Universitat de Girona

MOLECULAR INSIGHTS INTO MAMMALIAN SPERM PHYSIOLOGY: A COMPARATIVE STUDY OF GLUTATHIONE S-TRANSFERASES IN MALE REPRODUCTION

Marc Llavanera Bruguera

Universitat de Girona

Doctoral Thesis

Molecular insights into mammalian sperm physiology:
a comparative study of glutathione S-transferases in male reproduction

Marc Llavanera Bruguera

2023

Universitat de Girona

DOCTORAL THESIS

Molecular insights into mammalian sperm physiology: a comparative study of glutathione S-transferases in male reproduction

Marc Llavanera Bruguera

2023

Doctoral programme in Technology

Supervised by:

Dr. Marc Yeste Oliveras

Dr. Sergi Bonet Marull

Thesis Dissertation submitted to obtain the degree of PhD at the University of Girona

Universitat de Girona

Dr. **Marc Yeste Oliveras**, Associate Professor of Cell Biology at the Department of Biology, University of Girona,

Dr. **Sergi Bonet Marull**, Full Professor of Cell Biology at the Department of Biology, University of Girona,

DECLARE:

That the Thesis entitled "***Molecular insights into mammalian sperm physiology: a comparative study of glutathione S-transferases in male reproduction***", submitted by Mr. Marc Llavanera Bruguera to obtain the doctoral degree, has been completed under our supervision and meets the requirements for the International Doctorate mention.

And for all intents and purposes, we hereby sign the document.

This work was funded by the Ministry of Universities, Spain (Grants: FPU18/00666, EST19/00389 and EST22/00052), the Ministry of Science and Innovation, Spain (Grants: FPU18/00666, AGL2017-88329-R and PID2020-113320RB-I00) and the Department of Research and Universities, Regional Government of Catalonia, Spain (2017-SGR-1229).

“Every new discovery is just a reminder we're all small and stupid”

Everything Everywhere All at Once, 2022

ACKNOWLEDGEMENTS

Com no pot ser d'una altra manera, vull començar els agraïments expressant la meva profunda gratitud al Director i Tutor d'aquesta Tesi Doctoral, el Dr. Marc Yeste. Marc, des del primer dia que vaig entrar per la porta del laboratori has exercit no només de Director sinó també de mentor, guia i *pare científic*. Quan em vas dir tot el que podria aconseguir amb disciplina i esforç no m'ho vaig creure, però com tantes altres vegades, estava equivocat. Sense cap dubte has estat una peça clau durant la meva carrera investigadora; acompanyant-me, guiant-me, formant-me i motivant-me. La teva dedicació i generositat són contagioses. Gràcies per donar-me l'oportunitat de formar part de la gran família que és TechnoSperm.

De la mateixa manera voldria agrair al co-Director de la Tesi i Director del grup, el Dr. Sergi Bonet. TechnoSperm no existiria sense tu. Gràcies per la confiança i l'oportunitat, ha sigut un privilegi formar part d'aquest equip. També a la Dra. Elisabeth Pinart, per donar-me l'oportunitat de col·laborar i tenir-me en compte per a tants projectes. Sens dubte has contribuït a la meva formació com a investigador i docent.

Aquesta Tesi també va dedicada als becaris (i no tant becaris) de TechnoSperm: Ariadna, Yentel, Sandra, Estel i Ferran. Què seria de mi si no haguéssim fet pinya? Haver coincidit amb vosaltres ha sigut un plaer, tant a dins com a fora del laboratori. Em vaig trobar excel·lents companys i professionals, i m'he acabat emportant amics. Ariadna, m'has ajudat amb tot des del primer dia que vaig entrar al grup. Gràcies pels teus consells i bons moments al laboratori. Tot i la diferència generacional (una generació de dos anys i mig), has sigut molt important durant aquests anys. Yentel, m'has ensenyat molt, a molts nivells. Qui m'havia de dir que una noia de Cardedeu acabaria essent la millor companya d'estada i amiga que podria tenir. Treballar amb tu al laboratori ha sigut tot un privilegi, i estic segur que no s'acabarà aquí. A canvi, has après un accent millor; el gironí. Sandra, gràcies per no deixar-me sol en la impaciència i l'humor negre. Ara entenc per què ets fan de One Piece; comparteixes els mateixos valors que

transmet l'*anime*, i que ara també tindrà el petit *Rauliki*. Estel, ets una font d'alegria i motivació, per això ets integrant de l'*equipo happy*. Me n'alegro que decidissis fer la tesi amb nosaltres i no tinc cap dubte que aconseguiràs tot el que et proposis. Ferran, ets tan bon estudiant de TFG com company de laboratori, i espero haver estat a l'altura com a tutor. Tot i que en alguns moments hauràs d'intentar no mullar-te quan plou, estic segur que la teva nova etapa com a doctorand serà un èxit.

No em puc deixar d'agrair a tots els *postdocs* de TechnoSperm, que m'heu ajudat i ensenyat molt durant aquests anys. Isabel, fuiste una excelente tutora del TFM y más adelante compañera de laboratorio. Gracias por ayudarme siempre y tenerme en cuenta para tus proyectos. Bea, gracias por enseñarme a tener pensamiento crítico y que hay otras maneras de hacer ciencia. Jordi, estàs sempre disposat a donar un cop de mà, tant dins del laboratori com discutint els resultats. Gràcies pel temps dedicat i per haver-me ensenyat tantes tècniques. A canvi, només t'he pogut ensenyar la tècnica del con. Albert, un Reusenc Tota la Vida (RTV) i integrant de l'*equipo happy*. La teva alegria i positivisme són contagiosos. Gràcies per tenir-me en compte en els teus projectes, per ensenyar-me tant i estar sempre disposat a ajudar. A Carolina y Lorena, el dúo murciano. Ya ni me acuerdo de cómo era el ambiente del laboratorio cuando no estabais. A parte de ser dos veterinarias e investigadoras excelentes, vuestro desparpajo es un motivo para querer ir al laboratorio todos los días. Estela, vas ser la meva primera referència a un laboratori. Gràcies per la paciència i per haver-me ensenyat com es treballa a un laboratori.

També vull agrair aquesta Tesi als companys i companyes de la UAB (Jordi Miró, Joan Enric, Jaime, Iris, Tània, Meritxell, Sebastián i Marion). Especialment als professors Joan Enric i Jordi Miró, que amb cada visita vostra ens enduem un nou aprenentatge. També a l'Iris i en Jaime. Les pujades que feu al vostre grup de Girona són sempre un plaer. Sou excel·lents com a persones i científics, i estic segur que continuareu així en tots els projectes que emprengueu.

During my Doctoral Thesis, I had the privilege of visiting two distinguished research groups in the field of reproduction. Firstly, I would like to express my gratitude to Dr. Diego Bucci for providing me with the opportunity to conduct a

part of my Doctoral Thesis in your laboratory at the University of Bologna. I would also like to extend my heartfelt thanks to the entire team, including Dr. Carlo Tamanini, Dra. Giovanna Galeati, Dra. Marcella Spinaci, Dra. Beatrice Mislei, and Dra. Olga Blanco-Prieto, for their unwavering support, guidance, and invaluable knowledge throughout my stay. Secondly, I am immensely grateful for the confidence placed in me by Dr. Pedro F. Oliveira and Dr. Marco G. Alves during my research stay at the University of Aveiro. The autonomy to work independently, coupled with the assurance of having a supportive team to rely on, was crucial to my research stay. Your expertise, patience, and willingness to help have been invaluable.

En últim lloc, però no menys important, vull agrair aquesta Tesi als amics i la família. A en Gerard, Genís i Pol. Gràcies per tots els dimarts i els viatges; no hauria arribat fins aquí sense vosaltres i aquests moments de desconnexió i connexió amb el món real. Finalment, aquesta Tesi no hauria sigut possible sense l'ajuda dels meus pares i el meu germà. Gràcies pel vostre suport, paciència i comprensió. També per acompanyar-me sempre i proporcionar-me tot el que he necessitat.

A tots vosaltres, us dedico aquesta Tesi Doctoral.

LIST OF PUBLICATIONS

This Dissertation is presented as a compendium of the following publications:

PAPER I

Llavanera, M., Delgado-Bermúdez, A., Olives, S., Mateo-Otero, Y., Recuero, S., Bonet, S., Fernández-Fuertes, B., Yeste, M., & Barranco, I. (2020). **Glutathione S-Transferases Play a Crucial Role in Mitochondrial Function, Plasma Membrane Stability and Oxidative Regulation of Mammalian Sperm**. *Antioxidants* (Basel, Switzerland), 9(2), 100. https://doi.org/10.3390/antiox9020100.

- Impact factor: **6.313** (2020)
- JCR category: **Chemistry, medicinal**
- Category quartile: **Q1/D1**

PAPER II

Llavanera, M., Delgado-Bermúdez, A., Mateo-Otero, Y., Padilla, L., Romeu, X., Roca, J., Barranco, I., & Yeste, M. (2020). **Exploring Seminal Plasma GSTM3 as a Quality and In Vivo Fertility Biomarker in Pigs-Relationship with Sperm Morphology**. *Antioxidants* (Basel, Switzerland), 9(8), 741. https://doi.org/10.3390/antiox9080741.

- Impact factor: **6.313** (2020)
- JCR category: **Chemistry, medicinal**
- Category quartile: **Q1/D1**

PAPER III

Llavanera, M., Delgado-Bermúdez, A., Fernández-Fuertes, B., Recuero, S., Mateo, Y., Bonet, S., Barranco, I., & Yeste, M. (2019). **GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm**. *Journal of animal science and biotechnology*, 10, 61. https://doi.org/10.1186/s40104-019-0370-5.

- Impact factor: **4.167** (2019)
- JCR category: **Agriculture, dairy & animal science**
- Category quartile: **Q1/D1**

PAPER IV

Garriga, F.*, **Llavanera, M**.*, Viñolas-Vergés, E., Recuero, S., Tamargo, C., Delgado-Bermúdez, A., & Yeste, M. (2022) (* first co-authors). **Glutathione S-transferase Mu 3 is associated to in vivo fertility, but not sperm quality, in bovine**. *Animal: an international journal of animal bioscience*, 16(9), 100609. https://doi.org/10.1016/j.animal.2022.100609.

- Impact factor: **3.730** (2022)
- JCR category: **Veterinary sciences**
- Category quartile: **Q1/D1**

PAPER V

Llavanera, M., Mateo-Otero, Y., Delgado-Bermúdez, A., Recuero, S., Olives, S., Barranco, I., & Yeste, M. (2021). **Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality**. *Frontiers in cell and developmental biology*, 9, 627140. https://doi.org/10.3389/fcell.2021.627140.

- Impact factor: **6.081** (2021)
- JCR category: **Developmental biology**
- Category quartile: **Q1/D2**

PAPER VI

Llavanera, M., Ribas-Maynou, J., Mateo-Otero, Y., Benet, J., Bonet, S., & Yeste, M. (2023). **Evaluation of Glutathione S-transferase Mu 3 (GSTM3) levels in sperm as a simple method to predict oxidative DNA damage and seminogram alterations**. *Andrology* (under review).

PAPER VII

Llavanera, M., Mateo-Otero, Y., Recuero, S., Delgado-Bermúdez, A., Bonet, S., Ribas-Maynou, J. & Yeste, M. (2023). **Sperm Glutathione S-transferase Mu 3 is associated with sperm DNA integrity and in vitro fertilisation in pigs**. *Andrology* (under review).

LIST OF ABBREVIATIONS

ΔΨm	Mitochondrial membrane potential
$\cdot O_2^-$	Superoxide anion
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
ANOVA	One-way analysis of variance
ART	Assisted reproductive technologies
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
AU	Arbitrary units
AZF	Azoospermia factor
BCF	Beat-cross frequency
BE	Corpus epididymis
BSA	Bovine serum albumin
BTB	Blood-testis barrier
CA	Carbonic anhydrase
Ca^{2+}	Calcium
cAMP	Adenosine monophosphate
CASA	Computer-assisted sperm analysis
CAT	Catalase
CATSPER	Cation channel of sperm
CMA3	Chromomycin A3
CO_2	Carbon dioxide
COCs	Cumulus-oocyte complexes
CPAs	Cryoprotective agents
CRISPs	Cysteine-rich secretory proteins
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsSDF	Double-stranded sperm DNA fragmentation
EA	Ethacrynic acid
ELISA	Enzyme-linked immunosorbent assay
Em	Hyperpolarisation

ESHRE	European Society of Human Reproduction and Embryology
EV	Electronic volume
FN-2	Fibronectin type II
FR	Farrowing rate
FSC	Forward scatter
GFE	Good freezability ejaculate
GPI	Glycosylphosphatidylinositol
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSR	Glutathione reductase
GSSG	Oxidised glutathione
GST	Glutathione S-transferase
GSTA	Glutathione S-transferase alpha class
GSTM	Glutathione S-transferase mu class
GSTO	Glutathione S-transferase omega class
GSTP	Glutathione S-transferase pi class
GSTS	Glutathione S-transferase sigma class
GSTT	Glutathione S-transferase theta class
GSTZ	Glutathione S-transferase zeta class
H-42	Hoechst 33342
H_2DCFDA	2',7'-dichlorofluorescin diacetate
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotropin
HCO_3^-	Bicarbonate
HE	Hydroethidine
HE	Caput epididymis
HRP	Horseradish peroxidase
IAM	Inner acrosomal membrane
ICSI	Intracytoplasmic sperm injection
IgSF	Immunoglobulin superfamily
ISAC	International Society for Advancement of Cytometry
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
JC-1	1,1',3,3'-Tetraethyl-5,5',6,6'-tetrachloroimidacarbocyanine iodide
JNK	c-Jun N-terminal kinases

LDHC	Lactate dehydrogenase
LIN	Linearity
LS	Litter size
LS-MS/MS	Liquid chromatography–mass spectrometry
M540	Merocyanine 540
MAPEG	Membrane-Associated Proteins in Eicosanoid and Glutathione
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NRR	Non-return rate
O-GlcNAc	O-linked β-N-acetylglucosamine
O_2	Oxygen
O_2^{2-}	Peroxide
OAM	Outer acrosomal membrane
ODFs	Outer dense fibres
OH·	Hydroxyl radical
OS	Oxidative stress
OTM	Olive tail moment
P	Prostate
PAWP	Post-acrosomal WW-domain binding protein (also known as WBP2NL)
PBS	Phosphate-buffered saline
PFE	Poor freezability ejaculate
PGD_2	Prostaglandin D2
PI	Propidium iodide
PKA	cAMP-Dependent Protein Kinase A
PLCζ	Phospholipase Cζ
PMN	Polymorphonuclear cells
PMOT	Progressive motility
PNA-FITC	Peanut agglutinin conjugated with fluorescein isothiocyanate
PON1	Paraoxonase 1
PRx	Peroxiredoxins
PT	Perinuclear theca
pT	Threonine phosphorylation
PTM	Post-translational modifications
PUFA	Polyunsaturated fatty acids

PVDF	Polyvinylidene difluoride
pY	Tyrosine phosphorylation
R	Correlation coefficient
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SAB	SH3-domain binding protein 5
sADCY	Soluble adenylyl cyclase
SBC	Sodium bicarbonate cotransporter
SCs	Sertoli cells
SED	Squared Euclidean distance
SEM	Standard error of the mean
SOAF	Sperm-borne oocyte activating factors
SOD	Superoxide dismutase
SP	Seminal plasma
SSC	Side scatter
ssSDF	Single-stranded sperm DNA fragmentation
STR	Straightness
SV	Seminal vesicles
T	Testicle
TE	Cauda epididymis
TER	Terrapin 199 (also known as Ezatiostat)
TMOT	Total motility
VAP	Average pathway velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WHO	World Health Organisation
ZP	Zona pellucida

GENERAL CONTENTS

LIST OF FIGURES

Introduction

Paper I

Paper II

Paper III

Paper IV

Paper V

Paper VI

Paper VII

LIST OF TABLES

Introduction

Paper I

Paper II

Paper III

Paper VI

ABSTRACT/RESUM/RESUMEN

ABSTRACT

Worldwide, 20-30% of infertility cases are exclusively caused by a male factor, and sperm quality has been seen to progressively decline over the last decades. Moreover, the incidence of male infertility and subfertility is relevant for the animal breeding industry, as it exerts a negative impact on balance sheets and profits. Fertility preservation procedures, which include sperm storage, are combined with other assisted reproduction techniques in both humans and farm animals. Notwithstanding preservation methods, both liquid-storage and cryopreservation, may entail sperm damage in a species-specific manner, they are extensively used in humans and livestock. Examining the causes of male-factor infertility is, therefore, a requisite to develop novel strategies to predict related disorders as well as the sperm resilience to conservation. While the conventional seminogram is an excellent, rapid and cheap approach to assess semen quality - and is widely utilised by both fertility clinics and the animal breeding industry -, it leaves the cellular and molecular features of sperm aside, thus limiting its sensitivity and accuracy. It is noteworthy, moreover, that the multicausal nature of male infertility hinders its diagnosis and prediction in both humans and other animals. Under these circumstances, the interest on the exploration of novel molecular markers is progressively increasing. Molecular markers can provide relevant information about the physiological status of sperm with cost-effective protocols, thus overcoming seminogram limitations in the prediction of male infertility and the capability of sperm to withstand preservation. Besides, the molecular characterisation of sperm proteins may lead to the establishment of new therapeutic targets. Glutathione S-transferases (GSTs) are a group of ubiquitous antioxidant enzymes suggested to be linked to (in)fertility in humans and farm animals. Their role in male (in)fertility, however, has been understudied in mammals. Acknowledging the putative relevance of GSTs in male (in)fertility, their involvement in sperm physiology and usefulness as a molecular marker was investigated in the present Dissertation. For this purpose, immunological and pharmacological approaches were undertaken to: (i)

characterise GSTs in reproductive tissues, seminal plasma, and sperm of pigs, cattle and humans; (ii) investigate their physiological role in sperm detoxification and regulation of cellular stress-signalling; (iii) assess their usefulness as a biomarker of sperm quality and fertilising ability; and (iv) address its potential to predict the capacity of sperm to withstand preservation. Immunoblotting and immunostaining revealed that GSTs, and specifically GSTM3 and GSTP1, are present in the sperm of pigs, cattle and humans, exhibiting species-specific differences in terms of band-patterns and localisation. Specifically, in pigs, GSTM3 was identified in reproductive tissues, seminal plasma and sperm. In sperm, this antioxidant enzyme was subject to relocalisation in response to liquid-storage and cryopreservation, suggesting the adaptation of the cell to these changes. Subsequently, pharmacological inhibition of the antioxidant activity of GSTs by ethacrynic acid, strongly blocking the glutathione-binding site, was conducted to investigate their involvement in sperm detoxification. Thanks to this approach, GSTs were found to play a key role in mitochondrial activity, plasma membrane stability and oxidative regulation of pig sperm, thus being antioxidant enzymes essential for preserving their function. Furthermore, pharmacological dissociation of the GSTP1-JNK heterocomplex by ezatiostat evidenced that GSTP1 regulates sperm function by inhibiting JNK, thus preserving mitochondrial activity and membrane stability. Given the observed role of sperm GSTs in the detoxification and regulation of the cellular stress response, the potential use of GSTM3 as a biomarker of sperm quality and fertilising ability was also interrogated in humans and farm animals. GSTM3 levels were found to be associated to the quality and fertilising ability of porcine, bovine and human sperm. Finally, the ability of GSTM3 to predict the capacity of sperm to withstand preservation was also evaluated. This antioxidant enzyme was observed to predict the sperm resilience to liquid-storage and cryopreservation in pigs. In conclusion, the results of the present Dissertation warrant the use of sperm GSTM3 as a molecular marker of sperm quality, fertilising ability and capacity to withstand preservation. Although further research with larger cohorts and ROC analysis should be performed before this biomarker can be implemented in fertility clinics and the animal breeding industry, the findings shown herein

support the development of novel GSTM3-based predictive systems with cost-effective and accurate outcomes.

RESUM

A escala mundial, entre un 20 i un 30% dels casos d'infertilitat es deuen exclusivament a factors masculins. A més, durant les últimes dècades, s'ha observat una disminució progressiva de la qualitat espermàtica. Així mateix, cal destacar que la incidència de la infertilitat/subfertilitat masculina és summament important per a la indústria ramadera, atès que pot repercutir negativament en els seus indicadors econòmics. Tant en humans com en animals de granja, els mètodes de conservació de l'esperma i de preservació de la fertilitat s'utilitzen conjuntament amb altres tècniques de reproducció assistida. Tanmateix, els protocols de refrigeració i de criopreservació espermàtiques, emprats tant en humans com en espècies d'interès productiu, provoquen danys en els espermatozoides de manera específica a cada espècie. Per tant, examinar les causes de la infertilitat masculina és un requisit pel desenvolupament de noves estratègies que prediguin aquests trastorns, alhora que és fonamental determinar la capacitat de resistència dels espermatozoides a la seva conservació. Si bé el seminograma convencional és un mètode excel·lent, ràpid i econòmic per avaluar la qualitat del semen, i el seu ús s'ha estès tant en clíniques de fertilitat com en la indústria ramadera, no contempla les característiques cel·lulars i moleculars dels espermatozoides, la qual cosa en limita la seva sensibilitat i precisió. Endemés, la naturalesa multifactorial de la infertilitat masculina dificulta la seva diagnosi i prognosi, tant en humans com en animals domèstics. En aquest sentit, hi ha un interès creixent per explorar i establir nous marcadors moleculars. Aquests marcadors poden proporcionar informació addicional i rellevant sobre l'estat fisiològic de l'espermatozoide, superant així les limitacions del seminograma a l'hora de predir la infertilitat masculina i la capacitat de resistència de l'espermatozoide a la seva conservació. Addicionalment, la caracterització molecular de les proteïnes espermàtiques pot conduir a l'establiment de noves dianes terapèutiques. Les glutatió S-transferases (GST) són un grup d'enzims antioxidants ubics, i s'ha suggerit que poden estar relacionats amb la (in)fertilitat en éssers humans i animals d'interès productiu. Tanmateix, la seva relació amb la (in)fertilitat masculina s'ha estudiat poc en mamífers. Per això, en aquesta Tesi

Doctoral es va investigar la rellevància funcional de les GSTs als espermatozoides humans, porcins i bovins, i se'n va determinar la seva utilitat com a marcador moleculars. Es van dur a terme tècniques immunològiques i farmacològiques per (i) caracteritzar les GSTs en els teixits reproductius, el plasma seminal i els espermatozoides; (ii) determinar el seu paper fisiològic en la detoxificació dels espermatozoides i la resposta a l'estrès oxidatiu; (iii) avaluar el seu ús com a biomarcadors de qualitat espermàtica i capacitat fecundant; i (iv) abordar el seu potencial per predir la resistència dels espermatozoides a la conservació. Les anàlisis d'immunotransferència i immunofluorescència van revelar que les GSTs, específicament la GSTM3 i la GSTP1, es troben als espermatozoides porcins, bovins i humans, per bé que hi ha diferències entre aquestes espècies tant pel que fa al patró de bandes proteiques (immunotransferència) com a la localització cel·lular. També es va identificar, a l'espècie porcina, la presència de GSTM3 en els teixits reproductius, el plasma seminal i els espermatozoides. En aquests últims, es va observar que tant la refrigeració com la criopreservació indueixen la relocalització d'aquest enzim antioxidant, la qual cosa suggereix que els gàmetes masculins tenen la capacitat d'adaptar-se als canvis de l'entorn, també quant a l'homeòstasi redox. Posteriorment, es va inhibir l'activitat antioxidant de les GSTs mitjançant àcid etacrínic, que bloqueja el seu lloc de unió al glutatió, per investigar el seu paper en la detoxificació dels espermatozoides. Gràcies a aquesta aproximació, es va demostrar que les GSTs juguen un paper clau en l'activitat mitocondrial, l'estabilitat de la membrana plasmàtica i la regulació oxidativa de les cèl·lules espermàtiques, de tal manera que es pot considerar que són enzims antioxidants essencials per la funció de les mateixes. A més, la dissociació farmacològica de l'heterocomplex GSTP1-JNK mitjançant ezatiostat va posar en evidència que la funció de la GSTP1 està relacionada amb la inhibició de la JNK i que, d'aquesta manera, preserva l'activitat mitocondrial i l'estabilitat de la membrana. Considerant el paper de les GSTs dels espermatozoides en la seva detoxificació i la regulació de la resposta a l'estrès cel·lular, també es va investigar la utilitat de la GSTM3 com a biomarcador de qualitat espermàtica i capacitat fecundant en humans i espècies d'interès ramader. Es va trobar que els nivells de GSTM3 estan associats amb la qualitat i la capacitat fecundant dels

espermatozoides porcins, bovins i humans. Finalment, també es va avaluar la relació de la GSTM3 amb la resistència dels espermatozoides a la refrigeració i a la criopreservació. Es va observar que, en porcí, aquest enzim antioxidant pot predir la resiliència dels espermatozoides a ambdós mètodes de conservació. A tall de conclusió, els resultats d'aquesta Tesi Doctoral encoratgen la utilització de la GSTM3 com a marcador molecular de qualitat espermàtica, capacitat fecundant i resistència a la conservació. Malgrat que calen estudis complementaris amb cohorts més grans i corbes ROC abans d'implementar aquest biomarcador en clíniques de fertilitat i la indústria ramadera, les dades obtingudes en aquest treball recolzen el desenvolupament de nous sistemes predictius basats en la GSTM3.

RESUMEN

A escala mundial, entre un 20 y un 30% de los casos de infertilidad se deben exclusivamente al factor masculino. Además, durante las últimas décadas, se ha observado una disminución progresiva de la calidad espermática. Cabe destacar, asimismo, que la infertilidad y subfertilidad de los sementales es de gran importancia para la industria ganadera, dada su repercusión negativa en los indicadores económicos. Tanto en la reproducción humana cuanto en la de los animales de interés productivo, los métodos de conservación del semen y de preservación de la fertilidad se utilizan conjuntamente con otras técnicas de reproducción asistida. Sin embargo, dichos protocolos, tanto la refrigeración cuanto la criopreservación, producen daño en los espermatozoides de manera particular en cada especie. Con todo, el examen de las causas de la infertilidad masculina es un requisito para desarrollar nuevas estrategias que predigan los trastornos de infertilidad masculina y la capacidad de resistencia de los espermatozoides a la refrigeración y a la criopreservación. Si bien el seminograma convencional es una manera rápida, adecuada y económica de evaluar la calidad del semen - y su uso se ha extendido tanto en clínicas de fertilidad como en la producción animal -, tiene el inconveniente de que no contempla las características celulares y moleculares de los espermatozoides, lo que limita su sensibilidad y precisión. Por otra parte, cabe destacar que la naturaleza multifactorial de la infertilidad masculina dificulta su capacidad predictiva y de diagnóstico, tanto en humanos cuanto en otros animales. Por este motivo, el interés por hallar nuevos marcadores moleculares está incrementando progresivamente. Los marcadores moleculares pueden proporcionar información relevante sobre el estado fisiológico de los espermatozoides con protocolos sencillos, superando así las limitaciones del seminograma respecto a la predicción de la infertilidad masculina y a la capacidad de resistencia del espermatozoide a la refrigeración y a la criopreservación. De igual modo, la caracterización molecular de las proteínas espermáticas puede conducir al establecimiento de nuevas dianas terapéuticas. Las glutatión S-transferasas (GST) son un grupo de enzimas antioxidantes ubicuas que parecen estar

relacionadas con la (in)fertilidad en humanos y otros animales. Sin embargo, su papel en la (in)fertilidad masculina se ha estudiado poco en mamíferos. Teniendo en cuenta la posible relevancia de las GSTs en la (in)fertilidad masculina, en esta Tesis Doctoral se investigó su papel en la fisiología espermática, así como su utilidad como marcador molecular en cerdos, toros y humanos. Se llevaron a cabo técnicas inmunológicas y farmacológicas para (i) caracterizar las GST en los tejidos reproductivos, el plasma seminal y los espermatozoides, (ii) investigar su papel fisiológico en la detoxificación de las células espermáticas y la regulación de la señalización del estrés celular, (iii) evaluar su papel como biomarcador de calidad espermática y capacidad fecundante, y (iv) abordar su potencial para predecir la resistencia de los espermatozoides a la conservación. Los análisis de inmunotransferencia e inmunofluorescencia revelaron que las GSTs, y específicamente la GSTM3 y la GSTP1, están presentes en los espermatozoides de cerdo, toro y humano, observándose diferencias entre especies tanto en los patrones de bandas en la inmunotransferencia cuanto a la localización celular. Específicamente, en cerdo, se confirmó la presencia de la GSTM3 en los tejidos reproductivos, el plasma seminal y los espermatozoides. En estos últimos, se halló que esta enzima antioxidante se relocaliza en respuesta a la refrigeración y a la criopreservación espermáticas, lo que sugiere que el gameto masculino tiene la capacidad de adaptar su fisiología a los cambios que dichos métodos de conservación producen. Posteriormente, se inhibió la actividad antioxidante de las GSTs mediante ácido etacrínico, bloqueando el sitio de unión al glutatión, para investigar el papel de aquéllas en la detoxificación de los espermatozoides. Gracias a esta aproximación, se demostró que las GSTs desempeñan un papel clave en la actividad mitocondrial, la estabilidad de la membrana plasmática y la regulación oxidativa de las células espermáticas, siendo enzimas antioxidantes esenciales para la función de las mismas. Además, la disociación farmacológica del heterocomplejo GSTP1-JNK mediante ezatiostat evidenció que la GSTP1 regula la función de los espermatozoides mediante la inhibición de la JNK, manteniendo así la actividad mitocondrial y la estabilidad de la membrana. Considerando el papel de las GSTs de los espermatozoides en su detoxificación y la regulación de la respuesta al estrés

celular, también se investigó el uso de la GSTM3 como biomarcador de calidad espermática y capacidad fecundante en humanos y especies de interés productivo. Se encontró que los niveles de la GSTM3 están asociados con la calidad y la capacidad fecundante de los espermatozoides porcinos, bovinos y humanos. Por último, también se evaluó la utilidad de la GSTM3 para pronosticar la resistencia de los espermatozoides a la conservación. Se observó que esta enzima antioxidante puede predecir la capacidad de los espermatozoides de cerdo de resistir a la refrigeración y a la criopreservación. En conclusión, los resultados de la presente Tesis Doctoral justifican el uso de la GSTM3 como marcador molecular de calidad espermática, capacidad fecundante y resiliencia a la conservación. Aunque hacen falta aún estudios complementarios con cohortes más grandes y análisis mediante curvas ROC antes de implementar este biomarcador en las clínicas de fertilidad y la industria ganadera, los resultados de este trabajo respaldan el desarrollo de nuevos sistemas predictivos basados en la GSTM3, con resultados rentables y precisos.

INTRODUCTION

INTRODUCTION

1.1. Infertility disorders: a rising problematic

1.1.1. A global perspective of infertility

As defined by the World Health Organisation (WHO), clinical infertility is the inability to conceive after more than one year of unprotected intercourse (Zegers-Hochschild et al., 2017; Vander Borght and Wyns, 2018). From the cell viewpoint, infertility occurs when sperm cells are unable to fertilise the oocyte, whereas subfertility refers to any form of reduced fertility (Gnoth et al., 2005). Clinical infertility has been reported to be a major health problem, affecting 8-12% of couples at reproductive age, which represents about 48.5 million couples worldwide (Agarwal et al., 2015a, 2021; Vander Borght and Wyns, 2018), and increasing annually in both women and men (Sun et al., 2019). In some regions, such as South and Central Asia, Middle East and North Africa, and Central and Eastern Europe, clinical infertility is estimated to be around 30% of couples at reproductive age (Nachtigall, 2006; Mascarenhas et al., 2012). In the light of the above and accounting for the significant public health implications of fertility disorders, research on the causes and treatment of this continuing decline is urgently needed. Particularly, further implementing, strengthening and refining assisted reproductive technologies (ART) could be beneficial for addressing reproductive challenges (Ziebe and Devroey, 2008).

1.1.2.Assisted reproductive technology (ART)

ART encompasses medical procedures intended to rescue fertility, including diagnosis and treatment of both male and female subfertility/ infertility disorders. Since the birth of the first child conceived by *in vitro* fertilisation (IVF) in 1978[1] (Daily Mail Reporters, 1978), the utilisation and continuous innovation of ART has exploded. Forty years later, over 8 million children from infertile couples

[1] Louise Brown was the first child born by using *in vitro* fertilisation (IVF) on 25 July 1978 in Oldham, England. It was a highly mediated and visible event through the publication of a documentary in the British television that contributed to the normalisation of IVF (Dow, 2019).

worldwide had been born by using ART, as estimated by the European Society of Human Reproduction and Embryology (ESHRE) (European Society of Human Reproduction and Embryology, 2018). Nowadays, ART comprises a wide range of medical procedures, from initial fertility tests to intracytoplasmic sperm injection (ICSI), as well as the retrieval, cryopreservation and transfer of gametes and embryos, among many others. In humans, nevertheless, cumulative live-birth rates after IVF are around 50% (Moragianni and Penzias, 2010), so that many factors associated to both female and male infertility are still hindering fertilisation despite the use of ART. Moreover, ART procedures have been developed and optimised for both humans and farm animals. Indeed, they have extensively been used in livestock (cattle, pigs, and sheep), thus increasing the offspring of genetically selected animals, improving gamete preservation, and allowing simple genetic manipulation and the transport of germplasm across long distances, among other benefits (Hansen, 2020).

1.2. Male infertility

Worldwide, male infertility is responsible for about 50% of cases of unsuccessful pregnancy, which indicates that the contribution of the two sexes is similar. Remarkably, 20-30% of infertility cases reported are exclusively caused by a male factor (Agarwal et al., 2015a). Moreover, it is known that the male contribution to infertility widely differs between regions. While Sub-Saharan Africa and Asia show the lowest rate of infertile men, Central and Eastern Europe and the Middle East have the highest rates (**Figure 1**; Agarwal et al., 2015). Although the magnitude and prevalence of male infertility are challenging to calculate, it is clear that male infertility is a global health issue warranting further research.

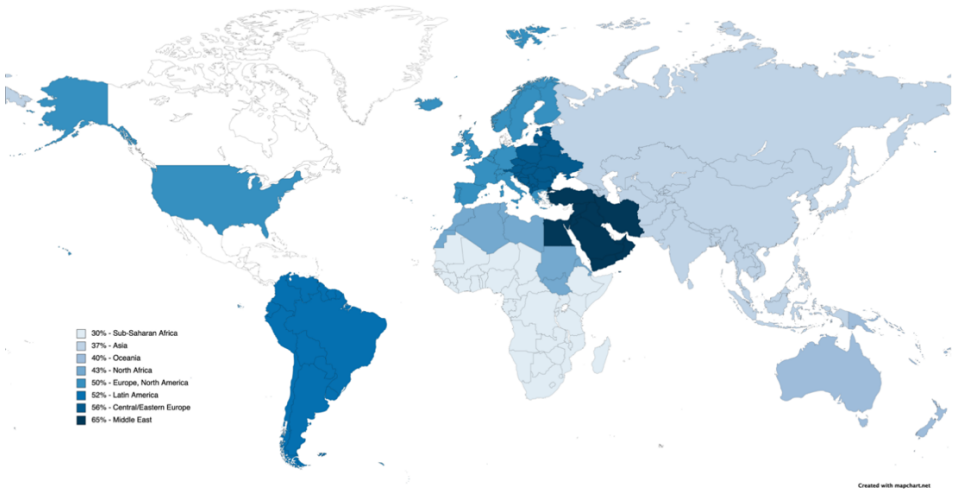

Figure 1*. *Male related infertility per region. *World map containing the percentages of male infertility cases per region studied (Sub-Saharan Africa, North Africa, Asia, Oceania, Middle East, Europe, Central/Eastern Europe, and North America). Data extracted from (Agarwal et al., 2015a).*

1.2.1. Aetiology of male infertility

Since the first study reporting a decrease in semen quality 48 years ago (Nelson and Bunge, 1974), several studies evidenced a clear decline in semen parameters over the last decades (Carlsen et al., 1992; Swan et al., 2000; Mishra et al., 2018). Specifically, a meta-regression analysis estimated a 50-60% reduction in sperm counts between 1973 and 2013 (Levine et al., 2017). More recently, this meta-regression analysis was updated with new data between 2014 and 2019 (Levine et al., 2022). All these findings concurred in a continuous drop of sperm concentration over the years, with an average global rate of -0.87 million sperm/mL/year (Levine et al., 2022). Examining the causes of the male infertility is, therefore, a requisite to develop novel strategies to predict and diagnose male infertility disorders as well as to improve semen quality. Related to this, one has to note the high complexity of the diagnosis and treatment of infertility as, because of its multicausal nature, many factors known to be related to male subfertility/infertility need to be envisaged (Ilacqua et al., 2018; Pillai and McEleny, 2021).

As summarised in **Figure 2**, a wide range of causes and factors contributing to infertility have been hitherto reported (Pillai & McEleny, 2021).

Lifestyle habits are modifiable factors highly associated to the decline of sperm quality. Poor nutrition, lack of physical exercise and psychological stress, among many others, are suspected to impair testicular function (Ilacqua et al., 2018). Likewise, other health problems such as urogenital infections are a source of acute inflammation that may also compromise spermatogenesis and male fertility (Purvis and Christiansen, 1993). In addition to this, many other contributing factors may reduce sperm quality and subsequent fertility rates. Environmental factors, such as hazardous chemical exposure like endocrine-disrupting chemicals (Sharma et al., 2020) or ionising radiation (Ahmad and Agarwal, 2017), are known to have deleterious effects upon spermatogenesis. Besides, congenital and genetic defects, especially azoospermia factor (AZF) deletions[2], are responsible for defective spermatogenesis, thus causing male infertility (Kuroda et al., 2020). All the aforementioned factors, together with many other health conditions involving hormonal dysregulation, sexual dysfunction or intake of certain types of drugs, may exert a negative impact upon semen quality, thus impairing the function of male gametes and reducing or preventing fertilisation (Pillai and McEleny, 2021). In this regard, research into the aetiology and diagnosis of male fertility disorders is much warranted, as it may contribute to reduce male sub-fertility and infertility.

[2] Azoospermia factor (AZF) deletions are genomic deletions in the euchromatic part of the long arm of the human Y chromosome (Yq11) associated with azoospermia or severe oligozoospermia (Vogt, 2005).

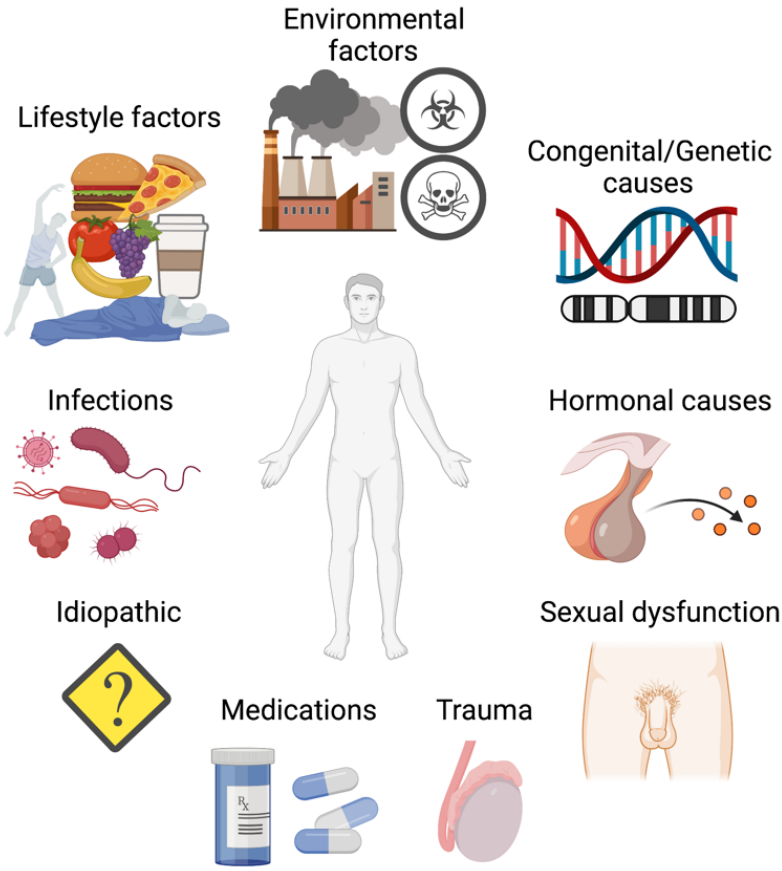

Figure 2. Causes and factors contributing to male infertility*. Data extracted from Pillai and McEleny (2021). Figure created with BioRender.*

1.2.2.Diagnostic of male infertility

The initial clinical evaluation of male (in)fertility covers a careful examination of (i) the medical history and physical status, (ii) hormonal imbalance, and (iii) semen quality (Pan et al., 2018). Remarkably, sperm quality analysis is acknowledged to be the mainstay of the initial evaluation for male factor (in)fertility.

1.2.2.1. Conventional semen analysis: the seminogram

Evaluation of semen quality has traditionally been performed through semen analysis, also known as conventional spermiogram or seminogram. In humans, the seminogram is performed using two semen samples collected about a month

apart, and after 2-5 days of abstinence from ejaculation (Male Infertility Best Practice Policy Committee of the American Urological Association and Practice Committee of the American Society for Reproductive Medicine, 2006). This is an excellent, rapid and cheap method to assess semen quality, that is extensively used in fertility clinics worldwide and consists of the evaluation of ejaculate volume, sperm concentration, morphology, motility, and viability (World Health Organisation, 2021). Ranges for normal semen parameters are established by WHO guidelines (World Health Organisation, 2021). The seminogram, however, leaves aside other cellular and molecular features of the sperm cell such as DNA integrity, oxidative status, or the presence of essential sperm proteins (Lewis, 2007; Altmäe and Salumets, 2011), thus not providing complete information of its function and fertilising ability. Despite the extensive utilisation of the seminogram to evaluate semen quality, its application for the prognosis and diagnosis of male (in)fertility is currently under debate due to its limited sensitivity and accuracy (Lewis, 2007; Altmäe and Salumets, 2011; Kwon et al., 2014).

Table 1. Cut-off reference values for semen characteristics as published in The World Health Organisation (WHO) guidelines in 2021 *(World Health Organisation, 2021).*

Semen parameter	Reference value
Volume (mL)	1.4
Sperm concentration ($\times 10^6$/mL)	16
Total sperm number ($\times 10^6$)	39
Total motility (%)	42
Progressive motility (%)	30
Viability (%)	54
Normal morphology (%)	4

Along these lines, male (in)fertility is extremely challenging to predict because of its multicausal nature, the complex physiology of sperm cells and the unknown details of how they interact with the female reproductive tract (Oehninger and Ombelet, 2019), consequently limiting the potential value of

seminogram. Hence, the exploration of new molecular markers with higher sensitivity and specificity in predicting male (in)fertility is of great interest for both fertility clinics and the livestock industry.

1.2.2.2. Molecular (in)fertility markers

Currently, there is a rising interest in the exploration of novel molecular markers due to their potential to overcome seminogram limitations in the prediction of male (in)fertility. Molecular markers can determine biochemical, metabolic and/or structural characteristics of semen samples by using accurate, rapid and cheap techniques, thus providing relevant information about sperm physiological status with cost-effective methods (Kovac et al., 2013; Carrell et al., 2016; Yadav, 2017).

The OMICs revolution, which refers to the high-throughput evaluation of genes (genomics), transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics), has transformed the scientific landscape and is fuelling the research of novel molecular markers (Kovac et al., 2013). Considering that OMICs allow for the determination of molecules playing a crucial role in sperm physiology, they are increasingly studied in the andrology field (Kovac et al., 2013; Egea et al., 2014; Carrell et al., 2016; Yadav, 2017). Indeed, OMIC technologies are in constant development and are the grounds of setting new markers associated to male (in)fertility (Egea et al., 2014; Yadav, 2017). Tackling this issue, an exhaustive systematic review intended to identify the most robust molecular biomarkers in semen for the diagnosis of male (in)fertility, and their potential clinical use, has been recently published (Llavanera et al., 2022). Noncoding ribonucleic acid in semen, such as miR-34c-5p, sperm and seminal plasma proteins, such as TEX101, and even their metabolomic profile, exhibit an excellent diagnostic potential. It is thus clear, as previously mentioned, that the novel application and improvement of molecular markers evaluating functional sperm parameters is of great interest for both assisted human reproduction and animal breeding industry. In this regard, the investigation of molecular markers in semen is necessary to improve the prognosis and diagnosis of sperm quality and (in)fertility, optimising ART procedures and ultimately increasing fertility rates.

1.3. Male reproductive function in mammals: general considerations

Semen is composed by the secretions of the male reproductive organs, consisting of sperm and seminal plasma (SP). Both components have been the subject of intensive research for several decades, due to the rise of reproductive disorders and the usage of ART. Thus, this section provides a comprehensive overview of the fundamentals of mammalian sperm and SP.

1.3.1. The sperm cell

During the mid-17th century, the technological advances in microscopy allowed Antony van Leeuwenhoek [3], and its student Johan Ham, the discovery of sperm, which were initially denominated "*animalcules spermatiques*" (Leeuwenhoek, 1679). They observed, for the first time, the liquefaction of semen, the motile ability of sperm and their basic morphological characteristics. After discovering the presence of numerous "*animalcules"* in human semen, Leeuwenhoek initiated an investigation on their biological and morphological properties in a wide range of species, including dogs, pigs, rabbits, fishes, birds and amphibians, among others (**Figure 3**; Castellani, 1973). As a result of these observations, Leeuwenhoek intuitively associated sperm with the male contribution to fertilisation, and thought that motility was a feature required for their survival in the female reproductive tract. In spite of this, the biological role of sperm was not understood and empirically confirmed until almost two centuries later, when the oocyte was discovered and the fertilisation process was described (Bon Baer, 1956; Andrade-Rocha, 2017). Since then, many studies have been carried out examining not only sperm physiology but also their role in the fertilisation process.

[3] Antony van Leeuwenhoek (1632-1723) was a Dutch businessman and scientist known as "*the Father of Microbiology*" for his pioneering work in Microscopy (Porter, 1976).

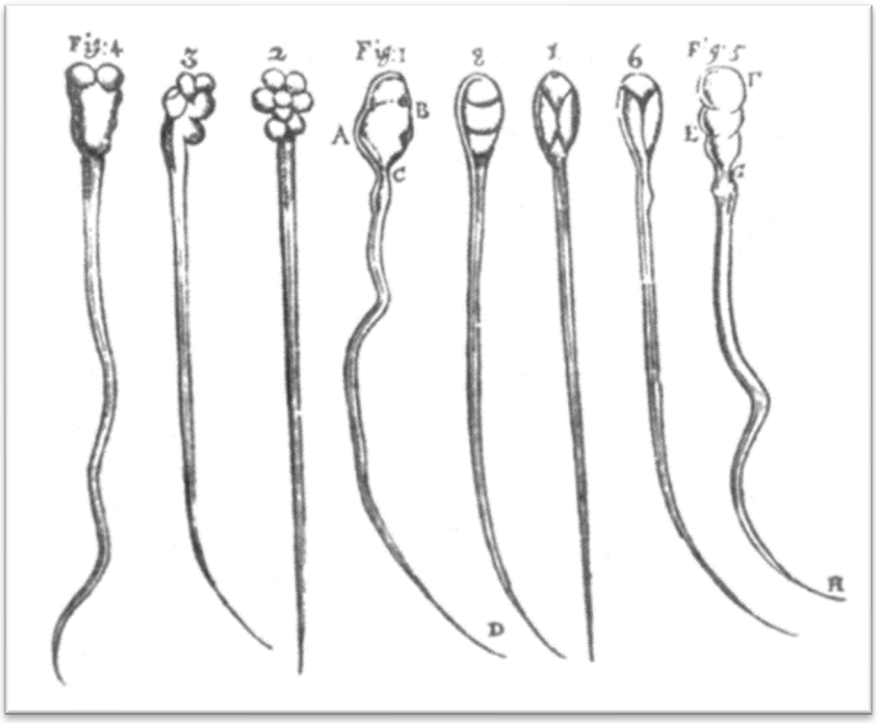

Figure 3. First published illustration of mammalian sperm *accompanying a letter from Leeuwenhoek to the Royal Society on March 18, 1678. Retrieved from Androutsos (2014).*

Sperm are known to be highly specialised cells forming the cellular fraction of semen. In general terms, after spermiogenesis, mammalian sperm are composed of two main segments: (i) the head at the proximal end, and (ii) the flagellum (or tail) at the distal end, united with each other by the connecting piece (or neck) (**Figure 4**; Chianese & Meccariello, 2018; de Jonge & Barratt, 2006).

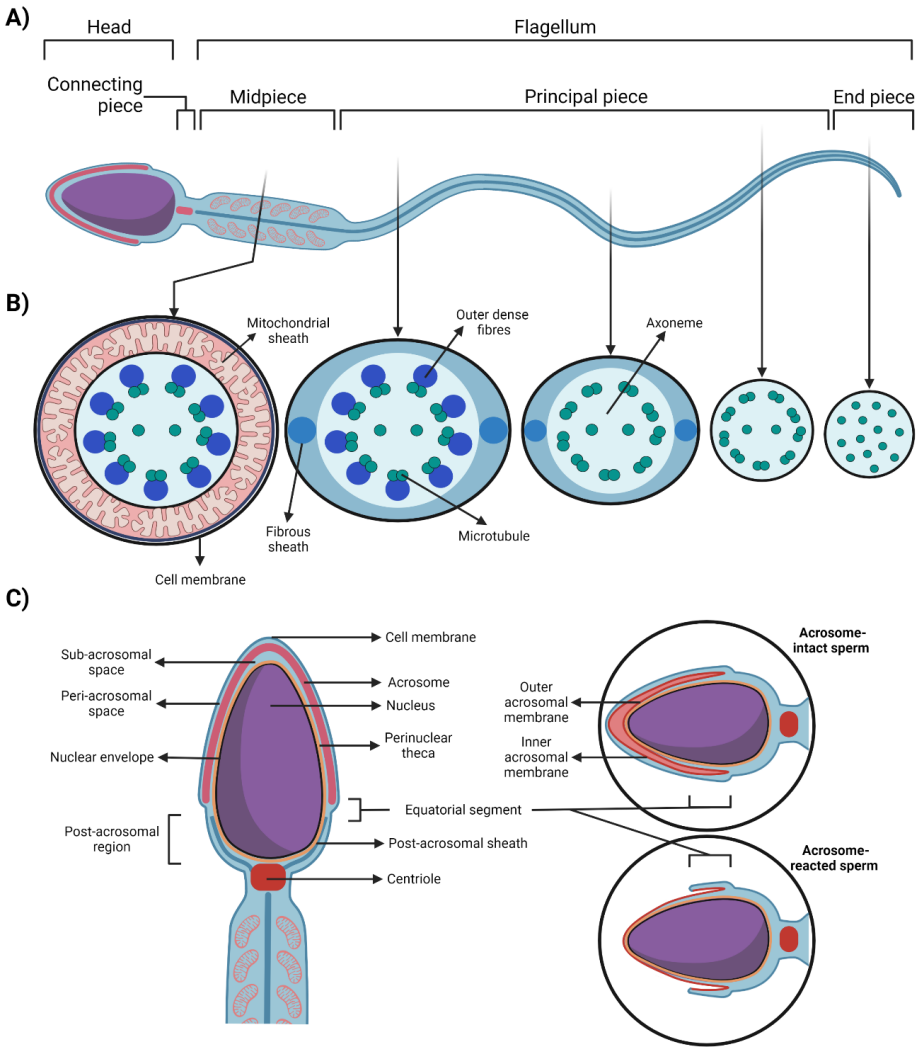

***Figure 4. Schematic representation of a mammalian sperm cell (parts and components**). The figure was adapted and modified from Kumar & Singh (2021) and Schoeller et al. (2020) , and created with BioRender.*

Regarding its ultrastructure, the sperm head is composed by (i) the nucleus, containing the genetic material delivered to the offspring, and (ii) a sperm-specific organelle known as acrosome, forming a cap on the proximal end of the head (**Figure 4C**). During spermiogenesis, the sperm nucleus undergoes an extensive chromatin remodelling, partially replacing histones by protamines, which generates a hypercondensed chromatin and silences gene expression

(Marcon and Boissonneault, 2004; Miller et al., 2010; Chianese and Meccariello, 2018).

The nucleus is covered by a nuclear envelope lacking nuclear pore complexes (de Jonge and Barratt, 2006). A rigid protective shell, named perinuclear theca (PT), surrounds the nucleus and its envelope. The PT is composed of proteins, some of them stabilised through disulphide bonds, which are understood to participate in cell signalling pathways in the oocyte upon gamete fusion (Oko, 1995; Sutovsky et al., 2003). The PT is divided into three major segments, each serving specific functions (de Jonge and Barratt, 2006): (i) the sub-acrosomal layer, which is fused with the inner acrosomal membrane; (ii) the equatorial segment, a folded-over complex of the PT, inner and outer acrosomal membranes that is involved in sperm–oocyte binding; and (iii) the post-acrosomal sheath, believed to content sperm-borne oocyte activating factors (SOAF) responsible for oocyte activation after sperm-oocyte fusion (Sutovsky et al., 2003). Indeed, several PT-specific proteins have been suggested to be involved in oocyte activation in mammals, such as phospholipase Cζ (PLCζ) and post-acrosomal WW-domain binding protein (PAWP; also known as WBP2NL) (Yeste et al., 2017).

The acrosome, on the other hand, is a Golgi apparatus-derived organelle that contains degradative enzymes and receptors required for sperm interaction with the zona pellucida (ZP) of the oocyte (Chianese and Meccariello, 2018). The acrosome holds a dense mixture of proteases and protein receptors, called acrosomal matrix (Yoshinaga and Toshimori, 2003; Buffone et al., 2009). The acrosome membrane is divided into two major segments (de Jonge and Barratt, 2006): the inner acrosomal membrane (IAM) and the outer acrosomal membrane (OAM). Whereas the OAM is lost after acrosomal exocytosis, the IAM remains anchored to the sub-acrosomal layer (**Figure 4C**).

The sperm flagellum has a complex molecular architecture and is crucial for intrinsic motility. It is divided into the (i) connecting, (ii) mid, (iii) principal, and (iv) end pieces (**Figure 4A and 4B**). The flagellum is supported by an axoneme along the entire length, surrounded by periaxonemal structures, except for the end piece, that is exclusively surrounded by the plasma membrane (Kumar and

Singh, 2021). The axoneme is composed of a specific "9 + 2" structure, consisting of nine microtubule doublets in the outer part, and two single microtubules in the centre (Schoeller et al., 2020). The central microtubules are linked with radial spokes and dynein arms, the latter being responsible for the sperm tail movement (Schoeller et al., 2020).

Several periaxonemal structures enclose the axoneme and are highly variable across flagellum parts. In the mid-piece, the mitochondrial sheath is arranged girdling the axoneme in a helical pattern known as gyres (Kumar and Singh, 2021). The outer dense fibres (ODFs) consist of nine columns composed of 14 polypeptides, variable across mammalian species, and present in the flagellum from the connecting piece to the end of the principal piece (Oko, 1988). Besides their essential role for sperm motility (Zhao et al., 2018), the ODFs protect sperm from the damage caused during the transport through the epididymis and ejaculation (Kumar & Singh, 2021). The fibrous sheath (FS) is another essential component for the structural integrity of the sperm tail. It consists of two longitudinal columns at the principal piece. In the mid and end pieces of the tail, the FS is replaced by ODFs (Lehti and Sironen, 2017). The FS contains enzymes and channels essential for sperm physiology, such as sperm-specific lactate dehydrogenase (LDHC), which is responsible for glycolytic energy production; cation channels (CatSper); and the calcium-binding tyrosine phosphorylation-regulated protein (CABYR) (Kumar & Singh, 2021). All these enzymes and ion channels play an essential role in sperm motility, capacitation and fertilisation.

1.3.2. The seminal plasma

The SP is the acellular portion of semen, and is made up of a mixture of secretions from the testes, epididymis, vas deferens and accessory sex glands (Plant and Zeleznik, 2014). The SP is a medium intended to nourish and protect sperm from ejaculation to fertilisation, as well as to modulate the uterine environment for proper embryo implantation and development (Morgan and Watkins, 2020).

1.3.2.1. Molecular composition of seminal plasma

The molecular composition of SP is highly species-specific, although it consistently contains certain types of molecules, such as inorganic ions, energy substrates, amino acids, cholesterol, peptides and proteins, and DNA and RNA (Juyena and Stelletta, 2012; Rodriguez-Martinez et al., 2021). The major components of SP are peptides and proteins, and the protein fraction of SP has a similar composition to blood plasma, containing pre-albumin, albumin, globulins, transferrin, enzymes and immunoglobulins. In addition to these molecules, other SP proteins are exclusively found in this fluid, as explained below (Rodriguez-Martinez et al., 2021).

In mammalian SP, the vast majority of proteins can be classified into three main groups: (i) spermadhesins, (ii) fibronectin type II module-containing (FN-2) proteins, and (iii) cysteine-rich secretory proteins (CRISPs) (Rodríguez-Martínez et al., 2011). Spermadhesins are multifunctional secretory glycoproteins, initially described in farm animals, that bind to the extracellular surface of horse, pig, and cattle sperm (Töpfer-Petersen et al., 1998). They are known to bind to a wide range of ligands, including carbohydrates, sulphated glycosamino-glycans and phospholipids, thus evidencing their role in sperm membrane stability, capacitation, acrosome reaction and fertilisation (Töpfer-Petersen et al., 1998). In humans, nevertheless, despite lacking spermadhesins, spermadhesin-like proteins have been reported (Kraus et al., 2005). On the other hand, FN-2 proteins are a family of sperm-binding proteins that interact with phosphorylcholine, a polar head group of some membrane phospholipids (Desnoyers and Manjunath, 1992, 1993). The FN-2 proteins are present in the SP of most mammalian species, and are known to be involved in sperm maturation, capacitation and fertilisation (Manjunath et al., 2009). Finally, CRISP family members are predominantly expressed along the male reproductive tract of mammalian species and are involved in capacitation-associated events (protein tyrosine phosphorylation, motility hyperactivation and acrosome reaction) and gamete interaction (cumulus penetration, sperm ZP-binding and penetration and gamete fusion; Gonzalez et al., 2021). In addition to these three major protein families, proteomic analyses have revealed many other proteins and peptides present in the SP of humans and

farm animals (Pilch and Mann, 2006; Druart et al., 2013). Indeed, more than 2,000 SP proteins have been comprehensively described in humans (Batruch et al., 2011). For this reason, SP proteins have been suggested as biomarkers, since they have been detected to be differentially abundant in the SP of men with good and poor sperm quality (Batruch et al., 2011; Sharma et al., 2013).

1.3.2.2. The multifunctional role of seminal plasma

In addition to the nourishing and protective functions of SP with regard to sperm, recent studies evidenced the multifunctional role of this fluid in sperm physiology, the fertilising process and the female reproductive tract. Specifically, proteins and peptides are recognised to be directly involved in a wide range of essential processes, such as prevention of premature sperm capacitation, modulation of the uterine immune response, transport of sperm within the female genital tract and sperm-oocyte interaction, among others (Rodríguez-Martínez et al., 2011).

1.3.2.2.1. Role of seminal plasma proteins on sperm

During the last years, intensive work has been carried out to characterise the role of SP proteins on sperm functionality. CRISP proteins are known to modulate ion channels, inhibiting Ca^{2+} influx via regulation of ryanodine receptors and subsequent flagellar activity (Gibbs et al., 2006; Koppers et al., 2011). Related to the aforementioned function, some CRISP members have also been reported to act as decapacitation factors by preventing early-capacitation events (Roberts et al., 2003). Moreover, CRISP proteins appear to be involved in the modulation of sperm–oocyte binding (Koppers et al., 2011). Specifically, some sperm membrane-adhered CRISP proteins migrate to the equatorial region during sperm capacitation and acrosome reaction, and are established to participate in binding to the ZP and oolemma (da Ros et al., 2004).

As far as the role of FN-2 proteins on sperm physiology is concerned, they have been suggested to stabilise the plasma membrane of sperm during their journey along the male reproductive tract (Manjunath et al., 2007). Once in the oviduct, FN-2 proteins facilitate capacitation by interaction with high-density lipoproteins and glycosaminoglycans. Furthermore, FN-2 proteins are thought to

participate in the formation of the sperm reservoir by promoting sperm binding to the oviductal epithelium (Gwathmey et al., 2003).

Spermadhesins have a mechanism of action similar to FN-2 proteins, as both groups of molecules are able to bind a wide range of ligands. Spermadhesins can form a layer coating sperm by binding phospholipid matrices. This sperm-coating ability acts as a stabilising factor to prevent premature capacitation and acrosome reaction (Töpfer-Petersen et al., 1998; Vadnais and Roberts, 2007). In addition, similar to FN-2 proteins, spermadhesins have also been reported to be involved in the formation of the sperm reservoir in some mammalian species, by mediating sperm-oviduct binding (Wagner et al., 2002; Ekhlasi-Hundrieser et al., 2005). During capacitation, most of these spermadhesins are released from the sperm membrane and act as cholesterol acceptors (Dostàlovà et al., 1994; Jonáková et al., 2000). Some spermadhesins, such as AWN and AQN1, remain attached to the sperm plasma membrane after capacitation and participate in the recognition and binding to the ZP (Jonáková et al., 2000; van Gestel et al., 2007).

Apart from the main groups described above, many other proteins have been shown to play an essential role for sperm function. Particularly important are the enzymatic antioxidants present in the SP, as they protect sperm cells from oxidative damage. In fact, a wide range of antioxidant enzymes, such as superoxide dismutase (SOD; Papas et al., 2019; Peeker, 1997), catalase (CAT; Jeulin et al., 1989; Papas et al., 2019), glutathione peroxidase (GPx; Kantola et al., 1988; Papas et al., 2019), glutathione reductase (GSR; Papas et al., 2019), glutathione S-transferase (GST; Raijmakers et al., 2003), peroxiredoxins (PRx; Gong et al., 2012) and thioredoxin reductase (TrxR; Moradi et al., 2018), have been identified in mammalian SP. It is important to note that the total antioxidant capacity relies upon both enzymatic and non-enzymatic antioxidants. Several non-enzymatic antioxidants have been reported in mammalian SP, such as albumin, vitamin E, ferritin, transferrin and reduced glutathione (Fischer-Hammadeh et al., 2007; Micheli et al., 2016). Indeed, quantification of total antioxidant capacity in SP, both enzymatic and non-enzymatic, has been

established as a reliable and simple method for the diagnosis and management of male infertility (Mahfouz et al., 2009).

1.3.2.2.2. Role of seminal plasma proteins on the female reproductive tract

Regarding the modulating effect of SP upon the female reproductive tract, recent studies evidenced its role in facilitating uterine remodelling, embryo implantation and foetal development (Morgan and Watkins, 2020), thus improving clinical pregnancy in women exposed to this fluid (Crawford et al., 2015) and modulating the offspring phenotype in mammals (Crean et al., 2012; Kekäläinen et al., 2020). Related to this, recent animal studies evidenced that supplementation of seminal doses with SP prior to AI increases the ability of sperm to penetrate the cervical mucus, and the subsequent pregnancy rates (Okazaki et al., 2012; Morrell et al., 2014; Rickard et al., 2014). Moreover, the complex protein composition and relevant physiological role of this fluid makes it a promising source of biomarker candidates for sperm quality and male (in)fertility. In spite of all the aforementioned, the overall understanding of the mechanisms underlying the modulation of SP to the offspring is still limited. Even so, a specific cellular mechanism driving the regulation of the uterine environment by SP has been identified in several mammalian species such as swine, cattle, horse and humans. Specifically, the recruitment of polymorphonuclear cells (PMN) and the subsequent release of DNA to form neutrophil extracellular traps (NETs) occurs in response to semen (Jorch and Kubes, 2017; Mateo-Otero et al., 2022). This process leads to the entrapment and successive removal of both pathogens and excessive sperm (Scott et al., 2006). Furthermore, a remodelling of the uterine environment occurs to facilitate the implantation of conceptus (O'Leary et al., 2004). On the other hand, the regulation of the female reproductive tract by SP shows species-specific effects. Although the SP of pigs was reported not to affect NET formation (Wei et al., 2020), that of cattle and donkeys indeed induces NET formation (Fichtner et al., 2020; Mateo-Otero et al., 2022). These data highlight the importance of SP in the regulation of the female environment in a species-specific manner.

1.4. Male reproductive function in mammals: from the testis to the oocyte

The male reproductive function is crucial for fertilisation, as it produces a gamete that delivers the paternal genome to the oocyte, thereby initiating the development of a new individual. In eutherian mammals, the journey of sperm from the testis to the oocyte is a complex process that requires the coordinated efforts of multiple organs and systems in both male and female bodies.

1.4.1.Spermatogenesis and spermiogenesis: cellular basis

Spermatogenesis is the process by which sperm cells are produced, and occurs in waves within the seminiferous tubules of the testis (**Figure 5**). It starts during puberty and continues throughout the male's life. This process involves the transformation of undifferentiated diploid cells (spermatogonia) into motile, highly differentiated haploid cells (sperm), via meiotic cell division (Nishimura and L'Hernault, 2017).

Spermatogenesis begins with the division of undifferentiated germ cells, which are maintained in a metabolically quiescent state (Cheng & Sun, 2021). To ensure synchronous spermatogenesis, cytoplasmic bridges are established between germ cells to allow those located in the same cross-section of the seminiferous tubule to develop together (Ventelä, 2006). These germ cells give rise to spermatogonial stem cells (type A spermatogonia), which divide through repeated cycles of mitosis, maintaining the cell reserve. These cells are characterised by a large, oval nucleus with condensed chromatin. The production of type A spermatogonia marks the beginning of spermatogenesis. Type A spermatogonia (2n) undergo mitosis to produce type B spermatogonia (2n), which are committed to produce tetraploid primary spermatocytes (4n) via one mitotic division. During the next stage, primary spermatocytes undergo meiosis I to produce diploid secondary spermatocytes (2n), which later divide into round spermatids (n) through meiosis II. During the process of cell division and differentiation that occurs from type A spermatogonia to spermatids, these cells migrate from the basement membrane to the luminal side of the seminiferous tubules (Nishimura and L'Hernault, 2017). Spermiogenesis is the last stage, during which round spermatids (n) are radically transformed into elongated

spermatids and then spermatozoa (n). It involves the formation of the acrosomal vesicle, rotation of the nucleus, formation of the flagellum, replacement of histones by protamines in the nucleus, as well as the condensation and flattening of the nucleus (Dadoune, 1994). Finally, the resulting sperm cells are released into the lumen of the seminiferous tubules through a process called spermiation (O'Donnell et al., 2011).

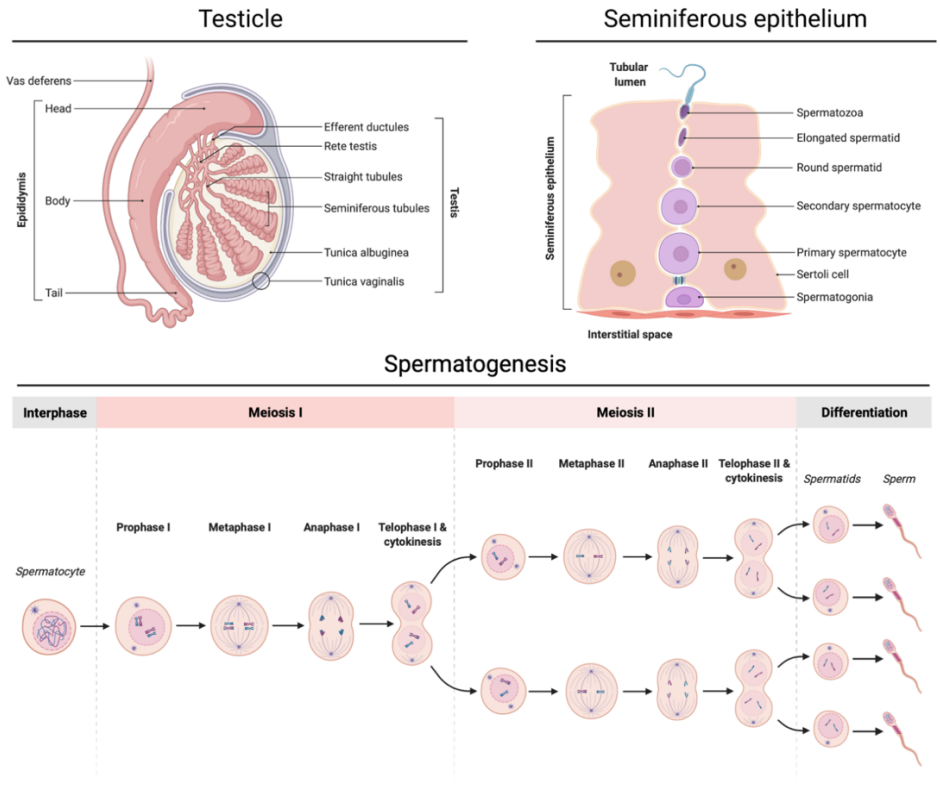

Figure 5. Schematic representation of spermatogenesis*. Anatomy of the human male testis, epididymis, and ductus deferens (upper left corner), the histological structure of the seminiferous epithelium, with the different cell types of spermatogenesis, (upper right corner), and the meiosis stages occurring during spermatogenesis (down) are schematically represented. Figure created with BioRender.*

During spermatogenesis, developing germ sperm cells are supported by Sertoli cells (SCs). These cells are located in the seminiferous tubules of the testes and extend from the basement membrane to the lumen (Oliveira and Alves, 2015a). SCs are in close contact with developing spermatogenic cells and

provide them with structural and metabolic support. Accordingly, SCs are commonly called as the “nurse cells” of the testis, since they supply an adequate environment for germ cell development during spermatogenesis, from the maintenance of spermatogonial stem cells to elongated spermatids (O’Donnell et al., 2022). The blood-testis barrier (BTB) formed by SCs controls which substances enter the seminiferous epithelium (Mruk and Cheng, 2015). Moreover, and in order to accomplish their nutritional role in support of germ cells through the BTB, SCs exhibit high metabolic plasticity, thus being able to degrade a variety of substrates through a wide range of catabolic pathways (Oliveira and Alves, 2015b). The majority of these catabolic pathways converge on lactate production, which is an essential substrate for germ cell metabolism and survival (Jutte et al., 1982; Erkkila, 2002; Boussouar and Benahmed, 2004).

1.4.2.Epididymal maturation

Upon leaving the testis, sperm cells have not yet acquired the capability to move and fertilise the oocyte. In order to become fertilising competent, they must undergo a maturation process in the epididymis. Epididymal maturation can be described as the changes that occur to sperm cells during their transit through the epididymis, which give them the ability to elicit capacitation in the female reproductive tract. This process involves a series of biochemical and physiological changes that require the incorporation of new molecules from the epididymal epithelium, as well as post-translational modifications of endogenous proteins synthesised during spermatogenesis (Gervasi and Visconti, 2017).

The epididymis is a highly convoluted duct of the male reproductive system that is involved in the maturation and storage of sperm (Cornwall, 2008). Its function has been studied mainly in rodents, which present anatomical and physiological differences with humans (Sullivan and Mieusset, 2016). In the latter, however, transcriptomic and proteomic studies showed that gene expression varies along the human epididymis, with the caput segment having the highest activity, which supports that the epididymis does play a role in sperm maturation (Sullivan and Mieusset, 2016; Björkgren and Sipilä, 2019). Moreover, the role of extracellular microvesicles, known as epididymosomes, in transferring proteins

and small RNAs to maturing sperm and regulating gene expression in the epididymis were also studied (Sullivan, 2015; Barrachina et al., 2022). Epididymosomes appear to play a role in post-testicular sperm maturation, embryonic development, offspring health and epigenetic changes (Paul et al., 2021).

One of the most thoroughly studied functional changes that occurs to sperm during epididymal maturation is the acquisition of progressive motility, which is a prerequisite for the ability to undergo hyperactivation during capacitation (Yeung and Cooper, 2002). Other potential modifications occurring to sperm during their epididymal transit include the ability to increase tyrosine phosphorylation of certain proteins, which is related to the capacitation process and is involved in the acrosome reaction, sperm binding to ZP, fusion with the oolemma and oocyte fertilisation (Gervasi and Visconti, 2017). Epididymal maturation can thus be defined as all the biochemical and physiological changes that sperm experience during their transit through the epididymis, which later allow them to undergo capacitation within the female reproductive tract.

1.4.3.Sperm capacitation

Before stepping into the sperm capacitation process, it is worth looking back into the genesis and definition of this term. The very first origin of the term "capacitation" lies in the studies reported in 1951 by two independent scientists: Colin Russell Austin[4] in Australia (Austin, 1951) and Min Chueh Chang[5] in the United States (Chang, 1951). They noticed that, in rats and rabbits, "[…] *sperms introduced into the Fallopian tubes shortly after ovulation seldom penetrated the eggs; but if sperms were introduced a few hours before ovulation, the majority of the eggs were later observed to be fertilized.*" (Austin, 1952). In accordance with these findings, the investigations of the two authors simultaneously and

[4] Colin Russell Austin (1914-2004) was an English-Australian scientist devoted to the study of the fertilisation process and early embryonic development in mammals, considered the founder of the modern study of embryology (Short, 2014).
[5] Min Chueh Chang (1908-1991) was a Chinese-American reproductive biologist known for his work on mammalian fertilisation and the development of the oral contraceptive pill (Caspi, 1991).

independently led to the same conclusion; "[…] *the sperm must undergo some form of physiological change or capacitation before it is capable of penetrating the egg.*" (Austin, 1952). Years later, this definition was extended to include that sperm must reside in the female genital tract in order to become capacitated, as observed by Austin and Bishop in 1958 (Austin and Bishop, 1958). The major advances in technology and the up-to-date knowledge in reproductive biology significantly updated the definition of sperm capacitation.

Sperm capacitation occurs within the female reproductive tract after ejaculation. At the cellular level, capacitation causes changes in sperm motility, known as hyperactivated movement, and prepares sperm to trigger the acrosome reaction, an exocytotic process. At the molecular level, capacitation is characterised by cholesterol loss from the sperm plasma membrane, increased membrane fluidity, changes in intracellular ion concentrations, hyperpolarisation of the sperm plasma membrane, increased activity of cAMP-Dependent Protein Kinase A (PKA), and protein tyrosine phosphorylation (Stival et al., 2016; Molina et al., 2018).

During mammalian sperm capacitation, two distinct signalling events can be differentiated: the fast and the slow events (Visconti, 2009; Ickowicz et al., 2012). The fast events of capacitation involve the activation of the flagella, which causes vigorous and asymmetric movement of the sperm tail. These events occur as soon as the sperm leave the epididymis and enter the female reproductive tract and are dependent on the activation of PKA. On the other hand, the slow events of capacitation involve changes in the movement pattern of the sperm tail, known as hyperactivation. The beginning of the slow events is marked by the removal of cholesterol from the membrane, which increases its fluidity. Additionally, protein tyrosine phosphorylation is another hallmark occurring during the late stages of capacitation on a different timescale from the fast events.

Although the interconnections between these events and their roles in regulating sperm motility and preparation for the acrosome reaction are not yet fully understood, it is known that the PKA pathway plays a key role in coordinating the majority of events related to capacitation (Stival et al., 2016). A simplified

model of signalling pathways and cellular changes involved in mammalian sperm capacitation is represented in **Figure 6**.

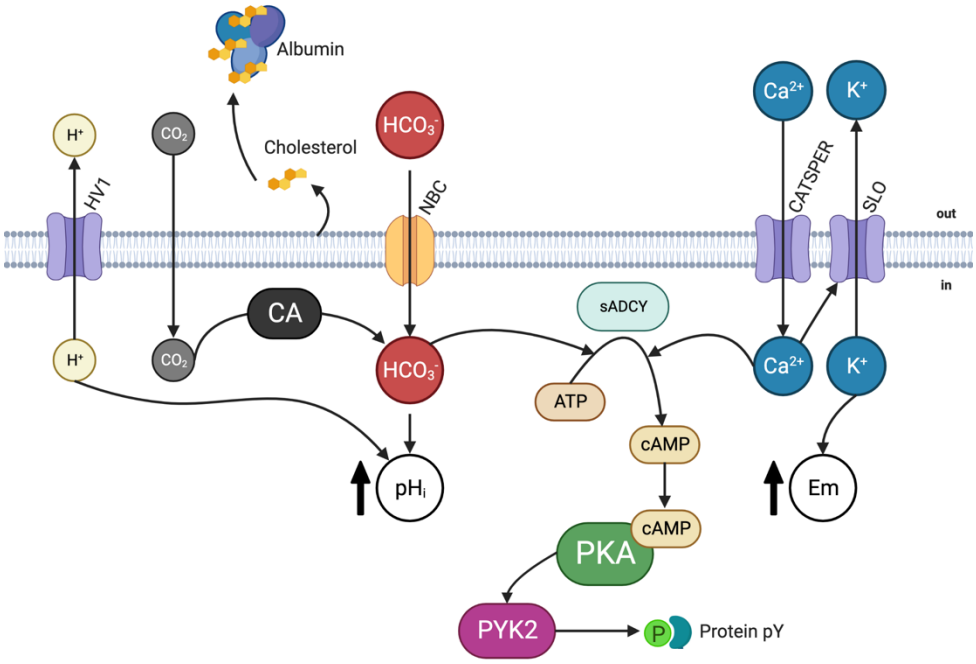

Figure 6. Molecular mechanisms regulating sperm capacitation*. During capacitation, removal of cholesterol from the sperm plasma membrane is induced by albumin, increasing its fluidity. Moreover, the influx of bicarbonate (HCO_3^-) by sodium bicarbonate cotransporter (NBC), the conversion of carbon dioxide (CO_2) to HCO_3^- by carbonic anhydrase (CA) and the efflux of protons (H^+) through the voltage-gated H^+ channel (HV1), contributes to the increase of intracellular pH (pH_i). On the other hand, the influx of calcium ions (Ca^{2+}) via the cation channel of sperm (CATSPER) and the efflux of potassium ions (K^+) through the sperm K^+ channel (SLO), induces the hyperpolarisation (Em) of plasma membrane, and activates enzymes such as the soluble adenylyl cyclase (sADCY), which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Ultimately, all these events contribute to the activation of the cAMP-Dependent Protein Kinase A (PKA), which promotes the tyrosine phosphorylation (protein pY) of certain sperm proteins. The figure was adapted and modified from Molina et al. (2018). Figure created with BioRender.*

One of the most significant changes that occurs during sperm capacitation is the loss of cholesterol from the sperm plasma membrane, which leads to an increase in membrane fluidity. In addition to changes in cholesterol and membrane fluidity, capacitation is also associated with alterations in intracellular ion concentrations, such as H^+, Ca^{2+} and K^+, and a particularly high

increase in cyclic adenosine monophosphate (cAMP), by the soluble adenylyl cyclase (sADCY). This increase in cAMP activates PKA, which plays a central role in coordinating the majority of events related to capacitation. An increase in the permeability of the membrane to K^+ leads to a decrease in membrane potential (hyperpolarisation). Finally, capacitation is also accompanied by protein tyrosine phosphorylation, which involves the addition of a phosphate group to specific tyrosine residues of certain proteins. This modification can alter the activity or function of the protein and may play a role in preparing sperm for fertilisation. Overall, these molecular changes that take place during capacitation are essential for sperm to acquire the ability to hyperactivate and trigger the acrosome reaction, which are critical steps for successful fertilisation (Stival et al., 2016; Molina et al., 2018). In mammals, hyperactivation is a particular motility pattern of sperm entailing an increase in flagellar bend amplitude and beat asymmetry, that allows their penetration through the ZP (Suarez and Ho, 2003; Suarez, 2008). The acrosome reaction, on the other hand, is an exocytotic process that releases the enzymatic content of the acrosome, helping sperm to pass through oocyte vestments and penetrate the ZP (Hirohashi and Yanagimachi, 2018). This process is thought to be activated by a rise in intracellular pH (pH_i) during sperm capacitation and is triggered by the presence of progesterone (Aldana et al., 2021).

1.4.4.Fertilisation

Fertilisation is a highly specialised process that requires molecular recognition events between the sperm plasma membrane, the ZP, and the oocyte plasma membrane (Wassarman, 1999). The ZP, in particular, plays a crucial role in species-specific sperm-oocyte recognition due to the carbohydrate moieties of its glycoproteins (Clark, 2014). The binding of capacitated sperm to these ZP glycoproteins is a crucial step in fertilisation, as defective sperm-oocyte binding is the most common cause of failure in clinical IVF (Liu and Baker, 2000). The characterisation of oocyte receptors on the sperm plasma membrane is, therefore, essential for practical applications of ART in both animals and humans.

According to Primakoff & Myles (2002), fertilisation in mammals can be divided into three main stages (**Figure 7**). (i) The first step consists of the penetration of capacitated sperm through the corona radiata via the use of surface hyaluronidases, hyperactivated motility and the acrosome reaction. It is noteworthy that cumulus cells release progesterone, promoted through the release of glycosylphosphatidylinositol (GPI)-anchored membrane proteins from sperm (Roldan et al., 1994). Cumulus cells-released progesterone can trigger the acrosome reaction of sperm, thus facilitating their penetration through the corona radiata (Hirohashi, 2016). Subsequently, (ii) intact sperm interact with the extracellular matrix surrounding the oocyte, known as the ZP. Although the vast majority of sperm have their acrosomes lost before coming into contact with the ZP, the biding of acrosome-intact sperm to ZP glycoproteins (ZP1-4), can also trigger their acrosome reaction (Hirohashi, 2016). Acrosome-contained hydrolytic enzymes also account for the penetration of sperm through the ZP. Finally, (iii) acrosome-reacted sperm adhere to the oolemma, leading to the fusion of the inner acrosomal membrane with the oolemma. This membrane fusion is known to be mediated by the interaction of the sperm protein IZUMO1 with its oocyte receptor JUNO (Bianchi et al., 2014), among other interactors.

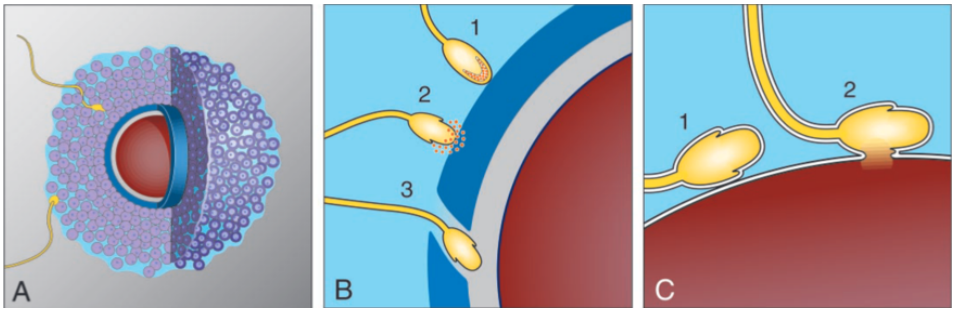

Figure 7. The fertilisation mechanism*. (**A**) Sperm penetration of the corona radiata (purple) to reach the zona pellucida (ZP; navy blue). (**B**) The oocyte is depicted with cumulus cells removed; (1) the spermatozoon binds to the ZP (navy blue); (2) the spermatozoon triggers the acrosome reaction (AR), releasing acrosomal contents (red); (3) the spermatozoon penetrates the ZP and enters the perivitelline space (grey). (**C**) Spermatozoon 1 binds to the oolemma by the inner acrosomal membrane; spermatozoon 2 fuses with the oocyte plasma membrane. Figure was extracted from Primakoff & Myles (2002).*

Upon the fusion of sperm and oocyte membranes, oocyte activation is initiated. Specifically, PLCζ, a SOAF residing in the sperm PT, triggers that process. PLCζ induces the release of Ca^{2+} from the endoplasmic reticulum of the oocyte, which activates a PKC-mediated pathway. The series of Ca^{2+} oscillations elicited by sperm alleviate the oocyte from meiosis II arrest, induce the exocytosis of cortical granules and eventually drive the onset of embryogenesis (Yeste et al., 2017).

1.5. Sperm preservation in humans and livestock

Storage of sperm, either for long or short term, is a common strategy in assisted reproduction in both humans and farm animals. The use of an appropriate preservation method allows sperm to maintain their quality and fertilising ability for a long period of time. The preferred sperm preservation method (i.e., cryopreservation or liquid storage) depends on the objective and species. Sperm preservation strategies have thus to envisage the species-specific differences and the influence of various factors, such as temperature, duration of storage and the medium employed.

1.5.1. Cryopreservation

The first documented attempts at cryopreserving mammalian sperm date back to the 18th century. In 1776, Lazzaro Spallanzani [6] used a microscope to study the *animalcules* present in the semen of various animals and men. He also examined the reaction of these *animalcules* when exposed to different temperatures, and observed that some were able to survive and continue swimming even after being threatened by low temperatures. This investigation into the effects of temperature on biological processes is often cited as one of the earliest examples of cryobiology, as Spallanzani was the first to systematically study the survival of sperm at ice-forming temperatures (Sztein et al., 2018). It was not until the 20th century that the use of glycerol as a cryoprotective agent allowed the successful

[6] Lazzaro Spallanzani (1729–1799) was an Italian scientist and catholic priest. He might be considered the founder of modern biology, by paving the way for the downfall of the leading theory of spontaneous generation. Spallanzani made relevant contributions to the study of animal physiology and reproduction (Ariatti and Mandrioli, 1993; Capanna, 1999).

cryopreservation of sperm from various species, including horses, cattle, and pigs (Yeste, 2016; Sztein et al., 2018). Nowadays, cryopreservation of gametes and reproductive tissues is a widely utilised practice in both humans and livestock for fertility preservation (Loren et al., 2013). Although this preservation strategy can cause cellular damage, the extent of the injuries varies greatly across species and is highly dependent on the sperm's resilience to freezing and thawing (Mazur et al., 2008; Kopeika et al., 2015).

1.5.1.1. Cryoprotective agents in sperm cryopreservation

Cryoprotective agents (CPAs) are substances used to protect cells and tissues from damage during the process of cryopreservation. They work by decreasing the rate at which ice crystals form, reducing the concentration of solutes, and stabilising the cell membrane. This helps reduce the severity of cryoinjuries. These agents are typically added to the cell suspension before cryopreservation, and the optimal concentration of CPA is reliant upon species (Sieme et al., 2016). These agents can be further classified as either nonpermeating or permeating, based on their ability to pass through the plasma membrane. Nonpermeating CPAs are substances that do not cross the cell membrane, but rather work extracellularly to prevent ice formation and stabilise proteins and cell membranes (Benson et al., 2012). While these solutes are not able to fully protect the cell on their own, they can increase the effectiveness of permeating CPAs, allowing a decrease in their concentration (Fahy, 1986). Nonpermeating CPAs commonly used in freezing extenders include sugars, particularly disaccharides such as lactose or trehalose, and egg yolk proteins (Yeste, 2016). When combined with a surfactant (i.e., Equex), egg yolk proteins show better protection because this chemical facilitates their interaction with the plasma membrane (Holt, 2000; Rodriguez-Martinez and Wallgren, 2011). On the other hand, permeating CPAs are substances that can pass through the plasma membrane and are relatively nontoxic (Yeste, 2016). These agents include glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, methanol, and propylene glycol. Permeating CPAs work by decreasing the concentration of intracellular electrolytes, which reduces osmotic shrinkage at low temperatures (Mazur, 1984; Gao and Critser, 2000). Yet,

they can also cause damage to the cell and induce osmotic volume changes at temperatures above 5°C. It is thus important that they are able to permeate the cell before freezing and be quickly removed upon thawing (Gao et al., 1995). For mammalian sperm cryopreservation, glycerol is currently the most effective permeating CPA (Yeste, 2015).

1.5.1.2. Sperm cryodamage

Despite the usage of CPAs, freezing and thawing are known to harm sperm because of the phase change of water in both intracellular and extracellular compartments (i.e., cryoinjuries; Gao & Critser, 2000). Cryoinjury is defined as the cell damage caused by the process of cryopreservation. This damage can be provoked by both high and low cooling rates, and the thawing process. High cooling rates can result in the formation of intracellular ice crystals, whereas low cooling rates can lead to cell dehydration and denaturation of macromolecules (Gao and Critser, 2000). Similarly, both slow and fast thawing rates can have negative effects, with slow rates heading to recrystallisation and fast rates producing osmotic stress (Mazur, 1990; Muldrew and McGann, 1994). Adding CPAs to freezing and thawing media, notwithstanding, reduces the extent of cryodamage (Gao and Critser, 2000).

Cryoinjuries have been widely described throughout the past decades, being found to exert a detrimental impact upon sperm physiology, such as a decrease in motility; induce changes in the composition and biophysical properties of the plasma membrane; cause alterations in the levels, localisation, function and tyrosine-phosphorylation of sperm proteins; impair mitochondrial function; and increase reactive oxygen species (ROS) levels, among many others. This wide range of harmful effects impair sperm function and survival, underlying a significant decrease in its reproductive performance after thawing (Yeste, 2016). It is known that the plasma membrane of mammalian sperm is highly sensitive to temperature changes, due to its abundance of unsaturated phospholipids and low amount of cholesterol molecules (Casas and Flores, 2013). Accordingly, temperatures less than or equal to 5ºC lead to the destabilisation of the sperm plasma membrane. As a result, some proteins are translocated and/or lose their

function during cryopreservation, thereby being a potential cause of subfertility in frozen-thawed sperm (Yeste, 2016). Additionally, these membrane changes may lead to capacitation-like changes (i.e., cryocapacitation), causing similar, but not completely analogous, events to those occurring during sperm capacitation; these changes, therefore, do not entail a true capacitation (Green and Watson, 2001). Furthermore, the sperm susceptibility to cryoinjuries is understood to be species-specific. In this regard, sperm of boars, rams, bulls and stallions are particularly sensitive to cryodamage, whereas those of men, rabbits and dogs appear to be less susceptible (Grötter et al., 2019).

On the other hand, the sperm resilience to cryopreservation procedures (i.e., freezability) shows a large variability between ejaculates, which supports their classification as "good" or "bad/poor" freezers (GFE and PFE, respectively) (Yeste, 2016). Post-thaw sperm quality and fertilising ability are higher in GFE than in PFE (Casas et al., 2009), probably because of variations in the content of proteins involved in the cell resistance against cryodamage. Although the mechanisms underlying the differences between GFE and PFE still remain unknown, it is of great interest predicting the freezability of ejaculates before undertaking the cryopreservation process.

1.5.2. Liquid preservation

As explained above, freezing and thawing can cause cryodamage to sperm cells, thus decreasing sperm quality and fertility. It is known, however, that the extent of cryoinjuries on sperm cells is species-specific. In order to mitigate the negative effects of cryopreservation upon sperm physiology, an alternative method consisting of the liquid preservation of sperm at temperatures ranging between 4°C and 25°C has been investigated over the last decades. While cryopreservation allows for the storage of sperm for an indefinite period, liquid preservation is able to maintain their viability for several days. This is achieved by decreasing the sperm metabolic rate, thus prolonging their lifespan. During liquid storage, nonetheless, the metabolic activity of sperm cells is not completely arrested, leading to the depletion of available nutrients and the accumulation of

metabolic by-products (Maxwell and Salamon, 1993; Waberski et al., 2011; Ribas-Maynou et al., 2021).

The first attempt to preserve sperm in liquid state can be traced back to the 1930s. The researchers diluted bovine semen with an egg yolk-phosphate extender at a temperature of 10°C, and reported that these cells maintained their motility for six days and even gave rise to post-storage successful pregnancies (Phillips, 1939). Over the following decades, the formulation of the extenders used for semen liquid storage was improved and adapted to different species. Nowadays, the majority of AIs in porcine and small ruminants is performed using liquid-stored semen preserved at temperatures ranging between 17-25°C (Waberski et al., 2019; Henning et al., 2022) and 4-10°C (Maxwell and Salamon, 1993; Falchi et al., 2018), respectively.

1.5.2.1. Extenders for semen liquid preservation

Liquid preservation consists of diluting semen in specific media and subsequent cooling. To mitigate the negative effects of semen storage, an optimal extender must maintain an appropriate pH and have a buffering capacity to protect sperm from osmotic and cooling stress as well as prevent the generation or scavenge of excessive ROS. Several compounds, including plant extracts, antioxidant enzymes, vitamins, seminal plasma fractions, sugars, fatty acids, and caffeine, among many others, have been found to improve sperm quality when added to semen extenders (Allai et al., 2018; Ribas-Maynou et al., 2021; Silvestre et al., 2021; Cheng et al., 2022). Currently, there are a large number of semen extenders available, but there is a significant variability on the period they are able to maintain sperm function and survival. Hence, it is essential to identify suitable extenders for liquid preservation of sperm that can maintain their fertilising potential for long time. Most inseminations using liquid-stored semen are carried out within 72 hours of storage (Bustani and Baiee, 2021). In this regard, most semen extenders were designed to maximise sperm quality and fertilising ability during the first three days of liquid storage. Yet, liquid storage of semen for longer periods (e.g., seven days after collection or even further) with optimal fertilisation rates can provide greater efficiencies. For this reason, novel semen extenders for

long- (4-7 days) and ultra-long- (>7 days) term storage were developed in pigs (Dziekońska et al., 2013). Long- and ultra-long-term preservation of semen in liquid state, nevertheless, requires special attention to microbial growth, especially at storage temperatures ranging between 17 and 25ºC. Particularly, the antimicrobial content of extenders needs to be considered (Hernández-Avilés et al., 2019; Pohjanvirta et al., 2020; Tvrdá et al., 2021).

1.5.2.2. Sperm physiology during liquid preservation

Liquid preservation is known to have multiple effects on sperm, most of which are not detectable when using conventional semen evaluation. Liquid storage has in effect been reported to exert a detrimental impact on sperm motility, plasma membrane integrity, lipid peroxidation and ROS generation (Waberski et al., 2011; Falchi et al., 2018), which is known to have a negative repercussion on fertilising ability. Special attention needs to be paid to unbalanced levels of ROS, which can cause significant oxidative stress (OS) to sperm during liquid preservation (Khoi et al., 2021). Mature sperm have limited capacity of balancing ROS due to their low concentration of scavenging enzymes in their residual cytoplasm (Cerolini et al., 2000; Lenzi et al., 2000; Gavella and Lipovac, 2013; Aitken et al., 2016). The SP possesses antioxidant properties, which can scavenge ROS and protect sperm against OS (Lewis et al., 1995; Barranco et al., 2015b). Diluting semen with extenders/preservation media, however, reduces the protective capacity of SP. Consequently, the quality of liquid-preserved semen is deteriorated over time, and fertilisation rates are known to decrease as storing periods increase (Waberski et al., 2011). It can thus be said that liquid storage causes a decline in sperm quality and fertilising ability due to an alteration in the balance between antioxidants and pro-oxidants, thereby leading to an overproduction of ROS and, thus, to OS and lipid peroxidation. Additionally, the high amount of polyunsaturated fatty acids (PUFA) in the sperm plasma membrane of some mammalian species, such as porcine and small ruminants (Gautier and Aurich, 2022), make them particularly susceptible to lipid peroxidation, in addition to cold shock.

Despite the advances made in preservation methods, the use of liquid-stored semen to overcome the challenges of distance and time has yet to meet some expectations. Cell-physiological differences between species and ejaculates, in terms of their ability to withstand preservation, are still challenging. In this regard, an evaluation of preservation-induced damage in sperm is of great interest to identify differences in their individual storage capacity, to assess the effects of different semen liquid preservation strategies, and to predict their fertilising ability.

1.6. Redox homeostasis: a double-edged sword

The physiological mechanisms of sperm are complex and not fully understood, with multiple factors contributing to their regulation (Thompson et al., 2013). As by-products of oxygen metabolism, ROS are highly reactive oxygen derivatives that can be toxic at levels beyond normal physiological concentrations (Ayaz et al., 2018; Bui et al., 2018). At physiological concentrations, ROS have been shown to mediate essential cellular functions (Thompson et al., 2013), not only facilitating intracellular signalling cascades necessary for proper sperm functions, including maturation, hyperactivation, capacitation and acrosome reaction, but also playing a crucial role in the fertilisation process (Agarwal et al., 2012; Thompson et al., 2013). In spite of this, overproduction of ROS has been identified as a potentially disruptor of sperm function, which can lead to OS when the generation of oxidative agents exceeds the total antioxidant capacity (Halliwell and Cross, 1994). The balance between oxidants and antioxidants within a cell is known as redox homeostasis, and is crucial for maintaining cellular function. An unbalance that turns into OS potentially contributes to the development of pathological conditions.

1.6.1.Reactive oxygen species (ROS)

Atoms or molecules that are missing one electron or have only one free electron in its outer shell are called radicals. Diatomic oxygen (O_2) is a diradical with two unpaired electrons in its outer shell, which confers chemical reactivity. Oxygen is required for cellular respiration and is essential for cell survival. When oxygen is reduced, it can generate the superoxide anion ($\bullet O_2^-$), which is a radical that can

interfere with various cellular functions. If the $\bullet O_2^-$ gains an electron, it becomes peroxide (O_2^{2-}), which is not a free radical (Ford, 2004). The $\bullet O_2^-$ dismutation can produce hydrogen peroxide (H_2O_2), an endogenous and non-charged oxidant, which is abundant and relatively less reactive (Dutta et al., 2020). In spite of this less reactivity, the uncharged nature of H_2O_2 makes it able to pass through the plasma membrane. $\bullet O_2^-$ and H_2O_2 can also undergo a chain of transformations through Fenton and Haber-Weiss reactions to form the highly reactive hydroxyl radical (OH•). ROS include all free radicals with an oxygen atom and, in some cases, H_2O_2, which is considered a reactive oxygen derivative but not a radical. As oxidants, these molecules capture electrons from adjacent cellular structures, leading to the disruption of cellular components (Thompson et al., 2013; Dutta et al., 2020).

1.6.2. Source of ROS in seminal plasma and sperm

Semen is known to contain several endogenous sources of ROS. A major source of ROS in semen are peroxidase-positive leukocytes, such as polymorphonuclear leukocytes and macrophages. In response to urogenital infections or inflammation, these cells are able to generate 100 times more ROS than basal conditions. This results in a rise in pro-inflammatory mediators and decreased antioxidant capacity, which may contribute to OS through the mechanism of respiratory burst (Ford et al., 1997). Dysfunctional sperm, especially those with cytoplasmic droplet retention and head morphology abnormalities, are recognised to be a major source of ROS in semen (Agarwal et al., 2014). Normal sperm are also able to generate ROS through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase residing in their plasma membrane, as well as the NADH-dependent oxidoreductase present in their mitochondria, with the Krebs cycle being a major participant in generating ATP and leading to ROS production (Gavella and Lipovac, 1992; du Plessis et al., 2015; Aitken, 2017). Finally, the presence of pathological reproductive conditions, such as varicocele, are thought to cause OS-induced sperm dysfunction. Indeed, a higher incidence of OS in semen samples from varicocele-affected patients compared to healthy donors has been reported (Agarwal et al., 2006; Cho et al., 2016). Although

endogenous sources of ROS are understood to induce oxidative damage in sperm, there is a range of exogenous sources of ROS that can alter redox balance in SP and sperm. Certain types of electromagnetic radiation have been suggested to induce ROS generation, leading to the impairment of sperm function (Agarwal et al., 2008; Kesari et al., 2018). On the other hand, lifestyle factors, such as smoking and alcohol drinking, can induce oxidative damage in sperm, compromising their fertilising ability (Dutta et al., 2020). Finally, exposure to certain toxins, such as endocrine disruptors or toxic metals, may lead to OS-induced sperm damage (Sengupta, 2013; Wang and Su, 2018).

1.6.3. Physiological role of ROS in sperm

Generation of ROS is a common feature of cells, including mammalian sperm. Sperm are mitochondria-containing cells (Henkel, 2011), which are a major source of ROS due to electron leakage, triggered by various factors that disrupt the electron transport chain and the NADH-dependent oxidoreductases. It is acknowledged, however, that physiological levels of ROS mediate essential sperm functions.

The formation of disulphide bonds in cysteine residues of sperm protamines is mediated by ROS to ensure chromatin stability and prevent DNA damage (Dutta et al., 2020). Moreover, ROS also play a role in the mitochondrial capsule formation, hindering proteolytic degradation of mitochondria (Fujii and Tsunoda, 2011; Thompson et al., 2013). Furthermore, epididymal maturation of sperm is mediated by cell signalling pathways modulated by ROS levels in the so-called fluid (Griveau and le Lannou, 1997; du Plessis et al., 2015). Indeed, ROS regulate the redox status of cysteine residues and the activation of sADCY and subsequent increase of cAMP levels (Thompson et al., 2013). Accordingly, ROS levels in sperm play a key function in the hyperactivation of their motility (Dutta et al., 2020). In this regard, ROS levels in sperm are especially important during sperm capacitation, by promoting the phosphorylation of tyrosine residues (Donà et al., 2011), increasing intracellular cAMP levels (Thompson et al., 2013) and activating PKA (Aitken et al., 2015; Aitken, 2017). Moreover, ROS exert a crucial task during the acrosome reaction by inducing phosphorylation of the Protein

kinase C (PKC) and activating acrosomal enzymes, leading to sperm-zona penetration (Thompson et al., 2013; du Plessis et al., 2015).

In short, ROS at physiological levels are recognised to be essential for sperm function, mediating intracellular signalling pathways and regulating key processes of sperm such as chromatin condensation, capacitation, hyperactivation and acrosome reaction. Excessive ROS generation, however, can disrupt redox homeostasis in sperm, exerting detrimental effects on their function. In this regard, the balance between ROS generation and the antioxidant sperm capacity is of outmost importance and highlights the essential mission of antioxidant enzymes in the male gamete.

1.7. Glutathione S-transferases (GSTs) and male (in)fertility

GSTs are a group of cytosolic and membrane-bound antioxidant isoenzymes. These ubiquitous enzymes are known to protect mammalian cells against OS by the catalysis of glutathione-dependent reactions intended to remove electrophilic substances (Hayes et al., 2005).

1.7.1.GSTs classifications, genetics, and protein structure

Since the discovery of the GST activity by Combes & Stakelum (1961), several antioxidant enzymes catalysing glutathione-dependent reactions have been identified and classified into three superfamilies: (i) canonical soluble GSTs, ubiquitously present in mammalian cells; (ii) distantly related soluble kappa class GSTs, mainly located in mitochondria and peroxisomes; and (iii) hydrophobic GSTs (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism; MAPEG), found in microsomal fractions (Deponte, 2013). The major representative superfamily in mammalian sperm is that of canonical soluble GSTs. Each GST isoform included within the canonical soluble GST superfamily is classified based on their variable C-terminal α-helical domain. This classification leads to the formation of seven classes: (i) alpha (GSTA), (ii) mu (GSTM), (iii) omega (GSTO), (iv) pi (GSTP), (v) sigma (GSTS), (vi) theta (GSTT) and (vii) zeta (GSTZ) (Morel et al., 2004; Mannervik et al., 2005). The classification of canonical soluble GTSs classes, and all their GST members, are represented in **Table 2**.

***Table 2**. Classification of canonical soluble GSTs. Nucleotide and amino acid lengths correspond to the canonical isoform. Data were extracted from (Mannervik et al., 2005; O'Leary et al., 2016; Bateman et al., 2017) and the Table was adapted from (Llavanera et al., 2020).*

Class	Member	Chromosome	Confirmed number of isoforms	Nucleotide length (bp)	Amino acid length (aa)
Alpha	GSTA1	6p12	2	1,218	222
	GSTA2	6p12	1	1,221	222
	GSTA3	6p12	2	908	222
	GSTA4	6p12	1	1,240	222
	GSTA5	6p12	1	845	222
Mu	GSTM1	1p13	2	1,155	218
	GSTM2	1p13	2	1,166	218
	GSTM3	1p13	1	4,144	225
	GSTM4	1p13	2	1,372	218
	GSTM5	1p13	1	1,561	218
Omega	GSTO1	10q24.3	3	813	241
	GSTO2	10q24.3	4	6,715	243
Pi	GSTP1	11q13	1	986	210
Sigma	GSTS1	4q22.3	1	1615	199
Theta	GSTT1	22q11.2	9	1,109	240
	GSTT2	22q11.2	1	1,231	244
Zeta	GSTZ1	14q24.3	4	1,186	216

At the genetic level, GSTs have a particular organisation. While the members of every GSTs class are encoded by individual genes, these are grouped into genetic clusters. This class-clustered genetic organisation of GSTs reveals their importance during evolutionary history (di Pietro et al., 2010). In this regard, GSTs are known to have homologs in both prokaryotes and eukaryotes, establishing their evolutionary origin more than 2.5 billion years ago (Pearson, 2005). Indeed, this ancient protein superfamily shows a wide taxonomic distribution that evidences its essential role along the evolution (Sheehan et al., 2001). Their particular genetic organisation also addresses a major characteristic

of GSTs: their tissue-specific expression pattern (Hayes and Pulford, 1995). It is worth mentioning that, despite GSTA2, GSTP1 and GSTMs having been found in human testis, GSTM3 is the most abundant isoform in this tissue. In effect, the testis has been reported to be, by far, the tissue with the highest levels of GSTM3 (Listowsky et al., 1998).

At the protein level, GST members have a molecular mass of 23-30 kDa, composed of 199-244 amino acids (O'Leary et al., 2016; Bateman et al., 2017). In the cell, GSTs are active as dimers, either homodimers of the same isoform or heterodimers of members of the same class (Mannervik and Jensson, 1982; Mannervik et al., 1988; di Pietro et al., 2010). The protein structure of each GST subunit consists of an N-terminal domain linked to a C-terminal domain by an α-helix (Sheehan et al., 2001). The C-terminal domain is known to be extremely variable, whereas the N-terminal domain is conserved among isoforms. The glutathione-binding site of GSTs (G site) is found within the N-terminal domain, thus being highly conserved across classes. The C-terminal domain, however, contains the modular binding site for electrophilic substances (H site), thus conferring a large heterogeneity to this domain (Sheehan et al., 2001; Deponte, 2013).

1.7.2.Molecular function of GSTs

Although one might assume that a phylogenetically ubiquitous protein is functionally conserved, GSTs are admitted to have an exceptional functional plasticity (Pearson, 2005). Whilst the principal molecular function of GSTs is known to be the cell protection against OS by glutathione-dependent reactions (Hayes et al., 2005), these enzymes have been reported to exert additional and essential roles in cell physiology.

The main mission of GSTs is the protection of macromolecules from the attack of reactive electrophiles via the oxidation of reduced glutathione (GSH) (Armstrong et al., 2017). GSH is a tripeptide that can be quickly used as an antioxidant. It exists in the reduced (GSH) and oxidised (GSSG) forms, and both forms can interact with GSH-related enzymes through the thiol group (–SH) of their cysteine (Knapen et al., 1999). The mechanism by which the cell prevents

OS through the equilibrium between the generation of electrophilic compounds and the antioxidant capacity of GSH is illustrated in **Figure 8**.

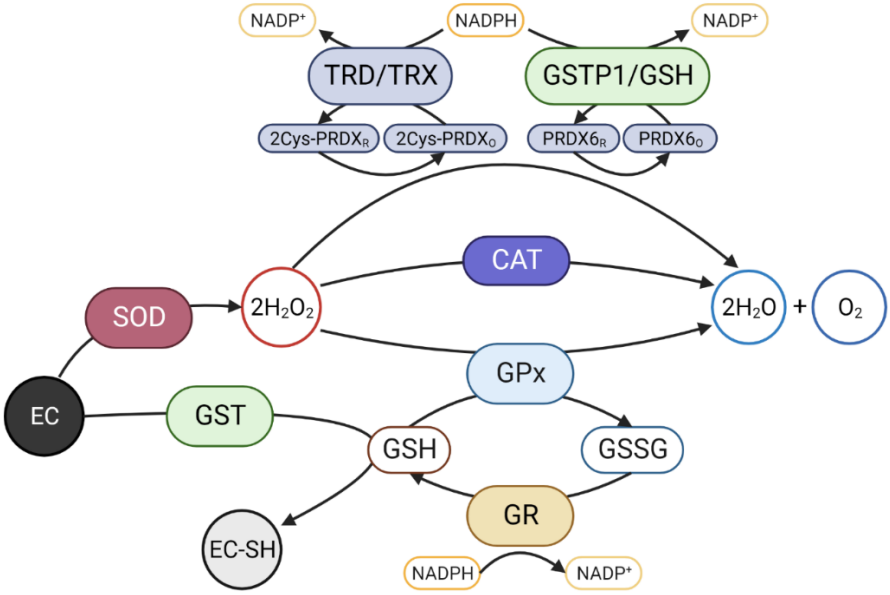

Figure 8. GSH-mediated antioxidant mechanism of sperm*. Internally and/or externally generated electrophilic compounds (EC) interact with the reduced nucleophilic thiol group (-SH) of extracellular glutathione (GSH) through a reaction catalysed by glutathione transferases (GSTs), thereby neutralising the EC and forming the reaction product (EC-SG). The superoxide anion (•O_2^-) can be directly eliminated by the GST/GSH system. Furthermore, superoxide dismutase (SOD) catalyses the dismutation of •O_2^- to hydrogen peroxide (H_2O_2), which is highly reactive and can lead to pathological conditions like lipid peroxidation. This molecule may subsequently be eliminated through various mechanisms, including: (1) catalysis by catalase (CAT) to transform H_2O_2 into H_2O and O_2, (2) reduction by peroxiredoxins (PRDX) to H_2O and O_2, with subsequent re-reduction thanks to thioredoxin reductase/thioredoxin (TRD/TRX) and GSTP1/GSH systems, (3) reduction by sperm glutathione peroxidases (GPx) via the oxidation of GSH to GSSG and subsequent re-reduction by glutathione reductase (GR), (4) formation of the hydroxyl radical (OH·) from H_2O_2 through the Fenton reaction, and (5) direct neutralisation by the GST/GSH system. The process requires reducing power (NADPH to NADP$^+$). Figure adapted from* Llavanera et al. (2020) and *created with BioRender.*

Apart from acting as antioxidant enzymes, GSTs show additional functionalities in eukaryotic cells. On the one hand, GSTs are known to be important modulators of cell signalling. GSTP is a direct regulator of the c-Jun N-terminal kinases (JNK) pathway in response to cellular stress (Adler, 1999).

Specifically, cellular stressors can induce the oligomerisation of GSTP and subsequent dissociation of the GSTP-JNK complex, thus providing protection against cellular stress via activation of specific kinases (Adler, 1999; Yin et al., 2000). Furthermore, GSTMs are acknowledged to modulate the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (ASK1), which can activate JNK (Cho et al., 2001). The C-terminal domain of GSTM1 directly binds the N-terminal region of ASK1, suppressing the stress-stimulated ASK1 activity, independently from the GSH-conjugating activity of this enzyme (Cho et al., 2001).

On the other hand, GSTs are also involved in the biosynthesis of prostaglandins and sex steroids. An isoform of the GSTS class was reported to function as a hematopoietic prostaglandin D synthase, implicated in the biosynthesis of prostaglandin D2 (PGD_2) (Kanaoka et al., 1997). Furthermore, GSTAs are involved in steroid hormone biosynthesis, since GSTA3-3 was found to catalyse the double-bond isomerisation of Δ5-androstene-3,17-dione and Δ5-pregnene-3,20-dione, which are hormonal precursors of testosterone and progesterone, respectively (Johansson and Mannervik, 2001).

The variety of molecular functions of GST classes in the cell, therefore, makes them critical enzymes for cellular homeostasis. Indeed, specific mutations or the absence of specific GSTs have been associated with human diseases such as Parkinson and Alzheimer diseases (Li et al., 2003), cardiovascular disorders (Maciel et al., 2009) and resistance to chemotherapeutic and carcinogenic compounds (Hayes and Pulford, 1995), thus serving as promising therapeutic targets and biomarkers for a wide range of human diseases (Townsend and Tew, 2003).

1.7.3.Expression and localisation of GSTs within the male reproductive tract

It is known that ROS scavengers are secreted by both sperm and male reproductive organs. Among them, several GST isoforms have been identified within the male reproductive tract. GSTs have been found in rat (Aravinda et al., 1995) and human (Klys et al., 1992) testes. As previously mentioned, even though GSTA2, GSTP1 and GSTMs are present, GSTM3 is the most abundant isoform

in this specific tissue. In effect, testis has thus far been identified the tissue with the highest levels of GSTM3 (Listowsky et al., 1998). In rats, SCs seem to be responsible for detoxification of germ cells through the secretion of GSTs and GSH (Aravinda et al., 1995; Mukherjee et al., 1999). In spite of this, the role of Leydig cells in detoxification through GSTs has not been studied. Germ cells, on the other hand, are known to synthesise, but do not secrete, GSTs (Mukherjee et al., 1999). In mice, *Gstm2* expression is high in spermatogonia, but is downregulated in mature germ cells (Yu et al., 2003), thus being downregulated during sperm development (Paz et al., 2006).

In addition to being highly expressed in the testis, there is increasing evidence for GSTs presence in the epididymis. Different GSTs have been found to be expressed in a region-specific manner in the human epididymis, with GSTP1 and GSTM3 being present at higher concentrations in the corpus and cauda, and GSTM2, GSTM5, and GSTO1 being highly secreted in the caput (Li et al., 2010). GSTM2-3 and GSTP1 have been characterised in epididymosomal membranes and proposed to bind the sperm surface during epididymal transport (Thimon et al., 2008). Another study also observed the incorporation of GSTMs into sperm during epididymal maturation, suggesting that epididymal secretions may contain GSTs that bind sperm as they migrate through the lumen, which may play a role in sperm maturation (Suryawanshi et al., 2011). It is believed that sperm-surface GSTs protect cells from OS, which could help improve the survival of sperm during epididymal storage (Dacheux et al., 2009).

The prostate has been found to secrete GSTP1 through prostasomes (Utleg et al., 2003), which are believed to support sperm motility and prevent premature acrosome reaction (Carlsson et al., 1997; Cross and Mahasreshti, 1997). Indeed, prostate GSTP1 serves as a prostate cancer biomarker due to the abnormal methylation of the gene encoding this antioxidant enzyme in over 90% of prostate tumours (Crocitto et al., 2004).

The presence of GSTs in the sperm of rat, mouse, and human is understood since 1978 (Mukhtar et al., 1978). Years later, in the 1990s, other researchers identified a member of the GSTM class, with GST activity and associated to the fibrous sheath, in rat and mouse sperm (Shaha et al., 1988;

Aravinda et al., 1995; Fulcher et al., 1995). In 1998, both Mu- and Pi-GST class members were identified on the surface of goat sperm (Gopalakrishnan et al., 1998), as attached by non-covalent interactions to the sperm plasma membrane (Hemachand et al., 2002). Both GSTs classes were also found to undergo relocalisation during epididymal maturation in goats, with GSTPs migrating from the post-equatorial segment to the anterior acrosome and post-equatorial regions, and GSTMs relocating from the entire sperm surface to the sperm head (Fulcher et al., 1995). Another GST class, GSTO, was observed in the post-acrosomal sheath of the perinuclear theca of bovine, porcine, and murine sperm (Hamilton et al., 2017; Protopapas et al., 2019). This enzyme has been shown to actively participate in nuclear decondensation in mouse sperm, possibly facilitated by the reducing power of GSH (Hamilton et al., 2019).

1.7.4.The role of GSTs in male (in)fertility

Albeit poorly studied, the presence and putative role of GSTs in maintaining oxidative homeostasis and physiological status of sperm suggest their relevance for male fertility. In this regard, numerous studies showed that *GSTM1*$^{-/-}$, *GSTP1*$^{-/-}$ and *GSTT1*$^{-/-}$ genotypes are more common in infertile than in fertile men (Aydos et al., 2009; Safarinejad et al., 2010; Vani et al., 2010; Lakpour et al., 2013; Kolesnikova et al., 2017). Additionally, three meta-analyses found that *GSTM1*$^{-/-}$ and *GSTT1*$^{-/-}$ genotypes are a risk factor for idiopathic male infertility (Tang et al., 2012; Kan et al., 2013; Song et al., 2013). Reduced levels of GSH and decreased GPx and GSR activity have been observed in *GSTT1*$^{-/-}$ sperm, supporting the notion that GSTs may play a crucial role for sperm function (Kolesnikova et al., 2017). There is also evidence suggesting that *GSTM1*$^{-/-}$ men with idiopathic infertility present increased levels of ROS in their SP and sperm (Aydemir et al., 2007). Based on these data, it has been posited that the infertility phenotype of *GSTM1*$^{-/-}$, *GSTT1*$^{-/-}$, and *GSTP1*$^{-/-}$ men may be due to the inability of their sperm to reduce OS and prevent DNA damage. This hypothesis is supported by the finding that *GSTM1*$^{-/-}$ men exhibit a higher susceptibility to sperm DNA damage when exposed to air pollution (Rubes et al., 2007) and that the *GSTM1*$^{-/-}$ genotype is associated with higher levels of PAH-DNA adducts in sperm, which are a direct

sign of DNA damage caused by exposure to polycyclic aromatic hydrocarbons (Paracchini et al., 2005). A range of proteomic studies have been conducted to evaluate sperm GSTM3 as a marker of infertility in humans and pigs. In addition, GSTM3 is known to be overexpressed in sperm from infertile men with unilateral varicocele (Agarwal et al., 2015b), suggesting a detoxification role for this enzyme in these patients. GSTM3 was also found to be increased in the SP from men with mitochondria-altered sperm (Intasqui et al., 2015). Finally, it was also reported that high levels of GSTM3 in pig sperm are associated with small litter sizes (Kwon et al., 2015).

In summary, sperm GSTs seem to be ubiquitously present in mammals, although their localisation pattern is suggested to be variable across species. The findings reported in the literature suggest that some GST classes in sperm, mainly GSTMs and GSTPs, are linked to infertility in humans and farm animals, and that the mechanisms through which GSTM3 is associated with (in)fertility may be linked to the regulation of redox homeostasis and cellular signalling. In spite of all the aforementioned, and although potentially critical for sperm physiology and subsequent male fertility, this group of antioxidant enzymes has been poorly studied in both humans and livestock, so that further research is needed to fully understand their physiological and molecular role.

OBJECTIVES

OBJECTIVES

Against the background stated in the previous section, the main aim of the present Doctoral Thesis was to evaluate the physiological role of Glutathione S-transferases (GSTs) in male (in)fertility in both livestock and humans. For this purpose, four specific objectives were set:

1) To characterise the presence and localisation of GSTs in sperm and seminal plasma as well as their putative species-specific differences in mammals.

2) To explore the role of sperm GSTs in detoxification and regulation of cellular signalling in sperm.

3) To evaluate the potential use of GSTs as molecular markers of sperm quality, DNA integrity and fertilising ability in humans and farm animals.

4) To address whether GSTs are able to predict sperm quality after undergoing liquid preservation and cryopreservation procedures.

PAPER COMPENDIUM

PAPER I

Glutathione S-Transferases Play a Crucial Role in Mitochondrial Function, Plasma Membrane Stability and Oxidative Regulation of Mammalian Sperm

Marc Llavanera, Ariadna Delgado-Bermúdez, Samuel Olives, Yentel Mateo-Otero, Sandra Recuero, Sergi Bonet, Beatriz Fernández-Fuertes, Marc Yeste & Isabel Barranco

Antioxidants

2020;9(2), 100

DOI: 10.3390/antiox9020100

antioxidants

MDPI

Article

Glutathione S-Transferases Play a Crucial Role in Mitochondrial Function, Plasma Membrane Stability and Oxidative Regulation of Mammalian Sperm

Marc Llavanera [1,2], Ariadna Delgado-Bermúdez [1,2], Samuel Olives [2], Yentel Mateo-Otero [1,2], Sandra Recuero [1,2], Sergi Bonet [1,2], Beatriz Fernández-Fuertes [1,2], Marc Yeste [1,2,*,†] and Isabel Barranco [1,2,*,†]

[1] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, E-17003 Girona Spain; marc.llavanera@udg.edu (M.L.); ariadna.delgado@udg.edu (A.D.-B.); yentel.mateo@udg.edu (Y.M.-O.); sandra.recuero@udg.edu (S.R.); sergi.bonet@udg.edu (S.B.); beatriz.fernandez@udg.edu (B.F.-F.)

[2] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, E-17003 Girona, Spain; samolives04@gmail.com

* Correspondence: marc.yeste@udg.edu (M.Y.); isabel.barranco@udg.edu (I.B.); Tel.: +34-972-419514 (M.Y. & I.B.)

† Both authors have contributed equally to the direction of this study.

Received: 29 December 2019; Accepted: 22 January 2020; Published: 24 January 2020

Abstract: Glutathione S-transferases (GSTs) are essential sperm antioxidant enzymes involved in cell protection against oxidative stress and toxic chemicals, preserving sperm function and fertilising ability. Artificial insemination (AI) in pigs is commonly carried out through the use of liquid-stored semen at 17 °C, which not only reduces sperm metabolic activity but also sperm quality and AI-farrowing rates within the 72 h of storage. While one may reasonably suggest that such enzymes are implicated in the physiology and maintenance of mammalian sperm function during liquid-storage, no previous studies conducted on any species have addressed this hypothesis. Therefore, the objective of the present work was to characterise the presence and function of sperm GSTs in mammalian sperm, using the pig as a model. In this regard, inhibition of such enzymes by ethacrynic acid (EA) during semen storage at 17 °C was performed to evaluate the effects of GSTs in liquid-preserved boar sperm by flow cytometry, immunofluorescence, and immunoblotting analysis. The results of this study have shown, for the first time in mammalian species, that the inhibition of GSTs reduces sperm quality and functionality parameters during their storage at 17 °C. These findings highlight the key role of such enzymes, especially preserving mitochondrial function and maintaining plasma membrane stability. In addition, this study has identified and localised GSTM3 in the tail and equatorial subdomain of the head of boar sperm. Finally, this study has set grounds for future investigations testing supplementation of semen extenders with GSTs, as this may improve fertility outcomes of swine AIs.

Keywords: Mammalian sperm; Ethacrynic acid; GSTs; GSTM3; Boar semen; Liquid-storage

1. Introduction

Pig breeding worldwide is fundamentally based on the use of artificial insemination (AI). Such a technique is commonly performed through the use of liquid-preserved semen diluted with a proper extender and stored at 15–20 °C, usually for 1 to 5 days [1]; in some cases, however, media may preserve sperm up to 12–15 days [2]. Extender composition and low temperatures contribute to the decrease of sperm metabolic activity, thus maintaining their function and fertilising ability [2]. However, AI-fertility rates using liquid-stored sperm are known to decline within 72 h of its storage [3].

A wide range of changes occurs during liquid-storage of boar semen, including a decrease in sperm motility, viability, and plasma membrane stability as well as an increase of oxidative stress (OS), lipid peroxidation, and apoptotic-like events [3,4].

Reactive oxygen species (ROS) include superoxide anion, hydrogen peroxide, hydroxyl radical, amongst others, and are naturally produced by the activity of the mitochondrial electron transport chain. Depending on their concentration, localisation, or exposure time, the effects of ROS may be both beneficial than harmful [5]. Moreover, OS reflects the imbalance between ROS production and antioxidant sperm capacity [6]. One of the major causes of the decreased sperm quality in liquid-preserved boar semen is the OS-related damage [7]. In comparison to somatic cells, mammalian sperm are highly sensitive to OS due to the high amount of polyunsaturated fatty acids in their plasma membrane phospholipids and their relatively low antioxidant capacity [8]. Along these lines, any factor that initiates OS in sperm, such as low levels of antioxidant protection or high levels of ROS, leads to the induction of upraised OS levels and cell damage as a result of a self-perpetuating redox cycle [9,10]. It has been demonstrated that many antioxidant systems, such as glutathione peroxidases (GPX), glutathione reductases (GSR), catalase (CAT), superoxide dismutase (SOD), and peroxinreductases (PRDX), are capable of regulating physiological levels of ROS in sperm, protecting them from OS (reviewed in [11]). However, the specific role of glutathione S-transferases (GSTs) on mammalian sperm physiology has not been investigated.

GSTs are postulated as important detoxifying enzymes that catalyse reduced glutathione-dependent reactions involved in cellular protection against OS and toxic chemicals [12]. The triple role of sperm GSTs is known to be (i) cell detoxification, which prevents lipid membrane peroxidation and subsequent OS; (ii) cellular signalling regulation involved in sperm capacitation; and (iii) fertilising ability, since GSTM3 is involved in sperm-zona pellucida binding events (reviewed in [13]). Experiments performed in goats evidenced that GSTMs are attached to the sperm plasma membrane by non-covalent interactions [14] and maintain their motility, viability, mitochondrial status, and fertilising ability by preventing lipid peroxidation, and OS [15]. Furthermore, GSTM3 has been recently established as a fertility [16] and cryotolerance [17] biomarker in boar sperm. Related to this, while GSTM3 seems to be the main GST family member in sperm and could play a crucial role in sperm physiology and the maintenance of their function and quality during liquid-storage, its role in sperm cells is yet to be investigated.

Ethacrynic acid (EA) is a well-known inhibitor of GSTs enzymatic activity that strongly and specifically inhibits GSTAs, GSTMs, and GSTPs by blocking their substrate-binding site [18,19]. This blocking effect can occur by direct binding of EA to GSTs as a non-substrate ligand [20] or by conjugation of EA to the thiol group of GSH (EA-GSH), which can be formed either spontaneously or through a GST-catalysed reaction [21].

Along these lines, mounting evidence suggests the essential role of GSTs, and specifically GSTM3, in sperm physiology of goat and boar [13,16,22]. Although OS is known to induce detrimental effects on boar sperm quality parameters during liquid-storage (e.g., impaired motility, viability, and fertilising capacity), the effects of GSTs during semen storage are yet to be investigated in any species. In this regard, understanding the effects and mechanisms of sperm GSTs in liquid-storage of boar semen is of utmost importance, since it may allow improving their preservation and fertilising ability. With this purpose, inhibition of sperm GSTs activity by EA was performed in order to evaluate the effects of GSTs in liquid-preserved boar sperm. Sperm quality and functionality parameters (including motility, mitochondrial membrane potential [i.e., $\Delta\Psi m$], viability, membrane lipid disorder, acrosome membrane integrity, apoptotic-like changes, intracellular calcium [Ca^{2+}] levels and superoxide [i.e., $O_2^{-}\bullet$], and peroxide [i.e., H_2O_2)] levels) were assessed at 0, 24, 48, and 72 h of storage at 17 °C. Additionally, the presence, localisation, and relative levels of GSTM3 in boar sperm were assessed in each treatment after 0 and 72 h of storage at 17 °C.

2. Materials and Methods

2.1. Reagents

Ethacrynic acid (EA; Sigma-Aldrich, Saint Louis, MO, USA) was reconstituted in dimethyl sulfoxide (DMSO) to a stock solution of 64 mM. Fluorochromes used for flow cytometry analysis were purchased from Life Technologies (ThermoFisher; Carlsbad, CA, USA) and reconstituted in DMSO, except for propidium iodide (PI) and peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC), which were diluted in phosphate-buffered saline 1× (PBS). The antibody against GSTM3 (ref. ARP53561_P050), as well as its specific blocking peptide (ref. AAP53561), were purchased from Aviva Systems Biology (San Diego, CA, USA). Secondary antibodies for immunoblotting analysis were goat anti-rabbit and rabbit anti-mouse conjugated with horseradish peroxidase (ref. P0448 and ref. P0260; Dako, Derkman A/S; Denmark, respectively), whereas that for immunofluorescence analysis was an anti-rabbit antibody conjugated with Alexa Fluor 488 (ref. A32731; ThermoFisher).

2.2. Animals and Ejaculates

Production of the seminal doses used in this study followed the ISO certification (ISO-9001:2008), and the authors did not manipulate any animal, but they purchased semen doses from a local farm, which operates under commercial, standard conditions. Ejaculates from ten healthy and sexually mature Piétrain boars (n = 10; 1–3 years-old) were provided by an authorised, AI-centre (Grup Gepork S.L., Masies de Roda, Spain). Boars were fed with a standard and balanced diet with water being provided ad libitum, and ejaculates were collected twice a week through the gloved-hand method. Ejaculates were diluted to a final concentration of 30×10^6 spermatozoa/mL with a commercial extender (Androstar® Plus, Minitüb Ibérica, S.L.; Tarragona, Spain), and transported at 17 °C to the laboratory within four hours post-collection. All ejaculates fulfilled the standards of quantity and quality ($>200 \times 10^6$ spermatozoa/mL, 70% motile spermatozoa, and 75% morphologically normal cells).

2.3. Experimental Design

Ten ejaculates (one per boar) were split into two aliquots containing 100 μmol/L of EA and the same volume of DMSO as a vehicle control group. The concentration of DMSO in all treatments was 0.15% (v:v). Inhibitor concentration was selected based on previous studies [23] and preliminary concentration tests performed at our laboratory. All samples were stored for 72 h in closed plastic containers with constantly and gently agitation at 17 °C. Sperm motility and flow cytometry parameters were evaluated at 0, 24, 48, and 72 h, whereas immunofluorescence and immunoblotting analysis against GSTM3 were assessed at 0 and 72 h of semen storage at 17 °C.

2.4. Sperm Motility

Prior to sperm motility analysis, 500 μL of each sample was incubated at 37 °C for 10 min. Subsequently, 5 μL of each sperm sample was placed onto a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Motility evaluation was performed through a commercial computer-assisted sperm analysis (CASA) system (Olympus BX41) connected to a computer equipped with ISAS software (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). The following sperm motility parameters were provided by the software: total motility, TMOT (%); progressive motility, PMOT (%); average pathway velocity, VAP (μm/s); curvilinear velocity, VCL (μm/s); straight-line velocity, VSL (μm/s); linearity, LIN (%); beat-cross frequency, BCF (Hz); amplitude of lateral head displacement, ALH (μm) and straightness, STR (%). The sperm motility variables used in the present study were recorded as the percentage of TMOT (average path velocity ≥ 10 μm/sec) and that of motile spermatozoa showing rapid and progressive movement (straightness ≥ 45%). Three replicates per sample, with a minimum of 1000 spermatozoa per replicate, were assessed.

2.5. Flow Cytometry Analyses

Sperm plasma membrane integrity (i.e., viability), membrane lipid disorder, acrosome integrity, apoptotic-like changes, ΔΨm, intracellular Ca^{2+} levels, and intracellular levels of O_2^-• and H_2O_2 were evaluated in each treatment and time. Samples were diluted with pre-warmed PBS to a final concentration of 2×10^6 sperm/mL in a final volume of 0.8 mL and subsequently stained. Flow cytometric analysis was conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter; Fullerton, CA, USA) equipped with an argon-ion laser (488 nm) set at a power of 22 mW. Laser voltage and rate were constant throughout the experiment. Unstained and single-stained samples for each fluorochrome were used for setting the electronic volume (EV) gain, FL-1, FL-2, and FL-3 PMT-voltages and for compensating spill over the other channels. EV was used to distinguish the sperm population from debris. Each sample was evaluated three times, with 10,000 events per replicate. Flow cytometric data analysis was performed using Flowing Software (Ver. 2.5.1; University of Turku, Finland), as recommended for the International Society for Advancement of Cytometry.

Sperm viability was evaluated using co-staining with SYBR14 (100 nmol/L) and PI (12 µmol/L) [24]. Membrane lipid disorder of sperm was assessed by merocyanine 540 (M540; 2.6 µmol/L) and YO-PRO-1 (25 nmol/L) co-staining, following the procedure described by Yeste et al. 2014 [25]. Acrosome membrane integrity was assessed by PNA-FITC (2.5 µg/mL), and PI (12 µmol/L) co-staining according to the modified procedure described by Nagy et al. [26]. Apoptotic-like changes in sperm were evaluated by Annexin V and PI co-staining, following the recommended procedure from the Annexin-V-FLUOS Staining Kit (11858777001; Roche Diagnostics, Germany). Levels of ΔΨm were evaluated through 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide (JC1; 0.3 µmol/L) staining [27]. Sperm head and mid-piece Ca^{2+} levels were evaluated through the staining with Fluo3-AM (1 µmol/L) and PI (12 µmol/L) [28,29]. On the other hand, Rhod5-AM (5 µmol/L) and YO-PRO-1 (25 nmol/L) co-staining was performed in order to evaluate head Ca^{2+} deposits exclusively [29–31]. Finally, sperm OS was evaluated by assessing intracellular levels of O_2^-• and H_2O_2, through staining with hydroethidine (HE; 4 µmol/L) and YO-PRO-1 (25 nmol/L) [32] and 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; 10 µmol/L) and PI (12 µmol/L) [32], respectively. All flow cytometry protocols are described in detail in the Additional File 1 in Supplementary Materials.

2.6. Immunofluorescence

Localisation of GSTM3 in boar sperm during liquid preservation was evaluated through immunofluorescence at 0 and 72 h of storage at 17 °C in each treatment. Samples containing 3×10^6 sperm/mL were fixed with 2% (w:v) paraformaldehyde and subsequently washed. The different slides containing two drops per sample were blocked and permeabilised with a blocking solution containing 0.25% (v:v) Triton X-100 and 3% (w:v) Bovine serum albumin (BSA) for 40 min. Then, samples were incubated with a primary anti-GSTM3 antibody (1:250; v:v) overnight. Following this, slides were washed and incubated with an anti-rabbit antibody (1:500; v:v). Then, 10 µL of Vectashield mounting medium containing 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was added. Finally, a coverslip was placed, and samples were sealed with nail varnish. In negative controls, the primary antibody was omitted. For the peptide competition assay, samples were incubated with GSTM3-specific blocking peptide, which was 20 times in excess with regard to the corresponding primary antibody. A confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon Corp., Tokyo, Japan) was used to evaluate all samples.

2.7. Immunoblotting

Boar sperm samples of all treatments at 0 and 72 h of storage at 17 °C were used for Western blot analysis. In brief, samples were centrifuged twice at 3000× *g* for 5 min and resuspended in lysis buffer (RIPA Buffer, Sigma-Aldrich) prior to incubation in agitation at 4 °C for 30 min. Triple sonication per sample was carried out, followed by centrifugation at 10,000× *g*, and the supernatant was stored

at −80 °C. A detergent compatible method (BioRad; Hercules, CA, USA) was used to quantify total protein. Ten micrograms of total protein were diluted 1:1 (v:v) in Laemmli reducing buffer 2× and boiled at 96 °C for 5 min before proteins were loaded onto the gel and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes using Trans-Blot® Turbo™ (BioRad) and blocked with 5% BSA. Blocked membranes were then incubated with the anti-GSTM3 primary antibody (1:20,000; v:v) overnight. Next, membranes were washed thrice and incubated with the secondary antibody for an hour with agitation (1:35,000; v:v). Finally, bands were visualised using a chemiluminescent substrate (Immobilion™ Western Detection Reagents, Millipore) and scanned with G:BOX Chemi XL 1.4 (SynGene, Frederick, MT, USA). Next, membranes were stripped and blocked prior to incubation with an anti-α-tubulin antibody (1:100,000, v:v) overnight. Subsequently, membranes were washed trice and incubated with an anti-mouse antibody (1:200,000, v:v) for 1 h. Finally, membranes were washed, visualised, and scanned as described previously. The specificity of the primary antibody was confirmed through peptide competition assays utilising GSTM3-immunising peptides, 20 times in excess with regard to the antibody. Bands of three technical replicates per samples were quantified using Quantity One software package (Version 4.6.2; BioRad), and pattern quantifications were normalized using α-tubulin.

2.8. Statistical Analysis

Results were analysed using a statistical package (IBM SPSS for Windows 25.0; Armonk, NY, USA). First, data were checked for normality and homogeneity of variances using Shapiro–Wilk and Levene tests, respectively. When required, data were transformed with arcsin $\sqrt{x}$ and then re-assessed for normality and homogeneity of variances. Each statistical case consisted of a separate biological replicate.

Sperm quality and functionality parameters, as well as the relative content of GSTM3, were compared between treatments (EA-treated and control spermatozoa) and throughout storage time (0, 24, 48 and 72 h) with a linear mixed model (repeated measures); within-subjects factor was the time of storage, between-subjects factor was the treatment, and the random-effects factor was the boar. The post-hoc Sidak test was used for pair-wise comparisons. Finally, Pearson correlation coefficients were calculated between the relative content of the GSTM3 band and quality and functionality parameters. Data are shown as mean ± SEM. For all analyses, the level of significance was set at $p \leq 0.05$.

3. Results

All sperm quality and functionality parameters (total and progressive motility, ΔΨm, viability, membrane lipid disorder, acrosome membrane integrity, apoptotic-like changes, intracellular Ca^{2+} levels, and total intracellular $O_2^{-}\bullet$ and H_2O_2 levels) of semen samples incubated with EA and the control group were assessed at 0, 24, 48 and 72 h of storage at 17 °C. No differences between groups were found in any sperm quality and functionality parameter at 0 h of storage at 17 °C.

3.1. Inhibition of GSTs Impairs Sperm Motility and ΔΨm

Motility was assessed by the percentage of total and progressively motile sperm and the VAP at 0, 24, 48, and 72 h of liquid-storage at 17 °C, whereas sperm mitochondrial function was assessed by the percentage of high ΔΨm resulting from the orange-stained populations ($JC1_{agg}$) (Figure 1).

Compared to the control group, total and progressive motilities and the VAP of EA-treated sperm samples dramatically decreased within the first 24 h of liquid-storage and remained low until 72 h of storage ($p < 0.05$). On the other hand, a dramatic decrease in the percentage of sperm showing high ΔΨm was observed in EA-treated samples compared to the control within the first 24 h of liquid-storage ($p < 0.05$). Moreover, a strong correlation between total motility and ΔΨm was observed (r = 0.873; $p < 0.01$).

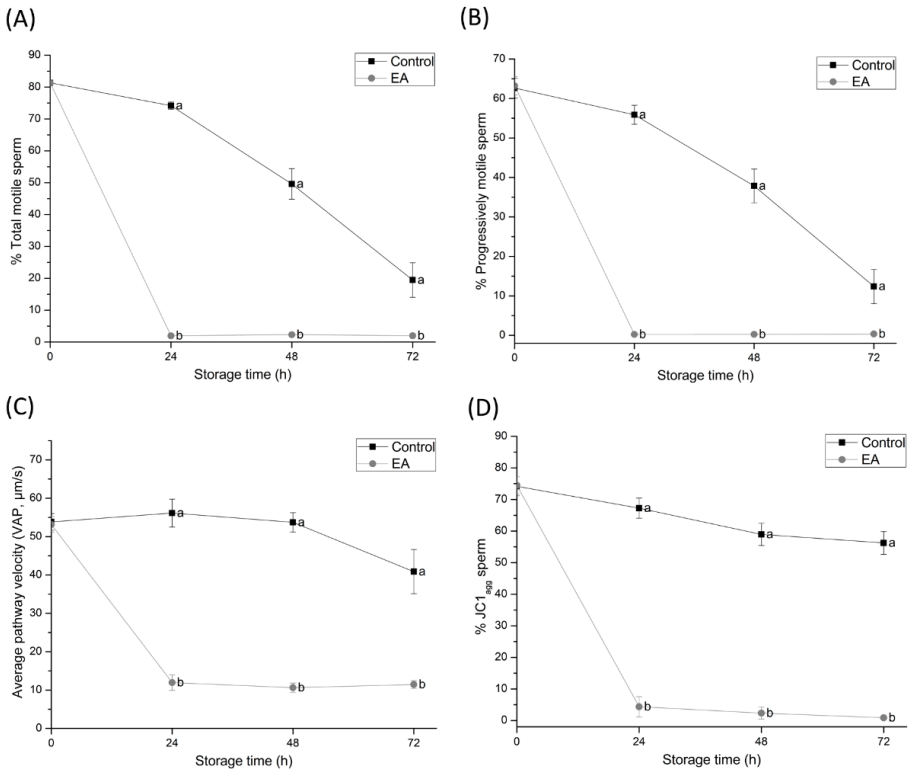

Figure 1. (**A**) percentages of total motile sperm, (**B**) percentages of progressive motile sperm, (**C**) average pathway velocity (VAP; µm/s), and (**D**) percentages of high ΔΨm sperm ($JC1_{agg}$ sperm) of semen samples treated with ethacrynic acid (EA), a glutathione S-transferases (GSTs) inhibitor, and the control group, assessed at different evaluation times during liquid storage at 17 °C (0, 24, 48, and 72 h). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within storage time.

3.2. Inhibition of GSTs Causes Sperm Plasma Membrane but not Acrosome Damage

Sperm plasma membrane status was characterised through SYBR14/PI, M540/YO-PRO-1, PNA-FITC/PI, and Annexin V/PI staining (Figure 2). Although no statistically significant differences in the percentage of viable spermatozoa ($SYBR14^+/PI^-$) were found between control and EA-treated samples at 0, 24, and 48 h of semen storage, a reduced viability was evidenced at 72 h ($p < 0.05$).

On the other hand, the percentage of sperm with high membrane lipid disorder ($M540^+$/YO-PRO-1^-) was higher in EA-treated samples at 24, 48, and 72 h of liquid-storage ($p < 0.05$). Related to this, the percentage of viable membrane-intact sperm ($PNA\text{-}FITC^-/PI^-$) was used to assess acrosome membrane intactness, whereas the percentage of viable Annexin V-positive sperm (Annexin V^+/PI^-) was used to assess apoptotic-like changes. EA-treated samples did not show either acrosome membrane damage or apoptotic-like changes at any time-point in comparison to the control group.

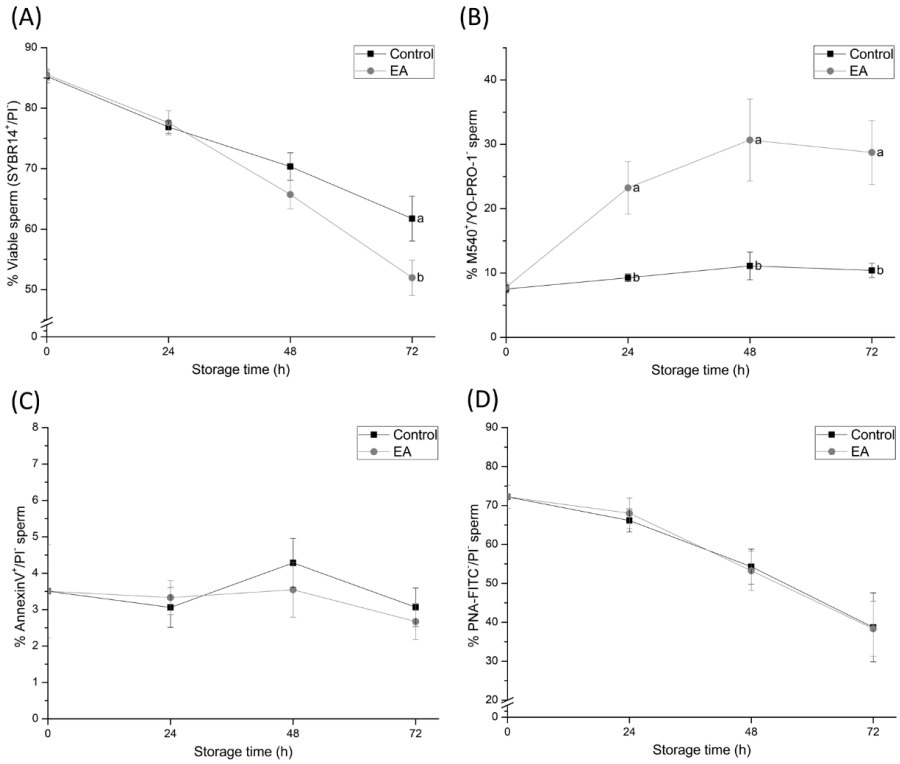

Figure 2. Percentages of (**A**) total viable sperm (SYBR14$^+$/PI$^-$), (**B**) viable sperm with high membrane lipid disorder (M540$^+$/YO-PRO-1$^-$), (**C**) viable apoptotic-like sperm (AnnexinV$^+$/PI$^-$) and (**D**) viable acrosome membrane-intact sperm (PNA-FITC$^-$/PI$^-$) of semen samples treated with ethacrynic acid (EA), a glutathione S-transferases (GSTs) inhibitor, and the control group, assessed at different evaluation times during liquid storage at 17 °C (0, 24, 48, and 72 h). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within storage time.

3.3. Sperm GSTs Are Involved in Ca^{2+} Homeostasis

The percentage and fluorescence intensity of viable spermatozoa showing high Ca^{2+} levels (Fluo3$^+$/PI$^-$ and Rhod5$^+$/YO-PRO-1$^-$) were used to assess sperm intracellular Ca^{2+} levels (Figure 3). Although no differences in the percentage of Fluo3$^+$/PI$^-$ sperm were found, Fluo3$^+$/PI$^-$ fluorescence intensity in EA-treated spermatozoa was higher in comparison to the control group after 24, 48 and 72 h of liquid preservation ($p < 0.05$), showing increased Ca^{2+} levels in GSTs-inhibited samples. On the other hand, percentages of Rhod5$^+$/YO-PRO-1$^-$ sperm and Rhod5$^+$-fluorescence intensity did not show differences between treatments at any time-point of semen storage at 17 °C.

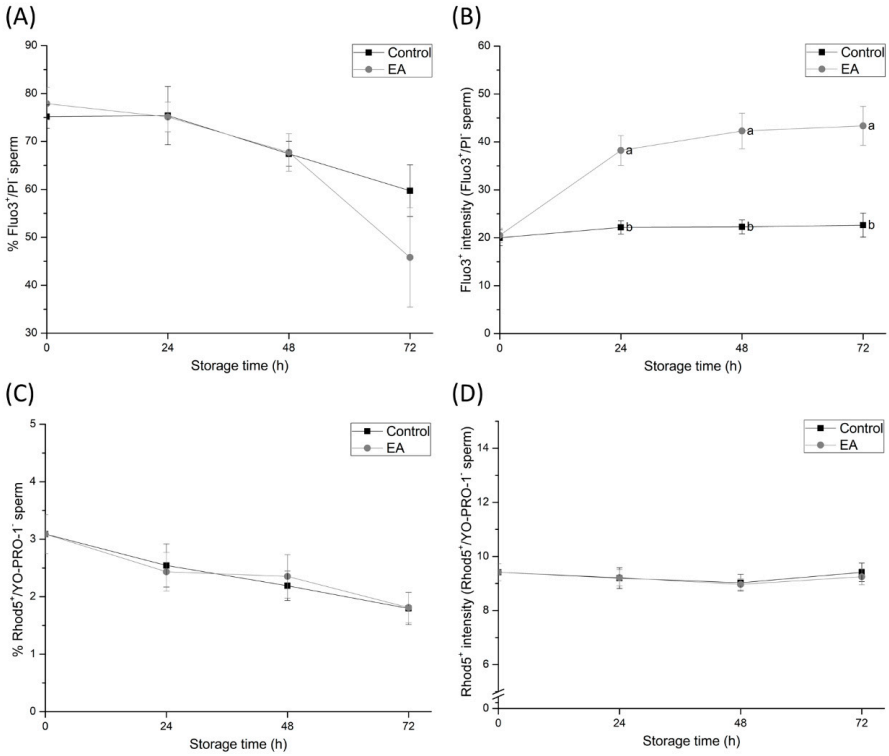

Figure 3. (**A**) Percentages of viable spermatozoa showing high intracellular calcium levels in the mid-piece and head ($Fluo3^+/PI^-$), (**B**) mean $Fluo3^+$ fluorescence intensity of viable spermatozoa showing high intracellular calcium levels in the mid-piece and head, (**C**) percentages of viable spermatozoa showing high intracellular calcium levels in the head ($Rhod5^+/YO\text{-}PRO\text{-}1^-$), and (**D**) mean $Rhod5^+$ fluorescence intensity of viable spermatozoa showing high intracellular calcium levels in the sperm head of semen samples treated with ethacrynic acid (EA), a glutathione S-transferases (GSTs) inhibitor, and the control group, assessed at different evaluation times during liquid storage at 17 °C (0, 24, 48, and 72 h). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within storage time.

3.4. Sperm GSTs are Involved in Intracellular ROS Regulation

Percentages of $E^+/YO\text{-}PRO\text{-}1^-$ and DCF^+/PI^- sperm and fluorescence intensities of E^+ and DCF^+ were assessed to evaluate intracellular levels of $O_2^{-}\bullet$ and H_2O_2 (Figure 4). An increase of $O_2^{-}\bullet$ in sperm cells due to GSTs inhibition was detected, since E^+-fluorescence intensity, although not the percentage of $E^+/YO\text{-}PRO\text{-}1^-$ sperm, in EA-treated samples was higher than the control at 24, 48, and 72 h of liquid-storage ($p < 0.05$). On the other hand, a decrease in the percentage of H_2O_2-positive sperm cells (DCF^+/PI^- sperm) was found due to GSTs inhibition at any evaluation time ($p < 0.05$), even though the mean fluorescence intensity of DCF^+ did not differ from the control group.

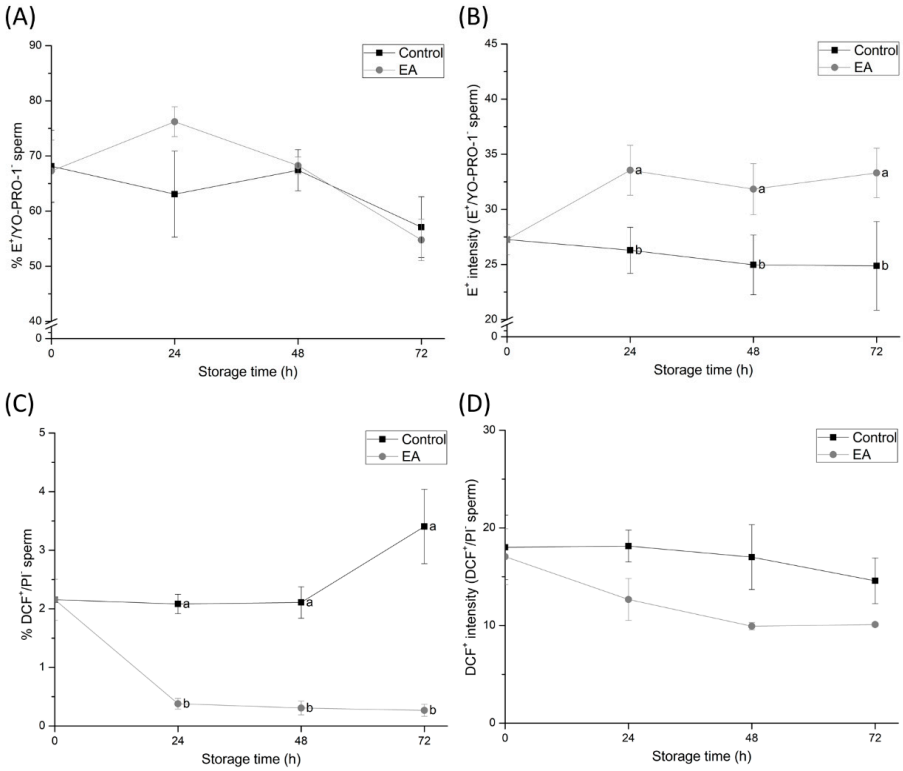

Figure 4. (**A**) Percentages of viable spermatozoa showing high superoxide (O_2^-•) levels (E^+/YO-PRO-1^-), (**B**) mean E^+ fluorescence intensity of viable spermatozoa showing high superoxide levels, (**C**) percentages of viable spermatozoa showing high peroxide (H_2O_2) levels (DCF^+/PI^-), and (**D**) mean DCF^+ fluorescence intensity of viable spermatozoa showing high peroxide levels of semen samples treated with ethacrynic acid (EA), a glutathione S-transferases (GSTs) inhibitor, and the control group, assessed at different evaluation times during liquid storage at 17 °C (0, 24, 48, and 72 h). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within storage time.

3.5. GSTM3 Partially Disappear from the Boar Sperm Mid-Piece during Liquid-Storage

Localisation of GSTM3 was resolved at 0 and 72 h of storage at 17 °C by immunofluorescence. Figure 5 shows representative localisation patterns of GSTM3 in GSTs-inhibited and non-inhibited sperm samples at 0 and 72 h of liquid-storage at 17 °C. All sperm cells showed the GSTM3 fluorescence signal, and the negative control and peptide competition assay confirmed the specificity of the GSTM3 antibody. GSTM3 was found to be localized in the mid, principal, and end pieces of the tail and the equatorial subdomain of the head in sperm samples at 0 h of liquid-storage. However, after 72 h of semen storage at 17 °C, the GSTM3 signal partially disappeared from the mid-piece in both the control and GSTs-inhibited samples.

3.6. Sperm GSTM3 Content Was Reduced During Sperm Liquid Preservation

Immunoblotting analysis of GSTM3 showed a triple-band pattern of ~25, ~28, and ~48 kDa in every experimental condition. Peptide competition assay utilising GSTM3 immunising peptide confirmed both ~25 (GSTM3-A) and ~28 (GSTM3-B) kDa-bands as GSTM3-specific (Figure 6).

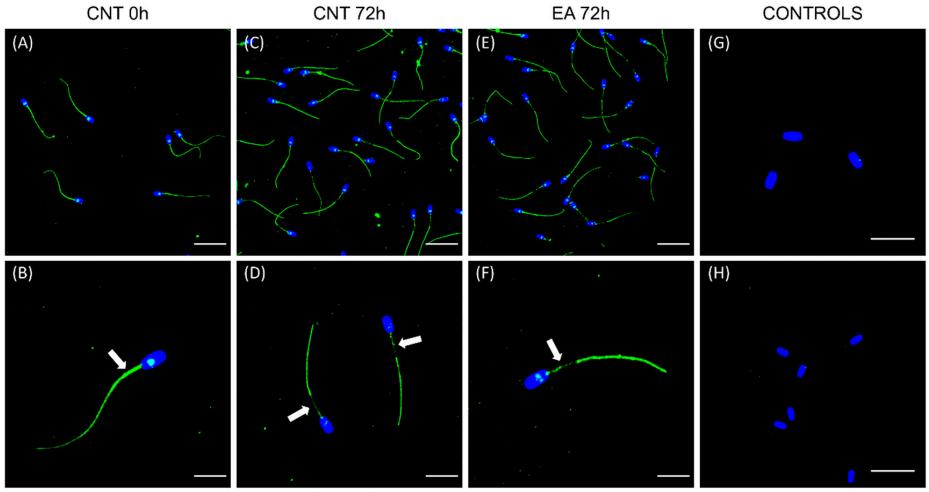

Figure 5. Immunolocalisation of sperm GSTM3. (**A**,**B**) control group at 0 h of storage at 17 °C, (**C**,**D**) control group at 72 h of liquid-storage, (**E**,**F**) Ethacrynic acid (EA)-treated spermatozoa at 72 h of liquid-storage, (**G**) negative control, and (**H**) peptide competition assay. White arrows indicate the sperm midpiece. The nucleus is shown in blue colour (DAPI), whereas GSTM3 is shown in green (fluorescein isothiocyanate, FITC). Scale bars: A, C, E, H: 30 µm; D, G: 15 µm; B, F: 10 µm.

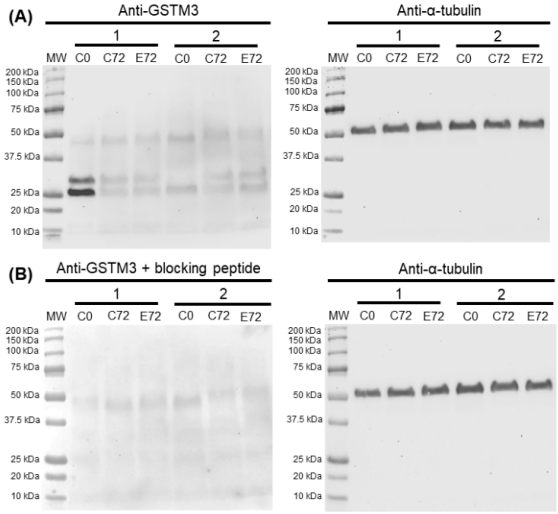

Figure 6. (**A**) Representative Western blots resulting from the incubation with the GSTM3 antibody (Anti-GSTM3) and its loading control (α-tubulin). (**B**) Western blots resulting from incubation with the GSTM3 antibody with GSTM3-blocking peptide (Anti-GSTM3 + blocking peptide) and its loading control (α-tubulin). Lanes MW: molecular weight. Lanes C0: control at 0 h of sperm liquid storage. Lanes C72: control at 72 h of sperm liquid storage. Lanes E72: EA-treated samples at 72 h of liquid storage.

As shown in Figure 7, normalised GSTM3-A content was found to be significantly higher than GSTM3-B after 0 h of storage at 17 °C ($p < 0.05$). Additionally, at 0 h, GSTM3-A was significantly higher than GSTM3-A and GSTM3-B after 72 h of storage in control and GSTs-inhibited groups ($p < 0.05$).

However, the relative abundance of GSTM3 in GSTs-inhibited samples at 72 h of liquid storage did not differ from the control.

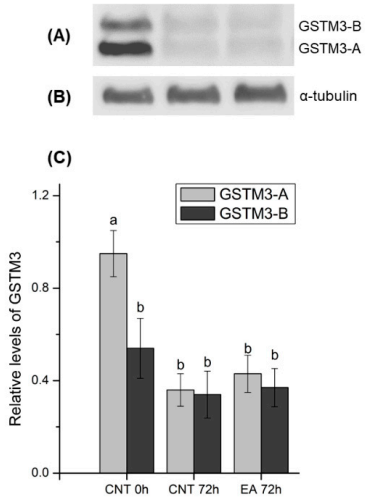

Figure 7. (**A**) Representative Western blot resulting from incubation with the GSTM3 antibody and (**B**) its loading control (α-tubulin). (**C**) Relative abundances of ~25 (GSTM3-A) and ~28 (GSTM3-B) kDa bands as mean ± standard error of the mean in all treatments. Values were normalised using the α-tubulin protein as an internal standard. Each sperm sample (n = 10) was evaluated two times. CNT 0 h: control at 0 h of sperm liquid storage; CNT 72 h: control at 72 h of sperm liquid storage; EA 72 h: Ethacrynic acid (EA)-treated samples at 72 h of liquid storage. Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments.

3.7. Relative Content of GSTM3 Was Highly Correlated with ΔΨm and Motility

Pearson correlation coefficients of relative content of GSTM3-A and GSTM3-B at 0 h with sperm quality and functionality parameters of liquid-stored sperm at 72 h are shown in Table 1. No correlation between the relative abundance of GSTM3-A relative and any sperm quality or functionality parameters was found. However, the relative abundance of GSTM3-B was negatively correlated with total and progressive sperm motility and ΔΨm ($JC1_{agg}$) ($p < 0.05$).

Table 1. Pearson correlation coefficients between the relative GSTM3-A and GSTM3-B abundance at 0 h and sperm quality and functionality parameters at 72 h of liquid storage at 17 °C.

Sperm Quality and Functionality Parameters.	GSTM3-A	GSTM3-B
% total motile sperm	0.27	−0.93 **
% progressively motile sperm	0.18	−0.92 **
% high mitochondrial membrane potential sperm ($JC1_{agg}$)	0.03	−0.87 *
% total viable sperm ($SYBR14^+/PI^-$)	−0.15	0.45
% viable lipid membrane-destabilised sperm ($M540^+/YO\text{-}PRO\text{-}1^-$)	0.29	−0.48
% viable membrane-intact sperm ($PNA\text{-}FITC^-/PI^-$)	−0.05	0.10
% viable apoptotic-like spermatozoa ($Annexin\ V\text{-}FITC^+/PI^-$)	−0.05	0.34
% viable high-Ca^{2+} sperm ($Fluo3^+/PI^-$)	−0.66	0.88
% viable high-Ca^{2+} sperm ($Rhod5^+/YO\text{-}PRO\text{-}1^-$)	0.29	0.86
% viable high- H_2O_2 sperm (DCF^{++}/PI^-)	0.02	−0.70

*$p < 0.05$; **$p < 0.01$.

4. Discussion

Preservation of boar semen in liquid storage at 17 °C leads to a decrease in sperm metabolic activity in order to maintain their function and fertilising ability [2]. However, sperm liquid-preservation may result in impaired motility, viability, membrane stability, OS, and apoptotic-like changes [3,4]. GSTs in sperm are membrane-attached, detoxifying enzymes [14], which have been considered to be fertility [16], and cryotolerance [17] biomarkers in boar sperm. Furthermore, previous studies have shown that extender supplementation with glutathione decreases OS and improves the quality of boar semen during liquid storage at 17 °C [33]. Such findings suggest that GSTs play a vital role in maintaining sperm physiology during liquid preservation. However, its effects upon quality and functionality parameters of sperm have never been investigated. Findings from this work are in accordance with the aforementioned studies since GSTs-inhibition during boar semen storage was found to decrease sperm quality and function parameters dramatically.

The most noticeable effect of GSTs-inhibition was evidenced by the complete loss of total and progressive motility and a significant reduction in VAP within the first 24 h of semen liquid-storage at 17 °C. Such motility impairment is in agreement with previous studies performed in the goat, where sperm motility is known to decrease due to GSTs-inhibition [23]. Furthermore, the fact that GSTM3 was localised along the principal piece of boar sperm supports these results. In addition, not only did JC1 staining show a dramatic decrease in $\Delta\Psi m$ due to GSTs-inhibition, which was also described in goat sperm by Hemachand and Shaha [34], but $\Delta\Psi m$ was strongly and positively correlated with total motility. Correlation between these factors has been extensively reported in the literature, as adenosine triphosphate (ATP) production [35] and controlled ROS levels [36] are known to be required for proper sperm motility. Together, these findings suggest that sperm GSTs play an essential role in regulating mitochondrial function and motility performance during liquid-storage of boar semen.

The results of the present study have also confirmed that sperm plasma membrane status is impaired by GSTs-inhibition. Although the percentage of viable sperm was not significantly affected until 72 h of semen liquid-storage at 17 °C, the percentage of viable sperm with high membrane lipid disorder dramatically increased within the first 24 h of semen storage. Such findings are in agreement with previously-reported studies confirming that GSTs function is mainly located in the sperm plasma membrane [34], and their inhibition causes sperm membrane damage in goat sperm [23]. Along these lines, the present study provides evidence confirming that membrane-bound GSTs prevent cholesterol efflux and membrane lipid disorder, and thus delay capacitation-like changes in liquid-stored boar sperm. However, further experiments regarding the specific role of GSTs in sperm capacitation should be performed in order to clarify their specific role in the changes in the sperm plasma membrane.

Although the inhibition of sperm GSTs during liquid-storage was found to cause sperm membrane destabilisation, the acrosome membrane remained intact. These findings suggest that despite GSTs-inhibition increasing the lipid disorder of the sperm plasma membrane and causing capacitation like-changes in sperm cells, GSTs do not exert a direct effect on the acrosome membrane. On the other hand, and in agreement with the maintenance of sperm viability during the first hours of semen storage, apoptotic-like changes in sperm do not increase due to GSTs inhibition. Such results suggest that GSTs are not involved in apoptotic-like processes in sperm during boar semen liquid-storage. Likewise, GSTs were found to be involved in sperm intracellular Ca^{2+} content release. Intracellular Ca^{2+} levels from the mid-piece and sperm head were observed to increase within 24 h of semen storage due to GSTs-inhibition, whereas Ca^{2+} levels in sperm head did not. These findings indicate that the inhibition of sperm GSTs augment Ca^{2+} levels in the sperm mid-piece rather than in the head. While mitochondrial Ca^{2+} signalling is not completely understood, such organelles are known to function as intracellular Ca^{2+} stores, since the negatively charged mitochondrial matrix can sequester Ca^{2+} ions [36]. The impairment of mitochondrial Ca^{2+} homeostasis due to GSTs-inhibition may be caused by the destabilisation of mitochondrial membranes. However, further research is required in order to elucidate this hypothesis. In spite of the aforementioned, these results evidence, altogether,

the crucial role of sperm GSTs in the regulation of mitochondrial Ca^{2+} homeostasis during liquid-storage of boar semen.

Our results also demonstrated that the inhibition of GSTs led to changes in the physiological ROS levels of sperm during storage at 17 °C. Although the percentage of O_2^{-}•-positive sperm increased because of GSTs-inhibition, intracellular levels of H_2O_2 decreased. The fact that the main ROS source in sperm is thought to reside in the mitochondria [37], which have been shown to be impaired by GSTs-inhibition, supports the apparent role of GSTs in sperm ROS production. Impaired mitochondrial activity by GSTs-inhibition may contribute to the formation, but not removal of the O_2^{-}• in sperm [38], which could explain the high percentage of O_2^{-}•-positive sperm in GSTs-inhibited samples. Interestingly, while H_2O_2 is generated by SOD using O_2^{-}• as substrate, H_2O_2 levels in GSTs-inhibited sperm were seen to decrease. This apparent contradiction can be easily addressed. In addition to GSTs, other relevant antioxidant systems in sperm, such as GPX, CAT, and PRDX, have been shown to modulate physiological H_2O_2 levels [39]. Furthermore, the formation of H_2O_2 by SOD could be reduced due to GSTs inhibition, since this NADPH-dependent enzyme is blocked by the lack of reducing power caused by mitochondrial impairment. Consequently, the arrest in H_2O_2 generation but the continuous removal of this electrophilic compound could explain its reduction during GSTs-inhibition. However, the analysis of NADPH generation would be required in order to confirm this hypothesis. Along these lines, the inhibition of the detoxification function in sperm GSTs was found to enhance the formation of O_2^{-}• and to reduce that of H_2O_2. These results unveil the essential role played by GSTs, which, together with other antioxidant systems, regulate physiological ROS levels in sperm and protect them from OS [11]. Therefore, our study could serve as a basis for further studies aimed at clarifying the specific role of GSTs during sperm capacitation and fertilisation, as physiological ROS levels are essential for both processes.

Results from the immunoblotting analysis of GSTM3 showed a specific two-band pattern consisting of ~ 25 (GSTM3-A) and ~ 28 (GSTM3-B) kDa-bands, and a non-specific band of ~48 kDa. The double-band pattern found in the present work could be caused as a result of post-translational modifications of GSTM3 such as phosphorylation, acetylation or glycosylation, among others, which are widely reported in the literature (reviewed by [40]). Quantification of both bands showed higher relative levels of GSTM3-A than GSTM3-B at 0 h of liquid storage. Furthermore, GSTM3-A at 0 h showed higher relative levels than GSTM3-A and GSTM3-B after 72 h of liquid storage in control and GSTs-inhibited samples. Therefore, the results shown herein indicate that a loss of GSTM3 content occurs during liquid storage at 17 °C. However, inhibition of GSTs does not induce changes in the GSTM3 content. In this regard, the preservation of boar semen in liquid storage could induce GSTM3 loss and, consequently, impairment of its function. Nevertheless, a specific assay confirming the presence of post-translational modifications should be performed to gain further insights into the molecular action of GSTM3 in sperm.

The localisation patterns of GSTs in boar sperm during liquid preservation has been established for the first time in the present study. Sperm GSTM3 was localised in the mid, principal, and end pieces of the tail and the equatorial subdomain of the head of samples at 0 h of liquid-storage. This localisation pattern is similar to that found in the boar [17] and other species, such as the buffalo [41]. Moreover, the localisation of GSTM3 in the sperm tail would contribute to explaining the dramatic effect of GSTs-inhibition upon sperm motility and mitochondrial function. Interestingly, the GSTM3 signal was found to be partially reduced from the mid-piece during boar semen liquid-storage in both the control and GSTs-inhibited samples. Since immunoblotting analysis found GSTM3 content to be reduced during semen storage, it becomes apparent that such enzyme is lost rather than relocalised from the mid-piece during liquid-storage. Contrary to the results of the present study, GSTM3 was reported to relocalise to the mid-piece following boar sperm cryopreservation [17].

Finally, the present study also attempted to find a relationship between sperm quality and functionality parameters after 72 h of liquid storage and the relative amounts of GSTM3 at 0 h. Interestingly, a negative correlation between relative levels of GSTM3-B at 0 h and motility and

mitochondrial function after 72 h of sperm preservation was observed. Mounting evidence in the literature supports the relationship between GSTM3 and mitochondrial function, since GSTM3 content is known to be higher in mitochondrial-altered sperm of men [42]. Moreover, recent studies demonstrated that GSTM3 content in fresh sperm is highly correlated to the mitochondrial activity of frozen-thawed sperm, and relocalisation of this enzyme from the entire tail to the mid-piece occurs during cryopreservation of boar [17] and buffalo [41] sperm. Therefore, the relationship between GSTM3 content and mitochondrial activity found in the present study strengthens the hypothesis of a tight molecular relationship between sperm GSTs and mitochondrial function. Moreover, GSTM3 is clearly related to sperm quality, as it has been established as a quality [42–44], fertility [16], and cryotolerance [17] biomarker in both boar and human sperm. Hence, one could suggest that the GSTM3 content in fresh boar semen may be used as a biomarker of sperm quality during liquid preservation.

5. Conclusions

In conclusion, the data reported in the present study revealed the essential role of membrane-attached sperm GSTs to preserve sperm function and quality in liquid-stored boar semen. Specifically, inhibition of sperm GSTs evidenced that these enzymes are highly related to the preservation of mitochondrial function and maintenance of the plasma membrane stability, thus preserving sperm motility, maintaining physiological ROS levels, and regulating mitochondrial Ca^{2+} homeostasis. In addition, this study identified and localised GSTM3 for the first time in boar sperm during storage at 17 °C for 72 h. GSTM3 was localised in the mid, principal and end pieces of the tail and the equatorial subdomain of the head, and was partially lost from the mid-piece after 72 h of liquid preservation. Matching with this, immunoblotting showed that the relative amounts of sperm GSTM3 decreased after 72 h of liquid storage at 17 °C. Additionally, relative GSTM3-content at 0 h of storage was negatively correlated to sperm mitochondrial function and motility after 72 h of storage, supporting the mitochondrial-protective role of GSTs and suggesting GSTM3 as a putative biomarker of sperm quality during semen liquid-storage. Finally, while the molecular role of GSTs on sperm physiology and specifically on mitochondrial function is yet to be elucidated, the findings reported in this study warrant further research testing the supplementation of boar semen extender with GSTs, as this may preserve sperm mitochondrial function and plasma membrane stability during liquid storage and improve subsequent reproductive performance of boar AI-doses.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/2/100/s1, Additional File 1: supplementary information for Materials and Methods.

Author Contributions: Conceptualisation—M.L., M.Y. and I.B.; methodology—M.L., M.Y. and I.B.; investigation—M.L, A.D.-B., S.O.; data curation—M.L. and M.Y.; writing—original draft preparation—M.L.; review and editing—M.Y., I.B., A.D.-B., Y.M.-O., S.R., S.B., B.F.-F.; funding acquisition—M.Y., B.F.-F., I.B., M.L. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge the support from the European Commission (H2020-MSCA-IF-79212); Ministry of Science, Innovation, and Universities, Spain (RYC-2014-15581, AGL2017-88329-R, FJCI-2017-31689, and FPU18/00666); and Regional Government of Catalonia, Spain (2017-SGR-1229).

Acknowledgments: The authors would like to thank the Technical Research Services, University of Girona for their technical support.

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

References

1. Johnson, L.A.; Weitze, K.F.; Fiser, P.; Maxwell, W.M.C. Storage of boar semen. *Anim. Reprod. Sci.* **2000**, *62*, 143–172. [CrossRef]
2. Yeste, M. State-of-the-art of boar sperm preservation in liquid and frozen state. *Anim. Reprod.* **2017**, *14*, 69–81. [CrossRef]

3. Waberski, D.; Henning, H.; Petrunkina, A.M. Assessment of storage effects in liquid preserved boar semen. *Reprod. Domest. Anim.* **2011**, *46*, 45–48. [CrossRef]
4. Kumaresan, A.; Kadirvel, G.; Bujarbaruah, K.M.; Bardoloi, R.K.; Das, A.; Kumar, S.; Naskar, S. Preservation of boar semen at 18 °C induces lipid peroxidation and apoptosis like changes in spermatozoa. *Anim. Reprod. Sci.* **2008**, *110*, 162–171. [CrossRef] [PubMed]
5. Di Meo, S.; Reed, T.T.; Venditti, P.; Victor, V.M. Harmful and beneficial role of ROS. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 1–2. [CrossRef] [PubMed]
6. Betteridge, D.J. What is oxidative stress? *Metabolism* **2000**, *49*, 3–8. [CrossRef]
7. Li, Q.; Shaoyong, W.; Li, Y.; Chen, M.; Hu, Y.; Liu, B.; Yang, G.; Hu, J. Effects of oligomeric proanthocyanidins on quality of boar semen during liquid preservation at 17 °C. *Anim. Reprod. Sci.* **2018**, *198*, 47–56. [CrossRef]
8. Brzezińska-Ślebodzińska, E.; Ślebodziński, A.B.; Pietras, B.; Wieczorek, G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol. Trace Elem. Res.* **1995**, *47*, 69–74. [CrossRef]
9. De Lamirande, E.; Lamothe, G. Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radic. Biol. Med.* **2009**, *46*, 502–510. [CrossRef]
10. Aitken, R.J.; Whiting, S.; De Iuliis, G.N.; McClymont, S.; Mitchell, L.A.; Baker, M.A. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J. Biol. Chem.* **2012**, *287*, 33048–33060. [CrossRef]
11. O'Flaherty, C. The enzymatic antioxidant system of human spermatozoa. *Adv. Androl.* **2014**, *2014*, 1–15.
12. Hayes, J.D.; Flanagan, J.U.; Jowsey, I.R. Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 51–88. [CrossRef] [PubMed]
13. Llavanera, M.; Mateo, Y.; Bonet, S.; Barranco, I.; Fernández-Fuertes, B.; Yeste, M. The triple role of glutathione S-transferases in mammalian male fertility. *Cell. Mol. Life Sci.* **2019**, *77*, 1–12. [CrossRef]
14. Hemachand, T.; Gopalakrishnan, B.; Salunke, D.M.; Totey, S.M.; Shaha, C. Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. *J. Cell Sci.* **2002**, *115*, 2053–2065. [PubMed]
15. Kodama, H.; Kuribayashi, Y.; Gagnon, C. Effect of sperm lipid peroxidation on fertilization. *J. Androl.* **1996**, *17*, 151–157. [PubMed]
16. Kwon, W.S.; Oh, S.A.; Kim, Y.J.; Rahman, M.S.; Park, Y.J.; Pang, M.G. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci. Rep.* **2015**, *5*, 1–10. [CrossRef] [PubMed]
17. Llavanera, M.; Delgado-Bermúdez, A.; Fernandez-Fuertes, B.; Recuero, S.; Mateo, Y.; Bonet, S.; Barranco, I.; Yeste, M. GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–11. [CrossRef]
18. Mathew, N.; Kalyanasundaram, M.; Balaraman, K. Glutathione S-transferase (GST) inhibitors. *Expert Opin. Ther. Pat.* **2006**, *16*, 431–444. [CrossRef]
19. Ploement, J.H.T.M.; Ommen, B.V.A.N.; Bogaards, J.J.P. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. *Xenobiotica* **1993**, *23*, 913–923. [CrossRef]
20. Yamada, T.; Kaplowitz, N. Binding of ethacrynic acid to hepatic glutathione-transferases in the rat. *Biochem. Pharmacol.* **1980**, *29*, 1205–1208. [CrossRef]
21. Awasthi, S.; Srivastava, S.K.; Ahmad, F.; Ahmad, H.; Ansari, G.A.S. Interactions of glutathione S-transferase-π with ethacrynic acid and its glutathione conjugate. *Biochim. Biophys. Acta* **1993**, *1164*, 173–178. [CrossRef]
22. Safarinejad, M.R.; Shafiei, N.; Safarinejad, S. The association of glutathione-S-transferase gene polymorphisms (GSTM1, GSTT1, GSTP1) with idiopathic male infertility. *J. Hum. Genet.* **2010**, *55*, 565–570. [CrossRef] [PubMed]
23. Gopalakrishnan, B.; Shaha, C. Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett.* **1998**, *422*, 296–300. [CrossRef]
24. Garner, D.L.; Johnson, L.A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **1995**, *53*, 276–284. [CrossRef] [PubMed]
25. Yeste, M.; Estrada, E.; Rivera del Álamo, M.-M.; Bonet, S.; Rigau, T.; Rodríguez-Gil, J.-E. The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17 °C is concomitant with a higher cryotolerance of boar spermatozoa. *PLoS ONE* **2014**, *9*, e90887. [CrossRef]
26. Nagy, S.; Jansen, J.; Topper, E.K.; Gadella, B.M. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol. Reprod.* **2003**, *68*, 1828–1835. [CrossRef]

27. Ortega-Ferrusola, C.; Sotillo-Galan, Y.; Varela-Fernandez, E.; Gallardo-Bolanos, J.M.; Muriel, A.; Gonzalez-Fernandez, L.; Tapia, J.A.; Pena, F.J. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. *J. Androl.* **2007**, *29*, 213–221. [CrossRef]
28. Harrison, R.A.P.; Mairet, B.; Miller, N.G.A. Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations. *Mol. Reprod. Dev.* **1993**, *35*, 197–208. [CrossRef]
29. Yeste, M.; Fernández-Novell, J.M.; Ramió-Lluch, L.; Estrada, E.; Rocha, L.G.; Cebrián-Pérez, J.A.; Muiño-Blanco, T.; Concha, I.I.; Ramírez, A.; Rodríguez-Gil, J.E. Intracellular calcium movements of boar spermatozoa during "in vitro" capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. *Andrology* **2015**, *3*, 729–747. [CrossRef]
30. Hayato, R.; Higure, Y.; Kuba, M.; Nagai, H.; Yamashita, H.; Kuba, K. β3-Adrenergic activation of sequential Ca^{2+} release from mitochondria and the endoplasmic reticulum and the subsequent Ca^{2+} entry in rodent brown adipocytes. *Cell Calcium* **2011**, *49*, 400–414. [CrossRef]
31. De la Fuente, S.; Fonteriz, R.I.; Montero, M.; Alvarez, J. Dynamics of mitochondrial [Ca^{2+}] measured with the low-Ca^{2+}-affinity dye rhod-5N. *Cell Calcium* **2012**, *51*, 65–71. [CrossRef] [PubMed]
32. Guthrie, H.D.; Welch, G.R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J. Anim. Sci.* **2006**, *84*, 2089–2100. [CrossRef] [PubMed]
33. Zhang, X.G.; Liu, Q.; Wang, L.Q.; Yang, G.S.; Hu, J.H. Effects of glutathione on sperm quality during liquid storage in boars. *Anim. Sci. J.* **2016**, *87*, 1195–1201. [CrossRef] [PubMed]
34. Hemachand, T.; Shaha, C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett.* **2003**, *538*, 14–18. [CrossRef]
35. Ford, W.C.L. Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round? *Hum. Reprod. Update* **2006**, *12*, 269–274. [CrossRef]
36. Amaral, A.; Lourenço, B.; Marques, M.; Ramalho-Santos, J. Mitochondria functionality and sperm quality. *Reproduction* **2013**, *146*, 163–174. [CrossRef]
37. Storey, B.T. Mammalian sperm metabolism: Oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* **2008**, *52*, 427–437. [CrossRef]
38. Han, D.; Williams, E.; Cadenas, E. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. *Biochem. J.* **2001**, *353*, 411–416. [CrossRef]
39. O'Flaherty, C.; Rico de Souza, A. Hydrogen peroxide modifies human sperm peroxiredoxins in a dose-dependent manner. *Biol. Reprod.* **2010**, *84*, 238–247. [CrossRef]
40. Samanta, L.; Swain, N.; Ayaz, A.; Venugopal, V.; Agarwal, A. Post-translational modifications in sperm proteome: The chemistry of proteome diversifications in the pathophysiology of male factor infertility. *Biochim. Biophys. Acta Gen. Subj.* **2016**, *1860*, 1450–1465. [CrossRef]
41. Kumar, R.; Singh, V.K.; Atreja, S.K. Glutathione-S-transferase: Role in buffalo (*Bubalus bubalis*) sperm capacitation and cryopreservation. *Theriogenology* **2014**, *81*, 587–598. [CrossRef] [PubMed]
42. Intasqui, P.; Camargo, M.; Antoniassi, M.P.; Cedenho, A.P.; Carvalho, V.M.; Cardozo, K.H.M.; Zylbersztejn, D.S.; Bertolla, R.P. Association between the seminal plasma proteome and sperm functional traits. *Fertil. Steril.* **2015**, *105*, 617–628. [CrossRef] [PubMed]
43. Botta, T.; Blescia, S.; Martínez-Heredia, J.; Lafuente, R.; Brassesco, M.; Ballescà, J.L.; Oliva, R. Identificación de diferencias proteómicas en muestras oligozoospérmicas. *Rev. Int. Androl.* **2009**, *7*, 14–19. [CrossRef]
44. Agarwal, A.; Sharma, R.; Durairajanayagam, D.; Ayaz, A.; Cui, Z.; Willard, B.; Gopalan, B.; Sabanegh, E. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reprod. Biol. Endocrinol.* **2015**, *13*, 1–22. [CrossRef]

PAPER II

Exploring Seminal Plasma GSTM3 as a Quality and In Vivo Fertility Biomarker in Pigs-Relationship with Sperm Morphology

Marc Llavanera, Ariadna Delgado-Bermúdez, Yentel Mateo-Otero, Lorena Padilla, Xavier Romeu, Jordi Roca, Isabel Barranco & Marc Yeste

Antioxidants

2020;9(8), 741

DOI: 10.3390/antiox9080741

antioxidants

MDPI

Article

Exploring Seminal Plasma GSTM3 as a Quality and In Vivo Fertility Biomarker in Pigs—Relationship with Sperm Morphology

Marc Llavanera [1,2], Ariadna Delgado-Bermúdez [1,2], Yentel Mateo-Otero [1,2], Lorena Padilla [3], Xavier Romeu [1], Jordi Roca [3], Isabel Barranco [1,2,*,†] and Marc Yeste [1,2,*,†]

1 Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain; marc.llavanera@udg.edu (M.L.); ariadna.delgado@udg.edu (A.D.-B.); yentel.mateo@udg.edu (Y.M.-O.); u1941676@campus.udg.edu (X.R.)

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

3 Department of Medicine and Animal Surgery, Veterinary Science, University of Murcia, ES-30100 Murcia, Spain; lorenaconcepcion.padilla@um.es (L.P.); roca@um.es (J.R.)

* Correspondence: isabel.barranco@udg.edu (I.B.); marc.yeste@udg.edu (M.Y.); Tel.: +34-972-419514 (I.B. & M.Y.)

† Both authors have contributed equally to the direction of this study.

Received: 1 July 2020; Accepted: 10 August 2020; Published: 12 August 2020

Abstract: Glutathione S-transferases Mu 3 (GSTM3) is an essential antioxidant enzyme whose presence in sperm has recently been related to sperm cryotolerance, quality and fertility. However, its role in seminal plasma (SP) as a predictor of the same sperm parameters has never been investigated. Herein, cell biology and proteomic approaches were performed to explore the presence, origin and role of SP-GSTM3 as a sperm quality and in vivo fertility biomarker. GSTM3 in SP was quantified using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit specific for *Sus scrofa*, whereas the presence of GSTM3 in testis, epididymis and accessory sex glands was assessed through immunoblotting analysis. Sperm quality and functionality parameters were evaluated in semen samples at 0 and 72 h of liquid-storage, whereas fertility parameters were recorded over a 12-months as farrowing rate and litter size. The presence and concentration of GSTM3 in SP was established for the first time in mammalian species, predominantly synthesized in the epididymis. The present study also evidenced a relationship between SP-GSTM3 and sperm morphology and suggested it is involved in epididymal maturation rather than in ejaculated sperm physiology. Finally, the data reported herein ruled out the role of this antioxidant enzyme as a quality and in vivo fertility biomarker of pig sperm.

Keywords: pig; fertility; GSTM3; quality; seminal plasma

1. Introduction

Artificial insemination (AI) is one of the major breakthroughs of pig reproductive biotechnology and has become the main technique for the breeding of this species worldwide, being an essential tool to achieve productivity challenges in swine industry [1]. Although AI can be performed using both frozen-thawed and liquid-stored sperm at 17 °C, the latter is used in the vast majority of pig AI [2,3]. While pig ejaculates are selected on the basis of sperm quality parameters prior to AI (i.e., motility, morphology and plasma membrane integrity), farrowing rates are often suboptimal after liquid-storage [3,4]. In this regard, it is estimated that about 6% of spermiogram-normal AI-pigs are subfertile individuals that remain "hidden," which could lead to reproductive and economic

losses [4,5]. Hence, exploring new sperm quality and fertility molecular biomarkers is essential to improve subfertility diagnosis and subsequent reproductive performance of pig AI-doses.

Pig seminal plasma (SP) is a complex mixture of secretions from testis, epididymis, seminal vesicles, bulbourethral glands and prostate that provides the physiological conditions for sperm during and after ejaculation [6]. As a result, SP is essential to preserve sperm metabolism and physiological status [7]. The complex composition of SP makes it likely to be a promising source of sperm quality and fertility biomarker candidates. Specifically, the protein fraction of SP has been found to be especially relevant for both sperm function and interaction with the female genital tract, even being essential for fertilization (reviewed from Reference [8]). Identification and quantification of differentially expressed proteins is known as comparative proteomics. The application of this emerging approach for the identification of novel SP quality and fertility biomarkers is currently flourishing [9]. In this regard, great efforts have been devoted to exploring new biomarkers in pig SP through comparative proteomics [10–13]. Specifically, recent studies uncovered the role of antioxidant enzymes in SP, such as glutathione peroxidase 5 (GPX5) and paraoxonase 1 (PON1), as sperm cryotolerance, quality and/or fertility biomarkers [14–16].

Glutathione S-transferases (GSTs) are essential antioxidant enzymes involved in cellular protection against oxidative stress, preserving sperm function and fertilizing ability (reviewed in Reference [17]). Antioxidant enzymes are known to be especially relevant for sperm cells since they are highly sensitive to oxidative stress due to the high amount of polyunsaturated fatty acids and their relatively low antioxidant capacity. Recent studies in pig and goat sperm evidenced the significance of GSTs in mammalian sperm physiology, highlighting their role on preserving mitochondrial function and maintaining plasma membrane stability [18,19]. Recently, the triple role of sperm GSTs has been well-established, being involved in cell detoxification, cellular signaling regulation and sperm-zona pellucida binding events [17]. In addition, previous research, including three recent meta-analysis, confirmed that some GSTs null-genotypes are a risk factor for male idiopathic subfertility or infertility in men [20–27]. Specifically, Aydemir et al. [28] reported that men with a specific GST Mu class null genotype showed increased oxidative stress in SP. Moreover, sperm GST Mu 3 (GSTM3) has been recently proposed as a quality [18], fertility [29] and cryotolerance [30] biomarker for pig sperm. Mounting evidence demonstrates the extracellular membrane-attached localization of sperm GSTs (reviewed from Reference [17]), surmising a potential direct relationship between sperm and SP-GSTM3. Therefore, SP-GSTM3 is a promising candidate to sperm quality and fertility biomarker.

In this regard, while sperm GSTM3 is well-characterized, the presence and putative function of seminal plasma GSTM3 (SP-GSTM3) on sperm function and fertility outcomes is yet to be investigated. Exploring the presence and role of pig SP-GSTM3 as a sperm quality and in vivo fertility biomarker is of utmost importance since it could improve and facilitate male subfertility diagnosis as well as give us some new insights into its molecular role as an antioxidant sperm enzyme.

2. Materials and Methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Fluorochromes were purchased from Molecular Probes (Thermo Fisher Scientific; Waltham, MA, USA). All reagents are listed in the Table S1.

2.2. Animals and Samples

Semen and tissue samples were provided by an AI Spanish Centre (AIM Ibérica; Topigs Norsvin Spain SLU; Spain registration number (ES300130640127; August 2006) and European Union registration number (ES13RS04P; July 2012)). Production of the seminal AI-doses used in this study followed the current Spanish and European legislation for both commercialization of pig semen and animal health and welfare. Entire ejaculates were collected from 36 healthy and sexually mature (1–3 years-old)

AI-pigs of four different breeds (Duroc (n = 8), Landrace (n = 13), Large White (n = 6) and Pietrain (n = 9)). A semi-automatic collection method was used (Collectis®, IMV Technologies, L'Aigle, France). Pigs were undergoing twice semen collections per week at the time of sample obtention.

2.3. Experimental Design

2.3.1. Relationship Between SP-GSTM3 Concentration and Sperm Quality and Functionality Parameters

Entire ejaculates from 20 AI-boars (one ejaculate per boar) were collected and split into three aliquots. The first aliquot was used to assess sperm concentration and morphology. The second one was extended like an AI-dose (30×10^6 sperm/mL in Biosem+; Magapor S.L., Ejea de los Caballeros, Spain) and used to evaluate sperm quality and functionality parameters immediately after ejaculate collection (0 h) and after 72 h of storage at 17 °C. The third aliquot was centrifuged twice at 1500× g for 10 min at room temperature (RT) to harvest SP. Thereafter, SP samples were examined under a microscope (Eclipse E400; Nikon, Tokyo, Japan) ensuring the absence of sperm. Finally, SP samples were immediately aliquoted (3 mL) and stored at −80 °C until thawed for SP-GSTM3 concentration analysis.

2.3.2. Expression of GSTM3 in Boar Testis, Epididymis and Accessory Sexual Glands

In order to uncover the putative contribution of the testis, epididymis and accessory sexual glands to SP-GSTM3 content, a total of three healthy AI-boars were slaughtered (slaughterhouse La Mata de los Olmos, Teruel, Spain) for genetic replacement reasons. Genital tracts (medial testis; caput, corpus and cauda of the epididymis; mid-areas of the prostate; seminal vesicles; and bulbourethral glands) were dissected out to collect tissue samples (1 cm × 1 cm and 1 mm thick) and immediately frozen into liquid nitrogen and stored at −80 °C until Western blot analysis.

2.3.3. Relationship Between SP-GSTM3 Content and In Vivo Fertility of Liquid-Stored Semen Samples

Three entire ejaculates from 16 AI-boars were collected over a 12-month period (one ejaculate every 4 months). These ejaculates were centrifuged for SP harvesting and the resulting SP-samples were stored at −80 °C for GSTM3 content analysis. For this 12-month period, weaned multiparous sows (1–7 litters produced) were cervically inseminated (2–3 times per estrus) using AI-doses (2400×10^6 of total spermatozoa in 80 mL Biosem+) from these 16 AI-boars. Sows (Landrace and Large White) were housed in different Spanish farms and subjected to the same housing and management conditions. Fertility parameters, that is, farrowing rate (the proportion of inseminated sows that farrowed) and litter size (the total number of piglets born per litter), were recorded for this 12-month period. The number of inseminated sows was 3017 (more than 100 sows per boar).

2.4. Sperm Quality and Functionality Assessment

Sperm concentration, morphology, total and progressive motility, viability and acrosome integrity were assessed as quality parameters, whereas sperm membrane lipid disorder and intracellular hydrogen peroxide levels were assessed as functionality parameters.

Sperm concentration was measured automatically using a cell counter (NucleoCounter® NC-100™; ChemoMetec, Allerod, Denmark). Sperm morphology was assessed in semen samples extended (1:1; *v:v*) with 0.12% formaldehyde saline solution (Panreac, Barcelona, Spain). Sperm morphology evaluation was performed under a phase contrast microscope at 1000× magnification coupled with a SCA® Production software (Sperm Class Analyzer Production, 2010; Microptic S.L., Barcelona, Spain). A total of 200 sperm per sample were evaluated and classified into the following categories: morphologically normal spermatozoa, acrosome abnormalities, folded and coiled tails, proximal and distal droplets and abnormal head size and shape. Sperm motility was assessed through a computer assisted sperm analyzer (CASA, ISASV1®, Proiser R+D S.L., Paterna, Spain). For this assessment, 5 µL of sperm at 20×10^6 sperm/mL was loaded onto a pre-warmed (38 °C)

Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Two replicates per sample, with a minimum of 600 sperm per each replicate, were assessed. The recorded sperm motility parameters were the percentage of motile sperm, with an average path velocity ≥20 μm/s and the percentage of sperm with progressive movement, showing a straight-line velocity ≥40 μm/s. The corresponding mean ± SEM was subsequently calculated.

Sperm viability, acrosome damage, membrane lipid disorder and intracellular hydrogen peroxide levels were assessed by flow cytometry. Sperm parameters were evaluated using a BD FACS Canto II flow cytometer (Becton Dickinson & Company, Franklin Lakes, NJ, USA) in semen samples extended at 30×10^6 sperm per mL in Biosem+. Three technical replicates with a minimum of 10,000 sperm events positive to Hoechst 33342 (H-42) dye per sample were evaluated. Plasma membrane (viability) and acrosome integrities were assessed by triple-staining using H-42, propidium iodide (PI) and fluorescein-conjugated peanut agglutinin (PNA-FITC). Sperm samples (100 μL) were incubated with 3 μL of H-42 (0.5 mg/mL in PBS 1×), 2 μL of PI (0.5 mg/mL in PBS 1×) and 2 μL of PNA-FITC (100 μg/mL in PBS1×) for 10 min at 38 °C in the dark. Following this, sperm samples were diluted in 400 μL of PBS and subsequently analyzed through flow cytometry. Results were presented as the percentage of viable sperm ($H\text{-}42^+/PI^-$) with intact acrosome membrane ($PNA\text{-}FITC^-$).

Sperm membrane lipid disorder was assessed by incubating semen samples (50 μL) with 2.5 μL of H-42 (0.05 mg/mL in PBS 1×) and 10 μL of Yo-Pro-1 (2.5 μM in dymetil sulfoxide (DMSO)) for 8 min at 38 °C in the dark. Next, 26 μL of Merocyanine 540 (M-540, 0.1 mM in DMSO) was added to each sample prior to incubation for 2 min at 38 °C in the dark. Results were presented as the percentage of viable sperm ($H\text{-}42^+/Yo\text{-}Pro\text{-}1^-$) with high plasma membrane lipid disorder ($M\text{-}540^+$). Finally, intracellular hydrogen peroxide levels were evaluated through the incubation of sperm samples (50 μL) with 1.5 μL of H-42 (0.05 mg/mL in PBS 1×), 1 μL of PI (0.05 mg/mL in PBS 1×) and 1 μL of 5- and 6-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester ($CM\text{-}H_2DCFDA$; 1 mM in DMSO) in 950 μL of PBS for 30 min at 38 °C in the dark. A sample of each semen samples was incubated with 1 μL of tert-butyl hydroperoxide solution (70% in distilled water) and used as a positive control. Results are presented as the percentage of viable sperm ($H\text{-}42^+/Yo\text{-}Pro\text{-}1^-$) with high intracellular hydrogen peroxide levels (DCF^+).

2.5. Western Blot Analysis

Tissue samples from testis and accessory glands were lysed through a hybrid method combining both chemical and mechanical lysis. A total of 50 mg of tissue was resuspended in 800 μL of lysis buffer (xTractor™ Buffer; Takara Bio, Mountain View, CA, USA) supplemented with 50 U DNase I (Takara Bio), 1% protease inhibitor cocktail and sodium orthovanadate (700 mM). Samples were vortexed and incubated for 10 min at 4 °C. Subsequently, samples were disrupted mechanically four times through a TissueLyser II (Qiagen, Hilden, Germany) set at 30 strokes/s for 5 min at 4 °C. Finally, samples were centrifuged at 12,000× g for 30 min at 4 °C and supernatants were stored at −80 °C prior to total protein quantification. Quantification of total protein was carried out in triplicate by a detergent compatible (DC) method (BioRad). Fifteen micrograms of total protein were resuspended in Laemmli reducer buffer 2× (BioRad) and heated at 95 °C for 7 min. Subsequently, samples were loaded onto a gradient (8–16%) polyacrylamide gel (Mini-PROTEAN® TGX Stain-FreeTM Precast Gels, Bio-Rad) and electrophoresed at 150 V for 90 min. Next, total protein was visualized by UV exposition and acquisition using a G:BOX Chemi XL system (SynGene, Frederick, MD, USA). Following this, proteins from gels were transferred onto polyvinylidene fluoride (PVDF) membranes using Trans-Blot® TurboTM (Bio-Rad), which were subsequently blocked in blocking buffer (10 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween-20; pH = 7.3 and 5% bovine serum albumin (Roche Diagnostics, S.L., Basel, Switzerland) for 1 h at room temperature (RT). Blocked membranes were incubated with primary anti-GSTM3 antibody (ref. ARP53561_P050; 0.05 μg/mL) for 1 h with agitation at RT. Subsequently, membranes were washed thrice with TBS1×-Tween20 (10 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween-20; pH = 7.3) and incubated with a secondary anti-rabbit antibody conjugated with HRP for 1 h with

agitation (ref. P0448; 0.025 µg/mL) at RT. Finally, membranes were washed five times and visualized with a chemiluminescence substrate (Immobilion^{TM} Western Detection Reagents, Millipore) prior to be scanned with G:BOX Chemi XL 1.4. A peptide competition assays utilizing 20-fold GSTM3 immunizing peptide with regard to the antibody was performed to confirm the specificity of the GSTM3-primary antibody. Three replicates per sample were evaluated.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Porcine GSTM3 in SP-samples was quantified using a porcine-specific competitive ELISA kit (MBS7260929; MyBioSource, San Diego, CA, USA) following the manufacturer's guidelines. In brief, 100 µL of GSTM3 standards (0, 10, 25, 50, 100, 250 ng of GSTM3/mL) were loaded onto the corresponding wells to obtain the standard curve. The same volume of SP was loaded onto their corresponding wells. Then, samples and standards were incubated together with 50 µL of HRP-conjugated GSTM3 for 1 h at 37 °C. Subsequently, wells were washed five times and incubated with the substrate of HRP enzyme for 15 min at 37 °C. Finally, stop solution was added and the color intensity was measured spectrophotometrically at 450 nm in a microplate spectrophotometer (BioTek Epoch; BioTek, Winooski, Vermont, USA). A standard curve relating the absorbance (ABS) to the GSTM3 concentration of standards was plotted. The logarithmic regression curve was subsequently calculated and the GSTM3 concentration (GSTM3) of each sample was interpolated from the following standard curve:

$$[\text{GSTM3}] = -109.5 \ln(\text{ABS}) + 95,587 \; R^2 = 0.962. \quad (1)$$

The ELISA kit was highly specific for porcine GSTM3, with a sensitivity of 1.0 ng/mL and a detection range of 94–103%. The blank control wells contained PBS 1× (pH = 7.0). All standards and samples were loaded in duplicate.

2.7. Statistical Analysis

Data were evaluated using a statistical package (IBM SPSS Statistics 25.0; IBM Corp., Armonk, NY, USA). First of all, normal distribution and homogeneity of variances were tested through Shapiro-Wilk and Levene tests, respectively.

Differences of GSTM3 concentration in SP (SP-GSTM3) between breeds were tested through one-way analysis of variance (ANOVA; factor: breed; independent variable: SP-GSTM3) followed by post-hoc Sidak for pair-wise comparisons. Correlations between SP-GSTM3 and sperm quality and functionality parameters evaluated upon ejaculation (0 h) were determined through Pearson coefficient. Correlations between SP-GSTM3 and the variations within total and progressive sperm motility, viability, acrosome damage, intracellular hydrogen peroxide levels and membrane stability throughout liquid-storage at 17 °C (i.e., 0 vs. 72 h) were also calculated through Pearson correlation coefficient.

Boar reproductive performance data were corrected for parameters related to farm and sow through a multivariate statistical model, as described in Broekhuijse et al. [31]. The resulting deviations in fertility parameters (farrowing rate deviation, FR; and litter size deviation, LS) were used to classify the 16-AI boars into two groups (high FR and LS; low FR and LS). This classification was conducted through a hierarchical cluster analysis based on the nearest neighbor approach and the squared Euclidean distance (SED). Following this, the SP-GSTM3 concentration evaluated with ELISA was compared between the two fertility groups (i.e., high and low FR/LS) through a *t*-test for independent samples.

The level of significance was set at $p < 0.05$ and data are shown as mean ± standard error of the mean (SEM).

3. Results

3.1. Characterisation of Porcine SP-GSTM3

The GSTM3 concentration in SP from pig ejaculates was 61.62 ± 2.18 ng/mL, ranging from 38.26 to 81.82 ng/mL. No differences in SP-GSTM3 concentration levels were found between breeds ($p > 0.05$): Duroc (60.64 ± 4.34 ng/mL), Landrace (60.24 ± 3.47 ng/mL) and Pietrain (64.49 ± 3.87 ng/mL) (Figure 1).

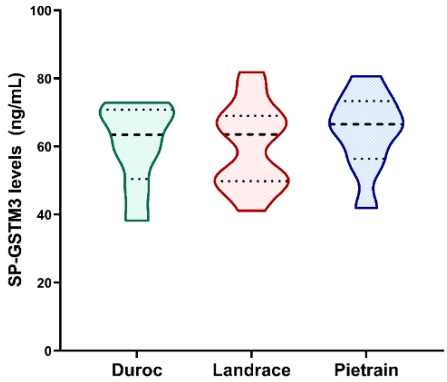

Figure 1. Violin plots showing seminal plasma GSTM3 (SP-GSTM3) concentration levels (ng/mL) and distribution from ejaculates of different pig breeds. Duroc, $n = 8$; Landrace, $n = 13$ and Pietrain, $n = 9$. Dashed line represents the median and dotted lines the 25 and 75% quartiles. No significant differences ($p > 0.05$) in SP-GSTM3 concentrations were found between breeds.

3.2. Correlation between SP-GSTM3 and Sperm Quality and Functionality Parameters of Semen Samples

Sperm quality and functionality parameters (mean ± SEM and range) of semen samples assessed immediately after ejaculation (0 h) are shown in Table 1.

Table 1. Sperm quality and functionality parameters of pig semen samples assessed immediately after ejaculation ($n = 20$).

Sperm Quality and Functionality Parameters	Mean ± SEM	Range (Min–Max)
Ejaculate volume (mL)	619.05 ± 21.28	357–729
Sperm concentration (106 sperm per mL)	171.93 ± 10.85	91.65–256
Viable sperm with intact acrosome (%)	84.73 ± 1.47	72.10–91.60
Motile sperm (%)	76.85 ± 2.03	51–90
Progressive motile sperm (%)	50 ± 2.36	26–66
Normal morphology (%)	77.95 ± 3.14	40–95
Coiled tails (%)	0.30 ± 0.13	0–2
Folded tails (%)	6.25 ± 1.26	0–19
Acrosome abnormalities (%)	3.32 ± 0.95	0–17
Proximal droplets (%)	6 ± 1.55	0–26
Distal droplets (%)	5.45 ± 1.57	0–29
Abnormal head size and shape (%)	0.90 ± 0.35	0–5
Viable sperm with high intracellular H2O2 (%)	30.69 ± 3.58	3.40–56.40
Viable sperm with high plasma membrane fluidity (%)	1.69 ± 0.19	0.50–3.50

Figure 2 shows Pearson correlation coefficients between SP-GSTM3 concentration and sperm quality and functionality parameters of semen samples immediately after ejaculation (0 h). No correlation between SP-GSTM3 concentration and sperm quality and functionality parameters were found ($p > 0.05$), except for morphology parameters. The SP-GSTM3 concentration was positively correlated with the percentage of sperm with normal morphology ($R = 0.501$; $p < 0.05$) and negatively

correlated with the percentages of sperm with proximal droplets (R = −0.454; $p < 0.05$), distal droplets (R = −0.604; $p < 0.05$) and coiled tails (R = −0.574; $p < 0.05$).

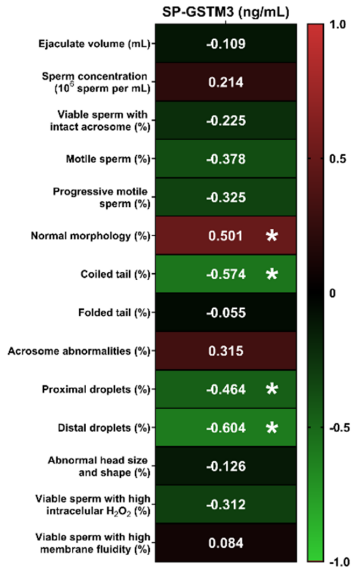

Figure 2. Heatmap of Pearson correlation coefficients between pig seminal plasma GSTM3 (SP-GSTM3) concentrations (ng/mL) and sperm quality and functionality parameters assessed in semen samples immediately after ejaculation ($n = 20$). * $p < 0.05$.

3.3. Relationship between SP-GSTM3 Concentration and Sperm Resilience to Withstand Liquid-Storage at 17 °C

Total and progressive sperm motility, viability, acrosome damage, intracellular hydrogen peroxide levels and membrane stability were assessed at 0 and 72 h of liquid-storage. The difference of the percentage in each sperm parameter between both evaluation time-points (0 and 72 h) was calculated to evaluate the putative relationship between the ability of semen samples to withstand liquid-storage at 17 °C and SP-GSTM3 concentration. Subsequently, the decline of each parameter between 0 and 72 h and SP-GSTM3 concentrations were compared through Pearson correlations (Figure 3). No significant correlations between SP-GSTM3 concentration and the difference in percentages of each sperm quality and functionality parameters between both evaluation time-points were found ($p > 0.05$).

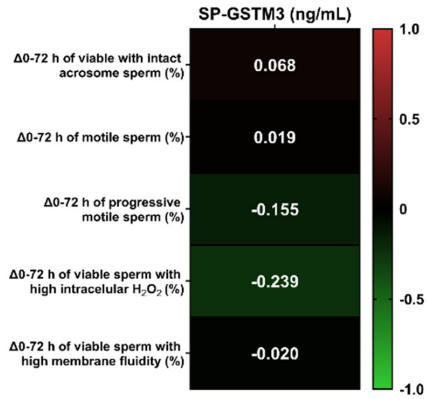

Figure 3. Heatmap of Pearson correlation coefficients between seminal plasma GSTM3 (SP-GSTM3) concentrations (ng/mL) and the differences of sperm quality and functionality parameters between 0 and 72 h of storage (n = 20). No significant correlations were found ($p > 0.05$).

3.4. Presence of GSTM3 in SP-Related Testis and Accessory Sexual Glands

Immunoblotting analysis were performed to elucidate the putative contribution of testis, epididymis and accessory glands on GSTM3 content in SP. As shown in Figure 4, immunoblotting of GSTM3 reported a single or double-band pattern of ~25 and ~75 kDa, depending on the tissue type. Specifically, the testis (T) and caput epididymis (HE) showed both ~25 and ~75 kDa-bands, whereas the corpus (BE) and caput (TE) of the epididymis, the prostate (P) and seminal vesicles (SV) showed a single ~75 kDa-band. Peptide competition assay confirmed the GSTM3-specificity of all bands. Remarkably, GSTM3 band-signal intensity was found to be higher in the caput epididymis than in other accessory sexual glands. However, no GSTM3 signal was found in bulbourethral glands (B).

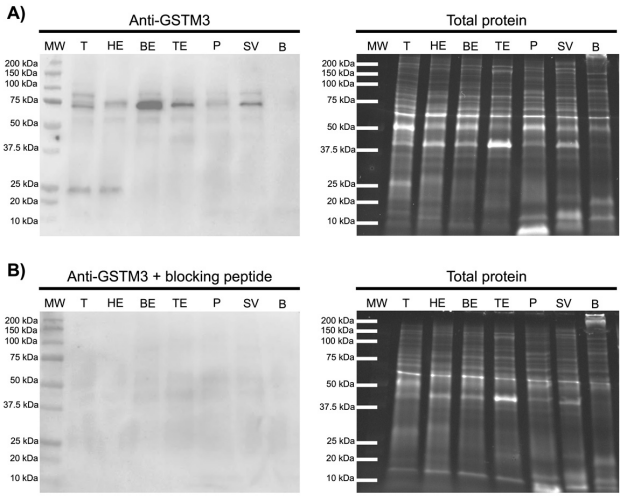

Figure 4. Representative Western blot resulting from incubation with the (**A**) GSTM3 antibody (Anti-GSTM3), (**B**) its corresponding peptide competition assay (Anti-GSTM3 + blocking peptide) and their loading controls (Total protein). MW: molecular weight. T: testis. HE: caput epididymis. BE: corpus epididymis. TE: cauda epididymis. P: prostate. SV: seminal vesicles. B: bulbourethral glands.

3.5. Relationship between SP-GSTM3 and In Vivo Fertility Outcomes

A total of 16-AI boars was classified through hierarchical clustering ($p < 0.001$) into two groups according to their farrowing rate and litter size deviation (low fertility and high fertility boars). The six AI-boars that exhibited the highest ($n = 3$) and lowest ($n = 3$) farrowing rate (FR) and litter size (LS) deviation were selected for SP-GSTM3 analysis (Figure 5A). A dot plot of FR and LS deviation showing the selected individuals from the 16 AI-boars is shown in Figure S1. Farrowing rate and litter size deviation significantly differed between fertility groups ($p < 0.05$; Figure 5A). The concentration of GSTM3 was assessed in SP-samples from three ejaculates of each boar. No differences were found when comparing SP-GSTM3 concentrations between SP-samples from low (64.66 ± 6.52 ng/mL) and high (64.66 ± 7.99 ng/mL) fertility groups ($p > 0.05$) (Figure 5B).

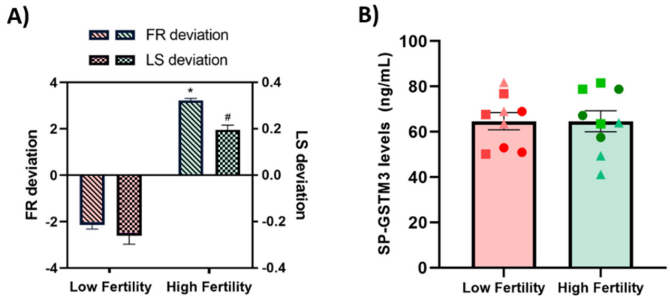

Figure 5. (**A**) Mean ± standard error of the mean (SEM) of farrowing rate (FR; lines) and litter size (LS; squares) deviation of samples classified as low fertility (red) and high fertility (green) boars. The six Artificial Insemination (AI)-boars were classified as having low ($n = 3$) or high ($n = 3$) fertility outcomes deviation (FR and LS). Different symbols (*, #) indicate significant differences ($p < 0.05$) between fertility groups. (**B**) Mean ± SEM of the concentration (ng/mL) of GSTM3 in seminal plasma (SP-GSTM3) were assessed in three ejaculates per boar, categorized as low (red) and high (green) fertility boars. Different symbols (●, ■, ▲) represent different ejaculates from the same boar within a fertility group. No significant differences in SP-GSTM3 content were found between fertility groups.

4. Discussion

Given the role of sperm GSTM3 as a sperm quality [18], fertility [29] and cryotolerance [30] biomarker and its extracellular membrane-attached localization in mammalian species (reviewed in Reference [17]), SP-GSTM3 is likely to be related to those sperm parameters. Therefore, it is reasonable to suggest that exploring SP-GSTM3 as a sperm quality and in vivo fertility biomarker may improve the evaluation of reproductive performance of pig AI-doses. To the best of our knowledge, this is the first report confirming the presence of GSTM3 in SP of mammals, which underpins the contribution of testis, epididymis and accessory sexual glands to GSTM3 content in SP and assessing the putative role of SP-GSTM3 as a molecular biomarker.

The results of the present study confirmed the presence and concentration of GSTM3 in SP for the first time in any species. The average SP-GSTM3 concentration was 61.62 ± 2.18 ng/mL, ranging from 38.26 to 81.82 ng/mL. As far as we are aware, no information regarding GSTs concentration in SP has been reported in the literature. However, the mean concentration of GSTM3 in pig SP was higher from other antioxidant enzymes such as glutathione peroxidase 5 [14] (GPX5; 9.63–30.13 ng/mL) and paraoxonase 1 [32] (PON1; 0.96–1.67 ng/mL). Another objective of the present study was to compare SP-GSTM3 levels between pig breeds. Although differences in ejaculate volume, sperm concentration and percentage of viable sperm have been extensively reported across pig breeds [33], our results did not show differences in the SP-GSTM3 content between Duroc, Landrace and Pietrain breeds. In this context, it is worth bearing in mind that the class-clustered organization of GST genes in both plant and animals reveals their importance during evolutionary history. Furthermore, GSTs are ubiquitous

and highly conserved enzymes among species (reviewed from Reference [17]). Therefore, the fact that GSTs are highly conserved proteins would support the similar GSTM3 concentrations found in the SP of these three pig breeds.

The putative relationship between SP-GSTM3 concentration and sperm quality and functionality parameters was measured using Pearson correlation coefficients. While sperm GSTM3 has been proposed as a quality [18] and cryotolerance [30] biomarker, the role of SP-GSTM3 as a predictor of quality and functionality of ejaculated sperm had never been explored. Interestingly, in the present study, no correlations of SP-GSTM3 with ejaculate volume, sperm concentration, motility, viability, acrosome damage, membrane lipid disorder and ROS levels were found. However, a clear relationship between SP-GSTM3 and sperm morphology was observed. Specifically, higher concentrations of GSTM3 in SP were significantly associated to a lower percentage of sperm exhibiting proximal and distal droplets and coiled tails. Concomitantly, higher SP-GSTM3 levels were related to a higher percentage of sperm with normal morphology. It is widely known that sperm malformations could have their origin in the testis (primary malformations) or in the epididymis (secondary malformations) [34,35]. All sperm abnormal morphologies related to SP-GSTM3 (proximal and distal droplets and coiled tails) are categorized as secondary malformations and therefore are a result of an inadequate or poor epididymal maturation. Cytoplasmatic droplets are originated in the testis and move distally during epididymal maturation [35,36]. Both distal and proximal droplets are considered as sperm malformations since they have been related to male infertility in domestic species and indicate the failure of epididymal maturation (reviewed from References [35,36]). On the other hand, coiled tails are formed during sperm epididymal maturation, probably because of the weakness of dense fibers [37]. Other studies in men showed significant correlations between sperm morphology and the content and/or activity of some antioxidant enzymes in SP such as superoxide dismutase (SOD), catalase (CAT) and GPX [38,39]. Against this background, it is suggested that GSTM3 in SP plays a key role during epididymal maturation and is proposed as a sperm morphology biomarker candidate. However, further research regarding its molecular role upon sperm epididymal maturation is required to confirm this hypothesis.

Immunoblotting analyses of the testis, epididymis and accessory glands were performed to elucidate their contribution to SP-GSTM3 secretion. The presence of GSTM3 was confirmed by a double-band pattern of ~25 and ~75 kDa in the testis and cauda epididymis and a single band of ~75 kDa in the corpus and cauda epididymis, the prostate and seminal vesicles. No GSTM3-signal was found in bulbourethral glands. Previous studies in pigs reported a single band of ~25 kDa in sperm samples [29,30], which corresponds to its molecular mass. The GSTM3-specific ~75 kDa-band reported herein in tissue samples could be attributed to either GSTM3 homo- or hetero-trimerization; however, further research to confirm this hypothesis is much warranted. Expression of GSTM3 was found to be higher in the epididymis than the testis and accessory glands. As aforementioned, SP is a mixture of secretions from the testis, epididymis and accessory sexual glands. In this regard, the testis, epididymis and accessory glands, except for bulbourethral glands, contribute to GSTM3 content in SP. Moreover, the fact that SP-GSTM3 is mainly synthesized in the epididymis is an evidence that would support the role of this enzyme during epididymal maturation and the occurrence of secondary sperm morphology abnormalities. Accordingly and based on the results of the present study, poor synthesis of GSTM3 in the epididymis could lead to an inadequate epididymal maturation of sperm, which could be detected in SP.

The ability of SP-GSTM3 of predicting sperm resilience to withstand liquid-storage at 17 °C was assessed for the first time in any mammalian species. Considering that other antioxidant enzymes in SP such as GPX5 or SOD have shown to be quality predictors of AI-doses during liquid-storage [14,40], GSTM3 would also be expected to be a good biomarker. Although recent reports showed sperm-GSTM3 as a biomarker of sperm resilience to withstand liquid-storage and cryopreservation [18,30], our findings did not find SP-GSTM3 to be a good predictor. In effect, the results reported herein did not show significant correlations between SP-GSTM3 concentrations and the decline in sperm quality and

functionality parameters during liquid-storage of semen at 17 °C. Different studies in goats and pigs showed the importance of sperm membrane-attached GSTM3 for mitochondrial function, plasma membrane stability and oxidative regulation [18,19], thus evidencing its molecular role in sperm physiology. The lack of correlation between SP-GSTM3 content and the sperm resilience to withstand liquid-storage would indicate the absence of molecular effects of this antioxidant enzyme upon sperm physiology. It is hypothesized that the presence of GSTM3 in SP could correspond to the remaining content of its activity in the epididymis during sperm maturation, rather than being physiological active upon ejaculated sperm.

Finally, the role of SP-GSTM3 to be an in vivo fertility biomarker was explored. The relevance of antioxidant enzymes from SP as fertility biomarkers is not clear, since it has been found to differ between molecular types. Recent studies showed the importance of SP-GPX5 as a relevant fertility biomarker of pig semen [14], whereas SOD turned out not to be related to sperm fertilizing ability [40]. Although sperm GSTM3 was stablished as an in vivo fertility biomarker in pigs [29], the results of the present study did not show any effect of SP-GSTM3 concentration upon in vivo fertility outcomes of AI-boars. However, the ejaculates used here were obtained from an AI-center, which selects their boars on the basis of their reproductive performance. Therefore, the good fertility of the boars used in this study could mask the real effects of SP-GSTM3 on its fertility. Conducting similar experiments using non-selected species, such as humans, is recommended to confirm our results in other mammalian species.

5. Conclusions

In conclusion, the data reported in the present study established the presence and concentration of GSTM3 in pig SP, remaining similar between boar breeds. On the other hand, SP-GSTM3 was reported to be predominantly synthesized in the epididymis and its concentration was found to be negatively correlated to abnormal sperm morphology. Indeed, low GSTM3 content in SP, mainly synthesized during sperm transport through the epididymis, was found to be related to increased percentage of secondary sperm malformations (coiled tails and proximal and distal droplets). Moreover, a lack of correlation between SP-GSTM3 content and the resilience of sperm to withstand liquid-storage was also observed. While SP-GSTM3 is thus suggested to have a molecular role during epididymal maturation rather than being involved in the physiology of ejaculated sperm, further studies using GSTs inhibitors are required in order to confirm this hypothesis. Finally, whilst the findings of the present study supported the use of SP-GSTM3 as a good sperm morphology predictor, they ruled out its relationship with other sperm quality parameters or with boar reproductive performance.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/8/741/s1, Figure S1: Dot plot representing farrowing rate (FR) and litter size (LS) deviation of all 16 fertility Artificial Insemination (AI)-boars. The three AI-boars selected for seminal plasma GSTM3 quantification and classified as having the lowest (n = 3) fertility outcomes deviation (FR and LS) are shown as red dots, whereas 3 AI-boars classified as having highest (n = 3) fertility outcomes deviation (FR and LS) are shown as green dots. Table S1: List of reagents, sources and identifiers used in the study.

Author Contributions: Conceptualization, M.L., M.Y., J.R. and I.B.; methodology, M.L., M.Y., L.P. and I.B.; investigation, M.L., L.P., Y.M.-O., A.D.-B., X.R.; data curation, M.L. and M.Y.; writing—original draft preparation, M.L.; review and editing, M.Y., I.B., A.D.-B., Y.M.-O., J.R., L.P.; supervision, M.Y., J.R. and I.B.; funding acquisition, M.Y., J.R., I.B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge the support from the Ministry of Science and Innovation (Spain), Ministry of Economy and Competitiveness (Spain) and FEDER funds (EU) (RYC-2014-15581, AGL2015-69738-R, AGL2017-88329-R, FJCI-2017-31689 and FPU18/00666); Seneca Foundation Murcia (Spain, 19892/GERM-15); and Regional Government of Catalonia, Spain (2017-SGR-1229).

Acknowledgments: The authors of this manuscript would like to thank AIM Iberica (Topigs Norsvin Iberica, Spain) for supplying the boar ejaculates.

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

Abbreviations

AI	Artificial insemination
SP	Seminal plasma
BE	Corpus epididymis
BE	Bulbourethral glands
CM-H_2DCFDA	5- and 6-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester
DMSO	Dymetil sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FR	Farrowing rate
GPX5	Glutathione peroxidase 5
GSTM3	Glutathione S-transferase Mu 3
GSTs	Glutathione S-transferases
H-42	Hoechst 33342
HE	Caput epididymis
LS	Litter size
M540	Merocyanine 540
P	Prostate
PI	Propidium iodide
PNA	Peanut agglutinin
PNA	Peanut agglutinin
PON1	Paraoxonase 1
PVDF	Polyvinylidene fluoride
RT	Room temperature

References

1. Waberski, D.; Riesenbeck, A.; Schulze, M.; Weitze, K.F.; Johnson, L. Application of preserved boar semen for artificial insemination: Past, present and future challenges. *Theriogenology* **2019**, *137*, 2–7. [CrossRef]
2. Johnson, L.A.; Weitze, K.F.; Fiser, P.; Maxwell, W.M.C. Storage of boar semen. *Anim. Reprod. Sci.* **2000**, *62*, 143–172. [CrossRef]
3. Yeste, M. State-of-the-art of boar sperm preservation in liquid and frozen state. *Anim. Reprod.* **2017**, *14*, 69–81. [CrossRef]
4. Roca, J.; Broekhuijse, M.; Parrilla, I.; Rodriguez-Martinez, H.; Martinez, E.A.; Bolarin, A. Boar Differences In Artificial Insemination Outcomes: Can They Be Minimized? *Reprod. Domest. Anim.* **2015**, *50*, 48–55. [CrossRef]
5. Roca, J.; Parrilla, I.; Bolarin, A.; Martinez, E.A.; Rodriguez-Martinez, H. Will AI in pigs become more efficient? *Theriogenology* **2016**, *86*, 187–193. [CrossRef] [PubMed]
6. Bonet, S.; Garcia, E.; Sepúlveda, L. The Boar Reproductive System. In *Boar Reproduction: Fundamentals and New Biotechnological Trends*; Springer: Heidelberg/Berlin, Germany, 2013; pp. 65–108.
7. Sancho, S.; Vilagran, I. The Boar Ejaculate: Sperm Function and Seminal Plasma Analyses. In *Boar Reproduction: Fundamentals and New Biotechnological Trends*; Springer: Heidelberg/Berlin, Germany, 2013; pp. 471–516.
8. Rodríguez-Martínez, H.; Kvist, U.; Ernerudh, J.; Sanz, L.; Calvete, J.J. Seminal plasma proteins: What role do they play? *Am. J. Reprod. Immunol.* **2011**, *66*, 11–22. [CrossRef]
9. Holland, A.; Ohlendieck, K. Comparative profiling of the sperm proteome. *Proteomics* **2015**, *15*, 632–648. [CrossRef]
10. González-Cadavid, V.; Martins, J.A.M.; Moreno, F.B.; Andrade, T.S.; Santos, A.C.L.; Monteiro-Moreira, A.C.O.; Moreira, R.A.; Moura, A.A. Seminal plasma proteins of adult boars and correlations with sperm parameters. *Theriogenology* **2014**, *82*, 697–707. [CrossRef] [PubMed]
11. Perez-Patiño, C.; Barranco, I.; Parrilla, I.; Valero, M.L.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. Characterization of the porcine seminal plasma proteome comparing ejaculate portions. *J. Proteomics* **2016**, *142*, 15–23. [CrossRef] [PubMed]

12. Pérez-Patiño, C.; Parrilla, I.; Barranco, I.; Vergara-Barberán, M.; Simó-Alfonso, E.F.; Herrero-Martínez, J.M.; Rodriguez-Martínez, H.; Martínez, E.A.; Roca, J. New In-Depth Analytical Approach of the Porcine Seminal Plasma Proteome Reveals Potential Fertility Biomarkers. *J. Proteome Res.* **2018**, *17*, 1065–1076. [CrossRef] [PubMed]
13. De Lazari, F.L.; Sontag, E.R.; Schneider, A.; Araripe Moura, A.A.; Vasconcelos, F.R.; Nagano, C.S.; Dalberto, P.F.; Bizarro, C.V.; Mattos, R.C.; Mascarenhas Jobim, M.I.; et al. Proteomic identification of boar seminal plasma proteins related to sperm resistance to cooling at 17 °C. *Theriogenology* **2020**, *147*, 135–145. [CrossRef] [PubMed]
14. Barranco, S.; Tvarijonaviciute, A.; Perez-Patiho, C.; Vicente-Carrillo, A.; Parrilla, N.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. Glutathione peroxidase 5 is expressed by the entire pig male genital tract and once in the seminal plasma contributes to sperm survival and in vivo fertility. *PLoS ONE* **2016**, *11*, e0162958. [CrossRef] [PubMed]
15. Barranco, I.; Tvarijonaviciute, A.; Perez-Patiño, C.; Alkmin, D.V.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality and in vivo fertility. *Andrology* **2015**, *3*, 315–320. [CrossRef] [PubMed]
16. Li, J.; Barranco, I.; Tvarijonaviciute, A.; Molina, M.F.; Martinez, E.A.; Rodriguez-Martinez, H.; Parrilla, I.; Roca, J. Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. *Theriogenology* **2018**, *107*, 27–35. [CrossRef] [PubMed]
17. Llavanera, M.; Mateo-Otero, Y.; Bonet, S.; Barranco, I.; Fernández-Fuertes, B.; Yeste, M. The triple role of glutathione S-transferases in mammalian male fertility. *Cell. Mol. Life Sci.* **2019**, *77*, 1–12. [CrossRef] [PubMed]
18. Llavanera, M.; Delgado-bermúdez, A.; Olives, S.; Mateo-otero, Y.; Recuero, S.; Bonet, S.; Fernández-Fuertes, B.; Yeste, M.; Barranco, I. Glutathione S-transferases play a crucial role in mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm. *Antioxidants* **2020**, *9*, 100. [CrossRef]
19. Hemachand, T.; Shaha, C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett.* **2003**, *538*, 14–18. [CrossRef]
20. Aydos, S.E.; Taspinar, M.; Sunguroglu, A. Association of CYP1A1 and glutathione S-transferase polymorphisms with male factor infertility. *Fertil. Steril.* **2009**, *92*, 541–547. [CrossRef]
21. Vani, G.T.; Mukesh, N.; Siva Prasad, B.; Rama Devi, P.; Hema Prasad, M.; Rani, P.U.; Reddy, P.P. Role of glutathione S-transferase Mu-1 (GSTM1) polymorphism in oligospermic infertile males. *Andrologia* **2010**, *42*, 213–217. [CrossRef]
22. Kolesnikova, L.I.; Kurashova, N.A.; Bairova, T.A.; Dolgikh, M.I.; Ershova, O.A.; Dashiev, B.G.; Korytov, L.I.; Koroleva, N.V. Role of Glutathione-S-Transferase Family Genes in Male Infertility. *Bull. Exp. Biol. Med.* **2017**, *163*, 643–645. [CrossRef]
23. Safarinejad, M.R.; Shafiei, N.; Safarinejad, S. The association of glutathione-S-transferase gene polymorphisms (GSTM1, GSTT1, GSTP1) with idiopathic male infertility. *J. Hum. Genet.* **2010**, *55*, 565–570. [CrossRef] [PubMed]
24. Lakpour, N.; Mirfeizollahi, A.; Farivar, S.; Akhondi, M.M.; Hashemi, S.B.; Amirjannati, N.; Heidari-Vala, H.; Sadeghi, M.R. The association of seminal plasma antioxidant levels and sperm chromatin status with genetic variants of GSTM1 and GSTP1 (Ile105Val and Ala114Val) in infertile men with oligoasthenoteratozoospermia. *Dis. Markers* **2013**, *34*, 205–210. [CrossRef] [PubMed]
25. Tang, M.; Wang, S.; Wang, W.; Cao, Q.; Qin, C.; Liu, B.; Li, P.; Zhang, W. The glutathione-S-transferase gene polymorphisms (GSTM1 and GSTT1) and idiopathic male infertility risk: A meta-analysis. *Gene* **2012**, *511*, 218–223. [CrossRef] [PubMed]
26. Kan, H.P.; Wu, F.L.; Guo, W.B.; Wang, Y.Z.; Li, J.P.; Huang, Y.Q.; Li, S.G.; Liu, J.P. Null genotypes of GSTM1 and GSTT1 contribute to male factor infertility risk: A meta-analysis. *Fertil. Steril.* **2013**, *99*, 690–696. [CrossRef] [PubMed]
27. Song, X.; Zhao, Y.; Cai, Q.; Zhang, Y.; Niu, Y. Association of the Glutathione S-transferases M1 and T1 polymorphism with male infertility: A meta-Analysis. *J. Assist. Reprod. Genet.* **2013**, *30*, 131–141. [CrossRef]
28. Aydemir, B.; Onaran, I.; Kiziler, A.R.; Alici, B.; Akyolcu, M.C. Increased oxidative damage of sperm and seminal plasma in men with idiopathic infertility is higher in patients with glutathione S-transferase Mu-1 null genotype. *Asian J. Androl.* **2007**, *9*, 108–115. [CrossRef]

29. Kwon, W.S.; Oh, S.A.; Kim, Y.J.; Rahman, M.S.; Park, Y.J.; Pang, M.G. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci. Rep.* **2015**, *5*, 1–10. [CrossRef]
30. Llavanera, M.; Delgado-Bermuúdez, A.; Fernandez-Fuertes, B.; Recuero, S.; Mateo, Y.; Bonet, S.; Barranco, I.; Yeste, M. GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–11. [CrossRef]
31. Broekhuijse, M.L.W.J.; Šoštarić, E.; Feitsma, H.; Gadella, B.M. The value of microscopic semen motility assessment at collection for a commercial artificial insemination center, a retrospective study on factors explaining variation in pig fertility. *Theriogenology* **2012**, *77*, 1466–1479. [CrossRef]
32. Barranco, I.; Roca, J.; Tvarijonaviciute, A.; Rubér, M.; Vicente-Carrillo, A.; Atikuzzaman, M.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H. Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Mol. Reprod. Dev.* **2015**, *82*, 58–65. [CrossRef]
33. Smital, J.; De Sousa, L.L.; Mohsen, A. Differences among breeds and manifestation of heterosis in AI boar sperm output. *Anim. Reprod. Sci.* **2004**, *80*, 121–130. [CrossRef]
34. Bonet, S.; Briz, M.D.; Yeste, M. A Proper Assessment of Boar Sperm Function May Not Only Require Conventional Analyses but Also Others Focused on Molecular Markers of Epididymal Maturation. *Reprod. Domest. Anim.* **2012**, *47*, 52–64. [CrossRef] [PubMed]
35. Briz, M.D.; Fàbrega, A. The boar spermatozoon. In *Boar Reproduction: Fundamentals and New Biotechnological Trends*; Springer: Heidelberg/Berlin, Germany, 2013; pp. 3–47.
36. Cooper, T.G. Cytoplasmic droplets: The good, the bad or just confusing? *Hum. Reprod.* **2005**, *20*, 9–11. [CrossRef]
37. Briz, M.D.; Bonet, S.; Pinart, B.; Camps, R. Sperm malformations throughout the boar epididymal duct. *Anim. Reprod. Sci.* **1996**, *43*, 221–239. [CrossRef]
38. Otasevic, V.; Kalezic, A.; Macanovic, B.; Jankovic, A.; Stancic, A.; Garalejic, E.; Korac, A.; Korac, B. Evaluation of the antioxidative enzymes in the seminal plasma of infertile men: Contribution to classic semen quality analysis. *Syst. Biol. Reprod. Med.* **2019**, *65*, 343–349. [CrossRef] [PubMed]
39. Macanovic, B.; Vucetic, M.; Jankovic, A.; Stancic, A.; Buzadzic, B.; Garalejic, E.; Korac, A.; Korac, B.; Otasevic, V. Correlation between sperm parameters and protein expression of antioxidative defense enzymes in seminal plasma: A pilot study. *Dis. Markers* **2015**, *2015*, 436236. [CrossRef]
40. Barranco, I.; Padilla, L.; Tvarijonaviciute, A.; Parrilla, I.; Martínez, E.A.; Rodriguez-Martinez, H.; Yeste, M.; Roca, J. Levels of activity of superoxide dismutase in seminal plasma do not predict fertility of pig AI-semen doses. *Theriogenology* **2019**, *140*, 18–24. [CrossRef]

PAPER III

GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm

Marc Llavanera, Ariadna Delgado-Bermúdez, Beatriz Fernández-Fuertes, Sandra Recuero, Yentel Mateo-Otero, Sergi Bonet, Isabel Barranco & Marc Yeste

Journal of animal science and biotechnology
2019;10, 61

DOI: 10.1186/s40104-019-0370-5

Journal of Animal Science and Biotechnology

RESEARCH Open Access

GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm

Marc Llavanera, Ariadna Delgado-Bermúdez, Beatriz Fernandez-Fuertes, Sandra Recuero, Yentel Mateo, Sergi Bonet, Isabel Barranco and Marc Yeste*

Abstract

Background: Cryopreservation is currently the most efficient method for long-term preservation of mammalian gametes and is extensively used in swine artificial insemination (AI) centres. However, it is well-known that cryopreservation procedures induce changes in the water phase in both intra and extracellular compartments, which alter the content and localisation of several proteins and ends up curtailing the structural integrity of functional sperm (i.e., cryoinjuries). Alterations and deficiencies of sperm-oocyte binding proteins during gamete recognition are one of the causes of reproductive failure both *in vitro* and *in vivo*. In this sense, characterisation of cryopreservation effects upon oocyte-binding proteins of sperm, such as IZUMO1 and GSTM3, is essential when assessing the impact of this technique in swine reproduction.

Results: Cryopreservation was found to induce changes in the localisation of IZUMO1 and GSTM3 in boar sperm. However, the relative content of both proteins was not altered after thawing. Furthermore, whereas IZUMO1 content was found not to be related to the cryotolerance of boar sperm, GSTM3 content was observed to be higher in poor (PFE) than in good (GFE) freezability ejaculates in both pre-frozen (1.00 INT·mm^2 ± 0.14 INT·mm^2 vs. 0.72 INT·mm^2 ± 0.15 INT·mm^2; $P < 0.05$) and post-thawed (0.96 INT·mm^2 ± 0.20 INT·mm^2 vs. 70 INT·mm^2 ± 0.19 INT·mm^2; $P < 0.05$) samples. Moreover, GSTM3 levels were found to be higher in those spermatozoa that exhibited low mitochondrial activity, high reactive oxygen species (ROS) production, and high membrane lipid disorder post-thaw ($P < 0.05$).

Conclusions: The difference in GSTM3 content between GFE and PFE, together with this protein having been found to be related to poor sperm quality post-thaw, suggests that it could be used as a cryotolerance marker of boar spermatozoa. Furthermore, both IZUMO1 and GSTM3 relocate during cryopreservation, which could contribute to the reduced fertilising capacity of frozen-thawed boar sperm.

Keywords: Boar, Cryopreservation, GSTM3, IZUMO1, ROS, Sperm

Introduction

Sperm cryopreservation is currently the most efficient method for long-term storage of mammalian gametes for artificial insemination (AI). In spite of this, freezing and thawing processes are known to harm spermatozoa (i.e., cryoinjuries) because of the phase change of water in both intracellular and extracellular compartments [1]. Cryoinjuries cause detrimental effects on sperm motility and plasma membrane integrity. They also lead to changes in sperm protein levels, localisation, function and tyrosine-phosphorylation; alterations of mitochondrial function, and high ROS production, among many others [2]. This wide range of cryoinjuries impair sperm function and survival, underlying a significant decrease in the reproductive performance after thawing [1]. Furthermore, boar sperm are more susceptible to damage by the freeze-thaw process than sperm from other species [3], which leads to a decrease in the use of this technique for swine sperm preservation.

It is well known that boar sperm plasmalemma is highly sensitive to temperature changes, due to the abundance of unsaturated phospholipids and to the low amount of cholesterol [4]. As a result, temperatures

* Correspondence: marc.yeste@udg.edu
Biotechnology of Animal and Human Reproduction (TechnoSperm), Unit of Cell Biology, Department of Biology, Institute of Food and Agricultural Technology, University of Girona, C/Maria Aurèlia Campany, 69, Campus Montilivi, E-17003 Girona, Spain

lower than or equal to 5 °C lead to the destabilisation of sperm plasma membrane [2]. In turn, this leads to protein translocation and/or loss of function, thereby being a potential cause of subfertility in frozen-thawed sperm [2]. In pigs, as in other species, differences in the ability to withstand freeze-thawing processes have been found between ejaculates. This has led ejaculates to be classified as good freezability ejaculates (GFE) or poor freezability ejaculates (PFE) [5, 6].

Among other factors, male subfertility has been associated to genetic abnormalities [7] and low levels of sperm-oocyte binding proteins [8]. For successful fertilisation to occur, sperm must be able to bind and penetrate the zona pellucida (ZP) of the oocyte, and then bind and fuse to the oocyte plasma membrane. It is evident then that any alteration that inhibits sperm from carrying out these processes would result in male subfertility or infertility. Several sperm proteins, such as ADAM family members [9], PH-20 [10], TMEM95 [11], IZUMO1 [12] and GSTM3 [13, 14], have been shown to play an essential role for oocyte binding. Thus, improper expression and/or localisation of such proteins due to cryopreservation procedures is likely to lead to subfertility because of a failure in oocyte recognition. Two essential fertility-related proteins of boar sperm, which may be altered by cryopreservation procedures and in consequence impair sperm fertilising ability, are IZUMO1 and GSTM3.

IZUMO1 is a member of the immunoglobulin superfamily (IgSF) which was discovered for the first time in mouse as an essential sperm-oocyte binding protein [12] through the interaction with its oocyte receptor JUNO (Folate receptor 4, FOLR4) after acrosome reaction [15]. While studies in bull sperm by Fukuda et al. [16] showed no changes in the relative IZUMO1-content in response to cryopreservation, they reported an aberrant translocation of this protein to the whole equatorial or acrosomal regions in acrosome-exocytosed sperm, resembling to the pattern observed in *in vitro* capacitated and acrosome-reacted sperm. In pigs, Kim et al. [17] reported that IZUMO1 is mainly located at the equatorial segment and inner acrosomal membrane of capacitated sperm.

On the other hand, glutathione S-transferase Mu 3 (GSTM3) is a member of a large group of cytosolic, membrane-bound multi-gene and multi-functional isoenzymes that catalyse a number of reduced glutathione-dependent reactions which are involved in cellular protection against oxidative stress and toxic chemicals [18]. It is known that Mu members of the glutathione S-transferase family are attached to the sperm plasma membrane via non-covalent interactions and their activity is mainly restricted to the plasma membrane rather than other compartments (e.g. mitochondria) [13, 19]. In mammalian sperm, membrane-bound GSTM3 is involved in the prevention of oxidative stress [20] and in the fertilisation of the oocyte through its interaction with ZP4 [14]. Kwon and colleagues [21] found that, in boar sperm, higher levels of GSTM3 are associated to smaller litter sizes. Moreover, Kumar et al. [22] showed that cryopreservation reduces GSTM3-content and induces its relocation from the connecting, mid, principal and end pieces, to the mid-piece in buffalo sperm after freeze-thawing.

Despite the clear role IZUMO1 and GSTM3 on sperm fertility, there is no literature available on their localisation pattern in fresh boar sperm, nor on the effects of cryopreservation upon their relative content and localisation. Thus, this work sought to elucidate the effects of cryopreservation on the presence, content and localisation of IZUMO1 and GSTM3 in boar sperm, as this may contribute to our understanding of the reduced fertility of frozen-thawed sperm. In addition, the ability of these proteins to serve as predictors of sperm cryotolerance was also explored.

Materials and methods

Boars and ejaculates

Twelve different ejaculates from different sexually mature Piétrain boars ($n = 12$) were purchased from an AI centre (Grup Gepork S.L., Masies de Roda, Spain). Ejaculates were collected using the gloved-hand method, diluted 1:2 (*v:v*) using a commercial extender (Vitasem LD; Magapor S.L., Zaragoza, Spain), packed in bags and transported at 17 °C to the laboratory within 5 h post-collection.

Upon arrival, each ejaculate was split into four aliquots. The first one was used to assess pre-frozen sperm quality, whereas the second and third aliquots were used for Western blot and immunofluorescence analysis, respectively. Finally, the fourth aliquot was stored at 17 °C until its cryopreservation the following day.

Sperm cryopreservation

The fraction intended for cryopreservation was processed following the protocol described by Prieto-Martínez et al. [23], with minor modifications. Briefly, samples were split into 50 mL tubes and centrifuged at 2400×*g* at 15 °C for 3 min. Then, supernatants were discarded and sperm pellets were pooled and resuspended to a final concentration of 1.5×10^9 spermatozoa per mL with lactose-egg yolk freezing medium (LEY; 80% (*v:v*) lactose [0.31 mol/L; Sigma-Aldrich, St. Louis, MO, USA], and 20% (*v:v*) egg yolk). Samples were cooled down to 5 °C for 120 min and diluted to a final concentration of 1×10^9 spermatozoa per mL with LEYGO medium (6% glycerol [Sigma-Aldrich] and 1.5% Orvus ES paste [Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA]). Then, sperm were loaded into 0.5 mL straws (Minitub Ibérica, S.L.; Tarragona, Spain) and

placed in a controlled-rate programmable freezer (Icecube 14SB; Minitub Ibérica, S.L.). Cooling rates and times were those used by Casas et al. [6]: 100 s from 5 °C to − 5 °C at a rate of − 6 °C per min, 113 s from − 5 °C to − 80 °C at a rate of − 39.82 °C per min, 30 s at − 80 °C (no temperature variation), and 70 s from − 80 °C to − 150 °C at a rate of − 60 °C per min. Finally, straws were plunged into liquid nitrogen (− 196 °C) and stored.

For thawing, three straws per ejaculate were placed into a water bath at 38 °C with vigorous shaking for 10 s. The straw content was diluted 1:3 with pre-warmed Beltsville Thawing Solution (BTS) [24]. Finally, frozen-thawed samples were placed at 38 °C and sperm quality was assessed after 30 and 240 min of incubation. Additionally, Western blot and immunofluorescence analysis of cryopreserved sperm were performed 30 min post-thaw.

Flow cytometry analyses

Four sperm parameters (plasma membrane integrity, sperm membrane lipid disorder, mitochondrial membrane potential and intracellular levels of superoxides [O_2^{-}▪]) were evaluated. All sperm samples were diluted with phosphate buffered saline 1× (PBS) to a final concentration of 5×10^6 cells per mL in a final volume of 0.6 mL before they were stained with the corresponding protocol. The flow cytometry assessments were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter; Fullerton, CA, USA), and samples were excited with an argon ion laser (488 nm) set at a power of 22 mW. A total of three technical replicates, with a minimum of 10,000 events per replicate, were evaluated for each ejaculate and sperm parameter. Flowing Software (Ver. 2.5.1; University of Turku, Finland) was used to perform flow cytometric data analysis, following the recommendations of the International Society for Advancement of Cytometry (ISAC). The corresponding mean ± standard error of the mean (SEM) was subsequently calculated. Sperm viability was evaluated by assessing their membrane integrity using the SYBR14/PI according to the protocol of Garner and Johnson [25]. Membrane lipid disorder of pre-frozen and frozen-thawed sperm was evaluated by M540 and YO-PRO-1 co-staining, following the procedure of Rathi et al. [26] with minor modifications by Yeste et al. [27]. Mitochondrial membrane potential of pre-frozen and frozen-thawed sperm was evaluated following a protocol modified from Ortega-Ferrusola et al. [28], assessed through JC-1 staining. Finally, sperm oxidative stress was evaluated by assessing intracellular levels of hydrogen superoxides (O_2^{-}▪) through co-staining with HE and YO-PRO-1, following a modification of the procedure described by Guthrie and Welch [29]. All protocols are described in detail in Additional file 1.

Sperm motility

Sperm motility was evaluated using a commercial computer assisted sperm analysis (CASA) system consisting of a phase contrast microscope (Olympus BX41) at 100× magnification (Olympus 10× 0.30 PLAN objective lens; negative phase-contrast field) connected to a computer equipped with ISAS software (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). Three replicates per sample, with a minimum of 1000 spermatozoa per replicate, were assessed placing 5 μL of each sperm sample onto a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). The recorded sperm motility parameters provided by the software were sperm progressive motility (PMOT, %); curvilinear velocity (VCL, μm/s); average path velocity (VAP, μm/s); straight line velocity (VSL, μm/s); amplitude of lateral head displacement (ALH, μm); beat cross frequency (BCF, Hz); linearity (LIN, %); and straightness (STR, %). A sperm cell was considered to be motile when VAP was higher than 10 μm/s. The corresponding mean ± SEM was subsequently calculated.

Western blot analysis

Pre-frozen and frozen-thawed boar sperm were used for Western blot analysis. Samples were centrifuged and re-suspended in lysis buffer. Following this, samples were incubated in agitation at 4 °C for 30 min. After incubation, all samples were sonicated thrice, centrifuged at 10,000×g and stored at − 80 °C prior to protein quantification. Quantification of total protein in all samples was carried out in triplicate by a detergent compatible (DC) method (BioRad).

Ten micrograms of total protein were resuspended in Laemmli reducer buffer 2× and boiled at 96 °C before proteins were loaded onto the upper stacking gel. After, electrophoretic protein separation, proteins from the gel were transferred onto polyvinyl fluoride membranes (Immobilion-P; Millipore, Darmstadt, Germany) using Trans-Blot® Turbo™ (BioRad). Blocked membranes were then incubated overnight with primary antibodies: anti-IZUMO1 polyclonal rabbit antibody (ref. NBP1–83086; Novus Biologicals, Littleton, CO, USA; 1:10,000; *v:v*) or anti-GSTM3 polyclonal rabbit antibody (ref. ARP53561_P050; Aviva Systems Biology, San Diego, USA; 1:20,000; *v:v*). Next, membranes were washed and incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Dako, Derkman A/S; Denmark) for an hour with agitation (1:15,000 (*v:v*) dilution for IZUMO1 and 1:25,000 (*v:v*) for GSTM3). Finally, membranes were washed and bands were visualised with a chemiluminescent substrate (ImmobilionTM Western Detection Reagents, Millipore) and scanned with G:BOX Chemi XL 1.4 (SynGene, Frederick, MT, USA).

Following these steps, the membranes were stripped by incubation with agitation at room temperature with a stripping buffer. Next, stripped membranes were blocked and then incubated overnight with anti-alpha-tubulin monoclonal mouse antibody (ref. MABT205, Millipore; 1:100,000, *v:v*). Thereafter, membranes were washed and incubated with secondary anti-mouse HRP–conjugated polyclonal rabbit antibody (ref. P0260; Dako; 1:150,000, *v:v*) for 1 h. Finally, membranes were washed, incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and scanned with G:BOX Chemi XL 1.4 (SynGene).

Three technical replicates per sample were evaluated and bands were quantified using Quantity One Version 4.6.2 software package (BioRad). Pattern quantifications were normalized using alpha-tubulin, and the corresponding mean ± SEM of each sample was subsequently calculated.

The specificity of primary antibodies was confirmed through peptide competition assays utilising IZUMO1- (ref. NBP1-83086PEP; Novus Biologicals) and GSTM3- (ref. AAP53561; Aviva Systems Biology) immunising peptides, 20 times in excess with regard to their respective antibodies (see Additional file 2). Detailed Western blot protocol is described in Additional file 1.

Immunofluorescence

Localisation of IZUMO1 and GSTM3 in pre-frozen and frozen-thawed boar sperm was evaluated through immunofluorescence. Sperm samples were diluted to a final concentration of 3×10^6 cells per mL, fixed with 1.5% (*w:v*) paraformaldehyde and washed with PBS 1×. Two drops per sample were placed onto different slides, and all slides were blocked and permeabilised with blocking solution. Then, all samples were incubated overnight with primary antibodies anti-IZUMO1 polyclonal rabbit antibody (ref. NBP1–83086; Novus Biologicals, Littleton, CO, USA; 1:250; *v:v*) and anti-GSTM3 polyclonal rabbit antibody (ref. ARP53561_P050; Aviva Systems Biology; 1:200; *v:v*). Following the primary antibody incubation, slides were washed and incubated with a secondary antibody anti-rabbit antibody conjugated with Alexa Fluor488 (Molecular Probes) diluted 1:250 (*v:v*) for IZUMO1 and 1:500 for GSTM3 in blocking solution. Finally, a drop of 10 μL of Vectashield mounting medium containing DAPI was added, and a coverslip was placed prior to sealing with nail varnish.

All samples were evaluated under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon Corp., Tokyo, Japan). In negative controls, the primary antibodies were omitted. Furthermore, the specificity of the primary antibodies was confirmed by separate peptide competition assays. Samples were incubated with GSTM3- (ref. AAP53561; Aviva Systems Biology) and IZUMO1-specific (ref. NBP1-83086PEP; Novus Biologicals) blocking peptides, which were 10 times in excess with regard to the corresponding primary antibody (see Additional file 3). The immunofluorescence protocol is described in detail in Additional file 1.

Statistical analysis

Data were analysed with a statistical package (IBM SPSS for Windows 25.0; Armonk, NY, USA). First, normality and homogeneity of variances were checked with Shapiro-Wilk and Levene tests, respectively. When needed, data were transformed with arcsin $\sqrt{x}$ and re-checked for normality and homogeneity of variances. Each biological replicate was considered as a statistical case.

Groups of ejaculates with good (GFE) and poor (PFE) freezability were set on the basis of their total and progressive sperm motilities, and sperm viability (% SYBR14+/PI- spermatozoa) at 30 min through a two-step hierarchical cluster analysis using the log-likelihood as a distance measure and the Bayesian Schwarz criterion to build the groups. After establishing the two groups of ejaculates (GFE and PFE), sperm function parameters (i.e. % Viable spermatozoa with low lipid disorder, % Viable spermatozoa with low superoxide levels…) and relative content of IZUMO1 and GSTM3 were compared between these two groups and before and after cryopreservation (pre-frozen, FT 30 min, FT 240 min) with a linear mixed model (repeated measures). In this model, the cryopreservation step was the within-subjects factor, the ejaculate group (GFE vs. PFE) was the between-subjects factor and the boar was the random-effects factor. Post-hoc Sidak test was used for pair-wise comparisons. Finally, Pearson correlation coefficients were calculated between relative content of IZUMO1 and GSTM3 in pre-frozen and frozen-thawed sperm and all quality parameters evaluated in pre-frozen and 30- and 240-min post-thaw sperm. Data are shown as mean ± SEM. For all analyses, the level of significance was set at $P \leq 0.05$.

Results

Classification of boar ejaculates in GFE and PFE groups

Sperm viability assessed at 30 min post-thaw was used to classify ejaculates as GFE and PFE. Although no differences were found between groups in pre-frozen samples ($P > 0.05$), sperm total and progressive motility, and viability were higher ($P < 0.05$) in GFE than in PFE at both 30 and 240 min post-thaw (Table 1).

Effects of cryopreservation on sperm quality parameters

Sperm quality parameters from boar ejaculates classified as GFE and PFE, were assessed before and after cryopreservation and are summarized in Table 1. Regarding

Table 1 Sperm quality parameters in pre-frozen (P-F) and frozen–thawed (F-T) sperm, 30 (F-T-30 min) and 240 (F-T-240 min) min after thawing (mean ± SEM)

Parameter	Classification	P-F	F-T-30 min	F-T-240 min
% Total motile spermatozoa	GFE	$72.52 \pm 4.49^{a,1}$	$30.10 \pm 1.91^{a,2}$	$8.14 \pm 1.29^{a,3}$
	PFE	$71.45 \pm 6.53^{a,1}$	$4.45 \pm 1.47^{b,2}$	$1.97 \pm 0.34^{b,2}$
% Progressive motile spermatozoa	GFE	$54.25 \pm 2.60^{a,1}$	$21.04 \pm 1.77^{a,2}$	$5.02 \pm 1.48^{a,3}$
	PFE	$50.09 \pm 5.01^{a,1}$	$1.83 \pm 0.99^{b,2}$	$0.28 \pm 0.09^{b,2}$
% SYBR-14^+ /PI^- spermatozoa	GFE	$81.20 \pm 1.65^{a,1}$	$28.77 \pm 2.85^{a,2}$	$21.56 \pm 3.46^{a,3}$
	PFE	$79.84 \pm 3.42^{a,1}$	$5.60 \pm 1.56^{b,2}$	$3.79 \pm 1.01^{b,2}$
% $M540^-$/YO-PRO-1^- spermatozoa	GFE	$75.13 \pm 3.23^{a,1}$	$24.36 \pm 2.33^{a,2}$	$14.59 \pm 2.49^{a,3}$
	PFE	$73.32 \pm 3.58^{a,1}$	$6.26 \pm 1.51^{b,2}$	$2.72 \pm 0.72^{b,2}$
% $JC1_{agg}$ spermatozoa	GFE	$79.14 \pm 3.04^{a,1}$	$34.29 \pm 2.36^{a,2}$	$27.57 \pm 5.51^{a,2}$
	PFE	$77.50 \pm 3.28^{a,1}$	$16.50 \pm 3.28^{b,2}$	$12.75 \pm 5.88^{a,2}$
% E^-/YO-PRO-1^- spermatozoa	GFE	$80.01 \pm 2.23^{a,1}$	$21.92 \pm 2.26^{a,2}$	$14.09 \pm 3.15^{a,3}$
	PFE	$76.10 \pm 2.79^{a,1}$	$8.86 \pm 1.10^{b,2}$	$3.39 \pm 1.82^{b,2}$

Each ejaculate was classified as having good (GFE) or poor freezability (PFE). Different superscript numbers (^{1,2,3}) indicate significant differences ($P < 0.05$) between conditions (pre-frozen (P-F), F-T-30 min and F-T-240 min). Different superscript letters (^{a,b}) indicate significant differences($P < 0.05$) between GFE and PFE in a given parameter

viable and total and progressive motile spermatozoa, no differences were found in pre-frozen samples between GFE and PFE ($P > 0.05$). Although motility and viability were decreased after cryopreservation in both GFE and PFE groups, this decrease was more dramatic in PFE than in GFE, at both 30 and 240 min after thawing ($P < 0.05$).

Concerning the evaluation of sperm membrane lipid disorder ($M540^-$/YO-PRO-1^-), PFE showed higher membrane lipid disorder than GFE at 30- and 240-min after thawing ($P < 0.05$). As expected, the percentage of viable spermatozoa with low membrane lipid disorder were lower 30 and 240 min post-thaw than samples before freezing in both GFE and PFE ($P < 0.05$).

Regarding mitochondrial membrane potential, GFE contained a higher percentage of sperm with high mitochondrial membrane potential ($JC\text{-}1_{agg}$), than PFE at 30 min post-thaw ($P < 0.05$). However, 240 min after thawing of the samples, no differences could be observed between groups. Finally, intracellular levels of superoxides ($O_2^{-}\bullet$) were higher in PFE than in GFE ($P < 0.05$). Moreover, a decrease of viable spermatozoa with high superoxide levels was observed after freeze-thawing in both GFE and PFE ($P < 0.05$).

Effects of cryopreservation on the localisation of IZUMO1 and GSTM3

Localisation of IZUMO1 and GSTM3 was determined in pre-frozen and frozen-thawed boar spermatozoa by immunofluorescence. No differences were found in IZUMO1- and GSTM3-localisation patterns between GFE and PFE groups.

Figure 1 shows the representative localisation patterns of IZUMO1 before and after cryopreservation of boar sperm. Two IZUMO1 localisation patterns were found in pre-frozen boar sperm: 1) fluorescence signal was located in the principal and end pieces of the tail of all spermatozoa, whereas 2) only some cells showed an IZUMO1-signal in the acrosome. With regard to frozen-thawed samples, IZUMO1 was exclusively localised in the equatorial segment. Interestingly, some pre-frozen and frozen-thawed sperm did not exhibit a positive IZUMO1-signal.

Figure 2 shows representative localisation patters of GSTM3 in pre-frozen and frozen-thawed boar sperm. Sperm GSTM3 was located in the equatorial subdomain of the head and in mid-, principal and end-pieces of the tail before cryopreservation. Frozen-thawed boar sperm showed an intense GSTM3 signal in the mid-piece area only, being absent from the principal and end pieces of the tail and from the equatorial subdomain of the head. As opposed to the IZUMO-1 stained samples, all spermatozoa showed GSTM3 fluorescence signal.

Relative abundances of IZUMO1 and GSTM3 during cryopreservation

Western blot analysis of IZUMO1 evidenced a single-band of ~ 48 kDa band in both pre-frozen and frozen-thawed boar sperm (see Additional file 4). Stripping of membranes and incubation with α-tubulin was performed in order to confirm the same amount of total protein was loaded in all samples. The results showed a band at ~ 50 kDa in every sample. Following quantifications of relative IZUMO1-conent, no differences were observed between groups (GFE vs. PFE) either before or after cryopreservation (Fig. 3).

Immunoblotting of GSTM3 showed a single-band pattern of ~ 25 kDa in pre-frozen and frozen-thawed boar

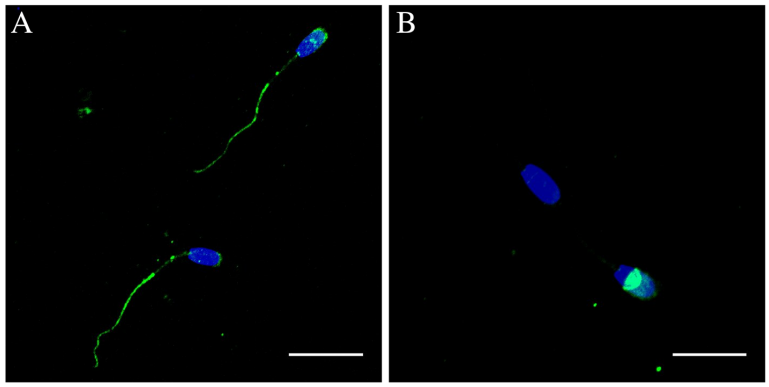

Fig. 1 Immunolocalisation of IZUMO1 in (**a**) pre-frozen and (**b**) frozen-thawed boar spermatozoa. Nucleus is shown in blue (DAPI), whereas IZUMO1 is shown in green (FITC). Scale bars: **A**: 20 μm; **B**: 14 μm

sperm (see Additional file 4). Weak-intensity bands at ~ 28 and ~ 48 kDa were also observed. The incubation with α-tubulin showed a ~ 50 kDa band in all samples. Quantification of relative levels of GSTM3 evidenced differences in protein content between GFE and PFE in both pre-frozen (0.72 INT·mm^2 ± 0.15 INT·mm^2 vs. 1.00 INT·mm^2 ± 0.14 INT·mm^2; $P < 0.05$) and frozen-thawed (0.70 INT·mm^2 ± 0.19 INT·mm^2 vs. 0.96 INT·mm^2 ± 0.20 INT·mm^2; $P < 0.05$) samples, with PFE showing higher relative GSTM3-levels than the GFE (Fig. 4).

Correlations between relative contents of IZUMO1 and GSTM3 and sperm quality parameters

Tables 2 and 3 show Pearson correlation coefficients between relative contents of IZUMO1 and GSTM3, respectively, and sperm quality parameters in pre-frozen and frozen-thawed sperm (30 and 240 min post-thaw). No correlation between the relative content of these proteins and pre-frozen sperm quality parameters was found, nor between relative IZUMO1-abundance and post-thaw sperm quality parameters. However, relative levels of pre-frozen GSTM3 were negatively correlated with percentages of viable spermatozoa (SYBR14$^+$/PI$^-$), viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO-1$^-$), viable spermatozoa with low levels of superoxides (E$^-$/YO-PRO-1$^-$), and spermatozoa with high mitochondrial membrane potential (JC1$_{agg}$) 30 min post-thaw ($P < 0.05$). In addition, relative levels of GSTM3 after cryopreservation were negatively correlated ($P < 0.05$) with percentages of viable spermatozoa with low levels of superoxides (E$^-$/YO-PRO-1$^-$) and

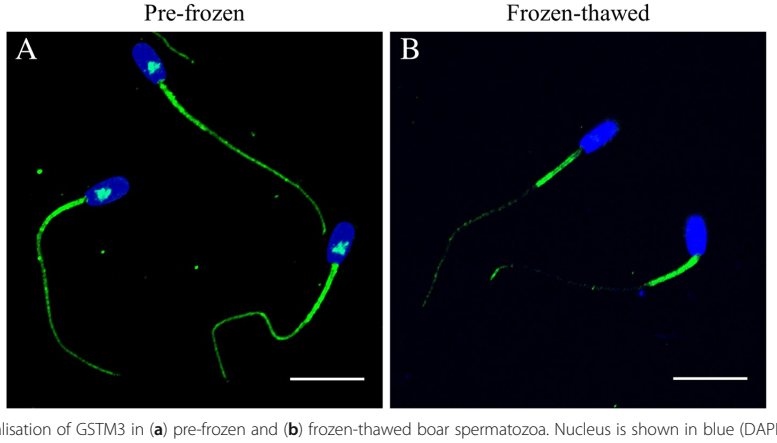

Fig. 2 Immunolocalisation of GSTM3 in (**a**) pre-frozen and (**b**) frozen-thawed boar spermatozoa. Nucleus is shown in blue (DAPI), whereas GSTM3 is shown in green (FITC). Scale bars: **A-B**: 14.1 μm

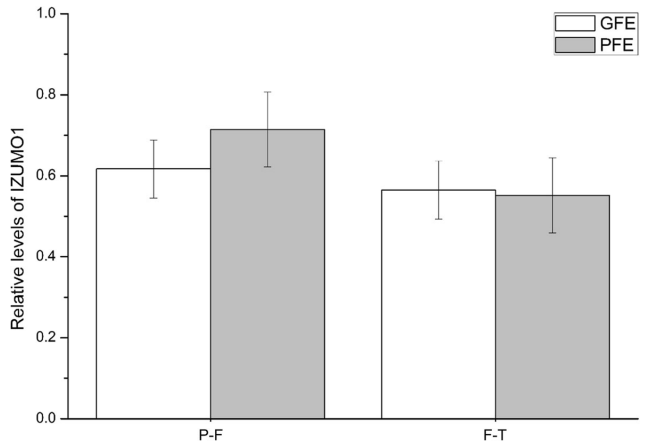

Fig. 3 Relative abundances of IZUMO1 as mean ± standard error of the mean (SEM) of GFE and PFE in pre-frozen (P-F) and frozen-thawed (F-T) boar spermatozoa. Values were normalized using α-tubulin protein as an internal standard. Each sperm sample were evaluated three times

high mitochondrial membrane potential ($JC1_{agg}$) 30 min post-thaw. Finally, percentages of viable spermatozoa ($SYBR14^+/PI^-$) and viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) at 240 min post-thaw were negatively correlated ($P < 0.05$) with the relative levels of GSTM3 evaluated in pre-frozen and frozen-thawed sperm.

Discussion

Alterations in the levels of membrane-bound fertility-related proteins, such as IZUMO1 and GSTM3, have been associated to male subfertility [8]. Moreover, cryopreservation is known to alter the content and localisation of several sperm proteins [2]. Based on these facts, the current work aimed to evaluate the localisation of IZUMO1 and GSTM3 and quantify their relative levels in boar spermatozoa before and after cryopreservation, comparing ejaculates with good (GFE) and poor (PFE) freezability. The results reported in this work show that: 1) both IZUMO1 and GSTM3 undergo relocation due to cryopreservation; however, 2) their relative abundance levels are not altered by this process; 3) remarkably, relative GSTM3-content in pre-frozen sperm is correlated with post-thaw sperm quality, and is higher in PFE than in GFE.

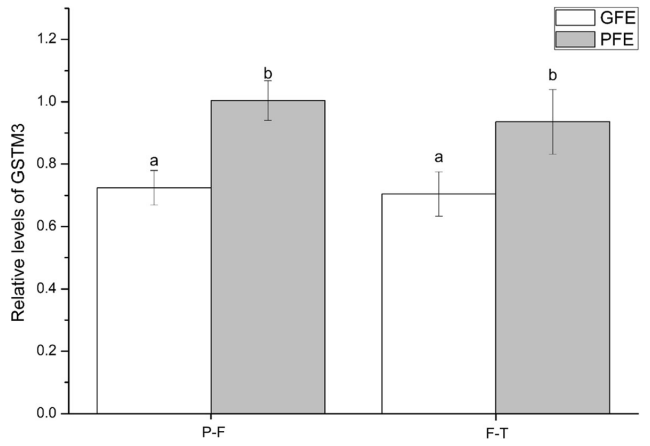

Fig. 4 Relative abundances of GSTM3 as mean ± standard error of the mean (SEM) of GFE and PFE in pre-frozen (P-F) and frozen-thawed (F-T) boar spermatozoa. Values were normalized using α-tubulin protein as an internal standard. Each sperm sample were evaluated two times

Table 2 Correlation coefficients between relative IZUMO1 abundances in both pre-frozen (P-F) and frozen thawed (F-T) spermatozoa and sperm quality parameters in pre-frozen (P-F) and frozen-thawed spermatozoa, evaluated 30 (F-T-30) and 240 (F-T-240) min post-thaw

		Relative levels of P-F IZUMO1	Relative levels of F-T IZUMO1
P-F	% Progressive motile spermatozoa	0.37	0.19
	% Total motile spermatozoa	0.12	0.38
	% SYBR-14$^+$/PI$^-$ spermatozoa	0.24	− 0.23
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	0.21	0.21
	% $JC1_{agg}$ spermatozoa	−0.27	− 0.51
	% E$^-$/YO-PRO-1$^-$ spermatozoa	0.22	− 0.55
F-T-30	% Progressive motile spermatozoa	0.26	−0.25
	% Total motile spermatozoa	0.23	−0.27
	% SYBR-14$^+$/PI$^-$ spermatozoa	−0.05	−0.08
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	−0.05	0.03
	% $JC1_{agg}$ spermatozoa	−0.11	−0.01
	% E$^-$/YO-PRO-1$^-$ spermatozoa	−0.25	− 0.03
F-T-240	% Progressive motile spermatozoa	0.25	−0.12
	% Total motile spermatozoa	0.20	−0.11
	% SYBR-14$^+$/PI$^-$ spermatozoa	−0.05	0.08
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	−0.15	0.11
	% $JC1_{agg}$ spermatozoa	−0.31	−0.41
	% E$^-$ / YO-PRO-1$^-$ spermatozoa	0.02	0.23

The presence of IZUMO1 in boar sperm was assessed by immunoblotting, and a single-band of ~ 48 kDa was found in both pre-frozen and frozen-thawed boar sperm. Although the predicted molecular mass for IZUMO1 is ~ 37 kDa, our results are in agreement with those reported in boar [17] and bull [16] spermatozoa. This data suggests that IZUMO1 could undergo post-translation modifications (such as glycosylation or phosphorylation) in both species during sperm maturation, and that these modifications increase its predicted molecular weight.

Regarding protein localisation, IZUMO1 was found in the acrosome and principal and end tail pieces of boar spermatozoa before freezing. Translocation from this location to the equatorial segment was observed in frozen-thawed sperm. This relocation is in agreement with the results previously reported by Fukuda, et al. [16], who found IZUMO1 to be located in the whole equatorial segment of frozen-thawed bull sperm. Moreover, a similar relocation has been reported in mouse sperm during acrosome reaction. Mice IZUMO1 relocates from both the inner and outer acrosome membranes to the equatorial segment due to the acrosome reaction [30]. It is worth noting that IZUMO1-staining was unexpectedly found in the sperm tail of pre-frozen, but not frozen-thawed, boar sperm. That being said, no differences in the localisation of this protein between GFE and PFE were observed, either before and after cryopreservation.

Our results demonstrate that IZUMO1 content was similar in pre-frozen and frozen-thawed sperm, without differences being observed between GFE and PFE. Our finding contrasts with those reported by Fukuda et al. [16], who showed a reduction of IZUMO1 in cryopreserved bull samples compared with pre-frozen sperm due to the loss of acrosome integrity during this procedure. However, it should be noted that Fukuda et al. [16] assessed this reduction of protein content through immunofluorescence analysis, whereas in this study, quantification of Western blot bands was carried out. In fact, similarly to what it is being reported in this study on boar sperm, relocation (rather than complete loss) of IZUMO1 occurs during capacitation and acrosome reaction in mouse sperm [31].

Western blot analysis showed a single band pattern of ~ 25 kDa in pre-frozen and frozen-thawed boar sperm when membranes were probed with an anti- GSTM3 antibody. Similarly, Kwon et al. [21] also reported a single band of ~ 27 kDa for GSTM3, the predicted molecular mass of boar GSTM3 being 26.6 kDa. Additionally, we also found an additional weak band of ~ 28 kDa in GSTM3 membranes that was not observed in the peptide competition assay. These slight differences in molecular weight could be again due to post-translational modifications of GSTM3. Moreover, another low-intensity band of ~ 48 kDa, which also disappeared in

Table 3 Correlation coefficients between relative GSTM3-abundances in both pre-frozen (P-F) and frozen thawed (F-T) spermatozoa and sperm quality parameters in pre-frozen (P-F) and frozen-thawed spermatozoa, evaluated 30 (F-T-30) and 240 (F-T-240) min post-thaw. *$P < 0.05$; **$P < 0.01$

		Relative levels of P-F GSTM3	Relative levels of F-T GSTM3
P-F	% Progressive motile spermatozoa	0.29	0.40
	% Total motile spermatozoa	0.21	0.31
	% SYBR-14$^+$/PI$^-$ spermatozoa	−0.23	−0.24
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	− 0.11	−0.03
	% JC1$_{agg}$ spermatozoa	0.06	0.09
	% E$^-$/YO-PRO-1$^-$ spermatozoa	−0.48	−0.27
F-T-30	% Progressive motile spermatozoa	−0.04	0.11
	% Total motile spermatozoa	−0.08	0.05
	% SYBR-14$^+$/PI$^-$ spermatozoa	−0.67*	−0.50
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	−0.70*	− 0.54
	% JC1$_{agg}$ spermatozoa	−0.80**	−0.70**
	% E$^-$/YO-PRO-1$^-$ spermatozoa	−0.74**	− 0.71**
F-T-240	% Progressive motile spermatozoa	0.08	0.19
	% Total motile spermatozoa	−0.08	0.04
	% SYBR-14$^+$/PI$^-$ spermatozoa	−0.75**	−0.64*
	% M540$^-$/YO-PRO-1$^-$ spermatozoa	−0.75**	− 0.71**
	% JC1$_{agg}$ spermatozoa	−0.56	0.56
	% E$^-$ / YO-PRO-1$^-$ spermatozoa	−0.45	−0.40

peptide competition assay, was present on GSTM3 membranes. The presence of that band may be due to the homodimerisation of this protein [32].

Since immunoblotting analysis did not show any loss of GSTM3 during cryopreservation, a relocation rather than a loss of this protein appears to occur due the freeze-thawing procedures. This was confirmed by immunofluorescence, where we observed that GSTM3 underwent relocation from the equatorial subdomain of the head, and mid-, principal and end pieces of the tail to the mid-piece during cryopreservation. In addition, no differences were observed between sperm from GFE and PFE in GSTM3-localisation. Previous studies in goat and human sperm [13, 14, 19] found GSTM3 at the apical region of the acrosome. These works also reported that this protein translocates to the equatorial and posterior acrosome regions during capacitation, and is lost from the acrosome upon acrosome reaction. On the other hand, Kumar et al. [22] showed that buffalo GSTM3 is localised over the connecting, mid-, principal, and end pieces of the tail in sperm before cryopreservation. These authors also demonstrated that after cryopreservation, GSTM3 migrated to the mid-piece. Remarkably, while this localisation pattern in pre-frozen and post-thawed buffalo sperm was similar to that observed in our study, it differed from that found in goat and human sperm.

Regarding GSTM3 content during cryopreservation, the results of the present study differ from those reported by Kumar et al. [22] in buffalo sperm. While these authors found a decrease in GSTM3 content after cryopreservation, we did not observe this effect. These differences might arise from the differences in techniques used between this study and the study of Kumar et al. [22]. Perhaps the most interesting result of our study, however, was the difference in relative GSTM3 content found between GFE and PFE, in both pre-frozen and post-thawed sperm. Ejaculates classified as PFE showed higher levels of GSTM3 than GFE. Taking into account that GSTM3 is involved in cell protection against oxidative stress [20], and PFE exhibit lower post-thaw sperm quality, it is reasonable to suggest that the higher levels of this protein in PFE could represent a mechanism to reduce oxidative stress.

The present study also attempted to find a relationship between sperm quality parameters and the relative amounts of IZUMO1 and GSTM3. Interestingly, a negative correlation between sperm quality parameters at 30 min post-thaw and relative levels of GSTM3 in pre-frozen spermatozoa was observed. Higher GSTM3-

content before cryopreservation was related to a lower percentage of viable spermatozoa (SYBR14$^+$/PI$^-$), viable spermatozoa with low membrane disorder, viable spermatozoa with low levels of intracellular superoxide levels, and spermatozoa with low mitochondrial membrane potential at post-thaw. Interestingly, other studies have demonstrated that an overexpression of GSTM3 in pre-frozen sperm is related with small litter sizes in boars [21] and with lower sperm quality in humans [33]. In addition, Hemachand and Shaha [20] reported that membrane-bound GSTMs eliminate ROS via extracellular glutathione and, consequently, prevent lipid membrane peroxidation, a process highly damaging to sperm membrane integrity [34]. This protection against oxidative stress exerted by this protein could preserve sperm motility, viability, mitochondrial status, oocyte binding capacity and fertilising ability. In fact, in the present study, a decrease of mitochondrial activity, and an increase in superoxide production and lipid disorder after cryopreservation were observed in both GFE and PFE. Thus, the relocation of GSTM3 to the mid-piece in response to cryopreservation reported in this study could be a mechanism of sperm to reduce oxidative stress during freeze-thawing.

Collectively, our results indicate that higher relative content of GSTM3 in pre-frozen sperm is related to lower sperm cryotolerance, and could be related to the fertility-associated issues of frozen-thawed sperm. While our findings demonstrate the reliability of GSTM3 as a sperm cryotolerance marker, further research including *in vitro* and *in vivo* fertilisation experiments is required to elucidate whether this protein is also a marker of their fertilising ability in both pre-frozen and frozen-thawed boar semen.

Conclusion

On the basis of immunofluorescence analysis and in accordance with studies in other species, relocation of IZUMO1 and GSTM3 occurs in response to cryopreservation. On the other hand, Western blot analysis shows no significant variations of IZUMO1 and GSTM3 content along the cryopreservation protocol. Nevertheless, although the content of IZUMO1 in pre-frozen boar sperm was found not to be related to their cryotolerance, sperm GSTM3 content before cryopreservation was higher in PFE than in GFE. These data indicate that GSTM3 could be used as a freezability marker in boar sperm. Finally, since no significant reduction of IZUMO1 and GSTM3 content has been reported during cryopreservation procedures, it is reasonable to suggest that the impaired fertilising ability of cryopreserved boar spermatozoa could be partially related to the abnormal translocation of both fertility-related proteins. However, additional *in vitro* and *in vivo* fertilisation essays are required to confirm this hypothesis.

Additional files

Additional file 1: Supplementary information for Materials and Methods. (DOC 53 kb)

Additional file 2: Western blots resulting from incubation with the (**A**) IZUMO1-antibody together with the IZUMO1-blocking peptide (IZUMO1 – blocking peptide) and its loading control (α-tubulin); and (**B**) GSTM3-antibody with GSTM3-blocking peptide (GSTM3 – blocking peptide) and its loading control (α-tubulin). Lanes P-F: pre-frozen sperm. Lanes F-T: frozen-thawed sperm. Lanes GFE: good freezability ejaculates. Lanes PFE: poor freezability ejaculates. (TIF 1214 kb)

Additional file 3: Immunofluorescence of (**A**) IZUMO1 negative control; (**B**) IZUMO1-antibody incubation with the IZUMO1-blocking peptide; (**C**) GSTM3 negative control and (**D**) GSTM3-antibody incubation with the GSTM3-blocking peptide. Nucleus is shown in blue (DAPI). Scale bars: A-B: 18 μm; C-D: 14 μm. (TIF 514 kb)

Additional file 4: Representative Western blot resulting from incubation with the (**A**) IZUMO1 antibody and its loading control (α-tubulin) and (**B**) GSTM3 antibody and its loading control (α-tubulin). Lanes P-F: pre-frozen sperm. Lanes F-T: frozen-thawed sperm. Lanes GFE: "good" freezability ejaculates. Lanes PFE: "poor" freezability ejaculates. (TIF 1479 kb)

Abbreviations

AI: Artificial insemination; ALH: Amplitude of lateral head displacement; ART: Assisted reproduction techniques; BCF: Beat cross frequency; GFE: Good freezability ejaculates; HE: Hydroethidine; IgSF: Immunoglobulin superfamily; ISAC: International Society for Advancement of Cytometry; IVF: *in vitro* fertilisation; JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide; LIN: Linearity; M540: Merocyanine 540; PFE: Poor freezability ejaculates; PI: Propidium iodide; PMOT: Progressive motility; ROS: Reactive oxygen species; SEM: Standard error of the mean; STR: Straightness; VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight line velocity

Acknowledgements

The authors would like to thank Estela Garcia and Technical Research Services of University of Girona for their technical support.

Authors' contributions

ML undertook the analysis and wrote the draft. AD performed cytometry analysis. IB, BF, SR, YM, SB contributed to the finalized manuscript. MY conceived the study. All authors read and approved the final manuscript.

Funding

The authors acknowledge the support from Ministry of Science, Innovation and Universities, Spain (Grants: RYC-2014-15581, AGL2017–88329-R and FJCI-2017-31689) and European Commission (H2020-MSCA-IF-79212).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 1 March 2019 Accepted: 5 June 2019
Published online: 05 August 2019

References

1. Gao D, Critser JK. Mechanisms of cryoinjury in living cells. ILAR J. 2000;41:187–96.
2. Yeste M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 2016;85:47–64.

3. Knox RV. The fertility of frozen boar sperm when used for artificial insemination. Reprod Domest Anim. 2015;50:90–7.
4. Casas I, Flores E. Gene banking: the freezing strategy. Boar Reprod. 1st ed. Heidelberg. Berlin: Springer; 2013. p. 551–88.
5. Waterhouse KE, Hofmo PO, Tverdal A, Miller RR. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction. 2006;131:887–94.
6. Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, et al. Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. Theriogenology. 2009;72:930–48.
7. Carrell DT, De Jonge C, Lam DJ. The genetics of male infertility: a field of study whose time is now. J Reprod Syst. 2006;52:269–74.
8. Parent S, Lefièvre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. Mol Reprod Dev. 1999;52:57–65.
9. Evans JP. The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. Hum Reprod Update. 2002;8:297–311.
10. Hunnicutt GR, Primakoff P, Myles DG. Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. Biol Reprod. 1996;55:80–6.
11. Pausch H, Kölle S, Wurmser C, Schwarzenbacher H, Emmerling R, Jansen S, et al. A nonsense mutation in TMEM95 encoding a nondescript transmembrane protein causes idiopathic male subfertility in cattle. PLoS Genet. 2014;10:1–11.
12. Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with egg. Nature. 2005;434:234–8.
13. Gopalakrishnan B, Aravinda S, Pawshe CH, Totey SM, Nagpal S, Salunke DM. Studies on glutathione S-transferase important for sperm function: evidence of catalytic activity-independent functions. Biochem J. 1998;329:231–41.
14. Petit FM, Serres C, Bourgeon F, Pineau C, Auer J. Identification of sperm head proteins involved in zona pellucida binding. Hum Reprod. 2013;0:1–14.
15. Bianchi E, Doe B, Goulding D, Wright GJ. Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature. 2014;508:483–7.
16. Fukuda M, Sakase M, Fukushima M, Harayama H. Changes of IZUMO1 in bull spermatozoa during the maturation, acrosome reaction, and cryopreservation. Theriogenology. 2016;86:2179–88 Elsevier Inc.
17. Kim E, Kim J-S, Lee Y, Song B-S, Sim B-W, Kim S-U, et al. Molecular cloning, characterization of porcine IZUMO1, an IgSF family member. Reprod Domest Anim. 2013;48:90–7.
18. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol. 2005;45:51–88.
19. Hemachand T, Gopalakrishnan B, Salunke DM, Totey SM, Shaha C. Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. J Cell Sci. 2002;115:2053–65.
20. Hemachand T, Shaha C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. FEBS Lett. 2003;538:14–8.
21. Kwon WS, Oh SA, Kim YJ, Rahman MS, Park YJ, Pang MG. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. Sci Rep. 2015;5:1–10.
22. Kumar R, Singh VK, Atreja SK. Glutathione-S-transferase: role in buffalo (Bubalus bubalis) sperm capacitation and cryopreservation. Theriogenology. 2014;81:587–98.
23. Prieto-Martínez N, Vilagran I, Morató R, Rivera del Álamo MM, Rodríguez-Gil JE, Bonet S, et al. Relationship of aquaporins 3 (AQP3), 7 (AQP7), and 11 (AQP11) with boar sperm resilience to withstand freeze-thawing procedures. Andrology. 2017;5:1153–64.
24. Pursel VG, Johnson LA. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. J Anim Sci. 1975;40:99–102.
25. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod. 1995;53:276–84.
26. Rathi R, Colenbrander B, Bevers MM, Gadella BM. Evaluation of *in vitro* capacitation of stallion spermatozoa. Biol Reprod. 2001;65:462–70.
27. Yeste M, Estrada E, Rivera del Álamo M-M, Bonet S, Rigau T, Rodríguez-Gil J-E. The increase in phosphorylation levels of serine residues of Ppotein HSP70 during holding time at 17°C is concomitant with a higher cryotolerance of boar spermatozoa. PLoS One. 2014;9:e90887.
28. Ortega-Ferrusola C, Sotillo-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, Gonzalez-Fernandez L, et al. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl. 2007;29:213–21.
29. Guthrie HD, Welch GR. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J Anim Sci. 2006;84:2089–100.
30. Aitken J, Young SM, Baker M. Phosphorylation of Izumo1 and its role in male infertility. Asian J Androl. 2015;17:708–10.
31. Satouh Y, Inoue N, Ikawa M, Okabe M. Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. J Cell Sci. 2012;125:4985–90.
32. Rowe JD, Patskovsky YV, Patskovska LN, Novikova E, Listowsky I. Rationale for reclassification of a distinctive subdivision of mammalian class mu glutathione S-transferases that are primarily expressed in testis. J Biol Chem. 1998;273:9593–601.
33. Botta T, Blescia S, Martínez-Heredia J, Lafuente R, Brassesco M, Ballescà JL, et al. Identificación de diferencias proteómicas en muestras oligozoospérmicas. Rev Int Androl. 2009;7:14–9.
34. Kodama H, Kuribayashi Y, Gagnon C. Effect of sperm lipid peroxidation on fertilization. J Androl. 1996;17:151–7.

PAPER IV

Glutathione S-transferase Mu 3 is associated to in vivo fertility, but not sperm quality, in bovine

Ferran Garriga*, Marc Llavanera*, Estel Viñolas-Vergés, Sandra Recuero, Carolina Tamargo, Ariadna Delgado-Bermúdez & Marc Yeste (* first co-authors)

Animal: an international journal of animal bioscience
2022;16(9), 100609

DOI: 10.1016/j.animal.2022.100609

Animal 16 (2022) 100609

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Glutathione S-transferase Mu 3 is associated to *in vivo* fertility, but not sperm quality, in bovine

Ferran Garriga [a,b,1], Marc Llavanera [a,b,1], Estel Viñolas-Vergés [a,b], Sandra Recuero [a,b], Carolina Tamargo [c], Ariadna Delgado-Bermúdez [a,b], Marc Yeste [a,b,d,*]

[a] *Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain*
[b] *Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain*
[c] *Department of Animal Selection and Reproduction, The Regional Agri-Food Research and Development Service of Asturias (SERIDA), ES-33394 Gijón, Spain*
[d] *Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain*

ARTICLE INFO

Article history:
Received 17 January 2022
Revised 7 July 2022
Accepted 11 July 2022

Keywords:
Biomarker
Dairy cattle
Flow cytometry
Non-return rate
Sperm quality

ABSTRACT

In the dairy breeding industry, pregnancy of dairy cows is essential to initiate milk production, so that high fertility rates are required to increase their productivity. In this regard, sperm proteins that are indicative of sperm quality and/or fertility have become an important target of study. Glutathione S-transferase Mu 3 (**GSTM3**) has been established as a fertility and sperm quality parameter in humans and pigs and, consequently, it might be a potential biomarker in cattle. For this reason, the present work aimed to determine if GSTM3 could predict sperm quality and *in vivo* fertility in this species. Sperm quality was assessed with flow cytometry and computer-assisted sperm analysis. Immunoblotting and immunofluorescence analysis were performed to determine the presence and localisation pattern of sperm GSTM3. This enzyme was found to be present in bovine sperm and to be localised along the sperm tail and the equatorial segment of the head. No significant associations between sperm GSTM3 and sperm quality parameters were observed, except a negative association with morphologically abnormal sperm having a coiled tail. In addition, and more relevant, higher levels of GSTM3 in sperm were seen in bulls showing lower *in vivo* fertility rates. In conclusion, our data evidenced the presence of GSTM3 in bovine sperm. Moreover, we suggest that, despite not being associated with sperm quality, GSTM3 might be an *in vivo* subfertility biomarker in cattle sperm, and that high levels of this protein could be an indicative of defective spermatogenesis and/or epididymal maturation.

Implications

In the dairy breeding industry, high fertility is required to increase productivity. The evaluation of fertility biomarkers is, therefore, of high relevance. Accordingly, the present work identifies and localises Glutathione S-transferase Mu 3 in bovine sperm and reveals that, despite not being associated to their quality, it could predict *in vivo* fertility in bulls. Although further research is required, sperm Glutathione S-transferase Mu 3 might be used as a molecular biomarker for *in vivo* fertility in cattle, which can maximise the efficiency and profitability of the dairy breeding industry.

Introduction

Over the years, the dairy breeding industry has selected males and females on the basis of their genetic traits for increasing milk production. Given that the lactation cycle is exclusively initiated by pregnancy, the prediction of bull sperm fertility is crucial to maximise the efficiency of the sector (Pryce et al., 2004; Miglior et al., 2017; Menezes et al., 2019). While the conventional spermiogram is the most commonly used method to evaluate male fertility, it is not always able to predict differences in fertility rates between males because it does not assess the physiological status of sperm (Krzyściak et al., 2020). Consequently, exploring novel sperm fertility biomarkers appears to be an interesting field of study.

Several molecular biomarkers have been reported as potential indicators of male fertility and subfertility (Krzyściak et al., 2020). Sperm proteins related to mitochondrial activity, such as Heat Shock Protein Family D Member 1, as well as antioxidant enzymes, such as glutathione peroxidases and glutathione S-transferases (**GSTs**), have been found to be relevant fertility

* Corresponding author.
E-mail address: marc.yeste@udg.edu (M. Yeste).
[1] These authors contributed equally to this work and share first authorship.

https://doi.org/10.1016/j.animal.2022.100609
1751-7311/

biomarkers in sperm (Kwon et al., 2015). Specifically, GSTs have been reported to play a crucial role in cell detoxification, catalysing the conjugation of electrophilic substances into reduced glutathione (**GSH**) (Hayes et al., 2004).

Alpha, Mu, Omega and Pi GST classes have been described to be present in mammalian sperm, playing a triple role (Llavanera et al., 2019b) consisting of (i) cell detoxification (Fafula et al., 2019), (ii) cell signalling regulation (Cho et al., 2001) and (iii) fertilisation (Petit et al., 2013). In addition, previous research supports that GSTs are essential to maintain sperm quality by protecting the male gamete from oxidative stress (Fafula et al., 2019) through the activation of the JNK signalling pathway (Llavanera et al., 2021a). Furthermore, GSTM3, a specific GST, has been shown to be specifically relevant in sperm cells. This protein belongs to the Mu class of canonical soluble GSTs and is active as a dimer (Armstrong, 1997). It is expressed both in the testis, during spermatogenesis, and throughout the male reproductive tract (Li et al., 2010), and appears to be essential for proper sperm-oocyte binding, interacting with the zona pellucida (Petit et al., 2013). In men, high amounts of sperm GSTM3 are related to low sperm quality in patients suffering from oligozoospermia and varicocele (Botta et al., 2009; Agarwal et al., 2015). In boars, high levels of sperm GSTM3 are joined with small litter sizes (Kwon et al., 2015). GSTM3 has also been established as a sperm quality biomarker in pigs, since its abundance negatively correlates to sperm motility and mitochondrial activity, although its presence is necessary to maintain sperm quality (Llavanera et al., 2020b). Moreover, GSTM3 has been found to be a cryotolerance marker in porcine sperm (Llavanera et al., 2019a).

In spite of all the aforementioned, and to the best of the authors' knowledge, no previous study has investigated the presence and role of GSTM3 in bovine sperm. Considering the relevant function of GSTM3 as a potential quality and fertility biomarker in mammalian sperm, the aim of the present work was to determine the presence and localisation of GSTM3 in bovine sperm, as well as to address whether it could be a sperm quality and *in vivo* fertility biomarker in cattle.

Material and methods

Unless otherwise indicated, chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, United States).

Animals and ejaculates

Seminal AI-doses used in this study were produced following the Spanish and European legislation for animal husbandry and welfare. Twelve healthy and sexually mature Holstein bulls from 1.5 to 2 years old were involved in this research. Animals were housed at Cenero AI centre in Gijón, Asturias (Spain), under standard feeding and housing conditions. Ejaculates were collected using an artificial vagina. A total of 2 087 heifers were inseminated, with an average number of 174 inseminated heifers per bull. Supplementary Fig. S1 shows the distribution of NRR among bulls. Ninety-day non-return rates (**NRRs**) to oestrus were used to assess *in vivo* fertility, through dividing the serviced heifers by the total number of inseminations.

Ejaculates having 2–8 ml of volume, sperm concentration >10^9 spermatozoa per ml and total motility greater than 85% were cryopreserved. First, the concentration of ejaculates was adjusted using a commercial extender (Bioxcell; IMV Technologies L'Aigle, France) to 92 × 10^6 sperm per ml and then packaged into 0.25-ml straws. The cryopreservation procedure was performed by using a controlled-rate freezer (Digit-cool; IMV Technologies). Straws were then stored in a nitrogen tank. The thawing procedure consisted of warming sperm samples at 38 °C for 20 s in a water bath. A total of six straws per bull were pooled together (biological replicate) before the assessment of sperm quality, using two straws from three independent ejaculates per bull.

Sperm motility

Samples were diluted 1:3 (v:v) with Phosphate-Buffered Saline (**PBS**) and subsequently evaluated. A Computer-Assisted Sperm Analysis (**CASA**) system was used to determine sperm motility parameters (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain) prewarmed at 38 °C. Sperm loaded into Leja chamber slides (Leja Products BV; Nieuw-Vennep, The Netherlands) were subsequently analysed by capturing 30 frames per second. The average path velocity at ≥10 μm/s was the threshold to consider a spermatozoon as motile, whereas the index of straightness at ≥70% was the threshold to consider a spermatozoon as progressively motile. A total of 1 000 sperm per replicate and two technical replicates per sample were assessed.

Sperm morphology

Sperm samples were diluted 1:3 (v:v) with PBS. Five μl of a diluted sample was used for each examination. Sperm samples were observed under an optical microscope (Olympus BX41) and evaluated using the SCA® Production software (Microptic S.L., Barcelona, Spain). Sperm were visually classified as morphologically normal or abnormal (abnormalities of the head size, shape and acrosome, isolated heads, folded and coiled tails, and proximal and distal droplets). Two hundred sperm per sample were examined.

Oxygen consumption rate

A SensorDish® Reader system (PreSens Gmbh; Regensburg, Germany) was used to evaluate oxygen consumption rate in sperm samples. A volume of 150 μl from each sperm sample was diluted in 850 μl of PBS and transferred onto Oxodish® OD24 plates before sealing them with Parafilm®. Plates were incubated at 38 °C for 3 h, and O_2 concentration was measured every 30 s. Oxygen consumption rate of each sample was subsequently calculated and normalised by the total number of viable sperm per well.

Flow cytometry analysis

Flow cytometry analysis was performed using a CytoFLEX cytometer (Beckman Coulter, California, USA). Sperm samples were diluted in PBS to a final concentration of 4 × 10^6 sperm per ml. Five sperm parameters were evaluated (sperm viability [**SYBR-14/PI**], intracellular calcium levels [**Fluo3-AM/PI**], intracellular Reactive Oxygen Species (**ROS**) levels [**H_2DCFDA/PI**], intracellular superoxide levels [**HE/Yo-Pro-1**], and chromatin (de)condensation [**CMA3/Yo-Pro-1**]). SYBR-14, Fluo3-AM, Yo-Pro-1 and H_2DCFDA were excited with the 488 nm laser, and their fluorescence was detected by the FITC channel (525/40). HE was excited with the 488 nm laser, and its fluorescence was collected through the PE channel (585/42). PI was excited with the 488 nm laser, and its fluorescence was detected by the PC5.5 channel (690/50). CMA3 was excited with the 405 nm laser, and its fluorescence was collected via the Violet610 channel (610/20).

Sperm viability was determined by double staining using SYBR-14 (32 nmol/l) and PI (7.5 μmol/l), based on the protocol of Garner and Johnson (1995). Intracellular calcium levels were evaluated through co-staining with Fluo3-AM (1.2 μmol/l) and PI (5.6 μmol/l), following the protocol described by Harrison et al. (1996). Overall ROS levels were determined after co-staining with H_2DCFDA (100 μmol/l) and PI (6 μmol/l), as described by Guthrie

and Welch (2006). The assessment of intracellular superoxide levels was performed by co-staining samples with Yo-Pro-1 (31.2 nmol/l) and HE (5 µmol/l), following a modification of the protocol of Guthrie and Welch (2006). Finally, chromatin (de)compaction was evaluated following double staining with CMA3 and Yo-Pro-1, as previously described by Llavanera et al. (2021b). Extended flow cytometry protocols are described in detail in Supplementary Material S1.

Immunoblotting analysis

Sperm samples were centrifuged twice at 3 000*g* for 5 min. Total protein content was extracted by mixing sperm samples with 400 µl of lysis buffer (RIPA buffer; Sigma), supplemented with 1% protease inhibitor cocktail (Sigma), followed by incubation on ice for 30 min. Samples were sonicated and subsequently centrifuged at 12 000*g* and 4 °C for 20 min. Finally, total protein of supernatants was assessed through a detergent-compatible method using a commercial kit (BioRad).

Ten µg of total protein was resuspended in Laemmli Reductor 4× buffer supplemented with 10% (v:v) β-mercaptoethanol and heated up to 95 °C for 5 min. Next, samples were loaded onto 12% Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) and electrophoresis was carried out at 200 V for 40 min at 4 °C. Total protein from the gel was quantified using the Stain-Free method and visualised using a G:BOX Chemi XL system (Syngene, Frederick, MD, United States). Following this, a Trans-Blot Turbo device (BioRad) was used to transfer proteins onto PVDF membranes. Membranes were blocked in blocking buffer (5% BSA in TBS) in agitation for 1 h. Thereafter, membranes were incubated with a primary rabbit anti-GSTM3 antibody (1:15 000; v:v) overnight at 4 °C and, subsequently, washed thrice with TBS-Tween-20 and incubated with an HRP-coupled secondary goat anti-rabbit antibody (1:30 000; v:v) for 1 h. Finally, membranes were washed five times, revealed with a chemiluminescent substrate and visualised with a G:BOX Chemi XL system (Syngene). To assess the specificity of the primary anti-GSTM3 antibody, a peptide competition assay was performed using an excess of the GSTM3 immunising peptide (20-fold regarding the primary antibody). Relative GSTM3 levels were quantified using Image Studio™ Lite v.3.1. (Licor) and normalised against the total protein from the blot. Two technical replicates per sample were analysed.

Immunofluorescence analysis

To determine the localisation of GSTM3 in bovine sperm, an immunofluorescence assay was performed. Sperm samples were adjusted to a final concentration of 5×10^6 sperm per ml, centrifuged at 600*g* for 5 min, resuspended in 2% paraformaldehyde and incubated for 30 min prior to being washed again. Then, 150 µl of diluted sperm was placed on a slide. Subsequently, slides were washed thrice and incubated with PBS supplemented with 1% Triton and 5% BSA for permeabilisation and blocking, respectively. Next, slides were incubated with the primary antibody, diluted at 1:250 (v:v) in blocking solution, at 4 °C overnight. For peptide competition assay, the GSTM3-specific immunising peptide was added 10 times in excess with regard to the primary antibody. Five rinses were performed before incubation with the secondary antibody, diluted 1:500 (v:v) in blocking solution, for 1 h in the dark. Finally, five rinses were performed, and samples were mounted with Vectashield mounting medium before coverage with a coverslip. Samples were observed under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon, Tokyo, Japan) with predetermined acquisition settings and analysed using the Fiji ImageJ software (Schindelin et al., 2012). Brightness and contrast were homogenously adjusted in all images.

Statistical analysis

Data were analysed with IBM SPSS Statistics 27.0 (IBM, Armonk, NY, USA) and plotted with GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, USA). First, normal distribution and homogeneity of variances were checked by running Shapiro-Wilk and Levene tests, respectively. Twelve biological replicates were used ($n = 12$), each one being considered a statistical case. The level of significance was set at $P \leq 0.05$.

Sperm samples were classified by their GSTM3 content in two groups on the basis of the median value. Sperm parameters were compared between the two GSTM3 content groups through a parametric *t*-test. Alternatively, a non-parametric Mann-Whitney U test was used when data did not meet normality and/or homoscedasticity assumptions. The Spearman's rank coefficient was used to determine the correlations of sperm GSTM3 levels with sperm quality and *in vivo* fertility parameters.

Results

Presence and localisation of Glutathione S-transferase Mu 3 in bovine sperm

Immunoblotting analysis was performed to determine the presence and relative content of GSTM3 in frozen-thawed bovine sperm. Immunoblotting analysis using an anti-GSTM3 antibody evidenced a single band of ~48 kDa in every assessed sample

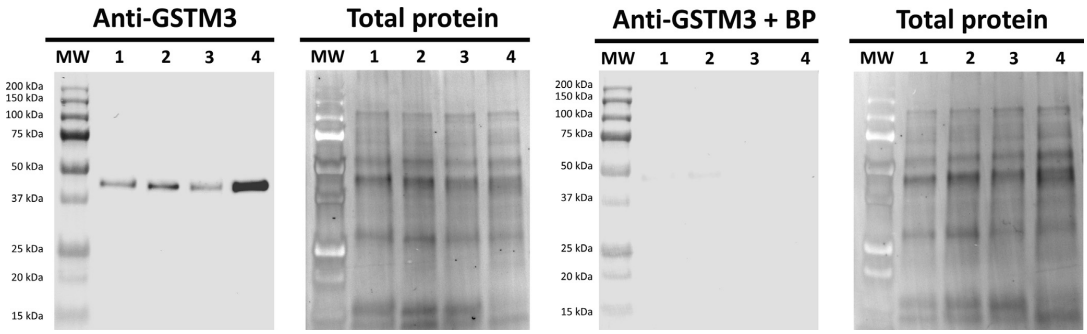

Fig. 1. Representative Western blots of GSTM3 in bovine sperm. (**A**) Incubation with the GSTM3 antibody (Anti-GSTM3) and its loading control (Total protein); (**B**) Incubation with the GSTM3 antibody and the corresponding immunising peptide (peptide competition assay), and its loading control (Total protein). MW: Molecular weight; 1–4 correspond to four independent sperm samples from different bulls. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; BP, blocking peptide.

(Fig. 1A). This ~48 kDa band was absent from the blot incubated with the blocking peptide (Fig. 1B).

The localisation pattern of GSTM3 in bovine sperm was assessed by immunofluorescence. Sperm GSTM3 was found to be localised along the principal, mid and end pieces of the tail, as shown in Fig. 2. Moreover, a weaker GSTM3-specific signal was also found in the equatorial segment of the head. The peptide competition assay did not show green fluorescence.

Correlations of sperm Glutathione S-transferase Mu 3 with sperm quality and fertility

Fig. 3 shows Spearman's rank correlation coefficient between relative GSTM3 content, sperm quality and *in vivo* fertility parameters. No correlation between sperm GSTM3 content and sperm quality parameters was found ($P > 0.05$), excepting a negative correlation between GSTM3 levels and the percentage of morpholog-

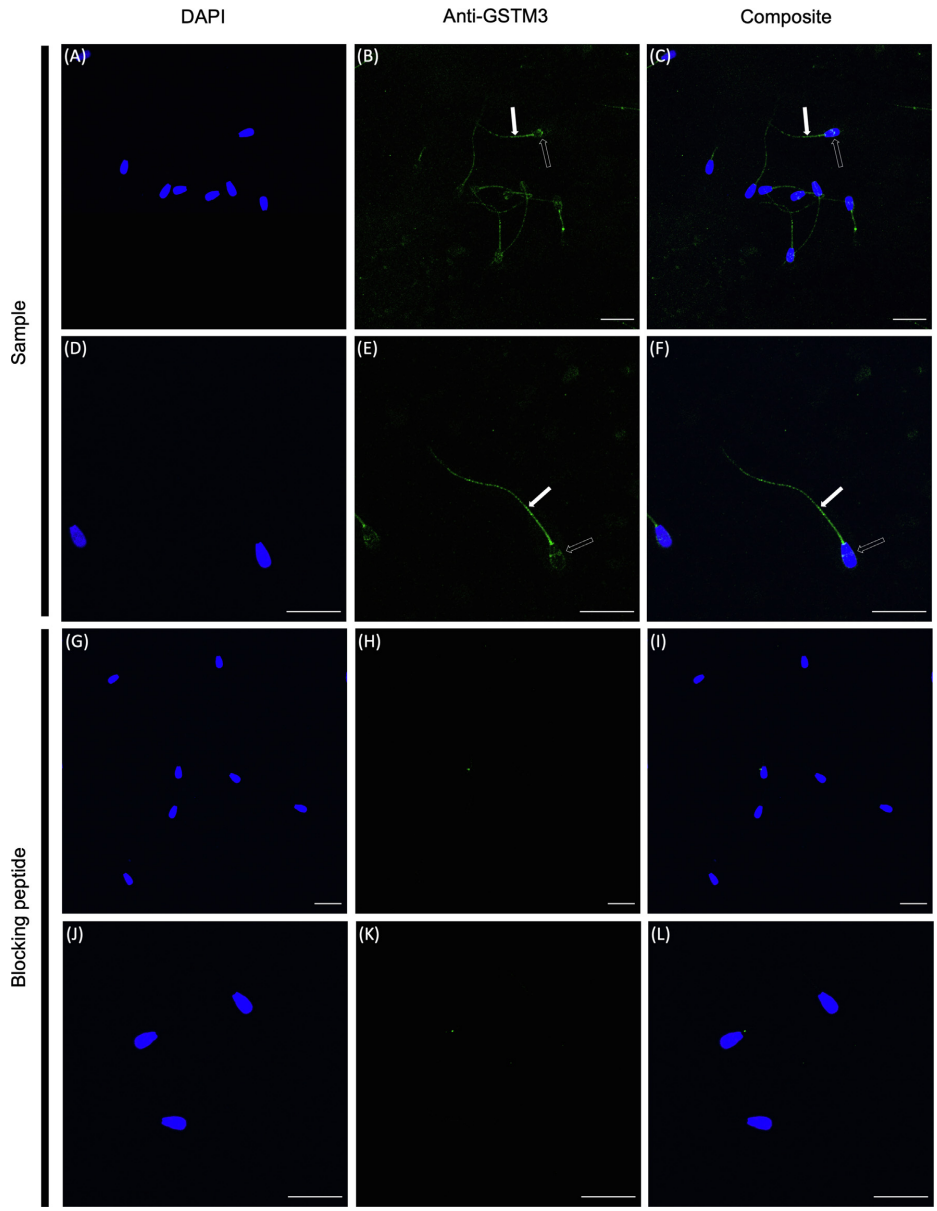

Fig. 2. Representative immunofluorescence analysis of GSTM3 in bovine sperm. (**A-F**) Representative bovine sperm sample (sample). (**G-L**) Peptide competition assay of the sample (blocking peptide). The nucleus is shown in blue (DAPI), whereas GSTM3 is shown in green. White arrows indicate GSTM3 in the sperm tail, whereas black arrows indicate GSTM3 within the equatorial subdomain of the head. Scale bars: 20 µm. Abbreviations: GSTM3, Glutathione S-transferase Mu 3.

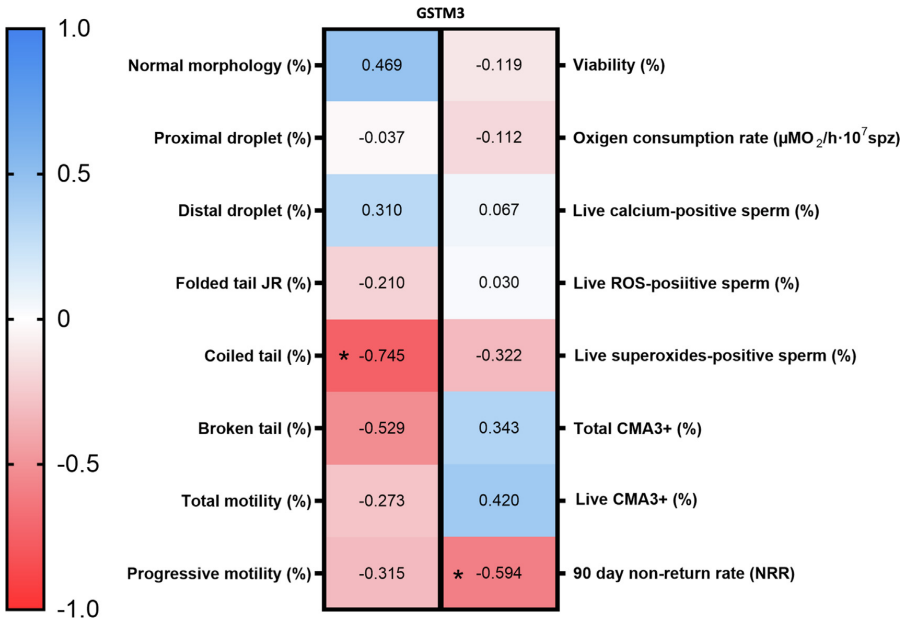

Fig. 3. Heatmap of the Spearman's rank correlation coefficients between relative GSTM3 content in bovine sperm and the different parameters evaluated to determine sperm parameters and *in vivo* fertility. (*) indicates significant correlations ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3; JR, Jansen's ring; ROS, reactive oxygen species; $CMA3^+$, chromomycin A3-positive cells.

ically abnormal sperm with coiled tail ($R = -0.75$; $P < 0.05$). Moreover, a significant negative correlation was observed between sperm GSTM3 content and NRR ($R = -0.60$; $P < 0.05$).

Comparison of sperm quality parameters between Glutathione S-transferase Mu 3 groups

Sperm viability, total and progressive motility, and morphology were analysed as conventional sperm quality parameters. Each sperm parameter was compared between GSTM3 groups (low and high GSTM3 content). No significant differences**** ($P > 0.05$) between GSTM3 groups were found when assessing sperm viability (Fig. 4C), total motility (Fig. 4A), progressive motility (Fig. 4B) or morphology (Fig. 4D-I). An increased percentage of morphologically abnormal sperm with coiled tails, however, was found in the low GSTM3 group ($P < 0.05$).

Comparison of sperm metabolic parameters between Glutathione S-transferase Mu 3 groups

Sperm metabolism was evaluated through the analysis of the following sperm parameters: O_2 consumption rate (Fig. 5A), percentage of calcium-positive viable sperm (Fig. 5B), percentage of overall ROS-positive viable sperm (Fig. 5C), and percentage of superoxide-positive viable sperm (Fig. 5D). No significant differences ($P > 0.05$) between GSTM3 groups were observed in any of these metabolism-related parameters.

Comparison of sperm chromatin condensation status between Glutathione S-transferase Mu 3 groups

The putative relationship between sperm GSTM3 content and sperm chromatin (de)condensation status was evaluated through flow cytometry with a double CMA3/Yo-Pro-1 staining. Fig. 6 represents the distribution of the percentages of CMA3-positive cells in total and viable sperm populations between GSTM3 groups (i.e. low and high GTSM3 content). No significant differences ($P > 0.05$) in sperm chromatin condensation were found between GSTM3 groups.

Relationship between sperm Glutathione S-transferase Mu 3 content and in vivo fertility

To evaluate the relationship between sperm GSTM3 content and *in vivo* fertility, NRRs were compared between the two GSTM3 groups (i.e. low and high GSTM3 content). Furthermore, Spearman's rank correlation coefficient between the two parameters was analysed. A negative correlation between sperm GTSM3 content and NRR was observed ($R = -0.60$; $P < 0.05$) (Fig. 7B). Moreover, significant differences in NRR between low and high sperm GSTM3 groups were found ($P < 0.05$). Specifically, higher NRRs were observed in samples showing low levels of GSTM3 (Fig. 7A). Raw data of NRR and sperm GSTM3 levels of each bull are available in Supplementary Table S1.

Discussion

Molecular biomarkers have become relevant tools to predict the physiological status of sperm, which is unachievable with conventional semen analysis (Krzyściak et al., 2020). GSTM3 is an antioxidant enzyme that has been reported to be a useful biomarker for male infertility or subfertility in humans and pigs, as well as a predictor of sperm quality in these species (Botta et al., 2009; Kwon et al., 2015). In this regard, it is reasonable to suggest that sperm GSTM3 could be a potential biomarker for sperm quality and *in vivo* fertility in bovine species.

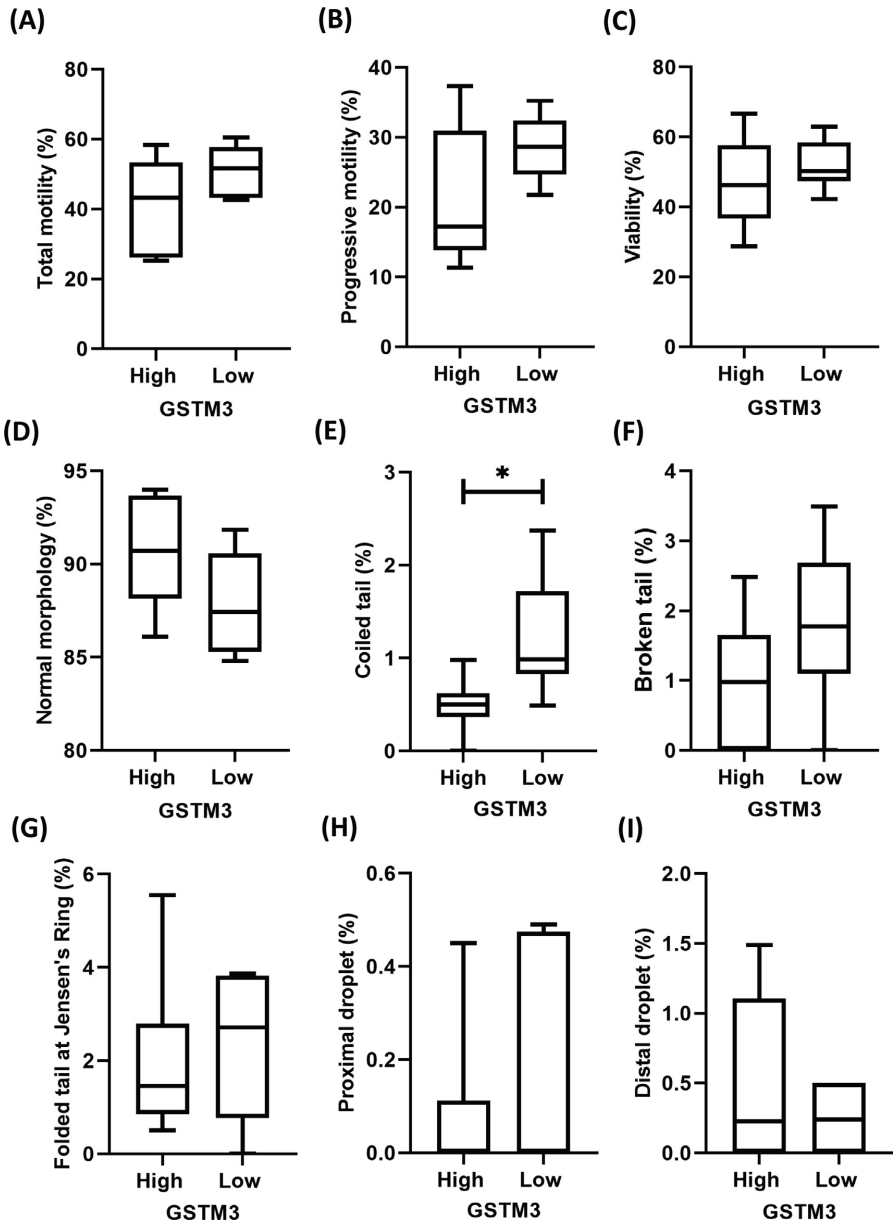

Fig. 4. Box plots representing the distribution of the percentages of (**A**) total motile sperm; (**B**) progressively motile sperm; (**C**) viable sperm; (**D**) morphologically normal sperm; (**E**) sperm with coiled tails; (**F**) sperm with broken tails; (**G**) sperm with folded tails at Jensen's ring; (**H**) sperm with proximal droplets; and (**I**) sperm with distal droplets, between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). (*) indicates significant differences between GSTM3 groups ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3; spz, spermatozoa.

Immunoblotting and immunofluorescence analysis allowed us to confirm the presence and localisation of GSTM3 in bovine sperm, which, to the best of our knowledge, has not been previously described. A single band of ~48 kDa was observed in immunoblotting analysis, which was confirmed to be GSTM3-specific in the peptide competition assay. These results are consistent with the molecular weight of GSTM3 as, while this protein is known to be ~26 kDa in its monomeric form, it has been described to be stable as a homodimer only (Armstrong, 1997) which would explain the presence of the ~48 kDa band in blots. Yet, the unexpected increase in the molecular weight of GSTM3 could also be caused by post-translational modifications, which have been

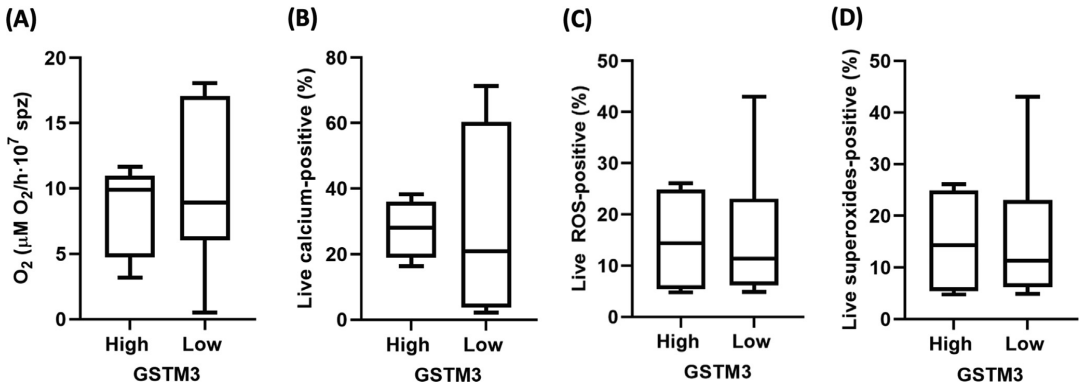

Fig. 5. Box plots representing the distribution of (**A**) O_2 consumption rate normalised against viable sperm (µM O_2/h × 10^7 sperm); (**B**) percentage of calcium-positive viable sperm; (**C**) percentage of ROS-positive viable sperm between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs); and (**D**) percentage of superoxide-positive viable sperm. No significant differences between groups were observed. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; spz, spermatozoa.

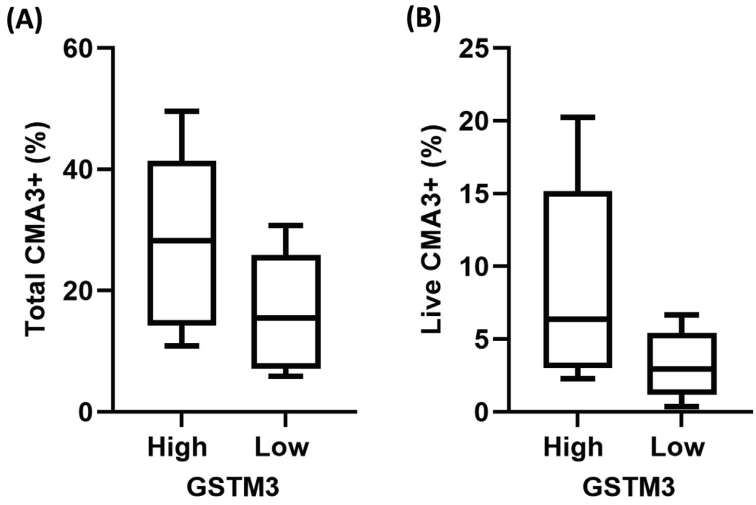

Fig. 6. Box plots representing the distribution of the percentage of (**A**) total and (**B**) viable CMA3-positive sperm between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). No significant differences between groups were observed. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; $CMA3^+$, chromomycin A3-positive cells.

extensively reported in sperm (Samanta et al., 2016), or covalent protein-protein interactions. In spite of this, while a ~25 kDa band corresponding to monomeric GSTM3 was observed in the sperm of other species (Kwon et al., 2015; Llavanera et al., 2019a), it was not detected herein. Thus, although the presence of GSTM3 was confirmed in the present study, whether its homodimerisation, post-translational modifications and/or covalent protein-protein interactions occur in bovine sperm remains unknown.

Immunofluorescence analysis allowed determining the specific localisation of GSTM3 in bovine sperm. Sperm GSTM3 was found to be present along the sperm tail, comprising mid, principal and end pieces. Interestingly, this pattern partially differs from that observed in frozen-thawed sperm from other species. In pigs, sperm GSTM3 is localised only in the midpiece of the tail (Llavanera et al., 2019a) after cryopreservation, whereas in humans and goats, it is comprised in the acrosome and the postequatorial region (Gopalakrishnan et al., 1998; Petit et al., 2013). Interestingly, the localisation of GSTM3 in frozen-thawed cattle sperm seen in this study was similar to that reported for fresh but not for frozen-thawed pig sperm, where it is found in the sperm tail and the equatorial segment of the head (Llavanera et al., 2020b). Remarkably, the literature, together with our results, suggests a highly variable localisation pattern of sperm GSTM3 among mammalian species.

The association between sperm GSTM3 levels and their quality parameters was also evaluated in the present study. GSTM3 in porcine and caprine sperm has been reported to be essential to maintain sperm motility and mitochondrial activity (Gopalakrishnan and Shaha, 1998; Llavanera et al., 2020b). Interestingly, in this study, no significant correlation between GSTM3 levels and sperm

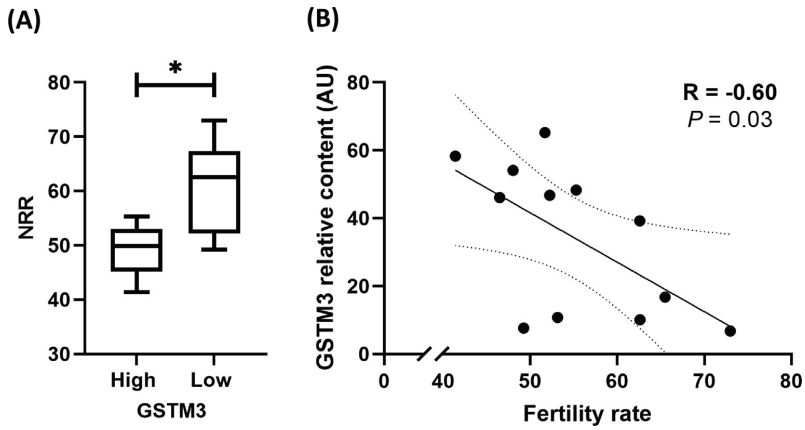

Fig. 7. (**A**) Box plot representing the distribution of non-return rates (NRRs) between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). (**B**) Scatter plot of Spearman's rank correlation coefficients between GSTM3 relative content and NRR. (*) indicates significant differences between GSTM3 groups ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3.

quality and metabolic parameters was observed. Moreover, no differences between high and low GSTM3 groups were seen. These results suggest that GSTM3 is not related to sperm quality and metabolism in cattle. Specifically, given the role of GSTM3 as an antioxidant enzyme, the lack of correlation between this enzyme and ROS levels in sperm is interesting. The fact that GSTM3 shows an association with sperm motility and mitochondrial activity in porcine and ovine, but not in bovine, indicates that the involvement of this protein on sperm physiology is variable among mammalian species. Moreover, sperm GSTM3 was not found to be associated to ROS levels in cattle, which concurs with previous research in humans and pigs and leads one to posit that this enzyme is not involved in cell detoxification in these species.

The results obtained in the present work suggest a putative relationship between sperm GSTM3 levels and sperm morphology, specifically in tail malformations. The percentage of coiled tail sperm was negatively associated with relative GSTM3 content, suggesting that low levels of this enzyme are associated to an increased percentage of sperm with a coiled tail. Accordingly, the percentage of sperm showing this abnormality was also found to be higher in the group with lower GSTM3 content. Sperm malformations can be originated during spermatogenesis (primary malformations) or sperm maturation along the epididymis (secondary malformations) (Briz and Fàbrega, 2013), and the coiled tail is known to be a secondary malformation (Nisa et al., 2018). The association between GSTM3 and secondary malformations has already been discussed in a previous study assessing the function of pig GTSM3 in seminal plasma (Llavanera et al., 2020a). In that study, a role of GSTM3 during epididymal maturation, but not in ejaculated sperm, was suggested (Llavanera et al., 2020a). These results agree with those reported herein, evidencing that GSTM3 might play a key role during epididymal maturation rather than in sperm antioxidant capacity.

Regarding the putative relationship between GSTM3 levels and sperm chromatin status, our work did not find any significant relationship between both parameters, suggesting that GSTM3 is not involved in sperm chromatin (de)condensation. In contrast, Tarozzi et al. (2009) reported a negative relationship between the antioxidant capacity of seminal plasma and the protamination status of sperm chromatin in humans. These differences could be explained because sperm GSTM3 might not play an antioxidant role on sperm, which agrees with the fact that no relationship between its levels and the parameters concerning oxidative stress was observed. Due to the low number of samples used in both studies, nevertheless, further research to elucidate the relationship between antioxidant enzymes and sperm chromatin status is required.

As previously described in other mammalian species, sperm GSTM3 could be related to male fertility, since it is a membrane-bound protein that interacts with the zona pellucida during fertilisation (Botta et al., 2009; Agarwal et al., 2015; Llavanera et al., 2019b). Our results evidenced a negative correlation between sperm GSTM3 content and *in vivo* fertility in bovine sperm. In effect, low sperm GSTM3 levels were found in highly fertile males when compared to subfertile individuals. These results agree with those reported by other authors assessing the relationship between sperm GSTM3 and *in vivo* fertility in humans and pigs, with lower levels of GSTM3 in highly fertile ejaculates (Botta et al., 2009; Kwon et al., 2015). The increased levels of sperm GSTM3 in males showing reduced fertility might be associated to an increased rate of defective spermatogenesis and/or epididymal maturation (Sabeti et al., 2016). Because the main role of GSTM3 is known to be cell detoxification, spermatogonia with high oxidative stress and/or inadequate spermatogenesis may enhance the expression of GSTM3 (Llavanera et al., 2019b). Consequently, sperm with defective spermatogenesis and subsequent impaired fertility might show higher levels of GSTM3, indicating increased oxidative stress during spermatogenesis (Gharagozloo and Aitken, 2011). Moreover, given the previously mentioned association between GSTM3 content and secondary malformations during epididymal maturation (Llavanera et al., 2020a), this antioxidant enzyme could also be implied in this process. Our results are consistent with those reported in men and boars showing sperm GSTM3 as a potential fertility biomarker in these species. In this regard, although further research is required, sperm GSTM3 may be used as a biomarker of *in vivo* fertility rates in bovine, which is of great interest for the dairy breeding industry. Furthermore, these results pave the way for future research regarding the role of sperm GTSM3 in other mammalian species.

In conclusion, immunoblotting analysis evidenced the presence of GSTM3 in bovine sperm for the first time. Immunofluorescence results confirmed the species-specific expression pattern of GSTM3 in bovine sperm, reported along the sperm tail and the equatorial segment, and differing from those observed in other mammalian

species. When analysing its potential role as a sperm quality biomarker, no significant association between GSTM3 and sperm quality and metabolism parameters, such as ROS levels and sperm chromatin (de)condensation, was observed. Interestingly, high GSTM3 levels were related to a lower percentage of sperm showing tail morphologic abnormalities, suggesting a putative function of this enzyme during epididymal maturation. Finally, we also assessed if GSTM3 could be a male *in vivo* fertility biomarker in cattle. As previously described in humans and pigs, high GSTM3 levels in bovine sperm were found to correlate with low fertility rates. Thus, although further studies involving a larger number of animals are required to confirm our results, this work suggests that sperm GSTM3 could be used as a fertility biomarker in bovine.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100609.

Ethical Approval

Not applicable.

Author ORCIDs

Ferran Garriga (0000-0002-9445-4438); **Marc Llavanera** (0000-0003-0685-7534), **Estel Viñolas-Vergés** (0000-0001-5286-3092); **Sandra Recuero** (0000-0001-9341-1164); **Carolina Tamargo** (0000-0002-4484-8981); **Ariadna Delgado-Bermúdez** (0000-0001-5655-5003); **Marc Yeste** (0000-0002-2209-340X).

Authors' contributions

ML and **MY** conceived the study. **CT** handled animals and conducted sperm cryopreservation and **AI**. **FG**, **ML**, **EV-V**, **SR** and **AD-B** conducted laboratory analysis. **FG**, **ML** and **MY** participated in the discussion of the results. **FG** and **ML** wrote the Manuscript. **MY** revised and edited the Manuscript. All authors contributed to the finalised Manuscript, read and approved the final version.

Declaration of interest

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

Acknowledgements

Not applicable.

Financial support statement

The authors acknowledge the support from the Ministry of Science and Innovation, Spain (Grants: AGL2017-88329-R, FPU18/00666 and PID2020-113320RB-I00), the Ministry of Universities, Spain (Grant: EST19/00389), the Catalan Agency for Management of University and Research Grants, Regional Government of Catalonia, Spain (Grant: 2017-SGR-1229), and the Catalan Institution for Research and Advanced Studies (ICREA).

Data and model availability statement

None of the data were deposited in an official repository. The datasets used and/or analyses during the current study are available from the corresponding author on reasonable request.

References

Agarwal, A., Sharma, R., Durairajanayagam, D., Ayaz, A., Cui, Z., Willard, B., Gopalan, B., Sabanegh, E., 2015. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. Reproductive Biology and Endocrinology 13, 1–22. https://doi.org/10.1186/S12958-015-0007-2.

Armstrong, R.N., 1997. Structure, Catalytic Mechanism, and Evolution of the Glutathione Transferases. Chemical Research in Toxicology 10, 2–18. https://doi.org/10.1021/TX960072X.

Botta, T., Blescia, S., Martínez-Heredia, J., Lafuente, R., Brassesco, M., Luis Ballescà, J., Oliva, R., 2009. Identificación de diferencias proteómicas en muestras oligozoospérmicas. Revista Internacional de Andrología 7, 14–19. https://doi.org/10.1016/S1698-031X(09)70257-2.

Briz, M., Fàbrega, A., 2013. The boar spermatozoon. In: Bonet, S., Casas, I., Holt, W., Yeste, M. (Eds.), Boar Reproduction: Fundamentals and New Biotechnological Trends. Springer-Verlag, Berlin, Germany, pp. 3–47. https://doi.org/10.1007/978-3-642-35049-8_1.

Cho, S.-G., Lee, Y.H., Park, H.-S., Ryoo, K., Kang, K.W., Park, J., Eom, S.-J., Kim, M.J., Chang, T.-S., Choi, S.-Y., Shim, J., Kim, Y., Dong, M.-S., Lee, M.-J., Kim, S.G., Ichijo, H., Choi, E.-J., 2001. Glutathione S-Transferase Mu Modulates the Stress-activated Signals by Suppressing Apoptosis Signal-regulating Kinase 1. Journal of Biological Chemistry 276, 12749–12755. https://doi.org/10.1074/jbc.M005561200.

Fafula, Paranyak, N.M., Besedina, A.S., Vorobets, D.Z., Iefremova, U.P., Onufrovych, O. K., Vorobets, Z.D., 2019. Biological significance of glutathione S-transferases in human sperm cells. Journal of Human Reproductive Sciences 12, 24. https://doi.org/10.4103/jhrs.JHRS_106_18.

Garner, D.L., Johnson, L.A., 1995. Viability Assessment of Mammalian Sperm Using SYBR-14 and Propidium Iodide. Biology of Reproduction 53, 276–284. https://doi.org/10.1095/biolreprod53.2.276.

Gharagozloo, P., Aitken, R.J., 2011. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Human Reproduction 26, 1628–1640. https://doi.org/10.1093/humrep/der132.

Gopalakrishnan, B., Aravinda, S., Pawshe, C.H., Totey, S.M., Nagpal, S., Salunke, D.M., Shaha, C., 1998. Studies on glutathione S-transferases important for sperm function: Evidence of catalytic activity-independent functions. Biochemical Journal 329, 231–241. https://doi.org/10.1042/bj3290231.

Gopalakrishnan, B., Shaha, C., 1998. Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. FEBS Letters 422, 296–300. https://doi.org/10.1016/S0014-5793(98)00032-5.

Guthrie, H.D., Welch, G.R., 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. Journal of Animal Science 84, 2089–2100. https://doi.org/10.2527/jas.2005-766.

Harrison, R.A.P., Ashworth, P.J.C., Miller, N.G.A., 1996. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Molecular Reproduction and Development 45, 378–391. https://doi.org/10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2004. Glutathione transferases. Annual Review of Pharmacology and Toxicology 45, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857.

Krzyściak, W., Papież, M., Bąk, E., Morava, E., Krzyściak, P., Ligęzka, A., Gniadek, A., Vyhouskaya, P., Janeczko, J., 2020. Sperm Antioxidant Biomarkers and Their Correlation with Clinical Condition and Lifestyle with Regard to Male Reproductive Potential. Journal of Clinical Medicine 9, 1785. https://doi.org/10.3390/jcm9061785.

Kwon, W.-S., Oh, S.-A., Kim, Y.-J., Rahman, M.S., Park, Y.-J., Pang, M.-G., 2015. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. Scientific Reports 5, 1–10. https://doi.org/10.1038/srep13821.

Li, J., Liu, F., Wang, H., Liu, X., Liu, J., Li, N., Wan, F., Wang, W., Zhang, C., Jin, S., Liu, J., Zhu, P., Liu, Y., 2010. Systematic Mapping and Functional Analysis of a Family of Human Epididymal Secretory Sperm-Located Proteins. Molecular & Cellular Proteomics 9, 2517–2528. https://doi.org/10.1074/MCP.M110.001719.

Llavanera, M., Delgado-Bermúdez, A., Fernandez-Fuertes, B., Recuero, S., Mateo, Y., Bonet, S., Barranco, I., Yeste, M., 2019a. GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. Journal of Animal Science and Biotechnology 10, 1–11. https://doi.org/10.1186/S40104-019-0370-5.

Llavanera, M., Delgado-Bermúdez, A., Mateo-Otero, Y., Padilla, L., Romeu, X., Roca, J., Barranco, I., Yeste, M., 2020a. Exploring Seminal Plasma GSTM3 as a Quality and In Vivo Fertility Biomarker in Pigs—Relationship with Sperm Morphology. Antioxidants 9, 741. https://doi.org/10.3390/antiox9080741.

Llavanera, M., Delgado-Bermúdez, A., Olives, S., Mateo-Otero, Y., Recuero, S., Bonet, S., Fernández-Fuertes, B., Yeste, M., Barranco, I., 2020b. Glutathione S-Transferases Play a Crucial Role in Mitochondrial Function, Plasma Membrane

Stability and Oxidative Regulation of Mammalian Sperm. Antioxidants 9, 100. https://doi.org/10.3390/antiox9020100.
Llavanera, M., Mateo-Otero, Y., Bonet, S., Barranco, I., Fernández-Fuertes, B., Yeste, M., 2019b. The triple role of glutathione S-transferases in mammalian male fertility. Cellular and Molecular Life Sciences 77, 2331–2342. https://doi.org/10.1007/S00018-019-03405-W.
Llavanera, M., Mateo-Otero, Y., Delgado-Bermúdez, A., Recuero, S., Olives, S., Barranco, I., Yeste, M., 2021a. Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality. Frontiers in Cell and Developmental Biology 9, 208. https://doi.org/10.3389/fcell.2021.627140.
Llavanera, M., Ribas-Maynou, J., Delgado-Bermúdez, A., Recuero, S., Muiño, R., Hidalgo, C.O., Tamargo, C., Bonet, S., Mateo-Otero, Y., Yeste, M., 2021b. Sperm chromatin condensation as an in vivo fertility biomarker in bulls: a flow cytometry approach. Journal of Animal Science and Biotechnology 12, 1–12. https://doi.org/10.1186/S40104-021-00634-7.
Menezes, E.B., Velho, A.L.C., Santos, F., Dinh, T., Kaya, A., Topper, E., Moura, A.A., Memili, E., 2019. Uncovering sperm metabolome to discover biomarkers for bull fertility. BMC Genomics 20, 1–16. https://doi.org/10.1186/S12864-019-6074-6.
Miglior, F., Fleming, A., Malchiodi, F., Brito, L.F., Martin, P., Baes, C.F., 2017. A 100-Year Review: Identification and genetic selection of economically important traits in dairy cattle. Journal of Dairy Science 100, 10251–10271. https://doi.org/10.3168/jds.2017-12968.
Nisa, L.C., Rahayu, S., Ciptadi, G., 2018. The Abnormality of Spermatozoa Goat after Freezing on -80°C Using Tris Diluent Added Combination Hatching Egg Yolk and Amnion Fluid. The Journal of Experimental Life Sciences 8, 133–138 https://doi.org/10.21776/ub.jels.2018.008.03.01.
Petit, F.M., Serres, C., Bourgeon, F., Pineau, C., Auer, J., 2013. Identification of sperm head proteins involved in zona pellucida binding. Human Reproduction 28, 852–865. https://doi.org/10.1093/humrep/des452.
Pryce, J.E., Royal, M.D., Garnsworthy, P.C., Mao, I.L., 2004. Fertility in the high-producing dairy cow. Livestock Production Science 86, 125–135. https://doi.org/10.1016/S0301-6226(03)00145-3.
Sabeti, P., Pourmasumi, S., Rahiminia, T., Akyash, F., Talebi, A.R., 2016. Etiologies of sperm oxidative stress. International Journal of Reproductive BioMedicine 14, 231–240 https://doi.org/10.29252/ijrm.14.4.231.
Samanta, L., Swain, N., Ayaz, A., Venugopal, V., Agarwal, A., 2016. Post-Translational Modifications in sperm Proteome: The Chemistry of Proteome diversifications in the Pathophysiology of male factor infertility. Biochimica et Biophysica Acta, General Subjects 1860, 1450–1465. https://doi.org/10.1016/j.bbagen.2016.04.001.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.
Tarozzi, N., Nadalini, M., Stronati, A., Bizzaro, D., Dal Prato, L., Coticchio, G., Borini, A., 2009. Anomalies in sperm chromatin packaging: Implications for assisted reproduction techniques. Reproductive BioMedicine Online 18, 486–495. https://doi.org/10.1016/S1472-6483(10)60124-1.

PAPER V

Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality

Marc Llavanera, Yentel Mateo-Otero, Ariadna Delgado-Bermúdez, Sandra Recuero, Samuel Olives, Isabel Barranco & Marc Yeste

Frontiers in cell and developmental biology
2021;9, 627140

DOI: 10.3389/fcell.2021.627140

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 25 February 2021
doi: 10.3389/fcell.2021.627140

Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality

Marc Llavanera[1,2], Yentel Mateo-Otero[1,2], Ariadna Delgado-Bermúdez[1,2], Sandra Recuero[1,2], Samuel Olives[1,2], Isabel Barranco[1,2†] and Marc Yeste[1,2*†]*

[1] *Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, Girona, Spain,* [2] *Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, Girona, Spain*

OPEN ACCESS

Edited by:
Ana Josefa Soler, University of Castilla–La Mancha, Spain

Reviewed by:
David Martin Hidalgo, University of Porto, Portugal
Olga Garcia-Alvarez, University of Castilla–La Mancha, Spain

***Correspondence:**
Isabel Barranco
isabel.barranco@udg.edu
Marc Yeste
marc.yeste@udg.edu

† These authors share senior authorship

Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *08 November 2020*
Accepted: *22 January 2021*
Published: *25 February 2021*

Citation:
Llavanera M, Mateo-Otero Y, Delgado-Bermúdez A, Recuero S, Olives S, Barranco I and Yeste M (2021) Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality. Front. Cell Dev. Biol. 9:627140. doi: 10.3389/fcell.2021.627140

Fifty percent of male subfertility diagnosis is idiopathic and is usually associated with genetic abnormalities or protein dysfunction, which are not detectable through the conventional spermiogram. Glutathione *S*-transferases (GSTs) are antioxidant enzymes essential for preserving sperm function and maintaining fertilizing ability. However, while the role of GSTP1 in cell signaling regulation via the inhibition of c-Jun N-terminal kinases (JNK) has been enlightened in somatic cells, it has never been investigated in mammalian spermatozoa. In this regard, a comprehensive approach through immunoblotting, immunofluorescence, computer-assisted sperm assessment (CASA), and flow cytometry analysis was used to characterize the molecular role of the GSTP1–JNK heterocomplex in sperm physiology, using the pig as a model. Immunological assessments confirmed the presence and localization of GSTP1 in sperm cells. The pharmacological dissociation of the GSTP1–JNK heterocomplex resulted in the activation of JNK, which led to a significant decrease in sperm viability, motility, mitochondrial activity, and plasma membrane stability, as well as to an increase of intracellular superoxides. No effects in intracellular calcium levels and acrosome membrane integrity were observed. In conclusion, the present work has demonstrated, for the first time, the essential role of GSTP1 in deactivating JNK, which is crucial to maintain sperm function and has also set the grounds to understand the relevance of the GSTP1–JNK heterocomplex for the regulation of mammalian sperm physiology.

Keywords: ezatiostat, GSTP1-JNK heterocomplex, mitochondria, sperm functionality, mammalian sperm

INTRODUCTION

In humans, about 30–50% of fertilizations fail because of male subfertility problems, usually related to abnormal sperm count, motility, and/or morphology (Ghuman and Ramalingam, 2017). However, over 50% of male subfertility diagnosis is of unknown etiology, since no abnormalities are detected in conventional semen analysis (sperm count, motility, and morphology) (Ghuman and Ramalingam, 2017). These patients are diagnosed as normozoospermic subfertile men (i.e., male idiopathic subfertility). Male idiopathic subfertility has been associated with genetic abnormalities (Carrell et al., 2006) and low levels of sperm-specific proteins (Parent et al., 1999; Bracke et al., 2018). While the general processes of sperm maturation, capacitation, and fertilization are well

described, the underlying molecular mechanisms that take place in mammalian sperm cells remain mostly unknown (Klinovska et al., 2014). The origin of male idiopathic subfertility may be explained by molecular defects in these processes, since they are not detectable through the conventional spermiogram (Bracke et al., 2018). For this reason, characterization of signaling pathways and posttranslational modifications in mammalian sperm cells are of utmost interest for the andrology field.

Several studies reported the association between male idiopathic subfertility or infertility and some null genotypes of glutathione *S*-transferases (GSTs) (Aydos et al., 2009; Safarinejad et al., 2010; Vani et al., 2010; Tang et al., 2012; Kan et al., 2013; Lakpour et al., 2013; Song et al., 2013; Kolesnikova et al., 2017). Moreover, recent studies have evidenced the essential role of these antioxidant enzymes in sperm protection against oxidative stress and preservation of sperm function and fertilizing ability (Llavanera et al., 2019b, 2020). The first evidence of GST activity in mammalian sperm dates back to 1978 in murine species (Mukhtar et al., 1978), and the first report confirming the presence of the Pi class of GSTs (GSTP1) was published in 1998 in goat sperm (Gopalakrishnan et al., 1998). Since then, several proteomic profiling studies have identified GSTP1 in the sperm cells of a wide range of mammalian species, including humans (Wang et al., 2013), mice (Vicens et al., 2017), pigs (Pérez-Patino et al., 2019), cattle (Peddinti et al., 2008), and coatis (Rodrigues-Silva et al., 2018). In somatic cells, the main well-defined function of GSTP1 is cell signaling regulation via inhibition of the c-Jun N-terminal kinase (JNK)–C-Jun pathway (Adler et al., 1999; Wang et al., 2001; Turella et al., 2005). In non-stressed cells, GSTP1 is able to inhibit JNK kinase activity by blocking the JNK-binding site to C-Jun, forming a GSTP1–JNK heterocomplex. However, under cellular stress conditions, a GSTP1 aggregation followed by its dissociation from the heterocomplex leads to an increase in JNK activity (Adler et al., 1999; Wang et al., 2001; Turella et al., 2005). Recently, the JNK signaling cascade has been reported to be involved in sperm capacitation and apoptosis (Luna et al., 2017), which may undercover the role of GSTP1 in sperm physiology. However, the role of GSTP1 and the JNK–C-Jun pathway in mammalian sperm still remains unknown.

Ezatiostat or Terrapin 199 (TER) is a specific inhibitor of the GSTP1–JNK heterocomplex, used as an anticancer drug (Wu and Batist, 2013). After intracellular de-esterification, which is a process that commonly occurs in sperm cells (Griveau and Le Lannou, 1997), the active form of TER binds to GSTP1, blocking its JNK-binding site and, therefore, inhibiting the formation of the GSTP1–JNK heterocomplexes (Mathew et al., 2006). This inhibition enables JNK phosphorylation and activation of the subsequent pathway.

Along these lines, understanding the molecular role of the GSTP1–JNK heterocomplex in mammalian sperm physiology is much warranted. Herein, cell biology and immunological approaches were performed through pharmacologically inhibiting the formation of the GSTP1–JNK heterocomplex, prior to analyzing sperm quality and functionality parameters, the presence and localization of GSTP1, and the activation of JNK. Therefore, the present study aimed to investigate the function of this heterocomplex in mammalian sperm physiology, using the pig as a model, which has recently been stablished as a suitable animal model for research in human reproduction (Zigo et al., 2020). Accordingly, we hypothesized that the dissociation of the GSTP1–JNK heterocomplex, known to occur under cellular stress conditions, enhances the JNK signaling pathway and disrupts sperm physiology. The results obtained in this study can be used as a starting point for further investigations seeking the molecular basis of sperm dysfunction and may contribute to shedding light into the diagnosis of idiopathic male infertility.

MATERIALS AND METHODS

Reagents

Chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, United States), unless otherwise indicated. TER was reconstituted in dimethyl sulfoxide (DMSO) to a stock solution of 64 mM. Fluorochromes [SYBR-14, propidium iodide (PI), merocyanine 540 (M540), Yo-Pro-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC1), Fluo3-AM (Fluo3), hydroethidine (HE), and fluorescein-conjugated peanut agglutinin/PI (PNA)] were purchased from Life Technologies (Thermo Fisher Scientific, Carlsbad, CA, United States). SYBR-14, M540, Yo-Pro-1, JC1, Fluo3, and HE were reconstituted in DMSO, whereas PI and PNA were diluted in phosphate-buffered saline (PBS) 1X. Antibody against GSTP1 (ref. MBS3209038) was purchased from MyBioSource (San Diego, CA, United States), whereas phospho-JNK (Thr183/Tyr185) antibody (pJNK, ref. 4668S) was purchased from Cell Signaling Technology (Danvers, MA, United States). Secondary anti-rabbit (ref. P0448) and anti-mouse (ref. P0260) antibodies conjugated with horseradish peroxidase for immunoblotting analysis were purchased from Dako (Derkman A/S, Denmark), whereas the secondary anti-rabbit antibody conjugated with Alexa Fluor 488 for immunofluorescence analysis was purchased from Thermo Fisher Scientific (ref. A32731).

Animals and Ejaculates

Semen samples, commercially sold as pig artificial insemination (AI) seminal doses, were purchased from an authorized local AI center (Grup Gepork S.L., Masies de Roda, Spain) that followed ISO certification (ISO-9001:2008) and operates under commercial, standard conditions. Thirteen ejaculates (one ejaculate per boar, n = 13) from healthy and sexually mature Piétrain boars (1–3 years old) were collected using the gloved-hand method and diluted (33×10^6 sperm/ml) using a commercial extender (Vitasem LD, Magapor S.L., Zaragoza, Spain). Packed ejaculates were transported at 17°C to the laboratory within 4 h after ejaculation. Since seminal doses were purchased from the aforementioned farm and the authors of this study did not manipulate any animal, no authorization from the institutional ethics committee was required.

Experimental Design

All semen samples (n = 13) were split into three aliquots. The first aliquot was used to assess initial sperm quality

and functionality (control-0h). The second and third aliquots were liquid-stored at 17°C for 72 h in the presence of (i) 100 μM ezatiostat (TER-72h) and (ii) the same volume of DMSO, as a vehicle control group (control-72h). Concentration of TER was selected based on the results obtained from a preliminary concentration test performed in our laboratory (**Supplementary Figure 1**), whereas storage time was decided following practical application criteria, considering that sows are artificially inseminated (two to three times per estrus) with AI doses stored until 72 h at 17°C. After 72 h, both groups were incubated at 38°C for 1 h prior to their analysis. All assessments were performed at every time point (control-0h, control-72h, and TER-72 h). Sperm motility, viability, plasma membrane stability, mitochondrial activity, intracellular calcium levels, intracellular superoxide levels, and acrosome membrane integrity were determined to evaluate sperm quality and functionality. The presence and localization of GSTP1 were explored by immunoblotting and immunofluorescence analyses, respectively. Finally, the activation of the JNK pathway was evaluated through immunoblotting analysis of JNK tyrosine and threonine phosphorylation. Raw data of sperm quality and functionality parameters of all treatments and time points are available as a data set (**Supplementary Table 1**).

Sperm Motility Analysis

Sperm motility assessment was performed through a computer-assisted sperm analysis (CASA) system, using an Olympus BX41 microscope (Olympus, Tokyo, Japan) with a negative phase-contrast field (Olympus 10 × 0.30 PLAN objective, Olympus) connected to a personal computer containing the ISAS software (Integrated Sperm Analysis System V1.0, Proiser S.L., Valencia, Spain). Semen samples were incubated for 15 min at 38°C prior to motility assessment. Once incubated, 5 μl of each sample was examined in a prewarmed (38°C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Three technical replicates of at least 500 sperm per replicate were examined in each sample. Total motility (TMOT), progressive motility (PMOT), and average path velocity (VAP, μm/s) were used to evaluate sperm motility. A sperm cell was considered motile when VAP was ≥10 μm/s and progressively motile when the coefficient of straightness (STR) was ≥45%.

Flow Cytometric Assessments

Sperm viability, plasma membrane stability, mitochondrial activity, intracellular calcium levels, intracellular superoxide levels, and acrosome membrane integrity assessments were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, United States) equipped with an argon-ion laser (488 nm) set at a power of 22 mW. Semen samples were diluted (2×10^6 sperm/ml) in prewarmed PBS to a final volume of 600 μl prior to staining with the corresponding protocol. Sperm viability (SYBR-14/PI) (Garner and Johnson, 1995), plasma membrane stability (M540/Yo-Pro-1) (Rathi et al., 2001), mitochondrial membrane potential (MMP; JC1) (Ortega-Ferrusola et al., 2007), intracellular calcium levels (Fluo3/PI) (Harrison et al., 1993), intracellular superoxide levels (HE/Yo-Pro-1) (Guthrie and Welch, 2006), and acrosome membrane integrity (fluorescein-conjugated PNA/PI) (Nagy et al., 2003) were assessed. Extended flow cytometry protocols are described in **Supplementary File 1**.

The electronic volume (EV) gain, PMT voltages of optical filters (FL-1, FL-2, and FL-3), and fluorescence overlapping were set using unstained and single-stained samples of each fluorochrome. Flow rate, laser voltage, and sperm concentration were constant throughout the experiment. Sperm cells from debris events were distinguished using EV. Three technical replicates of at least 10,000 sperm per replicate were examined for each sample. As recommended by the International Society for Advancement of Cytometry (ISAC), Flowing Software (Ver. 2.5.1, University of Turku, Finland) was used to analyze flow cytometry data.

Immunofluorescence Analysis

Semen samples were diluted in PBS (3×10^6 sperm/ml) and fixed in 2% paraformaldehyde (Alfa Aesar, Haverhill, MA, United States) and washed twice. Two 150-μl aliquots of each sample were placed in an ethanol prerinsed slide and subsequently blocked and permeabilized for 40 min at room temperature (RT) with a blocking solution containing 0.25% (v:v) Triton X-100 and 3% (w:v) bovine serum albumin (BSA). Samples were incubated with anti-GSTP1 antibody (1:200, v:v) overnight, washed thrice, and subsequently incubated with an anti-rabbit antibody (1:400, v:v). In negative controls, the primary antibody was omitted. Then, 10 μl of Vectashield mounting medium containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was added prior to being covered and sealed with nail varnish. Finally, each sample was evaluated using a confocal laser scanning microscope (CLSM, Nikon A1R, Nikon Corp., Tokyo, Japan).

Immunoblotting Analysis

Semen samples were centrifuged twice (3,000 × *g* for 5 min), and the sperm pellets were resuspended in lysis buffer (xTractor™ buffer, Takara Bio, Kusatsu, Japan) following the manufacturer's instructions. Then, samples were centrifuged (10,000 × *g* for 20 min at 4°C), and the supernatants were assessed for total protein quantification using a detergent-compatible method (Bio-Rad, Hercules, CA, United States). Finally, samples were stored at −80°C until analysis.

Twenty micrograms of total protein was diluted (1:1, v:v) in Laemmli reducing buffer 4X (Bio-Rad) and heated at 95°C for 7 min prior to being loaded onto a 12% polyacrylamide gel (Mini-PROTEAN® TGX Stain-Free™ Precast Gels, Bio-Rad) and electrophoresed for 2 h at 120 V. Total protein was visualized using a G:BOX Chemi XL system (Syngene, Frederick, MD, United States). Mini-PROTEAN® TGX Stain-Free™ Precast Gels contain a trihalo compound that allows fluorescent detection of tryptophan residues. Thereafter, proteins from the gel were transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot® Turbo™ (Bio-Rad). Transferred membranes were blocked using 5% BSA and incubated with the anti-GSTP1 (1:5,000, v:v) or anti-pJNK (1:2,000, v:v) antibodies for 1 h in agitation at RT. Next, membranes were rinsed thrice and incubated with the secondary

anti-rabbit antibody 1:10,000 (v:v) for GSTP1 and 1:4,000 (v:v) for pJNK. Then, membranes were washed five times, and bands were visualized through incubation with a chemiluminescent substrate (Immobilon^{TM} Western Detection Reagents, Millipore, United States) prior to scanning with G:BOX Chemi XL 1.4 (Syngene, India). Finally, membranes were stripped, and the process was repeated by replacing the primary antibody for the anti-α-tubulin antibody (1:100,000, v:v) and the secondary antibody for the anti-mouse antibody (1:150,000, v:v), as loading control and for normalization. In the pJNK assessment, Quantity One software package (Version 4.6.2, Bio-Rad) was used to quantify the bands of two technical replicates per sample, normalized using α -tubulin.

Statistical Analysis

Plotting and statistical analysis of the results were performed using GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, United States) and IBM SPSS for Windows v. 25.0 (IBM Corp., Armonk, NY, United States). Each biological replicate was considered a statistical case, and data were checked for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test). Sperm quality and functionality parameters, as well as normalized pJNK relative levels, were compared between treatments (control-0h, control-72h, and TER-72h) using a one-way ANOVA followed by Tukey's multiple-comparison test. Data are shown as mean ± standard error of the mean (SEM). The level of significance was set at $p \leq 0.05$.

RESULTS

GSTP1 Is Present in Sperm Cells and Is Localized in the Principal and End Pieces of the Tail

The presence and localization of GSTP1 in sperm samples are presented in **Figures 1**, **2**. Immunoblotting analysis of GSTP1 (**Figure 1A**) showed a single band of ∼48 kDa in all samples, whereas anti-α-tubulin (**Figure 1B**) showed a ∼50 kDa band. In **Figure 2**, a GSTP1 signal was observed in the posterior region of the head and the middle, principal, and end pieces of the tail of control-0h samples. In control-72h and TER-72h samples, the GSTP1 signal was observed only in the equatorial subdomain of the head and in the principal and end pieces of the tail.

Inhibition of GSTP1–JNK Heterocomplex Formation by TER Induces Thr183 and Tyr185 Phosphorylation of JNK

Immunoblotting analysis of Thr183 and Tyr185 phosphorylation of JNK revealed a double-band pattern showing both p46 and p54 splicing variants of JNK (**Figure 3**). Anti-α-tubulin immunoblot showed a single band of ∼50 kDa, which corresponds to α-tubulin. Subsequent band quantification analysis of pJNK normalized using α-tubulin showed a significant increase ($p < 0.05$) in the relative levels of Thr183 and Tyr185 phosphorylation of the p46 splicing variant of JNK in TER-72h samples when compared to control-0h and control-72h samples. However, no effects of TER were observed in Thr183 and Tyr185 phosphorylation of the p54 splicing variant ($p > 0.05$).

Sperm Viability Is Reduced by TER-Induced JNK Phosphorylation

The percentage of viable sperm was higher ($p < 0.05$) in the semen samples of the control-0h group than in those of the TER-72h and control-72h groups (**Figure 4**). In addition, sperm viability was lower ($p < 0.05$) in the semen samples of the TER-72h group compared to those of the control-72h group.

Phosphorylation of JNK by the Inhibition of GSTP1–JNK Binding Impairs Sperm Motility

As shown in **Figure 5**, the percentage of total and progressive motile sperm was higher ($p < 0.05$) in the semen samples of the control-0h group than in those of the control-72h and TER-72h groups. However, VAP was lower ($p < 0.05$) in the semen samples of the TER-72h group compared to those of the control groups (0 and 72 h). Interestingly, total and progressive motility and VAP were significantly lower ($p < 0.05$) in the semen samples of the TER-72h group than in those of the control-72h group.

Mitochondrial Activity Is Significantly Reduced by JNK Phosphorylation by the Inhibition of GSTP1–JNK Binding

The assessment of MMP is presented in **Figure 6**. The percentage of sperm showing high MMP differed ($p < 0.05$) among the three groups, with the semen samples of the control-0h and TER-72h groups showing the highest and lowest percentages, respectively. Thus, a dramatic reduction in MMP was observed in the TER-72h group when compared to the control groups.

Sperm Plasma Membrane Is Highly Destabilized by the Inhibition of GSTP1–JNK Binding and Subsequent JNK Phosphorylation

As shown in **Figure 7**, sperm membrane stability was presented as the percentage of membrane-destabilized cells within the total viable sperm population. The percentage of viable sperm showing plasma membrane destabilization was higher ($p < 0.05$) in the semen samples of the TER-72h group than in those of the control groups (0 and 72 h). On the other hand, the plasma membrane stability of the semen samples did not differ between control groups ($p > 0.05$).

Intracellular Superoxide Levels Were Increased by the Phosphorylation of JNK

Figure 8 shows the relative E+ fluorescence intensity of the viable sperm population. No differences ($p > 0.05$) were observed in intracellular superoxide levels between semen samples of control groups (0 and 72 h). However, our results showed a significant increase ($p < 0.05$) in superoxide levels in semen samples of the TER-72h group compared to those of the control groups (0 and 72 h).

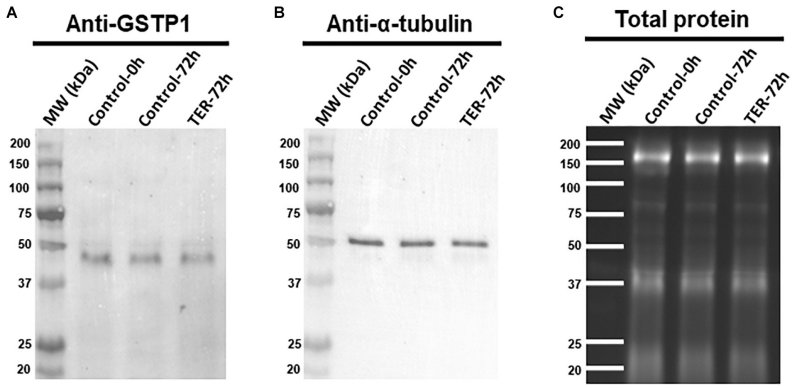

FIGURE 1 | Immunoblotting analysis of GSTP1. Pig sperm lysates were incubated with **(A)** anti-GSTP1 antibody, **(B)** anti-α-tubulin antibody, and **(C)** total protein bands. Lanes: MW (kDa), molecular weight; Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Alpha-tubulin (anti-tubulin) and TGX Stain-Free™ (total protein) were performed as complementary loading controls. These results are representative of three independent experiments (n = 3).

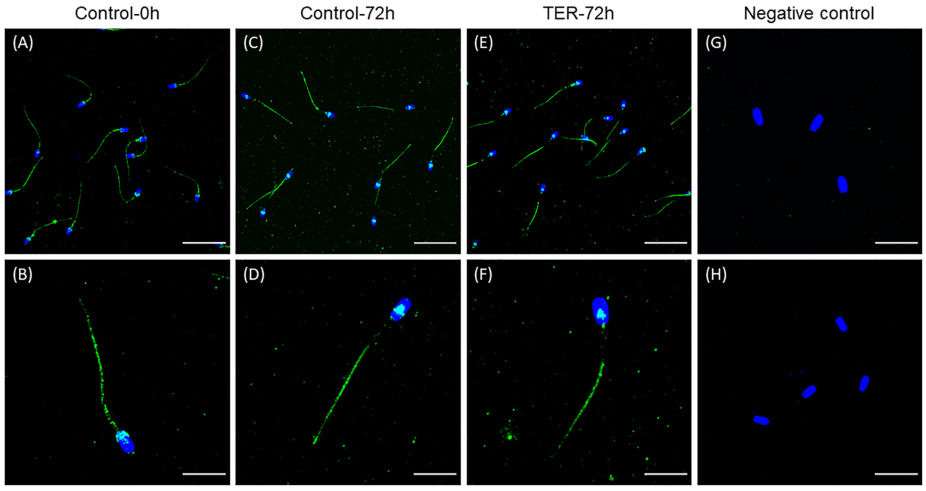

FIGURE 2 | Immunolocalization of GSTP1 in pig sperm. **(A,B)** Control-0h, semen samples at 0 h of storage. **(C,D)** Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C. **(E,F)** TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. **(G,H)** Negative control. The nucleus is shown in blue (DAPI), whereas GSTP1 is shown in green. Scale bars: **(A,C,E)**: 30 μm; **(B,D,F)**: 15 μm; **(G)**: 20 μm; **(H)**: 25 μm. These results are representative of three independent experiments (n = 3).

Sperm Intracellular Calcium Levels and Acrosome Membrane Integrity Are Not Affected by TER-Induced JNK Phosphorylation

The relative Fluo3 fluorescence intensity ($Fluo3^+$) of the viable sperm population (PI^-) is presented in **Figure 9**, whereas the percentage of viable sperm with an intact acrosome (PNA^-/PI^-) is shown in **Figure 10**. No differences ($p > 0.05$) in intracellular calcium nor acrosome membrane integrity was observed among groups.

DISCUSSION

Several studies have evidenced the essential role of GSTs as molecular regulators of mammalian sperm physiology and fertilizing capacity (Gopalakrishnan et al., 1998;

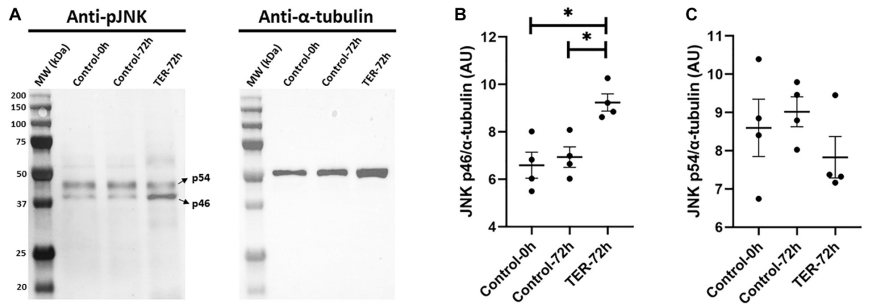

FIGURE 3 | (A) Immunoblotting of pig sperm lysate for pThr183 and pTyr185 of JNK (p46 and p54 splicing variants) and α-tubulin. Subsequent band intensity quantification and normalization of **(B)** p46 and **(C)** p54 splicing variants of JNK, normalized using α-tubulin, showed a significant increase in Thr183 and Tyr185 phosphorylation in the p46 variant ($p < 0.05$), but not in the p54 variant ($p > 0.05$), in TER-treated samples (TER-72h) compared to control samples (control-0h and control-72h). Lanes: MW (kDa), molecular weight; control-0h, semen samples at 0 h of storage; control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. These results are representative of four independent experiments ($n = 4$). *$p \leq 0.05$.

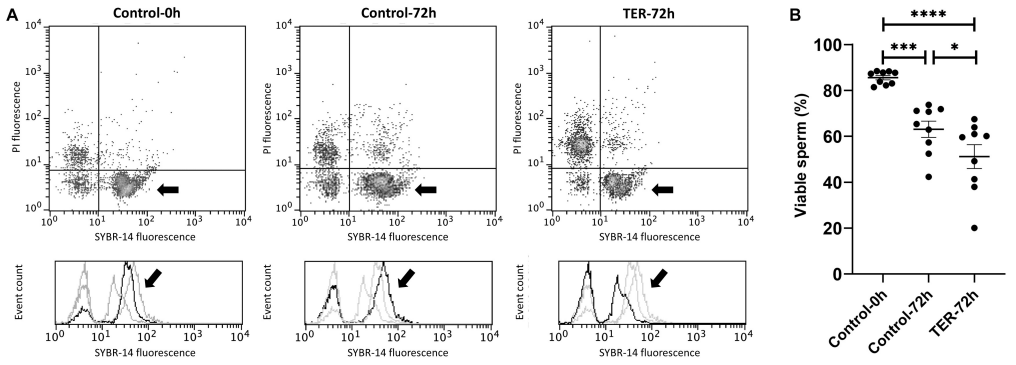

FIGURE 4 | Flow cytometry analysis of sperm viability. **(A)** Representative flow cytometry dot plots of propidium iodide (PI) and SYBR-14 fluorescence and histograms showing the event count of SYBR-14 fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the percentage of viable sperm in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size ($n = 9$). *$p \leq 0.05$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Aydos et al., 2009; Safarinejad et al., 2010; Vani et al., 2010; Tang et al., 2012; Kan et al., 2013; Lakpour et al., 2013; Song et al., 2013; Kolesnikova et al., 2017; Llavanera et al., 2019b, 2020). On the other hand, a recent study established JNK signaling cascade as a regulator of specific physiological parameters in sperm cells (Luna et al., 2017). In this regard, under physiological conditions, GSTP1 is a well-known regulator of the JNK singling pathway in somatic cells by inhibiting its kinase activity when forming a GSTP1–JNK heterocomplex (Adler et al., 1999; Turella et al., 2005). However, the effects of inhibiting GSTP1 upon JNK signaling regulation in male gametes have never been investigated. To the best of our knowledge, this is the first report uncovering the physiological role of JNK inhibited by GSTP1 in mammalian sperm physiology. To this end, a specific inhibitor of the GSTP1–JNK heterocomplex was used, leading to the subsequent activation of JNK.

While the presence of GSTP1 in the sperm was established by proteomic studies in several mammalian species such as human (Wang et al., 2013), murine (Vicens et al., 2017), porcine (Pérez-Patino et al., 2019), and bovine (Peddinti et al., 2008), its localization was determined for the first time in the present study. Immunoblotting analysis of the present study identified a single ∼48 kDa band corresponding to GSTP1. Although the molecular mass of GSTP1 is ∼24 kDa, it is known to exist intracellularly as homodimers (Okamura et al., 2015), which is likely to be responsible for the ∼48 kDa band found in immunoblots. Furthermore, immunofluorescence analysis found GSTP1 to be localized in the posterior region of the head and the middle,

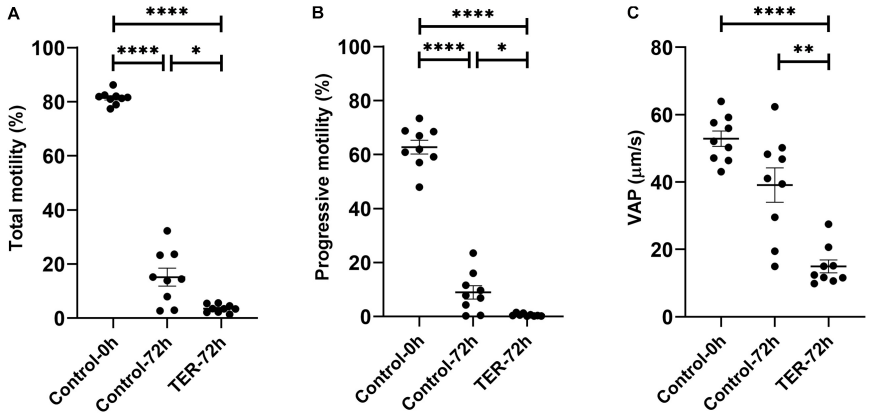

FIGURE 5 | Computer-assisted sperm analysis (CASA) of pig semen samples. Mean, standard error of the mean (SEM), and sample distribution of the percentage of **(A)** total motile sperm, **(B)** progressively motile sperm, and **(C)** average path velocity sperm (VAP; μm/s) in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$.

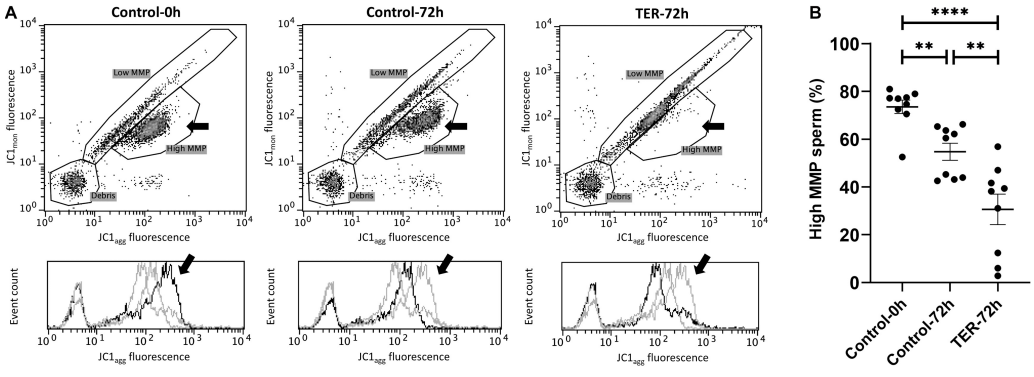

FIGURE 6 | Flow cytometry analysis of pig sperm mitochondrial activity. **(A)** Representative flow cytometry dot plots of JC1 monomers ($JC1_{mon}$) and JC1 aggregates ($JC1_{agg}$) fluorescence and histograms showing the event count of $JC1_{agg}$ fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the percentage of high mitochondrial membrane potential (MMP) sperm in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). **$p \leq 0.01$; ****$p \leq 0.0001$.

principal, and end pieces of the tail in fresh control samples. The localization pattern of GSTP1 in fresh samples was similar to that found for other GST family members such as GSTM3 in pig (Llavanera et al., 2020) and buffalo (Kumar et al., 2014) sperm, which is present in the entire sperm tail. Interestingly, liquid storage for 72 h rather than inhibition with TER was responsible for the alteration of the GSTP1 localization pattern. Contrary to fresh samples, GSTP1 was found to be localized in the equatorial subdomain of the head and the principal and end pieces of the tail. A similar modulation of the GSTP1 localization pattern due to liquid storage was observed in other GST family members such as GSTM3 (Llavanera et al., 2020). Contrary to that, GSTM3 was found to be relocalized to the middle piece during sperm cryopreservation (Llavanera et al., 2019a). According to the results of the present study, previous studies hypothesized that the GST localization pattern in the sperm tail and their relocalization from or to the middle piece during liquid storage or cryopreservation, respectively, could contribute to the explanation of their significant role in mitochondrial function, sperm motility, and membrane stability. Sperm GSTs are known to be membrane-anchored proteins, and thus, their localization is determined by membrane stability (Llavanera et al., 2019b).

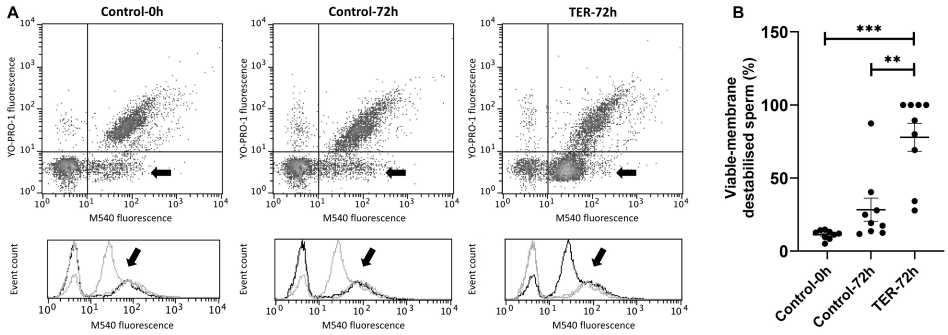

FIGURE 7 | Flow cytometry analysis of plasma sperm membrane stability. **(A)** Representative flow cytometry dot plots of Yo-Pro-1 and merocyanine (M540) fluorescence and histograms showing the event count of M540 fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the percentage of membrane-destabilized cells within the total viable sperm population in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

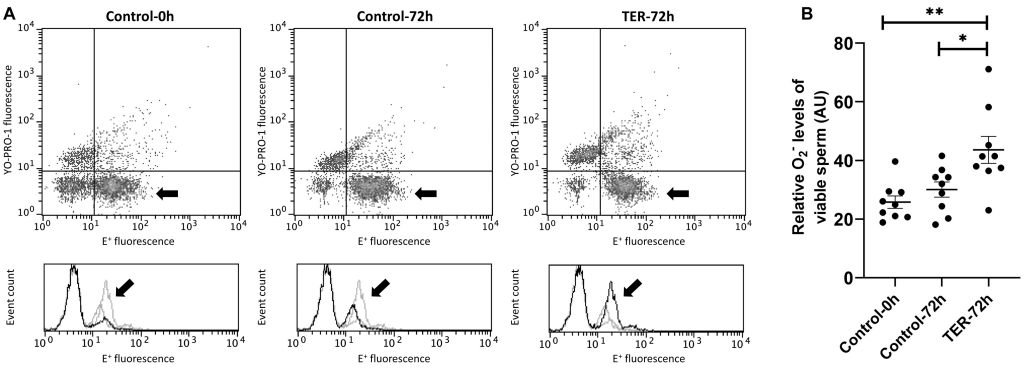

FIGURE 8 | Flow cytometry analysis of intracellular superoxide levels. **(A)** Representative flow cytometry dot plots of Yo-Pro-1 and ethidine (E^+) fluorescence and histograms showing the event count of E^+ fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the relative E^+ fluorescence intensity of the viable sperm population in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

The loss of GSTP1 from the middle piece suggests stronger membrane destabilization of this region. In this regard, the loss of GSTP1 in the middle piece would indicate a major membrane destabilization of this region due to preservation in liquid storage.

As has been previously reported in the literature, the JNK activation is regulated by GSTP1 in somatic cells (Adler et al., 1999; Wang et al., 2001). However, there were no studies regarding this molecular interaction in mammalian sperm cells. Immunoblotting analysis of phospho-JNK reported herein showed an intensified tyrosine and threonine phosphorylation of this protein in TER-treated samples, a specific blocker of the JNK-binding site in GSTP1. It is widely known that mitogen-activated protein kinases (MAPKs; e.g., JNKs) are activated via a dual phosphorylation upon tyrosine and threonine residues (Lawler et al., 1998). Hence, our results evidence, for the first time in mammalian sperm, the role of the GSTP1–JNK heterocomplex as an inhibitor of JNK activation by preventing the dual phosphorylation of tyrosine and threonine residues.

An interesting physiological effect of the activation of JNK was the significant decrease in sperm mitochondrial activity, viability, and motility. Activation of JNK has been reported in the literature to be related to mitochondrial dysfunction and cell death in somatic cells (Aoki et al., 2002; Heslop et al., 2020). Admittedly, a study conducted in ram sperm (Luna et al., 2017) showed that phosphorylation of sperm JNK increased apoptotic-like changes and DNA damage as

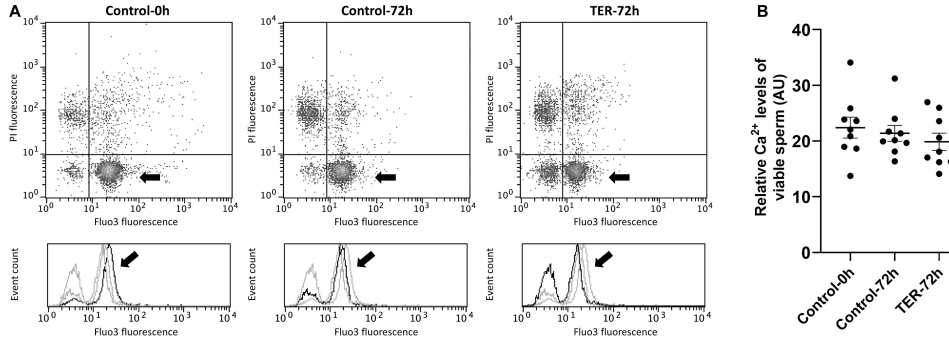

FIGURE 9 | Flow cytometry analysis of intracellular calcium levels. **(A)** Representative flow cytometry dot plots of propidium iodide (PI) and Fluo3-AM (Fluo3) fluorescence and histograms showing the event count of Fluo3 fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the relative $Fluo3^{+}$ fluorescence intensity of the viable sperm population in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). $p > 0.05$.

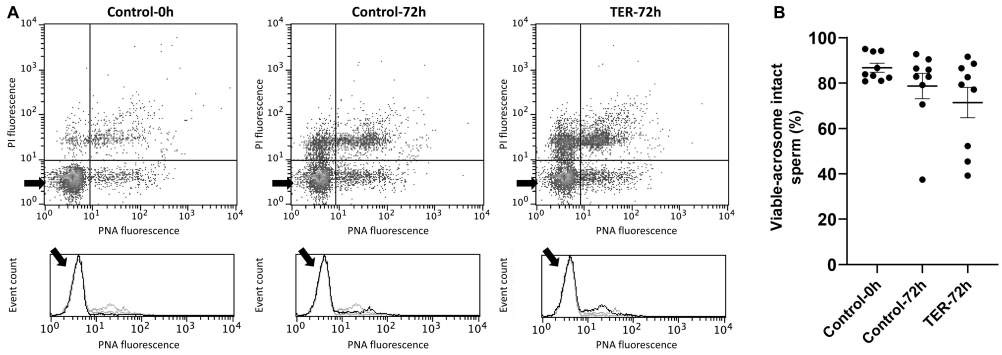

FIGURE 10 | Flow cytometry analysis of acrosome integrity. **(A)** Representative flow cytometry dot plots of propidium iodide (PI) and fluorescein-conjugated peanut agglutinin/propidium iodide (PNA) fluorescence and histograms showing the event count of PNA fluorescence intensity of all treatments. Black arrows show the flow cytometry population selected for analysis represented in **(B)**. Dark gray lines in histograms show the event count of its corresponding treatment, whereas light gray lines show the event count of the remaining treatments. **(B)** Mean, standard error of the mean (SEM), and sample distribution of the percentage of viable sperm with an intact acrosome in all treatments. Control-0h, semen samples at 0 h of storage; Control-72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER-72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C. Sample size (n = 9). $p > 0.05$.

well as capacitation-related events. These results would suggest that the GSTP1–JNK heterocomplex could prevent sperm to undergo early capacitation-related events or apoptotic-like changes during liquid storage. In this regard, the detrimental effects of JNK activation upon mitochondrial functionality in sperm cells showed herein are in agreement with the results reported in sperm and other cell types. Moreover, the reduction of mitochondrial activity is likely to be responsible for the loss of sperm motility, since mammalian sperm rely upon high levels of the adenosine triphosphate (ATP) required for axonemal dynein to drive sperm motility (Vívenes et al., 2009). Altogether, our findings suggest the role of the GSTP1–JNK heterocomplex in preserving sperm mitochondrial activity and subsequent viability and motility as well as in preventing capacitation-related events or apoptotic-like changes. Specific molecular mechanisms through which JNK activation may trigger sperm mitochondrial dysfunction in sperm cells remains to be determined. However, in somatic cells, JNK-mitochondrial SH3-domain binding protein 5 (SAB), a docking protein for JNK, has been suggested as a putative responsible for these processes, since it was found to lead to an intramitochondrial signal transduction pathway that impairs mitochondrial activity and enhances the production of reactive oxygen species (Win et al., 2018). In this regard, further investigations on the downstream effects of activated JNK upon mitochondrial activity should be performed.

Related to sperm mitochondrial dysfunction, the results of the present study showed an increase in intracellular superoxide levels triggered by the GSTP1–JNK heterocomplex dissociation and subsequent activation of JNK. Similar results were reported in somatic cells, where JNK activation was related to increased superoxide formation (Heslop et al., 2020). The main superoxide source in mammalian sperm cells is known to be the mitochondria, specifically, the electron transport chain (Storey, 2008; Brand, 2016). These results suggest that, in line with the previously mentioned results, the activation of JNK would lead to the disruption of the electron transport chain of sperm mitochondria. Moreover, previous studies in caprine and porcine evidenced the essential role of sperm GSTs in maintaining mitochondrial activity and physiological levels of reactive oxygen species (Hemachand and Shaha, 2003; Llavanera et al., 2020). Related with this, the results of the present study would indicate that the effects of GSTs upon sperm mitochondria would be mediated by a JNK signaling pathway. However, further research regarding the molecular mechanism of GSTs in regulating sperm mitochondrial function is required.

Our results showed that pharmacological dissociation of the GSTP1–JNK heterocomplex in sperm cells significantly impaired the stability of lipidic membranes, although it did not affect the acrosome membrane. Previous studies utilizing general GST inhibitors in goat and pig sperm reported high levels of plasma membrane damage and destabilization, although they did not find any effect on the acrosomal membrane (Gopalakrishnan and Shaha, 1998; Llavanera et al., 2020). These evidences reveal a significant role of these antioxidant enzymes on the stability of sperm plasma membrane but not on that of acrosome membrane. In accordance with the previously reported results, these findings could suggest a specific destabilization of the membranes located in the middle and principal pieces rather than from the sperm head, which could cause mitochondrial and motility impairment. However, the specific localization and molecular mechanisms by which GSTs are able to maintain membrane stability are currently unknown. The results of the present study shed some light on the mechanisms regulating destabilization of sperm membranes, suggesting that this process could be mediated by the activation of JNK signaling. However, the specific JNK downstream signaling proteins are yet to be determined. Uncovering the specific molecular signaling pathway through which sperm membrane stability is reduced is of utmost interest to develop new strategies for increasing sperm life span and quality.

Interestingly, although GSTP1–JNK dissociation caused severe mitochondrial damage and membrane destabilization in sperm cells, it did not have any effects upon intracellular calcium reservoirs. In this sense, previous studies in pig sperm showed that general GST inhibitors caused a significant increase in calcium levels, predominantly in the sperm middle piece (Llavanera et al., 2020). The present results suggested that, despite some specific GST classes being involved in the regulation of sperm calcium levels, the inhibition of GSTP1 upon JNK seems not to be related to calcium fluctuations. However, further research tackling calcium levels due to JNK activation should be performed in order to confirm this hypothesis.

In conclusion, immunological and cell biology analyses confirmed that, as schematized in **Figure 11**, the dissociation of the GSTP1–JNK heterocomplex results in the activation of JNK and significantly declines sperm viability, motility, mitochondrial activity, and plasma membrane stability and increased superoxide levels, without altering intracellular calcium

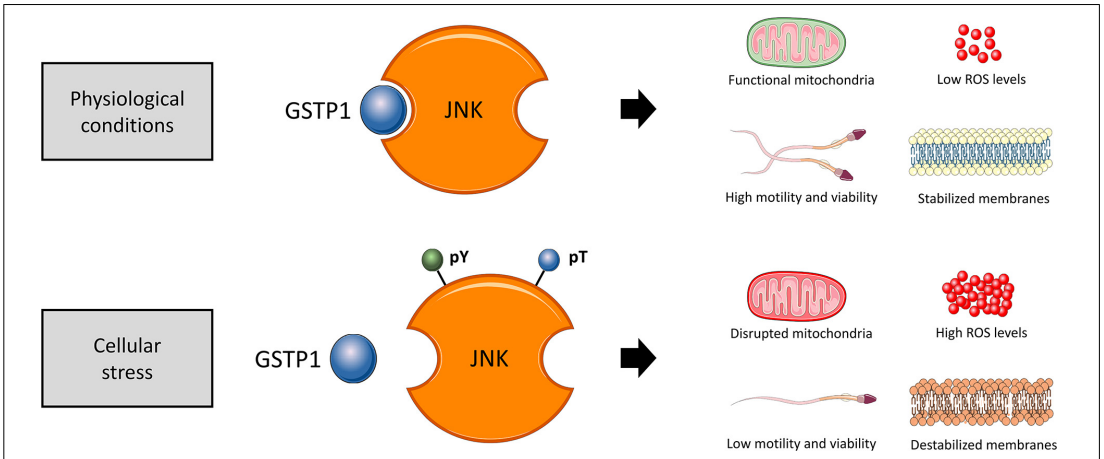

FIGURE 11 | Proposed diagram of the function of the GSTP1–JNK heterocomplex under physiological conditions and cellular stress in sperm cells. In physiological conditions, the GSTP1–JNK heterocomplex prevents JNK activation, maintaining sperm viability, motility, mitochondrial activity, and plasma membrane stability and increased superoxide levels. On the other hand, under cellular stress conditions, the GSTP1–JNK heterocomplex is dissociated, and thus, JNK is activated by tyrosine (pY) and threonine (pT) phosphorylation. The activation of JNK decreases sperm viability and motility, disrupts mitochondrial activity, causes plasma membrane destabilization, and increases intracellular superoxide levels.

levels and acrosome membrane integrity. Thus, the present study provides several evidences supporting the molecular role of JNK activation via dissociation of the GSTP1–JNK heterocomplex, uncovering the role of this protein in maintaining sperm functionality, especially with regard to the preservation of mitochondrial physiology. These findings set the grounds for understanding the relevance of GSTP1–JNK cell signaling regulation in mammalian sperm physiology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MY and ML: conceptualization and methodology. ML, SO, YM-O, AD-B, and SR: formal analysis and investigation. ML: writing-original draft preparation. MY and IB: writing-review and editing and supervision. MY: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledged the support from the Ministry of Science, Innovation and Universities, Spain (RYC-2014-15581, AGL2017-88329-R, FJCI-2017-31689, and FPU18/00666), and Regional Government of Catalonia, Spain (2017-SGR-1229).

ACKNOWLEDGMENTS

The authors would like to thank the Technical Research Services (University of Girona) for their technical support. The authors would also like to thank the Servier Medical Art for their image bank used to create all figures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.627140/full#supplementary-material

Supplementary Figure 1 | Preliminary concentration test. Mean and standard error of the mean (SEM) of the percentage of **(A)** viable sperm, **(B)** total motile sperm, **(C)** viable-membrane destabilized sperm,and **(D)** high mitochondrial membrane potential (high MMP) sperm. Different ezatiostat (TER) concentrations (10 μM, 100 μM, and 1000 μM) were tested (TER 72h; light gray bars), and the same volume of DMSO was added to Control-72h samples as a vehicle control group (Control 72h; medium gray bars). Semen samples at 0 h of storage were also analyzed (Control 0h; dark gray bars). Sample size ($n = 3$). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$. $^{***}p \leq 0.001$. A concentration of 10 μM of TER showed no effect on any sperm quality parameter compared to Control 0h and 72h samples ($p > 0.05$). Sperm samples treated with 100 μM showed a significant decrease in total sperm motility ($p < 0.001$) and MMP ($p < 0.01$), an increased sperm membrane destabilization ($p < 0.001$), and a minor but significant decrease in sperm viability ($p < 0.05$), compared to the Control 0h and 72h samples. Finally, samples treated with 1000 μM of TER showed significant ($p < 0.001$) detrimental effects upon all sperm quality parameters, especially on sperm viability, compared to the Control 0h and 72h samples. These results suggested that samples treated with 10 μM of TER did not exert any effect upon sperm physiology, whereas 1000 μM of TER caused cytotoxic effects on sperm and thus masking the physiological effects of the inhibitor upon sperm quality and functionality. Finally, samples treated with 100 μM of TER showed physiological effects upon sperm membranes, mitochondria, and motility without exerting critical cytotoxicity.

Supplementary Table 1 | Raw data. Raw data of sperm quality and functionality parameters of all treatments and time points. TMOT, percentage of total motile sperm; PMOT, percentage of progressively motile sperm; VAP, sperm average path velocity (μm/s); VIABILITY, percentage of viable sperm (SYBR-14^{+}/PI^{-}); M540, percentage of membrane-destabilized cells (M540^{+}) within the total viable sperm population (Yo-Pro-1^{-}); HE, relative fluorescence intensity of viable sperm with high levels of intracellular $O_2^{-\cdot}$ (E^{+}/PI^{-}); JC1, percentage of high mitochondrial membrane potential sperm ($\Delta\Psi$m) resulted from the orange-stained ($JC1_{agg}$) population; PNA, percentage of acrosome membrane-intact sperm (PNA-FITC^{-}) within the total viable sperm population (PI^{-}); FL3, relative Fluo3-AM fluorescence intensity of viable sperm with high levels of intracellular calcium (Fluo3-AM^{+}/PI^{-}); CNT 0h, semen samples at 0 h of storage; CNT 72h, vehicle control (dimethyl sulfoxide; DMSO) semen samples at 72 h of storage at 17°C; TER 72h, semen samples treated with 100 μM ezatiostat (TER) for 72 h of storage at 17°C.

Supplementary File 1 | Supplementary information for Materials and Methods.

REFERENCES

Adler, V., Yin, Z., Fuchs, S. Y., Benezra, M., Rosario, L., Tew, K. D., et al. (1999). Regulation of JNK signaling by GSTp. *EMBO J.* 18, 1321–1334.doi: 10.1093/emboj/18.5.1321

Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M., et al. (2002). Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. *J. Biol. Chem.* 277, 10244–10250. doi: 10.1074/jbc.M112355200

Aydos, S. E., Ph, D., Taspinar, M., Sc, M., Sunguroglu, A., and Ph, D. (2009). Association of CYP1A1 and glutathione S-transferase polymorphisms with male factor infertility. *Fertil. Steril.* 92, 541–547.doi: 10.1016/j.fertnstert.2008.07.017

Bracke, A., Peeters, K., Punjabi, U., Hoogewijs, D., and Dewilde, S. (2018). A search for molecular mechanisms underlying male idiopathic infertility. *Reprod. Biomed. Online* 36, 327–339. doi: 10.1016/j.rbmo.2017.12.005

Brand, M. D. (2016). Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic. Biol. Med.* 100, 14–31. doi: 10.1016/j.freeradbiomed.2016.04.001

Carrell, D. T., De Jonge, C., and Lam, D. J. (2006). The genetics of male infertility: a field of study whose time is now. *J. Reprod. Syst.* 52, 269–274.doi: 10.1080/01485010500503603

Garner, D. L., and Johnson, L. A. (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* 53, 276–284.doi: 10.1095/biolreprod53.2.276

Ghuman, N., and Ramalingam, M. (2017). Male infertility. *Obstet. Gynaecol. Reprod. Med.* 28, 7–14. doi: 10.1016/j.ogrm.2017.10.007

Gopalakrishnan, B., Aravinda, S., Pawshe, C. H., Totey, S. M., Nagpal, S., and Salunke, D. M. (1998). Studies on glutathione S-transferase important for sperm function: evidence of catalytic activity-independent functions. *Biochem. J.* 329, 231–241.doi: 10.1042/bj3290231

Gopalakrishnan, B., and Shaha, C. (1998). Inhibition of sperm glutathione S-transferase leads to functional impairment

due to membrane damage. *FEBS Lett.* 422, 296–300. doi: 10.1016/S0014-5793(98)00032-5

Griveau, J. F., and Le Lannou, D. (1997). Reactive oxygen species and human spermatozoa: physiology and pathology. *Int. J. Androl.* 20, 61–69.doi: 10.1046/j.1365-2605.1997.00044.x

Guthrie, H. D., and Welch, G. R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J. Anim. Sci.* 84, 2089–2100.doi: 10.2527/jas.2005-766

Harrison, R. A. P., Mairet, B., and Miller, N. G. A. (1993). Flow cytometric studies of bicarbonate-mediated Ca2+ influx in boar sperm populations. *Mol. Reprod. Dev.* 35, 197–208.doi: 10.1002/mrd.1080350214

Hemachand, T., and Shaha, C. (2003). Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett.* 538, 14–18. doi: 10.1016/S0014-5793(03)00103-0

Heslop, K. A., Rovini, A., Hunt, E. G., Fang, D., Morris, M. E., Christie, C. F., et al. (2020). JNK activation and translocation to mitochondria mediates mitochondrial dysfunction and cell death induced by VDAC opening and sorafenib in hepatocarcinoma cells. *Biochem. Pharmacol.* 171:113728.doi: 10.1016/j.bcp.2019.113728

Kan, H. P., Wu, F. L., Guo, W. B., Wang, Y. Z., Li, J. P., Huang, Y. Q., et al. (2013). Null genotypes of GSTM1 and GSTT1 contribute to male factor infertility risk: a meta-analysis. *Fertil. Steril.* 99, 690–696. doi: 10.1016/j.fertnstert.2012.10.037

Klinovska, K., Sebkova, N., and Dvorakova-Hortova, K. (2014). Sperm-egg fusion: a molecular enigma of mammalian reproduction. *Int. J. Mol. Sci.* 15, 10652–10668.doi: 10.3390/ijms150610652

Kolesnikova, L. I., Kurashova, N. A., Bairova, T. A., Dolgikh, M. I., Ershova, O. A., Dashiev, B. G., et al. (2017). Role of glutathione-S-transferase family genes in male infertility. *Bull. Exp. Biol. Med.* 163, 643–645.doi: 10.1007/s10517-017-3869-9

Kumar, R., Singh, V. K., and Atreja, S. K. (2014). Glutathione-S-transferase: role in buffalo (*Bubalus bubalis*) sperm capacitation and cryopreservation. *Theriogenology* 81, 587–598. doi: 10.1016/j.theriogenology.2013.11.012

Lakpour, N., Mirfeizollahi, A., Farivar, S., Akhondi, M. M., Hashemi, S. B., Amirjannati, N., et al. (2013). The association of seminal plasma antioxidant levels and sperm chromatin status with genetic variants of GSTM1 and GSTP1 (Ile105Val and Ala114Val) in infertile men with oligoasthenoteratozoospermia. *Dis. Markers* 34, 205–210.doi: 10.3233/DMA-120954

Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998). Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. *Curr. Biol.* 8, 1387–1391.doi: 10.1016/s0960-9822(98)00019-0

Llavanera, M., Delgado-bermúdez, A., Olives, S., Mateo-otero, Y., Recuero, S., Bonet, S., et al. (2020). Glutathione S-transferases play a crucial role in mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm. *Antioxidants* 9, 1–16. doi: 10.3390/antiox9020100

Llavanera, M., Delgado-Bermuùdez, A., Fernandez-Fuertes, B., Recuero, S., Mateo, Y., Bonet, S., et al. (2019a). GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. *J. Anim. Sci. Biotechnol.* 10:61. doi: 10.1186/s40104-019-0370-5

Llavanera, M., Mateo-Otero, Y., Bonet, S., Barranco, I., Fernández-Fuertes, B., and Yeste, M. (2019b). The triple role of glutathione S-transferases in mammalian male fertility. *Cell. Mol. Life Sci.* 77:2331–2342. doi: 10.1007/s00018-019-03405-w

Luna, C., Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J. A., and Muiño-Blanco, T. (2017). c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways link capacitation with apoptosis and seminal plasma proteins protect sperm by interfering with both routes. *Biol. Reprod.* 96, 800–815.doi: 10.1093/biolre/iox017

Mathew, N., Kalyanasundaram, M., and Balaraman, K. (2006). Glutathione S -transferase (GST) inhibitors. *Expert Opin. Ther. Pat.* 16, 431–444. doi: 10.1517/13543776.16.4.431

Mukhtar, H., Lee, I. P., and Bend, J. R. (1978). Glutathione S-transferase activities in rat and mouse sperm and human semen. *Biochem. Biophys. Res. Commun.* 83, 1093–1098. doi: 10.1016/0006-291X(78)91507-3

Nagy, S., Jansen, J., Topper, E. K., and Gadella, B. M. (2003). A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol. Reprod.* 68, 1828–1835. doi: 10.1095/biolreprod.102.011445

Okamura, T., Antoun, G., Keir, S. T., Friedman, H., Bigner, D. D., and Ali-Osman, F. (2015). Phosphorylation of glutathione s-transferase p1 (gstp1) by epidermal growth factor receptor (EGFR) promotes formation of the gstp1-c-jun n-terminal kinase (JNK) complex and suppresses JNK downstream signaling and apoptosis in brain tumor cells. *J. Biol. Chem.* 290, 30866–30878.doi: 10.1074/jbc.M115.656140

Ortega-Ferrusola, C., Sotillo-Galan, Y., Varela-Fernandez, E., Gallardo-Bolanos, J. M., Muriel, A., Gonzalez-Fernandez, L., et al. (2007). Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. *J. Androl.* 29, 213–221.doi: 10.2164/jandrol.107.003640

Parent, S., Lefièvre, L., Brindle, Y., and Sullivan, R. (1999). Bull subfertility is associated with low levels of a sperm membrane antigen. *Mol. Reprod. Dev.* 52, 57–65.doi: 10.1002/(SICI)1098-2795(199901)52:1<57::AID-MRD8<3.0.CO;2-U

Peddinti, D., Nanduri, B., Kaya, A., Feugang, J. M., Burgess, S. C., and Memili, E. (2008). Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst. Biol.* 2:1–13. doi: 10.1186/1752-0509-2-19

Pérez-Patino, C., Parrilla, I., Li, J., Barranco, I., Martínez, E. A., Rodriguez-Martínez, H., et al. (2019). The proteome of pig spermatozoa is remodeled during ejaculation. *Mol. Cell. Proteomics* 18, 41–50.doi: 10.1074/mcp.RA118.000840

Rathi, R., Colenbrander, B., Bevers, M. M., and Gadella, B. M. (2001). Evaluation of in vitro capacitation of stallion spermatozoa. *Biol. Reprod.* 65, 462–470. doi: 10.1095/biolreprod65.2.462

Rodrigues-Silva, H. V., Rodriguez-Villamil, P., de Magalhães, F. F., Nunes, T. G. P., de Freitas, L. A., Ribeiro, L. R., et al. (2018). Seminal plasma and sperm proteome of ring-tailed coatis (Nasua nasua, Linnaeus, 1766). *Theriogenology* 111, 34–42.doi: 10.1016/j.theriogenology.2017.12.036

Safarinejad, M. R., Shafiei, N., and Safarinejad, S. (2010). The association of glutathione-S-transferase gene polymorphisms (GSTM1, GSTT1, GSTP1) with idiopathic male infertility. *J. Hum. Genet.* 55, 565–570.doi: 10.1038/jhg.2010.59

Song, X., Zhao, Y., Cai, Q., Zhang, Y., and Niu, Y. (2013). Association of the Glutathione S-transferases M1 and T1 polymorphism with male infertility: a meta-analysis. *J. Assist. Reprod. Genet.* 30, 131–141.doi: 10.1007/s10815-012-9907-7

Storey, B. T. (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* 52, 427–437.doi: 10.1387/ijdb.072522bs

Tang, M., Wang, S., Wang, W., Cao, Q., Qin, C., Liu, B., et al. (2012). The glutathione-S-transferase gene polymorphisms (GSTM1 and GSTT1) and idiopathic male infertility risk: a meta-analysis. *Gene* 511, 218–223.doi: 10.1016/j.gene.2012.09.054

Turella, P., Cerella, C., Filomeni, G., Bullo, A., De Maria, F., Ghibelli, L., et al. (2005). Proapoptotic activity of new glutathione S-transferase inhibitors. *Cancer Res.* 65, 3751–3761. doi: 10.1158/0008-5472.CAN-04-3903

Vani, G. T., Mukesh, N., Siva Prasad, B., Rama Devi, P., Hema Prasad, M., Rani, P. U., et al. (2010). Role of glutathione S-transferase Mu-1 (GSTM1) polymorphism in oligospermic infertile males. *Andrologia* 42, 213–217.doi: 10.1111/j.1439-0272.2009.00971.x

Vicens, A., Borziak, K., Karr, T. L., Roldan, E. R. S., and Dorus, S. (2017). Comparative sperm proteomics in mouse species with divergent mating systems. *Mol. Biol. Evol.* 34, 1403–1416.doi: 10.1093/molbev/msx084

Vívenes, C. Y., Peralta-Arias, R. D., Camejo, M. I., Guerrero, K., Fernández, V. H., Piñero, S., et al. (2009). Biochemical identification of dynein-ATPase activity in human sperm. *Z. Naturforsch. C J. Biosci.* 64, 747–753.doi: 10.1515/znc-2009-9-1022

Wang, G., Guo, Y., Zhou, T., Shi, X., Yu, J., Yang, Y., et al. (2013). In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J. Proteomics* 79, 114–122.doi: 10.1016/j.jprot.2012.12.008

Wang, T., Arifoglu, P., Ronai, Z., and Tew, K. D. (2001). Glutathione S-transferase P1–1 (GSTP1–1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *J. Biol. Chem.* 276, 20999–21003.doi: 10.1074/jbc.M101355200

Win, S., Than, T. A., and Kaplowitz, N. (2018). The regulation of JNK signaling pathways in cell death through the interplay with mitochondrial SAB and upstream post-translational effects. *Int. J. Mol. Sci.* 19:3657.doi: 10.3390/ijms19113657

Wu, J. H., and Batist, G. (2013). Glutathione and glutathione analogues; therapeutic potentials. *Biochim. Biophys. Acta* 1830, 3350–3353.doi: 10.1016/j.bbagen.2012.11.016

Zigo, M., Maòásková-Postlerová, P., Zuidema, D., Kerns, K., Jonáková, V., Tùmová, L., et al. (2020). Porcine model for the study of sperm capacitation, fertilization and male fertility. *Cell Tissue Res.* 380, 237–262.

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

PAPER VI

Evaluation of Glutathione S-transferase Mu 3 (GSTM3) levels in sperm as a simple method to predict oxidative DNA damage and seminogram alterations

Marc Llavanera, Jordi Ribas-Maynou, Yentel Mateo-Otero, Jordi Benet, Sergi Bonet, Marc Yeste

Andrology
(Under review)

Evaluation of Glutathione S-transferase Mu 3 (GSTM3) levels in sperm as a simple method to predict oxidative DNA damage and seminogram alterations

Marc Llavanera[1,2]; Jordi Ribas-Maynou[1,2,*]; Yentel Mateo-Otero[1,2]; Jordi Benet[3]; Sergi Bonet[1,2]; Marc Yeste[1,2,4,*]

[1] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain

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

[3] Unit of Cell Biology and Medical Genetics, Department of Cell Biology, Physiology and Immunology, Faculty of Medicine, Autonomous University of Barcelona, ES-08193 Bellaterra (Cerdanyola del Vallès), Barcelona, Spain.

[4] Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain.

[*] Corresponding authors: jordi.ribasmaynou@udg.edu; marc.yeste@udg.edu.

Abstract

Background. Previous evidence suggested that sperm Glutathione S-transferase Mu 3 (GSTM3) is essential for proper mitochondrial function, plasma membrane stability and oxidative regulation in mammalian sperm. In humans, however, neither has this enzyme been related to semen alterations nor has it been reported to be associated to oxidative DNA damage and (in)fertility. Objectives. The aim of the present study was to assess the usefulness of GSTM3 to predict spermiogram alterations and oxidative DNA damage in sperm. Material and Methods. A total of 26 semen samples were collected, evaluated by conventional semen analysis, subsequently cryopreserved, and assessed for DNA fragmentation and the presence, localisation and content of GSTM3. Results. Sperm GSTM3 content was positively related to sperm quality parameters ($P < 0.05$). Specifically, lower levels of this antioxidant enzyme were observed in asthenoteratozoospermic/oligoasthenoteratozoospermic men ($P < 0.05$), but not idiopathic infertile patients ($P > 0.05$), when compared to normospermic samples. Moreover, sperm GSTM3 was negatively associated to oxidative DNA damage ($P < 0.05$). Finally, the ability to predict spermiogram alterations was determined by

ROC curve analysis using GSTM3 alone (AUC = 0.91; $P < 0.05$), and in combination with oxidative DNA damage (AUC = 0.92; $P < 0.05$). Discussion and conclusions. Although the limited number of samples of the present study warrants further research, including clinical trials with larger sample sizes, the results reported herein set the grounds for using GSTM3 as a novel biomarker and therapy target for male infertility and oxidative DNA damage in sperm.

1. Introduction

Infertility is defined as the inability of sperm to fertilise the oocyte, whereas subfertility refers to any form of reduced fertility (1). The prevalence of infertility has been reported to range between 8% and 12% of couples at reproductive age, involving more than 48.5 million couples worldwide (2–4). About 20-30% of the cases are known to be exclusively caused by a male factor (3). Related to this, worthy of notice is the progressive decline in semen quality registered over the last decades (5–8), estimating a decrease of 50-60% in sperm counts between 1973 and 2011 (9). Improving diagnosis and treatment of male infertility is, therefore, much warranted, especially due to the multicausal origin of the disease, which hinders the prediction of sperm quality and fertilising ability (10).

At present, the diagnosis of male reproductive disorders and the prediction of sperm fertilising ability in fertility clinics are mainly based on conventional semen analysis (i.e., sperm concentration, motility and morphology). For this purpose, Computer-Assisted Sperm Analysis (CASA) systems have become a useful and rapid tool to evaluate sperm quality (11). Despite these advances, conventional semen analysis still shows poor sensitivity and accuracy for the prognosis of male (in)fertility and assisted reproduction outcomes, limiting its value for clinical use (12,13). For this reason, whether the conventional spermiogram can predict male fertility is under debate (13,14). In the route towards improving sperm analysis, employing molecular biomarkers has been suggested as an interesting approach (15). For example, sperm DNA fragmentation (SDF) analysis has been proposed as a putative diagnostic parameter for fertility status (16). Yet, because the relationship between SDF and assisted reproduction

outcomes is still controversial, further efforts to set novel molecular markers are needed.

Glutathione S-transferases (GSTs) are antioxidant enzymes with a triple role in mammalian sperm: (i) detoxification of electrophilic compounds, (ii) regulation of cellular signalling and (iii) zona pellucida recognition (17). Many GST isoforms have been described in mammalian sperm and seminal plasma, such as GSTAs (18), GSTMs (18–22), GSTPs (18–20,22), and GSTOs (22–24). Specifically, in humans, impaired expression of GSTs has been associated with reduced male fertility. Several studies evidenced that null GSTM or GSTT genotypes are a risk factor causing idiopathic male infertility (25–32). Moreover, sperm from a GSTM null genotype showed increased reactive oxygen species (ROS) levels in sperm (33) and their DNA was more susceptible to be damaged when individuals were exposed to air pollution (34). In other mammalian species, sperm GSTs, and specifically GSTM3, were found to play a key role in sperm physiology. In goats, sperm GSTs were associated to lipid peroxidation and mitochondrial activity (35). In pigs, GSTM3 was established as a sperm quality (36), fertility (37), cryotolerance (38) and liquid-preservation (36) biomarker in sperm. Given the aforementioned association between GSTs, sperm physiology and male (in)fertility, exploring the putative role of sperm GSTM3 as a molecular biomarker for human male fertility is much warranted. To the best of the authors' knowledge, nevertheless, little evidence in the literature regarding the physiological role of GSTM3 in human sperm as well as their potential capacity to predict fertilising ability is available.

The objectives of the present study were to (i) determine the presence and localisation of GSTM3 in human sperm; (ii) explore the association of sperm GSTM3 levels with sperm quality and DNA damage; and (iii) address the potential use of this antioxidant enzyme to predict sperm quality and male (in)fertility.

2. Materials and Methods

2.1. Patients and samples

Healthy, fertile men and men referred for clinical infertility evaluation were included in the study. Infertile men were considered as those unable to conceive after one

year of regular unprotected intercourse, regardless of whether they had a normal (idiopathic) or an altered (asthenoteratozoospermia or oligoasthenoteratozoospermia) seminogram. Fertile men were considered to be those that conceived a child within a year prior to semen donation and had a normal seminogram. Semen samples were obtained in collaboration with the Autonomous University of Barcelona (UAB), Spain. All donors signed the informed consent, and the study was approved by the Ethics Committee of Parc Taulí Health Corporation, Spain (ref.: 2008532). A total of 34 semen samples were collected after 2–5 days of sexual abstinence and, following liquefaction at room temperature, they were evaluated on the basis of the conventional spermiogram (sperm concentration, ejaculate volume and pH, percentage of leucocytes, sperm motility and morphology) following the 5th Edition of the World Health Organisation Manual (39). All samples were free from epithelial cells. After semen quality evaluation, samples were cryopreserved following the protocol described in Ribas-Maynou et al. (40), and were stored in liquid nitrogen at -196°C until analysis. Samples showing >2% leukocytes (n=8) were excluded from the study in order to minimise non-sperm protein contamination. The remaining semen samples (n=26) were classified as: (i) fertile with a normal spermiogram (NSP; n=10); (ii) infertile with asthenoteratozoospermia or oligoasthenoteratozoospermia (AT/OAT; n=9); or (iii) idiopathic infertile (ID; n=7). Frozen-thawed sperm samples were subsequently assessed for DNA fragmentation through alkaline and neutral Comet assays, the presence and localisation of GSTM3 through immunoblotting and immunofluorescence, and the quantification of GSTM3 through an enzyme-linked immunosorbent assay (ELISA).

Table 1. Average age and semen characteristics in fertile normozoospermic (NSP), infertile asthenozooteratospermic/oligoastenoteratozoospermic (AT/OAT), and idiopathic infertile patients (IDNSP).

Parameters	NSP	AT/OAT	IDNSP
Individuals (n)	10	9	7
Age (years)	33.7 (30 - 35)[a]	37.9 (32 - 51)[a]	35.3 (23 - 41)[a]
Semen volume (mL)	3.3 (2.0 - 6.5)[a]	3.9 (1.8 - 7.5)[a]	4.1 (0.5 - 6.0)[a]
pH	7.6 (7.0 - 8.0)[a]	7.4 (7.0 - 8.0)[a]	7.3 (6.7 - 7.7)[a]
Sperm concentration (10^6/mL)	91.7 (39 - 210)[ab]	15.0 (1 - 195)[b]	203.0 (52 - 299)[a]
Total motility (a+b+c; %)	61.4 (16.6 - 82.9)[a]	32.1 (9 - 65)[b]	91.3 (72 - 99)[c]
Progressive motility (a+b; %)	37.7 (2.3 - 61)[a]	14.9 (6 - 29)[b]	69.7 (55 - 81)[c]
Normal forms (%)	6.0 (5 - 8)[a]	2.0 (0 - 4)[b]	6.0 (4 - 18)[a]

Sperm concentration and normal forms did not meet the parametric assumptions of normal distribution and homogeneity of variances and are expressed as median. Statistical comparisons were performed using the Kruskal-Walli's test. The other parameters following the parametric assumptions are expressed as mean (min – max). Statistical comparisons were performed by the one-way ANOVA. $P < 0.05$ was considered significant.

2.2. Evaluation of sperm quality

Sperm concentration, motility and morphology were determined as parameters defining sperm quality. For sperm concentration and motility, seven µL of liquefied semen were loaded into a 10-micron Leja Chamber (Leja Products B.V., Luzemestraat, Nieuw-Vennep, The Netherlands) prewarmed at 37°C. Videos were captured at 30 frames per second under an Olympus AX70 phase-contrast microscope (Olympus Corporation, Tokyo, Japan), using the Sperm Class Analyzer (SCA; Microptic S.L., Barcelona, Spain) CASA system. At least 500 spermatozoa from at least five homogeneous fields were evaluated, and sperm concentration (million sperm/mL), progressive motility (% type A + type B), non-progressive motility (% type C) and the percentage of immotile sperm (% type D) were recorded. For sperm morphology, pre-stained SpermBlue slides (Microptic S.L.) were used. Briefly, 10 µL of semen were dispensed on top of the slide, covered with a 22 × 22 coverslip and visualised under an Olympus AX70 microscope, using the morphology module of the SCA system. The percentage of sperm with abnormalities was assessed using Kruger's strict criteria, which includes cytoplasmic droplets and alterations in head, midpiece and tail.

2.3. Alkaline and neutral Comet assays

Both alkaline and neutral Comet assays were performed as previously described in Casanovas et al. (41), with minor modifications. Prior to the initiation of the Comet assay, glass slides were pre-treated for gel adhesion by submerging them into 1% low melting point agarose. First, sperm cells were diluted to a concentration of 1×10^6 sperm per mL. Twenty-five mL of diluted sperm were mixed with 50 mL of 1% low melting point agarose. Fifteen mL of the mixture was placed onto two pre-treated slides, covered with coverslips and incubated at 4°C for 5 min. Thereafter, coverslips were carefully removed, and slides were incubated in lysis solution 1 (0.8 M Tris-HCl, 0.8 M DTT, 1% SDS, pH=7.5) for 30 min, and then in lysis solution 2 (0.4 M Tris-HCl, 0.4 M DTT, 50 mM EDTA, 2 M NaCl, pH=7.5) for further 30 min. Next, all slides were washed in TBE buffer (0.445 M Tris-HCl, 0.445 M Boric acid, 0.01 M EDTA, pH=8) for 10 min. For the alkaline Comet assay, samples were incubated in denaturing solution (0.03 M NaOH, 1 M NaCl, pH=13) at 4°C for 2.5 min; electrophoresis was performed in 0.03 M NaOH buffer (pH=13) at 1 V/cm for 4 min. For the neutral Comet assay, electrophoresis was performed in TBE buffer at 1 V/cm for 12.5 min, and slides were further washed in 0.9% NaCl for 2 min. Subsequently, all slides were incubated in neutralising solution (0.4 M Tris-HCl, pH=7.5) for 5 min, followed by incubation in TBE for 2 min. Finally, all slides were dehydrated in an increasing series of ethanol (70%, 90% and 100%; 2 min each). Samples were visualised and captured at 100× magnification using an epifluorescence microscope (Axio Imager Z1, Carl Zeiss AG; Oberkochen, Germany) equipped with a camera (AxioCam, Carl Zeiss AG; Oberkochen, Germany).

Comet images of at least 100 sperm/sample were automatically analyzed using the CometScore v2.0 software (Rexhoover, http://rexhoover.com/cometscoredownload.php), quantifying the fluorescence intensity of Comet tails and heads. Background intensity was adjusted to correctly visualise the comets, and the automatic analysis was supervised by an expert to adjust head/tail detection and to remove overlapping and misanalyzed comets. The quantification of DNA breaks in sperm cells was determined by the Olive Tail Moment, which is an informative indicator of DNA damage in a specific cell (42,43)

defined by the following formula: (tail mean intensity – head mean intensity) × Tail DNA/100. Percentages of sperm with single- and double-stranded DNA fragmentation (ssSDF and dsSDF, respectively) were assessed following the criteria reported before by Casanovas et al. (41) and Ribas-Maynou et al. 44).

2.4. Immunoblotting analysis

Semen samples were centrifuged, supernatants were discarded, and sperm cells were resuspended in a cell lysis buffer (RIPA buffer). Next, samples were incubated in agitation at 4°C for 30 min and centrifuged at 12,000 × g and 4°C for 20 min. Finally, supernatants were quantified for total protein through a detergent compatible method (DCTM Protein Assay, Bio-Rad, Hercules, CA, USA). Fifteen µg of total protein from each pool was resuspended in reducing buffer and heated at 95°C for 5 min prior to loading onto a 12% polyacrylamide gel (Mini-PROTEAN® TGX Stain-Free™ Precast Gels, Bio-Rad). Next, electrophoresed proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using Trans-Blot® Turbo™ (Bio-Rad). Following this, and in order to get a loading control, blots were exposed to UV and total protein bands were acquired using a G:BOX Chemi XL system (SynGene, Frederick, MD, USA). Subsequently, blots were incubated in blocking solution (10 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20; pH=7.3, and 5% bovine serum albumin [Roche Diagnostics, S.L., Basel, Switzerland]) for 1 h at room temperature. After that, blocked blots were incubated with an anti-GSTM3 rabbit polyclonal antibody (ref. ARP53561_P050; 1:20,000, v:v) at 4°C overnight. A parallel peptide competition assay was conducted by incubating the anti-GSTM3 antibody along with the GSTM3 blocking peptide (ref. AAP53561), 10 times in excess regarding the antibody. Next, blots were rinsed thrice and incubated with an anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (ref. P0448, Agilent, Santa Clara, CA, USA; 1:40,000, v:v) at room temperature for 1 h. Finally, blots were rinsed thrice, and bands were visualised with a chemiluminescent substrate (Immobilon ECL Ultra Western HRP Substrate, Merk) and scanned using G:BOX Chemi XL.

2.5. Immunofluorescence analysis

Localisation of GSTM3 in sperm was determined by immunofluorescence. Ten million sperm per sample were washed with PBS. Sperm were then fixed with 2% (w:v) paraformaldehyde and washed twice. One hundred μL of sperm were extended on a slide and subsequently blocked and permeabilised through incubation with a solution containing 1% (v:v) Triton X-100 and 5% (w:v) bovine serum albumin (BSA) at room temperature for 1 h. Subsequently, samples were incubated with a primary anti-GSTM3 antibody (ref. ARP53561_P050; 1:250, v:v) for 2 h. For the peptide competition assay, the GSTM3-specific blocking peptide, which was 10 times in excess, was incubated along with the corresponding primary antibody. Next, samples were washed and incubated with a secondary anti-rabbit antibody conjugated with Alexa Fluor™ 488 (ref. A32731; 1:500, v:v) for 1 h, and further washed in PBS for 10 min. Finally, samples were mounted with ProLong™ Diamond Antifade Mountant containing DAPI (ref. P36962, ThermoFisher). Sperm samples were examined under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon Corp., Tokyo, Japan).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Human GSTM3 in sperm samples was quantified using a human-specific sandwich ELISA kit (ABK1-E6981; Abyntek Biopharma, Derio, Spain) following the manufacturer's guidelines. In brief, a standard curve was obtained by loading 100 μL of GSTM3 standards (0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 ng of GSTM3/mL) onto wells. The same volume of sperm lysate diluted to 1 mg of total protein per mL was loaded onto their corresponding wells. Following this, samples and standards were incubated together with detection reagents A and B at 37°C for 60 and 30 min, respectively. Afterwards, samples were washed five times and incubated with the substrate of the HRP enzyme at 37°C for 12 min, prior to adding the stop solution. Finally, colour intensity was measured spectrophotometrically at 450 nm in a microplate spectrophotometer (BioTek Epoch; BioTek, Winooski, Vermont, USA). A standard curve relating absorbance to the GSTM3 concentration of standards was plotted. The regression curve was subsequently calculated to interpolate the GSTM3 concentration of each sample; the R^2 of the

curve was 0.9988. The ELISA kit, which was designed for human GSTM3, showed a sensitivity of 1.15 ng/mL. Blank control wells contained PBS 1× (pH=7.0). All standards and samples were loaded in duplicate.

2.7. Statistical analysis

Data were analyzed and plotted with IBM SPSS Statistics v. 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v. 8 (GraphPad Software, La Jolla, CA, USA), respectively. Shapiro-Wilk and Levene tests were performed to check normal distribution and homogeneity of variances. Significant differences between groups were evaluated through an analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. When data did not meet normality and/or homogeneity of variances assumptions, Kruskal–Wallis test was used as a non-parametric alternative. Correlations between variables were determined through the analysis of Spearman's rank correlation coefficients. Finally, a receiver operating curve (ROC) analysis to discriminate between normal and abnormal spermiograms was run. Three variables were assessed: 1) content of GSTM3 in sperm, 2) percentage of sperm showing oxidative DNA damage, and 3) regression factors from the principal component analysis (PCA) of the previously mentioned variables. The level of significance was set at $P \leq 0.05$.

3. Results

3.1. GSTM3 is present in human sperm and is localised along in their tail

Immunoblotting analysis of GSTM3 evidenced a double-band pattern of ~ 48 and ~ 75 kDa. The GSTM3-specificity of both bands was confirmed by their absence in the peptide competition assay. Apparent GSTM3-specific bands were present in sperm samples from normospermic men, whereas undetectable levels were observed in samples showing an abnormal spermiogram. Moreover, immunofluorescence analysis allowed the localisation of GSTM3 along the tail of all sperm, as well as within the equatorial segment of some cells.

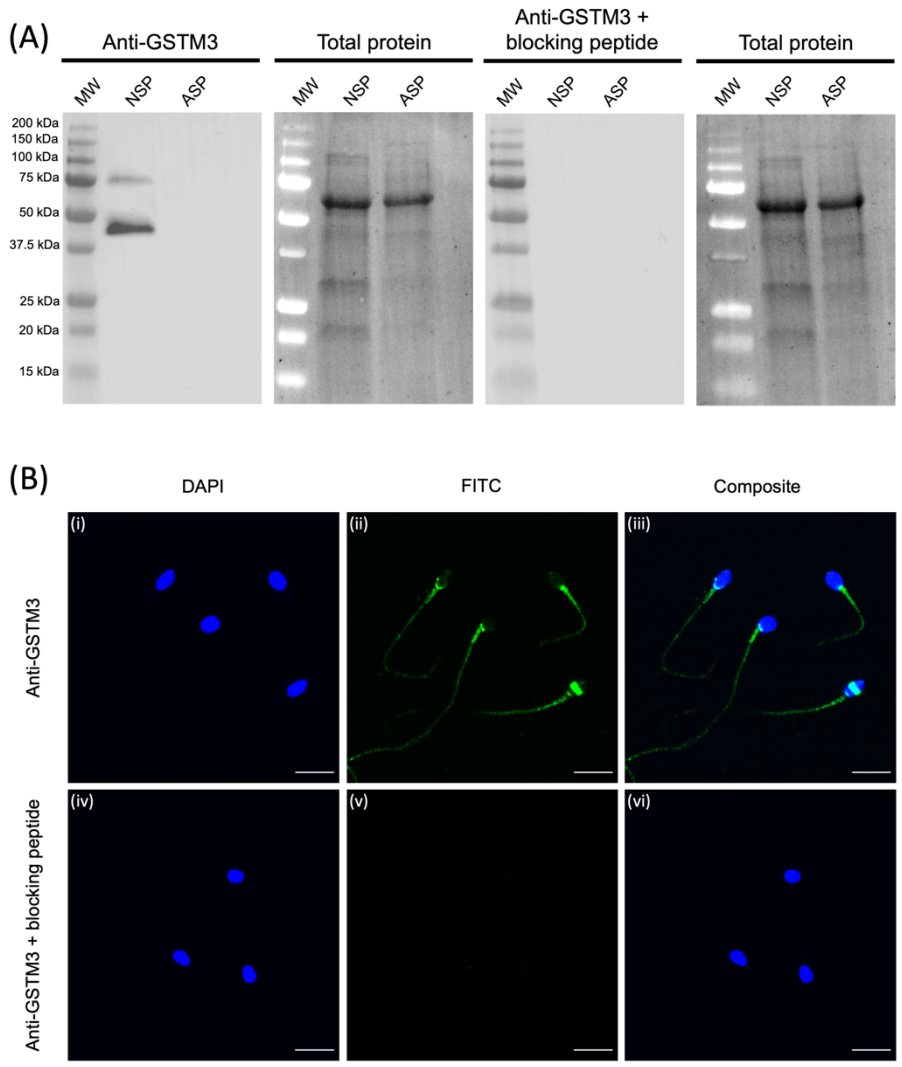

Figure 1. (**A**) Immunoblotting analysis of GSTM3 in human sperm. Blots resulting from incubation with the GSTM3 antibody (Anti-GSTM3) and its loading control (Total protein), and from incubation with the GSTM3 antibody plus the GSTM3 immunising peptide (Anti-GSTM3 + blocking peptide) and its loading control (Total protein) are shown. Lanes MW: molecular weight. Lanes NSP: pool of four sperm lysates from men with a normal spermiogram. Lanes ASP: pool of two sperm lysates from astenoteratozoospermic (AT) and oligoastenoteratozoospermic men (OAT). (**B**) Immunolocalisation of GSTM3 (i, ii, iii) and the corresponding peptide competition assay (iv, v, vi). Nucleus is shown in blue (DAPI), whereas GSTM3 is shown in green (FITC). Scale bars: A-B: 10 μm.

3.2. GSTM3 content in sperm is positively associated with their quality

Correlations between semen quality parameters and content of GSTM3 in sperm are shown in Figure 2. On one hand, semen volume and pH did not show statistically significant correlations with sperm GSTM3 content ($P > 0.05$). On the other hand, sperm quality parameters (sperm concentration, total motility, progressive motility, and the percentage of morphologically normal sperm) showed a positive and statistically significant correlation with GSTM3 levels in sperm ($P < 0.05$).

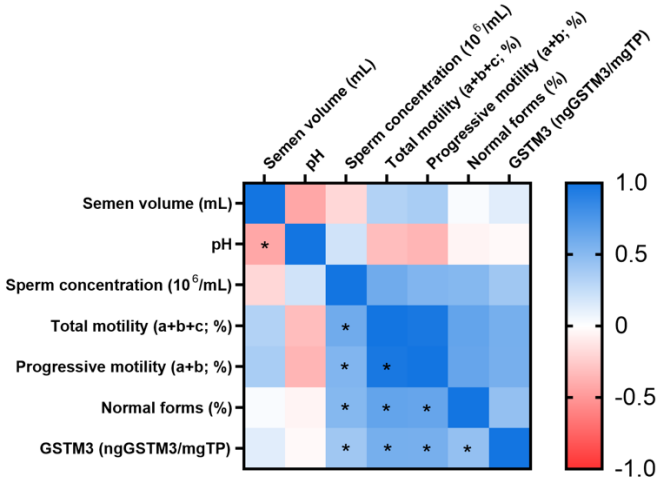

Figure 2. Heatmap of Spearman's rank correlation coefficients between sperm GSTM3 levels (ng GSTM3/mg total protein) and semen (semen volume and pH) and sperm (sperm concentration, total motility, progressive motility and normal forms) quality parameters. n = 26. *$P \leq 0.05$.

3.3. Lower GSTM3 content in sperm are associated to greater oxidative DNA damage

Spearman correlation coefficients between sperm DNA integrity, assessed by alkaline and neutral Comet, and GSTM3 content are shown in Figure 3. Levels of GSTM3 were found to be negatively and significantly correlated with the percentage of sperm with single-strand DNA breaks (ssDNA breaks; ssSDF [%]; R = -0.70; $P < 0.05$) and the incidence of DNA breaks (R = -0.47; $P < 0.05$) measured by the alkaline Comet. Interestingly, the proportion of sperm with double-strand DNA breaks (dsDNA breaks; dsSDF [%]) and the incidence of DNA

breaks assessed by the neutral Comet were not correlated to the GSTM3 content in sperm ($P > 0.05$).

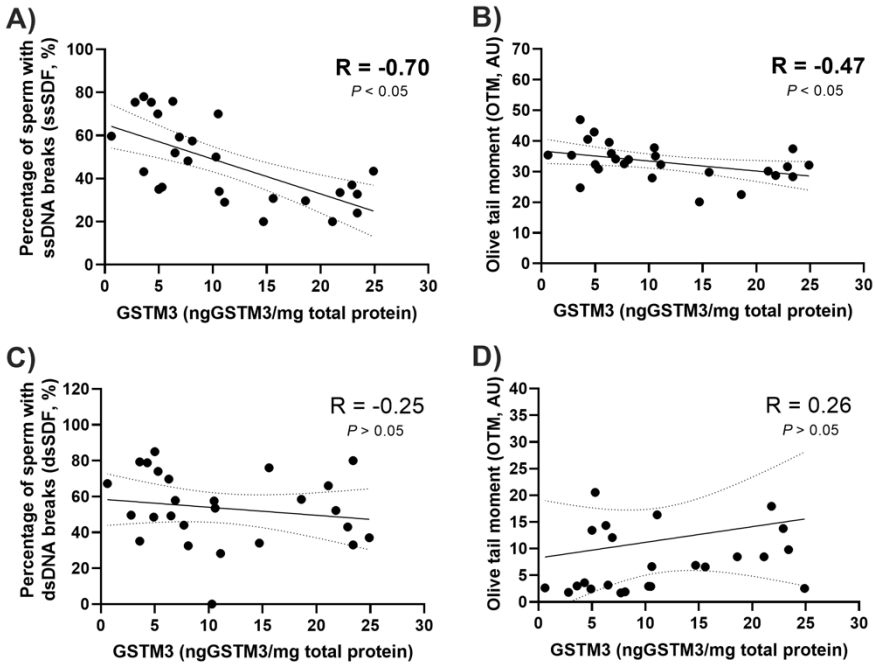

Figure 3. Spearman's rank correlation coefficients between sperm GSTM3 content (ngGSTM3/mg total protein) and (**A**) the percentage of sperm with single-strand DNA breaks (ssDNA breaks [ssSDF, %]), (**B**) the incidence of single-strand DNA breaks, (**C**) the percentage of sperm with double-strand DNA breaks (dsDNA breaks [dsSDF, %]), and (**D**) the incidence of double-strand DNA breaks. n = 26.

3.4. Poor quality ejaculates show lower levels of GSTM3 in sperm

Sperm GSTM3 content was compared between quality and fertility groups (Figure 4). Regarding sperm quality, GSTM3 levels were found to be significantly higher ($P < 0.05$) in men with NSP than in those with AT/OAT. As far as fertility groups are concerned, whereas fertile and idiopathic infertile men showed similar GSTM3 levels in sperm ($P > 0.05$), those levels were significantly lower in infertile men with an altered spermiogram ($P < 0.05$).

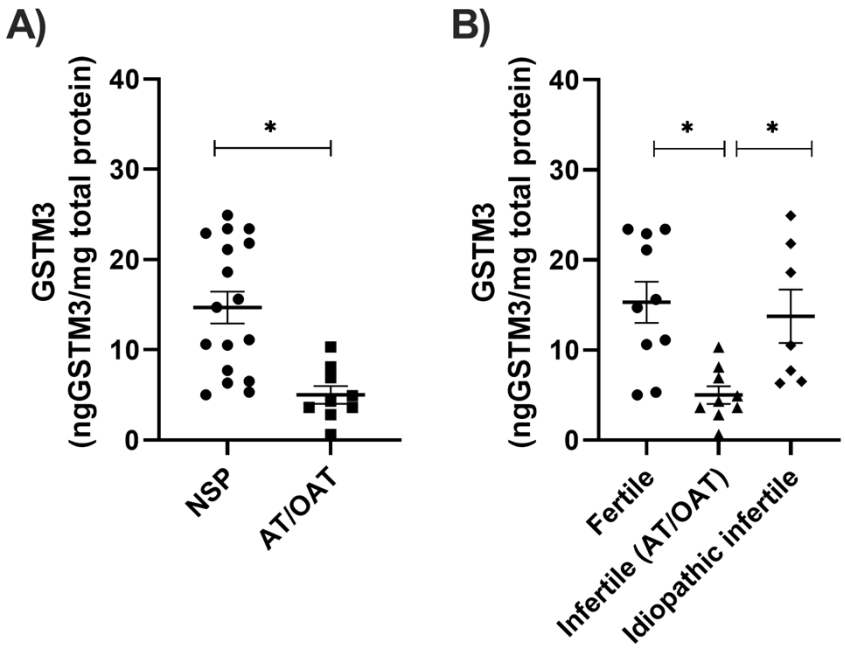

Figure 4. Mean ± standard error of the mean (SEM) of GSTM3 levels in sperm (ngGSTM3/mg total protein) between (**A**) sperm quality and (**B**) fertility groups. NSP: sperm samples with normal spermiogram (n=17). Fertile: sperm samples from proven fertility men (n=10). Infertile (AT/OAT): sperm samples from infertile men with asthenoteratozoospermia or oligoasthenoteratozoospermia (n=9). Idiopathic infertile: sperm samples from infertile men with a normal spermiogram (n=7). *$P \leq 0.05$.

3.5. GSTM3 is suggested as a sperm quality biomarker in men

Given the association between GSTM3 levels in sperm and spermiogram alterations, this antioxidant enzyme was explored as a putative sperm quality biomarker in men, through running ROC analysis (Figure 5 and Table 2). The incidence of ssDNA breaks and the levels of GSTM3 in sperm were capable to discriminate between normal and altered semen analysis ($P < 0.05$), with AUCs of 0.89 and 0.91, respectively. Interestingly, the combination of the two variables (GSTM3 levels and ssDNA breaks) exhibited a higher AUC (0.92).

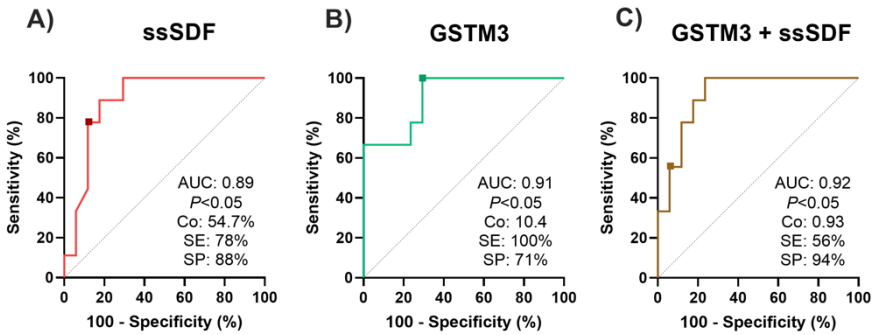

Figure 5. Receiver operating characteristic (ROC) curves of the percentage of sperm with single strand DNA fragmentation (ssSDF, %]), the levels of Glutathione S-transferase Mu3 levels in sperm (GSTM3 [ng GSTM3/mg total protein]) and the regression factors resulting from a principal component analysis (PCA) of both parameters for discriminating between semen samples with a normal (n=17; normozoospermia) and altered (n=9; asthenoteratozoospermia and oligoasthenoteratozoospermia) spermiogram.

Table 2. Receiver operating characteristic (ROC) curve analysis showing the area under the curve (AUC), standard deviation (SD), level of significance (P-value), cut-off value, sensitivity, specificity and likelihood ratio of the percentage of sperm with single strand DNA fragmentation (ssDNA breaks [SDF, %]), the sperm glutathione S-transferase mu3 levels (GSTM3 [ng GSTM3/mg total protein (TP)]) and the regression factors resulting from a principal component analysis (PCA) of both parameters for discriminating between semen samples with a normal (n=17; normozoospermia) and altered (n=9; asthenoteratozoospermia and oligoasthenoteratozoospermia) spermiogram.

	AUC	SD	*P*-value	Cut-off	Sensitivity	Specificity	Likelihood ratio
ssDNA breaks (SDF, %)	0.89 (0.76 – 1.00)	0.07	< 0.05	54.7	78%	88%	6.61
GSTM3 (ng/mg TP)	0.91 (0.79 – 1.00)	0.06	< 0.05	10.4	100%	71%	3.40
ssDNA breaks + GSTM3	0.92 (0.81 – 1.00)	0.05	< 0.05	0.93	56%	94%	9.44

4. Discussion

Male infertility is a rising disease worldwide (2–4). While much effort to standardise the prediction of sperm fertilising ability has been made, the multifactorial nature of this disease hinders the power of sperm quality to diagnose male infertility (10). Conventional semen analysis through a CASA system is the predominant method for the diagnosis and prognosis of male fertility disorders (11). Yet, the use of molecular biomarkers has been proposed to be helpful to identify infertility etiologies and predict male fertility with simple and sensitive tools (15). Although

glutathione S-transferases, and specifically GSMT3, have been shown to be related to sperm quality and male fertility in several mammalian species (17), little evidence is, to the best of the authors' knowledge, available regarding the physiological role of GSTM3 in human sperm and its potential utilisation as a novel molecular biomarker. Accordingly, the present study sought to address the presence of GSTM3 in human sperm and elucidate its ability to predict sperm quality and male (in)fertility.

GSTM3 is a small enzyme of ~ 25 kDa. In this study, however, GSTM3 in immunoblotting analysis was identified as a double-band pattern of ~ 48 and ~ 75 kDa. Despite not being detected in human sperm, the ~ 25 kDa band was observed in other species (37). This shift in the molecular mass of GSTM3 could be a result of its homodimerisation, covalent binding with other proteins, or posttranslational modifications. Because only few studies on the molecular modifications of sperm GSTM3 have been performed, this hypothesis needs further research. On the other hand, it is worth noting that although the percentage of immature germ cells in human semen is rather low (3% to 7% (46)), the GSTM3 quantified in the present study by ELISA could originate from both sperm and immature germ cells. GSTM3 is especially abundant in the testis (47) and epididymis (22), and incorporated and/or attached to sperm during epididymal maturation (48), thus indicating an important role of this antioxidant enzyme during both spermatogenesis and epididymal maturation. Hence, even though the major GSTM3 content quantified herein is likely to pertain to mature sperm, a minority could be attributed to immature germ cells. Moreover, immunofluorescence analysis determined the subcellular localisation of this antioxidant enzyme in human sperm. Herein, GSTM3 was found to be present along the entire tail, comprising mid, principal and end pieces, in a similar fashion to other species such as the bovine (45). In some sperm cells, however, GSTM3-specific fluorescence was also found in the postequatorial region of the sperm head, resembling to the pattern reported in porcine (36). These results suggest a species-specific localisation of GSTM3 in sperm.

In the present study, levels of GSTM3 were found to be positively correlated with sperm concentration, motility and morphology. This indicated that

the higher the GSTM3 content in sperm, the greater the quality of the ejaculate. Besides, and in line with these results, a relevant association between sperm GSTM3 and DNA integrity was observed. Sperm DNA damage has been shown to compromise embryo development in mammals (50), thus highlighting the relevance of the male factor on (in)fertility. Previous research suggested a putative influence of GSTs upon the protection of sperm DNA from oxidative damage (33,34). Specifically, individuals with a GSTM1 null genotype were found to exhibit higher susceptibility to the sperm DNA damage associated with exposure to air pollutants (34), and had increased ROS levels in sperm and seminal plasma (33). The present study also investigated the relationship between GSTM3 levels in sperm and DNA integrity analyzed through the Comet assay. The Comet assay is known to provide an accurate assessment of sperm DNA integrity, allowing the differentiation between single- and double-stranded DNA (ssDNA and dsDNA) breaks by the performance of alkaline and neutral Comet, respectively (51,52). The data reported herein evidenced a clear negative association between sperm GSTM3 content and ssSDF, but not dsSDF, breaks. Single-stranded DNA breaks are understood to be induced by oxidative damage, mainly as a result of endogenous and/or exogenous ROS (53–55). Remarkably, the current study found that individuals with lower levels of sperm GSTM3 exhibited greater oxidative DNA damage, thus supporting the essential role of this antioxidant enzyme in maintaining redox homeostasis and preventing oxidative DNA damage in human sperm. The association found herein, therefore, evidences the antioxidant role of GSTM3 in sperm. Related to this, it is worth mentioning that GSTM3 has been reported to be involved in sperm detoxification of reactive electrophiles, including environmental carcinogens and intracellular ROS (17). In fact, in other mammalian species, GSTM3 has been shown to prevent the lipid peroxidation of sperm membrane (35), as well as maintain mitochondrial function, plasma membrane stability and oxidative homeostasis (36). In the view of the above, reduced GSTM3 levels were found in sperm from infertile men with AT/OAT, but not with idiopathic infertility, when compared to normospermic men. These findings suggest that sperm samples with poor quality have a decreased GSTM3 antioxidant capacity, which is likely to unbalance their redox homeostasis, increasing oxidative DNA

damage and subsequently reducing their fertilising ability. Although one might assume that high levels of this antioxidant enzyme in sperm would protect them from OS, the results previously reported in the literature do not align with the current study. In effect, Botta et al. (57) reported higher levels of GSTM3 in oligospermic men, and Behrouzi et al. (58) associated high GSTM3 levels in sperm with sperm DNA damage. These apparently contradictory results could be explained by methodological dissimilarities between studies. While in the present work GSTM3 was quantified by ELISA, relative quantification by two-dimensional proteomics and LS-MS/MS was carried out in the other studies. Further research should, therefore, address these inconsistencies and investigate the molecular basis underlying the individual variation of GSTM3 levels in sperm and seminal plasma.

Finally, and given the clear association between sperm GSTM3 levels and the quality of sperm, the potential use of this protein as a sperm quality biomarker in men was interrogated. While this was the first study investigating whether the content of GSTM3 in human sperm may predict their quality, previous research reported that the alkaline Comet, which is associated to sperm motility (41), is a good predictor of sperm fertilising ability (59). In the present work, ROC analysis evidenced a good discrimination value of both the percentage of ssSDF breaks and the sperm levels of GSTM3 in differentiating normal from altered spermiograms (AUCs of 0.89 and 0.91, respectively). Accordingly, the two variables were combined through a principal component analysis (PCA), and the ROC analysis was re-conducted. The combination of the incidence of ssDNA breaks and GSTM3 levels in sperm increased the prediction potential, showing an AUC of 0.92, with a sensitivity of 56% and a specificity of 94%. These promising results encourage the utilisation of ssDNA breaks and sperm GSTM3 levels as reliable sperm quality biomarkers, even though further validation with larger sample sizes should be performed.

In conclusion, the present work evidenced that the content of GSTM3 in human sperm is associated with their quality and oxidative DNA damage, and it could diagnose spermiogram alterations (Figure 6). Data presented herein paves the way towards the use of GSTM3 as a molecular marker to predict sperm quality

and DNA integrity in humans, being a more robust, simpler, and faster alternative to conventional semen analysis. These results could be of great interest for the field of assisted reproduction.

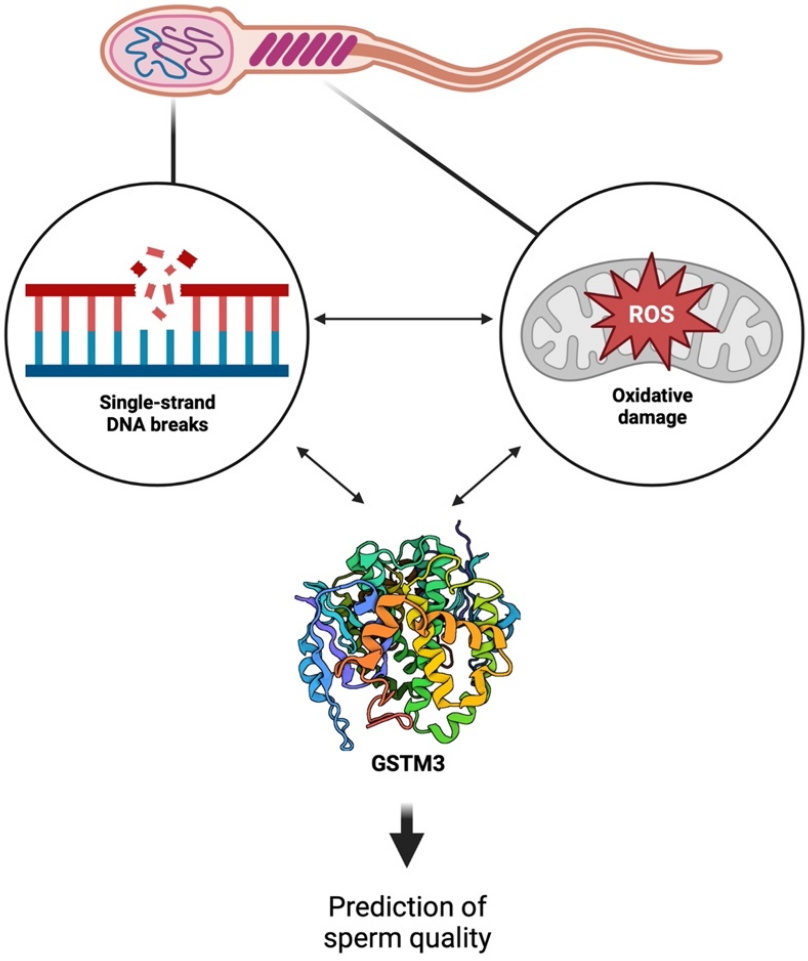

Figure 6. Sperm GSTM3 is associated with oxidative damage and single-strand DNA breaks, and it has been confirmed as a biomarker to predict sperm quality.

5. Ethics approval and consent to participate

All donors signed the informed consent, and the study was approved by the Ethics Committee of Corporació Sanitaria Parc Taulí, Spain (ref.: 2008532).

6. Consent for publication

Not applicable

7. Availability of data and materials

Due to privacy and ethical concerns, data generated herein cannot be made openly available. The dataset used during the current study is available from the corresponding author on reasonable request.

8. Competing interests

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

9. Funding

This study was supported by the Ministry of Science and Innovation, Spain (PID2020-113320RB-I00), the Ministry of Universities, Spain (Grant: FPU18/00666), the European Union's Horizon 2020 Research and Innovation scheme under the Marie Skłodowska-Curie grant agreement Nº 801342 (Tecniospring INDUSTRY; TECSPR-19-1-0003), the Catalan Agency for Management of University and Research Grants, Regional Government of Catalonia, Spain (Grant: 2017-SGR-1229 and 2020-FI-B-00412), and the Catalan Institution for Research and Advanced Studies (ICREA).

10. Authors' roles

ML, JR-M and MY conceived the study. JR-M and JB acquired the samples. ML and JR-M and YM-O conducted laboratory analysis. ML, JR-M, SB and MY participated in the critical discussion of the results. ML wrote the Manuscript. JR-M and MY revised and edited the final version of the Manuscript. All authors contributed to the finalised Manuscript, read, and approved the final version.

11. Acknowledgments

The authors would like to thank Technical Research Facility, University of Girona for their technical support. Graphical abstract was created by BioRender.com.

12. References

1. Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges J, Freundl G. Definition and prevalence of subfertility and infertility. *Human Reproduction*. 2005;20(5):1144–1147.

2. Vander Borght M, Wyns C. Fertility and infertility: Definition and epidemiology. *Clin Biochem*. 2018 Dec;62:2–10.

3. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology*. 2015 Dec 26;13(1):37.

4. Agarwal A, Baskaran S, Parekh N, Cho CL, Henkel R, Vij S, et al. Male infertility. *The Lancet*. 2021 Jan;397(10271):319–33.

5. Nelson CMK, Bunge RG. Semen Analysis: Evidence for Changing Parameters of Male Fertility Potential. *Fertil Steril*. 1974 Jun;25(6):503–7.

6. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *BMJ*. 1992 Sep 12;305(6854):609–13.

7. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect*. 2000 Oct;108(10):961–6.

8. Mishra P, Negi MPS, Srivastava M, Singh K, Rajender S. Decline in seminal quality in Indian men over the last 37 years. *Reproductive Biology and Endocrinology*. 2018 Dec 23;16(1):103.

9. Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. *Hum Reprod Update*. 2017 Nov 1;23(6):646–59.

10. Oehninger S, Ombelet W. Limits of current male fertility testing. *Fertil Steril*. 2019 May;111(5):835–41.

11. Tomlinson MJ, Naeem A. CASA in the medical laboratory: CASA in diagnostic andrology and assisted conception. *Reprod Fertil Dev*. 2018;30(6):850–9.

12. Altmäe S, Salumets A. A novel genomic diagnostic tool for sperm quality? *Reprod Biomed Online*. 2011 May;22(5):405–7.

13. Lewis SEM. Is sperm evaluation useful in predicting human fertility? *Reproduction*. 2007 Jul;134(1):31–40.

14. Kwon WS, Rahman MS, Pang MG. Diagnosis and prognosis of male infertility in mammal: the focusing of tyrosine phosphorylation and phosphotyrosine proteins. *J Proteome Res*. 2014 Nov;13(11):4505–17.

15. Llavanera M, Delgado-Bermúdez A, Ribas-Maynou J, Salas-Huetos A, Yeste M. A systematic review identifying fertility biomarkers in semen: a clinical approach through Omics to diagnose male infertility. *Fertil Steril*. 2022. 118(2), 291–313.

16. Ribas-Maynou J, Yeste M, Becerra-Tomás N, Aston KKI, James EER, Salas-Huetos A. Clinical implications of sperm DNA damage in IVF and ICSI: updated systematic review and meta-analysis. *Biological Reviews*. 2021 Aug;96(4):1284–300.

17. Llavanera M, Mateo Y, Bonet S, Barranco I, Fernández-Fuertes B, Yeste M. The triple role of glutathione S transferases in mammalian male fertility. *Cellular and Molecular Life Sciences*. 2019;77(1):1–12.

18. Klys HS, Whillis D, Howard G, Harrison DJ. Glutathione S-transferase expression in the human testis and testicular germ cell neoplasia. *Br J Cancer*. 1992;66(3):589–93.

19. Gopalakrishnan B, Aravinda S, Pawshe CH, Totey SM, Nagpal S, Salunke DM. Studies on glutathione S-transferase important for sperm function: evidence of catalytic activity-independent functions. *Biochemical Journal*. 1998;329:231–41.

20. Hemachand T, Gopalakrishnan B, Salunke DM, Totey SM, Shaha C. Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. *J Cell Sci*. 2002;115(10):2053–65.

21. Fulcher KD, Welch JE, Klapper DG, O'Brien DA, Eddy EM. Identification of a unique μ-class glutathione S-transferase in mouse spermatogenic cells. *Mol Reprod Dev*. 1995;42(4):415–24.

22. Li JY, Liu FJ, Wang HY, Liu X, Liu J, Li N, et al. Systematic mapping and functional analysis of a family of human epididymal secretory sperm-located proteins. *Molecular and Cellular Proteomics*. 2010;9(11):2517–28.

23. Hamilton LE, Acteau G, Xu W, Sutovsky P, Oko R. The developmental origin and compartmentalization of glutathione-s-transferase omega 2 isoforms in the perinuclear theca of eutherian spermatozoa†. *Biol Reprod*. 2017 Oct;97(4):612–21.

24. Protopapas N, Hamilton LE, Warkentin R, Xu W, Sutovsky P, Oko R. The perforatorium and postacrosomal sheath of rat spermatozoa share common developmental origins and protein constituents†. *Biol Reprod*. 2019 Jun;100(6):1461–72.

25. Aydos SE, Ph D, Taspinar M, Sc M, Sunguroglu A, Ph D. Association of CYP1A1 and glutathione S-transferase polymorphisms with male factor infertility. *Fertil Steril*. 2009;92(2):541–7.

26. Vani GT, Mukesh N, Siva Prasad B, Rama Devi P, Hema Prasad M, Rani PU, et al. Role of glutathione S-transferase Mu-1 (GSTM1) polymorphism in oligospermic infertile males. *Andrologia*. 2010;42(4):213–7.

27. Kolesnikova LI, Kurashova NA, Bairova TA, Dolgikh MI, Ershova OA, Dashiev BG, et al. Role of Glutathione-S-Transferase Family Genes in Male Infertility. *Bull Exp Biol Med*. 2017;163(5):643–5.

28. Safarinejad MR, Shafiei N, Safarinejad S. The association of glutathione-S-transferase gene polymorphisms (GSTM1, GSTT1, GSTP1) with idiopathic male infertility. *J Hum Genet*. 2010;55(9):565–70.

29. Lakpour N, Mirfeizollahi A, Farivar S, Akhondi MM, Hashemi SB, Amirjannati N, et al. The association of seminal plasma antioxidant levels and sperm chromatin status with genetic variants of GSTM1 and GSTP1 (Ile105Val and Ala114Val) in infertile men with oligoasthenoteratozoospermia. *Dis Markers*. 2013;34(3):205–10.

30. Tang M, Wang S, Wang W, Cao Q, Qin C, Liu B, et al. The glutathione-S-transferase gene polymorphisms (GSTM1 and GSTT1) and idiopathic male infertility risk: A meta-analysis. *Gene*. 2012;511(2):218–23.
31. Kan HP, Wu FL, Guo W bin, Wang YZ, Li JP, Huang YQ, et al. Null genotypes of GSTM1 and GSTT1 contribute to male factor infertility risk: A meta-analysis. *Fertil Steril*. 2013;99(3):690–6.
32. Song X, Zhao Y, Cai Q, Zhang Y, Niu Y. Association of the Glutathione S-transferases M1 and T1 polymorphism with male infertility: A meta-Analysis. *J Assist Reprod Genet*. 2013;30(1):131–41.
33. Aydemir B, Onaran I, Kiziler AR, Alici B, Akyolcu MC. Increased oxidative damage of sperm and seminal plasma in men with idiopathic infertility is higher in patients with glutathione S-transferase Mu-1 null genotype. *Asian J Androl*. 2007;9(1):108–15.
34. Rubes J, Selevan SG, Sram RJ, Evenson DP, Perreault SD. GSTM1 genotype influences the susceptibility of men to sperm DNA damage associated with exposure to air pollution. *Mutat Res*. 2007;625(1–2):20–8.
35. Hemachand T, Shaha C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett*. 2003;538(1–3):14–8.
36. Llavanera M, Delgado-Bermúdez A, Olives S, Mateo-Otero Y, Recuero S, Bonet S, et al. Glutathione S-transferases play a crucial role in mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm. *Antioxidants*. 2020;9(2).
37. Kwon WS, Oh SA, Kim YJ, Rahman MS, Park YJ, Pang MG. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci Rep*. 2015;5(13821):1–10.
38. Llavanera M, Delgado-Bermúdez A, Fernandez-Fuertes B, Recuero S, Mateo Y, Bonet S, et al. GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. *J Anim Sci Biotechnol*. 2019;10(61):1–11.
39. Organization WH. WHO laboratory manual for the examination and processing of human semen. 5th ed. World Health Organization; 2010: WHO labora.
40. Ribas-Maynou J, Fernández-Encinas A, García-Peiró A, Prada E, Abad C, Amengual MJ, et al. Human semen cryopreservation: A sperm DNA fragmentation study with alkaline and neutral Comet assay. *Andrology*. 2014;2(1):83–7.
41. Casanovas A, Ribas-Maynou J, Lara-Cerrillo S, Jimenez-Macedo AR, Hortal O, Benet J, et al. Double-stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates. *Fertil Steril*. 2019 Apr;111(4):699-707.e1.
42. Langie SAS, Azqueta A, Collins AR. The comet assay: past, present, and future. *Front Genet*. 2015 Aug 13;6:266.
43. Simon L, Aston KI, Emery BR, Hotaling J, Carrell DT. Sperm DNA damage output parameters measured by the alkaline Comet assay and their importance. *Andrologia*. 2017 Mar 1;49(2):e12608.
44. Ribas-Maynou J, García-Peiró A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod*. 2012 Mar;27(3):652–8.

45. Garriga F, Llavanera M, Viñolas-Vergés E, Recuero S, Tamargo C, Delgado-Bermúdez A, et al. Glutathione S-transferase Mu 3 is associated to in vivo fertility, but not sperm quality, in bovine. *animal*. 2022 Sep;16(9):100609.

46. Patil PS, Humbarwadi RS, Gune AR, Patil AD. Immature germ cells in semen - correlation with total sperm count and sperm motility. *J Cytol*. 2013;30(3):185.

47. Listowsky I, Rowe JD, Patskovsky Y v, Tchaikovskaya T, Shintani N, Novikova E, et al. Human testicular glutathione S-transferases: insights into tissue-specific expression of the diverse subunit classes. *Chem Biol Interact*. 1998 Apr;111–112:103–12.

48. Suryawanshi AR, Khan SA, Gajbhiye RK, Gurav MY, Khole V v. Differential proteomics leads to identification of domain-specific epididymal sperm proteins. *J Androl*. 2011;32(3):240–59.

49. Intasqui P, Camargo M, Antoniassi MP, Cedenho AP, Carvalho VM, Cardozo KHM, et al. Association between the seminal plasma proteome and sperm functional traits. *Fertil Steril*. 2015;105(3):617–28.

50. Mateo-Otero Y, Llavanera M, Recuero S, Delgado-Bermúdez A, Barranco I, Ribas-Maynou J, et al. Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs. *Biol Res*, 55(1), 15.

51. Ribas-Maynou J, Garca-Peir A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Human Reproduction*. 2012;27(3):652–8.

52. van Kooij RJ, de Boer P, de Vreeden-Elbertse JMT, Ganga NA, Singh N, te Velde ER. The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. *Int J Androl*. 2004 Jun;27(3):140–6.

53. Simon L, Carrell DT. Sperm DNA Damage Measured by Comet Assay. *Methods in Molecular Biology*. 2013;927:137–46.

54. Agarwal A, Prabakaran SA. Mechanism, Measurement, and Prevention of Oxidative Stress in Male Reproductive Physiology. *Indian J Exp Biol*. 2005;43(11):963–74.

55. Ribas-Maynou J, Benet J. Single and double strand sperm DNA damage: Different reproductive effects on male fertility. *Genes (Basel)*. 2019;10(2).

56. Agarwal A, Sharma R, Durairajanayagam D, Ayaz A, Cui Z, Willard B, et al. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reproductive Biology and Endocrinology*. 2015;13(1):1–22.

57. Botta T, Blescia S, Martínez-Heredia J, Lafuente R, Brassesco M, Ballescà JL, et al. Identificación de diferencias proteómicas en muestras oligozoospérmicas. *Rev Int Androl*. 2009;7(1):14–9.

58. Behrouzi B, Kenigsberg S, Alladin N, Swanson S, Zicherman J, Hong SH, et al. Evaluation of potential protein biomarkers in patients with high sperm DNA damage. *Syst Biol Reprod Med*. 2013 Jun 2;59(3):153–63.

59. Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJJ, Prada E, et al. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*, 2013, 1(5), 715–722.

PAPER VII

Sperm Glutathione S-transferase Mu 3 is associated with sperm DNA integrity and in vitro fertilisation in pigs

Marc Llavanera, Yentel Mateo-Otero, Sandra Recuero, Ariadna Delgado-Bermúdez, Sergi Bonet, Jordi Ribas-Maynou, Marc Yeste

Andrology

(Under review)

Sperm Glutathione S-transferase Mu 3 is associated with sperm DNA integrity and in vitro fertilisation in pigs

Marc Llavanera[1,2,‡], Yentel Mateo-Otero[1,2,‡], Sandra Recuero[1,2], Ariadna Delgado-Bermúdez[1,2], Sergi Bonet[1,2], Jordi Ribas-Maynou[1,2,*], Marc Yeste[1,2,3,*]

[1] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain

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

[3] Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain.

[‡] Joined first authors.

[*] Corresponding authors: jordi.ribasmaynou@udg.edu; marc.yeste@udg.edu.

Abstract

Background: The growing interest in exploring new molecular biomarkers providing additional functional information about sperm physiology aims to overcome the limitations of conventional semen analysis to predict male fertility. Recently, great efforts have been made towards the identification and characterisation of sperm proteins, as they are crucial for sperm physiology in both humans and farm animals. In this regard, protein content in sperm may be used as a non-invasive diagnostic and prognostic tool, thus complementing the conventional spermiogram. Objectives: As Glutathione S-transferases (GSTs) have been reported to regulate mammalian sperm physiology, this study aimed to determine whether the aforementioned protein could also predict sperm DNA integrity and fertilising ability. Material and Methods: With this purpose, and using the pig as an animal model, cell biology and immunological approaches were combined with *in vitro* fertilisation procedures. Results and Discussion: Two specific GSTM3 bands of ~26 and ~28 kDa were identified and quantified in immunoblotting analysis. A strong association was found between the ~26 kDa/Total GSTM3 ratio and single-strand, but not double-strand, DNA damage, evidencing its antioxidant role in the prevention of oxidative DNA damage. Moreover, the ~26 kDa/Total GSTM3 ratio in sperm conditioned oocyte in vitro fertilisation, but not embryo development. Conclusion: In conclusion, GSTM3 is

proposed as a biomarker to predict the quality, DNA integrity and *in vitro* fertilising ability of mammalian sperm.

1. Introduction

The prediction of male fertility potential has become increasingly important for both livestock and human reproduction (1–3). Particularly in humans, where infertility affects between 8% and 12 % of couples worldwide, the male factor is involved in 50 % of cases (3). Despite this rising reproductive health concern, there is still a lack of biomarkers able to predict male fertility with high accuracy and sensitivity that enable clinicians to undertake better reproductive approaches. Traditionally, the prognosis of male fertility has been performed through conventional semen analysis, providing a general information on quantitative parameters such as the ejaculate volume, sperm morphology, concentration and motility. Although the spermiogram is a simple, fast, and cheap evaluation, it does not provide functional information of sperm physiology, leaving essential molecular aspects such as DNA integrity, sperm oxidative status and sperm-oocyte binding proteins, aside (4,5). For this reason, the use of conventional semen analysis for the prognosis and diagnosis of male fertility has been under debate for many years (6,7). In this context, exploring new molecular biomarkers in sperm providing additional functional information on their physiological status is of great interest not only for assisted human reproduction but also for the animal breeding industry.

To overcome the limitations of conventional semen analysis, further molecular methods are required to precisely evaluate male fertility. Genomic, metabolomic and proteomic biomarkers hold great promise in the diagnosis of male infertility (8). Specifically, previous efforts have been made towards the identification and characterisation of specific sperm proteins, as they are known to play a vital role for mammalian sperm physiology (9). In effect, comparative proteomic studies have identified a wide range of proteins as potential biomarkers of sperm quality and fertility in both humans (10,11) and farm animals (12,13). Hence, the levels of certain proteins in sperm may be used as a non-invasive diagnostic and prognostic tool, complementing conventional semen analysis.

Related with this, recent studies highlighted the importance of glutathione S-transferases (GSTs) in sperm physiology.

Sperm GSTs are a large group of membrane-bound, multi-functional isoenzymes involved in cellular protection against oxidative stress, regulation of cell signalling and fertilisation (14). To the best of our knowledge, however, only GST Mu 3 (GSTM3; (15,16), GST Pi 1 (GSTP1; (16,17) and GST Omega 1 and 2 (GSTO1 and GSTO2; (18–20) have been identified in mammalian sperm. Specifically, previous studies reported that sperm GSTM3 is essential for proper mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm (21,22). Accordingly, a comparative proteomic study by Kwon et al. (23) revealed that, in pigs, relative GSTM3 content in sperm is higher in ejaculates giving birth to small than in those giving birth to large litters. Furthermore, sperm GSTM3 is known to be related to sperm quality (22,24–26) and cryotolerance (15) of mammalian sperm. All these evidences suggest that this enzyme could be used as a biomarker for prognosis and diagnosis of male fertility. To the best of the authors' knowledge, nevertheless, the direct association between sperm GSTM3 content and sperm fertilising potential is yet to be elucidated.

Against this background, cell biology and immunological approaches were performed to evaluate the relationship of sperm GSTM3 with sperm quality, DNA integrity and *in vitro* fertility, using an animal model (27), as it is of great interest for both assisted human reproduction and the animal breeding industry.

2. Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). All fluorochromes (SYBR-14, propidium iodide [PI], PNA-Fluorescein isothiocyanate [PNA] and Fluo-3) were purchased from Thermo Fisher Scientific (MA, USA).

2.1. Animals and processing of samples

Twelve pig ejaculates (biological replicates; n=12) from sexually mature Piétrain boars (1–3 years-old) were purchased from an authorised AI-centre (Grup Gepork

S.L., Masies de Roda, Spain), which operates under commercial, standard conditions. The authors did not manipulate any animal, and the AI-centre complied with the ISO certification (ISO-9001:2008) while producing the seminal doses. Ejaculates were diluted to 33 × 10^6 sperm per mL in a commercial extender (Androstar® Plus, Minitüb Ibérica, S.L.; Tarragona, Spain), and preserved at 17 °C during their transport to the laboratory within 4 h post-collection. Upon arrival, sperm motility, viability, acrosome membrane integrity, intracellular calcium levels and DNA integrity were assessed before conducting in vitro fertilisation (IVF). Twelve mL of each sperm sample was centrifuged twice at 3,000 g and 17 °C for 5 min and the resulting sperm pellet was stored at -80 °C prior to performing immunoblotting analysis.

Prepubertal gilt ovaries were obtained from a local abattoir (Frigoríficos Costa Brava, S.A., Riudellots de la Selva, Spain). Ovaries were preserved at 38 °C in physiological saline solution supplemented with 70 µg/mL kanamycin during their transport to the laboratory within 2 h post-collection. Upon arrival, cumulus-oocyte complexes (COCs) were retrieved from follicles, washed in Dulbecco's PBS (Gibco, ThermoFisher), and subjected to *in vitro* maturation (IVM) and IVF procedures, as described below.

2.2. *In vitro* maturation (IVM), fertilisation (IVF) and embryo culture

Groups of 40 COCs per well were in vitro matured for 22 h in a four-well multidish (Nunc, Roskilde, Denmark) containing 500 µL of maturation medium (TCM-199 [Gibco] supplemented with 0.57 mM cysteine, 0.1% (w:v) PVA, 10 ng/mL EGF, 75 µg/mL penicillin-G potassium, 50 µg/mL streptomycin sulphate, 10 IU/mL equine chorionic gonadotropin [eCG; Folligon; Intervet International B.V.; Boxmeer, The Netherlands] and 10 IU/mL human chorionic gonadotropin [hCG; Veterin Corion; Divasa Farmavic S.A.; Gurb, Barcelona, Spain]). Further, COCs were incubated for 20-22 h in hormone-free maturation medium. Maturation was performed at 38.5 °C in 5% CO_2 in air and 95% relative humidity.

In vitro matured oocytes were decumulated with 0.05% hyaluronidase in Dulbecco's PBS (Gibco, ThermoFisher) by mechanical pipetting. Then, groups of 20 oocytes were placed into 50-µL drops of fertilisation medium (modified from

[26]) supplemented with 1 mM caffeine and incubated at the same conditions. Next, 8 × 10^5 sperm were resuspended in 1 mL of fertilisation medium, and 50 µL of the resulting suspension was added to each group of 20 oocytes. Finally, oocytes and sperm were co-incubated for 5 h at the same incubation conditions. Presumptive zygotes were washed in embryo culture medium and transferred into a four-well dish, each well containing 500 µL of glucose-free embryo culture medium (NCSU23 medium (29), supplemented with 0.4% BSA, 0.3 mM sodium pyruvate and 4.5 mM sodium lactate). Sperm fertilising ability was calculated as the percentage of cleaved embryos at day 2 (IVF rates) and day 6 (embryo development) post-fertilisation.

2.3. Sperm morphology assessment

Sperm samples were diluted in 0.9 % (w:v) NaCl and 0.03 % formaldehyde. Sperm were subsequently evaluated at 200× magnification under a phase contrast microscope coupled with a SCA® Production software (Sperm Class Analyser Production, 2010; Microptic S.L., Barcelona, Spain). Two-hundred sperm were evaluated for each sample and classified into the following categories: abnormal head size and shape, acrosome abnormalities, folded and coiled tails, proximal and distal droplets, and isolated heads. The percentage of morphologically normal sperm was used to assess sperm morphology. Two technical replicates were counted.

2.4. Sperm motility analysis

Sperm motility was assessed using a computer-assisted sperm analysis (CASA) system. Sperm samples were incubated at 38 °C for 10 min and then loaded into pre-warmed 20-µm Leja chamber slides (Leja Products BV; Nieuw-Vennep, The Netherlands). Motility parameters were acquired using a negative phase-contrast field (Olympus BX41 with 10× 0.30 PLAN objective; Olympus, Tokyo, Japan) coupled to the ISAS software (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). Two technical replicates with at least 500 sperm per replicate were analysed. An average path velocity (VAP) higher than 10 µm/s was set as a cut-off for considering a sperm cell as motile, whereas an index of straightness

(STR) higher than 45 % was established to classify a sperm cell as progressively motile. The percentages of total and progressively motile sperm were used to evaluate sperm motility.

2.5. Flow cytometry assessment

Sperm viability, acrosome integrity and intracellular Ca^{2+} levels were determined by flow cytometry. Samples were diluted in pre-warmed PBS to a final concentration of 2×10^6 sperm per mL before staining with the corresponding protocol. Flow cytometric analysis was conducted using a CytoFLEX cytometer (Beckman Coulter; Fullerton, CA, USA). Laser voltage and flow rate were constant along the experiment. Forward scatter (FSC) and side scatter (SSC) were used to gate the sperm population. Each sample was evaluated on the basis of 10,000 sperm events. Fluorescence from SYBR-14, PNA-FITC and Fluo-3 was detected by the FITC channel (525/40), whereas PI fluorescence was collected through the PC5.5 channel (690/50).

Sperm viability was analysed with the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA). Samples were stained with SYBR-14 (final concentration: 32 nM) and PI (final concentration: 7.5 μM) at 38 °C in the dark for 15 min and subsequently analysed by flow cytometry. The percentage of viable sperm ($SYBR\text{-}14^+/PI^-$) was used to assess sperm viability. To determine acrosome membrane integrity, sperm samples were simultaneously incubated with PNA-FITC (final concentration: 1.2 μM) and PI (final concentration: 7.5 μM) at 38 °C in the dark for 15 min prior to analysis with the flow cytometer. The percentage of viable sperm with an intact acrosome (PNA^-/PI^-) was used as a measure of acrosome integrity. Sperm intracellular calcium was evaluated by incubating sperm samples with Fluo-3 (final concentration: 1.2 μM) and PI (final concentration: 7.5 μM) at 38 °C in the dark for 15 min, and subsequently analysed by flow cytometry. The percentage of viable sperm showing intracellular calcium ($Fluo3^+/PI^-$) was used to determine intracellular calcium levels. The percentage of particles within the double negative quadrant of every parameter described above was corrected using the debris particles found in the $SYBR\text{-}14^-/PI^-$ population, and the percentages of the other populations were recalculated (30).

2.6. Neutral and alkaline Comet assays

Neutral and alkaline Comet assays were performed to determine double- and single-strand DNA breaks, respectively. The Comet protocol for pig sperm was performed as described in Ribas-Maynou et al. (31). In short, sperm were diluted and mixed with low melting point agarose prior to be poured onto two agarose pre-treated slides (neutral and alkaline Comet) covered with a round coverslip. Following this, agarose jellified and coverslips were subsequently removed. All slides were sequentially incubated in lysis solutions. Thereafter, for neutral Comet, slides were electrophoresed in TBE buffer at 1 V/cm for 4 min and washed in 0.9% NaCl for 2 min. For alkaline Comet, slides were denatured in alkaline solution for 5 min, and electrophoresed in an alkaline buffer at 1 V/cm for 4 min. Then, all slides were incubated in neutralisation solution for 5 min, in 70%, 90% and 100% ethanol series for 2 min each, and dried in horizontal position. The composition of solutions and buffers were that described in Ribas-Maynou et al. (31). Finally, samples were stained with Safeview DNA (NBS biological, Huntingdon, UK). Imaging was conducted under an epifluorescence microscope (Zeiss Imager Z1, Carl Zeiss AG, Oberkochen, Germany). A minimum of 100 cells per sample were captured at 100×magnification with constant exposure time. Images were analyzed using the CometScore v2.0 software (Rexhoover, www.rexhoover.com) open-access software, and subsequently reviewed manually. Finally, the olive tail moment (OTM) of every sample was calculated as follows: (Tail mean intensity – Head mean intensity) ×Tail DNA/100. DNA integrity was reported as the OTM of ssDNA (alkaline) and dsDNA (neutral) breaks.

2.7. Immunoblotting analysis

Sperm pellet was resuspended in lysis buffer (xTractorTM buffer, Takara Bio, Kusatsu, Japan) following the manufacturer's instructions. In brief, samples were centrifuged at 10,000 g and 4 °C for 20 min. The total protein content in the supernatant was quantified through a detergent-compatible (DC) method (Bio-Rad, Hercules, CA, US), and samples were stored at -80 °C until immunoblotting analysis. Fifteen micrograms of total protein was diluted 1:1 (v:v) in Laemmli

reducing buffer 4× (Bio-Rad) and heated at 95 °C for 5 min prior to being loaded onto a 8-16% gradient polyacrylamide gel (Mini-PROTEAN TGX Stain-Free™ Precast Gels, Bio-Rad). Electrophoresis was run at 120 V for 1.5 h, and proteins from the gel were subsequently transferred onto polyvinylidene difluoride (PVDF) low fluorescence membranes using the Trans-Blot Turbo™ system (Bio-Rad). Total protein in membranes was determined using a trihalo compound present in the gels, which allows fluorescent detection of tryptophan residues, and a G:BOX Chemi XL system (Syngene, Frederick, MD, United States). Membranes were subsequently blocked with 5% BSA at room temperature for 1 h, and then incubated with an anti-GSTM3 primary antibody (1:6,000; v:v) at 4 °C overnight. Further, membranes were rinsed thrice and incubated with a secondary antibody (1:12,000; v:v) at room temperature for 1 h. Next, membranes were rinsed five times and bands were visualised. Membranes were subsequently incubated with a chemiluminescent substrate (Immobilon™ Western Detection Reagents, Millipore, United States) following the manufacturer's instructions, and scanned with G:BOX Chemi XL 1.4 (Syngene, India). Three technical replicates per sample were evaluated. Total protein and sperm GSTM3-bands levels were quantified using Image Studio™ Lite v.3.1 (Licor). Sperm GSTM3 levels were normalised against their respective total protein content. To confirm the specificity of the anti-GSTM3 primary antibody, a peptide competition assay utilising 10-fold of the GSTM3 immunising peptide was performed.

2.8. Statistical analysis

Results were analysed with IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA) and plotted with GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, USA). Data were tested for normality (Shapiro-Wilk's test) and homogeneity of variances (Levene's test). High and low GSTM3 groups were established using the median value of the 26 kDa/Total GSTM3 ratio levels. Significant differences between GSTM3 groups were tested through a t-test for independent measures. Correlations between GSTM3 levels and sperm quality and in vitro fertility parameters were determined through Pearson correlation coefficient. Each

biological replicate was considered as a statistical case (n = 12), and the level of significance was set at $P \leq 0.05$.

3. Results

3.1. GSTM3 is present in pig sperm with a double-band pattern

Immunoblotting analysis of sperm GSTM3 (Figure 1) showed a triple-band pattern of ~26, ~28, and ~48 kDa. The peptide competition assay utilising the GSTM3 immunising peptide, however, indicated that the ~48 kDa band was unspecific. Considering that both ~26 and ~28 GSTM3 kDa-bands were found to be specific for GSTM3 in sperm samples, the signal intensity of both was quantified. Sperm GSTM3 content was therefore determined as the relative intensity of each band (26 kDa-band and 28 kDa-band), the sum of the two bands (Total GSTM3), and the ratio between the 26 kDa band and the sum of the two bands (26 kDa/Total GSTM3).

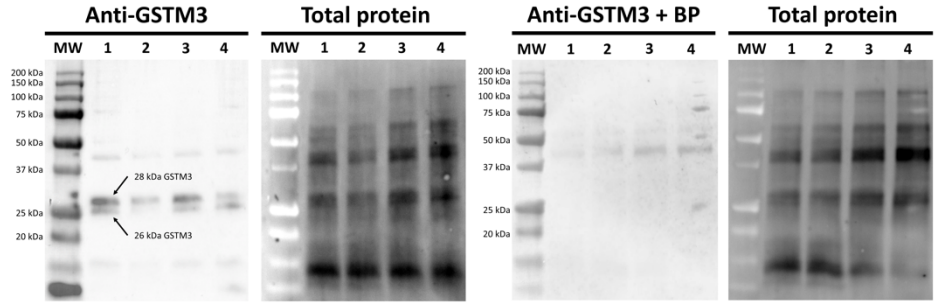

Figure 1. Representative blots resulting from the incubations with the Glutathione S-transferase Mu 3 (GSTM3) antibody (Anti-GSTM3) and the GSTM3 blocking peptide (Anti-GSTM3 + BP), and their corresponding loading controls (Total protein). Lanes: MW, molecular weight (kDa); Numbers 1 to 4 correspond to independent pig sperm samples.

3.2. Sperm 26 kDa/Total GSTM3, rather than individual 26 kDa and 28 kDa GSTM3 bands, are related to sperm quality

Pearson correlations between relative content of GSTM3 and sperm quality parameters are shown in Figure 2. Although the 26 kDa-band and Total GSTM3

did not show significant correlations with sperm quality variables ($P > 0.05$), the 28 kDa-band was positively correlated with the percentages of progressively motile sperm and viable sperm with an intact acrosome ($P < 0.05$). Interestingly, despite not significant, the 26 kDa and 28 kDa GSTM3 bands showed an inverse correlation pattern regarding sperm quality parameters. What is more, a very strong correlation was observed between sperm quality and 26 kDa/Total GSTM3 and 28 kDa/Total GSTM3 ratios; specifically, significant correlations with the percentages of total and progressively motile sperm, viable sperm, viable sperm with an intact acrosome and viable sperm with high intracellular Ca^{2+} levels were found ($P < 0.05$).

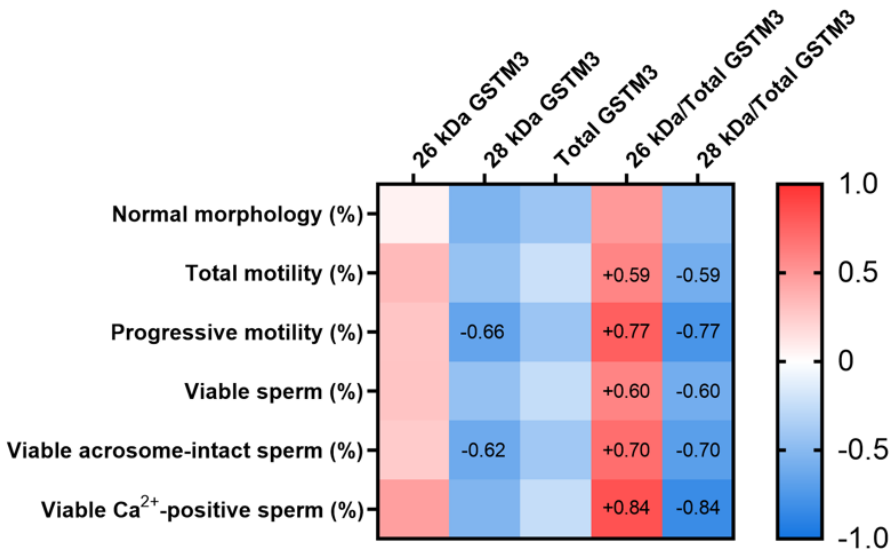

Figure 2. Heat map of Pearson correlation coefficients of Glutathione S-transferase Mu 3 (GSTM3) (~26 kDa band, ~28 kDa band, Total GSTM3 and the 26 and 28 kDa/Total GSTM3) ratio with sperm quality parameters (n = 12). Coefficients (R) of significant correlations ($P < 0.05$) are displayed within the heatmap.

3.3. Oxidative DNA damage is highly associated with sperm GSTM3

Considering the antioxidant nature of GSTM3 and the significant association of the 26 kDa/Total GSTM3 ratio with sperm quality, whether that ratio was correlated with sperm DNA damage was interrogated. As shown in Figure 3, the

26 kDa/Total GSTM3 ratio was negatively correlated with the incidence of ssDNA breaks (R = -0,86; $P < 0.01$), but not with that of the dsDNA ones (R = -0.09; $P > 0.05$). When comparing the olive tail moment (OTM) of alkaline and neutral Comet between samples showing high and low levels of the 26 kDa/Total GSTM3 ratio, the incidence of ssDNA breaks, but not that of the dsDNA ones, was significantly higher ($P < 0.05$) in the group showing low 26 kDa/Total GSTM3 ratios.

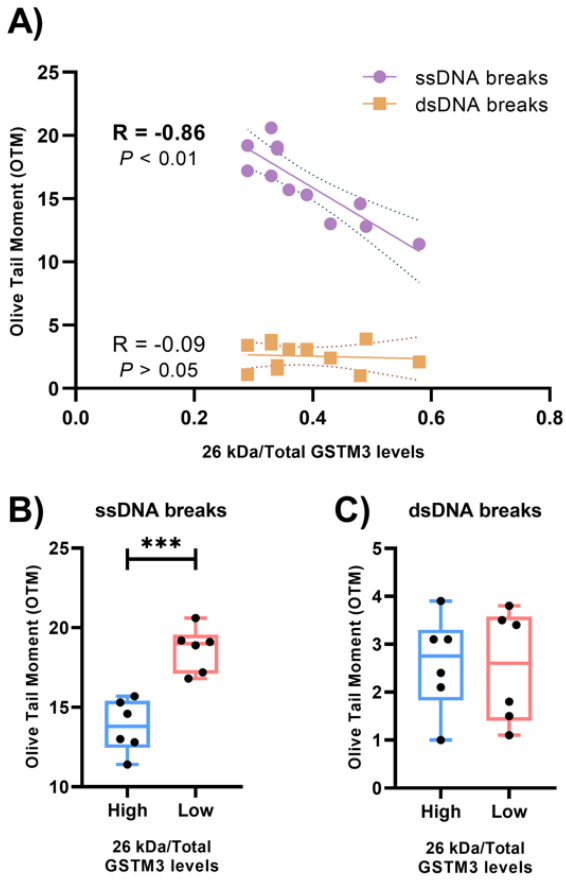

Figure 3. (**A**) Correlation dot plot between the ~26 kDa/Total GSTM3 ratio and (i) the olive tail moment (OTM) of single-strand DNA breaks (ssDNA breaks), and (ii) the OTM of double-strand DNA breaks (dsDNA breaks). Pearson correlation coefficients and P-values are represented in the plot. Box plots representing the distribution of the OTM of (**B**) ssDNA breaks and (**C**) dsDNA breaks between sperm samples exhibiting high and low levels of the 26 kDa/Total GSTM3 ratio (n = 12). *** $P < 0.001$.

3.4. GSTM3 is positively associated to higher sperm fertilising ability but not embryo development

The relationship of the 26 kDa/Total GSTM3 ratio with *in vitro* fertilisation and embryo development was also investigated (Figure 4). Remarkably, the 26 kDa/Total GSTM3 ratio was significantly and positively correlated with in vitro fertilisation rates (R = 0.73; $P < 0.05$), but not with embryo development ($P > 0.05$). Remarkably, while lower *in vitro* fertilisation rates were found in the group classified as having a low 26 kDa/Total GSTM3 ratio ($P < 0.05$), no significant differences in terms of embryo development were found between groups ($P > 0.05$).

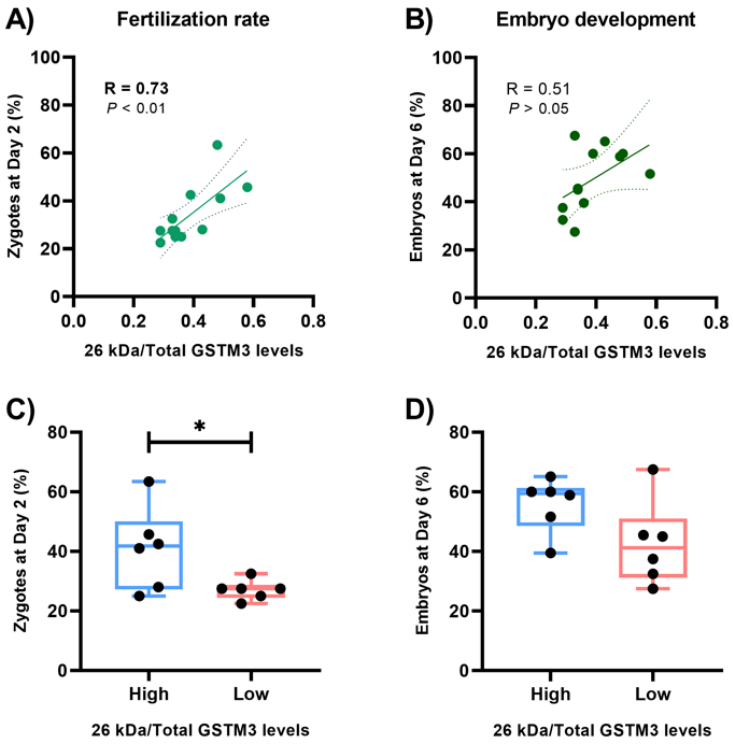

Figure 4. Correlation dot plot between the ~26 kDa/Total GSTM3 ratio and (**A**) the percentage of zygotes at day 2 post-fertilisation (Zygotes at Day 2), and (**B**) the percentage of embryos at day 6 post-fertilisation (Embryos at Day 6). Pearson correlation coefficients and P-values are represented in the plot. Box plots representing the distribution of (**C**) zygotes at day 2 and (**D**) embryos at day 6 between sperm samples exhibiting high and low levels of the 26 kDa/Total GSTM3 ratio (n = 12). * $P < 0.05$.

4. Discussion

The limitations of conventional semen analysis in predicting male fertility have led researchers to explore new molecular biomarkers with the aim to get a broader picture of the sperm physiological status (7,32,33). Recently, great efforts have been made towards the identification and characterisation of sperm proteins, as some are known to play a crucial role for sperm physiology in both humans and farm animals (9). In this regard, altered protein expression in sperm may be used as a non-invasive diagnostic and prognostic tool, extending the information provided by the conventional semen analysis. The GSTs, and notably GSTM3, have been reported to be essential for proper mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm (21,22). Moreover, although GSTM3 has been related to sperm quality (22,24,26,34) and cryotolerance (15), its association with DNA integrity and in vitro fertilisation has not yet been explored. This work, therefore, sought to determine whether this protein could also predict DNA integrity and fertilising ability of mammalian sperm.

While no transcript variants are known for GSTM3 (14), it has been reported to undergo post-translational modifications (PTM) (35). Accordingly, the immunoblotting analysis presented herein evidenced a GSTM3-specific double band pattern of ~26 and ~28 kDa. Indeed, the observed alteration in the GSTM3 molecular weight observed in this study is consistent with the expected shift caused by glycosylation. Actually, a previous study specifically identified a O-linked β-N-acetylglucosamine (O-GlcNAc) in GSTM3 (36). While O-GlcNAc has been associated with pathological conditions, such as type II diabetes and Alzheimer's disease (37,38), the specific function in cells is largely undefined. Moreover, glycosylation has been reported as an essential PTM for male fertility (39). Further research to uncover the molecular mechanism underlying the effect of PTMs, and specifically glycosylation, of GSTM3 in this cell type is thus warranted. On the other hand, and because of all the aforementioned, the association of sperm quality parameters with the unmodified GSTM3 (26 kDa GSTM3), the potentially post-translationally modified GSTM3 (28 kDa GSTM3), the total GSTM3 and the 26 and 28 kDa/Total GSTM3 ratios was explored.

The heat-mapped visualisation of the correlation coefficients between sperm GSTM3 parameters and quality variables displayed an interesting pattern: while the 26 kDa GSTM3 and the 26 kDa/Total GSTM3 ratio were positively correlated with sperm quality, the 28 kDa GSTM3, Total GSMT3 and the 28 kDa/Total GSTM3 ratio exhibited a negative correlation. In relation to these results, it is worth mentioning that previous research reported a negative association between the 28 kDa-GSTM3 and sperm quality of pig sperm (22), which is consistent with the results found herein.

When comparing GSTM3 levels with sperm physiological parameters, a lack of relationship was observed with the relative levels of 26 kDa, 28 kDa and the sum of the two band (total GSTM3). Interestingly, however, a very strong and positive association was found between the 26 kDa/Total GSTM3 ratio and total and progressive motility, viability, acrosome integrity and intracellular Ca^{2+} levels. The positive association of the 26 kDa/Total GSTM3 ratio with acrosome integrity suggests the involvement of this antioxidant enzyme in the maintenance of the acrosomal membrane. In agreement with the results found in this work, previous investigations in goat sperm reported the importance of sperm GSTM3 for the preservation of acrosome and plasma membrane intactness, as GSTs are known to be mainly located in sperm plasmalemma and the lack of GST activity leads to membrane damage (21,40). Similarly, the present work found lower 26 kDa/Total GSTM3 ratios in ejaculates having greater percentages of viable sperm with high intracellular Ca^{2+} levels. In this context, it is worth highlighting that a role of GSTs in sperm Ca^{2+} homeostasis was previously purported, since it was found to be dysregulated in GSTs-inhibited pig sperm (22). Taken together, these results indicate that the proportion between 26 and 28 kDa GSTM3, rather than the absolute GSTM3 content in sperm, is involved in the maintenance of sperm homeostasis. One could thus surmise that an increased proportion of PTM-GSTM3 would be a useful indicative of a reduced sperm quality.

Considering the relevance of PTMs in GSTM3 upon sperm quality, interrogating on its association with sperm DNA integrity is of much interest. The Comet assay is a widely used test for the assessment of sperm DNA integrity. Among its benefits, the Comet assay allows the differentiation between ssDNA

and dsDNA breaks (41,42). The data reported in the present study evidenced a clear negative association between the 26 kDa/Total GSTM3 ratio and ssSDF, but not dsSDF, breaks. ssDNA breaks are known to be generated by oxidative damage, mainly because of the presence of endogenous and/or exogenous reactive oxygen species (43–45). Thus, the absence of PTMs in GSTM3 could be associated to decreased oxidative DNA damage. The association of GSTM3 with sperm DNA integrity evidences the involvement of this antioxidant enzyme in the maintenance of redox balance, which would prevent oxidative DNA damage. In this regard, it is worth mentioning that GSTM3 has been reported to be involved in sperm detoxification of electrophilic compounds (14), prevention of lipid peroxidation (21), and maintenance of mitochondrial function, and oxidative homeostasis in sperm (22).

Finally, the relationship between PTMs of GSTM3 and *in vitro* fertility was also explored in the present study. A previous comparative proteomics study performed by Kwon et al. (23) identified GSTM3 as an *in vivo* fertility marker in pigs. In the present study, the 26 kDa/Total GSTM3 ratio was significantly correlated with *in vitro* fertilisation rates, but not embryo development. While these results point to an essential role of GSTM3 in *in vitro* sperm fertilising capacity, they do not support an involvement of this sperm protein in embryo development. Specifically, increased 26 kDa/Total GSTM3 ratios were found in samples with higher *in vitro* fertilisation rates, thus suggesting a negative effect of post-translational modifications of GSTM3. Related to this, previous studies in human sperm evidenced the binding capacity of sperm GSTM3 to zona pellucida (ZP) (46). In fact, GSTM3 has been proposed as an important molecule during the first steps of gamete recognition to allow fertilisation to occur (14). The present study suggests the importance of PTMs for the binding ability of sperm GSTM3 to ZP and the subsequent fertilising ability, although further studies are required to confirm this hypothesis.

In conclusion, the physiological role of sperm GSTM3 and the effects of its potential PTM were investigated in the present study. A strong association between the 26 kDa/Total GSTM3 ratio and sperm quality, DNA integrity and *in vitro* fertilisation was observed. The absence of PTMs in GSTM3, rather than the

total GSTM3 content, was found to be associated with better sperm quality, higher DNA integrity and increased ability to fertilise the oocyte, suggesting the relevance of PTMs in GSTM3 for the regulation of sperm physiology and fertilising ability. Accordingly, although further research is required to determine the specific PTMs present in sperm GSTM3, this study highlights its relevance in the maintenance of sperm homeostasis, DNA integrity, and subsequent fertilisation ability.

5. Ethics approval and consent to participate

Not applicable

6. Consent for publication

Not applicable

7. Availability of data and materials

Due to privacy and ethical concerns, data generated herein cannot be made openly available. The dataset used during the current study is available from the corresponding author on reasonable request.

8. Competing interests

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

9. Funding

The authors acknowledge the support from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 801342 (Tecniospring INDUSTRY; TECSPR-19-1-0003); the Ministry of Science, Innovation and Universities, Spain (AGL2017-88329-R and FPU18/00666); Regional Government of Catalonia, Spain (2017-SGR-1229 and 2020-FI-B-00412); and the Catalan Institution for Research and Advanced Studies (ICREA).

10. Authors' roles

ML and MY conceived the study. ML, YM-O, JR-M, SR and AD-B undertook laboratory analysis. ML, SB and MY participated in the discussion of the results. ML wrote the draft, which was later revised/edited by MY. YM-O, JR-M, SB and MY contributed to the finalised manuscript. All authors read and approved the final manuscript.

11. Acknowledgments

The authors would like to thank the slaughterhouse staff at Frigoríficos Costa Brava S.A for supplying ovaries.

12. References

1. Gadea J. Sperm factors related to in vitro and in vivo porcine fertility. *Theriogenology*. 2005 Jan;63(2):431–44.

2. Khatun A, Rahman MS, Pang MG. Clinical assessment of the male fertility. *Obstet Gynecol Sci*. 2018 Mar;61(2):179–91.

3. Agarwal A, Baskaran S, Parekh N, Cho CL, Henkel R, Vij S, et al. Male infertility. *The Lancet*. 2020;397(10271):319–33.

4. Lewis SEM. Is sperm evaluation useful in predicting human fertility? *Reproduction*. 2007 Jul;134(1):31–40.

5. Altmäe S, Salumets A. A novel genomic diagnostic tool for sperm quality? *Reprod Biomed Online*. 2011 May;22(5):405–7.

6. Kwon WS, Rahman MS, Pang MG. Diagnosis and prognosis of male infertility in mammal: the focusing of tyrosine phosphorylation and phosphotyrosine proteins. *J Proteome Res*. 2014 Nov;13(11):4505–17.

7. Lewis SEM. Is sperm evaluation useful in predicting human fertility? *Reproduction*. 2007 Jul;134(1):31–40.

8. Kovac JR, Pastuszak AW, Lamb DJ. The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertil Steril*. 2013 Mar;99(4):998–1007.

9. Kovac JR, Pastuszak AW, Lamb DJ. The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertil Steril*. 2013 Mar;99(4):998–1007.

10. Korbakis D, Schiza C, Brinc D, Soosaipillai A, Karakosta TD, Légaré C, et al. Preclinical evaluation of a TEX101 protein ELISA test for the differential diagnosis of male infertility. *BMC Med*. 2017;15(1):1–16.

11. Intasqui P, Agarwal A, Sharma R, Samanta L, Bertolla RP. Towards the identification of reliable sperm biomarkers for male infertility: A sperm proteomic approach. *Andrologia*. 2017;50(3):1–11.

12. Pérez-Patiño C, Parrilla I, Barranco I, Vergara-Barberán M, Simó-Alfonso EF, Herrero-Martínez JM, et al. New In-Depth Analytical Approach of the Porcine Seminal Plasma Proteome Reveals Potential Fertility Biomarkers. *J Proteome Res*. 2018;17(3):1065–76.

13. Muhammad Aslam MK, Sharma VK, Pandey S, Kumaresan A, Srinivasan A, Datta TK, et al. Identification of biomarker candidates for fertility in spermatozoa of crossbred bulls through comparative proteomics. *Theriogenology*. 2018 Oct;119:43–51.

14. Llavanera M, Mateo Y, Bonet S, Barranco I, Fernández-Fuertes B, Yeste M. The triple role of glutathione S transferases in mammalian male fertility. Cellular and Molecular Life Sciences. 2019;77(1):1–12.

15. Llavanera M, Delgado-Bermúdez A, Fernandez-Fuertes B, Recuero S, Mateo Y, Bonet S, et al. GSTM3, but not IZUMO1, is a cryotolerance marker of boar sperm. *J Anim Sci Biotechnol*. 2019;10(61):1–11.

16. Gopalakrishnan B, Aravinda S, Pawshe CH, Totey SM, Nagpal S, Salunke DM. Studies on glutathione S-transferase important for sperm function: evidence of catalytic activity-independent functions. *Biochemical Journal*. 1998;329:231–41.

17. Llavanera M, Mateo-Otero Y, Delgado-Bermúdez A, Recuero S, Olives S, Barranco I, et al. Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality. *Front Cell Dev Biol*. 2021;9(February):1–13.

18. Hamilton LE, Acteau G, Xu W, Sutovsky P, Oko R. The developmental origin and compartmentalization of glutathione-s-transferase omega 2 isoforms in the perinuclear theca of eutherian spermatozoa†. *Biol Reprod*. 2017 Oct 1;97(4):612–21.

19. Hamilton LE, Suzuki J, Aguila L, Meinsohn MC, Smith OE, Protopapas N, et al. Sperm-borne glutathione-S-transferase omega 2 accelerates the nuclear decondensation of spermatozoa during fertilization in mice†. *Biol Reprod*. 2019 Aug 1;101(2):368–76.

20. Protopapas N, Hamilton LE, Warkentin R, Xu W, Sutovsky P, Oko R. The perforatorium and postacrosomal sheath of rat spermatozoa share common developmental origins and protein constituents†. *Biol Reprod*. 2019 Jun 1;100(6):1461–72.

21. Hemachand T, Shaha C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett*. 2003;538(1–3):14–8.

22. Llavanera M, Delgado-Bermúdez A, Olives S, Mateo-Otero Y, Recuero S, Bonet S, et al. Glutathione S-transferases play a crucial role in mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm. *Antioxidants*. 2020;9(2).

23. Kwon WS, Oh SA, Kim YJ, Rahman MS, Park YJ, Pang MG. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci Rep*. 2015;5(13821):1–10.

24. Botta T, Blescia S, Martínez-Heredia J, Lafuente R, Brassesco M, Ballescà JL, et al. Identificación de diferencias proteómicas en muestras oligozoospérmicas. *Rev Int Androl*. 2009;7(1):14–9.

25. Agarwal A, Sharma R, Durairajanayagam D, Ayaz A, Cui Z, Willard B, et al. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reproductive Biology and Endocrinology*. 2015;13(1):1–22.

26. Intasqui P, Camargo M, Antoniassi MP, Cedenho AP, Carvalho VM, Cardozo KHM, et al. Association between the seminal plasma proteome and sperm functional traits. *Fertil Steril*. 2015;105(3):617–28.

27. Zigo M, Maňásková-Postlerová P, Zuidema D, Kerns K, Jonáková V, Tůmová L, et al. Porcine model for the study of sperm capacitation, fertilization and male fertility. Vol. 380, *Cell and Tissue Research*. Springer; 2020. p. 237–62.

28. Abeydeera LR, Day BN. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol Reprod*. 1997 Oct;57(4):729–34.

29. Peters JK, Milliken G, Davis DL. Development of porcine embryos in vitro: effects of culture medium and donor age. *J Anim Sci*. 2001 Jun 1;79(6):1578–83.

30. Petrunkina AM, Waberski D, Bollwein H, Sieme H. Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. *Theriogenology*. 2010 Apr 15;73(7):995–1000.

31. Ribas-Maynou J, Delgado-Bermúdez A, Garcia-Bonavila E, Pinart E, Yeste M, Bonet S. Complete Chromatin Decondensation of Pig Sperm Is Required to Analyze Sperm DNA Breaks With the Comet Assay. *Front Cell Dev Biol*. 2021 Jun 14;9.

32. Altmäe S, Salumets A. A novel genomic diagnostic tool for sperm quality? *Reprod Biomed Online*. 2011 May;22(5):405–7.

33. Kwon WS, Rahman MS, Pang MG. Diagnosis and prognosis of male infertility in mammal: the focusing of tyrosine phosphorylation and phosphotyrosine proteins. *J Proteome Res*. 2014 Nov;13(11):4505–17.

34. Agarwal A, Sharma R, Durairajanayagam D, Ayaz A, Cui Z, Willard B, et al. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reproductive Biology and Endocrinology*. 2015;13(1):1–22.

35. Hornbeck P v, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res*. 2015 Jan;43:D512-20.

36. Gurcel C, Vercoutter-Edouart AS, Fonbonne C, Mortuaire M, Salvador A, Michalski JC, et al. Identification of new O-GlcNAc modified proteins using a click-chemistry-based tagging. *Anal Bioanal Chem*. 2008 Apr 28;390(8):2089–97.

37. Robert J. Konrad, Jeffrey E. Kudlow. The role of O-linked protein glycosylation in β-cell dysfunction. *Int J Mol Med*. 2002;10(5):535–9.

38. Liu F, Iqbal K, Grundke-Iqbal I, Hart GW, Gong CX. O-GlcNAcylation regulates phosphorylation of tau: A mechanism involved in Alzheimer's disease. *Proceedings of the National Academy of Sciences*. 2004 Jul 20;101(29):10804–9.

39. Brohi RD, Huo LJ. Posttranslational Modifications in Spermatozoa and Effects on Male Fertility and Sperm Viability. *OMICS*. 2017 May;21(5):245–56.

40. Gopalakrishnan B, Shaha C. Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett*. 1998;422(3):296–300.

41. Ribas-Maynou J, Garca-Peir A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Human Reproduction*. 2012;27(3):652–8.
42. van Kooij RJ, de Boer P, de Vreeden-Elbertse JMT, Ganga NA, Singh N, te Velde ER. The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. *Int J Androl*. 2004 Jun;27(3):140–6.
43. Simon L, Carrell DT. Sperm DNA Damage Measured by Comet Assay. *Methods in Molecular Biology*. 2013;927:137–46.
44. Agarwal A, Prabakaran SA. Mechanism, Measurement, and Prevention of Oxidative Stress in Male Reproductive Physiology. Indian *J Exp Biol*. 2005;43(11):963–74.
45. Ribas-Maynou J, Benet J. Single and double strand sperm DNA damage: Different reproductive effects on male fertility. *Genes (Basel)*. 2019;10(2).
46. Petit FM, Serres C, Bourgeon F, Pineau C, Auer J. Identification of sperm head proteins involved in zona pellucida binding. *Human Reproduction*. 2013;28(4):852–65.

DISCUSSION

DISCUSSION

Male infertility accounts for half of unsuccessful pregnancies worldwide, with 20-30% of infertility cases purported to be caused by the male factor (Agarwal et al., 2015a). Exploring the aetiology and making an accurate diagnosis of male infertility is, therefore, much warranted in both humans and other animals. Despite the routine evaluation of semen with the seminogram, the multicausal nature of this condition limits its ability to predict reduced sperm fertility (Oehninger and Ombelet, 2019). For this reason, searching new molecular markers with high sensitivity and specificity to assess sperm quality and function, and predict fertilising ability is of great interest for both fertility clinics and the animal breeding industry. Moreover, while ROS at physiological levels are acknowledged to be essential for sperm function, excessive generation of these chemical species and/or insufficient antioxidant activity may head to disrupted redox homeostasis and have a detrimental effect upon sperm function (Thompson et al., 2013). In this scenario, and because of the reduced capacity of sperm to scavenge ROS, antioxidant enzymes are likely to play a major role. Glutathione S-transferases are among the ubiquitous antioxidant isoenzymes intended to protect mammalian cells from OS (Hayes et al., 2005). Specifically, GSTMs and GSTPs are suggested to be potentially critical for sperm physiology and male fertility (Gopalakrishnan and Shaha, 1998; Hemachand and Shaha, 2003; Kwon et al., 2015). In spite of their promising role in male reproduction, nevertheless, GSTs in sperm and SP are understudied in mammals. Accordingly, the present Dissertation aimed (1) to determine the presence of GSTs in male reproductive tissues, seminal plasma and sperm; (2) to uncover the role of this group of antioxidant enzymes in sperm physiology; and (3) to assess their putative use as molecular markers for mammalian sperm.

Characterisation of GSTs in reproductive tissues, seminal plasma and sperm

Despite data reported in the literature suggesting that GSTMs and GSTPs are associated to (in)fertility in mammals, the presence and localisation patterns of these antioxidant enzymes in reproductive tissues, SP and sperm has been

poorly studied in both humans and farm animals. For this reason, the present Doctoral Thesis addressed the presence of GSTM3 in reproductive tissues, SP and sperm of three mammalian species: pigs (**paper I, II and III**), cattle (**paper IV**) and humans (**paper VI**). Worthy of notice is that because of (i) the economic relevance of the swine industry in Spain and worldwide (FAO, 2022); (ii) the logistical ease as a consequence of the availability of pig semen; and (iii) the suitability of the porcine as a model for the study of sperm physiology and male (in)fertility (Zigo et al., 2020), the pig was selected as the main species to determine the presence of GSTM3 in male reproductive tissues, SP and sperm.

In the first approach to the subject, the contribution of the testis, epididymis, and accessory glands to the content of GSTM3 in sperm and SP was examined in porcine by immunoblotting. GSTM3 was found to be present as a double-band pattern of ~25 and ~75 kDa in the testis and caput epididymis, and a single band of ~75 kDa in the corpus and cauda epididymis, the prostate and seminal vesicles. In contrast, GSTM3 was absent from bulbourethral glands. Previous studies in pigs reported a single band of ~25 kDa in sperm samples (Kwon et al., 2015), which would correspond to its molecular mass and would agree with immunoblotting results of the testis reported herein. Related with this, it is worth mentioning that the testis is known to be the tissue with the highest levels of GSTM3 (Listowsky et al., 1998). Also matching with the results of this Dissertation, a study in humans established that GSTM3 is present at high concentrations in the corpus and cauda epididymis (Li et al., 2010). This could be linked to the incorporation and/or attachment of GSTMs to sperm during epididymal maturation (Suryawanshi et al., 2011), and would support a function for these proteins during the passage of sperm through the epididymis. In fact, sperm-attached GSTs have been reported to protect cells from OS during their storage within the epididymis (Dacheux et al., 2009). Regarding the prostate and seminal vesicles, no study previously investigated the presence of GSTM3 in these tissues. Because this Doctoral Thesis demonstrated, for the first time, that the prostate and seminal vesicles also express GSTM3, it is reasonable to hypothesise that this enzyme could contribute to the total antioxidant capacity of SP in pigs. In view of the above, accessory glands, except the bulbourethral ones,

and, particularly, testes and epididymes are likely to contribute to the GSTM3 present in sperm and SP. Based on the results of the present study, an inadequate synthesis of GSTM3 in the testis or the epididymis could lead to an inadequate spermatogenesis and/or epididymal maturation of sperm, which could be detected in the SP.

A wide range of antioxidant enzymes in SP has previously been identified in mammals (Kantola et al., 1988; Jeulin et al., 1989; Peeker, 1997; Raijmakers et al., 2003; Gong et al., 2012). Yet, and despite the importance of GSTs in maintaining cellular homeostasis (Armstrong et al., 2017), no information regarding their presence and abundance in SP has been reported in the literature. In **paper II**, the presence and abundance of GSTM3 in porcine SP was examined, its concentration ranging from 38.26 to 81.82 ng/mL (mean ± SEM: 61.62 ± 2.18 ng/mL). The concentration of GSTM3 in pig SP would, therefore, be higher than that of other antioxidant enzymes, such as glutathione peroxidase 5 (GPx5; 9.63-30.13 ng/mL; Barranco et al., 2016) and paraoxonase 1 (PON1; 0.96-1.67 ng/mL; I. Barranco et al., 2015). Moreover, and because of the previously revealed differences of semen quality between pig breeds (Smital et al., 2004), the concentration of GSTM3 in SP was compared between Duroc, Landrace and Pietrain breeds. No differences in terms of GSTM3 concentration in SP were found between breeds, which would suggest that this is a conserved feature within porcine species. In this regard, nonetheless, a previous study in pig sperm suggested that male-to-male variations could hinder breed differences (Waterhouse et al., 2006). All these findings support, in short, the relevance of GSTM3 in porcine SP, which is likely to confer a major antioxidant function to this fluid and ultimately protect sperm cells from oxidative damage.

Considering the nearly ubiquitous presence of GSTM3 in porcine reproductive tissues and SP, addressing its presence and localisation in sperm was also advised to be of great interest. GSTM3 was identified in pig sperm as a double-band pattern of ~25 kDa and ~28 kDa, which is in accordance with its predicted molecular mass in this species (Bateman et al., 2017). Regarding its localisation, GSTM3 was found along the entire length of the tail and the equatorial subdomain of the head in pig sperm. Interestingly, in other species,

previous investigations reported that GSTM3 is present in the apical region of the acrosome in goats (Gopalakrishnan et al., 1998; Hemachand et al., 2002) and over the sperm tail in buffaloes (Kumar et al., 2014). This species-specific localisation of GSTM3 suggests an adaptive response to the physiological requirements of each species. As a matter of fact, the subjection of sperm to preservation methods was seen to lead to the relocalisation of this antioxidant enzyme, pointing out to a potential adaptation of the cell during liquid-storage or cryopreservation. Remarkably, the localisation pattern of GSTs during liquid preservation of semen was established for the first time in the present Dissertation. Sperm GSTM3 was initially localised in the tail and the equatorial subdomain of the head. After 72 h of liquid-storage, however, it was found to partially disappear from the midpiece. Specifically, the loss of GSTM3 from the midpiece, rather than its relocalisation, appeared to occur during liquid storage, as immunoblotting analysis evidenced a decline in its abundance after preservation at 15º-20ºC for 72 h. Interestingly, alterations in the localisation pattern of this antioxidant enzyme were also observed to happen during cryopreservation of pig sperm. In effect, after freeze-thawing, sperm GSTM3 was present in the midpiece area only, being absent from the rest of the tail and the equatorial subdomain of the head. Yet, and in contrast to what observed during liquid preservation, similar amounts of this antioxidant enzyme were found before freezing and after thawing. Thus, a relocalisation rather than a loss of the protein was seen to take place in response to cryopreservation. These findings are in agreement with those previously reported in buffalos, where a similar relocalisation of GSTM3 during cryopreservation, with a migration of the enzyme from the entire sperm tail to the midpiece, occurs (Kumar et al., 2014). It is known that both liquid preservation (Waberski et al., 2011; Falchi et al., 2018) and cryopreservation (Yeste, 2016) may trigger the overproduction of ROS, thus unbalancing the redox homeostasis of sperm cells. This, together with the results reported herein and in line with that aforementioned, suggests that an adaptive response of sperm (i.e., relocalisation of GSTM3) to the different antioxidant requirements of the cell occurs during both liquid-storage and cryopreservation.

In **papers IV and VI**, the presence and localisation of GSTM3 in sperm were investigated in cattle and humans, respectively. Contrarily to pigs, where most AIs are carried out employing liquid-stored semen (Waberski et al., 2019), ART (AI/IUI, IVF and ICSI) in cattle (Ugur et al., 2019) and humans (di Santo et al., 2012) preferentially use cryopreserved sperm. For this reason, the identification of GSTM3 in the sperm of these two species was conducted in frozen-thawed cells. A single GSTM3-specific band of ~48 kDa was observed in cattle sperm, whereas a double band-pattern of ~48 kDa and ~75 kDa was identified in human sperm. These results differed from those found in pig sperm, which exhibited a double band-pattern of ~25 kDa and ~28 kDa. Furthermore, these findings also differed from those seen in immunoblots of pig reproductive tissues, which showed bands of ~25 kDa and ~75 kDa. Interestingly, a GSTM3 pattern of ~25 kDa, ~28 kDa, ~48 kDa and ~75 kDa bands was observed in the individual species in both reproductive tissues and sperm. According to UniProt (Bateman et al., 2017), the molecular mass of GSTM3 in porcine, bovine and human species is 26.93 kDa, 28.44 kDa and 26.56 kDa, respectively. The slight shift of the protein bands responsible for the double-band pattern of GSTM3 in pig sperm (~25 kDa and ~28 kDa) could be caused by post-translational modifications, such as glycosylation (Gurcel et al., 2008). Yet, the considerable difference between the expected and reported molecular mass of GSTM3 in reproductive tissues, and in bovine and human sperm (~25, ~48, and ~75 kDa), could more likely be a result of protein interactions. While GSTM3 is known to be ~ 26 kDa in its monomeric form, it has been described to be active and stable as a homo- or hetero-dimer (Mannervik and Jensson, 1982; Mannervik et al., 1988; di Pietro et al., 2010), which would explain the presence of the ~48 kDa band in the blots. The unexpected increase in the molecular mass of GSTM3 could, nevertheless, be a consequence of covalent interactions with other proteins. Although the presence of this antioxidant enzyme in pig, cattle and human sperm was confirmed in the present Dissertation, whether post-translational modifications, covalent protein-protein interactions and/or homodimerisation of GSTM3 take place in mammalian sperm remains unknown. Consequently, specific experiments confirming the occurrence of covalent protein-protein interactions and post-translational

modifications in the GSTM3 of sperm and reproductive tissues should be performed to gain further insights into the relevance of this protein for male fertility.

In spite of the fact that GSTM3 in sperm showed a different band pattern between mammalian species in the immunoblotting assay, immunofluorescence analysis revealed a similar localisation in cattle and humans, but not in pigs. In effect, while GSTM3 resided in the midpiece of frozen-thawed pig sperm, this enzyme was found in the tail and equatorial subdomain of the head in their cattle and human counterparts. This localisation pattern in cattle and human sperm resembled to that of fresh pig sperm. Again, these findings support an adaptive response of pig sperm to freeze-thawing procedures involving, among other changes, the relocalisation of GSTM3. Because, in this Dissertation, fresh and cryopreserved sperm were only compared in the case of pigs, specific experiments addressing the changes of localisation of GSTM3 during sperm cryopreservation in other mammalian species, such as bovine and human, are warranted.

Finally, and in addition to GSTM3, another GST class was characterised in pig sperm. Immunoblotting analysis confirmed that GSTP1 is present in pig sperm as a single ~48 kDa-band. Although the molecular mass of GSTP1 is ~25 kDa, similar to GSTM3, the presence of homo- and hetero-dimers or covalent protein-protein interactions could again account for the ~48 kDa-band observed in immunoblots (Okamura et al., 2015). Furthermore, while proteomic studies already identified GSTP1 in human (Wang et al., 2013), murine (Vicens et al., 2017), porcine (Pérez-Patino et al., 2019) and bovine (Peddinti et al., 2008) sperm, its particular localisation was determined for the first time in the present Dissertation. Immunofluorescence analysis found GSTP1 to reside in the posterior region of the head and the entire tail of fresh sperm. This localisation pattern of GSTP1 was similar to that found for other GSTs family members, such as GSTM3 in pig and buffalo (Kumar et al., 2014) sperm, which is present in the entire sperm tail. Interestingly, liquid-storage for 72 h was responsible for the alteration of GSTP1 localisation. After 72 h of liquid storage, sperm GSTP1 was observed to relocate to the equatorial subdomain of the head and the principal

and end pieces of the tail, being absent from the midpiece. A similar modification was observed in GSTM3, suggesting that the adaptive response of sperm to liquid-storage involves both GSTM3 and GSTP1.

To sum up, GSTs were characterised, in the present Doctoral Thesis, in three mammalian species. In porcine, the presence of GSTM3 was confirmed in reproductive tissues (testis, epididymis and accessory glands, except bulbourethral glands), SP and sperm. In pig sperm, GSTM3 was identified as a double band-pattern of ~25 kDa and ~28 kDa, likely caused by post-translational modifications in the latter case. Regarding its localisation pattern, GSTM3 was found along the entire length of the tail and the equatorial subdomain of the head. Relocalisation events or protein loss, notwithstanding, took place during sperm preservation. Specifically, not only was GSTM3 lost from the midpiece after 72 h of liquid storage, but the protein was confined to the midpiece after cryopreservation. Similarly, GSTP1 was identified in pig sperm as a single ~48 kDa-band, and relocalisation from the posterior region of the head and the entire tail to the equatorial subdomain of the head and the principal and end pieces of the tail occurred during liquid preservation. These findings suggest that GSTs differentially adapt to the specific requirements of the cell during preservation. On the other hand, GSTM3 appeared as a single ~48 kDa-band and two bands of ~48 and ~75 kDa in cattle and human sperm, respectively. Interestingly, this antioxidant enzyme was found to distribute along the tail and the equatorial subdomain of the head in both species. These results point out to an evolutionary conserved, adaptive response of sperm, entailing changes in GSTs in a species-specific manner. Specifically, sperm would modify their physiological status relocalising GSTs, and potentially inducing post-translational modifications and/or establishing interactions of GSTs with other proteins.

GSTs are involved in the detoxification of sperm and the regulation of cellular stress signalling

Mounting evidence suggests that GSTs play an essential role in the physiology of goat, buffalo, and pig sperm (Hemachand et al., 2002; Hemachand and Shaha, 2003; Safarinejad et al., 2010; Kumar et al., 2014; Kwon et al., 2015). In spite of

this, and although liquid preservation-induced OS is known to exert detrimental effects on pig sperm quality (Waberski et al., 2011; Falchi et al., 2018), whether GSTs could protect semen during storage was not investigated previously in this species. In this regard, it is worth addressing if and how GSTs perform this protective role during liquid-storage of pig semen, as this could help improve semen preservation. For this reason, the present Dissertation evaluated the antioxidant function of sperm GSTs (**paper I**) and the regulation of cellular stress-signalling by GSTP1 (**paper V**) during liquid preservation of pig semen.

Physiological role of GSTs in sperm detoxification

The antioxidant role of GSTs in sperm was evaluated using a specific inhibitor. Ethacrynic acid (EA) is an extensively used inhibitor of GSTs enzymatic activity that strongly blocks the GSH-binding site (Ploement et al., 1993; Mathew et al., 2006); this results in the hindrance of the antioxidant function of GSTs. The inhibition of GSTs in pig extended semen stored for 72 h resulted in a reduction of their quality and functionality parameters. The most evident effect of GSTs inhibition was the complete loss of total and progressive motility, as well as a significant reduction in VAP within the first 24 hours of storage. This impairment of motility would align with previous studies conducted in goats, where sperm motility was found to decrease as a result of GSTs inhibition (Gopalakrishnan and Shaha, 1998). Furthermore, the localisation of GSTM3 along the principal piece of pig sperm would support these findings, as it could act as an important detoxification enzyme during sperm motility. Additionally, JC-1 staining revealed a dramatic decrease in mitochondrial membrane potential ($\Delta\Psi m$) of sperm due to GSTs inhibition. This outcome is consistent with the reduced mitochondrial activity and motility reported in goat sperm (Hemachand and Shaha, 2003). Furthermore, a strong and positive correlation was observed between $\Delta\Psi m$ and total motility. This correlation has been well-documented in the literature, as the production of adenosine triphosphate (ATP) and appropriate levels of ROS (Ford, 2006; Amaral et al., 2013) are known to be essential for sperm motility. Taking these evidences together, one could reasonably posit that sperm GSTs play a

role in regulating mitochondrial function and subsequent motility performance during liquid-storage of semen.

The role of GSTs in sperm membrane stability was substantiated in **paper I**, as the inhibition of these enzymes was seen to compromise plasma membrane integrity. Although the proportions of viable sperm were not significantly altered until 72 h of liquid storage, a marked increase in the percentage of viable sperm with high membrane lipid disorder was noticed as early as 24 h. This finding concurs with previous studies reporting that GSTs are predominantly localised in the sperm plasma membrane (Hemachand and Shaha, 2003) and their inhibition leads to damage of these membranes, as observed in goat sperm (Gopalakrishnan and Shaha, 1998). Besides, this study provided evidence that membrane-bound GSTs prevent cholesterol efflux and membrane lipid disorder, thus contributing to the preservation of pig semen. Despite this observed membrane lipid disorder resulting from GSTs inhibition, the acrosome membrane remained intact. These findings imply that even though GSTs inhibition leads to an increased membrane lipid disorder, this does not entail a direct effect on the acrosome membrane. This may also be related to the absence of GSTM3 from the acrosome membrane. Furthermore, GSTs were found to regulate the Ca^{2+} content within sperm. In effect, total intracellular Ca^{2+} levels were observed to increase within 24 hours of semen storage when GSTs were inhibited, whereas Ca^{2+} levels in the sperm head did not. These observations would suggest that the inhibition of sperm GSTs increases Ca^{2+} levels in the sperm mid-piece rather than in the head. While the role of mitochondrial Ca^{2+} in sperm signalling pathways is not fully understood, it is assumed that these organelles function as intracellular Ca^{2+} stores, as the negatively charged mitochondrial matrix can sequester Ca^{2+} ions (Amaral et al., 2013). The impairment of mitochondrial Ca^{2+} homeostasis due to GSTs inhibition may be caused by the destabilisation of sperm membranes, although further research is necessary to verify this hypothesis. These results point out to the essential role of sperm GSTs in regulating mitochondrial Ca^{2+} homeostasis during liquid storage of pig sperm. Overall, the role of sperm GSTs in regulating membrane lipid disorder, mitochondrial function and Ca^{2+}

homeostasis suggests an active role of these antioxidant enzymes in maintaining sperm functionality during liquid-storage of pig sperm.

Given the antioxidant nature of GSTs, the intracellular levels of ROS in GSTs-inhibited sperm were evaluated during liquid preservation. The inhibition of GSTs led to changes in the physiological levels of ROS over the 72-h period of storage. Interestingly, although inhibition of GSTs resulted in an increase in the percentage of $\bullet O_2^-$-positive sperm, intracellular levels of H_2O_2 decreased. This is consistent with the knowledge that the main source of ROS in sperm is thought (i) to reside in the mitochondria (Storey, 2008), which were shown to be impaired by GSTs inhibition; and (ii) to result from the activity of a membrane attached-NADH oxidase (Aitken and Baker, 2020), which would share its localisation with GSTs in the sperm membrane. The impaired mitochondrial activity caused by GSTs inhibition could contribute to the formation of $\bullet O_2^-$ in sperm (Han et al., 2001), which could explain the high percentage of $\bullet O_2^-$-positive cells in GSTs-inhibited samples. Yet, while H_2O_2 is understood to be generated by SOD using $\bullet O_2^-$ as a substrate, H_2O_2 levels in GSTs-inhibited sperm were found to decrease. This apparent contradiction could be explained by considering that other antioxidant enzymes present in sperm, such as GPx, CAT, and PRDX, scavenge intracellular H_2O_2 levels more efficiently than GSTs do (O'Flaherty and Rico de Souza, 2011). In order to compensate the effects of the inhibition of GSTs, the activity of H_2O_2-specific scavenger enzymes could increase, which would in turn reduce the intracellular levels of H_2O_2 in GSTs-inhibited sperm. This possibility is, however, unclear, and further research analysing the interactions between antioxidant enzymes in sperm is needed before this hypothesis can be confirmed. The evidence collected thus far indicates that GSTs protect sperm from overproduction of ROS, and suggests that they may exert this function in conjunction with other antioxidant systems. As physiological ROS levels are essential for both capacitation and fertilisation, these findings could serve as a foundation for further studies aimed at clarifying the specific role of GSTs in these two processes.

Taking all these results into consideration, the present Dissertation demonstrated, for the first time, the crucial role of GSTs in maintaining sperm

function and quality during preservation of semen at 15ºC-20ºC. Specifically, the inhibition of these antioxidant enzymes impaired the mitochondrial function and stability of the plasma membrane which, in turn, led to reduced sperm motility, compromised redox homeostasis and dysregulated intracellular Ca^{2+} levels. In conclusion, while the molecular mechanisms underlying the effects of GSTs on sperm physiology, and specifically ROS scavenging, remain to be fully comprehended, the findings reported herein highlight the essential role of GSTs for sperm function.

Cellular stress-signalling regulation of GSTP1 in sperm

As discussed previously, GSTs were, in this Dissertation, observed to be essential for preserving sperm function during liquid preservation by their detoxification role using reduced GSH. In eukaryotic cells, however, some GST members are involved in important cell signalling activity, such as the regulation of the response to cellular stress (Adler, 1999) and apoptosis (Cho et al., 2001). Specifically, GSTP1 has been established as a direct regulator of the JNK pathway in response to cellular stress by inducing the oligomerisation of GSTP1 and the subsequent dissociation of the GSTP-JNK complex, thus protecting the cell from OS via activation of specific kinases (Adler, 1999; Yin et al., 2000). Despite the important role of GSTP1 in cell signalling regulation via the inhibition of JNK enlightened in somatic cells, this molecular function of GSTs has never been investigated in mammalian sperm. Worthy of notice is that a study in sheep demonstrated that phosphorylation of JNK in sperm cells leads to increased apoptotic-like changes, DNA damage, and events related to capacitation (Luna et al., 2017). The aforementioned results suggest a putative role of the GSTP1-JNK heterocomplex in preventing sperm to activate cellular stress-signalling pathways causing early capacitation-related events or apoptotic-like changes during liquid storage. For this purpose, in **paper V**, a pharmacological dissociation of the GSTP1–JNK heterocomplex in liquid-preserved pig semen was induced by Ezatiostat, also known as Terrapin 199 (TER) (Wu and Batist, 2013). TER can bind GSTP1, thus blocking the JNK-binding site, which in turn inhibits the formation of the GSTP1–JNK heterocomplex (Mathew et al., 2006). Consequently, the inhibition of

GSTP1–JNK binding enables the phosphorylation of JNK and the activation of the downstream cascade.

The experiments conducted in this study utilised immunoblotting analysis to investigate phospho-JNK, which revealed a heightened level of tyrosine (pY) and threonine (pT) phosphorylation in samples treated with TER after 72 h of liquid preservation. It is well established that mitogen-activated protein kinases (MAPKs; e.g., JNKs) are triggered through dual phosphorylation on Y and T residues (Lawler et al., 1998). These findings, therefore, provide the first evidence in mammalian sperm about the role of the GSTP1-JNK heterocomplex as an inhibitor of the JNK signalling pathway, by preventing the dual phosphorylation of its Y and T residues. Thus, pharmacological phosphorylation and subsequent activation of JNK were found to elicit a notable decrease of sperm mitochondrial function, viability and motility. This is in line with previous research establishing a link between JNK activation and mitochondrial dysfunction and cell death in somatic cells (Aoki et al., 2002; Heslop et al., 2020). This reduction of mitochondrial activity is likely to be responsible for the loss of sperm motility, due to the fact that high levels of ATP are required for axoneme dynein to drive sperm motility (Vívenes et al., 2009). Accordingly, the GSTP1-JNK heterocomplex plays a role in preserving sperm mitochondrial activity, sperm viability and motility. Still, the specific molecular mechanisms through which JNK activation leads to mitochondrial dysfunction in sperm cells are yet to be determined. In somatic cells, it has been suggested that JNK-mitochondrial SH3-domain binding protein 5 (SAB), a docking protein for JNK, may be responsible for these processes, as it has been found to stimulate an intramitochondrial signal transduction pathway that impairs mitochondrial activity and increases ROS generation (Win et al., 2018).

Related to sperm mitochondrial dysfunction, an increase in intracellular $\bullet O_2^-$ levels resulting from the pharmacological dissociation of the GSTP1-JNK heterocomplex was seen. These results align with previous research in somatic cells which demonstrated that JNK activation is related to increased $\bullet O_2^-$ formation (Heslop et al., 2020). Besides the membrane attached-NADH oxidase activity (Aitken and Baker, 2020), one of the main sources of $\bullet O_2^-$ in mammalian

sperm cells is believed to be the mitochondria, specifically the electron transport chain (Storey, 2008; Brand, 2016). These results suggest that the activation of JNK drives the disruption of the electron transport chain of sperm mitochondria. Further research is, however, needed to investigate the downstream effects of activated JNK on mitochondrial activity and $\bullet O_2^-$ formation.

Pharmacological stimulation of JNK appeared to reduce the stability of sperm plasma membrane without affecting acrosome integrity. Similarly, and as aforementioned, inhibiting the detoxification activity of GSTs also led to sperm membrane destabilisation, but did not affect the acrosome membrane. In spite of this, and in contrast to that observed when the detoxification activity of GSTs was inhibited, the dissociation of the GSTP1-JNK heterocomplex had no effect on intracellular Ca^{2+} levels. These findings suggest a specific destabilisation of plasma membrane in the sperm tail through activation of JNK, which could impair motility and mitochondrial activity without triggering Ca^{2+} fluctuations. Whilst these results suggest that this process could be mediated by the activation of JNK thanks to the dissociation of GSTP1-JNK heterocomplex, the specific molecular mechanisms by which GSTs maintain membrane stability remain unknown.

In conclusion, the dissociation of the GSTP1-JNK heterocomplex leads to the activation of JNK, which induces a significant decline in sperm viability, motility, mitochondrial activity and plasma membrane stability, and increases $\bullet O_2^-$ levels. These results support that the GSTP1-JNK heterocomplex regulates sperm physiology, specifically preserving mitochondrial function and membrane stability.

GSTM3 is a biomarker of sperm quality and fertilising ability in humans and other animals

As mentioned in the Introduction section, male infertility accounts for 50% of unsuccessful pregnancies, 20-30% of cases being exclusively caused by the male factor (Agarwal et al., 2015a). Traditionally, the prognosis of male (in)fertility has been achieved through conventional semen analysis, which is a simple, fast and cheap evaluation of semen quality. The seminogram, however, does not provide information about sperm physiology, leaving essential molecular aspects,

such as their DNA integrity and oxidative status, aside (Lewis, 2007; Altmäe and Salumets, 2011). Recently, there is a rising interest in the exploration of novel molecular markers to overcome seminogram limitations. Molecular markers are able to determine biochemical, metabolic and/or structural characteristics of semen samples in a cost-effective manner (Kovac et al., 2013; Carrell et al., 2016; Yadav, 2017). In this sense, searching novel molecular markers in semen is of great interest for ART of both fertility clinics and the animal breeding industry. Considering the essential role of GSTs in preserving sperm physiology, it is reasonable to presume that they can function as a biomarker of sperm physiology and fertilising ability in mammalian species. Based on this assumption, the present Dissertation evaluated the role of GSTM3 as a molecular marker of sperm quality and fertilising ability in pigs (**papers II and VII**), cattle (**paper IV**) and humans (**paper VI**).

In pigs, while previous experiments of this Doctoral Thesis demonstrated that GSTM3 is highly abundant in SP, the value of this antioxidant enzyme as a predictor of sperm function was not investigated. Although a previous study in humans associated reduced sperm quality with high amounts of GSTM3 in the SP (Intasqui et al., 2015), the results of the present Dissertation evidenced no association of GSTM3 levels in SP with sperm quality parameters, such as the ejaculate volume, sperm concentration, viability, motility, membrane lipid disorder, acrosome membrane damage and intracellular ROS levels, in pigs. Interestingly, however, a negative association of SP-GSTM3 with the percentage of sperm exhibiting proximal and distal droplets and coiled tails was detected. Sperm malformations are known to originate during spermatogenesis (primary malformations) and epididymal maturation (secondary malformations) (Bonet et al., 2012; Briz and Fàbrega, 2013). Proximal and distal droplets as well as coiled tails are likely to be secondary malformations resulting from an inadequate epididymal maturation (Cooper, 2005; Briz and Fàbrega, 2013). Furthermore, as discussed previously, immunoblotting analysis revealed a particularly high expression of GSTM3 in the epididymis, thus pointing out to an important role of this enzyme during epididymal maturation. Indeed, in humans, GSTMs are known to be incorporated and/or attached to sperm during epididymal maturation

(Suryawanshi et al., 2011), which suggests a function for these enzymes during sperm passage through the epididymis. Moreover, sperm-attached GSTs have been reported to protect sperm from OS and improve their storage within the epididymis (Dacheux et al., 2009). Taken together, these results indicate that higher amounts of GSTM3 in SP are associated with better epididymal maturation in terms of a lower occurrence of sperm secondary malformations, suggesting a putative role of this antioxidant enzyme during epididymal maturation. The molecular role of GSTM3 during this process has, nevertheless, been poorly studied and requires further research.

In addition to being identified in the SP, results of the current work also evidenced the presence of GSTs within the sperm cell. Remarkably, and as explained before, this group of antioxidant enzymes exhibited an important role in detoxification and cellular stress-signalling regulation. Consequently, the present Dissertation explored the potential use of sperm GSTM3 as a biomarker of sperm quality, functionality, and fertilising ability in pigs (**paper VII**), cattle (**paper IV**) and humans (**paper VI**). To compare the results obtained in each of these species, it is noteworthy that not only were dissimilarities of GSTM3 identified in the band patterns of blots (~25, ~28, ~48, and ~75 kDa), but the analytic procedure (immunoblotting or enzyme-linked immunosorbent assay, ELISA) and the source of semen (fresh/liquid-stored or cryopreservation) were also different. In effect, in pig sperm, the ~25 kDa and ~28 kDa GSTM3-specific bands, putatively caused by PTMs, were quantified as a whole (total GSTM3) and ratio (~25 kDa/total GSTM3) by immunoblotting analysis in fresh/liquid-stored samples. In cattle, however, the single ~48 kDa band observed in immunoblots was quantified in frozen-thawed sperm as total GSTM3. Finally, in humans, the limitations in sample availability restrained the quantification of this antioxidant enzyme to ELISA and cryopreserved sperm samples, thus simultaneously assessing both ~48 kDa and ~75 kDa GSTM3-specific bands.

Regarding sperm quality parameters, a positive association between the ~25 kDa/total GSTM3 ratio, but not total GSTM3, and sperm motility, normal morphology, acrosome membrane integrity and intracellular calcium levels was noticed in pigs. As discussed previously, the presence of ~25- and ~28-kDa

GSTM3-specific bands in pig sperm suggests the occurrence of PTMs in GSTM3. Indeed, tyrosine phosphorylation (Kumar et al., 2014) and O-linked glycosylation (Gurcel et al., 2008) have already been reported to regulate the activity of this enzyme in buffalos and humans, respectively. Taken together, these results indicate that greater amounts of post-translationally modified GSTM3 are associated with lower sperm quality in pigs. Thus, it is reasonable to hypothesise that a high proportion of post-translationally modified GSTM3 in sperm is linked to disrupted redox homeostasis during spermatogenesis and/or epididymal maturation, and underlies poor sperm quality. Given the association of GSTM3 and sperm quality in pigs, its relationship with DNA damage is worth of study. Sperm DNA damage was previously proposed to compromise embryo development in mammals (Mateo-Otero et al., 2022), thus highlighting the relevance of the male factor on (in)fertility, especially regarding DNA integrity. The experiments conducted in **paper VII** evidenced that single-stranded sperm DNA fragmentation (ssSDF), but not double-stranded SDF (dsSDF), was inversely associated with the ~25 kDa/total GSTM3 ratio in pig sperm. These observations were consistent with those reported for sperm quality, as a higher ~25 kDa/total GSTM3 ratio was associated with better sperm quality and lower DNA damage. The ssSDF is basically generated by oxidative damage, mainly as a result of endogenous and/or exogenous ROS (Agarwal and Prabakaran, 2005; Simon and Carrell, 2013; Ribas-Maynou and Benet, 2019). Accordingly, the results presented in this Dissertation support the essential role of GSTM3 in maintaining redox homeostasis and preventing oxidative DNA damage in pig sperm. Indeed, the putative influence of GSTs upon the protection of sperm DNA from oxidative damage was already observed. Previous studies proved that men with a *GSTM1*$^{-/-}$ genotype exhibited higher susceptibility to sperm DNA damage associated with exposure to air pollutants (Rubes et al., 2007), as well as increased ROS levels in sperm and SP (Aydemir et al., 2007). Related to this, it is important to bear in mind that, as discussed previously, GSTM3 would also be involved in sperm detoxification, thus preventing lipid peroxidation and maintaining mitochondrial function, plasma membrane stability and redox homeostasis (Hemachand and Shaha, 2003). Finally, given the association of sperm GSTM3 with sperm quality

and DNA integrity, it was reasonable to adumbrate that this antioxidant enzyme is also related to sperm fertilising ability. For this reason, further experiments using the pig as a model were subsequently run, demonstrating the association between sperm GSTM3 content and IVF outcomes. Specifically, a positive correlation between the ~25 kDa/Total GSTM3 ratio and *in vitro* fertilisation rates was seen. In support of these findings, a previous comparative proteomics study did report sperm GSTM3 as an *in vivo* fertility marker in pigs (Kwon et al., 2015). Specifically, those authors observed that a ~27 kDa-band GSTM3 was under-expressed in boars giving rise to large litter sizes. Noticeably, while that study identified a single GSTM3-band in blots, one could conjecture, based on the molecular weight, that it corresponded to the putative post-translationally modified GSTM3-specific band observed in this Dissertation. The results of Kwon et al., (2015) would, therefore, be in line with those of the present Doctoral Thesis, evidencing that higher levels of the post-translationally modified GSTM3 are present in poor quality sperm, with higher oxidative DNA damage and subsequent reduced IVF rates and litter sizes. In the view of the above, the ratio between unmodified GSTM3 and total content is strongly associated to sperm quality, DNA integrity and fertilising ability. Given the exploratory nature of these data, and despite further studies being needed before robust conclusions about the role of sperm GSTM3 as a molecular marker can be reached, these results warrant additional research to implement the practical use of this antioxidant enzyme as a quality and fertility biomarker in swine AI centres. Moreover, experiments addressing the identity of the PTMs responsible for the GSTM3 double band-pattern observed in pig sperm should be performed in the future, as they would provide relevant information on its regulation and relationship with sperm quality and fertility.

In spite of all the aforementioned for pigs, the relationship of GSTM3 with sperm quality and *in vivo* fertility was less obvious in the case of cryopreserved bovine sperm. Interestingly, although there was no relationship between total GSTM3 and sperm quality parameters, except for a negative correlation with the percentage of sperm with coiled tails, a strong, negative association with non-return fertility rates was noted. Similar to that previously hypothesised for pig

sperm, germ cells with high OS and/or inadequate spermatogenesis or epididymal maturation may enhance the expression of GSTM3, thus exhibiting higher levels of this antioxidant enzyme in mature sperm. Consequently, higher levels of GSTM3 in frozen-thawed bovine sperm could indicate an impaired spermatogenesis and/or epididymal maturation. This would be in agreement with the high levels of sperm GSTM3 found in ejaculates with lower *in vivo* fertility, despite the lack of a clear association between total GSTM3 content and sperm quality. Thus, although further research involving a larger cohort of animals is required, these data support that sperm GSTM3 could be used as an *in vivo* fertility biomarker in cattle.

The analysis of the association between GSTM3 content and sperm quality in cryopreserved human sperm painted a rather different picture. In effect, total levels of GSTM3 in sperm were found to be positively associated to their motility and normal morphology. Moreover, and in contrast to that detected in cattle, lower levels of GSTM3 were observed in infertile men with asthenozoospermia/oligozoospermia, but not with idiopathic infertility, when compared to fertile normospermic men. The lack of significant differences in sperm GSTM3 levels between idiopathic infertile and normospermic fertile men could indicate the association of this antioxidant enzyme with sperm quality rather than directly to fertility. In line with these results, a negative association between sperm GSTM3 and ssSDF, but not dsSDF, was also observed in humans in **paper VI**. In effect, high levels of GSTM3 were detected in sperm samples showing good quality and low oxidative DNA damage. Although one might assume that high levels of this antioxidant enzyme in sperm would protect them from OS, thus conferring better sperm quality and DNA integrity, these data are inconsistent with the results previously reported in the literature. Oppositely to these data, previous studies in humans evidenced high levels of GSTM3 in sperm of poor quality (Botta et al., 2009) and high DNA damage (Behrouzi et al., 2013). This apparent contradiction might, nevertheless, be explained by methodological dissimilarities between studies. While in the present work GSTM3 was measured with ELISA in control and oligozoospermic/ asthenozoospermic samples, pools of control and oligospermic samples were subject to two-dimensional proteomic

analysis in the former study and a proteomic approach through liquid chromatography–mass spectrometry (LC-MS/MS) of 11-30 kDa proteins was carried out in the latter. Yet, and despite these discrepancies, ROC analysis ran herein yielded a consistent, good discrimination value of sperm GSTM3 in differentiating normal from altered seminograms (AUC of 0.91). These data pave the way towards the use of GSTM3 as a molecular marker to predict sperm quality and DNA integrity in humans.

In conclusion, despite the high heterogeneity in GSTM3-band patterns, and the differences between analytic methods and semen sources, which hinder a direct comparison of sperm GSTM3 content between species, the data compiled in this Dissertation upholds the association of this antioxidant enzyme with sperm quality and fertility in mammalian sperm. In this regard, and albeit further research still being required, sperm GSTM3 could be used as a biomarker of sperm quality and fertility in mammals, which is of great interest for both fertility clinics and the animal breeding industry. Furthermore, these results warrant further validation with larger cohorts and ROC curve analysis prior to the commercial use of GSTM3 as a quality and fertility biomarker.

GSTM3 is able to predict the capacity of sperm to withstand preservation procedures

Whereas liquid storage entails a decrease in sperm metabolic activity to maintain their function and fertilising ability for a limited period (Waberski et al., 2019; Henning et al., 2022), cryopreservation can indefinitely safeguard the characteristics of sperm in a frozen state (Yeste, 2016). Despite the recent advances in the sperm conservation methods for humans and farm animals, the use of preserved sperm, in both liquid and frozen state, has yet to meet some expectations. Physiological differences between species and ejaculates, in terms of their resilience to preservation, are still challenging to anticipate. In this sense, the prediction of preservation-induced damage in sperm is of great interest for ART in both fertility clinics and the livestock industry. Considering the essential role of GSTM3 in keeping sperm intact and functional, and its association with sperm quality and fertility parameters, it is reasonable to presume that this

antioxidant enzyme can function as a predictor of sperm capacity to withstand preservation procedures. For this reason, the ability of GSTM3 to predict sperm quality after liquid storage and cryopreservation in pigs was evaluated in **papers I** and **III**, respectively.

Paper I sought to elucidate the relationship between the content of sperm GSTM3 prior to liquid storage and their quality and functionality after preservation for 72 h. Interestingly, a negative correlation was observed between relative levels of the putatively post-translationally modified GSTM3 before liquid preservation and their motility and mitochondrial activity after 72 h of storage. Accumulating evidence in the literature supports the relationship between GSTM3 and mitochondrial function (Gopalakrishnan and Shaha, 1998; Hemachand and Shaha, 2003). Indeed, previously discussed results proved the importance of GSTs activity in maintaining sperm motility and mitochondrial activity. The relationship between GSTM3 content and mitochondrial activity observed herein, therefore, strengthens the hypothesis of a tight relationship between GSTs activity and mitochondrial function. Specifically, these data suggest that the putative PTM of GSTM3, rather than its total content, is negatively associated to sperm quality during liquid preservation. Consequently, though validation assessments are required, these findings encourage the use GSTM3 as a predictor of the sperm capacity to withstand liquid preservation in pigs.

The present Dissertation also attempted to investigate the relationship of the content of GSTM3 in fresh sperm with their quality and functionality after cryopreservation. Cryopreservation is known to elicit detrimental effects on sperm physiology, such as a decrease in sperm motility, changes in the composition and biophysical properties of the plasma membrane, impairment of mitochondrial function and alterations in ROS generation (Yeste, 2016). Moreover, this Doctoral Thesis demonstrated the molecular role of GSTs in preventing sperm mitochondrial dysfunction, maintaining plasma membrane stability and promoting redox homeostasis. In this regard, it is reasonable to suggest that the antioxidant activity exerted by GSTM3 could be related to sperm motility, viability, mitochondrial status and redox homeostasis after cryopreservation. In fact, a previous study revealed that cryopreserved buffalo

sperm exhibit greater GST activity than their fresh counterparts (Kumar et al., 2014), thus backing the protective role of this antioxidant enzyme during cryopreservation. In fact, the relocalisation of GSTM3 to the mid-piece in response to freeze-thawing reported in the present Dissertation could be a protective mechanism to withstand cryopreservation. Furthermore, data evidenced a negative association between levels in fresh sperm of the putatively post-translationally modified GSTM3 and sperm viability, lipid membrane disorder, intracellular $\bullet O_2^-$ levels and mitochondrial activity at post-thawing. These results, together with the high levels of PTM-GSTM3 found in the fresh sperm of PFE ejaculates, suggest that this antioxidant enzyme is also a promising marker of pig sperm cryotolerance.

In short, and notwithstanding the fact that these results warrant the use of sperm GSTM3 as a molecular marker for the sperm resilience to liquid storage and cryopreservation, further studies with larger cohorts and ROC analysis should be performed to validate this antioxidant enzyme as a robust molecular predictor. Still, the results compiled in the present Dissertation are promising to develop novel predictive systems to anticipate sperm quality after preservation, in both liquid and frozen states. This may help identify poor quality ejaculates, unable to withstand preservation procedures, with a cost-effective, simple and accurate manner, thus improving the efficiency of this technology.

CONCLUSIONS

CONCLUSIONS

1. GSTs, and specifically GSTM3 and GSTP1, are present in human, pig and cattle sperm, and exhibit species-specific differences in terms of immunoblotting band-pattern and localisation.

2. In pigs, GSTM3 is present in reproductive tissues, seminal plasma and sperm, and, in the latter, is subject to loss or relocalisation in response to liquid-storage and cryopreservation.

3. GSTs play a key role in mitochondrial activity, plasma membrane stability and oxidative regulation, thus being essential antioxidant enzymes to preserve sperm function.

4. GSTP1 regulates sperm function by the formation of the GSTP1-JNK heterocomplex and subsequent inhibition of JNK, thus preserving mitochondrial activity and membrane stability.

5. GSTM3 in sperm can be used as a biomarker of sperm quality and *in vitro* fertility in pigs, of *in vivo* fertility in cattle, and of sperm quality in humans.

6. GSTM3 in sperm is able to predict their capacity to withstand both liquid-storage and cryopreservation in pigs.

REFERENCES

REFERENCES

Adler, V. (1999). Regulation of JNK signaling by GSTp. *EMBO J* 18, 1321–1334. doi: 10.1093/emboj/18.5.1321.

Agarwal, A., Aitken, R. J., and Alvarez, J. G. eds. (2012). *Studies on Men's Health and Fertility*. Totowa, NJ: Humana Press doi: 10.1007/978-1-61779-776-7.

Agarwal, A., Baskaran, S., Parekh, N., Cho, C.-L., Henkel, R., Vij, S., et al. (2021). Male infertility. *The Lancet* 397, 319–333. doi: 10.1016/S0140-6736(20)32667-2.

Agarwal, A., Deepinder, F., Sharma, R. K., Ranga, G., and Li, J. (2008). Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. *Fertil Steril* 89, 124–128. doi: 10.1016/j.fertnstert.2007.01.166.

Agarwal, A., Mulgund, A., Hamada, A., and Chyatte, M. R. (2015a). A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology* 13, 37. doi: 10.1186/s12958-015-0032-1.

Agarwal, A., and Prabakaran, S. A. (2005). Mechanism, Measurement, and Prevention of Oxidative Stress in Male Reproductive Physiology. *Indian J Exp Biol* 43, 963–974.

Agarwal, A., Prabakaran, S., and Allamaneni, S. S. (2006). Relationship between oxidative stress, varicocele and infertility: a meta-analysis. *Reprod Biomed Online* 12, 630–633. doi: 10.1016/S1472-6483(10)61190-X.

Agarwal, A., Sharma, R., Durairajanayagam, D., Ayaz, A., Cui, Z., Willard, B., et al. (2015b). Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reproductive Biology and Endocrinology* 13, 1–22. doi: 10.1186/s12958-015-0007-2.

Agarwal, A., Tvrda, E., and Sharma, R. (2014). Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. *Reproductive Biology and Endocrinology* 12, 45. doi: 10.1186/1477-7827-12-45.

Ahmad, G., and Agarwal, A. (2017). "Ionizing Radiation and Male Fertility," in *Male Infertility*, 185–196. doi: 10.1007/978-81-322-3604-7_12.

Aitken, R., Baker, M., and Nixon, B. (2015). Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? *Asian J Androl* 17, 633. doi: 10.4103/1008-682X.153850.

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol Reprod Dev* 84, 1039–1052. doi: 10.1002/mrd.22871.

Aitken, R. J., and Baker, M. A. (2020). The Role of Genetics and Oxidative Stress in the Etiology of Male Infertility—A Unifying Hypothesis? *Front Endocrinol* 11. doi: 10.3389/fendo.2020.581838.

Aitken, R. J., Gibb, Z., Baker, M. A., Drevet, J., and Gharagozloo, P. (2016). Causes and consequences of oxidative stress in spermatozoa. *Reprod Fertil Dev* 28, 1. doi: 10.1071/RD15325.

Aldana, A., Carneiro, J., Martínez-Mekler, G., and Darszon, A. (2021). Discrete Dynamic Model of the Mammalian Sperm Acrosome Reaction: The Influence of Acrosomal pH and Physiological Heterogeneity. *Front Physiol* 12. doi: 10.3389/fphys.2021.682790.

Allai, L., Benmoula, A., Marciane da Silva, M., Nasser, B., and el Amiri, B. (2018). Supplementation of ram semen extender to improve seminal quality and fertility rate. *Anim Reprod Sci* 192, 6–17. doi: 10.1016/j.anireprosci.2018.03.019.

Altmäe, S., and Salumets, A. (2011). A novel genomic diagnostic tool for sperm quality? *Reprod Biomed Online* 22, 405–407. doi: 10.1016/j.rbmo.2010.12.009.

Amaral, A., Lourenço, B., Marques, M., and Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction* 146, 163–174. doi: 10.1530/rep-13-0178.

Andrade-Rocha, F. (2017). On the origins of the semen analysis: A close relationship with the history of the reproductive medicine. *J Hum Reprod Sci* 10, 242. doi: 10.4103/jhrs.JHRS_97_17.

Androutsos, G. (2014). Anton van Leeuwenhoek (1632-1723) et la premiere description du spermatozoïde. *Histoire de l'Andrologie* 14, 336–342. doi: 10.1007/BF03034922.

Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M., et al. (2002). Direct Activation of Mitochondrial Apoptosis Machinery by c-Jun N-terminal Kinase in Adult Cardiac Myocytes. *J Biol Chem* 277, 10244–10250. doi: 10.1074/jbc.M112355200.

Aravinda, S., Gopalakrishnan, B., Dey, C. S., Totey, S. M., Pawshe, C. H., Salunke, D., et al. (1995). A testicular protein important for fertility has glutathione S-transferase activity and is localized extracellularly in the seminiferous tubules. *Journal of Biological Chemistry* 270, 15675–15685. doi: 10.1074/jbc.270.26.15675.

Ariatti, A., and Mandrioli, P. (1993). Lazzaro spallanzani: A blow against spontaneous generation. *Aerobiologia* 9, 101–107. doi: 10.1007/BF02066251.

Armstrong, R., Morgenstern, R., and Board, P. (2017). "Glutathione transferases," in *Comprehensive Toxicology*, ed. C. McQueen, 326–362.

Austin, C. (1951). Observations on the Penetration of the Sperm into the Mammalian Egg. *Aust J Biol Sci* 4, 581. doi: 10.1071/BI9510581.

Austin, C. R. (1952). The 'Capacitation' of the Mammalian Sperm. *Nature* 170, 326–326. doi: 10.1038/170326a0.

Austin, C. R., and Bishop, M. W. H. (1958). Capacitation of Mammalian Spermatozoa. *Nature* 181, 851–851. doi: 10.1038/181851a0.

Ayaz, A., Agarwal, A., Sharma, R., Kothandaraman, N., Cakar, Z., and Sikka, S. (2018). Proteomic analysis of sperm proteins in infertile men with high levels of reactive oxygen species. *Andrologia* 50, e13015. doi: 10.1111/and.13015.

Aydemir, B., Onaran, I., Kiziler, A. R., Alici, B., and Akyolcu, M. C. (2007). Increased oxidative damage of sperm and seminal plasma in men with idiopathic infertility is higher in patients with glutathione S-transferase Mu-1 null genotype. *Asian J Androl* 9, 108–15. doi: 10.1111/j.1745-7262.2007.00237.x.

Aydos, S. E., Ph, D., Taspinar, M., Sc, M., Sunguroglu, A., and Ph, D. (2009). Association of CYP1A1 and glutathione S-transferase polymorphisms with

male factor infertility. *Fertil Steril* 92, 541–547. doi: 10.1016/j.fertnstert.2008.07.017.

Barrachina, F., Battistone, M. A., Castillo, J., Mallofré, C., Jodar, M., Breton, S., et al. (2022). Sperm acquire epididymis-derived proteins through epididymosomes. *Hum Reprod* 37, 651–668. doi: 10.1093/humrep/deac015.

Barranco, I., Roca, J., Tvarijonaviciute, A., Rubér, M., Vicente-Carrillo, A., Atikuzzaman, M., et al. (2015a). Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Mol Reprod Dev* 82, 58–65. doi: 10.1002/mrd.22444.

Barranco, I., Tvarijonaviciute, A., Perez-Patiho, C., Vicente-Carrillo, A., Parrilla, N., Ceron, J. J., et al. (2016). Glutathione peroxidase 5 is expressed by the entire pig male genital tract and once in the seminal plasma contributes to sperm survival and in vivo fertility. *PLoS One* 11, 1–17. doi: 10.1371/journal.pone.0162958.

Barranco, I., Tvarijonaviciute, A., Perez-Patiño, C., Parrilla, I., Ceron, J. J., Martinez, E. A., et al. (2015b). High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Sci Rep* 5, 18538. doi: 10.1038/srep18538.

Bateman, A., Martin, M. J., O'Donovan, C., Magrane, M., Alpi, E., Antunes, R., et al. (2017). UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 45, D158–D169. doi: 10.1093/nar/gkw1099.

Batruch, I., Lecker, I., Kagedan, D., Smith, C. R., Mullen, B. J., Grober, E., et al. (2011). Proteomic Analysis of Seminal Plasma from Normal Volunteers and Post-Vasectomy Patients Identifies over 2000 Proteins and Candidate Biomarkers of the Urogenital System. *J Proteome Res* 10, 941–953. doi: 10.1021/pr100745u.

Behrouzi, B., Kenigsberg, S., Alladin, N., Swanson, S., Zicherman, J., Hong, S.-H., et al. (2013). Evaluation of potential protein biomarkers in patients with high sperm DNA damage. *Syst Biol Reprod Med* 59, 153–163. doi: 10.3109/19396368.2013.775396.

Benson, J. D., Woods, E. J., Walters, E. M., and Critser, J. K. (2012). The cryobiology of spermatozoa. *Theriogenology* 78, 1682–99. doi: 10.1016/j.theriogenology.2012.06.007.

Bianchi, E., Doe, B., Goulding, D., and Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* 508, 483–487. doi: 10.1038/nature13203.

Björkgren, I., and Sipilä, P. (2019). The impact of epididymal proteins on sperm function. *Reproduction* 158, R155–R167. doi: 10.1530/REP-18-0589.

Bon Baer, K. E. (1956). On the genesis of the ovum of mammals and of man; a letter to the Imperial Academy of Sciences of St. Petersburg. *Isis* 47, 121–153.

Bonet, S., Briz, M. D., and Yeste, M. (2012). A Proper Assessment of Boar Sperm Function May Not Only Require Conventional Analyses but Also Others Focused on Molecular Markers of Epididymal Maturation. *Reproduction in Domestic Animals* 47, 52–64. doi: 10.1111/j.1439-0531.2012.02033.x.

Botta, T., Blescia, S., Martínez-Heredia, J., Lafuente, R., Brassesco, M., Ballescà, J. L., et al. (2009). Identificación de diferencias proteómicas en muestras oligozoospérmicas. *Rev Int Androl* 7, 14–19. doi: 10.1016/S1698-031X(09)70257-2.

Boussouar, F., and Benahmed, M. (2004). Lactate and energy metabolism in male germ cells. *Trends in Endocrinology & Metabolism* 15, 345–350. doi: 10.1016/j.tem.2004.07.003.

Brand, M. D. (2016). Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic Biol Med* 100, 14–31. doi: 10.1016/j.freeradbiomed.2016.04.001.

Briz, M. D., and Fàbrega, A. (2013). "The boar spermatozoon," in *Boar Reproduction: Fundamentals and New Biotechnological Trends*, 3–47. doi: 10.1007/978-3-642-35049-8_1.

Buffone, M. G., Kim, K.-S., Doak, B. J., Rodriguez-Miranda, E., and Gerton, G. L. (2009). Functional consequences of cleavage, dissociation and exocytotic release of ZP3R, a C4BP-related protein, from the mouse sperm acrosomal matrix. *J Cell Sci* 122, 3153–3160. doi: 10.1242/jcs.052977.

Bui, A. D., Sharma, R., Henkel, R., and Agarwal, A. (2018). Reactive oxygen species impact on sperm DNA and its role in male infertility. *Andrologia* 50, e13012. doi: 10.1111/and.13012.

Bustani, G. S., and Baiee, F. H. (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Vet World*, 1220–1233. doi: 10.14202/vetworld.2021.1220-1233.

Capanna, E. (1999). Lazzaro Spallanzani: At the roots of modern biology. *J Exp Zool* 285, 178–96. doi: 10.1002/(sici)1097-010x(19991015)285:3<178::aid-jez2>3.3.co;2-r.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ* 305, 609–613. doi: 10.1136/bmj.305.6854.609.

Carlsson, L., Ronquist, G., Stridsberg, M., and Johansson, L. (1997). Motility stimulant effects of prostasome inclusion in swim-up medium on cryopreserved human spermatozoa. *Arch Androl* 38, 215–221. doi: 10.3109/01485019708994880.

Carrell, D. T., Aston, K. I., Oliva, R., Emery, B. R., and De Jonge, C. J. (2016). The "omics" of human male infertility: integrating big data in a systems biology approach. *Cell Tissue Res* 363, 295–312. doi: 10.1007/s00441-015-2320-7.

Casas, I., and Flores, E. (2013). "Gene Banking: The Freezing Strategy," in *Boar Reproduction* (Heidelberg, Berlin: Springer), 551–588. doi: 10.1007/978-3-642-35049-8.

Casas, I., Sancho, S., Briz, M., Pinart, E., Bussalleu, E., Yeste, M., et al. (2009). Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology* 72, 930–948. doi: 10.1016/j.theriogenology.2009.07.001.

Caspi, E. (1991). Letters to the editor. *Steroids* 56, 571–572. doi: 10.1016/0039-128X(91)90016-O.

Castellani, C. (1973). Spermatozoan biology from Leeuwenhoek to Spallanzani. *J Hist Biol* 6, 37–68. doi: 10.1007/BF00137298.

Cerolini, S., Maldjian, A., Surai, P., and Noble, R. (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid

storage. *Anim Reprod Sci* 58, 99–111. doi: 10.1016/S0378-4320(99)00035-4.

Chang, M. C. (1951). Fertilizing Capacity of Spermatozoa deposited into the Fallopian Tubes. *Nature* 168, 697–698. doi: 10.1038/168697b0.

Cheng, C. Y., and Sun, F. eds. (2021). *Molecular Mechanisms in Spermatogenesis*. doi: 10.1007/978-3-030-77779-1.

Cheng, Q., Li, L., Jiang, M., Liu, B., Xian, Y., Liu, S., et al. (2022). Extend the Survival of Human Sperm In Vitro in Non-Freezing Conditions: Damage Mechanisms, Preservation Technologies, and Clinical Applications. *Cells* 11, 2845. doi: 10.3390/cells11182845.

Chianese, R., and Meccariello, R. (2018). "Introductory Chapter: Spermatozoa - Facts and Perspectives," in *Spermatozoa - Facts and Perspectives* (London: IntechOpen). doi: 10.5772/intechopen.75674.

Cho, C.-L., Esteves, S., and Agarwal, A. (2016). Novel insights into the pathophysiology of varicocele and its association with reactive oxygen species and sperm DNA fragmentation. *Asian J Androl* 18, 186. doi: 10.4103/1008-682X.170441.

Cho, S.-G., Lee, Y. H., Park, H.-S., Ryoo, K., Kang, K. W., Park, J., et al. (2001). Glutathione S-Transferase Mu Modulates the Stress-activated Signals by Suppressing Apoptosis Signal-regulating Kinase 1. *Journal of Biological Chemistry* 276, 12749–12755. doi: 10.1074/jbc.M005561200.

Clark, G. F. (2014). A role for carbohydrate recognition in mammalian sperm-egg binding. *Biochem Biophys Res Commun* 450, 1195–1203. doi: 10.1016/j.bbrc.2014.06.051.

Combes, B., and Stakelum, G. S. (1961). A liver enzyme that conjugates sulfobromophthalein sodium with glutathione. *J Clin Invest* 40, 981–8. doi: 10.1172/JCI104337.

Cooper, T. G. (2005). Cytoplasmic droplets: The good, the bad or just confusing? *Human Reproduction* 20, 9–11. doi: 10.1093/humrep/deh555.

Cornwall, G. A. (2008). New insights into epididymal biology and function. *Hum Reprod Update* 15, 213–227. doi: 10.1093/humupd/dmn055.

Crawford, G., Ray, A., Gudi, A., Shah, A., and Homburg, R. (2015). The role of seminal plasma for improved outcomes during in vitro fertilization treatment: review of the literature and meta-analysis. *Hum Reprod Update* 21, 275–284. doi: 10.1093/humupd/dmu052.

Crean, A. J., Dwyer, J. M., and Marshall, D. J. (2012). Fertilization Is Not a New Beginning: The Relationship between Sperm Longevity and Offspring Performance. *PLoS One* 7, e49167. doi: 10.1371/journal.pone.0049167.

Crocitto, L. E., Korns, D., Kretzner, L., Shevchuk, T., Blair, S. L., Wilson, T. G., et al. (2004). Prostate cancer molecular markers GSTP1 and hTERT in expressed prostatic secretions as predictors of biopsy results. *Urology* 64, 821–825. doi: 10.1016/j.urology.2004.05.007.

Cross, N. L., and Mahasreshti, P. (1997). Prostasome Fraction of Human Seminal Plasma Prevents Sperm from Becoming Acrosomally Responsive to the Agonist Progesterone. *Arch Androl* 39, 39–44. doi: 10.3109/01485019708987900.

da Ros, V. G., Munuce, M. J., Cohen, D. J., Marín-Briggiler, C. I., Busso, D., Visconti, P. E., et al. (2004). Bicarbonate is required for migration of sperm epididymal protein DE (CRISP-1) to the equatorial segment and expression of rat sperm fusion ability. *Biol Reprod* 70, 1325–32. doi: 10.1095/biolreprod.103.022822.

Dacheux, J. L., Belleannée, C., Jones, R., Labas, V., Belghazi, M., Guyonnet, B., et al. (2009). Mammalian epididymal proteome. *Mol Cell Endocrinol* 306, 45–50. doi: 10.1016/j.mce.2009.03.007.

Dadoune, J. P. (1994). The cellular biology of mammalian spermatids: a review. *Bull Assoc Anat (Nancy)* 78, 33–40.

Daily Mail Reporters (1978). First Test-Tube Baby is Born and Medical History Made as a Mother's Dream Comes True. It's a girl. *Daily Mail*.

de Jonge, C., and Barratt, C. (2006). *The sperm cell: production, maturation, fertilization, regeneration*. 1st ed. , eds. C. de Jonge and C. Barratt New York: Cambridge University Press.

Deponte, M. (2013). Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochim Biophys Acta* 1830, 3217–66. doi: 10.1016/j.bbagen.2012.09.018.

Desnoyers, L., and Manjunath, P. (1992). Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. *J Biol Chem* 267, 10149–55.

Desnoyers, L., and Manjunath, P. (1993). Interaction of a Novel Class of Phospholipid-Binding Proteins of Bovine Seminal Fluid with Different Affinity Matrices. *Arch Biochem Biophys* 305, 341–349. doi: 10.1006/abbi.1993.1431.

di Pietro, G., Magno, L. A. v, and Rios-Santos, F. (2010). Glutathione S-transferases: an overview in cancer research. *Expert Opin Drug Metab Toxicol* 6, 153–170. doi: 10.1517/17425250903427980.

di Santo, M., Tarozzi, N., Nadalini, M., and Borini, A. (2012). Human Sperm Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. *Adv Urol* 2012, 1–12. doi: 10.1155/2012/854837.

Donà, G., Fiore, C., Tibaldi, E., Frezzato, F., Andrisani, A., Ambrosini, G., et al. (2011). Endogenous reactive oxygen species content and modulation of tyrosine phosphorylation during sperm capacitation. *Int J Androl* 34, 411–419. doi: 10.1111/j.1365-2605.2010.01097.x.

Dostàlovà, Z., Calvete, J. J., Sanz, L., and Töpfer-Petersen, E. (1994). Quantitation of boar spermadhesins in accessory sex gland fluids and on the surface of epididymal, ejaculated and capacitated spermatozoa. *Biochim Biophys Acta* 1200, 48–54. doi: 10.1016/0304-4165(94)90026-4.

Dow, K. (2019). Looking into the Test Tube: The Birth of IVF on British Television. *Med Hist* 63, 189–208. doi: 10.1017/mdh.2019.6.

Druart, X., Rickard, J. P., Mactier, S., Kohnke, P. L., Kershaw-Young, C. M., Bathgate, R., et al. (2013). Proteomic characterization and cross species comparison of mammalian seminal plasma. *J Proteomics* 91, 13–22. doi: 10.1016/j.jprot.2013.05.029.

du Plessis, S. S., Agarwal, A., Halabi, J., and Tvrda, E. (2015). Contemporary evidence on the physiological role of reactive oxygen species in human

sperm function. *J Assist Reprod Genet* 32, 509–520. doi: 10.1007/s10815-014-0425-7.

Dutta, S., Henkel, R., Sengupta, P., and Agarwal, A. (2020). "Physiological Role of ROS in Sperm Function," in *Male Infertility* (Cham: Springer International Publishing), 337–345. doi: 10.1007/978-3-030-32300-4_27.

Dziekońska, A., Fraser, L., Majewska, A., Lecewicz, M., Zasiadczyk, Ł., and Kordan, W. (2013). Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17°C. *Pol J Vet Sci* 16, 517–525. doi: 10.2478/pjvs-2013-0072.

Egea, R. R., Puchalt, N. G., Escrivá, M. M., and Varghese, A. C. (2014). OMICS: Current and future perspectives in reproductive medicine and technology. *J Hum Reprod Sci* 7, 73–92. doi: 10.4103/0974-1208.138857.

Ekhlasi-Hundrieser, M., Gohr, K., Wagner, A., Tsolova, M., Petrunkina, A., and Töpfer-Petersen, E. (2005). Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. *Biol Reprod* 73, 536–45. doi: 10.1095/biolreprod.105.040824.

Erkkila, K. (2002). Lactate inhibits germ cell apoptosis in the human testis. *Mol Hum Reprod* 8, 109–117. doi: 10.1093/molehr/8.2.109.

European Society of Human Reproduction and Embryology (2018). More than 8 million babies born from IVF since the world's first in 1978. *Science Daily*.

Fahy, G. M. (1986). The relevance of cryoprotectant "toxicity" to cryobiology. *Cryobiology* 23, 1–13. doi: 10.1016/0011-2240(86)90013-1.

Falchi, L., Galleri, G., Zedda, M. T., Pau, S., Bogliolo, L., Ariu, F., et al. (2018). Liquid storage of ram semen for 96 h: Effects on kinematic parameters, membranes and DNA integrity, and ROS production. *Livest Sci* 207, 1–6. doi: 10.1016/j.livsci.2017.11.001.

FAO (2022). FAOSTAT On-Line Database. *Food and Agriculture Organization of the United Nations*. Available at: https://www.fao.org/faostat/es/#home [Accessed January 24, 2023].

Fichtner, T., Kotarski, F., Gärtner, U., Conejeros, I., Hermosilla, C., Wrenzycki, C., et al. (2020). Bovine sperm samples induce different NET phenotypes in a

NADPH oxidase-, PAD4-, and Ca++-dependent process. *Biol Reprod* 102, 902–914. doi: 10.1093/biolre/ioaa003.

Fischer-Hammadeh, C., Spengler, K., Rosenbaum, P., Schmidt, W., and Hammadeh, M. E. (2007). Non enzymatic antioxidants in seminal plasma and their effect on IVF/ICSI outcome. *Fertil Steril* 88, S380. doi: 10.1016/j.fertnstert.2007.07.1264.

Ford, W. C. L. (2004). Regulation of sperm function by reactive oxygen species. *Hum Reprod Update* 10, 387–399. doi: 10.1093/humupd/dmh034.

Ford, W. C. L. L. (2006). Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round? *Hum Reprod Update* 12, 269–274. doi: 10.1093/humupd/dmi053.

Ford, W. C., Whittington, K., and Williams, A. C. (1997). Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of NADPH to protect sperm against their effects. *Int J Androl* 20 Suppl 3, 44–9.

Fujii, J., and Tsunoda, S. (2011). Redox regulation of fertilisation and the spermatogenic process. *Asian J Androl* 13, 420–423. doi: 10.1038/aja.2011.10.

Fulcher, K. D., Welch, J. E., Klapper, D. G., O'Brien, D. A., and Eddy, E. M. (1995). Identification of a unique μ-class glutathione S-transferase in mouse spermatogenic cells. *Mol Reprod Dev* 42, 415–424. doi: 10.1002/mrd.1080420407.

Gao, D., and Critser, J. K. (2000). Mechanisms of cryoinjury in living cells. *ILAR J* 41, 187–96. doi: 10.1093/ilar.41.4.187.

Gao, D. Y., Liu, J., Liu, C., McGann, L. E., Watson, P. F., Kleinhans, F. W., et al. (1995). Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Hum Reprod* 10, 1109–22. doi: 10.1093/oxfordjournals.humrep.a136103.

Gautier, C., and Aurich, C. (2022). "Fine feathers make fine birds" – The mammalian sperm plasma membrane lipid composition and effects on assisted reproduction. *Anim Reprod Sci* 246, 106884. doi: 10.1016/j.anireprosci.2021.106884.

Gavella, M., and Lipovac, V. (1992). Nadh-Dependent Oxidoreductase (Diaphorase) Activity and Isozyme Pattern of Sperm in Infertile Men. *Arch Androl* 28, 135–141. doi: 10.3109/01485019208987691.

Gavella, M., and Lipovac, V. (2013). Protective effects of exogenous gangliosides on ROS-induced changes in human spermatozoa. *Asian J Androl* 15, 375–381. doi: 10.1038/aja.2012.144.

Gervasi, M. G., and Visconti, P. E. (2017). Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology* 5, 204–218. doi: 10.1111/andr.12320.

Gibbs, G. M., Scanlon, M. J., Swarbrick, J., Curtis, S., Gallant, E., Dulhunty, A. F., et al. (2006). The cysteine-rich secretory protein domain of Tpx-1 is related to ion channel toxins and regulates ryanodine receptor Ca2+ signaling. *J Biol Chem* 281, 4156–63. doi: 10.1074/jbc.M506849200.

Gnoth, C., Godehardt, E., Frank-Herrmann, P., Friol, K., Tigges, J., and Freundl, G. (2005). Definition and prevalence of subfertility and infertility. *Human Reproduction* 20, 1144–1147. doi: 10.1093/humrep/deh870.

Gong, S., Gabriel, M. C. S., Zini, A., Chan, P., and O'Flaherty, C. (2012). Low Amounts and High Thiol Oxidation of Peroxiredoxins in Spermatozoa From Infertile Men. *J Androl* 33, 1342–1351. doi: 10.2164/jandrol.111.016162.

Gonzalez, S. N., Sulzyk, V., Weigel Muñoz, M., and Cuasnicu, P. S. (2021). Cysteine-Rich Secretory Proteins (CRISP) are Key Players in Mammalian Fertilization and Fertility. *Front Cell Dev Biol* 9. doi: 10.3389/fcell.2021.800351.

Gopalakrishnan, B., Aravinda, S., Pawshe, C. H., Totey, S. M., Nagpal, S., and Salunke, D. M. (1998). Studies on glutathione S-transferase important for sperm function: evidence of catalytic activity-independent functions. *Biochemical Journal* 329, 231–241. doi: 10.1042/bj3290231.

Gopalakrishnan, B., and Shaha, C. (1998). Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett* 422, 296–300. doi: 10.1016/S0014-5793(98)00032-5.

Green, C., and Watson, P. (2001). Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction*, 889–898. doi: 10.1530/rep.0.1220889.

Griveau, J. F., and le Lannou, D. (1997). Reactive oxygen species and human spermatozoa: Physiology and pathology. *Int J Androl* 20, 61–69. doi: 10.1046/j.1365-2605.1997.00044.x.

Grötter, L. G., Cattaneo, L., Marini, P. E., Kjelland, M. E., and Ferré, L. B. (2019). Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. *Reproduction in Domestic Animals* 54, 655–665. doi: 10.1111/rda.13409.

Gurcel, C., Vercoutter-Edouart, A.-S., Fonbonne, C., Mortuaire, M., Salvador, A., Michalski, J.-C., et al. (2008). Identification of new O-GlcNAc modified proteins using a click-chemistry-based tagging. *Anal Bioanal Chem* 390, 2089–2097. doi: 10.1007/s00216-008-1950-y.

Gwathmey, T. M., Ignotz, G. G., and Suarez, S. S. (2003). PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. *Biol Reprod* 69, 809–15. doi: 10.1095/biolreprod.102.010827.

Halliwell, B., and Cross, C. E. (1994). Oxygen-derived species: their relation to human disease and environmental stress. *Environ Health Perspect* 102, 5–12. doi: 10.1289/ehp.94102s105.

Hamilton, L. E., Acteau, G., Xu, W., Sutovsky, P., and Oko, R. (2017). The developmental origin and compartmentalization of glutathione-s-transferase omega 2 isoforms in the perinuclear theca of eutherian spermatozoa. *Biol Reprod* 97, 612–621. doi: 10.1093/biolre/iox122.

Hamilton, L. E., Suzuki, J., Aguila, L., Meinsohn, M.-C., Smith, O. E., Protopapas, N., et al. (2019). Sperm-borne glutathione-S-transferase omega 2 accelerates the nuclear decondensation of spermatozoa during fertilization in mice. *Biol Reprod* 101, 368–376. doi: 10.1093/biolre/ioz082.

Han, D., Williams, E., and Cadenas, E. (2001). Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the

intermembrane space. *Biochemical Journal* 353, 411–416. doi: 10.1042/bj3530411.

Hansen, P. J. (2020). Implications of Assisted Reproductive Technologies for Pregnancy Outcomes in Mammals. *Annu Rev Anim Biosci* 8, 395–413. doi: 10.1146/annurev-animal-021419-084010.

Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005). Glutathione transferases. *Annu Rev Pharmacol Toxicol* 45, 51–88. doi: 10.1146/annurev.pharmtox.45.120403.095857.

Hayes, J. D., and Pulford, D. J. (1995). The Glutathione S-Transferase Supergene Family: Regulation of GST and the Contribution of the Isoenzymes to Cancer Chemoprotection and Drug Resistance. *Crit Rev Biochem Mol Biol* 30, 521–600. doi: 10.3109/10409239509083492.

Hemachand, T., Gopalakrishnan, B., Salunke, D. M., Totey, S. M., and Shaha, C. (2002). Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. *J Cell Sci* 115, 2053–2065.

Hemachand, T., and Shaha, C. (2003). Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett* 538, 14–18. doi: 10.1016/S0014-5793(03)00103-0.

Henkel, R. R. (2011). Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian J Androl* 13, 43–52. doi: 10.1038/aja.2010.76.

Henning, H., Nguyen, Q. T., Wallner, U., and Waberski, D. (2022). Temperature limits for storage of extended boar semen from the perspective of the sperm's energy status. *Front Vet Sci* 9. doi: 10.3389/fvets.2022.953021.

Hernández-Avilés, C., Love, C. C., Serafini, R., Teague, S. R., and Varner, D. D. (2019). Supplemental Antibiotics in a Commercial Extender for Stallion Semen. *J Equine Vet Sci* 80, 33–35. doi: 10.1016/j.jevs.2019.06.014.

Heslop, K. A., Rovini, A., Hunt, E. G., Fang, D., Morris, M. E., Christie, C. F., et al. (2020). JNK activation and translocation to mitochondria mediates mitochondrial dysfunction and cell death induced by VDAC opening and

sorafenib in hepatocarcinoma cells. *Biochem Pharmacol* 171, 113728. doi: 10.1016/j.bcp.2019.113728.

Hirohashi, N. (2016). “Site of Mammalian Sperm Acrosome Reaction,” in *Sperm Acrosome Biogenesis and Function During Fertilization. Advances in Anatomy, Embryology and Cell Biology* (Switzerland: Springer, Cham), 145–158. doi: 10.1007/978-3-319-30567-7_8.

Hirohashi, N., and Yanagimachi, R. (2018). Sperm acrosome reaction: its site and role in fertilization†. *Biol Reprod* 99, 127–133. doi: 10.1093/biolre/ioy045.

Holt, W. v (2000). Basic aspects of frozen storage of semen. *Anim Reprod Sci* 62, 3–22. doi: 10.1016/s0378-4320(00)00152-4.

Ickowicz, D., Finkelstein, M., and Breitbart, H. (2012). Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. *Asian J Androl* 14, 816–821. doi: 10.1038/aja.2012.81.

Ilacqua, A., Izzo, G., Emerenziani, G. Pietro, Baldari, C., and Aversa, A. (2018). Lifestyle and fertility: the influence of stress and quality of life on male fertility. *Reproductive Biology and Endocrinology* 16, 115. doi: 10.1186/s12958-018-0436-9.

Intasqui, P., Camargo, M., Antoniassi, M. P., Cedenho, A. P., Carvalho, V. M., Cardozo, K. H. M., et al. (2015). Association between the seminal plasma proteome and sperm functional traits. *Fertil Steril* 105, 617–628. doi: 10.1016/j.fertnstert.2015.11.005.

Jeulin, C., Soufir, J. C., Weber, P., Laval-Martin, D., and Calvayrac, R. (1989). Catalase activity in human spermatozoa and seminal plasma. *Gamete Res* 24, 185–196. doi: 10.1002/mrd.1120240206.

Johansson, A.-S., and Mannervik, B. (2001). Human Glutathione Transferase A3-3, a Highly Efficient Catalyst of Double-bond Isomerization in the Biosynthetic Pathway of Steroid Hormones. *Journal of Biological Chemistry* 276, 33061–33065. doi: 10.1074/jbc.M104539200.

Jonáková, V., Manásková, P., Kraus, M., Liberda, J., and Tichá, M. (2000). Sperm surface proteins in mammalian fertilization. *Mol Reprod Dev* 56, 275–7. doi: 10.1002/(SICI)1098-2795(200006)56:2+<275::AID-MRD13>3.0.CO;2-G.

Jorch, S. K., and Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* 23, 279–287. doi: 10.1038/nm.4294.

Jutte, N. H. P. M., Jansen, R., Grootegoed, J. A., Rommerts, F. F. G., Clausen, O. P. F., and van der Molen, H. J. (1982). Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. *Reproduction* 65, 431–438. doi: 10.1530/jrf.0.0650431.

Juyena, N. S., and Stelletta, C. (2012). Seminal Plasma: An Essential Attribute to Spermatozoa. *J Androl* 33, 536–551. doi: 10.2164/jandrol.110.012583.

Kan, H. P., Wu, F. L., Guo, W. bin, Wang, Y. Z., Li, J. P., Huang, Y. Q., et al. (2013). Null genotypes of GSTM1 and GSTT1 contribute to male factor infertility risk: A meta-analysis. *Fertil Steril* 99, 690–696. doi: 10.1016/j.fertnstert.2012.10.037.

Kanaoka, Y., Ago, H., Inagaki, E., Nanayama, T., Miyano, M., Kikuno, R., et al. (1997). Cloning and Crystal Structure of Hematopoietic Prostaglandin D Synthase. *Cell* 90, 1085–1095. doi: 10.1016/S0092-8674(00)80374-8.

Kantola, M., Saaranen, M., and Vanha-Perttula, T. (1988). Selenium and glutathione peroxidase in seminal plasma of men and bulls. *Reproduction* 83, 785–794. doi: 10.1530/jrf.0.0830785.

Kekäläinen, J., Jokiniemi, A., Janhunen, M., and Huuskonen, H. (2020). Offspring phenotype is shaped by the nonsperm fraction of semen. *J Evol Biol* 33, 584–594. doi: 10.1111/jeb.13592.

Kesari, K. K., Agarwal, A., and Henkel, R. (2018). Radiations and male fertility. *Reproductive Biology and Endocrinology* 16, 118. doi: 10.1186/s12958-018-0431-1.

Khoi, H. X., Shimizu, K., Yoneda, Y., Minagawa, I., Abe, Y., Kuwabara, Y., et al. (2021). Monitoring the reactive oxygen species in spermatozoa during liquid storage of boar semen and its correlation with sperm motility, free thiol content and seasonality. *Andrologia* 53. doi: 10.1111/and.14237.

Klys, H. S., Whillis, D., Howard, G., and Harrison, D. J. (1992). Glutathione S-transferase expression in the human testis and testicular germ cell neoplasia. *Br J Cancer* 66, 589–593. doi: 10.1038/bjc.1992.319.

Knapen, M. F., Zusterzeel, P. L., Peters, W. H., and Steegers, E. A. (1999). Glutathione and glutathione-related enzymes in reproduction. A review. *Eur J Obstet Gynecol Reprod Biol* 82, 171–84. doi: 10.1016/s0301-2115(98)00242-5.

Kolesnikova, L. I., Kurashova, N. A., Bairova, T. A., Dolgikh, M. I., Ershova, O. A., Dashiev, B. G., et al. (2017). Role of Glutathione-S-Transferase Family Genes in Male Infertility. *Bull Exp Biol Med* 163, 643–645. doi: 10.1007/s10517-017-3869-9.

Kopeika, J., Thornhill, A., and Khalaf, Y. (2015). The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update* 21, 209–227. doi: 10.1093/humupd/dmu063.

Koppers, A. J., Reddy, T., and O'Bryan, M. K. (2011). The role of cysteine-rich secretory proteins in male fertility. *Asian J Androl* 13, 111–7. doi: 10.1038/aja.2010.77.

Kovac, J. R., Pastuszak, A. W., and Lamb, D. J. (2013). The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertil Steril* 99, 998–1007. doi: 10.1016/j.fertnstert.2013.01.111.

Kraus, M., Tichá, M., Železná, B., Pěknicová, J., and Jonáková, V. (2005). Characterization of human seminal plasma proteins homologous to boar AQN spermadhesins. *J Reprod Immunol* 65, 33–46. doi: 10.1016/j.jri.2004.10.001.

Kumar, N., and Singh, A. K. (2021). The anatomy, movement, and functions of human sperm tail: an evolving mystery. *Biol Reprod* 104, 508–520. doi: 10.1093/biolre/ioaa213.

Kumar, R., Singh, V. K., and Atreja, S. K. (2014). Glutathione-S-transferase: Role in buffalo (Bubalus bubalis) sperm capacitation and cryopreservation. *Theriogenology* 81, 587–598. doi: 10.1016/j.theriogenology.2013.11.012.

Kuroda, S., Usui, K., Sanjo, H., Takeshima, T., Kawahara, T., Uemura, H., et al. (2020). Genetic disorders and male infertility. *Reprod Med Biol* 19, 314–322. doi: 10.1002/rmb2.12336.

Kwon, W. S., Oh, S. A., Kim, Y. J., Rahman, M. S., Park, Y. J., and Pang, M. G. (2015). Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci Rep* 5, 1–10. doi: 10.1038/srep13821.

Kwon, W.-S., Rahman, M. S., and Pang, M.-G. (2014). Diagnosis and prognosis of male infertility in mammal: the focusing of tyrosine phosphorylation and phosphotyrosine proteins. *J Proteome Res* 13, 4505–4517. doi: 10.1021/pr500524p.

Lakpour, N., Mirfeizollahi, A., Farivar, S., Akhondi, M. M., Hashemi, S. B., Amirjannati, N., et al. (2013). The association of seminal plasma antioxidant levels and sperm chromatin status with genetic variants of GSTM1 and GSTP1 (Ile105Val and Ala114Val) in infertile men with oligoasthenoteratozoospermia. *Dis Markers* 34, 205–210. doi: 10.3233/DMA-120954.

Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998). Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. *Current Biology* 8, 1387–1391. doi: 10.1016/s0960-9822(98)00019-0.

Leeuwenhoek, A. Van (1679). Observationes D. Anthonii Lewenhoeck, de natis'e semine genitali animalculis. *Philos Trans R Soc Lond* 12, 1040–1046. doi: 10.1098/rstl.1677.0068.

Lehti, M. S., and Sironen, A. (2017). Formation and function of sperm tail structures in association with sperm motility defects. *Biol Reprod* 97, 522–536. doi: 10.1093/biolre/iox096.

Lenzi, A., Gandini, L., Maresca, V., Rago, R., Sgro, P., Dondero, F., et al. (2000). Fatty acid composition of spermatozoa and immature germ cells. *Mol Hum Reprod* 6, 226–231. doi: 10.1093/molehr/6.3.226.

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Jolles, M., et al. (2022). Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. *Hum Reprod Update*. doi: 10.1093/humupd/dmac035.

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., et al. (2017). Temporal trends in sperm count: a systematic review

and meta-regression analysis. *Hum Reprod Update* 23, 646–659. doi: 10.1093/humupd/dmx022.

Lewis, S. E. M. (2007). Is sperm evaluation useful in predicting human fertility? *Reproduction* 134, 31–40. doi: 10.1530/REP-07-0152.

Lewis, S. E. M., Boyle, P. M., McKinney, K. A., Young, I. S., and Thompson, W. (1995). Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil Steril* 64, 868–870. doi: 10.1016/S0015-0282(16)57870-4.

Li, J. Y., Liu, F. J., Wang, H. Y., Liu, X., Liu, J., Li, N., et al. (2010). Systematic mapping and functional analysis of a family of human epididymal secretory sperm-located proteins. *Molecular and Cellular Proteomics* 9, 2517–2528. doi: 10.1074/mcp.M110.001719.

Li, Y.-J., Oliveira, S. A., Xu, P., Martin, E. R., Stenger, J. E., Scherzer, C. R., et al. (2003). Glutathione S-transferase omega-1 modifies age-at-onset of Alzheimer disease and Parkinson disease. *Hum Mol Genet* 12, 3259–67. doi: 10.1093/hmg/ddg357.

Listowsky, I., Rowe, J. D., Patskovsky, Y. v, Tchaikovskaya, T., Shintani, N., Novikova, E., et al. (1998). Human testicular glutathione S-transferases: insights into tissue-specific expression of the diverse subunit classes. *Chem Biol Interact* 111–112, 103–112. doi: 10.1016/S0009-2797(97)00154-3.

Liu, D. Y., and Baker, H. W. G. (2000). Defective sperm–zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. *Human Reproduction* 15, 702–708. doi: 10.1093/humrep/15.3.702.

Llavanera, M., Delgado-Bermúdez, A., Ribas-Maynou, J., Salas-Huetos, A., and Yeste, M. (2022). A systematic review identifying fertility biomarkers in semen: a clinical approach through Omics to diagnose male infertility. *Fertil Steril*. doi: 10.1016/j.fertnstert.2022.04.028.

Llavanera, M., Mateo-Otero, Y., Bonet, S., Barranco, I., Fernández-Fuertes, B., and Yeste, M. (2020). The triple role of glutathione S-transferases in mammalian male fertility. *Cellular and Molecular Life Sciences* 77, 2331–2342. doi: 10.1007/s00018-019-03405-w.

Loren, A. W., Mangu, P. B., Beck, L. N., Brennan, L., Magdalinski, A. J., Partridge, A. H., et al. (2013). Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol* 31, 2500–10. doi: 10.1200/JCO.2013.49.2678.

Luna, C., Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J. A., and Muiño-Blanco, T. (2017). c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways link capacitation with apoptosis and seminal plasma proteins protect sperm by interfering with both routes. *Biol Reprod* 96, 800–815. doi: 10.1093/biolre/iox017.

Maciel, S. S., Pereira, A. da C., Silva, G. J. J., Rodrigues, M. v., Mill, J. G., and Krieger, J. E. (2009). Association between glutathione S-transferase polymorphisms and triglycerides and HDL-cholesterol. *Atherosclerosis* 206, 204–208. doi: 10.1016/j.atherosclerosis.2009.02.011.

Mahfouz, R., Sharma, R., Sharma, D., Sabanegh, E., and Agarwal, A. (2009). Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. *Fertil Steril* 91, 805–811. doi: 10.1016/j.fertnstert.2008.01.022.

Male Infertility Best Practice Policy Committee of the American Urological Association, and Practice Committee of the American Society for Reproductive Medicine (2006). Report on optimal evaluation of the infertile male. *Fertil Steril* 86, S202–S209. doi: 10.1016/j.fertnstert.2006.08.029.

Manjunath, P., Lefebvre, J., Jois, P. S., Fan, J., and Wright, M. W. (2009). New Nomenclature for Mammalian BSP Genes. *Biol Reprod* 80, 394–397. doi: 10.1095/biolreprod.108.074088.

Mannervik, B., Board, P. G., Hayes, J. D., Listowsky, I., and Pearson, W. R. (2005). “Nomenclature for Mammalian Soluble Glutathione Transferases,” in, 1–8. doi: 10.1016/S0076-6879(05)01001-3.

Mannervik, B., Helena Danielson, U., and Ketterer, B. (1988). Glutathione Transferases—Structure and Catalytic Activit. *Critical Reviews in Biochemistry* 23, 283–337. doi: 10.3109/10409238809088226.

Mannervik, B., and Jensson, H. (1982). Binary combinations of four protein subunits with different catalytic specificities explain the relationship

between six basic glutathione S-transferases in rat liver cytosol. *J Biol Chem* 257, 9909–12.

Marcon, L., and Boissonneault, G. (2004). Transient DNA Strand Breaks During Mouse and Human Spermiogenesis: New Insights in Stage Specificity and Link to Chromatin Remodeling. *Biol Reprod* 70, 910–918. doi: 10.1095/biolreprod.103.022541.

Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., and Stevens, G. A. (2012). National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. *PLoS Med* 9, e1001356. doi: 10.1371/journal.pmed.1001356.

Mateo-Otero, Y., Llavanera, M., Recuero, S., Delgado-Bermúdez, A., Barranco, I., Ribas-Maynou, J., et al. (2022). Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs. *Biol Res* 55, 15. doi: 10.1186/s40659-022-00386-2.

Mateo-Otero, Y., Zambrano, F., Catalán, J., Sánchez, R., Yeste, M., Miro, J., et al. (2022). Seminal plasma, and not sperm, induces time and concentration-dependent neutrophil extracellular trap release in donkeys. *Equine Vet J* 54, 415–426. doi: 10.1111/evj.13457.

Mathew, N., Kalyanasundaram, M., and Balaraman, K. (2006). Glutathione S - transferase (GST) inhibitors. *Expert Opin Ther Pat* 16, 431–444. doi: 10.1517/13543776.16.4.431.

Maxwell, W., and Salamon, S. (1993). Liquid storage of ram semen: a review. *Reprod Fertil Dev* 5, 613. doi: 10.1071/RD9930613.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *Am J Physiol* 247, C125-42. doi: 10.1152/ajpcell.1984.247.3.C125.

Mazur, P. (1990). Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. *Cell Biophys* 17, 53–92. doi: 10.1007/BF02989804.

Mazur, P., Leibo, S. P., and Seidel, G. E. (2008). Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biol Reprod* 78, 2–12. doi: 10.1095/biolreprod.107.064113.

Micheli, L., Cerretani, D., Collodel, G., Menchiari, A., Moltoni, L., Fiaschi, A. I., et al. (2016). Evaluation of enzymatic and non-enzymatic antioxidants in seminal plasma of men with genitourinary infections, varicocele and idiopathic infertility. *Andrology* 4, 456–464. doi: 10.1111/andr.12181.

Miller, D., Brinkworth, M., and Iles, D. (2010). Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* 139, 287–301. doi: 10.1530/REP-09-0281.

Mishra, P., Negi, M. P. S., Srivastava, M., Singh, K., and Rajender, S. (2018). Decline in seminal quality in Indian men over the last 37 years. *Reproductive Biology and Endocrinology* 16, 103. doi: 10.1186/s12958-018-0425-z.

Molina, L. C. P., Luque, G. M., Balestrini, P. A., Marín-Briggiler, C. I., Romarowski, A., and Buffone, M. G. (2018). Molecular basis of human sperm capacitation. *Front Cell Dev Biol* 6, 72. doi: 10.3389/fcell.2018.00072.

Moradi, M., Karimi, J., Khodadadi, I., Amiri, I., Karami, M., Saidijam, M., et al. (2018). Evaluation of the p53 and Thioredoxin reductase in sperm from asthenozoospermic males in comparison to normozoospermic males. *Free Radic Biol Med* 116, 123–128. doi: 10.1016/j.freeradbiomed.2017.12.038.

Moragianni, V. A., and Penzias, A. S. (2010). Cumulative live-birth rates after assisted reproductive technology. *Curr Opin Obstet Gynecol* 22, 189–192. doi: 10.1097/GCO.0b013e328338493f.

Morel, F., Rauch, C., Petit, E., Piton, A., Theret, N., Coles, B., et al. (2004). Gene and Protein Characterization of the Human Glutathione S-Transferase Kappa and Evidence for a Peroxisomal Localization. *Journal of Biological Chemistry* 279, 16246–16253. doi: 10.1074/jbc.M313357200.

Morgan, H. L., and Watkins, A. J. (2020). The influence of seminal plasma on offspring development and health. *Semin Cell Dev Biol* 97, 131–137. doi: 10.1016/j.semcdb.2019.06.008.

Morrell, J. M., Georgakas, A., Lundeheim, N., Nash, D., Davies Morel, M. C. G., and Johannisson, A. (2014). Effect of heterologous and homologous seminal plasma on stallion sperm quality. *Theriogenology* 82, 176–183. doi: 10.1016/j.theriogenology.2014.03.020.

Mruk, D. D., and Cheng, C. Y. (2015). The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocr Rev* 36, 564–591. doi: 10.1210/er.2014-1101.

Mukherjee, S. B., Aravinda, S., Gopalakrishnan, B., Nagpal, S., Salunke, D. M., and Shaha, C. (1999). Secretion of glutathione S-transferase isoforms in the seminiferous tubular fluid, tissue distribution and sex steroid binding by rat GSTM1. *Biochem J* 340 (Pt 1), 309–20.

Mukhtar, H., Lee, I. P., and Bend, J. R. (1978). Glutathione S-transferase activities in rat and mouse sperm and human semen. *Biochem Biophys Res Commun* 83, 1093–1098. doi: 10.1016/0006-291X(78)91507-3.

Muldrew, K., and McGann, L. E. (1994). The osmotic rupture hypothesis of intracellular freezing injury. *Biophys J* 66, 532–41. doi: 10.1016/s0006-3495(94)80806-9.

Nachtigall, R. D. (2006). International disparities in access to infertility services. *Fertil Steril* 85, 871–875. doi: 10.1016/j.fertnstert.2005.08.066.

Nelson, C. M. K., and Bunge, R. G. (1974). Semen Analysis: Evidence for Changing Parameters of Male Fertility Potential. *Fertil Steril* 25, 503–507. doi: 10.1016/S0015-0282(16)40454-1.

Nishimura, H., and L'Hernault, S. W. (2017). Spermatogenesis. *Curr Biol* 27, R988–R994. doi: 10.1016/j.cub.2017.07.067.

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., and Stanton, P. G. (2011). Spermiation: The process of sperm release. *Spermatogenesis* 1, 14–35. doi: 10.4161/spmg.1.1.14525.

O'Donnell, L., Smith, L. B., and Rebourcet, D. (2022). Sertoli cells as key drivers of testis function. *Semin Cell Dev Biol* 121, 2–9. doi: 10.1016/j.semcdb.2021.06.016.

Oehninger, S., and Ombelet, W. (2019). Limits of current male fertility testing. *Fertil Steril* 111, 835–841. doi: 10.1016/j.fertnstert.2019.03.005.

O'Flaherty, C., and Rico de Souza, A. (2011). Hydrogen Peroxide Modifies Human Sperm Peroxiredoxins in a Dose-Dependent Manner. *Biol Reprod* 84, 238–247. doi: 10.1095/biolreprod.110.085712.

Okamura, T., Antoun, G., Keir, S. T., Friedman, H., Bigner, D. D., and Ali-Osman, F. (2015). Phosphorylation of glutathione s-transferase p1 (gstp1) by epidermal growth factor receptor (EGFR) promotes formation of the gstp1-c-jun n-terminal kinase (JNK) complex and suppresses JNK downstream signaling and apoptosis in brain tumor cells. *Journal of Biological Chemistry* 290, 30866–30878. doi: 10.1074/jbc.M115.656140.

Okazaki, T., Akiyoshi, T., Kan, M., Mori, M., Teshima, H., and Shimada, M. (2012). Artificial Insemination With Seminal Plasma Improves the Reproductive Performance of Frozen-Thawed Boar Epididymal Spermatozoa. *J Androl* 33, 990–998. doi: 10.2164/jandrol.111.015115.

Oko, R. (1988). Comparative Analysis of Proteins from the Fibrous Sheath and Outer Dense Fibers of Rat Spermatozoa1. *Biol Reprod* 39, 169–182. doi: 10.1095/biolreprod39.1.169.

Oko, R. (1995). Developmental expression and possible role of perinuclear theca proteins in mammalian spermatozoa. *Reprod Fertil Dev* 7, 777. doi: 10.1071/RD9950777.

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016). Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res* 44, D733–D745. doi: 10.1093/nar/gkv1189.

O'Leary, S., Jasper, M. J., Warnes, G. M., Armstrong, D. T., and Robertson, S. A. (2004). Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* 128, 237–247. doi: 10.1530/rep.1.00160.

Oliveira, P. F., and Alves, M. G. (2015a). *Sertoli Cell Metabolism and Spermatogenesis*. Cham: Springer International Publishing doi: 10.1007/978-3-319-19791-3.

Oliveira, P. F., and Alves, M. G. (2015b). "Testicular Metabolic Cooperation," in *Sertoli Cell Metabolism and Spermatogenesis* (Cham: Springer International Publishing), 41–56. doi: 10.1007/978-3-319-19791-3_5.

Pan, M. M., Hockenberry, M. S., Kirby, E. W., and Lipshultz, L. I. (2018). Male Infertility Diagnosis and Treatment in the Era of In Vitro Fertilization and

Intracytoplasmic Sperm Injection. *Medical Clinics of North America* 102, 337–347. doi: 10.1016/j.mcna.2017.10.008.

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

Paracchini, V., Chang, S. S., Santella, R. M., Garte, S., Pedotti, P., and Taioli, E. (2005). GSMT1 deletion modifies the levels of polycyclic aromatic hydrocarbon-DNA adducts in human sperm. *Mutat Res Genet Toxicol Environ Mutagen* 586, 97–101. doi: 10.1016/j.mrgentox.2005.06.008.

Paul, N., Talluri, T. R., Nag, P., and Kumaresan, A. (2021). Epididymosomes: A potential male fertility influencer. *Andrologia* 53. doi: 10.1111/and.14155.

Paz, M., Morín, M., and del Mazo, J. (2006). Proteome profile changes during mouse testis development. *Comp Biochem Physiol* 1, 404–415. doi: 10.1016/j.cbd.2006.10.002.

Pearson, W. R. (2005). “Phylogenies of Glutathione Transferase Families,” in, 186–204. doi: 10.1016/S0076-6879(05)01012-8.

Peddinti, D., Nanduri, B., Kaya, A., Feugang, J. M., Burgess, S. C., and Memili, E. (2008). Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst Biol* 2, 1–13. doi: 10.1186/1752-0509-2-19.

Peeker, R. (1997). Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa. *Mol Hum Reprod* 3, 1061–1066. doi: 10.1093/molehr/3.12.1061.

Pérez-Patino, C., Parrilla, I., Li, J., Barranco, I., Martínez, E. A., Rodriguez-Martínez, H., et al. (2019). The proteome of pig spermatozoa is remodeled during ejaculation. *Molecular and Cellular Proteomics* 18, 41–50. doi: 10.1074/mcp.RA118.000840.

Phillips, P. (1939). Preservation of bull semen. *Journal of biological chemistry* 130, 415.

Pilch, B., and Mann, M. (2006). Large-scale and high-confidence proteomic analysis of human seminal plasma. *Genome Biol* 7, R40. doi: 10.1186/gb-2006-7-5-r40.

Pillai, R. N., and McEleny, K. (2021). Management of male infertility. *Obstet Gynaecol Reprod Med* 31, 192–198. doi: 10.1016/j.ogrm.2021.05.003.

Plant, T., and Zeleznik, A. (2014). *Knobil and Neill's Physiology of Reproduction*. 4th ed. Massachussetts: Elsevier.

Ploement, J. H. T. M., Ommen, B. V. A. N., and Bogaards, J. J. P. (1993). Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. *Xenobiotica* 23, 913–923. doi: 10.3109/00498259309059418.

Pohjanvirta, T., Vähänikkilä, N., Simonen, H., Pelkonen, S., and Autio, T. (2020). Efficacy of Two Antibiotic-Extender Combinations on Mycoplasma bovis in Bovine Semen Production. *Pathogens* 9, 808. doi: 10.3390/pathogens9100808.

Porter, J. R. (1976). Antony van Leeuwenhoek: tercentenary of his discovery of bacteria. *Bacteriol Rev* 40, 260–269. doi: 10.1128/br.40.2.260-269.1976.

Primakoff, P., and Myles, D. G. (2002). Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science (1979)* 296, 2183–2185. doi: 10.1126/science.1072029.

Protopapas, N., Hamilton, L. E., Warkentin, R., Xu, W., Sutovsky, P., and Oko, R. (2019). The perforatorium and postacrosomal sheath of rat spermatozoa share common developmental origins and protein constituents†. *Biol Reprod* 100, 1461–1472. doi: 10.1093/biolre/ioz052.

Purvis, K., and Christiansen, E. (1993). Infection in the male reproductive tract. Impact, diagnosis and treatment in relation to male infertility. *Int J Androl* 16, 1–13. doi: 10.1111/j.1365-2605.1993.tb01146.x.

Raijmakers, M. T. M., Roelofs, H. M. J., Steegers, E. A. P., Steegers-Theunissen, R. égine P. M., Mulder, T. P. J., Knapen, M. F. C. M., et al. (2003). Glutathione and glutathione S-transferases A1-1 and P1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. *Fertil Steril* 79, 169–172. doi: 10.1016/S0015-0282(02)04404-7.

Ribas-Maynou, J., and Benet, J. (2019). Single and double strand sperm DNA damage: Different reproductive effects on male fertility. *Genes (Basel)* 10. doi: 10.3390/genes10020105.

Ribas-Maynou, J., Mateo-Otero, Y., Delgado-Bermúdez, A., Bucci, D., Tamanini, C., Yeste, M., et al. (2021). Role of exogenous antioxidants on the performance and function of pig sperm after preservation in liquid and frozen states: A systematic review. *Theriogenology* 173, 279–294. doi: 10.1016/j.theriogenology.2021.07.023.

Rickard, J. P., Pini, T., Soleilhavoup, C., Cognie, J., Bathgate, R., Lynch, G. W., et al. (2014). Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. *Reproduction* 148, 469–478. doi: 10.1530/REP-14-0285.

Roberts, K. P., Wamstad, J. A., Ensrud, K. M., and Hamilton, D. W. (2003). Inhibition of capacitation-associated tyrosine phosphorylation signaling in rat sperm by epididymal protein Crisp-1. *Biol Reprod* 69, 572–81. doi: 10.1095/biolreprod.102.013771.

Rodríguez-Martínez, H., Kvist, U., Ernerudh, J., Sanz, L., and Calvete, J. J. (2011). Seminal Plasma Proteins: What Role Do They Play? *American Journal of Reproductive Immunology* 66, 11–22. doi: 10.1111/j.1600-0897.2011.01033.x.

Rodriguez-Martinez, H., Martinez, E. A., Calvete, J. J., Peña Vega, F. J., and Roca, J. (2021). Seminal Plasma: Relevant for Fertility? *Int J Mol Sci* 22, 4368. doi: 10.3390/ijms22094368.

Rodriguez-Martinez, H., and Wallgren, M. (2011). Advances in Boar Semen Cryopreservation. *Vet Med Int* 2011, 1–5. doi: 10.4061/2011/396181.

Roldan, E., Murase, T., and Shi, Q. (1994). Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science* 266, 1578–1581. doi: 10.1126/science.7985030.

Rubes, J., Selevan, S. G., Sram, R. J., Evenson, D. P., and Perreault, S. D. (2007). GSTM1 genotype influences the susceptibility of men to sperm DNA damage associated with exposure to air pollution. *Mutat Res* 625, 20–28. doi: 10.1016/j.mrfmmm.2007.05.012.

Safarinejad, M. R., Shafiei, N., and Safarinejad, S. (2010). The association of glutathione-S-transferase gene polymorphisms (GSTM1, GSTT1, GSTP1) with idiopathic male infertility. *J Hum Genet* 55, 565–570. doi: 10.1038/jhg.2010.59.

Schoeller, S. F., Holt, W. v., and Keaveny, E. E. (2020). Collective dynamics of sperm cells. *Philosophical Transactions of the Royal Society B: Biological Sciences* 375, 20190384. doi: 10.1098/rstb.2019.0384.

Scott, J. L., Ketheesan, N., and Summers, P. M. (2006). Leucocyte population changes in the reproductive tract of the ewe in response to insemination. *Reprod Fertil Dev* 18, 627. doi: 10.1071/RD05165.

Sengupta, P. (2013). Environmental and occupational exposure of metals and their role in male reproductive functions. *Drug Chem Toxicol* 36, 353–368. doi: 10.3109/01480545.2012.710631.

Shaha, C., Suri, A., and Talwar, G. P. (1988). Identification of sperm antigens that regulate fertility. *Int J Androl* 11, 479–491. doi: 10.1111/j.1365-2605.1988.tb01022.x.

Sharma, A., Mollier, J., Brocklesby, R. W. K., Caves, C., Jayasena, C. N., and Minhas, S. (2020). Endocrine-disrupting chemicals and male reproductive health. *Reprod Med Biol* 19, 243–253. doi: 10.1002/rmb2.12326.

Sharma, R., Agarwal, A., Mohanty, G., Jesudasan, R., Gopalan, B., Willard, B., et al. (2013). Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. *Reproductive Biology and Endocrinology* 11, 38. doi: 10.1186/1477-7827-11-38.

Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochemical Journal* 360, 1. doi: 10.1042/0264-6021:3600001.

Short, R. v. (2014). Colin Russell Austin 1914–2004. *Hist Rec Aust Sci* 25, 264. doi: 10.1071/HR14024.

Sieme, H., Oldenhof, H., and Wolkers, W. F. (2016). Mode of action of cryoprotectants for sperm preservation. *Anim Reprod Sci* 169, 2–5. doi: 10.1016/j.anireprosci.2016.02.004.

Silvestre, M. A., Yániz, J. L., Peña, F. J., Santolaria, P., and Castelló-Ruiz, M. (2021). Role of Antioxidants in Cooled Liquid Storage of Mammal Spermatozoa. *Antioxidants* 10, 1096. doi: 10.3390/antiox10071096.

Simon, L., and Carrell, D. T. (2013). Sperm DNA Damage Measured by Comet Assay. *Methods in Molecular Biology* 927, 137–146. doi: 10.1007/978-1-62703-038-0_13.

Smital, J., de Sousa, L. L., and Mohsen, A. (2004). Differences among breeds and manifestation of heterosis in AI boar sperm output. *Anim Reprod Sci* 80, 121–130. doi: 10.1016/S0378-4320(03)00142-8.

Song, X., Zhao, Y., Cai, Q., Zhang, Y., and Niu, Y. (2013). Association of the Glutathione S-transferases M1 and T1 polymorphism with male infertility: A meta-Analysis. *J Assist Reprod Genet* 30, 131–141. doi: 10.1007/s10815-012-9907-7.

Stival, C., Puga Molina, L. del C., Paudel, B., Buffone, M. G., Visconti, P. E., and Krapf, D. (2016). "Sperm Capacitation and Acrosome Reaction in Mammalian Sperm," in, 93–106. doi: 10.1007/978-3-319-30567-7_5.

Storey, B. T. (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. *International Journal of Developmental Biology* 52, 427–437. doi: 10.1387/ijdb.072522bs.

Suarez, S., and Ho, H.-C. (2003). Hyperactivated Motility in Sperm. *Reproduction in Domestic Animals* 38, 119–124. doi: 10.1046/j.1439-0531.2003.00397.x.

Suarez, S. S. (2008). Control of hyperactivation in sperm. *Hum Reprod Update* 14, 647–657. doi: 10.1093/humupd/dmn029.

Sullivan, R. (2015). Epididymosomes: a heterogeneous population of microvesicles with multiple functions in sperm maturation and storage. *Asian J Androl* 17, 726–9. doi: 10.4103/1008-682X.155255.

Sullivan, R., and Mieusset, R. (2016). The human epididymis: its function in sperm maturation. *Hum Reprod Update* 22, 574–587. doi: 10.1093/humupd/dmw015.

Sun, H., Gong, T.-T., Jiang, Y.-T., Zhang, S., Zhao, Y.-H., and Wu, Q.-J. (2019). Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: results from a

global burden of disease study, 2017. *Aging* 11, 10952–10991. doi: 10.18632/aging.102497.

Suryawanshi, A. R., Khan, S. A., Gajbhiye, R. K., Gurav, M. Y., and Khole, V. v. (2011). Differential proteomics leads to identification of domain-specific epididymal sperm proteins. *J Androl* 32, 240–259. doi: 10.2164/jandrol.110.010967.

Sutovsky, P., Manandhar, G., Wu, A., and Oko, R. (2003). Interactions of sperm perinuclear theca with the oocyte: Implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. *Microsc Res Tech* 61, 362–378. doi: 10.1002/jemt.10350.

Swan, S. H., Elkin, E. P., and Fenster, L. (2000). The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect* 108, 961–966. doi: 10.1289/ehp.00108961.

Sztein, J. M., Takeo, T., and Nakagata, N. (2018). History of cryobiology, with special emphasis in evolution of mouse sperm cryopreservation. *Cryobiology* 82, 57–63. doi: 10.1016/j.cryobiol.2018.04.008.

Tang, M., Wang, S., Wang, W., Cao, Q., Qin, C., Liu, B., et al. (2012). The glutathione-S-transferase gene polymorphisms (GSTM1 and GSTT1) and idiopathic male infertility risk: A meta-analysis. *Gene* 511, 218–223. doi: 10.1016/j.gene.2012.09.054.

Thimon, V., Frenette, G., Saez, F., Thabet, M., and Sullivan, R. (2008). Protein composition of human epididymosomes collected during surgical vasectomy reversal: A proteomic and genomic approach. *Human Reproduction* 23, 1698–1707. doi: 10.1093/humrep/den181.

Thompson, A., Agarwal, A., and du Plessis, S. (2013). “Physiological Role of Reactive Oxygen Species in Sperm Function: A Review. ,” in *Antioxidants in Male Infertility: A Guide for Clinicians and Researchers*, eds. SJ. Parekatil and A. Agarwal (New York: Springer Science+Business Media), 69–89.

Töpfer-Petersen, E., Romero, A., Varela, P. F., Ekhlasi-Hundrieser, M., Dostàlovà, Z., Sanz, L., et al. (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* 30, 217–24. doi: 10.1111/j.1439-0272.1998.tb01163.x.

Townsend, D. M., and Tew, K. D. (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* 22, 7369–7375. doi: 10.1038/sj.onc.1206940.

Tvrdá, E., Bučko, O., Rojková, K., Ďuračka, M., Kunová, S., Kováč, J., et al. (2021). The Efficiency of Selected Extenders against Bacterial Contamination of Boar Semen in a Swine Breeding Facility in Western Slovakia. *Animals* 11, 3320. doi: 10.3390/ani11113320.

Utleg, A. G., Yi, E. C., Xie, T., Shannon, P., White, J. T., Goodlett, D. R., et al. (2003). Proteomic analysis of human prostasomes. *Prostate* 56, 150–161. doi: 10.1002/pros.10255.

Vadnais, M. L., and Roberts, K. P. (2007). Effects of seminal plasma on cooling-induced capacitative changes in boar sperm. *J Androl* 28, 416–22. doi: 10.2164/jandrol.106.001826.

van Gestel, R. A., Brewis, I. A., Ashton, P. R., Brouwers, J. F., and Gadella, B. M. (2007). Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. *Mol Hum Reprod* 13, 445–54. doi: 10.1093/molehr/gam030.

Vander Borght, M., and Wyns, C. (2018). Fertility and infertility: Definition and epidemiology. *Clin Biochem* 62, 2–10. doi: 10.1016/j.clinbiochem.2018.03.012.

Vani, G. T., Mukesh, N., Siva Prasad, B., Rama Devi, P., Hema Prasad, M., Rani, P. U., et al. (2010). Role of glutathione S-transferase Mu-1 (GSTM1) polymorphism in oligospermic infertile males. *Andrologia* 42, 213–217. doi: 10.1111/j.1439-0272.2009.00971.x.

Ventelä, S. (2006). “Cytoplasmic Bridges as Cell-Cell Channels of Germ Cells,” in *Cell-Cell Channels* (New York, NY: Springer New York), 208–216. doi: 10.1007/978-0-387-46957-7_15.

Vicens, A., Borziak, K., Karr, T. L., Roldan, E. R. S., and Dorus, S. (2017). Comparative sperm proteomics in mouse species with divergent mating systems. *Mol Biol Evol* 34, 1403–1416. doi: 10.1093/molbev/msx084.

Visconti, P. E. (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences* 106, 667–668. doi: 10.1073/pnas.0811895106.

Vívenes, C. Y., Peralta-Arias, R. D., Camejo, M. I., Guerrero, K., Fernández, V. H., Piñero, S., et al. (2009). Biochemical identification of dynein-ATPase activity in human sperm. *Z Naturforsch C J Biosci* 64, 747–753. doi: 10.1515/znc-2009-9-1022.

Vogt, P. H. (2005). AZF deletions and Y chromosomal haplogroups: history and update based on sequence. *Hum Reprod Update* 11, 319–336. doi: 10.1093/humupd/dmi017.

Waberski, D., Henning, H., and Petrunkina, A. (2011). Assessment of storage effects in liquid preserved boar semen. *Reproduction in Domestic Animals* 46, 45–48. doi: 10.1111/j.1439-0531.2011.01836.x.

Waberski, D., Riesenbeck, A., Schulze, M., Weitze, K. F., and Johnson, L. (2019). Application of preserved boar semen for artificial insemination: Past, present and future challenges. *Theriogenology* 137, 2–7. doi: 10.1016/j.theriogenology.2019.05.030.

Wagner, A., Ekhlasi-Hundrieser, M., Hettel, C., Petrunkina, A., Waberski, D., Nimtz, M., et al. (2002). Carbohydrate-based interactions of oviductal sperm reservoir formation-studies in the pig. *Mol Reprod Dev* 61, 249–57. doi: 10.1002/mrd.1154.

Wang, G., Guo, Y., Zhou, T., Shi, X., Yu, J., Yang, Y., et al. (2013). In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics* 79, 114–122. doi: 10.1016/j.jprot.2012.12.008.

Wang, M., and Su, P. (2018). The role of the Fas/FasL signaling pathway in environmental toxicant-induced testicular cell apoptosis: An update. *Syst Biol Reprod Med* 64, 93–102. doi: 10.1080/19396368.2017.1422046.

Wassarman, P. M. (1999). Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell* 96, 175–183. doi: 10.1016/S0092-8674(00)80558-9.

Waterhouse, K. E., Hofmo, P. O., Tverdal, A., and Miller, R. R. (2006). Within and between breed differences in freezing tolerance and plasma membrane fatty

acid composition of boar sperm. *Reproduction* 131, 887–894. doi: 10.1530/rep.1.01049.

Wei, Z., Yu, T., Wang, J., Wang, C., Liu, X., Han, Z., et al. (2020). Swine sperm induces neutrophil extracellular traps that entangle sperm and embryos. *Reproduction* 160, 217–225. doi: 10.1530/REP-19-0327.

Win, S., Than, T. A., and Kaplowitz, N. (2018). The regulation of JNK signaling pathways in cell death through the interplay with mitochondrial SAB and upstream post-translational effects. *Int J Mol Sci* 19, 1–14. doi: 10.3390/ijms19113657.

World Health Organization (2021). *WHO laboratory manual for the examination and processing of human semen, Sixth Edition*. Geneva: World Health Organization.

Wu, J. H., and Batist, G. (2013). Glutathione and glutathione analogues; Therapeutic potentials. *Biochim Biophys Acta* 1830, 3350–3353. doi: 10.1016/j.bbagen.2012.11.016.

Yadav, S. (2017). Multi-omics and male infertility status integration and future prospects. *Frontiers in Bioscience* 9, 493. doi: 10.2741/s493.

Yeste, M. (2015). Recent Advances in Boar Sperm Cryopreservation: State of the Art and Current Perspectives. *Reprod Domest Anim* 50 Suppl 2, 71–9. doi: 10.1111/rda.12569.

Yeste, M. (2016). Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology* 85, 47–64. doi: 10.1016/j.theriogenology.2015.09.047.

Yeste, M., Jones, C., Amdani, S. N., and Coward, K. (2017). “Oocyte Activation and Fertilisation: Crucial Contributors from the Sperm and Oocyte,” in, 213–239. doi: 10.1007/978-3-319-44820-6_8.

Yeung, CH., and Cooper, TG. (2002). “Acquisition and development of sperm motility upon maturation in the epididymis,” in *The Epididymis: From Molecules to Clinical Practice*, eds. B. Robaire and BT. Hinton (New York: Kluwer Academic/Plenum Publishers), 417–434.

Yin, Z., Ivanov, V. N., Habelhah, H., Tew, K., and Ronai, Z. (2000). Glutathione S-transferase p elicits protection against H2O2-induced cell death via coordinated regulation of stress kinases. *Cancer Res* 60, 4053–7.

Yoshinaga, K., and Toshimori, K. (2003). Organization and modifications of sperm acrosomal molecules during spermatogenesis and epididymal maturation. *Microsc Res Tech* 61, 39–45. doi: 10.1002/jemt.10315.

Yu, Z., Guo, R., Ge, Y., Ma, J., Guan, J., Li, S., et al. (2003). Gene Expression Profiles in Different Stages of Mouse Spermatogenic Cells During Spermatogenesis. *Biol Reprod* 69, 37–47. doi: 10.1095/biolreprod.102.012609.

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., et al. (2017). The International Glossary on Infertility and Fertility Care, 2017. *Fertil Steril* 108, 393–406. doi: 10.1016/j.fertnstert.2017.06.005.

Zhao, W., Li, Z., Ping, P., Wang, G., Yuan, X., and Sun, F. (2018). Outer dense fibers stabilize the axoneme to maintain sperm motility. *J Cell Mol Med* 22, 1755–1768. doi: 10.1111/jcmm.13457.

Ziebe, S., and Devroey, P. (2008). Assisted reproductive technologies are an integrated part of national strategies addressing demographic and reproductive challenges. *Hum Reprod Update* 14, 583–592. doi: 10.1093/humupd/dmn038.

Zigo, M., Maňásková-Postlerová, P., Zuidema, D., Kerns, K., Jonáková, V., Tůmová, L., et al. (2020). Porcine model for the study of sperm capacitation, fertilization and male fertility. *Cell Tissue Res* 380, 237–262. doi: 10.1007/s00441-020-03181-1.