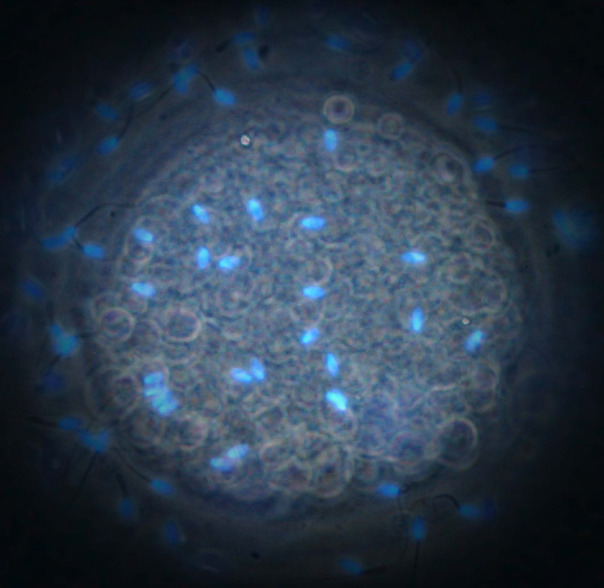
UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL



ESTRÉS OXIDATIVO DURANTE LA CONSERVACIÓN DEL SEMEN EQUINO

JOSÉ MANUEL ORTIZ RODRÍGUEZ

PROGRAMA DE DOCTORADO EN BIOMARCADORES DE SALUD Y ESTADOS PATOLÓGICOS

2021

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Estrés oxidativo durante la conservación del semen equino

Oxidative stress during the conservation of equine semen

José Manuel Ortiz Rodríguez

PROGRAMA DE DOCTORADO EN BIOMARCADORES DE SALUD Y ESTADOS PATOLÓGICOS (R012)

Conformidad de los directores

La conformidad de los directores de la tesis consta en el original en papel de esta Tesis Doctoral

Fdo. Fernando Juan Peña Vega **Fdo.** Cristina Ortega Ferrusola

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En Cáceres,

Fdo. José Manuel Ortiz Rodríguez

2021

Asunto: Rtdo. Informe sobre las publicaciones que componen la Tesis.
Destinatario: Sr. Coordinador de la Comisión Académica del Programa de Doctorado:

BIOMARCADODES DE SALUD Y ESTADOS PATOLOGICOS

Como Director/es de la Tesis doctoral titulada:

Estrés oxidativo durante la conservación del semen equino

Cuyo autor es D./Dª. JOSE MANUEL ORTIZ RODRIGUEZ,

INFORMO/INFORMAMOS

A la **Comisión Académica del Programa de Doctorado** que:

- La tesis está compuesta por un compendia de **6 publicaciones ya publicadas** en revistas indexadas **y un artículo enviado (en revisión en este momento).** Las revistas en las que se han publicado esta tesis doctoral son:
- **BIOLOGY OF REPRODUCTION** Q1 (Reproductive Biology) IF 4.3. dos publicaciones
- **REPRODUCTION** Q2 (Reproductive Biology) IF 3.9. una publicación
- **THERIOGENOLOGY** Q1 (Veterinary Sciences) IF 2.74 una publicación
- **PLOS ONE** Q2 (Multidisciplinary) IF 3.24 dos publicaciones

En todas estas publicaciones el doctorando figura como primer autor, lo que certifica su participación preferente en todas ellas

SOLICITO/SOLICITAMOS

de la **Comisión Académica del Programa de Doctorado** que autorice la presentación de la Tesis a la Comisión de Doctorado.

Cáceres a 18 Octubre de 2021

Fdo: Fernando J Peña Vega

Cristina Ortega Ferrusola

COLLEGE OF ENGINEERING, SCIENCE AND ENVIRONMENT

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Friday 17 September 2021

Professor Fernando Peña
Laboratory of Equine Reproduction
Department of Medicine
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Dear Professor Peña,

I have been asked to write a report on the quality of the thesis of José Manuel Ortiz Rodríguez, entitled 'Estrés oxidativo durante la conservación del semen equino', so that he may be considered for the title of 'International Doctorate' following his PhD conferral.

I have read the six manuscripts (five published, one submitted) which comprise Mr Ortiz Rodríguez's thesis, and find them to be of the utmost possible quality. These manuscripts have been published in the **highest impact factor journals in the field**, and present novel data, which is not only of an extremely high quality from molecular standpoint, but is also useful from an applied, clinical perspective. Detail of the merits of Mr Ortiz Rodríguez's individual manuscripts, are outlined below.

The first manuscript, entitled 'Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase', was published in *PLoS One* in 2019. Cryopreserved stallion spermatozoa have a significantly reduced longevity post-thaw, and this study aimed to improve the quality of those cells which survive the freeze-thaw process by enhancing their use of glycolysis (and decreasing their dependence on oxidative phosphorylation – a process which generates substantial quantities of reactive oxygen species) using the antidiabetic drug, rosiglitazone. Following thawing, spermatozoa were incubated with or without rosiglitazone, with the effects on mitochondrial integrity, motility, Akt phosphorylation, oxidation-reduction potential and apoptotic-like changes being assessed. Furthermore, Mr Ortiz-Rodriguez utilised computational flow cytometry – a technique which has not previously been utilised for equine fertility studies, and is just one example of Mr Ortiz-Rodriguez's use of sophisticated analytical techniques which differential him from other researchers at the same career stage. My team at UON were the first to reveal the pro-survival effects of rosiglitazone on stallion spermatozoa, and this study further delves into the mechanisms behind this phenomenon, **revealing for the first time that rosiglitazone increases Akt phosphorylation in stallion spermatozoa**, thereby inhibiting apoptosis. This is important, as the process of cryopreservation induces apoptotic-like changes which are responsible for the accelerated senescence in these cells.

The second manuscript, entitled 'The incorporation of cystine by the soluble carrier family 7

member 11 (SLC7A11) is a component of the redox regulatory mechanism in stallion spermatozoa' was published in *Biology of Reproduction* in 2019. This study confirmed a longstanding, but poorly evidenced paradigm; that the removal of antioxidant-rich seminal plasma results in an increase in sperm oxidation, and by extension, an increase in oxidative stress under certain conditions. However, the main aim of this study was to explore the connection between aberrant expression of the SLC7A11 gene and male subfertility, a phenomenon which was first observed using a knockout mouse model in 2017 (Hamashima et al., FREE RADICAL RESEARCH, 2017 VOL. 51, NO. 9-10, 851–860). However, Mr Ortiz Rodríguez was the **first to demonstrate the role of the SLC7A11 antiporter in redox regulation of mammalian spermatozoa**; a mechanism that is of utmost importance to stallion spermatozoa which produce vast quantities of reactive oxygen species as a by-product of their use of oxidative phosphorylation. This study revealed that stallion sperm are largely depend on the incorporation of exogenous cystine for the synthesis of an essential antioxidant, glutathione, and therefore the maintenance of essential redox homeostasis. It was demonstrated that cystine is transported into the cells via the SLC7A11 antiporter, a phenomenon which Mr Ortiz Rodríguez confirmed through the use of a specific inhibitor of the SLC7A11 antiporter.

In the third manuscript, 'The SLC7A11: sperm mitochondrial function and non-canonical glutamate metabolism', published in *Reproduction* in 2020, it was revealed that SLC7A11 also plays a role in glutamate metabolism via the exchange of extracellular cysteine with intracellular glutamate. Various inhibitors of the SLC7A11 uniporter were employed, with paradoxical effects: less potent inhibitors reduced sperm viability which could be reversed by co-incubation with cysteine, while a highly potent inhibitor actually increased mitochondrial membrane potential via the retention of glutamate which was fed into the non-canonical pathway to recycle GSSG to GSH and maintain redox homeostasis and mitochondrial function.

The fourth manuscript, entitled 'The inhibition of spermatic cystine/glutamate antiporter xCT (SLC7A11) influences the ability of cryopreserved stallion sperm to bind to heterologous zonae pellucidae', published in *Theriogenology* in 2021, further explores the functional effects of SLC7A11 uniporter inhibition on stallion sperm fertilising capacity through the use of a heterologous zona-pellucida/sperm binding assay, along with other measures of fertilising capacity. Interestingly, while zona-binding capacity was indeed reduced following SLC7A11 inhibition of frozen-thawed spermatozoa, no other sperm parameters (including tyrosine phosphorylation) were affected, suggesting an alternate mechanism of action in the frozen-thawed cells utilised in this study.

Mr Ortiz Rodríguez's fifth manuscript, entitled 'Low glucose and high pyruvate reduce the production of 2-oxoaldehydes, improving mitochondrial efficiency, redox regulation, and stallion sperm function', was published in *Biology of Reproduction* in 2021. **This is the first study to reveal toxic effects of supraphysiological glucose concentrations on stallion spermatozoa during in vitro storage**. In recent years, research from my group at UON and Mr Ortiz Rodríguez's group at UOE has revealed that stallion spermatozoa preferentially utilise

oxidative phosphorylation for the production of energy for motility, with a paucity of literature around the relative importance and implications of glycolysis in the spermatozoa of this species. As such, this study aimed to explore the possibility that commercial semen extenders which were formulated prior to our current knowledge of stallion sperm metabolism, may in fact contain levels of glucose which are toxic to stallion spermatozoa via the production of 2-oxoaldehydes. The study revealed that glycolysis is an important contributor to GSH reduction via the pentose phosphate pathway, thereby conveying important antioxidant properties to these cells.

In his final landmark study, 'Sperm cryopreservation impacts the early development of equine embryos by downregulating specific transcription factors' (under review), Mr Ortiz Rodríguez has utilised Next Generation Sequencing to investigate differences in transcriptome of equine embryos derived using fresh, or cryopreserved spermatozoa. His findings provide strong evidence that insemination with cryopreserved spermatozoa may compromise early embryo development, probable due to cryopreservation-induced modifications to sperm proteins. Mr Ortiz Rodríguez found a dramatic difference in the number of transcripts upregulated in embryos derived from fresh spermatozoa compared to embryos derived from inseminations with cryopreserved spermatozoa in the transition from 8 to 10 days, this **explains the well-recognised phenomenon that embryos obtained with cryopreserved spermatozoa experience a delay in development in this particular interval**. This highly powerful study provides further evidence for the need to improve current cryopreservation protocols, or indeed, to explore alternative methods of sperm storage for use in assisted reproductive technologies.

Mr Ortiz Rodríguez's research utilises a wide range of techniques and advanced analytical skills which are rarely seen in the veterinary field, making his body of research of **absolute world class quality**, and as such, I am recommending that Mr Ortiz Rodríguez be bestowed with the title of 'International Doctorate'.

Regards,

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Les meilleurs soins pour la vie
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Montréal October 20, 2021

Professor Fernando Peña Vega
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Dear Professor Peña Vega,

I was asked to evaluate the PhD Thesis of Mr. Jose Martin Ortiz Rodriguez entitled: "Estrés oxidativo durante la conservación del semen equino".

Seven manuscripts comprise the thesis. Six were published in high-impact factor peer-review journals in the biology of reproduction, and one manuscript is under review. The research presented in these papers is of high quality with meaningful data for the animal reproduction field, mainly equine reproduction. These studies are a combination of basic research with the translational potential to improve equine semen cryopreservation. Thus, the clinical value of these studies is extremely important since it is still challenging cryopreservation of many animal species of economic value, such as the equine. After reading all the manuscripts carefully, I can attest to the quality of Mr. Ortiz Rodriguez's research work.

Three manuscripts focused on the soluble carrier family 7 member 11 (SLC7A11) antiporter in equine spermatozoa. In the first manuscript entitled "*The incorporation of cystine by the soluble carrier family 7 member 11 (SLC7A11) is a component of the redox regulatory mechanism in stallion spermatozoa. Biol Reprod, 2019*", Mr. Ortiz Rodriguez demonstrated, for the first time, the role of SLC7A11 antiporter in the redox regulation of equine spermatozoa. He used a combination of molecular biology techniques (e.g. immunoblotting, flow cytometry, immunocytochemistry, and the use of the specific inhibitor of SLC7A11 (sulfasalazine) to characterize the location and function of the antiporter in equine spermatozoa. These findings are important for the field of equine reproduction since stallion spermatozoa produce significant amounts of ROS through the oxidative phosphorylation in mitochondria that must be controlled by levels of antioxidant compounds such as reduced glutathione (GSH). These findings are useful to re-think the way to cryopreserve mammalian spermatozoa and, in particular, for the equine species. Mammalian spermatozoa have low levels of GSH and the addition of cysteine to the semen extender will provide extra protection against oxidative stress generated by the cryopreservation process.

In the second manuscript, "*The SLC7A11: sperm mitochondrial function and non-canonical glutamate metabolism. Reproduction, 2020*", Mr. Ortiz Rodriguez demonstrated that SLC7A11 antiporter is important to maintain active mitochondria, fundamental organelles for equine spermatozoa that primarily obtained energy through oxidative phosphorylation. Mr. Ortiz Rodriguez demonstrated that the role of the antiporter is to ensure sufficient GSH production is taken place intracellularly by a non-canonical pathway that recycles oxidized glutathione into GSH to protect spermatozoa against the ROS levels produced in mitochondria.

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In the third manuscript on SLC7A11, entitled "*The inhibition of spermatic cystine/glutamate antiporter xCT (SLC7A11) influences the ability of cryopreserved stallion sperm to bind to heterologous zonae pellucidae. Theriogenology, 2021*", Mr. Ortiz Rodriguez provide strong evidence on the role of the antiporter in equine sperm fertilizing ability using the heterologous zona-pellucida-sperm binding assay. The inhibition of SLC7A11 inhibited the zona pellucida binding without altering other parameters important for fertilizing capacity, such as sperm motility and capacitation. With these findings, Mr. Ortiz Rodriguez opened a new avenue for research on the role of redox regulation in the sperm and egg interaction to ensure fertilization.

In the manuscript entitled "Low glucose and high pyruvate reduce the production of 2-oxoaldehydes, improving mitochondrial efficiency, redox regulation, and stallion sperm function. Biol Reprod 2021", Mr. Ortiz Rodriguez hypothesized that supraphysiological concentration of glucose found in commercial semen extenders are detrimental for equine spermatozoa because of the excessive production of 2-oxoaldehydes. He found that inhibition of glycolysis improves sperm viability associated with increased reduced glutathione production and improvement of mitochondrial activity. Moreover, low glucose concentrations improve motility and antioxidant capacity in spermatozoa showing lower levels of 2-oxo aldehydes compared to controls. This is the first report demonstrating that supraphysiological levels of glucose impairing equine sperm function. These studies are important as they highlight the need for basic research to improve cryopreservation methods based on the physiology of the spermatozoa of a given species.

Mr. Ortiz Rodriguez's research was dedicated not only to studying sperm metabolism but to elucidating ways to modulate sperm metabolism to increase the survival of these cells after cryopreservation. Equine spermatozoa are very sensitive to cryopreservation that promotes oxidative stress and apoptotic-like changes in thawed sperm cells. Thus, efforts to improve protocols to preserve horse spermatozoa are essential for the equine industry. In the manuscript entitled: "*Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase 3. PLOS ONE, 2019",* Mr. Ortiz Rodriguez treated equine spermatozoa with rosiglitazone, a drug used to treat diabetes, to improve sperm survival after cryopreservation. He found that rosiglitazone improves sperm survival by activating pro-survival pathways maintaining phosphorylation of AKT, and reducing caspase 3 activation. The treatment also maintains mitochondrial membrane potential and low reactive oxygen species levels, which is important to ensure normal sperm function. This is the first evidence reported that the cryopreservation of equine spermatozoa can be improved by supplementing semen extenders with rosiglitazone.

Because cryopreserved spermatozoa are altered by the frozen-thawing process, and that sperm quality is necessary for normal embryo development, the manuscript entitled: *"Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. PLOS ONE, 2019",* Mr. Ortiz Rodriguez compared the transcriptome of cryopreserved and fresh equine spermatozoa. He found that the transcriptome profile of embryos produced using frozen-thawed equine spermatozoa was significantly different from that of embryos obtained using fresh equine spermatozoa. Mr. Ortiz Rodriguez found that genes to produce proteins involved in oxidative phosphorylation, DNA binding and replication, and immune response were down-regulated in the former

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embryos. These findings demonstrated for the first time that sperm cryopreservation alters the paternal genome promoting changes in the embryos produced and can account for the embryo losses that it is significantly higher when frozen-thawed spermatozoa are used. Indeed, many genes found by Mr. Ortiz Rodriguez are orthologs of genes known to be embryonic lethal in mice.
In the last manuscript (under review), entitled:" *Sperm cryopreservation impacts the early development of equine embryos by downregulating specific transcription factors,"* Mr. Ortiz Rodriguez used Next Generation Sequencing to characterize the transcriptome of early embryos produced with either frozen-thawed or fresh equine spermatozoa. He found 12 mRNAs significantly downregulated in embryos produced with frozen-thawed spermatozoa. Among others, these mRNA encoding for the chromatin-remodelling ATPase INO80, Lipase maturation factor 1 (LMF1), the mitochondrial mRNA pseudouridine synthase RPUSD3, LIM and cysteine-rich domains protein 1, LMCD1. This study provides evidence of altered proteins that can be used to evaluate embryo quality for animal production.

In Conclusion, Mr. Ortiz Rodriguez's research is focused on understanding molecular mechanisms of equine spermatozoa with well-planned experimental designs that are uncommon to see in animal reproduction science. The use of different cellular and advanced molecular biology techniques and critical analysis of the data evidence Mr. Ortiz Rodriguez's scientific rigor and research quality. Moreover, it is evidenced by Mr. Ortiz Rodriguez's excellent expertise in mastering these techniques and conducting these experiments. From these seven manuscripts, I can attest that Mr. Ortiz Rodriguez's studies are an outstanding contribution to our knowledge on sperm physiology in equine and help to improve the cryopreservation methods for animal production. Thus, basic and translational science researchers are benefiting by his research.

I want to emphasize that these studies are of an outstanding scientific quality that reinforces the world leadership of Dr. Peña Vega's laboratory in equine spermatology. Based on my assessment, I strongly recommend Mr. Ortiz Rodriguez for the title of "International Doctorate".

Sincerely,

Cristian O'Flaherty, D.V.M, Ph.D.

Professor of Surgery (Urology)

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October 25th 2021

Comments on the thesis by Jose´ M. Ortiz-Rodriguez

The project of José M. Ortiz-Rodriguez includes a series of experiments with the results contributing considerably to the recent knowledge on equine semen physiology and pathophysiology. The results for the first time provide evidence that fertilization with frozen-thawed sperm alters the gene expression during early development of horse embryos. This result is also of great interest with regard to many other species where cryopreserved semen is used for insemination. In the main part of the project, the significance of SLC7A11, a member of the soluble carrier family 7, for mitochondrial function and redox regulation in stallion sperm was investigated in detail. The results of this research project have been summarized in a total of six publications of outstanding quality with José M. Ortiz-Rodriguez being the first author of all of them. The manuscripts have been published in PLoS ONE (two publications, impact factor 3.240), Reproduction (one publication, impact factor 3.906), Biology of Reproduction (two publications, impact factor 4.285) and Theriogenology (one publication, impact factor 2.740). These journals have a very high reputation among scientists involved in research in animal reproduction. The international relevance of the research conducted by José M. Ortiz-Rodriguez is thus clearly reflected.

The results published by José M. Ortiz-Rodriguez' provide important information and have the potential to advance the standards of stallion semen preservation considerably. The research published in thesis is thus highly likely to contribute to improved fertility rates and a reduction of early embryonic loss not only in equines but most likely also in other species. The results are therefore of high relevance for both the scientific community interested in research on semen preservation and the underlying mechanisms of sperm function as well as the horse breeding industry.

A.Univ.-Prof. Dr. Christine Aurich

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Friday 22 October 2021

Evaluation of Dr. José Manuel Ortiz Rodríguez's PhD thesis

Dear Professor Peña,

In this letter, I give my assessment of the PhD thesis by José Manuel Ortiz Rodríguez entitled "Estrés oxidativo durante la conservación del semen equino", in order to allow him to be considered for the title of "International Doctorate" after thesis defense.

The thesis presents original research on the impact of oxidative stress on stallion spermatozoa during semen storage and on the development of new strategies for sperm conservation and treatment of male factor infertility. The studies resulted in the publication of six, almost seven, valuable manuscripts in high impact factor journals in reproduction. Undoubtedly, his research is of superb quality and below I retrace the most significant milestones in his PhD.

In the first manuscript "Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase", José Manuel Ortiz Rodríguez and his co-authors investigated the mechanisms behind the positive effect of Rosiglitazone on frozen stallion semen. For the first time, they reveal that Rosiglitazone can activate a pro survival pathways by increasing Akt phosphorylation in stallion spermatozoa, providing a starting point for improving current sperm biotechnology.

The research of his team on the role of spermatic cystine/glutamate antiporter xCT (SLC7A11) produced three papers: (1) "The incorporation of cystine by the soluble carrier family 7member 11 (SLC7A11) is a component of the redox regulatory mechanism in stallion spermatozoa"; (2) "The SLC7A11: sperm mitochondrial function and non-canonical glutamate metabolism"; (3) "The inhibition of spermatic cystine/glutamate antiporter xCT (SLC7A11) influences the ability of cryopreserved stallion sperm to bind to heterologous zonae pellucidae". In the first two papers, the role of SLC7A11 in redox regulation and in the glutamate metabolism of mammalian spermatozoa was demonstrated. Afterwards, the supplementation and the inhibition of CysS incorporation by SLC7A11 of thawed semen was tested. The results suggest that CysS incorporation by SLC7A11 can influence the ability of spermatozoa to bind the heterologous zona pellucidae.

Moreover, in the manuscript "Low glucose and high pyruvate reduce the production of 2-oxoaldehydes, improving mitochondrial efficiency, redox regulation, and stallion sperm function", the research group of José Manuel Ortiz Rodríguez reveal that high glucose concentration are toxic for stallion spermatozoa during *in vitro* storage and low glucose concentrations may permit more efficient sperm metabolism and redox regulation. Furthermore, this finding can be useful in the development of new extenders.

Another line of research of José Manuel Ortiz Rodríguez focuses on the effect of fertilization with cryopreserved semen on early embryo development, that is a challenging topic in many species, including human. The manuscript "Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse" provides strong evidence of altered transcription in embryos resulting from fertilization with cryopreserved spermatozoa. The revelation that insemination with cryopreserved spermatozoa can compromise viability and early development of embryo, probably linked to sperm proteins modification induced by cryopreservation, is reported in the manuscript entitled "Sperm cryopreservation impacts the early development of equine embryos by downregulating specific transcription factors" (under review).

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Meanwhile, all research conducted during the PhD used most advanced techniques and equipment for semen evaluation including flow cytometry, computer assisted sperm analysis and fluorescence microscopy and for the assessment of sperm biology (e.g. liquid chromatography-tandem mass spectrometry). Published manuscripts demonstrated the abilities of José Manuel Ortiz Rodríguez in semen evaluation and in analysis and interpretation of results, as well as his ability to collaborate with local and international research groups.

In conclusion, the research of José Manuel Ortiz Rodríguez presents original research results of great importance in the field of animal reproduction and I recommend without hesitation that the candidate is awarded the "International doctorate" degree.

Dr. Natascia Cocchia
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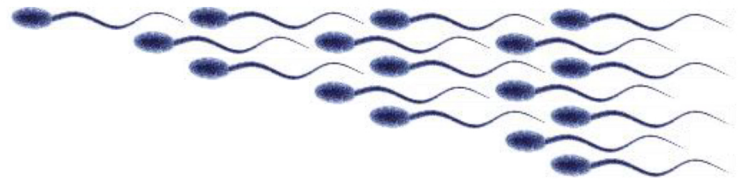
AGRADECIMIENTOS



"La gratitud se da cuando la memoria se almacena en el corazón y no en la mente"
(Lionel Hampton)

Seis son los años transcurridos desde mi graduación como veterinario y el comienzo de esta etapa, la Tesis Doctoral, que indudablemente me ha aportado un gran crecimiento personal y profesional. Crecimiento dependiente de todas y todos los que me habéis acompañado en el camino. Muchos serían los folios necesarios para agradecer individualmente, por lo que me disculpo si alguien no queda reflejado en estas palabras. Mis agradecimientos van para todas las personas que en algún momento de este trayecto me regalasteis alguna sonrisa, pues sonreír no solo cambia la expresión de la cara, sino que también hace que produzcamos endorfinas, reduciendo el dolor físico o emocional y provocando sensación de bienestar.

Agradezco a mis directores de tesis, Fernando y Cristina, la oportunidad de introducirme en la investigación. Después de cuatro años como alumno interno y la realización de mi Trabajo Fin de Grado con vosotros, me permitisteis continuar con mi formación. A ti Fernando, agradecerte tu gran sabiduría, la dirección constante de mi trabajo y todos los consejos, incluso aquellos que dices desde el silencio. Son muchos los conocimientos adquiridos y no puedo dejar de nombrar los de clínica; Cristina muchas gracias por confiar en mí desde el principio y enseñarme tanto sobre reproducción equina.

A mi familia, en especial a mis padres, por vuestro apoyo incondicional y la ayuda infinita. Muchas gracias por ser, estar y construirme, por la transmisión de vuestros valores, por esa bondad que os caracteriza y por vuestras fuerzas para mantener siempre la lucha. Indudablemente, nada de todo lo que hoy soy hubiera sido posible sin vosotros. Y a ti, Cristinita, la mejor de las hermanas, aún te recuerdo sentada en mi regazo el día que partía de casa para iniciar mis estudios. No dejes nunca que tu esencia se pierda, consérvate y cuídate, porque todos los que te rodeamos somos mejores por el simple hecho de tenerte. Gracias también por ese angelito que nos has traído, mi pequeño Jaime, que solo con mirarlo irradia toda la luz y ternura propia de su madre.

A mis amigos de siempre, Raquel, Emilio, Helena, Luis, Sara y Rocío, gracias por todos y cada uno de los momentos que hemos compartido. En especial a mis pilares, Lucía y Elsa, por vuestra eterna amistad y sinceridad; por decir incluso aquello que sabéis que ayudará, aunque no lo quiera escuchar. Gracias también a las amistades que quedaron de los estudios de veterinaria y que estarán por siempre. Pilar, muchas gracias por tu personalidad característica, por tu cariño y por todo lo que con tu amistad regalas. Y a ti, Rocío, mi compañera de prácticas, la sonrisa dulce del grupo, agradecerte tu sensibilidad y amabilidad, tu escucha y tus consejos.

Agradecer también a mi grupo de las HAD@S, por todos los ratos, los cafelitos a media mañana, las cervezas de los viernes y los viajes inolvidables. En estos últimos años os eché mucho en falta. Particularmente, tengo que agradecer a mis GG's, Gemma y Gara, mis profes de las prácticas de farmacología, que os convertisteis en grandes amigas. Me faltan las palabras de agradecimiento y es que, si no hubiera sido porque estabais, seguramente no hubiera llegado hasta aquí hoy. Gemma, son infinitas las cualidades que se pueden destacar de ti, dulzura, gratitud, amabilidad, bondad... Y no son menos las tuyas Gara, espontaneidad, alegría y superación, entre otras muchas. Sois mis ángeles encontrados, gracias por existir y compartir, por vuestros abrazos, por poner vuestro hombro, pero sobre todo por la alegría que siempre me transmitís, que como dice Paulo Coelho, la alegría es contagiosa y siempre consigue descubrir una solución donde la lógica sólo encontró una explicación para el error.

Antonio, a ti gracias por aconsejarme, enseñarme, apoyarme y tranquilizarme. Son casi cuatro años compartidos. Experiencias en Rusia, Italia, Alemania, Portugal, Dinamarca y España. Espero que siempre multipliquemos. Muchas gracias por escuchar, por las reflexiones infinitas y por estar siempre presente.

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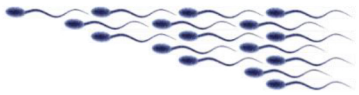
A mis padres

ÍNDICE

RESUMEN

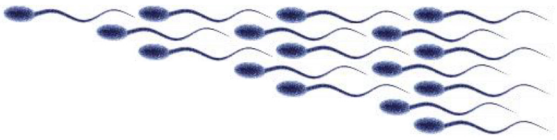


Las principales biotecnologías reproductivas utilizadas en la industria equina son las técnicas de conservación de semen, refrigeración y congelación; sin embargo, persisten problemas que limitan su uso. Estas técnicas se basan en la reducción o detención del metabolismo para prolongar la vida de los espermatozoides tras la eyaculación, pero el metabolismo bajo las condiciones de conservación está lejos de ser completamente entendido. El metabolismo se compone de un conjunto de reacciones de oxidación-reducción (redox) que generan especies reactivas de oxígeno (EROs) como subproductos. Muchas funciones biológicas son reguladas a través de la señalización redox producida por EROs, como la capacitación, la hiperactivación y la reacción acrosómica de los espermatozoides. No obstante, las EROs deben mantenerse en niveles fisiológicos mediante los complejos antioxidantes para preservar la homeostasis redox. La pérdida o desregulación de esta homeostasis da lugar al estrés oxidativo, ocasionando una disminución de la viabilidad y la motilidad, así como daño en el ADN y peroxidación lipídica, entre otras consecuencias. Diferentes estudios describen que las biotecnologías de conservación ocasionan estrés oxidativo, bien sea por un incremento en la producción de EROs o por una reducción significativa de los antioxidantes. Este estrés oxidativo puede dar lugar a la pérdida de la función espermática o afectar al material genético, aún sin una disminución apreciable de la fertilidad, ocasionando alteraciones en la descendencia. Por tanto, el estudio de la relación entre el metabolismo y la regulación redox es un enfoque prometedor para mejorar las técnicas actuales de conservación. En la presente Tesis Doctoral planteamos como objetivo incrementar el conocimiento sobre la biología del espermatozoide equino, su implicación en el desarrollo embrionario y la posibilidad de mejorar la calidad espermática tras la optimización de las biotecnologías de conservación. En nuestros estudios se establecieron como hipótesis que la cistina es interiorizada desde el medio extracelular para su reducción en dos moléculas de cisteína y la síntesis de glutatión (GSH), principal antioxidante intracelular; que la calidad espermática tras la descongelación puede ser mejorada, que los diluyentes de refrigeración pueden ser optimizados y que la congelación de semen tiene un gran impacto en el desarrollo embrionario temprano. Con los resultados obtenidos concluimos que los espermatozoides equinos presentan el transportador SLC7A11 que intercambia una molécula de cistina extracelular por una de glutamato intracelular, cuya funcionalidad es crucial para mantener la actividad mitocondrial, la homeostasis redox y la capacidad de fecundación. La suplementación con cistina reestablece la depleción de los grupos tiol que ocurre durante la conservación seminal y la calidad de las muestras seminales descongeladas pueden ser mejorada gracias a la alta plasticidad metabólica de los espermatozoides. En cuanto a la refrigeración, los diluyentes pueden ser optimizados con menores concentraciones de glucosa, pues a elevadas concentraciones ocasiona toxicidad. Por último, la inseminación con espermatozoides descongelados compromete el desarrollo embrionario, probablemente debido a las modificaciones oxidativas inducidas en las proteínas espermáticas durante la congelación-descongelación.

SUMMARY

The main reproductive biotechnologies used in the equine industry are techniques for conservation of semen, including refrigeration and freezing; however, problems which limit their use persist. These techniques are based on slowing down or pausing spermatic metabolism to prolong the life of sperm after ejaculation, but metabolism under conditions used for artificial conservation is far from completely understood. Metabolism is composed of a series of oxidation-reduction (redox) reactions which form reactive oxygen species (ROS) as by products. Many biological functions are regulated by redox signalling by ROS, such as capacitation, hyperactivation and the acrosome reaction in sperm. However, ROS need to be maintained at physiological levels via antioxidant complexes to preserve redox homeostasis. The loss or dysregulation of this homeostasis gives place to oxidative stress, causing a reduction in viability and motility, as well as damage to DNA and lipidic peroxidation, among other consequences. Various studies note that biotechnologies used for conservation cause oxidative stress, either due to an increase in production of ROS or a significant reduction in antioxidants. This oxidative stress can give rise to the loss of spermatic function or affect genetic material, even when there is not an appreciable reduction in fertility, causing changes in offspring. Therefore, the study of the relationship between metabolism and redox regulation is a promising focus to improve current techniques used for conservation. This doctoral thesis aims to contribute to knowledge regarding the biology of the equine spermatozoa, its involvement in embryo development and the possibility of improving spermatic quality by optimizing conservation biotechnologies. It was hypothesised that cystine is internalised from extracellular media for subsequent reduction into two cysteine molecules and for the synthesis of glutathione (GSH), the most important intracellular antioxidant; that sperm quality post thaw could be improved, that refrigeration diluents can be optimised, and the cryopreservation of semen has a significant impact on early embryo development. The results obtained led to the conclusion that equine spermatozoa contain the SLC7A11 transporter which exchanges an extracellular cystine molecule for an intracellular glutathione molecule, whose functionality is key in order to maintain mitochondrial activity, redox homeostasis and the capacity for fertilization. Supplementation with cystine replenishes the depletion of the thiol groups that occurs during semen conservation and the quality of thawed semen samples can be improved as a result of the high metabolic plasticity of spermatozoa. Regarding refrigeration, diluents can be optimised with lower glucose concentrations, as high concentrations cause toxicity. Lastly, insemination with thawed spermatozoa compromises embryo development, probably due to the oxidative modifications induced in sperm proteins during freezing and thawing.

INTRODUCCIÓN



Antoine van Leeuwenhoek describió por primera vez el gameto masculino o espermatozoide en 1677 y el ovocito o gameto femenino se identificó en el año 1827 por Karl Ernst von Baer. No obstante, la necesidad de la fusión de ambos gametos para que la fecundación se produzca de forma exitosa no fue descrita hasta 1876 por Oscar Hertwig. Estos conocimientos desencadenaron el desarrollo de la reproducción asistida, conjunto de técnicas que facilitan o sustituyen los procesos naturales de fecundación. La inseminación artificial, la conservación seminal, la transferencia de embriones o la fecundación *in vitro* son algunas de las técnicas de reproducción asistida utilizadas en la actualidad, tanto en humanos como en animales. En el sector equino, la conservación seminal tiene gran importancia, constituye la base del comercio de semen y permite la obtención de una amplia descendencia de un determinado semental[1-3].

El espermatozoide es una célula haploide, encargada de transportar el genoma masculino. El semen se deposita tras la eyaculación en el tracto reproductor femenino, variando el lugar de deposición entre las diferentes especies; en primates la eyaculación es intravaginal profunda, en cerdos ocurre en el cérvix y en équidos es intrauterina[4]. Los espermatozoides, gracias a la motilidad y a las contracciones uterinas, recorren el aparato reproductor hasta alcanzar el oviducto, atraviesan el istmo tubárico y permanecen unidos al epitelio oviductal en un estadio de quietud relativa. Con la ovulación se produce la hiperactivación, los espermatozoides adquieren una motilidad de alta frecuencia, el flagelo presenta un movimiento asimétrico de gran amplitud que facilita la liberación del epitelio oviductal, el ascenso hasta la ampolla tubárica y la penetración en la zona pelúcida (ZP) del ovocito[5, 6]. De forma simultánea, ocurre la capacitación, que incrementa la fluidez de la membrana plasmática[7, 8] y prepara al espermatozoide para la reacción acrosómica, en la que la membrana celular se fusiona con la membrana externa del acrosoma y se liberan enzimas que digieren las proteínas de la ZP[9, 10]. Todos los procesos necesarios para la fecundación (motilidad, capacitación, hiperactivación y reacción acrosómica) son dependientes de energía, obtenida tras la hidrólisis del adenosín trifosfato (ATP) a adenosín difosfato (ADP) más fósforo inorgánico (Pi). El ATP se obtiene mediante el metabolismo energético y la fecundación da lugar al consumo del 70% de ATP producido por el espermatozoide[11].

1. Conservación seminal

Las técnicas de conservación seminal se desarrollaron en la segunda mitad del siglo XX. Desde entonces se han producido nuevos avances como la mejora de los diluyentes y el descubrimiento de nuevos agentes crioprotectores[12, 13]. Estas técnicas son importantes para el sector ganadero, la conservación de especies en peligro de extinción y la medicina clínica reproductiva. Permite el intercambio nacional e internacional de diferentes líneas genéticas de una especie, el almacenamiento de eyaculados previamente tratados y seleccionados de animales con una mala calidad espermática inicial, el mantenimiento de líneas genéticas superiores (establecimiento de un banco genético) o la preservación de semen recolectado en situaciones no fisiológicas (espermatozoides epididimarios en situaciones post-mortem o tras una castración).

La conservación seminal requiere una reducción o detención del metabolismo de los espermatozoides, prolongando así su vida media. La combinación de la temperatura de almacenamiento y la composición química del diluyente, claves para la supervivencia de las células espermáticas, ha dado lugar al desarrollo de dos métodos, la refrigeración y la congelación[14]. A pesar de que estas técnicas conforman la base del comercio de semen equino, persisten problemas que limitan su uso. En primer lugar, existe una elevada variabilidad entre los sementales en cuanto a la refrigerabilidad y/o congelabilidad de sus eyaculados y, en segundo lugar, se carece de estandarización de los protocolos[15]. Además, un tercer factor de gran importancia es que muchos sementales alcanzan una mayor demanda de dosis seminales a edades avanzadas, cuando la calidad seminal empieza a decaer y la tolerancia de los eyaculados a los procesos de conservación es aún más reducida.

La refrigeración de los eyaculados es la biotecnología más utilizada para la conservación seminal, pero la supervivencia de los espermatozoides se limita a periodos cortos de tiempo (24-48 horas). Como se ha mencionado, los espermatozoides dependen del metabolismo energético para la síntesis de ATP y cabe destacar que, aunque investigaciones recientes indican que la glucólisis no es la principal vía de producción de ATP en el espermatozoide equino[16], los diluyentes comerciales para el semen de esta especie están formulados con concentraciones muy altas de glucosa[17]. Por otro lado, la congelación de semen induce la mortalidad del 50% de la población espermática inicial[18, 19] y los espermatozoides que sobreviven al proceso no son completamente funcionales, experimentan una senescencia acelerada, lo cual requiere un manejo más intenso y caro de las yeguas inseminadas con semen congelado-descongelado para compensar la reducida vida media de los espermatozoides[20].

Una de las estrategias más utilizadas para la mejora de las técnicas de conservación de semen ha sido la adición de antioxidantes a los diluyentes, sin embargo, los resultados han sido inconcluyentes[21, 22]. La razón de ello es una aproximación empírica al problema

y un conocimiento superficial de la biología redox (conjunto de reacciones biológicas de oxidación-reducción) del espermatozoide equino, asumiendo que la producción de especies reactivas de oxígeno (EROs) es siempre perjudicial. Aunque en la última década se han desarrollado avances significativos, el metabolismo de los espermatozoides bajo las condiciones de refrigeración y congelación está lejos de ser completamente entendido. En relación con este aspecto, el conocimiento del papel de las EROs está evolucionando drásticamente[23-28]; en equinos la mayoría de los espermatozoides fértiles se caracterizan por una mayor producción de EROs[23, 29]. Además, estudios recientes de proteómica están ampliando nuestro conocimiento sobre el metabolismo espermático[30-33] y el estudio de la relación entre el metabolismo y la regulación redox es un enfoque prometedor para mejorar las técnicas actuales de conservación seminal[29, 34, 35].

2. Metabolismo del espermatozoide

Todas las células llevan a cabo el metabolismo energético mediante una serie de reacciones biológicas redox, generando energía en forma de ATP a partir de nutrientes. Químicamente, la oxidación consiste en la pérdida de electrones (e^-), mientras que la reducción es la ganancia de e^-. Esta nomenclatura refleja la tendencia del oxígeno, átomo altamente electronegativo, a la captación parcial o total de e^- de otras moléculas. En el metabolismo, las biomoléculas se oxidan a moléculas simples y la energía liberada en estos procesos, termodinámicamente favorables, se aprovecha para la fosforilación del ADP[36]. Los espermatozoides de mamíferos producen ATP principalmente a través de dos vías metabólicas, la glucólisis y la fosforilación oxidativa, aunque existen evidencias científicas de la existencia de otras rutas metabólicas como la vía de las pentosas fosfatos y la β-oxidación de ácidos grasos[11, 30, 37].

2.1. Glucólisis

Ruta o vía metabólica encargada de oxidar la glucosa ($C_6H_{12}O_6$) a través de diez reacciones enzimáticas consecutivas, produciendo piruvato ($C_3H_4O_3$), que sigue otras rutas metabólicas y continua con la producción de energía. En esta vía, por cada molécula de glucosa oxidada, se obtienen dos moléculas netas de ATP. La glucólisis se divide en dos fases, en la primera se produce un gasto energético mediante la transformación de la glucosa en dos triosas, gliceraldehído-3-fosfato (G3P) y dihidroxiacetona-fosfato (DHAP), pues se desfosforilan dos moléculas de ATP. La molécula de DHAP se isomeriza a G3P gracias a la enzima triosa fosfato isomerasa y las dos moléculas de G3P son transformadas a dos moléculas de piruvato en la segunda fase o fase de beneficio energético, con la obtención de cuatro moléculas de ATP (Figura 1). Además, en la fase de beneficio energético se obtienen dos moléculas de nicotinamida adenina dinucleótido reducida (NADH) tras la liberación de poder reductor en la

oxidación de las dos moléculas de G3P a dos moléculas de 1,3-bifosfatoglicerato. El NADH participará en la fosforilación oxidativa, donde será oxidado y transferirá sus electrones al oxígeno molecular (O_2), formándose de nuevo la nicotinamida adenina dinucleótido oxidada (NAD^+)[38, 39].

No obstante, debemos indicar que el metabolismo de la glucosa no es un proceso perfecto. Mediante auto oxidación, la glucosa puede formar glioxal y las triosas (G3P y DHAP) pueden ser degradadas por una vía no enzimática generando metilglioxal. Estas moléculas, también generadas en el metabolismo de los lípidos, son 2-oxoaldehidos que se caracterizan por una alta capacidad electrofílica. Reaccionan rápidamente con nucleófilos de proteínas, lípidos y ácido desoxirribonucleico (ADN), formando productos finales de glicación avanzada (AGEs) potencialmente citotóxicos y mutagénicos (Figura 1). En las células somáticas, la producción de estos 2-oxoaldehídos es proporcional a la concentración de glucosa presente[40-44].

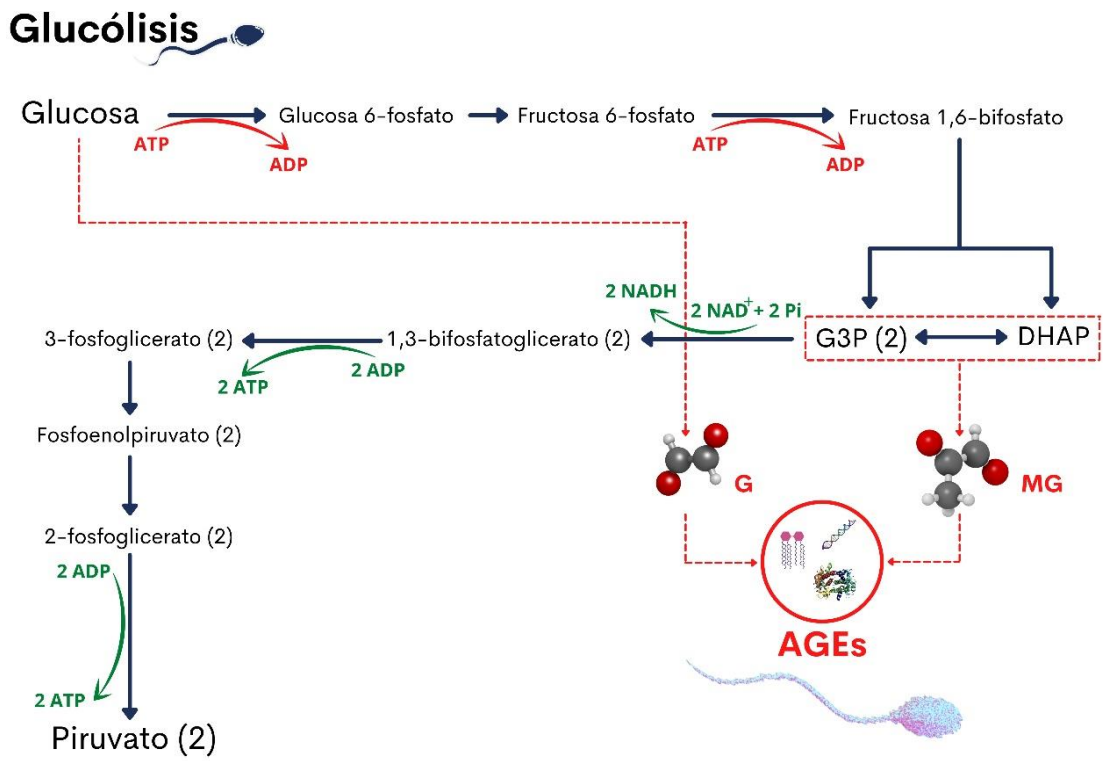


Figura 1. Oxidación de glucosa a piruvato, con la obtención de 2 moléculas netas de ATP. Como subproductos de esta ruta metabólica se obtienen 2 moléculas de NADH, así como Glioxal (G) y metilglioxal (MG) que interaccionan con biomoléculas (ADN, proteínas o lípidos) y dan lugar a productos finales de glicación avanzada (AGEs).

Los espermatozoides son células muy diferenciadas sin apenas reserva energética, presentan un citoplasma pequeño y dependen de la internalización de azúcares como la glucosa, la fructosa o la manosa como combustible energético, aunque también pueden usar otros metabolitos como el lactato y el citrato. Estos azúcares son incorporados al interior celular a través de la membrana plasmática, gracias a transportadores proteicos específicos denominados GLUTs, localizados en la región del acrosoma, la línea ecuatorial de la cabeza y a lo largo de la cola de los espermatozoides de mamíferos. Diferentes estudios, han descrito que la obtención de ATP mediante glucólisis ocurre en la cabeza y en la pieza principal del flagelo[11, 16, 45-50].

El piruvato obtenido en la glucólisis puede ser transformado en lactato por la enzima lactato deshidrogenasa (LDH) o puede entrar en la matriz mitocondrial, donde el complejo piruvato deshidrogenasa (PDH) lo transforma en acetil coenzima A (actil-CoA), generando una molécula de NADH[51]. Esta reacción irreversible es la interconexión entre la glucólisis y el ciclo del ácido cítrico, también denominado ciclo de los ácidos tricarboxílicos o ciclo de Krebs (Figura 2).

2.2. Ruta de las pentosas fosfatos

La glucosa también puede ser metabolizada a través de la ruta de las pentosas fosfato. La enzima glucosa 6-fosfato deshidrogenasa (G6PDH) es clave en esta vía metabólica, se localiza tanto en la cabeza como en la pieza intermedia de los espermatozoides[52]. Aunque la nicotinamida adenina dinucleótido fosfato (NADPH) puede resultar de la degradación de productos del ciclo de Krebs, de la oxidación de ácidos grasos y de la utilización de cuerpos cetónicos, la ruta de las pentosas fosfatos es la principal fuente de NADPH[53-56]. Esta ruta comprende dos ramas, la rama oxidativa que genera NADPH y ribonucleótidos, y la rama no oxidativa que recluta metabolitos intermediarios de la glucólisis para convertirlos en pentosas fosfatos de manera reversible[54]. En la rama oxidativa, la primera reacción es la deshidrogenación de la glucosa-6-fosfato por la acción de la G6PDH para producir NADPH y 6-fosfogluconolactona, que luego es hidrolizada por la enzima fosfoglucolactonasa en 6-fosfogluconato. La 6-fosfogluconato deshidrogenasa cataliza la descarboxilación oxidativa del 6-fosfogluconato y produce NADPH nuevamente y ribulosa-5-fosfato que se convierte en ribosa-5-fosfato[54]. Mediante las enzimas transcetolasa y transaldolasa, la ribosa-5-fosfato se convierte en G3P y fructosa-6-fosfato, estableciéndose una relación reversible entre la ruta de las pentosas fosfatos y la glucólisis[39].

El NADPH junto con el glutatión reducido (GSH) es esencial para mantener la homeostasis redox[57], proteger a los espermatozoides del estrés oxidativo y mantener niveles fisiológicos de EROs involucrados en vías de señalización que dan lugar a la capacitación, la motilidad progresiva y la fusión de los gametos[52, 58-63].

2.3. Ciclo de Krebs

Ruta metabólica que consiste en una serie de reacciones que ocurren en un ciclo cerrado y que está interconectada con la fosforilación oxidativa. El ciclo se inicia con la acción de la citrato sintasa que cataliza la unión del acetil-CoA (2C) y una molécula de oxalacetato (4C) para generar una molécula de citrato (6C). El citrato (6C) es convertido en su isómero isocitrato (6C) y el ciclo continúa con dos reacciones de descarboxilación oxidativa; primero el isocitrato es descarboxilado a α-cetoglutarato (5C) y éste a succinil coenzima A (succinil-CoA) (4C), liberándose dos moléculas de dióxido de carbono (CO_2). Posteriormente, se forma una molécula de succinato (4C) y se libera coenzima A y una molécula de guanosín trifosfato (GTP), análogo de ATP. El ciclo continúa formando fumarato, malato y, por último, oxalacetato, que puede unirse a otro acetil-CoA e iniciar un nuevo ciclo (Figura 2). A lo largo del ciclo se liberan cuatro pares de átomos de hidrógeno en cuatro reacciones de oxidación, reduciéndose tres moléculas de NAD^+ a NADH y una molécula de flavina adenina dinucleótido oxidada (FAD) a $FADH_2$[39, 64].

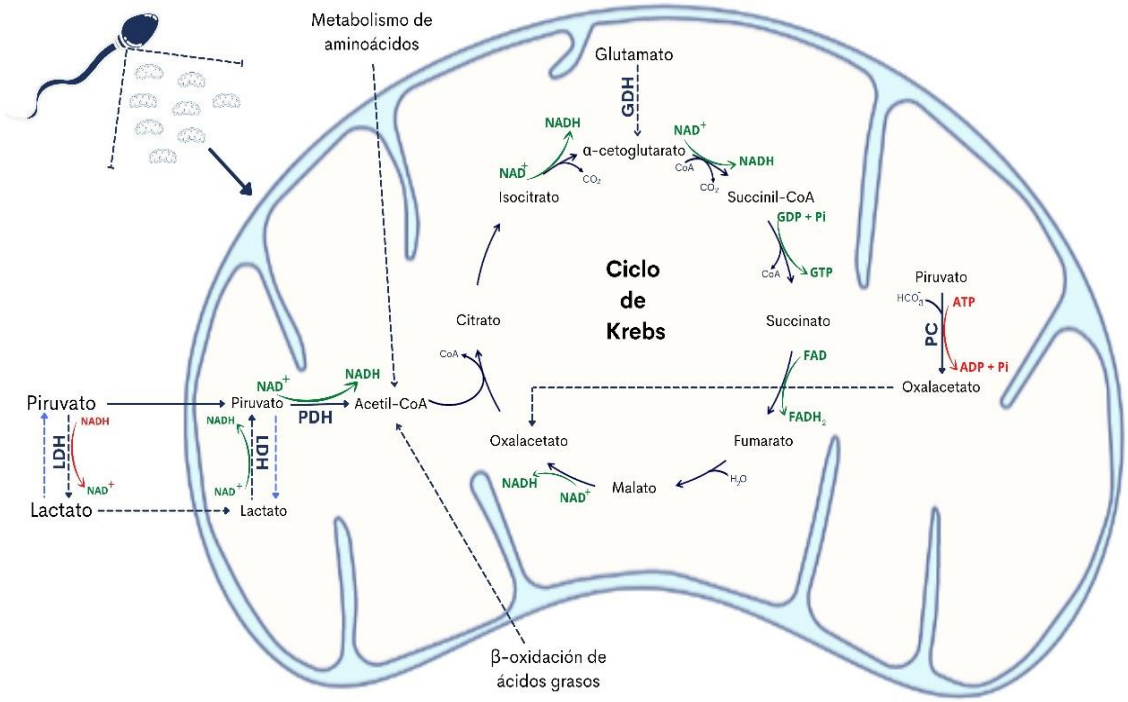


Figura 2. Metabolismo de los ácidos tricarboxílicos en la matriz mitocondrial para la obtención de energía en forma de GTP y poder reductor (NADH y $FADH_2$).

El acetil-CoA puede proceder de la oxidación del piruvato, de ácidos grasos o del metabolismo de aminoácidos como la leucina, isoleucina o triptófano. Investigaciones recientes basadas en proteómica indican que el metabolismo de lípidos y aminoácidos también se encuentra presente en el espermatozoide[30, 33]. Además, el ciclo de Krebs se activa en diferentes puntos, incluyendo la conversión del piruvato a oxalacetato por la enzima piruvato carboxilasa (PC), y la glutaminolisis, que es la conversión de la glutamina a glutamato (Glu), que puede ser convertida a α-cetoglutarato por la enzima glutamato deshidrogenasa (GDH). La oxidación del lactato también constituye una reacción importante para iniciar el ciclo de Krebs (Figura 2)[65, 66]. La importancia del metabolismo energético del lactato ha sido descrita en espermatozoides de conejos, bovinos y equinos. La LDH, responsable de la conversión del lactato en piruvato, se encuentra presente en la matriz mitocondrial de los espermatozoides[33, 67, 68].

Las moléculas reducidas NADH y $FADH_2$ que se forman en la glucólisis, la β-oxidación de ácidos grasos y el ciclo de Krebs son ricas en energía. Cada molécula contiene un par de electrones potencialmente transferibles que se utilizan para reducir el O_2 a agua (H_2O), liberándose gran cantidad de energía que puede ser utilizada para generar ATP[39]. Esta producción de energía en forma de ATP ocurre mediante la fosforilación oxidativa y tiene lugar en la membrana mitocondrial interna.

2.4. Fosforilación oxidativa

En espermatozoides, la producción de ATP a través de esta ruta metabólica ocurre en la pieza intermedia, constituida por un conjunto de mitocondrias reorganizadas helicoidalmente alrededor de la porción anterior del axonema[69-72]. La fosforilación oxidativa involucra a dos componentes de la membrana interna mitocondrial, la cadena respiratoria o cadena mitocondrial de transporte de electrones (CMTE) y la ATP sintasa. La CMTE está formada por cuatro complejos proteicos que son el complejo I o NADH deshidrogenasa, el complejo II o Succinato deshidrogenasa, que a su vez es la encargada de formar el fumarato a partir del succinato en el ciclo de Krebs, el complejo III o Citocromo C reductasa y el complejo IV o Citocromo C oxidasa. El complejo I oxida una molécula de NADH a NAD^+ y el complejo II oxida una molécula de $FADH_2$ a FAD. Ambos complejos transfieren protones (H^+) al espacio intermembrana de la mitocondria y dos e^- al complejo III. Los e^- aceptados por el complejo III se transfieren al complejo IV, a la vez que se expulsan H^+ al espacio intermembrana. Por último, el complejo IV cede los e^- a la matriz mitocondrial, reduciendo una molécula de O_2 a dos moléculas de H_2O, expulsando H^+ al espacio intermembrana. Con el paso de estos H^+ disminuye la concentración de H^+ en la matriz mitocondrial y se establece un gradiente electroquímico, denominado potencial de membrana mitocondrial (PMM). Los H^+ regresan a la matriz mitocondrial a través de la ATP sintasa, que utiliza la energía suministrada por el flujo de H^+ para sintetizar ATP mediante la fosforilación del ADP (Figura 3). Por cada molécula de glucosa oxidada completamente a CO_2 y H_2O por la

glucólisis y el ciclo de Krebs, se obtiene un total de 32 moléculas de ATP, 2 correspondientes al ATP neto producido en la glucólisis, 2 de las dos moléculas de GTP producidas en el ciclo de Krebs y 28 sintetizadas por la ATP sintasa en la fosforilación oxidativa, tras la oxidación de las 10 moléculas de NADH y las 2 de $FADH_2$ obtenidas en las rutas metabólicas descritas[38, 39].

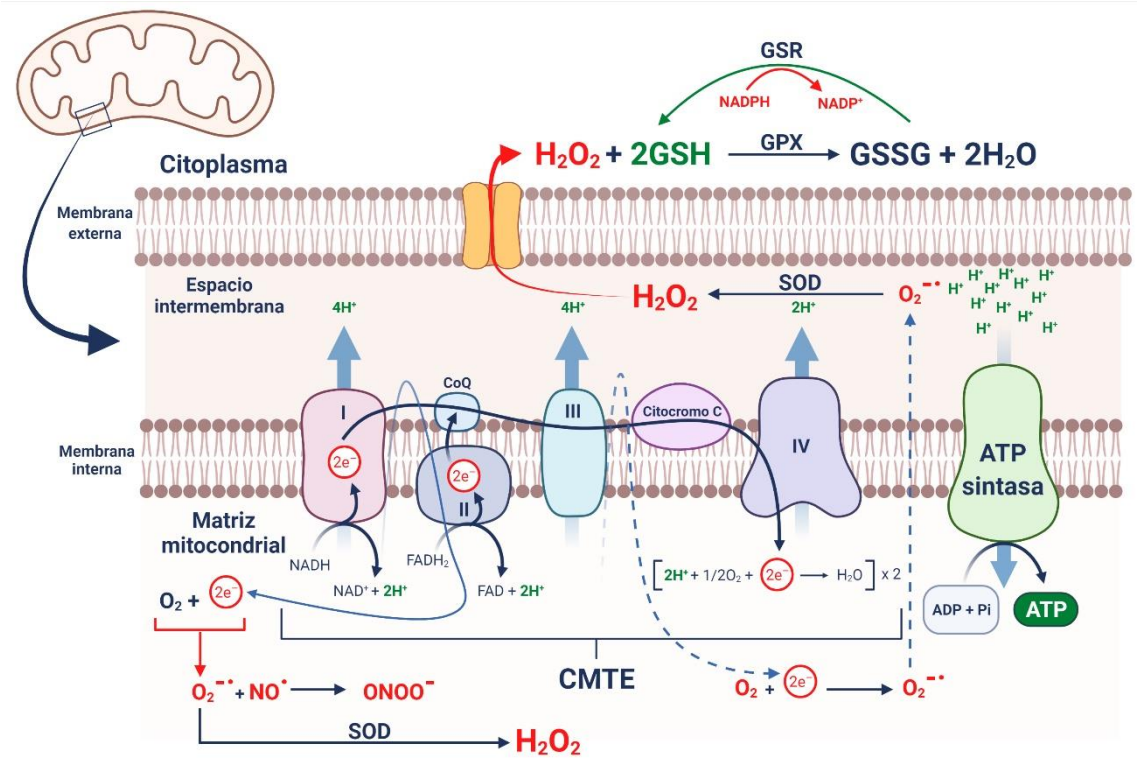


Figura 3. Síntesis de ATP y producción de EROs en la fosforilación oxidativa.

A pesar de que la obtención de ATP en la mitocondria a través del ciclo de Krebs y la fosforilación oxidativa es 15 veces superior al ATP generado por la glucólisis, la preferencia de los espermatozoides por una u otra vía metabólica para producir energía es muy dependiente de la especie. En cerdos, ratones y humanos se ha descrito que la principal vía de obtención de energía en forma de ATP es la glucólisis, mientras que en toros y caballos es la fosforilación oxidativa[16, 73-76]. No obstante, la producción de energía se puede restringir a la glucólisis, a la respiración mitocondrial o a una combinación de ambas según el estado metabólico y la función espermática para la que se requiere esta energía[11, 77]. En humanos y cerdos, la ruta glucolítica es la principal fuente de energía para mantener la motilidad progresiva, así como la capacitación y la hiperactivación espermática, pero la maduración y diferenciación depende del ATP producido en las mitocondrias. Además, tras la capacitación, la actividad mitocondrial se incrementa[11, 78-80]. En el caso de ratones, la motilidad depende de la glucólisis, pero al

igual que en toros, requieren de la fosforilación oxidativa para la capacitación y la hiperactivación[16, 46, 69, 81]. En cambio, la hiperactivación, la reacción acrosómica y la unión a la zona pelúcida depende de la ruta glucolítica en macacos[82]. Por último, en équidos, la fosforilación oxidativa es el principal mecanismo productor de energía para la motilidad y el mantenimiento de la integridad de la membrana plasmática. Los espermatozoides equinos presentan una actividad glucolítica limitada, aunque la glucólisis participa en la motilidad y es responsable de la velocidad espermática gracias a las enzimas glucolíticas presentes en el flagelo[29, 83-85]. En definitiva, los espermatozoides de mamíferos dependen de ambas vías metabólicas para llevar a cabo su principal función, la fecundación.

Las mitocondrias son responsables de la producción de aproximadamente el 90% de la energía celular, pero también intervienen en otras funciones como el mantenimiento de la homeostasis del calcio, el metabolismo del hierro, la apoptosis celular y la formación de especies reactivas de oxígeno (EROs). Una disfunción mitocondrial por daños de la estructura, el genoma, el transcriptoma o el proteoma mitocondrial, así como por una disminución del PMM o un consumo alterado de O_2, causa la alteración de la funcionalidad espermática[23, 29, 83, 85-89].

3. Producción y regulación de EROs

El metabolismo de los organismos aeróbicos está compuesto por reacciones biológicas redox que generan subproductos como las EROs, un grupo de moléculas ubicuas que contienen oxígeno y son consideradas metabolitos parcialmente reducidos, con una fuerte capacidad oxidante que varía según las diferentes especies. El término de especies reactivas de nitrógeno (ERNs) se ha utilizado para aquellas moléculas parcialmente reducidas que contienen nitrógeno, pero actualmente se utiliza EROs como término general, debido a la estrecha relación entre las EROs y las ERNs. Entre ellas se distinguen especies reactivas que son radicales libres, que contienen uno o más e^- que se encuentran solos en el orbital molecular, y otras que no son radicales[90-93]. En general, los radicales libres son más inestables y reactivos que los no radicales. Sus reacciones con moléculas diana favorecen un comportamiento de reacción en cadena, que consiste en iniciación, propagación y terminación. Normalmente, el paso de iniciación es la generación de radicales libres a partir de iniciadores mediante procesos térmicos, de radiación o de transferencia de electrones. Dentro de las células, el O_2 es el iniciador más común, que a través del proceso de transferencia de electrones catalizado por varios generadores de EROs endógenos produce radicales de partida. En la fase de iniciación hay un aumento neto de radicales libres. En la propagación o segunda reacción en cadena, los radicales de partida pueden reaccionar con una molécula estable sin electrones desapareados y formar un enlace covalente, por lo que

la molécula diana pierde un e^- y se convierte en el nuevo radical, sin incremento neto de radicales libres. Por último, la fase de terminación comprende la reacción de los radicales formados en la propagación con antioxidantes fenólicos como la vitamina E, flavonoides y ácido ascórbico, o con otro radical libre para formar un enlace covalente, generándose una disminución neta de radicales libres. La reactividad relativa de los radicales libres se puede especificar en voltios (V) de acuerdo con el potencial de reducción de un electrón (E^o), que refleja la tendencia de una especie química a recibir electrones y reducirse. La reactividad relativa de los no radicales se basa en su constante de velocidad de segundo orden (*k*), un parámetro químico cinético para la velocidad de reacción con GSH. Las EROs pueden reaccionar con una gran diversidad de moléculas, incluidas el sulfuro de hierro (Fe-S), otras EROs, residuos de proteínas como cisteína (Cys), metionina (Met), tirosina (Tyr) y triptófano (Trp) y cualquier macromolécula (ADN, ARN o ácido ribonucleico, proteínas y lípidos)[93, 94].

La producción de EROs en las células se describió por primera vez en espermatozoides. J. Tosic y A. Walton demostraron que los espermatozoides de bovinos producían peróxido de hidrógeno (H_2O_2) como consecuencia de la respiración celular y que, a su vez, la producción de H_2O_2 inhibía la respiración celular, por lo que concluyeron que los espermatozoides debían poseer mecanismos de eliminación del H_2O_2 para mantenerlo a niveles fisiológicos[95]. Las EROs presentes en el semen pueden ser intrínsecas o extrínsecas de los espermatozoides. Las EROs extrínsecas son producidas principalmente por los leucocitos (macrófagos y neutrófilos) presentes en el eyaculado, procedentes de las glándulas sexuales accesorias. Los leucocitos producen EROs con una tasa 1000 veces superior que los espermatozoides, pero los caballos reproductivamente sanos tienen baja concentración de leucocitos en sus eyaculados y, como consecuencia, la producción de EROs por estas células no resulta perjudicial para la calidad espermática[96-99]. Otras fuentes de EROs extrínseca son los xenobióticos y las radiaciones electromagnéticas[100, 101]. En el caso de las EROs intrínsecas, la principal fuente son las mitocondrias a través de la CMTE, aunque el sistema NADPH oxidasa, así como otras oxidasas, también pueden ser una fuente adicional de EROs[102, 103]. Las formas más comunes de EROs en espermatozoides son el anión superóxido ($O_2^{-\bullet}$) y el H_2O_2. En la CMTE, en los complejos I y III, el 1-2% del O_2 sufre una reducción univalente durante la fosforilación oxidativa, generando así $O_2^{-\bullet}$. El $O_2^{-\bullet}$ creado por el complejo III se libera al espacio intermembrana y por dismutación espontánea o gracias a la enzima superóxido dismutasa (SOD) mitocondrial se convierte en H_2O_2, difundiendo al citoplasma mediante canales aniónicos dependientes de voltaje[104, 105]. Por el contrario, el $O_2^{-\bullet}$ producido por el complejo I se libera directamente a la matriz mitocondrial, dificultándose su escape y produciendo estrés oxidativo cuando su concentración supera a los mecanismos antioxidantes en el interior mitocondrial (Figura 3)[72, 106]. El radical hidroxilo ($OH^\bullet$) también es una ERO intrínseca y la mayor parte de este radical se genera a partir de $O_2^{-\bullet}$ y H_2O_2, en una reacción catalizada por un ion metálico como el hierro (Fe), conocida

como la reacción de Haber-Weiss. Esta reacción ocurre en dos pasos, primero Fe^{+3} es reducido a Fe^{+2} ($Fe^{+3} + O_2^{-\bullet} \rightarrow Fe^{+2} + O_2$) y en segundo lugar ocurre la reacción de Fenton ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^\bullet + OH^-$). El $OH^\bullet$ puede reaccionar con los lípidos, extrayendo un átomo de hidrógeno y formando un radical alcoxilo lipídico ($LO^\bullet$), que puede reaccionar rápidamente con O_2 y generar peróxido lipídico ($LOO^\bullet$), que es un oxidante moderado capaz de abstraer un hidrógeno de los lípidos cercanos para generar hidroperóxido lipídico (LOOH) y un nuevo $LO^\bullet$[107]. Otras EROs intrínsecas son el óxido nítrico ($NO^\bullet$) y el peroxinitrito ($ONOO^-$), esta última formada por la combinación de $NO^\bullet$ y $O_2^{-\bullet}$. $NO^\bullet$ se genera a partir del aminoácido L-arginina, en una reacción catalizada por la enzima óxido nítrico sintasa[28, 93].

3.1. Radicales libres

Los radicales libres son aquellas moléculas capaces de existir de forma independiente con uno o más e^- desapareados en su orbital más externo, por lo que presentan alta capacidad de reacción con otras moléculas y ocasionan cambios en la composición química o estructura de algunos componentes celulares[91, 92]. Entre ellos encontramos:

- **Anión superóxido ($O_2^{-\bullet}$)**. Radical débil ($E^o \sim 0.94$ V), debido en parte a que su carga aniónica impide su acceso a centros ricos en e^-. Su vida media útil es relativamente corta, ya que dismuta rápidamente a H_2O_2, de forma espontánea o gracias a la enzima SOD. El $O_2^{-\bullet}$ reacciona preferentemente con $NO^\bullet$ o con metales de transición, particularmente con el grupo Fe-S. Este radical libre es considerado la principal ERO porque puede reaccionar con un mayor número de moléculas y formar otras EROs secundarias[92, 108].
- **Óxido nítrico ($NO^\bullet$)**. Oxidante muy débil ($E^o \sim 0.80$ V) que presenta la vida media útil más larga de las EROs identificadas. Tiene alta solubilidad y se difunde libremente a través de las membranas. El $NO^\bullet$ es relativamente inerte para la mayoría de las biomoléculas, prefiere reaccionar con radicales libres o metales, y tiende a oxidarse en lugar de reducirse. Cuando reacciona con $O_2^{-\bullet}$ se genera $ONOO^-$ que es un oxidante más potente e induce estrés oxidativo. En cambio, cuando reacciona con radicales libres lipídicos como $LO^\bullet$ o $LOO^\bullet$, se forma LONO y LOONO respectivamente, ejerciendo una acción protectora, finalizando la peroxidación lipídica[93]. Además, el $NO^\bullet$ puede reaccionar con aminoácidos y formar proteínas nitrosiladas, así como reaccionar con metales[109].
- **Dióxido de nitrógeno ($NO_2^\bullet$)**. Moderadamente reactivo ($E^o \sim 1.04$ V), oxida los grupos tiol (-SH) a radicales libres de tiilo ($S^\bullet$), que posteriormente reaccionan con $NO^\bullet$ y genera S-nitrosotioles (RSNO). También puede oxidar el residuo de Tyr a un radical tirosilo, pero la reacción es más lenta que su reacción con los -SH[110, 111].

- **Trióxido de carbono ($CO_3^{-\bullet}$)**. Muy reactivo ($E^o \sim 1.78$ V) con una vida media útil muy corta. Este radical puede oxidar los -SH de las biomoléculas, el ion metálico de una proteína, residuos proteicos (Cys, Met, Tyr, Trp e histidina) y restos de guanina de ADN o ARN. Este radical libre se ha propuesto como mediador clave del daño oxidativo generado por $ONOO^-$ [112, 113].
- **Radicales alcoxilos ($RO^\bullet$) y peroxilos ($ROO^\bullet$)**. Son muy reactivos ($E^o > 1.78$ V). No obstante, la reactividad de estos radicales puede verse afectada por las diferentes moléculas, aquellas que atraen e^- aumentan la reactividad, pero aquellas moléculas donantes de e^- la disminuyen. En la peroxidación de lípidos, $LO^\bullet$/$LOO^\bullet$ pueden abstraer rápidamente el hidrógeno de los lípidos cercanos, generando nuevos radicales libres de lípidos y propagando la peroxidación en cadena[114].
- **Radical hidroxilo ($OH^\bullet$)**. El más reactivo ($E^o \sim 2.31$ V). Puede reaccionar con la mayoría de las biomoléculas, incluidos lípidos, proteínas, carbohidratos y ADN. Debido a su reactividad extremadamente alta, tiene una vida media útil muy corta. El $OH^\bullet$ reacciona con sus moléculas diana a través del mecanismo de abstracción, adición y transferencia de electrones del hidrógeno. Al reaccionar con compuestos orgánicos (proteínas y lípidos) genera un radical alcoxilo ($RO^\bullet$) y cuando reaccionan con el ácido araquidónico dan lugar a isoprostanos, provocando estrés oxidativo. En el caso de los compuestos aromáticos (ADN y ARN) produce aductos de radicales hidroxilados como la 8-hidroxi-2'-desoxiguanosina (8-OHdG)[114, 115].

3.2. EROs no radicales

Moléculas pequeñas, más estables que los radicales libres, pero con la capacidad de generarlos y ocasionar efecto tóxico a largo plazo[116]. Entre las EROs no radicales destacan:

- **Oxígeno (O_2)**. Oxidante biológico fuerte, generado a través de procesos dependientes de la luz o independientes, mediante la quimio-excitación catalizada por enzimas como las peroxidasas y oxigenasas. Las principales reacciones de oxidación iniciadas por O_2 pueden dar lugar a modificaciones en las dianas celulares clave, incluida la guanina de los ácidos nucleicos, los lípidos insaturados y los aminoácidos[117-119].
- **Peroxinitrito ($ONOO^-$)**. Altamente reactivo con una vida media corta, reacciona selectivamente con pocas moléculas, entre ellas CO_2, -SH, compuestos de selenio y centros metálicos. La reacción con CO_2 da como resultado la generación de los radicales libres $CO_3^{-\bullet}$ y $NO_2^\bullet$, radicales tóxicos que pueden oxidar los -SH a $S^\bullet$. La reacción de $ONOO^-$ con -SH da como resultado la generación del correspondiente disulfuro[120, 121].

- **Peróxido de hidrógeno (H_2O_2)**. Tiene una vida media relativamente larga y puede reaccionar con centros ferrosulfurados (Fe-S), metales débilmente unidos, GSH y residuos de cisteína libre (Cys) y metionina (Met). Su velocidad de reacción con el residuo Cys depende del estado de ionización, la forma de anión tiolato (Cys-S-) favorece una reacción más rápida que la forma protonada (Cys-SH) del residuo de Cys. En condiciones fisiológicas, el residuo de Cys suele existir en forma de anión tiolato y niveles bajos de H_2O_2 pueden oxidar la Cys-S- a la forma sulfénica (Cys-SOH). La Cys-SOH se puede reducir de nuevo a Cys-S- mediante GSH y tiorredoxina (TRX), una proteína que actúa como antioxidante, pero niveles altos de H_2O_2 pueden oxidar aún más la Cys-SOH a la forma sulfínica (Cys-SO_2H) o sulfónica (Cys-SO_3H), ambas irreversibles. Las peroxidasas catalizan el H_2O_2 rápidamente a H_2O antes de oxidar la Cys-S-[122].

La generación de las EROs por los organismos aeróbicos se mantiene a niveles fisiológicos gracias a la presencia de complejos antioxidantes enzimáticos y no enzimáticos[123, 124]. Estos sistemas antioxidantes están presentes tanto en los espermatozoides como en el plasma seminal. En el plasma seminal de humanos y équidos, como antioxidante no enzimático, se encuentra el ácido úrico y, como principales enzimas antioxidantes, la SOD, la catalasa (CAT) y la glutatión peroxidasa (GPX). Por otro lado, las defensas antioxidantes intrínsecas del espermatozoide incluyen el GSH como antioxidante no enzimático y enzimas como la paraoxonasa (PON), la tiorredoxina (TRX) y la familia de las proteínas peroxirredoxinas (PRDX)[25, 28, 125, 126].

El GSH es el antioxidante intracelular más abundante y ubicuo. Entre los espermatozoides de mamíferos, los de la especie equina presentan la mayor cantidad de GSH[27, 127, 128]. El GSH es un tripéptido compuesto por Cys, Glu y glicina (Gly), sintetizado mediante dos reacciones. La primera es catalizada por la enzima γ-glutamilcisteína sintasa, que une Cys y Glu, y resulta limitante en la formación de GSH. La segunda reacción es catalizada por la enzima glutatión sintasa y comprende la unión covalente de γ-glutamilcisteína y Gly, formando GSH. Estas enzimas han sido descritas en los espermatozoides de caballos por nuestro grupo de investigación[27]. La síntesis de GSH ocurre exclusivamente en el citosol, pero es distribuido al interior mitocondrial a través de transportadores específicos para equiparar la concentración de GSH de la matriz mitocondrial con la del citosol[127]. El grupo sulfhidrilo o tiol (-SH) en el residuo de Cys del GSH explica su fuerte característica de donación de e^-, por lo que GSH forma parte del mecanismo de defensa ante la producción de EROs en la respiración celular. El GSH se oxida cuando confiere poder reductor y forma el glutatión oxidado o disulfuro de glutatión (GSSG). El GSH también participa en la detoxificación de LOOH, así como en la regulación de la permeabilidad mitocondrial y muerte celular[93, 129, 130]. Una deficiencia de GSH produce una reducción de la motilidad y la alteración de la morfología

espermática, así como la alteración de los perfiles hormonales[131]. Además, el GSH puede usarse como reductor de las enzimas glutatión peroxidasas (GPXs) y otros antioxidantes oxidados, como la vitamina C y la vitamina E, o puede ser utilizado por enzimas glutatión-S-transferasas para la detoxificación de xenobióticos[93].

Las GPXs son una familia de peroxidasas que transforman el H_2O_2 en H_2O, o convierten el hidroperóxido orgánico (ROOH) en el alcohol correspondiente (ROH)[132]. De esta familia de enzimas, GPX4 se encuentra en espermatozoides y GPX5 en plasma seminal. La deficiencia de estas enzimas da lugar al estrés oxidativo y, como consecuencia, a la disminución de la funcionalidad espermática[133]. En caballos se han observado correlaciones positivas de la actividad total de GPX con la calidad y fertilidad del eyaculado fresco[134]. El contenido relativo de estas enzimas se correlaciona positivamente con la tolerancia a la congelación de los espermatozoides de humanos y cerdos[135, 136]. La GPX se oxida al llevar a cabo la reducción de las EROs y requiere de una molécula de GSH para reducirse, oxidando el GSH a GSSG[137]. Por otro lado, la enzima glutatión reductasa (GSR) también está asociada con la protección antioxidante intrínseca y extrínseca de los espermatozoides, ya que está presente en el plasma seminal y en el interior de los espermatozoides[138, 139]. La GSR cataliza la reducción de GSSG a GSH, utilizando NADPH y manteniendo de esta forma la concentración de GSH reducido (Figura 3)[133, 140].

Entre los antioxidantes enzimáticos cabe destacar la SOD, que se encuentra tanto en espermatozoides como en plasma seminal de équidos, perros, rumiantes, cerdos y humanos[133]. Esta enzima presenta varias isoformas (Cu/Zn-SOD y Mn-SOD) que tienen una alta actividad, por lo que se consideran los antioxidantes enzimáticos más importantes. Son los principales responsables de catalizar la dismutación de $O_2^{-\bullet}$ a H_2O_2 y O_2[138, 141, 142]. Una deficiencia de SOD causa estrés oxidativo y peroxidación lipídica, disminuyendo la calidad espermática (viabilidad, motilidad, morfología e integridad del acrosoma)[143, 144]. En caballos la deficiencia de SOD en el plasma seminal da lugar a una menor tolerancia de los espermatozoides a la congelación[145]. La actividad antioxidante de la SOD es complementada por la CAT, una peroxidasa que reduce el H_2O_2 en H_2O y O_2[132]. La CAT está presente tanto en espermatozoides como en plasma seminal de équidos, rumiantes, cerdos y humanos. Al igual que en el caso de SOD, una deficiencia de CAT causa disminución de la funcionalidad espermática[133]. Varios estudios han demostrado que la adición de CAT previene los efectos adversos del estrés oxidativo en los espermatozoides de humano, ratón, cerdos y caballos[146-148]. Estos estudios apoyan la importancia de la actividad de la CAT para la captación de EROs y la subsiguiente modulación del estrés oxidativo. Sin embargo, hay que mencionar que se han reportado resultados contradictorios con respecto a la fertilidad. En humanos se ha descrito que la baja actividad total de la CAT está asociada con infertilidad masculina[149], mientras que la actividad total de esta enzima antioxidante se ha correlacionado negativamente con la fertilidad en caballos[134].

La reducción de H_2O_2 a H_2O también puede ser catalizada por las peroxirredoxinas (PRDXs), familia de enzimas tiol peroxidasas. Estas enzimas antioxidantes también catalizan la reducción de ROOH a ROH, así como la eliminación de $ONOO^-$. En mamíferos, se han identificado seis isoformas (PRDX1-6). Todas las isoformas contienen algún residuo de Cys que, al reaccionar con H_2O_2 o ROOH, se oxida a su forma sulfénica (Cys-SOH), que puede reaccionar con otro residuo de Cys y formar un enlace disulfuro[93, 150]. Estudios recientes establecen que la expresión de PRDX es un biomarcador de fertilidad sensible en cerdos[151] y que PRDX6 se presenta en menor cantidad en hombres infértiles que fértiles, pues juega un papel vital en la reorganización y compactación del ADN de los espermatozoides durante la espermatogénesis[152]. En el grupo de investigación de Reproducción y Espermatología Equina de la Universidad de Extremadura hemos descrito que la baja expresión de PRDX en el plasma seminal de los sementales influye en la refrigerabilidad, los eyaculados con menor tolerancia a la refrigeración presentan una expresión 4 veces menor que los eyaculados de sementales que toleran bien esta técnica[153]. La actividad de las PRDXs se mantiene gracias a las tiorredoxinas (TRXs) que se encargan de reducir a estas enzimas una vez que han sido oxidadas tras catalizar la reducción de H_2O_2 a H_2O. En mamíferos, las TRXs se expresan de forma ubicua y hay dos tipos, TRX1 que es citosólica o extracelular y TRX2 que es específica de las mitocondrias[93, 154]. Por tanto, PRDX/TRX conforma un sistema antioxidante de vital importancia para proteger a los espermatozoides del estrés oxidativo[155].

Otras enzimas con actividad peroxidasa son las paraoxonasas (PONs), familia de enzimas (PON1, PON2 y PON3) funcionalmente ligadas al colesterol que, además de degradar el H_2O_2 producido bajo el estrés oxidativo, reducen los LOOH[156]. Las PONs protegen las lipoproteínas de baja densidad hidrolizando fosfolípidos oxidados; tienen un papel importante en la desintoxicación de contaminantes ambientales como los metales pesados y organofosforados[157-159]. La PON1 se ha identificado en plasma seminal de humanos, toros, cerdos y gallos[160-163]. En humanos los niveles de PON1 son relativamente bajos, pero sus cantidades se correlacionan negativamente con el estrés oxidativo en el semen y la infertilidad[160, 161]. En eyaculados de cerdos se ha descrito la presencia de PON1 en plasma seminal y PON2 en los espermatozoides. La actividad de PON1 se correlaciona positivamente con la calidad y la funcionalidad de los espermatozoides refrigerados, presentando además una asociación positiva con la fertilidad *in vivo*[164].

Entre los antioxidantes no enzimáticos, la vitamina E o α-tocoferol es el principal antioxidante que se encuentra en la bicapa lipídica de la membrana plasmática, ya que es soluble en lípidos. Reacciona con los radicales lipídicos e impide la peroxidación lipídica de la membrana. Este antioxidante ha sido descrito en eyaculados de caballos, cerdos, humanos y rumiantes. Su deficiencia da lugar al estrés oxidativo y a la disminución de la motilidad[93, 165]. Por otro lado, la vitamina C o ácido ascórbico es capaz

de reaccionar con una amplia variedad de EROs, incluidos $OH^{\bullet}$, $R^{\bullet}$, $ROO^{\bullet}$, $O_2^{-\bullet}$ y $ONOO^{-}$, y ha sido descrita en el plasma seminal de cerdos, hombres y conejos. Tiene un papel clave en la protección de los espermatozoides frente al daño oxidativo. Además, ayuda a reducir la vitamina E oxidada durante la peroxidación lipídica[93, 142, 166, 167]. La deficiencia de ácido ascórbico conduce a una disminución del recuento de espermatozoides y a un aumento de las anomalías morfológicas, junto con un daño oxidativo en el ADN[168, 169]. No obstante, en alguna situación, la vitamina C puede ser un prooxidante, ya que puede reducir el metal de transición de alta valencia a una forma de baja valencia, lo que impulsará la reacción de Fenton y aumentará la generación de radicales $OH^{\bullet}$ tóxicos[93].

Por último, el ácido úrico es el producto final del catabolismo de las purinas (guanosina y adenosina) y se encuentra presente en plasma seminal. Los niveles de ácido úrico se correlacionan positivamente con la funcionalidad espermática, pero a elevadas concentraciones resulta desfavorable para mantener la motilidad[170]. Varios estudios han demostrado que es un potente antioxidante y que constituye, junto a la vitamina C y la tirosina, el 37% de la actividad antioxidante seminal en humanos[171, 172]. Es el principal eliminador de $ONOO^{-}$ en los espermatozoides, aunque también elimina $OH^{\bullet}$ y $O_2^{-\bullet}$, mejorando de este modo la funcionalidad y la capacidad fecundante, ya que estas EROs pueden producir la oxidación de ADN, proteínas y lípidos, pudiendo ocasionar daño y muerte espermática[173-175].

4. Estrés oxidativo

Durante mucho tiempo, las EROs se consideraban únicamente productos tóxicos del metabolismo celular, determinándose como estrés oxidativo a una elevada producción de las mismas. Sin embargo, actualmente se conoce que muchas funciones biológicas son reguladas por EROs (señalización redox). Por ejemplo, $NO^{\bullet}$ interviene en la erección del pene, la ovulación, la espermatogénesis, la motilidad y la capacitación[176-178]. Por tanto, el concepto actual de estrés oxidativo se define en términos de regulación de la señalización redox, estableciéndose como la pérdida o desregulación de la homeostasis redox. La regulación redox es similar a la regulación del pH, variando entre compartimentos celulares, por lo que el estado redox no es un estado general, sino que varía entre los diferentes compartimentos de los espermatozoides. Por ello, la regulación del estado redox resulta un área de investigación importante en la biología espermática[179-181].

Los espermatozoides son células metabólicamente muy activas, requieren de una gran producción de ATP para el mantenimiento de su funcionalidad. La mayoría de los espermatozoides fértiles presentan una alta actividad mitocondrial y, como consecuencia, una elevada producción de EROs. Además, el espermatozoide equino presenta mayor actividad mitocondrial que otros mamíferos[23, 24, 29, 84]. La función

espermática depende del control del nivel de las EROs, ya que juegan un papel esencial en las vías de señalización celular. Un nivel adecuado de EROs es esencial para mantener la motilidad y llevar a cabo los procesos de capacitación, hiperactivación, reacción acrosómica y fecundación[180]. No obstante, las EROs pueden resultar patológicas en los gametos masculinos si se producen de forma excesiva o si hay un desequilibrio con las defensas antioxidantes disponibles, causando una disminución de la viabilidad, motilidad, PMM, y un incremento de anomalías espermáticas, daño en el ADN y peroxidación lipídica, dando lugar a fenómenos similares a la apoptosis[103, 182, 183]. La disfunción mitocondrial puede exacerbar la pérdida de e^-, dando lugar al incremento de EROs a niveles tóxicos, alterando la homeostasis redox[91].

El potencial redox en estado estacionario (E_h) se puede estimar usando la ecuación de Nerst:

$$E_h = E^o + RT/nF \times Ln\ [\text{moléculas oxidadas/moléculas reducidas}],$$

donde E^o es el potencial de reducción estándar, R es la constante de los gases, T es la temperatura absoluta, n el número de electrones transferidos y F es la constante de Faraday[124].

Recientemente, se ha puesto a disposición de los laboratorios de investigación y de las clínicas de reproducción asistida un sistema de diagnóstico para medir fácilmente el E_h en el semen (RedoxSYS®). Esta tecnología mide el potencial de oxidación-reducción estático (sORP), determinando la transferencia de e^- de un reductor a un oxidante en milivoltios (mV), y la reserva de capacidad antioxidante (cORP) calculada en microculombios (µC), que establece la cantidad presente de moléculas fácilmente oxidables[184]. En los eyaculados, la capacidad antioxidante total cuantifica la cantidad de antioxidantes presente en plasma seminal. Estudios recientes muestran una correlación positiva entre la capacidad antioxidante y la fertilidad *in vivo* e *in vitro*. Este parámetro depende de la edad del animal, la temporada reproductiva y la frecuencia de eyaculación[185-189]. Una baja capacidad antioxidante implica un incremento en la cantidad de EROs, que justifica la disminución de la fertilidad de los eyaculados. La deficiencia de antioxidantes en animales puede deberse a muchas razones. Una menor cantidad de antioxidantes enzimáticos se puede asociar a una deficiencia nutricional, enfermedades, intoxicaciones, mutación genética o a una modificación postraduccional incorrecta. En el caso de los antioxidantes no enzimáticos, el déficit se debe en la mayoría de las ocasiones a intoxicaciones y deficiencias nutricionales, que pueden interferir con la absorción de varios micronutrientes[133, 190].

5. Señalización redox y fisiopatología del estrés oxidativo

Recientes avances científicos en espermatología han mejorado el conocimiento sobre la intervención de las EROs en la regulación de muchas funciones biológicas[191, 192]. No obstante, el estrés oxidativo conlleva a la presencia de una elevada cantidad de EROs que reaccionan con los lípidos de las membranas, proteínas espermáticas y el material genético, impidiendo el mantenimiento de las características funcionales de los espermatozoides[193].

5.1. Maduración espermática

La maduración espermática ocurre en el epidídimo. Comprende la condensación de la cromatina, que consiste en la formación de enlaces disulfuro entre los -SH de las protaminas para proteger al ADN[194-196], y la adquisición de motilidad progresiva, gracias a la formación de puentes disulfuro entre los residuos de Cys de proteínas presentes en las fibras densas externas que envuelven al axonema de los espermatozoides, contribuyendo a que los flagelos constituyan estructuras más rígidas[197-199]. La formación de estos enlaces disulfuro ocurre gracias a la oxidación de los -SH por las EROs, que a su vez activan a los antioxidantes enzimáticos como las GPXs y PRDXs[200-202]. La actividad de GPX4 se relaciona con el aumento progresivo de la compactación del ADN durante la transición desde la cabeza a la cola del epidídimo[203, 204] y GPX5 juega un papel importante en la modificación flagelar, así como en la prevención de la reacción acrosómica prematura[205-207]. Por otro lado, el papel principal de las PRDXs se asocia especialmente con H_2O_2 y actúan como reguladoras de la señalización redox[208, 209]. La PRDX6 juega un papel importante en la fertilidad de los espermatozoides, a través de la regulación de la condensación del ADN, la prevención de la peroxidación lipídica y la reparación de los lípidos peroxidados de la membrana plasmática durante la maduración. Además, la exposición a niveles altos de EROs regula al alza la expresión de las PRDXs en los espermatozoides presentes en el epidídimo durante la maduración, con el fin de reducir el daño oxidativo[210-212]. Aunque las EROs son esenciales para la maduración, el estrés oxidativo puede inducir la peroxidación lipídica, desencadenar una capacitación prematura, dañar el ADN previo a la condensación y reducir la fertilidad de los espermatozoides. Por tanto, mantener la homeostasis redox es primordial para que se lleve a cabo una maduración óptima[213, 214].

5.2. Capacitación, hiperactivación y reacción acrosómica

Los espermatozoides desarrollan la capacitación y la hiperactivación una vez que alcanzan el oviducto. Sufren modificaciones moleculares que aseguran la reacción acrosómica y la fusión del espermatozoide con la membrana del ovocito[215]. Estas modificaciones incluyen la alcalinización del pH intracelular, la activación de rutas dependientes del adenosín monofosfato cíclico (AMPc), la eliminación de colesterol de

la membrana espermática y la fosforilación de proteínas en residuos de serina, treonina y tirosina por quinasas dependientes de AMPc. Las EROs juegan un papel importante en la regulación de estas modificaciones[215, 216].

Diferentes estudios describen la importancia del equilibrio redox en la regulación de la capacitación. La inhibición de la síntesis de H_2O_2 con CAT da lugar a la supresión de la capacitación y la incubación de los eyaculados con bajas concentraciones de H_2O_2 resulta en una mayor tasa de espermatozoides capacitados[217-219]. La importancia de las EROs en la regulación de la vía del AMPc, que implica la activación de la proteína quinasa A (PKA) y la fosforilación de sustratos de PKA, también ha sido descrita. De hecho, una alta tasa de fosforilación de proteínas y un aumento de los niveles de AMPc y Calcio (Ca^{2+}) se asocian con la síntesis de EROs durante la capacitación. Asimismo, la generación de EROs es sensible al pH intracelular[216]. La eliminación de bicarbonato del medio de incubación afecta a la síntesis de EROs y a la tasa de fosforilación de proteínas, lo que imposibilita la capacitación espermática. Las EROs, además de mediar la activación de las quinasas, suprimen las fosfatasas al oxidar sus residuos de Cys, esenciales para su activación. Por tanto, las EROs aumentan la tasa de fosforilación de proteínas, modulando la activación de las quinasas y la inhibición de las fosfatasas[197], y producen la oxidación del colesterol y su posterior salida de la membrana plasmática, esencial para que se lleve a cabo la reacción acrosómica[192, 220].

Durante la capacitación se produce un cambio en el patrón de motilidad, ocurre la hiperactivación. Los espermatozoides muestran un movimiento flagelar de gran amplitud no lineal, con un desplazamiento lateral de la cabeza significativamente mayor. La hiperactivación ayuda a la propulsión de los espermatozoides y a la penetración a través de las células del cúmulo que rodean el ovocito, por tanto, facilita la fecundación[213]. El papel de $O_2^{-\bullet}$ en la modulación de la hiperactivación se describe en un estudio *in vitro* que revela que la incubación de los espermatozoides con SOD inhibe la hiperactivación[221]. La fosforilación de proteínas de la vaina fibrosa del flagelo también ocurre durante la capacitación, lo que sugiere que la vía del AMPc-PKA, dependiente de EROs, también interviene en la regulación de la hiperactivación[222, 223]. Además, el Ca^{2+} regula la motilidad de los espermatozoides a través de los canales de cationes permeables al Ca^{2+}, localizados en la membrana de los flagelos, que a su vez regulan la captación extracelular de Ca^{2+} y la movilización del Ca^{2+} almacenado en la porción intermedia del espermatozoide. Un aumento del Ca^{2+} intracelular desencadena el cambio de la motilidad del estado progresivo al hiperactivado, mejorando la capacidad de penetrar a través de la ZP[224].

Los espermatozoides capacitados son los únicos capaces de experimentar la reacción acrosómica, lo que explica por qué estos dos procesos comparten los mecanismos moleculares. La fosforilación de las proteínas asociadas con la capacitación se extiende a las proteínas relacionadas con la reacción acrosómica, como la proteína quinasa C

(PKC) y la fosfolipasa A2 (PLA2)[213, 215]. El inicio de este proceso está mediado por la liberación de Ca^{2+} durante la capacitación, que induce la escisión de fosfatidilinositol-4,5-bisfosfato (PIP2) en diacilglicerol (DAG) e inositol trifosfato (IP3). El IP3 promueve la fusión de las membranas acrosómica y plasmática, dando como resultado la exocitosis acrosómica. Posteriormente, DAG activa PKC resultando en una mayor afluencia de Ca^{2+} y activación de PLA2. La PLA2 activada cataliza la escisión de los ácidos grasos secundarios del esqueleto de la bicapa lipídica de la membrana y, por tanto, aumenta la fluidez de la membrana plasmática de los espermatozoides, esencial para la fusión con el ovocito[213]. Diferentes estudios describen que bajas concentraciones de H_2O_2 y $O_2^{-\bullet}$ también intervienen en la reacción acrosómica, así como el $NO^{\bullet}$ a través de la síntesis del segundo mensajero Guanosín Monofosfato cíclico (GMPc) y la activación de quinasas[215, 225, 226].

5.3. Apoptosis

La apoptosis consiste en el desencadenamiento de una cascada de eventos que tienen como fin la muerte celular programada en ausencia de inflamación. Está compuesta por dos vías interconectadas, la vía intrínseca o mitocondrial y la vía extrínseca o de los receptores de muerte celular de la familia del factor de necrosis tumoral (TNF), que se encuentran anclados a la membrana plasmática. La vía extrínseca es activada por estímulos extracelulares y produce la activación de las cisteinil aspartato proteasas (caspasas). En primer lugar se activa la caspasa 8, que a su vez activará a las caspasas efectoras 3 y 7, dando lugar a la proteólisis de sustratos y finalmente a la muerte celular. En la vía intrínseca, el citocromo C es liberado al citoplasma tras la alteración de la integridad de la membrana externa mitocondrial, activando a la caspasa 9, que da lugar a la activación de las caspasas efectoras 3, 6 y 7, ocasionando la muerte celular. Los factores que pueden desestabilizar la membrana mitocondrial e iniciar la vía intrínseca de la apoptosis son las proteasas, la presencia de Ca^{2+} o las EROs[227-233]. En espermatozoides, la exposición a estímulos que conducen a estrés oxidativo como la cogelación, la radiación electromagnética de radiofrecuencia y la adición directa de H_2O_2 inducen la apoptosis[88, 192, 234].

Las EROs promueven modificaciones en numerosas proteínas, siendo la succinato deshidrogenasa o complejo II de la CMTE una de las principales dianas mitocondriales sensibles al estrés oxidativo. La alteración de la funcionalidad de esta enzima facilita la fuga de e^{-}, generandose $O_2^{-\bullet}$ en el interior mitocondrial[105]. Esta producción excesiva de EROs da lugar a la formación de un poro de transición que altera la permeabilidad mitocondrial[235]. La apertura transitoria de este poro tiene funciones esenciales en la homeostasis de las células al permitir que el exceso de EROs y Ca^{2+} salga de la mitocondria en condiciones normales. Las EROs liberadas al citosol inducen una cascada de aperturas transitorias del poro, fenómeno conocido como liberación de EROs inducida por EROs[236]. La inhibición de la formación del poro usando ciclosporina A da

como resultado un aumento de las EROs mitocondriales[237], lo que demuestra la importancia del poro en la homeostasis redox. En condiciones de estrés oxidativo, el poro permanece abierto durante períodos prolongados, permitiendo que el citocromo C también pase desde la mitocondria al citosol en cantidad suficiente para inducir la cascada apoptótica como resultado de la activación de las caspasas[238]. Por tanto, si bien las EROs pueden ser consideradas como desencadenantes de la vía mitocondrial de la apoptosis, al mismo tiempo, la apoptosis está asociada con el incremento del estrés oxidativo. La liberación del citocromo C al citosol representa un evento pro-oxidativo porque está asociado con la alteración de la CMTE, lo que conduce a una mayor producción de $O_2^{-\bullet}$. Esto explica la producción espermática de EROs por las mitocondrias junto a los cambios apoptóticos relacionados con la caspasa 9 en aquellas condiciones que promueven la pérdida del PMM[72, 239-241].

Los marcadores principales de apoptosis incluyen la externalización de fosfatidilserina al exterior de la membrana plasmática y la activación de las caspasas. La presencia de caspasas activas se correlaciona con una pobre calidad espermática y una menor capacidad de fecundación por la disminución del PMM, la pérdida de la motilidad y la alteración del ADN. No obstante, una interconexión entre la capacitación y la vía de señalización de apoptosis ha sido propuesta, así como que los espermatozoides poseen marcadores de apoptosis que pueden estar involucrados en la eliminación de espermatozoides que portan ADN anormal en el tracto reproductor femenino[242-249].

Aunque la apoptosis da lugar a la muerte celular, un ejemplo de los efectos beneficiosos de la apoptosis en espermatozoides se describe después de la inseminación, cuando el exceso de espermatozoides debe eliminarse de forma silente del tracto reproductivo femenino por los neutrófilos y fagocitos, un proceso que está mediado por la presencia de marcadores apoptóticos como la fosfatidilserina en la superficie espermática[216].

5.4. Peroxidación lipídica

Los espermatozoides contienen en la membrana plasmática ácidos grasos saturados y ácidos grasos poliinsaturados de cadena larga (PUFAs) que aportan elasticidad y fluidez a la membrana, característica importante para que la fecundación ocurra con éxito. Los PUFAs son susceptibles a la oxidación por las EROs porque presentan gran cantidad de dobles enlaces carbono-carbono, lo que ocasiona la peroxidación lipídica de la membrana[191, 250, 251]. Por tanto, la desregulación o pérdida de la homeostasis redox da lugar a la peroxidación lipídica, originando hidroperóxidos lipídicos inestables que se descomponen en compuestos secundarios más estables y menos reactivos. La peroxidación lipídica ocurre en tres fases, en la fase de iniciación una ERO abstrae el hidrógeno de una cadena lipídica y origina $LO^{\bullet}$, que en la fase de propagación reacciona con oxígeno y genera $LOO^{\bullet}$. Por último, $LOO^{\bullet}$ reacciona con otro lípido y abstrae su hidrógeno, formándose un $LO^{\bullet}$ y un LOOH. Estas moléculas son inestables y generan

nuevos radicales peroxilo y alcoxilo hasta descomponerse finalmente en productos secundarios[252-254]. Entre los compuestos secundarios de la peroxidación lipídica de los PUFAs de las membranas de los espermatozoides hay que destacar por su toxicidad a los aldehídos reactivos como 4-hidroxinonenal (4-HNE), acroleína y malondialdehído. Estos compuestos se originan en función de la oxidación de diferentes PUFAs. El malondialdehído se origina a partir de la oxidación de PUFAs que contengan al menos tres dobles enlaces, como el ácido araquidónico, mientras que el 4-HNE se origina de la oxidación de ácidos grasos ω-6[255-258]. El ácido docosapentaenoico (C22: 5ω-6) es el PUFA predominante entre los fosfolípidos que componen la membrana de los espermatozoides de équidos y, como consecuencia, el 4-HNE es el mayor aldehído producido con la peroxidación lipídica[259]. Si bien, todos los aldehídos reactivos producen daño celular, el 4-HNE es muy perjudicial en los espermatozoides de sementales, carneros, ratones y humanos[27, 257, 260-263]. Los aldehídos son electrofílicos y se unen de forma covalente a residuos de lisina, histidina y cisteína de proteínas diana, ocasionando alteración en su función[191, 216]. Diferentes estudios describen que las biotecnologías de conservación seminal incrementan la cantidad de 4-HNE y malondialdehído en los espermatozoides[264-267].

Entre los efectos producidos por la peroxidación lipídica podemos destacar el incremento de la permeabilidad de la membrana plasmática, la disminución de la motilidad progresiva, el incremento en la fragmentación de ADN y efectos nocivos sobre la morfología espermática[268-272]. No obstante, GSH presenta un papel importante en la peroxidación lipídica al reaccionar con 4-HNE, eliminando su toxicidad. Esta reacción puede ocurrir de forma espontánea, pero en la mayoría de los casos es catalizada por glutatión-S-transferasas. La enzima aldehído deshidrogenasa también produce la desintoxicación del 4-HNE, oxidándolo a ácido 4-hidroxinonenoico[252, 261]. Un estudio reciente de congelación seminal en peces ha descrito que la suplementación de los diluyentes con GSH y ATP disminuye la peroxidación lipídica de los espermatozoides descongelados[273].

5.5. Ubiquitinización

La ubiquitina es un péptido regulador que se encuentra en la mayoría de los tejidos de los organismos eucariotas. Este péptido se une covalentemente a proteínas y modula interacciones proteína-proteína, coordina la localización de las proteínas o degrada la proteína diana. Este proceso se denomina ubiquitinación y está asociado con el estrés oxidativo[274-281]. En eyaculados de ratones y humanos la producción prolongada del aldehído citotóxico 4-HNE inicia la ubiquitinación y la posterior degradación proteolítica[279, 282]. En el sistema reproductivo, la ubiquitinación tiene efectos durante la espermatogénesis, la maduración espermática, la formación de los axonemas, la fecundación y el desarrollo embrionario[283, 284]. En los testículos, la ubiquitina se une a las membranas de las mitocondrias espermáticas dañadas o mal formadas, actuando

eficazmente como un marcador de espermatozoides defectuosos a través de la presencia de una etiqueta de ubiquitina expuesta. Los procesos de ubiquitinación en el epidídimo conducen al reconocimiento y posterior unión a estas etiquetas, provocando la fagocitosis de los espermatozoides anormales[285]. Por tanto, el proceso de ubiquitinación es un mecanismo protector para preservar la calidad del eyaculado, reduciendo el número de espermatozoides defectuosos[286].

En espermatozoides de toro, el estrés térmico ocasiona una regulación positiva de PRDX5, GPX5 y proteínas ubiquitinadas, en respuesta al daño oxidativo[287]. Investigaciones recientes se centran en el uso de la ubiquitinación como marcador de la fertilidad en toros, cerdos, sementales y hombres[288-293]. En cerdos, la ubiquitinación de los espermatozoides afecta negativamente al desarrollo de los embriones obtenidos mediante la inyección intracitoplasmática de espermatozoides (ICSI)[290]. Estos hallazgos revelan los efectos negativos de grandes cantidades de ubiquitinación sobre la calidad del espermatozoide y el desarrollo embrionario, así como la posibilidad de enfoques terapéuticos de precisión para abordar la infertilidad o la subfertilidad masculina, basados en una comprensión molecular de estos procesos.

5.6. Impacto de las EROs en las proteínas

Entre las modificaciones proteicas inducidas por el estrés oxidativo podemos destacar la oxidación de grupos tiol (-SH), la S-glutationilación, la nitración de Tyr, S-nitrosilación, la sulfonación o introducción de un grupo ácido sulfónico ($-SO_3H$) y la formación de aductos proteicos 4-HNE tras la peroxidación lipídica[26]. El aminoácido Cys es un fuerte nucleófilo en condiciones fisiológicas gracias a su -SH, que puede ser oxidado formando puentes disulfuro (-SS-). Las moléculas proteicas mantienen relaciones específicas -SS-/-SH, esenciales para mantener sus funciones. El estrés oxidativo puede oxidar el -SH libre, impidiendo la formación de -SS- donde y cuando sea necesario en un proceso fisiológico, ocasionando el deterioro de la función proteica. Las enzimas antioxidantes de la familia peroxirredoxinas contienen una Cys (PRDX6) o dos (PRDX1-5) en su sitio activo, altamente reactivas con H_2O_2 y otros peróxidos, por lo que en presencia de altas concentraciones de H_2O_2 se saturan estos centros activos y se ocasiona el estrés oxidativo[294]. El H_2O_2 también puede oxidar el -SH de la enzima gliceraldehído-3-fosfato deshidrogenasa, inactivándola y alterando la ruta glucolítica, disminuyendo la motilidad de los espermatozoides[295-297]. La α-tubulina es una proteína estructural del flagelo y también contiene un -SH, que puede ser oxidado por H_2O_2, alterándose la polimerización de los microtúbulos y, por tanto, el funcionamiento del flagelo[298]. Otra modificación significativa de los -SH por EROs es la formación de aductos de proteínas con electrófilos como el 4-HNE y la acroleína[299]. Entre otros efectos, el 4-HNE promueve la inactivación de la succinato deshidrogenasa, enzima que interviene tanto en el ciclo de Krebs como en la CMTE, alterándose por tanto la actividad mitocondrial y promoviendo la mayor producción de EROs, ocasionando una reducción de la motilidad y un incremento del

daño en el material genético, así como la apoptosis. Otro ejemplo de la disfunción espermática por 4-HNE es la oxidación del complejo proteico HSPA2/ARSA/SPAM, esencial para el reconocimiento y la adhesión de los espermatozoides al ovocito[300, 301]. La acroleína también forma aductos con proteínas y ADN, ocasionando inactivación enzimática y mutagénesis. Además, existen estudios que describen que la acroleína altera la actividad de las TRX y PRDX, lo que disminuye la capacidad antioxidante y promueve el estrés oxidativo en los espermatozoides[105, 257, 302-304].

La S-nitrosilación de las proteínas y enzimas estructurales es producida por las EROs que contienen nitrógeno, como $NO^{\bullet}$ y $ONOO^{-}$, y puede modificar la función celular. En espermatozoides se han identificado modificaciones por S-nitrosilación en enzimas implicadas en la producción de energía, la motilidad, los canales iónicos y la función antioxidante, lo que altera la homeostasis redox[305, 306]. La reacción de un residuo de Tyr con estas EROs (nitración de Tyr) produce un radical $NO_2^{\bullet}$, alterando la función proteica. En espermatozoides de humanos astenozoospérmicos se han descrito niveles elevados de nitración de Tyr en enzimas que intervienen en la glucólisis (gliceraldehído 3-fosfato deshidrogenasa y fosfopiruvato hidratasa) y el ciclo de Krebs (α-cetoglutarato deshidrogenasa, aconitato hidratasa, malato deshidrogenasa y dihidrolipoamida deshidrogenasa). Esto provoca una reducción drástica de la producción de ATP y, como consecuencia, la disminución de la motilidad[307-309]. Además, el funcionamiento del flagelo puede alterarse porque la α-tubulina también puede modificarse mediante nitración de Tyr[310].

Otro de los efectos del estrés oxidativo sobre las proteínas es la carbonilación, que implica la modificación covalente e irreversible de residuos de cisteína, histidina, lisina y treonina mediante los productos finales de la peroxidación lipídica avanzada. Los efectos deletéreos de la carbonilación sobre la fertilidad se han descrito recientemente en toros y cerdos. La carbonilación de glutatión-S-transferasas y de proteínas de la fibra densa de los espermatozoides imposibilita la capacitación, reduciendo de este modo la fecundación[311, 312]. Además, las proteínas nucleares que contienen -SH, especialmente protaminas, también pueden sufrir carbonilación, alterándose completamente el plegamiento y la función de la cromatina[313]. La desregulación de la homeostasis redox también aumenta la metilación y acetilación de las histonas, ocasionando fragmentación en la doble hélice de ADN y mala calidad espermática, lo que puede alterar el desarrollo embrionario[314].

Recientemente, en el grupo de investigación de Reproducción y Espermatología Equina de la Universidad de Extremadura hemos descrito una reducción significativa de las enzimas antioxidantes como consecuencia de la congelación de semen. Este hallazgo apoya firmemente la teoría de que la alteración de la regulación redox y el estrés oxidativo es un factor importante en el daño inducido por la congelación y

descongelación, sugiriendo que el control de la regulación redox debe ser un objetivo importante para mejorar los procedimientos actuales de conservación seminal[315].

5.7. Impacto de las EROs en el material genético

Durante la espermatogénesis, las espermátidas cambian el plegamiento de su genoma, reemplazando las histonas por proteínas de transición y luego por protaminas, consiguiéndose una mayor compactación del ADN. No obstante, aunque la mayor parte del genoma se encuentra unido a protaminas, un pequeño porcentaje del ADN (5-10%) se mantiene organizado en nucleosomas por histonas residuales, resultando vulnerable al estrés oxidativo. Debido a que la localización de los cromosomas en el núcleo espermático no es aleatoria, determinados autosomas son más vulnerables a las EROs[316-321].

El daño oxidativo del ADN incluye la introducción de sitios abásicos, que aumentan el riesgo de errores de replicación como la 6-O-metilguanina, la introducción de bases oxidadas como la 8-OHdG y la fragmentación del ADN por roturas monocatenarias y bicatenarias. La 8-OHdG es uno de los principales productos de la oxidación del ADN y puede ocasionar una alteración de la transcripción de genes[322]. Como se ha mencionado anteriormente, el estrés oxidativo interfiere en la fertilidad espermática, pues ocasiona daños en proteínas y lípidos que son esenciales para la motilidad, la capacitación y la unión de los espermatozoides a los ovocitos, entre otras funciones. Sin embargo, un grado de estrés oxidativo insuficiente para impedir dichas funciones puede alterar el material genético del espermatozoide, sin una disminución apreciable de la fertilidad, especialmente cuando la cromatina está mal compactada o hay deficiencia de antioxidantes nucleares[191, 323]. Estos efectos se ven agravados por el escaso citoplasma de los espermatozoides, por lo que sufren de la falta de enzimas esenciales, o de un sistema completamente funcional de reparación de ADN, de la ausencia de protección de su ADN nuclear por la entrada de nucleasas y de la incapacidad de la activación transcripcional de genes que codifican para las enzimas antioxidantes involucradas[324].

Los espermatozoides, además del genoma paterno haploide, contienen información epigenética importante (ARN no codificante, metilación del ADN, modificaciones postraduccionales (PTMs) de histonas, plegamiento de la cromatina), con funciones reguladoras para el desarrollo embrionario temprano[325, 326]. El estrés oxidativo también afecta a esta información epigenética[327]. Las EROs pueden, por tanto, inducir alteraciones en la expresión génica a través de mecanismos genéticos, como mutaciones espontáneas de la línea germinal, o mediante mecanismos epigenéticos, como alteraciones en la metilación global del ADN, en los ARNs no codificantes o por PTMs de las histonas[328-331]. Las alteraciones epigenéticas inducidas por EROs pueden surgir en respuesta a factores dietéticos (desnutrición, deficiencias de micronutrientes o proteínas, toxinas dietéticas o dietas ricas en grasas), estrés fisiológico relacionado con

la obesidad o exposición a toxinas como pesticidas, productos farmacéuticos o contaminantes presentes en el aire[191, 331].

Nuestro grupo de investigación ha demostrado que los espermatozoides sometidos a las biotecnologías de conservación seminal presentan oxidación del ADN[332]. Actualmente, los protocolos de congelación siguen presentando inconvenientes y la fertilidad obtenida con esta biotecnología es menor que cuando se realizan inseminaciones con semen fresco, probablemente debido a la mayor tasa de muerte embrionaria, entre otras consecuencias[15, 333]. No obstante, lo alarmante del efecto del estrés oxidativo en el material genético y epigenético es que el espermatozoide puede mantener la capacidad de fecundación, lo que puede implicar importantes consecuencias para el desarrollo embrionario y la descendencia[234]. En humanos, el incremento del daño oxidativo del ADN espermático se ha correlacionado con cánceres infantiles y trastornos cerebrales como el autismo y la esquizofrenia, entre otras patologías de la descendencia[334, 335]. En animales, la fecundación de ovocitos con espermatozoides que presentan daño oxidativo en el ADN implica un compromiso de la calidad embrionaria, así como una disminución en las tasas de escisión embrionaria y un incremento significativo de la cantidad de células apoptóticas en los blastocistos resultantes[336, 337]. Además, estudios en roedores indican que la fecundación con ADN dañado puede producir alteraciones en la descendencia a largo plazo[338].

OBJETIVOS

Basándonos en la importancia de la homeostasis redox y en el efecto de las actuales biotecnologías de conservación seminal sobre ésta y la calidad espermática, en la presente Tesis Doctoral nos propusimos incrementar nuestro conocimiento sobre la biología del espermatozoide equino, su implicación en el desarrollo embrionario y la posibilidad de mejorar la calidad espermática tras la optimización de las biotecnologías de conservación. Para ello nos planteamos los siguientes objetivos específicos:

2.1. Profundizar en el conocimiento de la regulación redox en el funcionamiento del espermatozoide equino fresco y sometido a procesos de conservación.

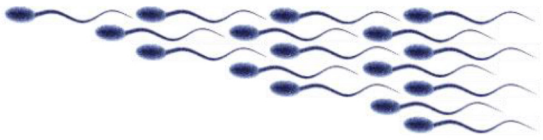
2.1.1. Determinar si la calidad del espermatozoide equino congelado puede ser mejorada tras la descongelación.

2.1.2. Establecer el papel de los grupos tiol en la funcionalidad espermática y comprobar si la concentración de GSH se puede incrementar para restablecer la depleción de los grupos tiol que ocurre durante la conservación.

2.2. Estudiar la plasticidad metabólica del espermatozoide equino, en relación con la regulación redox, y su potencial uso en la mejora de los diluyentes de conservación seminal.

2.3. Estudiar el impacto del proceso de congelación de semen equino en la expresión génica durante el desarrollo embrionario temprano.

JUSTIFICACIÓN



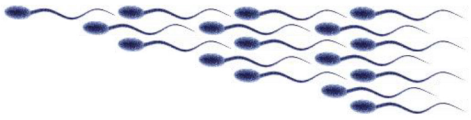
El comercio de semen es un elemento básico en la economía de la industria equina. No obstante, su uso está limitado por la variabilidad en la capacidad de refrigeración y/o congelación de los eyaculados de los diferentes sementales, y la falta de estandarización de los protocolos de conservación seminal[15]. Estos protocolos requieren de una reducción o detención del metabolismo de los espermatozoides para incrementar la supervivencia de los mismos[14]. No obstante, existen pocos estudios que se centren en el metabolismo bajo las condiciones de conservación seminal.

Los espermatozoides presentan un metabolismo muy activo y producen una elevada cantidad de ATP, energía requerida para mantener su funcionalidad. Por tanto, la fertilidad está correlacionada con la actividad mitocondrial y, como consecuencia, los espermatozoides fértiles producen una elevada cantidad de EROs[23, 24]. Nuevos avances científicos describen la intervención de las EROs en la regulación de funciones biológicas[191, 192]. No obstante, el estrés oxidativo conlleva a la presencia de una elevada cantidad de EROs, impidiendo el mantenimiento de las características funcionales de los espermatozoides[193]. La homeostasis redox resulta esencial para impedir el estrés oxidativo. Los antioxidantes son los responsables de regular los niveles de las EROs, siendo el GSH el principal antioxidante intrínseco[27]. Por esta razón, nos planteamos la hipótesis de que la cistina (CysS) es incorporada desde el medio extracelular al interior espermático para su posterior reducción en dos moléculas de cisteína (Cys) y el uso de estos aminoácidos para la síntesis de GSH. Por ello, en los tres primeros artículos de esta Tesis Doctoral estudiamos la existencia del transportador transmembrana SLC7A11 y sistema xCT y su funcionalidad, un complejo proteico encargado de interiorizar una molécula de CysS y exteriorizar una de glutamato (Glu). Este transportador ha sido descrito en las membranas plasmáticas de células del sistema nervioso central y del sistema inmune, donde presenta un papel importante en la regulación de la homeostasis redox[339, 340]. Nuestros resultados localizan al SLC7A11 en la región postacrosómica de los espermatozoides y describen la correlación de la funcionalidad de este transportador con la funcionalidad espermática, especialmente con la síntesis de GSH, la actividad mitocondrial y el metabolismo del glutamato.

El estudio de la relación entre el metabolismo y la regulación redox es un enfoque prometedor para mejorar las técnicas actuales de conservación seminal[29, 34, 35]. Teniendo en cuenta los estudios recientes de proteómica que amplían nuestro conocimiento sobre el metabolismo espermático[30-33] y la plasticidad observada en el metabolismo del glutamato, en el cuarto artículo nos propusimos mejorar la calidad espermática mediante la adición de rosiglitazona en los eyaculados descongelados. Por otro lado, en el quinto artículo estudiamos la posibilidad de mejorar los diluyentes de refrigeración teniendo en cuenta la biología espermática. Nuestro estudio consistió principalmente en reducir la concentración de glucosa en los medios de conservación, pues la glucólisis es utilizada para mantener las motilidades y velocidades espermáticas, pero la principal vía de producción de ATP en el espermatozoide equino es la fosforilación oxidativa[84].

Por último, en los dos últimos artículos de la Tesis Doctoral, estudiamos el impacto de la congelación-descongelación en el desarrollo embrionario temprano. Esta técnica de conservación seminal provoca un desequilibrio de la homeostasis redox en la población espermática que sobrevive[27, 255], pudiendo ocasionar daños importantes sin pérdida de la capacidad de fecundación, lo que implica importantes consecuencias para el desarrollo embrionario y la descendencia[234].

RESULTADOS



Artículo 1

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OXFORD

Research Article

The incorporation of cystine by the soluble carrier family 7 member 11 (SLC7A11) is a component of the redox regulatory mechanism in stallion spermatozoa†

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Abstract

Oxidative stress is considered a major mechanism causing sperm damage during cryopreservation and storage, and underlies male factor infertility. Currently, oxidative stress is no longer believed to be caused only by the overproduction of reactive oxygen species, but rather by the deregulation of redox signaling and control mechanisms. With this concept in mind, here, we describe for the first time the presence of the soluble carrier family 7 member 11 (SLC7A11) antiporter, which exchanges extracellular cystine (Cyss) for intracellular glutamate, in stallion spermatozoa, as well as its impact on sperm function using the specific inhibitor sulfasalazine. Spermatozoa incubated with Cyss exhibited an increased intracellular GSH content compared with controls ($P < 0.01$): 50% in fresh extended stallion spermatozoa and 30% in frozen-thawed spermatozoa. This effect was prevented by the addition of sulfasalazine to the media. Cystine supplementation also reduced the oxidation–reduction potential of spermatozoa, with sulfasalazine only preventing this effect on fresh spermatozoa that were incubated for 3 h at 37°C, but not in frozen-thawed spermatozoa. While sulfasalazine reduced the motility of frozen-thawed spermatozoa, it increased motility in fresh samples. The present findings provide new and relevant data on the mechanism regulating the redox status of spermatozoa and suggest that a different redox regulatory mechanism exists

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in cryopreserved spermatozoa, thus providing new clues to improve current cryopreservation technologies and treat male factor infertility.

Summary Sentence

The SLC7A11 antiporter that exchanges cystine by intracellular glutamate is present and functional in stallion spermatozoa, but cryopreserved spermatozoa may present altered functionality.

Key words: stallion, spermatozoa, GSH, flow cytometry, cysteine, cystine, oxidation, reduction.

Introduction

Spermatozoa are redox-regulated cells [1, 2], and deregulation of their redox status is considered to affect male fertility and to reduce their fertilizing capacity following biotechnological procedures, such as cryopreservation or sex sorting [3]. Redox homeostasis in spermatozoa depends on an adequate balance between oxidants and reductants. Spermatozoa maintain this balance and the physiological levels of reactive oxygen species (recently termed oxidative eustress [4]) via a number of antioxidant defenses that are present both in seminal plasma and in the sperm cell itself, including enzymatic and nonenzymatic systems [5–9]. However, due to their limited cytoplasm, intracellular defenses may be rapidly exhausted under conditions of intense oxidative stress [10]. This factor may be particularly critical when spermatozoa are cryopreserved, a process in which most of the seminal plasma, which is rich in antioxidants, is customarily removed by centrifugation [11–13]. Among the intracellular antioxidant systems, thiols and particularly glutathione (GSH) play a key role in maintaining redox homeostasis in spermatozoa [14]. A significant correlation between the thiol content and stallion sperm functionality has been reported [10]. Notably, spermatozoa surviving cryopreservation show compromised functionality and reduced intracellular thiol contents. Glutathione is present at millimolar quantities in somatic cells, where it is considered the major natural antioxidant [15]. Glutathione is also present in spermatozoa; interestingly, the stallion has the highest GSH levels among reported mammals [14, 16]. Glutathione is synthesized in two steps: first, cysteine and glutamate are linked in a reaction catalyzed by γ-glutamylcysteine synthase, which is the rate-limiting step in GSH formation. The second step is catalyzed by GSH synthase and comprises covalent linkage of glycine to γ-glutamylcysteine [15]. Both enzymes are present in stallion spermatozoa [14]. The availability of cysteine/cystine (Cys/Cyss) is the rate-limiting step in GSH synthesis, with Cys incorporated via the Na^+-dependent alanine-serine-cysteine (ASC) group or transporters such as ASC1 (SLC7A10) [17–20] and Cyss incorporated via the soluble family 7 carrier member 11 (SLC7A11) antiporter, the Na^+-independent exchange system for Cyss/glutamate [18]. SLC7A11 is constitutively expressed in a limited number of organs, such as the thymus, spleen, and brain [21], but SLC7A11 mRNA is expressed in testis and SLC7A11 knockout male mice display reduced reproductive performance [22]. Moreover, Cys is also synthetized from methionine by the enzymes cystathionine beta-synthase (CBS) and cystathionase. Cysteine is rapidly and spontaneously oxidized to Cyss to become the predominant form in the extracellular space. Once incorporated, Cyss is rapidly reduced to Cys intracellularly and used for GSH synthesis [23, 24]. In addition, the Cys/Cyss couple is considered a redox node controlling cellular functions [25] in a similar manner to the GSH/GSSG or thioredoxin nodes. Since the function of spermatozoa is regulated by redox pathways, we hypothesized that stallion spermatozoa may incorporate Cyss (to be reduced in the cell) as a component of the redox regulation machinery to maintain intracellular GSH levels and/or may constitute a redox node itself with critical roles in sperm functionality. We aimed to determine (i) the presence of the SLC7A11 antiporter as an exchanger of extracellular Cyss for intracellular glutamate in stallion spermatozoa and (ii) the effects of SLC7A11 on sperm motility, velocities, and overall oxidation–reduction status in fresh and frozen-thawed stallion spermatozoa. We also investigated the presence of the enzymes involved in the synthesis of Cys from methionine: CBS and cystathionase.

Materials and methods

Reagents and media

The anti-CBS antibody (ab54883), anti-cystathionase antibody (ab 151769), and anti-SLC7A11 antibody (ab99059) were purchased from Abcam (Cambridge, UK). Anti-rabbit and anti-goat IgG horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence detection reagents were obtained from Pierce (Rockford, IL). Goat anti-rabbit IgG antibody conjugated with Alexa Fluor 546 was from Thermo Fisher (Waltham, MA). Hyperfilm ECL was purchased from Amersham (Arlington Heights, IL), and the nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH), while L-Cyss, monochlorobimane, sulfasalazine (SS), and all other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

Semen collection and processing

Semen was collected from 11 Purebred Spanish horses (PRE) (at least three ejaculates from each stallion) that were individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. Stallions were maintained according to institutional and European animal care regulations (Law 6/2913 June 11 and European Directive 2010/63/EU), and semen was collected on a regular basis (two collections/week) throughout the 2018 breeding season. All procedures performed in this study were approved by the ethical committee of the University of Extremadura. Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. After collection, the semen was extended 1:2 in an INRA-96 extender (IMV L'Aigle, France) and was immediately transported to the laboratory for evaluation and processing. The experimental design employed a split sample approach, with one ejaculate divided into two subsamples (fresh and frozen-thawed experimental groups). Upon arrival at the laboratory, the semen was centrifuged at 600 × g for 10 min to remove the bulk of the seminal plasma and then resuspended in Tyrode media (fresh extended semen) or resuspended in freezing media and frozen using standard procedures that have been previously described by our laboratory (frozen-thawed semen) [26]. Samples were thawed in a water bath at 37°C for at least 30 s. Fresh and frozen-thawed samples were washed and resuspended in Tyrode media [27]

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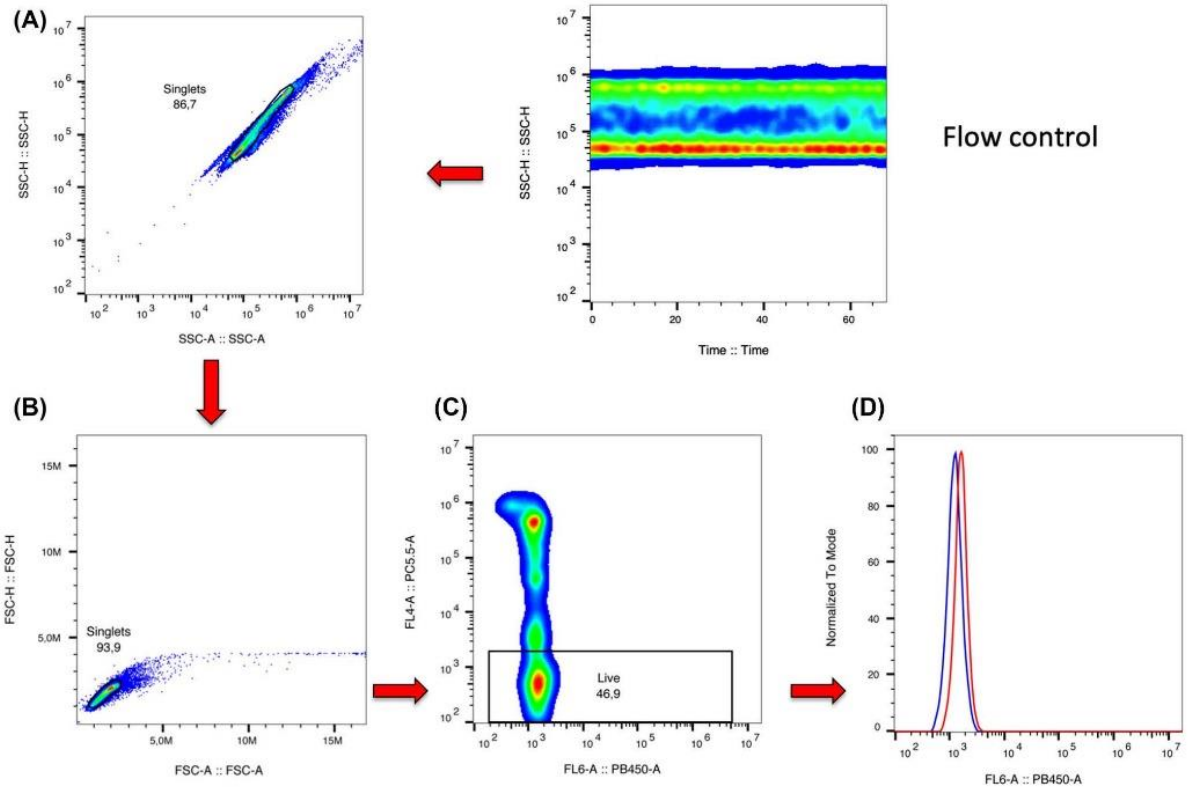

Figure 1. Gating strategy for the flow cytometry-based determination of GSH concentrations in spermatozoa. Stallion spermatozoa were processed and stained as described in the Materials and methods section. Flow control. (A and B) Identification of doublets and cell clumps that are gated and excluded from the analysis. (C) Dot plot of spermatozoa stained with monochlorobimane (FL6-A) and Eth-1 (FL4-A), dead spermatozoa were gated out. (D) Fluorescence of live spermatozoa.

to a final concentration of 50×10^6 spermatozoa/mL for incubation. The sperm suspensions (fresh and frozen-thawed samples) were divided into subsamples for control and experimental treatments and incubated in a water bath at 37°C.

Experimental design

Initially, the presence of the SLC7A11 antiporter and the enzymes involved in the synthesis of Cys from methionine was investigated using western blotting (WB) and image cytometry. The function of SLC7A11 was investigated using the specific inhibitor SS, with the sperm GSH content, motility, velocities, and overall oxidation–reduction status as end points. Moreover, the contribution of seminal plasma to the overall oxidation–reduction status of the stallion spermatozoa was evaluated after removing most of the seminal plasma through colloidal centrifugation [28, 29]

Samples of fresh and frozen-thawed stallion spermatozoa were incubated in a 37°C water bath for up to 6 h for the fresh samples or up to 3 h for the frozen-thawed samples to study the effects of the SLC7A11 antiporter on sperm GSH content, motility, velocities, and oxidation–reduction status. Split samples were supplemented with Cyss 0.5 mM (0.05 M stock solution in 1 M HCl); SS 100, 200, or 500 μM, (stock solution: 0.5 M in DMSO); and the combination of 0.5 mM Cyss and 100, 200, or 500 μM SS. The vehicle control HCl was included, and the pH was evaluated. The control DMSO was also included; DMSO concentrations in all samples remained below 0.001%. After a 3- or 6-h incubation for the fresh samples, and after a 1- or 3-h incubation for the frozen-thawed samples, aliquots were removed to evaluate the GSH content, sperm motility and velocities, and oxidation–reduction status.

Computer-assisted sperm analysis

Sperm motility was assessed using a computer-assisted sperm analysis system (ISAS Proiser, Valencia, Spain) according to standard protocols from our center [30]. Semen samples were loaded in a Leja® chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 37°C. The analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10× negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with a VAP (average velocity) < 15 μm/s were considered immotile, spermatozoa with a VAP > 35 μm/s were considered motile, and spermatozoa with VAP between 15 and 35 μm/s were considered locally motile spermatozoa. Spermatozoa deviating < 45% from a straight line were classified as linearly motile. The following parameters were measured: percentages of total and linear motile spermatozoa, circular velocity (VCL) in μm/s, straight-line velocity (VSL) in μm/s, and average path velocity (VAP) in μm/s.

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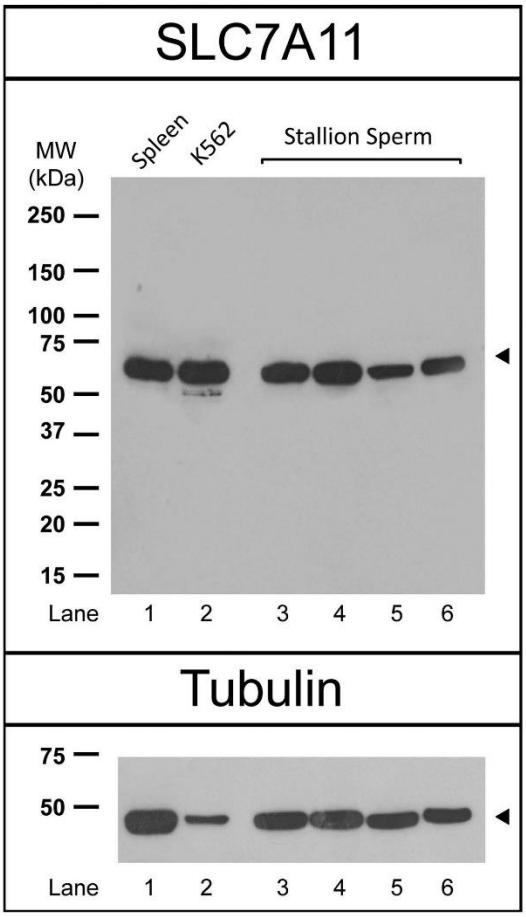

Figure 2. Identification of the SLC7A11 system. Stallion sperm lysates were immunoblotted with specific antibodies against SLC7A11, as described in the Materials and methods section. Lane 1–2 controls, lanes 3–6 stallion sperm lysates.

Flow cytometry

Flow cytometry analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter) equipped with violet, blue, and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowJoV 10.4.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One controls were used to determine compensations and positive and negative events, as well as to establish regions of interest, as described in previous publications from our laboratory [31–33].

Flow cytometry-based determination of the GSH content in stallion spermatozoa

The intracellular GSH content was determined by adapting previously published protocols [34, 35] optimized for GSH detection [36] using flow cytometry. Briefly, sperm aliquots (1–5 × 10^6 sperm/mL) were stained with 10 μM monochlorobimane and ethidium homodimer (Eth-1, 0.5 μM) for exactly 5 min at 22°C. The applied gating strategy is depicted in Figure 1. Briefly, after an assessment of the quality of the flow, doublets and debris were gated out, and monochlorobimane was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, while Eth-1 was detected at a peak excitation of 488 nm and emission of 610/20 nm BP. Dead cells (Eth-1+) were excluded, and only live spermatozoa were analyzed.

Measurement of the oxidation–reduction potential

The oxidation–reduction potential (ORP) was measured using a RedoxSYS® diagnostic system (Englewood CO, USA). According to the manufacturer, the ORP measures the transfer of electrons from a reductant to an oxidant in mV. This novel technology measures the static oxidation–reduction potential (sORP), the potential of an electrochemical cell under static conditions, and the antioxidant capacity reserve (cORP), which is the total amount of readily oxidizable molecules [37], in 4 min. Briefly, 30 μL of a sperm suspension was loaded in the sample port of the pre-inserted disposable sensor, and the measurement was immediately initiated. After 4 min, the sORP is provided in millivolts (mV). According to the manufacturer, the sORP is measured while applying a low oxidizing current (1 nA) to the sample. After allowing 1 min and 50 s for equilibration, the reader measures the difference in mV potential between the working and the reference electrode twice per second and over a period of 10 s. Subsequently, the cORP is determined by applying a linearly increasing oxidizing current until a rapid change in the charge between working and reference electrodes is observed, indicating that all readily oxidizable molecules are oxidized. The time interval is used to calculate the number of electrons needed to cause charge changes, reported in μCoulombs (μC).

Western blotting

SDS-PAGE was performed to separate the proteins according to their apparent molecular masses, as previously described [38, 39], with modifications in the analysis of SLC7A11 due to the highly hydrophobic nature of this protein [40]. Briefly, proteins were extracted and denatured by boiling for 10 min at 70°C in a loading buffer supplemented with 5% mercaptoethanol. The protein content was calculated using the Bradford assay [41]. Ten micrograms of sperm protein extract was loaded on a 10% polyacrylamide gel and resolved using SDS-PAGE. Immunoblotting was performed by incubating the membranes with blocking buffer containing primary antibodies (CBS, cystathionase, and SLC7A11 diluted 1.5, 0.26, and 1 μg/mL, respectively) overnight at 4°C. Secondary antibodies were used at 0.27 μg/mL (anti-mouse) or 0.16 μg/mL (anti-rabbit) depending on the primary antibody used. Proteins from whole rat pancreas and liver were used as positive controls for CBS, liver for cystathionase, and spleen and K562 cells for SLC7A11 [42]. Irrelevant IgG controls were used for all the primary antibodies used and presented in Supplementary Figure S3.

Immunocytochemistry

Indirect immunofluorescence staining was performed using previously described methods [32, 39, 43]. Spermatozoa were fixed with 4% formaldehyde in PBS for 15 min at room temperature (22°C), and permeabilized with 0.2% Triton X-100 v/v in PBS at room temperature (22°C) for 5 min. Samples were washed three times with PBS and incubated with PBS supplemented with 5% BSA to block nonspecific binding sites. After blocking, cells were incubated with the following primary antibodies diluted in PBS containing 5% BSA (w/v) overnight at 4°C: 2.6 μg/mL anti-cystathionase, 7.5 μg/mL anti-CBS, and 5 μg/mL anti-SLC7A11. On the following day, cells were washed with PBS and further incubated for 45 min at 22°C with a 1/500 dilution of a goat anti-rabbit IgG antibody

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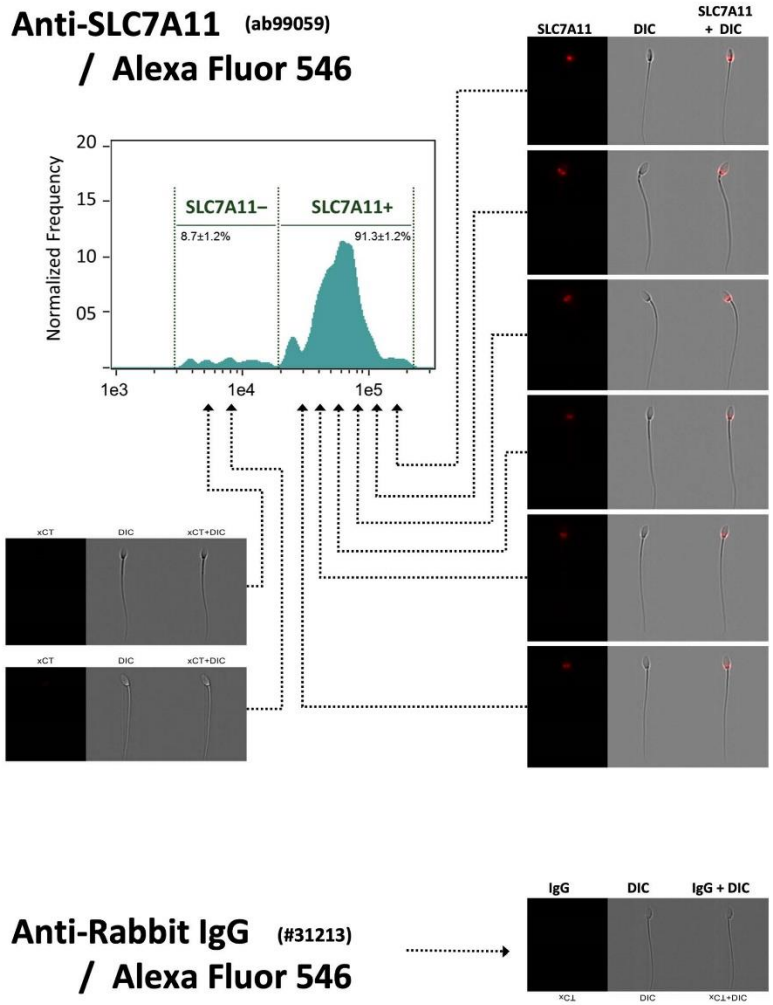

Figure 3. Subcellular distribution of SLC7A11 in spermatozoa. Stallion spermatozoa were processed for image cytometry as described in the Materials and methods section; 10 000 events were analyzed and classified. The histogram represents the percentage of positive spermatozoa expressing the antiporter. Right panel, representative images of stallion spermatozoa expressing the SLC7A11 antiporter; its expression is restricted to the postacrosomal region. Left panel, representative images of spermatozoa lacking the SLC7A11 antiporter. Bottom panel, irrelevant antibody control.

conjugated with Alexa Fluor 546 in PBS containing 5% BSA (w/v). Finally, cells were thoroughly washed with PBS. A total of 5000 cells were analyzed with the image flow cytometer. Image flow cytometry was performed using an ImageStream X Mark II Imaging Flow Cytometer (Merck Millipore) with a laser at a 488 nm wavelength, an intensity set to 100 mW, and 60× magnification. The raw images were analyzed using IDEAS1software (Version 6.0.309). The absence of nonspecific staining was determined by processing the samples without primary antibody (secondary antibody only) and using an irrelevant IgG control.

Statistical analysis

The normality of the data was assessed using the Kolmogorov–Smirnov test. Paired t-tests for changes in sORP in spermatozoa after removal of seminal plasma and one-way ANOVA followed by the Dunnett multiple comparisons test for all other experiments involving more than two groups were performed using GraphPad Prism software version 7.00 for Mac (La Jolla, CA, USA, www.graphpad.com). Differences were considered significant when $P < 0.05$. Results are displayed as means ± SEM.

Results

The SLC7A11 CSSC/L-glutamate antiporter is expressed in stallion spermatozoa

Systems designed to import extracellular Cyss are necessary for stallion spermatozoa to use exogenous Cyss to synthesize GSH. Since the main route of transport of L-Cyss into the cell is the SLC7A11 antiporter [44], its levels were investigated using WB and its location was assessed using immunocytochemistry (ICC) and image cytometry. The SLC7A antiporter was detected in stallion spermatozoa (Figure 2), and its subcellular distribution was restricted to the postacrosomal region (Figure 3).

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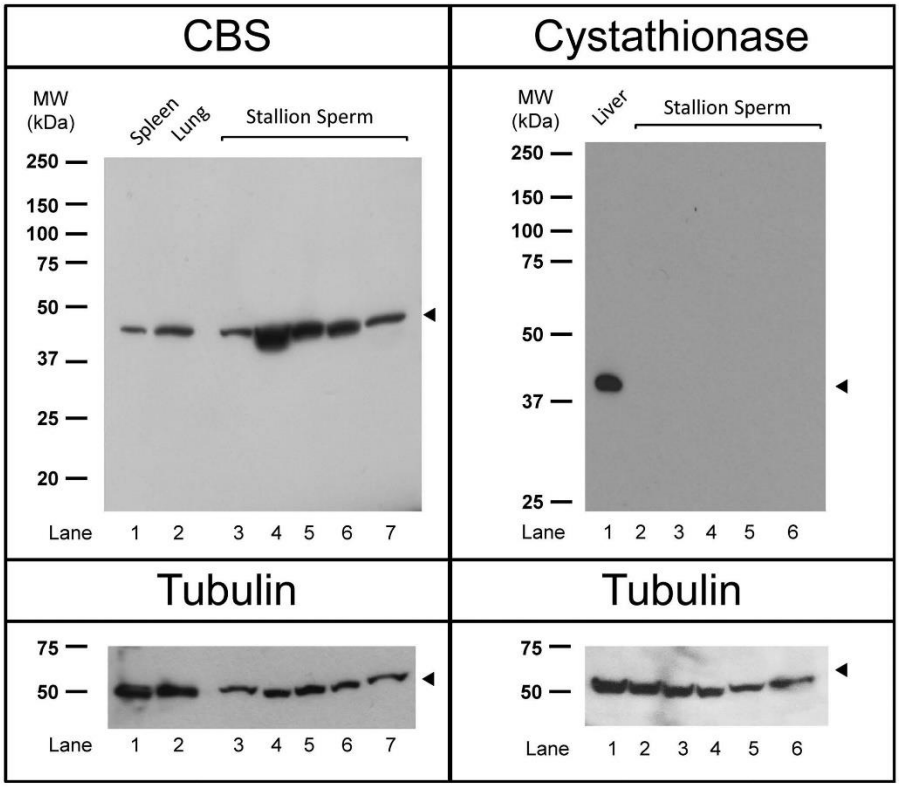

Figure 4. Investigation of the presence of cystathionine beta synthase (CBS) and cystathionase in stallion spermatozoa. Sperm lysates were subjected to western blotting using specific monoclonal antibodies, as indicated in the Materials and methods section. CBS: lanes 1–2, controls, lanes 3–6 stallion sperm lysates. Cystathionase: lane 1 control, lanes 2–6 stallion sperm lysates. Only CBS was identified in stallion sperm lysates.

Stallion spermatozoa express cystathionine-β-synthase, but not cystathionine γ ligase

L-cysteine is produced by enzymes in the trans-sulfuration pathway, notably CBS and CGL (cystathionine γ ligase). The levels of these enzymes were investigated using WB and ICC to determine if stallion spermatozoa contain the enzymatic machinery required to synthesize Cys. CBS was detected in lysates of stallion spermatozoa (Figure 4), and immunofluorescence staining revealed that this enzyme was distributed in the postacrosomal region and principally in the midpiece, but also was present in the rest of the tail (Figure 5). CGL was not detected in stallion spermatozoa (Figure 4).

Cystine supplementation increases intracellular GSH concentrations in fresh stallion spermatozoa

Split sperm samples, either freshly extended or frozen-thawed, were incubated with Cyss (0 and 0.5 mM) or Cyss with the inhibitor SS (0, 100, 200, or 500 μM) to assess the function of the SLC7A11 transporter and to determine if the incorporation of Cyss is required for GSH synthesis in stallion spermatozoa. The GSH content was evaluated using monochlorobimane, which reacts with GSH by a reaction catalyzed by glutathione S transferase [36, 45], and thus is highly specific for this thiol [36]. Fresh samples were incubated for up to 6 h at 37°C, and after 3 and 6 h of incubation, aliquots were collected to measure the GSH concentrations. In freshly extended stallion spermatozoa, Cyss supplementation increased the intracellular GSH content by 50% compared to controls ($P < 0.01$) at 3 and 6 h of incubation (Figure 6A and B). This effect was prevented by the SLC7A11 inhibitor SS in a dose-dependent manner. While the presence of 100 μM SS had no effect after 6 h of incubation, 200 and 500 μM SS inhibited the Cyss-induced increase in GSH levels. Moreover, at concentrations greater than 200 μM, SS reduced GSH concentrations, but only after 3 h of incubation (200 μM $P < 0.01$ and 500 μM $P < 0.01$ Figure 6A), whereas no differences were observed in samples treated with SS for 6 h compared with controls (Figure 6B).

Cystine supplementation increases intracellular GSH concentrations in frozen-thawed stallion spermatozoa

In frozen-thawed spermatozoa, Cys supplementation also increased intracellular GSH concentrations (by 30% compared with controls, $P < 0.01$, Figure 6C), an effect that was also prevented by the SLC7A11 inhibitor in a dose-dependent manner (Figure 6C and D). After 1 h of incubation, supplementation with 500 μM SS reduced GSH concentrations compared to controls ($P < 0.01$) and samples treated with Cyss ($P < 0.01$). An incubation with SS at doses > 200 μM for 3 h reversed the effect of Cyss supplementation (Figure 6C).

Cystine reduces the total oxidation–reduction potential and increases the antioxidant capacity of freshly extended stallion spermatozoa

The sORP and total antioxidant capacity (cORP) were measured to determine whether the effects of Cyss were linked to a reduction in

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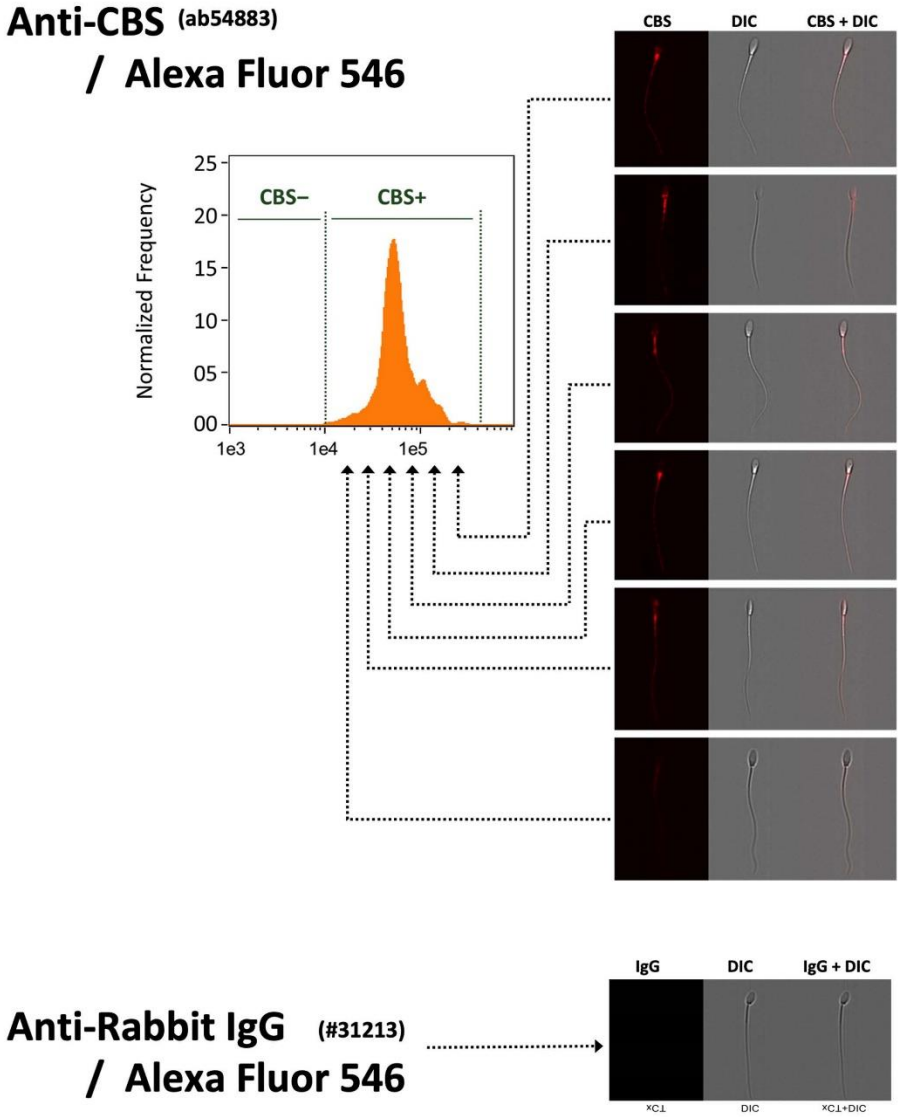

Figure 5. Subcellular location of CBS in stallion spermatozoa. Stallion spermatozoa were processed for image cytometry as described in the Materials and methods section; 10 000 events were analyzed and classified. The histogram represents the percentage of spermatozoa expressing CBS, which was 100% of the spermatozoa analyzed in this case Immunoreactivity with the anti-CBS antibody indicated that CBS was located in the postacrosomal region and along the midpiece and tail. Right panel, representative images of stallion spermatozoa expressing CBS; bottom panel, irrelevant antibody control.

total sperm oxidation. In freshly extended sperm suspensions, Cyss supplementation reduced the sORP after 3 (6.9 ± 1.0 to 6.1 ± 0.9 mV/10^6 spm (Figure 7A) and 6 h (6.7 ± 0.9 to 6.2 ± 0.8 mV/10^6 spm) (Figure 7B) of incubation ($P < 0.01$). This effect was prevented by SS, but only after 3 h of incubation (Figure 7A). The cORP increased after 3 ($P < 0.01$) (Figure 7C) and 6 h of incubation (Figure 7D) ($P < 0.05$) in the presence of Cyss.

Cystine reduces the total oxidation–reduction potential and increases the antioxidant capacity of frozen-thawed stallion spermatozoa

In frozen-thawed spermatozoa, Cyss reduced the sORP of the samples (8.5 ± 0.7 to 7.2 ± 0.7 mV/10^6 spm) (Figure 7E and F) ($P < 0.01$) both after 1 ($P < 0.01$) (Figure 7E) and 3 h of incubation (7.8 ± 0.7 to 7.2 ± 0.7 mV/10^6 spm) ($P < 0.01$) (Figure 7D). The cORP increased after 1 ($P < 0.01$) (Figure 7G) and 3 h of incubation (Figure 7H) ($P < 0.05$) with Cyss.

The removal of seminal plasma increases the oxidation status of stallion spermatozoa

In an attempt to determine the contribution of the seminal plasma to the ORP of the spermatozoa, the sORP was measured in raw semen and in samples from which the seminal plasma had been removed by colloidal centrifugation. The static sperm ORP increased from 0.746 mV/10^6 spermatozoa in raw semen to 9 mV/10^6 spermatozoa after the removal of seminal plasma ($P < 0.001$) (Figure 8A).

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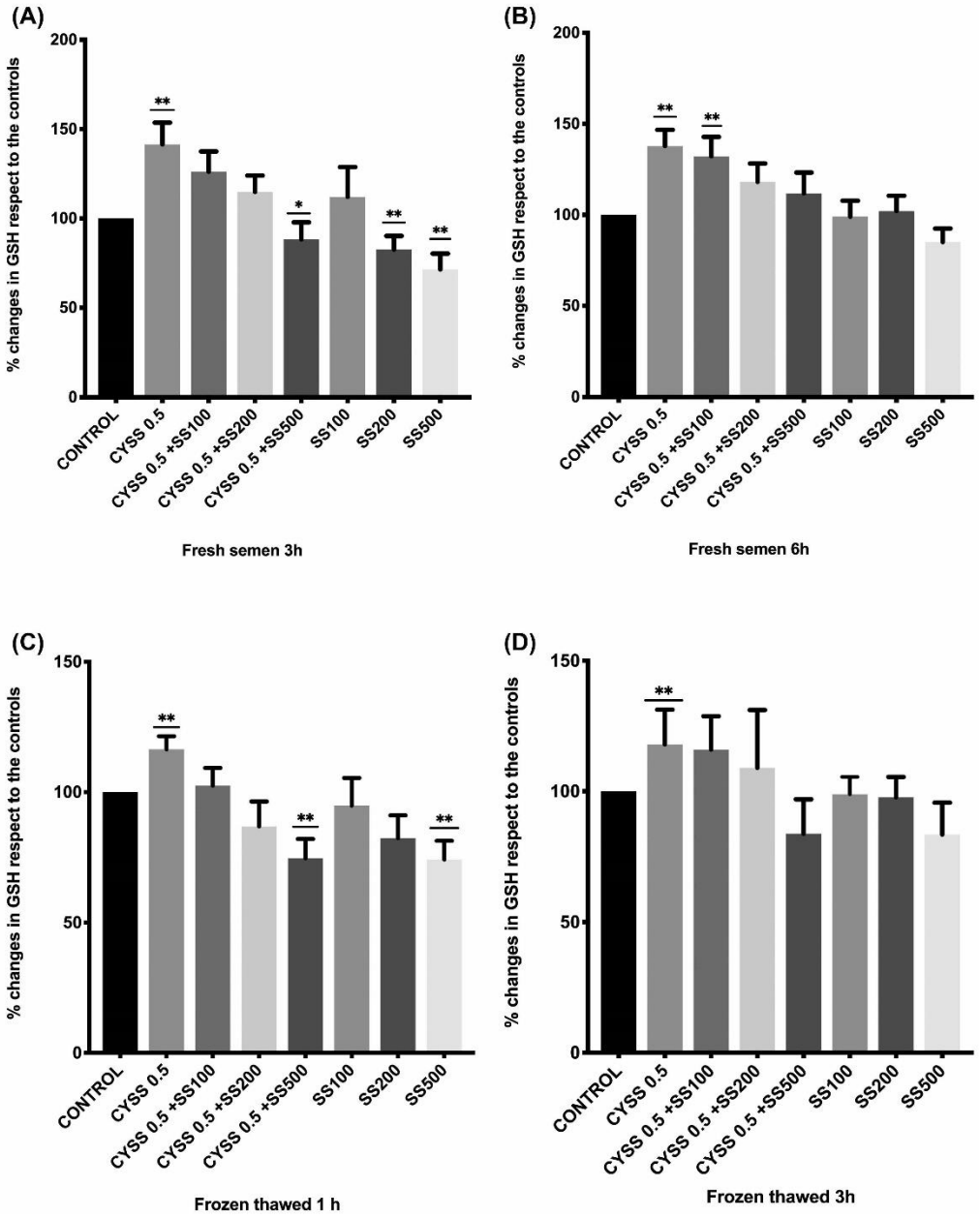

Figure 6. Effects of cystine and sulfasalazine on the GSH content in fresh and frozen-thawed spermatozoa stallion spermatozoa. Semen was obtained, processed as described in the Materials and methods section, and incubated with 0 or 0.5 mM Cyss; 100, 200, or 500 μM sulfasalazine; or the combination of 0.5 mM Cyss and 100, 200, or 500 μM sulfasalazine at 37°C. Results are presented as mean percent changes with respect to controls ± SEM. (A) Fresh extended semen after 3 h of incubation. (B) Fresh extended semen after 6 h of incubation. (C) Frozen-thawed semen after 1 h of incubation. (D) Frozen-thawed semen after 3 h of incubation * $P < 0.05$, ** $P < 0.01$, comparisons are made against controls.

Significant, although less dramatic, changes in cORP were also observed ($P < 0.05$) (Figure 8B).

Sulfasalazine increases sperm motility in fresh extended semen

In freshly extended spermatozoa, SS exerted different dose-dependent effects. After treatment with 500 μM SS for 1 to 3 h, the motility decreased from 51.6 ± 4.9 to 37.5 ± 3.4% ($P < 0.05$) (Figure 9A). However, a treatment with 200 and 500 μM SS in the presence of Cyss increased motility, an effect that was more evident in the percentages of linear motile spermatozoa (Figure 9B). The 100 and 200 μM dosages increased these percentages from 30.2 ± 3.6 to 38.5 ± 3.4 and 37.1 ± 3.4, respectively ($P < 0.01$ and $P < 0.05$). A similar effect was observed in the presence of Cyss 0.5 mM and SS at

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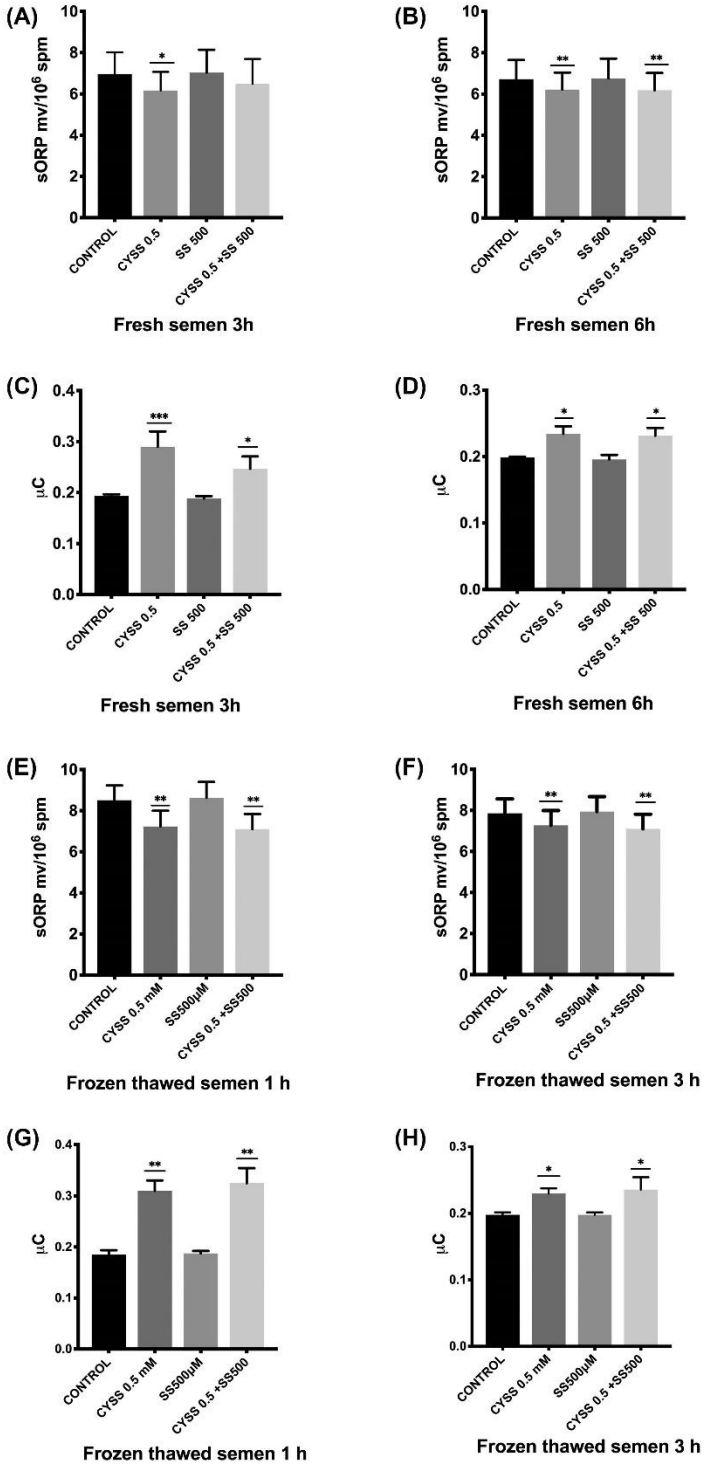

Figure 7. Static oxidation–reduction potential (sORP) and capacity oxidation–reduction potential (cORP) of freshly extended stallion spermatozoa incubated for 3 and 6 h at 37°C and of frozen-thawed stallion semen incubated for 1 or 3 h at 37°C with cystine, sulfasalazine, or both reagents. Results are presented as means ± SEM. (A, B) Static ORP in fresh extended semen after 3 and 6 h of incubation. (C, D) Capacity ORP in fresh extended semen after 3 and 6 h of incubation. (E, F) Static ORP in frozen-thawed spermatozoa after 1 and 3 h of incubation with cystine, sulfasalazine, or both reagents. (G, H) Capacity ORP in frozen-thawed spermatozoa after 1 and 3 h of incubation with cystine, sulfasalazine, or both reagents. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, comparisons are made against controls.

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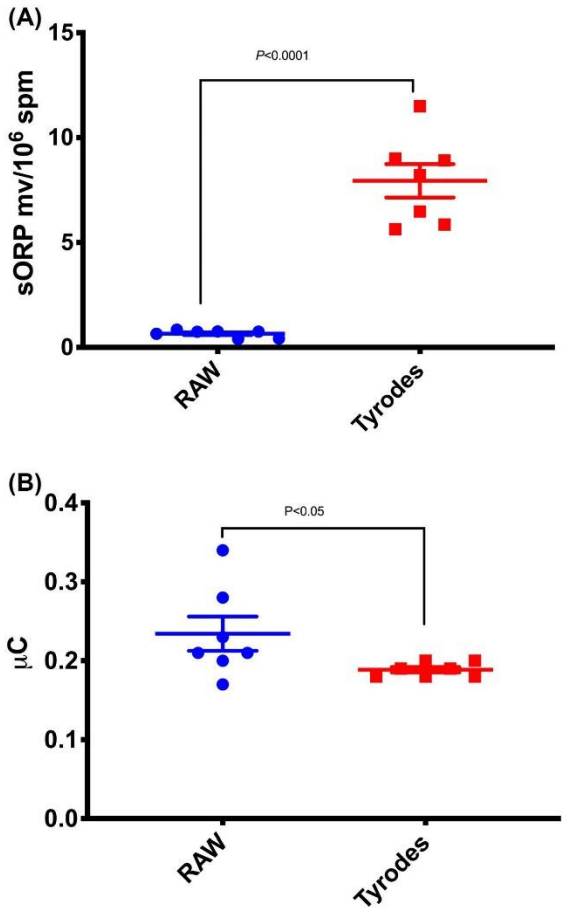

Figure 8. Static oxidation–reduction potential (sORP) (A) and capacity oxidation reduction potential (cORP) (B) of semen (spermatozoa and seminal plasma) and after the removal of most of the seminal plasma by colloidal centrifugation.

200 μM (Figure 9B). After 6 h of incubation, similar findings were obtained (Supplementary Figure S1A and B), with 100 and 200 μM SS increasing motility ($P < 0.05$) and 100 μM SS increasing the percentage of linear motile spermatozoa from 19.9 ± 3.8 to 31.5 ± 2.4 ($P < 0.01$) (Supplementary Figure S1B). Regarding sperm velocities, the VLC was reduced by a treatment with 100 μM SS for 6 h (Supplementary Figure S1C, $P < 0.01$); none of the other concentrations tested exerted an effect.

Sulfasalazine reduces sperm motility in frozen-thawed semen

Fresh and frozen-thawed sperm suspensions responded differently to the SLC7A11 inhibitor SS. In frozen-thawed samples, following 1 h of incubation at 37°C, treatments with 100 and 200 μM for 1 h at 37°C reduced the total sperm motility (Figure 10A) (27.6 ± 4.1 to 19.5 ± 2 and 17.9 ± 2%, respectively), an effect that was inhibited by Cyss. The percentage of linear motile spermatozoa was also reduced in the presence of 100 μM SS, an effect also inhibited by Cyss (Figure 10B). After 3 h of incubation, all concentrations of SS tested reduced motility ($P < 0.05$) (21.4 ± 3.3 to 14 ± 2.5, 13.1 ± 2.8 and 13.9 ± 2.9% respectively) (Supplementary Figure S2A), a reduction that was prevented by Cyss, with the exception of samples treated with 500 μM SS (Supplementary Figure S2A). The percentage of linear motile spermatozoa was reduced by 200 μM SS ($P < 0.05$), an effect that was blocked by Cyss (Supplementary Figure S2B). Sperm velocities were also affected by the treatments after 1 h of incubation (Figure 10C–E), with 500 μM SS reducing all velocities ($P < 0.01$). In the presence of Cyss ($P < 0.05$), 200 and 500 μM SS also reduced the velocities ($P < 0.05$). After 3 h of incubation, the effect was particularly evident on samples treated with Cyss (Supplementary Figure S2C–E)

Discussion

In this study, the expression and role of the SLC7A11 antiporter was investigated in stallion spermatozoa. We also investigated the expression of enzymes involved in the synthesis of Cys from methionine. Only CBS was detected, and thus stallion spermatozoa do not appear to contain the trans-sulfuration pathway. Based on this finding, these cells largely depend on the incorporation of exogenous Cys for GSH synthesis and the maintenance of essential redox homeostasis. Using WB and ICC, the SLC7A11 antiporter was identified for the first time in spermatozoa. This finding is noteworthy since this transporter is expressed constitutively in a limited number of tissues, basically the central nervous system and the immune system [21], and points to a sophisticated mechanism regulating redox homeostasis in these highly specialized cells. Furthermore, its activity in spermatozoa was investigated using the specific inhibitor SS [46–53]. Since spermatozoa are considered highly vulnerable to oxidative stress [54–57], the constitutive expression of the SLC7A11 in stallion spermatozoa is not unexpected. We also investigated how cryopreservation of spermatozoa may affect the function of SLC7A11.

Supplementation with Cyss increased intracellular GSH concentrations (Figure 6), suggesting that the SLC7A11 antiporter functions in stallion spermatozoa. We used the probe monochlorobinane, a probe with sufficient sensitivity for GHS, to detect GSH concentrations in individual cells using optimized protocols that have validated its specificity for GSH [36]. Since Cys is rapidly and spontaneously oxidized to the disulfide Cyss, generating H_2O_2, O_2^-, and $OH^\bullet$ during the process [58, 59], we hypothesized that systems mediating the incorporation of Cyss may be present. The oxidation of Cys to Cyss may explain the negative outcomes of trials in which semen was supplemented with *N*-acetylcysteine or cysteamine [60, 61], and emphasizes the need for basic research to improve current sperm biotechnologies and identify the molecular basis of male factor infertility. As indicated above and to further explore this hypothesis, the levels of the SLC7A11 antiporter were investigated using WB and ICC, revealing, for the first time, the expression of this transporter in stallion spermatozoa (Figures 2 and 3); interestingly, image cytometry revealed that the SLC7A11 antiporter is present in the postacrosomal region of most cells (Figure 3). This finding is remarkable, since the constitutive expression of this protein in absence of disease is rather restricted [21] and limited to lymphoid organs and the CNS [62]. However, the expression of the SLC7A11 antiporter is upregulated in different types of cancers, including gliomas, lymphomas, and pancreatic and hepatocellular carcinomas [52]. Researchers have postulated that the upregulation of SLC7A11 enables cancer cells to resist ROS and underlies the chemoresistence to anticancer drugs. A similar situation may be occurring in the stallion spermatozoa, in which ATP generation largely depends on oxidative phosphorylation [63–65], substantially increasing mitochondrial activity and ROS production [26, 33, 55, 63, 66–68]. Thus, in the course of

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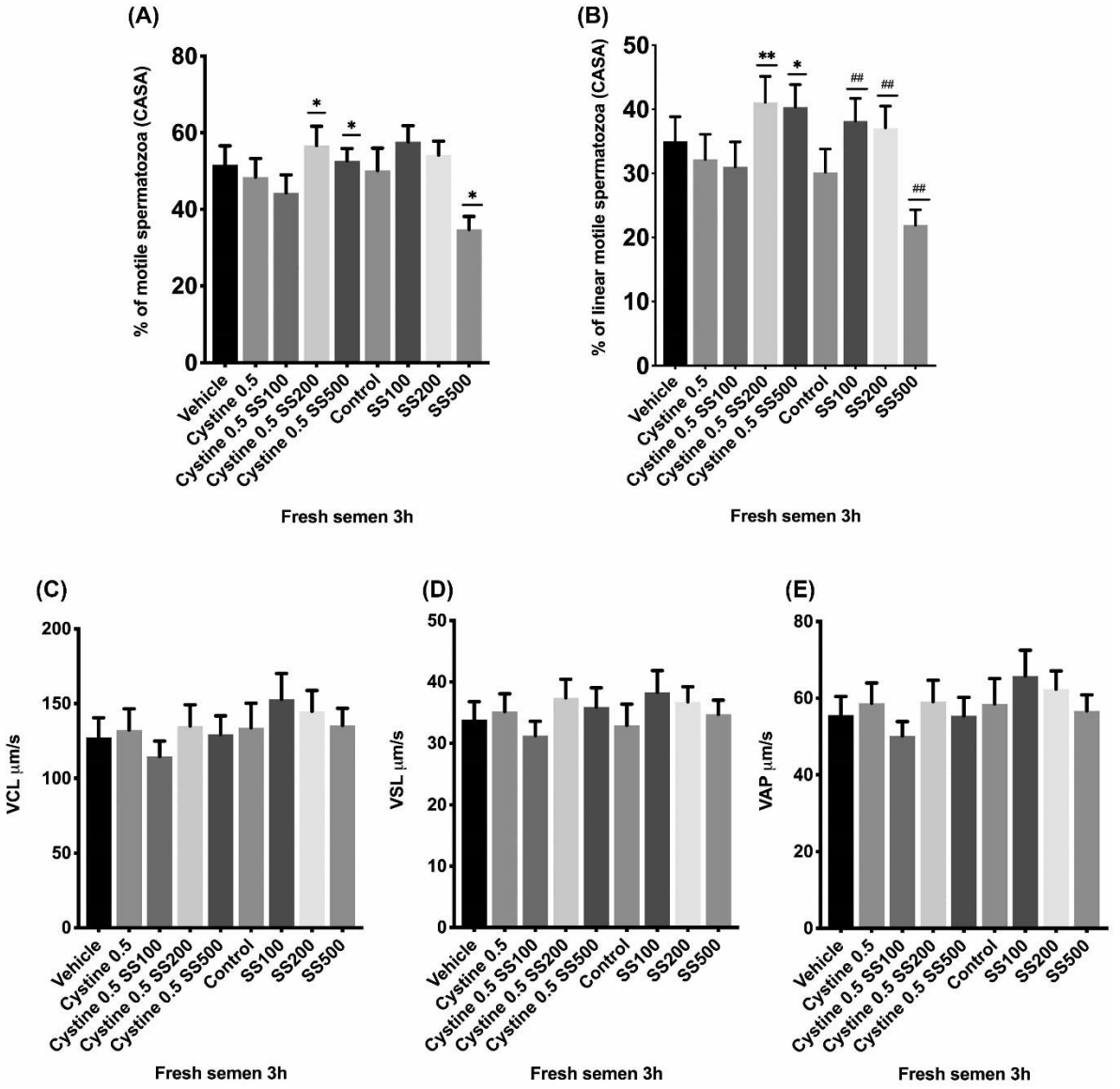

Figure 9. Effects of cystine and sulfasalazine on the functionality of freshly extended stallion spermatozoa after a 3-h incubation. Freshly extended spermatozoa were obtained, processed as described in the Materials and methods section, and incubated with 0 or 0.5 mM Cyss; 100, 200, or 500 μM sulfasalazine; or the combination of 0.5 mM Cyss and 100, 200, or 500 μM sulfasalazine at 37°C. CASA, computer-assisted sperm analysis; VCL, circular velocity (μm/s); VSL, straight line velocity (μm/s); VAP, average path velocity (μm/s). * $P < 0.05$, ** $P < 0.01$, comparisons are made against vehicle, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ comparisons are done against controls.

evolution, sophisticated antioxidant systems have likely developed in this species. Additional evidence supporting this hypothesis is based on the finding that intracellular GSH concentrations are in the micromolar range per billion spermatozoa in this species, but in the nanomolar range in other species [16].

The GSSG/GSH redox pair depends on the import of the amino acid Cyss, which is the oxidized form of Cys, via the SLC7A11 antiporter into cells with a 1:1 counter transport of glutamate [62, 69, 70]. Cystine, in addition to being the rate-limiting molecule for GSH synthesis, forms a key redox couple of its own with Cys [69, 70].

We investigated the function of the SCL7A11 antiporter by supplementing fresh and frozen-thawed stallion spermatozoa with Cyss and/or SS, the specific inhibitor of the SLC7A11 antiporter channel [46, 48–51]. Sulfasalazine reduced GSH concentrations in spermatozoa treated with or without Cyss, an effect that was observed on both freshly extended and frozen-thawed samples (Figure 6). The findings provide evidence that Cyss is incorporated in stallion spermatozoa through the SLC7A11 system, based on the reduction in GSH concentrations in presence of the inhibitor SS and the inhibition of the increase in GSH concentrations after addition of Cyss in spermatozoa treated with SS. Reports of increased concentrations of

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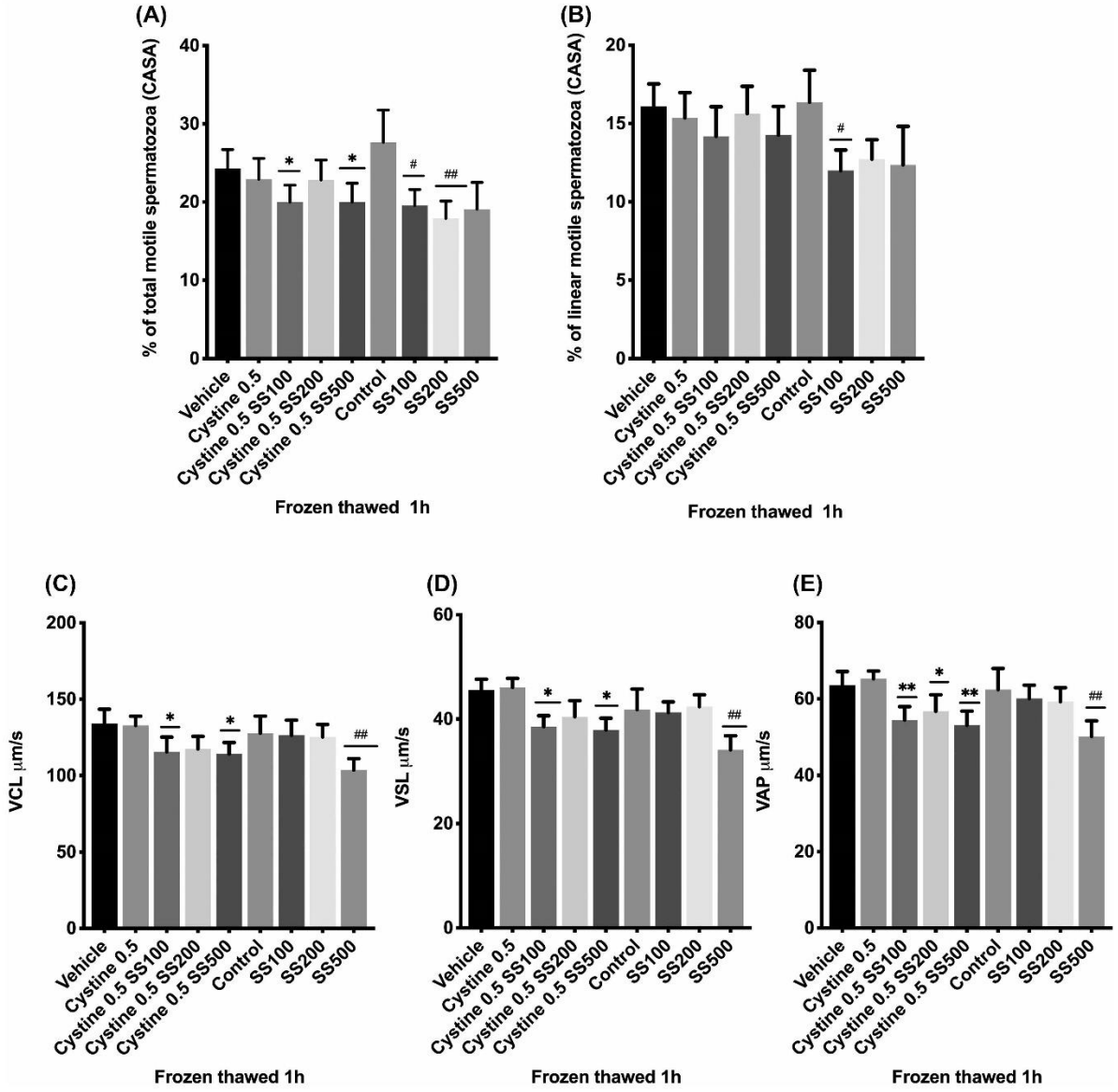

Figure 10. Effects of cystine and sulfasalazine on functionality of frozen-thawed stallion spermatozoa after a 1-h incubation at 37° C. Frozen-thawed spermatozoa were obtained, processed as described in the Materials and methods section, and incubated with 0 or 0.5 mM Cyss; 100, 200, or 500 μM sulfasalazine, or the combination of 0.5 mM Cyss and 100, 200, or 500 μM sulfasalazine at 37°C. CASA, computer-assisted sperm analysis; VCL, circular velocity (μm/s); VSL, straight line velocity (μm/s); VAP, average path velocity (μm/s). * $P < 0.05$, ** $P < 0.01$, comparisons are made against vehicle, # $P < 0.05$, ## $P < 0.01$ comparisons are done against controls.

glutamate in the media during the incubation of human spermatozoa may support the existence of an active SLC7A11 antiporter that, interestingly, appears to be more active in samples of better quality [71].

We measured the sORP of spermatozoa to investigate the overall effect of Cyss and the function of the SLC7A11 system in maintaining the redox balance of spermatozoa. This experiment analyzes all known and unknown oxidants and antioxidants in the spermatozoa and does not rely on a single biomarker of oxidative stress [37, 72]. Thus, this technique provides a global view of the redox status of the cell. As expected, Cyss significantly reduced the sORP in supplemented samples of both fresh and frozen-thawed sperm, and increased the total antioxidant capacity (cORP) (Figure 7). However, at doses that reduced intracellular GSH concentrations, SS did not prevent the effect of Cys on frozen-thawed samples or affect the sORP. In fresh spermatozoa, SS prevented the reduction in sORP induced by Cyss after 3 h of incubation, but, interestingly, these changes did not occur in frozen-thawed samples. These facts are

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easily explained by the finding that Cyss may be used for GSH synthesis and functions as a redox node in the Cys/Cyss couple [25], providing antioxidant capacity independently of its incorporation into GSH. The presence of a functional Cys/Cyss redox node in spermatozoa would explain this apparent paradox. Both sORP and cORP were measured in raw semen and spermatozoa to determine the contribution of seminal plasma to the ORP. The elimination of seminal plasma resulted in a dramatic increase in sperm oxidation, and changes in cORP were also observed. This finding is consistent with the hypothesis that seminal plasma has a substantial contribution to antioxidant defenses in spermatozoa [73, 74], although the changes observed in total antioxidant capacity were not as dramatic, suggesting that the spermatozoa also possess potent antioxidant mechanisms. This result is particularly relevant in the context of biotechnology, where the removal of seminal plasma is the usual practice in cryopreservation protocols. Moreover, after ejaculation, and once in the mare reproductive tract, spermatozoa lose contact with the seminal plasma and become more dependent on their own redox regulatory mechanisms. The finding that cORP was apparently less dependent on the presence of seminal plasma argues in favor of the presence of intrinsic cellular defenses in the sperm cell.

The functionality of the spermatozoa was affected by the treatments and was significantly improved in fresh extended semen, with apparently contradictory yet explainable outcomes taking into account the duality of Cyss, as a source of Cys for GSH synthesis and its participation in the Cys/Cyss redox node [25]. For example, notable improvements in sperm motility occurred in fresh extended samples supplemented with Cyss and SS, while 500 μM SS significantly reduced motility. The latter is potentially explained by the reduced intracellular GSH concentration observed in this sample. On the other hand, lower dosages of SS, and the combination of SS and Cys, may have improved sperm motility due to a compensatory increase in the activity of the Cyss/Cys node. Data on the total oxidation–reduction potential (sORP) of the sample support this hypothesis, since the combination of SS and Cyss also reduced the total oxidation of the sperm sample. One advantage of this node is that Cyss may serve as an intermediate disulfide to oxidize proteins by the thiol/disulfide exchange, with the advantage of decreasing the risk of oxidizing proteins to higher nonreversible oxidation states [25]. Here, we provide the first evidence of the functionality of this node in spermatozoa.

However, in frozen-thawed samples, SS impaired sperm function, a change that only was partially prevented by the simultaneous presence of Cyss (Figure 10). Differences observed between fresh and frozen-thawed samples suggest that a different redox regulatory mechanism is employed by cryopreserved semen and possibly a compromised function of the SLC7A11 antiporter in thawed semen. Supporting this hypothesis, previous research from our laboratory indicates altered functions of membrane channels in cryopreserved spermatozoa [26].

In conclusion, we have provided the first evidence that the SLC7A11 antiporter is present and functional in stallion spermatozoa, likely by incorporating Cyss for GSH synthesis. Furthermore, a Cys/Cyss redox node may also be functional in stallion spermatozoa. Moreover, changes in the mechanism regulating redox homeostasis in spermatozoa as a consequence of cryopreservation may underlie the reduced functionality of thawed spermatozoa. The findings reported here may have implications for improving our understanding of male fertility and practical applications in the field of sperm biotechnology.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Figure S1. Effects of cystine and sulfasalazine on the functionality of freshly extended stallion spermatozoa after a 6-h incubation. Freshly extended spermatozoa were obtained, processed as described in the Materials and methods section, and incubated with 0 or 0.5 mM Cyss, 100, 200, or 500 μM sulfasalazine or the combination of 0.5 mM Cyss and 100, 200, or 500 μM sulfasalazine at 37°C. CASA, computer-assisted sperm analysis; VCL, circular velocity (μm/s), VSL, straight line velocity (μm/s); VAP, average path velocity (μm/s). $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ comparisons are done against controls.

Supplementary Figure S2. Effects of cystine and sulfasalazine on the functionality of frozen-thawed stallion spermatozoa after a 3-h incubation at 37°C. Frozen-thawed spermatozoa were obtained, processed as described in the Materials and methods section, and incubated with 0 or 0.5 mM Cyss; 100, 200, or 500 μM sulfasalazine; or the combination of 0.5 mM Cyss and 100, 200, or 500 μM sulfasalazine at 37°C. CASA, computer-assisted sperm analysis; VCL, circular velocity (μm/s); VSL, straight line velocity (μm/s); VAP, average path velocity (μm/s). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, comparisons are made against vehicle, $^{\#}$ $P < 0.05$, comparisons are done against controls.

Supplementary Figure S3. Irrelevant IgG controls for all the primary antibodies used in this study.

Conflict of interest

The authors have no conflicts of interest that could be perceived to prejudice the reported research to declare.

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Artículo 2

REPRODUCTION
RESEARCH

The SLC7A11: sperm mitochondrial function and non-canonical glutamate metabolism

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Abstract

Spermatozoa are redox-regulated cells, and stallion spermatozoa, in particular, present an intense mitochondrial activity in which large amounts of reactive oxygen species (ROS) are produced. To maintain the redox potential under physiological conditions, sophisticated mechanisms ought to be present, particularly in the mitochondria. In the present study, we investigated the role of the SLC7A11 antiporter. This antiporter exchanges intracellular glutamate for extracellular cystine. In the spermatozoa, cystine is reduced to cysteine and used for GSH synthesis. The importance of the antiporter for mitochondrial functionality was studied using flow cytometry and UHPLC/MS/MS approaches. Intracellular GSH increased in the presence of cystine, but was reduced in the presence of Buthionine sulphoximine (BSO), a γ-glutamylcysteine synthetase inhibitor ($P < 0.001$). Inhibition of the SLC7A11 antiporter with sulfasalazine caused a dramatic drop in intracellular GSH ($P < 0.001$) and in the percentage of spermatozoa showing active mitochondria ($P < 0.001$). These findings suggest that proper functionality of this antiporter is required for the mitochondrial function of spermatozoa. We also describe that under some conditions, glutamate may be metabolized following non-conventional pathways, also contributing to sperm functionality. We provide evidences, that the stallion spermatozoa have important metabolic plasticity, and also of the relation between redox regulation and metabolic regulation. These findings may have important implications for the understanding of sperm biology and the development of new strategies for sperm conservation and treatment of male factor infertility.

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Introduction

Insemination with refrigerated semen is common practice in the equine industry (Vidament *et al.* 2012). In spite of the importance of this technique, unresolved questions still persist. In particular, the metabolism of the spermatozoa under this and other conditions is far from being fully understood. Nevertheless, significant advances have occurred in the past decade that have deepened our knowledge of stallion sperm physiology. Regarding this particular aspect, knowledge of the role of reactive oxygen species (ROS) has dramatically evolved (Salicioni *et al.* 2007, Gibb *et al.* 2014, O'Flaherty 2015, Lee *et al.* 2017, O'Flaherty & Matsushita-Fournier 2017, Griffin *et al.* 2019, Ortega-Ferrusola *et al.* 2019, Pena *et al.* 2019). For a long time, the predominant belief was that ROS were merely toxic byproducts of metabolism that should be kept to a minimum with the use of antioxidants. However, paradoxical findings showing that more fertile spermatozoa were characterized by more intense ROS production, have challenged this paradigm (Gibb *et al.* 2014, Darr *et al.* 2016). Recent proteomic studies have widened our knowledge of sperm metabolism and have put assumed paradigms in spermatology into doubt (Amaral *et al.* 2013, 2014, Paiva *et al.* 2015, Swegen *et al.* 2015). In line with these recent findings, the relationship between metabolism and redox regulation is unveiled as a promising approach to improve current techniques for sperm conservation (Gibb *et al.* 2015, Darr *et al.* 2016, Swegen *et al.* 2016).

Reactive oxygen species are formed by incomplete reduction of molecular oxygen and are atoms or molecules with a single unpaired electron. These include, among others, superoxide ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$) and the lipid peroxide radical ($LOO^{\cdot}$). Although hydrogen peroxide (H_2O_2) itself is not a free radical, it is a precursor of free radicals. UV radiation and the presence of metal ions (Fe^{2+}, Fe^{3+} or Cu^{2+}) generate hydroxyl radicals (Pena

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et al. 2019). All aerobic organisms depend on the generation of ATP from the four-electron reduction of molecular oxygen into water. During this process, the mitochondrial transport chain may lose electrons leading to the formation of ROS. Mitochondrial dysfunction may exacerbate the loss of electrons and thus increase the production of reactive oxygen species (Kalyanaraman 2013). Stallion spermatozoa are characterized by an intense mitochondrial activity in comparison with other mammals (Gibb *et al.* 2014, Darr *et al.* 2016, Griffin *et al.* 2019). Recent research points to sophisticated regulation of production and elimination of ROS, with important roles in stallion sperm physiology. The reversible oxidation of thiol-containing residues in specific amino acids of regulatory proteins is of particular importance (Ortega-Ferrusola *et al.* 2019, Ortiz-Rodriguez *et al.* 2019*b*). Mitochondria plays a major role in the production of reactive oxygen and nitrogen species in spermatozoa (Ortega Ferrusola *et al.* 2009, Davila *et al.* 2016), and thus also play a role in redox regulation. Recently, it was found that the SLC7A11 antiporter has a role in redox regulation in the spermatozoa; incorporating cystine (the oxidized form of cysteine) in exchange for intracellular glutamate (Ortiz-Rodriguez *et al.* 2019*b*). This is a mechanism which maintains intracellular GSH at physiological levels (Ortega-Ferrusola *et al.* 2019). Recycling oxidized GSH (GSSG) requires reducing equivalents that are produced in energy metabolism. Glutathione is synthesized exclusively in the cytosol, but is distributed to the mitochondria through specific carriers with the end result that GSH concentration in the mitochondrial matrix is equal to that of the cytosol (Ribas *et al.* 2014). Here GSH acts as an electron donor as part of a defense mechanism against respiration induced ROS and also participates in the detoxification of lipid hydroperoxides. Moreover, mitochondrial GSH is a regulator of mitochondrial permeability, and thus of cell death (Coppola & Ghibelli 2000, Circu *et al.* 2008). However, inhibition of the antiporter may lead both to positive and negative effects in stallion spermatozoa (Ortiz-Rodriguez *et al.* 2019*b*). This apparently paradoxical effect may relate to other functions of the antiporter, not only as a source of extracellular cystine used for GSH synthesis. Recently SLC7A11 has been described as having a possible role in glutamate metabolism. In some cellular models, disruption of the SLC7A11 antiporter greatly improves cell viability after glucose withdrawal, because conservation of glutamate enables cells to maintain mitochondrial respiration (Shin *et al.* 2017). To test if a similar picture may be occurring in stallion spermatozoa, we investigated the SLC7A11 antiporter, paying special attention to the effects on mitochondrial function due to the central role of these organelles in redox regulation and metabolism.

Materials and methods

Reagents and media

L-Cystine (Cyss), L-buthionine-sulfoximine (BSO), monochlorobimane (MCB), sulfasalazine (SS), α-methyl-4-carboxyphenilglycine (mCPG), R162 (GDH1 inhibitor), methyl α-ketoglutarate, Erastin, RSL3, liproxstatin-1 and all other chemicals were purchased from Sigma Aldrich. All other reagents for flow cytometry were purchased from Thermofisher. Anti-GDH1 antibody was purchased from Abcam (ab89967).

Semen collection and processing

Semen was collected from 11 stallions maintained according to institutional and European animal care regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). All procedures used in this study received approbation from the ethical committee of the University of Extremadura (Ref# AGL201783149R). Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina following standard veterinary practices. After collection, the ejaculate was immediately evaluated and processed in the adjacent laboratory. Colloidal centrifugation (Morrell *et al.* 2011) was performed to remove dead spermatozoa and contaminating particles from the ejaculate. The pellet was re-extended to a final concentration of 40 x 10^6 spermatozoa/ml in Tyrodes media (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2{\cdot}2H_2O$, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.3 mM KH_2PO_4, 20 mM HEPES, 5 mM glucose, 21.7 mM Na lactate, 1 mM Na Pyruvate, 15 mM $NaHCO_3$, 3% v/v BSA, 50 mg/mL kanamicin) pH 7.4 (Balao da Silva *et al.* 2014). The sperm suspension was then split into sub-samples for control and experimental treatments. For UHPLC/MS/MS experiments ejaculates were also processed through colloidal centrifugation to remove dead and contaminating cells and separate seminal plasma. Pellets obtained were washed three times in PBS (1500 ***g*** x 10′ at 4°C) suspended in 1 mL of PBS and stored at −80°C until further use.

Flow cytometry

Flow cytometry analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter) equipped with violet, blue, yellow and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowjoV 10.6.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory (Gallardo Bolanos *et al.* 2014, Martin Munoz *et al.* 2015).

Measurement of GSH, viability and mitochondrial membrane potential in stallion spermatozoa

Intracellular GSH was measured by adapting previously published protocols optimized for GSH detection using flow

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cytometry (Capek *et al.* 2017) adapted to equine spermatozoa in our laboratory (Ortiz-Rodriguez *et al.* 2019*b*). The mitochondrial membrane potential and sperm viability were also simultaneously assessed. In brief, sperm aliquots (1–5 x 10^6 sperm/mL) were stained with JC-1 1 µM (30 min in the darkness at r.t.), DRAQ7 3 µM and monochlorobimane (MCB) 10 µM (10 min in the darkness at r.t). The applied gating strategy is depicted in Fig. 1. Briefly, after assessment of the quality of the flow, doublets and debris were gated out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates) and DRAQ7, at a peak excitation of 640, and emission of 690 nm.

Computational flow cytometry (t-SNE)

The data from each multi-parametric assay described in the 'Material and methods' section were exported as FCS files from the flow cytometer and loaded and analyzed using FlowjoV 10.6.2 Software (Ashland, OR, USA). For computational analysis, data from all the replicates for each treatment were concatenated and single cell events analyzed. The files obtained after concatenation were exported as FCS files. Flow cytometry files were then analyzed using non-linear dimensional reduction techniques (t-SNE) and automatic classification of cellular expression by non-linear stochastic embedding (ACCENSE) accessible at http://www.cellaccense.com/index.html. The Barnes–Hut implementation of t-SNE was used for low dimensional embedding to perform dimensional reduction of cytometry data, classification of cells was based on K means techniques with the significance level set at $P=0.0001$. ACCENSE identifies clusters within multidimensional data without losing single cell resolution (Shekhar *et al.* 2014), allowing automatic gating of cells.

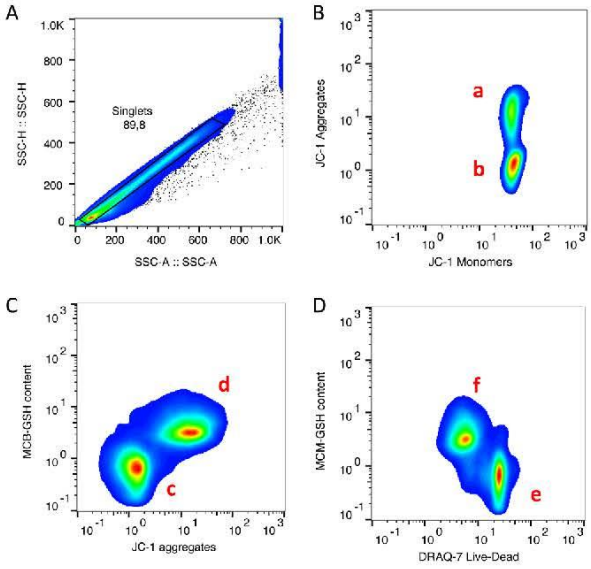

Figure 1 Gating strategy for flow cytometry-based determination of changes in GSH, mitochondrial activity and percentages of live cells in stallion spermatozoa. After verification of the quality of the flow, single cells were identified confronting SSC-H vs SSC-A gating out doublets (A). Then the percentages of cells showing high (a) and low (b) mitochondrial activity (B), the relative GSH content (C) and the percentage of live spermatozoa (D) were identified and quantified. Interestingly most the population of spermatozoa showing high mitochondrial activity (a) was also characterized by high GHS content (d) showing the close link between mitochondrial activity and GSH.

UHPLC-MS/MS detection of reduced and oxidized glutathione

The reduced (GSH) and oxidized (GSSG) forms of glutathione were analyzed by UHPLC-MS/MS following previously published protocols (Ortega-Ferrusola *et al.* 2019). In brief, a chromatography separation was performed using a Thermo Ultimate 3000 Ultra (Thermo Fisher Scientific) and separations were carried out on a C18 column (100 × 21 mm; 1.7 µm, Bruker Intensity Solo, Billerica, MA, USA). The analytes eluted from the UHPLC column were directly introduced in an ion trap mass spectrometer (Amazon SL, Bruker Daltonik GmbH, Bremen, Germany). The mass spectrometer was run in MS/MS mode, in which the protonated reduced glutathione (308 m/z) and protonated oxidized glutathione (613 *m/z*) were isolated and subjected to collision-induced dissociation, using Helium gas and radio frequency (RF) with fragmentation amplitudes of 0.45 and 0.65 V, respectively. The full scan MS/MS or MS2 spectra was obtained under these conditions, in which the 178.6 *m/z* ion was selected as the quantification ion for reduced glutathione and 484+355 *m/z* was selected as the quantification ion for oxidized glutathione.

Metabolomics

The sperm pellets were redissolved in 1 mL milliQ water and sonicated for 3 s. Immediately afterwards, samples were centrifuged at 4,000 ***g*** at 4°C for 3 min and the supernatant was injected into the UHPLC-MS/MS. Separation and analysis of the samples were performed with a UHPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies) equipped with an automated multisampler module and a High Speed Binary Pump, coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the HPLC and Q-TOF was through the MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). Samples were injected onto an Agilent C18 HPLC column (4.6 mm, 100 mm × 2.1 µm, Agilent technologies), thermostatted at 40°C, at a flow rate of 0.4 ml/min. The injection volume was 5 µL. For gradient elution, solvent A was: 0.2% (v/v) acetic acid in Milli Q water and solvent B was 0.2% acetic acid in acetonitrile. In the beginning, solvent B was maintained at 2% for 3 min. Solvent B was increased from 0 to 98% from 4 to 12 min and held at 98% for an additional 2 min. Then solvent B was returned to the initial conditions up to min 15. The mass spectrometer was operated in positive and negative modes. The best results were obtained in negative mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to

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12 L/min at a temperature of 150°C, and the sheath gas flow was set to 15 L/min at a temperature of 350°C. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500, 360 and 750 V, respectively. Centroid data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range was 50–950 *m/z* and scan rates were 5 spectra/s for MS and 2 spectra/s for MS/MS. All Ions mode was used without precursor selection in two experiments or channels at the same time, one of low energy (0 V collision energy) and one of high energy (20 V collision energy). The low value produces the precursor ions (MS scan) for the compounds and the high value generates the precursors plus their product ions (MS/MS scan). Data processing and analysis were performed using MassHunter Qualitative Analysis Software (Rev B.07.00.201, Agilent Technologies). The chromatography peaks were qualified by accurate mass precursor ions and accurate mass product ions from the MS/MS spectrum. Furthermore, some compounds were also identified using the METLIN metabolites Personal Compounds Database Library (PDCL) (Agilent Technologies), in which mass peak in the low energy channel are first searched for against this PDCL for compounds which have the same *m/z* values. In addition, the fragment ions in the high energy channel for these compounds were compared with MS/MS spectra from PDCL.

Measurement of oxidation–reduction potential

Oxidation–reduction potential (ORP) was measured using the RedoxSYS® diagnostic system (Englewood CO, USA), following previously published protocols adapted for equine spermatozoa (Ortiz-Rodriguez *et al.* 2019*a*).

Western blotting (WB)

To separate the proteins according to their apparent molecular masses, SDS-PAGE was performed as previously described (Aparicio *et al.* 2016). In brief, proteins were extracted and denatured by boiling for 10 min at 70°C in a loading buffer supplemented with 5% mercaptoethanol. Ten micrograms of sperm protein extract were loaded and resolved by SDS-PAGE on a 10% polyacrylamide gel. Immunoblotting was performed by incubating the membranes in blocking buffer at 4°C overnight with of 1 µg/mL anti GDH-1 primary antibody.

Immunocytochemistry (ICC)

Indirect immunofluorescence was performed as previously described (Tapia *et al.* 2012). After blocking, cells were incubated with primary antibody anti GDH-1, overnight at 4°C diluted 1/200 in PBS containing 5% BSA (w/v). The following day, cells were washed with PBS and further incubated for 45 min at r.t. with goat anti-goat IgG antibody conjugated with Alexa Fluor 647 diluted to 1/500 in PBS containing 5% BSA (w/v). Finally, cells were thoroughly washed with PBS. A total of 5000 cells were analyzed in the ImageStream X Mark II Imaging Flow Cytometer (Merck Millipore) using a 642 nm line laser with intensity set to 100 mW, at 60× magnification. Data analysis of the raw images was accomplished using IDEAS1software (Version 6.0.309). The absence of non-specific staining was measured by processing the samples without primary antibody (secondary antibody only).

Computer-assisted sperm analysis (CASA)

Sperm motility and velocity were assessed using a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to standard protocols used at our center (Ortega-Ferrusola *et al.* 2009). Semen samples were loaded in a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37°C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10× negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa VAP > 35 µm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

Assessment of caspase 3 activity and phosphatidylserine (PS) translocation

Annexin V 647 conjugated and CellEvent® Caspase 3/7 Green Detection Reagent were combined in a multi-parametric test and evaluated by FC (Ortega-Ferrusola *et al.* 2017). Samples were loaded with Hoechst 33342 (0.3 µM) and CellEvent (2 µM) and incubated at room temperature for 15 min. Following this, the samples were washed by a short centrifugation spin for 12″ and suspended in 200 µL of Annexin binding-buffer (solution in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Five microliters of Annexin V was added to 200 µL of sample. After 15 min of incubation, in the dark at room temperature, 400 µL of 1× Annexin binding-buffer was added before reading in the flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). To gate dead spermatozoa samples were stained with 0.3 µM of Eth-1 and incubated for 5 min before they were immediately evaluated in a flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). CellEvent staining was validated as previously described (Ortiz-Rodriguez *et al.* 2019*a*).

Statistical analysis

Sperm samples were obtained from 11 different stallions. All experiments were repeated at least three times with independent samples (three separate ejaculates from each of the donor stallions). The normality of the data was assessed using the Kolmogorov–Smirnoff test. Paired *t*-tests and one-way ANOVA followed by Dunnett's multiple comparisons test and were performed using GraphPad Prism version 7.00 for www.graphpad.com. Differences were considered significant when $P < 0.05$. Results are displayed as means ± S.E.M.

Results

Stallion spermatozoa synthetize GSH from cystine

In order to confirm changes in intracellular glutathione in both reduced (GSH) and oxidized (GSSG) forms, concentrations were monitored using UHPLC/MS/MS.

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Incubation of stallion spermatozoa in the presence of 0.5 mM Cyss caused an increase in intracellular GSH that was prevented with coincubation in the presence of buthionine sulphoximine (BSO 50 μM) a γ-glutamylcysteine synthetase inhibitor that is the limiting enzyme for GSH synthesis (Fig. 2A). At the same time, levels of oxidized glutathione (GSSG) were observed to be reduced after 1 h of incubation in Cyss treated samples (Fig. 2B).

Inhibition of the SLC7A11 antiporter reduces stallion sperm viability

The incubation of stallion spermatozoa in the presence of the SLC7A11 antiporter inhibitor sulfasalazine (SS) reduced sperm viability at 200 μM ($P < 0.01$) and 500 μM ($P < 0.001$). The percentages of live spermatozoa were reduced from 71.0 ± 4.4% in controls to 64.1 ± 4.4 and 55.5 ± 5.4%, respectively, in the presence of SS at 200 and 500 μM. The simultaneous presence of Cyss and SS 200 and 500 μM also caused reduced sperm viability ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 3A) (68.5 ± 3.7 in controls to 53.0 ± 5.4 in Cyss-SS 500 μM supplemented sub-samples). When stallion spermatozoa were incubated in the presence of the inhibitor α-methyl-4-carboxyphenilglycine (mCPG), all concentrations tested reduced viability ($P < 0.05$) (Fig. 3B), from 63.0 ± 5.9% in controls to 59.7 ± 7.0, 57.7 ± 6.5 and 54.8 ± 7.0 in samples treated with 100, 200 and 500 μM mCPG, respectively, an effect that was prevented by Cyss (0.5mM) when mCPG was present at 100 and 200 μM (Fig. 3B).

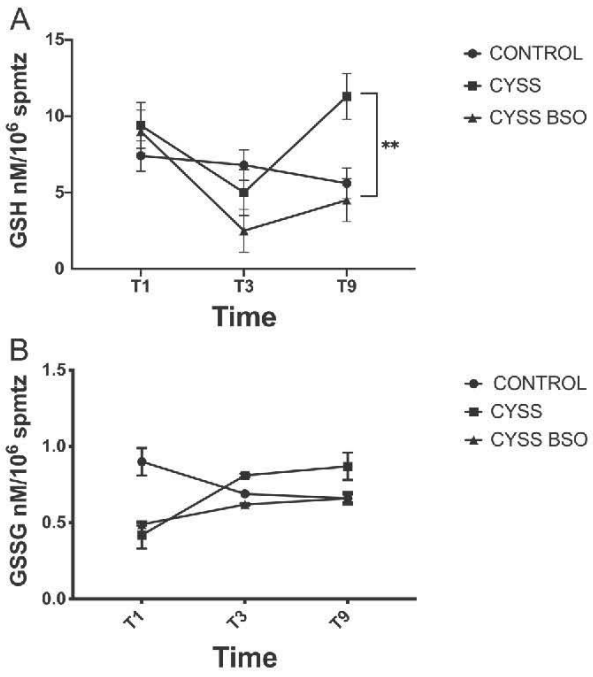

Figure 2 Stallion spermatozoa incorporate Cyss for GSH synthesis. In order to determine the functionality of the SLC7A11 antiporter, stallion spermatozoa were incubated in the presence of Cys or Cys and BSO (inhibitor of γ-glutamylcysteine synthetase a key enzyme in glutathione biosynthesis). Then the content of reduced (GSH) and oxidized (GSSG) glutathione was investigated using mass spectrometry (MS) as described in the 'Materials and methods' section. Incubation in the presence of Cyss increased intracellular GSH ($P < 0.01$), effect that was prevented in the presence of BSO (A). The changes in GSSG are presented in B. Data are presented as means ± S.E.M. of five different replicate experiments, $^{**}P < 0.01$.

Inhibition of the SLC7A11 antiporter with sulfasalazine reduces mitochondrial membrane potential (ΔΨm)

Semen samples incubated in the presence of SS experimented a drop in the percentage of spermatozoa showing high ΔΨm from 46.3 ± 5% of the spermatozoa in controls to 23.1 ± 4.5% in the presence of SS 500 μM ($P < 0.01$). Cyss showed a synergistic effect, reducing the percentage of spermatozoa with high ΔΨm in samples incubated in the presence of 0.5 mM Cyss and 500 μM SS ($P < 0.001$) to 12.6 ± 2%. Also, SS 200 μM in the presence of Cyss resulted in a reduced percentage of spermatozoa with high ΔΨm (35.5 ± 4 $P < 0.01$) (Fig. 4-1A). When the second inhibitor was used no effect was observed, to the contrary, a combination of 0.5 mM Cyss and 100 μM mCPG resulted in an increased percentage of spermatozoa with high ΔΨm ($P < 0.001$), from 40.0 ± 1.4 in controls to 50.1 ± 1.8 in samples supplemented with 0.5 mM Cyss and mCPG 100 μM (Fig. 4-2A).

Sulfasalazine reduces intracellular GSH, while carboxyphenylglicine increases intracellular GSH and reduces the steady state redox potential (Eh) in presence of Cyss

Since a close relationship has been established between GSH content and sperm viability, changes in intracellular GSH were monitored. Incubation of stallion spermatozoa in the presence of 500 μM SS reduced intracellular GSH content to 72% of the initial values ($P < 0.01$) (Fig. 5A), an effect that was also seen in the presence of 0.5 mM Cyss. To the contrary, 500 μM mCPG induced a 28.81% increase in GSH. This effect was even more evident in the presence of 0.5 mM Cyss and 100 μM mCPG with an increase of 48,22% with respect to initial values (Fig. 5B). Concomitantly with increased intracellular GSH the combination of 0.5 mM Cyss and 100 μM mCPG reduced Eh ($P < 0.05$) (Fig. 5C). The t-SNE maps built after computational analysis of the data, showed how the treatments modified GSH content in the whole population of spermatozoa at the single cell level. As seen in Fig. 5E, 0.5 mM Cyss supplementation caused an increase in GSH content. At the right of the

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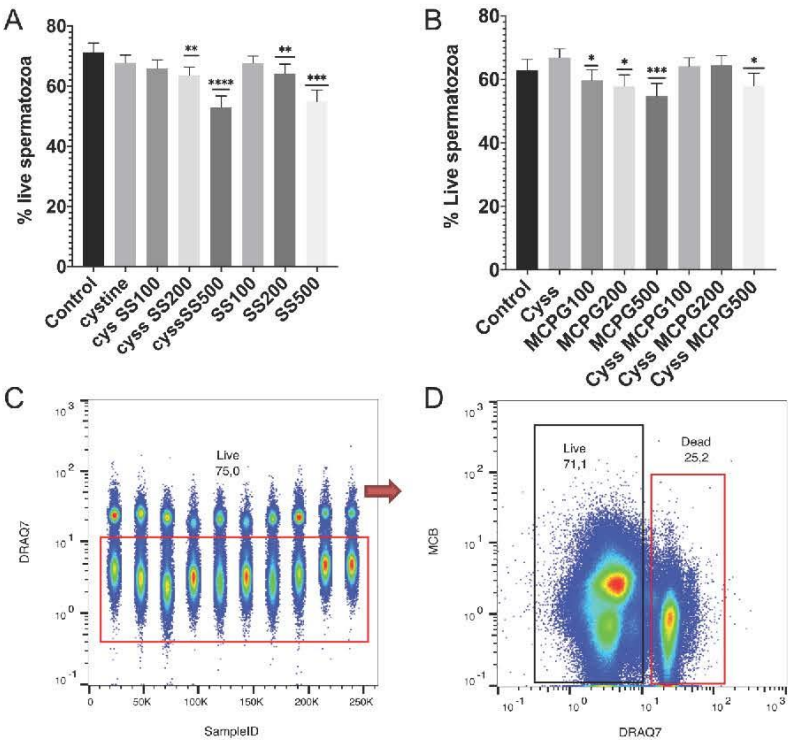

Figure 3 Inhibition of the SLC7A11 antiporter reduces sperm viability. To determine the impact of SLC7A11 on viability, stallion spermatozoa were incubated in the presence of SS and mCPG. SS reduced viability at 200 and 500 μM (A). The incubation in the presence of mCPG reduced viability that was prevented by 0.5 mM Cyss when mCPG was present at 100 and 200 μM (B). Representative cytograms of the assay are presented in C and D. In C data from ten different replicates are concatenated (50,000 events per replicate), in D the cytogram represents all the events gathered in the ten replicates in a single 2D plot identifying live and dead cells. Data are means ± S.E.M., ***$P < 0.001$. *$P < 0.05$. **$P < 0.01$.

t-SNE map the color code for the heat map is depicted. Blue areas represent low expression, red, yellow and green increased expression. Supplementation with 0.5 mM Cyss and 100 μM mCPG caused an overall increase in the content of GSH (Fig. 5F) seen as a reduction of the blue areas in the t-SNE map and an increase in yellow-green and red areas. To the contrary, SS at 500 μM caused a reduction of GSH content as seen in the t SNE map (dominance of the blue color as seen in Fig. 5G).

Erastin reduces sperm viability that is prevented by the ferroptosis inhibitor liproxstatin-1

In order to determine if ferroptosis can be induced in ejaculated stallion spermatozoa, spermatozoa were treated with erastin 40 μM, a known inductor of ferroptosis, RSL3 (10 μM) (an inhibitor of GPX-4) and SS 500 μM. Moreover, the specific ferroptosis inhibitor, liproxstatin-1 (5 and 10 μM) was also used. These concentrations were chosen on the basis of literature currently available in germinal cells (Bromfield *et al.* 2019). Incubation of stallion spermatozoa in the presence of erastin caused a significant reduction in the percentage of live spermatozoa after incubation for 3 h at 37°C. From 37.4 ± 2.8 in controls to 25.6 ± 2.0 in erastin treated spermatozoa ($P < 0.001$) (Fig. 6A). This reduction was prevented by the presence of the ferroptosis inhibitor liproxstatin-1. We also determined the potential involvement of caspase 3 in sperm death. The simultaneous presence of liproxstatin-1 with erastin and SS reduced the percentage of caspase 3 positive cells (Fig. 6B) ($P < 0.05$). The percentage of dead cells were higher in the presence of erastin, liproxstatin-1 and in the simultaneous presence of erastin and liproxstatin-1 (Fig. 6C) ($P < 0.05$, $P < 0.01$).

Erastin reduces sperm motility, velocities and beat cross frequency (BCF), while RSL reduces velocities and BCF

A significant reduction was observed in the percentage of motile sperm, from 80.1 ± 1.25 in controls to 61.2 ± 4.1 in the presence of erastin ($P < 0.001$) (Fig. 7A). The simultaneous presence of liproxstatin-1 had a synergistic inhibitory effect, further reducing motility to 49.9 ± 3.9% ($P < 0.001$). RSL3 and SS had no effect, although RSL3 in the presence of liproxstatin-1 caused a reduction in motility ($P < 0.001$). A similar picture was observed when referring to percentages of linear motile spermatozoa (Fig. 7B). Interestingly, and in contrast to what was observed regarding motilities, both inducers of ferroptosis (erastin and RSL3) but not SS, reduced sperm velocities and BCF (Fig. 7D, E and F). Circular velocity was reduced from 213 ± 5.4 μm/s in controls to 124.3 ± 4.53 μm/s in samples treated with erastin ($P < 0.001$), and to 184.7 ± 6.8 μm/s ($P < 0.05$) in samples treated with RSL3 ($P < 0.05$). Liproxstatin-1 had no effects in the presence of erastin, but had a

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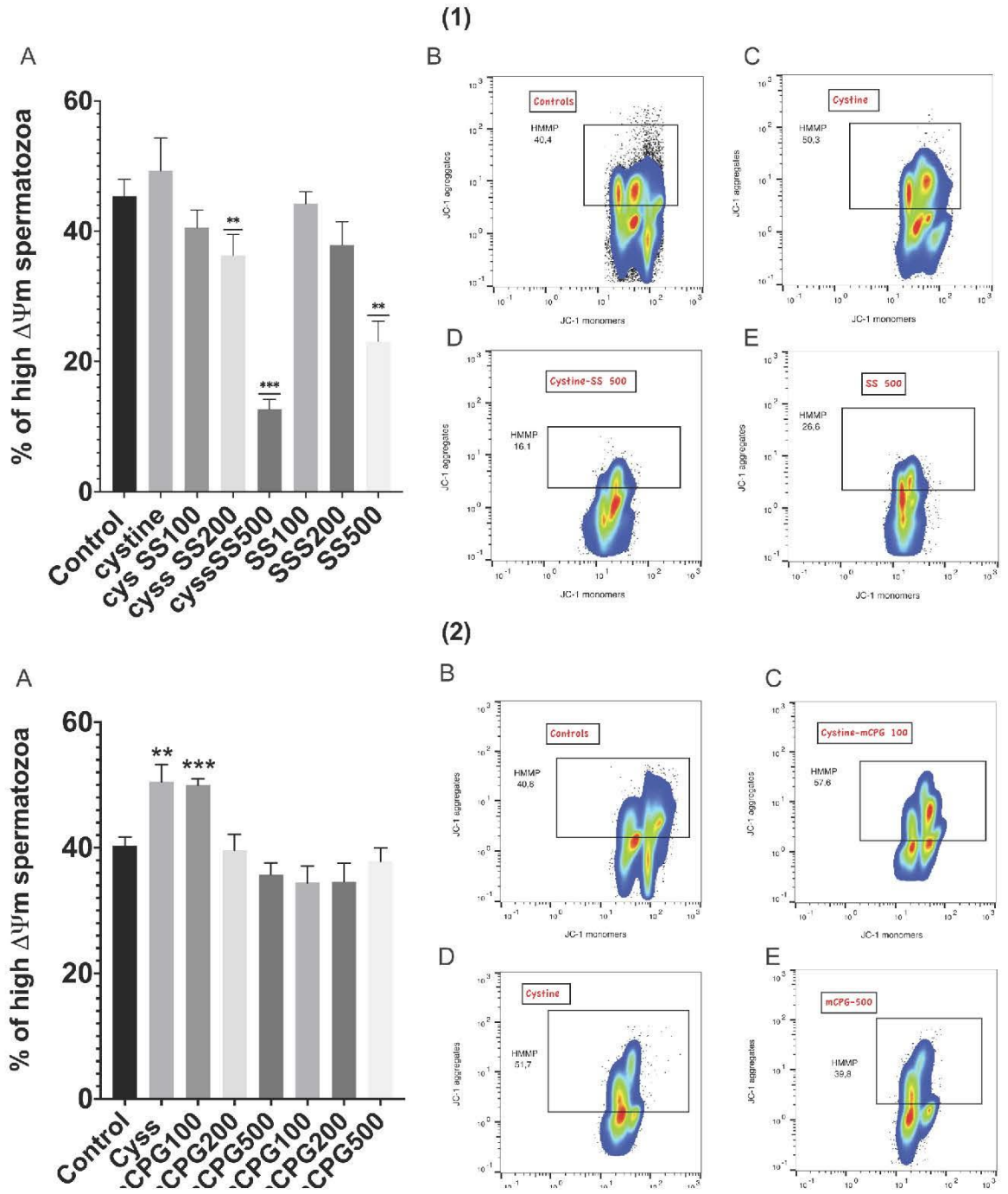

Figure 4 (1) (A) Sulfasalazine reduces mitochondrial membrane potential. Incubation of stallion spermatozoa in the presence of 500 μM SS reduced mitochondrial membrane potential. Simultaneous presence of Cyss caused further decrease in mitochondrial membrane potential. (B, C and D) representative 2D plots of concatenated replicates depicting the effect of the different treatments in the percentages of spermatozoa showing high mitochondrial membrane potential. (2) (A) mCPG does not reduce mitochondrial membrane potential. Stallion spermatozoa were incubated in presence of mCPG or mCPG and Cyss, and mitochondrial membrane potential evaluated after 3 h of incubation at 37°C. (B, C, D and E) 2D plots after concatenation of all the replicates showing changes in the mitochondrial activity. In (C) (0.5 mM Cyss and mCPG 100 μM supplemented samples) and in (D) (samples supplemented with 0.5 mM Cyss) there is an increase in mitochondrial membrane potential. (B) are controls and (E) are samples supplemented with mCPG 500 μM. Data are means ± S.E.M. *** $P < 0.001$. ** $P < 0.01$.

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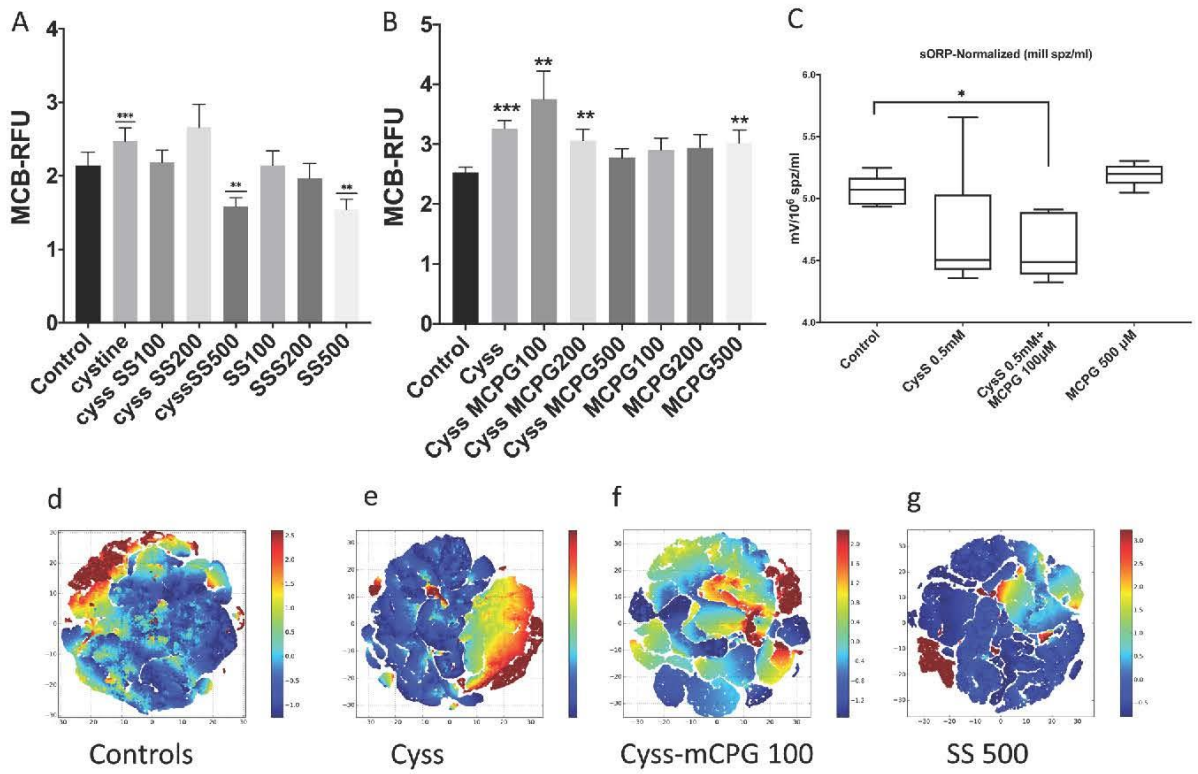

Figure 5 Sulfasalazine reduces intracellular GSH. Stallion spermatozoa were incubated in the presence of SS and/or Cyys and mCPG as described in the 'Materials and methods' section. SS caused a marked drop in GSH content at 500 µM even in the presence of Cyss ($P < 0.01$) (A). The supplementation with mCPG caused increases in GSH ($P < 0.01$) that were even bigger in the presence of 0.5 mM Cyss ($P < 0.001$) (B). The combination of 0.5 mM Cyss and 100 µM mCPG also reduced the steady state redox potential (Eh) (C). A t-SNE map shows the increase in GSH content in samples supplemented with only Cyss (e), mCPG and Cyss (f), evidenced by changes in the heat map overlay (more yellow-green-red areas) when compared with controls (d) (predominance of blue areas) and samples supplemented with SS 500 µM (g) (predominance of blue areas). The color code for the heat map is depicted at the right of every t-SNE plot. Data are means ± S.E.M. ***$P < 0.001$. **$P < 0.01$; *$P < 0.05$.

synergistic effect in the presence of RSL3 ($P < 0.001$) (Fig. 7C). Sulfasalazine increased VCL ($P < 0.01$), and liproxstatin-1 was able to revert the effect of SS. The straight line and average velocities showed the same behavior as that of the VCL (Fig. 7D and E). We also studied the effect on BCF; once again, both inducers of ferroptosis reduced this parameter (Fig. 7F) $P < 0.001$ for erastin and $P < 0.05$ for RSL3. Liproxstatin-1 reverted the effect of RSL3 but not of erastin (Fig. 7F).

Inhibition of glutamate dehydrogenase (GDH1) increases intracellular GSH and improves mitochondrial function and motility

The increase in GSH after inhibition of the antiporter with mCPG may be explained by increased availability for intracellular glutamate that can be used for GSH synthesis. Alternatively, glutamate can be transformed into α-ketoglutarate through glutamate dehydrogenase 1 (GDH1) and incorporated into de TCA cycle, providing reducing equivalents to recycle GSSG. This enzyme has been detected in proteomic studies in our laboratory and confirmed using western blotting and immunofluorescence (Fig. 8-1). The expression of GDH1, as expected, was restricted to the mid piece (Fig. 8-1). If GDH1 is inhibited, glutamate cannot be transformed into α-ketoglutarate and intracellular concentrations will increase. This approach mimics the effect of SLC7A11 inhibition, resulting in increased intracellular glutamate. In order to test this hypothesis, we performed a series of experiments. Stallion spermatozoa were incubated in the presence of the specific inhibitor of GDH1; R162 (20–80 µM), and after three and 6 h of incubation at 37°C, intracellular GSH and mitochondrial activity ($\Delta\Psi m$) were evaluated. Inhibition of GDH1 resulted in an increase of >50% of the initial levels of GSH after 3 h of incubation ($P < 0.01$) (Fig. 8-2A). The percentage of spermatozoa showing high $\Delta\Psi m$ increased after 3 and 6 h of incubation (Fig. 8-2C and D); from 68.1 ± 5.4 in controls to 76.5 ± 6.5

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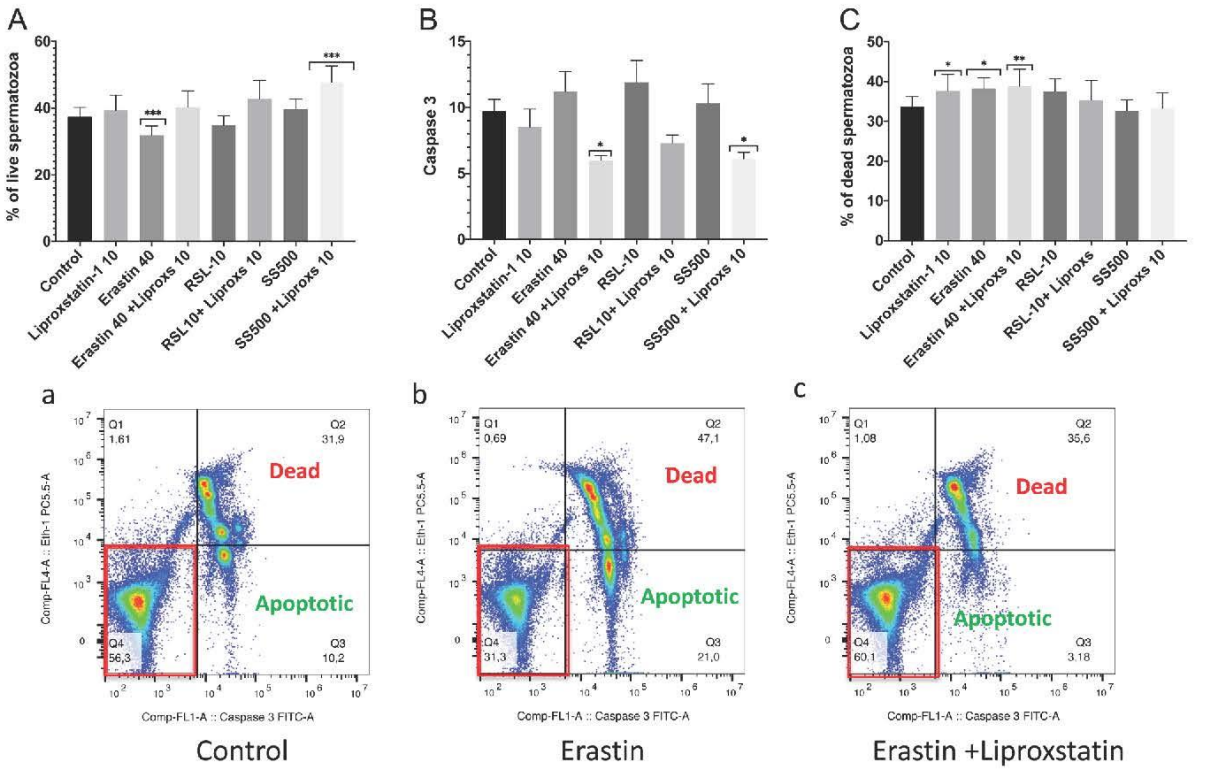

Figure 6 Effect of two different inductors of ferroptosis in stallion sperm viability. Stallion spermatozoa were incubated for 3 h at 37°C and at the final of the incubation period viability was assessed using flow cytometry.Panel A shows sperm viability, B) Caspase 3 and C) dead spermatozoa.Split samples from the same ejaculate were incubated in the presence of the inductors of ferroptosis erastin 40 μM (b) and RSL3 10 μM, and the inhibitor of ferroptosis liproxstatin-1 10 μM (b) compared with controls (a). Erastin caused a significant reduction of the percentage of viable spermatozoa that was prevented in presence of liproxstatin-1. Data are means ± S.E.M. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

in samples treated with 20 μM R162 ($P < 0.001$) and to 76.4 ± 5.6 in samples treated with 40 μM R162 ($P < 0.01$) after 3 h of incubation (Fig. 8C). After 6 h of incubation the percentage of spermatozoa showing high ΔΨm increased from 64.7 ± 7.8 in controls to 71.5 ± 4.8 in samples treated with 80 μM R162 ($P < 0.05$) (Fig. 8-2D). Also, mitochondrial activity increased both after three ($P < 0.05$) (Fig. 8-2E) and 6 h ($P < 0.01$) (Fig. 8-2F) of incubation at 37°C. Concomitantly due to increased intracellular GSH, the steady state oxidation potential was reduced in samples supplemented with the GDH1 inhibitor ($P < 0.05$) (Fig. 8-2b). The GDH1 inhibitor also caused increases in the percentage of motile spermatozoa (Fig. 8-3A) ($P < 0.05$) and sperm velocity (Fig. 8-3C and E). ($P < 0.05$).

Metabolomics inhibition of GDH1 increases succinate, fumarate, and malate

Inhibition of GDH1, thus blocking the transformation of glutamate into α-ketoglutarate, caused an unexpected increase in the levels of succinate (Fig. 9-1) and of fumarate. This finding suggests that an alternative route for the formation of succinate and fumarate from glutamate is present in the stallion spermatozoa.

Effect α-ketoglutarate on stallion sperm function

To determine the importance of α-ketoglutarate on sperm function stallion spermatozoa were incubated in the presence of the product of GDH1, dimethyl-α ketoglutarate (a cell permeable form of α ketoglutarate) (0 μM, 20 μM, 100 μM and 0.5 mM). After 3 h of incubation at 37°C mitochondrial function (ΔΨm), the percentage of live cells and GSH content were measured. Supplementation with dimethyl-α ketoglutarate did not modify the relative amount of GSH, percentage of live spermatozoa (Fig. 9-2C), or the percentage of spermatozoa showing high mitochondrial activity (Fig. 9-2B).

Discussion

In the present study, we evaluated the relationship between the SLC7A11 antiporter and mitochondrial function in the stallion spermatozoa. The major findings

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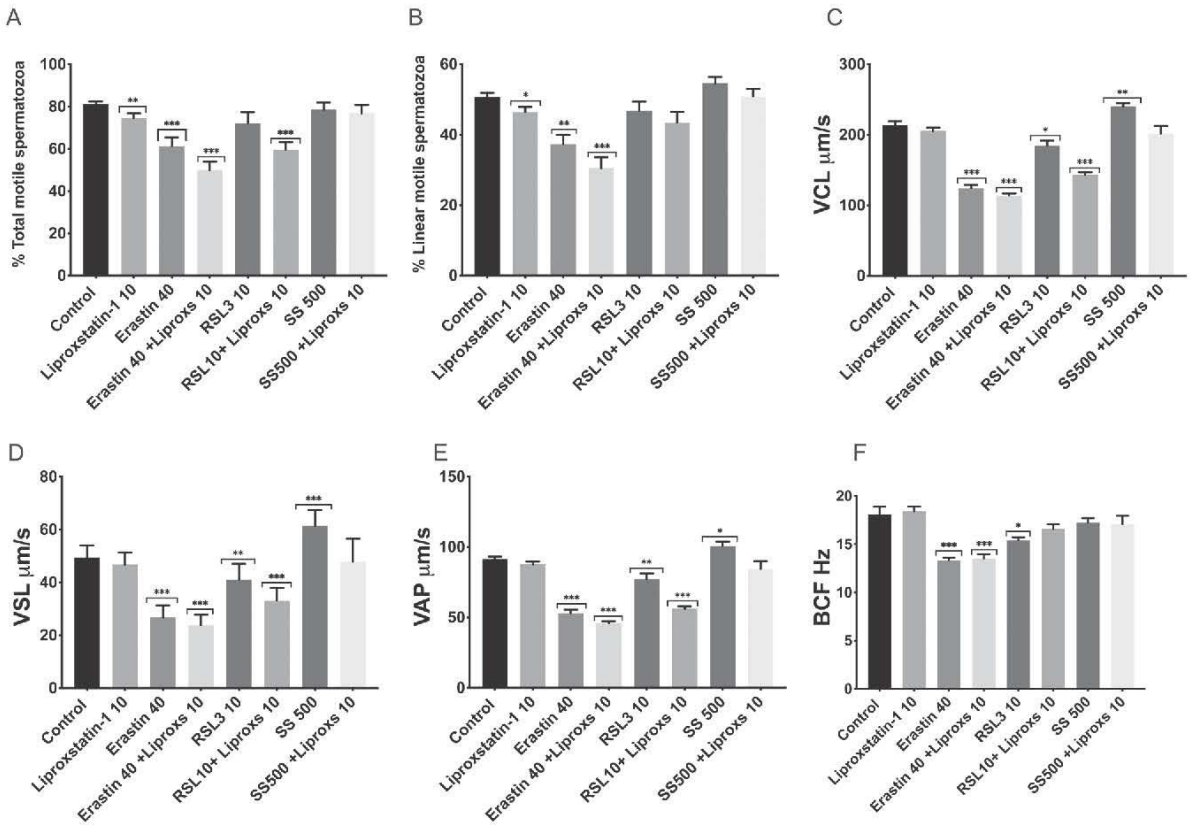

Figure 7 Effect of two different inductors of ferroptosis in stallion sperm motility, velocities and beat cross frequency (BCF). Stallion spermatozoa were incubated for 3 h at 37°C and at the final of the incubation period motility was assessed using computer-assisted sperm analysis (CASA). Split samples from the same ejaculate were incubated in the presence of the inductors of ferroptosis erastin 40 μM and RSL3 10 μM, and the inhibitor of ferroptosis liproxstatin-1 10 μM. Erastin reduced sperm motility (A) and (B). Erastin and RSL3 reduced velocities (C, D and E). BCF was reduced by erastin and RSL3, the reduction induced by RSL3 was prevented by liproxstatin-1 (F). Data are means ± S.E.M. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

of this study are summarized in the diagram provided in Fig. 10. First, we provide additional evidence that this antiporter participates in redox regulation in spermatozoa, in addition to presenting findings suggesting that it may also have an important role in glutamate metabolism. We showed that supplementation with Cyss increases intracellular GSH, and this increase is inhibited in the presence of BSO. This finding is evidence of the incorporation of Cyss, that after reduction to Cys is used for GSH synthesis in the spermatozoa. The findings reported here underline the importance of GSH for proper mitochondrial function in these particular cells. Inhibition of the SLC7A11 antiporter with SS caused a dramatic decrease in the percentage of spermatozoa with high mitochondrial membrane potential. This effect was even more evident with the simultaneous presence of Cyss. Changes described in the percentages of mitochondrial membrane potential in samples incubated in the presence of SS were accompanied by equivalent reductions in the percentage of live spermatozoa and intracellular glutathione. The impact of reduced availability of GSH in mitochondrial function is expected; GSH is synthetized only in the cytosol and then transported to the mitochondria were mitochondrial GSH (mGSH) is present at concentrations similar to those in the cytosol (10–14 mM) (Lluis *et al.* 2005, Mari *et al.* 2009, 2013). Although reported concentrations of GSH in spermatozoa in most domestic species is low, around 0.3 mM (Li 1975), concentrations reported in stallions are estimated in the range of 30 mM (Ortega-Ferrusola *et al.* 2019). Since mitochondria are the primary intracellular site of oxygen consumption and thus, the major source of intracellular ROS, mGSH is essential to balance the activity of Mn-SOD (the mitochondrial form of SOD) and the GSH redox node, to maintain hydrogen peroxide within physiological limits. Metabolism of H_2O_2 in the mitochondria depends on mGSH with the participation of GSH peroxidases and peroxiredoxins (Mari *et al.* 2009, 2013). Reduced GSH may be due to increased ROS production and/or to reduced activity

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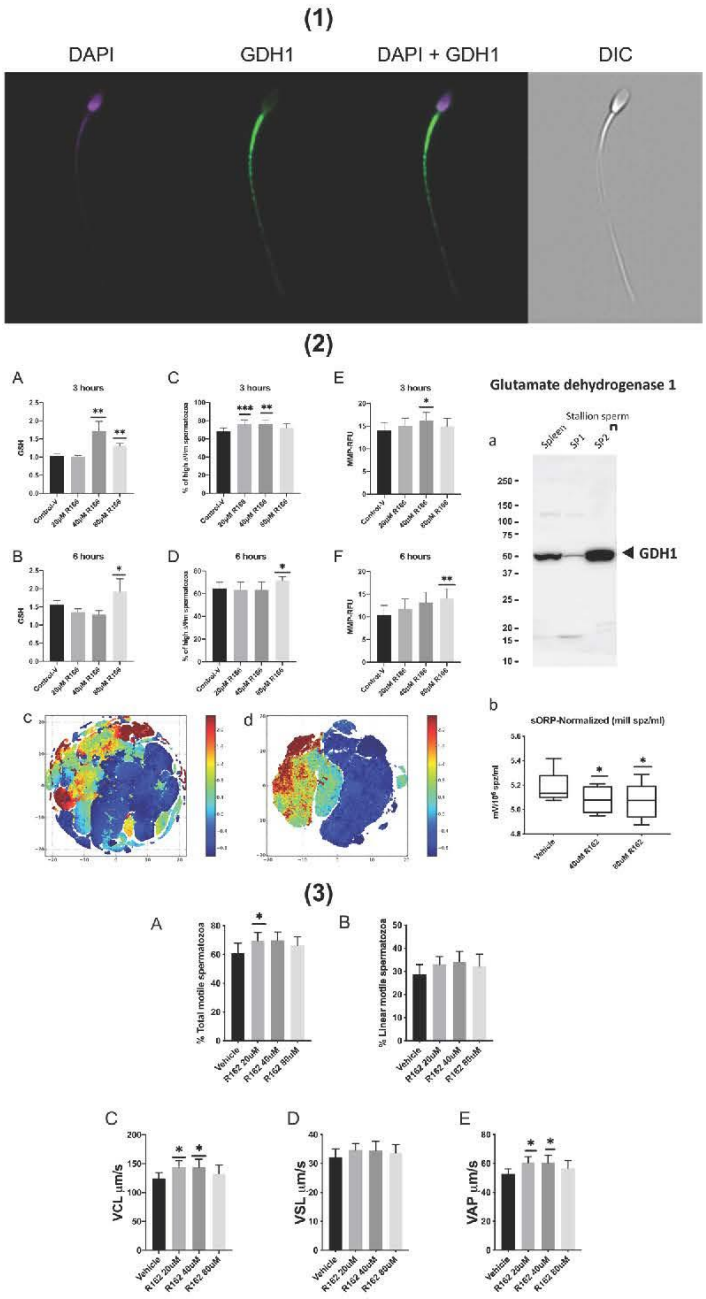

Figure 8 (1) Glutamate dehydrogenase 1 (GDH1) is present in stallion spermatozoa. Stallion spermatozoa were processed and stained as described in material and methods and the presence and distribution of the enzyme was assessed using image flow cytometry. GDH1 was present in the mid piece. (2) Inhibition of glutamate dehydrogenase 1 (GDH1) increases intracellular GSH. Stallion spermatozoa were incubated in presence of the specific GDH1 inhibitor R166 and intracellular GSH and the mitochondrial function evaluated after 3 and 6 h of incubation at 37°C. (A) Inhibition of GDH1 increased GSH content after 3 and after 6 h of incubation (B). (C and D) Inhibition of GDH1 increased the percentage of spermatozoa with high mitochondrial membrane potential after 3 (C) and 6 h of incubation (D). (E and F) Also inhibition of GDH1 increased mitochondrial activity. The presence of Glutamate dehydrogenase 1 was also confirmed using Western blotting (a). R162 also reduced the steady state redox potential (Eh) (b). c-d tSNE maps showing changes in GSH in controls (c) and R166 treated samples. Data are means ± S.E.M. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (3) Inhibition of glutamate dehydrogenase 1 (GDH1) increases sperm motility and velocity. Stallion spermatozoa were incubated in the presence of the GDH1 inhibitor R166 during 3 h at 37°C and after the incubation period sperm motility (% of motile spermatozoa) (A) and velocities (VCL µm/s, VAPµm/s and VSLµm/s) (B) were measured using CASA analysis. Increases in motility VCL and VAP were detected in samples incubated in the presence of GDH1. Data are means ± S.E.M. *$P < 0.05$.

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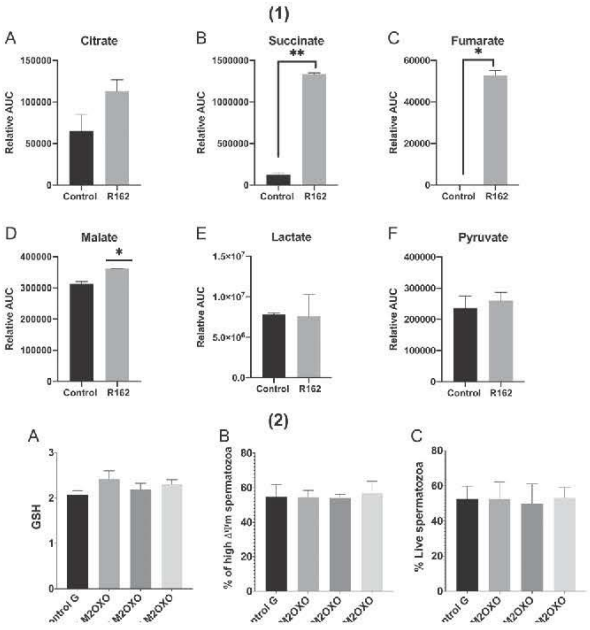

Figure 9 (1) Inhibition of glutamate dehydrogenase 1 (GDH1) modifies the stallion sperm metabolome. A) Citrate B) Succinate C) Fumarate D) Malate E) Lactate F) Pyruvate. Succinate and fumarate increased after GDH1 inhibition indicating that glutamate may be metabolized, even more efficiently, through pathways alternative to glutamate dehydrogenase. (2) Effect of supplementation with dimethyl α-ketoglutarate for 3 h at 37°C on GSH content, mitochondrial membrane potential and viability of stallion spermatozoa. A) GSH B) High Mitochondrial Membrane Potential C) Viability. Data are means ± S.E.M. $^{**}P < 0.01$; $^{*}P < 0.05$.

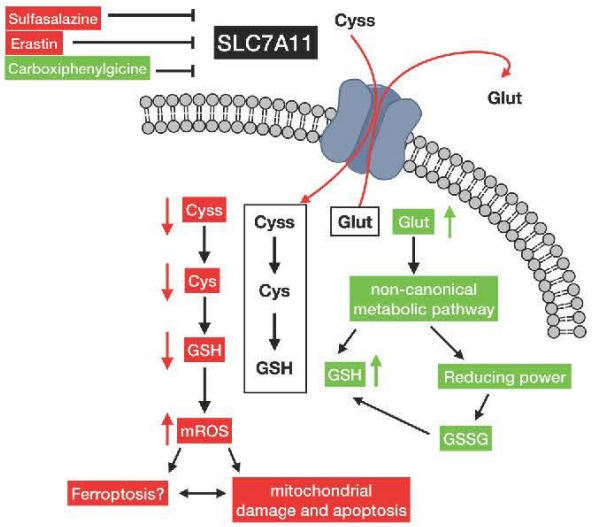

Figure 10 Schematic representation of the major hypothesis and findings of this study. The SLC7A11 antiporter contributes to redox regulation in stallion spermatozoa through the exchange of extracelular cystine (Cyss) for intracelullar glutamate (Glut). Cyss intracellularly is reduced to Cys that is used for GSH synthesis. Inhibition of this antiporter may lead to two major situations. In red, inhibition of the SLC7A11 antiporter with sulfasalazine leads to reduced intracellular Cys and then reduction in intracellular GSH. This causes redox deregulation and mitochondrial damage. Erastin also causes inhibition of the SLC7A11 antiporter. The deregulation of redox homeostasis may lead to increase of lipoperoxides (LPO) and the induction of ferroptosis. Alternatively (in green), the use of a much more potent inhibitor of the antiporter, may lead to greater increases of intracellular glutamate that may be metabolized following an alternative pathway. This alternative pathway produces reducing power to recycle GSSG to GSH and maintain redox homeostasis and mitochondrial function.

of the SLC7A11 antiporter and depletion of glutathione peroxidase 4 (GPX4) (Yu *et al.* 2019). This induces a form of programed cell death termed ferroptosis, that has been reported in the male germ line (Bromfield *et al.* 2019). GPX-4 forms the mitochondrial sheath of the spermatozoa, being crucial for the maintenance of sperm functionality (Brigelius-Flohe & Flohe 2019). To test the possibility that a ferroptotic mechanism is activated after SLC7A11 inhibition, we performed experiments comparing the effect of SS with known inducers of ferroptosis, such as erastin and RSL3. Erastin reduced the percentage of live spermatozoa, an effect that was combated by the ferroptosis inhibitor liproxstatin-1. This finding supports the existence of ferroptosis in ejaculated stallion spermatozoa. Interestingly, the simultaneous presence of SS and liproxtatin-1 resulted in increased viability and reduced expression of caspase 3, suggesting different mechanisms of cell death caused by erastin and sulfasalazine. However, although erastin and RSL3 reduced motility and velocities in spermatozoa, their effects were not prevented by liproxstatin-1. We also investigated effects on BCF, that is related to increased sperm functionality and ATP content (Mannowetz *et al.* 2012). This parameter was reduced in the presence of both inductors of ferroptosis, but liproxstatin-1 only reverted the effect of RSL3. The differential effect on viability and motility of erastin could relate to the effect of this drug on the voltage-dependent anion channel in the mitochondria (VDAC) that regulates the metabolic flux across the outer mitochondrial membrane (Blachly-Dyson & Forte 2001). Overall these results may indicate that different forms of cellular demise activated under redox deregulation may be present in ejaculated spermatozoa. However, further research is warranted to fully characterize these mechanisms and the crosstalk between them. It is also worth noting that the best spermatozoa from the ejaculate were selected through colloidal centrifugation; different responses in different sperm subpopulations ought to be considered.

The more potent inhibitor methyl S-4-carboxyphenylglycine (mCPG), increased the percentage of spermatozoa with high mitochondrial membrane potential ($\Delta\Psi m$) when Cyss was also present. Incubation in the presence of mCPG resulted in reduced percentages of live spermatozoa that was prevented in

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the presence of Cyss. The percentage of spermatozoa with high mitochondrial membrane potential and intracellular GSH increased when Cyss was also present, and in this case, without impact on sperm viability. The improvement in $\Delta\Psi m$ was in parallel with increased intracellular GSH and thus can be explained through this mechanism.

It is paradoxical that both increases or decreases in GSH may occur using different inhibitors of the SLC7A11 antiporter. One possible explanation may relate to the dual effects of the antiporter. This is, as a regulator of the availability of Cys for GSH synthesis and that it may also participate in the regulation of glutamate metabolism (Shin *et al.* 2017). The mCPG is a very potent inhibitor of the SLC7A11 antiporter, rapidly leading to increased intracellular glutamate accumulation (Patel *et al.* 2004). In this model, SLC7A11 inhibition blocks the exchange of intracellular glutamate for extracellular Cyss that leads to increased intracellular glutamate, an effect that may be enhanced in the presence of extracelullar Cyss. After transformation to α-ketoglutarate, glutamate may feed the mitochondrial TCA cycle (Jin *et al.* 2015). In fact, glutamate, supports the production of molecules such as GSH and NDPH after transformation into α-ketoglutarate by the enzyme glutamate dehydrogenase 1 (GDH1) (Wise & Thompson 2010, Jin *et al.* 2015). This mechanism provides a plausible explanation for our findings. Further supporting this hypothesis antioxidant effects of α-ketoglutarate supplementation have been previously reported in spermatozoa (Li *et al.* 2010). Recent reports in rabbit spermatozoa, showing increased GSH after glutamine supplementation also support this hypothesis (Zhu *et al.* 2017). To increase intracellular glutamate, we blocked the conversion of glutamate to α-ketoglutarate through the inhibition of the glutamate dehydrogenase 1 (GDH1) enzyme. Inhibition of GDH1, blocking the conversion of glutamate into α-ketoglutarate and incorporation into the TCA cycle, caused increased mitochondrial membrane potential and intracellular GSH (Fig. 8). This may indicate that excess glutamate is not necessarily metabolized through the GDH1 pathway, and that alternative routes are present in stallion spermatozoa. Alternative routes have been reported in tumoral cells (DeBerardinis & Cheng 2010, Son *et al.* 2013, Boroughs & DeBerardinis 2015). Whereas in most cells GDH1 converts glutamate into α-ketoglutarate, some tumors use a distinct pathway involving transaminases (GOT1), in which glutamate is converted to pyruvate increasing the NADPH/$NADP^+$ ratio and maintaining the cellular redox state (Son *et al.* 2013). A similar mechanism has recently been described. Under impaired mitochondrial metabolism cytosolic reductive carboxylation of glutamine regenerates NADH via malate dehydrogenase (Gaude *et al.* 2018). This is a very similar metabolic landscape to the one observed in our study in stallion spermatozoa after GDH1 inhibition. Interestingly transaminase activity has been reported in mammalian spermatozoa (Flipse 1960). We conducted metabolomic studies using UHPLC/MS/MS after inhibition of GDH1, focusing on metabolites of the TCA cycle. Treatment with R162, the specific GDH1 inhibitor, caused a dramatic increase in succinate, that was unexpected, since succinate is produced downstream of α-ketoglutarate in the TCA cycle. There was also a dramatic increase in fumarate in R162 treated samples. This observation suggests, as indicated above, that glutamate can also be metabolized through alternative pathways, probably by transaminases (DeBerardinis *et al.* 2007). In this model, the secretion of amino groups from glutamate is used to generate NADPH. Fumarate is also produced in the process of ammonia detoxification, through the cleavage of arginine succinate to form fumarate and arginine. Fumarate returns to the citric acid cycle (Shambaugh 1977). This mechanism may explain our findings since glutamate can be metabolized to arginine (Yelamanchi *et al.* 2016). Moreover, and further supporting this hypothesis a functional urea cycle has been described in mammalian spermatozoa (Dietz & Flipse 1969). As previously mentioned, transaminase activity has been reported in mammalian spermatozoa (Flipse 1960), and also in a recent proteomic study from our laboratory (Martin-Cano *et al.* 2020). These findings may indicate that there is a non-canonical pathway for glutamate metabolism, producing reducing power to recycle GSSG into GSH. This provides an explanation for our findings of increased GSH and mitochondrial membrane potential after GDH1 inhibition. A similar pathway has been described in pancreatic ductal adenocarcinoma cells (PADC) in which glutamate in the cytoplasm forms aspartate and then oxaloacetate that is converted to malate and then pyruvate increasing the NADPH/NAD+ ratio and in this way maintains cellular redox (Son *et al.* 2013). Increased malate was also observed in our study after GDH-1 inhibition. In this model, dimethyl α-ketoglutarate does not restore cell growth after glutamate deprivation, indicating a glutamate metabolism different from canonical models. As in the aforementioned study and in ours, supplementation with dimethyl α-ketoglutarate did not improve stallion sperm function, also suggesting alternative glutamate metabolic pathways in spermatozoa. This provides a plausible explanation to our findings of improved sperm function after GDH1 inhibition, and could also provide an explanation to increased GSH after SLC7A11 inhibition. Interestingly, for the first time, our findings suggest, that the stallion spermatozoa show an important metabolic plasticity. This is an interesting finding that could open new routes for the development of novel strategies for the management of male factor infertility, and development of new strategies for sperm conservation.

In conclusion, the results reported here describe the importance of the activity of the SLC7A11 antiporter for

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proper mitochondrial function in stallion spermatozoa, providing Cys for GSH synthesis (Martensson *et al.* 1990). They also suggest that different forms of programmed cell death may be present in the spermatozoa that warrant further research. Our findings provide evidence of a high plasticity in the metabolism of the spermatozoa and in the regulation of their redox potential. In addition, an active and complex glutamate metabolism may be present in the stallion spermatozoa (Dietz & Flipse 1967, 1969, Flipse & Dietz 1969, Flipse *et al.* 1969, DeBerardinis *et al.* 2007, Jin *et al.* 2015, Yelamanchi *et al.* 2016) that also warrants further research. The findings reported here are in line with the growing body of scientific evidence indicating that mitochondria are crucial for stallion sperm functionality and fertility, being a hallmark of fertile spermatozoa (Gibb *et al.* 2014, Davila *et al.* 2016).

Declaration of interest

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

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Author contribution statement

J M O-R, F E M-C, A S, M C G and C O-F performed experiments, contributed to data analysis. G G-P performed experiments and writing edition (native English speaker). J A T, E R and J M performed experiments. F J P conceived the study, involved in supervision, data analysis and interpretation, writing and funding acquisition.

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The inhibition of spermatic cystine/glutamate antiporter xCT (SLC7A11) influences the ability of cryopreserved stallion sperm to bind to heterologous zonae pellucidae

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ABSTRACT

Sperm are redox-regulated cells, and deregulation of their redox status is considered to affect male fertility and to reduce their fertilizing ability following biotechnological procedures, such as cryopreservation. Cystine (CysS), after incorporation in sperm via SLC7A11 antiporter, has been demonstrated to increase intracellular GSH content, the most important non enzymatic antioxidant. This study was aimed at investigating the role of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to *in vitro* capacitating environment after post-thaw incubation with CysS and/or Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter. Viability, motility, immunolocalization of tyrosine phosphorylated proteins and the ability to bind to heterologous zonae pellucidae were evaluated. Thawed sperm from seven stallions (2 ejaculates/stallion) was washed and resuspended in Tyrodes media; each thawed ejaculate was divided in Control (CTR) and 3 samples supplemented with: 0.5 mM Cystine (CysS), 500 μM Sulfasalazine (SS) and 0.5 mM CysS + 500 μM SS (CysS + SS). After 1 h of incubation at 37 °C, samples were washed twice, resuspended in capacitating BWW medium and incubated at 38 °C under 5% CO_2. After 30 and 60 min, sperm motility, viability and tyrosine phosphorylated protein immunolocalization, used as capacitation status index, were evaluated. After 30 min of capacitation, 4×10^5 sperm were co-incubated with denuded pig oocytes in capacitation medium for 30 min for the heterologous binding assay. None of the sperm parameters studied (motility, viability and tyrosine phosphorylation) showed any difference respective to control. The number of sperm bound per oocyte (mean ± SEM) tended to increase in CysS group (44.0 ± 12.3) respect CTR (40.8 ± 10.8) while decreased in SS group (32.4 ± 7.8) ($p < 0.01$). Moreover, CysS + SS group showed a lower binding rate (32.0 ± 10.0) compared to CysS ($p < 0.001$). Our results suggest that CysS supplementation of thawed stallion sperm can influence their ability to bind to heterologous zona pellucidae as the inhibition of CysS incorporation by SLC7A11 reduced the number of sperm bound per oocyte. This effect does not seem to be ascribed to a modification of sperm motility, membrane integrity and tyrosine phosphorylation.

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1. Introduction

Sperm are redox regulated cells. Controlled and physiological reactive oxygen species (ROS) amounts are required as signalling molecules for sperm crucial functions such as capacitation, acrosome reaction and sperm-oocyte interactions. However high ROS levels are considered the main cause of cellular damage induced by many sperm biotechnologies [1–5].

Most of the ROS formation takes place at the mitochondrial transport chain level during the process of oxidative phosphorylation (OXPHOS). Stallion sperm produce high amounts of ROS as they are highly dependent on OXPHOS for ATP production and are characterized by an unusually intense mitochondrial activity in

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comparison with those of other mammals [6–10]. Degradation of ROS depends on different enzymatic and non-enzymatic systems present in sperm and seminal plasma [11,12]. In human and equine seminal plasma, superoxide dismutase, catalase and glutathione peroxidase have been described as the primary enzymatic antioxidants, while the exact contribution of non-enzymatic antioxidants to the total antioxidant capacity has not yet been established [13–17]. On the other hand, intrinsic sperm antioxidant defences include enzymes (such as paraoxonase, thioredoxin and the peroxiredoxin families of proteins) and glutathione (GSH), the most important non enzymatic antioxidant [11,18–20]. Stallion sperm are characterized by higher GSH levels compared to other mammals and a significant correlation between the thiol content and stallion sperm functionality has been reported [5,21,22]. However, intrinsic sperm antioxidant defences, due to the limited amount of cytoplasm, offers only little protection. These sperm antioxidant defences can be rapidly overwhelmed in conditions of oxidative stress such as those occurring during sperm cryopreservation procedures that expose sperm to physical and chemical stressors that generate an increase of ROS, while seminal plasma, endowed with high radical scavenging activity, is preventively removed by centrifugation from the sample. For this reason, the addition of several antioxidants to sperm freezing media has been tested in different species in order to mitigate the adverse effects of ROS during cryopreservation and to better maintain frozen-thawed sperm quality and function [23–26]. Several studies demonstrated that a further approach is to supplement semen extender with antioxidant compounds during thawing; post-thaw treatment of boar sperm with resveratrol and epigallocatechin-3-gallate, alone or in combination, has been demonstrated to improve *in vitro* fertilization (IVF) outcome [25,27] while MnTBAP (a synthetic metalloporphyrin) exerts a positive effect on sperm motility, viability, lipid peroxidation and DNA integrity of frozen-thawed stallion sperm [28].

Recently, the incubation of frozen-thawed stallion sperm with cystine (CysS) has been demonstrated to induce an increase of intracellular GSH content and of total antioxidant capacity of frozen-thawed stallion sperm. In fact, extracellular CysS is exchanged for intracellular glutamate via the SLC7A11 antiporter, recently discovered in stallion sperm [29] and, once in the cell, CysS is reduced to cysteine (Cys) and used to synthesize GSH that exerts a fundamental role in maintaining redox homeostasis in sperm [30–33]. Moreover, Cys/Cyss redox node has been suggested to be functional in stallion sperm and CysS may provide antioxidant capacity independently of its incorporation into GSH [29].

Cryodamage has been described to induce structural modifications in several proteins, such as membrane transporters [34], and it has been suggested that SLC7A11 antiporter may present altered functionality in stallion cryopreserved sperm [29].

On these bases, this study was aimed at investigating the influence of CysS and the role of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to *in vitro* capacitating environment after post-thaw incubation with CysS and/or Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter. Sperm viability, motility, immunolocalization of tyrosine phosphorylated proteins, and the ability to bind to heterologous zonae pellucidae were evaluated.

2. Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.1. Semen processing

Frozen-thawed semen (two ejaculates from seven different stallions of proven fertility) housed at the National Institute of Artificial Insemination (AUB-INFA), University of Bologna, were used. Straws were thawed in a water bath at 37 °C for at least 30 s and then diluted (v/v) in pre-warmed Tyrodes medium pH 7.4 (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM $NaHCO_3$, 1 mM Na-Pyruvate, 21.6 Na-Lactate, 2 mM $CaCl_2$*$2H_2O$, 3.1 mM KCl, 0.4 mM $MgSO_4$*$7H_2O$, 0.3 mM NaH_2PO_4*H_2O, 0.3% BSA). Samples were centrifuged (600 g × 10') and resuspended in Tyrodes medium to a final concentration of 50 × 10^6 sperm/mL.

2.2. Experimental design

Each frozen-thawed ejaculate was divided in Control (CTR) and 3 samples supplemented with: 0.5 mM Cystine (CysS), 500 μM Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter, and 0.5 mM CysS + 500 μM SS (CysS + SS) and incubated in a water bath at 37 °C for 1 h. CysS and SS concentrations have been chosen based on previous studies [29].

Samples were then washed twice in Biggers, Whitter and Whittingham (BWW) medium pH 7.4 (20 mM HEPES, 5.6 mM D-Glucose, 95 mM NaCl, 25 mM $NaHCO_3$, 0.275 mM Na-Pyruvate, 3.7 μL/mL Sodium Lactate 60% w/v, 1.7 mM $CaCl_2$*$2H_2O$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$*$7H_2O$, 0.1% PVA) and resuspended in a capacitating BWW medium (BWW supplemented with 5 mM Dibutyryl cyclic AMP, 0.5 mM Methyl-β-cyclodextrin and 3 mM Caffeine) to a final concentration of 10 × 10^6 sperm/mL and incubated at 38 °C in 95% humidity and 5% CO_2 in air, as described by Bromfield et al., 2014 [35].

After 30 min of capacitation, 4 × 10^5 sperm were co-incubated with 20 denuded pig oocytes for the heterologous binding assay.

Sperm viability, motility and tyrosine phosphorylated protein immunolocalization were evaluated in each sample after 1 h of incubation in Tyrodes medium and after 30 min and 1 h of incubation in capacitating condition.

2.3. Sperm viability assessment

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

2.4. Sperm motility assessment

Sperm motility was measured by a computer-assisted sperm analysis system, using the open source Image J BGM plugin as described by Giaretta et al., 2014 [36]. Briefly, a Leitz diaplan microscope (Wild LeitzGmbH, D6330, Wetzlar, Germany) with a 10x plan objective with negative phase-contrast was used. The microscope was equipped with a Z31 Ascon technologic heated stage (Ascon technologic, PV–IT). The video camera, a 3.1-megapixel CMOS USB 2.0 Infinity 1–3 Camera (Lumenera corporation, Ottawa, ON, Canada), was coupled with the microscope using a c-mount adapter and videos were recorded for 3 s at a resolution of 800 × 600 pixels and 60 frames/sec (fps). Images were saved on a

hard drive using the Infinity analysing and capture software 6.4 (Lumenera corporation) and converted to avi format for subsequent analysis using the BGM Image J plugin.

Prior to any observation, sperm were loaded on a fixed height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of separate fields, each lasting 3 s, were recorded per sperm sample and the central second of each video was analysed. Sperm motility endpoints assessed were: percent of total motile sperm ©, percent of progressive sperm (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The setting parameters used by the program were the following: 60 frames per second, number of frames 45, threshold path minimum; minimum VAP for motility 20 μm/s; minimum VCL for motility 30 μm/s; VAP cut off for progressive cells 15 μm/s; STR cut off for progressive cells 45%.

2.5. Immunolocalization of tyrosine phosphorylated proteins

Samples of each experimental group, after 1 h of incubation in Tyrodes medium and at 30 min and 1 h of capacitation, were washed twice in PBS and aliquots of 80 μL were placed on poly-L-lysine-coated slides and fixed with cold methanol for 15 min at −20 °C and then with acetone for 30 s. Slides were washed with PBS, allowed to dry and stored at −20 °C until staining. Unspecific bindings were blocked with PBS with 10% foetal calf serum (FCS) for 1 h at room temperature in the dark. Analysis of tyrosine phosphorylation status was performed with an anti-phosphotyrosine antibody (1:150 clone 4G10, Millipore, Italy) incubated overnight at 4 °C. After three washes in PBS, slides were incubated with a sheep anti-mouse IgG FITC conjugated (1:850, BioFx, Italy) for 60 min at room temperature in the dark. Slides were washed three times in PBS and mounted with a drop of Vectashield (Vector Laboratories, Burlingame, CA, USA). Oil immersion at 400 × magnification was used utilizing the microscope described above, and at least 200 cells per sample were scored. For negative control samples, the same treatment was applied, omitting the primary antibody.

2.6. Heterologous binding assay

2.6.1. Pig oocyte in vitro maturation (IVM)

Ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported (in 0.9% wt/vol NaCl solution) to the laboratory within 2 h. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles, 3–6 mm in diameter, with a 18-gauge needle fixed to a 10-mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 supplemented with 5 μg/mL insulin, 1 mM glutamine, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 μM β-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 μL of the same medium per well and cultured at 39 °C in a humidified atmosphere of 5% CO_2 in air [37]. For the first 22 h of *in vitro* maturation the medium was supplemented with 1 mM Dibutyryl cyclic AMP and 0.12 IU/mL Pluset (CALIER, Italia); for the last 22 h, COCs were transferred to fresh maturation medium [38]. At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting, transferred in hypertonic solution (1.5 M $MgCl_2$, 40 mM HEPES, 0.1% PVP) and stored at 4 °C until use. Before use, stored pig oocytes were washed twice in PBS.

2.6.2. Heterologous binding assay

For the binding assay, control semen and semen previously treated with CysS, SS or CysS + SS and then incubated for 30 min in capacitating condition, were co-incubated (1×10^6 sperm/mL) with 20 denuded oocytes in 400 μL of capacitating BWW medium at 38 °C in 95% humidity and 5% CO_2 in air. After 30 min of gamete co-incubation, the oocytes were washed four times in PBS 0.4% BSA with a wide bore glass pipette to remove the sperm loosely attached to the zonae pellucidae (ZP). The oocytes were then incubated with 8.9 μM Hoechst 33342 for 10 min in PBS 0.4% BSA in the dark, washed twice in the same medium, and individually placed in droplets of Vectashield (Vector Laboratories) on a slide and covered with a coverslip. The number of sperm attached to the ZP of each oocyte was assessed using the above-described microscope. A total of 1000 oocytes were evaluated (240 for CTR, 255 for CysS, 262 for SS and 243 for CysS + SS).

2.7. Statistical analysis

Statistical analyses were performed using R (version 3.5.2). Values are expressed as mean ± SEM, unless otherwise specified and the level of significance used was $p \leq 0.05$. Motility and post thawing parameters were tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests. Then, a mixed effect model was set to determine treatment and time effects (1 h post-incubation in Tyrodes medium, 30 min post-capacitation and 1 h post-capacitation) and their interaction, with the ejaculate and repetition as random effects.

As for oocyte binding assays, the results are expressed as the number of sperm bound per oocyte normalized to the daily standard deviation. The variable was analysed using a general linear model with Poisson distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.

3. Results

3.1. Sperm viability and motility assessment

No significant differences were found in viability and motility parameters (TM, PM, VCL, VAP, VSL, STR, LIN, BCF ALH, and WOB) among experimental groups after incubation in Tyrodes medium (Fig. 1) (Supplementary file 1). Sperm incubation in capacitating condition induced a significant decrease in viability, total motility and progressive motility independently form the experimental group ($p < 0.05$) (Fig. 1).

3.2. Detection of tyrosine phosphorylated proteins

Different patterns were detected as described by Balao da Silva et al., 2013 [39]. In this study, sperm with immunoreactivity either on the tail or on the tail and head (equatorial region and/or acrosome) were classified as capacitated (Fig. 2).

A significant increase in the percentage of immunoreactive sperm for tyrosine-phosphorylated proteins was observed after 30 min of capacitation ($p < 0.05$) in all the experimental groups with the exception of CysS group, in which a non-significant tendency to an increase of sperm tyrosine-phosphorylation was observed (Fig. 3).

After 1 h in capacitating condition the percentage of immunoreactive cells in each experimental group decreased to levels similar to those observed after Tyrodes incubation (Fig. 3).

No significant differences in the percentage of positive cells were recorded among the different experimental groups at the same time points of the experiment.

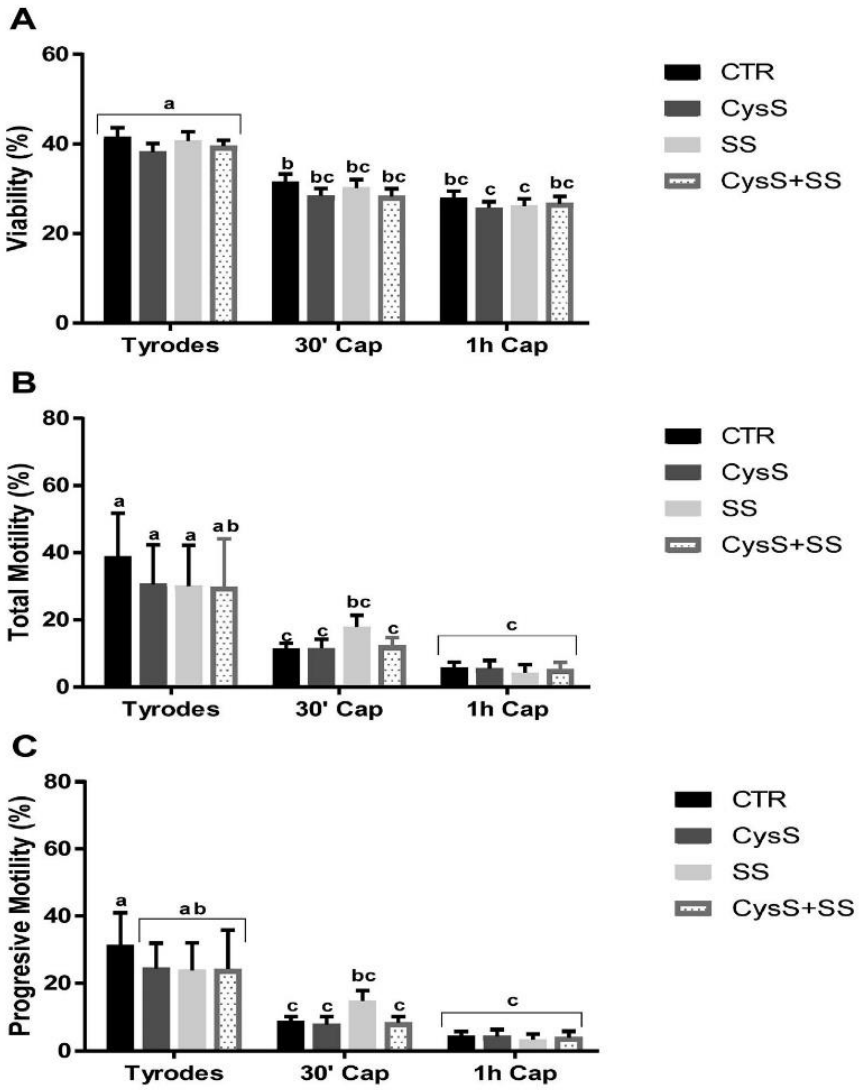

Fig. 1. Effect of 0.5 mM CysS, 500 μM SS and 0.5 mM CysS + 500 μM SS supplementation during post-thaw incubation at 37 °C for 1 h on Viability (A), Total Motility (B) and Progressive Motility (C) of frozen-thawed stallion sperm, before (Tyrodes) and after 30 min (30′ Cap) and 1 h (1 h Cap) under capacitating condition. Different letters indicate a significant difference between incubation times ($p < 0.05$). Data represent the mean ± SEM.

3.3. Heterologous binding assay

No significant difference was observed between the number of sperm bound per oocyte (mean ± SEM) in CTR and CysS groups (40.8 ± 10.8 *vs* 44.0 ± 12.3). The inhibition of SCL7A11 antiporter with SS (SS and CysS + SS experimental groups) significantly decreased ($p < 0.001$) the number of sperm bound to the ZP compared to CTR and CysS groups (Fig. 4).

4. Discussion

In this study, the effect of CysS addition and the functionality of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to *in vitro* capacitating environment was investigated.

Recently, Ortiz-Rodriguez et al. have described SLC7A11 presence and activity in stallion sperm and they have demonstrated that supplementing CysS, which in the cell is reduced to Cys and used in GSH synthesis, lead to an increased intracellular GSH concentration; this effect was prevented by SS, the specific SLC7A11 inhibitor [29,30].

The results obtained from the evaluation of frozen-thawed stallion sperm viability and motility did not evidence any significant effect due to either the addition of CysS or the inhibition of SLC7A11 transporter, both in presence and in absence of CysS, at the end of 1 h post-thaw incubation in Tyrodes medium. It is well known that sperm cryopreservation induces alterations in membrane stability and integrity [40,41]. In agreement with other studies on frozen-thawed semen, which recorded a viability after thawing around of 40–50% in stallions that have good freezability [42,43], the percentage of live cells recorded in this study after thawing semen for each stallion was around 40%, without differences between experimental groups. Nevertheless, it cannot be excluded that the incubation time of 1 h with CysS and SS may be too short to induce evident modification in sperm motility and

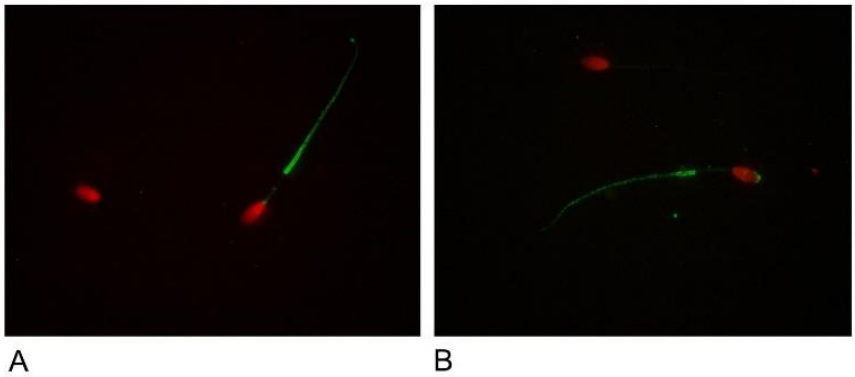

Fig. 2. Representative micrographs of protein tyrosine immunostaining (green). (A) Negative (left) and positive spermatozoon (right; tail positivity); (B) Negative (upward) and positive spermatozoon (downward; tail and head positivity). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

viability or that the functionality of SLC7A11 antiporter may be partially altered in cryopreserved spermatozoa as previously suggested by Ortiz-Rodriguez et al. [29].

A significant decrease in viability, total motility and progressive motility was recorded when frozen-thawed sperm of all experimental groups were incubated in capacitating condition. A reduction in sperm viability associated with *in vitro* sperm capacitation process has been described in numerous studies [28,44]. Capacitation is a process that sperm must undergo in order to fertilize the oocyte, requiring large quantities of energy in the form of adenosine triphosphate (ATP), which is synthesized during OXPHOS in the mitochondria. OXPHOS is responsible for ROS production and these molecules, at very low and controlled concentrations, have been recognized to participate in signal transduction mechanisms involved in sperm activation and capacitation [7,45–48]. Sperm capacitation, the process that sperm must undergo in order to fertilize the oocyte, is characterized by increase in membrane fluidity, cholesterol depletion and lipid raft aggregation of the sperm plasma membrane, in addition to protein tyrosine phosphorylation [46]. Therefore, the decrease in sperm viability recorded when samples have been *in vitro* capacitated may be due to membrane instability induced by the capacitating events. Moreover, it has been reported that a subpopulation of cryopreserved sperm shows capacitation-like changes/characteristics after thawing, since cryopreservation induces damages of the sperm plasma membrane, producing spermptosis [22,49–51]. On these bases, the reduction of sperm viability and motility recorded after incubation of frozen-thawed stallion sperm in capacitation conditions may be due to a high sensitivity of cryopreserved sperm to capacitating stimuli that explains their limited life span and the loss of

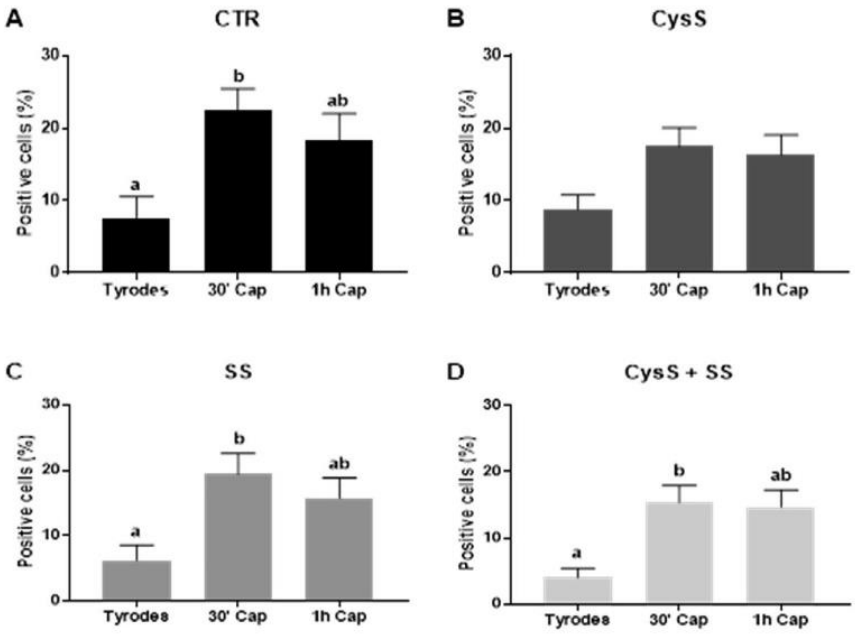

Fig. 3. Percentages of cells displaying tyrosine phosphorylation immunoreactivity before (Tyrodes) and after incubation in capacitating condition for 30 min (30′ Cap) and 1 h (1 h Cap) in control group (A) and in groups supplemented during post-thaw incubation at 37 °C for 1 h with 0.5 mM CysS (B), 500 μM SS (C) and 0.5 mM CysS + 500 μM SS (D). Different letters represent a significant difference for $p < 0.05$ in the same treatment between incubation times. Data represent the mean ± SEM.

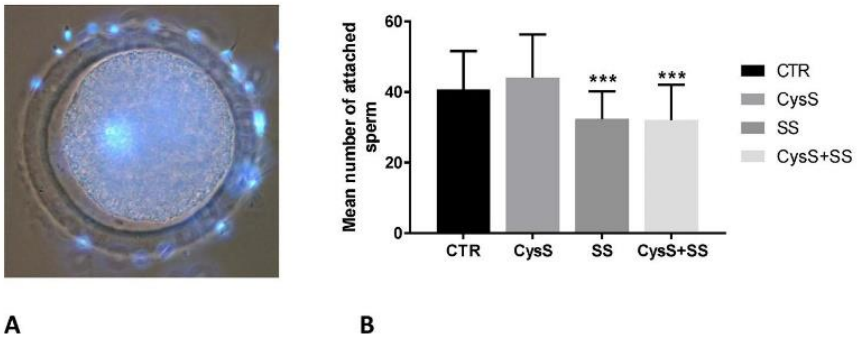

Fig. 4. (A) Epifluorescence and phase contrast microscopy overlapped images of a pig oocyte with bound stallion sperm stained with Hoechst 33342. (B) Effect of CysS (0.5 mM), SS (500 μM) and CysS + SS (0.5 mM CysS + 500 μM SS) supplementation during post-thaw incubation at 37 °C for 1 h on the number of bound sperm per porcine oocyte after incubation in capacitating condition. A total of 1000 oocytes were evaluated (numbers of oocytes analysed in the different groups were: 240 for CTR, 255 for CysS, 262 for SS and 243 for CysS + SS).***$p < 0.001$. Data represent the mean ± SEM.

functionality as observed in small ruminants [52,53]. Anyway, the addition of CysS, SS or a combination of CysS and SS did not induce any difference in sperm viability and motility between experimental groups under capacitation conditions suggesting that the effect of CysS and SS observed on oocyte binding ability is not due to an impact on those two sperm parameter. Phosphorylation of tyrosine residues on sperm proteins is considered a suitable marker of capacitation. As previously described in the equine spermatozoa [39,54], we observed three main patterns of phospho-tyrosine immunopositivity on stallion sperm: tail, tail and head, or none. As previously mentioned, cryopreservation can induce capacitation-like changes after thawing, so it was not surprising to observe that, after 1 h post-thawing incubation in Tyrodes prior to induction of capacitation, in all the experimental groups a subpopulation of sperm displayed tyrosine phosphorylation immunoreactivity. After incubation in capacitating condition, all experimental groups showed a significant increase in the percentage of sperm displaying protein tyrosine phosphorylation, with the exception of CysS group that showed only a not significant tendency towards higher levels of protein tyrosine phosphorylation. As already mentioned, CysS enters the cytosol of stallion sperm through the SLC7A11 antiporter and is used for the synthesis of GSH that plays an important role as an intrinsic spermatic antioxidant, causing a decrease of sperm ROS levels [11,29]. Protein tyrosine phosphorylation is also a redox-regulated process [45,55,56] as ROS inactivates tyrosine phosphatases (PTPs) permitting the up-regulation of tyrosine phosphorylation that characterizes sperm capacitation [53]. Therefore, it can be hypothesized that the lower increase in protein tyrosine phosphorylation in the CysS experimental group we observed after *in vitro* induction of capacitation, may be due to the increase in intracellular GSH induced by CysS supplementation, as described by Ortiz-Rodriguez et al. [29], that may have induced a decrease in ROS levels and in turn a lower inhibition PTPs.

In this study, the influence of CysS and SLC7A11 functionality on stallion sperm ability to interact with oocyte ZP has been tested using heterologous ZP binding assay. This assay has been developed to address difficulties associated with the acquisition of horse oocytes [45,57,58] and was demonstrated to be as reliable as homologous one [44]. The supplementation of CysS during 1 h post-thaw of incubation in Tyrodes was unable to increase ZP binding after *in vitro* induction of capacitation. Interestingly, both the experimental groups in which SLC7A11 antiporters were inhibited by SS (SS and CysS + SS) showed a decrease in the number of sperm bound per oocyte. The inhibitory effect exerted by SS on sperm binding ability firstly suggest that the incorporation of CysS by SLC7A11 may exert a role in allowing sperm acquisition to interact with oocyte ZP after capacitation and, secondly, suggest that SLC7A11 functionality is maintained, at least in part, in frozen-thawed stallion sperm.

As previously mentioned, CysS incorporated into the sperm is used to synthetize GSH which is implicated in processes such as neutralization of superoxide and detoxification of metabolites [59–61]. GSH can also interact with its associated enzymes (glutathione peroxidases, glutathione reductases and glutathione S-transferases), to provide protection against oxidative stress. It has been reported that sperm glutathione S-transferases play an important role in fertilization, as specific glutathione S-transferase members are involved in sperm-oocyte binding [62,63]. It can be hypothesized that the reduction of CysS incorporation induced by SLC7A11 inhibition carried out by SS may have affected sperm-ZP binding through an impairment of glutathione S-transferases function.

In conclusion, the incorporation of CysS through the SLC7A11 antiporter in frozen-thawed stallion sperm may influence the sperms ability to bind to heterologous zonae pellucidae. The mechanism by which this effect is exerted remains to be elucidated as sperm motility, membrane integrity and protein tyrosine phosphorylation were not affected.

Authors contribution

OR JM, MS and GG designed the work, conducted the experiments and wrote the manuscript. CN and BM conducted the experiments. DB conducted the experiments and performed the statistical analysis. CT and FP critically revised the work. All authors discussed the results and contributed to the final manuscript.

Declaration of competing interest

None of the authors have conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2021.03.002.

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Artículo 4

PLOS ONE

RESEARCH ARTICLE

Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase 3

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Abstract

Background

The population of stallion spermatozoa that survive thawing experience compromised mitochondrial functionality and accelerated senescence, among other changes. It is known that stallion spermatozoa show very active oxidative phosphorylation that may accelerate sperm senescence through increased production of reactive oxygen species. Rosiglitazone has been proven to enhance the glycolytic capability of stallion spermatozoa maintained at ambient temperature.

Objectives

Thus, we hypothesized that thawed sperm may also benefit from rosiglitazone supplementation.

Materials and methods

Thawed sperm were washed and resuspended in Tyrodes media, and the samples were divided and supplemented with 0 or 75 μM rosiglitazone. After one and two hours of incubation, mitochondrial functionality, Akt phosphorylation and caspase 3 activity were evaluated. Additional samples were incubated in the presence of an Akt1/2 inhibitor, compound C (an AMPK inhibitor) or GW9662 (an antagonist of the PPARγ receptor).

Results

Rosiglitazone maintained Akt phosphorylation and reduced caspase 3 activation ($p<0.01$), both of which were prevented by incubation in the presence of the three inhibitors. Rosiglitazone also enhanced mitochondrial functionality ($P<0.01$).

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

Conclusion

We provide the first evidence that the functionality of frozen stallion spermatozoa can be potentially improved after thawing through the activation of pro survival pathways, providing new clues for improving current sperm biotechnology.

Introduction

Stallion spermatozoa can be stored in a liquid state for short periods, or it can be frozen for longer-term storage. Storing spermatozoa in a frozen state has numerous advantages; however, its widespread use is still constrained by a number of weaknesses [1]. Among them are high stallion-to-stallion variability and insufficient standardization of the freezing and thawing procedures. While cryopreservation induces mortality for an average 50% of the initial population of spermatozoa [1–4], the surviving spermatozoa are not completely functional; on the contrary, they experience accelerated senescence that requires more intense and costly mare management for insemination to compensate for this reduced lifespan. Most of the studies on sperm cryopreservation have aimed to increase the number of spermatozoa surviving the procedure, but studies aiming to improve the quality of the surviving population are scarce. Although the changes induced by cryopreservation have been extensively investigated, mostly focusing on cryopreservation induced necrosis [5–7], few studies have addressed the physiology of spermatozoa surviving freezing and thawing. There are not many studies that have tried to develop measures to increase the quality of frozen spermatozoa after thawing, with the exception of procedures to remove dead and damaged spermatozoa from the cryopreserved sample [8–10].

The population of spermatozoa surviving freezing and thawing experience changes that were recently termed spermptosis [11]. These changes basically represent acceleration of the apoptotic pathway to death that most spermatozoa undergo after ejaculation [12, 13]. In brief, osmotic shock induces membrane and mitochondrial damage [14], then the mitochondrial damage causes impairment of redox regulation, leading to lipid, protein and DNA modifications in the spermatozoa, resulting in decreased motility and viability [11, 15, 16]. Other changes recently described in relation to cryopreservation include increased intracellular Na+ and membrane depolarization due to the compromised functionality of the Na+-K+ATPase pump [17]. Akt (also called protein kinase B) plays a major role in the regulation of sperm survival. When this kinase remains phosphorylated, spermatozoa are viable, but upon dephosphorylation of Akt, pro-caspase 3 is cleaved and the spermatozoa rapidly enter a default apoptotic pathway and finally lose their ability to maintain motility [18, 19]. The cryopreserved spermatozoa show impaired mitochondrial activity due to oxidative stress and the osmotic damage that occurs during thawing [20–23]; further, cryopreserved spermatozoa present diminished mitochondrial oxygen consumption [24]. This damage compromises the capability of thawed stallion spermatozoa to produce ATP through oxidative phosphorylation [15, 25, 26]. Thus, the cryopreserved spermatozoa have lower motility and lower ATP content compromising their functionality and finally their fertilizing ability. Rosiglitazone can improve the glycolytic activity of stallion spermatozoa maintained at ambient temperature for extended periods [27]; moreover, human [28] and porcine [29] studies indicate that rosiglitazone activates pro-survival pathways in spermatozoa. In view of these facts, our hypothesis was that thawed stallion spermatozoa may also benefit from rosiglitazone supplementation.

Materials and methods

Animals

The ethical committee of the University of Extremadura approved the study AGL-2017-83149-R. The only manipulation of the animals was semen collection under regular veterinary practices. Six pure Spanish horses were used in this study (Table 1); the animals were fed with hay and grain, given water ad libitum and exercised daily in a horse exerciser. Ejaculates were collected using a prewarmed lubricated Missouri Model artificial vagina (Minutüb Ibérica, Tarragona, Spain) with an in line filter to eliminate the gel fraction. After collection, the semen was extended at 1:2 in INRA 96 (IMV L'Aigle France) and immediately transported to the laboratory for evaluation and processing.

Reagents and media

Hoechst 33342 (excitation: 350 nm, emission: 461 nm) (Ref: H3570); 5,5',6,6'–tetrachloro-1,1',3,3'tetraethylbenzymidazolyl carbocianyne iodine (JC-1) (excitation: 488 nm, emission: 530 nm, monomer form) (excitation: 561 nm, emission: 591 nm, aggregate form) (Ref: T3168); CellRox Deep Red Reagent (excitation: 644 nm, emission: 655 nm) (Ref: C10422); Cell Event Caspase-3/7 Green Detection Reagent (excitation, 502 nm, emission: 530 nm) (Ref: C10423); Annexin V Alexa Fluor 647 conjugate (excitation: 650 nm, emission: 665 nm) (Ref: A23204); and ethidium homodimer (excitation, 528 nm, emission, 617 nm) (Ref: E1169) were purchased from ThermoFisher Scientific (Molecular Probes) (Waltham, Massachusetts, USA). Anti-phospho-Akt (Ser 473) (D9E) XP Rabbit mAb (Alexa Fluor 488 conjugate was acquired from Cell Signalling Technology (Danvers, Massachusetts, USA). Rosiglitazone, dorsomorphin, GW9662 and an Akt I-II kinase inhibitor and all other reagents unless otherwise specified were purchased from Sigma-Aldrich (Madrid, Spain).

Experimental design

Frozen doses of spermatozoa (triplicate ejaculates from 6 different stallions) stored in our center were used in this study. The samples were previously obtained from stallions housed in our center as described in the section "Animals" following Institutional and European Animal care regulations (Law 6/2923 June 11th and European Directive 2010/63/EU), and collected and processed following the same protocol described in previous publications of our laboratory [11, 15, 30, 31]. Straws were thawed in a water bath at 37˚C for at least 30 s and were then diluted in prewarmed INRA-96 (Humeco, Huesca, Spain) extender to a final concentration of 50×10^6 spermatozoa/ml. The samples were centrifuged (600 g × 10') and resuspended in

Table 1. Summary of the stallions used in this study.

Stallion	Age	Breed	Motility % (CASA)	Morphology (% of normal spermatozoa)
1	11	PSH	89 ± 1.32	74 ± 1.33
2	12	PSH	83 ± 1.51	59 ± 1.75
3	11	PSH	71 ± 3.09	51 ± 2.1
4	8	PSH	80 ± 4.76	80 ± 1.51
5	6	PSH	87 ± 1.01	70 ± 1.26
6	13	PSH	86 ± 1.39	58 ± 1.19

Data presented as the means ± SEM

PSH Pure Spanish Horse

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Tyrode's media [32] to a final concentration of 50×10^6 spermatozoa/ml. The semen was split into subsamples for control and experimental treatments and incubated in a water bath at 37˚C. The doses of rosiglitazone were selected based on a previously published work [27], and incubation of the stallion spermatozoa was performed in the presence of three different concentrations (0: vehicle control DMSO 1:1000, 50, 75 and 100 μM) (Fig 1). Then, the rest of the experiments were conducted in the presence of 75 μM rosiglitazone and in the presence of specific inhibitors. Sperm functions studied included motility and kinematics, mitochondrial membrane potential, production of superoxide and live spermatozoa, caspase 3, phosphorylation of Akt and determination of the oxidation reduction potential.

Sperm motility

Sperm motility was assessed using a Computer Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain). Semen was loaded in a 20 μm deep Leja chamber (Leja, Amsterdam,

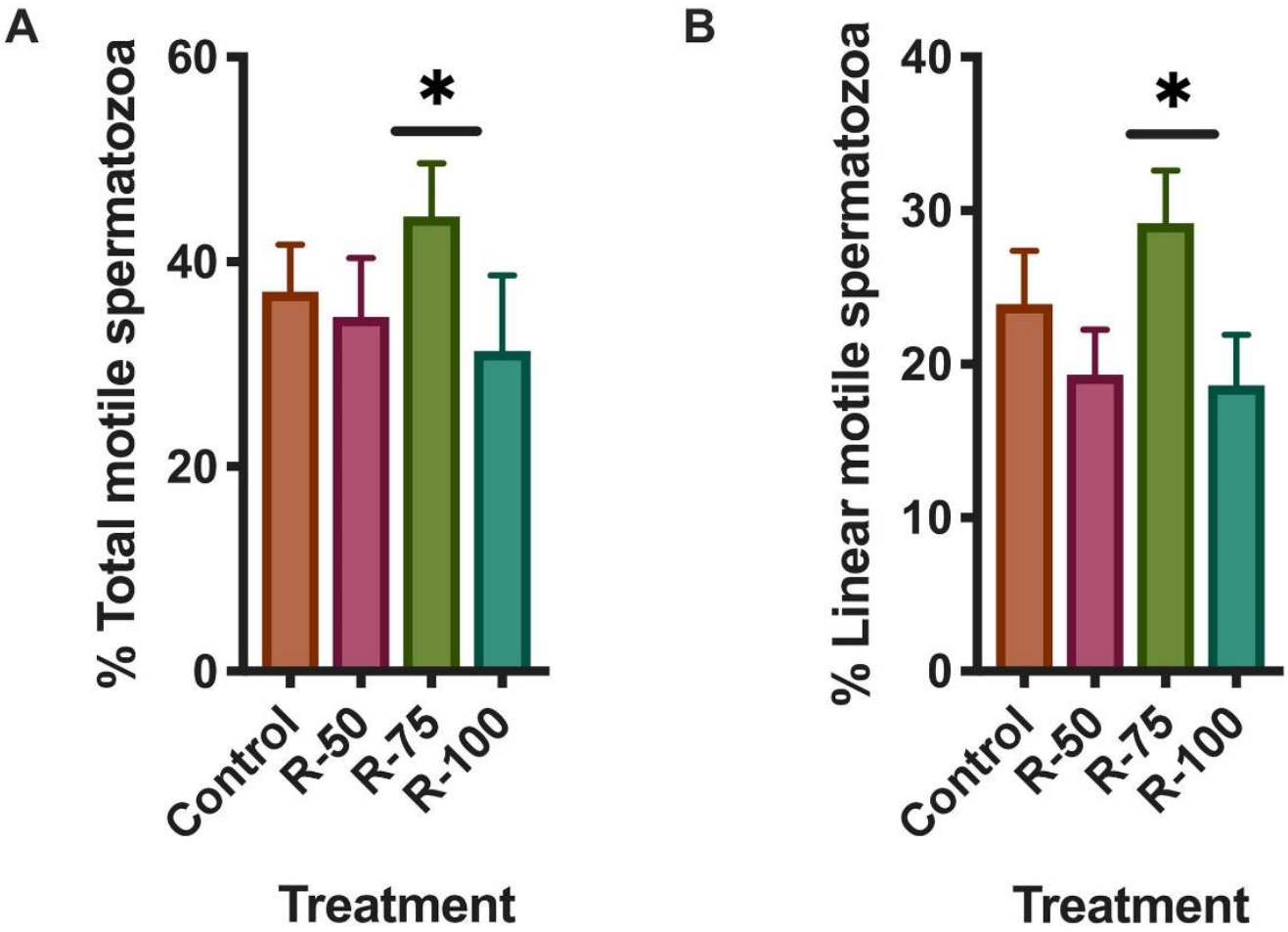

Fig 1. Effect of rosiglitazone added to the thawing media on stallion sperm motility. Samples were processed as described in the Materials and Methods and supplemented in the thawing media with rosiglitazone (0, 50, 75 and 100 μM) and then incubated at 37˚C for two hours; then the motility was evaluated using a computer assisted system (CASA). Rosiglitazone at 75 μM increased the percentage of total (A) linearly motile (B) spermatozoa ($P<0.01$) (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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The Netherlands) and placed on a warmed stage at 37˚C. The analysis was based on an evaluation of 60 consecutive digitalized images obtained using a 10× negative phase-contrast objective (Olympus CX 41). At least three different fields were recorded to ensure that at least 300 spermatozoa were analyzed per sample. Spermatozoa with a VAP (average velocity) <15 μm/s were considered immotile, while spermatozoa with a VAP > 35 μm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

Simultaneous detection of mitochondrial membrane potential and superoxide production

Aliquots of thawed spermatozoa were loaded with JC-1 (90 nM), CellROX deep red (2.5 μM) and Hoechst 33342 (0.25 μM) in the dark for 30 min at 22˚C [33]. The samples were then run in a flow cytometer (MACSQuant VYB Miltenyi Biotech) provided with yellow laser excitation; mitochondrial membrane potential and superoxide production were investigated only in live cells using H 33342 as a viability indicator [34] and a tool to gate out non sperm events. Positive controls consisted of samples supplemented with 10 μM oligomycin to inhibit the ATP synthase a prevent incorporation of H^+ in the mitochondrial matrix, while the negative controls were samples treated with 5 μM of the mitochondrial uncoupler CCCP.

Measurement of oxidation-reduction potential

Oxidation-reduction potential was measured using a RedoxSYS Diagnostic system (Englewood CO, USA). This is a novel technology that measures in 4 min the static oxidation reduction potential (sORP) by measuring the potential of an electrochemical cell under static conditions, followed by measuring the capacity of oxidation reduction potential (cORP), which is the total amount of readily oxidizable molecules [35]. This technique has already been validated to determine the oxidation reduction potential of the semen and spermatozoa [36–42]. In brief, 30 μL of spermatozoa was loaded in the sample port of the pre-inserted disposable sensor, and the measurement began at the moment of loading. After 4 minutes, the static oxidative-reduction potential (sORP) is provided in millivolts (mV). ORP is calculated with the Nerst equation $ORP = E^o - RT/nF \ln[Red]/[Ox]$, being E^o = standard reduction potential, R = universal gas constant, T = absolute temperature, n = number of moles of exchanged electrons, F = Faraday constant, [Red] = concentration of reduced species, [Ox] = concentration of oxidized species[41].

According to the manufacturer, sORP is measured while applying a low oxidizing current (1 nA) to the sample. After allowing 1 min and 50 s for equilibration, the reader measures twice per second over 10 s the difference in potential between the working and reference electrode in mV. Subsequently, cORP was measured by applying a linearly increasing oxidizing current until the charge rapidly changes between the working and reference electrode, indicating that all readily oxidizable molecules are fully oxidized. The time until the charge changes was used to calculate the number of electrons needed to cause charge changes and is reported in μCoulomb (μC). As controls, we used seminal plasma (rich in antioxidants)[1, 43–48], saline solution (lack of known antioxidants) and samples treated with vitamin E and menadione.

Staining for determination of live and dead cells and caspase 3 and 7 activity

This protocol has been developed in the laboratory where the present research was conducted and has been extensively described in previous publications [15, 16, 18, 49, 50]. In brief,

spermatozoa (5×10^6/mL) in 1 mL of PBS were stained with CellEvent 2 μM and 0.5 μM Hoechst 33342 and incubated for 25' in the dark at 22˚C. Then, 0.3 μM of Eth-1 was added to each sample and after incubation for 5 additional minutes the samples were immediately evaluated in a flow cytometer (Cytoflex flow cytometer, Beckman Coulter). CellEvent staining was validated using western blotting as described in [50]. Positive controls were obtained by incubating stallion spermatozoa at 37˚C for 3 hours in the presence of three known inductors of apoptosis [51–55], staurosporine 10 μM, thapsigargin 50 μM and betulinic acid 50 μM.

Simultaneous assessment of caspase 3 activity and phosphatidylserine (PS) translocation

Spermptotic changes were detected in spermatozoa with the use of Annexin V 674 conjugate (Molecular Probes, Leiden Holland), which detects the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane and is associated with membrane changes related to sperm processing and with the CellEvent Caspase 3/7 Green Detection Reagent. This consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. The activation of caspase 3 and caspase 7 proteins enables them to cleave the caspase 3/7 recognition sequence that is encoded in the DEVD peptide. Cleavage of the recognition sequence and binding of the DNA by the reagent labels apoptotic cells[56]. Both stains were combined in a multiparametric test and evaluated by FC[57]. A final concentration of 5×10^6 spermatozoa/ml was obtained by adding 10 μL of diluted spermatozoa to 990 μL of Annexin Binding Buffer. Then, the samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent (2 μM) and incubated at room temperature for 15 minutes. Next, the samples were washed by a short centrifugation spin for 12"and suspended in 200 μl of Annexin binding-buffer (solution in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). To 200 μL of sample per assay, 5 μL of Annexin V was added. After 15 minutes of incubation in the dark at room temperature, 400 μL of 1 × Annexin binding-buffer was added before reading it in the flow cytometer (Cytoflex flow cytometer, Beckman Coulter).

Detection of phosphorylated AKT (Ser ^{473}) in stallion spermatozoa

Samples were washed in PBS and fixed with 2% paraformaldehyde in PBS for 10 minutes at 4˚C; after fixation, the cells were washed (centrifuged at 473 ×g for 8 minutes at room temperature) twice with PBS and once with PBS-1% BSA, and permeabilized for 30 min in 0.1% saponin in PBS-1% BSA. Then, the samples were stained with 2 μL/ml of phospho-AKT Alexa fluor 488 conjugate (cat n˚ 4071, Cell Signalling Technology) and incubated in PBS-1% BSA for 30 min in the dark at room temperature. Samples were then washed again in PBS and analyzed in the flow cytometer (Cytoflex flow cytometer (Beckman Coulter). This flow cytometry protocol has been previously validated in our laboratory [18].

Flow cytometry

Flow cytometry analyses were conducted using a Cytoflex flow cytometer (Beckman Coulter) equipped with violet (405 nm), blue (488 nm) and red lasers (635 nm) and a MACSQuant VYB (Miltenyi Biotech) flow cytometer equipped with yellow (561 nm), violet (405 nm), and blue lasers (488 nm) as sources of excitation. The quadrants or regions used to quantify the frequency of each sperm subpopulation depended on the particular assay. Forward and sideways light scatter were recorded for a total of 50,000 events per sample. Gating the spermatozoa population after Hoechst 33342 staining eliminated nonsperm events. The instruments were calibrated daily using specific calibration beads provided by the manufacturers. A compensation overlap was performed before each experiment. Files were exported as FCS files and

analyzed using FlowjoV 10.4.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications from our laboratory [18, 58, 59].

Computational flow cytometry (t-SNE analysis)

Flow cytometry data are usually analyzed using a series of 2D plots and manual gating; however, the increase in the number of parameters measured increased the number of 2D plots to display for every marker combination. For example, a combination of four colors requires 30 2D plots. To overcome these problems, computational methods to automatically identify populations in multidimensional flow cytometry data have been developed [60]. Using Flowjo v 10.5.3 software (Ashland, OR, USA) compensated data of each multiparametric assay as described in the material and methods, the data were exported as FCS files from the flow cytometer and loaded into Flowjo for computational analysis. Data were downsampled, concatenated and single cell events analyzed. Flow cytometry data were analyzed using non-linear dimensional reduction techniques (t-SNE). This technique identifies clusters within multidimensional data without losing single cell resolution [61, 62], allowing for automatic gating of cells. Within the t-SNE maps generated, heat maps were applied to identify the expression of specific markers.

Statistical analysis

Frozen semen samples were obtained from 6 different stallions. All experiments were repeated at least three times with independent samples (three separate ejaculates from each of the donor stallions). The normality of the data was assessed using the Kolmogorov-Smirnoff test. Since the data were normally distributed, paired t tests and one-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Overton cumulative histogram subtraction was also performed [63] to determine positivity in selected cytometry analysis; in brief, this method determines the percent of the events that are considered to have positive florescence for the selected parameter by subtracting out the florescence of the control. Differences were considered significant when $P < 0.05$ and are indicated as the following: * $P<0.05$ and ** $P<0.01$. Results are displayed as the means ± SEM.

Results

Rosiglitazone in the thawing media improves sperm motility

Post thaw incubation with rosiglitazone (75 μM) showed significant improvements in motility after two hours of incubation ($P<0.05$, one way ANOVA, n = 18). Both the percentages of total motile spermatozoa (36.0 ± 5.0 in controls vs 45.0±5.2% in samples supplemented with rosiglitazone 75 μM) and linear motile spermatozoa showed significant improvements ($P<0.05$, one way ANOVA n = 18) with the treatment (Fig 1A and 1B). Other concentrations of rosiglitazone tested had no effect.

Rosiglitazone enhances mitochondrial function in thawed stallion spermatozoa

Mitochondrial impairment is a hallmark of thawed stallion spermatozoa [11, 22, 64, 65] and is also considered an early event in spermptosis [11]. To determine if rosiglitazone is able to improve mitochondrial function, thawed stallion spermatozoa were incubated in the presence of rosiglitazone (75 μM), and after one and two hours of incubation the mitochondrial

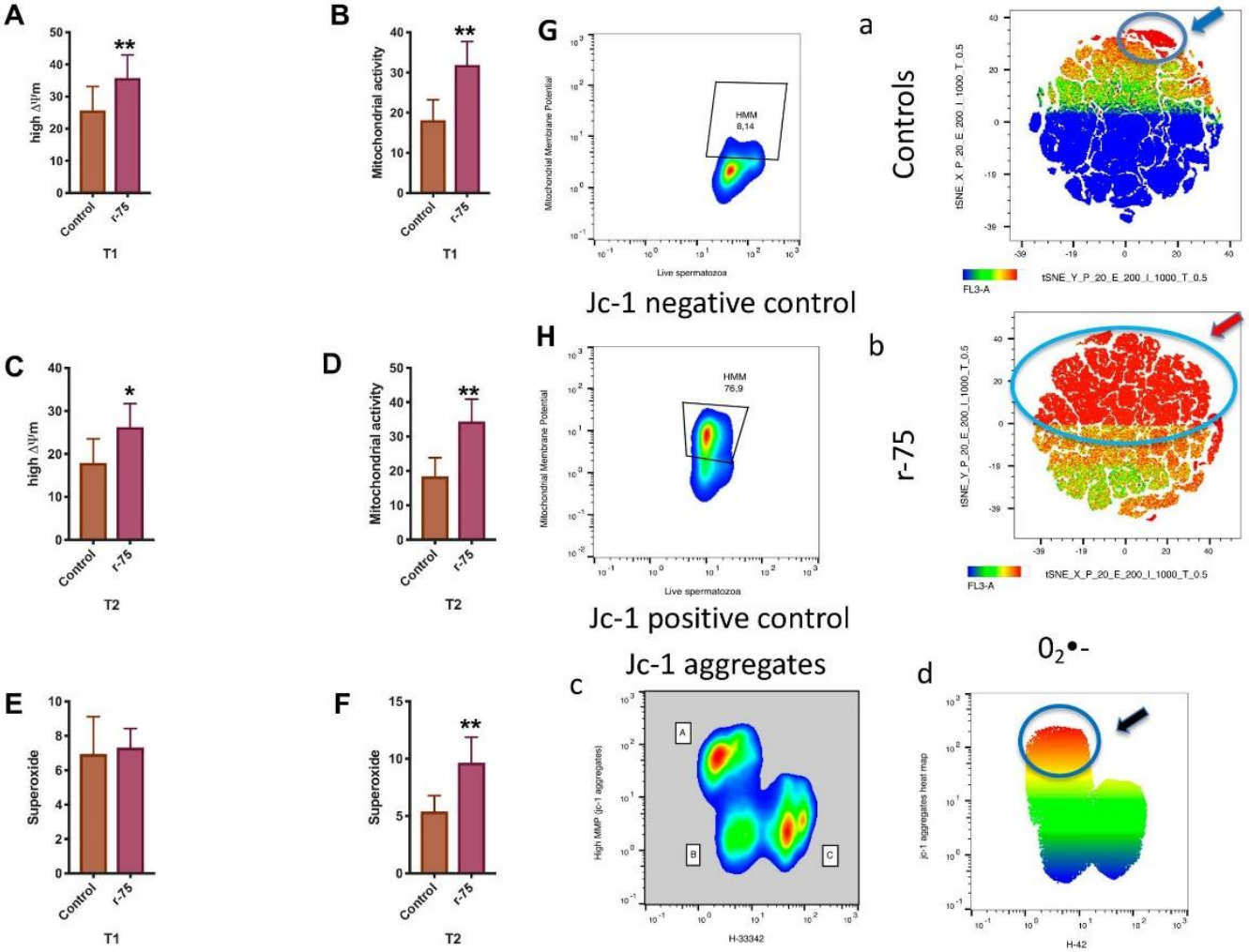

Fig 2. Effects of rosiglitazone added to the thawing media on mitochondrial function of stallion spermatozoa after thawing. Frozen stallion semen was thawed and processed as described in the Materials and Methods. Split samples were supplemented with rosiglitazone (0 and 75 μM) and mitochondrial functionality was investigated after 1 and 2 hours of incubation. A and C, percentage of spermatozoa showing orange fluorescence after JC-1 staining, B and D, mitochondrial functionality expressed as the mean fluorescence intensity in the PE channel indicative of JC-1 aggregates (high mitochondrial potential), E and F production of superoxide after 1 and 2 hours of incubation at 37˚C. The results are presented as the means ± SEM. * $P<0.05$, ** $P<0.01$. In a and b, the t-SNE map after computational analysis is shown; in the t-SNE map, each point represents individual spermatozoa in the sample, and the heat map applied to the t-SNE map shows increased PE fluorescence (jc-1 aggregates) in the rosiglitazone treated samples. Circles identify the populations of spermatozoa showing high ΔΨm. In c a representative 2D plot after JC-1/H33342 is presented, A live spermatozoa with high ΔΨm, B live spermatozoa, C dead spermatozoa. In d the same plot is presented but a heat map overlay of the APC channel (production of superoxide) is shown over the 2D plot depicted in c, maximum production of superoxide is present in live sperm showing high ΔΨm (orange events in the plot), and this population is also circled (black arrow). Controls for the JC-1 are presented; G negative controls that are samples treated with the mitochondrial uncoupler CCCP 5 μM. Positive controls are presented in H; they are samples treated with oligomycin 10 μM to inhibit the passage of H^+ to the mitochondrial matrix (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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function of the surviving spermatozoa was investigated using JC-1. Since the production of the superoxide anion (0_2•$^-$) is a byproduct of oxidative phosphorylation in the mitochondria [66, 67], the production of 0_2•$^-$ was concurrently investigated. Rosiglitazone significantly increased ($P<0.05$ and $P<0.001$, two tails paired t test, n = 18, after 1 and two hours of incubation, respectively) the mitochondrial potential of the surviving spermatozoa at both timepoints examined (Fig 2A–2D). Increased mitochondrial activity estimated as an increased presence of

JC-1 aggregates [56] also occurred ($P<0.05$ two tails paired t test n = 18) without concomitant increases in the production of $O_2\bullet^-$ after 1 hour of incubation (Fig 2E), but there was a significant increase ($P<0.01$, two tails paired t test, n = 18) in $O_2\bullet^-$ in the supplemented samples after 2 hours of incubation at 38˚C (Fig 2F). When the analysis was performed on a cell by cell basis of the whole sperm population, the heat map generated after the t-SNE analysis showed evident changes indicating that rosiglitazone increased mitochondrial activity (estimated as the number of JC-1 aggregates) compared with controls (Fig 2A and 2B) in the whole sperm population, although the changes varied in degree. Additionally, to identify the major source of $O_2\bullet^-$, a heat map was generated for superimposing the APC channel (CellRox deep red) over the JC-1/H33342 2D plot (Fig 2C), showing that the major production of $O_2\bullet^-$ occurred in the more active mitochondria (Fig 2D, blue circle and black arrow).

Effect of rosiglitazone on the oxidation reduction potential (sORP)

To determine if the increased production of superoxide is just caused by intense mitochondrial activity [66] or is a sign of oxidative stress, the oxidation-reduction status of the samples was investigated. No changes were observed in the static oxidation reduction potential (sORP) or in the total antioxidant capacity in the supplemented samples (Fig 3) (n.s., two tails paired t test, n = 18).

Rosiglitazone reduces caspase 3 activation without changes in phosphatidylserine transposition

Since it has been reported that caspase activation triggers sperm senescence [18], we studied the effect of rosiglitazone on caspase 3 activation and phosphatidylserine (PS) transposition; when thawing media was supplemented with 75 μM rosiglitazone, a significant decrease of >25% in respect to the initial values in controls after 1 and 2 hours of incubation occurred (two tails paired t test, n = 18, $P<0.05$, $P<0.01$, respectively) (Fig 4A and 4B). Study of the t-SNE map also showed a decrease in caspase 3 activation (Fig 5A and 5B). No changes induced by rosiglitazone were observed in PS (Fig 4C and 4D). The combined 2D dot plot and heat map of the Annexin-V fluorescence intensity revealed that most of the caspase 3 positive spermatozoa were also Annexin-V positive (Fig 4E, red circle).

Rosiglitazone phosphorylates Akt and increases the percentage of live non apoptotic spermatozoa

Previous findings from our laboratory linked the dephosphorylation of Akt to the activation of caspase 3 in ejaculated stallion spermatozoa [18]. We hypothesized that rosiglitazone may be linked to Akt phosphorylation. Incubation of thawed stallion spermatozoa in the presence of rosiglitazone maintained phosphorylated Akt after two hours of incubation at 37˚C in comparison with untreated controls (two tails paired t test, $P<0.05$, n = 18) (Figs 6 and 7). Furthermore, samples incubated in the presence of 75 μM rosiglitazone showed an increased percentage of live non apoptotic spermatozoa, 23.0 ± 1.9 in controls vs 35.0± 1.4% in 75 μM rosiglitazone supplemented samples ($P<0.05$, two tails paired t test, n = 18).

Inhibition of Akt, PPARγ and AMPK abolished the reduction of caspase 3 activation induced by rosiglitazone

The effects of rosiglitazone can be mediated by the PPARγ receptor and/or by the phosphorylation of the AMPK[68–70]. In both cases, Akt can be phosphorylated [29]. To determine if the effects observed from supplementing with rosiglitazone could be reverted by inhibiting

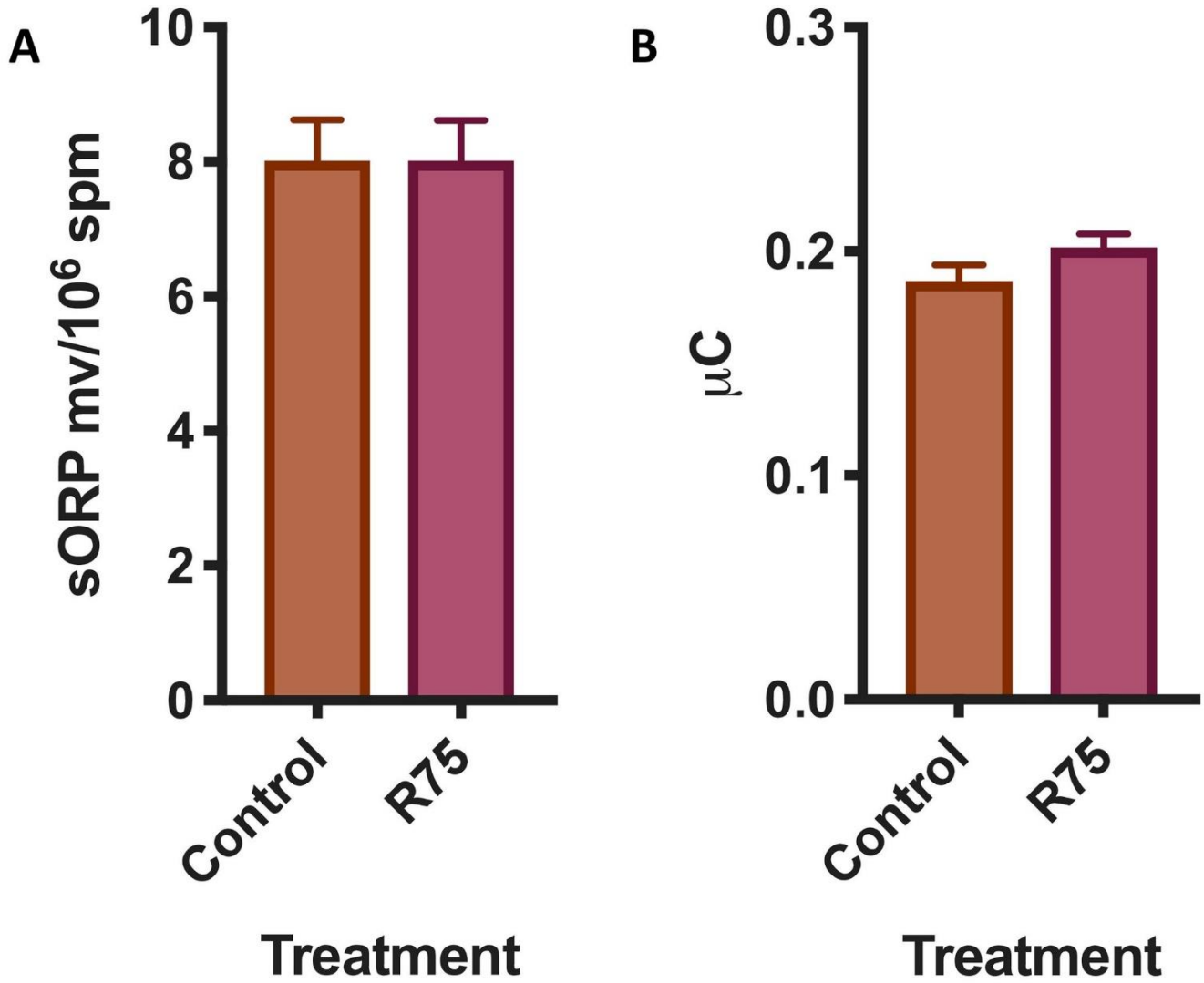

Fig 3. Effect of rosiglitazone added to the thawing media on sORP (mV/10^6 sperm) (A) that is the integrated measure of the existing balance between oxidants and reductants and (B) antioxidant capacity reserve cORP (μC) (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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PPARγ, pAMPK and AKT phosphorylation, samples were incubated in the presence of an Akt1/2 inhibitor (30 μM)[18], GW9662 (inhibitor of PPARγ, 10 μM)[27] and dorsomorphin (an inhibitor of AMPK, 100μM)[27] and then incubated in the presence of rosiglitazone 75 μM. As seen in the previous experiment, rosiglitazone reduced caspase 3 activation (one way ANOVA, n = 18, $P<0.01$); however, when the samples were incubated in the presence of rosiglitazone and the three inhibitors, at one and two hours of incubation the reduced caspase 3 activation induced by the rosiglitazone was no longer present (Fig 8A and 8B).

Discussion

In this study, we aimed to determine whether the quality of frozen stallion spermatozoa can be improved after thawing. Traditional approaches to improve sperm survival after freezing and

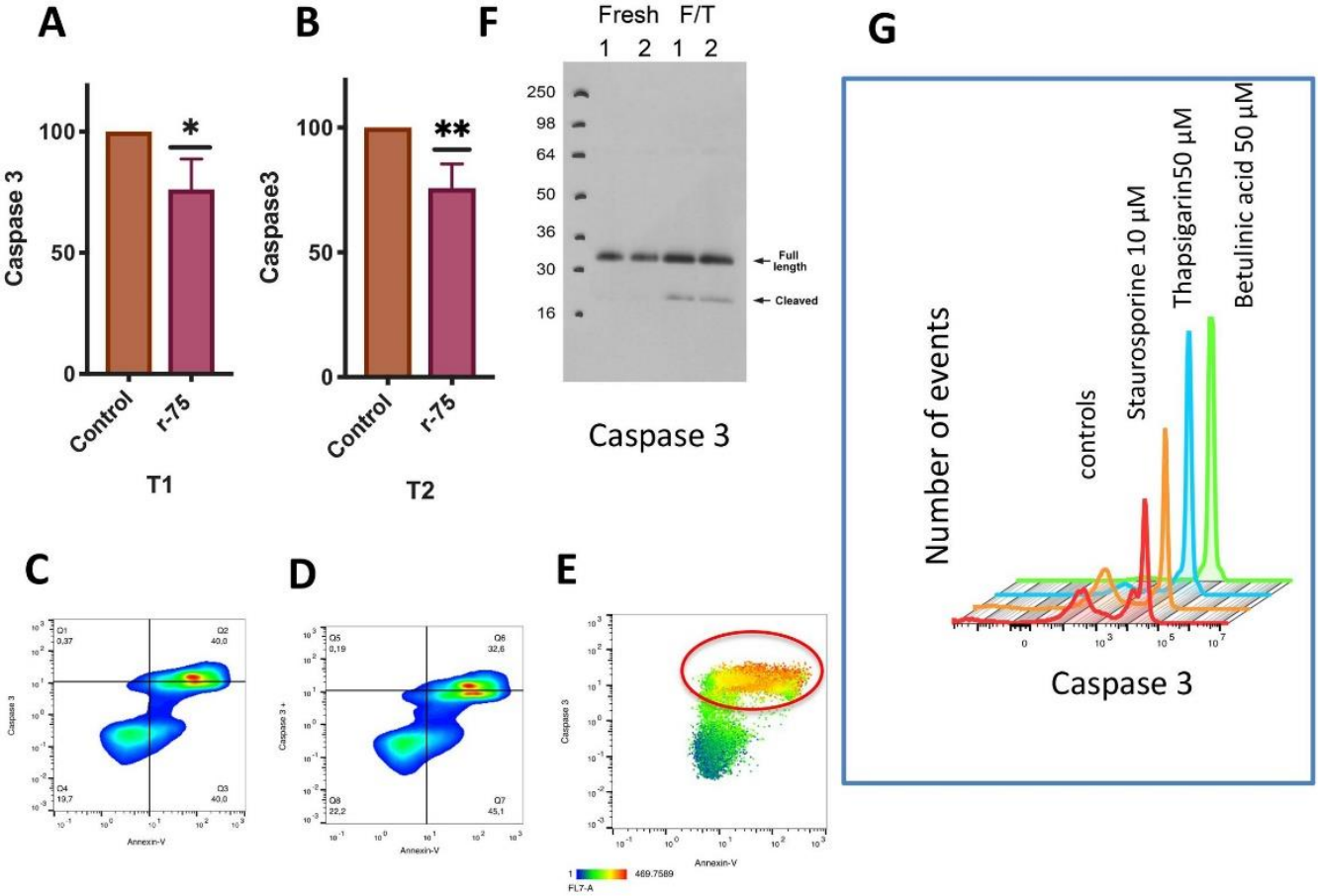

Fig 4. Changes in caspase 3 and phosphatidylserine (PS) transposition after rosiglitazone supplementation of the thawing media. Commercial frozen doses of stallion sperm were thawed and processed as described in the Material and Methods. Split samples were incubated in the presence of rosiglitazone 0 and 75 μM and caspase 3 activity was determined by flow cytometry. Data represent percent changes with respect the controls after 1 hour (A) and two hours (B) of incubation and are expressed as the means ± SEM, observed (* $P<0.05$, results are derived from three independent frozen ejaculates from 6 different stallions n = 18). F) Western blot (WB) controls for caspase 3 using frozen and thawed stallion spermatozoa as positive controls; semen was processed and analyzed as described in reference 50. (G) Further controls were obtained after incubating stallion spermatozoa at 37°C for 3 hours in the presence of three known inductors of apoptosis, staurosporine 10 μM, thapsigargin 50 μM and betulinic acid 50 μM. In C and D, representative cytograms of the simultaneous detection of active caspase 3 and PS transposition are presented where Q2 and Q3 represent events positive both for caspase 3 and Annexin-V. Q2 represents events with higher caspase 3 expression. No significant changes were detected. In E a heat map showing the intensity of Annexin-V staining demonstrates that PS is preferentially expressed in caspase 3 positive cells.

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thawing have focused on the improvement of extenders, sperm selection prefreezing or post thawing, and freezing and thawing rates [8, 71–77]. Few studies have focused on the development of methods to improve sperm quality after the thawing phase; moreover, few studies have addressed the biology of thawed spermatozoa. Our results show that the functionality of thawed stallion spermatozoa can be improved through the activation of pro-survival pathways; in particular, its mitochondrial function can be significantly improved using this strategy.

Recent studies point to stallion spermatozoa as highly dependent on intracellular thiols for their proper functionality [15, 30, 78] and highly dependent on oxidative phosphorylation in the mitochondria as the main source of ATP for motility, but they act mainly in maintenance of membrane functionality [21, 65, 67, 79, 80]. These facts have important implications for the selection of more fertile spermatozoa [67] and sperm conservation [20, 79–81]. The thawed

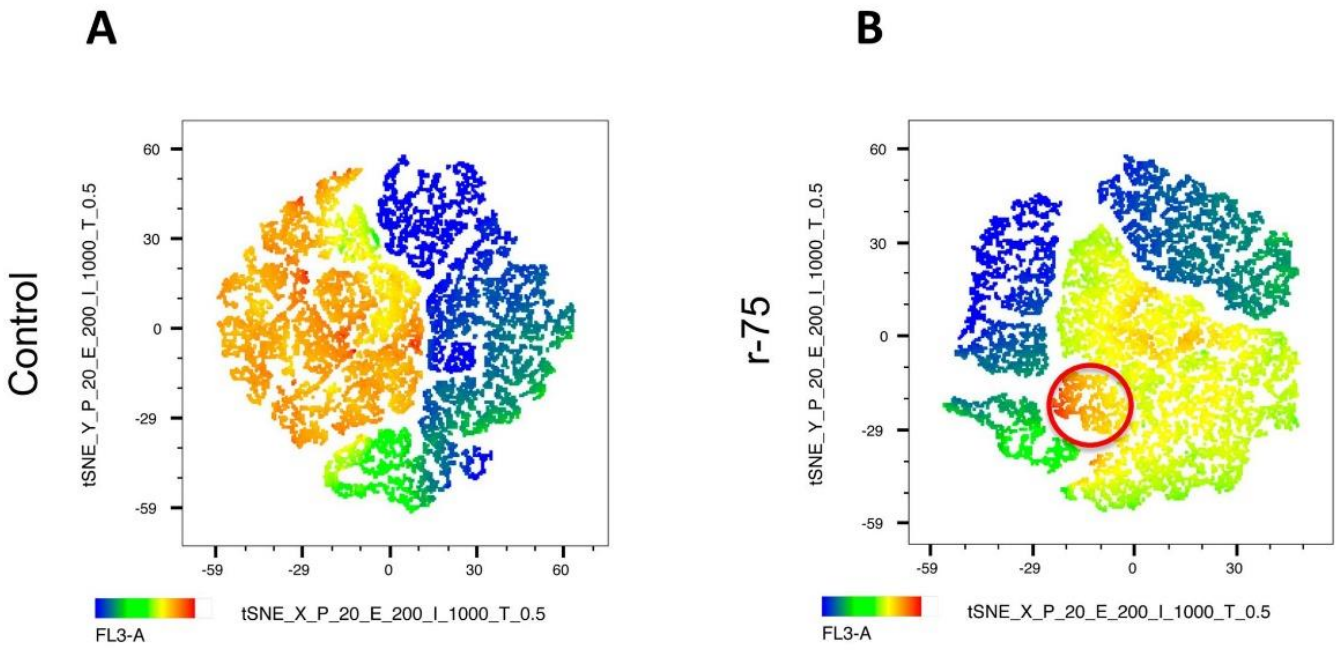

Fig 5. Computational cytometry analysis (t-SNE) graphics. Heat maps are presented showing the effect of 75 μM rosiglitazone supplementation on caspase 3 activity in thawed stallion spermatozoa. A, Control samples, each point represents an individual spermatozoa, and as seen in the heat map, almost half of the population shows high caspase 3 expression (orange color). B, Samples supplemented with 75 μM rosiglitazone, and as seen in the heat map, caspase 3 expression is reduced with only a small population with high caspase 3 (red circle) (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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spermatozoa are characterized by compromised mitochondrial function [11, 17] and an unstable redox status, leading rapidly to oxidative stress [1, 17, 48]. We aimed to induce metabolic flexibility to improve the functionality of thawed spermatozoa, a strategy that has proven successful in the conservation of stallion spermatozoa kept at ambient temperature for long periods [27]. Moreover, we studied the potential mechanisms behind this improvement. The PPAR γ agonist rosiglitazone induced clear improvements in mitochondrial function and reduced caspase 3 activity and these effects were also linked to increased phosphorylation of Akt. Previous reports indicate the importance of Akt phosphorylation in sperm function [18, 19, 82, 83] and recently a link between PPARγ agonists and Akt phosphorylation in human [28] and pig spermatozoa [29] has been reported. Moreover, strategies to maintain Akt phosphorylation in spermatozoa have proven to be successful in human sperm cryopreservation [82, 84].

The approach described in our work allowed us to maintain p-Akt (the phosphorylated form) in thawed spermatozoa. Moreover, the use of specific Akt inhibitors provided further support to the proposed relationship between p-Akt and proper sperm function, as reported for human [19, 28] and equine spermatozoa [18, 85]. It may be of practical importance to underscore the fact that phosphorylated Akt can be maintained in thawed sperm through the use of rosiglitazone; this fact may indicate that after thawing spermatozoa, in spite of the dramatic osmotic stress occurring during the procedure, the spermatozoa may maintain mechanisms to regulate their lifespan. This is an interesting finding, since it opens a new approach to develop strategies to improve the quality of frozen spermatozoa after thawing. The balance between survival or death pathways activation may depend on the capability to regulate redox homeostasis [16]. In different cellular models, Akt regulates mitochondrial function, and this

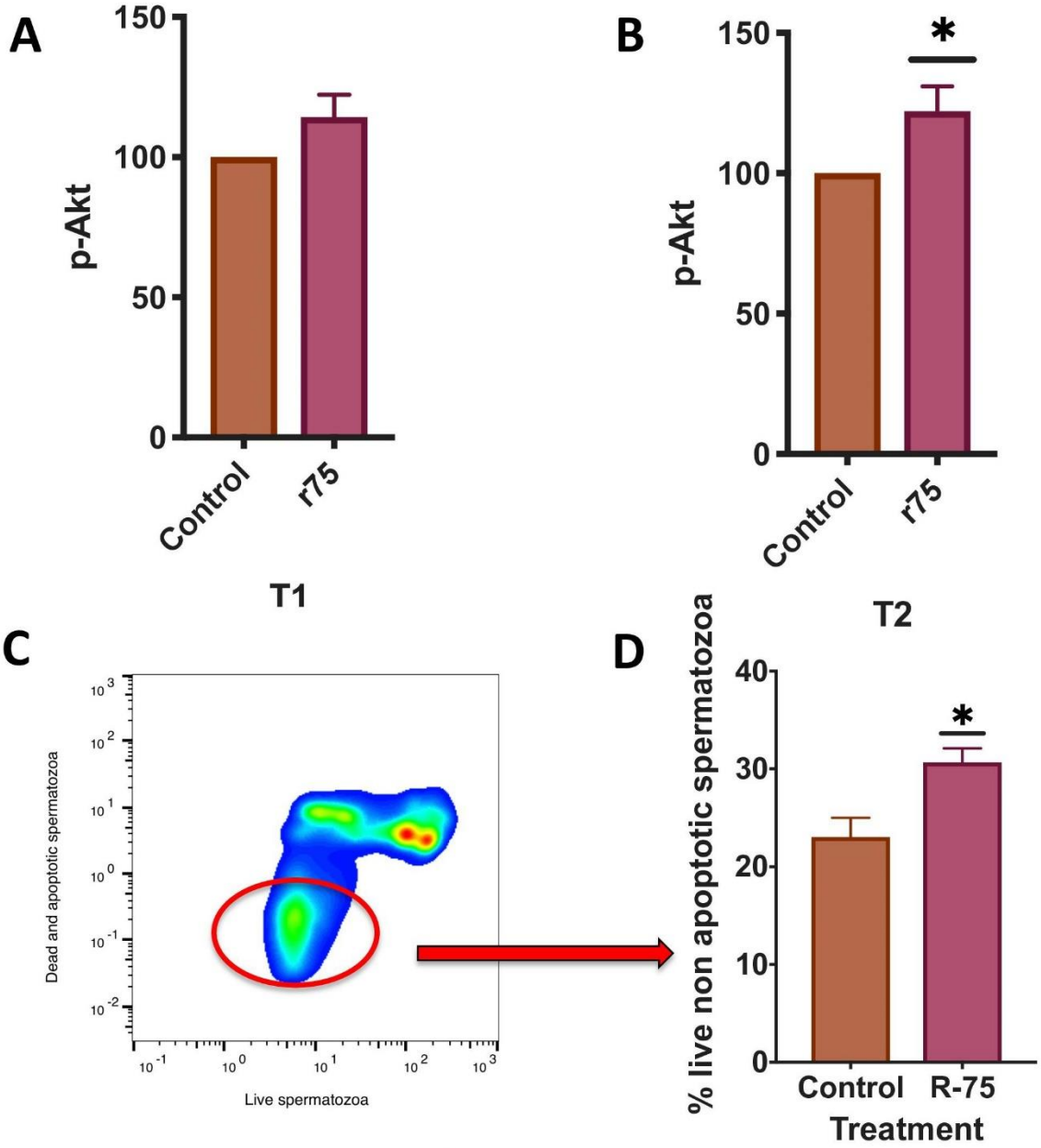

Fig 6. Effect of rosiglitazone on Akt phosphorylation (Ser ^{473}) on stallion spermatozoa and on the percentage of live non apoptotic spermatozoa (caspase 3 negative). Commercial frozen doses of stallion sperm were thawed and processed as described in the Materials and Methods. Split samples were incubated in the presence of rosiglitazone 0 and 75 μM and Akt phosphorylation was measured after 1 (A) and 2 hours (B) of incubation at 37˚C. Data represent percent changes with respect to the controls and are expressed as the means ± SEM * $P<0.05$. In C, a representative cytogram showing the identification of live non apoptotic spermatozoa is shown. Live spermatozoa are identified by the red circle. In D the effect of the incubation of stallion spermatozoa in the presence of rosiglitazone 75 μM is presented. Data represent percent changes with respect to controls and are expressed as the means ± SEM * $P<0.05$ (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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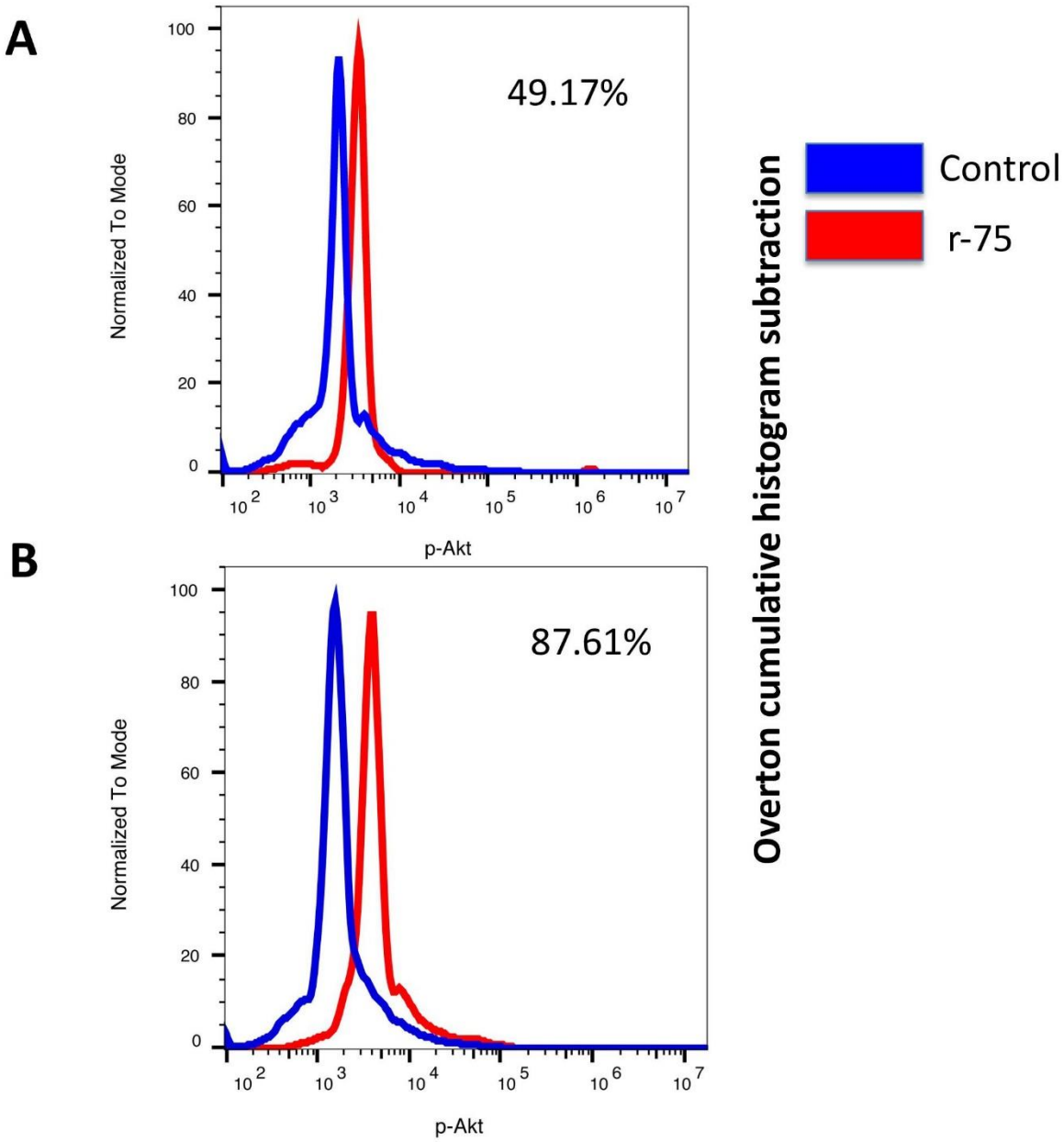

Fig 7. Representative overlay cytograms of the p-Akt assay after 1 hour (A) and 2 hours of incubation (B). To calculate the expression of the different germ cell markers we used the population comparison analysis available in FlowJo, version 10.4.1 (TreeStar, OR, USA). This analysis uses the Overton cumulative histogram subtraction algorithm (Overton, 1988) and overlaps histograms of the control (isotype control) and sample, allowing for subtraction of the control to calculate the percentage of positive cells in the sample (percentage of cells showing increased expression with respect to the controls) (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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regulation is not necessarily dependent on transcriptional activity [86], supporting the proposed mechanism described here of enhanced mitochondrial function after PPARγ agonist treatment in spermatozoa. Further supporting this hypothesis, treatment of stallion spermatozoa with Akt inhibitors prevented improvements after rosiglitazone treatment, as did

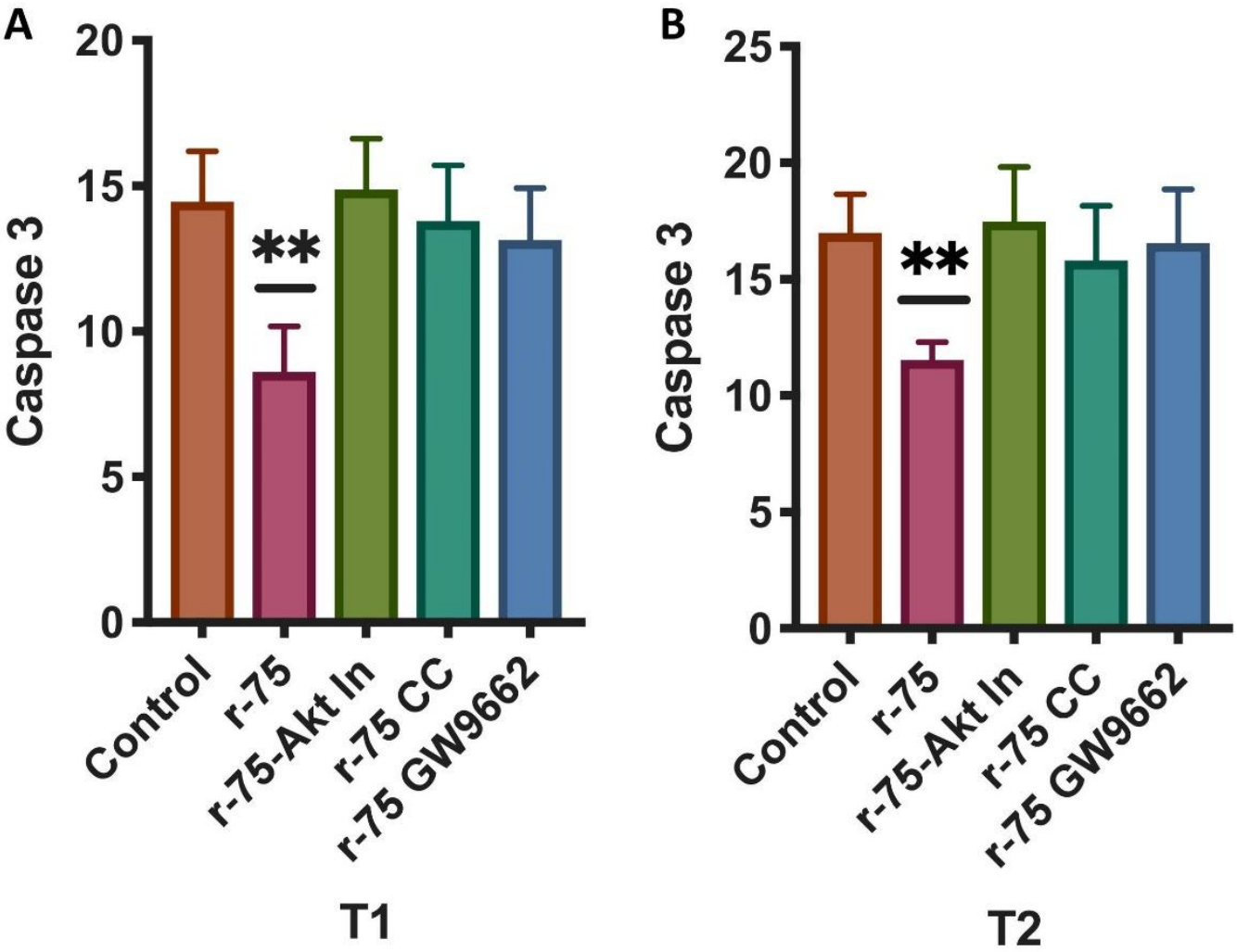

Fig 8. Effects of the Akt1/2 kinase inhibitor, dorsomorphin (AMPK inhibitor) and GW9662 (PPAR γ inhibitor) on caspase 3 inhibition after rosiglitazone treatment. Thawed semen doses were processed as described in the Materials and Methods and were incubated in the presence of rosiglitazone (0 and 75 μM) or rosiglitazone 75 μM plus an Akt kinase inhibitor 30 μM, rosiglitazone 75 μM plus GW9662 10 μM or rosiglitazone 75 μM plus dorsomorphin 100 μM. After 1 and 2 hours of incubation caspase 3 activity was determined using flow cytometry. The results are presented as the means ± SEM. * $P<0.05$ A) changes after 1 hour of incubation, B) Changes after 2 hours of incubation (results are derived from three independent frozen ejaculates from 6 different stallions n = 18).

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inhibition of PPARγ and AMPK, although a negative effect of the inhibitor in the absence of rosiglitazone cannot be excluded. However, the inhibitor treatments indicated that in stallions most of the effect of rosiglitazone may be related to AMPK activation, since this effect has been previously reported in stallion spermatozoa maintained in the liquid state [27]; in addition, the PPARγ inhibitor was less efficient at reverting rosiglitazone's effects. More interestingly, rosiglitazone enhanced mitochondrial function while maintaining redox homeostasis; although increased superoxide production was observed after two hours, the oxidation reduction potential sORP did not change, suggesting that, as previously reported [24], increased production of superoxide may be an indicator of intense mitochondrial activity. However, although superoxide production is somewhat mitochondrial specific, it can also be produced by NADPH oxidases in the sperm head. Additionally, there is some evidence that mitochondrial ribosomes in

the spermatozoa are both transcriptionally and translationally active [87], so the possibility that rosiglitazone may be acting through translational pathways warrants further investigation.

Cryopreservation depletes the thiols in spermatozoa, causing an unstable redox status that rapidly evolves to redox deregulation [88]. This situation induces caspase 3 activation and sperm death. The results reported here show that this form of sperm death can be delayed. In fact, we observed that supplemented samples showed a higher percentage of live non-apoptotic spermatozoa after two hours of incubation (Fig 6D). The positive outcome of rosiglitazone supplementation reported here can be attributed to the activation of metabolic flexibility. In this way, spermatozoa may be more effective at using glycolysis [27] and β oxidation of fatty acids [89] for energy production. Additionally, as revealed in our experiment, this pathway improves the efficiency of mitochondrial function. Mitochondrial function is considered a hallmark of functional spermatozoa [90–92], and more fertile stallion samples show more active mitochondria [67]; these reports support the concept that the quality and fertilizing ability of thawed samples can be modulated after thawing.

In conclusion, thawed stallion spermatozoa can be improved post thaw through mechanisms that maintain Akt in the phosphorylated state, which is a process that may involve AMPK and PPARγ activation. Moreover, these findings may have practical applications to improve the quality of thawed samples independently of the initial freezing protocol.

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PRESENTATION AT SCIENTIFIC CONFERENCE

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https://www.sciencedirect.com/science/article/pii/S073708061830251X?via%3Dihub

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Resources: Fernando J. Peña.

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Writing – original draft: Fernando J. Peña.

Writing – review & editing: Fernando J. Peña.

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Artículo 5

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Research Article

OXFORD

Research Article

Low glucose and high pyruvate reduce the production of 2-oxoaldehydes, improving mitochondrial efficiency, redox regulation, and stallion sperm function†

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Abstract

Energy metabolism in spermatozoa is complex and involves the metabolism of carbohydrate fatty acids and amino acids. The ATP produced in the electron transport chain in the mitochondria appears to be crucial for both sperm motility and maintaining viability, whereas glycolytic enzymes in the flagella may contribute to ATP production to sustain motility and velocity. Stallion spermatozoa seemingly use diverse metabolic strategies, and in this regard, a study of the metabolic proteome showed that Gene Ontology terms and Reactome pathways related to pyruvate metabolism and the Krebs cycle were predominant. Following this, the hypothesis that low glucose concentrations can provide sufficient support for motility and velocity, and thus glucose concentration can be significantly reduced in the medium, was tested. Aliquots of stallion semen in four different media were stored for 48 h at 18°C; a commercial extender containing 67 mM glucose was used as a control. Stallion spermatozoa stored in media with low glucose (1 mM) and high pyruvate (10 mM) (LG-HP) sustained better motility and velocities than those stored in the commercial extender formulated with very high glucose (61.7 ± 1.2% in INRA 96 vs 76.2 ± 1.0% in LG-HP media after 48 h of incubation at 18°C; $P < 0.0001$). Moreover, mitochondrial activity was superior in LG-HP extenders (24.1 ± 1.8% in INRA 96 vs 51.1 ± 0.7% in LG-HP of spermatozoa with active mitochondria after 48 h of storage at 18°C; $P < 0.0001$). Low glucose concentrations may permit more efficient sperm metabolism and redox regulation when substrates for an efficient tricarboxylic acid cycle are provided. The improvement seen using low glucose extenders is due to reductions in the levels of glyoxal and methylglyoxal, 2-oxoaldehydes

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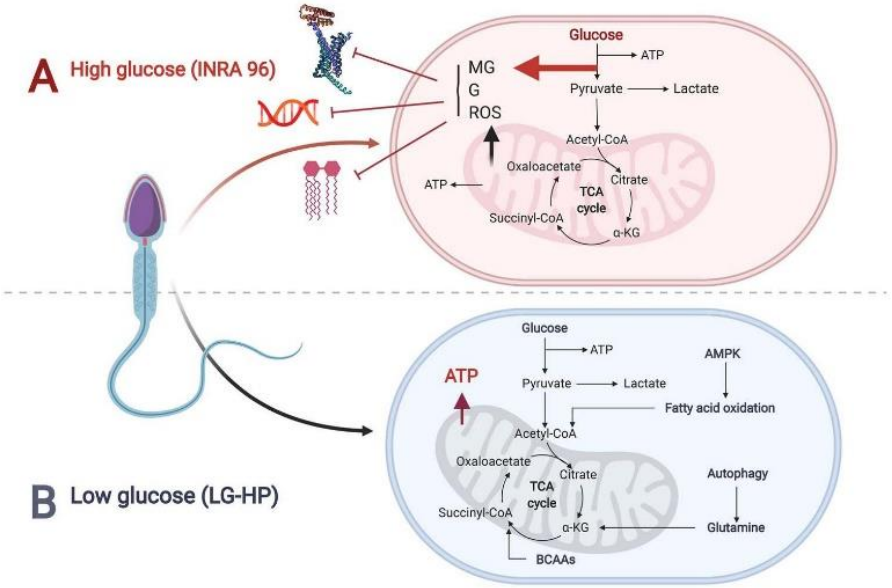
formed during glycolysis; these compounds are potent electrophiles able to react with proteins, lipids, and DNA, causing sperm damage.

Summary sentence

Hiigh glucose in the extender damages spermatozoa through formation of 2-oxoaldehydes.

Graphical Abstract



Key words: stallion, semen, flow cytometry, metabolism, ROS, GSH, glyoxal, methylglyoxal, glucose, pyruvate.

Introduction

For a long time, glucose metabolized through a purely glycolytic pathway has been considered the main source of energy for stallion spermatozoa, and thus, most commercial extenders have been formulated containing supraphysiological concentrations of glucose [1], with use recommended under anaerobic conditions. However, sperm metabolism is complex, as are the interactions between glycolysis, the Krebs cycle, and oxidative phosphorylation (OXPHOS) [2–5]. The application of proteomics and metabolomics to the study of spermatozoa reveals that this highly specialized cell has complex metabolic pathways and a much higher level of metabolic plasticity than previously thought [2, 6, 7]. Moreover, the link between metabolism and redox regulation arises as a key factor underlining sperm biology, as occurs in somatic cells [8, 9].

This recent research disputes the paradigm of the predominantly glycolytic metabolism of spermatozoa and indicates that lipids, together with carbohydrate and amino acid metabolism, contribute to the production of substrates for the Krebs cycle in male gametes [2, 10, 11]. Although glycolysis occurs in the cytosol, the final product of glycolysis, pyruvate, enters the mitochondria and is decarboxylated to acetyl coenzyme A by the pyruvate dehydrogenase complex, feeding the Krebs cycle in the mitochondrial matrix. The Krebs cycle then produces NADH and succinate, which are oxidized in the inner mitochondrial membrane during OXPHOS. The importance of OXPHOS for stallion spermatozoa is now well described, composing the principal source of ATP for sperm motility and the maintenance of membranes [12].

Mitochondria are thus key organelles in sperm metabolism, and they are also the source of important signaling molecules for proper sperm functionality, particularly regarding physiological levels of reactive oxygen species (ROS), which, if deregulated, may trigger the release of death signals [13–17]. New studies in somatic cells indicate that other molecules originating in the mitochondria play an important regulatory role, particularly through the release of mitochondrial DNA and tricarboxylic acid cycle (TCA) cycle metabolites into the cytosol [18]. Glycolysis is not a perfect process; the Emden–Meyerhof–Parmas pathway includes a series of steps for elimination of phosphates from the trioses phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [19], and during this process, glyoxal (G) and methylglyoxal (MG) are produced continuously, and their production is proportional to the concentration of glucose present [20–22]. These products are also generated during lipid metabolism; chemically, both are 2-oxoaldehydes, and due to their adjacent carbonyl groups, 2-oxoaldehydes are strong electrophiles that react rapidly and spontaneously with nucleophiles from proteins, lipids, and DNA, forming advanced glycation end products. These compounds are potentially cytotoxic and mutagenic.

Our hypothesis is that since current research indicates that stallion spermatozoa have limited glycolytic capacity, glucose levels in storage media for stallion spermatozoa can be significantly reduced. Moreover, we hypothesize that supraphysiological concentrations of glucose present in commercial extenders are in fact detrimental to spermatozoa due to increased production of 2-oxoaldehydes.

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Material and methods

Reagents and media

Monochlorobimane (MCB), methyl alpha-ketoglutarate, and all other chemicals were purchased from Sigma Aldrich (Madrid, Spain). All other reagents for flow cytometry were purchased from Thermo Fisher (Carlsbad, CA, USA). ViaKrome 808 Fixable Viability Dye was purchased from Beckman Coulter (Indianapolis, IN, USA). For mass spectrometry analysis, O-benzylhydroxylamine hydrochloride (O-BHA) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), glyoxal, and methylglyoxal were supplied by Sigma Aldrich (St. Louis, MO, USA). LC–MS grade formic acid and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Ultrapure deionized water (>18.2 MΩ·cm) was produced from a Millipore Milli-Q Gradient system (Millipore, Bedford, MA, USA).

Semen collection and processing

Semen was collected from five stallions of different breeds individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain, and maintained according to institutional and European animal care regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). All procedures used in this study received approbation from the ethical committee of the University of Extremadura. Ejaculates were collected using a prewarmed, lubricated Missouri model artificial vagina following standard protocols used at our center. After collection, the semen was immediately evaluated and processed in the adjacent laboratory. The ejaculate was extended 1:2 in INRA 96 extender (IMV, L'Aigle, France), centrifuged at $600 \times g$ for 10 min to remove the bulk of the seminal plasma, and then resuspended in basic Tyrode's media (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$.2H2O, 0.4 mM $MgSO_4.7H_2O$, 0.3 mM KH_2PO4, 20 mM HEPES, 5 mM glucose, 21.7 mM Na-L-Lactate, 1 mM Na Pyruvate, 15 mM NaH_2CO_3, 0.3% v/v bovine serum albumin) pH 7.4 [23] and various experimental media as outlined below. The original medium was named G, and the medium in which glucose was substituted by 2-deoxy-d-glucose was named 2-DG. All samples were adjusted to a concentration of 25×10^6 total spermatozoa/ml.

Experimental design

The sperm suspension was split into subsamples for control and experimental treatments: one glucose was substituted with 5 mM 2-DG, and two further experimental groups were treated with 5 mM 2-DG plus the permeable analog of oxoglutarate, dimethyl-oxoglutarate, at 20 or 100 µM. Samples were then incubated at 37°C, and after 1 and 3 h of incubation, aliquots were removed for measurement of sperm motility and velocities using computer-assisted sperm analysis (CASA), and viability, mitochondrial membrane potential, and Glutathione (GSH) content were measured using flow cytometry.

In a second set of experiments, aliquots of the same ejaculates were split into control and experimental samples. The commercial extender INRA 96 and Tyrode's media were used as controls, and aliquots of stallion spermatozoa were incubated in two modified versions of Tyrode's media: low glucose high pyruvate (LG-HP) and LG-HP supplemented with 100 mM methyl-oxoglutarate (LG-HP-OXO). The components of these media are given in Supplementary Table S1. Split samples of stallion spermatozoa were incubated at a concentration of 25×10^6 spermatozoa at 18°C for 48 h. Motility, velocities, viability, mitochondrial membrane potential, GSH content, Reactive Oxygen Species (ROS), static oxidation reduction potential (sORP), and total antioxidant capacity were measured after 24 and 48 h of storage at 18°C; this temperature was chosen to avoid cold shock to the membranes [24]. Additional independent ejaculates from the same stallions were collected and processed for the proteomic study. Finally, the levels of the 2-oxoaldehydes glyoxal (G) and methylglyoxal (MG) were measured using mass spectrometry (HPLC/MS) in ejaculates conserved in the media described in all the control and experimental samples. For all experiments, at least three independent ejaculates from each of the five stallions were collected and processed ($n = 15$ replicates).

Flow cytometry

Two different flow cytometers were used in this study. A Cytoflex LX flow cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with ultraviolet, violet, blue, yellow, red, and infrared lasers and a Cytoflex flow cytometer equipped with violet, blue, yellow, and red lasers were used. The instruments were calibrated daily using specific calibration beads provided by the manufacturer. Compensation for spectral overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowJo V 10.7 Software (Ashland, OR, USA). Unstained, single stained, and fluorescence minus one controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications from our laboratory [25–27].

Measurement of GSH, viability, and mitochondrial membrane potential

Intracellular GSH was measured using 10 µM MCB adapted to previously published protocols [28, 29] and optimized for GSH detection by flow cytometry [30], which was also adapted to equine spermatozoa by our laboratory [31, 32]. Mitochondrial membrane potential (JC-1) and sperm viability (DRAQ7) were also simultaneously assessed. In brief, sperm aliquots (2.5×10^6 sperm/ml) were stained with JC-1 to measure the mitochondrial membrane potential at 1 µM (20 min in the dark at room temperature (r.t.)), 10 µM MCB for the measurement of GSH, and 3 µM DRAQ7 for the measurement of live spermatozoa (10 min in the dark at r.t.). Briefly, after assessment of flow quality, doublets and debris were gated out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates), and DRAQ7 at a peak excitation of 640 nm and emission at 690 nm. Unstained and single-stained spermatozoa were used to set compensations and regions of interest as previously described in our laboratory [33].

Measurement of GSH, viability, mitochondrial membrane potential, and ROS

In this protocol, stallion spermatozoa washed in phosphate-buffered saline (PBS) (2.5×10^6 sperm/ml) were stained with 1 µM JC-1 for the measurement of mitochondrial membrane potential, 10 µM MCB for the measurement of GSH, ViaKrome 808 for measurement of live and dead spermatozoa (2.5 µl of the reconstituted solution as indicated by the manufacturer), and CellRox Deep Red at 5 µM for the measurement of ROS and were incubated at 37°C for 30 min (JC-1, CellRox Deep Red, and ViaKrome 808) and 10 min at r.t. with MCB. Briefly, after assessing flow quality, doublets, and debris were gated out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates), and 488 nm excitation

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and 530 nm emission (monomers), CellRox Deep Red at 644 nm excitation and 655 nm emission, and Viakrome 808 at 854 nm excitation and 878 nm emission. After each incubation period, the samples were washed and resuspended in PBS before they were run through a flow cytometer. Unstained, single-stained, and stimulated (positive controls) spermatozoa were used to set compensations and regions of interest as previously described in our laboratory [34, 35]; the use of a different laser for excitation of every probe (and two for excitation of JC-1) allowed a five-color experiment with minimal spectral overlap. For all the probes used, the percentages of positive spermatozoa were measured, except for MCB (GSH measurement), for which the relative fluorescence units (r.f.u) were measured. The gating strategy is depicted and fully described in Figure 6.2. In brief, after measurement of flow quality and elimination of debris and doublets, live spermatozoa were identified (Figure 6.2B), and for mitochondrial membrane potential (Figure 6.2F–H) and GSH content (Figure 6.2I–L), measurements were only performed on the live population, whereas analysis of ROS production (Figure 6.2M–P) was performed on the whole population (live and dead spermatozoa). FCS files were exported to FlowJo V 10.7 Software (Ashland, OR, USA) for further analysis. All of the events from every single replicate for each experimental group were concatenated together in a single FCS and analyzed; dot plots are presented as density plots comprising 1×10^6 events (spermatozoa) each.

Analysis of the proteins with metabolic function

Independent ejaculates were used to study the proteome of stallion spermatozoa, paying special attention to proteins involved in the regulation of sperm metabolism. Proteomic analysis of stallion spermatozoa was performed as described in previous publications [10, 36]. In brief, proteins were extracted from stallion spermatozoa and analyzed using UHPLC/MS/MS (Agilent 1290 Infinity II Series UHPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high-speed binary pump coupled to an Agilent 6550 Q-TOF mass spectrometer. Data processing and analysis were performed using the Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA).

Identification of proteins with metabolic function in stallion spermatozoa

Due to the increased detail available for the human proteome in terms of annotation, the equine annotations obtained in the previous step were transformed to their human orthologs using g: Profiler (https: //biit.cs.ut.ee/gprofiler/orth). Pathway enrichment analysis and visualization were also performed using g: Profiler (https: //biit.cs.ut.ee/gprofiler/gost), and Cytoscape analysis was performed using Reactome (https: //reactome.org).

Network analysis

The Cytoscape (https: //cytoscape.org) plug-in CLueGO was used to identify functionally grouped Gene Ontology terms in sperm proteins as previously described [37, 38]. A kappa score of 0.52 was used, and pathways were set at $P < 0.01$.

Measurement of oxidation–reduction potential

Oxidation–reduction potential was measured using the MioxSYS diagnostic system (Englewood, CO, USA) [39–41]. This technology measures the sORP over 4 min, measuring the potential of an electrochemical cell under static conditions, followed by measurement of the antioxidant capacity reserve (cORP), the total amount of readily oxidizable molecules. In brief, 30 µl of the sperm suspension was loaded onto the sample port of the preinserted disposable sensor, at which point measurement began. After 4 min, the sORP is provided in millivolts. According to the manufacturer, sORP was measured while applying a low oxidizing current (1 nA) to the sample. After allowing 1 min and 50 s for equilibration, the reader took two measurements per second over a 10 s period of the difference in potential between the working and reference electrodes in millivolts. Subsequently, cORP was measured by applying a linearly increasing oxidizing current until the charge rapidly changed between the working and reference electrodes, indicating that all readily oxidizable molecules were oxidized and providing a measurement of antioxidant capacity reserve (cORP) [41]. The time until the charge changed was used to calculate the number of electrons needed to cause charge changes and is reported in microCoulombs.

UHPLC/MS measurement of glyoxal and methylglyoxal

The sperm pellet containing 100×10^6 spermatozoa was resuspended in 300 µl of Milli-Q water and sonicated for 5 s. Immediately afterwards, it was centrifuged at $6272 \times g$ at 4°C for 3 min, and the supernatant was subjected to a derivatization procedure. The derivatization reagent was O-BHA coupled to EDC, which catalyzed the derivatization reaction. This reagent is suitable for derivatizing acids, ketones, and aldehyde groups. The derivatization was carried out following the procedure for the analysis of short-chain fatty acids [42] with modifications. Specifically, 100 µl of supernatant was incubated with 20 µl of 0.1 M BHA in MeOH and 20 µl of 0.25 M EDC in MeOH at 35°C for 1 h. After incubation, 50 µl was diluted 20-fold in 50% methanol, and 500 µl of dichloromethane was added to liquid–liquid extraction. Finally, a volume of dichloromethane (containing the derivatized analytes) was evaporated until dry. The residue was reconstituted in 100 µl of 50% aqueous MeOH and vortexed briefly, and 5 µl was injected on UHPLC–MS/MS. The effectiveness of the process was evaluated. Calibration was carried out by a standard addition technique. Therefore, the standards for glyoxal and methylglyoxal were prepared in supernatant samples spiked with increasing amounts of each, following the same derivatization procedure. The derivatized glyoxal and methylglyoxal (BHA-Gly and BHA-MGly) were analyzed by UHPLC–MS/MS. An Agilent 1290 Infinity II UHPLC coupled with 6470 triple quadrupole (QqQ) (Agilent Technologies, Waldbronn, Germany) was used. The UHPLC was equipped with a built-in autodegasser, binary pump, and column thermostat. A Zorbax C18 column, 100 × 2.1 mm, 1.8 µm (Agilent, CA, USA) was used for the separation step at 25°C. The LC–MS interface was ESI with jet stream. Nitrogen was used as the nebulizing gas, drying gas, sheath gas, and collision gas. The mobile phase was composed of two solutions: A, aqueous with 0.5% formic acid, and B, acetonitrile with 0.5% formic acid. A binary gradient was applied with a flow rate of 0.4 ml/min: 0–5 min 20% B, 5–10 min linear increase from 20 to 100% B, and maintained until minute 12, followed by re-equilibration of the column up to minute 15. BHA-Gly and BHA-MGly were eluted at 6.4 and 6.8 min, respectively. The ionization source parameters, operating in positive polarity, were optimized by injecting 3 mg/l BHA-Gly and BHA-MGly. The best sensitivity was obtained with the following ionization source parameters: drying gas temperature at 200°C, nebulizer at 25 psi, drying gas flow at 12 l/min, sheath gas temperature and flow rate at 350°C and 10 l/min, respectively, capillary voltage at 3500 V and fragmentor to 110 V. The MRM conditions were optimized by

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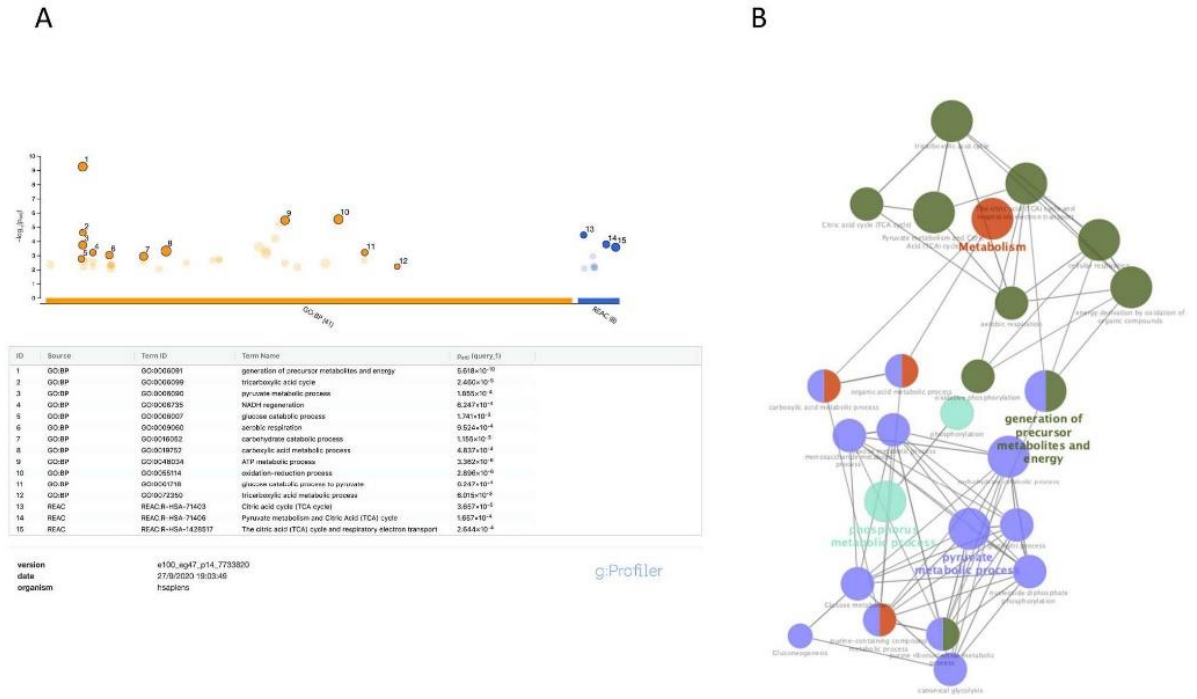

Figure 1. (A) g: GOST multiquery Manhattan plot showing comparative enrichment analysis of sperm proteins involved in metabolism. Gene Ontology (GO) for biological processes (BP) in orange and Reactome pathways in dark blue (all using human orthologs) are given. The *P* values are depicted on the *y*-axis and in more detail in the results table below the image. (B) CLueGO network analysis of proteins in stallion spermatozoa involved in metabolism. To reduce the redundancy of GO terms, the fusion option was selected. Reactome pathways of functionally grouped networks with terms are indicated as nodes (Benjamini–Hochberg *P* value < 0.01), linked by their kappa score level (≥ 0.52), where only the label for the most significant term per group is shown.

injecting the same solution at different collision energies (CE). The transitions were from 269 to 91 (at 20 eV CE) and to 144.1 (at 10 eV CE) for BHA-Gly and from 283 to 91 (at 25 V CE) and to 181.1 (at 10 eV CE) for the selected MRMs to determine sensitivity and selectivity. The quantification transitions were 269–91 and 283–91 for BHA-Gly and BHA-MGly, respectively.

Statistical analysis

Sperm samples were obtained from ejaculates from five different stallions. All experiments were repeated at least three times with independent samples (three separate ejaculates from each of the donor stallions). The normality of the data was assessed using the Kolmogorov–Smirnoff test. One-way analysis of variance followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.00 for Mac, La Jolla, CA, USA (www.graphpad.com); in this way, every treatment was compared with each sample's own controls and all other treatments. Differences were considered significant when $P < 0.05$, and the results are displayed as the mean ± standard error of mean.

Results

Characterization of the metabolic proteome of stallion spermatozoa

Analysis of Gene Ontology of biological processes and Reactome pathways enriched in proteins with metabolic function of stallion spermatozoa using human orthologs revealed the key role of pyruvate and the Krebs cycle (Figure 1A and B). The Reactome pathways most significantly enriched were the citric acid cycle REAC:R-HAS-71403, pyruvate metabolism and the citric acid (TCA) cycle REAC:R-HAS-71406, and the citric acid (TCA) cycle and respiratory electron transport REAC:R-HSA-1428517 (Figure 1A). Network analysis using Cytoscape and the CLueGO app also revealed the major role of pyruvate metabolism (Figure 1B).

Inhibition of glycolysis improves the percentage of live spermatozoa

Split samples from the same stallion and ejaculate were incubated for up to 3 h in the presence of 5 mM glucose (G) and 5 mM 2-DG, a nonmetabolizable glucose analog, and the percentage of live spermatozoa was evaluated by flow cytometry. When G was substituted by 2-DG, a significant increase in the percentage of live spermatozoa was observed after 1 and 3 h of incubation at 37°C, from 59.7 ± 3.6% in samples incubated in the presence of glucose to 70.5 ± 0.63% in samples incubated in the presence of 2-DG after 1 h of incubation ($P < 0.05$; Figure 2A).

Inhibition of glycolysis modifies intracellular GSH and improves mitochondrial function

Since recycling of oxidized glutathione (GSSG) depends on the availability of reducing power in the form of NADPH, which can be produced in the pentose phosphate pathway (PPP), we investigated whether inhibition of glycolysis affected intracellular GSH.

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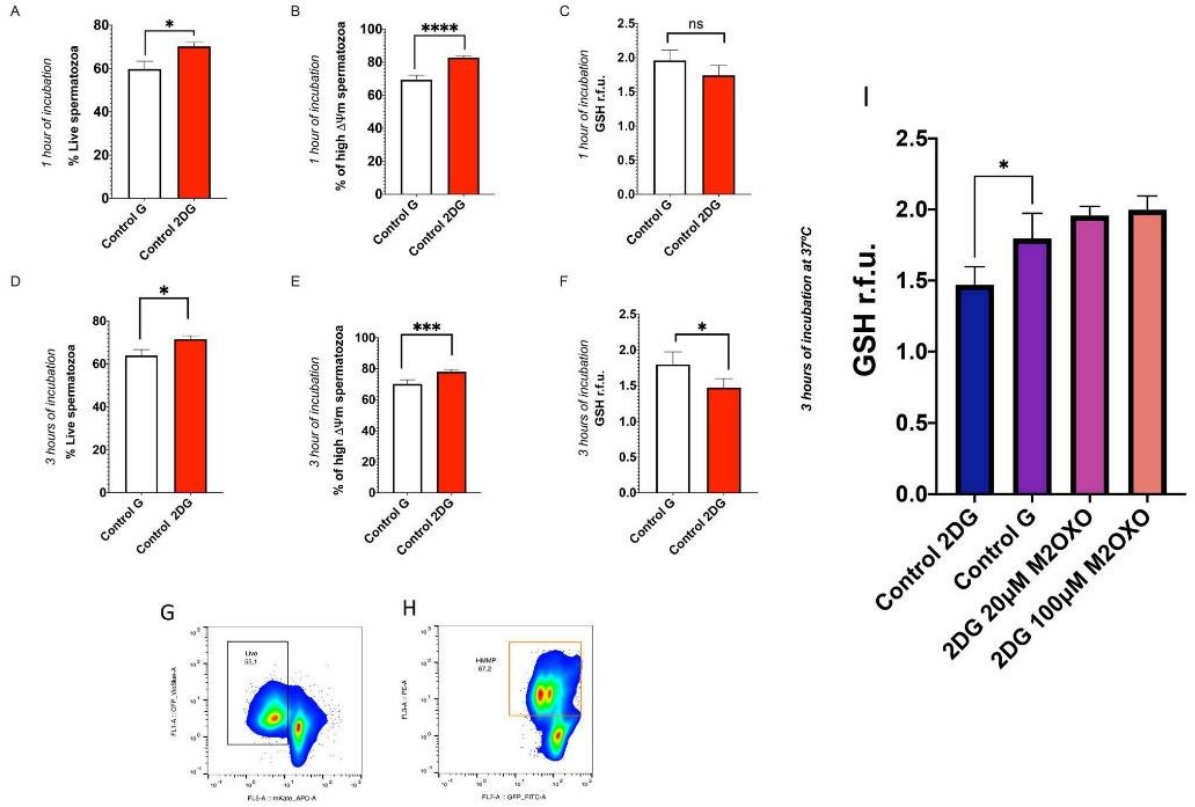

Figure 2. Effect of the inhibition of glycolysis on the viability, mitochondrial membrane potential, and relative GSH content in stallion spermatozoa. Stallion spermatozoa were processed as described in the Materials and Methods, and split aliquots were incubated in the presence of 5 mM glucose or 5 mM 2-deoxyglucose, a glycolysis inhibitor. After 1 h of incubation at 37°C, inhibition of glycolysis resulted in improved viability (A) ($P < 0.05$), better mitochondrial membrane potential (B) ($P < 0.0001$), and no changes in relative GSH content (C). After 3 h of incubation, inhibition of glycolysis resulted in more viable spermatozoa (D) ($P < 0.05$), improved mitochondrial membrane potential (E) ($P < 0.001$), and reduced GSH content (F) ($P < 0.05$). G and H are representative cytograms of the assays. (I) Effect of oxoglutarate on GSH content. The addition of 20 and 100 µM methyl-oxoglutarate reverted the reduction in GSH when glycolysis was inhibited.

It was observed that substitution of G by 2-DG did not modify intracellular GSH in stallion spermatozoa after 1 h of incubation (Figure 2C), but reduced intracellular GSH after 3 h of incubation, from 1.8 ± 0.17 in G samples to 1.5 ± 0.12 in 2-DG samples ($P < 0.05$; Figure 2F). Interestingly, this difference was due to the maintenance of the amount of GSH in glucose samples, since the amount of GSH remained fairly constant in glucose-treated aliquots (Figure 2C). The percentage of spermatozoa with high mitochondrial membrane potential ($\Delta\Psi m$) increased in samples containing 2-DG, both after 1 and 3 h of incubation. After 1 h, the percentage of spermatozoa with high $\Delta\Psi m$ was increased from 67.1 ± 6.5 in samples containing glucose to $82.6 \pm 2.2\%$ in samples containing 2-DG ($P < 0.0001$; Figure 2B). After 3 h of incubation, the percentage of high $\Delta\Psi m$ increased from 70.1 ± 2.6 to $78.3 \pm 1.4\%$ ($P < 0.001$; Figure 2E).

Oxoglutarate maintains GSH in the absence of glucose

Since reducing equivalents can be obtained via different metabolic pathways, we investigated whether oxoglutarate can rescue the drop in GSH in the absence of glucose. Stallion spermatozoa were incubated for 3 h at 37°C in the presence of glucose, 2-DG, and in the presence of 2-DG with 20 or 100 µM methyl-oxoglutarate, a cell permeable form of oxoglutarate. The GSH content (as the mean florescence intensity units) was 1.8 ± 0.17 in samples supplemented with glucose, and this dropped to 1.4 ± 0.12 in media containing 2-DG ($P < 0.05$). Methyl-oxoglutarate prevented the drop in GSH when glucose was substituted by 2-DG, where the GSH content was 2.0 ± 0.9 r.f.u at 100 µM (Figure 2I).

Inhibition of glycolysis reduces sperm motility and velocity

When G was substituted by 2-DG after 1 h of incubation at 37°C, there was a drop in the percentage of motile spermatozoa from 84.5 ± 2.0 to $65.8 \pm 3.2\%$ ($P < 0.001$; Figure 3A and B). The velocities of motile spermatozoa were also reduced. In samples incubated in media containing glucose, after 1 h of incubation at 37°C, circular velocity (VCL) was 200.7 ± 9.9 µm/s, whereas in samples in which glucose was substituted by 2-DG, VCL dropped to 102.2 ± 2.7 µm/s ($P < 0.001$; Figure 3C–E).

Low glucose concentrations maintain motility for longer periods of time

Previous experiments showed that stallion spermatozoa can survive without glucose, although motility and especially velocity were

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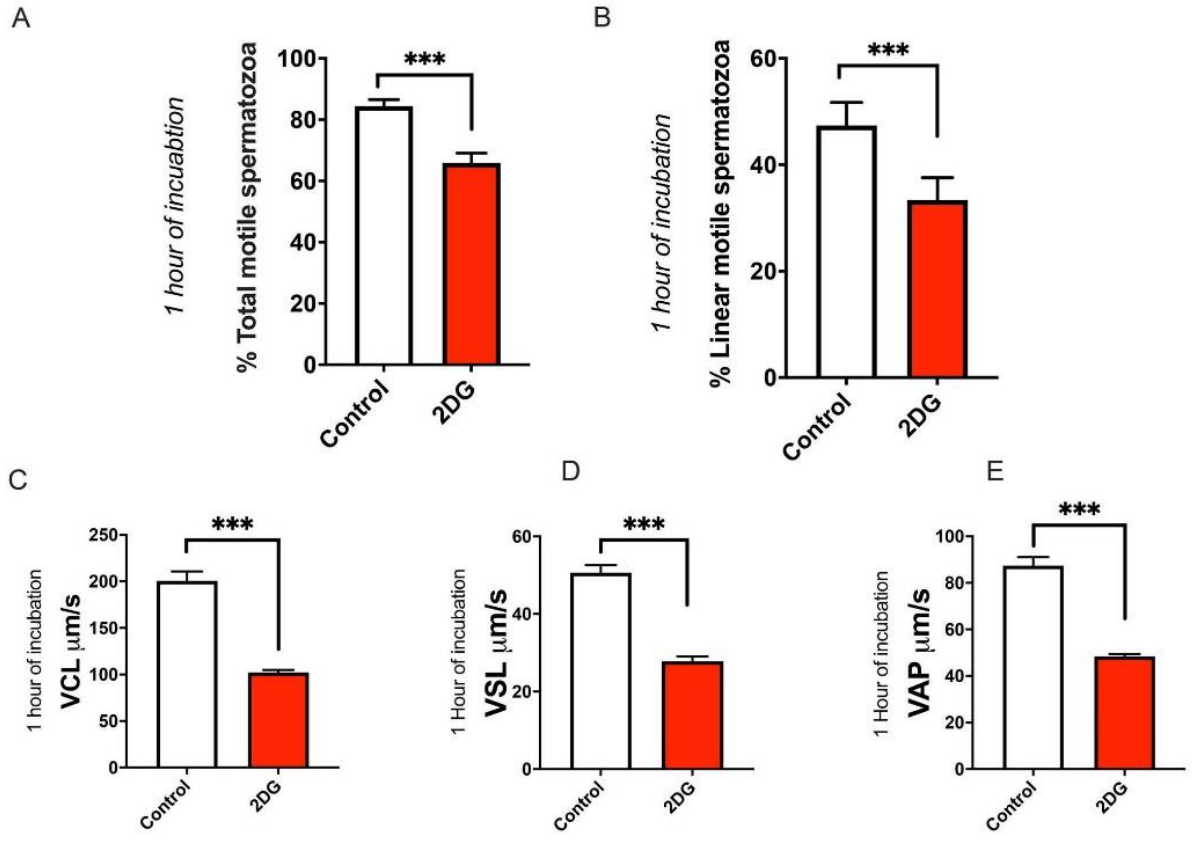

Figure 3. Effect of glycolysis inhibition on stallion sperm motility and velocities. Inhibition of glycolysis by 2-DG resulted in reduced percentages of total motile A ($P < 0.001$) and linearly motile B ($P < 0.001$) spermatozoa. Sperm velocities were also reduced; circular velocity (VCL) C, straight-line velocity (VSL) D, and average path velocity (VAP) E were reduced (*** $P < 0.001$).

reduced. To test whether a reduced amount of glucose is sufficient to maintain motility, we developed low glucose media supplemented with pyruvate and oxoglutarate that readily feed the TCA cycle. The commercial extender INRA 96 was used as a control, and aliquots of stallion spermatozoa were incubated in Tyrode's media and two modified versions of Tyrode's media: LG-HP and LG-HP supplemented with 100 µM methyl-oxoglutarate (LG-HP-OXO). The components of these media are given in Supplementary Table S1. Split samples of stallion spermatozoa were incubated at a concentration of 25×10^6 spermatozoa at 18°C for 48 h. Motility and velocities were measured after 24 and 48 h. Motility was better in all variants of Tyrode's media than in INRA 96 after 24 and 48 h. After 24 h of storage at 18°C, total motility was 68.4 ± 1.11% in INRA 96, 77.9 ± 1.1% in Tyrode's ($P < 0.0001$), and 79.2 ± 1.0% ($P < 0.0001$) and 76.7 ± 1.2% ($P < 0.01$) in LG-HP and LG-HP-OXO, respectively (Figure 4A-B). The percentage of linear motile spermatozoa followed the same tendency, and the most significant differences with respect to the INRA 96 control were observed in the LG-HP and LG-HP-OX groups (Figure 4B and D). Aliquots extended to INRA 96 and Tyrode's showed percentages of linear motility of 53.0 ± 1.6 and 58.0 ± 1%, respectively ($P < 0.01$), and samples extended to LG-HP and LG-HP-OX showed significantly higher linear motility: 60.3 ± 0.86 and 60.0 ± 1.1%, respectively ($P < 0.01$ and $P < 0.0001$) (Figure 4B). After 48 h of storage, better motilities were observed in spermatozoa stored in the LG-HP groups. Total motility was 61.7 ± 1.2% in the INRA 96 group and 76.2 ± 1.0% in samples stored in the LG-HP extender ($P < 0.0001$; Figure 4C). Interestingly, the motility values were more homogeneous in the LG-HP extender (Figure 4C, red circle). The percentage of linear motile spermatozoa after 48 h of storage followed the same tendency (Figure 4D).

Low glucose concentration improves sperm velocities in stallion spermatozoa stored for long periods

VCL was significantly higher in all of the Tyrode's-based extenders than in INRA 96 at every time point considered (Figure 5A). Circular motility after 24 h of incubation at 18°C was 112.3 ± 2.2 µm/s in aliquots conserved in INRA 96, whereas it was 214.5 ± 6.9 ($P < 0.001$), 226.7 ± 7.6 ($P < 0.0001$), and 212.3 ± 6.8 µm/s ($P < 0.0001$) in samples conserved in Tyrode's, LG-HP, and LG-HP-OX, respectively. A similar pattern was observed in the straight-line (VSL) and average path (VAP) velocities after 24 h of conservation at 18°C (Figure 5B and C). After 48 h of storage at 18°C, the tendency was maintained with better velocities in all Tyrode's-based extenders; VCL was 111.6 ± 4.3 µm/s in the INRA 96 extender, 191.1 ± 3.57 µm/s in Tyrode's ($P < 0.0001$), and 203 ± 3.2 µm/s in LG-HP ($P < 0.0001$), which was also significantly different from Tyrode's ($P < 0.01$). In samples extended in LG-HP-OX, the VCL after 48 h of storage was 168.7 ± 6.4 µm/s ($P < 0.001$). The VSL

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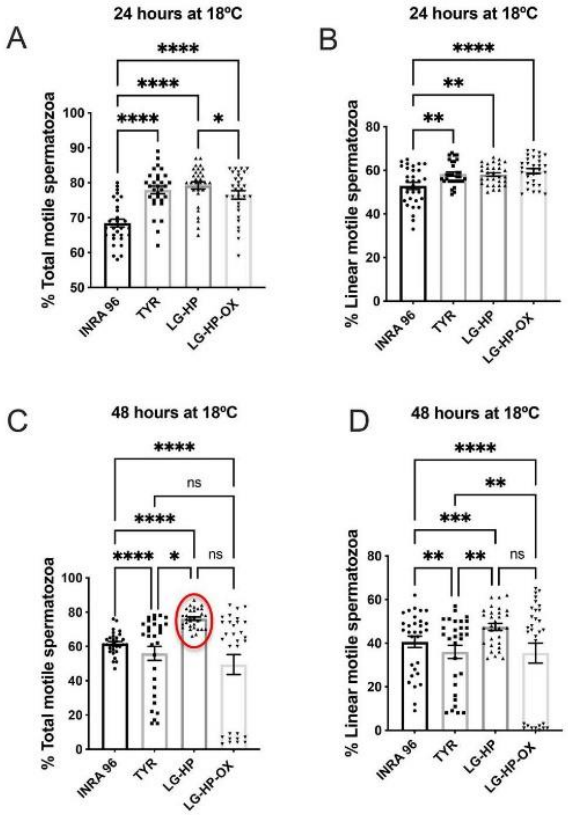

Figure 4. Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP), both supplemented (LG-HP-OX) and unsupplemented with oxoglutarate, sustains motility. Aliquots from the same ejaculate were stored at 18°C for 48 h in a commercial extender, INRA 96, in Tyrode's media (Tyr), and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10 mM) LG-HP and a modified LG-HP supplemented with 100 µM methyl-oxoglutarate, LG-HP-OX. (A–C) Percentages of total motile spermatozoa after 24 and 48 h of storage at 18°C. Interestingly, the motility values were more homogeneous in the LG-HP extender (C, red circle). (B–D) Percentages of linear motile spermatozoa after 24 and 48 h of storage at 18°C. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

and VAP followed the same trend as VCL (Figure 5C–E), but in this case, VSL and VAP were also significantly improved in LG-HP ($P < 0.001$) with respect to samples stored in Tyrode's basal medium.

Low glucose concentration improves mitochondrial membrane potential in stallion spermatozoa stored for long periods

Media formulated with low glucose showed an improved capacity to sustain high mitochondrial activity in stallion spermatozoa. After 24 h of storage at 18°C, the percentage of spermatozoa with high mitochondrial membrane potential was 25.4 ± 2.0% in samples extended in INRA 96, whereas this was 58.4 ± 4.0% ($P < 0.0001$), 63.5 ± 4.9% ($P < 0.0001$), 64.6 ± 5.9% ($P < 0.0001$), and 62.7 ± 529% ($P < 0.0001$) in samples stored in Tyrode's, LG-HP, and LG-HP-OX media, respectively. The same effect was maintained after 48 h of storage (Figure 6), but at this point in time, samples stored in LG-HP media also showed better mitochondrial membrane potential than samples stored in Tyrode's medium (51.1 ± 0.7 vs 43.6 ± 1.5%; $P < 0.01$). After 24 h of storage at 18°C, samples stored in Tyrode's medium showed lower viability than those stored in INRA 96 medium ($P < 0.001$). After 48 h of storage, samples stored in LG-HP-OX medium showed lower viability ($P < 0.05$).

Low glucose concentrations reduce ROS and increase GSH content in stallion spermatozoa

In low glucose extenders, the percentages of live cells producing significant amounts of ROS were lower than in samples extended in INRA 96 or Tyrode's media. After 24 h of storage at 18°C, the percentage of cells producing ROS was 61.1 ± 1.4 and 64.3 ± 1.3% in aliquots stored in INRA 96 and Tyrode's medium, respectively. In samples stored in LG-HP and LG-HP-OX, the percentage of ROS-positive cells dropped to 45.4 ± 4.7% ($P < 0.01$) and to 40.1 ± 5.9% ($P < 0.01$; Figure 6D). At the same time, GSH content increased in LG-HP extenders, from 10 162 ± 731.7 r.f.u in INRA 96 to 15 553 ± 912 and 15 242 ± 1327 r.f.u in samples stored in LG-HP and LG-HP-OX, respectively ($P < 0.001$, $P < 0.01$). ROS production and GSH content followed the same trend after 48 h of storage (Figure 6G and H). GSH content was higher in Tyrode's and LG-HP-OX extended samples after 48 h of incubation (Figure 6G).

Total antioxidant capacity reserve is higher in LG-containing media

The sORP and the antioxidant capacity reserve (cORP) were measured after 48 h of storage at 18°C. Samples stored in LG-HP-OX showed a slight, although significant, increase in the sORP with respect to those stored in INRA 96 (12.3 ± 0.1 vs 11.7 ± 0.0 mV/10^6 spermatozoa; $P < 0.001$). On the other hand, samples stored in Tyrode's and LG-HP media showed an increased antioxidant capacity reserve ($P < 0.05$; Supplementary Figure S1B).

Production of glyoxal and methylglyoxal is reduced in LG-HP media

The production of glyoxal and methylglyoxal 2-oxoaldehydes was measured after 24 and 48 h of conservation at 18°C in stallion spermatozoa extended in different media with varying glucose concentrations. After 24 h of incubation, the concentration of G was 1.7 ± 0.1 ng/100 × 10^6 spermatozoa in aliquots extended in INRA 96 and 1.1 ± 0.1 ng/100 × 10^6 spermatozoa in samples extended in LG-HP medium ($P < 0.01$; Figure 7A). The same effect was observed after 48 h of storage at 18°C ($P < 0.01$; Figure 7B). The production of methylglyoxal was also reduced in media with low glucose; after 24 h of incubation, the concentration of MG was 2.3 ± 0.2 ng/100 × 10^6 spermatozoa in aliquots stored in INRA 96, whereas it was 1.3 ± 0.2 ng/100 × 10^6 spermatozoa in aliquots stored in LG-HP and LG-HP-OXO ($P < 0.05$; Figure 7C). After 48 h of incubation, the production of MG was reduced in all media with respect to INRA 96, and the concentration of MG was 2.7 ± 0.3 ng/100 × 10^6 spermatozoa in samples stored in INRA 96, 1.8 ± 0.3 ng/100 × 10^6 spermatozoa in aliquots stored in Tyrode's ($P < 0.05$), 1.6 ± 0.2 ng/100 × 10^6 spermatozoa when stored in LG-HP ($P < 0.05$), and 1.8 ± 0.4 ng/100 × 10^6 spermatozoa in aliquots extended in LG-HP-OX ($P < 0.05$; Figure 7D).

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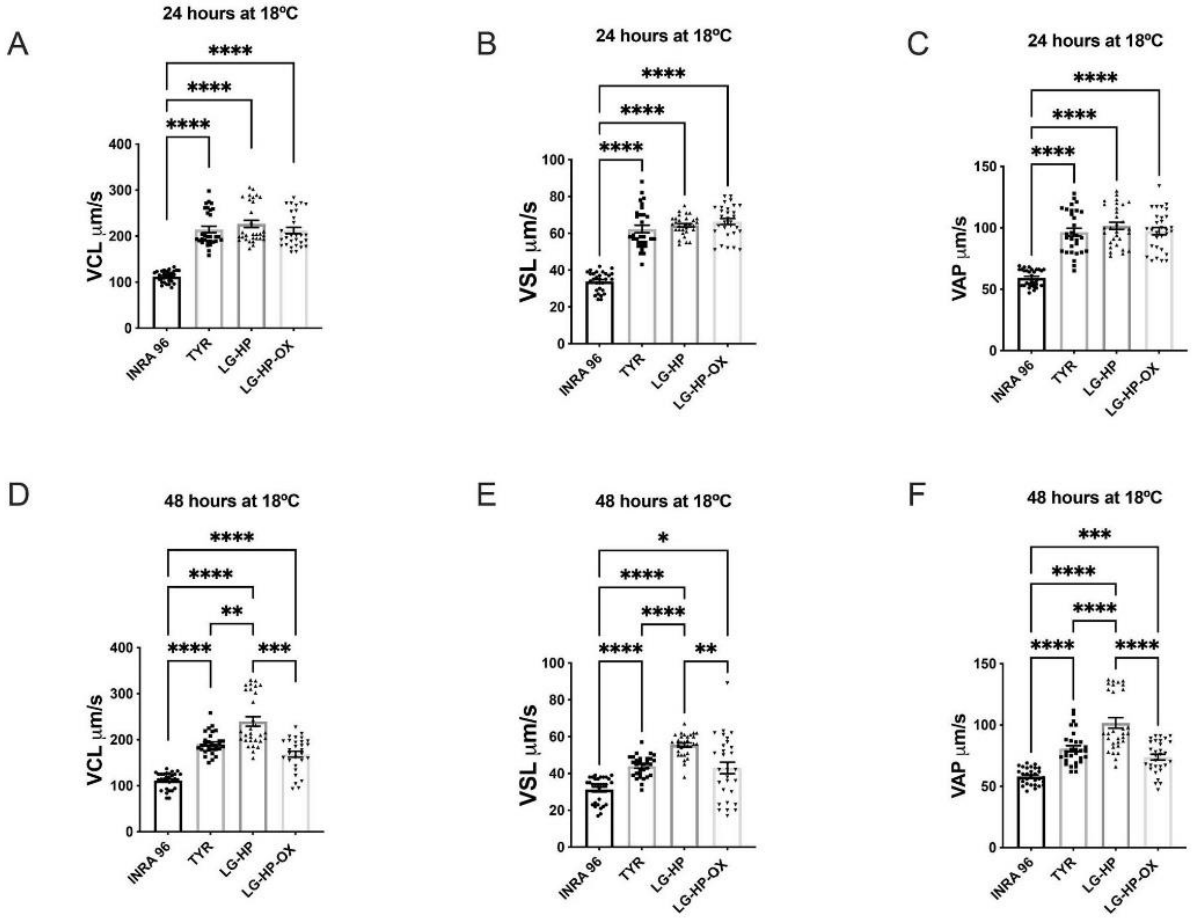

Figure 5. Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP), both supplemented (LG-HP-OX) and unsupplemented with oxoglutarate, sustains sperm velocities. Aliquots from the same ejaculate were stored at 18°C for 48 h in a commercial extender, INRA 96, in Tyrode's media (Tyr), and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10 mM) LG-HP and a modified LG-HP supplemented with 100 µM methyl-oxoglutarate, LG-HP-OX. (A–D) Circular velocity (µm/s) (VCL) after 24 and 48 h of storage at 18°C. (B–E) Straight-line velocity (µm/s) (VSL) after 24 and 48 h of storage at 18°C. (C–F) Average path velocity (µm/s) (VAP) after 24 and 48 h of storage at 18°C; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Discussion

Although current research indicates that glycolysis is not the main pathway producing ATP in stallion sperm metabolism, commercial extenders for stallion spermatozoa are formulated with very high glucose concentrations [1]. The main role of glycolysis is to provide precursors for the Krebs cycle under aerobiosis [43]. However, the role of glycolysis in supporting glycolytic enzymes all along the flagella should also be considered [4, 5].

In this study, the major pathways in the metabolic proteome of stallion spermatozoa were examined, showing the predominance of the Krebs cycle and pyruvate metabolism. Moreover, in the present study, inhibition of glycolysis had no effect on sperm viability or mitochondrial activity; in fact, it caused significant improvements in both parameters. Despite the fact that motility and velocity parameters were reduced under this condition, these findings argue in favor of the importance of the numerous glycolytic enzymes localized in the flagella [5], supporting sperm motility through glycolysis [12]. However, the use of 2-DG may cause ATP depletion due to the action of the sperm hexokinase phosphorylating 2-DG, making it difficult to establish the real cause of low ATP levels.

In a previous study [16], we showed that reduced motility and velocities in the presence of 2-DG were not present in media without glucose, suggesting that reduced motility and velocities caused by 2-DG were most likely due to ATP depletion due to futile 2-DG phosphorylation. Although after 1 h of incubation there were no changes in GSH, inhibition of glycolysis caused reduced GSH content after 3 h of incubation at 37°C. This finding is probably explained by reduced flux to the PPP and thus diminished generation of reduced power in the form of NADPH to recycle GSSG into GSH [44]. However, NADPH can be generated in the Krebs cycle. To test whether this occurs in the spermatozoa, aliquots were incubated in the presence of the cell permeable analog of oxoglutarate, methyl-oxoglutarate. This compound was able to restore GSH to levels comparable with those observed when glycolysis was not inhibited.

Overall, these results may suggest that important plasticity exists in stallion spermatozoa and underlines the close interplay between oxidant species and metabolism [44]. In view of these previous findings, we tested the hypothesis that extenders with a low glucose concentration could be effective for the long-term preservation of spermatozoa. For this, aliquots of stallion ejaculates were extended

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in different low glucose media at 18°C for 48 h. A commercial extender for equine semen that has been successfully used at this temperature [24] and contains 67 mM glucose in HEPES-supplemented Hank's solution [1] was used as a control. The composition of this extender differs from that of Tyrode's base used in the modified extenders, not only in terms of glucose concentration. The main difference, in addition to a much higher glucose concentration, is the presence in the commercial media of purified milk fractions to provide membrane protection to the spermatozoa during storage at 4°C. However, samples were stored at 18°C, and this difference may not be substantial at this temperature.

The composition and concentration of salts and HEPES buffer are comparable in all extenders used; this, in addition to the fact that the commercial extender is also used at temperatures ranging from 15 to 20°C [24] in horses considered "poor coolers," makes it a good control, especially considering that it is widely used in Europe, and the hypothesis being tested was that the high glucose concentrations found in commercial extenders can be substantially reduced.

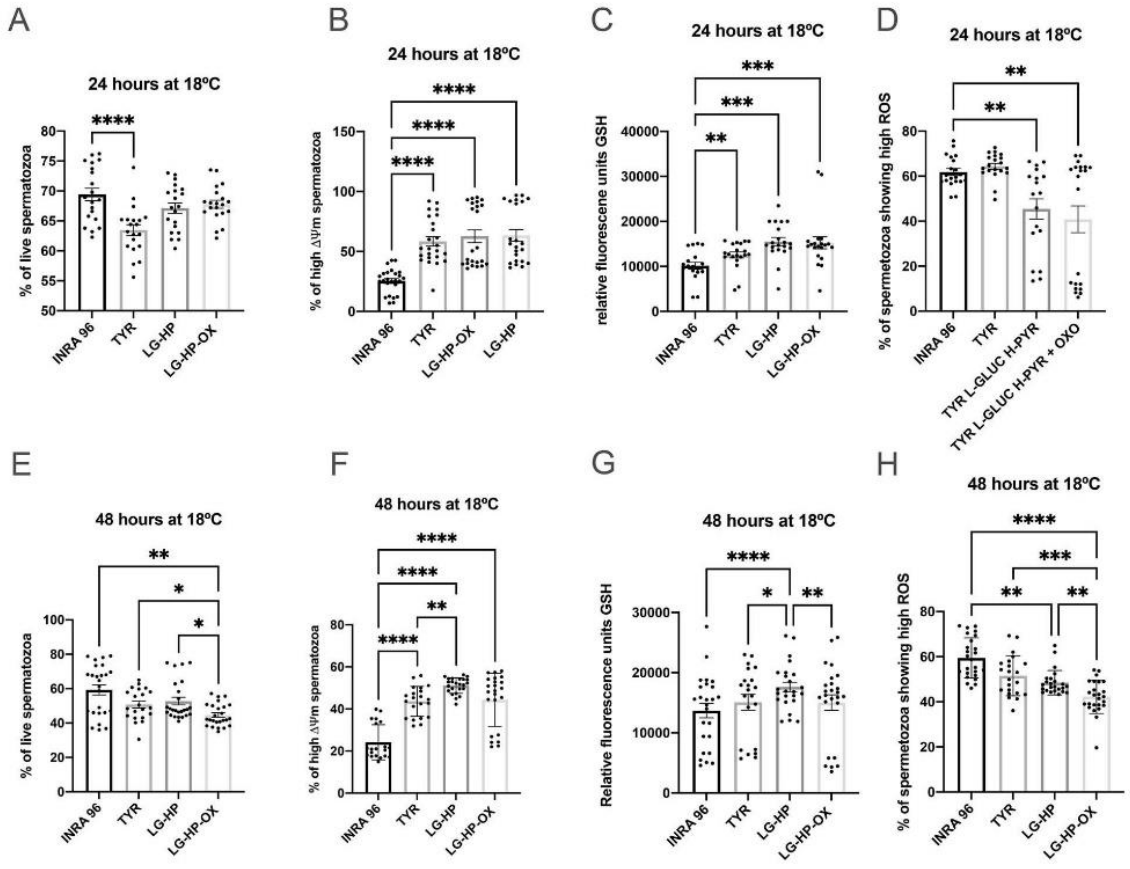

Figure 6. Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP), both supplemented (LG-HP-OX) and unsupplemented with oxoglutarate, sustains viability and mitochondrial activity. Aliquots from the same ejaculate were stored at 18°C for 48 h in a commercial extender, INRA 96, in Tyrode's medium (Tyr), and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10 mM) LG-HP and a modified LG-HP supplemented with 100 μM methyl-oxoglutarate, LG-HP-OX. (A–E) Percentage of viable spermatozoa after 24 (A) and 48 (E) h of storage at 18°C. (B–F) Percentage of spermatozoa showing high mitochondrial activity after 24 (B) and 48 h (F) of storage at 18°C. (C–G) Relative GSH content of spermatozoa after 24 (C) and 48 h (G) of storage at 18°C. (D–H) Percentage of viable cells showing significant production of reactive oxygen species (ROS) after 24 (D) and 48 h (H) of storage at 18°C. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. (6.2) Gating strategy and density dot plots showing the flow cytometry analysis performed. Samples were stained and processed as described in the Materials and Methods. Monochlorobimane was used to measure GSH at a peak excitation of 405 nm and emission of 450/45 nm BP. Mitochondrial membrane potential was measured using JC-1, and this probe was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates representing mitochondria with high membrane potential) and 488 nm excitation and 530 nm emission (monomers, representing spermatozoa with low mitochondrial membrane potential). The amount of reactive oxygen species was measured using CellRox Deep Red at 644 nm excitation and 655 nm emission. Finally, the percentages of live and dead spermatozoa were measured using Viakrome 808 at 854 nm excitation and 878 nm emission. The use of five different lasers, one for each probe and two (blue and yellow) to excite the JC-1 probe, allowed the development of a five-color panel with minimal spectral overlap. In the first step and after assessing the flow quality, doublets and clumps were identified and gated out comparing SSC-H versus SSC-A (6.2A). Then, cells in this gate were analyzed for the measurement of live and dead spermatozoa (6.2B), dead cells were excluded, and live cells were used to determine the percentage of spermatozoa showing high mitochondrial membrane potential (MMP; 6.2E–H) and the GSH content expressed as relative fluorescence units (r.f.u; 6.2I–L). Finally, the percentage of stallion spermatozoa showing high production of reactive oxygen species was measured in the whole sperm population (live and dead) (6.2M–P).

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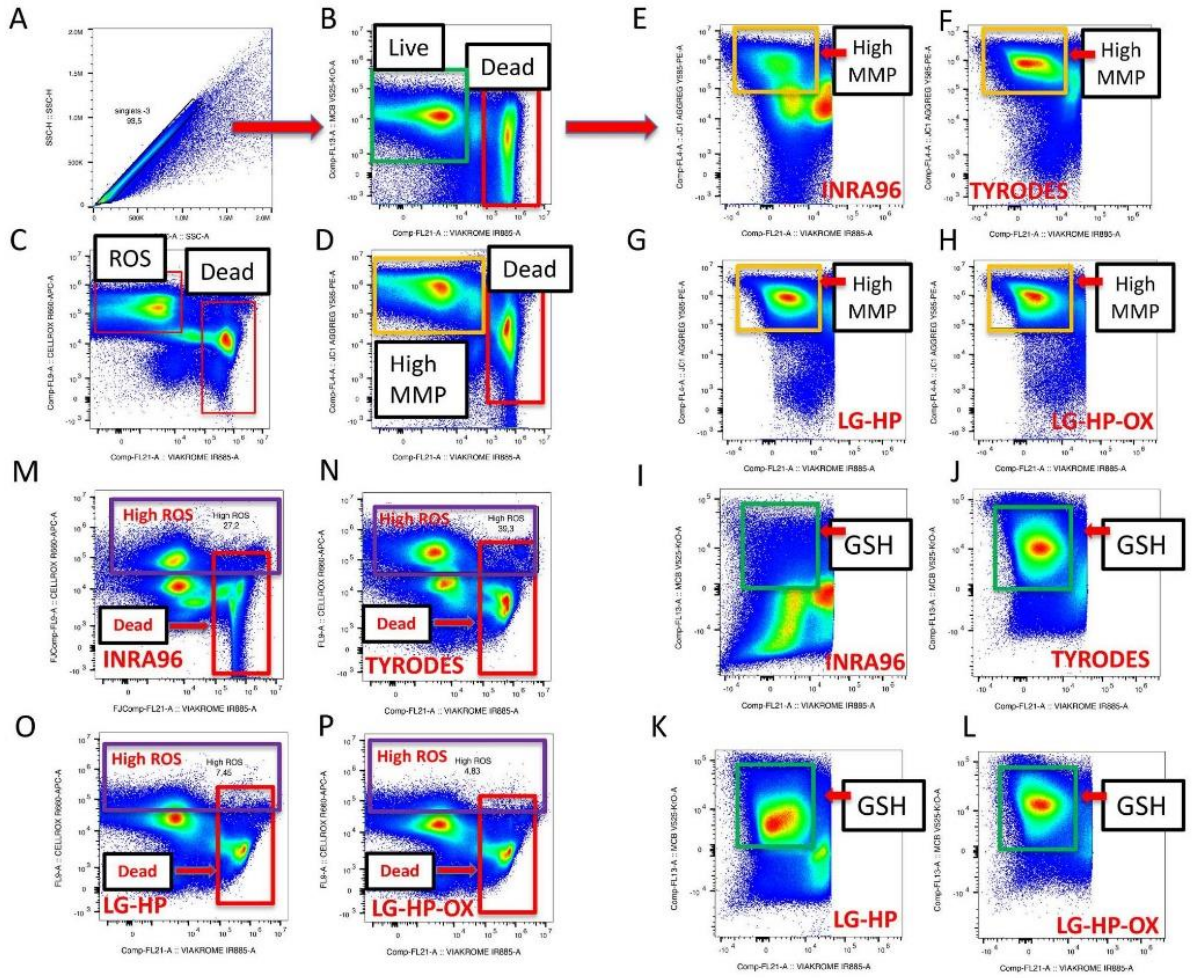

Figure 6. Continued.

All media used in the experiment had the same pH and osmolality. Two low glucose extenders (1 mM) in which pyruvate was present at 10 mM were formulated. The Reactome pathways identified in our study confirmed previous studies and proteomic research, indicating that this metabolite is used very efficiently by stallion spermatozoa [10, 14, 45]. Methyl-oxoglutarate was also tested in one of the defined media. CASA showed significant improvements in all the parameters studied in the low glucose media. The percentage of total motile spermatozoa was significantly higher in all defined media, with significant improvements in the LG-HP and LG-HP-OX media with respect to both total and progressive motility after 48 h of storage (Figure 4C and D). These improvements were manifested even more in sperm velocities. These findings can be easily explained; low glucose concentrations are sufficient to feed the glycolytic enzymes of the flagella [46] while reducing glucose toxicity. The localization of the high-affinity glucose transporter GLUT 1 throughout the flagellum in stallion spermatozoa supports this hypothesis [47].

Another important point to consider when explaining our results is that during glycolysis, the production of toxic 2-oxoaldehydes is unavoidable [48, 49]. These compounds, particularly methylglyoxal, are produced during elimination of the phosphate groups from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [19]. These 2-oxoaldehydes are potent electrophiles that may oxidize proteins, lipids, and nucleic acids, and thus are potentially cytotoxic and mutagenic. In addition, high glucose may have a direct effect, inducing cell toxicity through mitochondrial toxicity and increased production of ROS [50–54]. To measure if reduced production of 2-oxoaldehydes occurs in low glucose media, levels of glyoxal and methylglyoxal were measured at 24 and 48 h of storage at 18°C. The amount of glyoxal was significantly reduced in LG-HP media in comparison with INRA 96, both after 24 and 48 h storage; methylglyoxal was reduced in both LG-HP and LG-HP-OXO after 24 h of storage with respect to INRA 96 and was reduced in all media with respect to INRA 96 after 48 h of storage. These findings indicate that 2-oxoaldehydes are produced in higher amounts during storage of stallion spermatozoa in media containing high levels of glucose and cause sperm damage. This is the first time that sperm toxicity induced by high glucose concentrations in the extender has been described. This finding challenges current procedures in use for the storage of stallion spermatozoa in refrigeration and provides new clues that can be used to improve this technology.

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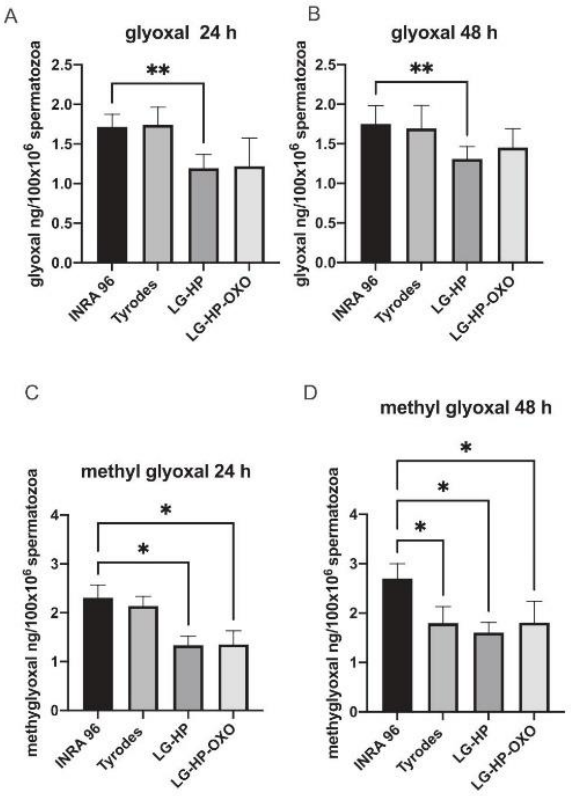

Figure 7. The production of 2-oxoaldehydes, glyoxal, and methylglyoxal was measured using UHPLC/MS as described in the Materials and Methods section after 24 and 48 h of conservation at 18°C in stallion spermatozoa extended in different media with different glucose concentrations. Changes in the amount of glyoxal in ng/100 × 10^6 spermatozoa after 24 h (A) and 48 h (B) of storage at 18°C and changes in the amount of methylglyoxal in ng/100 × 10^6 spermatozoa after 24 h (C) and 48 h (D) of storage at 18°C. *$P < 0.05$; **$P < 0.01$.

Reduced percentages of ROS-positive spermatozoa extended in LG-HP extenders were found, which favors this hypothesis. In addition, the role of glycolysis in stallion spermatozoa seems to be a mechanism that may support motility, feeding glycolytic enzymes in the flagella [5, 12, 45], although the main source of ATP in stallion spermatozoa is OXPHOS [7, 14, 17, 45]; thus, low concentrations may suffice to accomplish this function while reducing glucose toxicity, as demonstrated by the levels of MG and G in our study. The improved velocities seen in all LG media also work in favor of this hypothesis. Low glucose media also improved other sperm functions. In particular, mitochondrial activity was enhanced in all Tyrode's-based media throughout the storage time periods. The percentage of viable spermatozoa (those with intact membranes) was more constant in low glucose media, and at the same time, low glucose extenders showed lower percentages of cells showing significant ROS production linked to higher GSH content. Since detoxification of 2-oxoaldehydes, especially methylglyoxal, is linked to GSH [22, 55, 56], lower production of MG explains why GSH levels can remain high in low glucose media.

Flow cytometry indicated that efficient mitochondrial activity was linked to LG-HP extenders, as was increased GSH content. Recent reports link sperm mitochondrial activity and fertility [14, 17, 57, 58]. In addition, the concomitant increase in GSH indicates that redox homeostasis was maintained in these spermatozoa, and thus oxidative stress did not occur [8]. Currently, oxidative stress is defined as a disruption of redox signaling and control rather than as increased production of ROS [59].

Overall, our findings support previous research indicating the importance of pyruvate metabolism and provide the first published evidence of glucose toxicity due to increased levels of the 2-oxoaldehydes glyoxal and mainly methylglyoxal found in stallion spermatozoa stored in commercial extenders with high glucose concentrations. Low glucose concentrations may permit more efficient mitochondrial activity, boosting sperm metabolism when substrates for an efficient TCA cycle are provided. For the first time, we demonstrated that excess glucose in the media increases the amount of unmetabolized glucose and causes increases in the levels of 2-oxoaldehydes formed during glycolysis. Unmetabolized glucose may also stimulate direct ROS production in the mitochondria [60], causing mitochondrial malfunction, further increasing ROS production with entry into a self-exacerbating loop of ROS production. The findings reported here are readily applicable to the development of new extenders for semen conservation and stress the importance of further research into sperm metabolism and the relationship between energetic metabolism and redox regulation.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Artículo 6

PLOS ONE

RESEARCH ARTICLE

Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse

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Abstract

Artificial insemination with cryopreserved spermatozoa is a major assisted reproductive technology in many species. In horses, as in humans, insemination with cryopreserved sperm is associated with lower pregnancy rates than those for fresh sperm, however, direct effects of sperm cryopreservation on the development of resulting embryos are largely unexplored. The aim of this study was to investigate differences in gene expression between embryos resulting from fertilization with fresh or cryopreserved sperm. Embryos were obtained at 8, 10 or 12 days after ovulation from mares inseminated post-ovulation on successive cycles with either fresh sperm or frozen-thawed sperm from the same stallion, providing matched embryo pairs at each day. RNA was isolated from two matched pairs (4 embryos) for each day, and cDNA libraries were built and sequenced. Significant differences in transcripts per kilobase million (TPM) were determined using (i) genes for which the expression difference between treatments was higher than 99% of that in the random case ($P < 0.01$), and (ii) genes for which the fold change was ≥ 2, to avoid expression bias in selection of the candidate genes. Molecular pathways were explored using the DAVID webserver, followed by network analyses using STRING, with a threshold of 0.700 for positive interactions. The transcriptional profile of embryos obtained with frozen-thawed sperm differed significantly from that for embryos derived from fresh sperm on all days, showing significant down-regulation of genes involved in biological pathways related to oxidative phosphorylation, DNA binding, DNA replication, and immune response. Many genes with reduced expression were orthologs of genes known to be embryonic lethal in mice. This study, for the first time, provides evidence of altered transcription in embryos resulting from fertilization with cryopreserved spermatozoa in any species. As sperm cryopreservation is

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

commonly used in many species, including human, the effect of this intervention on expression of developmentally important genes in resulting embryos warrants attention.

Introduction

Cryopreservation is a common procedure in assisted reproductive technology, in both humans and the animal breeding industry [1–3]. Cryopreserved sperm are routinely used for artificial insemination (AI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). However, it is clear that sperm cryopreservation methods are currently sub-optimal, as pregnancy rates with cryopreserved sperm are lower than those with fresh sperm in humans and horses [4–6], among other species. Cryopreservation leads to extensive damage of sperm cell membranes and causes metabolic and functional alteration of sperm [7, 8], particularly of their mitochondria [9–11]. Cryopreservation may alter sperm DNA [12]; recently, specific cryodamage to sperm genes and transcripts have been reported [13, 14], even in samples with good sperm motility post thaw and in the absence of detectable DNA fragmentation. The sperm DNA is epigenetically programmed to regulate embryonic gene expression, and changes to this epigenome cause developmental disregulation [15]. Cryopreservation has been found to significantly change the sperm DNA methylome, as well as to alter expression of epigenetic-related genes such as methyltransferases [12, 16]. Cryopreservation of sperm imposes oxidative stress and redox deregulation in spermatozoa, leading to the presence of toxic adduct-forming compounds such as 4- hydroxynonenal (4-HNE) in sperm membranes [17]. Moreover, mitochondria of spermatozoa surviving cryopreservation show increased production of reactive oxygen species [9, 10, 18]. Signaling pathways crucial to normal embryo development are sensitive to perturbations of endogenous redox state, and are also susceptible to modulation by reactive oxygen species [19]. Thus, fertilization by damaged spermatozoa may impact early embryo development and even have effects that appear later in the life of the offspring [20].

Moreover, appreciation of the contribution of sperm to embryo development has evolved from the concept that the only role of sperm at fertilization is to introduce the male genome into the egg. Sperm carry a myriad of small noncoding RNAs with potential roles in early embryo development [21, 22]. Notably, sperm carry the activating factor PLCζ, which triggers calcium oscillations that induce oocyte activation [23, 24], and it has been shown in mouse and rabbit that alterations in frequency and amplitude of post-fertilization calcium oscillations can affect the phenotype of the resulting embryo into post-implantation development and adulthood [25, 26]. Thus, there are extensive pathways by which cryopreservation of sperm could alter the development of the fertilized oocyte and embryo.

Despite the widespread use of cryopreserved sperm, and the known decrease in pregnancy rates with its use, little direct information is available on the effect of sperm cryopreservation on development of the resulting embryo. Recent advances in transcriptome amplification and next-generation sequencing provide the ability to obtain the full transcriptome of individual embryos [27], thus offering a basis for studies on differences in gene expression associated with fertilization with cryopreserved sperm. In the present study, we analyzed the transcriptome of equine embryos produced with fresh or frozen-thawed sperm, to determine the impact of sperm cryopreservation on gene expression during early equine embryo development.

Material and methods

Animals and experimental design

Animals belonging to and housed in our institution were maintained according to European laws and regulations, and all experimental procedures were reviewed and approved by the Ethical committee of the University of Extremadura, Cáceres, Spain. Six mares were used for this study; they were inseminated with the same stallion of known fertility to reduce genetic variability [28]. Each mare was assigned a day of embryo recovery (8, 10 or 12 days post ovulation) and on successive cycles was assigned to be inseminated with fresh or frozen-thawed sperm from the same stallion, to provide a matched embryo pair for that day of embryo development. The mares were treated with a prostaglandin analogue to shorten the luteal phase and were monitored daily by transrectal ultrasonography. When a follicle of at least 35 mm diameter was detected in the absence of luteal tissue, with marked uterine edema and low cervical tone, mares received 2,500 IU of hCG i.v.. The follicle was monitored by transrectal ultrasonography every 6 h thereafter to detect the time of ovulation. Mares were inseminated immediately once ovulation was detected, with a minimum of 100 million either fresh sperm or frozen-thawed sperm, from the same stallion and ejaculate. For this, semen was collected, and half of the ejaculate was processed as fresh semen for the immediate insemination of the mare. The other half was frozen following the standard protocol in our center [17, 29, 30], and stored in LN for the next insemination of the same mare. Following this protocol, each mare was inseminated with the same ejaculate, first with the fresh extended aliquot and on a second cycle with the frozen-thawed aliquot.

Embryos were obtained by uterine lavage on the designated day after ovulation. For each embryo day, 2 embryos produced with fresh sperm, designated FRSH embryos, and 2 embryos produced with frozen-thawed sperm, designated CRYO embryos were obtained. Embryos were snap-frozen in liquid N_2 and stored at -80˚C until analysis. Previous clinical reports indicated that there is no a significant effect in the rate of embryonic vesicle growth between mares inseminated with fresh or frozen-thawed sperm if both are inseminated post-ovulation [31].

Isolation of RNA

Total RNA was isolated from the embryos using the PicoPure RNA Isolation Kit (Catalog number KIT0204, Thermofisher) following the manufacturer's instructions. RNA concentration and quality were assessed by automatic electrophoresis using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RNA-seq analysis

cDNA libraries were built using an IonTorrent S5/XL sequencer (Thermo Fisher Scientific, Waltham, MA USA). The raw reads were aligned to a horse transcriptome generated using ENSEMBL (Equ Cab 2 version) in the Torrent server with proprietary ThermoFisher algorithms. Then, custom scripts were used to transform reads into transcript counts, and transcripts per kilobase million (TPM) scores for each gene were retrieved. A gene was considered expressed if the reads per kilobase or transcript model per million mapped reads was > 0.4. In order to evaluate gene expression differences between treatments (FRSH or CRYO embryos), we calculated two thresholds: first, we calculated the random TPM differences between FRSH and CRYO embryos by permutation of the TPM gene scores. Then we chose the genes whose expression difference between the two conditions was higher than in 95% ($P<0.05$) or in 99% ($P<0.01$) of the random cases. As a second score, we used a fold change ≥ 2 as a threshold in order to avoid expression biases in the selection of the candidate genes.

Gene ontology and pathway analysis

The annotations of the candidate genes selected after the RNA-seq analyses were explored to detect significant differences in molecular pathways between treatments. Specifically, the DAVID webserver [32] was used to retrieve the terms (gene ontology, up-expressed tissues, KEGG and reactome pathways, protein-protein interactions, etc.) with significant over-presence of the candidate genes, using a false discovery rate (FDR) < 0.05. We used the human genome as reference for the analysis because of its increased depth in terms of annotation.

Network analysis

STRING [33] was used to analyze the internal structure of the functional network obtained using the candidate genes. Data included co-expression, genetic fusion, co-occurrence or protein-protein interactions, among others. A high threshold (0.700) was selected for positive interaction between a pair of genes.

Results

A total of 12 conceptuses were analysed (2 FRSH and 2 CRYO at each day). An average of 29,196 transcripts per embryo were obtained.

Day-8 embryos

In Day-8 CRYO embryos, 100 transcripts showed increased abundance and 157 transcripts showed decreased abundance in respect to FRSH embryos of the same age from the same stallion and mare (Fig 1).

Of the 100 transcripts showing increased abundance in CRYO embryos, 23 could be aligned to the genome build (S1 Table). These included the progesterone receptor membrane component (8PGRMC1). Enriched biological processes (Fig 2A) included extracellular region genes, defensing beta 119, insulin like 3, prostaglandin D2 synthase and uteroglobin; genes associated with negative regulation of cysteine type endopeptidase activity involved in apoptotic processes including nuclear receptor subfamily 4 group A member and paired box 2; and genes involved in skeletal muscle cell differentiation including activating transcription factor 3 and nuclear receptor subfamily 4 group 4 A member. STRING analysis revealed no significant enrichments in functional networks for transcripts with increased abundance.

Transcripts showing decreased abundance in CRYO embryos provided more information, with 129 transcripts annotated in the equine database. The complete list of transcripts is presented in S2 Table. Due to the large number of genes retrieved, the threshold was reset at $P< 0.001$ and 62 transcripts were then retrieved (Table 1). Related gene ontology terms are shown in Fig 2B. Enriched terms in KEGG (Kyoto encyclopedia of gene and genomes) pathways included ribosome, Parkinson disease and oxidative phosphorylation (Fig 3). STRING analysis, performed using a threshold of 0.700, obtained a protein-protein interaction (PPI) enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 4). The complete list of genes in this network with their clustering is presented in S3 Table. Enriched biological processes included cellular process, iron ion transport, cellular iron ion homeostasis, metabolic process, response to inorganic substance, biological regulation, single-organism process, cellular macromolecule metabolic process, single organism cellular process, cellular metabolic process, response to stimulus, cellular response to zinc ion, transport, regulation of biological process, oxidation-reduction process, cellular component disassembly, cellular nitrogen compound metabolic process, translation, single organism transport, gene expression, positive regulation of nitrogen compound

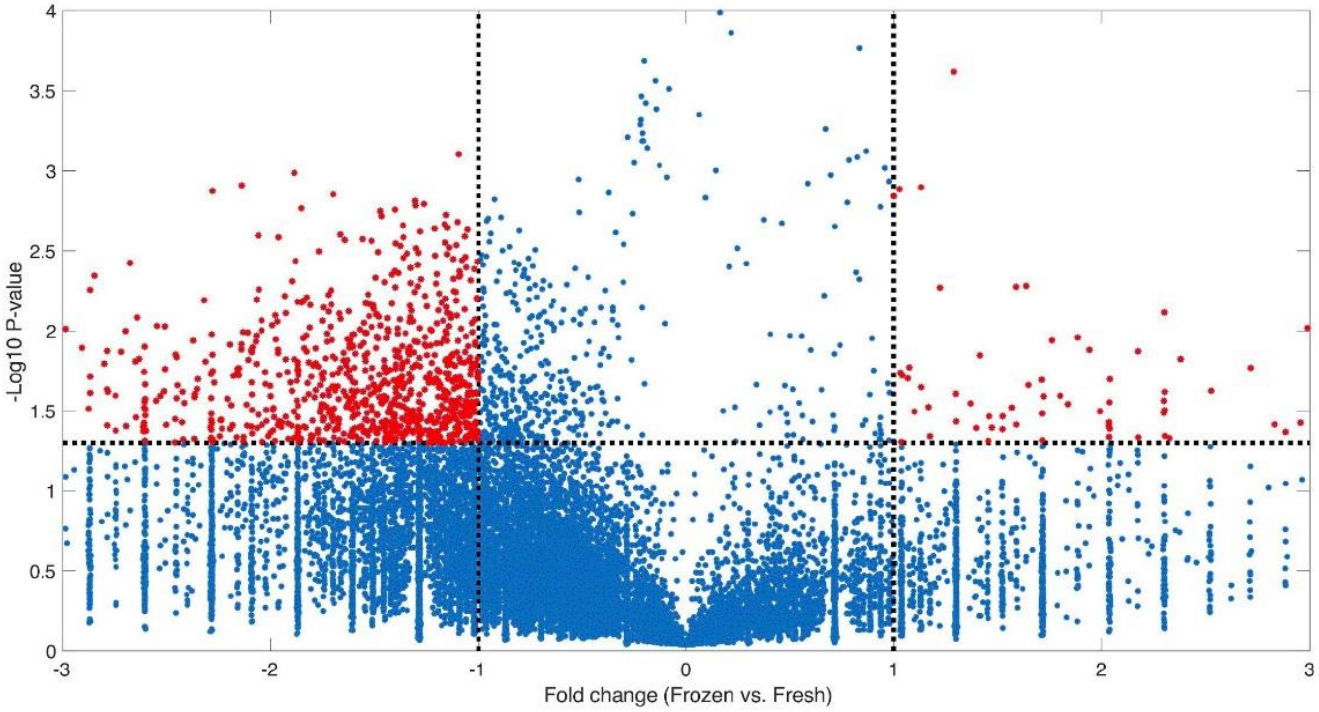


Fig 1. Volcano plot representing the RNA-seq results for Day-8 equine embryos conceived using fresh sperm (FRSH) or frozen-thawed sperm (CRYO). Each point represents a gene. On the X-axis, the fold change was calculated as the log2-ratio between the average gene expression in CRYO embryos and the average gene expression in FRSH embryos. Therefore, positive values indicate genes in which expression is higher in CRYO embryos, while negative values indicate genes whose expression is higher in FRSH embryos. On the Y-axis, the statistical significance for the difference in gene expression (see Methods) is represented as the (-log10) of the P-value. Dashed lines indicate the thresholds for significance on the two axes (-1 and +1 in the case of the X-axis for up-regulated and down-regulated genes, respectively, in CRYO embryos; and 1.30 (equal to a P-value = 0.05) in the case of the Y-axis). Red points mark differentially expressed genes.

https://doi.org/10.1371/journal.pone.0213420.g001

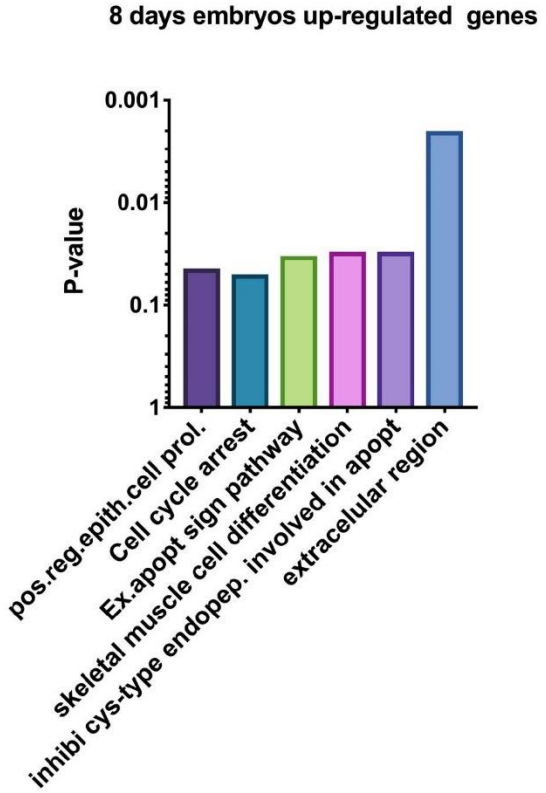
metabolic process, biological process, protein folding, cellular component organization, regulation of cell proliferation, and primary metabolic process.

Day-10 embryos

In Day-10 embryos 239 transcripts showed increased abundance ($P < 0.01$), and 206 showed decreased abundance, in CRYO embryos in comparison with FRSH embryos.

Of the 239 transcripts showing increased abundance in CRYO embryos, 53 aligned to the genome build (S4 Table). Functional annotation revealed these genes to be related to the GO terms and KEGG pathways nucleosome, systemic lupus erythematatosus, DNA replication-dependent nucleosome assembly, protein heterodemerization, alcoholism, nuclear chromosome, telomeric region, regulation of gene silencing, nucleosomal DNA binding, membrane, translation, poly (A) RNA binding, viral carcinogenesis, negative regulation of megakaryocyte differentiation, DNA replication independent nucleosome assembly, extracellular exosome, DNA-templated transcription, xenophagy, ribosome, positive regulation of defense to virus by host, DNA binding, mitochondrion, cytosolic large ribosomal subunit, extracellular space, transcriptional misregulation in cancer, innate immune response in mucosa, U1 snRNP, antibacterial humoral response, telomerase RNA binding and mitochondrial small ribosomal subunit (Fig 5A). STRING analysis revealed a PPI enrichment P value of $< 1.0 \times 10^{-16}$. Functional enrichment included the PFAM protein domain Core histone H2A/H2B/H3/HA and the

A


8 days embryos up-regulated genes
P-value
0.001
0.01
0.1
1
pos.reg.epith.cell prol.
Cell cycle arrest
Ex.apopt sign pathway
skeletal muscle cell differentiation
inhibi cys-type endopep. involved in apopt
extracelular region

B

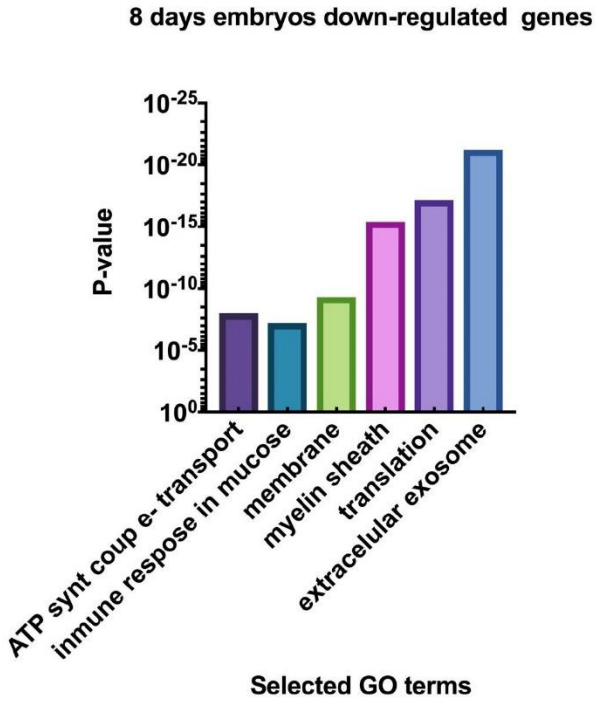

8 days embryos down-regulated genes
P-value
10-25
10-20
10-15
10-10
10-5
100
ATP synt coup e- transport
immune respose in mucose
membrane
myelin sheath
translation
extracelular exosome
Selected GO terms

Fig 2. Selected enriched GO terms differentially regulated in Day-8 equine embryos obtained using fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated in 8-Day CRYO embryos, (B) transcripts up regulated in 8-Day CRYO embryos.

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INTERPRO protein domains, including Histone fold, Histone H3/CNEP-A, Histone H2A/H2B/H3, Histone H4, Histone H4 conserved site, TATA box binding protein associated factor (TAF) and ribosomal protein L23/L15e core domain.

Of the 206 transcripts showing decreased abundance in CRYO embryos at Day 10, 115 were aligned. Enriched KEGG pathways that were also detected in Day-8 embryos (Table 2) included oxidative phosphorylation, Parkinson disease, Alzheimer disease, Hungtington disease, Metabolic pathways, Ribosome, cardiac muscle contraction, and non-alcoholic fatty liver disease. Three new KEGG enriched pathways, protein processing in endoplasmic reticulum, systemic lupus erythematosus and phagosome, were detected (Fig 6). More significantly represented GO terms were ATP synthesis coupled proton transport, translation, nucleosome assembly, cell redox homeostasis, extracellular exosome, myelin sheath, respiratory chain, mitochondrion, extracellular space, NADH dehydrogenase (ubiquinone) activity, structural constituent of ribosome, and proton transporting ATP synthase activity rotational mechanism (Fig 5B). A complete list of enriched GO terms retrieved are given in Table 3. STRING analysis revealed functional networks with a PPI enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 7). Functional enrichment included the PAFM domains core histone H2A/H2B/H3/H4, thiorredoxin, NADH deshidrogenase, NADH-Ubiquinone and plastoquinone (Complex I), various chains.

Day-12 embryos

In Day-12 embryos, 149 transcripts showed increased abundance and 157 showed decreased abundance in CRYO embryos. Of the 149 transcripts with increased abundance, 61 were annotated (S5 Table). Enriched KEGG pathways included ribosome and Parkinson disease and the GOterms extracellular exosome, translation, structural constituent of ribosome, nuclear nucleosome, mitochondrial respiratory chain complex I, cytosolic large ribosomal subunit, nucleosome assembly, methylosome, and catalytic step 2 spliceosome (Fig 8A). On STRING analysis, the KEGG pathways Ribosome (Pathway ID 03010) and Parkinson disease (Pathway ID 05012) showed a PPI enrichment P value of $< 8 \times 10^{-10}$.

Of the 157 transcripts showing decreased abundance in Day-12 CRYO embryos, 60 transcripts aligned to the genome build (S6 Table). Enriched KEGG pathways, also detected in 8- and 10-day embryos, included oxidative phosphorylation, Parkinson disease, metabolic pathways, Alzheimer disease, Huntington disease, non-alcoholic fatty acid liver disease and cardiac muscle contraction. In addition a new pathway, folate biosynthesis, was enriched (Fig 9). GO terms enriched annotations (Fig 8B) were NADH dehydrogenase (ubiquinone) activity, mitochondrial respiratory chain complex I, nucleosome, DNA replication dependent nucleosome assembly, protein heterotetramerization, mitochondrion, respiratory chain, negative regulation of megakaryocyte differentiation, DNA template transcription initiation, ATP synthesis coupled electron transport, nuclear chromosome telomeric region, DNA binding, oxireductase activity, mitochondrial inner membrane, integral component of membrane, mitochondrial electron transport NADH to ubiquinone, and extracellular exosome. The complete list is given in Table 4

STRING analysis revealed functional networks with a PPI enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 10). Functional enrichment included the PFAM protein domains core histone H2A/H2B/H3/H4, NADH dehydrogenase, and NADH-ubiquinone/plastoquinone (complex I various chains).

Table 1. Enriched biological processes from DEGs (downregulated) in 8 days embryos obtained after AI with frozen thawed sperm, as identified by DAVID functional annotation analysis.

Functional terms of overrepresented biological processes [a]	P value [b]
Chromosome (21, 35.78)	7.1×10^{-26}
Nucleosome core (19, 42.08)	9.2×10^{-25}
Extracellular exosome (62, 3.56)	5.7×10^{-22}
Histone fold (19, 30.02)	7.5×10^{-22}
Structural constituent of ribosome (24, 13.82)	5.8×10^{-20}
Ribosome (24, 12.89)	1.21×10^{-19}
Histone core (15, 36.91)	1.69×10^{-18}
Translation (21, 14.65)	7.15×10^{-18}
Mylein sheath (18, 16.63)	3.87×10^{-16}
Nucleosome (13, 31.72)	4.20×10^{-15}
Ribonucleoprotein (14, 24.48)	1.15×10^{-14}
Ribosomal protein (13, 29.28)	1.39×10^{-14}
Nucleosome assembly (13, 23.18)	2.14×10^{-13}
Poly (A) RNA binding (34, 4.29)	4.69×10^{-13}
Nuclear nucleosome (10, 40.67)	1.37×10^{-12}
Parkinson's disease (18, 9.52)	2.60×10^{-12}
Cytosolic small ribosomal subunit (10, 33.98)	8.65×10^{-12}
Cytosolic large ribosomal subunit (11, 24.85)	1.46×10^{-11}
Systemic lupus erythematosus (16, 10.14)	2.51×10^{-11}
Hungtinton's disease (19, 7.38)	4.19×10^{-11}
H2B (8, 52.06)	7.18×10^{-11}
Nucleus (26, 4.79)	8.79×10^{-11}
Oxidative phosphorylation (16, 9.01)	$1{,}43 \times 10^{-10}$
Histone H2B (8, 48.75)	1.48×10^{-10}
DNA binding (21, 6.06)	1.81×10^{-10}
Membrane (27, 4.21)	4.82×10^{-10}
Alzheimer disease (17, 7.38)	6.32×10^{-10}
Alcoholism (16, 7.17)	3.64×10^{-9}
ATP synthesis coupled proton transport (7, 41.39)	1.0×10^{-8}
Innate immune response in mucose (6, 51.80)	5.89×10^{-8}
Antibacterial humoral response (6, 48.15)	9.1×10^{-8}
Focal adhesion (15, 6.10)	1.38×10^{-7}
DNA binding (18, 4.18)	1.02×10^{-6}
DNA replication dependent nucleosome assembly (6, 30.64)	1.13×10^{-6}
Hydrogen ion transport (5, 55.38)	1.38×10^{-6}
Protein heterotetramerizacion (6, 29.31)	1.44×10^{-6}
Proton transporting ATP synthase activity, rotational mechanism (5, 51.26)	1.66×10^{-6}
Cytoplasm (11, 5.91)	1.60×10^{-5}
Cytoplasmatic translation (5, 29.56)	2.00×10^{-5}
H4 (4, 60.63)	2.86×10^{-5}
Viral carcinogenesis (13, 4.39)	3.09×10^{-5}
Cardiac muscle contraction (8, 8.53)	3.57×10^{-5}
Histone H4 (4, 56.81)	3.68×10^{-5}
Histone H4 conserved site (4, 56.81)	3.68×10^{-5}
TAF (4, 56.88)	5.56×10^{-5}
Defense response to gram positive bacterium (6, 49.69)	6.14×10^{-5}

(*Continued*)

Table 1. (Continued)

Functional terms of overrepresented biological processes [a]	P value [b]
Tata Box binding protein associated factor (TAF) (4, 14.04)	7.14×10^{-5}
Negative regulation of megakaryocite differentiation (4, 46.53)	1.04×10^{-4}
ATP hydrolysis coupled ion transport (5, 40.85)	1.14×10^{-4}
Acetylation (6, 19.37)	1.32×10^{-4}
H2A (4, 12.08)	3.67×10^{-4}
Mitochondrial electron transport, cytochrome c to oxygen (3, 27.33)	4.57×10^{-4}
Ribosomal large subunit assembly (4, 84.27)	4.94×10^{-4}
DNA replication independent nucleosome assembly (4, 24.96)	4.94×10^{-4}
Histone H2A (4, 24.37)	5.44×10^{-4}
V-ATPase proteolipid subunit C-like domain (3, 76.78)	5.86×10^{-4}
DNA templated transcription, initiation (4, 22.47)	6.81×10^{-4}
Nuclear chromosome, telomeric region (6, 8.22)	7.79×10^{-4}
Non alcoholic fatty liver disease (NAFLD) (9, 4.40)	8.75×10^{-4}
Lactate/malate dehydrogenase (3, 63.99)	8.74×10^{-4}
Lactate malate dehydrogenase, N–terminal (3, 63.99)	8.74×10^{-4}
Mitochondrial proton transporting ATP synthase complex (3, 61.00)	9.60×10^{-4}

[a] Values in parenthesis represent the number of genes involved in and the fold enrichment of the corresponding functional terms

[b] EASE score examine the significance of gene term enrichment with a modified Fisher's exact test

https://doi.org/10.1371/journal.pone.0213420.t001

Comparison of downregulated genes with the mouse genome database

In order to explore mechanisms that may relate to reduced viability in embryos obtained using cryopreserved semen, the Mouse Genome Database [34, 35] was queried to determine whether genes downregulated in CRYO equine embryos were orthologs to mouse genes with known associations with embryo lethality.

Day-8 embryos

In Day-8 CRYO embryos, transcripts of genes associated with the following terms were found to be of low abundance: failure of zygotic division, decreased embryo size, abnormal embryo size, embryonic growth arrest, embryonic growth retardation, embryonic lethality before implantation-complete penetrance, embryonic lethality between implantation and somite formation-complete penetrance, embryonic lethality between somite formation and embryo turning-complete penetrance, embryonic lethality prior to tooth bud stage, abnormal embryonic tissue morphology, abnormal extraembryonic tissue morphology, delayed allantois development, perinatal lethality incomplete penetrance, prenatal lethality-complete penetrance, preweaning lethality-complete penetrance, abnormal male germ cell apoptosis, abnormal spermatogenesis, azoospermia, male infertility, and female infertility.

Day 10 embryos

In Day-10 CRYO embryos, the following gene associations cited above for Day-8 embryos were found: decreased embryo size, abnormal embryo size, failure of zygotic cell division, embryonic lethality between implantation and somite formation, embryonic lethality between implantation and somite formation-complete penetrance, embryonic lethality prior to tooth bud stage, prenatal lethality-complete penetrance, perinatal lethality-incomplete penetrance,

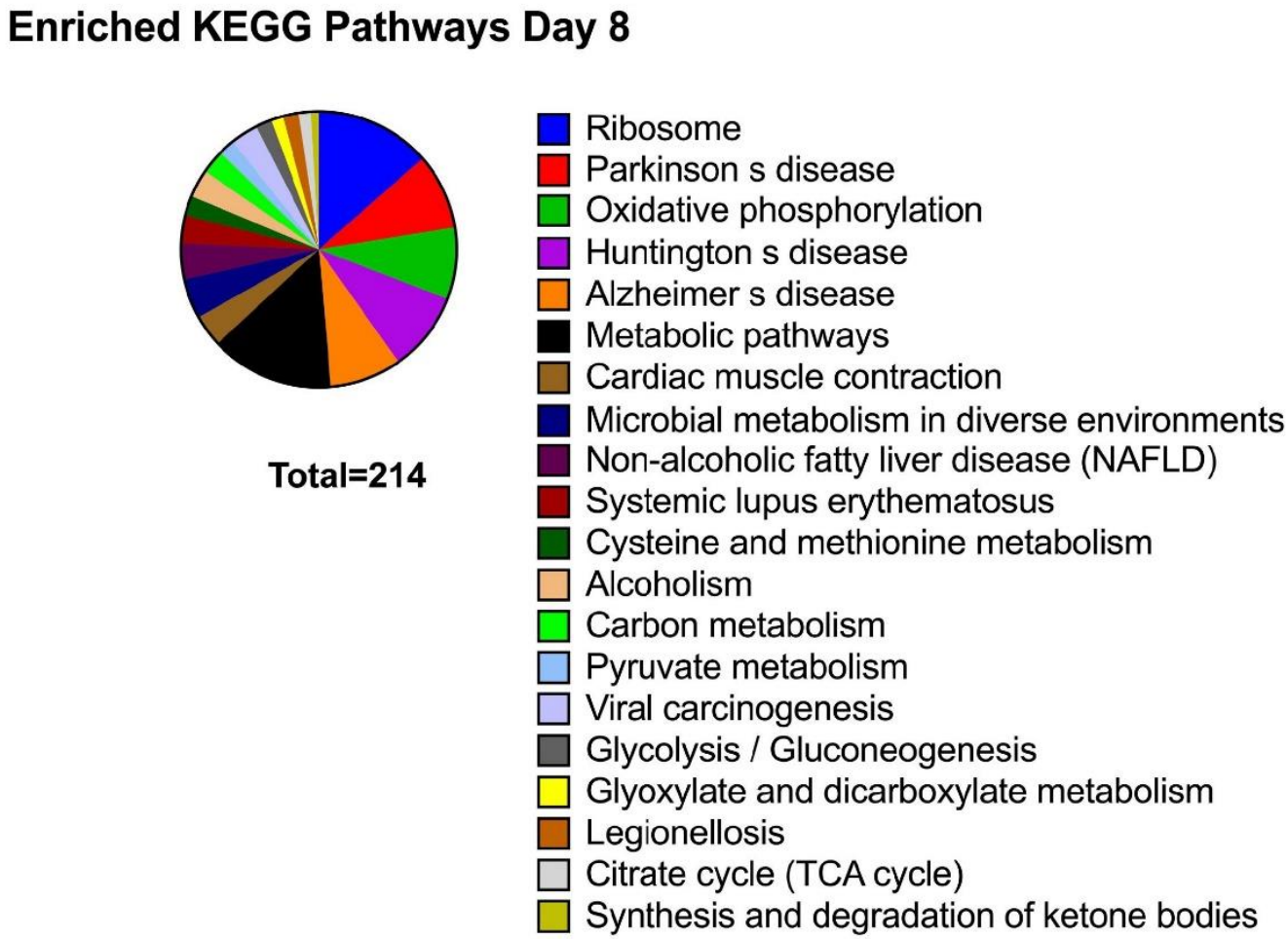

Fig 3. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa.

https://doi.org/10.1371/journal.pone.0213420.g003

preweaning lethality, preweaning lethality-complete penetrance, and abnormal spermatogenesis.

In addition, the following associations were found: abnormal blastocyst morphology, absent blastocele, abnormal inner cell mass morphology, absent inner cell mass proliferation, empty decidua capsularis, embryonic growth retardation, failure of blastocyst to hatch from the zona pellucida, abnormal preimplantation embryo development, failure to gastrulate, embryonic lethality prior to embryogenesis, failure of embryo implantation, abnormal decidua basalis morphology, abnormal extraembryonic endoderm formation, prenatal lethality prior to heart atrial septation, decreased fetal size, preweaning lethality incomplete penetrance, abnormal gametogenesis, abnormal spermatid morphology, abnormal vas deferens morphology, decreased mature ovarian follicle number, reduced female fertility and small ovary.

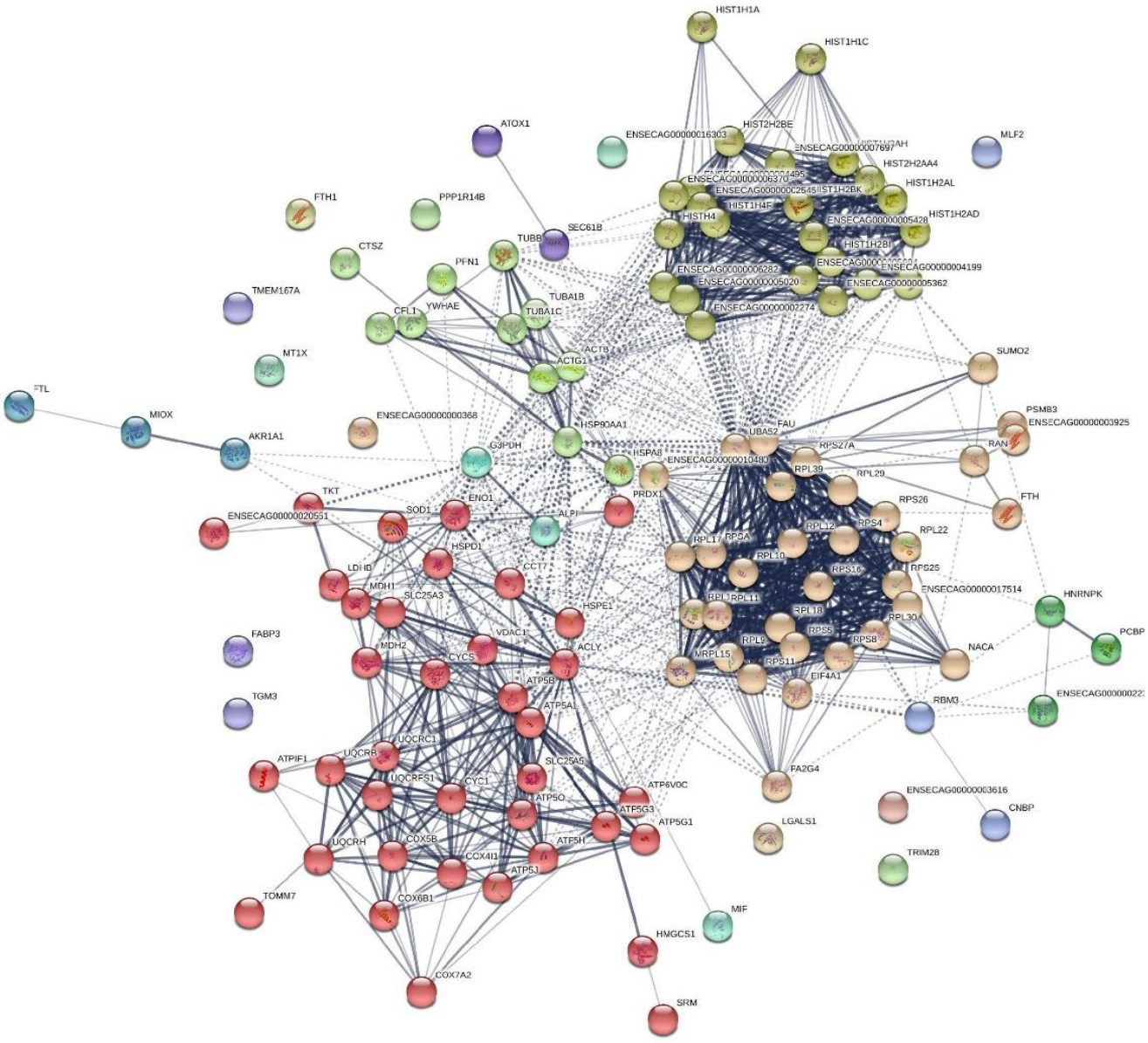

Fig 4. Functional networks (STRING) of transcripts downregulated in 8-Day equine embryos obtained with frozen-thawed sperm (CRYO embryos). Functional networks apply to histones and mitochondrial proteins. Controls are 8-Day embryos from the same mare, obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos. A list of the transcripts in each cluster obtained after STRING analysis are presented. Colors for each cluster are given in S3 Table.

https://doi.org/10.1371/journal.pone.0213420.g004

Day 12 embryos

In 12-day CRYO embryos the following gene associations cited above were found: abnormal embryo size, decreased embryo size, prenatal lethality prior to heart atrial septation, embryonic lethality prior to tooth bud stage, preweaning lethality-complete penetrance, male infertility, female infertility, and small ovary.

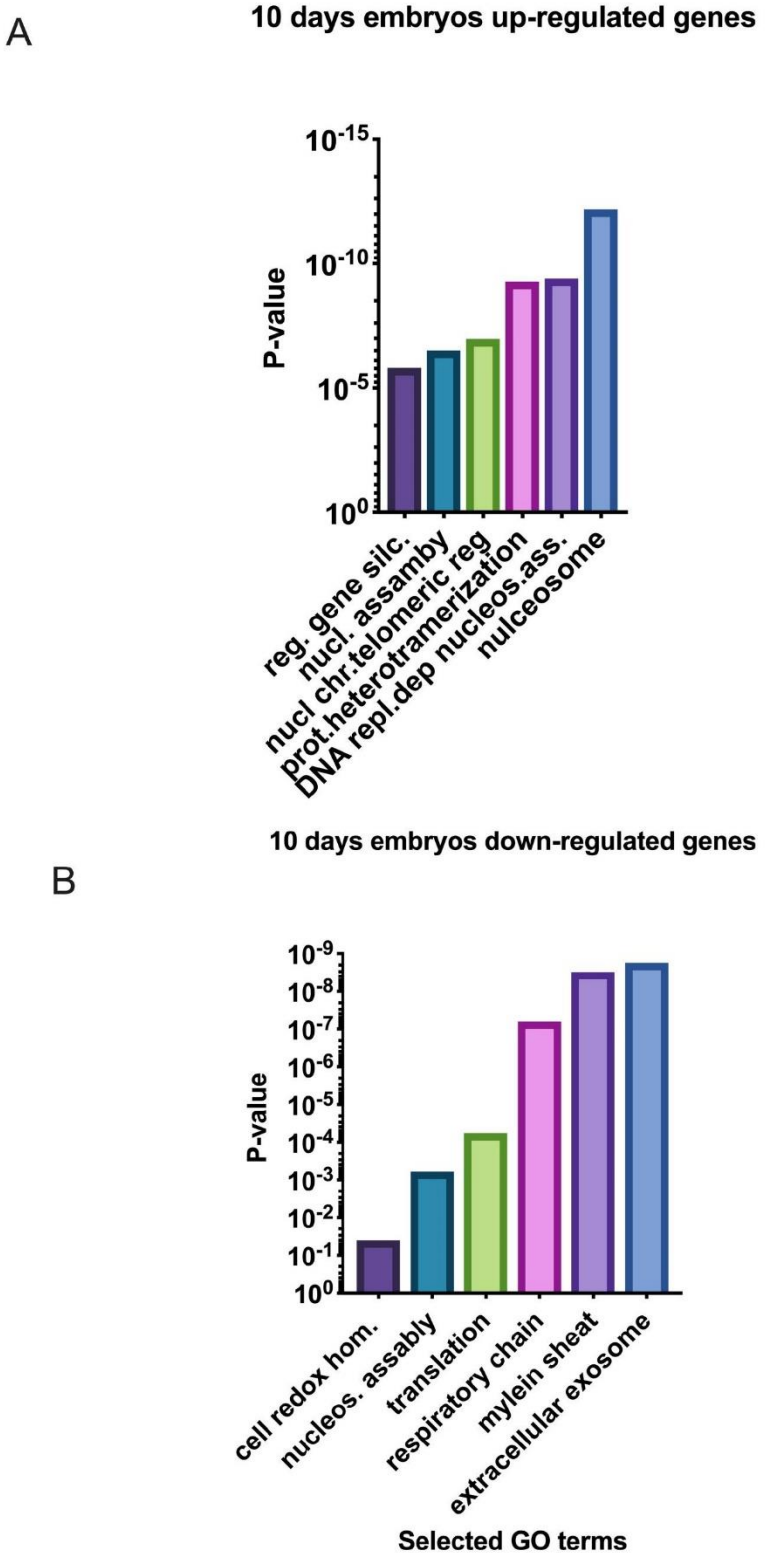
A
10 days embryos up-regulated genes
P-value
10-15
10-10
10-5
100
reg. gene silc.
nucl. assamby
nucl chr.telomeric reg
prot.heterotramerization
DNA repl.dep nucleos.ass.
nulceosome
B
10 days embryos down-regulated genes
P-value
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
cell redox hom.
nucleos. assably
translation
respiratory chain
mylein sheat
extracellular exosome
Selected GO terms

Fig 5. Selected enriched GO terms differentially regulated in 10-Day equine embryos obtained with fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated transcripts 10-Day CRYO embryos, (B) transcripts up regulated in 10-Day CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g005

In addition, the following associations were found: incomplete embryo turning, embryonic lethality prior to organogenesis, embryonic lethality during organogenesis-complete penetrance, decreased FSH level, small seminal vesicle, small seminiferous tubules, small testis, absent mature ovarian follicles, abnormal ovulation, abnormal corpus luteum morphology, uterus hypoplasia, and vaginal atresia.

Discussion

Here we report, for the first time, evidence that procedures performed during handling of sperm, such as freezing and thawing, have a significant impact on critical aspects of the early embryo transcriptome. The equine model used in our study has a number of advantages, including a long pre-attachment embryonic period in which the embryo remains spherical, which facilitates embryo collection, and the possibility of repeated embryo collections from the same animals over successive estrus cycles. Additionally, the stallion serves as an excellent model for the human male, as stallions are typically not selected for sperm quality nor the ability of semen to be cryopreserved, in contrast to males in production species, such as the bull. Moreover, since many stallions reach advanced age, the horse can be used as a model to study the impact of paternal age on embryo quality.

Our study, focused on three embryo ages (8, 10 and 12 days post ovulation), revealed a significant impact of sperm cryopreservation on the transcriptome of the resulting embryo. Importantly, transcripts with decreased abundance reflected genes related to DNA replication and assembly, and oxidative phosphorylation. Exploration of differentially-expressed genes at the molecular and cellular level revealed alterations in important functions including ATP synthesis, regulation of transcription, nucleosome assembly, chromatin silencing, protein synthesis, and redox regulation. Alterations in these genes help to explain the reduced fertility observed with cryopreserved sperm attributable to increased early embryo mortality [11, 12].

The pre-implantation period is a period of rapid embryo growth, requiring a ready supply of ATP. The equine embryo appears to have a significant capacity for glycolysis, but also uses oxidative phosphorylation [36]. The KEGG pathways analysis of downregulated genes revealed enriched annotations for oxidative phosphorylation, pyruvate metabolism, glycolysis, and the TCA cycle, suggesting compromised energy metabolism in CRYO embryos. A similar picture was observed in Day-10 and Day-12 embryos, with the pathways for oxidative

Table 2. Selected enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in downregulated transcripts of in 10 days embryos obtained after AI with frozen thawed sperm.

KEGG pathway	Pathway description	observed gene count	false discovery rate
190	Oxidative phosphorylation	21	2,45E-23
5012	Parkinson s disease	20	4,64E-21
5010	Alzheimer s disease	15	1,05E-12
5016	Huntington s disease	15	3,42E-12
1100	Metabolic pathways	28	4,29E-09
3010	Ribosome	11	8,96E-09
4260	Cardiac muscle contraction	7	2,56E-06
4932	Non-alcoholic fatty liver disease (NAFLD)	9	3,96E-06
4141	Protein processing in endoplasmic reticulum	9	1,61E-05

https://doi.org/10.1371/journal.pone.0213420.t002

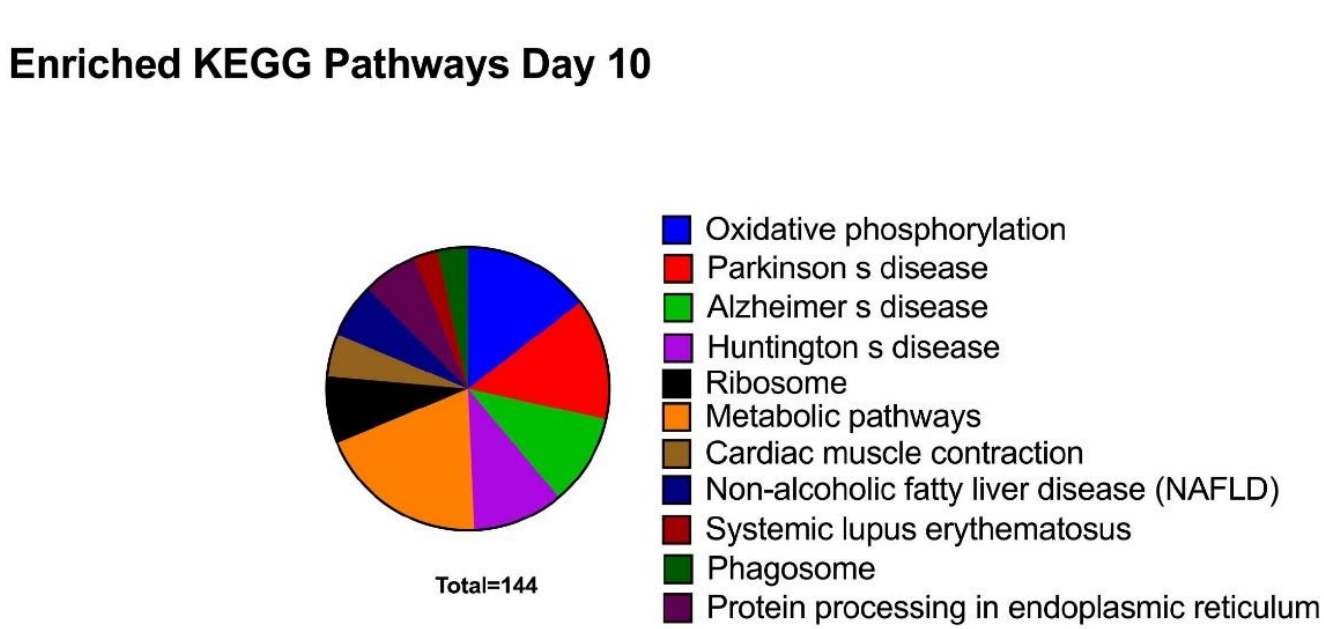

Fig 6. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 10-Day embryos obtained with frozen-thawed spermatozoa.

https://doi.org/10.1371/journal.pone.0213420.g006

phosphorylation, metabolic pathways, and non alcoholic fatty liver disease significantly over-represented in transcripts with reduced abundance of all CRYO embryos obtained.

When we evaluated low-abundance equine transcripts for their mouse orthologs, we found that many of the genes downregulated in CRYO embryos have knockout database annotation terms related to reduced embryonic viability. This finding opens the possibility that not only genes related to the metabolism and thus growth of embryos, but also genes directly related to embryo organogenesis, embryo survival, and offspring health are affected by the use of cryopreserved sperm, and thus these genes warrant further investigation.

While the mechanisms behind the effects reported here are as yet unclear, a major factor may be the well-documented oxidative damage that the genome and epigenome experiences during cryopreservation and thawing [11–14]. Cryopreservation is a major cause of oxidative stress [37] and lipid peroxidation in stallion spermatozoa [10, 17, 38, 39]. Lipid peroxidation in spermatozoa surviving cryopreservation [37] is associated with increased levels of 4-hydroxinonenal (4-HNE) [17]. This compound is able to interact with DNA to form adducts that have been related directly to increased rates of mutation in important cell-cycle regulators [40, 41]. The production of 4-HNE during cryopreservation of stallion spermatozoa is well documented [10, 17, 39], and it is possible that significant amounts of 4-HNE and other toxic lipid aldehydes are incorporated to the oocyte, potentially causing alterations in embryo development. In addition to DNA damage, 4-HNE can alkylate the sperm centrioles, and in horses, as in humans, paternal centrioles are inherited by the embryos. Damaged centrioles may cause disrupted cytoskeletal protein organization during early cleavage [42].

Supporting this line of reasoning, recent reports have linked abnormal early cleavage events and changes in embryo transcript abundance to fertilization with spermatozoa showing oxidative stress. Macaque embryos obtained after fertilization with ROS-treated sperm showed significantly lower rates of development to the four- and eight-cell stages, and changes in transcript abundance for genes related to actin cytoskeleton organization, cell junction

Table 3. Gene ontology annotations enriched in downregulated transcripts of 10 days embryos obtained after AI with frozen thawed sperm.

Term	P Value
GO:0070062~extracellular exosome	1,72E-09
GO:0043209~myelin sheath	3,03E-09
GO:0070469~respiratory chain	6,30E-08
GO:0022625~cytosolic large ribosomal subunit	5,33E-07
GO:0008137~NADH dehydrogenase (ubiquinone) activity	8,07E-07
GO:0005747~mitochondrial respiratory chain complex I	3,12E-06
GO:0015986~ATP synthesis coupled proton transport	4,78E-06
GO:0003735~structural constituent of ribosome	1,66E-05
GO:0000788~nuclear nucleosome	2,25E-05
GO:0046933~proton-transporting ATP synthase activity, rotational mechanism	2,90E-05
GO:0005925~focal adhesion	4,41E-05
GO:0006412~translation	5,75E-05
GO:0046961~proton-transporting ATPase activity, rotational mechanism	1,59E-04
GO:0000786~nucleosome	1,73E-04
GO:0042773~ATP synthesis coupled electron transport	2,22E-04
GO:0006334~nucleosome assembly	5,99E-04
GO:0005743~mitochondrial inner membrane	0,001576512
GO:0004129~cytochrome-c oxidase activity	0,002995144
GO:0005739~mitochondrion	0,00444262
GO:0006336~DNA replication-independent nucleosome assembly	0,005364717
GO:0045261~proton-transporting ATP synthase complex, catalytic core F(1)	0,011191606
GO:0015991~ATP hydrolysis coupled proton transport	0,013628447
GO:0006457~protein folding	0,017943471
GO:0051603~proteolysis involved in cellular protein catabolic process	0,019505148
GO:0003677~DNA binding	0,022056606
GO:0006123~mitochondrial electron transport, cytochrome c to oxygen	0,024396132
GO:0044822~poly(A) RNA binding	0,032707627
GO:0005753~mitochondrial proton-transporting ATP synthase complex	0,0332053
GO:0005615~extracellular space	0,040768468
GO:0005687~U4 snRNP	0,044030068
GO:0045454~cell redox homeostasis	0,047994139
GO:0006122~mitochondrial electron transport, ubiquinol to cytochrome c	0,048204941
GO:1902166~negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	0,054067016
GO:0006120~mitochondrial electron transport, NADH to ubiquinone	0,059893472
GO:0045653~negative regulation of megakaryocyte differentiation	0,065684522
GO:0030330~DNA damage response, signal transduction by p53 class mediator	0,065684522
GO:0016020~membrane	0,071097195
GO:0004185~serine-type carboxypeptidase activity	0,07400431
GO:0034719~SMN-Sm protein complex	0,075791838
GO:0005685~U1 snRNP	0,075791838
GO:0002227~innate immune response in mucosa	0,077161252
GO:0007569~cell aging	0,077161252
GO:0005682~U5 snRNP	0,080983251
GO:0071157~negative regulation of cell cycle arrest	0,082847353
GO:0019731~antibacterial humoral response	0,082847353
GO:0005975~carbohydrate metabolic process	0,083234703

(*Continued*)

Table 3. (Continued)

Term	P Value
GO:0005686~U2 snRNP	0,086145885
GO:0030970~retrograde protein transport, ER to cytosol	0,088498889
GO:0000784~nuclear chromosome, telomeric region	0,089088094
GO:0045787~positive regulation of cell cycle	0,094116067

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assembly and cell adhesion [43]. Although seen at a much later stage of development, in our study we also found that genes for cytoskeleton components tubulin alpha 1 a, tubulin beta 2

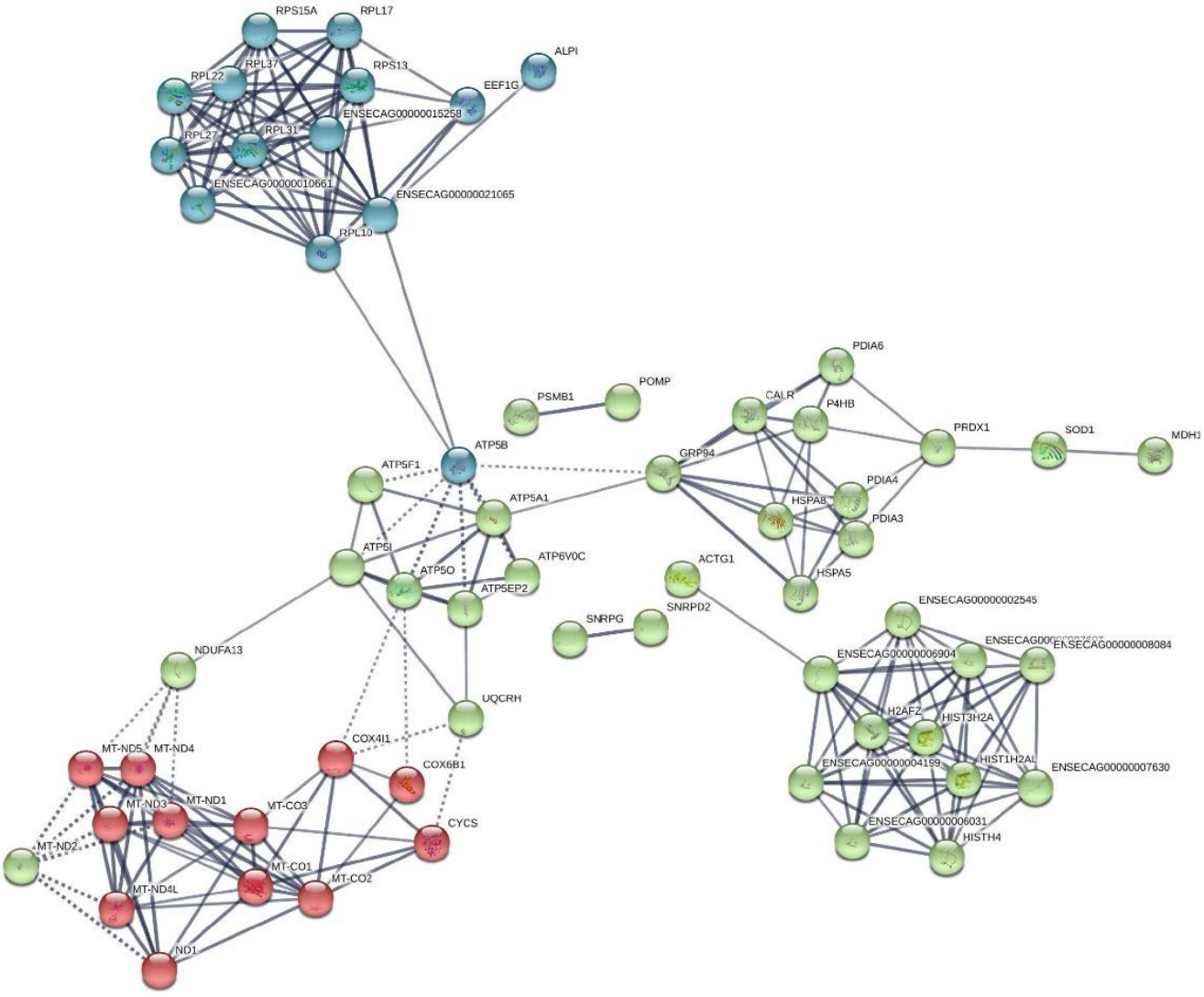

Fig 7. Functional networks (STRING) of transcripts downregulated in 10-Day equine embryos obtained with frozen-thawed sperm (CRYO embryos). Functional networks apply to histones and mitochondrial proteins. Controls are same-age embryos from the same mare, obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g007

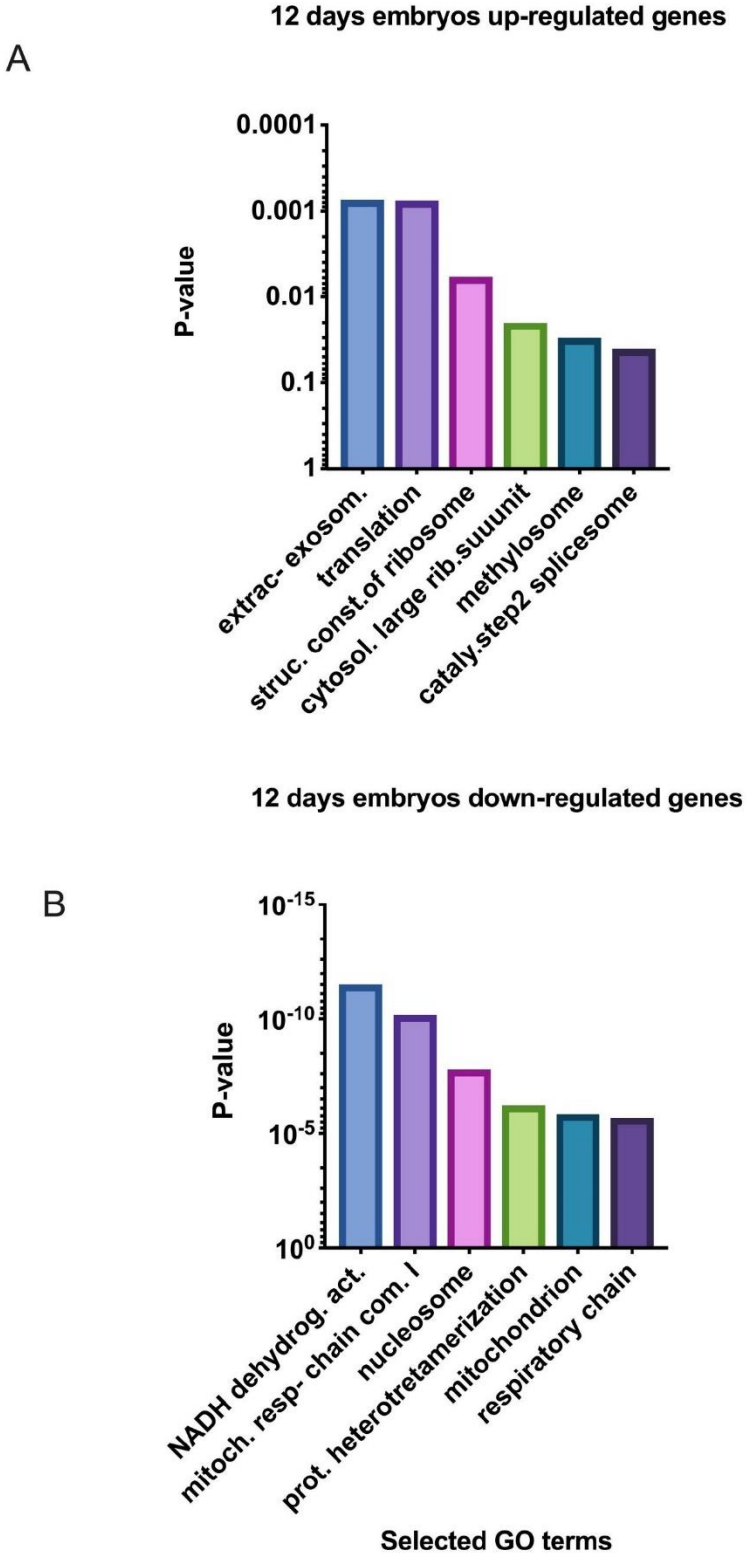
A
12 days embryos up-regulated genes
P-value
0.0001
0.001
0.01
0.1
1
extrac- exosom.
translation
struc. const.of ribosome
cytosol. large rib.suuunit
methylosome
cataly.step2 splicesome
B
12 days embryos down-regulated genes
P-value
10-15
10-10
10-5
100
NADH dehydrog. act.
mitoch. resp- chain com. I
nucleosome
prot. heterotretamerization
mitochondrion
respiratory chain
Selected GO terms

Fig 8. Selected enriched GO terms differentially regulated in 12-Day equine embryos obtained with fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated in 12-Day CRYO embryos, (B) transcripts up regulated in 12-Day CRYO embryos.

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class II a and actin, cytoplasmic 1, N-terminally processed were downregulated in 8-day CRYO embryos.

Cryopreservation may also directly affect the epigenome of the paternal DNA; recent studies have shown that cryopreservation increases the level of DNA methylation in equine sperm [12] and the expression of genes important to intracellular regulation of epigenetic status [16]. Notably, we also found significant reduction in abundance of transcripts for histones in CRYO embryos.

The finding that many differentially regulated genes in CRYO embryos are orthologs of mouse genes that have knockout database annotation terms related to reduced embryonic viability provides further evidence linking cryopreserved sperm to reduced embryonic viability. These annotations consistently appeared on analysis of low-abundance transcripts in all CRYO embryos, and included genes related to embryonic growth retardation and embryo lethality. Interestingly, annotations related to male and female infertility were also present; this warrants further investigation on the effect of sperm origin on the fertility of resulting offspring.

In summary, the present study provides for the first time transcriptomic analysis of equine embryos in relation to the handling of semen used for their production, however we acknowledge the preliminary and descriptive nature of this report but our data provide strong evidence that cryopreservation of sperm exerts a profound impact on the transcriptome of early

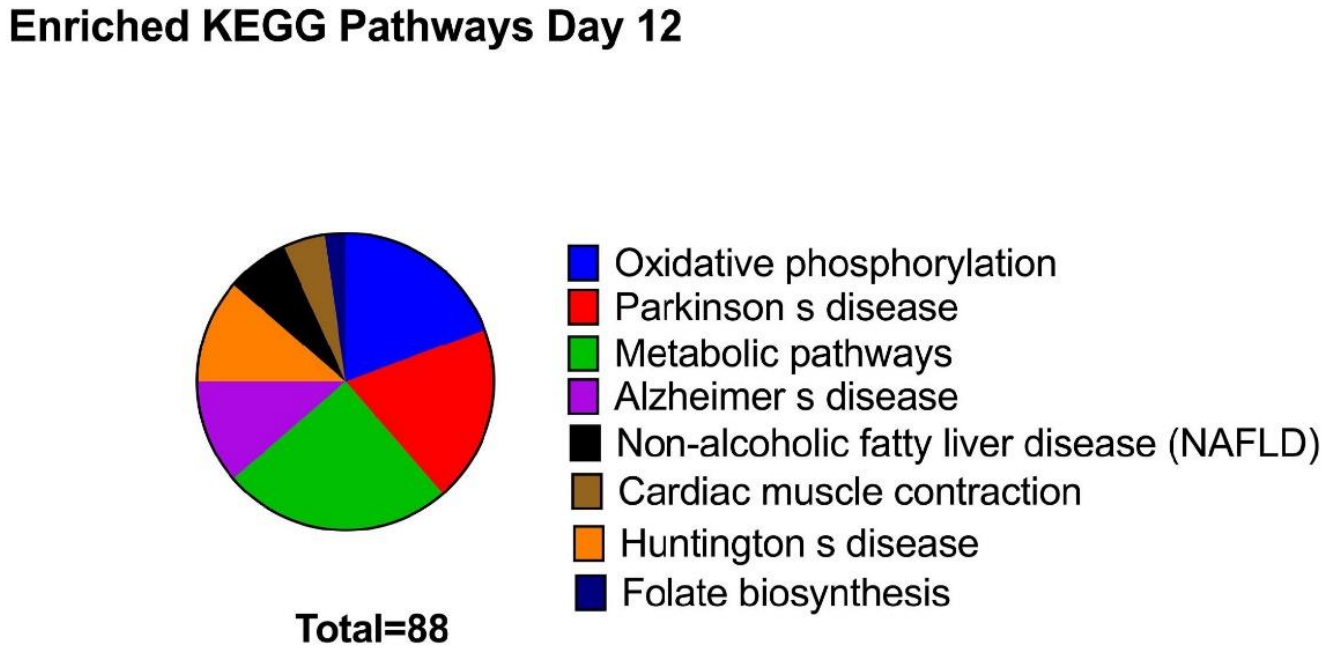

Fig 9. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 12-Day embryos obtained with frozen thawed spermatozoa.

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Table 4. Functional annotation chart of differentially expressed genes (downregulated) in Day-12 equine embryos obtained after AI with frozen-thawed sperm.

Category	Term	Count	PValue
UP_KEYWORDS	Membrane	22	0,035165176
GOTERM_CC_DIRECT	GO:0016021~Integral component of membrane	19	0,023827798
KEGG_PATHWAY	ecb01100:Metabolic pathways	18	8,33E-05
GOTERM_CC_DIRECT	GO:0005739~Mitochondrion	14	1,40E-06
KEGG_PATHWAY	ecb00190:Oxidative phosphorylation	13	1,69E-12
KEGG_PATHWAY	ecb05012:Parkinson's disease	13	3,57E-12
GOTERM_CC_DIRECT	GO:0070062~Extracellular exosome	13	0,047249394
UP_KEYWORDS	Chromosome	10	4,30E-12
UP_KEYWORDS	Mitochondrion	10	1,63E-10
UP_KEYWORDS	DNA-binding	10	2,09E-05
UP_KEYWORDS	Transport	10	3,60E-05
UP_KEYWORDS	Nucleus	10	6,21E-04
UP_KEYWORDS	Respiratory chain	9	7,16E-16
UP_KEYWORDS	Electron transport	9	3,97E-14
UP_KEYWORDS	Nucleosome core	9	2,19E-11
INTERPRO	IPR009072:Histone-fold	9	2,07E-10
UP_KEYWORDS	Ubiquinone	8	3,96E-16
GOTERM_MF_DIRECT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	8	3,09E-12
GOTERM_CC_DIRECT	GO:0005747~Mitochondrial respiratory chain complex I	8	6,67E-11
GOTERM_MF_DIRECT	GO:0003677~DNA binding	8	5,87E-04
UP_SEQ_FEATURE	Transmembrane region	8	9,18E-04
GOTERM_CC_DIRECT	GO:0000786~Nucleosome	7	1,55E-08
UP_KEYWORDS	NAD	7	5,92E-08
KEGG_PATHWAY	ecb05322:Systemic lupus erythematosus	7	3,03E-05
UP_KEYWORDS	Oxidoreductase	7	4,97E-05
KEGG_PATHWAY	ecb05034:Alcoholism	7	2,10E-04
GOTERM_CC_DIRECT	GO:0016020~Membrane	7	0,047593153
UP_KEYWORDS	Mitochondrion inner membrane	6	2,81E-07
KEGG_PATHWAY	ecb05010:Alzheimer's disease	6	0,001939005
KEGG_PATHWAY	ecb05016:Huntington's disease	6	0,003145256
GOTERM_BP_DIRECT	GO:0006335~DNA replication-dependent nucleosome assembly	5	4,77E-07
GOTERM_BP_DIRECT	GO:0051290~Protein heterotetramerization	5	5,76E-07
INTERPRO	IPR007125:Histone core	5	2,52E-05
GOTERM_CC_DIRECT	GO:0000784~Nuclear chromosome, telomeric region	5	2,30E-04
GOTERM_CC_DIRECT	GO:0005743~Mitochondrial inner membrane	5	0,001683893
SMART	SM00417:H4	4	6,25E-07
SMART	SM00803:TAF	4	1,22E-06
GOTERM_CC_DIRECT	GO:0070469~Respiratory chain	4	2,03E-06
INTERPRO	IPR019809:Histone H4, conserved site	4	2,80E-06
INTERPRO	IPR001951:Histone H4	4	2,80E-06
GOTERM_BP_DIRECT	GO:0045653~Negative regulation of megakaryocyte differentiation	4	4,00E-06
INTERPRO	IPR004823:TATA box binding protein associated factor (TAF)	4	5,47E-06
GOTERM_BP_DIRECT	GO:0006336~DNA replication-independent nucleosome assembly	4	1,95E-05
GOTERM_BP_DIRECT	GO:0006352~DNA-templated transcription, initiation	4	2,71E-05
INTERPRO	IPR020904:Short-chain dehydrogenase/reductase, conserved site	4	1,57E-04
INTERPRO	IPR002347:Glucose/ribitol dehydrogenase	4	6,29E-04
GOTERM_BP_DIRECT	GO:0006334~Nucleosome assembly	4	8,65E-04
GOTERM_MF_DIRECT	GO:0016491~Oxidoreductase activity	4	0,001585835

(*Continued*)

Table 4. (Continued)

Category	Term	Count	PValue
INTERPRO	IPR016040:NAD(P)-binding domain	4	0,016019866
KEGG_PATHWAY	ecb04932:Non-alcoholic fatty liver disease (NAFLD)	4	0,045179763
INTERPRO	IPR001750:NADH:ubiquinone/plastoquinone oxidoreductase	3	3,19E-05
GOTERM_BP_DIRECT	GO:0042773~ATP synthesis coupled electron transport	3	5,21E-05
GOTERM_CC_DIRECT	GO:0000788~Nuclear nucleosome	3	0,004620686
KEGG_PATHWAY	ecb04978:Mineral absorption	3	0,022822957
GOTERM_CC_DIRECT	GO:0000790~Nuclear chromatin	3	0,073903618

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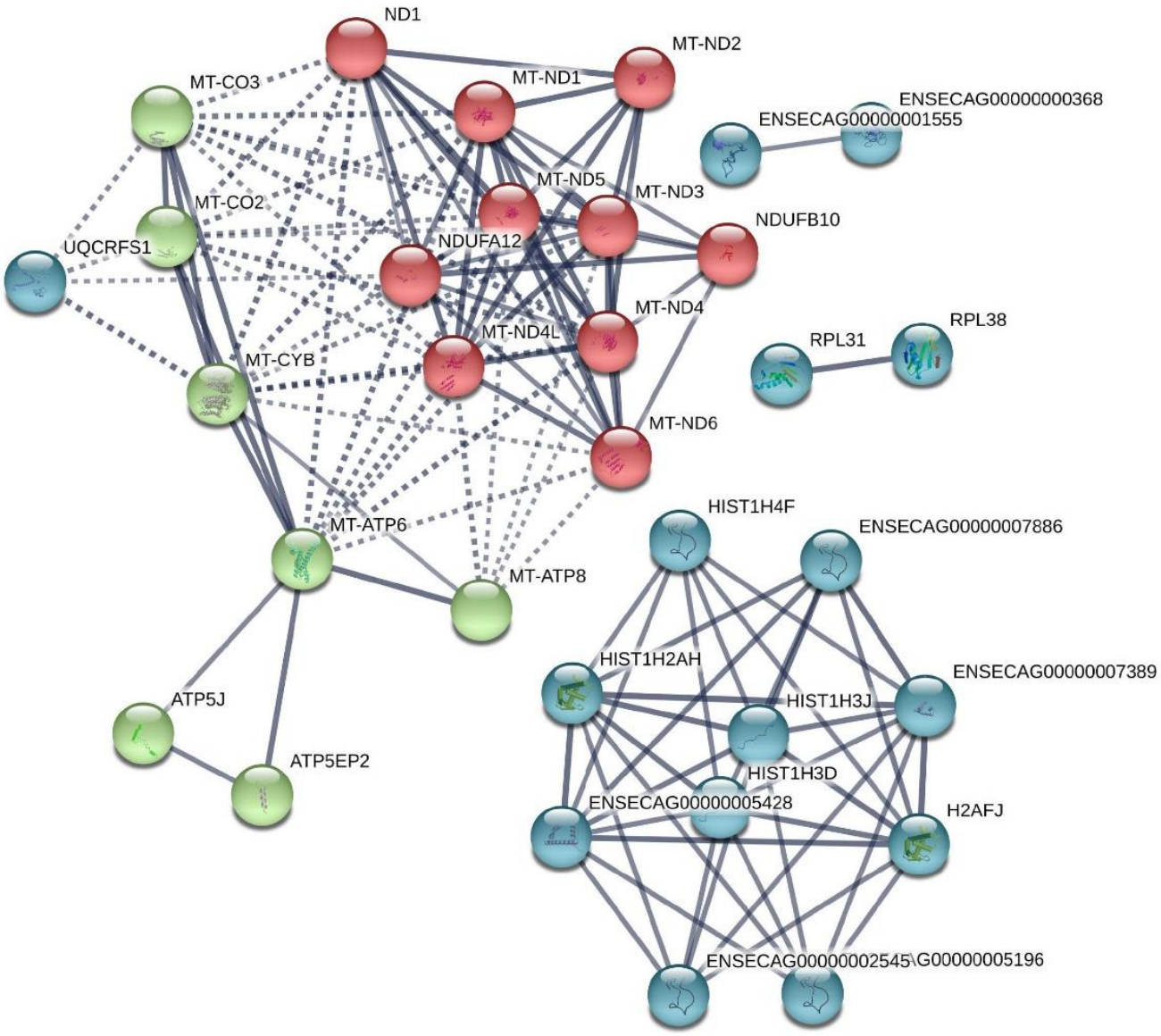

Fig 10. Functional networks (STRING) of transcripts downregulated in 12-Day equine embryos obtained with frozen thawed sperm (CRYO). Functional networks apply to histones and mitochondrial proteins. Controls are same-age embryos from the same mare obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g010

embryos. Our findings may stimulate new lines of research to improve this biotechnology in humans and animals.

Supporting information

S1 Table. Transcripts upregulated in 8-Day embryos obtained with frozen-thawed spermatozoa with respect to controls obtained with fresh semen.
(XLSX)

S2 Table. Transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S3 Table. Network analysis of transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa. List of transcripts in each cluster obtained after STRING analysis; genes in each cluster are presented and colors for each cluster are given. Network is presented in Fig 4.
(XLSX)

S4 Table. Transcripts upregulated in 10-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S5 Table. Transcripts upregulated in 12-day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S6 Table. Transcripts downregulated in 12-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

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Supervision: Katrin Hinrichs, Fernando J. Peña.

Validation: Ángel Román.

Visualization: Ángel Román.

Writing – original draft: Katrin Hinrichs, Fernando J. Peña.

Writing – review & editing: Heriberto Rodríguez-Martínez, Katrin Hinrichs, Fernando J. Peña.

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Artículo 7

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Sperm cryopreservation impacts the early development of equine embryos by downregulating specific transcription factors

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ABSTRACT

Equine embryos were obtained by insemination with either fresh or frozen-thawed spermatozoa at 8, 10 and 12 h post spontaneous ovulation, maintaining the pairs mare-stallion for the type of semen used. Next generation sequencing (NGS) was performed in all embryos and bioinformatic and enrichment analysis performed on the 21,058 identified transcripts. A total of 165 transcripts were downregulated in embryos obtained with cryopreserved spermatozoa respect embryos resulting from an insemination with fresh spermatozoa (p=0.021, q=0.1). The enrichment analysis using human orthologs using g:profiler on the downregulated transcripts marked an

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enrichment in transcription factors (TFs) in mRNAs downregulated in embryos obtained after insemination with cryopreserved spermatozoa. The 12 mRNAs (discriminant variables) most significantly downregulated in these embryos included among others, the *chromatin-remodeling ATPase INO80, Lipase maturation factor 1 LMF1, the mitochondrial mRNA pseudouridine synthase RPUSD3, LIM and cysteine-rich domains protein 1, LMCD1*. Sperm cryopreservation also caused a significant impact on the embryos at 8 to 10 days of development, but especially in the transition from 10 to 12 days. Overall, our findings provide strong evidence that insemination with cryopreserved spermatozoa poses a major impact in embryo development that may compromise its growth and viability, probably due to modifications in sperm proteins induced by cryopreservation. Identification of specific factors in the spermatozoa causing these changes may improve cryopreservation.

Key words: horse, embryo, semen, cryopreservation, NGS, mRNAs, transcriptome, INO80, sperm proteins

INTRODUCTION

Cryopreservation is widely used due to their importance for the international commerce of equine semen of superior sires, and as a safety measure to avoid loses of genetic material in case of accidental death of a stallion. However, this technology still is suboptimal and numerous drawbacks persist (Pena et al. 2011). The fertility obtained with this technology is overall considered reduced compared with fresh-extended spermatozoa, and one of the causes is related to increased embryo loss when frozen semen is used (Panzani et al. 2014; Ortiz-Rodriguez et al. 2019b). In addition, delayed embryo development is associated with insemination of cryopreserved spermatozoa (Stout 2006). A growing body of scientific evidence indicates that the spermatozoa have important regulatory roles in the early embryo development (Jodar et al. 2020). These findings underpin the importance of potentially damaged sperm factors controlling early embryo development (Castillo et al. 2018). Processing the ejaculate for cryopreservation implies the removal of seminal plasma,

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extension in media containing cryoprotectants cooling, freezing, storage in liquid nitrogen, and thawing. All these procedures impact the spermatozoa; numerous mechanisms explaining cryodamage have been described, including toxicity of cryoprotectants (Macias Garcia et al. 2012), osmotic shock at freezing, and specially at thawing damaging membranes and the mitochondria (Garcia et al. 2012; Pena et al. 2015). This mitochondrial damage causes redox imbalance and accelerates different forms of sperm death, including necrotic, apoptotic (Aitken and Koppers 2011; Aitken and Baker 2013) and probably ferroptotic sperm mortality (Ortiz-Rodriguez et al. 2020). In addition to protein degradation during cryopreservation, redox deregulation may induce oxidative damage to proteins causing notable modifications of the sperm proteome (Bogle et al. 2017; Martin-Cano et al. 2020a; Gaitskell-Phillips et al. 2021b). All these modifications may affect proteins participating in the regulation and or survival of the early embryo (Castillo et al. 2018). Thus, we tested the hypothesis that early embryos, product of insemination with cryopreserved spermatozoa display detectable transcriptomic alterations that could jeopardize their development, taking in account that RNA seq is considered a relevant tool to evaluate embryo competence (Groff et al. 2019).

MATERIAL AND METHODS

Embryo collection and experimental design

Animals were maintained according to European regulations, and all experimental procedures were reviewed and approved by the Ethical committee of the University of Extremadura, Cáceres, Spain. Mares were treated with a prostaglandin analogue to shorten the luteal phase and were monitored daily for follicular development, degree of uterine edema and cervical tone using transrectal ultrasonography (US). When a follicle at least 35 x35 mm was detected in absence of luteal tissue, with good uterine edema and low cervical tone, mares received 2.500 IU of hCG. Follicular development was thereafter closely monitored by US and mares were inseminated immediately once ovulation was detected either with fresh sperm or frozen-thawed sperm from the same stallion (two mares were paired per stallion, one for each type of sperm used). A

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total of 18 conceptuses were obtained by uterine lavage at 8, 10, or 12 days post ovulation; embryos were snap frozen in LN_2 and stored at -80ºC until analysis.

Isolation of RNA

Total RNA was isolated from the embryos using the kit PicoPure™ RNA Isolation Kit (Catalog number:KIT0204, Thermofisher) following manufacturer instructions. RNA concentration and quality were assessed by automatic electrophoresis using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RNA-seq analysis

Libraries were built for RNA-seq analysis in an IonTorrent S5/XL sequencer (Thermo Fisher Scientific, Waltham, MA USA). The raw reads were aligned to a horse transcriptome generated using ENSEMBL (Equ Cab 3 version) in Torrent server with the proprietary ThermoFisher algorithms. Then, BAM files were imported into Qlucore Omics Explorer ver 3.7 (https://www.qlucore.com) for analysis.

Bioinformatic Analysis

Variance filtering and PCA

Transcripts were normalized to TPM, and then aligned data were normalized and log_2 transformed using Qlucore Omics Explorer (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score (Fontes and Soneson 2011) was used to determine the optimal filtering threshold.

Identifying discriminating variables

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Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables with significant differences between transcripts in embryos resulting from inseminations with fresh or cryopreserved spermatozoa, and in embryos of different ages also either obtained with fresh or frozen thawed spermatozoa. The identification of significantly different variables between the different subgroups of embryos was performed by fitting a linear model for each variable semen used in the insemination (fresh or frozen thawed) and age of the embryo. P-values were adjusted for multiple testing using the Benjamini-Hochberg method (Viskoper et al. 1989; Tamhane et al. 1996) and variables with adjusted p-values equal or below 0.1 were considered significant.

Gene Ontology and pathway analysis

PANTHER (http://www.pantherdb.org/pathway/pathwayList.jsp) and KEGG pathway (https://www.genome.jp/kegg/) (Ogata et al. 1999; Altermann and Klaenhammer 2005; Du et al. 2014; Mi et al. 2019) analysis was used to identify biological pathways likely to be active in the proteins enriched in each group. Reactome (https://reactome.org) (Ogata et al. 1999; Altermann and Klaenhammer 2005; Du et al. 2014; Mi et al. 2019) analysis were used to identify biological pathways likely to be active in the transcripts enriched in each group, g:Profiler was also used to perform an enrichment analysis (Raudvere et al. 2019). Due to the increased depth of the human proteome in terms of annotation, the equine annotations were transformed to their human orthologs using g:Profiler (https://biit.cs.ut.ee/gprofiler/orth) and a pathway enrichment analysis and visualization was performed again using g:Profiler and Cytoscape analysis using Reactome (https://reactome.org).

Network analysis

Cytoscape (https://cytoscape.org) plug in ClueGo was used to identify functionally grouped gene ontology terms in equine seminal plasma as described in (Bindea et al. 2009; Mlecnik et al. 2018). STRING (https://version-10-5.string-db.org) was used to identify potential functional partners of specific proteins.

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RESULTS

Overall impact of cryopreserved spermatozoa on the transcriptome of equine embryos

A total of 21058 transcripts were identified. In a first step we constructed volcano plots to have a general overview of the impact of the type of semen (fresh or cryopreserved) on the transcriptome of the equine embryos (Figure 1 B). Then we compared the overall change induced by insemination with frozen and thawed spermatozoa on the embryonic transcriptome; we found that 165 transcripts were downregulated in embryos obtained with cryopreserved spermatozoa respect embryos resulting from an insemination with fresh spermatozoa (Figure 1AC; p=0.021, q=0.1). We performed enrichment analysis, using human orthologs, on the transcripts downregulated in embryos using g profiler, and significant enrichment was detected in different gene ontology (GO) terms; particularly notable was the enrichment in transcription factors (TFs) in transcripts downregulated in embryos obtained after insemination with cryopreserved spermatozoa. A total of 84 TFs were identified (Figs 2 and supplementary figure 1), including as most significantly enriched the TF NF-1 ($p=1.040x10^{-14}$), TF KLF13 ($p=2.734x10^{-14}$), TF CPBP ($p=1.450x\ 10^{-12}$), TF BTEB3 ($p=1.139x10^{-11}$), TF TCF7L1 ($p=1.398x\ 10^{-11}$) and TF KLF3 ($p=5.8x\ 10^{-11}$).

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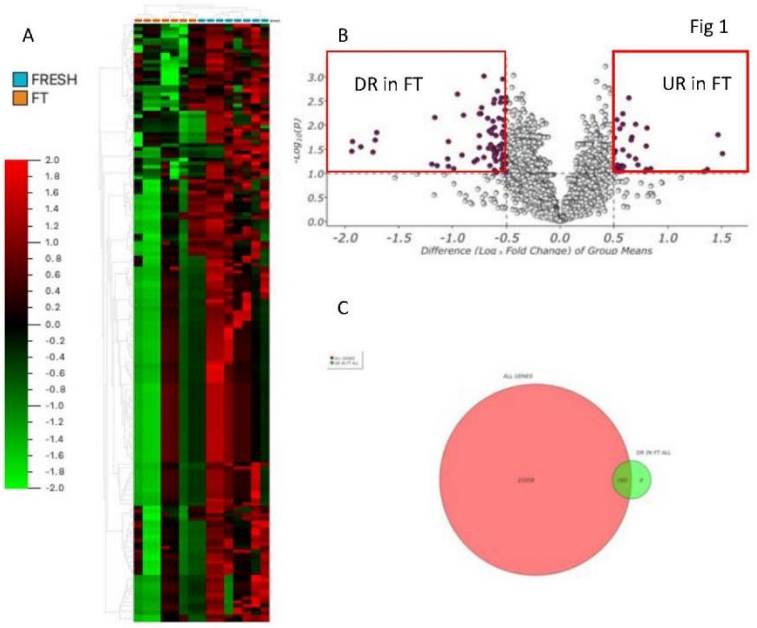

Figure 1.- Changes in the embryo transcriptome depending of the type of semen used, fresh or cryopreserved. A) Heat map showing different expression of transcripts in embryos produced with fresh semen (right part), and those obtained with cryopreserved sperm (left part). In B, a Volcano plot showing transcripts differentially expressed in embryos obtained with fresh or cryopreserved spermatozoa. C) Venn diagram showing transcripts downregulated in embryos obtained with cryopreserved spermatozoa.

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Fig 2

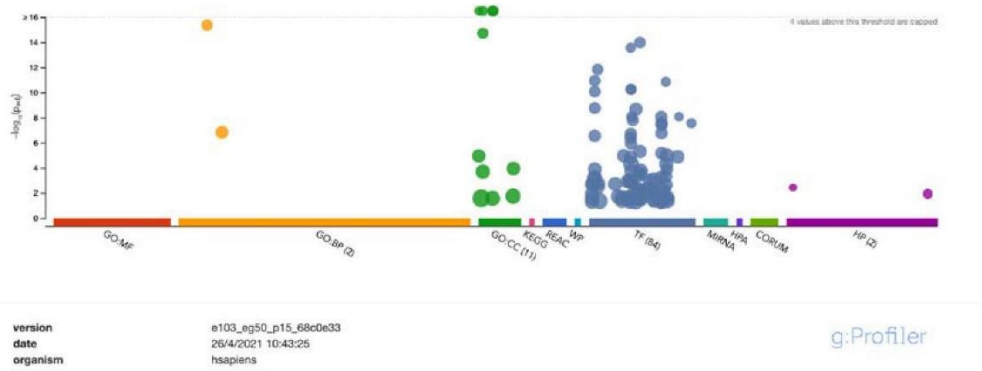

Figure 2.- g:GOST Manhattan plot showing terms enriched in downregulated transcripts in embryos obtained with cryopreserved spermatozoa (human orthologs were used due to the deeper annotation of the human genome). Interestingly, 84 transcription factors appeared downregulated. Detailed information appears in supplementary figures 1 and 2

Network visualization analysis

The visualization of integration networks using the Cytoscape app Clue go revealed annotations like regulation of nuclear division, mitotic DNA damage checkpoint and positive regulation of molecular mediator of immune response, and interleukine 4 and 13 signalling were significantly affected by sperm cryopreservation (Fig 3)

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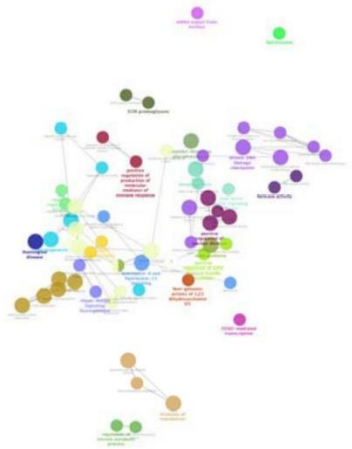

Figure 3.- ClueGo network analysis of proteins upregulated in embryos obtained with fresh semen compared wth respect to those in embryos obtained with cryopreserved spermatozoa. To reduce the redundancy of GO terms, the fusion option was selected. GO/KEGG/REACTOME pathways functionally grouped networks with terms are indicated as nodes (Benjamini-Hockberg P value <0.01) and a minimum of 5 genes per group, linked by their kappa score level >0.4 where only the label of the most significant term per group is shown.

Identification of discriminant variables between fresh and cryopreserved spermatozoa derived embryos

To reduce the number of variables, we applied stricter statistical criteria to reduce the number of variables differing between both conditions. We identified 12 discriminant variables significantly downregulated in embryos obtained with cryopreserved spermatozoa including among others, the *chromatin-remodeling ATPase INO80, Lipase maturation factor 1 LMF1, the mitochondrial mRNA pseudouridine synthase RPUSD3, LIM and cysteine-rich domains protein 1, LMCD1* (Figure 4).

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Fig 4

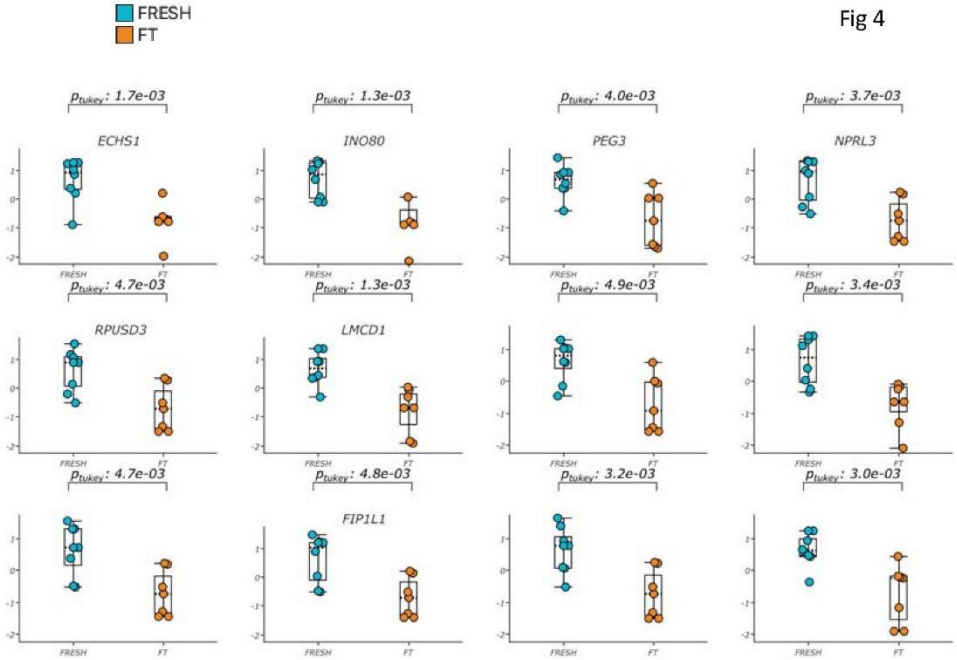

Figure 4.- Differences in the amounts of specific transcripts in embryos obtained with inseminations with fresh or cryopreserved spermatozoa.

Impact of cryopreservation on the development of equine embryos from 8 to 10 days post ovulation

We investigated if the use of frozen thawed spermatozoa impacted embryo development in two developmental stages 8 to 10 days and 10 to 12 days after ovulation. We identified numerous genes differentially expressed in both stages. On the first stage studied, transition 8 to 10 days, 202 upregulated expressed transcripts were detected (Fig 5A; p=0.04 q=0.1) in embryos obtained after insemination with fresh semen, while in embryos obtained after insemination with frozen thawed semen only 39 transcripts were upregulated from day 8 to day 10 (p=0.04 q=0.03; Figure 5 B). A significant enrichment was only detected in embryos derived from insemination with fresh spermatozoa (Figure 5). In this stage the TFs E2F-4 $8.1x10^{-3}$, C/EBPgamma $2.57X10^{-2}$ and ZGPAT $2.69x10^{-2}$were significantly enriched, as were the reactome

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pathways C6 deamination of adenosine, formation of editosomes by ADAR proteins and mRNA editing Ato I conversion ($2.39x10^{-2}$) (Figure 6)

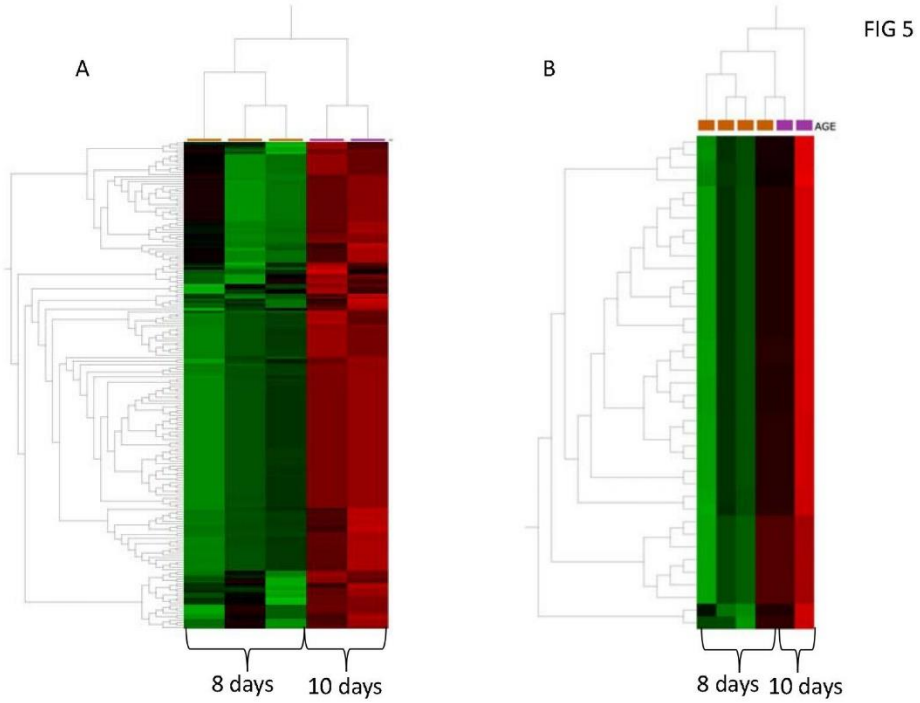

Figure 5.- Heat maps showing the transition from 8 to 10 days of embryo development, A) 202 upregulated expressed transcripts were detected (p=0.04 q=0.1) in embryos obtained after insemination with fresh semen. B) In embryos obtained after insemination with frozen thawed semen only 39 transcripts were upregulated from day 8 to day 10 (p=0.04 q=0.03)

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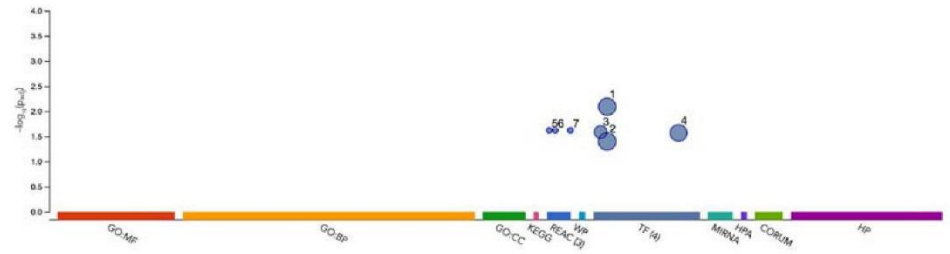
FIGURE 6

UR FRESH 8-10 dias



ID	Source	Term ID	Term Name	P_{adj} (query_1)
1	TF	TF:M11535	Factor: E2F-4; motif: TTTTGGCGCCAWWN	8.093×10^{-3}
2	TF	TF:M09894_1	Factor: E2F-4; motif: SNGGGCGGGAANN; match class: 1	3.993×10^{-2}
3	TF	TF:M11324_1	Factor: C/EBPgamma; motif: NNTTGCGYMANN; match class: 1	2.576×10^{-2}
4	TF	TF:M09739	Factor: ZGPAT; motif: GRGGCWGNGGNG	2.694×10^{-2}
5	REAC	REAC:R-HSA-75102	C6 deamination of adenosine	2.392×10^{-2}
6	REAC	REAC:R-HSA-77042	Formation of editosomes by ADAR proteins	2.392×10^{-2}
7	REAC	REAC:R-HSA-75064	mRNA Editing: A to I Conversion	2.392×10^{-2}

version	e103_eg50_p15_68c0e33
date	3/5/2021 14:20:38
organism	hsapiens

g:Profiler

Figure 6.- g:GOST multiquery Manhattan plot showing enrichment analysis of the transcripts upregulated of embryos obtained with fresh spermatozoa in the transition from 8 to 10 days after ovulation. Three Reactome pathways and four transcription factors were significantly enriched. The *P* values are depicted on the y axis and in more detail in the results table below present the image.

Impact of cryopreservation on the development of equine embryos from 10 to 12 days post ovulation

On the other hand, when we compared 10 versus 12 days old embryos it was evident that some genes were differentially expressed, with a group of transcripts upregulated and another downregulated (Fig 7 C). This stage was characterized by a dramatic change in the transcriptome of equine embryos irrespective of the kind of spermatozoa used, either fresh or frozen-thawed.

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A total of 18270 transcripts were differential expressed in the transition from 10 to 12 days in all embryos (Fig 7 C and D; p=0.02; q=0.03); 19651 transcripts were upregulated in embryos resulting from cryopreserved spermatozoa (p=0.04; q=0.07), while, only 25 transcripts were upregulated in the transition from 10 to 12 days in embryos obtained with fresh semen (p=0.049; q=0.09; Figure 7 D-E). Enrichment analysis of these transcripts, after conversion to their human orthologs, revealed dramatic differences between both conditions, with two terms (MIRNAs) enriched in the transition from 10 to 12 days in fresh spermatozoa (has-miR-3150a-3p; $4.2x10^{-2}$ and has-miR 6763-5p $4.2x10^{-2}$; Fig 6). On the contrary, embryos derived from cryopreserved spermatozoa showed significant enrichment in many gene ontology terms; 10 for molecular function (MF) and 58 for biological process (BP) (Figure 8 and suppl figures 3-5). The reactome pathway metabolism was significantly enriched (4.48×10^{-17}), as was the TF TEF-3:EBPbeta ($9.48x10^{-3}$). Detailed description for all the enrichment is provided in supplementary figures. To better disclose the meaning of the increase in the number of annotations observed, we performed PANTHER overrepresentation tests, and found significantly underrepresented annotations in the categories "Protein class" (Table 1) and "Molecular Function" (Table 2). Interestingly this analysis showed many annotations underrepresented, including the protein classes gene-specific transcriptional regulator (PC00264; p=3.52E-03, FDR=3.77E-02), DNA-binding transcription factor (PC00218: p=2.52E-03; FDR=2.52E-03), zinc finger transcription factor (PC00244; p=9.42E-05, FDR=2.60E-03) and chromatin/chromatin-binding, or -regulatory protein (PC00077; p=2.94E-04, FDR=6.31E-03). Among the GO terms (MF) underrepresented in transcripts obtained from embryos obtained with cryopreserved spermatozoa were transmembrane receptor protein kinase activity (GO:0019199; p=2.35E-05, FDR=7.99E-03) and transmembrane receptor protein tyrosine kinase activity (GO:0004714; p=2.08E-05, FDR=7.62E-03).

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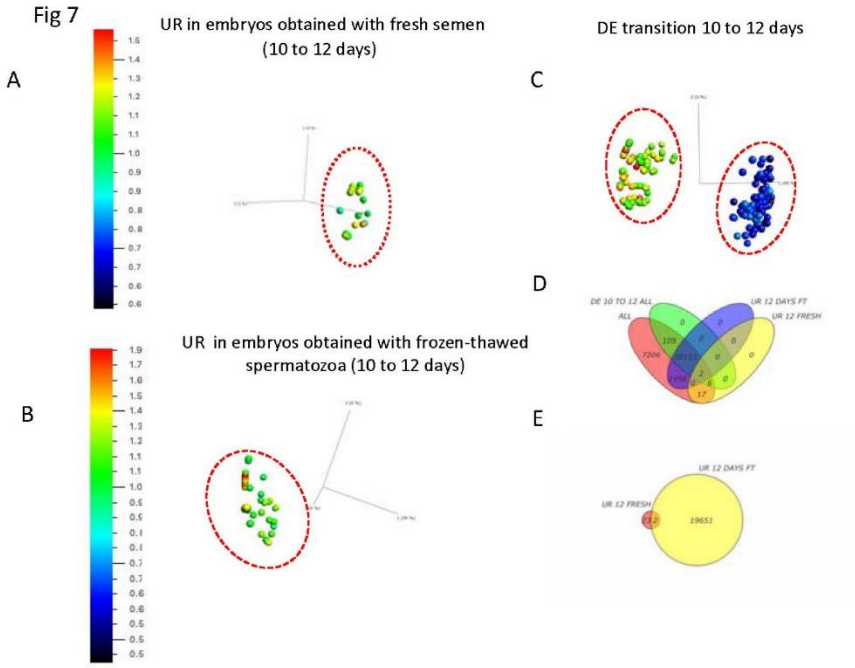

Fig 7.- 3D Principal component analysis of the changes in the transcriptome in embryos derived from fresh or cryopreserved spermatozoa in the transition from 10 to 12 days of embryo development. A total of 18,270 transcripts were differential expressed in the transition from 10 to 12 days in all embryos (C and D; p=0.02; q=0.03). B) 19651 transcripts were upregulated in embryos resulting from cryopreserved spermatozoa (p=0.04; q=0.07), while only 25 transcripts were upregulated in the transition from 10 to 12 days in embryos obtained with fresh semen (p=0.049; q=0.09; A-D).

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Figure 8

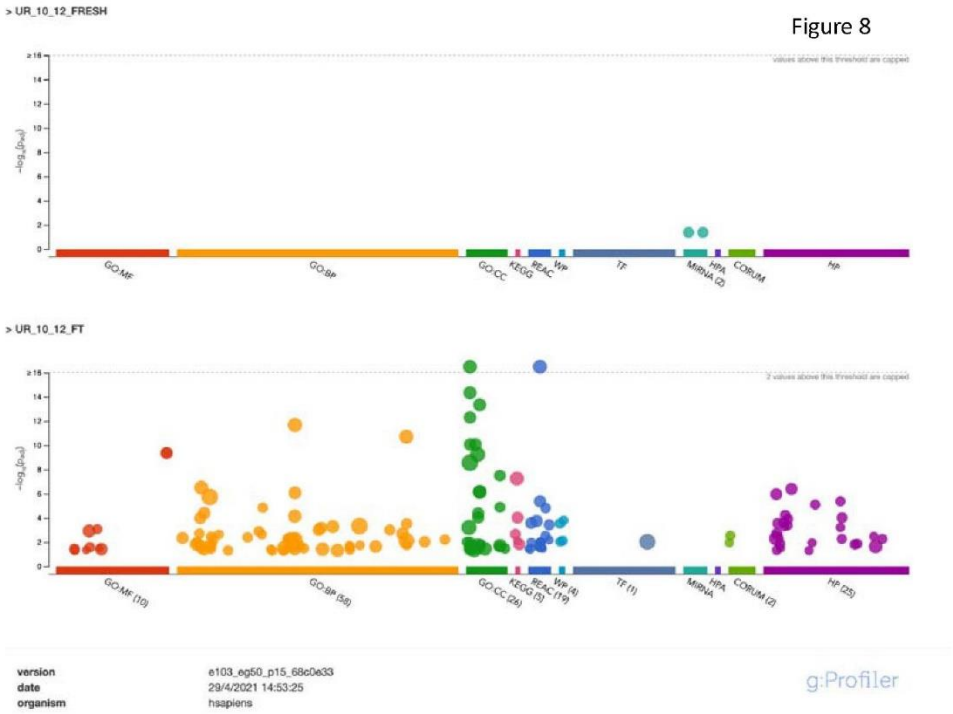

Fig 8.- g:GOST multiquery Manhattan plot showing comparative enrichment analysis of equine embryo transcripts under two different experimental conditions; embryos derived from inseminations with fresh or cryopreserved spermatozoa. Gene Ontology terms (GO) for molecular function (MF) are in red, for Biological Process (BP) in orange, and for cellular component (CC) in green. The P values are depicted in the y axis and more detailed in the result table below the image. Enrichment analysis was performed using human orthologs

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Table 1.- Panther overrepresentation test (Protein class) of transcripts upregulated in the transition from 10 to 12 days in embryos derived from cryopreserved spermatozoa.

PANTHER Protein Class	Homo sapiens - REFLIST (20595)	upload_1 (7809)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
transferase (PC00220)	665	343	252.15	+	1.36	3.48E-06	1.68E-04
metabolite interconversion enzyme (PC00262)	1843	925	698.81	+	1.32	5.02E-13	9.70E-11
oxidoreductase (PC00176)	553	276	209.68	+	1.32	2.02E-04	4.88E-03
protein class (PC00000)	12043	4727	4566.34	+	1.04	1.64E-03	2.26E-02
Unclassified (UNCLASSIFIED)	8552	3082	3242.66	-	.95	1.64E-03	2.44E-02
gene-specific transcriptional regulator (PC00264)	1280	414	485.34	-	.85	3.52E-03	3.77E-02
DNA-binding transcription factor (PC00218)	1194	381	452.73	-	.84	2.52E-03	2.86E-02
zinc finger transcription factor (PC00244)	541	144	205.13	-	.70	9.42E-05	2.60E-03
C2H2 zinc finger transcription factor (PC00248)	460	115	174.42	-	.66	3.16E-05	1.02E-03
chromatin/chromatin-binding, or -regulatory protein (PC00077)	284	67	107.68	-	.62	2.94E-04	6.31E-03
immunoglobulin (PC00123)	207	48	78.49	-	.61	1.48E-03	2.38E-02
extracellular matrix protein (PC00102)	162	33	61.43	-	.54	6.82E-04	1.20E-02
GTPase-activating protein (PC00257)	139	28	52.70	-	.53	1.65E-03	2.12E-02
G-protein modulator (PC00022)	262	52	99.34	-	.52	5.73E-06	2.21E-04
guanyl-nucleotide exchange factor (PC00113)	113	21	42.85	-	.49	1.78E-03	2.15E-02
cell adhesion molecule (PC00069)	203	32	76.97	-	.42	4.67E-07	3.00E-05
microtubule binding motor protein (PC00156)	69	8	26.16	-	.31	4.56E-04	8.80E-03
cadherin (PC00057)	113	4	42.85	-	.09	1.07E-11	1.03E-09

Analysis Type: PANTHER Overrepresentation Test (Released 20210224)
Annotation Version and Release Date: PANTHER version 16.0 Released 2020-12-01
Analyzed List: upload_1 (Homo sapiens)
Reference List: Homo sapiens (all genes in database)
Test Type: FISHER
Correction: FDR

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Table 2. Panther overrepresentation test (Molecular Function) of transcripts upregulated in the transition from 10 to 12 days in embryos derived from cryopreserved spermatozoa.

GO molecular function complete	Homo sapiens - REFLIST (20595)	upload_1 (7809)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
oxidoreductase activity (GO:0016491)	759	367	287.79	+	1.28	1.17E-04	2.95E-02
identical protein binding (GO:0042802)	2079	906	788.29	+	1.15	2.49E-04	4.76E-02
protein binding (GO:0005515)	14300	5901	5422.13	+	1.09	9.34E-25	1.48E-21
catalytic activity (GO:0003824)	5778	2376	2190.84	+	1.08	8.78E-05	2.46E-02
binding (GO:0005488)	16484	6614	6250.23	+	1.06	6.78E-20	8.08E-17
molecular_function (GO:0003674)	18235	7265	6914.16	+	1.05	8.53E-31	4.07E-27
DNA binding (GO:0003677)	2483	791	941.48	-	.84	4.66E-06	2.22E-02
adenyl nucleotide binding (GO:0030554)	1572	468	596.05	-	.79	1.28E-06	7.64E-04
adenyl ribonucleotide binding (GO:0032559)	1559	463	591.13	-	.78	1.16E-06	7.89E-04
ATP binding (GO:0005524)	1495	437	566.86	-	.77	4.52E-07	3.59E-03
nucleoside-triphosphatase regulator activity (GO:0060589)	522	132	197.93	-	.67	1.64E-05	6.52E-02
GTPase regulator activity (GO:0030695)	478	114	181.24	-	.63	3.09E-06	1.64E-03
Unclassified (UNCLASSIFIED)	2360	544	894.84	-	.61	8.53E-31	2.03E-27
GTPase activator activity (GO:0005096)	277	60	105.03	-	.57	3.65E-05	1.09E-01
ATPase activity (GO:0016887)	488	92	185.03	-	.50	3.45E-11	3.30E-07
helicase activity (GO:0004386)	159	29	60.29	-	.48	1.08E-04	2.85E-02
calcium ion transmembrane transporter activity (GO:0015085)	135	24	51.19	-	.47	2.45E-04	4.87E-02
motor activity (GO:0003774)	131	18	49.67	-	.36	7.81E-06	3.39E-03
microtubule motor activity (GO:0003777)	69	7	26.16	-	.27	1.47E-04	3.50E-02
transmembrane receptor protein kinase activity (GO:0019199)	81	8	30.71	-	.26	2.35E-05	7.99E-03
transmembrane receptor protein tyrosine kinase activity (GO:0004714)	62	4	23.51	-	.17	2.08E-05	7.62E-03
extracellular matrix structural constituent conferring tensile strength (GO:0030020)	41	2	15.55	-	.13	2.29E-04	4.76E-02
microfilament motor activity (GO:0000146)	29	0	11.00	-	< 0.01	1.91E-04	4.14E-02
ATP-dependent microtubule motor activity (GO:1990939)	35	0	13.27	-	< 0.01	2.58E-05	8.22E-03
glutamate receptor activity (GO:0008066)	28	0	10.62	-	< 0.01	1.75E-04	3.97E-02

Analysis Type PANTHER Overrepresentation Test (Released 20210224)

Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.4495804 Released 2021-02-01

Analyzed List: upload_1 (Homo sapiens)

Test Type: FISHER

Correction: FDR

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DISCUSSION

In the present study we investigated the overall impact of the use of cryopreserved spermatozoa on the transcriptome of equine embryos, and whether the use of frozen-thawed spermatozoa may impact the development of equine embryos at two stages, 8 to 10 days, and 10 to 12 days after ovulation. Cryopreservation is known to cause significant sperm death (Pena et al. 2011; Munoz et al. 2016; Ortega Ferrusola et al. 2017), and the surviving population experiences accelerated senescence characterized by mitochondrial malfunction and redox deregulation leading to reduced lifespan of cryopreserved spermatozoa (Martin Munoz et al. 2015; Ortega-Ferrusola et al. 2019; Ortiz-Rodriguez et al. 2019a; Ortiz-Rodriguez et al. 2021). One important aspect is the role of the spermatozoa on embryo development (Teperek et al. 2016). A recent study of the human sperm proteome (Castillo et al. 2018), identified 103 proteins with known roles in fertilization and 93 with roles in early embryo development. Additionally, 560 sperm proteins were found as involved in modulating gene expression by regulation of transcription, DNA methylation, histone post-translational modifications and non-coding RNA biogenesis (Castillo et al. 2015; Castillo et al. 2018). Some of these proteins may be critical for gene expression regulation after embryo genome activation; the integrative analysis of the sperm, oocyte and blastocyst proteomes and transcriptomes revealed a set of embryo proteins with an exclusive paternal origin, some of which are crucial for correct embryogenesis and, possibly, for modulation of the offspring phenotype (Castillo et al. 2018). In relation to this, cryopreservation is known to impact the sperm proteome (Bogle et al. 2017; Martin-Cano et al. 2020b; Gaitskell-Phillips et al. 2021b; Gaitskell-Phillips et al. 2021a). Taking all these findings in account, is likely that embryos produced with cryopreserved spermatozoa may experience alterations resulting of changes induced by cryopreservation in the proteome of the spermatozoa. Overall, we observed a downregulation of numerous transcripts in embryos resulting from inseminations with

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cryopreserved spermatozoa in relation to those originated after insemination with fresh spermatozoa. This fact can be easily explained due to the damage induced by cryopreservation to sperm proteins with regulatory roles in embryo development; interestingly and further supporting this hypothesis, all these downregulated transcripts were transcription factors considered essential for embryo development from a very early stage (Godini and Fallahi 2019). Many of the transcription factors found downregulated in embryos obtained using cryopreserved spermatozoa have crucial functions in embryo development, explaining the delayed development and increased embryo mortality attributed to inseminations with cryopreserved semen (Jia et al. 2015). In relation to this, factors found downregulated in embryos derived from insemination with cryopreserved spermatozoa included the Krüppel-like transcription factor3 (KLF)13. This is a member of the Krüppel- like family of transcription factors that controls many growth and developmental processes (Zhou et al. 2007). KLF13 has an important role sensitizing the endometrium to progesterone being crucial for pregnancy initiation and maintenance (Martin et al. 2001; Pabona et al. 2010; Heard et al. 2012; Chen et al. 2015; Grasso et al. 2018). Other members of this family of Kruppel-like factors, including KLF3, KLF14, KLF15 and KLF17 were found downregulated in embryos derived from cryopreserved spermatozoa. Among the discriminant variables detected we observed the proteins the *chromatin-remodeling ATPase INO80,* that has important roles in transcriptional regulation, and DNA replication and repair (Jin et al. 2005; Bakshi et al. 2006; Cai et al. 2007). This gene has a specific regulatory effect on the viability, migration and invasion of trophoblast cells and the knockout mouse results in embryonic lethality, this gene has other important functions in embryonic development (Wang et al. 2014; Qiu et al. 2016; Xian et al. 2021). Another transcript identified was *The mitochondrial mRNA pseudouridine synthase RPUSD3* that catalyzes uridine to pseudouridine isomerization, that is essential for specific mitochondrial mRNAs translation *(Antonicka et al. 2017)*. Downregulation of these proteins argues in favor of a compromise in the development of embryos obtained with cryopreserved spermatozoa; further supporting this hypothesis the visualization of integration networks using the Cytoscape app Clue go revealed annotations like regulation of nuclear division, mitotic DNA damage

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checkpoint and positive regulation of molecular mediator of immune response significantly affected by cryopreservation.

When we investigated whether embryo development was affected when cryopreserved spermatozoa was used, we found a dramatic difference in the number of transcripts upregulated in embryos derived from fresh spermatozoa, respect embryos derived from inseminations with cryopreserved spermatozoa in the transition from 8 to 10 days, this fact may help to explain why embryos obtained with cryopreserved spermatozoa experience a delay in development in this particular interval (Stout 2006). However, from 10 to 12 days, a higher number of transcripts were upregulated in embryos obtained with cryopreserved spermatozoa. These data may indicate that embryos derived from cryopreserved spermatozoa may lose control of transcription control, as has been described in low quality human embryos (Groff et al. 2019). One of the possible explanations for this finding may be delayed development of these embryos, representing late activation of transcripts. While the enrichment of transcripts related to metabolism may argue in favor of this argument, the GO terms involved in post transcriptional gene silencing were highly enriched. Moreover, the human phenotype ontology (https://hpo.jax.org/app/) terms were enriched in embryos obtained with cryopreserved spermatozoa, including terms related to abnormal metabolism, which may suggest an abnormal gene activation in these embryos. To further explore the biological meaning of these findings we also conducted Panther overrepresentation tests, to found a significant underrepresentation of protein classes involved in transcriptional regulation, and different transcription factors. Moreover, underrepresentations of protein classes related to immune functions were also observed which argues in favor of abnormal gene activation, reinforced by the molecular function GO terms related to cellular signaling like receptor protein kinase and protein tyrosine kinase activities highly underrepresented.

In sum, cryopreservation seriously influences the transcriptome of early embryos, having a direct impact in all embryos derived from cryopreserved spermatozoa, characterized by downregulation of numerous transcription factors. In addition,

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cryopreservation also impacts embryo development in the transition from 8 to 10 days, and from 10 to 12 days. Overall, our findings provide strong evidence that insemination with cryopreserved spermatozoa may compromise early embryo development, probable due to cryo-induced modifications in sperm proteins. Identification of which specific factors may cause such changes in the sperm proteome induced by cryopreservation is expected.

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DISCUSIÓN

En la presente Tesis Doctoral describimos la presencia y funcionalidad del transportador SLC7A11 en los espermatozoides equinos frescos y congelados-descongelados. Este transportador se localiza en la zona postacrosómica y se encarga de interiorizar una molécula de CysS y exteriorizar una de Glu[340]. Mediante la adición de CysS y el uso de dos inhibidores específicos de SLC7A11, sulfasalazina (SS) y α-metil-4-carboxifenilglicina (mCPG)[341, 342], hemos comprobado el papel del transportador en la viabilidad, los parámetros cinemáticos, la actividad mitocondrial y la capacidad de unión heteróloga de los espermatozoides con ovocitos de cerda, así como su relación con el metabolismo del glutamato. Por otro lado, estudiamos la flexibilidad metabólica y la relación entre el metabolismo y la regulación redox para la optimización de las técnicas actuales de conservación seminal, suplementando con rosiglitazona los espermatozoides descongelados y modificando los diluyentes de refrigeración, reduciendo la concentración de glucosa e incrementando la de piruvato. Finalmente, observamos el impacto de la congelación-descongelación espermática en el desarrollo embrionario temprano, ya que los espermatozoides pueden mantener la capacidad de fecundación, a pesar del estrés oxidativo provocado por esta técnica de conservación[234].

El potencial de oxidación-reducción estática (sORP), y la capacidad antioxidante (cORP) fueron medidos con RedoxSYS® en semen puro, y en una suspensión de espermatozoides tras la centrifugación y eliminación del plasma seminal, determinándose la contribución del plasma seminal a la regulación de la homeostasis redox. Dicha eliminación resultó en un incremento drástico de la oxidación, lo que está en concordancia con el aporte sustancial de defensas antioxidantes extracelulares por el plasma seminal[343, 344]. Por el contrario, los cambios observados en la cORP no fueron tan drásticos, justificado por la presencia de antioxidantes intrínsecos. El GSH es el principal antioxidante intracelular y las concentraciones de GSH espermáticas reportadas en la mayoría de los mamíferos es baja, en torno a 0.3 mM, pero en sementales se ha estimado en torno a 30 mM[27, 128]. Estos resultados son particularmente relevantes en el contexto de las biotecnologías de conservación seminal, donde la eliminación del plasma seminal es una práctica usual, principalmente en los protocolos de congelación. Las diferentes biotecnologías de conservación utilizadas en la clínica

reproductiva equina conllevan al incremento del estrés oxidativo espermático[255, 345, 346]. Además, debemos destacar la importancia fisiológica de los antioxidantes intracelulares, pues tras la eyaculación, una vez que los espermatozoides alcanzan el tracto reproductivo de la yegua, el plasma seminal y el exceso de espermatozoides son eliminados[347] y los espermatozoides que permanecen con capacidad fecundante son dependientes de su propia regulación redox.

Un estudio reciente de nuestro grupo de investigación describe la presencia de las enzimas encargadas de la síntesis de GSH a partir de Cys, Glu y Gly en los espermatozoides equinos[27]. El Glu puede ser obtenido gracias a la GDH1 que convierte el α-cetoglutarato proveniente del ciclo de Krebs en Glu[348]. En el segundo artículo de la Tesis Doctoral, identificamos la presencia de GDH1 en espermatozoides equinos mediante *Western blotting* y con inmunocitoquímica determinamos que se localiza en la porción intermedia. Sin embargo, la obtención de Cys resulta el paso limitante para la síntesis de GSH, en el primer artículo observamos que los espermatozoides no cuentan con las enzimas necesarias para la síntesis de Cys mediante la vía de transulfuración, principal vía para la obtención de este aminoácido en los seres vivos[349]. En la región postacrosómica, la porción intermedia y a lo largo del flagelo se encuentra presente la enzima cistationina-β-sintasa (CBS), pero los espermatozoides equinos no presentan la enzima cistationina-γ-ligasa, responsable de catalizar el paso de cistationina a Cys. Según lo descrito, la Cys se obtiene por su internalización desde el medio extracelular, pero la Cys es rápida y espontáneamente oxidada a CysS[350, 351]. Por esta razón, estudiamos la incorporación de CysS mediante el SLC7A11, para su posterior reducción en dos moléculas de Cys y su uso para la síntesis de GSH. Este transportador ha sido definido en las membranas plasmáticas de células del sistema nervioso central y del sistema inmune, donde presenta un papel importante en la regulación de la homeostasis redox[339, 340]. En diferentes tipos de cáncer ocurre una sobreexpresión de SLC7A11, incluyendo gliomas, linfomas y carcinomas. Esta sobreexpresión permite que las células cancerígenas resistan a las EROs, resistiendo a las quimioterapias, drogas anticancerígenas que principalmente se centran en inducir estrés oxidativo en las células tumorales[352-355]. Mediante *Western blotting* e inmunocitoquímica, hemos sido pioneros en identificar la presencia del transportador SLC7A11 en las membranas plasmáticas de la zona postacrosómica de los espermatozoides equinos. Asimismo, nuestros resultados muestran que la suplementación con CysS incrementa la concentración de GSH intracelular, un 50% en el semen fresco y un 30% en el semen descongelado. Esta diferencia se explica por la alteración que sufren las membranas plasmáticas tras la descongelación, afectándose a su vez la funcionalidad del SLC7A11, así como por el incremento del estrés oxidativo y la disminución de la viabilidad en el semen

descongelado[267, 356]. Tras la congelación-descongelación, los eyaculados equinos que muestran buena congelabilidad presentan un 40-50% de viabilidad. Los espermatozoides muertos son una fuente importante de EROs, que reaccionan con GSH y lo oxidan a GSSG[357, 358]. Cuando bloqueamos el SLC7A11 con SS, la concentración de GSH se redujo tanto en las muestras suplementadas como en las no suplementadas con CysS. Esto provee evidencia de que la CysS es incorporada en los espermatozoides equinos a través del SLC7A11, reduciéndose a Cys para la síntesis de GSH. La existencia y funcionalidad de este transportador también son respaldadas por algunos estudios en humanos. Durante la incubación de los espermatozoides se produce un incremento de las concentraciones de Glu en los medios extracelulares, presentándose mayor cantidad de Glu extracelular en las muestras espermáticas de mejor calidad[359]. En nuestros estudios, la concentración de GSH se midió principalmente por citometría de flujo con el uso de la sonda monoclorobrimano, sensible al GSH y cuya especificidad ha sido validada, detecta la concentración de GSH en células individuales[360]. No obstante, también hemos determinado la concentración de GSH y GSSG mediante la espectrometría de masas en cromatografía líquida de alto rendimiento (UHPLC-MS/MS), observando que la concentración de GSSG es menor en las muestras tratadas con CysS. Por otro lado, los resultados obtenidos con el uso de butionina sulfoximina (BSO), un inhibidor de la enzima γ-glutamilcisteína sintasa, también apoyaron la evidencia de la síntesis de GSH a partir de la CysS incorporada mediante el SLC7A11. La BSO redujo la concentración de GSH en presencia de CysS.

La relación entre la homeostasis redox y la funcionalidad del transportador la estudiamos mediante RedoxSYS®, técnica que da una visión global del estado redox de la muestra[361, 362]. La CysS redujo significativamente el sORP e incrementó la cORP, tanto en semen fresco como descongelado. En cambio, a pesar de la reducción de la concentración de GSH, tras la inhibición del SLC7A11 con SS, el sORP y la cORP no mostraron diferencias significativas con las muestras control. Del mismo modo, la SS solo previno la reducción del sORP inducida por CysS en los eyaculados frescos después de 3 horas de incubación, efecto que no se observó a las 6 horas, ni en las muestras descongeladas. Esto se explica por el hecho de que la CysS puede ser utilizada para la síntesis de GSH o presentar funciones como nodo redox junto a Cys (Cys/CysS)[363], aportando capacidad antioxidante independientemente de su incorporación a GSH.

En cuanto a los parámetros cinemáticos de los espermatozoides, los resultados obtenidos fueron diferentes en los eyaculados frescos y congelados. En semen fresco observamos un incremento significativo de la motilidad total (MT) y progresiva (MP) en las muestras tratadas con CysS más SS 200 mM y con CysS más SS 500 mM. Por el contrario, en las muestras exclusivamente suplementadas con SS 100 mM o SS 200 mM

se observó un incremento exclusivo de la MP y en las muestras SS 500 mM una disminución significativa tanto de la MT como de la MP. Esta disminución es explicable por la reducida concentración de GSH observada en estas muestras. Por otro lado, la mejoría de las motilidades espermáticas con las dosis bajas de SS puede ser explicada por la dualidad de la CysS, es decir, las motilidades pueden haber mejorado por el incremento compensatorio en la actividad del nodo Cys/CysS. Los datos del sORP apoyan esta hipótesis, la combinación de CysS y SS también redujo la oxidación total de la muestra. Una ventaja de este nodo es que la CysS puede servir como un intermediario disulfuro al oxidarse proteínas por el intercambio tiol/disulfuro, con la ventaja de disminuir el riesgo de la oxidación proteica a estados mayores de oxidación, no reversibles ante el estrés oxidativo[363]. Estos resultados aportan la primera evidencia de la funcionalidad del nodo Cys/CysS en espermatozoides equinos. Por otro lado, en las muestras de semen congelado, la SS disminuye tanto las motilidades como las velocidades espermáticas, una alteración que fue parcialmente prevenida con la presencia simultánea de CysS y SS. Estas diferencias observadas entre los eyaculados frescos y congelados-descongelados sugieren el uso de un mecanismo redox diferente y la posibilidad de una función comprometida del transportador SLC7A11 en el semen congelado. Previas investigaciones de nuestro grupo de investigación apoyan estos hallazgos, describen la alteración de las funciones de diferentes canales de membrana en espermatozoides descongelados[356].

La correlación entre la función del SLC7A11 y la capacidad de fecundación en semen congelado fue estudiada con un ensayo de unión heteróloga entre los espermatozoides de caballo y ovocitos de cerda. El estudio fue realizado con ovocitos de cerda por la dificultad de adquirirlos de yegua, teniendo en cuenta que diferentes grupos de investigación han contrastado la fiabilidad de la técnica de unión heteróloga entre espermatozoides equinos y ovocitos de otras especies[364-368]. Para llevar a cabo el experimento, los espermatozoides fueron incubados en un medio capacitante y luego se incubaron con los ovocitos, para el posterior estudio de la cantidad de espermatozoides unidos a la ZP. Un marcador de capacitación bien reconocido es la fosforilación de los residuos de tirosina de las proteínas espermáticas[369], que se estudió mediante inmunocitoquímica. Tras la incubación de las diferentes muestras en el medio capacitante, el grupo control, el grupo suplementado con SS y el grupo con CysS y SS, pero no el grupo suplementado solo con CysS, mostraron un incremento significativo en el número de espermatozoides con tirosina fosforilada en comparación con las muestras previas a la capacitación. Como se ha mencionado, la CysS entra en el citosol y se sintetiza GSH, importante antioxidante intracelular que causa la disminución de los niveles espermáticos de EROs[28]. La fosforilación de tirosina proteica es un proceso

dependiente de la señalización redox[216, 234], es decir, las EROs inactivan las tirosina fosfatasas permitiendo la regulación al alza de la fosforilación de tirosina que caracteriza la capacitación espermática[370]. Por ello, podemos decir que el menor incremento en la fosforilación de tirosina tras la capacitación en el grupo experimental suplementado con CysS se debe al incremento intracelular de GSH.

En cuanto a la habilidad de interacción de los espermatozoides con la ZP, la CysS no incrementó la cantidad de espermatozoides en comparación con la muestra control. En cambio, es interesante señalar que las muestras incubadas con SS, tanto en presencia como en ausencia de CysS, mostraron una disminución significativa en el número de espermatozoides unidos a los ovocitos. Este efecto inhibitorio del transportador sugiere que la funcionalidad del SLC7A11 se mantiene tras la congelación, al menos en parte, y que la incorporación de la CysS desempeña un papel importante en la fecundación. El GSH está implicado en procesos como la neutralización del $O_2^{-\bullet}$, así como en la detoxificación de algunos metabolitos[371-373], y la suplementación con SS disminuye las concentraciones de GSH, tanto en eyaculados frescos como descongelados. Además, GSH puede interactuar con sus enzimas asociadas (glutatión peroxidasas, glutatión reductasas y glutatión S-transferasas), proveyendo protección ante el estrés oxidativo. Diferentes estudios han descrito la importancia de las enzimas glutatión S-transferasas de los espermatozoides en la fecundación[374, 375]. Por ello, el deterioro de la función de las enzimas glutatión S-transferasas, como consecuencia de la inhibición del transportador y la disminución de la concentración de GSH, puede ser la razón de la disminución de la unión heteróloga entre espermatozoides y ovocitos. La funcionalidad del SLC7A11 en el espermatozoide equino es relevante para que se mantenga la capacidad de fecundación.

Según los resultados obtenidos con la SS, incremento de la motilidad espermática en semen fresco y disminución de la capacidad de fecundación en semen congelado, podemos determinar que este transportador ejerce diferentes funciones, es decir, no se trata única y exclusivamente de una fuente de CysS extracelular para la síntesis de GSH. De hecho, estudios recientes han descrito la importancia del SLC7A11 en el metabolismo del Glu. En algunos modelos celulares, el bloqueo del SLC7A11 mejora en gran medida la viabilidad celular en ausencia de glucosa, porque la conservación de Glu intracelular permite a la célula mantener la respiración mitocondrial[376]. Para determinar si una situación similar puede estar ocurriendo en espermatozoides equinos, investigamos la relación entre la funcionalidad del SLC7A11 y la actividad mitocondrial, ya que las mitocondrias presentan un papel central en la regulación redox y el metabolismo. Tras inhibir la función del transportador con SS se produjo una disminución significativa en el porcentaje de espermatozoides con alto PMM. Esta disminución del PMM fue

acompañada de un descenso de la concentración de GSH intracelular y de la viabilidad espermática. El impacto de la disponibilidad de GSH reducido en la función mitocondrial es esperado. El GSH se sintetiza en el citosol y es transportado al interior mitocondrial para igualar las concentraciones del GSH mitocondrial (GSHm) con las del citosol[377-379]. Debido a que la mitocondria es el principal sitio intracelular de consumo de O_2, este orgánulo es la mayor fuente de EROs intracelulares, por lo que el GSHm es esencial para el equilibrio de la actividad de la SOD mitocondrial y el nodo redox GSH/GSSG, para mantener el H_2O_2 a niveles fisiológicos. El metabolismo del H_2O_2 en la mitocondria depende del GSHm con la participación de las GPXs y las PRDXs[378, 379]. La disminución del GSH intracelular puede deberse a un incremento de la producción de EROs y/o a la actividad reducida del transportador SLC7A11, así como al agotamiento de la enzima GPX4[380]. Dicho agotamiento induce una forma de muerte celular programada denominada ferroptosis, descrita en la línea germinal masculina[381]. Además, la GPX4 presenta un papel crucial para el mantenimiento de la funcionalidad espermática[382]. En esta Tesis Doctoral realizamos experimentos que comparan el efecto de la SS con inductores conocidos de la ferroptosis, tales como la erastina y el RSL3[381]. La suplementación con erastina redujo el porcentaje de espermatozoides vivos, un efecto que fue impedido con el inhibidor de la ferroptosis liproxstatina-1 (LIP1). Estos hallazgos apoyan la existencia de ferroptosis en los espermatozoides equinos. Por otro lado, la presencia simultánea de SS y LIP1 resultó en un incremento de la viabilidad y la reducción de la apoptosis, disminuyendo la actividad de las caspasas, lo que sugiriere diferentes mecanismos de muerte celular causados por la erastina y la SS. Sin embrago, la LIP1 no previene la reducción de las motilidades y velocidades espermáticas producidas por la erastina y el RSL3. La frecuencia de batido de los espermatozoides (BCF), parámetro relacionado con el incremento de la funcionalidad espermática y el contenido de ATP[383], disminuye en presencia de ambos inductores de ferroptosis y la LIP1 solo revierte el efecto del RSL3. El efecto diferencial en la viabilidad y la motilidad producido por la erastina podría relacionarse con el efecto de esta droga en el canal aniónico dependiente de voltaje en las mitocondrias, que regula el flujo metabólico a través de la membrana mitocondrial externa[384]. En general, estos resultados indican que en los espermatozoides pueden estar presente diferentes formas de muerte celular activadas bajo la desregulación redox, pero se necesitan más investigaciones para caracterizar completamente estos mecanismos y la diafonía entre ellos.

Como se ha citado, la funcionalidad de este sistema xCT también fue estudiada con el uso de mCPG, otro inhibidor que da lugar a un incremento de la acumulación de Glu intracelular[341]. La presencia de mCPG resultó en la reducción del porcentaje de espermatozoides vivos, efecto que no ocurre en presencia de CysS. La suplementación

conjunta de mCPG y CysS también incrementó el porcentaje de espermatozoides con alto PMM y la concentración intracelular de GSH. Es una paradoja que un aumento o una disminución en GSH pueda ocurrir usando diferentes inhibidores del SLC7A11. Una posible explicación puede relacionarse con el doble efecto del transportador, es decir, regulador de la disponibilidad de Cys para la síntesis de GSH y participante en la regulación del metabolismo del Glu[376]. El mCPG bloquea el intercambio de Glu por CysS, dando lugar a un incremento del Glu intracelular. El Glu puede sustentar al ciclo de Krebs tras su transformación a α-cetoglutarato por la enzima GDH1, así como incrementar la producción de moléculas como GSH y NADPH[348, 385]. Esta vía metabólica del Glu puede explicar de forma plausible nuestros hallazgos. Apoyando aún más esta hipótesis, en espermatozoides se ha descrito que la suplementación con α-cetoglutarato tiene efecto antioxidante[128], así como que la suplementación con glutamina en espermatozoides de conejo incrementa la concentración de GSH[386].

Con el uso de un inhibidor específico de la GDH1 (R162) bloqueamos la conversión del Glu a α-cetoglutarato y, como consecuencia, su incorporación en el ciclo de Krebs. El R162 causa un aumento del Glu intracelular que da lugar al incremento del PMM y del GSH intracelular. Esto indica que el exceso de Glu no es necesariamente metabolizado a través de la vía de la GDH1, por lo que en el espermatozoide equino existen rutas alternativas para el metabolismo del Glu, también descrito en células tumorales[387]. Mientras que en la mayoría de las células la GDH1 convierte el Glu a α-cetoglutarato, algunos tumores usan distintas vías que involucran a las transaminasas (GOT1) y convierten el Glu a piruvato, incrementando la ratio $NADPH/NADP^+$ y manteniendo la homeostasis redox[387-389]. En espermatozoides de mamíferos también ha sido descrita la actividad de las transaminasas[390]. Tras la inhibición de la GDH1, realizamos el estudio metabolómico con UHPLC/MS/MS, centrándonos en los metabolitos del ciclo de Krebs. El tratamiento con R162 causa un incremento drástico e inesperado de succinato, ya que el succinato se produce después del α-cetoglutarato en el ciclo de Krebs. Además, hallamos un gran incremento en fumarato, lo que sugiere una vez más que el Glu también puede ser metabolizado a través de vías alternativas[391]. El fumarato también es producido en el proceso de desintoxicación de amoniaco, a través de la escisión del succinato de arginina para formar fumarato y arginina, y el fumarato regresa al ciclo de Krebs[392]. Este mecanismo puede explicar nuestros hallazgos, ya que el Glu puede ser metabolizado a arginina[393]. Asimismo, y reforzando esta hipótesis, un ciclo de urea funcional ha sido descrito en espermatozoides de mamíferos[394], también hallado en équidos en un estudio reciente mediante proteómica de nuestro grupo de investigación[395]. Además, también obtuvimos un incremento de malato tras la inhibición de la GDH1. Todo esto indica que el espermatozoide equino cuenta con una vía no

canónica para el metabolismo del Glu, produciendo poder reductor para reciclar el GSSG a GSH, lo que explica nuestros hallazgos del incremento de GSH y del PMM tras la inhibición de la GDH1. En células de adenocarcinoma ductal pancreático se ha descrito una ruta similar, donde el Glu citoplasmático forma aspartato y luego oxalacetato, que es convertido a malato y luego a piruvato, incrementando el ratio $NADPH/NAD^+$ y manteniendo de esta forma el equilibrio redox celular. En este estudio, la adición de dimetil-2-oxoglutarato (OXO) no restauró el crecimiento celular después de la privación de Glu, indicando un metabolismo diferente del Glu con respecto a modelos canónicos[389]. Del mismo modo, en nuestros experimentos, la suplementación con OXO no mejoró la función espermática, lo que también sugiere la existencia de vías metabólicas alternativas para el Glu. Esto proporciona una explicación plausible al incremento de GSH tras la inhibición del SLC7A11 con mCPG. Por primera vez se sugiere que el espermatozoide equino muestra una plasticidad metabólica importante, hallazgo que puede abrir nuevas líneas de investigación para el desarrollo de estrategias novedosas en el manejo de la infertilidad de factor masculino.

Asumiendo la plasticidad metabólica de los espermatozoides equinos, nos propusimos optimizar la calidad espermática en las biotecnologías de conservación seminal. Debido a la gran existencia de dosis comerciales de semen congeladas, planteamos mejorar la calidad espermática mediante la adición de rosiglitazona tras la descongelación y, por otro lado, estudiamos la posibilidad de mejora de los diluyentes de refrigeración, teniendo en cuenta la biología espermática y los resultados hasta ahora descritos.

Tradicionalmente los enfoques para mejorar la supervivencia espermática tras la congelación- descongelación se han focalizado en la suplementación de antioxidantes y en la selección espermática, sin centrarse en la biología de los espermatozoides descongelados, que presentan una función mitocondrial comprometida y un estado redox inestable[13, 356, 396-402]. Nosotros nos propusimos incrementar la funcionalidad de los espermatozoides descongelados induciendo flexibilidad metabólica, una estrategia que ha sido probada con éxito en la conservación espermática a temperatura ambiente[35]. La rosiglitazona, agonista del receptor activado por proliferadores de peroxisomas gamma (PPARγ), induce claras mejoras en la función mitocondrial y reduce la apoptosis espermática. El PPARγ actúa como un factor de transcripción activado por ligando y regula procesos como la homeostasis energética, la proliferación y la diferenciación celular[403]. Los efectos de rosiglitazona se relacionan con una mayor fosforilación de la proteína quinasa B (Akt), importante para mantener la funcionalidad espermática[404-407]. Recientemente, se ha descrito una relación entre agonistas del PPARγ y la fosforilación de la Akt en espermatozoides de humanos y cerdos[408, 409]. Algunas estrategias para mantener esta fosforilación se han probado en

espermatozoides descongelados de humanos[406, 410]. La rosiglitazona mantiene la Akt fosforilada, lo que indica que el espermatozoide mantiene mecanismos para regular su supervivencia a pesar del estrés osmótico que ocurre durante la descongelación. El balance entre la activación de las vías de supervivencia y muerte espermática depende de la capacidad de regulación de la homeostasis redox[255]. En diferentes modelos celulares, la Akt regula la función mitocondrial y esta regulación no es necesariamente dependiente de la actividad transcripcional[411], apoyando la propuesta del mecanismo aquí descrito de incrementar la función mitocondrial tras el tratamiento con un agonista del PPARγ en espermatozoides. El uso de inhibidores específicos de la Akt en nuestros experimentos brindaron mayor apoyo a la propuesta de la relación entre la Akt fosforilada y la función propia del espermatozoide, tal y como ha sido reportado en espermatozoides de humanos y equinos[404, 405, 408, 412]. Reforzando esta hipótesis, el tratamiento con inhibidores de PPARγ y AMPK (proteína quinasa activada por AMP), que inhiben a la Akt, previno la mejora del tratamiento con rosiglitazona. Sin embargo, el tratamiento con el inhibidor indicó que, en sementales, el mayor de los efectos de rosiglitazona puede estar relacionado con la activación de la AMPK, efecto previamente reportado en espermatozoides conservados a temperatura ambiente[35]. Además, el inhibidor del PPARγ fue menos eficiente en revertir los efectos de la rosiglitazona. Este agonista del PPARγ mejoró la función mitocondrial mientras mantuvo la homeostasis redox. Aunque incrementó la producción de $O_2^{-\bullet}$, el sORP no se vio afectado, pues un incremento en la producción de $O_2^{-\bullet}$ es indicativo de una actividad mitocondrial intensa[83]. No obstante, aunque esta producción es específica de las mitocondrias, también puede ser producido por las enzimas NADPH. Adicionalmente, hay evidencias de que los ribosomas mitocondriales en el espermatozoide son activos transcripcionalmente y translacionalmente[413], por lo que la posibilidad de que rosiglitazona pueda ser activa a través de vías traslacionales requiere una mayor investigación.

La congelación espermática agota los grupos tiol, causando un estado redox inestable que rápidamente provoca la desregulación o pérdida de la homeostasis redox[255]. Esta situación induce la activación de caspasas, es decir, la apoptosis y muerte espermática. Nuestros resultados muestran que esta forma de muerte celular puede ser retrasada. De hecho, observamos que las muestras suplementadas con rosiglitazona tienen un mayor porcentaje de espermatozoides vivos no apoptóticos tras dos horas de incubación. El resultado positivo de esta suplementación puede ser atribuido a la activación de flexibilidad metabólica. En este sentido, los espermatozoides pueden ser más efectivos al usar la glucólisis y la β-oxidación de ácidos grasos para la producción de energía en forma de ATP[33, 35]. Adicionalmente, como se reveló en nuestro experimento,

estas vías incrementan la eficiencia de la función mitocondrial y, con ella, la funcionalidad espermática[414-416]. Por tanto, la calidad y la habilidad de fecundación de las muestras seminales congeladas pueden ser moduladas tras la descongelación.

En cuanto a la refrigeración, los diluyentes comerciales están formulados con concentraciones muy altas de glucosa, a pesar de que investigaciones recientes indican que la glucólisis no es la principal vía de producción de ATP en el metabolismo espermático equino[17]. En el quinto artículo, teniendo en cuenta los resultados obtenidos sobre la plasticidad metabólica de los espermatozoides, estudiamos el proteoma metabólico y observamos que las vías metabólicas que predominan en el espermatozoide son el ciclo de Krebs y el metabolismo del piruvato. En función de estos resultados realizamos un experimento donde se indujo la inhibición de la glucólisis mediante la adición de un análogo de la glucosa no metabolizable en las muestras seminales, la 2-desoxiglucosa (2-DG). Dicha inhibición no tuvo efecto negativo ni en la viabilidad ni en la actividad mitocondrial de los espermatozoides, por el contrario, se produjo un incremento significativo en ambos parámetros. No obstante, la motilidad y las velocidades espermáticas se redujeron bajo estas circunstancias, hallazgos que apoyan la importancia de la degradación de la glucosa por las enzimas glucolíticas localizadas en el flagelo[16]. Una explicación de la disminución en la cantidad de ATP y, con ello, de los parámetros cinemáticos es que 2-DG puede ser fosforilada por las hexoquinasas, impidiendo la entrada de la glucosa en la vía glucolítica y reduciéndose dicha producción de energía. Estudios previos de nuestro grupo de investigación mostraron que la motilidad y las velocidades reducidas en presencia de 2-DG no ocurre en medios sin glucosa[85]. Por otro lado, la adición de 2-DG causó una disminución del GSH, que puede ser explicado por la reducción del flujo a la vía de las pentosas fosfatos, lo que disminuye la generación de poder reductor en forma de NADHP para reducir el GSSG a GSH[36]. Sin embargo, el poder reductor se puede generar en el ciclo de Krebs. Para determinar si esto ocurre en el espermatozoide, se incubaron muestras seminales en presencia de OXO, análogo de α-cetoglutarato permeable en la membrana plasmática. Este compuesto fue capaz de restaurar los niveles de GSH en las muestras suplementadas con 2-DG, comparables con los observados cuando la glucólisis no había sido inhibida. En general, estos resultados sugieren una vez más que existe una plasticidad importante en el metabolismo del espermatozoide equino y resalta la estrecha interacción entre las EROs y el metabolismo[36].

En vista de estos resultados, analizamos la hipótesis de que los diluyentes con una concentración menor de glucosa pueden ser efectivos para la conservación seminal. Diluimos diferentes muestras espermáticas en distintos medios con baja concentración de glucosa y utilizamos como control un diluyente comercial que contiene gran cantidad

de glucosa (INRA96). Se prepararon dos diluyentes con 1 mM de glucosa (LG) en los que añadimos 10 mM de piruvato (HP), ya que las vías metabólicas identificadas en estudios previos y en nuestro estudio mediante proteómica indicaron que este metabolito es utilizado de manera eficiente por los espermatozoides de caballos[29, 34, 395]. Estos dos medios diferían en que uno era LG-HP y el otro fue suplementado con OXO (LG-HP-OXO).

Mediante el sistema computarizado de análisis espermático (CASA) observamos que las motilidades y velocidades espermáticas fueron significativamente mayores en los medios LG-HP y LG-HP-OXO en comparación con INRA96. Estos resultados determinan que concentraciones bajas de glucosa son suficientes para sustentar las enzimas glucolíticas del flagelo, mientras reducimos la toxicidad de la glucosa[47, 417]. Las células sintetizan glioxal y metilglioxal a partir de glucosa[418, 419], electrófilos potentes que pueden oxidar proteínas, lípidos y ácidos nucleicos, resultando citotóxico y mutagénico. Además, una alta concentración de glucosa puede tener un efecto tóxico directo, induciendo toxicidad mitocondrial e incrementando la producción de EROs[420-424]. Los niveles de glioxal y metilglioxal fueron medidos mediante UHPLC-MS/MS y determinamos que en los medios LG hay menor producción de estos 2-oxoaldehídos en comparación con INRA96. Por primera vez, describimos que las altas concentraciones de glucosa incrementan la producción de 2-oxoaldehidos y causan toxicidad durante el almacenamiento de los espermatozoides equinos. Por tanto, nuestros resultados desafían los procedimientos de refrigeración en uso para el almacenamiento de espermatozoides de sementales y proporcionan nuevos indicios que pueden usarse para mejorar esta técnica de conservación seminal.

En cuanto al estrés oxidativo, se hallaron porcentajes reducidos de EROs en los espermatozoides diluidos en los medios LG-HP, lo cual apoya nuestra hipótesis. Además, el papel de la glucólisis en el espermatozoide equino parece ser un mecanismo necesario para la motilidad, alimentando las enzimas glucolíticas en el flagelo[16, 34, 84], aunque la principal fuente de ATP sea la fosforilación oxidativa[23, 29, 35]; por tanto, concentraciones bajas de glucosa pueden ser suficientes para lograr esta función mientras se reduce su toxicidad. Los medios LG también aumentan otras funciones espermáticas como la actividad mitocondrial. El porcentaje de espermatozoides vivos fue más constante en los medios LG, y al mismo tiempo, estos diluyentes mostraron bajos porcentajes de células que muestran una producción significativa de EROs, vinculado a un mayor contenido de GSH. Debido a que la detoxificación de los 2-oxoaldehidos, especialmente metilglioxal, está ligada al GSH[43, 44, 425], la baja producción de metilglioxal explica el por qué los niveles de GSH pueden permanecer altos en los medios LG. Por último, en cuanto a la actividad mitocondrial, se observó un mayor PMM. La actividad mitocondrial espermática es indispensable para mantener la fertilidad[23, 24, 29, 191]. Además, el

incremento concomitante en GSH indica que la homeostasis redox se mantuvo en estos espermatozoides y, por tanto, no ocurre el estrés oxidativo[28].

Finalmente, siguiendo el último objetivo de la Tesis Doctoral, estudiamos el impacto de la congelación seminal en la expresión génica durante el desarrollo embrionario temprano. Los resultados obtenidos en los objetivos previos de la Tesis muestran que la población espermática que sobrevive a esta biotecnología reproductiva experimenta una senescencia acelerada, caracterizada por un mal funcionamiento mitocondrial y el desequilibrio de la homeostasis redox. Esto da lugar a una supervivencia reducida de los espermatozoides descongelados, resultados apoyados por otros estudios de nuestro grupo de investigación[27, 255]. Los espermatozoides presentan un papel importante en el desarrollo embrionario[325]. Un estudio reciente del proteoma del espermatozoide humano identificó 103 proteínas con funciones conocidas en la fertilización y 93 con funciones en el desarrollo embrionario temprano[426]. Además, se encontraron 560 proteínas implicadas en la modulación de la expresión génica mediante la regulación de la transcripción, la metilación del ADN, las PTMs de histonas y la biogénesis del ARN no codificante[426, 427]. El análisis de los proteomas y transcriptomas de espermatozoides, ovocitos y blastocistos ha revelado en los embriones un conjunto de proteínas de origen paterno exclusivo, algunas de las cuales son cruciales para una correcta embriogénesis y, posiblemente, para la modulación del fenotipo de la descendencia[426]. Asimismo, diferentes investigaciones describen cómo afecta la congelación al proteoma espermático[315, 395, 428, 429]. Teniendo en cuenta estos hallazgos, es probable que los embriones producidos con espermatozoides descongelados puedan experimentar alteraciones resultantes de cambios inducidos en el proteoma espermático. Debemos destacar que los embriones equinos presentan numerosas ventajas como modelo de investigación traslacional, pues presentan un periodo de pre-implantación largo, en el cual la vesícula embrionaria permanece esférica[430], por lo que la recuperación del embrión resulta fácil y es posible repetir las recuperaciones embrionarias en los mismos animales en sus sucesivos ciclos estrales.

Nuestro estudio se centró en la recuperación de embriones a los 8, 10 y 12 días de la ovulación, resultantes de la inseminación artificial con semen fresco o descongelado del mismo semental, revelándose un impacto significativo de la congelación seminal en el transcriptoma de los embriones. Los embriones obtenidos con semen descongelado presentaron una menor expresión de los genes relacionados con la replicación y el ensamblaje del ADN, la transcripción y síntesis proteica, el silenciamiento de la cromatina, la fosforilación oxidativa, la glucólisis y el ciclo de Krebs, así como con la regulación redox, sugiriendo un metabolismo energético comprometido en los embriones procedentes de semen congelado. Estas alteraciones genéticas ayudan a

explicar la fertilidad reducida que se observa con la utilización de semen congelado, atribuible al incremento de la mortalidad embrionaria[338, 431]. Muchos ARN mensajeros (ARNm) reguladas negativamente en embriones procedentes de inseminaciones con semen congelado, son factores de transcripción considerados esenciales para el desarrollo embrionario desde una etapa muy temprana[432, 433]. En relación con esto, los genes que se encuentran regulados a la baja en los embriones derivados de la inseminación con semen congelado-descongelado incluyen el factor de transcripción similar a Krüppel (KLF) 13. Los KLF controlan muchos procesos de crecimiento y desarrollo[434]. El KLF13 tiene un papel importante en la sensibilización del endometrio a la progesterona, crucial para el inicio y el mantenimiento de la gestación[435-439]. Otros miembros de esta familia de factores de transcripción, incluidos KLF3, KLF14, KLF15 y KLF17, se encontraron regulados negativamente en embriones derivados de espermatozoides descongelados. Entre las variables discriminantes detectadas entre los embriones obtenidos con semen fresco y congelado se observaron las proteínas ATPasa INO80 remodeladoras de cromatina, que tienen papeles importantes en la regulación transcripcional, y en la replicación y reparación del ADN[440-442]. Este gen tiene un efecto regulador específico sobre la viabilidad, migración e invasión de las células del trofoblasto. El ratón *knockout* para este gen presenta letalidad embrionaria[443-445]. Otra transcripción identificada fue la pseudouridina sintasa de ARNm mitocondrial RPUSD3 que cataliza la isomerización de uridina a pseudouridina, esencial para la traducción de ARNm mitocondriales específicos[446]. La regulación a la baja de estas proteínas argumenta a favor del compromiso en el desarrollo de embriones obtenidos con espermatozoides descongelados.

Si bien los mecanismos causantes de los efectos aquí reportados no están todavía claros, un factor importante puede ser el daño oxidativo que el genoma y el epigenoma espermático experimenta durante la congelación[338, 431, 447, 448]. La congelación-descongelación es una de las mayores causas de estrés oxidativo[402] y peroxidación lipídica en el espermatozoide equino[255, 401, 449, 450]. La peroxidación lipídica está relacionada con el incremento de 4-HNE[255], compuesto capaz de interactuar con el ADN para formar aductos que han sido relacionados directamente con un incremento de mutaciones en reguladores importantes del ciclo celular[451, 452]. Es posible que cantidades significativas de 4-HNE y otros aldehídos lipídicos tóxicos sean incorporados al ovocito, causando alteraciones en el desarrollo embrionario. Además del daño en el ADN, el 4-HNE puede producir la alquilación de los centriolos espermáticos, y en caballos, como en humanos, los centriolos paternos son interiorizados por los embriones. Los centriolos dañados pueden causar una interrupción de la organización de la proteína citoesquelética durante la escisión temprana[453]. Apoyando esta línea de razonamiento,

estudios recientes han vinculado los eventos de escisión temprana anormal y los cambios en la abundancia del transcriptoma embrionario con la fecundación con espermatozoides que muestran estrés oxidativo. Embriones obtenidos de macacos tras la fecundación con espermatozoides tratados con EROs mostraron significativamente tasas más bajas de desarrollo a los estadios de 4 y 8 células, y cambios en la abundancia del transcriptoma para genes relacionados con la organización del citoesqueleto de actina, el ensamblaje de unión y la adhesión celular[454]. Aunque se ve en una etapa de desarrollo más tardía, en nuestro estudio también encontramos que los genes para los componentes del citoesqueleto tubulina alfa 1 a, tubulina beta 2 clase II a y actina, citoplasmático 1 y los procesos N-terminalmente se regularon a la baja en embriones de 8 días obtenidos con semen descongelado.

Cuando investigamos si el desarrollo embrionario se vio afectado con el uso de semen congelado-descongelado, encontramos una diferencia drástica en el número de transcripciones reguladas al alza en embriones derivados de espermatozoides frescos en la transición de 8 a 10 días. Este hecho puede ayudar a explicar por qué los embriones obtenidos con espermatozoides descongelados experimentan un retraso del desarrollo en este intervalo en particular[455]. Sin embargo, en la transición de 10 a 12 días obtuvimos un mayor número de transcritos con regulación al alza en embriones obtenidos con espermatozoides descongelados. Estos datos pueden indicar que estos embriones pierden el control de la transcripción, como se ha descrito en embriones humanos de baja calidad[456]. Una de las posibles explicaciones de este hallazgo puede ser el retraso en el desarrollo embrionario, lo que representa la activación tardía de las transcripciones. Si bien el enriquecimiento de las transcripciones relacionadas con el metabolismo puede argumentar a favor de esto, los términos de *Gene Ontology* implicados en el silenciamiento génico postranscripcional estaban muy enriquecidos.

En comparación con la ontología del fenotipo humano (https://hpo.jax.org/app/), los embriones obtenidos con semen descongelado presentaron enriquecimiento de términos relacionados con el metabolismo anormal, lo que puede sugerir una activación genética anormal en estos embriones. Para explorar más a fondo el significado biológico de estos hallazgos, realizamos pruebas de sobrerrepresentación de *PANTHER* y encontramos anotaciones significativamente subrepresentadas en proteínas involucradas en la regulación transcripcional y en diferentes factores de transcripción. Además, también se observaron subrepresentaciones de proteínas relacionadas con las funciones inmunes, lo que aboga a favor de la activación anormal de genes, reforzada por los términos de *Gene Ontology* de función molecular relacionados con la señalización celular, como las actividades de proteína quinasa receptora y proteína tirosina quinasa, que se hallaron altamente subrepresentadas.

Por tanto, la biotecnología reproductiva de congelación seminal influye seriamente en el transcriptoma embrionario. Los embriones derivados de la inseminación con espermatozoides descongelados presentan una regulación a la baja de numerosos factores de transcripción. Además, la congelación-descongelación también afecta al desarrollo del embrión en la transición de 8 a 10 días y de 10 a 12 días. En general, los hallazgos de la presente Tesis Doctoral proporcionan una fuerte evidencia de que la fecundación de los espermatozoides descongelados puede comprometer el desarrollo temprano del embrión, probablemente debido a modificaciones inducidas por la congelación-descongelación en las proteínas espermáticas. Futuras investigaciones son necesarias para la identificación de los factores específicos que causan tales cambios inducidos por la congelación en el proteoma del espermatozoide.

CONCLUSIONES

1. El transportador SLC7A11 se encuentra en la membrana plasmática del espermatozoide equino y su funcionalidad es crucial para mantener la actividad mitocondrial, la homeostasis redox y, como consecuencia, la capacidad de fecundación del espermatozoide.

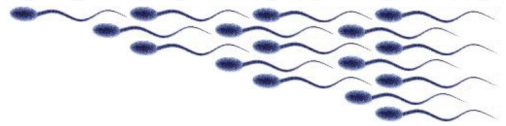
2. La suplementación con CysS reestablece la depleción de los grupos tiol que ocurre durante la conservación seminal, incrementando la concentración de GSH y manteniendo la homeostasis redox.

3. La congelación de semen provoca cambios en el mecanismo de regulación redox, lo que explica la funcionalidad reducida de los espermatozoides descongelados. No obstante, la alta plasticidad metabólica del espermatozoide equino permite mejorar la calidad de las muestras seminales tras la descongelación.

4. El exceso de glucosa en los diluyentes de conservación seminal incrementa la cantidad de glucosa no metabolizada provocando un desequilibrio en la homeostasis redox y toxicidad en los espermatozoides. Las técnicas de conservación seminal pueden ser mejoradas con el desarrollo de nuevos diluyentes con bajas concentraciones de glucosa.

5. La inseminación con espermatozoides descongelados puede comprometer el desarrollo embrionario, probablemente debido a modificaciones inducidas en las proteínas de los espermatozoides durante el proceso de congelación-descongelación. Nuevas líneas de investigación son necesarias para la identificación de qué factores específicos pueden causar tales cambios en el proteoma espermático.

CONCLUSIONS



1. The SLC7A11 transporter is found in the plasma membrane of equine sperm and its function is essential to maintain mitochondrial activity, redox homeostasis and, as a consequence, the fertilization capacity of sperm.

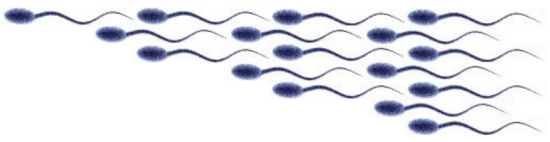
2. Supplementation with CysS counteracts the depletion of thiol groups that occurs during seminal conservation, increasing the concentration of GSH and maintaining redox homeostasis.

3. Freezing semen causes changes in the redox regulation mechanism, which explains the reduced functionality of thawed sperm. However, the high metabolic plasticity of equine sperm makes it possible to improve the quality of seminal samples after thawing.

4. Excess glucose in seminal preservation diluents increases the amount of non-metabolized glucose causing an imbalance in redox homeostasis and sperm toxicity. Seminal preservation techniques could be improved with the development of new diluents with low glucose concentrations.

5. Insemination with thawed sperm can compromise embryonic development, probably due to induced modifications in sperm proteins during the freeze-thaw process. New lines of research are necessary to identify specific factors that cause such changes in the sperm proteome.

ABREVIATURAS

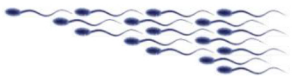


2-DG: 2-desoxiglucosa
4-HNE: 4-hidroxinonenal
8-OHdG: 8-hidroxi-2'-desoxiguanosina
µC: microculombios
ADN: Ácido desoxirribonucleico
ADP: Adenosín difosfato
AGEs: Productos finales de glicación avanzada
Akt: Proteína quinasa B
AMPc: Adenosín monofosfato cíclico
AMPK: Proteína quinasa activada por AMP
ARN: Ácido ribonucleico
ARNm: Ácido ribonucleico mensajero
ATP: Adenosín trifosfato
BCF: Frecuencia de batido de los espermatozoides
BSO: Butionina sulfoximina
Ca^{2+}: Calcio
Caspasas: Cisteinil aspartato proteasas
CASA: Sistema computarizado de análisis espermático
CAT: Catalasa
CBS: Cistationina-β-sintasa
CMTE: Cadena mitocondrial de transporte de electrones
CO_2: Dióxido de carbono
CO_3^-: Trióxido de carbono
CoA: Coenzima A
cORP: Capacidad antioxidante
Cys: Cisteína
CysS: Cistina
DAG: Diacilglicerol
DHAP: Dihidroxiacetona-fosfato
e^-: Electrón
E_h: Potencial redox en estado estacionario
E^o: Potencial de reducción de un electrón
ERNs: Especies reactivas de nitrógeno
ERO(s): Especie(s) reactiva(s) de oxígeno
FAD: Flavina adenina dinucleótido oxidada
$FADH_2$: Flavina adenina dinucleótido reducida
Fe: Hierro
Fe-S: Sulfuro de hierro
G3P: Gliceraldehído-3-fosfato
G6PDH: Glucosa 6-fosfato deshidrogenasa
GDH: Glutamato deshidrogenasa
Glu: Glutamato

Gly: Glicina
GMPc: Guanosín monofosfato cíclico
GPX(s): Glutatión peroxidasa(s)
GSH: Glutatión reducido
GSHm: Glutatión mitocondrial
GSSG: Glutatión oxidado o
GTP: Guanosín trifosfato
HEPES: Ácido 4-(2-hidroxietil)piperazin-1-iletanosulfónico
HP: Alta concentración de piruvato (10 mM)
H^+: Protón
H_2O: Agua
H_2O_2: Peróxido de hidrógeno
ICSI: Inyección intracitoplasmática de espermatozoides
IP3: Inositol trifosfato
***K*:** Constante de velocidad de segundo orden
KLF: Factor de transcripción similar a Krüppel
LDH: Lactato deshidrogenasa
LG: Baja concentración de glucosa (1 mM)
LIP1: Liproxstatina-1
$LO^\bullet$: Alcoxilo lipídico
$LOO^\bullet$: Peróxido lipídico
LOOH: Hidroperóxido lipídico
mCPG: α-metil-4-carboxifenilglicina
Met: Metionina
mM: Milimolar
MP: Porcentaje de espermatozoides motiles progresivos
MT: Porcentaje de espermatozoides motiles totales
mV: Milivoltios
NAD^+: Nicotinamida adenina dinucleótido oxidada
NADH: Nicotinamida adenina dinucleótido reducida
$NADP^+$: Nicotinamida adenina dinucleótido fosfato oxidada
NADPH: Nicotinamida adenina dinucleótido fosfato reducida
$NO^\bullet$: Óxido nítrico
$NO_2^\bullet$: Dióxido de nitrógeno
O_2: Oxígeno
$O_2^{-\bullet}$: Anión superóxido
$OH^\bullet$: Radical hidroxilo
$ONOO^-$: Peroxinitrito
OXO: Dimetil-2-oxoglutarato o dimetil α-cetoglutarato
PDH: Piruvato deshidrogenasa
Pi: Fosfato inorgánico
PIP2: Fosfatidilinositol-4,5-bisfosfato
PKA: Proteína quinasa A

PKC: Proteína quinasa C
PLA2: Fosfolipasa A2
PMM: Potencial de membrana mitocondrial
PON(s): Paraoxonasa(s)
PPARγ: Receptor de peroxisoma-proliferador-activado gamma
PRDX(s): Peroxirredoxina(s)
PTMs: Modificaciones postraduccionales
PUFA(s): Ácido(s) graso(s) poliinsaturado(s) de cadena larga
R162: Inhibidor de la enzima GDH1
Redox: Oxidación-reducción
RO•: Radical alcoxilo
ROO•: Radical peroxilo
ROH: Alcohol orgánico
ROOH: Hidroperóxido orgánico
RSNO: S-nitrosotioles
S•: Radical libre de tiilo
SOD: Superóxido dismutasa
sORP: Potencial de oxidación-reducción estática
-SH: Grupo tiol
SS: Sulfasalazina
-SS-: disulfuro
TNF: Factor de necrosis tumoral
Trp: Triptófano
TRX(s): Tiorredoxina(s)
Tyr: Tirosina
UHPLC-MS/MS: Espectrometría de masas en cromatografía líquida de alto rendimiento
V: Voltios
ZP: Zona pelúcida

ANEXOS



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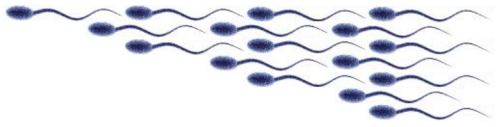

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