integrity, and positively ($p < 0.05$) to the percentage of viable sperm with high plasma membrane lipid disorder. In conclusion, enzymatic (PON1 and SOD) and non-enzymatic (TEAC) antioxidants of SP are related to horse sperm cryotolerance. In addition, our results suggest that PON1 could be one of the main antioxidant enzymes involved in the control of ROS in this species. Further investigation is needed to confirm the potential use of these SP-antioxidants and OSI to predict sperm cryotolerance in horses.

**Keywords:** antioxidants; cryopreservation; horse; oxidative stress; reactive oxygen species; seminal plasma; sperm

## 1. Introduction

Sperm cryopreservation has acquired great importance in recent decades, due to the benefits offered by its use [1–3]. Freeze-thawing procedures, however, impact negatively on sperm motility, morphology, functionality, and survival [4,5], as well as on their fertilizing capacity [6]; this leads to lower pregnancy rates after artificial insemination compared to fresh or cooled semen [7,8]. During cryopreservation, a stressful environment is generated for sperm [9], mainly associated to an excess of reactive oxygen species (ROS) [6] that overwhelms the sperm antioxidant defense capacity. This situation drives the oxidative/antioxidant balance to shift towards the oxidative state [10,11]. In this circumstance, oxidative stress underlies several sperm injuries at the level of plasma membrane (lipoperoxidation) [12–14] and DNA (fragmentation) [11,15,16], and affects functional parameters (such as motility, viability, acrosome membrane integrity and mitochondrial membrane potential) [9,17,18].

Horse sperm are particularly susceptible to oxidative stress due to the large amount of polyunsaturated fatty acids (PUFA) in plasma membrane phospholipids [13,19,20], and their low intracellular antioxidant activity [21–24]. Under this scenario, seminal plasma (SP) represents the main antioxidant defense source, as it is endowed with enzymatic and non-enzymatic antioxidants that scavenge exceeding ROS [25–28]. Yet, current equine semen cryopreservation protocols remove SP by centrifugation [14,29]. While this step is carried out to improve post-thaw sperm quality [1,30], it entails the removal of antioxidants thus increasing further the susceptibility of sperm to oxidative damage [24,31].

In the horse, as in other mammalian species, differences in sperm cryotolerance between breeds [32], individuals [33] and even between ejaculates from the same animal [6] have been reported. Because these differences, which allow ejaculates to be classified as with "good" (GFE) or "poor" freezability (PFE) [14,28,34], are related to the composition of SP [35,36], elucidating the function of antioxidants is very relevant. In horses, some SP antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT) and the glutathione peroxidase/glutathione reductase system (GPX/GSR)) are responsible for reducing oxidative stress [14,26,37]. Of these enzymes, total and specific activities of SOD in horse SP are related to sperm cryotolerance [14]. Moreover, paraoxonase type 1 (PON1) is another antioxidant enzyme that has been found in the SP of humans [38,39], pigs [40] and donkeys [28]. This enzyme could also play a function, as its activity levels are known to be positively associated to several sperm quality and functionality parameters in liquid-stored pig semen [41], and frozen-thawed pig [42] and donkey [28] sperm. Regarding the non-enzymatic antioxidant capacity of SP, it has been analyzed in other mammalian species using different methodological approaches, such as cupric-reducing antioxidant capacity (CUPRAC), ferric-reducing ability of plasma (FRAP) and Trolox equivalent antioxidant capacity (TEAC). Recently, these non-enzymatic antioxidant capacities were found to be directly involved in the resilience of pig [42] and donkey [28] sperm to freeze-thawing.

A balance between ROS production and antioxidants is crucial for sperm function and survival [9,43]. In this sense, it has been established that the measurement of the oxidative stress index (OSI) accurately shows the oxidant/antioxidant ratio in a biological sample; hence, an increase in this ratio would indicate the risk of oxidative stress due to an increase in ROS production or a decrease in antioxidants [44]. This new concept has been applied to SP in other species, such as pigs [45] and donkeys [28]. In both cases, the levels of SP-OSI appear to be related to the sperm ability to withstand liquid storage in pigs [45] and cryopreservation in donkeys [28].

Against this background, the present study aimed to evaluate whether OSI, activities of enzymatic antioxidants (including PON1, SOD, CAT, and GPX) and capacity of non-

enzymatic antioxidants (measured in terms of: CUPRAC, FRAP, and TEAC) are related to horse sperm cryotolerance.

## 2. Materials and Methods

### 2.1. Stallions and samples

Ejaculates were collected from 21 stallions (one ejaculate per individual) of different breeds, mature (between 5 and 15 years old) and of proven fertility. All stallions were clinically healthy and their standard diet included mixed hay and basic concentrate, without antioxidant supplementation and with *ad libitum* water availability. They were housed in individual paddocks at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), which is an equine semen collection center approved by the European Union (authorization number: ES09RS01E) that operates under rigorous health and animal welfare protocols. This Service already works under the approval of the Regional Government of Catalonia, Spain; given that no manipulation on the animals beyond the collection of semen was conducted, the Ethics Committee of our institution indicated that no further ethical approval to carry out this study was necessary. Likewise, the health guidelines established by the Council of the European Communities in Directive 82/894/CEE of December 21, 1982 were complied, as stallions were free from equine viral arteritis, equine infectious anemia and equine contagious metritis.

The collection was performed using a Hannover model artificial vagina (Minitüb GmbH, Tiefenbach, Germany), previously heated to a temperature between 48 °C and 50 °C and coupled with an in-line nylon filter to remove the gel fraction. Once the ejaculate was obtained, the gel fraction was removed and 10 µL were taken to evaluate the sperm concentration using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). After that, each ejaculate was divided into two aliquots. The first was used to harvest the SP (see section 2.2 for more details), and the second was diluted 1:5 (v:v) in a commercial extender based on skimmed milk [46] and subsequently cryopreserved (see section 2.5 for more details). Before cryopreservation, this second aliquot was used for sperm quality analysis (which included sperm motility, morphology and viability). Sperm motility was analyzed using a Computer-Aided Sperm Analysis (CASA) system (see section 2.6 for more details), and sperm viability and morphology through an eosin-nigrosine staining [47]. All semen samples used in this study fulfilled the standard thresholds for sperm quality before freezing (> 80 % total motile sperm, > 60 % viable sperm, and > 70 % morphologically normal sperm).

### 2.2. Seminal plasma (SP) collection

Right after collection, ejaculates were centrifuged five times at 1500× *g* and 4 °C for 10 min (Medifriger BL-S; JP Selecta S.A., Barcelona, Spain). The supernatant was examined under a phase contrast microscope (Olympus Europe; Hamburg, Germany) at 200× to verify the absence of sperm. Finally, 5 mL of each SP sample was stored at -80 °C until analysis. Once all SP samples were procured and stored, they were thawed on ice to measure the activity levels of enzymatic and non-enzymatic antioxidants at the same time.

### 2.3. Measurement of enzymatic and non-enzymatic antioxidant levels in SP

Enzymatic antioxidants assessed in SP were PON1, SOD, CAT, and GPX. Activity levels of PON1 were measured following the protocol described by Barranco et al. [40] adapted to horse SP, by measuring the hydrolysis of p-nitrophenyl acetate into p-nitrophenol. For the measurement of SOD, CAT, and GPX activity, commercially available assays were used following the manufacturer's instructions (CAT: Merck, Darmstadt, Germany; GPX and SOD: Randox, Crumlin, UK). Determination of PON1, SOD, and GPX activity was conducted using an Olympus AU400 automated chemistry analyzer (Olympus Europe GmbH, Hamburg, Germany), whereas that of CAT was performed using a

115

microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA). Activity levels of PON1 and GPX were expressed as IU/L, whereas those of SOD and CAT were expressed as IU/mL.

Non-enzymatic antioxidant capacity of SP was measured in terms of CUPRAC, FRAP, and TEAC. All of them were assessed following the protocol described by Li et al. [42], adapted to horse SP. These assays are based on the reduction of $Cu^{2+}$ to $Cu^+$ (CU-PRAC) [48] and of $Fe^{3+}$ to $Fe^{2+}$ (FRAP) [49], as well as a color change by 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate (TEAC) [10]. All these determinations were performed using an Olympus AU400 automated chemistry analyzer. Activity levels of CUPRAC, FRAP, and TEAC were expressed as mmol Trolox equivalent/L.

Measurements of all analytes were performed in duplicate for each SP sample. For all analytes, each test showed intra- and inter-assay coefficient variations below 10%.

### 2.4. Measurement of oxidative stress index (OSI) in SP

The levels of SP-OSI were calculated as follows: OSI (arbitrary unit) = Total oxidative status (TOS, μmol $H_2O_2$ equivalent/L) / TEAC (mmol Trolox equivalent/L) [28,50]. TOS was measured following the protocol described by Erel [51], adapted to horse SP. This assay is based on the oxidation of $Fe^{2+}$ to $Fe^{3+}$ in the presence of several oxidizing species in an acid medium and the measurement of $Fe^{3+}$ by xylenol orange. This assessment was performed using an Olympus AU400 automated chemistry analyzer. TOS results were expressed as μmol $H_2O_2$ equivalent/L.

### 2.5. Sperm cryopreservation

Prior to cryopreservation, each extended semen sample was centrifuged at 660× *g* and 20 °C for 15 min. Thereafter, the supernatant was discarded and the pellet was resuspended in a commercial freezing extender containing 1 % glycerol and 4 % methylformamide as permeable cryoprotectants (BotuCRIO®; Botupharma Animal Biotechnology, Botucatu, Brazil). Subsequently, sperm concentration and viability were analyzed in each sample, and the same freezing medium was subsequently added to obtain a final concentration of $200 \times 10^6$ viable sperm/mL. Finally, samples loaded into 0.5-mL straws were cooled/frozen using an automatic controlled-rate freezer (Ice-Cube 14S; Minitüb GmbH, Tiefenbach, Germany); the freezing curve included the following three stages: (1) cooling of 20 °C to 5 °C for 60 min at a rate of –0.25 °C/min, (2) freezing of 5 °C to –90 °C for 20 min at a rate of –4.75 °C/min, and (3) freezing from –90 °C to –120 °C for 2.7 min at a rate of –11.11 °C/min. Once this process was completed, straws were plunged into liquid nitrogen at –196 °C and stored in appropriate tanks for conservation. Thawing was carried out in a circulating water bath at 37 °C for 30 s; the content of each straw was poured into a 10-mL conical tube and further diluted (1:2 ,v/v) with Kenney extender [46], pre-warmed at 37 °C. Sperm quality and functionality parameters assessed in each frozen-thawed sample were: (1) motility, (2) plasma membrane and (3) acrosome integrity, (4) membrane lipid disorder, (5) mitochondrial membrane potential (MMP), and intracellular levels of (6) overall ROS and (7) superoxides (see sections 2.6 and 2.7 for more details).

### 2.6. Sperm motility analysis

Sperm motility was analyzed using the CASA-Mot module of the ISAS® v1.2 system (Proiser R + D, Valencia, Spain) equipped with a high-resolution digital camera model MQ003MG-CM (Proiser R + D) capable of capturing up to 100 frames per second (fps). Briefly, 2 μL of each sample was placed onto a reusable Spermtrack®10 chamber (Spk 10; Proiser R + D), pre-warmed at 37 °C, and a minimum of 500 spermatozoa were counted per analysis with a 10× negative phase contrast microscope model UOP200i (Proiser R + D). The analysis included total (TM, %) and progressive (PM, %) motility, together with the kinematic parameters that define sperm movement: curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity coefficient

(LIN = [VSL/VCL] × 100, %), straightness coefficient (STR = [VSL/VAP] × 100, %), wobble                     203
coefficient (WOB = [VAP/VCL] × 100, %), amplitude of lateral head displacement (ALH,                          204
μm), and beat-cross frequency (BCF, Hz). In all the analyses, the CASA-Mot settings rec-                       205
ommended by the manufacturer were used (particle area > 4 and < 75 μm$^2$, connectivity =                      206
6, minimum number of images to calculate ALH = 10) and cut-off values were taken for                          207
total (VAP ≥ 10 μm/s) and progressive (STR ≥ 75%) motility. Three replicates per sample                       208
were examined.                                                                                                 209

*2.7. Sperm functionality analysis*                                                                            210

Sperm functionality parameters were analyzed by flow cytometry and included:                                  211
plasma membrane integrity (SYBR14/PI), acrosome integrity (*Arachis hypogaea* (peanut)                         212
agglutinin-fluorescein isothiocyanate (PNA-FITC)/Propidium iodide (PI)), mitochondrial                         213
membrane potential (MMP; 5,5′,6,6′-tetrachloro-1,1′3,3′tetraethyl-benzimidazolylcarbo-                         214
cyanine iodide (JC-1)), intracellular levels of ROS (2,7-dichlorodihydrofluorescein and di-                    215
acetate (H$_2$DCFDA)/PI) and superoxides (hydroethidine (HE)/1-(4-[3-methyl-2,3-dihydro-                       216
(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethylammonium propane dio-                              217
dide (YO-PRO-1)), and plasma membrane lipid disorder (Merocyanine 540 (M540)/YO-                               218
PRO-1). The flow cytometer used was a CytoFLEX (Beckman Coulter Fullerton, CA, USA)                           219
with a sheath flow rate set at 10 μL/min. Fluorochromes were purchased from Molecular                          220
Probes® (Thermo Fisher Scientific, Waltham, MA, USA) and were resuspended in dime-                             221
thyl sulfoxide (DMSO; Merck). Analyses were performed following the recommendations                            222
of the International Society for Advance Cytometry (ISAC) [52]. Prior to staining, sperm                       223
concentration was adjusted to 1 × 10$^6$ sperm/mL. In each sample, a total of 10,000 events                    224
were analyzed and three technical replicates were evaluated.                                                   225

Samples were excited with an argon ion laser (488 nm) at a power of 50 mW. Distri-                            226
butions of two different dot plots were used to exclude (1) cellular aggregates, based on                      227
the dot plot distribution of forward scatter height (FSC-H) and altitude (FSC-A), and (2)                      228
cellular debris, as a function of FSC-A distribution and side scatter altitude (SSC-A) dot                     229
plots. Four different optical filters were used: (1) FITC with a band-pass of 525-540 nm for                   230
analysis of SYBR14, PNA-FITC, JC-1 monomers (JC-1$_{mon}$), dichlorofluorescein (DCF$^+$) and                  231
(YO-PRO-1), (2) PE with a band-pass of 585-542 nm for analysis of JC-1 aggregates (JC-                         232
1$_{agg}$) and fluorescent ethidium (E$^+$), (3) ECD with a band-pass of 610-620 nm for analysis               233
of M540, and (4) PC5.5 with a band-pass of 690-650 nm for analysis of PI. The information                     234
obtained regarding each event (FSC-A, FSC-H, SSC-A, FITC, PE and PC5.5) was collected                         235
in xit files and analyzed using the CytExpert analysis software (Beckman Coulter Fuller-                      236
ton) to quantify sperm populations. For each parameter, the corresponding mean and                            237
standard error of the mean (SEM) were calculated.                                                             238

2.7.1. Plasma membrane integrity (SYBR14/PI)                                                                   239

Plasma membrane integrity of sperm was analyzed using the LIVE/DEAD sperm vi-                                 240
ability kit (SYBR14/PI), according to the protocol described by Garner and Johnson [53],                       241
adapted to horse sperm. Briefly, semen samples were incubated with SYBR14 (final con-                          242
centration: 31.8 nM) for 10 min and then with PI (final concentration: 7.6 μM) for 5 min in                    243
the dark at 37 °C. Three sperm populations were identified: (1) sperm with an intact                           244
plasma membrane (SYBR14$^+$/PI$^-$; viable sperm), (2) sperm with a damaged plasma mem-                        245
brane (SYBR14$^-$/PI$^+$), and (3) sperm with a damaged plasma membrane (SYBR14$^+$/PI$^+$). Par-               246
ticles without staining (SYBR14$^-$/PI$^-$) were considered as non-sperm debris and were used                 247
to correct the data in the other assessments. SYBR14 overflow in channel PC5.5 (8.34%)                         248
was compensated.                                                                                               249

2.7.2. Acrosome integrity (PNA-FITC/PI)                                                                        250

Acrosome integrity of sperm was analyzed using the combination of PNA-FITC and                                251
PI, according to the protocol described by Rathi et al. [54]. Briefly, semen samples were                      252

incubated in the dark at 37 °C for 10 min with PNA conjugated with FITC (final concentration: 1.17 μg/mL) and with PI (final concentration: 5.6 μM). Four sperm populations were identified: (1) viable sperm with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$), (2) sperm with a damaged plasma membrane that exhibited an outer acrosome membrane that could not be completely intact (PNA-FITC$^+$/PI$^+$), (3) sperm with damaged plasma membrane together with fully-lost outer acrosome membrane (PNA-FITC$^-$/PI$^+$), and (4) sperm with damaged plasma membrane (PNA-FITC$^+$/PI$^-$). No compensation was needed.

### 2.7.3. Mitochondrial membrane potential (JC-1)

Mitochondrial membrane potential (MMP) of sperm was analyzed using JC-1, according to the protocol described by Ortega-Ferrusola et al. [55]. In brief, semen samples were incubated in the dark at 37 °C for 30 min with JC-1 (final concentration: 750 nM). JC-1 molecules remain as green fluorescent monomers (JC-1$_{mon}$) in the presence of low MMP, while forming orange fluorescent aggregates (JC-1$_{agg}$) in the presence of high MMP. Two sperm populations were identified: (1) sperm exhibiting low MMP (JC-1$_{mon}$ fluorescence intensity higher than JC-1$_{agg}$), and (2) sperm exhibiting high MMP (JC-1$_{agg}$ fluorescence intensity higher than JC-1$_{mon}$). In each population, the fluorescence intensity of JC-1$_{mon}$ and JC-1$_{agg}$ was recorded and the ratio between them was calculated. Data were not compensated.

### 2.7.4. Intracellular reactive oxygen species: total ROS (H$_2$DCFDA/PI) and O$_2^-$ (HE/YO-PRO-1)

Intracellular ROS levels of sperm were analyzed using oxidation-sensitive fluorescent probes: H$_2$DCFDA to assess overall ROS and HE to measure superoxide anion (O$_2^-$) [56]. The differentiation of viable and non-viable spermatozoa was performed using PI (for H$_2$DCFDA) or YO-PRO-1 (for HE), following the modified protocol of Guthrie and Welch [57].

For overall ROS measurement, semen samples were incubated with H$_2$DCFDA (final concentration: 50 μM) at 37 °C in the dark for 20 min, and then with PI (final concentration: 6 μM) for 5 min. In presence of ROS, there is a de-esterification and oxidation of H$_2$DCFDA to DCF$^+$, which is a highly fluorescent molecule. Four sperm populations were identified: (1) viable sperm with high ROS levels (DCF$^+$/PI$^-$), (2) non-viable sperm with high ROS levels (DCF$^+$/PI$^+$), (3) viable sperm with low ROS levels (DCF$^-$/PI$^-$), and (4) non-viable sperm with low ROS levels (DCF$^-$/PI$^+$). DCF$^+$ fluorescence intensity was recorded in all sperm populations. Data were not compensated.

For O$_2^-$ analysis, semen samples were incubated with HE (final concentration: 5 μM) and YO-PRO-1 (final concentration: 31.25 nM) at 37 °C in the dark for 30 min. In presence of O$_2^-$, HE is oxidized into E$^+$. Four sperm populations were identified: (1) viable sperm with high O$_2^-$ levels (E$^+$/YO-PRO-1$^-$), (2) non-viable sperm with high O$_2^-$ levels (E$^+$/YO-PRO-1$^+$), (3) viable sperm with low O$_2^-$ levels (E$^-$/YO-PRO-1$^-$), and (4) non-viable sperm with low O$_2^-$ levels (E$^-$/YO-PRO-1$^+$). Fluorescence intensity of E$^+$ was recorded in all sperm populations. The overflow of E$^+$ to the FITC channel (3.62%) was compensated.

### 2.7.5. Plasma membrane lipid disorder (M540/YO-PRO-1)

Plasma membrane lipid disorder of sperm was analyzed using the combination of M540/YO-PRO-1, according to the protocol described by Rathi et al. [54] with minor modifications [58], adapted to horse sperm. Briefly, semen samples were incubated in the dark at 37 °C for 10 min with M540 (final concentration: 2.5 μM) and YO-PRO-1 (final concentration: 25 nM). Four sperm populations were identified: (1) viable sperm with high plasma membrane lipid disorder (M540$^+$/YO-PRO-1$^-$), (2) non-viable sperm with high plasma membrane lipid disorder (M540$^+$/YO-PRO-1$^+$), (3) viable sperm with low plasma membrane lipid disorder (M540$^-$/YO-PRO-1$^-$), and (4) non-viable sperm with low plasma membrane lipid disorder (M540$^-$/YO-PRO-1$^+$). Data were not compensated.

*2.8. Statistical analysis* 303

Data were analyzed with R statistical package (V 4.0.3, R Core Team; Vienna, Austria) 304
and graphs were made with the GraphPad Prism software (V 8.4.0, GraphPad Software 305
LLC; San Diego, CA, USA). The first step was to check the normality of data using the 306
Shapiro-Wilk test, as well as the homogeneity of variances using the Levene test. When 307
necessary, arcsine $\sqrt{x}$ was applied to transform data and thus obtain a normal distribution. 308
In all cases, the minimum level of statistical significance was set at $p \leq 0.05$. 309

2.8.1. Classification of ejaculates based on their cryotolerance 310

Classification of the 21 horse ejaculates based on their cryotolerance (GFE and PFE) 311
was performed following the procedure described by Morató et al. [34]. In brief, post- 312
thaw percentages of TM and of sperm with an intact plasma membrane (sperm viability, 313
SYBR14$^+$/PI$^-$) recorded in each sample were taken to perform a complete linkage hierar- 314
chical cluster analysis using Euclidean distances from the mentioned parameters. Results 315
in the text are expressed as means ± SEM. 316

2.8.2. Comparison of antioxidant variables measured in SP between good (GFE) and 317
poor freezability ejaculates (PFE) 318

Comparison between GFE and PFE regarding activity levels of enzymatic (PON1, 319
SOD, CAT, and GPX) and non-enzymatic (CUPRAC, FRAP, and TEAC) antioxidants in 320
SP, and SP-OSI was performed using a *t*-test for independent samples. When, even after 321
transforming the data, a normal distribution was not obtained, the Mann-Whitney test 322
was used as a non-parametric alternative. Results in the text were expressed as means ± 323
SEM. 324

2.8.3. Correlations of antioxidant variables with post-thaw semen parameters 325

The relationship of activity levels of enzymatic antioxidants (PON1, SOD, CAT, and 326
GPX) and non-enzymatic antioxidants (CUPRAC, FRAP, and TEAC) in SP, and SP-OSI 327
with motility parameters (TM, PM, VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) and 328
with sperm functionality parameters (SYBR14$^+$/PI$^-$, PNA-FITC$^-$/PI$^-$, JC-1$_{agg}$, DCF$^+$/PI$^-$, 329
E$^+$/YO-PRO-1$^-$, and M540$^+$/YO-PRO-1$^-$) was evaluated by calculating Pearson's correlation 330
coefficients. 331

**3. Results** 332

*3.1. Classification of horse ejaculates based on their cryotolerance* 333

Classifying the 21 horse ejaculates through hierarchical clusters ($p < 0.001$) based on 334
post-thaw TM and sperm viability (SYBR14$^+$/PI$^-$) separated 13 GFE from 8 PFE. Figure 1 335
shows that the ejaculates classified as GFE exhibited significantly higher values ($p < 0.001$) 336
of both TM and sperm viability (SYBR14$^+$/PI$^-$) compared to those classified as PFE (66.44 337
± 2.61 % vs. 35.54 ± 4.36 %, and 67.90 ± 1.54 % vs. 42.03 ± 2.49 %, respectively). The ranges 338
of TM and sperm viability (SYBR14$^+$/PI$^-$) in both groups of ejaculates are shown in Tables 339
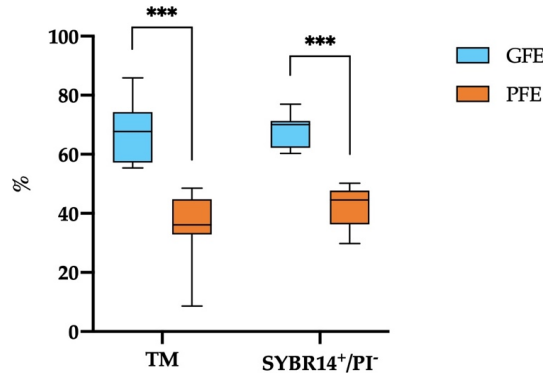S1 and S2, respectively. 340

**Figure 1.** Box-whisker plot showing the percentages of total motile (TM) and viable (SYBR14+/PI−) sperm after thawing in horse ejaculates classified as with good (GFE, *n* = 13; blue) or poor freezability (PFE, *n* = 8; orange). The boxes enclose the 25th and 75th percentiles, the whiskers extend to the 5th and 95th percentiles, and the line indicates the median. (***) $p \le 0.001$.
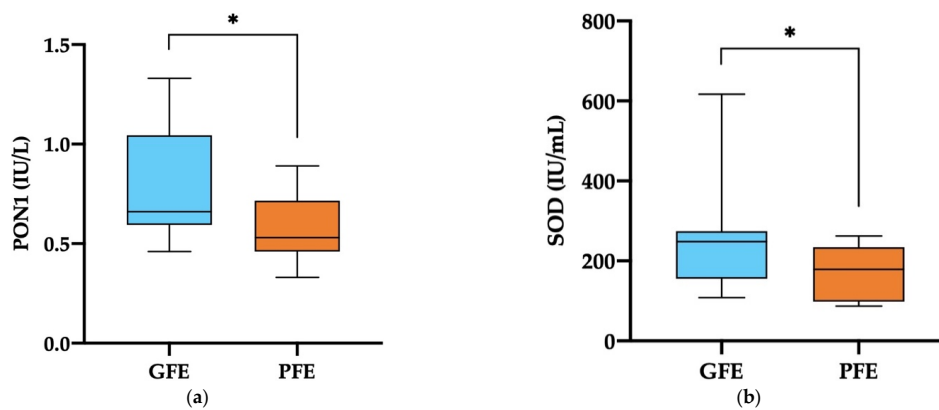
*3.2. Activity levels of enzymatic antioxidants in the SP of horse ejaculates classified as GFE and PFE*

The ejaculates classified as GFE showed significantly higher activity levels ($p < 0.05$) of PON1 and SOD in their SP than those classified as PFE ($0.80 \pm 0.08$ IU/L vs. $0.58 \pm 0.06$ IU/L, and $245.23 \pm 35.52$ IU/mL vs. $171.38 \pm 24.19$ IU/mL, respectively, Figure 2a,b). No differences regarding the activity levels of CAT and GPX in SP (Figure 2c,d) were found between GFE and PFE. The mean ± SEM and ranges of the activity levels of enzymatic antioxidants measured in the SP of all horse ejaculates are shown in Table S3.
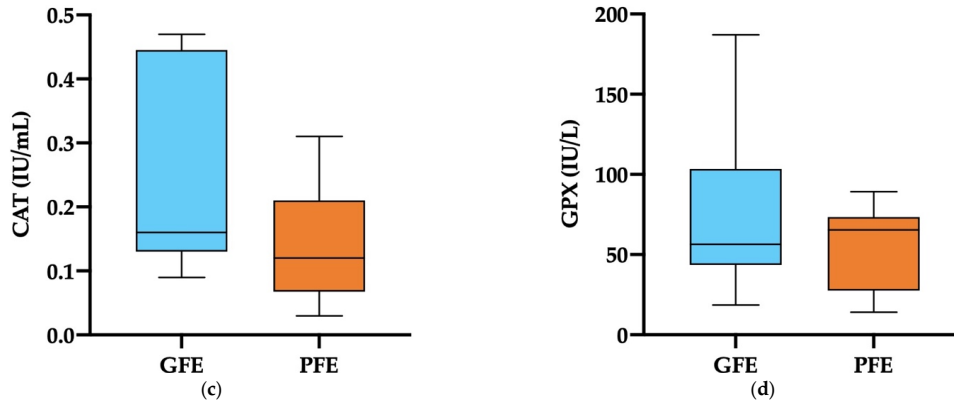


(**a**)       (**b**)

(c)



(d)

**Figure 2.** Box-whisker plot showing the activity levels (IU/mL) of enzymatic antioxidants measured in the seminal plasma of horse ejaculates classified as with good (GFE, $n = 13$; blue) or poor freezability (PFE, $n = 8$; orange): paraoxonase type 1 (PON1, **a**), superoxide dismutase (SOD, **b**), catalase (CAT, **c**), and glutathione peroxidase (GPX, **d**). The boxes enclose the 25th and 75th percentiles, the whiskers extend to the 5th and 95th percentiles, and the line indicates the median. (*) $p \leq 0.05$.
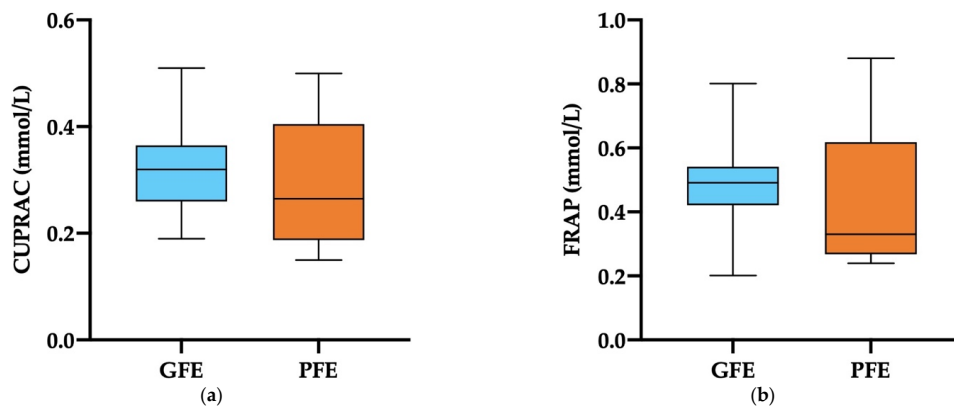
*3.3. Activity levels of non-enzymatic antioxidants in the SP of horse ejaculates classified as GFE and PFE*

The SP-levels of non-enzymatic antioxidants measured in terms of CUPRAC and FRAP did not differ between GFE and PFE (Figure 3a,b). The levels of TEAC in SP, however, were significantly higher ($p < 0.001$) in ejaculates classified as GFE than in those classified as PFE ($0.83 \pm 0.06$ mmol/L vs. $0.47 \pm 0.05$ mmol/L, respectively; Figure 3c). The mean $\pm$ SEM and ranges of non-enzymatic antioxidants measured in the SP of all ejaculates are shown in Table S3.
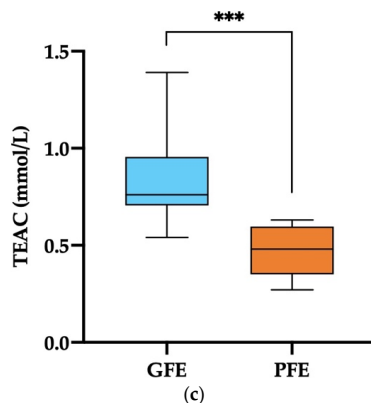


(a)



(b)

**(c)**

**Figure 3.** Box-whisker plot showing non-enzymatic antioxidants in the seminal plasma of horse ejaculates classified as with good (GFE, *n* = 13; blue) or poor freezability (PFE, *n* = 8; orange), measured as cupric-reducing antioxidant capacity (CUPRAC, **a**), ferric-reducing ability of plasma (FRAP, **b**), and Trolox equivalent antioxidant capacity (TEAC, **c**). The boxes enclose the 25th and 75th percentiles, the whiskers extend to the 5th and 95th percentiles, and the line indicates the median. (***) $p \leq 0.001$.

*3.4. Oxidative stress index in the SP of horse ejaculates classified as GFE and PFE*

Ejaculates classified as GFE showed significantly lower SP-OSI levels ($p < 0.001$) compared to those classified as PFE ($6.35 \pm 0.48$ vs. $13.03 \pm 1.80$; Figure 4). The SP levels of TOS in ejaculates classified as GFE were $4.99 \pm 0.18$ µmol/L, and in those classified as PFE were $5.53 \pm 0.31$ µmol/L ($p > 0.05$). The mean ± SEM and ranges of levels OSI and TOS measured in the SP of all horse ejaculates are shown in Table S3.
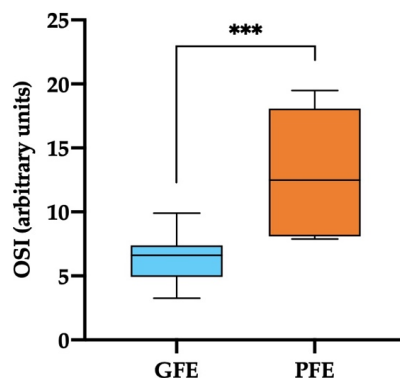


**Figure 4.** Box-whisker plot showing oxidative stress indexes (OSI) (arbitrary units) measured in the seminal plasma of horse ejaculates classified as with good (GFE, *n* = 13; blue) or poor freezability (PFE, *n* = 8; orange). The boxes enclose the 25th and 75th percentiles, the whiskers extend to the 5th and 95th percentiles, and the line indicates the median. (***) $p \leq 0.001$.

*3.6. Correlations between SP antioxidants (enzymatic, non-enzymatic and OSI) and post-thaw sperm motility parameters*

Figure 5 shows the correlations of enzymatic and non-enzymatic antioxidants, and OSI levels measured in the SP of horse ejaculates with post-thaw sperm motility parameters. Activity levels of four enzymatic antioxidants (PON1, SOD, CAT, and GPX) were positively correlated ($p < 0.05$) with PM (PON1: r = 0.46; SOD: r = 0.47; CAT: r = 0.53; GPX: r = 0.48). In addition, activity levels of PON1 and CAT were also positively correlated with

TM (r = 0.50, $p < 0.05$; and r = 0.61, $p < 0.01$, respectively) and with several kinematic parameters such as: VCL (r = 0.44, $p < 0.05$; and r = 0.56, $p < 0.01$, respectively), VAP (r = 0.50, $p < 0.05$; and r = 0.51, $p < 0.05$, respectively), VSL in the case of PON1 (r = 0.45, $p < 0.05$), and ALH in the case of CAT (r = 0.53, $p < 0.05$). For non-enzymatic antioxidants, only TEAC was positively correlated with TM (r = 0.65, $p < 0.01$) and PM (r = 0.62, $p < 0.01$), as well as with the following parameters kinematic: VCL (r = 0.62, $p < 0.01$), VSL (r = 0.51, $p < 0.05$), VAP (r = 0.54, $p < 0.05$), and ALH (r = 0.62, $p < 0.01$). Finally, OSI was negatively correlated with TM (r = –0.72, $p < 0.001$) and PM (r = –0.65, $p < 0.01$), and the following kinematic parameters: VCL (r = – 0.68, $p < 0.001$), VSL (r = –0.60, $p < 0.01$), VAP (r = –0.64, $p < 0.01$), ALH (r = –0.67, $p < 0.001$), and BCF (r = –0.43, $p < 0.05$). The mean ± SEM and ranges of each sperm motility parameter in GFE and PFE recorded post-thaw are shown in Table S1.
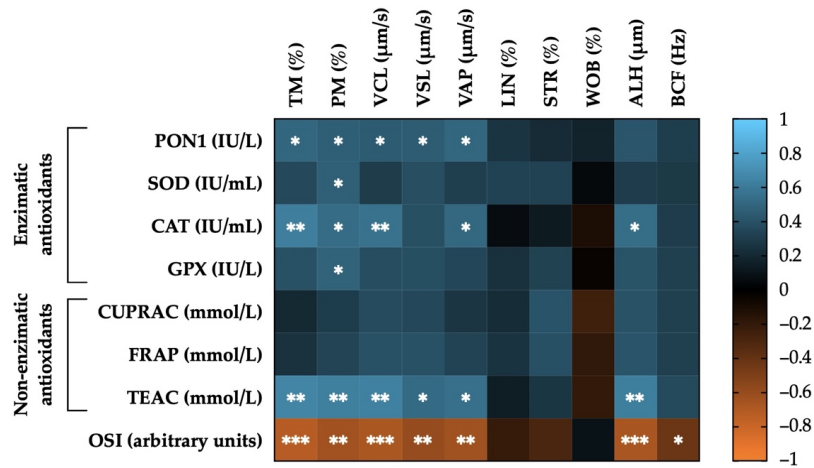


**Figure 5.** Heat map showing the correlations of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX) and non-enzymatic antioxidants (measured in terms of: cupric-reducing antioxidant capacity, CUPRAC; ferric-reducing ability of plasma, FRAP; and Trolox equivalent antioxidant capacity, TEAC), as well as of oxidative stress index (OSI) in the seminal plasma of horse ejaculates ($n = 21$) with post-thaw sperm motility parameters (total motility, TM; progressive motility, PM; curvilinear velocity, VCL; straight line velocity, VSL; average path velocity, VAP; linearity coefficient, LIN; straightness coefficient, STR; wobble coefficient, WOB; amplitude of lateral head displacement, ALH; and beat-cross frequency, BCF). The colors on the scale (1 to –1) indicate whether the correlation is positive (blue) or negative (orange). (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

*3.7. Correlations between SP antioxidants (enzymatic, non-enzymatic and OSI) levels and post-thaw sperm functionality parameters*

Figure 6 shows the correlations of enzymatic and non-enzymatic antioxidants, and OSI measured in the SP of horse ejaculates with post-thaw sperm functionality parameters. Enzymatic antioxidant activity levels were positively correlated with several sperm functionality parameters: PON1 and CAT with plasma membrane integrity (SYBR14+/PI–; r = 0.46, $p < 0.05$; r = 0.45, $p < 0.05$, respectively), SOD and GPX with acrosome and plasma membrane integrity (PNA-FITC–/PI–; r = 0.59, $p < 0.01$; and r = 0.67, $p < 0.001$, respectively), and GPX with the proportion of viable sperm with high MMP (JC-1agg; r = 0.46, $p < 0.05$). Similarly, non-enzymatic antioxidants were positively correlated with several sperm functionality parameters: TEAC with plasma membrane integrity (SYBR14+/PI–; r = 0.57, $p < 0.01$), and CUPRAC and TEAC with acrosome and plasma membrane integrity (PNA-FITC–/PI–; r = 0.52, $p < 0.05$; and r = 0.46, $p < 0.05$, respectively). Finally, OSI was negatively correlated with plasma membrane integrity (SYBR14+/PI–; r = –0.70, $p < 0.001$) and with

acrosome and plasma membrane integrity (PNA-FITC⁻/ PI⁻; r = –0.46, $p < 0.05$), and positively with the proportion of viable sperm with high plasma membrane lipid disorder (M540⁺/YO-PRO-1⁻; r = 0.41, $p < 0.05$). The mean ± SEM and ranges of each sperm functionality parameter in GFE and PFE recorded post-thaw are shown in Table S2.
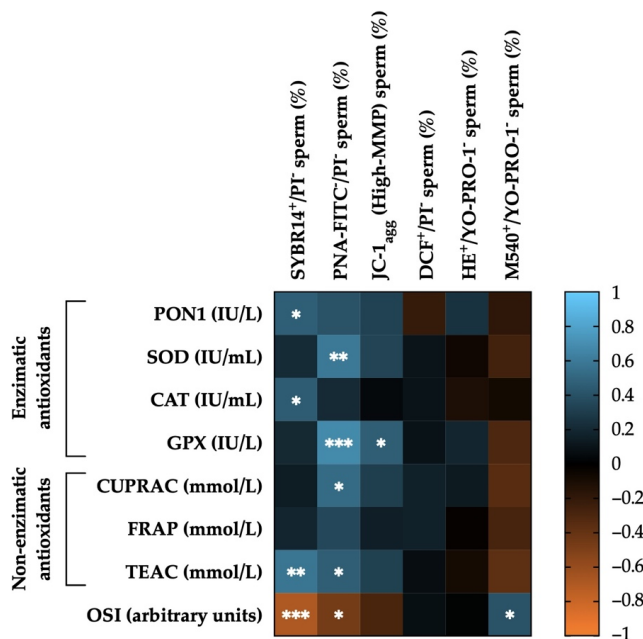


**Figure 6.** Heat map showing the correlations of enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX) and non-enzymatic antioxidants (measured in terms of: cupric-reducing antioxidant capacity, CUPRAC; ferric-reducing ability of plasma, FRAP; and Trolox equivalent antioxidant capacity, TEAC), as well as of oxidative stress index (OSI) in the seminal plasma of horse ejaculates (*n* = 21) with post-thaw sperm functionality parameters (plasma membrane integrity, SYBR14⁺/PI⁻; acrosome membrane integrity, PNA-FITC⁻/PI⁻; mitochondrial membrane potential, MMP, JC-1agg; intracellular ROS levels, DCF⁺/PI⁻; intracellular superoxide levels, E⁺/YO-PRO-1⁻; and plasma membrane lipid disorder, M540⁺/YO-PRO-1⁻). The colors on the scale (1 to –1) indicate whether the correlation is positive (blue) or negative (orange). (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

*3.8. Correlations between seminal plasma antioxidants (enzymatic, non-enzymatic and OSI) in horse ejaculates*

Figure 7 shows the correlations between OSI, enzymatic and non-enzymatic antioxidants measured in the SP of horse ejaculates. Levels of enzymatic and non-enzymatic antioxidants showed positive correlations with each other: PON1 and CAT with GPX (r = 0.57, $p < 0.01$; and r = 0.48, $p < 0.05$, respectively) and with TEAC (r = 0.51, $p < 0.05$, and r = 0.50, $p < 0.05$, respectively); SOD with GPX (r = 0.86, $p < 0.001$) and with non-enzymatic antioxidants (CUPRAC: r = 0.71, $p < 0.001$; FRAP: r = 0.62, $p < 0.01$, and TEAC: r = 0.75, $p < 0.001$); GPX with non-enzymatic antioxidants (CUPRAC: r = 0.72, $p < 0.001$; FRAP: r = 0.64, $p < 0.01$, and TEAC: r = 0.75, $p < 0.001$); CUPRAC with FRAP (r = 0.92, $p < 0.001$) and with TEAC (r = 0.62, $p < 0.01$); and FRAP with TEAC (r = 0.54, $p < 0.05$ ). On the other hand, OSI was negatively correlated with PON1 (r = –0.49, $p < 0.05$), SOD (r = –0.49, $p < 0.05$), GPX (r = –0.54, $p < 0.05$), and with TEAC (r = –0.85, $p < 0.001$). The mean ± SEM and ranges of each enzymatic and non-enzymatic antioxidant's levels, as well as OSI measured in the SP of all horse ejaculates are shown in Table S3.
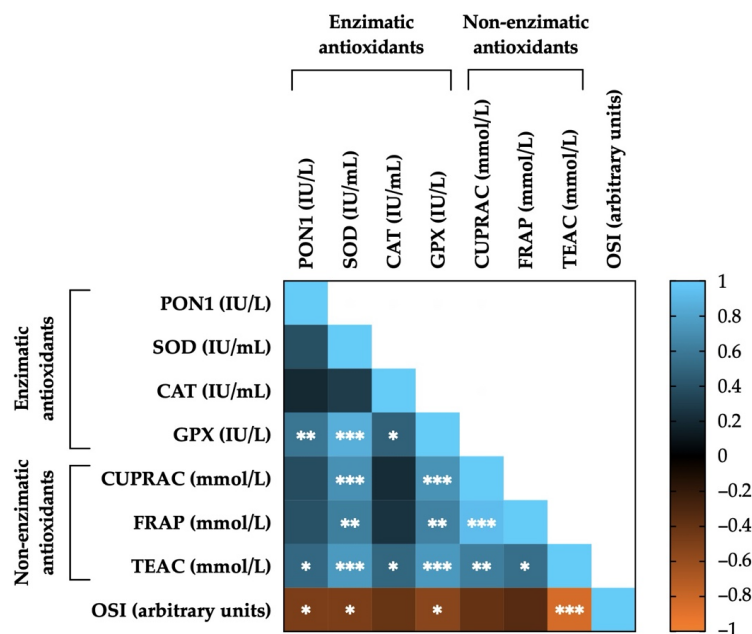
**Figure 7.** Heat map showing the correlations between OSI, enzymatic (paraoxonase type 1, PON1; superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX) and non-enzymatic antioxidants (measured in terms of: cupric-reducing antioxidant capacity, CUPRAC; ferric-reducing ability of plasma, FRAP; and Trolox equivalent antioxidant capacity, TEAC) measured in the seminal plasma of horse ejaculates (*n* = 21). The colors on the scale (1 to -1) indicate whether the correlation is positive (blue) or negative (orange). (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

## 4. Discussion

The present study showed that some SP-components, such enzymatic (PON1 and SOD) and non-enzymatic (measured in terms of TEAC) antioxidants, and SP-OSI, are related to sperm cryotolerance in the horse.

Exposure of sperm to a stressful environment during cryopreservation is associated with an overproduction of ROS [6]. While low levels of ROS have been reported to be beneficial for physiological sperm processes, such as sperm motility, capacitation, and acrosome reaction [59], excessive ROS lead to an imbalance in cellular antioxidant defense and, ultimately, to oxidative stress [26,60]. Due to their limited antioxidant capacity, sperm cannot counteract high levels of ROS [23,61], meaning that the antioxidant responsibility relies on SP, which is removed before freezing [14,29]. It seems, however, that the brief contact between sperm and SP before its removal could be enough for SP antioxidants to exert a beneficial effect upon sperm cryotolerance [14,27,28]. In this sense, the great variability in sperm cryotolerance between stallions could be related to the composition of their SP [14]. Our results agree with this hypothesis, as there were differences between enzymatic, non-enzymatic antioxidants and SP-OSI between GFE and PFE, and these SP components were even correlated to some post-thaw sperm quality and functionality parameters.

Regarding enzymatic antioxidants, we quantified, for the first time in horses, the activity levels of PON1 in SP and we evaluated its relationship with sperm cryotolerance. The results evidenced that the activity levels of this enzyme were higher in the SP of GFE than in that of PFE. These results agree with previous studies conducted in pigs [42] and, more recently, in donkeys [28]. It is also worth noting that the activity levels of PON1 in horse SP were higher (approximately more than two-fold) than those reported in pigs and donkeys, which could represent a species-specificity regarding the antioxidant function

125

of this enzyme. In this regard, this enzyme could play a key role as an antioxidant in horse semen. This hypothesis would be supported by the positive relationship found between PON1, some sperm motility parameters and plasma membrane integrity. In fact, the negative effect of ROS on horse sperm motility is well-known, mainly due to the plasma membrane damage resulting from lipoperoxidation [9,17,18]. In addition, Aitken et al. [62] associated the loss of sperm motility with an increase in the availability of homocysteine thiolactone, a cyclic congener of homocysteine, in sperm. Homocysteine is a non-protein $\alpha$-amino acid that differs from cysteine by having an additional methylene bridge; elevated levels of both homocysteine and homocysteine thiolactone may induce oxidative stress [63]. As a product of plasma membrane lipoperoxidation, lipid aldehydes, such as 4-hydroxynonenal (4-HNE) [64,65], are generated; this favors the accumulation of homocysteine thiolactone in sperm, thus inhibiting PON1 activity [62]. Blockade of PON1 facilitates the direct interaction of homocysteine thiolactone with the $\epsilon$-amino group of lysine residues in sperm proteins, triggering a series of changes that end up with a reduction of sperm motility [12]. Bearing this in mind, it is reasonable to suggest that PON1 in SP may be an essential ROS scavenger in horse semen.

Similarly, our results confirmed that the SOD present in SP is relevant for the cryotolerance of horse sperm, because its activity levels were higher in GFE than in PFE. Furthermore, a positive relationship between SOD activity in SP and some post-thaw sperm quality parameters, such as progressive motility and acrosome membrane integrity, was observed. These results are consistent with those previously reported in horse [14], donkey [27,28], pig [42], buffalo [66], and human SP [67]. SOD is an essential antioxidant to avoid oxidative stress because it initiates a cascade of reactions intended to neutralize the most harmful ROS [42,68]. Specifically, SOD neutralizes $O_2^-$, which, in excess, triggers a dangerous oxidative chain reaction leading to lipoperoxidation of plasma membrane and thereby leads to the loss of motility and sperm death [15]. This would explain the positive relationship of SOD activity with sperm motility and acrosome integrity, as well as the differences between GFE and PFE observed herein and in previous studies with frozen-thawed horse [14] and donkey [27,28] sperm. In addition, it is important to mention that the activity levels of SOD in horse SP observed in this work were lower than those reported in a recent study in donkey SP [28], but higher than those observed in pigs [42], which would indicate a great inter-species variability in SP composition.

As far as CAT and GPX are concerned, we did not find differences in their activity levels between GFE and PFE. This would be in agreement with previous findings in horses [14] and donkeys [27]. In our study, nevertheless, a positive relationship between the activity levels of these antioxidant enzymes and some post-thaw sperm quality and functionality parameters was observed. Supplementing the freezing medium with CAT has a positive impact on post-thaw sperm quality in pigs [69], bulls [70], and humans [71]. Similarly, the addition of other antioxidant enzymes, such as GPX, to the freezing media increases the cryotolerance of buffalo [66], dog [72], and cattle sperm [73]. Dismutation of $O_2^-$ by SOD ends in the formation of hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$); CAT detoxifies the excess of $H_2O_2$ and GPX maintains $H_2O_2$ balance [37,74]. The CAT present in horse SP, therefore, seems to play a key role in preventing the accumulation of $H_2O_2$, which is known to negatively affect sperm in this species [75]. This would be in agreement with our results, as CAT activity in SP was positively related to the percentage of sperm with intact plasma membrane. On the other hand, the $H_2O_2$ initially generated after the action of SOD enters the oxidative/reductive balance system mediated by GPX, which is able maintain this ROS within the physiological levels without altering sperm function and survival [37,74]. This would be in accordance with our results because a positive relationship between GPX activity and acrosome integrity was found. Moreover, the link of CAT with sperm motility parameters has been observed in the liquid-stored semen of other mammalian species [61], such as dogs [76] and sheep [77], and GPX activity has been found to be positively related to the quality of fresh horse semen [78], and frozen-thawed cattle [79] and donkey [28] semen. Curiously, in our study, a positive relationship

between GPX activity levels and mitochondrial membrane potential was observed, which could contribute to explain the relationship between GPX and sperm motility.

Regarding non-enzymatic antioxidants in horse SP (measured in terms of CUPRAC, FRAP, and TEAC), only TEAC had a significant relevance for sperm cryotolerance, showing higher levels in the SP of GFE than in that of PFE. These data are in agreement with previous research in frozen-thawed pig [42] and donkey sperm [28]. TEAC evaluates the antioxidant effect of low molecular weight molecules (such as uric acid, $\alpha$-tocopherol, bilirubin and ascorbic acid (AA)), and of those that contain sulfhydryl groups (-SH) in their structure (such as albumin) [80,81]. The antioxidant molecules containing -SH exert protection from the oxidative damage caused by ROS during cryopreservation, because they act on disulfide bonds between chromatin fibers, thus maintaining the integrity of the nucleoprotein structure [82–84]. In this study, we also found that TEAC in SP was positively related to post-thaw sperm plasma membrane integrity, which was in agreement with previous observations in frozen-thawed donkey sperm [28]. In the present study, we also found that TEAC in SP was positively related to acrosome integrity of post-thaw sperm. Consequently, non-enzymatic antioxidants of SP (assessed by TEAC) could be key to maintain the functional integrity of cryopreserved horse sperm, as the positive relationship between TEAC in SP and post-thaw sperm motility parameters demonstrated. This would be also in line with the results of the aforementioned studies in frozen-thawed pig [42] and donkey [28] sperm. It is also worth noting that the fact that the CUPRAC of SP did not differ between GFE and PFE was in agreement with previous results obtained in frozen-thawed pig sperm [42]. On the contrary, SP-FRAP in donkey [28] and pig semen [42], and SP-CUPRAC in donkey semen [28] were found to differ between GFE and PFE. These inconsistent results between species could be explained by differences in the composition of their SP and their sperm cryotolerance [14,27,28,37,85], as well as by the contribution of individual antioxidants to FRAP (such as AA, $\alpha$-tocopherol, and mainly uric acid) and CUPRAC (such as thiols (including GSH)) [86].

Maintaining a balance between ROS and antioxidant levels is essential for optimal sperm function [9]. While an excess of ROS production can lead to the generation of oxidative stress, controlled ROS levels are essential for sperm physiological processes [59]. In this sense, OSI analysis is considered an accurate method to measure the oxidant/antioxidant ratio in biological samples [44]. Our results reveled that SP-OSI values, which were lower in GFE than in PFE, were negatively related to post-thaw sperm motility, plasma membrane and acrosome membrane integrity. These findings demonstrate that an adequate balance between oxidants and antioxidants in SP is crucial for the ability of horse sperm to withstand freeze-thawing. Similar evidence was reported in liquid-stored pig semen [45] and recently in frozen-thawed donkey sperm [28]. It is well known that an excess of ROS has a negative impact on the activity of some essential enzymes for sperm motility, such as glucose-6-phosphate dehydrogenase [87]. In addition, a ROS/antioxidants imbalance may cause mitochondrial dysfunction, leading to adenosine triphosphate depletion; this decreases in the energy available to sperm results in a reduction of their motility [88]. Interestingly, OSI values in horse SP were higher than those reported in donkey [28] and in pig SP [45]. One may, therefore, posit that the control of oxidative stress is highly complex and species-specific [14]. This hypothesis would be supported by the lack of relationship between the different antioxidants (enzymatic and non-enzymatic), SP-OSI with intracellular ROS levels in post-thaw sperm observed in this study, which differs from that previously reported in donkey [28] and pig [42] semen. Differences in SP-composition, sperm metabolism and reproductive strategy between species could also contribute to explain these distinct outcomes.

Finally, we observed significant correlations between SP-OSI, enzymatic and non-enzymatic antioxidants; this demonstrates, also in horse semen, the great complexity of the oxidative stress control and maintenance of redox balance [89].

**5. Conclusions**

In conclusion, this research reported the relevance of enzymatic and non-enzymatic SP antioxidants, and OSI for horse sperm cryotolerance. This study quantified, for the first time in horse SP, PON1 levels, non-enzymatic antioxidant capacity (in terms of: CUPRAC, FRAP, and TEAC) and OSI. Our results revealed that the levels of some enzymatic (PON1 and SOD) and non-enzymatic (TEAC) antioxidants were higher in the SP of horse ejaculates classified as GFE than in those graded as PFE. A positive relationship between these SP components and some post-thaw sperm quality parameters was also observed (motility and plasma membrane integrity: PON1 and TEAC; acrosome membrane integrity: SOD and TEAC). In contrast, OSI in horse SP, which was higher in PFE than in GFE, was negatively associated to some sperm motility parameters, and plasma membrane and acrosome integrity, and positively to the percentage of viable sperm with high membrane lipid disorder. These findings support how important an adequate balance between oxidants and antioxidants in SP is for the ability of horse sperm to withstand freeze-thawing. In sum, the present work suggests that PON1, SOD, TEAC, and OSI in SP could be used as putative sperm cryotolerance biomarkers in the horse. Measuring these enzymatic and non-enzymatic antioxidants could allow us (1) to select those ejaculates that are likely to withstand better cryopreservation and (2) to identify which semen samples may need antioxidant supplements for their storage.

**References**

1. Alvarenga, M.A.; Papa, F.O.; Ramires Neto, C. Advances in stallion semen cryopreservation. *Vet. Clin. North Am. Equine Pract.* **2016**, *32*, 521–530, doi:10.1016/J.CVEQ.2016.08.003.

2. Aurich, J.; Kuhl, J.; Tichy, A.; Aurich, C. Efficiency of semen cryopreservation in stallions. *Animals* **2020**, *10*, 1033, doi:10.3390/ANI10061033.

3. Palmer, E.; Chavatte-Palmer, P. Contribution of reproduction management and technologies to genetic progress in horse breeding. *J. Equine Vet. Sci.* **2020**, *89*, 103016, doi:10.1016/J.JEVS.2020.103016.

4. Holt, W. V. Basic aspects of frozen storage of semen. *Anim. Reprod. Sci.* **2000**, *62*, 3–22, doi:10.1016/S0378-4320(00)00152-4.

5. Blottner, S.; Warnke, C.; Tuchscherer, A.; Heinen, V.; Torner, H. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. *Anim. Reprod. Sci.* **2001**, *65*, 75–88, doi:10.1016/S0378-4320(00)00214-1.

6. Yeste, M.; Estrada, E.; Rocha, L.G.; Marín, H.; Rodríguez-Gil, J.E.; Miró, J. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology* **2015**, *3*, 395–407, doi:10.1111/andr.291.

7. Vidament, M.; Vincent, P.; Martin, F.X.; Magistrini, M.; Blesbois, E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. *Anim. Reprod. Sci.* **2009**, *112*, 22–35, doi:10.1016/j.anireprosci.2008.03.016.

8. Canisso, I.F.; Carvalho, G.R.; Davis Morel, M.; Ker, P.G.; Rodrigues, A.L.; Silva, E.C.; Coutinho Da Silva, M.A. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Vet. J.* **2011**, *43*, 179–183, doi:10.1111/j.2042-3306.2010.00130.x.

9. Bansal, A.K.; Bilaspuri, G.S. Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med. Int.* **2011**, *686137*, 1–7, doi:10.4061/2011/686137.

10. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **2004**, *37*, 277–285, doi:10.1016/J.CLINBIOCHEM.2003.11.015.

11. Peña, F.J.; O'Flaherty, C.; Ortiz Rodríguez, J.M.; Martín Cano, F.E.; Gaitskell-Phillips, G.L.; Gil, M.C.; Ortega Ferrusola, C. Redox regulation and oxidative stress: the particular case of the stallion spermatozoa. *Antioxidants* **2019**, *8*, 567, doi:10.3390/antiox8110567.

12. Aitken, R.J.; De Iuliis, G.N.; Drevet, J.R. Role of oxidative stress in the etiology of male infertility and the potential therapeutic value of antioxidants. In *Oxidants, Antioxidants, and Impact of the Oxidative Status in Male Reproduction*; Henkel, R., Samanta, L., Agarwal, A., Eds.; Academic Press: London, UK, 2018; pp. 91–100 ISBN 9780128125014.

13. Aurich, C.; Ortega Ferrusola, C.; Peña Vega, F.J.; Schrammel, N.; Morcuende, D.; Aurich, J. Seasonal changes in the sperm fatty acid composition of Shetland pony stallions. *Theriogenology* **2018**, *107*, 149–153, doi:10.1016/J.THERIOGENOLOGY.2017.11.004.

14. Papas, M.; Catalán, J.; Fernandez-Fuertes, B.; Arroyo, L.; Bassols, A.; Miró, J.; Yeste, M. Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance. *Antioxidants* **2019**, *8*, 539, doi:10.3390/antiox8110539.

15. Agarwal, A.; Virk, G.; Ong, C.; du Plessis, S.S. Effect of oxidative stress on male reproduction. *World J. Mens. Health* **2014**, *32*, 1–17, doi:10.5534/WJMH.2014.32.1.1.

16. Dutta, S.; Sengupta, P.; Slama, P.; Roychoudhury, S. Oxidative stress, testicular inflammatory pathways, and male reproduction. *Int. J. Mol. Sci.* **2021**, *22*, 10043, doi:10.3390/IJMS221810043.

17. Baumber, J.; Ball, B.A.; Gravance, C.G.; Medina, V.; Davies-Morel, M.C.G. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *J. Androl.* **2000**, *21*, 895–902, doi:10.1002/j.1939-4640.2000.tb03420.x.

18. Baumber, J.; Vo, A.; Sabeur, K.; Ball, B.A. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology* **2002**, *57*, 1025–1033, doi:10.1016/S0093-691X(01)00710-5.

19. Wathes, D.C.; Abayasekara, D.R.E.; Aitken, R.J. Polyunsaturated fatty acids in male and female reproduction. *Biol. Reprod.* **2007**, *77*, 190–201, doi:10.1095/BIOLREPROD.107.060558.

20. Macías García, B.; González Fernández, L.; Ortega Ferrusola, C.; Morillo Rodríguez, A.; Gallardo Bolaños, J.M.; Rodríguez Martinez, H.; Tapia, J.A.; Morcuende, D.; Peña, F.J. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. *Theriogenology* **2011**, *75*, 811–818, doi:10.1016/J.THERIOGENOLOGY.2010.10.021.

21. Ball, B.A. Role of oxidative stress in normal and abnormal function of equine spermatozoa. *Rev. Bras. Reprodução Anim.* **2009**, *33*, 20–24.

22. Bollwein, H.; Fuchs, I.; Koess, C. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reprod. Domest. Anim.* **2008**, *43*, 189–195, doi:10.1111/J.1439-0531.2007.00876.X.

23. Amidi, F.; Pazhohan, A.; Shabani Nashtaei, M.; Khodarahmian, M.; Nekoonam, S. The role of antioxidants in sperm freezing: a review. *Cell Tissue Bank.* **2016**, *17*, 745–756, doi:10.1007/S10561-016-9566-5.

24. Dutta, S.; Majzoub, A.; Agarwal, A. Oxidative stress and sperm function: a systematic review on evaluation and management. *Arab J. Urol.* **2019**, *17*, 87–97, doi:10.1080/2090598X.2019.1599624.

25. Muiño-Blanco, T.; Pérez-Pé, R.; Cebrián-Pérez, J.A. Seminal plasma proteins and sperm resistance to stress. *Reprod. Domest. Anim.* **2008**, *43*, 18–31, doi:10.1111/j.1439-0531.2008.01228.x.

26. Papas, M.; Arroyo, L.; Bassols, A.; Catalán, J.; Bonilla-Correal, S.; Gacem, S.; Yeste, M.; Miró, J. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology* **2019**, *140*, 180–187, doi:10.1016/j.theriogenology.2019.08.032.

27. Papas, M.; Catalan, J.; Barranco, I.; Arroyo, L.; Bassols, A.; Yeste, M.; Miró, J. Total and specific activities of superoxide dismutase (SOD) in seminal plasma are related with the cryotolerance of jackass spermatozoa. *Cryobiology* **2020**, *92*, 109–116, doi:10.1016/j.cryobiol.2019.11.043.

28. Catalán, J.; Yánez-Ortiz, I.; Tvarijonaviciute, A.; González-Arostegui, L.G.; Rubio, C.P.; Yeste, M.; Miró, J.; Barranco, I. Impact of seminal plasma antioxidants on donkey sperm cryotolerance. *Antioxidants* **2022**, *11*, 417, doi:10.3390/ANTIOX11020417/S1.

29. Catalán, J.; Llavanera, M.; Bonilla-Correal, S.; Papas, M.; Gacem, S.; Rodríguez-Gil, J.E.; Yeste, M.; Miró, J. Irradiating frozen-thawed stallion sperm with red-light increases their resilience to withstand post-thaw incubation at 38 °C. *Theriogenology* **2020**, *157*, 85–95, doi:10.1016/J.THERIOGENOLOGY.2020.07.027.

30. Sieme, H.; Katila, T.; Klug, E. Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. *Theriogenology* **2004**, *61*, 769–784, doi:10.1016/S0093-691X(03)00251-6.

31. Yeste, M.; Estrada, E.; Casas, I.; Bonet, S.; Rodríguez-Gil, J.E. Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. *Theriogenology* **2013**, *79*, 929–939, doi:10.1016/j.theriogenology.2013.01.008.

32. Alvarenga, M.A.; Leao, K.M.; Papa, F.O.; Landim--Alvarenga, F.C.; Medeiros, A.S.L.; Gomes, G.M. The use of alternative cryoprotectors for freezing stallion semen. In Proceedings of the Workshop on Transporting Gametes and Embryos; Brewster, MA, USA, 2003; pp. 74–76.

33. Loomis, P.R.; Graham, J.K. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. *Anim. Reprod. Sci.* **2008**, *105*, 119–128, doi:10.1016/J.ANIREPROSCI.2007.11.010.

34. Morató, R.; Prieto-Martínez, N.; Muiño, R.; Hidalgo, C.O.; Rodríguez-Gil, J.E.; Bonet, S.; Yeste, M. Aquaporin 11 is related to cryotolerance and fertilising ability of frozen–thawed bull spermatozoa. *Reprod. Fertil. Dev.* **2018**, *30*, 1099–1108, doi:10.1071/RD17340.

35. Mogielnicka-Brzozowska, M.; Kordan, W. Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. *Pol. J. Vet. Sci.* **2011**, *14*, 489–499, doi:10.2478/V10181-011-0074-Z.

36. Bromfeld, J.J. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* **2016**, *152*, R223–R232, doi:10.1530/REP-16-0313.

37. Baumber, J.; Ball, B.A. Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. *Am. J. Vet. Res.* **2005**, *66*, 1415–1419, doi:10.2460/AJVR.2005.66.1415.

38. Verit, F.F.; Verit, A.; Ciftci, H.; Erel, O.; Çelik, H. Paraoxonase-1 activity in subfertile men and relationship to sperm parameters. *J. Androl.* **2009**, *30*, 183–189, doi:10.2164/JANDROL.108.004929.

39. Marsillach, J.; Lafuente, R.; Checa, M.A.; Maestre-Martínez, C.; Fabián, E.; Brassesco, M.; Beltrán-Debón, R.; Aragonès, G.; Carreras, R.; Pedro-Botet, J.; et al. Paraoxonase-1 is only present in traceable amounts in seminal fluid and does not show any relationship with male subfertility. *BJU Int.* **2011**, *108*, 566–570, doi:10.1111/J.1464-410X.2010.09888.X.

40. 4Barranco, I.; Roca, J.; Tvarijonaviciute, A.; Rubér, M.; Vicente-Carrillo, A.; Atikuzzaman, M.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H. Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Mol. Reprod. Dev.* **2015**, *82*, 58–65, doi:10.1002/MRD.22444.

41. Barranco, I.; Tvarijonaviciute, A.; Perez-Patiño, C.; Alkmin, D. V.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality, and in vivo fertility. *Andrology* **2015**, *3*, 315–320, doi:10.1111/ANDR.309.

42. Li, J.; Barranco, I.; Tvarijonaviciute, A.; Molina, M.F.; Martinez, E.A.; Rodriguez-Martinez, H.; Parrilla, I.; Roca, J. Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. *Theriogenology* **2018**, *107*, 27–35, doi:10.1016/J.THERIOGENOLOGY.2017.10.035.

43. Wagner, H.; Cheng, J.W.; Ko, E.Y. Role of reactive oxygen species in male infertility: an updated review of literature. *Arab J. Urol.* **2018**, *16*, 35, doi:10.1016/J.AJU.2017.11.001.

44. Abuelo, A.; Hernández, J.; Benedito, J.L.; Castillo, C. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal* **2013**, *7*, 1374–1378, doi:10.1017/S1751731113000396.

45. Barranco, I.; Rubio, C.P.; Tvarijonaviciute, A.; Rodriguez-Martinez, H.; Roca, J. Measurement of oxidative stress index in seminal plasma can predict in vivo fertility of liquid-stored porcine artificial insemination semen doses. *Antioxidants* **2021**, *10*, 1203, doi:10.3390/ANTIOX10081203/S1.

46. Kenney, M.R. Minimal contamination techniques for breeding mares: techniques and priliminary findings. *Proc. Am. Assoc. Equine Pract.* **1975**, 327–336.

47. Bamba, K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology* **1988**, *29*, 1245–1251, doi:10.1016/0093-691X(88)90004-0.

48. Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, Á. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: the CUPRAC–BCS assay. *Anal. Biochem.* **2009**, *392*, 37–44, doi:10.1016/J.AB.2009.05.024.

49. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76, doi:10.1006/ABIO.1996.0292.

50. Wu, R.; Feng, J.; Yang, Y.; Dai, C.; Lu, A.; Li, J.; Liao, Y.; Xiang, M.; Huang, Q.; Wang, D.; et al. Significance of serum total oxidant/antioxidant status in patients with colorectal cancer. *PLoS One* **2017**, *12*, e0170003, doi:10.1371/JOURNAL.PONE.0170003.
51. Erel, O. A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* **2005**, *38*, 1103–1111, doi:10.1016/J.CLINBIOCHEM.2005.08.008.
52. Lee, J.A.; Spidlen, J.; Boyce, K.; Cai, J.; Crosbie, N.; Dalphin, M.; Furlong, J.; Gasparetto, M.; Goldberg, M.; Goralczyk, E.M.; et al. MIFlowCyt: the minimum information about a flow cytometry experiment. *Cytometry* **2008**, *73*, 926–930, doi:10.1002/cyto.a.20623.
53. Garner, D.L.; Johnson, L.A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **1995**, *53*, 276–284, doi:10.1095/biolreprod53.2.276.
54. Rathi, R.; Colenbrander, B.; Bevers, M.M.; Gadella, B.M. Evaluation of in vitro capacitation of stallion spermatozoa. *Biol. Reprod.* **2001**, *65*, 462–470, doi:10.1095/biolreprod65.2.462.
55. Ortega-Ferrusola, C.; Sotillo-Galán, Y.; Varela-Fernández, E.; Gallardo-Bolaños, J.M.; Muriel, A.; González-Fernández, L.; Tapia, J.A.; Peña, F.J. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. *J. Androl.* **2008**, *29*, 213–221, doi:10.2164/jandrol.107.003640.
56. Catalán, J.; Papas, M.; Trujillo-Rojas, L.; Blanco-Prieto, O.; Bonilla-Correal, S.; Rodríguez-Gil, J.E.; Miró, J.; Yeste, M. Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light. *Front. Cell Dev. Biol.* **2020**, *8*, 588621, doi:10.3389/fcell.2020.588621.
57. Guthrie, H.D.; Welch, G.R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J. Anim. Sci.* **2006**, *84*, 2089–2100, doi:10.2527/jas.2005-766.
58. Yeste, M.; Estrada, E.; Rivera del Álamo, M.M.; Bonet, S.; Rigau, T.; Rodríguez-Gil, J.E. The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17°C is concomitant with a higher cryotolerance of boar spermatozoa. *PLoS One* **2014**, *9*, 90887, doi:10.1371/journal.pone.0090887.
59. Zan-Bar, T.; Bartoov, B.; Segal, R.; Yehuda, R.; Lavi, R.; Lubart, R.; Avtalion, R.R. Influence of visible light and ultraviolet irradiation on motility and fertility of mammalian and fish sperm. *Photomed. Laser Surg.* **2005**, *23*, 549–555, doi:10.1089/PHO.2005.23.549.
60. Sies, H. Oxidative stress: a concept in redox biology and medicine. *Redox Biol.* **2015**, *4*, 180–183, doi:10.1016/J.REDOX.2015.01.002.
61. Silvestre, M.A.; Yániz, J.L.; Peña, F.J.; Santolaria, P.; Castelló-Ruiz, M. Role of antioxidants in cooled liquid storage of mammal spermatozoa. *Antioxidants* **2021**, *10*, 1096, doi:10.3390/ANTIOX10071096.
62. Aitken, R.J.; Flanagan, H.M.; Connaughton, H.; Whiting, S.; Hedges, A.; Baker, M.A. Involvement of homocysteine, homocysteine thiolactone, and paraoxonase type 1 (PON-1) in the etiology of defective human sperm function. *Andrology* **2016**, *4*, 345–360, doi:10.1111/ANDR.12157.
63. Sibrian-Vazquez, M.; Escobedo, J.O.; Lim, S.; Samoei, G.K.; Strongin, R.M. Homocystamides promote free-radical and oxidative damage to proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 551–554, doi:10.1073/PNAS.0909737107/SUPPL_FILE/APPENDIX.PDF.
64. 6Martin Muñoz, P.; Ortega Ferrusola, C.; Vizuete, G.; Plaza Dávila, M.; Rodriguez Martinez, H.; Peña, F.J. Depletion of intracellular thiols and increased production of 4-hydroxynonenal that occur during cryopreservation of stallion spermatozoa lead to caspase activation, loss of motility, and cell death. *Biol. Reprod.* **2015**, *93*, 1–11, doi:10.1095/BIOLREPROD.115.132878/2434430.
65. Gibb, Z.; Lambourne, S.R.; Curry, B.J.; Hall, S.E.; Aitken, R.J. Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. *Biol. Reprod.* **2016**, *94*, 133–134, doi:10.1095/BIOLREPROD.116.140509/2863959.
66. Waheed, M.M.; Gouda, E.M.; Khalifa, T.A.A. Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. *Anim. Reprod. Sci.* **2013**, *142*, 126–130, doi:10.1016/J.ANIREPROSCI.2013.09.008.
67. Buffone, M.G.; Calamera, J.C.; Brugo-Olmedo, S.; De Vincentiis, S.; Calamera, M.M.; Storey, B.T.; Doncel, G.F.; Alvarez, J.G. Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa. *Fertil. Steril.* **2012**, *97*, 293–298, doi:10.1016/J.FERTNSTERT.2011.11.012.
68. Kowalowka, M.; Wysocki, P.; Fraser, L.; Strzezek, J. Extracellular superoxide dismutase of boar seminal plasma. *Reprod. Domest. Anim.* **2008**, *43*, 490–496, doi:10.1111/J.1439-0531.2007.00943.X.
69. Roca, J.; Rodríguez, M.J.; Gil, M.A.; Carvajal, G.; Garcia, E.M.; Cuello, C.; Vazquez, J.M.; Martinez, E.A. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J. Androl.* **2005**, *26*, 15–24, doi:10.1002/J.1939-4640.2005.TB02867.X.
70. Paudel, K.P.; Kumar, S.; Meur, S.K.; Kumaresan, A. Ascorbic acid, catalase and chlorpromazine reduce cryopreservation-induced damages to crossbred bull spermatozoaa. *Reprod. Domest. Anim.* **2010**, *45*, 256–262, doi:10.1111/J.1439-0531.2008.01278.X.
71. Moubasher, A.E.; El Din, A.M.E.; Ali, M.E.; El-Sherif, W.T.; Gaber, H.D. Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. *Andrologia* **2013**, *45*, 135–139, doi:10.1111/J.1439-0272.2012.01310.X.
72. Chatdarong, K.; Chaivechakarn, A.; Thuwanut, P.; Ponglowhapan, S. Effects of cold storage prior to freezing on superoxide dismutase, glutathione peroxidase activities, level of total reactive oxygen species and sperm quality in dogs. *Reprod. Domest. Anim.* **2012**, *47*, 274–277, doi:10.1111/RDA.12009.

73. Losano, J.D.A.; Angrimani, D.S.R.; Rui, B.R.; Bicudo, L.C.; Dalmazzo, A.; Silva, B.C.S.; Viana, C.H.C.; Mendes, C.M.; Assumpção, M.E.O.A.; Barnabe, V.H.; et al. The addition of docosahexaenoic acid (DHA) and antioxidants (glutathione peroxidase and superoxide dismutase) in extenders to epididymal sperm cryopreservation in bulls. *Zygote* **2018**, *26*, 199–206, doi:10.1017/S0967199418000096.

74. Papas, M.; Catalan, J.; Bonilla-Correal, S.; Gacem, S.; Miró, J.; Yeste, M. Seminal plasma has limited counteracting effects following induction of oxidative stress in donkey spermatozoa. *Reprod. Fertil. Dev.* **2020**, *32*, 619–628, doi:10.1071/RD19192.

75. Ball, B.A.; Gravance, C.G.; Medina, V.; Baumber, J.; Liu, I.K.M. Catalase activity in equine semen. *Am. J. Vet. Res.* **2000**, *61*, 1026–1030, doi:10.2460/AJVR.2000.61.1026.

76. Michael, A.J.; Alexopoulos, C.; Pontiki, E.A.; Hadjipavlou-Litina, D.J.; Saratsis, P.; Ververidis, H.N.; Boscos, C.M. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim. Reprod. Sci.* **2009**, *112*, 119–135, doi:10.1016/J.ANIREPROSCI.2008.04.007.

77. Câmara, D.R.; Mello-Pinto, M.M.C.; Pinto, L.C.; Brasil, O.O.; Nunes, J.F.; Guerra, M.M.P. Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. *Small Rumin. Res.* **2011**, *100*, 44–49, doi:10.1016/j.smallrumres.2011.05.010.

78. Waheed, M.M.; El-Bahr, S.M.; Al-haider, A.K. Influence of seminal plasma antioxidants and osteopontin on fertility of the Arabian horse. *J. Equine Vet. Sci.* **2013**, *33*, 705–709, doi:10.1016/J.JEVS.2012.11.006.

79. Bilodeau, J.F.; Blanchette, S.; Gagnon, C.; Sirard, M.A. Thiols prevent $H_2O_2$-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* **2001**, *56*, 275–286, doi:10.1016/S0093-691X(01)00562-3.

80. Rubio, C.P.; Hernández-Ruiz, J.; Martinez-Subiela, S.; Tvarijonaviciute, A.; Ceron, J.J. Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update. *BMC Vet. Res.* **2016**, *12*, 1–7, doi:10.1186/S12917-016-0792-7/FIGURES/3.

81. Cecchini Gualandi, S.; Giangaspero, B.; Di Palma, T.; Macchia, G.; Carluccio, A.; Boni, R. Oxidative profile and protease regulator potential to predict sperm functionality in donkey (*Equus asinus*). *Sci. Rep.* **2021**, *11*, 20551, doi:10.1038/s41598-021-99972-9.

82. Irvine, D.S. Glutathione as a treatment for male infertility. *Rev. Reprod.* **1996**, *1*, 6–12, doi:10.1530/ROR.0.0010006.

83. 8Luberda, Z. The role of glutathione in mammalian gametes. *Reprod. Biol.* **2005**, *5*, 5–17.

84. Yeste, M.; Flores, E.; Estrada, E.; Bonet, S.; Rigau, T.; Rodríguez-Gil, J.E. Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-thawing by stabilising disulfide bonds. *Reprod. Fertil. Dev.* **2013**, *25*, 1036–1050, doi:10.1071/RD12230.

85. Jonakova, V.; Jonak, J.; Ticha, M. Proteomics of male seminal plasma. In *Reproductive Genomics in Domestic Animals*; Jiang, Z., Ott, T.L., Eds.; Wiley-Blackwell: Oxford, UK, 2010; pp. 339–366 ISBN 9780813817842.

86. Rubio, C.P.; Martínez-Subiela, S.; Hernández-Ruiz, J.; Tvarijonaviciute, A.; Cerón, J.J.; Allenspach, K. Serum biomarkers of oxidative stress in dogs with idiopathic inflammatory bowel disease. *Vet. J.* **2017**, *221*, 56–61, doi:10.1016/J.TVJL.2017.02.003.

87. Aitken, R.J.; Fisher, H.M.; Fulton, N.; Gomez, E.; Knox, W.; Lewis, B.; Irvine, S. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol. Reprod. Dev* **1997**, *47*, 468–482, doi:10.1002/(SICI)1098-2795(199708)47:4.

88. de Lamirande, E.; Gagnon, C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J. Androl.* **1992**, *13*, 379–386, doi:10.1002/J.1939-4640.1992.TB03328.X.

89. Gadea, J.; Sellés, E.; Marco, M.A.; Coy, P.; Matás, C.; Romar, R.; Ruiz, S. Decrease in glutathione content in boar sperm after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology* **2004**, *62*, 690–701, doi:10.1016/J.THERIOGENOLOGY.2003.11.013.

# CAPÍTULO 4:

# DISCUSIÓN

Los resultados obtenidos en esta Tesis Doctoral mostraron la elevada tolerancia de los espermatozoides de burro al estrés oxidativo, que fue superior a la resistencia al estrés reductivo. Asimismo, se observó que los espermatozoides producen una elevada cantidad de ROS (medida en niveles de $H_2O_2$) cuando interactúan con las células del sistema inmunitario (PMN). Además, se determinó que la adición de GSH al medio de congelación modula la producción de ROS por parte de los espermatozoides de burro congelados-descongelados. Finalmente, se evidenció la importancia que tienen los antioxidantes enzimáticos y no enzimáticos y el OSI, medidos en el plasma seminal, para la resistencia de los espermatozoides de burro y caballo a los procesos de congelación y descongelación.

La combinación de los espermatozoides con el plasma seminal convierte al semen en un complejo sistema oxidante/antioxidante (Gadea et al., 2004). Mientras que los espermatozoides generan ROS como producto de su metabolismo (Peña et al., 2019), el plasma seminal constituye el principal sistema de defensa antioxidante (Muiño-Blanco et al., 2008; Papas et al., 2019a; Papas et al., 2019b; Papas et al., 2020a). Los espermatozoides de burro presentan una velocidad y progresividad significativamente superiores a las de los espermatozoides de caballo (Gacem et al., 2021), debido a que los primeros presentan un mayor potencial de membrana mitocondrial (Catalán et al., 2020c; Catalán et al., 2020d) que los segundos (Catalán et al., 2020b), lo cual podría sugerir que la producción de ROS en los espermatozoides de burro es aún mayor. Relacionado con esto, en el primer estudio de esta Tesis Doctoral se observó que la exposición de los espermatozoides de burro a concentraciones altas (hasta 10 mM) de GSH (estrés reductivo) y $H_2O_2$ (estrés oxidativo) no afectó a la motilidad total, manteniéndose sus valores incluso hasta los 60 y 120 minutos de incubación, mientras que la motilidad progresiva se redujo significativamente solo con la concentración de 10 mM de GSH. Del mismo modo, la viabilidad espermática no se vio afectada por las dosis altas de GSH y $H_2O_2$, pero sí por el tiempo de incubación, disminuyendo a partir de los 30 minutos. Esto indicaría que los espermatozoides de burro tienen una elevada resistencia a la toxicidad que producen los niveles elevados de GSH y que su capacidad antioxidante es muy alta en comparación con los espermatozoides de otras especies como el humano (Panner Selvam et al., 2020; Uribe et al., 2022), lo que junto al plasma seminal, les permite mantener el equilibrio oxidativo/reductivo después de la exposición a niveles elevados de ROS.

Además, y sorprendentemente, se observó que la producción de ROS extracelular aumentó de forma significativa al añadir GSH en concentraciones iguales o superiores a 8 mM (estrés reductivo), contrariamente a lo que podría esperarse una vez que el sistema de defensa antioxidante se mejoró exógenamente mediante la adición de GSH, lo cual se podría explicar por la presencia de la enzima LAAO en el acrosoma de los espermatozoides de varias especies como el caballo (Aitken et al., 2015) y el humano (Houston et al., 2015). La LAAO es capaz de producir ROS a través de la desaminación de L-amino o aminoácidos aromáticos (fenilalanina, tirosina y triptófano) (Upreti et al., 1998). Por otro lado, la presencia de espermatozoides no viables, e incluso de los morfológicamente anormales, es una fuente potencial de producción de ROS que terminan afectando a los espermatozoides que permanecen viables (Aitken y Koppers, 2011; Roca et al., 2016). Por su parte, la producción de ROS extracelular no difirió al utilizar dosis altas de $H_2O_2$ (estrés oxidativo). En este momento, no existe una explicación clara acerca de cómo los espermatozoides de burro adquieren esta elevada resistencia al estrés oxidativo, ya que de forma general, y de acuerdo con lo observado en otras especies, estas células son particularmente susceptibles al estrés oxidativo por sus inadecuados sistemas de reparación celular y su reducida capacidad de defensa antioxidante debido a su bajo contenido citoplasmático (Bollwein et al., 2008; Ball, 2009; Amidi et al., 2016; Dutta et al., 2019). Dentro de este contexto, el semen de burro parece tener un mejor sistema de defensa antioxidante, fundamentalmente enzimático, para combatir las ROS, en comparación con otras especies como el caballo (Papas et al., 2019a). Además, los espermatozoides de burro podrían tener la capacidad de detoxificar las ROS al reconfigurar su actividad metabólica, favoreciendo así su supervivencia en un ambiente oxidante y evitando la activación de las vías de muerte celular conocidas (cambios de tipo apoptótico y necrosis después de un estrés severo) (Guthrie y Welch, 2005), situación que se ha visto en las células cancerosas (Fiaschi y Chiarugi, 2012).

Aunque la producción de ROS intracelular por los espermatozoides de burro puede ser evaluada por citometría de flujo mediante el uso de distintas sondas fluorescentes (Catalán et al., 2020c; Catalán et al., 2020d), la cantidad de estas especies generadas durante la reacción endometrial después la IA aún no se ha determinado. A este respecto, en el primer estudio de esta Tesis Doctoral también se observó que la NETosis desencadenada por la interacción de los PMN con los

espermatozoides de burro, y en ausencia de plasma seminal, llevó aparejada la producción de una gran cantidad de ROS extracelular después de 30 minutos de incubación; estos niveles de ROS fueron cuatro veces más elevados que los observados en el caballo (Baumber et al., 2002). Por otro lado, los PMN solos demostraron ser capaces de generar de cinco a seis veces más ROS extracelular que su valor inicial a lo largo de la incubación, lo que se debe a un proceso de apoptosis constitutivo o espontáneo que padecen los PMN como parte de su mecanismo para mantener la homeostasis del sistema inmunitario (Savill et al., 1989). Curiosamente, el medio para centrifugación de Kenney (Kenney, 1975) evitó que los PMN reaccionaran, manteniendo constante la producción de ROS extracelular incluso después de 2 horas de incubación, condición que se podría atribuir a la presencia de glucosa, que es capaz de retrasar la apoptosis espontánea en los PMN (Manosudprasit et al., 2017).

Dada la gran tolerancia de los espermatozoides de burro a la toxicidad que producen los niveles altos de GSH, así como la gran cantidad de ROS extracelular que se produce en la NETosis de los PMN, el segundo estudio de esta Tesis Doctoral consideró la adición de GSH en distintas concentraciones al medio de congelación. El GSH ha sido utilizado ampliamente como suplemento antioxidante al medio de congelación de espermatozoides en varias especies como caballos (Oliveira et al., 2013), cerdos (Gadea et al., 2005; Yeste et al., 2013b; Estrada et al., 2014), ovejas (Silva et al., 2011), bovinos (Gangwar et al., 2018) y perros (Ogata et al., 2015), pero con resultados contradictorios, llegándose incluso a observar que las concentraciones altas de GSH (superiores a 2,5 mM) pueden ser citotóxicas (Oliveira et al., 2013). En el caso del burro, la suplementación al medio de congelación con GSH a una concentración tan alta como 10 mM no afectó la viabilidad, la integridad de las membranas plasmática y del acrosoma, y el potencial de membrana mitocondrial de los espermatozoides después de la descongelación. Esto demuestra, una vez más, la sorprendente resistencia de los espermatozoides de burro a dosis altas de GSH, incluso después de ser sometidos a congelación-descongelación.

Por otra parte, si bien las dosis de GSH iguales o superiores a 8 mM produjeron una disminución significativa de la motilidad total y progresiva, así como una modificación en la estructura de las subpoblaciones de espermatozoides

móviles después de la descongelación, el espermatozoide de burro demostró una gran tolerancia al GSH respecto al de caballo, en el que se observó una disminución de la motilidad total y progresiva con solo 2,5 mM de GSH añadidos al medio de congelación (Oliveira et al., 2013). Relacionado con esto, en el espermatozoide de cerdo se detectó que la adición de GSH al medio de congelación a una concentración de 2 mM modificaba la estructura de las subpoblaciones de espermatozoides móviles (Estrada et al., 2017). Este hecho destaca la criotolerancia de los espermatozoides de burro congelado-descongelado a dosis altas de GSH, más aún, considerando que los niveles intracelulares de ROS disminuyeron significativamente con la suplementación al medio de congelación de concentraciones altas de GSH (8 mM y 10 mM). La modificación de los grupos tiol proteicos sería el mecanismo más probable para mantener el equilibrio oxidativo/reductivo (Bilodeau et al., 2001; Gadea et al., 2005) y, de esta forma, revertir el estrés oxidativo generado durante la congelación-descongelación de los espermatozoides de burro.

La disminución de los niveles intracelulares de ROS observada en los espermatozoides de burro criopreservados con GSH tendría mucha relación con lo observado en el primer estudio de esta Tesis Doctoral, en el cual se observó que la NETosis de los PMN libera una gran cantidad de ROS al espacio extracelular como respuesta a la presencia de los espermatozoides, los cuales están expuestos a un entorno oxidante en el útero de la burra (Miró y Papas, 2018a). En esta circunstancia, sería lógico pensar que la suplementación del medio de congelación con GSH entre 2 mM y 6 mM podría ser utilizada como una estrategia contra el estrés oxidativo en esta especie, especialmente cuando se realiza la IA con semen congelado-descongelado. Sin embargo, el mecanismo de regulación de las ROS es complejo e implica la interacción de varias moléculas antioxidantes, presentes principalmente en el plasma seminal (Baumber y Ball, 2005; Barranco et al., 2015b; Li et al., 2018; Papas et al., 2019a; Papas et al., 2019b; Papas et al., 2020a; Tirpák et al., 2021). Es por este motivo que el objetivo del tercer y cuarto estudio de esta Tesis Doctoral fue determinar la relación de los antioxidantes enzimáticos y no enzimáticos y del OSI del plasma seminal con la criotolerancia de los espermatozoides de burro y caballo.

Si bien algunas enzimas antioxidantes del plasma seminal de burro y caballo como la SOD, la CAT, la GPX y la GSR han sido descritas anteriormente y se ha relacionado su actividad con algunos parámetros de calidad espermática (Papas et al., 2019a), ésta es la primera vez que los niveles de actividad de la SOD, la CAT y la GPX se relacionaron con algunos parámetros de calidad espermática y niveles intracelulares de ROS analizados mediante citometría de flujo en espermatozoides de burro y caballo congelados-descongelados. Además, es la primera vez que se evaluó la presencia de la PON1 en el plasma seminal de estas especies y se relacionó su nivel de actividad con la calidad seminal después de la descongelación. Los niveles de actividad de PON1 fueron significativamente más altos en los eyaculados de burro y caballo clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad". Estos valores encontrados en el plasma seminal de burro y caballo fueron superiores a los publicados previamente en cerdos (Barranco et al., 2015b; Li et al., 2018) e inferiores a los observados en humanos (Verit et al., 2009). No obstante, en el caballo los niveles de actividad de PON1 fueron aproximadamente más del doble que en el burro, lo cual podría representar una especificidad de especie en cuanto a la acción antioxidante esta enzima. La PON1 podría tener un rol más importante para la criotolerancia espermática en el semen de caballo que otras enzimas antioxidantes, como la CAT o la GPX, principalmente actuando como modulador en la detoxificación del $H_2O_2$. De esta forma, la PON1 podría intervenir en la protección de la membrana plasmática del espermatozoide, ya que es capaz de evitar la acción negativa de los aldehídos lipídicos, como el 4-hidroxinonenal (4-HNE), que se generan como producto de la peroxidación lipídica (Martin Muñoz et al., 2015; Aitken et al., 2016a; Gibb et al., 2016; Aitken et al., 2018). Este efecto positivo se ve reflejado en las correlaciones positivas de esta enzima antioxidante con la integridad de la membrana plasmática de los espermatozoides de burro y caballo después de la descongelación, coincidiendo con lo observado en el semen de cerdo refrigerado (Barranco et al., 2015b) y congelado-descongelado (Li et al., 2018).

La SOD también presentó niveles de actividad significativamente más altos en los eyaculados de burro y caballo clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad". Es importante destacar que la magnitud de esta diferencia era aproximadamente diez veces mayor en el burro que en el caballo, lo cual podría estar asociado con una mayor producción de ROS

por parte los espermatozoides de burro (valores mostrados en el primer estudio de esta Tesis Doctoral). La SOD protege a los espermatozoides contra el $O_2^-$ mediante la dismutación de este anión en el $H_2O_2$ (Silvestre et al., 2021). Por lo tanto, es lógico pensar que una menor cantidad de $O_2^-$ podría repercutir favorablemente sobre la motilidad y viabilidad de los espermatozoides después de la criopreservación, lo que se ha observado en estudios previos acerca de la actividad específica de esta enzima antioxidante en semen de burro (Papas et al., 2020a) y caballo congelado-descongelado (Papas et al., 2019b). Esto explicaría las diferencias de congelabilidad entre los eyaculados de estos animales, así como las correlaciones encontradas con algunos parámetros de motilidad y funcionalidad espermáticas después de la descongelación, lo cual concuerda con lo observado en otras especies como cerdo (Li et al., 2018), búfalo (Waheed et al., 2013) y humano (Buffone et al., 2012).

Los niveles de actividad de la CAT mostraron comportamientos distintos en la criotolerancia de los espermatozoides de burro y caballo, esto pese a ser especies filogenéticamente cercanas. Mientras en el burro los niveles de actividad de esta enzima antioxidante fueron significativamente más bajos en los eyaculados clasificados como de "buena congelabilidad" respecto a los clasificados como de "mala congelabilidad", en el caballo no se encontraron diferencias. Esto indicaría que la CAT en el semen de burro podría ser menos relevante como modulador de la detoxificación del $H_2O_2$ que otras enzimas antioxidantes, como la PON1 o la GPX, lo cual difiere de lo observado en estudios previos con semen de burro fresco (Papas et al., 2019a) y congelado-descongelado (Papas et al., 2020a). A pesar de esta menor relevancia, en este estudio se observó un efecto positivo de la CAT sobre el desorden lipídico de la membrana plasmática, parámetro que no fue evaluado en los estudios mencionados. Por el contrario, la CAT en el plasma seminal de caballo podría ser crucial para evitar la acumulación de $H_2O_2$, de modo que la integridad y funcionalidad del espermatozoide en esta especie se mantendrían mejor (Ball et al., 2000). Esta hipótesis se sustenta en las correlaciones positivas observadas con algunos parámetros de motilidad espermática y con la integridad de la membrana plasmática después de la descongelación.

La GPX no mostró diferencias en sus niveles de actividad en los eyaculados de burro y caballo clasificados como de "buena congelabilidad" y "mala

congelabilidad", lo cual está de acuerdo con lo observado previamente en estas especies (Papas et al., 2019b; Papas et al., 2020a). Sin embargo, se observó una relación positiva de esta enzima antioxidante con algunos parámetros de motilidad espermática después de la descongelación, a diferencia de estudios previos realizados en semen fresco de burro, caballo (Papas et al., 2019a), y carnero (Kasimanickam et al., 2006), así como en semen de burro (Papas et al., 2020a) y caballo congelado-descongelado (Papas et al., 2019b). También se observó una relación positiva de la GPX con algunos parámetros de funcionalidad espermática después de la descongelación, así como con los niveles intracelulares de ROS en el caso del burro, y con el potencial de membrana mitocondrial y con la integridad de la membrana del acrosoma en el caso del caballo. Por lo tanto, es razonable pensar que el sistema de equilibrio oxidativo/reductivo del $H_2O_2$ mediado por la GPX es importante para el mantenimiento de la funcionalidad y supervivencia espermáticas en estas especies.

Con respecto a los antioxidantes no enzimáticos (medidos en términos de CUPRAC, FRAP y TEAC), también es la primera vez que se describen en el plasma seminal de burro y caballo, y que sus niveles de actividad se relacionan con la criotolerancia de los espermatozoides de estas especies. En el burro, la CUPRAC, la FRAP y la TEAC, y en el caballo únicamente la TEAC, presentaron niveles de actividad significativamente más altos en los eyaculados clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad". Esto concuerda con lo observado en semen de cerdo congelado-descongelado para la FRAP y la TEAC, pero difiere en el caso de la CUPRAC (Li et al., 2018), lo cual podría estar relacionado con la distinta composición del plasma seminal y el papel de los antioxidantes en cada especie (Baumber y Ball, 2005; Papas et al., 2019b; Papas et al., 2020a), la estacionalidad (Papas et al., 2019a) y la edad (Waheed et al., 2013). En cuanto a los antioxidantes no enzimáticos, los resultados obtenidos en esta Tesis Doctoral indican que éstos podrían jugar un papel determinante en el mantenimiento de la estructura de los espermatozoides de burro y caballo contra el daño oxidativo causado por las ROS durante la criopreservación. De hecho, se evidenció, además, que la CUPRAC, la FRAP y la TEAC en el burro, y solo la TEAC en el caballo, se correlacionaban de forma positiva con algunos parámetros de motilidad espermática, así como con la integridad de la membrana plasmática y del acrosoma.

La resistencia de los espermatozoides de burro y caballo al estrés oxidativo que genera la congelación-descongelación puede estar determinada por los altos niveles de actividad de los antioxidantes enzimáticos (PON1 y SOD) y no enzimáticos (principalmente TEAC) presentes en el plasma seminal. Al parecer el breve contacto de los espermatozoides con el plasma seminal durante el tiempo transcurrido entre la eyaculación y la congelación; es decir, antes de su eliminación (Barranco et al., 2015c; Papas et al., 2019b; Papas et al., 2020a), podría ser suficiente para ejercer el efecto beneficioso de los antioxidantes sobre la criotolerancia de los espermatozoides de estas especies, principalmente en lo que concierne a la regulación de las ROS. Por lo tanto, la existencia de un equilibrio adecuado entre los niveles de ROS y de antioxidantes durante la criopreservación es esencial para una función espermática óptima (Bansal y Bilaspuri, 2011; Wagner et al., 2018). En este sentido, se observó que los niveles del OSI del plasma seminal fueron significativamente más bajos en los eyaculados de burro y caballo clasificados como de "buena congelabilidad" que en los clasificados como de "mala congelabilidad". Este estudio es el primero que ha valorado el OSI en el plasma seminal de estas especies y ha determinado su relación con la criotolerancia espermática. No obstante, los niveles del OSI del plasma seminal de caballo resultaron ser aproximadamente tres veces más altos que en el burro, tanto en los eyaculados clasificados como de "buena congelabilidad" (6,35 ± 0,48 vs. 2,65 ± 0,36, respectivamente) como en los clasificados como de "mala congelabilidad" (13,03 ± 1,80 vs. 4,68 ± 0,87, respectivamente).

Las diferencias en los niveles del OSI del plasma seminal observadas entre el burro y el caballo indicarían que el control del estrés oxidativo por parte del sistema de defensa antioxidante del plasma seminal es altamente complejo y específico de cada especie, pudiendo estar relacionado, no solo con los componentes (Morte et al., 2008; El-Badry et al., 2016) y niveles de actividad de los antioxidantes del plasma seminal (Papas et al., 2019a; Papas et al., 2019b; Papas et al., 2020a), sino también con el metabolismo espermático y la estrategia reproductiva de cada especie. Esto explicaría la falta de correlaciones de los antioxidantes enzimáticos y no enzimáticos, así como del OSI del plasma seminal de caballo con los niveles intracelulares de ROS después de la descongelación, lo que difiere de lo observado en el burro. Sin embargo, la existencia de un OSI bajo en el plasma seminal de burro y caballo representa una mejora en la calidad

espermática después de la descongelación, lo cual se ve reflejado en las correlaciones negativas encontradas entre el OSI con los parámetros de motilidad y funcionalidad, similar a lo observado también en semen de cerdo almacenado a 17 ºC (Barranco et al., 2021). Por el contrario, una consecuencia del mayor OSI encontrado en el plasma seminal de caballo podría justificar la relación entre un OSI elevado y un mayor porcentaje de espermatozoides viables con desorden lipídico de la membrana plasmática alto, lo cual no se produjo en el burro.

Finalmente, se observó que la gran mayoría de antioxidantes, tanto enzimáticos como no enzimáticos, medidos en el plasma seminal de burro y caballo estaban correlacionados positivamente entre sí, a excepción de la CAT en el caso de burro. Esto demuestra la gran complejidad que implica el control del estrés oxidativo en el semen y que el mantenimiento del equilibrio oxidativo/reductivo constituye un mecanismo integral, en el que todos los componentes del plasma seminal juegan un papel importante. Por otro lado, el OSI se correlacionó negativamente con los antioxidantes enzimáticos y no enzimáticos en las dos especies, lo cual era esperable debido a que la disminución de la actividad antioxidante repercute en un mayor nivel de oxidación.

# CAPÍTULO 5:

# CONCLUSIONES

Las conclusiones de esta Tesis Doctoral son:

1. El espermatozoide de burro presenta una mayor tolerancia al estrés oxidativo que al estrés reductivo, ya que la producción de ROS extracelular es mayor a concentraciones altas de GSH.

2. La NETosis de los PMN genera una gran cantidad de ROS extracelular, que aumenta cuando los espermatozoides de burro entran en contacto con los PMN.

3. La suplementación del medio de congelación con GSH a una concentración de entre 2 mM y 6 mM podría ayudar a controlar los niveles intracelulares de ROS producidos durante la criopreservación de los espermatozoides de burro.

4. Los niveles de actividad de la PON1, de la SOD y de la TEAC, así como del OSI del plasma seminal están relacionados con la resistencia de los espermatozoides de burro y caballo a la criopreservación.

5. En el burro, pero no en el caballo, los niveles de actividad de la CAT, de la CUPRAC y de la FRAP del plasma seminal también están relacionados con la resistencia de los espermatozoides a la criopreservación.

6. Los niveles de actividad de los antioxidantes enzimáticos (excepto la CAT) y no enzimáticos del plasma seminal se correlacionaron negativamente con los niveles intracelulares de ROS de los espermatozoides después de la descongelación, en el burro pero no en el caballo.

7. La regulación de las ROS por parte del sistema de defensa antioxidante, fundamentalmente del plasma seminal, constituye un mecanismo muy complejo que es específico de la especie, y en el que lo más importante es mantener el equilibrio oxidante/antioxidante para una funcionalidad espermática óptima.

# CAPÍTULO 6:

# REFERENCIAS BIBLIOGRÁFICAS

Abuelo, A., Hernández, J., Benedito, J. L., Castillo, C. (2013). Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal*, *7*(8), 1374–1378.

Adeoye, O., Olawumi, J., Opeyemi, A., Christiania, O. (2018). Review on the role of glutathione on oxidative stress and infertility. *JBRA Assisted Reproduction*, *22*(1), 61–66.

Agarwal, A., Aponte-Mellado, A., Premkumar, B. J., Shaman, A., Gupta, S. (2012). The effects of oxidative stress on female reproduction: a review. *Reproductive Biology and Endocrinology*, *10*(1), 1–31.

Agarwal, A., Virk, G., Ong, C., du Plessis, S. S. (2014). Effect of oxidative stress on male reproduction. *The World Journal of Men's Health*, *32*(1), 1–17.

Aitken, J. B., Naumovski, N., Curry, B., Grupen, C. G., Gibb, Z., Aitken, R. J. (2015). Characterization of an L-amino acid oxidase in equine spermatozoa. *Biology of Reproduction*, *92*(5), 1–13.

Aitken, R. J., Baker, M. A. (2006). Oxidative stress, sperm survival and fertility control. *Molecular and Cellular Endocrinology*, *250*(1–2), 66–69.

Aitken, R. J., De Iuliis, G. N., Drevet, J. R. (2018). Role of oxidative stress in the etiology of male infertility and the potential therapeutic value of antioxidants. In *Oxidants, Antioxidants, and Impact of the Oxidative Status in Male Reproduction* (pp. 91–100). R. Henkel, L. Samanta, A. Agarwal (Eds.), Academic Press.

Aitken, R. J., Drevet, J. R., Moazamian, A., Gharagozloo, P. (2022). Male infertility and oxidative stress: a focus on the underlying mechanisms. *Antioxidants*, *11*(2), 306.

Aitken, R. J., Flanagan, H. M., Connaughton, H., Whiting, S., Hedges, A., Baker, M. A. (2016a). Involvement of homocysteine, homocysteine thiolactone, and paraoxonase type 1 (PON-1) in the etiology of defective human sperm function. *Andrology*, *4*(2), 345–360.

Aitken, R. J., Gibb, Z., Baker, M. A., Drevet, J., Gharagozloo, P. (2016b). Causes and consequences of oxidative stress in spermatozoa. *Reproduction, Fertility and Development*, *28*(2), 1–10.

Aitken, R. J., Koppers, A. J. (2011). Apoptosis and DNA damage in human spermatozoa. *Asian Journal of Andrology*, *13*(1), 36–42.

Al-Essawe, E. M., Johannisson, A., Wulf, M., Aurich, C., Morrell, J. (2018a). Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma. *Cryobiology, 81*, 145–152.

Al-Essawe, E. M., Wallgren, M., Wulf, M., Aurich, C., Macías-García, B., Sjunnesson, Y., Morrell, J. M. (2018b). Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. *Theriogenology*, *115*, 99–107.

Alghamdi, A. S., Foster, D. N. (2005). Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. *Biology of Reproduction*, *73*(6), 1174–1181.

Alghamdi, A. S., Foster, D. N., Troedsson, M. H. T. (2004). Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Reproduction*, *127*(5), 593–600.

Alghamdi, A. S., Lovaas, B. J., Bird, S. L., Lamb, G. C., Rendahl, A. K., Taube, P. C., Foster, D. N. (2009). Species-specific interaction of seminal plasma on sperm–neutrophil binding. *Animal Reproduction Science*, *114*(4), 331–344.

Alvarenga, M. A., Leao, K. M., Papa, F. O., Landim-Alvarenga, F. C., Medeiros, A. S. L., Gomes, G. M. (2003). The use of alternative cryoprotectors for freezing stallion semen. In *Workshop on Transporting Gametes and Embryos*, 74–76.

Alvarenga, M. A., Papa, F. O., Ramires Neto, C. (2016). Advances in stallion semen cryopreservation. *Veterinary Clinics of North America: Equine Practice*, *32*(3), 521–530.

Álvarez, C., González, N., Luño, V., Martínez, F., Gil, L. (2019). Alternatives in donkey semen cryopreservation: mare vs. jenny colostrum. *Reproduction in Domestic Animals*, *54*(S4), 94–97.

Amann, R. P. (2011). Physiology and endocrinology. In *Equine Reproduction* (2nd Ed., pp. 881–908). A. O. McKinnon, E. L. Squires, W. E. Vaala, D. D. Varner (Eds.), Wiley-Blackwell.

Amann, R. P., Pickett, B. W. (1987). Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *Journal of Equine Veterinary Science*, *7*(3), 145–173.

Amidi, F., Pazhohan, A., Shabani Nashtaei, M., Khodarahmian, M., Nekoonam, S. (2016). The role of antioxidants in sperm freezing: a review. *Cell and Tissue Banking*, *17*(4), 745–756.

Attia, Y. A., El-Naggar, A. S., Abou-Shehema, B. M., Abdella, A. A. (2019). Effect of supplementation with trimethylglycine (betaine) and/or vitamins on semen quality, fertility, antioxidant status, DNA repair and welfare of roosters exposed to chronic heat stress. *Animals*, *9*(8), 547.

Aurich, C., Ortega Ferrusola, C., Peña Vega, F. J., Schrammel, N., Morcuende, D.,

Aurich, J. (2018). Seasonal changes in the sperm fatty acid composition of Shetland pony stallions. *Theriogenology*, *107*, 149–153.

Aurich, J., Aurich, C. (2006). Developments in European horse breeding and consequences for veterinarians in equine reproduction. *Reproduction in Domestic Animals*, *41*(4), 275–279.

Aurich, J., Kuhl, J., Tichy, A., Aurich, C. (2020). Efficiency of semen cryopreservation in stallions. *Animals*, *10*(6), 1033.

Bailey, J. L., Bilodeau, J. F., Cormier, N. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon minireview. *Journal of Andrology*, *21*(1), 1–7.

Ball, B. A. (2008). Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal Reproduction Science*, *107*(3–4), 257–267.

Ball, B. A. (2009). Role of oxidative stress in normal and abnormal function of equine spermatozoa. *Revista Brasileira de Reprodução Animal*, *33*(Suppl. 6), 20–24.

Ball, B. A., Gravance, C. G., Medina, V., Baumber, J., Liu, I. K. M. (2000). Catalase activity in equine semen. *American Journal of Veterinary Research*, *61*(9), 1026–1030.

Ball, B. A., Vo, A. T., Baumber, J. (2001). Generation of reactive oxygen species by equine spermatozoa. *American Journal of Veterinary Research*, *62*(4), 508–515.

Bansal, A. K., Bilaspuri, G. S. (2011). Impacts of oxidative stress and antioxidants on semen functions. *Veterinary Medicine International*, *2011*, 686137.

Bao, J., Bedford, M. T. (2016). Epigenetic regulation of the histone-to-protamine transition during spermiogenesis. *Reproduction*, *151*(5), R55–R70.

Barbagallo, F., La Vignera, S., Cannarella, R., Crafa, A., Calogero, A. E., Condorelli, R. A. (2021). The relationship between seminal fluid hyperviscosity and oxidative stress: a systematic review. *Antioxidants*, *10*(3), 356.

Barker, C. A. V., Gandier, J. C. C. (1957). Pregnancy in a mare resulting from frozen epididymal spermatozoa. *Canadian Journal of Comparative Medicine and Veterinary Science*, *21*(2), 47–51.

Barranco, I., Roca, J., Tvarijonaviciute, A., Rubér, M., Vicente-Carrillo, A., Atikuzzaman, M., Ceron, J. J., Martinez, E. A., Rodriguez-Martinez, H. (2015a). Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Molecular Reproduction and Development*, *82*(1), 58–65.

Barranco, I., Rubio, C. P., Tvarijonaviciute, A., Rodriguez-Martinez, H., Roca, J. (2021). Measurement of oxidative stress index in seminal plasma can predict in vivo fertility of liquid-stored porcine artificial insemination semen doses. *Antioxidants*, *10*(8), 1203.

Barranco, I., Tvarijonaviciute, A., Perez-Patiño, C., Alkmin, D. V., Ceron, J. J., Martinez, E. A., Rodriguez-Martinez, H., Roca, J. (2015b). The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality, and in vivo fertility. *Andrology*, *3*(2), 315–320.

Barranco, I., Tvarijonaviciute, A., Perez-Patiño, C., Parrilla, I., Ceron, J. J., Martinez, E. A., Rodriguez-Martinez, H., Roca, J. (2015c). High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Scientific Reports*, *5*(1), 1–9.

Baumber, J., Ball, B. A. (2005). Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. *American Journal of Veterinary Research*, *66*(8), 1415–1419.

Baumber, J., Ball, B. A., Gravance, C. G., Medina, V., Davies-Morel, M. C. G. (2000). The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology*, *21*(6), 895–902.

Baumber, J., Sabeur, K., Vo, A., Ball, B. A. (2003). Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. *Theriogenology*, *60*(7), 1239–1247.

Baumber, J., Vo, A., Sabeur, K., Ball, B. A. (2002). Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology*, *57*(3), 1025–1033.

Benedetti, S., Tagliamonte, M. C., Catalani, S., Primiterra, M., Canestrari, F., De Stefani, S., Palini, S., Bulletti, C. (2012). Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reproductive BioMedicine Online*, *25*(3), 300–306.

Benzie, I. F. F., Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76.

Bilodeau, J. F., Blanchette, S., Gagnon, C., Sirard, M. A. (2001). Thiols prevent $H_2O_2$-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*,

*56*(2), 275–286.

Björndahl, L., Mortimer, D., Barratt, C. L. R., Castilla, J. A., Menkveld, R., Kvist, U., Alvarez, J. G., Haugen, T. B. (2010). Basic physiology. In *A Practical Guide to Basic Laboratory Andrology* (pp. 5–32). Cambridge University Press.

Blottner, S., Warnke, C., Tuchscherer, A., Heinen, V., Torner, H. (2001). Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. *Animal Reproduction Science, 65*(1–2), 75–88.

Blount, J. D., Vitikainen, E. I. K., Stott, I., Cant, M. A. (2016). Oxidative shielding and the cost of reproduction. *Biological Reviews, 91*(2), 483–497.

Bollwein, H., Fuchs, I., Koess, C. (2008). Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reproduction in Domestic Animals, 43*(2), 189–195.

Branzk, N., Lubojemska, A., Hardison, S. E., Wang, Q., Gutierrez, M. G., Brown, G. D., Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nature Immunology, 15*(11), 1017–1025.

Brinkmann, V. (2018). Neutrophil extracellular traps in the second decade. *Journal of Innate Immunity, 10*(5–6), 414–421.

Brinsko, S. P., Crockett, E. C., Squires, E. L. (2000). Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenology, 54*(1), 129–136.

Brito, L. F. C. (2007). Evaluation of stallion sperm morphology. *Clinical Techniques in Equine Practice, 6*(4), 249–264.

Bucak, M. N., Sariözkan, S., Tuncer, P. B., Sakin, F., Ateşşahin, A., Kulaksiz, R., Çevik, M. (2010). The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Research, 89*(1), 24–30.

Buffone, M. G., Calamera, J. C., Brugo-Olmedo, S., De Vincentiis, S., Calamera, M. M., Storey, B. T., Doncel, G. F., Alvarez, J. G. (2012). Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa. *Fertility and Sterility, 97*(2), 293–298.

Cabrera, P., Sánchez, R., Risopatrón, J. (2014). Selección espermática en semen congelado/descongelado de equino: evaluación de las membranas plasmática,

acrosomal y potencial de membrana mitocondrial. *International Journal of Morphology*, *32*(2), 725–731.

Campos, C., Guzmán, R., López-Fernández, E., Casado, Á. (2009). Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: the CUPRAC–BCS assay. *Analytical Biochemistry*, *392*(1), 37–44.

Camps, J., Marsillach, J., Joven, J. (2009). The paraoxonases: role in human diseases and methodological difficulties in measurement. *Critical Reviews in Clinical Laboratory Sciences*, *46*(2), 83–106.

Canisso, I. F., Carvalho, G. R., Davis Morel, M., Ker, P. G., Rodrigues, A. L., Silva, E. C., Coutinho Da Silva, M. A. (2011). Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Veterinary Journal*, *43*(2), 179–183.

Canisso, I. F., Panzani, D., Miró, J., Ellerbrock, R. E. (2019). Key aspects of donkey and mule reproduction. *Veterinary Clinics of North America - Equine Practice*, *35*(3), 607–642.

Canisso, I. F., Segabinazzi, L. G. T. M., Fedorka, C. E. (2020). Persistent breeding-induced endometritis in mares—A multifaceted challenge: from clinical aspects to immunopathogenesis and pathobiology. *International Journal of Molecular Sciences*, *21*(4), 1432.

Canisso, I. F., Souza, F. A., Ortigoza Escobar, J. M., Ribeiro de Carvalho, G., Davies Morel, M. C., Capistrano da Silva, E., Domingos Guimarães, J., Linhares Lima, A. (2008). Congelamiento de semen de burro (*Equus asinus*). *Revista de Investigaciones Veterinarias Del Perú*, *19*(2), 113–125.

Canisso, I. F., Stewart, J., Coutinho da Silva, M. A. (2016). Endometritis: managing persistent post-breeding endometritis. *Veterinary Clinics of North America: Equine Practice*, *32*(3), 465–480.

Carvalho, L. E., Silva Filho, J. M., Palhares, M. S., Sales, A. L. R., Gonczarowska, A. T., Oliveira, H. N., Resende, M., Rossi, R. (2016). Physical and morphological characteristics of the first three jets of Pêga jackasses sperm-rich fraction. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *68*(4), 845–852.

Casas, I., Sancho, S., Briz, M., Pinart, E., Bussalleu, E., Yeste, M., Bonet, S. (2009). Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology*, *72*(7), 930–948.

Castagné, V., Lefèvre, K., Natero, R., Becker, D. A., Clarke, P. G. H. (1999). An optimal

redox status for the survival of axotomized ganglion cells in the developing retina. *Neuroscience*, *93*(1), 313–320.

Castiglione Morelli, M. A., Ostuni, A., Giangaspero, B., Cecchini, S., Carluccio, A., Boni, R. (2021). Relationships between seminal plasma metabolites, semen characteristics and sperm kinetics in donkey (*Equus asinus*). *Animals*, *11*(1), 201.

Catalán, J., Llavanera, M., Bonilla-Correal, S., Papas, M., Gacem, S., Rodríguez-Gil, J. E., Yeste, M., Miró, J. (2020a). Irradiating frozen-thawed stallion sperm with red-light increases their resilience to withstand post-thaw incubation at 38 °C. *Theriogenology*, *157*, 85–95.

Catalán, J., Papas, M., Gacem, S., Mateo-Otero, Y., Rodríguez-Gil, J. E., Miró, J., Yeste, M. (2020b). Red-light irradiation of horse spermatozoa increases mitochondrial activity and motility through changes in the motile sperm subpopulation structure. *Biology*, *9*(9), 1–17.

Catalán, J., Papas, M., Gacem, S., Noto, F., Delgado-Bermúdez, A., Rodríguez-Gil, J. E., Miró, J., Yeste, M. (2020c). Effects of red-light irradiation on the function and survival of fresh and liquid-stored donkey semen. *Theriogenology*, *149*, 88–97.

Catalán, J., Papas, M., Trujillo-Rojas, L., Blanco-Prieto, O., Bonilla-Correal, S., Rodríguez-Gil, J. E., Miró, J., Yeste, M. (2020d). Red LED light acts on the mitochondrial electron chain of donkey sperm and its effects depend on the time of exposure to light. *Frontiers in Cell and Developmental Biology*, *8*, 588621.

Cecchini Gualandi, S., Giangaspero, B., Di Palma, T., Macchia, G., Carluccio, A., Boni, R. (2021). Oxidative profile and protease regulator potential to predict sperm functionality in donkey (*Equus asinus*). *Scientific Reports*, *11*, 20551.

Cecchini, S., Fazio, F. (2020). Assessment of total antioxidant capacity in serum of heathy and stressed hens. *Animals*, *10*(11), 2019.

Celi, P. (2011). Biomarkers of oxidative stress in ruminant medicine. *Immunopharmacology and Immunotoxicology*, *33*(2), 233–240.

Christoffersen, M., Söderlind, M., Rudefalk, S. R., Pedersen, H. G., Allen, J., Krekeler, N. (2015). Risk factors associated with uterine fluid after breeding caused by *Streptococcus zooepidemicus*. *Theriogenology*, *84*(8), 1283–1290.

Christoffersen, M., Troedsson, M. H. T. (2017). Inflammation and fertility in the mare. *Reproduction in Domestic Animals*, *52*(Suppl. 3), 14–20.

Christoffersen, M., Woodward, E., Bojesen, A. M., Jacobsen, S., Petersen, M. R., Troedsson, M. H. T., Lehn-Jensen, H. (2012). Inflammatory responses to

induced infectious endometritis in mares resistant or susceptible to persistent endometritis. *BMC Veterinary Research*, *8*(1), 1–14.

Climent, F., Vilés, K., Miró, J. (2012). Vaginoplastia in two young infertile Catalan jennies. A clinical case. *Reproduction in Domestic Animals*, *47*(Suppl. 3), 113.

Contreras, M. J., Treulen, F., Arias, M. E., Silva, M., Fuentes, F., Cabrera, P., Felmer, R. (2020). Cryopreservation of stallion semen: effect of adding antioxidants to the freezing medium on sperm physiology. *Reproduction in Domestic Animals*, *55*(2), 229–239.

Da Costa, C. M., Dos Santos, R. C. C., Lima, E. S. (2006). A simple automated procedure for thiol measurement in human serum samples. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, *42*(5), 345–350.

Darr, C. R., Varner, D. D., Teague, S., Cortopassi, G. A., Datta, S., Meyers, S. A. (2016). Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility, and ROS production. *Biology of Reproduction*, *95*(2), 34–34.

Davila Plaza, M., Muñoz Martin, P., Bolaños Gallardo, J. M., Stout, T. A. E., Gadella, B. M., Tapia, J. A., Balao da Silva, C., Ortega Ferrusola, C., Peña, F. J. (2016). Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. *Reproduction*, *152*(6), 683–694.

de Andrade, A. F. C., Zaffalon, F. G., Celeghini, E. C. C., Nascimento, J., Tarragó, O. F. B., Martins, S. M. M. K., Alonso, M. A., Arruda, R. P. (2011). Addition of seminal plasma to post-thawing equine semen: what is the effect on sperm cell viability? *Reproduction in Domestic Animals*, *46*(4), 682–686.

De Leeuw, F. E., Chen, H. C., Colenbrander, B., Verkleij, A. J. (1990). Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology*, *27*(2), 171–183.

Desai, N. R., Mahfouz, R., Sharma, R., Gupta, S., Agarwal, A. (2010). Reactive oxygen species levels are independent of sperm concentration, motility, and abstinence in a normal, healthy, proven fertile man: a longitudinal study. *Fertility and Sterility*, *94*(4), 1541–1543.

Dutta, S., Majzoub, A., Agarwal, A. (2019). Oxidative stress and sperm function: a systematic review on evaluation and management. *Arab Journal of Urology*, *17*(2), 87–97.

Dutta, S., Sengupta, P., Slama, P., Roychoudhury, S. (2021). Oxidative stress,

testicular inflammatory pathways, and male reproduction. *International Journal of Molecular Sciences*, *22*(18), 10043.

El-Badry, D. A., El Sisy, G. A., Abo El-Maaty, A. M. (2016). Seminal plasma hormonal profile of Arabian stallions that are classified 'good' or 'poor' for semen freezing. *Asian Pacific Journal of Reproduction*, *5*(6), 453–458.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry*, *37*(4), 277–285.

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry*, *38*(12), 1103–1111.

Estrada, E., Rivera del Álamo, M. M., Rodríguez-Gil, J. E., Yeste, M. (2017). The addition of reduced glutathione to cryopreservation media induces changes in the structure of motile subpopulations of frozen-thawed boar sperm. *Cryobiology*, *78*, 56–64.

Estrada, E., Rodríguez-Gil, J. E., Rocha, L. G., Balasch, S., Bonet, S., Yeste, M. (2014). Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. *Andrology*, *2*(1), 88–99.

Ezzati, M., Shanehbandi, D., Hamdi, K., Rahbar, S., Pashaiasl, M. (2020). Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. *Cell and Tissue Banking*, *21*(1), 1–15.

Fiaschi, T., Chiarugi, P. (2012). Oxidative stress, tumor microenvironment, and metabolic reprogramming: a diabolic liaison. *International Journal of Cell Biology*, *2012*, 762825.

Fickel, J., Wagener, A., Ludwig, A. (2007). Semen cryopreservation and the conservation of endangered species. *European Journal of Wildlife Research*, *53*(2), 81–89.

Flesch, F. M., Gadella, B. M. (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes*, *1469*(3), 197–235.

Flores, C., Vilanova, L. (2015). Metabolismo espermático. *Gaceta de Ciencias Veterinarias*, *20*(1), 23–32.

Foster, A. P., Cunningham, P. M. (1997). Differential superoxide anion generation by equine eosinophils and neutrophils. *Veterinary Immunology and Immunopathology*, *59*(3–4), 225–237.

Fumuso, E. A., Aguilar, J., Giguère, S., Rivulgo, M., Wade, J., Rogan, D. (2007). Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: effects of immunomodulation. *Veterinary Immunology and Immunopathology*, *118*(1–2), 30–39.

Gacem, S., Valverde, A., Catalán, J., Yánez-Ortiz, I., Soler, C., Miró, J. (2021). A new approach of sperm motility subpopulation structure in donkey and horse. *Frontiers in Veterinary Science*, *8*, 651477.

Gadea, J., García-Vazquez, F., Matás, C., Gardón, J. C., Cánovas, S., Gumbao, D. (2005). Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. *Journal of Andrology*, *26*(3), 396–404.

Gadea, J., Sellés, E., Marco, M. A., Coy, P., Matás, C., Romar, R., Ruiz, S. (2004). Decrease in glutathione content in boar sperm after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, *62*(3–4), 690–701.

Gangwar, C., Saxena, A., Patel, A., Singh, S. P., Yadav, S., Kumar, R., Singh, V. (2018). Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. *Animal Reproduction Science*, *192*, 171–178.

Gibb, Z., Blanco-Prieto, O., Bucci, D. (2021). The role of endogenous antioxidants in male animal fertility. *Research in Veterinary Science*, *136*, 495–502.

Gibb, Z., Lambourne, S. R., Aitken, R. J. (2014). The paradoxical relationship between stallion fertility and oxidative stress. *Biology of Reproduction*, *91*(3), 77–78.

Gibb, Z., Lambourne, S. R., Curry, B. J., Hall, S. E., Aitken, R. J. (2016). Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. *Biology of Reproduction*, *94*(6), 133–134.

González-Arostegui, L. G., Muñoz-Prieto, A., Tvarijonaviciute, A., Cerón, J. J., Rubio, C. P. (2022). Measurement of redox biomarkers in the whole blood and red blood cell lysates of dogs. *Antioxidants*, *11*(2), 424.

Gosalvez, J., Tvrda, E., Agarwal, A. (2017). Free radical and superoxide reactivity detection in semen quality assessment: past, present, and future. *Journal of Assisted Reproduction and Genetics*, *34*(6), 697–707.

Grešáková, L., Tokarčíková, K., Čobanová, K. (2021). Bioavailability of dietary zinc sources and their effect on mineral and antioxidant status in lambs. *Agriculture*, *11*(11), 1093.

Gupta, S., Finelli, R., Agarwal, A., Henkel, R. (2021). Total antioxidant capacity— Relevance, methods and clinical implications. *Andrologia*, *53*(2), e13624.

Guthrie, H. D., Welch, G. R. (2005). Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. *Reproduction, Fertility and Development*, *17*(4), 467–477.

Guthrie, H. D., Welch, G. R. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology*, *78*(8), 1700–1708.

Gutjahr, S., Paccamonti, D. L., Pycock, J. F., Taverne, M. A. M., Dieleman, S. J., Van der Weijden, G. C. (2000). Effect of dose and day of treatment on uterine response to oxytocin in mares. *Theriogenology*, *54*(3), 447–456.

Hammerstedt, R. H., Graham, J. K. (1992). Cryopreservation of poultry sperm: the enigma of glycerol. *Cryobiology*, *29*(1), 26–38.

Hezavehei, M., Sharafi, M., Kouchesfahani, H. M., Henkel, R., Agarwal, A., Esmaeili, V., Shahverdi, A. (2018). Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. *Reproductive BioMedicine Online*, *37*(3), 327–339.

Hitit, M., Ugur, M. R., Dinh, T. T. N., Sajeev, D., Kaya, A., Topper, E., Tan, W., Memili, E. (2020). Cellular and functional physiopathology of bull sperm with altered sperm freezability. *Frontiers in Veterinary Science, 7*, 581137.

Hoffmann, N., Oldenhof, H., Morandini, C., Rohn, K., Sieme, H. (2011). Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing. *Animal Reproduction Science*, *125*(1–4), 112–118.

Holt, W. V. (2000). Basic aspects of frozen storage of semen. *Animal Reproduction Science*, *62*(1–3), 3–22.

Houston, B., Curry, B., Aitken, R. J. (2015). Human spermatozoa possess an IL4I1 L-amino acid oxidase with a potential role in sperm function. *Reproduction*, *149*(6), 587–596.

Iaffaldano, N., Paventi, G., Pizzuto, R., Passarella, S., Cerolini, S., Zaniboni, L., Marzoni, M., Castillo, A., Rosato, M. P. (2013). The post-thaw irradiation of avian spermatozoa with He–Ne laser differently affects chicken, pheasant and turkey sperm quality. *Animal Reproduction Science*, *142*(3–4), 168–172.

Ighodaro, O. M., Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their

fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287–293.

Irvine, D. S. (1996). Glutathione as a treatment for male infertility. *Reviews of Reproduction*, *1*(1), 6–12.

Jasko, D. J., Moran, D. M., Farlin, M. E., Squires, E. L. (1991). Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. *Theriogenology*, *35*(6), 1059–1067.

Juárez-Rojas, L., Casillas, F., López, A., Betancourt, M., Ommati, M. M., Retana-Márquez, S. (2022). Physiological role of reactive oxygen species in testis and epididymal spermatozoa. *Andrologia*, *54*(4), e14367.

Kamp, G., Schmidt, H., Stypa, H., Feiden, S., Mahling, C., Wegener, G. (2007). Regulatory properties of 6-phosphofructokinase and control of glycolysis in boar spermatozoa. *Reproduction*, *133*(1), 29–40.

Kapusta, A., Kuczyńska, B., Puppel, K. (2018). Relationship between the degree of antioxidant protection and the level of malondialdehyde in high-performance Polish Holstein-Friesian cows in peak of lactation. *PLOS ONE*, *13*(3), e0193512.

Kareskoski, A. M., Reilas, T., Sankari, S., Andersson, M., Güvenc, K., Katila, T. (2010). Alkaline and acid phosphatase, β-glucuronidase and electrolyte levels in fractionated stallion ejaculates. *Reproduction in Domestic Animals*, *45*(6), e369–e374.

Kareskoski, A. M., Rivera del Alamo, M. M., Güvenc, K., Reilas, T., Calvete, J. J., Rodriguez-Martinez, H., Andersson, M., Katila, T. (2011). Protein composition of seminal plasma in fractionated stallion ejaculates. *Reproduction in Domestic Animals*, *46*(1), e79–e84.

Kareskoski, M., Katila, T. (2008). Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. *Animal Reproduction Science*, *107*(3–4), 249–256.

Kargin, R., Emiroglu, M. Y., Evlice, M., Celik, M., Toprak, A. E., Avci, A., Ayturk, M., Kulahcioglu, S., Bulut, M., Caliskan, M. (2018). Role of the oxidative stress index, myeloperoxidase, catalase activity for cardiac allograft vasculopathy in heart transplant recipients. *Clinical Transplantation*, *32*(7), e13273.

Kasimanickam, R., Pelzer, K. D., Kasimanickam, V., Swecker, W. S., Thatcher, C. D. (2006). Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. *Theriogenology*, *65*(7), 1407–1421.

Katila, T. (1995). Onset and duration of uterine inflammatory response of mares after insemination with fresh semen. *Biology of Reproduction*, *52*, 515–517.

Katila, T. (2012). Post-mating inflammatory responses of the uterus. *Reproduction in Domestic Animals*, *47*(Suppl. 5), 31–41.

Kenney, M. R. (1975). Minimal contamination techniques for breeding mares: techniques and priliminary findings. *Proceedings American Association of Equine Practitioners*, 327–336.

Kodama, H., Kuribayashi, Y., Gagnon, C. (1996). Effect of sperm lipid peroxidation on fertilization. *Journal of Andrology*, *17*(2), 151–157.

Koppers, A. J., De Iuliis, G. N., Finnie, J. M., McLaughlin, E. A., Aitken, R. J. (2008). Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *The Journal of Clinical Endocrinology y Metabolism*, *93*(8), 3199–3207.

Kotilainen, T., Huhtinen, M., Katila, T. (1994). Sperm-induced leukocytosis in the equine uterus. *Theriogenology*, *41*(3), 629–636.

Kowalowka, M., Wysocki, P., Fraser, L., Strzezek, J. (2008). Extracellular superoxide dismutase of boar seminal plasma. *Reproduction in Domestic Animals*, *43*(4), 490–496.

Kumar, P., Kumar, R., Mehta, J. S., Chaudhary, A. K., Ravi, S. K., Chandra Mehta, S., Ansari, M. M., Legha, R. A., Tripathi, B. N., Talluri, T. R. (2019). Ameliorative effect of ascorbic acid and glutathione in combating the cryoinjuries during cryopreservation of exotic jack semen. *Journal of Equine Veterinary Science*, *81*, 102796.

Ladha, S. (1998). Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *The Journal of Membrane Biology*, *165*(1), 1–10.

Leahy, T., Gadella, B. M. (2011). Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction*, *142*(6), 759–778.

LeBlanc, M. M. (2010). Advances in the diagnosis and treatment of chronic infectious and post–mating-induced endometritis in the mare. *Reproduction in Domestic Animals*, *45*(Suppl. 2), 21–27.

Len, J. A., Jenkins, J. A., Eilts, B. E., Paccamonti, D. L., Lyle, S. K., Hosgood, G. (2010). Immediate and delayed (after cooling) effects of centrifugation on equine sperm. *Theriogenology*, *73*(2), 225–231.

Li, J., Barranco, I., Tvarijonaviciute, A., Molina, M. F., Martinez, E. A., Rodriguez-Martinez, H., Parrilla, I., Roca, J. (2018). Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. *Theriogenology*, *107*, 27–35.

Loomis, P. R. (2001). The equine frozen semen industry. *Animal Reproduction Science*, *68*(3–4), 191–200.

Loomis, P. R. (2006). Advanced methods for handling and preparation of stallion semen. *Veterinary Clinics of North America - Equine Practice*, *22*(3), 663–676.

Loomis, P. R., Graham, J. K. (2008). Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. *Animal Reproduction Science*, *105*(1–2), 119–128.

Luberda, Z. (2005). The role of glutathione in mammalian gametes. *Reproductive Biology*, *5*(1), 5–17.

Lushchak, V. I. (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions*, *224*, 164–175.

Macías-García, B., Gonzalez-Fernandez, L., Loux, S. C., Rocha, A. M., Guimarães, T., Peña, F. J., Varner, D. D., Hinrichs, K. (2015). Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. *Reproduction*, *149*(1), 87–99.

Macías García, B., González Fernández, L., Ortega Ferrusola, C., Morillo Rodríguez, A., Gallardo Bolaños, J. M., Rodríguez Martinez, H., Tapia, J. A., Morcuende, D., Peña, F. J. (2011). Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. *Theriogenology*, *75*(5), 811–818.

Magistrini, M., Lindeberg, H., Koskinen, E., Beau, P., Seguin, F. (2000). Biophysical and 1H magnetic resonance spectroscopy characteristics of fractionated stallion ejaculates. *Journal of Reproduction and Fertility Supplement*, *56*, 101–110.

Maitan, P., Bromfield, E. G., Stout, T. A. E., Gadella, B. M., Leemans, B. (2021). A stallion spermatozoon's journey through the mare's genital tract: *in vivo* and *in vitro* aspects of sperm capacitation. *Animal Reproduction Science*, 106848.

Mann, T., Lutwak-Mann, C. (1981). Male reproductive function and the composition of semen: general considerations. In *Male Reproductive Function and Semen* (pp. 1–37). Springer-Verlag.

Mann, T., Minotakis, C. S., Polge, C. (1963). Semen composition and metabolism in the stallion and jackass. *Journal of Reproduction and Fertility, 5*(1), 109–122.

Manosudprasit, A., Kantarci, A., Hasturk, H., Stephens, D., Van Dyke, T. E. (2017). Spontaneous PMN apoptosis in type 2 diabetes and the impact of periodontitis. *Journal of Leukocyte Biology*, *102*(6), 1431–1440.

Mari, G., Castagnetti, C., Rizzato, G., Mislei, B., Iacono, E., Merlo, B. (2011). Density gradient centrifugation of sperm from a subfertile stallion and effect of seminal plasma addition on fertility. *Animal Reproduction Science*, *126*(1–2), 96–100.

Marsillach, J., Lafuente, R., Checa, M. A., Maestre-Martínez, C., Fabián, E., Brassesco, M., Beltrán-Debón, R., Aragonès, G., Carreras, R., Pedro-Botet, J., Joven, J., Camps, J. (2011). Paraoxonase-1 is only present in traceable amounts in seminal fluid and does not show any relationship with male subfertility. *BJU International*, *108*(4), 566–570.

Marth, C. D., Firestone, S. M., Hanlon, D., Glenton, L. Y., Browning, G. F., Young, N. D., Krekeler, N. (2018). Innate immune genes in persistent mating-induced endometritis in horses. *Reproduction, Fertility and Development, 30*(3), 533–545.

Martin Muñoz, P., Ortega Ferrusola, C., Vizuete, G., Plaza Dávila, M., Rodriguez Martinez, H., Peña, F. J. (2015). Depletion of intracellular thiols and increased production of 4-hydroxynonenal that occur during cryopreservation of stallion spermatozoa lead to caspase activation, loss of motility, and cell death. *Biology of Reproduction*, *93*(6), 1–11.

Marzano, G., Moscatelli, N., Di Giacomo, M., Martino, N. A., Lacalandra, G. M., Dell'aquila, M. E., Maruccio, G., Primiceri, E., Chiriacò, M. S., Zara, V., Ferramosca, A. (2020). Centrifugation force and time alter CASA parameters and oxidative status of cryopreserved stallion sperm. *Biology, 9*(2), 22.

Mateo-Otero, Y., Zambrano, F., Catalán, J., Sánchez, R., Yeste, M., Miro, J., Fernandez-Fuertes, B. (2021). Seminal plasma, and not sperm, induces time and concentration-dependent neutrophil extracellular trap release in donkeys. *Equine Veterinary Journal*, *54*(2), 415–426.

Mazur, P., Leibo, S. P., Chu, E. H. Y. (1972). A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. *Experimental Cell Research*, *71*(2), 345–355.

Mesa, M. A., Vasquez, G. (2013). NETosis. *Autoimmune Diseases, 2013*, 651497.

Meseguer, M., Martinez-Conejero, J. A., Muriel, L., Pellicer, A., Remohi, J., Garrido, N. (2007). The human sperm glutathione system: a key role in male fertility and successful cryopreservation. *Drug Metabolism Letters, 1*(2), 121–126.

Meyers, S. A. (2009). Sperm physiology. In J. C. Samper (Ed.), *Equine Breeding Management and Artificial Insemination* (2nd Ed., pp. 47–55). Saunders Elsevier.

Miró, J., Gutiérrez-Reinoso, M., Aguiar da Silva, J., Fernandes, C., Rebordão, M. R., Alexandre-Pires, G., Catalán, J., Ferreira-Dias, G. (2020a). Collagen and eosinophils in jenny's endometrium: do they differ with endometrial classification? *Frontiers in Veterinary Science*, *7*, 631.

Miró, J., Marín, H., Catalán, J., Papas, M., Gacem, S., Yeste, M. (2020b). Seminal plasma, sperm concentration, and sperm-PMN interaction in the donkey: an in vitro model to study endometrial inflammation at post-insemination. *International Journal of Molecular Sciences*, *21*(10), 3478.

Miró, J., Papas, M. (2018a). Post–Artificial Insemination endometrial inflammation and its control in donkeys. *Journal of Equine Veterinary Science*, *65*, 38–43.

Miró, J., Papas, M. (2018b). Improvement of cryopreservation protocol in both purebred horses including Spanish horses. *Spanish Journal of Agricultural Research*, *16*(4), e0406–e0406.

Miró, J., Taberner, E., Rivera, M., Peña, A., Medrano, A., Rigau, T., Peñalba, A. (2009). Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. *Theriogenology*, *72*(8), 1017–1022.

Moore, T., Wilcke, J., Chilcoat, C., Eyre, P., Crisman, M. (1997). Functional characterization of equine neutrophils in response to calcium ionophore A23187 and phorbol myristate acetate ex vivo. *Veterinary Immunology and Immunopathology*, *56*(3–4), 233–246.

Morató, R., Prieto-Martínez, N., Muiño, R., Hidalgo, C. O., Rodríguez-Gil, J. E., Bonet, S., Yeste, M. (2018). Aquaporin 11 is related to cryotolerance and fertilising ability of frozen–thawed bull spermatozoa. *Reproduction, Fertility and Development*, *30*(8), 1099–1108.

Morrell, J. M., Rodriguez-Martinez, H. (2009). Biomimetic techniques for improving sperm quality in animal breeding: a review. *The Open Andrology Journal*, *1*, 1–9.

Morris, G. J., Acton, E., Murray, B. J., Fonseca, F. (2012). Freezing injury: the special case of the sperm cell. *Cryobiology*, *64*(2), 71–80.

Morris, L. H. A., McCue, P. M., Aurich, C. (2020). Equine endometritis: a review of challenges and new approaches. *Reproduction*, *160*(5), R95–R110.

Morte, M. I., Rodrigues, A. M., Soares, D., Rodrigues, A. S., Gamboa, S., Ramalho-

Santos, J. (2008). The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. *Animal Reproduction Science*, *106*(1–2), 36–47.

Moya, C., Rivera-Concha, R., Pezo, F., Uribe, P., Schulz, M., Sánchez, R., Hermosilla, C., Taubert, A., Gärtner, U., Zambrano, F. (2022). Adverse effects of single neutrophil extracellular trap-derived components on bovine sperm function. *Animals*, *12*(10), 1308.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián-Pérez, J. A. (2008). Seminal plasma proteins and sperm resistance to stress. *Reproduction in Domestic Animals*, *43*(Suppl.4), 18–31.

Neuhauser, S., Gösele, P., Handler, J. (2019). Postthaw addition of autologous seminal plasma improves sperm motion characteristics in fair and poor freezer stallions. *Journal of Equine Veterinary Science*, *72*, 117–123.

O'Brien, X. M., Biron, B. M., Reichner, J. S. (2017). Consequences of extracellular trap formation in sepsis. *Current Opinion in Hematology*, *24*(1), 66–71.

Ogata, K., Sasaki, A., Kato, Y., Takeda, A., Wakabayashi, M., Sarentonglaga, B., Yamaguchi, M., Hara, A., Fukumori, R., Nagao, Y. (2015). Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. *Journal of Reproduction and Development*, *61*(2), 116–122.

Oliveira, J. V., Papa, F. O., Melo-Oña, C. M., Monteiro, G. A., Puoli-Filho, J. N. P., Alvarenga, M. A. (2012). New procedures to freeze donkey semen and its influence on mares and jennies fertility. *Journal of Equine Veterinary Science*, *32*(8), 503–504.

Oliveira, R. A., Piersanti, R. L., Wolf, C. A., Viu, M. A. O., Gambarini, M. L. (2018). Glutathione for the freezing of cooled equine semen, using different protocols. *Animal Reproduction*, *11*(2), 104–109.

Oliveira, R. A., Wolf, C. A., Viu, M. A. O., Gambarini, M. L. (2013). Addition of glutathione to an extender for frozen equine semen. *Journal of Equine Veterinary Science*, *33*(12), 1148–1152.

Oliveira, S. N., Andrade, L. R. P., Silva, L. F. M. C., Araujo, E. A. B., Rayashi, R. M., Segabinazzi, L. G. T. M., Alvarenga, M. A., Dell'Aqua, C. P. F., Dell'Aqua, J. A., Papa, F. O. (2020). Fractionated semen collection as a tool to rescue fertility in stallions with seminal vesiculitis. *Theriogenology*, *157*, 110–120.

Olivera, M., Ruiz, T., Tarazona, A., Giraldo, C. (2016). El espermatozoide, desde la eyaculación hasta la fertilización. *Revista Colombiana de Ciencias Pecuarias*, *19*(4), 426–436.

Ortega-Ferrusola, C., Martin Muñoz, P., Ortiz-Rodriguez, J. M., Anel-López, L., Balao da Silva, C., Álvarez, M., de Paz, P., Tapia, J. A., Anel, L., Silva-Rodríguez, A., Aitken, R. J., Gil, M. C., Gibb, Z., Peña, F. J. (2019). Depletion of thiols leads to redox deregulation, production of 4-hydroxinonenal and sperm senescence: a possible role for GSH regulation in spermatozoa. *Biology of Reproduction*, *100*(4), 1090–1107.

Ortega Ferrusola, C., González Fernández, L., Salazar Sandoval, C., Macías García, B., Rodríguez Martínez, H., Tapia, J. A., Peña, F. J. (2010). Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. *Theriogenology*, *74*(3), 458–465.

Palmer, E., Chavatte-Palmer, P. (2020). Contribution of reproduction management and technologies to genetic progress in horse breeding. *Journal of Equine Veterinary Science*, *89*, 103016.

Panner Selvam, M. K., Agarwal, A., Henkel, R., Finelli, R., Robert, K. A., Iovine, C., Baskaran, S. (2020). The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa. *Free Radical Biology and Medicine*, *152*, 375–385.

Papas, M., Arroyo, L., Bassols, A., Catalán, J., Bonilla-Correal, S., Gacem, S., Yeste, M., Miró, J. (2019a). Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology*, *140*, 180–187.

Papas, M., Catalan, J., Barranco, I., Arroyo, L., Bassols, A., Yeste, M., Miró, J. (2020a). Total and specific activities of superoxide dismutase (SOD) in seminal plasma are related with the cryotolerance of jackass spermatozoa. *Cryobiology*, *92*, 109–116.

Papas, M., Catalan, J., Bonilla-Correal, S., Gacem, S., Miró, J., Yeste, M. (2020b). Seminal plasma has limited counteracting effects following induction of oxidative stress in donkey spermatozoa. *Reproduction, Fertility and Development*, *32*(6), 619–628.

Papas, M., Catalán, J., Fernandez-Fuertes, B., Arroyo, L., Bassols, A., Miró, J., Yeste, M. (2019b). Specific activity of superoxide dismutase in stallion seminal

plasma is related to sperm cryotolerance. *Antioxidants*, *8*(11), 539.

Papas, M., Catalán, J., Recuero, S., Morrell, J. M., Yeste, M., Miró, J. (2020c). Single layer centrifugation improves the quality of fresh donkey semen and modifies the sperm ability to interact with polymorphonuclear neutrophils. *Animals*, *10*(11), 2128.

Pegg, D. E. (2007). Principles of cryopreservation. In *Cryopreservation and freezing-drying protocols* (2nd Ed., pp. 39–57). J. G. Day, G. N. Stacey (Eds.), Humana Press.

Peña, F. J., Macías García, B., Samper, J. C., Aparicio, I. M., Tapia, J. A., Ortega Ferrusola, C. (2011). Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? *Theriogenology*, *76*(7), 1177–1186.

Peña, F. J., O'Flaherty, C., Ortiz Rodríguez, J. M., Martín Cano, F. E., Gaitskell-Phillips, G. L., Gil, M. C., Ortega Ferrusola, C. (2019). Redox regulation and oxidative stress: the particular case of the stallion spermatozoa. *Antioxidants*, *8*(11), 567.

Pérez, D. D., Acosta, M., Restrepo, G., Camacho, C., Pérez, J. (2017). Congelación de semen equino bajo dos esquemas de adición de dimetilformamida. *Revista de Investigaciones Veterinarias Del Perú*, *28*(4), 918–927.

Pesch, S., Bergmann, M. (2006). Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron*, *37*(7), 597–612.

Pintus, E., Ros-Santaella, J. L. (2021). Impact of oxidative stress on male reproduction in domestic and wild animals. *Antioxidants*, *10*(7), 1154.

Pommer, A. C., Rutllant, J., Meyers, S. A. (2002). The role of osmotic resistance on equine spermatozoal function. *Theriogenology*, *58*(7), 1373–1384.

Poyato-Bonilla, J., Sánchez-Guerrero, M. J., Cervantes, I., Gutiérrez, J. P., Valera, M. (2021). Genetic parameters for canalization analysis of morphological traits in the Pura Raza Español horse. *Journal of Animal Breeding and Genetics*, *138*(4), 482–490.

Prieto-Martínez, N., Morató, R., Muiño, R., Hidalgo, C. O., Rodríguez-Gil, J. E., Bonet, S., Yeste, M. (2017). Aquaglyceroporins 3 and 7 in bull spermatozoa: identification, localisation and their relationship with sperm cryotolerance. *Reproduction, Fertility and Development*, *29*(6), 1249–1259.

Pugliesi, G., Fürst, R., Carvalho, G. R. (2014). Impact of using a fast-freezing technique and different thawing protocols on viability and fertility of frozen equine spermatozoa. *Andrologia*, *46*(9), 1055–1062.

Rebordão, M. R., Amaral, A., Lukasik, K., Szóstek-Mioduchowska, A., Pinto-Bravo, P., Galvão, A., Skarzynski, D. J., Ferreira-Dias, G. (2018). Constituents of neutrophil extracellular traps induce in vitro collagen formation in mare endometrium. *Theriogenology*, *113*, 8–18.

Rebordão, M. R., Carneiro, C., Alexandre-Pires, G., Brito, P., Pereira, C., Nunes, T., Galvão, A., Leitão, A., Vilela, C., Ferreira-Dias, G. (2014). Neutrophil extracellular traps formation by bacteria causing endometritis in the mare. *Journal of Reproductive Immunology*, *106*, 41–49.

Renner-Martin, T. F. P., Forstenpointner, G., Weissengruber, G. E., Eberhardt, L. (2009). Gross anatomy of the female genital organs of the domestic donkey (*Equus asinus* Linné, 1758). *Anatomia, Histologia, Embryologia*, *38*(2), 133–138.

Restrepo Betancur, G., Usuga Suarez, A., Montoya Páez, J. D., Celis, Á. D., Henao, A. A. (2014). Evaluación de dos diluyentes para la criopreservación de semen de caballos de la raza criollo colombiano. *Revista Lasallista de Investigación*, *11*(2), 63–70.

Riesco, M. F., Alvarez, M., Anel-Lopez, L., Neila-Montero, M., Palacin-Martinez, C., Montes-Garrido, R., Boixo, J. C., de Paz, P., Anel, L. (2021). Multiparametric study of antioxidant effect on ram sperm cryopreservation—From field trials to research bench. *Animals*, *11*(2), 283.

Rigby, S., Derczo, S., Brinsko, S., Blanchard, T., Taylor, T., Forrest, D. W., Varner, D. (2000). Oviductal sperm numbers following proximal uterine horn or uterine body insemination. *Proceedings American Association of Equine Practitioners*, *46*, 332–334.

Robert, K. A., Sharma, R., Henkel, R., Agarwal, A. (2021). An update on the techniques used to measure oxidative stress in seminal plasma. *Andrologia*, *53*(2), e13726.

Roca, J., Parrilla, I., Gil, M. A., Cuello, C., Martinez, E. A., Rodriguez-Martinez, H. (2016). Non-viable sperm in the ejaculate: lethal escorts for contemporary viable sperm. *Animal Reproduction Science*, *169*, 24–31.

Rodríguez-Martínez, H., Kvist, U., Ernerudh, J., Sanz, L., Calvete, J. J. (2011). Seminal plasma proteins: what role do they play? *American Journal of Reproductive Immunology*, *66*(Suppl. 1), 11–22.

Rodriguez-Martinez, H., Saravia, F., Wallgren, M., Martinez, E. A., Sanz, L., Roca, J., Vazquez, J. M., Calvete, J. J. (2010). Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. *Journal of Reproductive Immunology*, *84*(1), 57–65.

Rota, A., Panzani, D., Sabatini, C., Camillo, F. (2012). Donkey jack (*Equus asinus*) semen cryopreservation: studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. *Theriogenology*, *78*(8), 1846–1854.

Rubio, C. P., Hernández-Ruiz, J., Martinez-Subiela, S., Tvarijonaviciute, A., Ceron, J. J. (2016). Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update. *BMC Veterinary Research*, *12*(1), 1–7.

Rubio, C. P., Mainau, E., Cerón, J. J., Contreras-Aguilar, M. D., Martínez-Subiela, S., Navarro, E., Tecles, F., Manteca, X., Escribano, D. (2019). Biomarkers of oxidative stress in saliva in pigs: analytical validation and changes in lactation. *BMC Veterinary Research*, *15*(1), 1–7.

Rubio, C. P., Martínez-Subiela, S., Hernández-Ruiz, J., Tvarijonaviciute, A., Cerón, J. J., Allenspach, K. (2017). Serum biomarkers of oxidative stress in dogs with idiopathic inflammatory bowel disease. *The Veterinary Journal*, *221*, 56–61.

Saalu, L. C. (2010). The incriminating role of reactive oxygen species in idiopathic male infertility: an evidence based evaluation. *Pakistan Journal of Biological Sciences*, *13*(9), 413–422.

Sabeur, K., Ball, B. A. (2006). Detection of superoxide anion generation by equine spermatozoa. *American Journal of Veterinary Research*, *67*(4), 701–706.

Samper, J. C. (2007). Techniques for artificial insemination. In *Current Therapy in Large Animal Theriogenology* (2nd Ed., pp. 37–42). R. S. Yougquist, W. R. Threlfall (Eds.), Saunders Elsevier.

Samper, J. C., Morris, C. A. (1998). Current methods for stallion semen cryopreservation: a survey. *Theriogenology*, *49*(5), 895–903.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *Journal of Clinical Investigation*, *83*(3), 865–875.

Schulte, R. T., Ohl, D. A., Sigman, M., Smith, G. D. (2010). Sperm DNA damage in male infertility: etiologies, assays, and outcomes. *Journal of Assisted Reproduction and Genetics*, *27*(1), 3–12.

Sicherle, C. C., de Souza, F. F., Freitas-Dell'Aqua, C. P., Mothé, G. B., Padovani, C. R., Papa, F. O., Lopes, M. D. (2020). Effects of the cryopreservation process on dog sperm integrity. *Animal Reproduction*, *17*(1), 1–10.

Šichtař, J., Bubeníčková, F., Sirohi, J., Šimoník, O. (2019). How to increase post-thaw

semen quality in poor freezing stallions: preliminary results of the promising role of seminal plasma added after thawing. *Animals*, *9*(7), 414.

Sieme, H., Harrison, R. A. P., Petrunkina, A. M. (2008). Cryobiological determinants of frozen semen quality, with special reference to stallion. *Animal Reproduction Science*, *107*(3–4), 276–292.

Sies, H. (1993). Strategies of antioxidant defense. *European Journal of Biochemistry*, *215*(2), 213–219.

Sies, H. (2015). Oxidative stress: a concept in redox biology and medicine. *Redox Biology*, *4*, 180–183.

Sikka, S. C., Rajasekaran, M., Hellstrom, W. J. G. (1995). Role of oxidative stress and antioxidants in male infertility. *Journal of Andrology*, *16*(6), 464–468.

Silva, S. V., Soares, A. T., Batista, A. M., Almeida, F. C., Nunes, J. F., Peixoto, C. A., Guerra, M. M. P. (2011). *In vitro* and *in vivo* evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. *Reproduction in Domestic Animals*, *46*(5), 874–881.

Silvestre, M. A., Yániz, J. L., Peña, F. J., Santolaria, P., Castelló-Ruiz, M. (2021). Role of antioxidants in cooled liquid storage of mammal spermatozoa. *Antioxidants*, *10*(7), 1096.

Skarzynski, D. J., Szóstek-Mioduchowska, A. Z., Rebordão, M. R., Jalali, B. M., Piotrowska-Tomala, K. K., Leciejewska, N., Łazarczyk, M., Ferreira-Dias, G. M. (2020). Neutrophils, monocytes and other immune components in the equine endometrium: friends or foes? *Theriogenology*, *150*, 150–157.

Sotgia, S., Taras, A., Zinellu, A., Cherchi, R., Mangoni, A. A., Carru, C., Bogliolo, L. (2020). Hercynine, ergothioneine and redox state in stallion's seminal plasma. *Antioxidants*, *9*(9), 855.

Szczykutowicz, J., Kałuza, A., Kaźmierowska-Niemczuk, M., Ferens-Sieczkowska, M. (2019). The potential role of seminal plasma in the fertilization outcomes. *BioMed Research International*, *2019*, 5397804.

Tirpák, F., Halo, M., Tokárová, K., Binkowski, L. J., Vašíček, J., Svoradová, A., Błaszczyk-Altman, M., Kováčik, A., Tvrdá, E., Chrenek, P., Lukáč, N., Massányi, P. (2021). Composition of stallion seminal plasma and its impact on oxidative stress markers and spermatozoa quality. *Life*, *11*(11), 1238.

Töpfer-Petersen, E., Ekhlasi-Hundriescr, M., Kirchhoff, C., Leeb, T., Sieme, H. (2005). The role of stallion seminal proteins in fertilisation. *Animal Reproduction Science*, *89*(1–4), 159–170.

Töpfer-Petersen, E., Romero, A., Varcla, P. F., Ekhlasi-Hundriescr, M., Dostàlovà, Z., Sanz, L., Calvete, J. J. (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia*, *30*(4–5), 217–224.

Tosic, J., Walton, A. (1946). Formation of hydrogen peroxide by spermatozoa and its inhibitory effect on respiration. *Nature*, *158*(4014), 485–485.

Tremellen, K. (2008). Oxidative stress and male infertility—a clinical perspective. *Human Reproduction Update*, *14*(3), 243–258.

Troedsson, M. H. T., Loset, K., Alghamdi, A. M., Dahms, B., Crabo, B. G. (2001). Interaction between equine semen and the endometrium: the inflammatory response to semen. *Animal Reproduction Science*, *68*(3–4), 273–278.

Troedsson, M. H. T., Woodward, E. M. (2016). Our current understanding of the pathophysiology of equine endometritis with an emphasis on breeding-induced endometritis. *Reproductive Biology*, *16*(1), 8–12.

Trotter, G. W., McKinnon, A. O. (1988). Surgery for abnormal vulvar and perineal conformation in the mare. *Veterinary Clinics of North America: Equine Practice*, *4*(3), 389–405.

Turner, R. M. (2005). Moving to the beat: a review of mammalian sperm motility regulation. *Reproduction, Fertility and Development*, *18*(2), 25–38.

Tvrdá, E., Schneidgenová, M., Jambor, T., Paál, D., Szabó, C., Lukáč, N. (2016). Comparison of two colorimetric antioxidant capacity assessment methods in bovine semen fractions. *Journal of Microbiology, Biotechnology and Food Sciences*, *5*(1), 47–49.

Ugur, M. R., Saber Abdelrahman, A., Evans, H. C., Gilmore, A. A., Hitit, M., Arifiantini, R. I., Purwantara, B., Kaya, A., Memili, E. (2019). Advances in cryopreservation of bull sperm. *Frontiers in Veterinary Science*, *6*, 268.

Upreti, G. C., Jensen, K., Munday, R., Duganzich, D. M., Vishwanath, R., Smith, J. F. (1998). Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. *Animal Reproduction Science*, *51*(4), 275–287.

Uribe, P., Meriño, J., Matus, C. E., Schulz, M., Zambrano, F., Villegas, J. V., Conejeros, I., Taubert, A., Hermosilla, C., Sánchez, R. (2022). Autophagy is activated in human spermatozoa subjected to oxidative stress and its inhibition impairs sperm quality and promotes cell death. *Human Reproduction*, *37*(4), 680–695.

Varner, D. D., Gibb, Z., Aitken, R. J. (2015). Stallion fertility: a focus on the spermatozoon. *Equine Veterinary Journal*, *47*(1), 16–24.

Varner, D. D., Johnson, L. (2011). From a sperm's eye view: revisiting our perception of this intriguing cell. In *Equine Reproduction* (2nd Ed., pp. 909–990). A. O. McKinnon, E. L. Squires, W. E. Vaala, D. D. Varner (Eds.), Wiley-Blackwell.

Vendramini, O. M., Guintard, C., Moreau, J., Tainturier, D. (1998). Cervix conformation: a first anatomical approach in baudet du poitou jenny asses. *Animal Science*, *66*(3), 741–744.

Verit, F. F., Verit, A., Ciftci, H., Erel, O., Çelik, H. (2009). Paraoxonase-1 activity in subfertile men and relationship to sperm parameters. *Journal of Andrology*, *30*(2), 183–189.

Verit, F. F., Verit, A., Kocyigit, A., Ciftci, H., Celik, H., Koksal, M. (2006). No increase in sperm DNA damage and seminal oxidative stress in patients with idiopathic infertility. *Archives of Gynecology and Obstetrics*, *274*(6), 339–344.

Vernet, P., Fulton, N., Wallace, C., Aitken, R. J. (2001). Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biology of Reproduction*, *65*(4), 1102–1113.

Vidament, M. (2005). French field results (1985–2005) on factors affecting fertility of frozen stallion semen. *Animal Reproduction Science*, *89*(1–4), 115–136.

Vidament, M., Dupere, A. M., Julienne, P., Evain, A., Noue, P., Palmer, E. (1997). Equine frozen semen: freezability and fertility field results. *Theriogenology*, *48*(6), 907–917.

Vidament, M., Vincent, P., Martin, F. X., Magistrini, M., Blesbois, E. (2009). Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. *Animal Reproduction Science*, *112*(1–2), 22–35.

Vilés, K., Rabanal, R., Rodríguez-Prado, M., Miró, J. (2013). Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology*, *79*(7), 1019–1026.

Wagner, H., Cheng, J. W., Ko, E. Y. (2018). Role of reactive oxygen species in male infertility: an updated review of literature. *Arab Journal of Urology*, *16*(1), 35–43.

Waheed, M. M., Gouda, E. M., Khalifa, T. A. A. (2013). Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. *Animal Reproduction Science*, *142*(3–4), 126–130.

Walczak-Jedrzejowska, R., Wolski, J. K., Slowikowska-Hilczer, J. (2013). The role of oxidative stress and antioxidants in male fertility. *Central European Journal of Urology*, *66*(1), 60–67.

Wathes, D. C., Abayasekara, D. R. E., Aitken, R. J. (2007). Polyunsaturated fatty acids in male and female reproduction. *Biology of Reproduction*, *77*(2), 190–201.

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, *60–61*, 481–492.

Woodward, E. M., Christoffersen, M., Campos, J., Betancourt, A., Horohov, D., Scoggin, K. E., Squires, E. L., Troedsson, M. H. T. (2013). Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding-induced endometritis. *Reproduction*, *145*(3), 289–296.

Woodward, E. M., Troedsson, M. H. T. (2015). Inflammatory mechanisms of endometritis. *Equine Veterinary Journal*, *47*(4), 384–389.

Wu, R., Feng, J., Yang, Y., Dai, C., Lu, A., Li, J., Liao, Y., Xiang, M., Huang, Q., Wang, D., Du, X. B. (2017). Significance of serum total oxidant/antioxidant status in patients with colorectal cancer. *PLOS ONE*, *12*(1), e0170003.

Yeste, M. (2016). Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology*, *85*(1), 47–64.

Yeste, M. (2017). State-of-the-art of boar sperm preservation in liquid and frozen state. *Animal Reproduction*, *14*(1), 69–81.

Yeste, M., Estrada, E., Casas, I., Bonet, S., Rodríguez-Gil, J. E. (2013a). Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. *Theriogenology*, *79*(6), 929–939.

Yeste, M., Estrada, E., Pinart, E., Bonet, S., Miró, J., Rodríguez-Gil, J. E. (2014). The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability. *Cryobiology*, *68*(2), 251–261.

Yeste, M., Estrada, E., Rocha, L. G., Marín, H., Rodríguez-Gil, J. E., Miró, J. (2015). Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. *Andrology*, *3*(2), 395–407.

Yeste, M., Flores, E., Estrada, E., Bonet, S., Rigau, T., Rodríguez-Gil, J. E. (2013b). Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-thawing by stabilising disulfide bonds. *Reproduction, Fertility and Development*, *25*(7), 1036–1050.

Žaja, I. Ž., Samardžija, M., Vince, S., Sluganović, A., Strelec, S., Šuran, J., DelVechio, I., Đuričić, D., Ostović, M., Valpotić, H., Milinković-Tur, S. (2016). Antioxidant protection and lipid peroxidation in testes and different parts of epididymis in boars. *Theriogenology*, *86*(9), 2194–2201.

Zambrano, F., Carrau, T., Gärtner, U., Seipp, A., Taubert, A., Felmer, R., Sanchez, R., Hermosilla, C. (2016). Leukocytes coincubated with human sperm trigger classic neutrophil extracellular traps formation, reducing sperm motility. *Fertility and Sterility*, *106*(5), 1053-1060.

Zorrilla García, A. E., Eirez Izquierdo, M., Izquierdo Expósito, M. (2004). Papel de los radicales libres sobre el ADN: carcinogénesis y terapia antioxidante. *Revista Cubana de Investigaciones Biomédicas*, *23*(1), 51–57.

Zribi, N., Chakroun, N. F., Elleuch, H., Abdallah, F. B., Ben Hamida, A. S., Gargouri, J., Fakhfakh, F., Keskes, L. A. (2011). Sperm DNA fragmentation and oxidation are independent of malondialdheyde. *Reproductive Biology and Endocrinology*, *9*(1), 1–8.