Universitat de Girona

PHYSIOLOGICAL ROLE OF SPERM AND SEMINAL PLASMA PROTEINS ALONG THE FEMALE REPRODUCTIVE TRACT IN LIVESTOCKa

Sandra Recuero Cobos

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DOCTORAL THESIS

Physiological role of sperm and seminal plasma proteins along the female reproductive tract in livestock

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Supervised by:
Dr. Marc Yeste Oliveras
Dr. Beatriz Fernández Fuertes

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Marc Yeste Oliveras, Professor Agregat de l'Àrea de Biologia Cel·lular del Departament de Biologia de la Universitat de Girona.

Beatriz Fernández Fuertes, Investigadora del programa Juan de la Cierva Incorporación del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA).

CERTIFIQUEM:

Que la tesi titulada **"Physiological role of sperm and seminal plasma proteins along the female reproductive tract in livestock",** presentada per Sandra Recuero Cobos per optar al grau de Doctor per la Universitat de Girona, s'ha realitzat sota la nostra direcció i, considerant-la acabada, autoritzem la seva presentació perquè sigui jutjada per la Comissió corresponent.

I, perquè així consti als efectes oportuns, signem aquest document.

Dr. Marc Yeste Oliveras Dra. Beatriz Fernández Fuertes

Girona, 8 de febrer del 2022.

Tatakae

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List of Publications

This Dissertation is presented as compendium of four publications:

Paper I:

Noto, F., Recuero, S., Valencia, J., Saporito, B., Robbe, D., Bonet, S., Carluccio, A., & Yeste, M. (2021) **Inhibition of potassium channels affects the ability of pig spermatozoa to elicit capacitation and trigger the acrosome exocytosis induced by progesterone**. *International Journal of Molecular Sciences*, 22(4), 1–17 (doi: 10.3390/ijms22041992).

- **Impact factor:** 5.924
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- **Category Quartile:** Q1

Paper II:

Recuero, S., Delgado-Bermúdez, A., Mateo-Otero, Y., Garcia-Bonavila, E., Llavanera, M., & Yeste, M. (2021) **Parkinson Disease Protein 7 (PARK7) is related to the ability of mammalian sperm to undergo in vitro capacitation**. *International Journal of Molecular Sciences*, 22(19) (doi: 10.3390/ijms221910804).

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Paper III:

Pavaneli, A. P. P., Recuero, S., Chaves, B. R., Garcia-Bonavila, E., Llavanera, M., Pinart, E., Bonet, S., De Andrade, A. F. C., & Yeste, M. (2020) **The presence of seminal plasma during liquid storage of pig spermatozoa at 17°c modulates their ability to elicit in vitro capacitation and trigger acrosomal exocytosis**. *International Journal of Molecular Sciences*, 21(12) (doi: 10.3390/ijms21124520).

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Paper IV:

Recuero, S., Sánchez, J. M., Mateo-Otero, Y., Bagés-Arnal, S., McDonald, M., Behura, S. K., Spencer, T. E., Kenny, D. A., Yeste, M., Lonergan, P., & Fernandez-Fuertes, B. (2020) **Mating to intact, but not vasectomized, males elicits changes in the endometrial transcriptome: insights from the bovine model**. *Frontiers in Cell and Developmental Biology*, 8 (doi: 10.3389/fcell.2020.00547)

- **Impact Factor:** 6.684
- **JCR Category:** Developmental Biology
- **Category Quartile:** Q1

List of Abbreviations

ACTB	Actin Cytoplasmic 1
AG	Accessory sex glands
AI	Artificial insemination
AKR1C4	3α-hydroxysteroid dehydrogenase
ALH	Amplitude of lateral head displacement
AQN-1	Alanine–Glutamine–Asparagine-1
AQN-3	Alanine–Glutamine–Asparagine-3
AR	Acrosome reaction
aSFP	Acidic seminal fluid protein
ATP	Adenosine triphosphate
AWN	Alanine–Tryptophan–Asparagine
BCF	Beat-cross frequency
BLA-DQB	Bovine lymphocyte antigen class II, DQ beta
BLAST	Basic local alignment search tool
BOLA-DQB	Bovine lymphocyte antigen class II, DQ beta
BSA	Bovine serum albumin
BSP-1	Binder of sperm protein-1
BSP-3	Binder of sperm protein-3
BSP-30 KDa	Bovine seminal plasma protein-30 KDa
BSP-5	Binder of sperm protein-5
BSP-A1/A2	Bovine seminal plasma protein A1/A2
BSP-A3	Bovine seminal plasma protein-A3
C4BPA	Complement component 4-binding protein alpha
C9	Component 9 of complement system
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAP1	Contraception associated protein-1
CASA	Computer-assisted sperm analysis
CCDC196	Coiled-coil domain containing 196
CD59	Complement regulatory protein CD59
CEBPB	CCAAT enhancer binding protein beta
CEBPD	CCAAT enhancer binding protein delta
CFTR	Cystic fibrosis transmembrane conductance regulator
CL	Corpus luteum
CLSM	Confocal laser-scanning microscope
CM	Capacitating medium
COX2	Eicosanoid-synthesising enzyme cyclo-oxygenase-2
CRISP	Cysteine-rich secretory proteins
CSF2	Colony-stimulating factor 2
CSF3	Colony-stimulating factor 3
CX3CL1	C-X3-C motif chemokine ligand 1
CXCL1	C-X-C motif chemokine ligand-1
CXCL10	C-X-C motif chemokine ligand-10
CXCL2	C-X-C motif chemokine ligand-2
DANCE	Forward movement of the head
DAPI	4′,6-diamidino-2-phenylindole
DDT	Dithiothreitol
DEG	Differentially expressed gene
DJ-1	Protein deglycase-1
DNA	Deoxyribonucleic acid
DRLP	Dichroic/Splitter
E2	Oestradiol
EEJ	Electroejaculation

EGF	Epidermal growth factor
EthD-1	3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide
EU	European Union
EV	Electronic volume
FDR	False discovery rate
FSC	Forward scatter
FSH	Follicle-stimulating hormone
GADPH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDP	Gross Domestic Product
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMFI	Geometric mean of fluorescence intensity
GnRH	Gonadotropin-releasing hormone
GPX6	Glutathione peroxidase 6
GSG1L	GSG1 like
GSK3α	Glycogen synthase kinase-3 isoform α
GSK3β	Glycogen synthase kinase-3 isoform β
HCO_3^-	Bicarbonate
HE	Hydroethidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRA	Health Products Regulatory Authority
HRP	Horseradish peroxidase
IFI47	Interferon gamma inducible protein 47
IFNE	Interferon epsilon
IFNT	Interferon-tau
IL17A	Interleukin 17A
IL17F	Interleukin 17F
IL1A	Interleukin-1A
IL1β	Interleukin-1β
IL6	Interleukin-6
IL8	Interleukin-8
ISAC	International Society for Advancement of Cytometry
ISAS	Integrated sperm analysis system
IVC	In vitro capacitation
JC-1_{agg}	5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide aggregates
JC-1_{mon}	5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide monomers
K_{2P}	Tandem pore domain potassium channel
Kca	Calcium-activated potassium channels
KCNB2	Potassium voltage-gated channel subfamily B member 2
KCNE1	Potassium voltage-gated channel subfamily E regulatory subunit 1
KCNK18	Potassium two pore domain channel subfamily K member 18
KCNMA1	Potassium calcium-activated channel subfamily M alpha 1
Kir	Inwardly rectifying potassium channels
Kv	Voltage-gated potassium channels
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LIN	Linearity
M540	Merocyanine-540
MAC	Membrane attack complex
MAD	Mean angular displacement
MCP-1	Monocyte chemoattractant protein-1

MHC	Major histocompatibility complex
MMP	Mitochondrial membrane potential
mRNA	Messenger ribonucleic acid
NBC	Na+/HCO3- cotransporter
NCBI	National Center for Biotechnology Information
OXT	Oxytocin/neurophysin I prepropeptide
P4	Progesterone
PARK7	Parkinson disease protein 7
PAX	Paxilline
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDC-109	Bovine seminal plasma protein A1/A2
PGE	Prostaglandin E
PGES	Prostaglandin E synthase
PGF2α	Prostaglandin 2Fα
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Phospholipase A2
PLA2G10	Group 10 secretory phospholipase A2
PMOT	Progressive motility
PMSF	Phenylmethanesulfonyl fluoride
PNA-FITC	Peanut agglutinin conjugated with fluorescein isothiocyanate
PON-1	Paraxonase-1
PPIA	Peptidyl-Prolyl Cis-Trans Isomerase A
PRID	8-day intravaginal device
PRSS2	Serine protease 2
PSF	Pre-sperm fraction
PSP-I	Porcine seminal plasma protein-I
PSP-II	Porcine seminal plasma protein-II
PSRF	Post-sperm-rich fraction
PTGS2	Prostaglandin-endoperoxide synthase 2
p-Tyr-GSK3α	Tyrosine-phosphorylated GSK3α
p-Tyr-GSK3β	Tyrosine-phosphorylated GSK3β
PVDF	Polyvinylidene fluoride membranes
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL18	60S Ribosomal Protein L18
RT-qPCR	Real-time quantitative polymerase chain reaction
sAC-PKA	Soluble adenylyl-cyclase, PKA-dependent signalling pathway
sACY	Soluble adenyl cyclase
SCARA5	Scavenger receptor class A, member 5
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser21	Serine residue 21
Ser9	Serine residue 9
SLC24A2	Solute carrier family 24 member 2
SLO1	Potassium channel member of SLO family
SLO3	Potassium channel member of SLO family
SNARE	Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor
SOD1	Superoxide dismutase 1
SP	Seminal plasma

SP1	Motile sperm subpopulation 1
SP2	Motile sperm subpopulation 2
SP3	Motile sperm subpopulation 3
SP22	Sperm protein 22
SRF	Sperm-rich fraction
SSC	Side scatter
STR	Straightness
TBS	Tris-buffered saline
TGFβ	Transforming growth factor beta
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TMOT	Total motility
TNF	Tumor necrosis factor
TNFA	Tumor necrosis factor alpha
Tyr216	Tyrosine residue 216
Tyr279	Tyrosine residue 279
UGT2A1	UDP glucuronosyltransferase family 2-member A1 complex locus
VAP	Average path velocity
VCAM-1	Vascular cell adhesion molecule 1
VCL	Curvilinear velocity
VSL	Straight linear velocity
WOB	Wobble coefficient
ZP	Zona pellucida

List of Figures

Introduction

Paper I

Supplementary Material

Paper II

Supplementary Material

Paper III

Supplementary Material

List of Tables

General contents

Abstract / Resum / Resumen

Abstract

Semen is composed by sperm and seminal plasma, the fluid that mixes with those cells during ejaculation. Physiological processes that take place after ejaculation are mediated by proteins, including sperm capacitation and modulation of maternal tract environment by paternal factors. Understanding the regulation of these processes is crucial to improve the reproductive performance of livestock, such as pigs and cattle. In this context, the present Dissertation sought to investigate the regulatory role of sperm and seminal plasma proteins in sperm capacitation and interaction with female tract. For this purpose, the first three studies were focused on *in vitro* capacitation of pig sperm. In these, sperm were incubated in capacitation medium, and subsequently, sperm motility and a variety of cell parameters indicative of capacitation (plasma and acrosome membrane integrity, lipid membrane disorder, intracellular calcium levels, and mitochondrial membrane potential) were evaluated by flow cytometry. Analyses were performed after 0, 120, and 240 min of incubation, as well as 5, 30, and 60 min after induction of acrosome exocytosis by progesterone exposure. On the one hand, the first study evaluated the impact of blocking potassium channels on these parameters. The second one aimed to study whether relative levels of PARK7, measured by Western Blot, as well as its localisation assessed by immunofluorescence, were related to the indicated parameters, in addition to intracellular levels of peroxides and superoxides. The third study of this Dissertation investigated how prolonged exposure (48 or 72 h) to seminal plasma (15% or 30%) during liquid storage affects the ability of pig sperm to elicit capacitation by evaluating the aforementioned parameters and the tyrosine phosphorylation levels of GSK3α/β by Western Blot. Finally, the fourth study sought to elucidate the effects of seminal plasma and/or sperm on the modulation of the female tract environment in species with intravaginal ejaculation, using the cattle as a model. To this end, heifers were mated to intact or vasectomised bulls and, 24 h after mating, changes in the endometrial transcriptome were analysed by RNA sequencing. Additionally, the expression of a selection of genes (*IL6, IL1A, IL8, TNFA, PLA2G10, CX3CL1, C4BPA, PRSS2, BLA-DQB*, and *CEBPD*) was also assessed by real-time PCR in vagina and oviduct tissues. Results obtained in this Dissertation indicated that potassium channels play a key role in the regulation of mitochondrial activity, sperm motility, and intracellular calcium levels during capacitation. In addition, lower mortality, higher tolerance to superoxide generation, and a more progressive capacitation process were observed in sperm with higher relative levels of PARK7. Moreover, seminal plasma exposure reduced the capacity of sperm to undergo acrosomal exocytosis, lower mitochondrial activity, and a

decrease in phosphorylation levels of GSK3α/β. With regard to the effects of seminal plasma on the female tract, whereas no changes after mating to vasectomised bulls were observed, mating to intact bulls resulted in 24 and 22 differentially expressed genes compared to unmated animals and heifers mated to vasectomised bulls, respectively. Despite the lack of differences between treatments, mating to intact and vasectomised bulls induced an increase in the expression of *IL1A* and *TNFA* in the vagina compared with the oviduct. Taken collectively, it can be concluded that sperm proteins, as well as those present in the seminal plasma, play a crucial role in the modulation of sperm capacitation. Conversely, in species with intravaginal ejaculation, sperm seem to play a more relevant function in the modulation of the female tract environment. It is, however, needed to determine whether this function is performed by intrinsic proteins or those acquired from seminal plasma. Although further studies are still needed to fully understand the modulatory role of sperm and seminal plasma proteins in order to give them a practical application, the results obtained in this Dissertation positively contribute with new insights that could be useful in assisted reproduction field.

Resum

El semen es composa d'espermatozoides i plasma seminal, que es barregen quan durant l'ejaculació. Els processos fisiològics que tenen lloc després de l'ejaculació, que inclou la capacitació espermàtica i la modulació de l'ambient del tracte matern per part dels factors paterns, són mediats per proteïnes. Comprendre la regulació d'aquests processos és crucial per la millora del rendiment reproductiu en espècies d'interès ramader com la porcina o bovina. Per aquest motiu, aquesta Tesi Doctoral va tenir per objectiu investigar el paper regulador de les proteïnes espermàtiques i del plasma seminal sobre la capacitació espermàtica i la interacció amb el tracte reproductor femení. Amb aquest objectiu, el tres primers estudis es van enfocar en la capacitació *in vitro* del semen porcí. En aquests estudis, els espermatozoides es van incubar en medi de capacitació i es va avaluar la motilitat espermàtica i diferents paràmetres cel·lulars indicatius de capacitació (integritat de la membrana plasmàtica i acrosòmica, desordre lipídic de membrana, nivells de calci intracel·lular i potencial de membrana mitocondrial). Les anàlisis es van realitzar després de 0, 120 i 240 min d'incubació, així com 5, 30 i 60 min després d'afegir la progesterona per tal d'induir la reacció acrosòmica. Per una banda, el primer estudi va avaluar l'impacte del bloqueig dels canals de potassi sobre aquests paràmetres. El segon va estudiar la relació dels nivells relatius de la proteïna PARK7, avaluats mitjançant Western Blot, així com la seva localització mitjançant immunofluorescència, amb els paràmetres mencionats prèviament, a més dels nivells intracel·lulars de peròxids i superòxids. El tercer estudi va investigar els efectes de l'exposició prolongada (48 o 72 h) a plasma seminal (15% o 30%) durant la refrigeració (17°C) del semen porcí sobre la capacitació, avaluant els paràmetres mencionats, a més d'analitzar els nivells de fosforilació de tirosines de la proteïna GSK3α/β mitjançant Western Blot. Finalment, el quart estudia va tenir l'objectiu de dilucidar els efectes del plasma seminal i/o l'espermatozoide en la modulació de l'ambient del tracte femení en espècies amb ejaculació vaginal, utilitzant l'espècie bovina com a model. Amb aquesta finalitat, les vedelles van ser muntades per toros intactes o vasectomitzats i 24 h després es van analitzar els canvis en el transcriptoma de l'endometri mitjançant la seqüenciació de RNA. A més, es van analitzar, mitjançant PCR a temps real, els nivells d'expressió d'una selecció de gens (*IL6, IL1A, IL8, TNFA, PLA2G10, CX3CL1, PRSS2, BLA-DQB* i *CEBPD*) a la vagina i l'oviducte. Els resultats obtinguts en aquesta Tesi Doctoral van indicar que els canals de potassi juguen un paper fonamental sobre la regulació de l'activitat mitocondrial, motilitat espermàtica i nivells de calci intracel·lular durant la capacitació. A més, també es va observar una menor mortalitat, major tolerància a la

generació de superòxids i un procés de capacitació més progressiu en espermatozoides amb majors nivells relatius de PARK7. D'altra banda, l'exposició a plasma seminal va resultar en una menor capacitat de l'espermatozoide de dur a terme l'exocitosi de l'acrosoma, una menor activitat mitocondrial i una disminució dels nivells de fosforilació de GSK3α/β. Respecte als efectes sobre el tracte femení, la munta amb toros vasectomitzats no va produir cap canvi sobre el transcriptoma endometrial, mentre que la munta amb toros intactes va resultar en 24 i 22 gens diferencialment expressats comparat amb els animals muntats per toros vasectomitzats i els no muntats, respectivament. Per una altra banda, tot i que no es van observar diferències entre tractaments, la munta amb toros intactes i vasectomitzats va provocar un augment de l'expressió de *IL1A* i *TNFA* a la vagina comparat amb l'oviducte. En conjunt, es pot concloure que tant les proteïnes de l'espermatozoide, com les que es troben al plasma seminal, juguen un paper essencial en la modulació de la capacitació. En canvi, en espècies amb ejaculació vaginal, sembla ser l'espermatozoide el que juga un paper rellevant en la modulació de l'ambient del tracte reproductor femení. No obstant això, és necessari determinar si son les seves proteïnes intrínseques o les adquirides del plasma seminal les que realitzen aquesta funció. Tot i que encara son necessaris altres estudis per tal d'aprofundir en el paper modulador de les proteïnes espermàtiques i del plasma seminal i poder donar-los-hi una aplicació pràctica, els resultats obtinguts en aquesta Tesi Doctoral contribuiran positivament en aquest àmbit, aportant nous coneixements que poden ser d'utilitat pel camp de la reproducció assistida.

Resumen

El semen se compone de espermatozoides y plasma seminal, que se mezclan durante la eyaculación. Los procesos fisiológicos que tienen lugar después de la eyaculación, incluyendo la capacitación espermática y la modulación del ambiente del tracto materno por parte de factores paternos, son mediados por proteínas. Comprender la regulación de estos procesos es crucial para la mejora del rendimiento reproductivo en especies de interés productivo como el porcino y el bovino. Por ello, la presente Tesis Doctoral ha pretendido investigar el papel regulador de las proteínas espermáticas y del plasma seminal en la capacitación espermática e interacción con el tracto reproductor femenino. Con este objetivo, los tres primeros estudios se enfocaron en la capacitación *in vitro* del semen porcino. En estos estudios los espermatozoides se incubaron en medio de capacitación y se evaluaron la motilidad espermática y distintos parámetros celulares indicativos de capacitación (integridad de la membrana plasmática y acrosómica, desorden lipídico de membrana, niveles de calcio intracelular y potencial de membrana mitocondrial) mediante citometría de flujo. Los análisis se realizaron tras 0, 120 y 240 min de incubación, así como 5, 30 y 60 min después de añadir la progesterona para inducir la reacción acrosómica. Por una parte, el primer estudio evaluó el impacto del bloqueo de los canales de potasio sobre estos parámetros. El segundo estudió la relación de los niveles relativos de la proteína PARK7, evaluados mediante Western Blot, así como su localización mediante inmunofluorescencia, con los parámetros mencionados previamente, además de los niveles intracelulares de peróxidos y superóxidos. El tercer estudio investigó los efectos de una exposición prolongada (48 o 72 h) al plasma seminal (15% o 30%) durante la refrigeración (17°C) del semen porcino sobre su posterior capacitación, evaluando los parámetros mencionados, además de analizar los niveles de fosforilación de tirosinas de la proteína GSK3α/β mediante Western Blot. Finalmente, el cuarto estudio tuvo por objetivo esclarecer los efectos del plasma seminal y/o el espermatozoide en la modulación del ambiente del tracto femenino en especies con eyaculación vaginal, usando la especie bovina como modelo. Para ello, las novillas fueron montadas por toros intactos o vasectomizados y 24 h después se analizaron los cambios en el transcriptoma del endometrio mediante secuenciación de RNA. Además, se analizaron, mediante PCR en tiempo real, los niveles de expresión de una selección de genes (*IL6, IL1A, IL8, TNFA, PLA2G10, CX3CL1, C4BPA, PRSS2, BLA-DQB y CEBPD*) en la vagina y el oviducto. Los resultados obtenidos en la presente Tesis Doctoral indicaron que los canales de potasio juegan un papel fundamental en la regulación de la actividad mitocondrial, motilidad

espermática y niveles de calcio intracelular durante la capacitación. Asimismo, también se observó una menor mortalidad, mayor tolerancia a la generación de superóxidos y un proceso de capacitación más progresivo en los espermatozoides con mayores niveles relativos de PARK7. Por otra parte, la exposición al plasma seminal resultó en una menor capacidad del espermatozoide para llevar a cabo la exocitosis del acrosoma, una menor actividad mitocondrial y una disminución de los niveles de fosforilación de GSK3α/β. Respecto a los efectos en el tracto femenino, la monta con toros vasectomizados no produjo ningún cambio sobre el transcriptoma endometrial, mientras que la monta con toros intactos resultó en 24 y 22 genes diferencialmente expresados comparado con los animales montados por toros vasectomizados y los no montados, respectivamente. Aunque no se observaron diferencias entre tratamientos, la monta con toros intactos y vasectomizados provocó un aumento en la expresión de *IL1A* y *TNFA* en la vagina comparado con el oviducto. En conjunto, se puede concluir que tanto las proteínas del espermatozoide, como las que se encuentran en el plasma seminal, juegan un papel esencial en la modulación de la capacitación. En cambio, en especies con eyaculación vaginal, parece ser el espermatozoide el que juega un papel relevante en la modulación del ambiente del tracto femenino. No obstante, es necesario determinar si son sus proteínas intrínsecas o las adquiridas del plasma seminal las que llevan a cabo esta función. Aunque aún son necesarios otros estudios para profundizar en el papel modulador de las proteínas espermáticas y del plasma seminal y poder darles una aplicación práctica, los resultados obtenidos en la presente Tesis Doctoral van a contribuir positivamente aportando nuevos conocimientos que pueden ser de utilidad para el campo de la reproducción asistida.

Introduction

1. Livestock industry

Primary agricultural production is a big and important sector in the European Union (EU), as it is essential for the downstream food and beverages processing industry. Indeed, in 2019, the contribution of the agricultural industry to the EU economy was EUR 176.4 billion, which represented 1.3% of the overall Gross Domestic Product (GDP) (Eurostat, 2020). In that year, the value of the total output produced by the agricultural industry in the EU was an estimated EUR 418.0 billion, a substantial part of which corresponded to the livestock sector (38.6%; EUR 161.4 billion; Figure 1). Related to that sector, the population of livestock in the EU comprised 143 million pigs, 77 million cattle, and 74 million sheep and goats in 2019 (Eurostat, 2020).

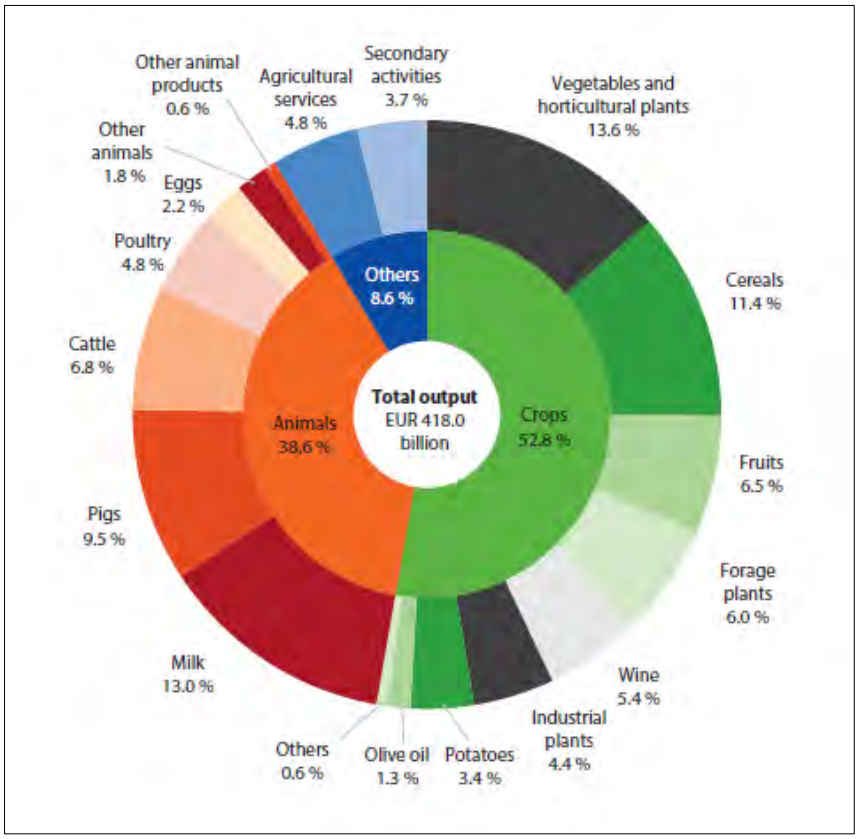

Figure 1: Output of the agricultural industry (% of total output), EU-27, 2019. Source: Eurostat 2020.

Swine, together with cattle, are the most important species in the context of the livestock industry. On the one hand, pigs represent 9.5% of the total output of the agricultural industry in the EU (*Figure 1*), with a production of 22.8 million tonnes in 2019 (Eurostat, 2020). On the other hand, beef and milk represent 6.8% and 13.0%, respectively, of the total output of the European agricultural industry (*Figure 1*). In 2019, the annual production of beef reached 6.9 million tonnes, whereas that of raw milk in EU farms was 158.2 million tonnes, 96% of which came from cows (*Figure 2*). As shown in

Figure 2, the vast majority of milk produced is intended for fresh and manufactured products (Eurostat, 2020).

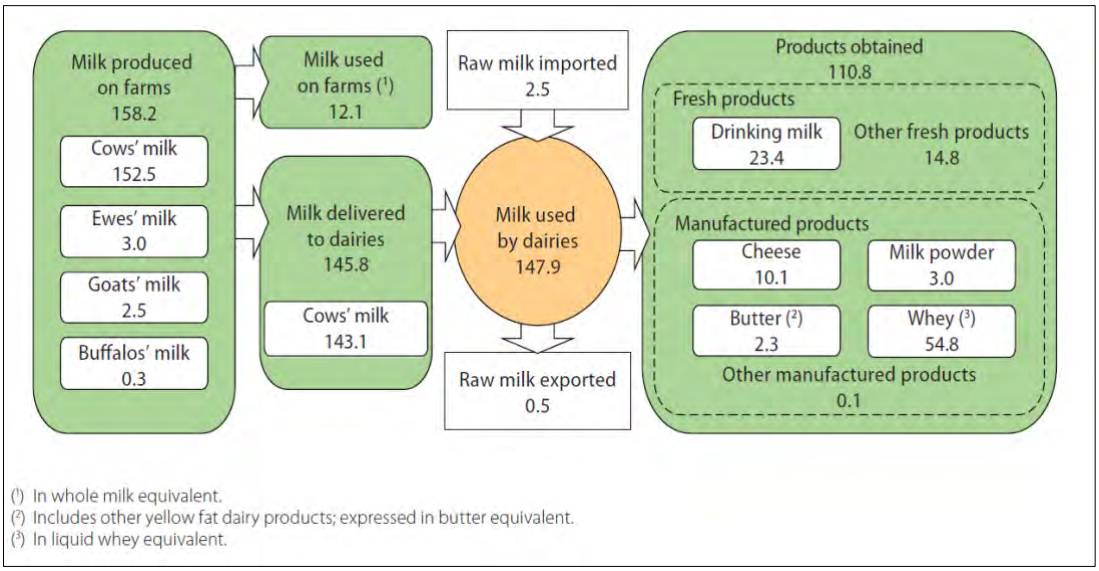

Figure 2: Production and use of milk (million tonnes), EU-27, 2019. Source: Eurostat 2020.

At a national level, these two species are also of economic relevance in livestock industry. Spain is the second producer of pork meat in the EU (Eurostat, 2020) and figures released by the Spanish Ministry of Agriculture, Fisheries and Food show our country in the fourth position of worldwide pig meat production, after China, USA, and Germany (MAPA, 2020a). In 2020, the production of pork had a value of EUR 8,655.5 million, which represented 42.8% of total value of livestock production. During this year, the production of pig meat increased 8.24%, reaching 5 million tonnes, and in addition, exportations also experimented an increase of 20.6% compared to the previous year (MAPA, 2020a).

Total production of raw cow milk in Spain was 7,221,934 tonnes in 2019, representing an increase of 1.4% in comparison to 2018; the same increase from 2017 to 2018 was observed (MAPA, 2020b). On the other hand, beef production had a value of EUR 3,092.6 million in 2020, with a production of 677,296 tonnes that represented 15.3% of the total value of livestock sector in Spain (MAPA, 2020c). While it seems that the consumption of beef in our country is decreasing, it is increasing in other countries, as reflected in the value of exports which increased by 4.4% from 2018 to 2019 (MAPA, 2020c).

Considering the importance and economic relevance of porcine and bovine species in our country and EU, ensuring proper breeding and productivity is crucial to the livestock industry and the European economy.

2. Anatomy and physiology of the male reproductive tract

The male reproductive system produces millions of sperm daily, through an organized process known as spermatogenesis. An appropriate hormonal control is essential not only to regulate this process, but also for the secretion of accessory fluids that accompany sperm during and after ejaculation (Knobil & Neill, 2015). In bovine and porcine species, the anatomic components of the male reproductive tract are the two testis, spermatic cord, scrotum, deferent ducts, two epididymides, penis, and the accessory sex glands (AG), the latter including: the ampulla (in bulls), the bulbourethral or Cowper's glands, the seminal vesicles, and the prostate (*Figure 3*, Senger, 2003).

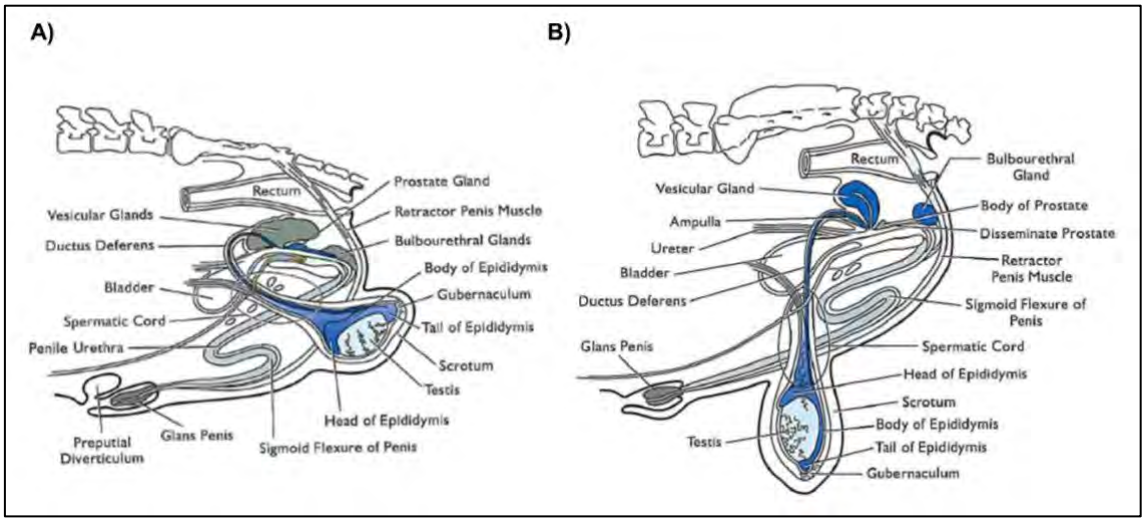

*Figure 3: Diagram of the boar (**A**) and bull (**B**) reproductive tract (adapted from Senger, 2003).*

The main functions of the testis are spermatogenesis and production of steroidal hormones, mainly testosterone. The testes are ovoid-like organs that descend outside of the abdominal wall, connected to the body by the spermatic cord and housed and supported by the scrotum, which is crucial to maintain a suitable temperature for sperm production (Senger, 2003). The testis consists of seminiferous tubules and the interstitial tissue between them, surrounded by a thick capsule of connective tissue. The interstitial tissue is composed of connective tissue, nerves, blood and lymph vessels, and clusters of Leydig cells, which are responsible for steroid hormone synthesis and secretion (Bonet et al., 2013). The seminiferous tubules contain the germinal or seminiferous epithelium, which consists of developing germ cells and Sertoli cells that support sperm production. In the basal membrane of the epithelium, spermatogonia, the undifferentiated male germ

cells, are located. The differentiation of germ cells takes place along the epithelial layer and sperm are finally released into the lumen of the tubule (Hopper, 2015; Senger, 2003). Spermatogonia undergo diverse mitotic divisions forming primary spermatocytes. Then, meiotic divisions occur, transforming from diploid to haploid cells and forming secondary spermatocytes. Each secondary spermatocyte divides into two smaller cells called spermatids. Spermatids experience a set of changes to finally become completely differentiated spermatozoa, a process known as spermiogenesis, which includes the formation of the acrosome, the head, the midpiece, and the tail (Ball & Peters, 2004; Bonet et al., 2013).

The regulation of spermatogenesis and testosterone production are under control of hormones from two regions of the brain: the hypothalamus and the adenohypophysis or anterior pituitary gland. The gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and induces the secretion of the follicular stimulating hormone (FSH) and luteinising hormone (LH) by the anterior pituitary gland. Pulsatile release of LH controls testosterone production by Leydig cells. In turn, elevated levels of testosterone inhibit GnRH and thus, FSH and LH secretion. The process of spermatogenesis is mainly under FSH control, which acts on Sertoli cells, but proper levels of testosterone are also required (Ball & Peters, 2004; Pinart & Puigmulé, 2013).

Although spermatozoa are fully differentiated once they exit the testis, motility and fertilising ability have not yet been acquired. Sperm pass through a set of interconnected tubules, the rete testis, and then through the efferent ducts in order to enter the epididymis, where sperm maturation takes place (i.e. acquisition of fertilising ability and motility; Senger, 2003). In the epididymis, three different anatomic regions can be distinguished: the caput or head, the corpus or body, and the cauda or tail. Histologically, this organ is composed of a pseudostratified epithelium with secretory and absorptive activity, surrounded by a smooth muscular layer, which facilitates sperm transport along the duct and expels the semen upon ejaculation (Bonet et al., 2013; Senger, 2003).

Sperm enter the epididymis highly diluted in the fluid derived from the rete testis. Along the epididymal transit, the composition of this fluid (ions, low weight molecules, lipids, proteins, and enzymes), as well as sperm concentration, changes along the separate regions in response to different processes, including sperm metabolic activity, secretion and reabsorption of different substances by epithelial cells, especially in the head and the body regions of the epididymis (Bonet et al., 2013; Senger, 2003). During epididymal maturation, endogenous sperm proteins undergo post-translational modifications and the sperm surface is remodelled by the removal and adsorption of

sperm-coating proteins, as well as changes in external sugars and lipids (Gervasi & Visconti, 2017). Since sperm are transcriptionally and translationally inactive cells, it is an essential step to acquire proteins involved in oocyte binding and recognition and, thus, fertilising ability (Gadella, 2017). As mentioned above, during epididymal passage sperm also acquire capacity for motility (Bonet et al., 2013). Despite this, activation of sperm motility takes place after ejaculation, as a consequence amongst other, of changes in pH and activation of signalling pathways involving cAMP and protein tyrosine phosphorylation (Lindemann & Kanous, 1989).

With an environment suitable for preserving sperm survival in a quiescent state, the epididymal tail is the main site of sperm storage, containing 70% of the total spermatozoa in the excurrent ducts, whilst the vas deferens, the continuation of the epididymis, only contains 2%. The extragonadal reserves include spermatozoa from the head to the tail of the epididymis, although only sperm from the distal section of the tail are ejaculated (Hafez & Hafez, 2000). Finally, the muscle contraction of the tail of epididymis is under the influence of sexual stimulation, inducing the release of sperm from the reproductive tract (Senger, 2003).

Apart from the secretions of the rete testis and epididymis, at the time of ejaculation, spermatozoa are bathed with those derived from the AG (see section 1.5.), whose proper development and function also depend on testosterone activity (Senger, 2003). In bulls, the ductus deferens presents enlargements, as a result of the increase in the mucosa layer, which constitutes the ampulla (Hopper, 2015). In both porcine and bovine species, the seminal vesicles or vesicular glands are the largest glands and the major contributors to the final ejaculate volume. These paired glands, in bulls, are compact and lobulated, whereas in boars they present a larger development (Bonet et al., 2013; Senger, 2003). The prostate gland is located near the bladder and pelvic urethra and comprises two structural forms: the corpus or body and the disseminated prostate, the latter being the major portion of this gland in pigs (Senger, 2003). Paired bilateral bulbourethral glands lie on each side of the pelvic urethra. In bulls, bulbourethral or Cowper's glands are small and ovoid, whereas in boars they are significantly larger and produce a highly viscous secretion (Bonet et al., 2013; Senger, 2003). Finally, all these glands discharge their secretions to the urethra. The fluid derived from these glands, together with the secretions from the epididymis and testis, is known as seminal plasma (SP) and it accompanies spermatozoa during and after ejaculation (Hopper, 2015; Senger, 2003).

3. Anatomy and physiology of the female reproductive tract

The main functions of the female reproductive system are the production of oocytes, their transport towards the site of fertilisation, and the support of embryo development, gestation and parturition. The reproductive tract of cows and sows consist of the external genitalia or vulva, the vagina, the cervix, the uterus, the oviducts and the ovaries (*Figure 4*, Senger, 2003). In general, the female reproductive tract presents a tubular structure organized in concentric layers: (i) the mucosa is the inner layer, consisting of the epithelium lining the lumen of the reproductive tract; (ii) the submucosa; (iii) the muscular layer or muscularis; and (iv) the serosa layer covering the surface of reproductive tract (Senger, 2003).

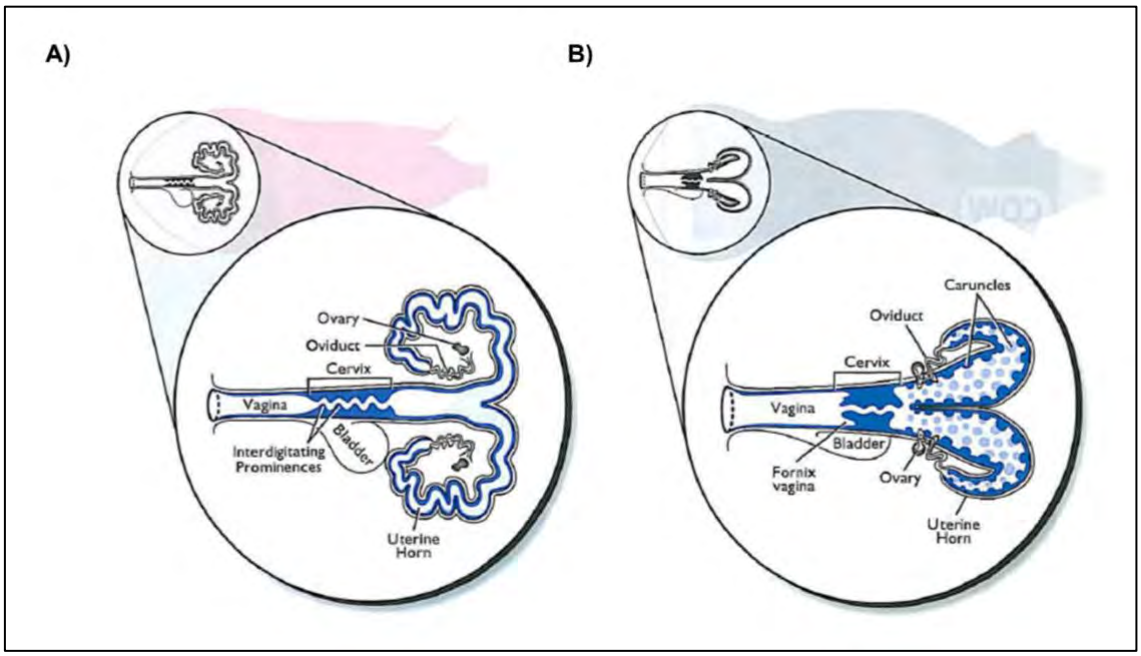

*Figure 4: Schematic illustration of sow's (**A**) and cow's (**B**) reproductive tracts (adapted from Senger, 2003)*

The vagina is the copulatory organ, which coincides with the site of ejaculate deposition in the cow (Hawk, 1983). By contrast, in porcine species ejaculation takes place in the narrow canal of the cervix, which connects the vagina with the uterine body (Rodríguez-Martínez et al., 2005). The cervix presents a lubricated thick wall and a narrow lumen with multiple folds. Epithelial cells of the cervix secrete a mucus, the viscosity of which varies during the reproductive cycle, causing periodic changes in the permeability to sperm cells through the cervical canal. During ovulation and sexual receptivity, the mucus presents the optimal properties for sperm transport (Hafez & Hafez, 2000). On the other hand, during luteal phase, the cervical mucosa produces a highly viscous mucus, isolating the uterus from the external environment and preventing the entry of pathogens (Senger, 2003; Turner, 2014).

The uterus consists of a common body, which is relatively small, and two longer horns, where embryo implantation and development takes place (Hopper, 2015; Senger, 2003). The endometrium, which comprises the mucosa and submucosa layers, contain glands, whose secretions undergo changes during the oestrous cycle and its composition is believed to be crucial to maintaining embryo viability during the preimplantation period (Hopper, 2015; Senger, 2003). In contrast to sows, the endometrium of cows is divided into two areas: caruncular and intercaruncular. Whereas uterine glands are located in the intercaruncular areas, the caruncles are non-glandular, small highly vascularised protuberances, which are the attachment sites to the placenta during pregnancy (Senger, 2003).

The oviduct can be divided into three anatomical regions: the infundibulum, the ampulla and the isthmus. The isthmus directly connects with the horn of the uterus; before fertilisation, a sperm reservoir is stablished in this region (Rodriguez-Martinez, 2007; Suarez, 2007). Fertilisation takes place in the ampulla, the longest and most dilated region of the oviduct (Hunter, 2012). The infundibulum is the ovarian end of the oviduct and presents structures called fimbriae. These projections increase the contact surface in order to capture the oocyte once ovulated (Senger, 2003).

Cows and sows possess two ovaries located in the abdominal cavity, near the terminal part of the oviduct (*Figure 4*). The ovary is the primary female reproductive organ and consists of follicles containing oocytes at different stages of maturation. Apart from producing female gametes, the synthesis and secretion of oestradiol (E2) and progesterone (P4), key reproductive female hormones, take place in the ovary (Senger, 2003).

Under hormonal control, the female reproductive tract undergoes cyclic changes, which is known as the oestrous cycle. The length of the oestrous cycle is approximately 21 – 22 days in cows and 19 – 20 in sows. Whereas the duration of oestrus (sexual receptivity) ranged from 18 to 19 hours in cows and from 48 to 72 in sows; ovulation in cows occurs 10 – 11 hours after the end of the oestrus and 35 – 45 hours from the beginning of the oestrus in sows (Hafez & Hafez, 2000). The oestrous cycle consists of two major phases: the follicular and the luteal. Furthermore, the follicular phase can be divided into proestrus and oestrus, and the luteal phase into metoestrus and dioestrus (*Figure 5*; Senger, 2003):

- Oestrus (Day 0): sexual receptivity
- Metoestrus (Days 1-4): postovulatory period

- Dioestrus (Days 5-18): presence of an active CL
- Pro-oestrus (Days 18-20): period prior to oestrus

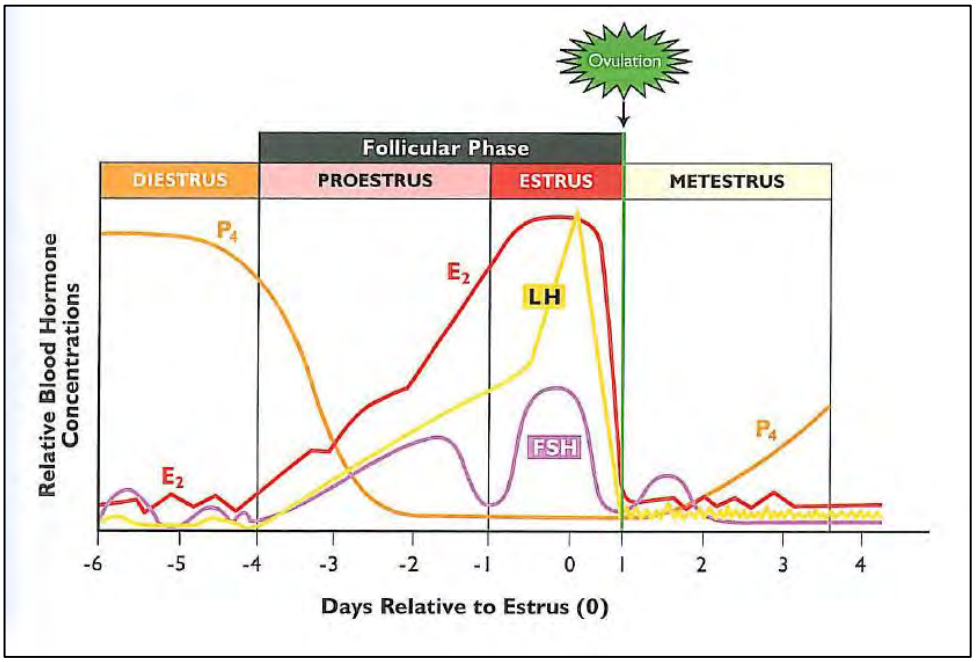

Figure 5: Hormonal changes and phases of the oestrous cycle (Senger, 2003)

During the follicular phase, hypothalamic GnRH induces the secretion of FSH by the anterior pituitary gland, stimulating follicular growth. Growing follicles secrete E2, which triggers the onset of the oestrus behaviour and causes a surge of GnRH, which in turn, induces the LH peak that triggers ovulation. In the following 2 – 4 days after ovulation, at the beginning of the luteal phase, under the stimulus of LH, the remaining follicular cells proliferate and become luteal cells, forming the corpus luteum (CL). This structure is responsible for P4 secretion, which blocks the secretion of GnRH and LH, preventing further follicular maturation. Besides this, the main role of P4 is to prepare the uterus for embryo reception. P4 stimulates the activity of endometrial glands, the secretory products of which are responsible for creating an appropriate environment that supports embryo development prior to implantation (Forde et al., 2009; Jindal et al., 1996). At the end of the luteal phase, around days 17 – 18, the secretion of uterine prostaglandin F2α ($PGF_{2\alpha}$), which is responsible for CL regression or luteolysis in non-pregnant cows or sows, takes place. In turn, P4 concentration decreases to basal levels, allowing GnRH secretion and the events that lead to the onset of a new oestrus cycle. If pregnancy occurs, the secretion of $PGF_{2\alpha}$ is blocked, thus maintaining elevated levels of P4 to support pregnancy (Ball & Peters, 2004; Senger, 2003).

4. Sperm interaction with female reproductive tract and capacitation

Mammalian sperm, once ejaculated, are unable to fertilise an oocyte, despite being mature and motile. In the 50's, Austin and Chang independently discovered that mammalian sperm must reside and interact with the female reproductive tract to acquire fertilising ability, in a process known as sperm capacitation (Austin, 1951, 1952; Chang, 1951).

4.1. Establishment of the oviductal reservoir

During natural mating, after ejaculate deposition, spermatozoa are exposed to different environments through the female reproductive tract before arriving at the ampullary-isthmic junction, where fertilisation takes place (Hunter, 2012). Whilst semen deposition occurs in the vagina in cattle, the ejaculate is deposited in the narrow cervical canal, near the uterine cavity, in pigs. Notwithstanding, sperm transport along female tract is comparable in both species, including three different phases: (i) fast transport through the uterus, immediately after ejaculate deposition, (ii) sperm reservoir formation in the lower oviduct until ovulation time, and (iii) gradual release from sperm reservoir towards the fertilisation site (Rodriguez-Martinez, 2007).

The main function of the sperm reservoir is to ensure sperm viability and fertilising ability, as well as protect sperm from the reaction of the female immune system and delay sperm capacitation (Rodriguez-Martinez, 2005, 2007). It has also been proposed that storage of sperm in the isthmus may diminish the risk of polyspermic fertilisation, releasing low numbers of sperm cells towards the ampulla (Suarez, 2007).

The formation of the sperm reservoir entails the interaction and adhesion of spermatozoa to the oviductal epithelial cells in the isthmus, near the utero-tubal junction, the region connecting the uterus to the isthmus (Yeste, 2013a). Oligosaccharides present in the surface of both sperm and epithelial cells play a crucial role in the formation of the oviductal reservoir. Sperm binding to epithelial cells is mediated by species-specific mechanisms involving carbohydrate recognition, in which fucose in bovine and mannose residues in porcine play a critical role (Yeste, 2013a). Several surface proteins of the oviductal cells have been shown to be involved in the sperm-epithelial cell interaction, including annexins (Ignotz et al., 2007; Teijeiro et al., 2009) and heat shock protein A8 (Elliott et al., 2009; Holt et al., 2015) in both cattle and pigs, and fibronectin in bovine species (Osycka-Salut et al., 2017). On the other hand, regarding sperm surface, bovine binder of sperm proteins (BSP) -1, -2, -3 (Gwathmey et al., 2003, 2006) and the porcine spermadhesin AQN-1 (Ekhlasi-Hundrieser et al., 2005), which are SP proteins

that bind the sperm plasma membrane upon ejaculation (see also sections 1.5.1. and 1.5.2.) have been identified to participate in the binding to oviductal cells (*Figure 6*).

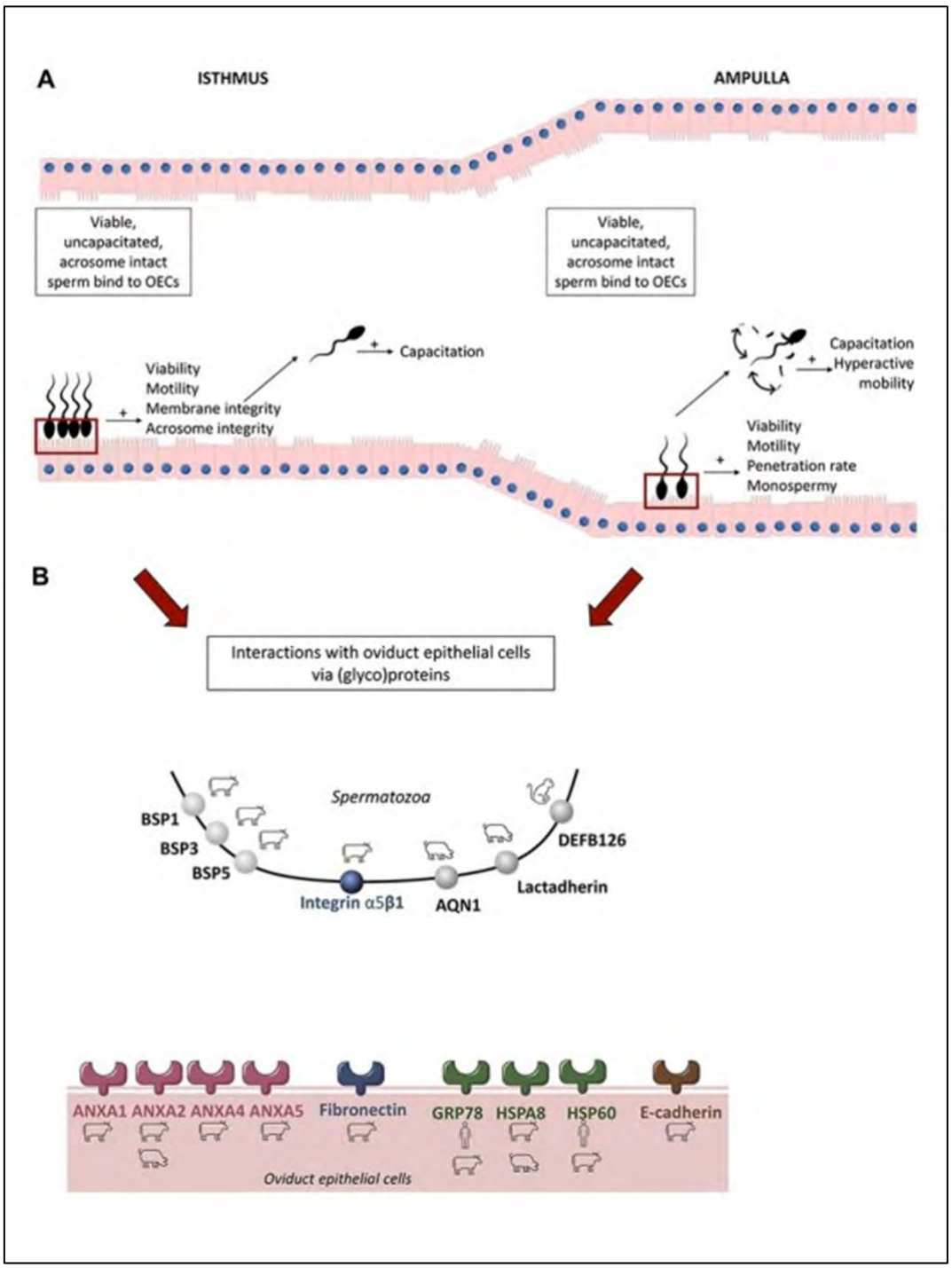

*Figure 6: Events taking place during the sperm passage through the mammalian oviduct (**A**) and mechanisms and proteins involved in the sperm-oviductal cells interaction (**B**) (Mahé et al., 2021).*

The microenvironment of the oviduct provides suitable conditions for gamete support and transport, sperm capacitation, fertilisation and early embryonic development (Yeste, 2013a). The oviductal fluid volume and composition change depending on the stage of the oestrus cycle, influenced by hormonal regulation, along with species-specific

variations (Rodriguez-Martinez, 2007). Around the time of ovulation, sperm are gradually detached from the oviductal reservoir and, during their migration towards ampullary-isthmic junction, when sperm are exposed to increasing concentrations of bicarbonate, capacitation takes place (Rodriguez-Martinez et al., 2001; Tienthai et al., 2004).

4.2. Sperm capacitation

Sperm capacitation is a complex and gradual process in which spermatozoa undergo an assortment of physiological and biochemical changes, including modifications in the composition and fluidity of the plasma membrane (Harrison & Miller, 2000; van Gestel et al., 2005), acrosome surface remodelling (Tsai et al., 2007), hyperactivation of motility (García Herreros et al., 2005; Suarez & Ho, 2003), a significant raise in intracellular Ca^{2+} and pH (Costello et al., 2009; Yeste et al., 2015), and the phosphorylation of certain proteins (Flesch et al., 1999; Tardif et al., 2001). This regulated signal transduction-mediated process gives sperm, once capacitated, the ability to bind and interact with the oocyte vestments (i.e., cumulus cells and the zona pellucida (ZP), a specialized extracellular matrix surrounding the oocyte), and subsequently to trigger the acrosome reaction, an essential step for successful fertilisation that allows gamete membrane fusion (Flesch & Gadella, 2000). Thus, capacitated sperm exhibit, at least, the following characteristics: (i) motility hyperactivation (vigorous and high amplitude flagellar beating), (ii) chemoattractant responsiveness under signal transduction regulation, and (iii) the ability to interact with the oocyte and to undergo acrosome reaction (Zigo et al., 2020).

Sperm capacitation can be performed *in vitro*, by incubating spermatozoa in a species-specific defined medium, mimicking the oviductal environment. In addition to being an essential step to achieve successful *in vitro* fertilisation, inducing capacitation *in vitro* (IVC) has become a valuable technique to unravel the specific molecular events underlying capacitation. It is well established that several molecules are required for successful IVC, including a protein source, which is usually bovine serum albumin (BSA), and an assortment of ions, including bicarbonate (HCO_3^-) and calcium (Ca^{2+}) (Salicioni et al., 2007; Visconti, 2009). BSA is associated with the removal of cholesterol from the plasma membrane, which destabilises the protein-lipid organization of sperm plasmalemma and increases its fluidity for the posterior membrane fusion with the oocyte (Langlais et al., 1988). Nevertheless, the study conducted by Tardif et al., (2003) suggested that porcine sperm can be *in vitro* capacitated in a BSA-free medium or with other non-albumin cholesterol acceptors replacing BSA but need the presence of Ca^{2+} and HCO_3^-, the key effectors of mammalian capacitation (Bailey, 2010). This contrasts

with Chaves et al., (2021) who indicated that, *in vitro* and under an atmosphere of 5% CO_2, BSA rather than HCO_3^- is indispensable for the pig sperm capacitation.

It is widely accepted that capacitation can be divided into two phases: (i) early/fast events, including changes in plasma membrane and sperm motility activation and (ii) late/slow events, which comprises motility hyperactivation, protein phosphorylation, and the ability of sperm to trigger the acrosome reaction (Ickowicz et al., 2012; Visconti, 2009). Despite some controversy existing as to whether early/fast events must be considered as part of the capacitation process, they are indispensable for further events and achieving a complete capacitation status (Signorelli et al., 2012).

Fast/early events of sperm capacitation are initiated immediately after ejaculation, when sperm are exposed to the high concentrations of Ca^{2+} and HCO_3^- present in SP (Yeste, 2013b). Once inside the sperm cell, HCO_3^- induces an intracellular increase of pH and stimulates the activity of the atypical soluble adenyl cyclase (sACY), which increases cAMP levels, stimulating the cAMP-dependent protein kinase (PKA) pathway (Chen et al., 2000; Harrison, 2004). In turn, the activation of PKA pathway by HCO_3^- induces lipid scrambling from plasma membrane, causing changes in membrane lipid architecture (Gadella & Harrison, 2002; Harrison & Miller, 2000), and further cholesterol depletion mediated by acceptor proteins such as albumin (Flesch et al., 2001). At this point, the activation of PKA pathway also stimulates the entry of Ca^{2+} through the opening of channels such as CatSper, a pH-sensitive and voltage-gated Ca^{2+} channel, inducing an increase in intracellular Ca^{2+} and changes in membrane potential (Wennemuth et al., 2003). Molecular events triggered by the entry of Ca^{2+} and HCO_3^- underlie the activation of sperm motility during the fast/early events of capacitation (*Figure 7*; Signorelli et al., 2012).

On the other hand, late/slow capacitation events require longer incubation times. It is considered that late/slow events begin with cholesterol depletion of sperm membrane mediated by BSA (Yeste, 2013b). This cholesterol efflux causes the reorganisation of lipid-raft and increases membrane fluidity, which also allows sperm to maintain high levels of HCO_3^- (Signorelli et al., 2012). It is interesting to note that early/fast and late/slow events are both dependent of HCO_3^- and Ca^{2+} levels, and are thus under the regulation of cAMP/PKA signalling pathway (Visconti, 2009). During the late/slow phase, through PKA activation, the phosphorylation of several serine and threonine protein residues takes place, which in turn induces the activation of other kinases and/or inhibition of phosphatases, finally leading to an increase in the protein phosphorylation of tyrosine residues (Ickowicz et al., 2012; Visconti, 2009). This signal

transduction pathway elicits sperm to achieve a complete capacitation status, manifested by the aforementioned events: motility hyperactivation, chemoattractant responsiveness, and the ability to trigger the acrosome reaction (*Figure* 7; Signorelli et al., 2012).

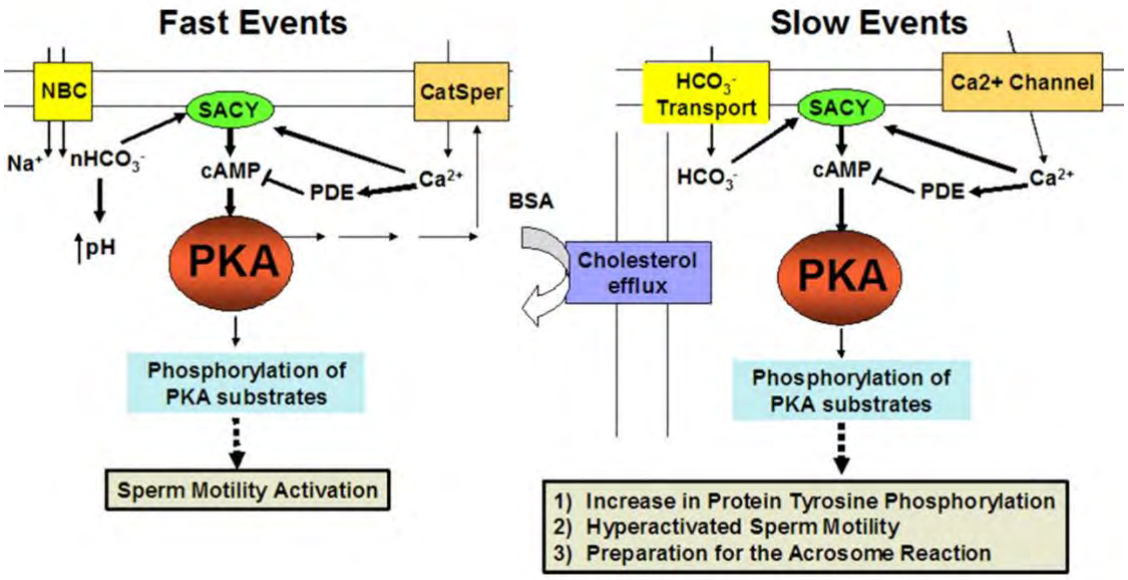

Figure 7: Molecular basis of principal capacitation-associated events. During fast events, HCO_3^- (through Na^+/HCO_3^- cotransporter, NBC) and calcium entrance (CatSper channel) stimulate the sACY and PKA pathway, inducing the activation of sperm motility. During slow events, the tyrosine phosphorylation of certain proteins takes place and sperm acquire hyperactivated motility and the ability to trigger the acrosome reaction (Visconti, 2009).

Membrane lipid reorganisation during late/slow events of capacitation promotes the interaction/apposition of the apical plasma and the outer acrosome membranes, inducing the formation of bilamellar structures (Tsai et al., 2007, 2010). The SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor) protein complex has been proposed to participate in the formation of these bilamellar structures and in the acrosome exocytosis, in a calcium-dependent manner (Roggero et al., 2007; Tsai et al., 2010).

It is worth mentioning that mechanisms exist to prevent premature sperm capacitation and maintain the uncapacitated status and the acrosome intact until sperm encounter the oocyte. The term “decapacitation factors” is generally referred to SP proteins that bind the sperm surface, stabilising sperm membrane and preventing sperm from premature capacitation (Bailey, 2010). In cattle, BSP proteins are the best characterised decapacitation factors, which bind to phospholipids thus stabilising sperm membrane and allowing the formation of the sperm reservoir (see sections 1.4.1. and 1.5.2.; Gwathmey et al., 2003, 2006; Manjunath & Thérien, 2002). Similarly in pigs, the

heterodimer PSP-I/PSP-II has been also suggested to play a role in the stabilisation of sperm membrane and the delay of capacitation events (Caballero et al., 2008).

4.2.1. Acrosome reaction

As explained in the previous section, physiological and biochemical changes during capacitation prepare spermatozoa to undergo the acrosome reaction (AR), which is essential to penetrate and fertilise the oocyte (Yeste, 2013b). Acrosome reaction was first described for Austin and Bishop in 1958, and the same authors defined the mammalian acrosome as “the characteristic cap-like structure over the anterior part of the sperm head”. Indeed, the acrosome is a large secretory vesicle positioned at the anterior end of the sperm head (Nicander & Bane, 1962; Yanagimachi, 1998). This Golgi-derived vesicle contains a variety of enzymes responsible for the digestion of glycoprotein layers surrounding the oocyte (Aguas & Pinto da Silva, 1985).

The calcium-dependent exocytosis process consists in the fusion between the outer acrosomal membrane and the sperm plasma membrane, resulting in the release of the acrosome content (Yanagimachi, 2011). Once the enzymatic content is released, acrosome-specific enzymes such as acrosin, trypsin, and hyaluronidase mediate the digestion of oocyte’s vestments, which finally allows sperm to penetrate and fertilise the oocyte (Georgadaki et al., 2016; Osman et al., 1989). The elevation of intracellular pH during capacitation, as well as the increase in intracellular calcium levels, are extremely important to trigger the AR (Beltrán et al., 2016). While the classical approach envisaged that the union between the capacitated spermatozoon and the zona pellucida triggered this process (Darszon et al., 1999), an accumulating body of evidence suggests that the presence of progesterone in the oviductal fluid may lead to the AR occurring before sperm interact with the oocyte vestments (La Spina et al., 2016; Yeste et al., 2017).

In a similar way to sperm capacitation, the AR can be induced *in vitro* in capacitated spermatozoa through incubation with calcium ionophores (Sirivaidyapong et al., 2001) or P4 (Jiménez et al., 2003). In addition to CL secretion of P4, cumulus cells also produce P4 and is physiologically involved in triggering the AR, inducing calcium influx and changes in sperm motility (Publicover et al., 2007; Witte & Schäfer-Somi, 2007). In this sense, P4 might be the best method to mimic what occurs *in vivo,* and has been demonstrated to induce successfully *in vitro* acrosome exocytosis in mammalian sperm (Jiménez et al., 2003; Wu et al., 2006).

4.2.2. Relevance of ion channels during sperm capacitation and acrosome reaction

Ion channels allow communication between spermatozoa, the surrounding environment, and the oocyte, playing critical roles in regulating, amongst others, sperm motility, capacitation, chemotaxis and the AR (La Spina et al., 2016; Salicioni et al., 2007; Santi et al., 2013; Visconti et al., 2011). Moreover, channels are efficient transporters, rapidly moving millions of ions flowing per second across the lipid bilayer, which affects the electrical potential of the cell membrane (Darszon et al., 1999). Thus, ion pumps and channels allow cells either to maintain or modify ion concentration gradients, regulating intracellular levels of secondary messengers, such as Ca^{2+}, and pH (Lishko et al., 2012).

As aforementioned, an increase in calcium influx, intracellular pH, and changes in membrane potential, which are also essential to trigger the AR, takes place during capacitation (Beltrán et al., 2016). Despite existing differences between species (Miller et al., 2015), these capacitation-associated events are orchestrated by ion pumps and channels, such as HCO_3^- membrane transporters, Na^+/H^+ exchangers and voltage-gated proton channels (Bernardino et al., 2019; Lishko & Kirichok, 2010; Mishra et al., 2019).

In mice (De La Vega-Beltran et al., 2012; Santi et al., 2010; X.-H. Zeng et al., 2011), it is well stablished that potassium channels play a pivotal role in membrane hyperpolarisation, which is essential for sperm to trigger the AR in this species (De La Vega-Beltran et al., 2012). While the mechanisms underlying this hyperpolarisation are not yet well understood, this phenomenon could be due to the activation of potassium channels, which raises K^+ permeability, and/or decreases the activity of sodium channels (Santi et al., 2013). Uncapacitated mouse spermatozoa exhibit a negative resting membrane potential (from -35 to -45 mV). During capacitation, the sperm plasma membrane hyperpolarises, due to an increase in K^+ permeability through potassium channels, and membrane potential becomes more negative (to approximately -70 mV) (Arnoult et al., 1999). With regard to this, Muñoz-Garay et al., (2001) found that intracellular alkalinisation during capacitation triggers the activation of certain potassium channels, leading to the hyperpolarisation of the plasma membrane and, subsequently, the regulation of downstream signalling pathways. Similarly, Navarro et al., (2007) reported that the intracellular pH increase produces a fast hyperpolarisation of the plasma membrane, mediated by an activation of the outwardly rectifying potassium current originated from the principal piece of the sperm flagellum. This pH-sensitive potassium current controls sperm membrane potential in mouse allowing K^+ ions to flow out of the cell leading to membrane hyperpolarisation (Navarro et al., 2007).

In pigs, the role of ion channels in sperm capacitation has not been fully studied and further characterisation of these channels is needed to elucidate their role during this physiological process. In spite of this, it is well known that CatSper channels modulate sperm motility during capacitation (Vicente-Carrillo et al., 2017), and recently, it has been reported that HVCN1 channels are also involved in the regulation of motility kinematics and calcium influx into the sperm head during that process (Yeste et al., 2020). Regarding potassium channels, SLO1 has been shown to be required to trigger acrosome exocytosis, induced by P4 *in vitro* (Yeste et al., 2019).

5. Seminal plasma

5.1. Composition

Seminal plasma is a complex fluid made up of the secretions from the rete testis, the epididymis and the AG, which accompanies spermatozoa during and after ejaculation. Seminal plasma contains a great variety of biochemical constituents: ions (Na^+, K^+, Zn^+, Ca^{2+}, Mg^{2+}, Cl_2), energy substrates, especially fructose in cattle and pigs; and organic compounds, including citric acid, amino acids, peptides, low– and high—molecular weight proteins, lipids, hormones, and cytokines (Juyena & Stelletta, 2012; Sancho & Vilagran, 2013).

Specific SP composition in different species is defined by the different size and contribution of AG to the ejaculate (Aumüller & Seitz, 1990). In rams and bulls, the seminal vesicles are the main contributors to SP volume, whereas the prostate, ampulla, and bulbourethral glands have a minor contribution, which results in concentrated ejaculates of low volume (Leahy & de Graaf, 2012). In contrast, boar and stallion ejaculates have a major contribution of the prostate and bulbourethral glands, resulting in larger ejaculates expelled in different fractions (i.e., sperm-rich and sperm-poor fractions; Rodríguez-Martínez et al., 2009). In this regard, whilst boars and stallions are intrauterine depositors, bulls and rams deliver the ejaculate into the vagina of the female, far from the fertilisation site. Remarkably, the low volume of the ejaculate reduces sperm backflow, ensuring that sperm cells reach the fertilisation site (Leahy & de Graaf, 2012).

Not only does ejaculate composition vary between species, but also between individuals and even between ejaculates of the same individual (Gürler et al., 2015; Valverde et al., 2016). In addition, semen collection methods can also have an impact (Marco-Jiménez et al., 2008; Rego et al., 2015). In the bull, two methods of semen collection are routinely used: artificial vagina and electroejaculation (EEJ). As these two techniques differentially stimulate AG (Mattner & Voglmayr, 1962), the impact on SP

composition has been interrogated. Interestingly, ejaculates collected with an artificial vagina exhibited higher sperm concentration and greater amounts of SP proteins, mainly from epididymal origin, compared with EEJ (Rego et al., 2015). These data agree with those reported in sheep (Marco-Jiménez et al., 2008), where the comparison of these two harvesting methods showed that sperm concentration was significantly lower for the EEJ method, without variations in ejaculate volume, and a moderate increase in sodium concentration. Besides this, two specific protein spots identified by 2-D electrophoresis were found to be significantly increased when semen was recovered by EEJ, whereas one precise protein spot was only identified in SP obtained by artificial vagina (Marco-Jiménez et al., 2008).

Moreover, intra-ejaculate differences among fractions have been reported (Mateo-Otero et al., 2020; Perez-Patiño et al., 2016; Saravia et al., 2009). As stated before, boar ejaculate is expelled in different fractions. Apart from the pre-sperm fraction (PSF), three well-defined fractions can be collected: (i) the first 10 mL of the sperm-rich fraction (SRF-P1), (ii) the rest of the sperm-rich portion (SRF-P2), and (iii) the post-sperm-rich fraction (PSRF); the SRF-P2 and the PSRF being those which present higher protein content (Rodríguez-Martínez et al., 2009, 2005). Regarding the contribution of AG to SP composition, clear differences exist among these fractions: whereas SRF-P1 is mostly composed by epididymis secretions, SRF-P2 mainly originates from the prostate, and the PSRF comprises the largest amount of SP, essentially originating from seminal vesicle secretions (Einarsson, 1971; Rodríguez-Martínez et al., 2009, 2005; Saravia et al., 2009). Considering such different contribution, besides differences in sperm concentration, these fractions also differ at molecular level, including protein (Perez-Patiño et al., 2016) and metabolic content (Mateo-Otero et al., 2020). The study of Perez-Patiño et al. (2016) revealed 34 proteins differentially expressed comparing the SP proteome of the SRF and the PSRF. Such variations in the protein content of SP seem to have an important impact in sperm physiology since different responses have been observed in kinematic parameters (Saravia et al., 2009) or cryotolerance (Alkmin et al., 2014) after sperm exposure to the SP of different ejaculate fractions. The effect of SP composition in sperm physiology, especially regarding SP-proteins, will be explained in more detail in the next section (1.5.2.).

Proteins are the major components of SP, and have been reported to modulate sperm function and physiology (Caballero et al., 2012; Rodríguez-Martínez et al., 2011). In mammals, three major families of SP proteins have been described: cysteine-rich secretory proteins (CRISP), spermadhesins, and fibronectin-2 type proteins (Rodríguez-

Martínez et al., 2011). CRISP proteins have been identified in the male reproductive tract of humans, rats, mice, horses and pigs. The spermadhesin family exhibits a great diversity of members that are detected in the SP of bull, stallion, ram, buck, and boar, representing more than 90% of SP proteins in the latter (Caballero et al., 2012). Fibronectin-2 type proteins were initially identified in bovine SP, but since homologous proteins were found in other mammalian species, they were renamed as binder of sperm proteins (BSP; Manjunath et al., 2009) . Both BSP and spermadhesins are low molecular weight proteins with the ability to bind different ligands, which may be related to their different roles during fertilisation (Caballero et al., 2012).

Studies conducted in bulls revealed that most of the SP proteins are derived from the seminal vesicles (Westfalewicz et al., 2017a), and to a lesser extent from the epididymis (Westfalewicz et al., 2017b). Three BSP derived from seminal vesicles, BSP-1, BSP-3 and BSP-5 (previously known as PDC-109 or BSP-A1/A2, BSP-A3 and BSP-30 kDa, respectively; Manjunath et al., 2009), are the major proteins in bovine SP, representing 50 - 70% of total proteins in this fluid (Nauc & Manjunath, 2000). On the other hand, the most studied and abundant proteins (>90%) of boar SP are the multifunctional spermadhesins, including porcine seminal plasma protein (PSP)-I, PSP-II, Alanine–Glutamine–Asparagine proteins (AQN)−1, AQN−3, and Alanine–Tryptophan–Asparagine proteins (AWNs) (Rodriguez-Martinez et al., 2021). More than 50% of the total protein content correspond to the heterodimer formed by PSP-I and PSP-II (Caballero et al., 2008).

Since proteins are the major components of SP, in the last years, the proteomes of both bovine (Kelly et al., 2006; Rego et al., 2015; Viana et al., 2018; Westfalewicz et al., 2017a, 2017b) and porcine (González-Cadavid et al., 2014; Mills et al., 2020; Novak et al., 2010; Pérez-Patiño et al., 2018, 2019; F. Zeng et al., 2021) SP have been extensively studied. These proteomic studies have been conducted in the attempt to establish potential protein fertility markers in bovine SP (Jobim et al., 2004; Kasimanickam et al., 2019; Moura et al., 2006; Viana et al., 2018), aiming to predict the success of reproductive performance and identify high fertility animals, which is of especial interest due to the extensive use of artificial insemination (AI) for livestock breeding. For example, the aforementioned proteins, BSP-1, -3, and -5, were detected in greater amounts in sperm and SP samples of high fertility bulls compared to low fertility animals (Kasimanickam et al., 2019). Furthermore, osteopontin, a multifunctional protein secreted by ampullae and seminal vesicles, has also been associated with bull fertility indexes (Moura, 2018; Moura et al., 2006). In addition, SP proteins have been also linked

to the resilience of sperm to withstand the damage induced during cryopreservation procedures (known as freezability or cryotolerance). For example, acidic seminal fluid protein (aSFP) and clusterin were identified as potential markers of high freezability bulls, since they were found to be more abundant in the SP of bulls classified with high freezability compared to those with low freezability; in contrast, lipocaline-like prostaglandin D synthase showed lower abundance in the former than in the latter (Jobim et al., 2004).

Similarly, the study of boar SP proteome revealed correlation between protein abundance of certain proteins and different fertility phenotype. Among these proteins, the abundance of porcine seminal protein (PSP-I) precursor was associated with high reproductive performance boars (Mills et al., 2020). Conversely, other proteins with antioxidant properties, including superoxide dismutase (SOD1) and glutathione peroxidase 6 (GPX6) were lower in animals with a subfertile phenotype (Mills et al., 2020). Pérez-Patiño and colleagues (2018) found associations between the expression of certain SP proteins and *in vivo* fertility outcomes; specifically, the differential expression of 11 proteins was associated to farrowing rate, whereas that of four proteins was related to litter size.

5.2. Effects of seminal plasma on sperm function

In addition to acting as a vehicle and nourishing medium for spermatozoa, ensuring their survival in both male and female reproductive tracts, SP plays key roles modulating some aspects of sperm physiology and fertilisation (Juyena & Stelletta, 2012; Rodríguez-Martínez et al., 2011).

Since sperm maturation takes place in the epididymis, the importance of the epididymal fluid, which contributes to the final composition of SP, is evident. Sperm undergo sequential changes during the maturation process, as a result of different secretion activity and composition that can be found along the different regions of the epididymis, reaching a maximum concentration of proteins and spermatozoa in the last portion: the tail or cauda (Belleannée et al., 2011). The proteomic study of bovine cauda epididymis fluid revealed the presence of proteins involved in sperm maturation by facilitating membrane remodelling, transporting lipophilic substances and ions, and protecting sperm against oxidative damage and immune attack (Moura et al., 2010). Similarly in pigs, proteomics studies of epididymal fluid also revealed proteins associated to sperm protection, including structural and functional preservation, antioxidant activity, immune response, and energy metabolism (Weber et al., 2020). Further, the study of

ligand-binding abilities of the boar epididymal fluid showed proteins capable of binding the ZP glycoproteins of the oocyte, as well as cholesterol or hyaluronic acid, suggesting a role in sperm capacitation (Maňásková-Postlerová et al., 2011).

Upon ejaculation, spermatozoa are exposed to AG secretions and the first cholesterol efflux from sperm membrane takes place; that efflux has been proposed to be induced by BSP-1, causing a reorganisation of the plasma membrane in bull sperm. Afterwards, BSP proteins coat the sperm surface by binding the choline phospholipids of plasma membrane. This stabilises sperm membrane during the transit of male gametes through the female tract and acts as a decapacitation factor, thus preventing the free movement of phospholipids and premature sperm capacitation (Manjunath & Thérien, 2002). Moreover, when sperm reach the isthmic oviduct, the oviductal sperm reservoir is formed, which is of special importance for successful fertilisation as secretions and protein factors of the epithelial cells of bovine oviduct have been demonstrated to maintain sperm viability and motility (Abe et al., 1995; Boilard et al., 2002). In this regard, SP proteins BSP-1, -3, and -5, which are bound to the sperm plasma membrane, promote the interaction between sperm and the lining oviductal epithelial cells (Gwathmey et al., 2003, 2006). These three BSP proteins are involved in the maintenance of sperm motility, while spermatozoa are attached to oviductal epithelial cells *in vitro* (Gwathmey et al., 2006).

On the other hand, BSP proteins have been suggested to interact with heparin and high-density lipoproteins (capacitation factors in bull sperm; Parrish, 2014) present in the female tract, inducing a second cholesterol efflux (Manjunath & Thérien, 2002). In turn, there is a further reorganisation of sperm plasma membrane, which is thought to trigger some downstream signal transduction pathways that lead spermatozoa to reach their capacitated status (Manjunath & Thérien, 2002). In addition, BSP-1 has been identified as an inhibitor of protein kinase C (PKC) (Yu et al., 2003), which is involved in the events of the signal transduction cascade that take place during capacitation and are needed for acrosome reaction to occur (Breitbart & Naor, 1999; Ickowicz et al., 2012). Thus, BSP proteins seem to be key regulators of sperm capacitation in bovine species.

Moreover, in cattle, several proteins identified in the AG fluid have been associated to important functions for sperm physiology and oocyte fertilisation (*Figure 8;* Moura et al., 2007). The aforementioned proteins aSFP and clusterin, which were identified in the bull AG fluid (Moura et al., 2007), have been proposed as potential markers of high freezability bulls (Jobim et al., 2004) and are known to protect sperm from oxidative damage (Reyes-Moreno et al., 2002; Schöneck et al., 1996). Moreover, multifunctional

proteins such as phospholipase A_2 (PLA_2) or osteopontin have been also identified in the AG fluid (Moura et al., 2007), both displaying higher expression in the SP of bulls classified as high fertility compared to their low fertility counterparts (Moura et al., 2006). Whereas osteopontin has been proposed to participate in the sperm-oocyte interaction, PLA_2 has been related to acrosome reaction and membrane fusion during fertilisation (Moura et al., 2007).

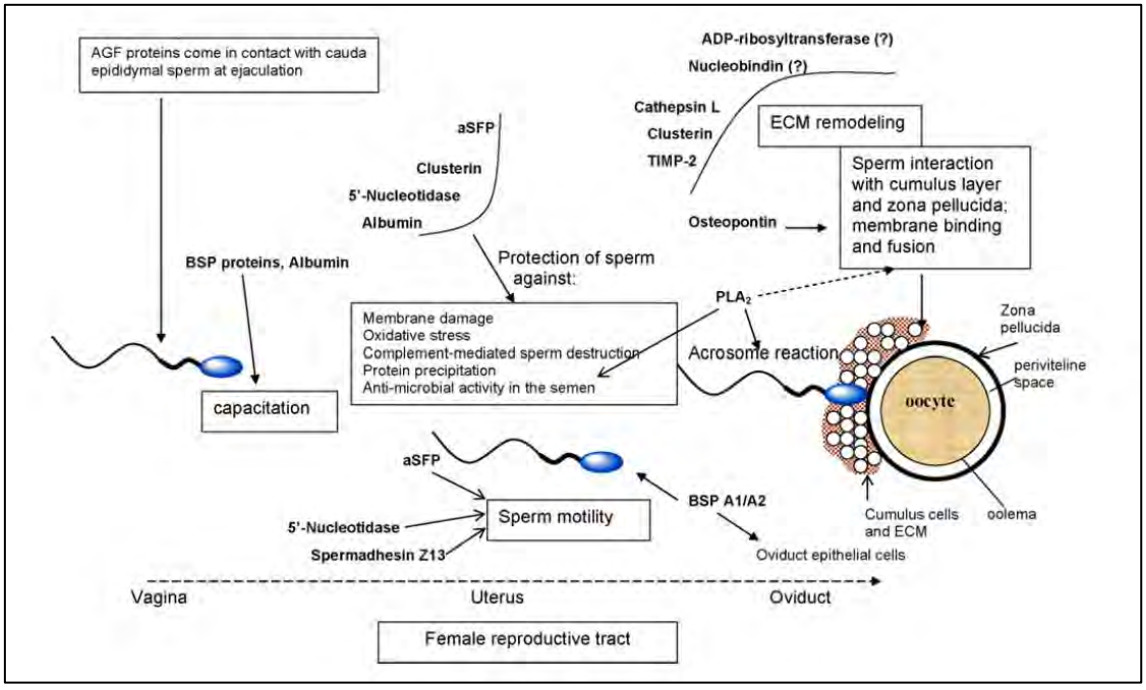

Figure 8: Schematic representation of sperm physiology and fertilisation processes modulated by proteins derived from SP (Moura et al., 2007).

In pigs, the heterodimer PSP-I/PSP-II has been proposed to modulate the capacitation process, acting as a transient decapacitation factor (Caballero et al., 2009), as well as to preserve the viability, motility and mitochondrial activity of liquid-stored sperm (Caballero et al., 2006; Centurion et al., 2003). In addition, it has been also suggested to participate in the protection of sperm against the female immune system (González-Cadavid et al., 2014). Moreover, the spermadhesins AQN-1, -3, and AWN-1 have also been determined to participate in the sperm capacitation (Dostàlovà et al., 1994) and oviductal sperm reservoir formation (Ekhlasi-Hundrieser et al., 2005).

Due to its lipid characteristics, particularly rich in polyunsaturated acids, pig sperm membrane is especially sensitive to oxidative damage (Cerolini et al., 2000). In this context, porcine SP exerts a protective effect from oxidative stress since it contains several antioxidant enzymes, including superoxide dismutase, catalase, glutathione

peroxidases, and paraoxonase type 1, which display higher levels in sperm-rich ejaculate portions (Rodriguez-Martinez et al., 2021).

Despite its implication in sperm physiology, the majority of SP is usually removed during sperm handling and prior to AI, in order to concentrate sperm or dilute in conservation media. In the last years, SP has emerged as a potential biotechnological tool to ameliorate some harmful effects on sperm, derived from its handling or treatment. In boars, bulls, and rams, the addition of SP to frozen-thawed sperm has been reported to have beneficial effects on sperm quality, which is significantly compromised during cryopreservation (Recuero et al., 2019).

5.3. Effects of seminal plasma on the female reproductive tract

In the last years, it has been demonstrated that not only does SP modulate sperm function, but it also interacts with female tissues, influencing maternal physiology during early pregnancy in some species, including mice and pigs (Bromfield, 2016; Robertson, 2005). In humans (Sharkey et al., 2012), rodents (Johansson et al., 2004; Schjenken et al., 2015; Song et al., 2016) and livestock species such as pigs (O'Leary et al., 2004; Rozeboom et al., 1999), horses (Tunon et al., 2000), and sheep (Scott et al., 2006), insemination is known to induce an inflammatory response in the female reproductive tract. Such an inflammatory response was initially thought to only serve to clear the uterus from microorganisms and excess sperm (Pandya & Cohen, 1985; Thompson et al., 1992). Nonetheless, mounting evidence suggests that the inflammatory response, driven by the exposure of the female tract to semen, primes the maternal immune system to paternal antigens, facilitating subsequent embryo development and implantation. Since embryos express semi-allogenic antigens, from the beginning of pregnancy, the maternal immune system must become tolerant to paternal antigens in order to allow successful embryo development and implantation (Robertson et al., 2013; Schjenken & Robertson, 2014).

The effects of SP modulating the environment of the female reproductive tract have been extensively studied in mice. In this species, an array of endometrial cytokines and chemokines (including C-X-C motif chemokine ligand-10 (*CXCL10*), -1 (*CXCL1*), -2 (*CXCL2*), colony-stimulating factor 3 (*CSF3*), interleukin-1A (*IL1A*), -6 (*IL6*), leukaemia inhibitory factor (*LIF*), and tumour necrosis factor (*TNF*)) have been shown to be modulated in response to SP exposure, leading to leukocyte recruitment into the uterus (Glynn et al., 2017; Schjenken et al., 2015). Some of the SP components identified as the triggering agents of such inflammatory reaction include transforming growth factor

beta (TGFβ), prostaglandin E (PGE; in humans) and ligands of toll-like receptor 4 (TLR4), (Schjenken & Robertson, 2020). After mating, synthesis of cytokines and chemokines triggered by SP induces the recruitment and infiltration of diverse leukocytes, and leads to the expansion and differentiation of T regulatory (Treg) cells (Guerin et al., 2011; Robertson et al., 2009; Shima et al., 2015). On the one hand, macrophages and T cells play an important role developing receptivity for embryo implantation by remodelling the endometrial tissue (Schjenken & Robertson, 2020). Besides this, Treg cells drive immune tolerance by modulating the immune response of other cells (Sakaguchi et al., 2001), thus mediating the immune tolerance toward the embryo and facilitating its implantation and development (*Figure 8*, Guerin et al., 2011; Robertson et al., 2009, 2018).

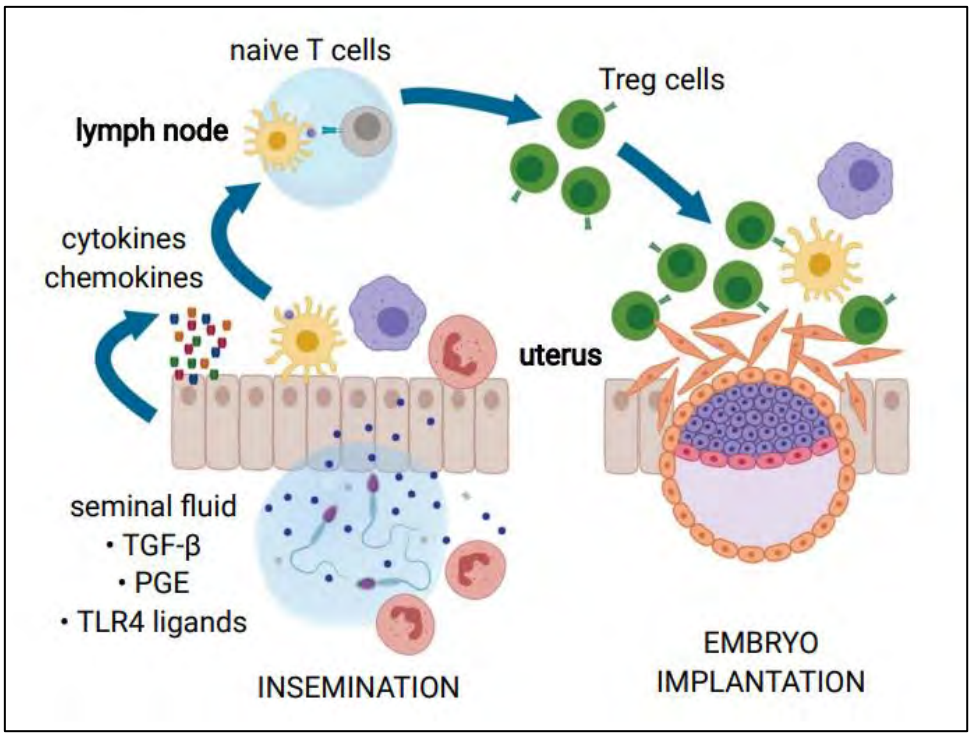

Figure 9: Model of immune response in the female tract driven by SP exposition (Schjenken & Robertson, 2020)

Similarly, in pigs, intra-uterine infusion of SP modifies the expression of endometrial cytokines, specifically granulocyte macrophage colony-stimulating factor (*GM-CSF*) or *CSF2*, monocyte chemoattractant protein-1 (*MCP-1*), eicosanoid-synthesising enzyme cyclo-oxygenase-2 (*COX2*), and *IL6* (O'Leary et al., 2004). In addition, these changes are accompanied by leukocyte recruitment into the endometrium, mainly the infiltration of macrophages and major histocompatibility complex (MHC) class II activated macrophages and dendritic cells (O'Leary et al., 2004). As observed in mice, SP infusion leads to a beneficial impact on embryo viability and development (Martinez et al., 2019; O'Leary et al., 2004).

In the mare, SP has also been shown to modulate the post-insemination inflammatory response, increasing the expression of interleukin-1β (*IL1β*) and interleukin-8 (*IL8*), whereas suppressing that of *TNF* (Fedorka et al., 2017). In addition, equine SP reduces sperm binding to polymorphonuclear neutrophils in the inflamed uterus, protecting spermatozoa and improving fertility in this condition (Alghamdi et al., 2004; Troedsson et al., 2002).

To the best of our knowledge*, in vivo* human studies describing the effects of seminal plasma on the endometrium have not yet been conducted. Notwithstanding, *in vitro* exposure of SP induces mRNA expression of *IL1β*, *IL6* and *LIF* in cultured endometrial epithelial cells (Gutsche et al., 2003). Similar effects have been observed after incubation of SP with cervical and vaginal epithelial cells, inducing the synthesis and secretion of several cytokines (Sharkey et al., 2007), as well as in ectocervical explants (Introini et al., 2017). Indeed, changes in cytokine expression and leukocyte recruitment were observed in cervical biopsies after unprotected coitus but not in condom-protected intercourse, demonstrating that the immune response was induced by seminal fluid rather than by the mechanical stimulus produced during coitus (Sharkey et al., 2012).

It has been reported that not only does SP act in the uterus, near the natural site of ejaculate deposition, but its effects also extend to more distal regions such as the oviduct and the ovary. In both mice (Gangnuss et al., 2004) and pigs (O'Leary et al., 2006), macrophage infiltration increases in the ovary after exposing the uterus to SP; furthermore, in pigs, there is a rise in CL weight and plasma progesterone concentration (O'Leary et al., 2006).

In addition to modulating the maternal environment and embryo viability, paternal factors in mice have been found to impact the health and metabolic status of the offspring (Bromfield et al., 2014; Watkins et al., 2018). Moreover, mating in absence of SP impairs embryo development and placentation, which results from the downregulation of embryotrophic cytokines, *Lif*, *Csf2*, *Il6*, and epidermal growth factor (*Egf*) in the oviduct (Bromfield et al., 2014).

In contrast, in cattle, similar evidence supporting the beneficial effects of SP exposure in the regulation of maternal environment is conflicting. The uterine infusion of SP, in the absence or presence of spermatozoa, induces changes in the expression of several inflammatory mediators in both ipsi- and contralateral uterine horns (Ibrahim et al., 2019). In spite of this, no effects on pregnancy rates are observed when SP is infused

into the uterus at the time of AI (Odhiambo et al., 2009; Ortiz et al., 2019), nor when heifers are exposed to vasectomised bulls for 21 days prior to AI (Pfeiffer et al., 2012).

In vitro experiments reported that live, but not dead, spermatozoa bind to endometrial cells and then induce a pro-inflammatory response (Elweza et al., 2018; Ezz et al., 2019). Incubation of bovine endometrial explants with spermatozoa, in the absence of SP, induces an increase in the expression of the inflammatory mediators *IL1β*, *IL8*, *TNF-α*, prostaglandin E synthase (*PGES*), and *TLR2* (Elweza et al., 2018; Ezz et al., 2019). In addition, while incubation of endometrial explants with SP alters the expression of *CSF2*, *IL1β*, *IL6*, *IL17A*, *TGFβ1*, interferon epsilon (*IFNE*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and 3α-hydroxysteroid dehydrogenase (*AKR1C4*), incubation with washed sperm free from AG secretions does not induce such changes (Ibrahim et al., 2019).

On the other hand, in the study of Fernandez-Fuertes et al. (2019), neither cauda epididymal sperm (which are never in contact with AG secretions) nor washed ejaculated sperm were found to induce changes in the expression of *IL1A, IL1β, IL6, IL8, PTGS2, TNF-α,* and *LIF* in endometrial explants. Surprisingly, this study also reported a detrimental effect of SP exposure on the RNA integrity of endometrial explants due to the presence of a ribonuclease in SP. The abundance of this ribonuclease depends on the method of semen collection, and is more abundant when artificial vagina, rather than EEJ, is used (Fernandez-Fuertes et al., 2019). Thus, caution must be paid when interpreting some specific data, since factors such as the method of collection can alter seminal plasma composition (Fernandez-Fuertes et al., 2019; Rego et al., 2015).

Finally, it is also worth taking into account that SP effects might be different considering the ejaculate deposition site during natural mating. Thus, uterine infusions in animals with vaginal deposition, like cattle, may not mimic the physiological conditions. A clear example is the study of Badrakh et al. (2020), in which SP infusion into the vagina, but not into the uterus, modified endometrial levels of epidermal growth factor (EGF).

Objectives

The main objective of this Dissertation was to explore the modulatory role of sperm and seminal plasma proteins in the events that take place after ejaculation and during sperm passage through the female tract, including sperm capacitation and regulation of female tract environment. The specific objectives, therefore, were the following:

1. To explore the role of calcium-activated potassium channels during *in vitro* capacitation and progesterone-induced acrosomal exocytosis in pig spermatozoa, using general and specific inhibitors.

2. To determine whether PARK7 relative content and localisation pattern are related to the sperm ability to undergo *in vitro* capacitation and the acrosomal exocytosis induced by progesterone in porcine species.

3. To investigate the effects of a prolonged exposure to seminal plasma during liquid storage (17°C) on the sperm ability to elicit *in vitro* capacitation and trigger acrosomal exocytosis induced by progesterone.

4. To elucidate whether changes in the transcriptome of the female reproductive tract are elicited by seminal plasma in the presence or absence of sperm, using cattle as a model of species with intravaginal ejaculation.

Paper compendium

PAPER I

Inhibition of potassium channels affects the ability of pig spermatozoa to elicit capacitation and trigger the acrosome exocytosis induced by progesterone.

Federico Noto, Sandra Recuero, Julián Valencia, Beatrice Saporito, Domenico Robbe, Sergi Bonet, Augusto Carluccio & Marc Yeste

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Article

Inhibition of Potassium Channels Affects the Ability of Pig Spermatozoa to Elicit Capacitation and Trigger the Acrosome Exocytosis Induced by Progesterone

Federico Noto [1,2,3,†], Sandra Recuero [1,2,†], Julián Valencia [2,4], Beatrice Saporito [3], Domenico Robbe [3], Sergi Bonet [1,2], Augusto Carluccio [3] and Marc Yeste [1,2,*]

1 Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; fnoto83@gmail.com (F.N.); sandra.recuero@udg.edu (S.R.); sergi.bonet@udg.edu (S.B.)
2 Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain; jvalencia21@uan.edu.co
3 Faculty of Veterinary Medicine, University of Teramo, Località Piano D'Accio, IT-64100 Teramo, Italy; beatrice.saporito.93@gmail.com (B.S.); drobbe@unite.it (D.R.); acarluccio@unite.it (A.C.)
4 University Antonio Nariño, Calle 53 #9-35, Popayán CO-190002, Colombia
* Correspondence: marc.yeste@udg.edu
† These authors contributed equally to this work.

Abstract: During capacitation, sperm undergo a myriad of changes, including remodeling of plasma membrane, modification of sperm motility and kinematic parameters, membrane hyperpolarization, increase in intracellular calcium levels, and tyrosine phosphorylation of certain sperm proteins. While potassium channels have been reported to be crucial for capacitation of mouse and human sperm, their role in pigs has not been investigated. With this purpose, sperm samples from 15 boars were incubated in capacitation medium for 300 min with quinine, a general blocker of potassium channels (including voltage-gated potassium channels, calcium-activated potassium channels, and tandem pore domain potassium channels), and paxilline (PAX), a specific inhibitor of calcium-activated potassium channels. In all samples, acrosome exocytosis was induced after 240 min of incubation with progesterone. Plasma membrane and acrosome integrity, membrane lipid disorder, intracellular calcium levels, mitochondrial membrane potential, and total and progressive sperm motility were evaluated after 0, 120, and 240 min of incubation, and after 5, 30, and 60 min of progesterone addition. Although blocking potassium channels with quinine and PAX prevented sperm to elicit in vitro capacitation by impairing motility and mitochondrial function, as well as reducing intracellular calcium levels, the extent of that inhibition was larger with quinine than with PAX. Therefore, while our data support that calcium-activated potassium channels are essential for sperm capacitation in pigs, they also suggest that other potassium channels, such as the voltage-gated, tandem pore domain, and mitochondrial ATP-regulated ones, are involved in that process. Thus, further research is needed to elucidate the specific functions of these channels and the mechanisms underlying its regulation during sperm capacitation.

Keywords: pigs; spermatozoa; capacitation; potassium channels; quinine; paxilline

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1. Introduction

Ion channels are crucial for sperm physiology and are involved in the sperm transport throughout the male and female reproductive tracts, epididymal maturation, motility activation, chemotaxis and thermotaxis, capacitation, and acrosome reaction [1–5]. These ion channels allow the rapid movement of millions of ions flowing per second across the lipid bilayer and, together with ion pumps, they allow sperm cells to regulate concentration gradients, which affects intracellular calcium levels, cell volume, and pH [6–9]. Chloride, Cystic fibrosis transmembrane conductance regulator (CFTR), potassium, and calcium

channels are the main ion channels in mammalian spermatozoa; specifically, potassium channels are involved in the regulation of sperm volume and underlie the hyperpolarization of sperm plasma membrane, which is involved in the regulation of sperm motility and acrosome reaction [2,10,11].

Potassium channels have been detected in spermatogenic cells and mature spermatozoa [12] and, based on its functionality and structure [13], they are classified into four classes [13]: (a) Voltage-gated potassium channels (Kv); (b) calcium-activated potassium channels (KCa); (c) inwardly rectifying potassium channels (Kir); and (d) tandem pore domain potassium channels (K_{2P}). The first two types can have overlapping properties as happens with SLO3, a voltage-gated potassium channel modulated by intracellular pH [14,15], and with SLO1, a calcium voltage-gated potassium channel [4]. While the role of ligand-activated potassium channels during pig sperm capacitation has not been studied much, these ion channels have been found to play an instrumental role for sperm membrane hyperpolarization in humans and mice [16–20].

Upon ejaculation, mammalian spermatozoa are not yet ready to fertilize the oocyte, but rather need to undergo a series of changes that occur within the female reproductive tract and are known as sperm capacitation [21–23]. Elucidating the role of potassium channels during capacitation of pig spermatozoa may be conducted through the use of blocking agents, in a similar fashion to previous studies [4,24,25]. Quinine is an alkaloid extracted from cinchona bark, which was the first effective antimalarial drug [26]. Quinine inhibits many K^+ channels including KCa, Kv (e.g., Kv2.2, which are encoded by KCNB2) and K_{2P} channels (e.g., Kv18.1, encoded by KCNK18), which have been reported to be involved in the regulation of cell volume [27,28], including mammalian sperm [29]. While incubation of human sperm with quinine has been found to increase their volume and induce kinematic alterations, which are crucial for sperm transport, including penetration and migration throughout the cervical mucus [11,30], there is no information about their implication in pig sperm capacitation. On the other hand, another inhibitor is paxilline, which specifically blocks KCa channels (e.g., KCa1.1, encoded by KCNMA1) [31,32]. Neither quinine nor paxilline have any effect on Na^+ and Ca^{2+} channels.

Against this background, this study sought to investigate the role of calcium-activated potassium channels during in vitro capacitation and acrosome reaction of pig spermatozoa. With this purpose, we tested the effects of general (quinine) and specific (paxilline) inhibitors of calcium-activated potassium channels at two concentrations (0.1 mM and 1 mM) on the integrity and lipid disorder of plasma membrane, acrosome exocytosis, intracellular calcium levels, mitochondrial membrane potential, and sperm motility. Our hypothesis is that because calcium-activated potassium channels have been demonstrated to be crucial for mammalian sperm physiology, their inhibition should modify the sperm ability to elicit in vitro capacitation and trigger the acrosome reaction induced by progesterone.

2. Results

2.1. Effects of Quinine and Paxilline on Plasma Membrane Integrity

Figure 1 shows the effects of quinine and PAX on plasma membrane integrity during sperm capacitation. Incubation of pig sperm with capacitation medium led, as expected, to a reduction of sperm membrane integrity ($SYBR14^+/PI^-$) in all treatments ($p < 0.05$). However, when quinine was present, the extent of that reduction was lower in a dose-dependent manner. Therefore, after 120 min and 240 min of incubation and after progesterone addition, percentages of sperm with an intact plasma membrane in the treatment containing 1 mM quinine were significantly higher ($p < 0.05$) than in the control. Furthermore, after 120 and 240 min of incubation and after 5 min of progesterone addition (245 min), percentages of sperm with an intact plasma membrane in the treatment containing 0.1 mM quinine were significantly higher ($p < 0.05$) than in the control. Conversely, the presence of PAX either had no effect on plasma membrane integrity (0.1 mM) or significantly ($p < 0.05$) reduced the percentages of spermatozoa with an intact plasma membrane (1 mM) after 240 min

of incubation and after 30 min of progesterone addition (270 min), when compared to the control.

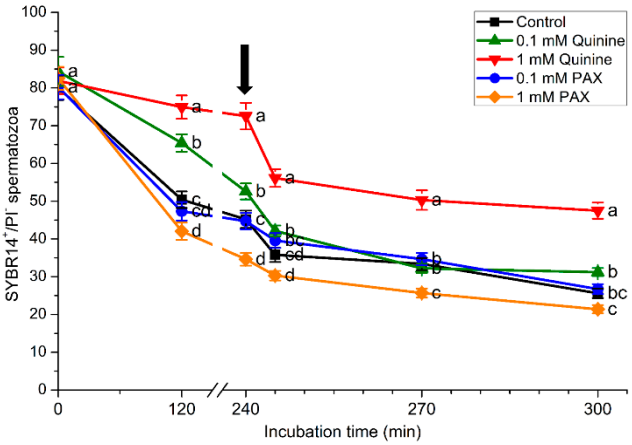

Figure 1. Percentages of spermatozoa with an intact plasma membrane ($SYBR14^+/PI^-$) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the control and treatments containing 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX), or 1 mM PAX. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a–d) indicate significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments.

2.2. Effects of Quinine and Paxilline on Acrosome Integrity

Percentages of viable spermatozoa with an intact acrosome membrane ($PNA^+/EthD\text{-}1^-$) significantly ($p < 0.05$) decreased throughout incubation in capacitation medium (Figure S1a). This decrease was more apparent in the control and samples incubated with 0.1 mM quinine, 0.1 mM PAX, and 1 mM PAX than in those containing 1 mM quinine. After 30 min and 60 min of the addition of progesterone (i.e., 270 min and 300 min), percentages of viable spermatozoa with an intact acrosome were significantly ($p < 0.05$) higher in samples incubated with 1 mM quinine than in the control and in samples containing 0.1 mM quinine, 0.1 mM PAX, or 1 mM PAX. After 60 min of progesterone addition (300 min), all samples containing quinine or PAX showed significantly ($p < 0.05$) higher percentages of viable spermatozoa with an intact acrosome membrane than the control. Furthermore, spermatozoa showing an exocytosed acrosome within the viable sperm population (PNA^-/viable spermatozoa) were significantly ($p < 0.05$) lower in the treatment containing 1 mM quinine than in the control and samples with 0.1 mM quinine, 0.1 mM PAX, or 1 mM PAX after 60 min of progesterone addition (300 min; Figure 2a).

2.3. Effects of Quinine and Paxilline on Membrane Lipid Disorder

Figure 2b, Figures S1b and S2 show the effects of blocking potassium channels with quinine or PAX on the percentages of spermatozoa with low membrane lipid disorder. Although there was a significant ($p < 0.05$) decrease in the percentages of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) throughout in vitro capacitation and after progesterone addition, no significant ($p > 0.05$) differences between treatments and the control were observed (Figure S1b).

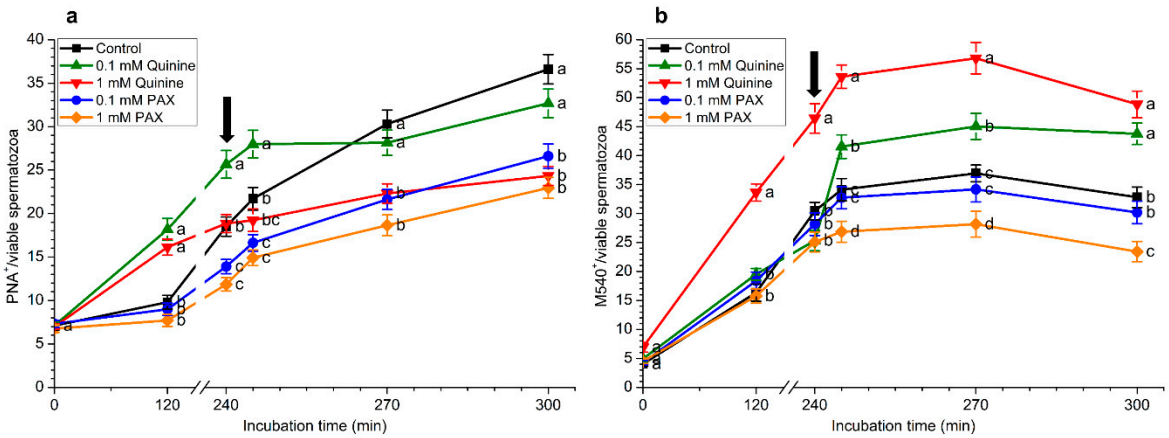

Figure 2. (**a**) Percentages of spermatozoa with an exocytosed acrosome (PNA^-) within the viable sperm population and (**b**) percentages of spermatozoa with high membrane lipid disorder ($M540^-$) within the viable sperm population during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline, (PAX), and 1 mM PAX. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a–c) mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments.

In contrast, and as depicted in Figure 2b, incubation with capacitation medium significantly ($p < 0.05$) increased the percentages of spermatozoa with high membrane lipid disorder ($M540^+$) within the viable sperm population. However, these percentages were significantly ($p < 0.05$) higher in the presence of 1 mM quinine than in the control after 120 min of incubation and until the end of the incubation period. Conversely, the percentages of spermatozoa with high membrane lipid disorder ($M540^+$) within the viable sperm population in the treatment containing 1 mM PAX were significantly ($p < 0.05$) lower than in the control and the other treatments after 5 min, 30 min, and 60 min of progesterone addition (i.e., 245 min, 270 min, and 300 min).

2.4. Effects of Quinine and Paxilline on Intracellular Calcium Levels

Figure 3a shows the effects of quinine and PAX on the percentages of viable spermatozoa with high intracellular calcium levels stained by Fluo3 ($Fluo3^+/PI^-$). In all treatments, incubation in capacitation medium significantly ($p < 0.05$) increased the percentages of $Fluo3^+/PI^-$ spermatozoa. The extent of that increase was, however, significantly ($p < 0.05$) higher in the control than in the treatments containing 0.1 mM quinine, 1 mM quinine, or 1 mM PAX after 120 min and 240 min incubation and after the addition of progesterone. No significant differences ($p > 0.05$) between treatments containing 0.1 quinine, 1 mM quinine, or 1 mM PAX were observed, except after 5 min of progesterone addition (245 min).

As Figure 3b depicts, geometric mean of $Fluo3^+$-intensity in the $Fluo3^+/PI^-$ sperm population was significantly ($p < 0.05$) higher in the control than in treatments containing 1 mM quinine and 1 mM PAX after 120 min and 240 min of incubation and after progesterone addition. Furthermore, geometric mean of $Fluo3^+$-intensity in the $Fluo3^+/PI^-$ sperm population was also significantly ($p < 0.05$) higher in the control than in the treatment containing 0.1 mM quinine at 120 min and after 30 min of the addition of progesterone (270 min).

In a similar fashion to that described for Fluo3-staining and as Figure 3c shows, percentages of viable spermatozoa with high intracellular calcium levels stained by Rhod5 ($Rhod5^+/YO\text{-}PRO\text{-}1^-$) significantly ($p < 0.05$) increased following incubation with capacitation medium. Again, the control presented significantly ($p < 0.05$) higher percentages of $Rhod5^+/YO\text{-}PRO\text{-}1^-$ spermatozoa than the treatment containing 1 mM quinine after 120 min and 240 min of incubation and 5 min after progesterone addition (245 min), and higher than the treatment with 1 mM PAX after 5 min of progesterone addition (245 min).

In the case of geometric mean of $Rhod5^+$-intensity in the $Rhod5^+/YO\text{-}PRO\text{-}1^-$ sperm population (Figure 3d), spermatozoa incubated in the control medium showed significantly

($p < 0.05$) higher values of this parameter than those containing 1 mM quinine or 1 mM PAX after 240 min of incubation and following progesterone addition (i.e., 245, 270, and 300 min).

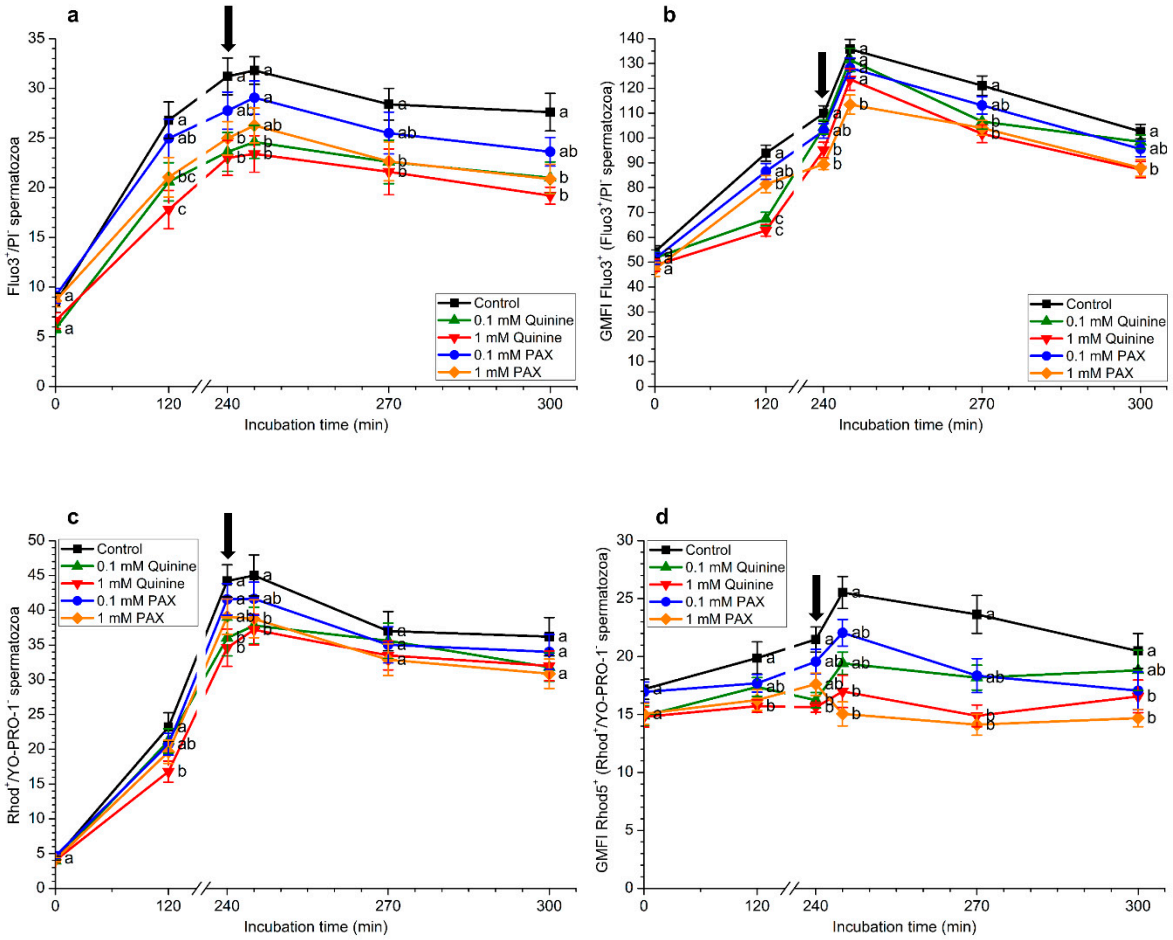

Figure 3. (**a**) Percentages of viable spermatozoa with high intracellular calcium levels evaluated with Fluo3 ($Fluo3^+/PI^-$), (**b**) geometric mean of fluorescence intensity (GMFI) of Fluo3 in $Fluo3^+/PI^-$ sperm population, (**c**) percentages of viable spermatozoa with high intracellular calcium levels evaluated with Rhod5 ($Rhod5^+/YO\text{-}PRO\text{-}1^-$) and (**d**) geometric mean of fluorescence intensity (GMFI) of Rhod5 in $Rhod5^+/YO\text{-}PRO\text{-}1^-$ sperm population during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX), and 1 mM PAX. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a,b) mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments.

2.5. Effects of Quinine and Paxilline on Mitochondrial Membrane Potential

As shown in Figure 4a and Figure S3, after 120 min of incubation and until the end of the experiment, percentages of spermatozoa with high mitochondrial membrane potential (MMP) were significantly ($p < 0.05$) higher in the control than in the treatments containing 1 mM quinine and 1 mM PAX. In addition, percentages of spermatozoa with high MMP were significantly ($p < 0.05$) higher in the treatment containing 0.1 mM quinine than in that with 1 mM quinine after progesterone addition (i.e., 245, 270 min, and 300 min). Moreover, the percentages of spermatozoa with high MMP were significantly ($p < 0.05$) higher in the treatment containing 0.1 mM PAX than in that with 1 mM PAX at 120 min, and after 30 min and 60 min of progesterone addition (270 min and 300 min).

The results observed for the percentages of spermatozoa with high MMP were similar to those found in their $JC1_{agg}/JC1_{mon}$ ratios (Figure 4b). In effect, these ratios were significantly ($p < 0.05$) lower in the control than in the treatments containing 1 mM quinine or 1 mM PAX at 120 min and 240 min, and after the addition of progesterone. Furthermore,

$JC1_{agg}/JC1_{mon}$ ratios of the sperm population with high MMP were significantly ($p < 0.05$) lower in the treatment containing 0.1 mM quinine than in the control at 240 min and after 30 min of progesterone addition (270 min). In contrast, no significant differences between the control and the treatment containing 0.1 mM PAX were observed.

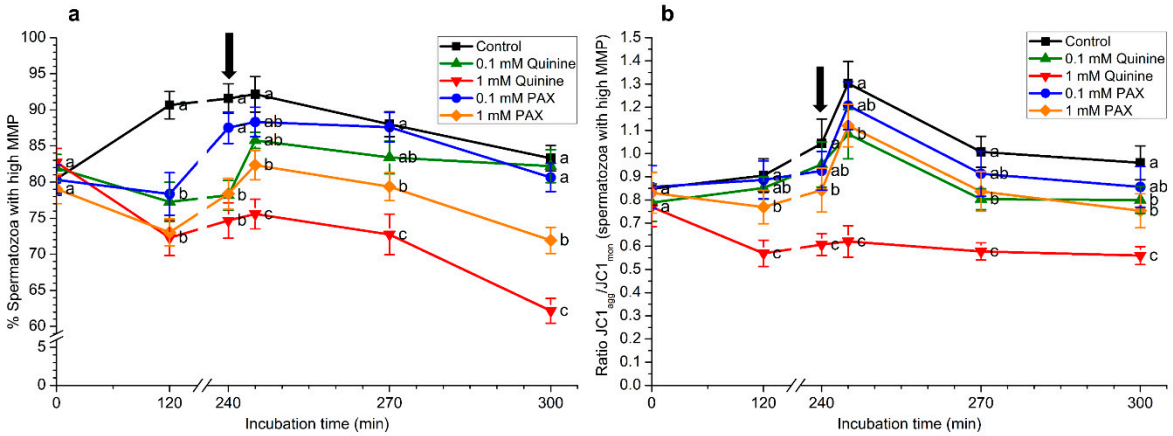

Figure 4. (**a**) Percentages of spermatozoa with high mitochondrial membrane potential (MMP) and (**b**) their $JC1_{agg}/JC1_{mon}$ ratios during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX), and 1 mM paxilline. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a–c) mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments.

2.6. Effects of Quinine and Paxilline on Sperm Motility

Inhibition of potassium channels with 1 mM quinine led to a dramatic drop in the percentages of total (Figure 5a) and progressively motile spermatozoa (calculated over motile cells) at the beginning of the experiment (Figure 5b). Thus, percentages of total and progressively motile spermatozoa were significantly ($p < 0.05$) lower in the treatment containing 1 mM quinine than in the control and the other treatments (0.1 mM quinine, 0.1 mM PAX, and 1 mM PAX) throughout the entire incubation period and following the addition of progesterone. Moreover, percentages of total and progressively motile spermatozoa were significantly ($p < 0.05$) higher in the control than in the treatment containing 0.1 mM quinine after progesterone addition (i.e., 245, 270, and 300 min). On the other hand, no significant differences between the control and treatments containing 0.1 mM and 1 mM PAX were observed in the percentages of progressively motile spermatozoa throughout the entire incubation period, which contrasted with that observed for the percentages of total motile spermatozoa in the treatment with 1 mM PAX after the addition of progesterone (245, 270, and 300 min).

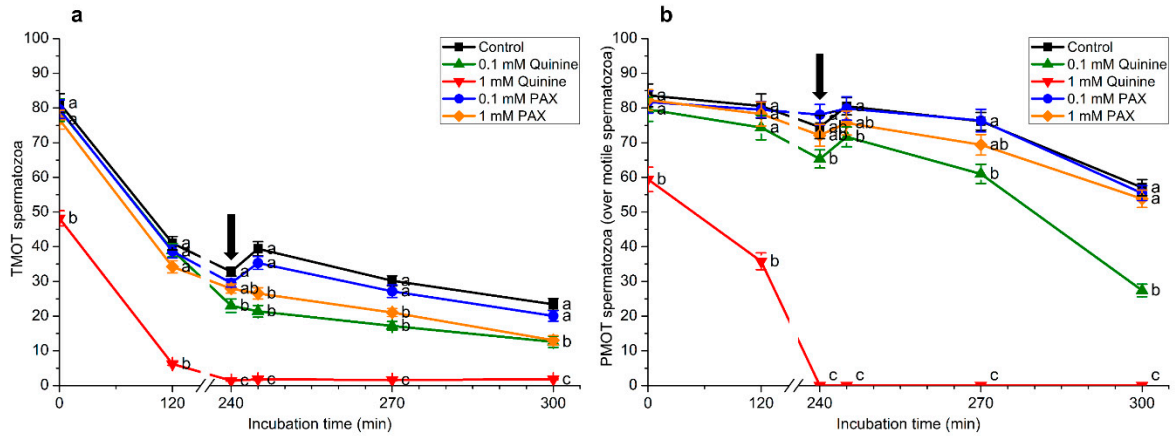

Figure 5. (**a**) Percentages of total motile (TMOT) and (**b**) of progressively motile spermatozoa (calculated over motile cells; PMOT) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX), and 1 mM paxilline. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a–c) mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments.

3. Discussion

While calcium-activated potassium channels are known to have a crucial role for mouse [17,31] and human sperm physiology [19,20], their role during pig sperm capacitation has not yet been studied. Taking this into account, the present study aimed at determining the contribution of potassium channels during in vitro capacitation and progesterone-induced acrosome exocytosis of pig spermatozoa through their inhibition with quinine, an inhibitor of a wide range of potassium channels (including voltage-gated potassium channels, calcium-activated potassium channels, and tandem pore domain potassium channels), and paxilline (PAX), which specifically blocks calcium-activated potassium channels. Paxilline is an indole diterpen that binds to KCa1.1 at an intracellular site that is involved in channel gating and seems to be coupled to the calcium binding site, whereas quinine blocks opening of KCa1.1 but also of other subtypes of potassium channels, including KCa3.1, Kv and K_{2P} [32–36].

Capacitation occurs within the female reproductive tract and consists of a series of changes that prepare sperm to fertilize the oocyte [21–23]. While plasma membrane integrity indicates that sperm are viable [37,38], its destabilization, which involves changes in its architecture and eventually leads to death, is one of the features of sperm capacitation [39]. The mechanisms underlying these changes are driven by a soluble adenylyl-cyclase, PKA-dependent signaling pathway (sAC-PKA), and are reliant upon the presence of cholesterol acceptors, such as proteins like bovine serum albumin (BSA) [40,41].

As expected, our results showed that whereas plasma membrane and acrosome integrity decreased throughout incubation with capacitated medium, the presence of 1 mM quinine, which inhibits several potassium channels, did partially counteract that reduction. This contrasted with the results of PAX, since at the highest concentration, this blocking agent of calcium-gated potassium channels reduced plasma membrane integrity. While these data suggest that quinine reduces the sperm ability to elicit in vitro capacitation and trigger progesterone-induced acrosome exocytosis, the increase in the percentages of spermatozoa with high membrane lipid disorder in samples incubated with 1 mM quinine does not seem to support that hypothesis. Again, the effects of PAX were the opposite, as the treatment with 1 mM PAX showed significantly lower percentages of spermatozoa with high membrane lipid disorder within the viable sperm population. Our results are in agreement with previous reports indicating that the inhibition of KCa1.1 channels, such as SLO1, prevents sperm capacitation in pigs without altering membrane lipid disorder [4]. Therefore, since quinine, which inhibits voltage-gated, calcium-activated, and tandem pore domain, potassium channels increased the lipid disorder of sperm plasma membrane, but

the use of PAX, which specifically inhibits calcium-activated potassium channels did not have such an effect, we suggest that potassium channels other than the calcium-activated ones regulate this increase in the membrane lipid disorder, which is linked to cholesterol efflux, during in vitro capacitation. In addition to this, it is worth bearing in mind that quinine prevents the regulation of sperm volume in different species, including the pig [29]. Thus, one could suggest that alterations in the regulation of sperm volume could affect the architecture of sperm plasma membrane, which would be featured by an increase in membrane lipid disorder regulated by the aforementioned channels. Nevertheless, further research is needed to understand the precise relationship between potassium channels and membrane lipid architecture.

Capacitation is also characterized by changes in sperm motility, including hyperactivation [42]. When progesterone was added to control samples to induce acrosome exocytosis, we observed an increase in both total and progressive sperm motility. However, samples treated with quinine and 1 mM PAX did not exhibit that increase. In fact, the highest concentration of quinine (1 mM) significantly abolished sperm motility from the beginning of the experiment and until the end of the incubation period. This dramatic effect on sperm motility could be explained by the inability of sperm cells to regulate their volume [29,30]. In fact, our results are in agreement with previous studies in humans, which reported that quinine induces sperm to swell and that the resulting motility alterations lead sperm cells to fail to migrate and penetrate the cervical mucus [30]. In addition, even though not to the same extent as 1 mM quinine, the presence of 0.1 mM quinine and 1 mM PAX also decreased total and progressive motility. Therefore, our findings support the role of a wide variety of potassium channels, not only the calcium-activated but also the voltage-gated and the tandem pore domain potassium ones in the modulation of motility during sperm capacitation [33,43]. Remarkably, the fact that more than one type of channels could be involved in the modulation of sperm motility could not only explain why the effects observed were dose-dependent but also why the extent of the impact of quinine, which blocks many potassium channels, was larger than that of PAX.

An appropriate regulation of calcium influx is essential for mammalian sperm function, as motility, chemotaxis, capacitation, and acrosome reaction are governed by changes in intracellular levels of this secondary messenger [44,45]. Intracellular calcium has been reported to be stored in both the sperm head, specifically in the acrosomal region, and the region of the sperm neck and mid-piece in mammals, suggesting that head calcium could be involved in acrosomal exocytosis, and mid-piece calcium could be related to sperm motility and mitochondria energy production [46,47]. In the present study, these two calcium deposits were assessed by two separate fluorochromes: Fluo3, which has affinity for head and mid-piece stores, and Rhod5, which has more affinity for the head store. In both cases, we observed that intracellular calcium levels were significantly lower in treatments containing quinine and PAX, especially when added at the highest concentrations (i.e., 1 mM), than in the control. Hence, these results suggest that potassium channels, particularly the calcium-activated ones, play a pivotal role in regulating calcium influx during sperm capacitation in pigs. Moreover, progesterone is known to act as a potent acrosomal exocytosis inducer, rising intracellular calcium levels in both the sperm head and the mid-piece [48]. Herein, we found that while the addition of progesterone to control samples led to an increase in intracellular calcium levels, this rise was not as high as that found in the control when potassium channels were inhibited with 1 mM quinine or 1 mM PAX. Moreover, in control samples, intracellular calcium levels evaluated through Fluo3-staining were higher than those evaluated through Rhod5. Nevertheless, when potassium channels were inhibited with quinine or PAX, there was a much apparent decrease in the intracellular calcium levels stained by Fluo3, which targets the calcium residing in the mid-piece. This finding underpins the relevance of potassium-conductance in triggering calcium influx to the flagellum during in vitro sperm capacitation and progesterone-induced acrosome exocytosis. Moreover, CatSper channels in pigs have been shown to be essential for the regulation of sperm motility [25]. As aforementioned, not only does the general inhibition

of potassium channels with quinine and the specific inhibition of calcium-activated potassium channels with PAX decrease calcium influx, but also reduces sperm motility. This suggest that, in pigs, calcium-activated potassium channels also regulate calcium influx by CatSper channels, reinforcing the hypothesis that potassium and calcium conductances are closely related.

Sperm mitochondria are located in the mid-piece and play an important role in maintaining appropriate energy levels for sperm function [49–51]. In control samples, mitochondrial membrane potential increased progressively during in vitro capacitation and after the induction of acrosome exocytosis with progesterone, which is in agreement with previous studies [47,52,53]. However, inhibiting potassium channels with quinine and PAX led sperm to exhibit lower MMP, especially at the highest quinine concentration. To the best of our knowledge, no previous study has investigated whether calcium-activated potassium channels are involved in the regulation of MMP during sperm capacitation in mammals. Since both the percentages and $JC1_{agg}/JC1_{mon}$ ratios of the sperm population with high MMP concurred in the same effect in the treatment containing 1 mM quinine but not in that with 1 mM PAX, our results suggest that in addition to calcium-activated potassium channels, the voltage-gated and the tandem pore domain potassium ones are involved in the regulation of mitochondrial activity during in vitro sperm capacitation. In this context, it is worth mentioning that, in bovine myocardium cells, quinine has also been reported to inhibit mitochondrial ATP-regulated potassium channel [54]. Therefore, the involvement of these mitochondrial channels could also contribute to explain the different effects observed in quinine- and PAX-blocked samples.

4. Materials and Methods

4.1. Reagents

Unless stated otherwise, all reagents were of analytic grade and were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Fluorochromes were acquired from Molecular Probes (Thermofisher Scientific; Waltham, MA, USA).

4.2. Semen Samples

A total of 15 semen samples, each coming from a separate boar, were used in this study. These samples were provided by a local farm (ServiciosGeneticosPorcinos, S.L.; Roda de Ter, Barcelona, Spain), which operates under standard, commercial conditions. Animals were housed in controlled conditions of temperature and humidity and fed with a standard and balanced diet; water was provided ad libitum. Ejaculates were collected twice a week through the gloved-hand method and the sperm-rich fraction was diluted to a final concentration of 30×10^6 spermatozoa/mL with a commercial extender (Duragen, Magapor; Ejea de los Caballeros, Zaragoza, Spain). Diluted semen was cooled down to 17 °C and transported to the laboratory within four hours post-collection. All ejaculates fulfilled the following quality thresholds: >80% viable spermatozoa, 75% motile spermatozoa, and >80% morphologically normal spermatozoa.

Since authors did not manipulate any animal, but ejaculates were purchased from a commercial farm that operates under standard regulations, no specific authorization from an Ethics Committee was required. In addition, the aforementioned farm confirmed that they handle animals in accordance with the Animal Welfare Law issued by the Regional Government of Catalonia (Generalitat de Catalunya, Spain).

4.3. In Vitro Sperm Capacitation

Semen samples were centrifuged at $600\times g$ at 17 °C for 10 min. Pellets were resuspended with capacitation medium to a final concentration of 20×10^6 spermatozoa/mL. This capacitation medium consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 4.5 mM $CaCl_2$, 21.7 mM L-lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$, and 5 mg/mL of bovine serum albumin (BSA). The osmolarity was 305 ± 7 mOsm/Kg, and

the pH was adjusted to 7.4. Sperm were incubated at 38.5 °C and 5% CO_2 for 300 min in a Heracell 150 incubator (Heraeus Instruments GmbH, Osterode, Germany), as described in Rocco et al. [55]. After 240 min, progesterone was added to a final concentration of 10 μg/mL to induce the acrosome exocytosis. At the beginning of the experiment, after 120 min and 240 min of incubation, and after 5, 30 min, and 60 min of progesterone addition (i.e., 245, 270 min, and 300 min), an aliquot was taken to evaluate sperm motility, plasma membrane and acrosome integrity, membrane lipid disorder, intracellular calcium levels, and mitochondrial membrane potential.

4.4. Inhibition of Calcium-Activated Potassium Channels with Quinine and Paxilline

In addition to the control, which resulted from incubating sperm with capacitating medium, sperm samples, previously resuspended in the same medium, were added with 0.1 quinine, 1 mM quinine, 0.1 mM PAX, or 1 mM PAX. These concentrations were set based on previous studies [4,11,30,56] and preliminary experiments conducted in our laboratory in a 10-fold series. The two chosen concentrations were the lowest one at which significant differences with regard to the control were observed, and the highest one that showed the clearest effect without being cytotoxic. Sperm were incubated for 300 min and added with 10 μg/mL progesterone after 240 min of incubation, as described previously.

4.5. Flow Cytometry Analyses

Information on flow cytometry experiments is provided following the recommendations of the International Society for Advancement of Cytometry (ISAC) [57]. Flow cytometry was used to evaluate the integrities of plasma membrane and acrosome, membrane lipid disorder, intracellular calcium levels, and mitochondrial membrane potential. Prior to staining, sperm concentration was adjusted to 1×10^6 spermatozoa/mL, as described in Yeste et al. [58]. Sperm cells were subsequently stained with the appropriate combinations of fluorochromes, as described below.

Spermatozoa were excited through an argon ion laser (488 nm; power: 22 mW) using a Cell Laboratory Quanta^{TM} SC cytometer (Beckman Coulter, Fullerton, CA, USA). In this equipment, electronic volume (EV) of particle is evaluated using the Coulter principle and the forward scatter (FS) is replaced by the EV. In addition, the EV channel was periodically calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size of the bead at channel 200 on the volume scale. Three different optical filters were used to evaluate sperm samples; these filters had the following characteristics: FL1 (green fluorescence): Dichroic/splitter long pass, DRLP: 550 nm, band pass, BP: 525 nm, detection width: 505–545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP: 575 nm, detection width: 560–590 nm); FL3 (red fluorescence): Long pass, LP: 670 nm, detection width: 655–685 nm. Signals were logarithmically amplified, and photomultiplier settings were adjusted to particular staining methods. FL1 was used to detect green fluorescence (YO-PRO-1; Fluo3; and JC1 monomers, $JC1_{mon}$), FL2 was utilized to detect JC1 aggregates ($JC1_{agg}$) and FL3 was used to detect red fluorescence (Merocyanine-540, M540; and Rhod5). Sheath flow rate was set at 4.17 μL/min in all analyses, and EV and side-scatter (SS) were recorded in a linear mode (in EV vs. SS dot plots) for a minimum of 5000 events per replicate. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 μm) and cell aggregates (particle diameter > 12 μm). Therefore, sperm-specific events were positively gated on the basis of their EV/SS distribution, whereas the others were gated out. In some protocols, compensation was used to minimize spill-over of green fluorescence into the red channel, as described below. Dot-plots (FL1 vs. FL3; FL2 vs. FL3) were analyzed through Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). In addition, data obtained from flow-cytometry experiments were corrected according to the procedure described by Petrunkina et al. [59], using the percentage of debris particles detected in the SYBR14/PI staining (see Section 4.5.1). Each sample and parameter were evaluated in triplicate using independent tubes, and the mean ± SEM was subsequently calculated.

4.5.1. Plasma Membrane Integrity

Plasma membrane integrity was evaluated using the LIVE/DEAD® sperm viability kit (Molecular Probes, Thermo Fisher Scientific; Waltham, MA, USA). Briefly, spermatozoa, previously diluted to a final concentration of 1×10^6 sperm/mL, were stained with SYBR14 (final concentration: 100 µM) for 10 min at 38 °C in the dark. Following this, spermatozoa were incubated with propidium iodide (PI; final concentration: 12 µM) for 5 min at the aforementioned conditions. Fluorescence of SYBR14 was detected through FL1, whereas that of PI was collected through FL3. Each spermatozoon was classified as with an intact ($SYBR14^+/PI^-$, green) or non-intact plasma membrane ($SYBR14^+/PI^+$, orange; or $SYBR14^-/PI^+$, red). Unstained and single-stained samples were used for setting the EV-gain, and FL1 and FL3 PMT voltages. Spillover from FL1 into the FL3 channel was compensated (2.45%). Percentages of non-sperm, debris particles appearing in the lower left quadrant ($SYBR14^-/PI^-$) were used to correct percentages of particles corresponding to sperm in this and other tests.

4.5.2. Acrosome Integrity

Acrosome integrity was evaluated following the protocol set by Cooper and Yeung [60] and adapted to pig spermatozoa by Rocco et al. [55]. In this protocol, sperm are first stained with ethidium homodimer (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1), then permeabilized and finally stained with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocyanate (FITC). Briefly, samples were incubated with EthD-1 (final concentration: 2.5 µg/mL) at 38 °C for 5 min in the dark. Following centrifugation at 2000× *g* and 17 °C for 30 s, spermatozoa were resuspended with PBS containing BSA (4 mg/mL). Thereafter, spermatozoa were again centrifuged at 2000× *g* and 17 °C for 30 s; the pellet was resuspended with 100 µL of ice-cold 100% methanol and incubated for 30 s. After centrifugation at 2000× *g* and 17 °C for 30 s, samples were resuspended with 250 µL PBS, and then added with 0.8 µL PNA-FITC (final concentration: 2.5 µM). Samples were incubated at 25 °C for 15 min in the dark and then centrifuged at 2000× *g* and 17 °C for 30 s. Pellets were resuspended with 0.6 µL PBS and centrifuged 2000× *g* and 17 °C for 30 s. This step was repeated two times. Finally, spermatozoa were evaluated with the flow cytometer and classified into one of the following populations: (1) Viable spermatozoa with an intact acrosome ($PNA^+/EthD\text{-}1^-$); (2) viable spermatozoa with an exocytosed acrosome ($PNA^-/EthD\text{-}1^-$); (3) non-viable spermatozoa with an intact acrosome ($PNA^+/EthD\text{-}1^+$); and (4) non-viable spermatozoa with an exocytosed acrosome ($PNA^-/EthD\text{-}1^+$). FL3 was used to detect red fluorescence from EthD-1, and FL1 to detect that of PNA-FITC. Unstained and single-stained samples were used for setting the EV gain, FL1 and FL3 PMT-voltages, and for compensation of FL1 spill over into the FL3 channel (2.70%). Percentages of PNA^-/PI^-spermatozoa were corrected with the percentages non-sperm debris particles ($SYBR14^-/PI^-$) and the other sperm proportions were recalculated.

4.5.3. Membrane Lipid Disorder

Membrane lipid changes were determined through staining with Merocyanine-540 (M540) and YO-PRO-1, as described by Harrison et al. [61]. In brief, spermatozoa were incubated with M540 (final concentration: 2.6 µM) and YO-PRO-1 (final concentration: 25 nM) at 38 °C for 10 min in the dark. A total of four sperm populations were identified: (1) Non-viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$), (2) non-viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$), (3) viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$), and (4) viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$). Fluorescence from M540 was detected through FL3, and that from YO-PRO-1 was collected through FL1. Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL1, and FL3 PMT-voltages. Data were not compensated. Percentages of debris particles found in SYBR14/PI staining ($SYBR14^-/PI^-$) were subtracted from the percent-

ages of M540$^-$/YO-PRO-1$^-$spermatozoa; the percentages of the other sperm populations were recalculated.

4.5.4. Intracellular Calcium Levels

Intracellular calcium levels of spermatozoa were determined by using two different fluorochromes, Fluo3 and Rhod5. On the one hand, Fluo3 staining was performed following the protocol described by Harrison et al. [62] and modified by Kadirvel et al. [63]. Briefly, spermatozoa were incubated with Fluo3-AM (final concentration: 1 µM) and PI (final concentration: 12 µM) at 38 °C for 10 min in the dark. Fluorescence emitted by Fluo3 was collected through FL1, whereas that of PI was detected with FL3. When samples were analyzed with the flow cytometer, four populations were identified: (1) Viable spermatozoa with low intracellular calcium levels (Fluo3$^-$/PI$^-$); (2) viable spermatozoa with high intracellular calcium levels (Fluo3$^+$/PI$^-$); (3) non-viable spermatozoa with low intracellular calcium levels (Fluo3$^-$/PI$^+$); and (4) non-viable spermatozoa with high intracellular calcium levels (Fluo3$^+$/PI$^+$). Unstained and single-stained samples were used for setting the EV-gain, FL1 and FL3 PMT voltages, and for compensating FL1 spill over into the FL3-channel (2.45%) and FL3 spill over into the FL1-channel (28.72%). Percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from the percentages of Fluo3$^-$/PI$^-$spermatozoa; the percentages of the other sperm populations were recalculated.

On the other hand, intracellular calcium levels were also evaluated with Rhod5 labelling, following the protocol described by Yeste et al. [47]. Spermatozoa were incubated with Rhod5N-AM (final concentration: 5 µM) and YO-PRO-1 (final concentration: 25 nM) and incubated at 38 °C for 10 min in the dark. Rhod5 fluorescence was collected through FL3, whereas that of YO-PRO-1 was detected by FL1. Spermatozoa were classified into one of the following categories: (1) Viable spermatozoa with low intracellular calcium levels (Rhod5$^-$/YO-PRO-1$^-$); (2) viable spermatozoa with high intracellular calcium levels (Rhod5$^+$/YO-PRO-1$^-$); (3) non-viable spermatozoa with low intracellular calcium levels (Rhod5$^-$/YO-PRO-1$^+$); and (4) non-viable spermatozoa with high intracellular calcium levels (Rhod5$^+$/YO-PRO-1$^+$). Unstained and single-stained samples were used for setting the EV-gain, FL1 and FL3 PMT voltages, and for compensating FL3 spill over into the FL1-channel (3.16%) and FL3 spill over into the FL1-channel (28.72%). Percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from the percentages of Rhod5$^-$/YO-PRO-1$^-$spermatozoa; the percentages of the other sperm populations were recalculated.

4.5.5. Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was determined following the protocol described by Guthrie and Welch [64]. Spermatozoa were incubated with JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide) at room temperature for 30 min the dark. JC1 molecules remain as monomers ($JC1_{mon}$) at low MMP, and form aggregates ($JC1_{agg}$) when they detect high MMP. Two different emission filters (FL1 and FL2) were used to differentiate two sperm populations: (a) Spermatozoa with high MMP ($JC1_{agg}$), and (b) spermatozoa with low MMP ($JC1_{mon}$). Percentages of spermatozoa with high MMP corresponded to the orange-stained spermatozoa, which appeared in the upper half of the diagram in FL1 vs. FL2 dot-plots. FL1 spill-over into the FL2 channel was compensated (51.70%). Percentages of debris particles found in SYBR14/PI staining (SYBR14$^-$/PI$^-$) were subtracted from the percentages of spermatozoa appearing in the lower left part of the diagram; percentages of the two sperm populations were recalculated.

4.6. Analysis of Sperm Motility

Sperm motility was assessed through a computerized assisted sperm analysis (CASA) system (ISAS version 1.2; Proiser R+D, Valencia, Spain) under a phase-contrast microscope (Olympus BX41; Olympus Europa GmbH, Hamburg, Germany) at 100× magnification.

Following this, 5 µL of each sample was placed onto a pre-warmed (38 °C) Makler counting chamber (Sefi-Medical Instruments; Haifa, Israel) and a minimum of 1000 spermatozoa per replicate were evaluated. The analyzed parameter ranges were: Curvilinear velocity (VCL), which is the mean path velocity of the sperm head along its actual trajectory (µm/s); linear velocity (VSL), which is the mean path velocity of the sperm head along a straight line from its first to its last position (µm/s); mean velocity (VAP), which is the mean velocity of the sperm head along its average trajectory (µm/s); linearity coefficient (LIN), which results from VSL/VCL × 100 (%); straightness coefficient (STR), which results from VSL/VAP × 100 (%); wobble coefficient (WOB), which results from VAP/VCL × 100 (%); mean amplitude of lateral head displacement (ALH), which is the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (µm); and frequency of head displacement (BCF), which is the frequency with which the actual sperm trajectory crosses the average path trajectory (Hz). A spermatozoon was considered to be motile when its VAP was ≥10 µm/s, and progressively motile when STR was ≥45%.

4.7. Statistical Analyses

Statistical analyses were performed using IBM SPSS 25.0 for Windows (IBM Corp., Armonk, NY, USA). Sperm quality and function parameters (plasma membrane and acrosome integrity, membrane lipid disorder, intracellular calcium levels, mitochondrial membrane potential, and sperm motility) were considered as dependent variables, whereas each experiment and incubation treatments using seminal samples from different boars were treated as biological replicates. All the variables were first tested for normality (Shapiro–Wilk test) and homoscedasticity (Levene test); when needed, data (x) were transformed with arcsin $\sqrt{x}$. Results were then evaluated with a mixed model (intra-subject factor: Incubation time; inter-subject factor: Treatment) followed by the post-hoc Sidak test for pair-wise comparisons. Because each sperm sample came from a separate boar, the animal could not be included as a random-effects factor in the model. The level of significance was set at $p \leq 0.05$ in all cases.

5. Conclusions

In conclusion, our results indicate that potassium channels are essential for sperm to elicit sperm capacitation and trigger acrosome exocytosis. Specifically, blocking potassium channels with quinine prevents sperm to achieve the capacitated status by decreasing their motility and mitochondrial function. Moreover, inhibition of these channels reduces calcium influx to both the sperm head and the mid-piece and hinders sperm to trigger acrosome exocytosis. However, in the case of quinine, these effects were unexpectedly observed together with a concomitant increase in membrane lipid disorder. On the other hand, quinine-induced effects, which targets a wide range of potassium channels, were larger than those observed with PAX, a specific blocker of calcium-activated potassium channels. While this indicates that potassium channels, other than the calcium-activated ones, which comprise voltage-gated, tandem pore domain, and mitochondrial ATP-regulated potassium channels are involved in sperm capacitation, further research should address how these channels alter the cAMP/PKA signaling pathway related to sperm capacitation, and the relationship of calcium-activated potassium channels with other ion channels, including CatSper.

Supplementary Materials: The following are available online at https://www.mdpi.com/1422-0067/22/4/1992/s1, Figure S1: (a) Percentages of viable spermatozoa with an intact acrosome ($PNA^+/EthD\text{-}1^-$) and (b) percentages of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX) and 1 mM PAX. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a-c) mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments; Figure S2: Representative dot-plots for M540/YO-PRO-1 staining in control and quinine- and PAX-

blocked samples; Figure S3: Representative dot-plots for JC1 staining in control and quinine- and PAX-blocked samples.

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Abbreviations

ALH	Amplitude of Lateral Head Displacement
BCF	Beat Cross Frequency
BSA	Bovine Serum Albumin
CASA	Computer-Assisted Sperm Analysis
CFTR	Cystic Fibrosis Transmembran Conductance Regulator
CM	Capacitating Medium
DANCE	Forward Movement of the Head
EV	Electronic Volume
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GSK3a	Glycogen Synthase Kinase-3 isoform α
GSK3ß	Glycogen Synthase Kinase-3 isoform β
ISAC	International Society for Advancement of Cytometry
ISAS	Integrated Sperm Analysis System
JC1	5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide
LIN	Linearity
M540	Merocyanine-540
MAD	Mean Angular Displacement
MMP	Mithocondrial membrane potential
PCA	Principal Component Analysis
PI	Propidium Iodide
PMOT	Progressive Motility
PNA	Peanut Agglutinin
SEM	Standard Error of the Mean
SP	Motile subpopulation
SRF	Sperm-Rich Fraction
SSC	Side Scatter
STR	Straightness
TMOT	Total Motility
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Linear Velocity
WOB	Wobble Coefficient

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Supplementary Material

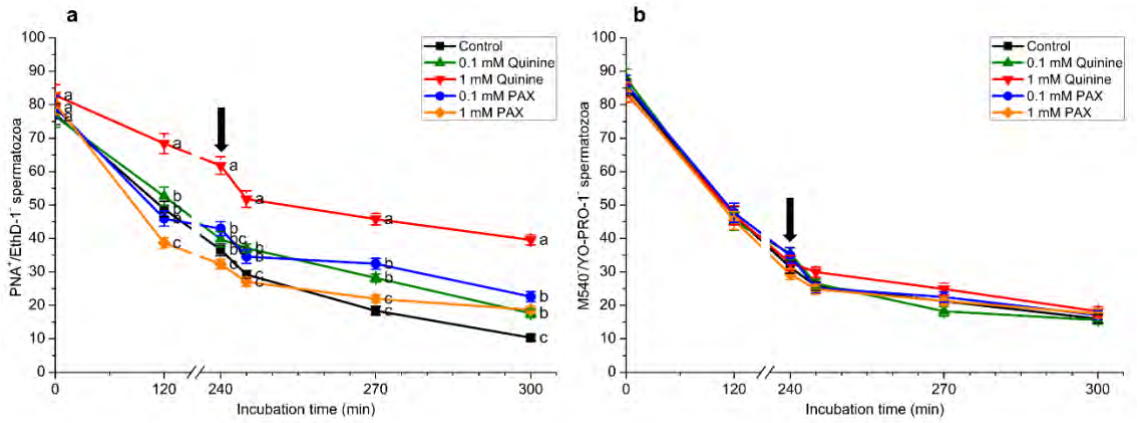

Figure S1: (a) Percentages of viable spermatozoa with an intact acrosome (PNA+/EthD-1−) and (b) percentages of viable spermatozoa with low membrane lipid disorder (M540−/YO-PRO-1−) during *in vitro* capacitation and progesterone-induced acrosomal exocytosis (300 min) in the presence of 0.1 mM quinine, 1 mM quinine, 0.1 mM paxilline (PAX) and 1 mM PAX. Black arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters (a-c) mean significant (p < 0.05) differences between treatments at a given time point. Data are shown as mean ± SEM for 15 independent experiments

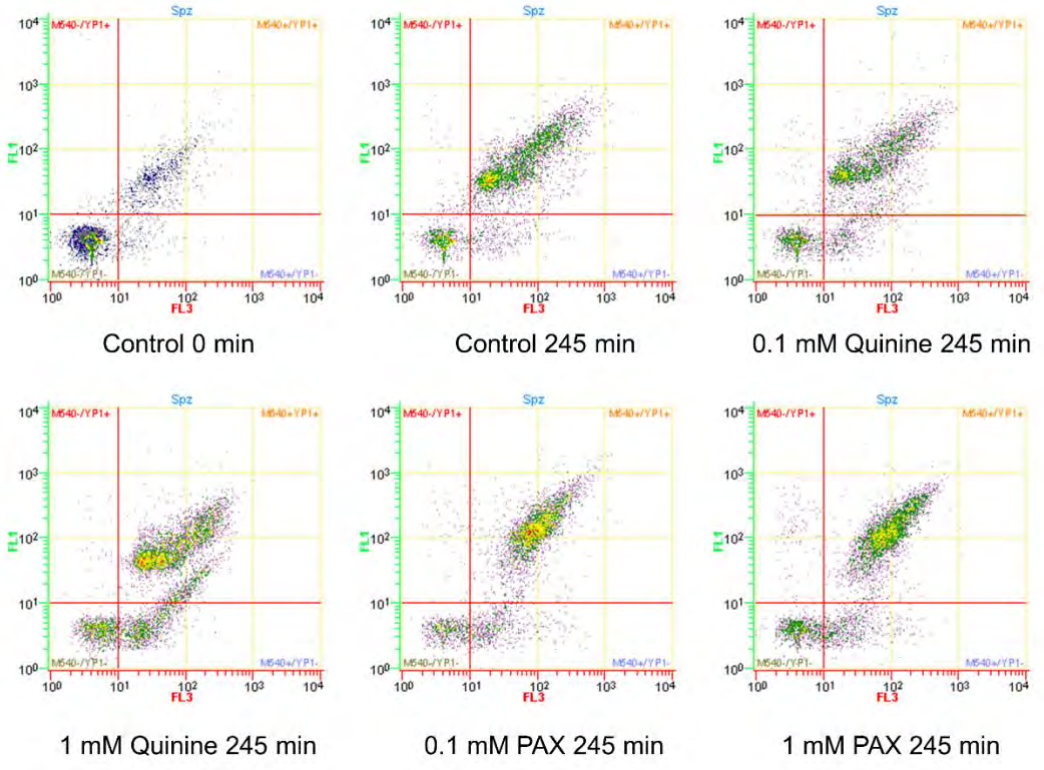

Figure S2: Representative dot-plots for M540/YO-PRO-1 staining in control and quinine- and PAX-blocked samples

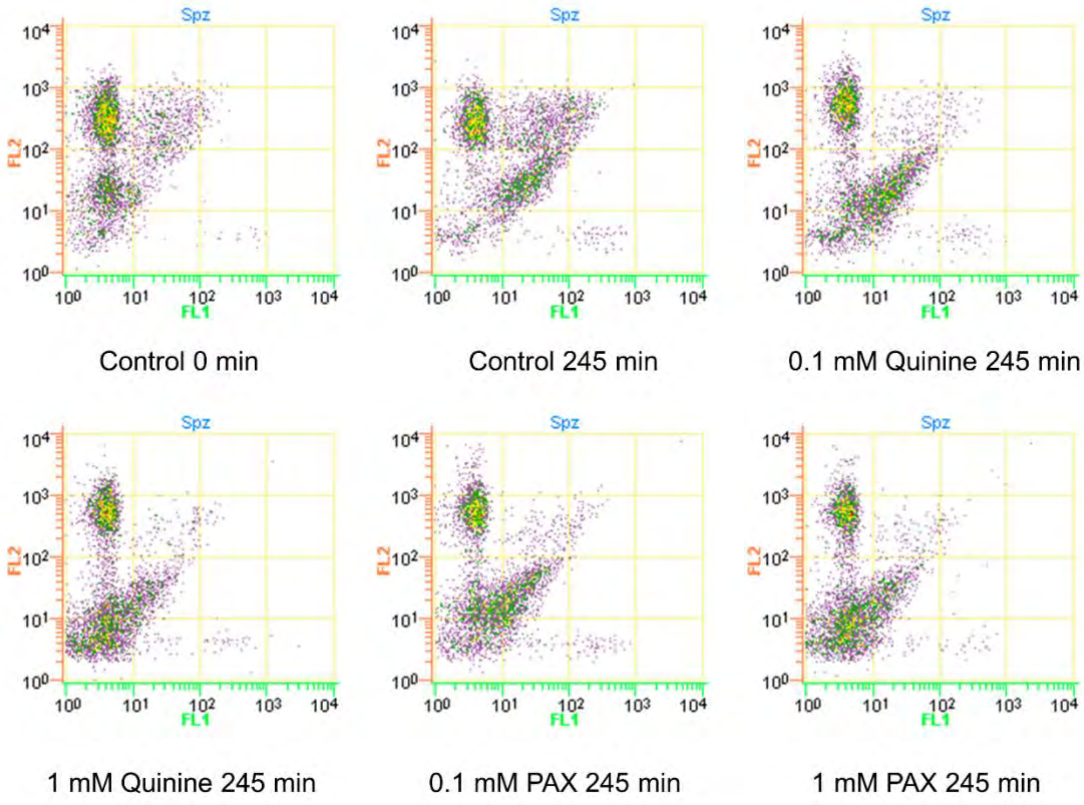

Figure S3: Representative dot-plots for JC1 staining in control and quinine- and PAX-blocked samples.

PAPER II

Parkinson Disease Protein 7 (PARK7) is related to the ability of mammalian sperm to undergo in vitro capacitation

Sandra Recuero, Ariadna Delgado-Bermúdez, Yentel Mateo-Otero, Estela Garcia-Bonavila, Marc Llavanera & Marc Yeste

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Article

Parkinson Disease Protein 7 (PARK7) Is Related to the Ability of Mammalian Sperm to Undergo In Vitro Capacitation

Sandra Recuero [1,2], Ariadna Delgado-Bermúdez [1,2], Yentel Mateo-Otero [1,2], Estela Garcia-Bonavila [1,2], Marc Llavanera [1,2] and Marc Yeste [1,2,*]

[1] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; sandra.recuero@udg.edu (S.R.); ariadna.delgado@udg.edu (A.D.-B.); yentel.mateo@udg.edu (Y.M.-O.); estela.garcia@udg.edu (E.G.-B.); marc.llavanera@udg.edu (M.L.)
[2] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain
* Correspondence: marc.yeste@udg.edu; Tel.: +34-972-419514

Abstract: Parkinson disease protein 7 (PARK7) is a multifunctional protein known to be involved in the regulation of sperm motility, mitochondrial function, and oxidative stress response in mammalian sperm. While ROS generation is needed to activate the downstream signaling pathways required for sperm to undergo capacitation, oxidative stress has detrimental effects for sperm cells and a precise balance between ROS levels and antioxidant activity is needed. Considering the putative antioxidant role of PARK7, the present work sought to determine whether this protein is related to the sperm ability to withstand in vitro capacitation. To this end, and using the pig as a model, semen samples were incubated in capacitation medium for 300 min; the acrosomal exocytosis was triggered by the addition of progesterone after 240 min of incubation. At each relevant time point (0, 120, 240, 250, and 300 min), sperm motility, acrosome and plasma membrane integrity, membrane lipid disorder, mitochondrial membrane potential, intracellular calcium and ROS were evaluated. In addition, localization and protein levels of PARK7 were also assessed through immunofluorescence and immunoblotting. Based on the relative content of PARK7, two groups of samples were set. As early as 120 min of incubation, sperm samples with larger PARK7 content showed higher percentages of viable and acrosome-intact sperm, lipid disorder and superoxide levels, and lower intracellular calcium levels when compared to sperm samples with lower PARK7. These data suggest that PARK7 could play a role in preventing sperm from undergoing premature capacitation, maintaining sperm viability and providing a better ability to keep ROS homeostasis, which is needed to elicit sperm capacitation. Further studies are required to elucidate the antioxidant properties of PARK7 during in vitro capacitation and acrosomal exocytosis of mammalian sperm, and the relationship between PARK7 and sperm motility.

Keywords: sperm; in vitro capacitation; PARK7; pig; ROS levels

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1. Introduction

Despite being mature and motile once ejaculated, mammalian sperm must reside in the female reproductive tract to acquire their fertilizing ability, in a process known as capacitation [1–3]. During capacitation, sperm undergo an assortment of physiological and biochemical changes, including alterations in plasma membrane permeability and fluidity [4,5], acrosome integrity [6], intracellular pH and calcium levels [7,8], mitochondrial activity [9], and the generation of reactive oxygen species (ROS) [10]. Oxidative stress, caused by excessive ROS formation, has detrimental consequences for sperm cells and fertility, including a decrease in sperm motility and their ability to interact with the oocyte, an increase in plasma membrane lipid peroxidation, protein damage and DNA fragmentation, and even the activation of apoptotic-like pathways [11]. Notwithstanding, moderate levels of ROS are needed to perform some physiological processes, such as sperm

capacitation [10,12]. Indeed, ROS generation during early capacitation events induces a rise of cAMP levels and the activation of protein kinases, which, in turn, trigger the phosphorylation of tyrosine residues of certain proteins, stimulating specific downstream pathways [13,14]. Thus, proper ROS homeostasis is required to avoid sperm damage.

Parkinson disease protein 7 (PARK7) or protein deglycase DJ-1 is a multifunctional protein encoded by the *PARK7* gene in chromosome 6, which has been found to be highly expressed in a large variety of mammalian tissues [15]. Although its biochemical function in sperm has not been fully studied, PARK7 and its rat homolog SP22/CAP1 have been reported to be involved in spermatogenesis [16,17], fertilization [18], motility [19], mitochondrial function [20], and oxidative stress response [20,21]. In human sperm, PARK7 is localized in the surface of the sperm head, the anterior part of the midpiece, and in the principal piece, reinforcing its putative role for oocyte binding during fertilization and for flagellar movement [22]. Similarly, PARK7 has also been detected on the surface of the equatorial segment of sheep [17], horse [23], rat, hamster, rabbit, and cattle sperm [18]. However, the precise localization of PARK7 in pig sperm is yet to be determined. With regard to the response to oxidative stress, PARK7 has been positively correlated with variables affected by ROS generation in human sperm, such as membrane integrity [17], total motility and superoxide dismutase (SOD) activity [19]. In patients with asthenozoospermia, characterized by a reduction in sperm motility, the relative content of PARK7 is lower in both ejaculated sperm [19,24] and seminal plasma [25]. In addition, PARK7 has been identified as a target of miR-4485-3, whose downregulation has also been associated to asthenozoospermia [26]. Under oxidative stress conditions associated with asthenozoospermia, PARK7 has been observed to be translocated from the equatorial segment to the midpiece, which has been proposed as a possible protective mechanism for sperm to maintain their mitochondrial function [20]. In addition to men suffering from asthenozoospermia, PARK7 is downregulated in patients with varicocele [27] and oligospermia [28], thus emphasizing its importance for male fertility.

Sperm handling and storage, such as cryopreservation or liquid conservation, have detrimental effects on sperm cells, including a reduction in viability and plasma membrane integrity, alterations in motility and mitochondrial function, and generation of oxidative stress [29,30]. Some of these perturbations share similarities with the events that take place during sperm capacitation and, for this reason, some authors refer to them as capacitation-like changes [31]. Besides that stated before, it is well known that changes in the levels, localization or function of specific proteins also occur [29]; for example, PARK7 is lost during liquid storage and cryopreservation of equine [23] and porcine sperm [32]. However, and to the best of our knowledge, whether or not PARK7 is involved in sperm capacitation has not been investigated in any species. Considering all the aforementioned, the present study sought to determine if PARK7 is related to the sperm ability to undergo in vitro capacitation and trigger the acrosomal exocytosis induced by progesterone using the pig as a model. Specifically, changes in the localization of this protein during in vitro capacitation were overseen, and whether the relative content of PARK7 in fresh sperm is related to their ability to withstand in vitro capacitation was interrogated.

2. Results

2.1. Classification of Sperm Samples Based on Their Relative PARK7 Content

Based on the relative content of PARK7 (PARK7/α-tubulin ratio), determined using immunoblotting analysis at the beginning of the experiment (0 min), two different groups of sperm samples, with high (n = 4; 1.55 ± 0.56; mean ± SD) and low relative levels of PARK7 (n = 4; 0.45 ± 0.13), were distinguished. The sperm samples with high relative PARK7 content at 0 min showed significantly ($p < 0.05$) higher levels of PARK7 than the group with low relative PARK7 content in all the time points assessed, except at 240 min when no statistical differences were observed (Figure 1). As shown in Figure 1, PARK7 levels decreased along incubation in capacitation medium, especially in sperm with high relative PARK7 content. Figure 2a,b show representative blots for α-tubulin and PARK7,

respectively. The peptide competition assay for the anti-PARK7 antibody demonstrated that the band at 25 kDa was specific for PARK7 (Figure 2c).

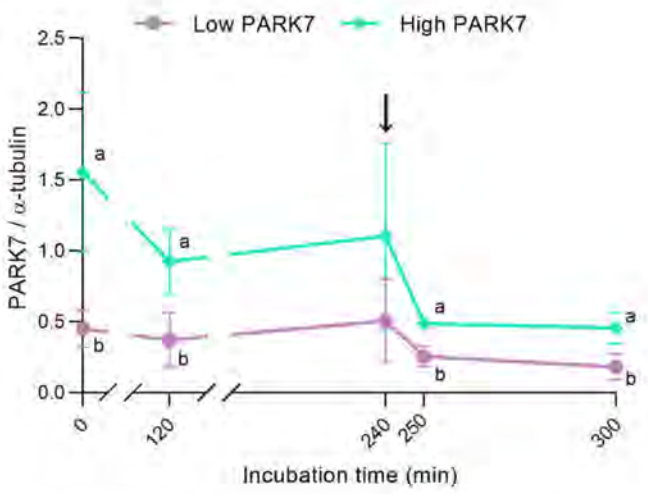

Figure 1. Relative PARK7 content (standardized against α-tubulin levels) during in vitro capacitation and acrosomal exocytosis induced by progesterone. The black arrow indicates the addition of progesterone at a final concentration of 10 µg/mL (240 min). Results are shown as the mean of PARK7/α-tubulin ratio ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples with high and low PARK7 content.

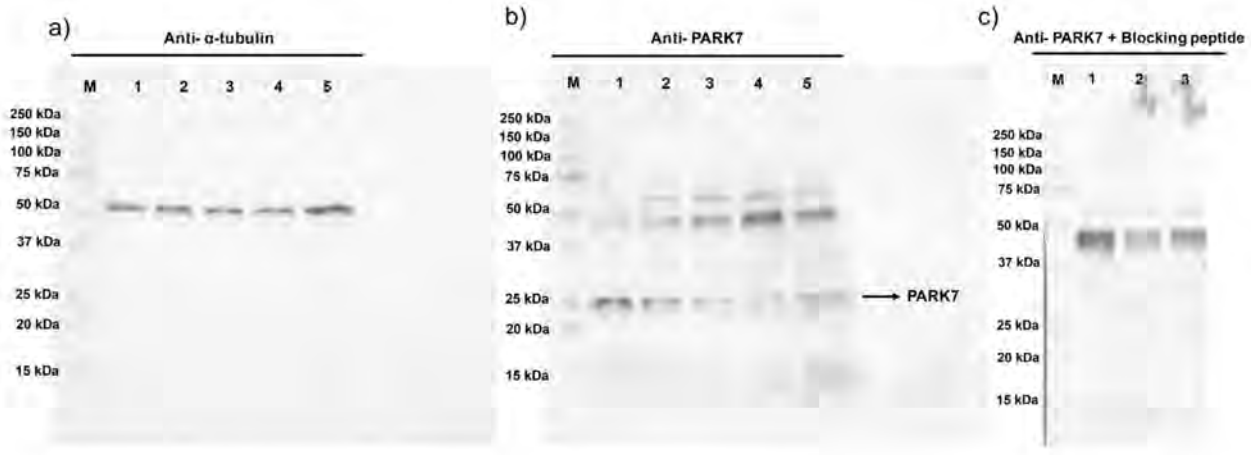

Figure 2. Representative immunoblots for α-tubulin (**a**), PARK7 (**b**), and peptide competition assay (anti-PARK7 + blocking peptide; (**c**). Lanes (M): protein ladder; (1) 0 min; (2) 120 min; (3) 240 min; (4) 250 min; and (5) 300 min of incubation.

2.2. Sperm Motility

Regarding the total (Figure 3a) and progressive (Figure 3b) motility, no significant differences ($p > 0.05$) between the sperm samples with high and low relative PARK7 levels were observed during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min). Although no significant differences were observed between the different time points in the group of samples with low relative PARK7, total motility also tended to decrease along incubation (0 min vs. 300 min; $p = 0.060$).

In relation to kinetic parameters, shown in Table 1, curvilinear velocity (VCL, µm/s) presented a tendency to be higher in samples with high PARK7 compared to those with low PARK7 after 250 min of incubation ($p = 0.052$). The same tendency was observed regarding straight-line velocity (VSL, µm/s; $p = 0.061$) and linearity (LIN, %; $p = 0.068$). Whereas the amplitude of lateral head displacement (ALH, µm) was significantly higher in samples

with high relative PARK7 content than in those with low levels of this protein at 240 min ($p < 0.05$), straightness (STR, %) was significantly higher in the sperm with high relative amount of PARK7 compared to those with low PARK7 after 250 min of incubation ($p < 0.05$).

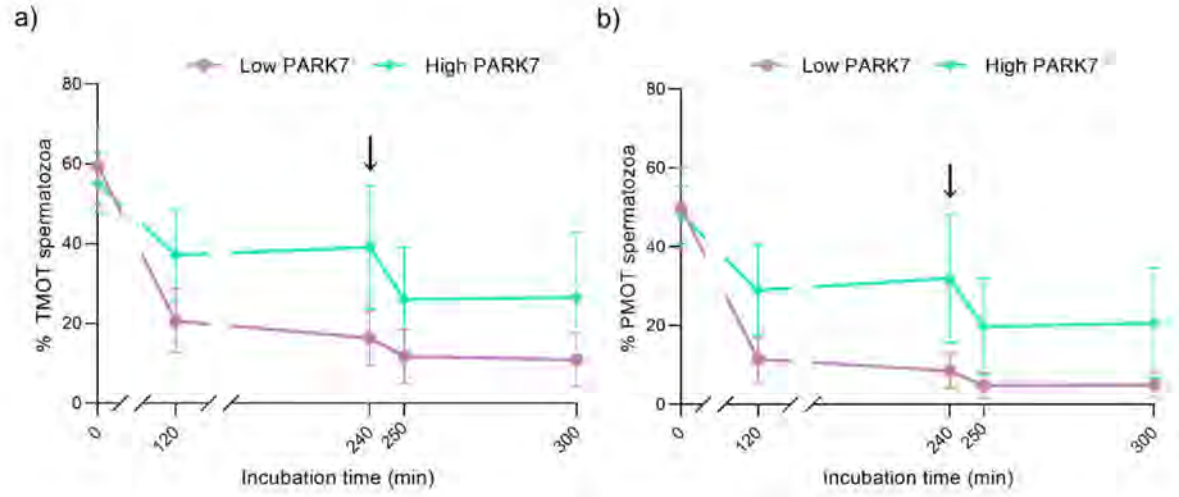

Figure 3. Percentages of total (**a**) and progressively (**b**) motile sperm during in vitro capacitation and acrosomal exocytosis induced by progesterone (300 min). The black arrows indicate the addition of progesterone at a final concentration of 10 µg/mL (240 min). Data are represented as means ± SD. Abbreviations: TMOT, total motility; PMOT, progressive motility.

Table 1. Values of kinematic parameters of two groups of sperm samples, clustered on the basis of their relative PARK7 content at 0 min, during in vitro capacitation and progesterone-induced acrosomal exocytosis (0, 120, 240, 250, 300 min). Results are shown as means ± SD. VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; WOB: wobble coefficient; STR: straightness.

		Incubation Time				
		0 min	**120 min**	**240 min**	**250 min**	**300 min**
VCL (µm/s)	Low PARK7	119.52 ± 15.09	76.15 ± 14.81	67.58 ± 4.59	65.99 ± 6.50	60.98 ± 11.27
	High PARK7	85.24 ± 35.07	77.30 ± 16.43	78.79 ± 8.14	84.73 ± 9.96	77.69 ± 7.49
VSL (µm/s)	Low PARK7	81.33 ± 16.15	42.75 ± 13.69	34.58 ± 14.31	31.09 ± 7.95	27.65 ± 11.34
	High PARK7	55.55 ± 30.51	43.37 ± 12.79	50.34 ± 6.14	55.83 ± 14.50	45.56 ± 13.55
VAP (µm/s)	Low PARK7	95.54 ± 16.06	55.03 ± 13.70	44.99 ± 12.07	42.59 ± 8.39	38.37 ± 12.66
	High PARK7	63.62 ± 34.78	51.67 ± 15.08	58.14 ± 7.50	63.39 ± 14.17	53.76 ± 12.33
LIN (%)	Low PARK7	67.53 ± 6.21	54.76 ± 8.91	50.30 ± 18.59	46.81 ± 8.89	43.36 ± 14.15
	High PARK7	62.81 ± 8.27	55.74 ± 10.33	63.97 ± 6.18	65.13 ± 9.19	57.77 ± 11.60
STR (%)	Low PARK7	84.69 ± 2.90 [a]	75.97 ± 7.09 [a]	74.23 ± 13.42 [a]	71.41 ± 6.16 [a]	68.56 ± 11.46 [a]
	High PARK7	87.03 ± 0.46 [a]	83.82 ± 3.70 [a]	86.67 ± 3.80 [a]	87.55 ± 3.87 [b]	83.75 ± 6.63 [a]
WOB (%)	Low PARK7	79.57 ± 4.84	71.49 ± 5.46	65.94 ± 14.16	64.34 ± 8.50	61.53 ± 11.69
	High PARK7	72.09 ± 9.24	66.26 ± 9.86	73.72 ± 4.80	74.15 ± 7.61	68.51 ± 8. 90
ALH (µm)	Low PARK7	3.71 ± 0.27 [a]	2.57 ± 0.40 [a]	2.23 ± 0.18 [a]	2.52 ± 0.24 [a]	2.23 ± 0.65 [a]
	High PARK7	2.90 ± 0.61 [a]	2.87 ± 0.42 [a]	2.68 ± 0.14 [b]	2.82 ± 0.03 [a]	2.78 ± 0.06 [a]
BCF (Hz)	Low PARK7	9.94 ± 0.52	8.08 ± 1.71	6.89 ± 2.16	6.53 ± 1.77	6.50 ± 2.03
	High PARK7	10.59 ± 0.44	9.88 ± 1.07	9.88 ± 0.70	9.65 ± 1.47	9.67 ± 1.21

[a,b] indicate significant differences ($p < 0.05$) between sperm samples containing high or low levels of PARK7.

2.3. Sperm Viability

The percentage of viable sperm (i.e., sperm with intact plasma membrane, SYBR14$^+$/PI$^-$) was similar between the groups along in vitro capacitation and progesterone-induced acrosomal exocytosis (Figure 4a). However, the viability of sperm samples with high relative PARK7 content presented a clear tendency to be higher compared to those with low relative PARK7 levels after 120 min of incubation (p = 0.057). On the other hand, although no significant differences in sperm viability between the different time points were observed in the group of samples with low relative amounts of PARK7, the percentage of viable sperm was significantly lower ($p < 0.05$) after 120 min of incubation than at the beginning of the experiment (0 min).

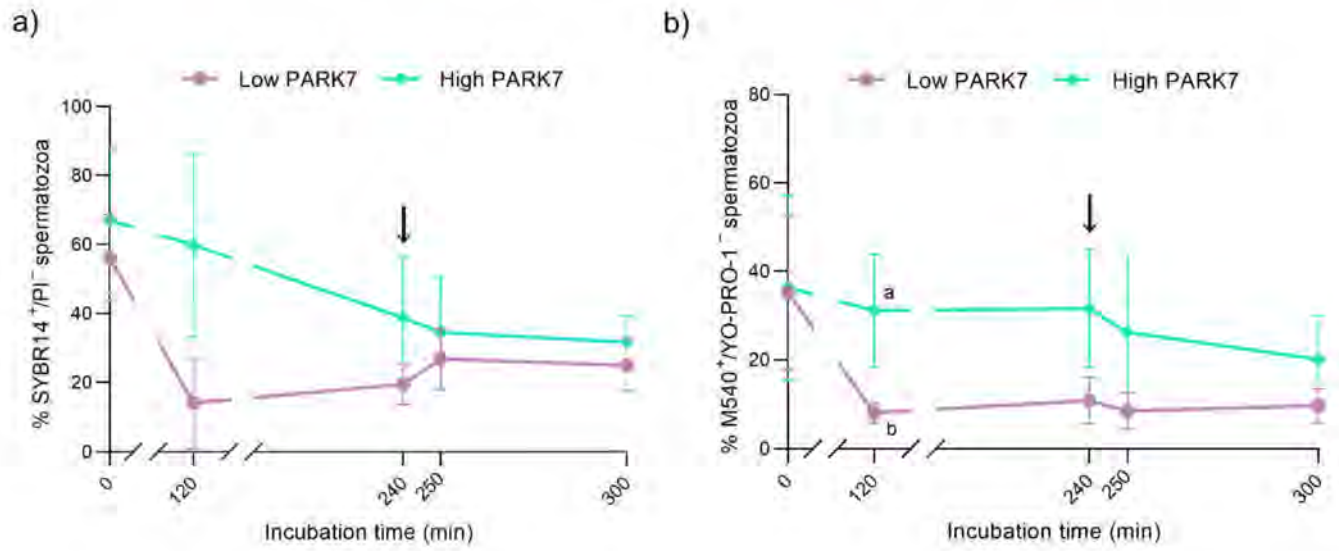

Figure 4. Percentages of viable sperm (SYBR14$^+$/PI$^-$ population) (**a**) and viable sperm with high lipid membrane disorder (M540+/YO-PRO-1- population) (**b**) during in vitro capacitation and acrosomal exocytosis induced by progesterone (300 min). The black arrows indicate the addition of progesterone at a final concentration of 10 µg/mL (240 min). Data are represented as means ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples containing high and low relative levels of PARK7.

2.4. Membrane Lipid Disorder

After 120 min of incubation in capacitation medium, the percentage of viable sperm with high lipid membrane disorder (M540$^+$/YO-PRO$^-$) was higher in the sperm samples with high relative PARK7 content than in those with lower levels of this protein ($p < 0.05$) (Figure 4b). Within the group of samples with high relative amounts of PARK7, the percentage of viable sperm with high membrane lipid disorder significantly decreased after 300 min of incubation compared to 120 min ($p < 0.05$). Regarding the population of viable sperm with low lipid membrane disorder (M540$^-$/YO-PRO$^-$), the percentage was higher in the sperm samples with high relative PARK7 content than in those with low relative PARK7 levels ($p < 0.05$) after 120 min of incubation (Figure S1). As far as the population of non-viable sperm with high membrane lipid disorder (M540$^+$/YO-PRO$^+$) is concerned, the percentage was higher in the sperm samples with low relative PARK7 content compared to those with high relative PARK7 amounts after 120 and 240 min of incubation ($p < 0.05$) (Figures S2–S5).

2.5. Acrosome Membrane Integrity

With regard to the integrity of the acrosome membrane, the percentage of viable sperm with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$) was higher in the samples with high relative PARK7 content than in those with low relative PARK7 levels after 120 and 240 min of incubation ($p < 0.05$) (Figure 5a). Conversely, the percentage of non-viable

sperm with an outer acrosome membrane that could not be fully intact (PNA-FITC$^+$/PI$^+$) was significantly lower in the sperm with high relative PARK7 content than in those with low relative PARK7 levels after 120 and 240 min of incubation ($p < 0.05$) (Figure S6). In samples with low relative amounts of PARK7, this percentage exhibited a prompt increase at 120 min compared to the beginning of the experiment. This increase was maintained until the end of the incubation in a capacitation medium (300 min).

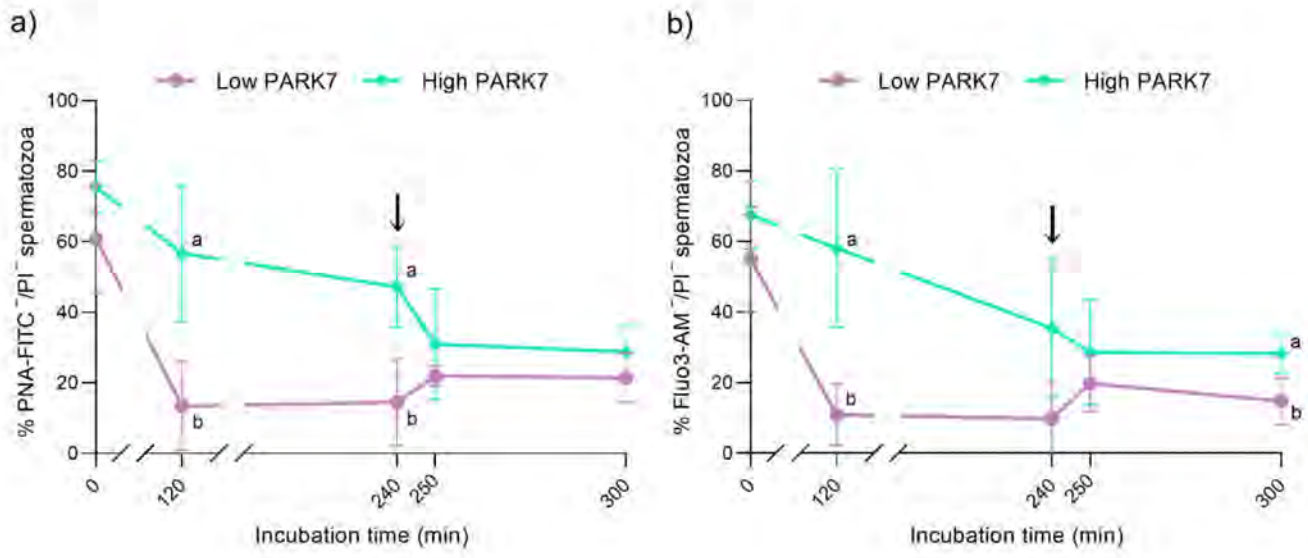

Figure 5. Percentages of viable sperm with an intact acrosome membrane (PNA$^-$FITC-/PI$^-$ population) (**a**) and viable sperm with low intracellular calcium levels (Fluo3-/PI- population) (**b**) during in vitro capacitation and acrosomal exocytosis induced by progesterone (300 min). The black arrows indicate the addition of progesterone at a final concentration of 10 µg/mL (240 min). Data are represented as means ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples containing high and low relative levels of PARK7.

2.6. Intracellular Calcium Levels

The percentage of viable sperm with low intracellular calcium levels (Fluo3$^-$/PI$^-$) was significantly higher in the samples with high relative PARK7 content compared to those with low relative PARK7 after 120 min of incubation in capacitation medium ($p < 0.05$) (Figure 5b). Although this difference was not statistically significant, this percentage presented a tendency to be higher in the sperm with high relative amounts of PARK7 than in those with low relative PARK7 levels after 300 min of incubation ($p = 0.056$).

2.7. Intracellular ROS Levels

After 120 and 240 min of incubation in capacitation medium, the percentage of viable sperm with high O_2^- levels (E$^+$/YO-PRO-1$^-$) was significantly higher in the sperm samples with high relative PARK7 content than in those with low relative PARK7 levels ($p < 0.05$) (Figure S7). Considering the viable sperm population only, the percentage of cells with high intracellular superoxide levels (E$^+$/viable sperm population) was found to be significantly higher in the sperm with high than in those with low relative PARK7 content after 120 min of incubation ($p < 0.05$) (Figure 6a). In contrast, no significant differences between the samples with high and low relative PARK7 levels were found regarding intracellular peroxide levels during in vitro capacitation and progesterone-induced acrosomal exocytosis ($p > 0.05$).

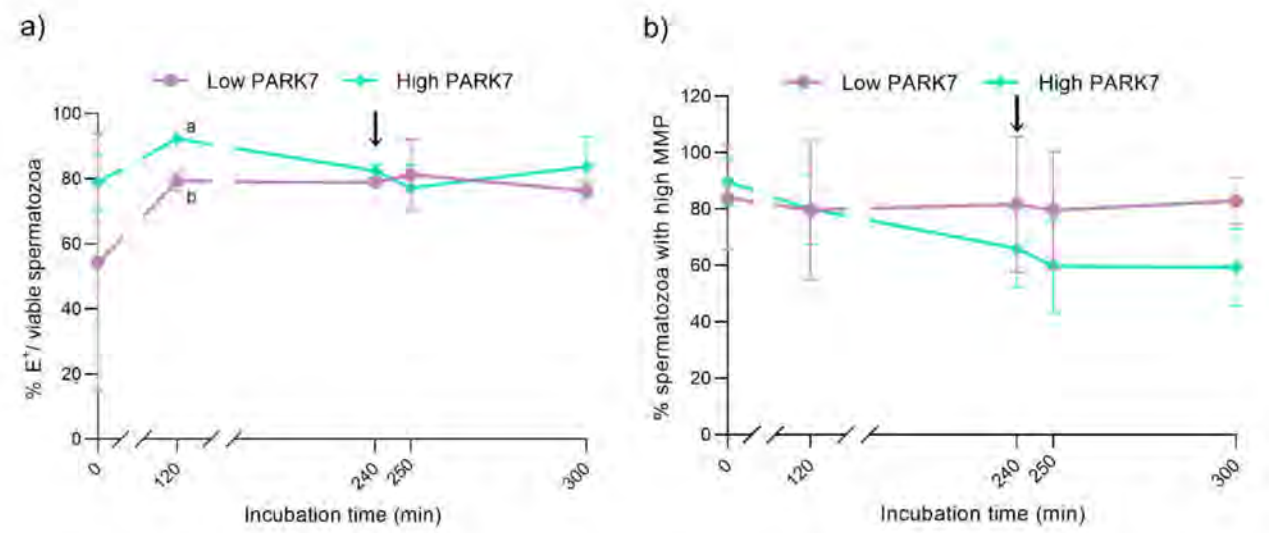

Figure 6. Percentages of sperm with high intracellular O_2^- levels in relation to viable sperm (E^+/viable sperm) (**a**) and sperm exhibiting high mitochondrial membrane potential (MMP) (**b**) during in vitro capacitation and acrosomal exocytosis induced by progesterone (300 min). The black arrows indicate the addition of progesterone at a final concentration of 10 µg/mL (240 min). Data are represented as means ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples containing high and low relative levels of PARK7.

2.8. Mitochondrial Membrane Potential (MMP)

The differences in the initial relative levels of PARK7 in the sperm did not affect MMP during in vitro capacitation and progesterone-induced acrosomal exocytosis. However, although not significant, the percentage of sperm exhibiting high MMP tended to be lower in sperm samples with high relative PARK7 content than in those with low relative levels of this protein after 300 min of incubation in capacitation medium ($p = 0.063$) (Figure 6b).

2.9. Localization of PARK7 in Sperm during In Vitro Capacitation and Acrosomal Exocytosis

Localization of PARK7 protein in sperm was assessed through immunofluorescence. As shown in Figure 7, green fluorescence was detected in the sperm flagellum, midpiece, the anterior part of the head and the post-acrosomal region. After antibody specificity assay (Figure 8), fluorescence staining of the flagellum and the post-acrosomal region disappeared, which confirmed these two regions as specific localizations of PARK7. On the other hand, PARK7-immunolocalization was also evaluated at each relevant time point (0, 120, 240, 250 and 300 min) (Figure 7) in order to determine whether PARK7 relocalized during in vitro capacitation and acrosomal exocytosis. As shown in Figure 7, the localization pattern of PARK7 in sperm was maintained along the incubation in capacitation medium (300 min).

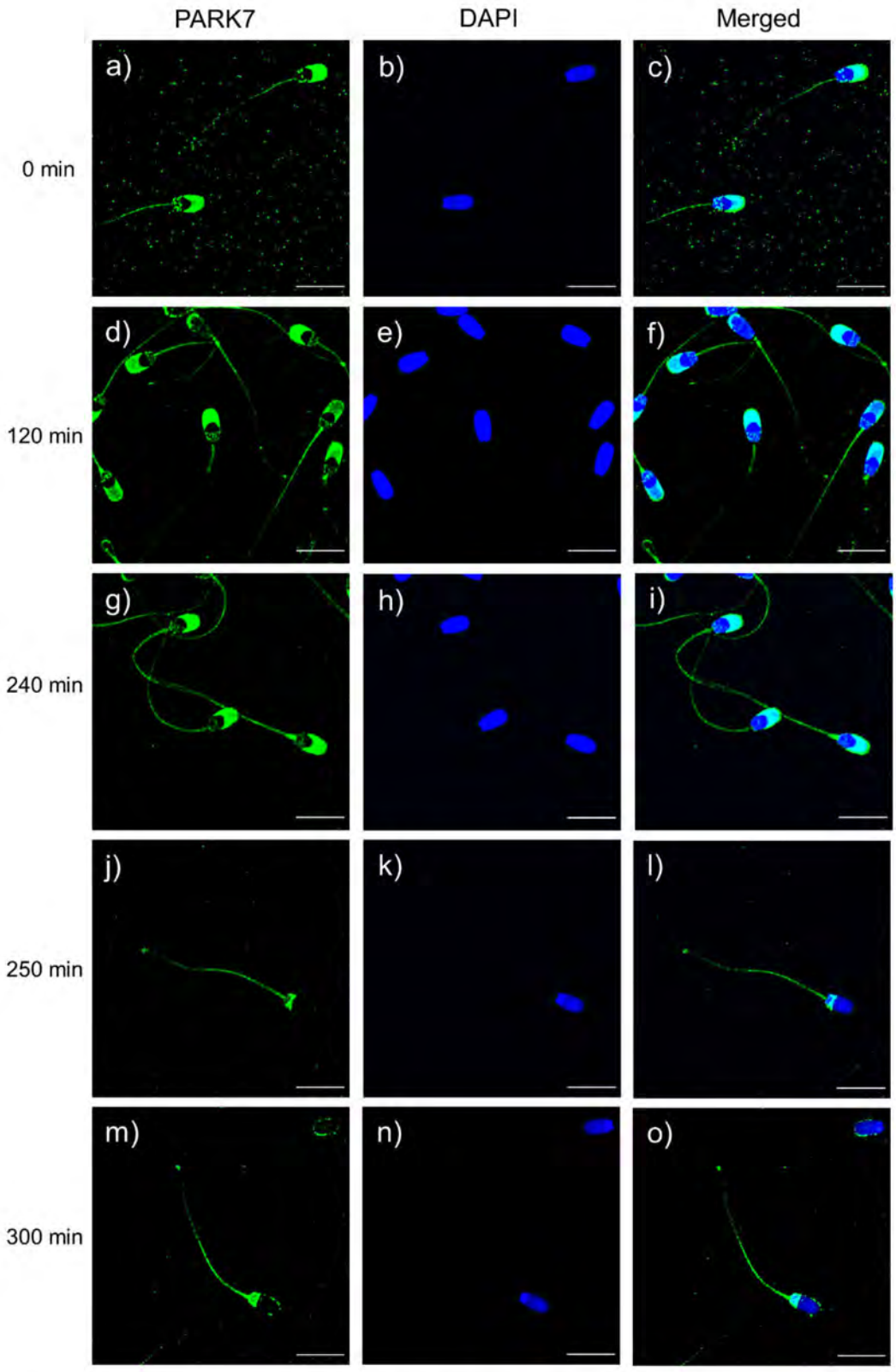

Figure 7. Immunolocalization of PARK7 protein at each relevant time point during in vitro sperm capacitation and acrosomal exocytosis: 0 min (**a**–**c**); 120 min (**d**–**f**); 240 min (**g**–**i**); 250 min (**j**–**l**); and 300 min (**m**–**o**). PARK7 fluorescence is shown in green, and nuclei are shown in blue (DAPI). Scale bar: 13.2 μm.

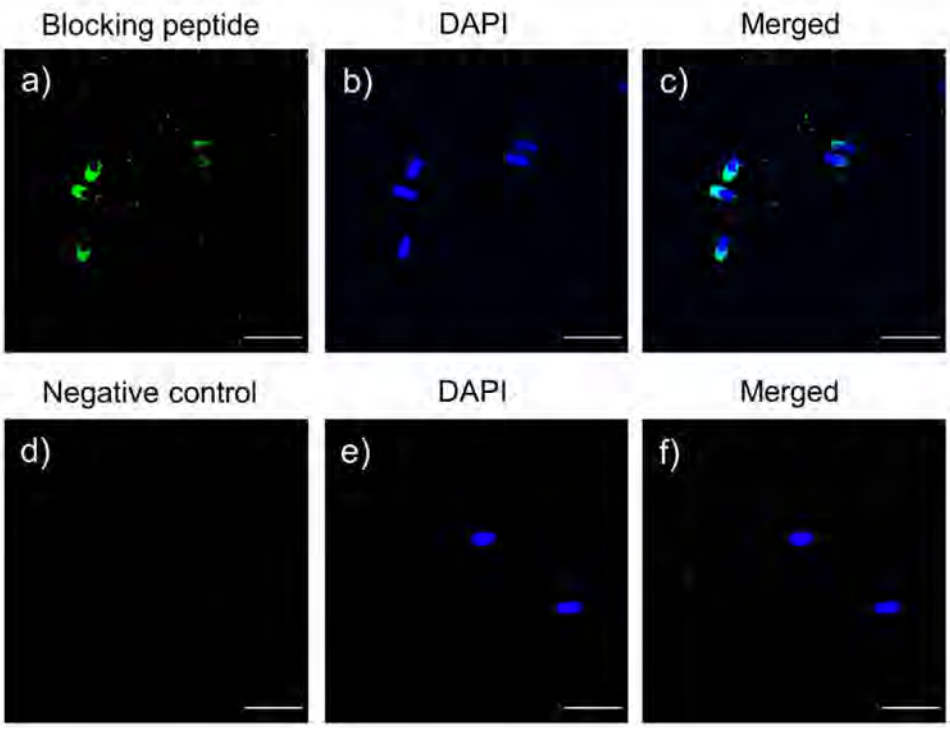

Figure 8. Immunofluorescence of the peptide competition assay for the anti-PARK7 antibody (**a**–**c**) and negative control (**d**–**f**). Green fluorescence indicates unspecific PARK7 staining, and nuclei are shown in blue (DAPI). Scale bar: 20 μm.

3. Discussion

During the physiological process through which a spermatozoon acquires the ability to penetrate the zona pellucida and fuse with the oocyte, namely capacitation, the sperm cells undergo a variety of changes including modifications in the fluidity of plasma and acrosome membranes [4–6], increase in intracellular calcium and ROS levels [7,8,10], and tyrosine phosphorylation of certain proteins [33,34]. In fact, the ROS generated during early capacitation events are related to calcium compartmentalization [35] and tyrosine phosphorylation [10], which are both required for triggering the downstream signaling pathways that lead sperm to achieve the capacitated status. In this regard, a balance between ROS formation and antioxidant activity is essential to avoid the harmful effects caused by oxidative stress.

Although PARK7 protein has been extensively studied in the context of neurodegenerative disorders (reviewed by [36]), it is known to be expressed not only in the brain but also in other tissues and organs, including male epididymis and testis [22]. Although its precise function in sperm is not fully understood, PARK7 is associated with the oxidative stress response [20,21]. In humans, PARK7 has been positively correlated with sperm plasma membrane integrity [17], total motility and SOD activity [19]. Additionally, the relevance of PARK7 for male fertility has been previously investigated. In effect, PARK7 has been found downregulated in patients with asthenozoospermia [19,24], varicocele [27], or oligozoospermia [28]. To the best of our knowledge, however, whether or not PARK7 is related to the sperm ability to undergo in vitro capacitation and trigger the progesterone-induced acrosomal exocytosis has not been investigated in any mammalian species, including humans. The present study demonstrated, for the first time and using the pig as an animal model, that the ability of mammalian sperm to undergo in vitro capacitation is related to their relative PARK7 content. Herein, the relative content of PARK7 was shown to decrease along the incubation of sperm in capacitation medium. This is in agreement with an earlier study that also found that the relative PARK7 content decreases after capacitation of pig sperm [37]. Moreover, the statistical approach performed in this work allowed distin-

guishing two different groups of sperm samples, based on the relative levels of PARK7 at the beginning of the experiment (0 min). Following this, the parameters associated with sperm capacitation were compared between these two groups, and how such potential changes were influenced by the relative PARK7 content was interrogated. Moreover, the localization pattern of PARK7 in pig sperm was not previously investigated, nor were the potential changes in that localization during in vitro capacitation and acrosomal exocytosis in any mammalian species addressed. The immunofluorescence assay revealed that the localization pattern of PARK7 is maintained during in vitro capacitation and acrosomal exocytosis and that this protein is located in the post-acrosomal region and tail of pig sperm. Similarly, in humans, PARK7 is present on the surface of the posterior part of the head, the midpiece and the flagellum [22].

Regarding the integrity of sperm plasma membrane, clear differences between samples with high and low PARK7 levels were found. Samples with low relative PARK7 content at the onset of the experiment experienced a severe decline in sperm viability during the first 120 min of incubation. In addition, non-viable sperm with high membrane lipid disorder were significantly higher in the group with low PARK7 levels. This high asymmetry in membrane lipids is likely to induce the destabilization of the plasma membrane, leading to sperm death. Remarkably, while a decrease in membrane integrity and, therefore, sperm viability, was expected during in vitro capacitation [38,39], such a reduction occurred more progressively in sperm samples that contained higher PARK7 levels. As mentioned earlier, PARK7 levels have been positively correlated with plasma membrane integrity [17]. Thus, in mammalian sperm that present a plasma membrane particularly sensitive to oxidative stress due to its polyunsaturated fatty acid composition [40], PARK7 could maintain membrane integrity through its antioxidant function. Along these lines, the integrity of the acrosome membrane was also highly altered in sperm samples with low relative PARK7 content at the beginning of the experiment. Whereas sperm samples with high PARK7 levels maintained their acrosome intact during the first 240 min of incubation, those with low PARK7 presented a significant decline in the integrity of both plasma and acrosome membrane. One possible explanation is that such a loss of acrosome integrity was a direct consequence of the aforementioned plasma membrane destabilization. In addition, in samples with low PARK7 content, viable sperm with low intracellular calcium levels promptly decreased, concomitantly with the loss of acrosomal and plasma membrane integrity during the first 120 min of incubation. Bearing in mind that intracellular calcium is essential to trigger acrosomal exocytosis [41] and considering that membrane destabilization could lead to alterations in ion permeability, an increase in calcium influx could provoke a premature acrosome exocytosis.

As pointed out earlier, high amounts of ROS have detrimental effects on sperm cells and, thus, a precise control of their levels is necessary during sperm capacitation. It is known that antioxidant molecules have the potential to modulate the capacitation status of sperm [35,42]. The antioxidant enzyme glutathione peroxidase 6 (GPX6) has been posited to prevent sperm from premature capacitation by removing the excess of ROS [43]. Similarly, another enzyme with antioxidant properties, paraoxonase 1 (PON-1), can hinder sperm from spontaneous acrosome reaction [44]. In this sense, PARK7 could also play a role modulating ROS levels during sperm capacitation. Strikingly, superoxide levels were higher in samples containing high relative PARK7 content; those samples also showed higher viability than the ones with low levels of PARK7. Furthermore, in humans, concentration of PARK7 in ejaculated sperm has been positively correlated with SOD activity, which catalyzes the conversion of superoxides into hydrogen peroxide [19]. Considering that sperm with high PARK7 content showed higher superoxide levels and that no significant differences were observed with regard to peroxide levels, PARK7 does not seem to be associated with SOD activity during in vitro capacitation. Therefore, the data obtained herein suggest that PARK7 could maintain sperm viability when superoxide levels are elevated, thus providing sperm with a higher tolerance to ROS generation needed for sperm capacitation, among other physiological processes.

Finally, and regarding motility, it has been proposed that mammalian sperm, including the porcine, display a specific motility pattern under capacitation conditions [45]. In humans, PARK7 levels have been positively correlated with sperm motility [19]. In spite of this, in the present study significant differences in total or progressive motility were not observed. Notwithstanding, significant differences or tendencies were observed in several kinematic sperm parameters, including VCL, VSL, LIN, ALH, and STR. Thus, it is reasonable to reckon that PARK7 plays a role in the regulation of sperm motility during capacitation. Taking this into consideration, future studies including more animals are needed to elucidate the relation of PARK7 with motility regulation in porcine sperm.

4. Materials and Methods

Unless otherwise stated, all reagents used in the present study were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

4.1. Semen Samples

Ejaculates from 32 different sexually mature Piétrain boars were provided by an authorized artificial insemination (AI) center (Grup Gepork S.L., Masies de Roda, Spain). Boars were fed with a standard and balanced diet with water being provided ad libitum. According to the farm records, all boars were fertile. Ejaculates were manually collected through the gloved-hand method. The sperm-rich fraction was diluted in a commercial extender for liquid storage (Vitasem LD; Magapor S.L., Zaragoza, Spain) at a final concentration of 3.3×10^7 sperm/mL. Seminal doses of 90 mL and 3 billion sperm per dose were transported at 17 °C to the laboratory within three hours post-collection.

All procedures involving animals were performed according to the EU Directive 2010/63/EU for animal experiments, the Animal Welfare Law and the current regulation on Health and Biosafety issued by the Department of Agriculture, Livestock, Food and Fisheries (Regional Government of Catalonia, Spain). Production of seminal doses by the AI center followed the ISO certification (ISO-9001:2008). As seminal doses were directly purchased from the local AI center, the authors did not manipulate any animal and no approval from an ethics committee was required.

4.2. In Vitro Capacitation and Progesterone-Induced Acrosomal Exocytosis

Four seminal doses from different boars were randomly pooled in each experiment (n = 8 different experiments; 32 boars), in order to avoid variability between males. Pooled semen samples were washed with PBS (centrifugation at 600× g and 17 °C for 5 min) as indicated by Yeste et al. [8], and pellets were finally resuspended in 40 mL of capacitation medium (CM) at a final concentration of 2.7×10^7 sperm/mL. The CM consisted of 20 mM HEPES (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 130 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 4.5 mM $CaCl_2 \cdot 2H_2O$, 15 mM $NaHCO_3$, and 5 mg/mL of bovine serum albumin (BSA). The osmolality was checked to be in the range of 290–310 mOsm/kg, and the pH was adjusted to 7.4. Sperm were incubated at 38 °C and 5% CO_2 for 300 min (Heracell 150; Heraeus Instruments GmbH, Osterode, Germany). After 240 min of incubation, progesterone was added at a final concentration of 10 µg/mL, in order to induce the acrosomal exocytosis [46,47].

At each relevant time point (0, 120, 240, 250, and 300 min), which were set based on previous studies [8,9], the following sperm parameters were evaluated: sperm viability, membrane lipid disorder, acrosome integrity, mitochondrial membrane potential, and intracellular levels of calcium, peroxides, and superoxides. In addition, two different aliquots were taken for immunoblotting and immunofluorescence analysis. Samples for protein extraction were washed twice (PBS, pH = 7.3) at 300× g for 5 min (room temperature) to remove the CM; sperm pellets were stored at −80 °C for further processing.

4.3. Sperm Motility

Evaluation of total and progressive sperm motility was performed using a commercial computer assisted sperm analysis (CASA) system. This system consisted of a phase-contrast microscope (Olympus BX41; Olympus, Hamburg, Germany) connected to a computer equipped with the Integrated Semen Analysis System (ISAS®) V1.0 software (Proiser S.L., Valencia, Spain). Briefly, 5 µL of each semen sample was placed onto a pre-warmed (37 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Then, samples were observed under a negative phase-contrast objective at 100× magnification (Olympus 10× 0.30 PLAN objective lens). Three replicates, with a minimum of 500 sperm per replicate, were counted in each sample per time point. The corresponding mean ± standard deviation (SD) was calculated. The sperm motility parameters recorded using the software were the following: total motility (TMOT, %); 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, %); wobble coefficient (WOB, %); and straightness (STR, %). Total motility was defined as the percentage of sperm showing VAP $\geq$ 10 µm/s, and progressive motility was defined as the percentage of motile sperm showing STR $\geq$ 45%. These parameters are described in more detail in Verstegen et al. [48].

4.4. Flow Cytometry Analyses

The following seven sperm variables were evaluated using flow cytometry at each relevant time point: sperm viability (i.e., plasma membrane integrity), acrosome integrity, membrane lipid disorder, acrosome membrane integrity, and intracellular levels of superoxides (O_2^-), peroxides (H_2O_2), and calcium. Samples were diluted in PBS (pH = 7.3) to a final concentration of 2 × 10^6 sperm/mL prior to fluorochrome staining. Unless otherwise stated, all fluorochromes were acquired from ThermoFisher Scientific (Waltham, MA, USA). Flow cytometry analyses were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA). Samples were excited with an argon ion laser (488 nm) set at power of 22 mW, and laser voltage and rate were constant along the experiment. For each event, the cytometer provided the electronic volume (EV) and side scatter (SS). Three different optical filters (FL-1, FL-2, and FL-3) were used with the following optical properties: FL-1: Dichroic/Splitter (DRLP): 550 nm, BP filter: 525 nm, detection width 505–545 nm. FL-1 detected green fluorescence (SYBR-14; YO-PRO-1; 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide monomers, JC-1 monomers, JC-1_{mon}; peanut agglutinin conjugated with fluorescein isothiocyanate, PNA-FITC; 2′,7′-dichlorofluorescin diacetate, H_2DCFDA; and Fluo-3-AM). FL-2: DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm. FL-2 allowed detecting orange fluorescence (JC-1 aggregates, JC-1_{agg}), and FL-3: LP filter: 670, detection width: 670 ± 30 nm. FL-3 was used to collect red fluorescence (merocyanine 540, M540; hydroethidine, HE; and propidium iodide, PI). For each staining protocol, photomultiplier settings were adjusted, and signals were logarithmically amplified. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 µm) and cell aggregates (particle diameter >12 µm).

Flow rate was set at 4.17 µL/min in all analyses and three technical replicates, with a minimum of 10,000 events per replicate, were evaluated for each sample and sperm parameter. Flow cytometry data analysis was performed using Flowing Software (Ver. 2.5.1; Turku Bioscience, Turku, Finland), according to the recommendations of the International Society for Advancement of Cytometry (ISAC). Following this, the corresponding mean ± SD was calculated.

4.4.1. Sperm Viability (SYBR-14/PI)

Evaluation of sperm viability was performed by assessing plasma membrane integrity, using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Eugene, OR, USA), following the protocol set by Garner and Johnson [49]. In brief, sperm were stained with SYBR-14

(100 nmol/L) for 10 min at 38 °C in the dark, and next, with PI (12 µmol/L) for 5 min at the same conditions. Flow cytometry dot-plots were generated based on the combination of SYBR-14 and PI, resulting in the following three sperm populations: (i) viable, green-stained sperm (SYBR-14^{+}/PI^{-}); (ii) non-viable, red-stained sperm (SYBR-14^{-}/PI^{+}); and (iii) non-viable sperm stained in both green and red (SYBR-14^{+}/PI^{+}). SYBR-14 spill over into the FL-3 channel was compensated (2.45%). The percentage of non-sperm particles, corresponding to non-stained debris particles (SYBR-14^{-}/PI^{-}), was used to correct the percentage of particles within the double negative quadrant of every described parameter. The percentages of the other three populations were also recalculated. This percentage of non-sperm particles was also used to correct the data from the other tests.

4.4.2. Membrane Lipid Disorder (M540/YO-PRO-1)

The assessment of membrane lipid disorder was performed using M540 and YO-PRO-1 fluorochromes, based on the protocol from Rathi et al. [50], as modified by Yeste et al. [51]. Since M540 uptake increases with high membrane destabilization, this fluorochrome has been established as a marker of sperm membrane destabilization in different mammalian species [52]. Sperm were incubated with M540 (2.6 µmol/L) and YO-PRO-1 (25 nmol/L) for 10 min at 38 °C in the dark. Four populations were identified in flow cytometry dot-plots as a result of the combination of both fluorochromes: (i) non-viable sperm with low membrane lipid disorder (M540^{-}/YO-PRO-1^{+}); (ii) non-viable sperm with high membrane lipid disorder (M540^{+}/YO-PRO-1^{+}); (iii) viable sperm with low membrane lipid disorder (M540^{-}/YO-PRO-1^{-}); and (iv) viable sperm with high membrane lipid disorder (M540^{+}/YO-PRO-1^{-}). Membrane lipid disorder was assessed as the percentage of viable, red-stained sperm with high membrane lipid disorder (M540^{+}/YO-PRO-1^{-}). Data were not compensated.

4.4.3. Intracellular Levels of Calcium (Fluo3-AM/PI)

Evaluation of intracellular calcium levels was conducted following a protocol modified from Harrison et al. [53]. Samples were incubated with Fluo3-AM (1 µmol/L) and PI (12 µmol/L) for 10 min at 38 °C in the dark. The following four populations were distinguished in the dot-plots: (i) viable sperm with low levels of intracellular calcium (Fluo3^{-}/PI^{-}); (ii) viable sperm with high levels of intracellular calcium (Fluo3^{+}/PI^{-}); (iii) non-viable sperm with low levels of intracellular calcium (Fluo3^{-}/PI^{+}); and (iv) non-viable sperm with high levels of intracellular calcium (Fluo3^{+}/PI^{+}). The population of viable sperm with high intracellular calcium levels was used to assess intracellular calcium. Compensations for Fluo3 spill over into the FL-3 channel (2.45%) and for PI into the FL-1 channel (28.72%) were performed.

4.4.4. Mitochondrial Membrane Potential (JC-1)

Evaluation of mitochondrial membrane potential (MMP) was determined through JC-1 staining, following the procedure set by Ortega-Ferrusola et al. [54] with few modifications. Samples were incubated with JC-1 (0.3 µmol/L) at 38 °C in the dark for 30 min. High MMP causes JC-1 aggregates (JC-1$_{agg}$) that emit orange fluorescence, which is detected by FL-2. Otherwise, when MMP is low, JC-1 forms monomers (JC-1$_{mon}$), emitting green fluorescence, which is collected through the FL-1 filter. Flow cytometry dot-plots allowed identifying the following three different populations: (i) green-stained sperm with low MMP (JC-1$_{mon}$); (ii) orange-stained sperm with high MMP (JC-1$_{agg}$); and (iii) double-stained sperm in both green and orange, which indicates sperm with heterogeneous mitochondria. Only orange-stained populations with high MMP were considered to assess the MMP.

4.4.5. Acrosome Membrane Integrity (PNA-FITC/PI)

The integrity of acrosome membrane was assessed with the PNA-FITC fluorochrome, following the procedure of Nagy et al. [55] with minor modifications. Peanut agglutinin (PNA) lectin binds the sugar moieties, which are uniquely present in the inner leaflet of

the outer acrosomal membrane [56]. Briefly, samples were incubated with PNA-FITC (2.5 µg/mL) for 5 min at 38 °C in the dark, and next, with PI (12 µmol/mL) for 5 min at 38 °C. Since sperm were not previously permeabilized, the following four populations were identified: (i) viable membrane-intact sperm (PNA-FITC$^-$/PI$^-$); (ii) viable sperm with a damaged plasma membrane (PNA-FITC$^+$/PI$^-$); (iii) non-viable sperm with a damaged plasma membrane and fully lost outer acrosome membrane (PNA-FITC$^-$/PI$^+$); and (iv) non-viable sperm with a damaged plasma membrane that presented an outer acrosome membrane that could not be fully intact (PNA-FITC$^+$/PI$^+$). Viable sperm with an intact acrosomal membrane (PNA-FITC$^-$/PI$^-$) were used to evaluate acrosome integrity. Compensation of PNA-FITC spill over into the PI channel (2.45%) was applied.

4.4.6. Intracellular O_2^- Levels (HE/YO-PRO-1)

Evaluation of intracellular superoxide (O_2^-) levels in sperm was performed by co-staining with HE and YO-PRO-1, according to the protocol described by Guthrie and Welch [57]. Sperm were incubated with HE (4 µmol/L) and YO-PRO-1 (40 nmol/L) for 20 min at 38 °C in the dark. Green fluorescence from YO-PRO-1 was detected by FL-1 and the oxidation of HE to ethidium (E$^+$) by O_2^- was detected as red fluorescence through FL-3. The following four different populations were distinguished: (i) non-viable sperm with high O_2^- levels (E$^+$/YO-PRO-1$^+$); (ii) non-viable sperm with low O_2^- levels (E$^-$/YO-PRO-1$^+$); (iii) viable sperm with high O_2^- levels (E$^+$/YO-PRO-1$^-$); and (iv) viable sperm with low O_2^- levels (E$^-$/YO-PRO-1$^-$).

4.4.7. Intracellular H_2O_2 Levels (H_2DCFDA/PI)

Assessment of intracellular peroxides (H_2O_2) levels in sperm was performed following a protocol modified from Guthrie and Welch [57]. Sperm samples were incubated with H_2DCFDA (10 µmol/L) for 30 min at 38 °C in the dark. Further, samples were stained with PI (12 µmol/L) for 10 min at 38 °C in the dark. The oxidation of H_2DCFDA to DCF$^+$ by H_2O_2 was collected by FL-1 as green fluorescence, and the red fluorescence from PI was detected by FL-3. The following four sperm populations were distinguished in dot-plots: (i) non-viable sperm with high H_2O_2 levels (DCF$^+$/PI$^+$); (ii) non-viable sperm with low H_2O_2 levels (DCF$^-$/PI$^+$); (iii) viable sperm with high H_2O_2 levels (DCF$^+$/PI$^-$); and (iv) viable sperm with low H_2O_2 levels (DCF$^-$/PI$^-$). The population of viable sperm with high H_2O_2 levels (DCF$^+$/PI$^-$) was used to assess H_2O_2 levels.

4.5. Immunoblotting

In order to perform total protein extraction, pellets were resuspended in lysis buffer (xTractor™ Buffer; Takara Bio, Mountain View, CA, USA), supplemented with a commercial proteases inhibitor cocktail (1:100, *v:v*; ref. P8340) and 0.1 M phenyl-methanesulfonylfluoride (PMSF). Samples were incubated at 4 °C for 30 min in agitation and then sonicated three times (5 × 1 s pulses at 20 KHz, 1 s pause between individual pulses). Thereafter, samples were centrifuged at 10,000× *g* for 15 min at 4 °C and supernatants were collected for subsequent protein quantification. Protein quantification was carried out in duplicate using a detergent compatible (DC) method (ref. 5000116, BioRad, Hercules, CA, USA).

A total of 10 µg of protein was mixed with 4× Laemmli reducing buffer containing 5% β-mercaptoethanol (BioRad). Protein samples and molecular weight marker (Precision Plus Protein All Blue Standards, Bio-Rad) were incubated at 95 °C for 5 min and then loaded onto gradient commercial SDS-PAGE gels (8–16% Mini-Protean TGX Stain-Free gels; BioRad). Gels were run at 20 mA and 120–150 V using an electrophoretic system (IEF Cell Protean System, Bio-Rad). Subsequently, separated proteins were transferred onto polyvinylidene fluoride membranes (Immobilion-P; Millipore, Darmstadt, Germany) using a Trans-Blot® Turbo™ device (BioRad).

Membranes were incubated overnight at 4 °C in agitation with a blocking solution consisting of 10 mM Tris (Panreac, Barcelona, Spain), 150 mM NaCl (LabKem, Barcelona,

Spain), 0.05% Tween20 (Panreac, Barcelona, Spain), and 5% BSA (pH adjusted at 7.3; Roche Diagnostics, S.L.; Basel, Switzerland). Afterwards, membranes were incubated with a primary anti-PARK7 antibody (ref. LS-C353340, LifeSpan BioSciences, Seattle, WA, USA) diluted 1:5,000 (*v*:*v*) in blocking solution for 1 h at room temperature in agitation. Membranes were then washed five times with TBS1×-Tween20 (10 mM Tris, 150 mM NaCl, and 0.05% Tween20), 5 min each, and incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (ref. P0448, Agilent, Santa Clara, CA, USA) diluted 1:10,000 (*v*:*v*) in blocking solution for 1 h at room temperature in agitation. Subsequently, membranes were rinsed eight times with TBS1×-Tween20. Reactive bands were visualized with a chemiluminescent substrate (Immobilion^{TM} Western Detection Reagents, Millipore, Darmstadt, Germany) and blots were scanned with the G:BOX Chemi XL 1.4 device (SynGene). Next, membranes were washed twice with a stripping solution containing 0.02 mM Glycine, 0.1% SDS and 1% Tween20 (pH adjusted at 2.2) for 10 min. This was followed by four washes with TBS1×-Tween20 (10, 10, 5 and 5 min). Membranes were blocked and then incubated with a primary anti-α-tubulin antibody (ref. 05-829, Millipore) diluted at 1:100,000 (*v*:*v*) in blocking solution, for 1 h at room temperature with agitation. Thereafter, membranes were rinsed three times with TBS1×-Tween20 and incubated with a secondary rabbit anti-mouse antibody conjugated with horseradish peroxidase (ref. P0260, Agilent, Santa Clara, CA, USA) diluted 1:150,000 (*v*:*v*) in blocking solution, for 1 h at room temperature in agitation. Further, membranes were washed five times with TBS1×-Tween20, reactive bands were visualized with a chemiluminescent substrate (Immobilion^{TM} Western Detection Reagents, Millipore, Darmstadt, Germany) and blots were scanned with G:BOX Chemi XL 1.4 (SynGene).

The specificity of the primary anti-PARK7 antibody was also assessed by incubation with the corresponding PARK7-blocking peptide (ref. LS-E42782, LifeSpan BioSciences) concentrated 20 times in excess in relation to the primary antibody. The intensity of protein bands was quantified using Quantity One 1-D Analysis Software (BioRad), evaluating two technical replicates per sample. Quantifications of PARK7 protein were normalized using α-tubulin of each lane, and the corresponding mean ± SD of ratios were calculated.

4.6. Immunofluorescence

Immunofluorescence was performed to determine the localization of PARK7 in sperm along the incubation period (0, 120, 240, 250, and 300 min). To this end, samples collected for immunofluorescence were centrifuged at 600× *g* for 5 min to remove the CM, and sperm pellets were resuspended in PBS. Thereafter, sperm cells were fixed through incubation with 2% paraformaldehyde (*v*:*v*) for 30 min at room temperature, and were further washed twice with PBS. After fixation, sperm were placed and sedimented onto slides for 1 h at room temperature, and then permeabilized through incubation in 1% Triton X-100 in PBS for 1 h at room temperature. After permeabilization, antigen unmasking was performed exposing sperm to an acidic Tyrode's solution for 20 s. Acid was then neutralized through washing three times with neutralization buffer, following Kashir et al. [58]. Slides were washed three times with PBS, and subsequently incubated with blocking solution (5% BSA in PBS) for 1 h at room temperature. Primary anti-PARK7 antibody (ref. LS-C353340, LifeSpan BioSciences) was diluted 1:25 (*v*:*v*) in 5% BSA in PBS (*v*:*v*) and incubated with slides for 1 h at room temperature. Subsequently, samples were incubated with a secondary antibody goat anti-rabbit Alexa Fluor^{TM} Plus 488 (ref. A32731, Invitrogen, USA) diluted 1:50 in 5% BSA in PBS (*v*:*v*) for 60 min in the dark at room temperature. Slides were then washed five times with PBS and mounted with 10 µL of ProLong^{TM} Glass Antifade Mountant with NucBlue^{TM} (Hoechst 33342; ref. P36985, Invitrogen) in the dark.

The specificity of the primary anti-PARK7 antibody was also assessed by incubation with the corresponding PARK7-blocking peptide (ref. LS-E42782, LifeSpan BioSciences) concentrated 20 times in excess in relation to the primary antibody.

Finally, all slides were observed under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon Corp., Tokyo, Japan). To localize the nuclei stained with Hoechst 33342,

samples were excited at 405 nm, whereas the localization of PARK7 was performed at an excitation wavelength of 496 nm.

4.7. Statistical Analyses

Results were analyzed using a statistical package (IBM for Windows 27.0; Armonk, NY, USA). Data were first checked for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test). When data did not fit with parametric assumptions, they were linearly transformed with arcsin $\sqrt{x}$. A two-step cluster analysis (likelihood distance and Bayesian information criterion) using the relative PARK7 content at 0 min was used to set two separate groups (sperm samples with (a) high and (b) low PARK7). Based on that classification, two groups of four samples each were identified. Following this, sperm quality and functionality parameters of these groups were compared with a linear mixed model followed by post hoc Sidak test for pair-wise comparisons (within subjects factor: incubation time; between subjects factor: sperm samples with high and low relative PARK7 content). When, even after linear transformation, data did not fit with parametric assumptions, Scheirer-Ray-Hare and Wilcoxon tests were used as alternatives. The level of significance was set at $p < 0.05$ and the confidence level was established at 95%.

5. Conclusions

Considering the results obtained in the present study, it can be concluded that sperm with higher levels of PARK7 at the onset of the experiment display a more progressive capacitation process with less sperm mortality. This denotes a better resilience to ROS levels, which are needed for sperm to undergo capacitation. Thus, in this context, PARK7 could play a role in preventing mammalian sperm from undergoing premature capacitation and degenerative acrosomal exocytosis. However, additional studies are needed to better understand the antioxidant properties of PARK7 during the capacitation of mammalian sperm. On the other hand, looking upon PARK7 rat homologue has been surmising to play a role in egg penetration and membrane fusion [18], performing IVF assays in other species or using knockout models could be an interesting focus for future studies. Finally, as this study was conducted in an animal model, further research using in vitro capacitated human sperm is warranted.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221910804/s1. Figure S1. Percentage of viable spermatozoa with low lipid membrane disorder (M540-/YO-PRO-1-) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between samples containing high or low levels of PARK7 at 0 min of incubation. Figure S2. Percentage of non-viable spermatozoa with high lipid membrane disorder (M540+/YO-PRO-1+) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between samples containing high or low levels of PARK7 at 0 min of incubation. Figure S3. Representative flow cytometry YO-PRO (a) and M540 (b) histograms of sperm samples at the beginning of the in vitro capacitation experiment (0 min of incubation). (c) Dot plots of M540 and YO-PRO staining showing four distinct populations: M540-/YO-PRO+, M540+/YO-PRO-, M540-/YO-PRO-, and M540+/YO-PRO+. Figure S4. Representative flow cytometry YO-PRO (a) and M540 (b) histograms of sperm samples after 240 min of incubation in capacitation medium. (c) Dot plots of M540 and YO-PRO staining showing four distinct populations: M540-/YO-PRO+, M540+/YO-PRO-, M540-/YO-PRO-, and M540+/YO-PRO+. Figure S5: Representative flow cytometry YO-PRO (a) and M540 (b) histograms of sperm samples after 300 min of incubation in capacitation medium. (c) Dot plots of M540 and YO-PRO staining showing four distinct populations: M540-/YO-PRO+, M540+/YO-PRO-, M540-/YO-PRO-, and M540+/YO-PRO+. Figure S6: Percentage of non-viable spermatozoa with an acrosome that could not be fully intact (PNA-FITC+/PI+) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time

at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples with high or low PARK7 levels at 0 min of incubation. Figure S7: Percentage of viable sperm with high intracellular O2- levels (E+/YO-PRO-1-) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples with high or low PARK7 levels at 0 min of incubation.

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Supplementary material (Recuero et al.)

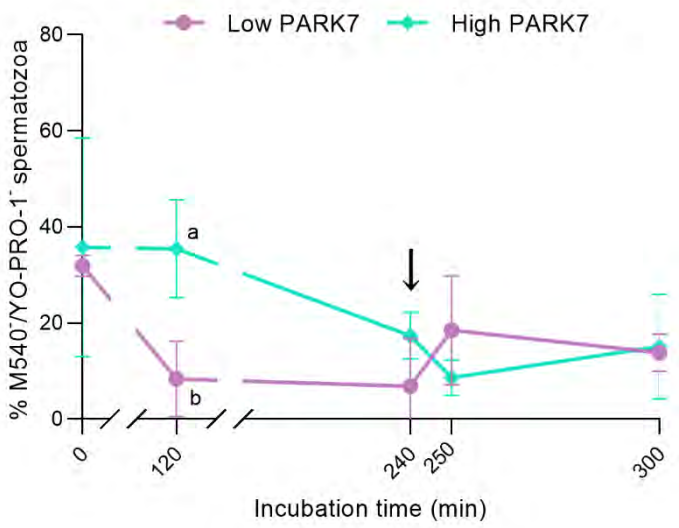

Figure S1. Percentage of viable spermatozoa with low lipid membrane disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 µg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between samples containing high or low levels of PARK7 at 0 min of incubation.

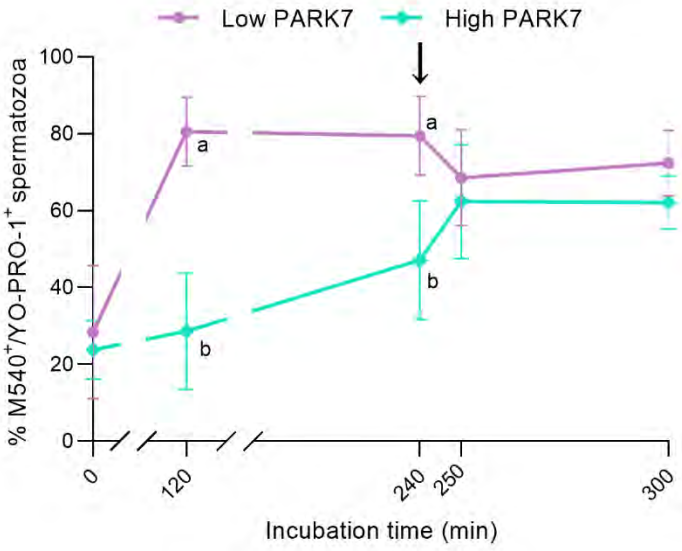

Figure S2. Percentage of non-viable spermatozoa with high lipid membrane disorder ($M540^+/YO\text{-}PRO\text{-}1^+$) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 µg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between samples containing high or low levels of PARK7 at 0 min of incubation.

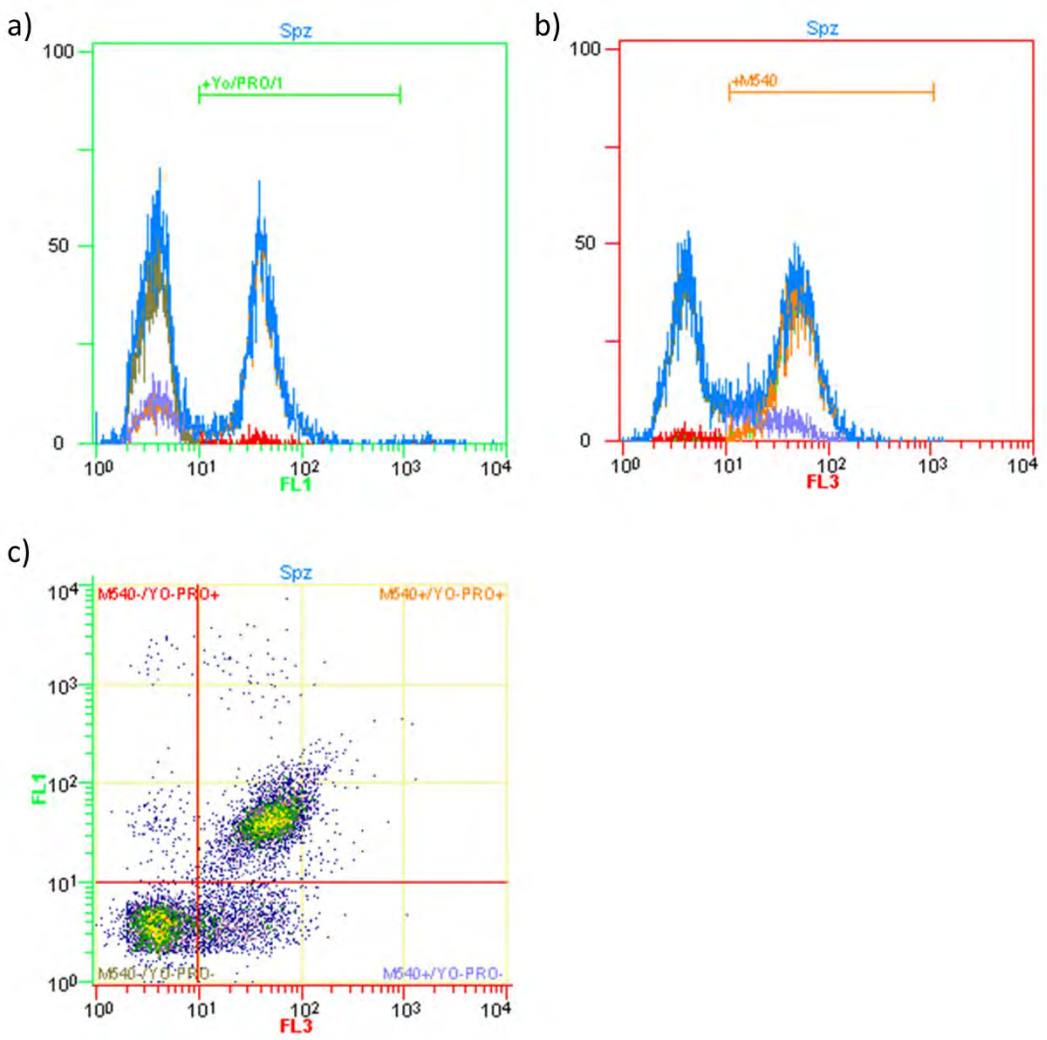

Figure S3. Representative flow cytometry YO-PRO (**a**) and M540 (**b**) histograms of sperm samples at the beginning of the in vitro capacitation experiment (0 min of incubation). **(c)** Dot plots of M540 and YO-PRO staining showing four distinct populations: $M540^-/YO\text{-}PRO^+$, $M540^+/YO\text{-}PRO^-$, $M540^-/YO\text{-}PRO^-$, and $M540^+/YO\text{-}PRO^+$.

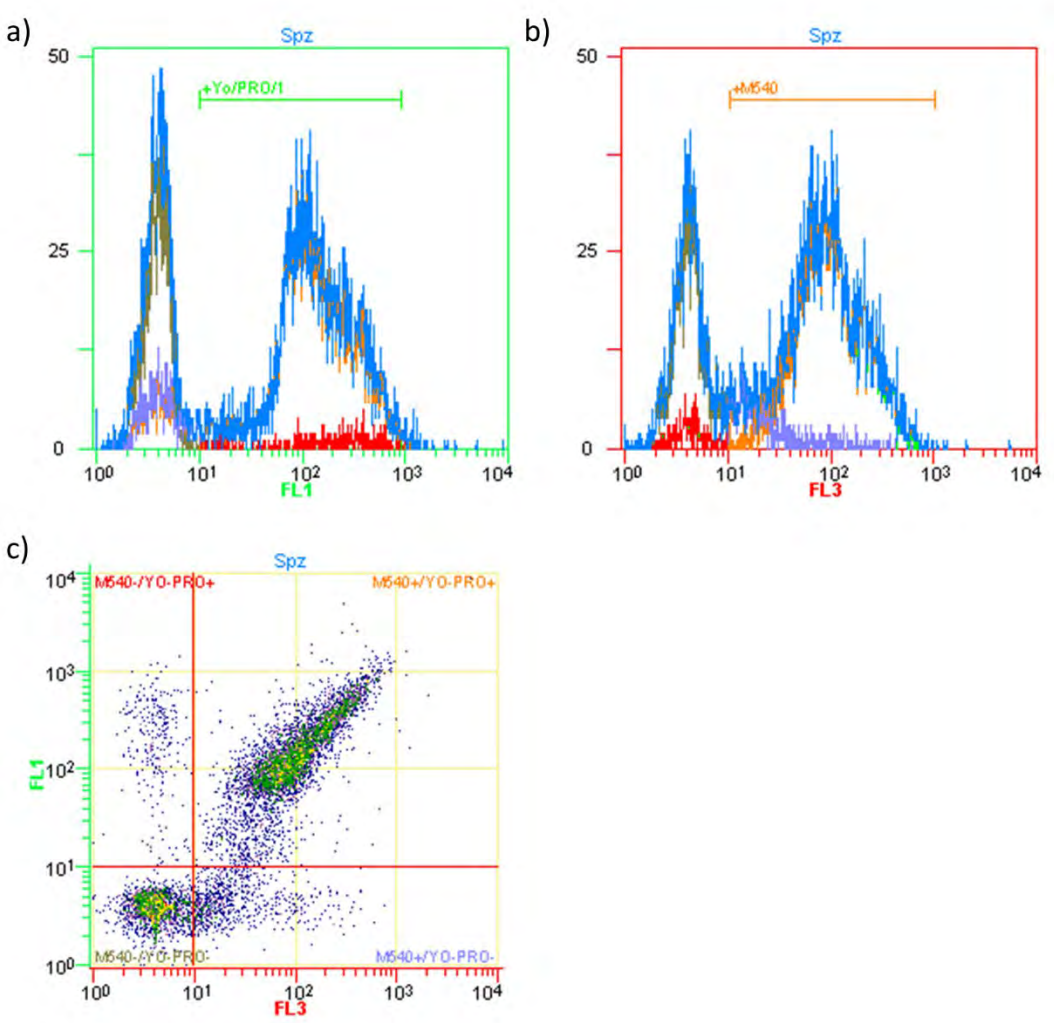

Figure S4. Representative flow cytometry YO-PRO (**a**) and M540 (**b**) histograms of sperm samples after 240 min of incubation in capacitation medium. **(c)** Dot plots of M540 and YO-PRO staining showing four distinct populations: $M540^{-}/YO\text{-}PRO^{+}$, $M540^{+}/YO\text{-}PRO^{-}$, $M540^{-}/YO\text{-}PRO^{-}$, and $M540^{+}/YO\text{-}PRO^{+}$.

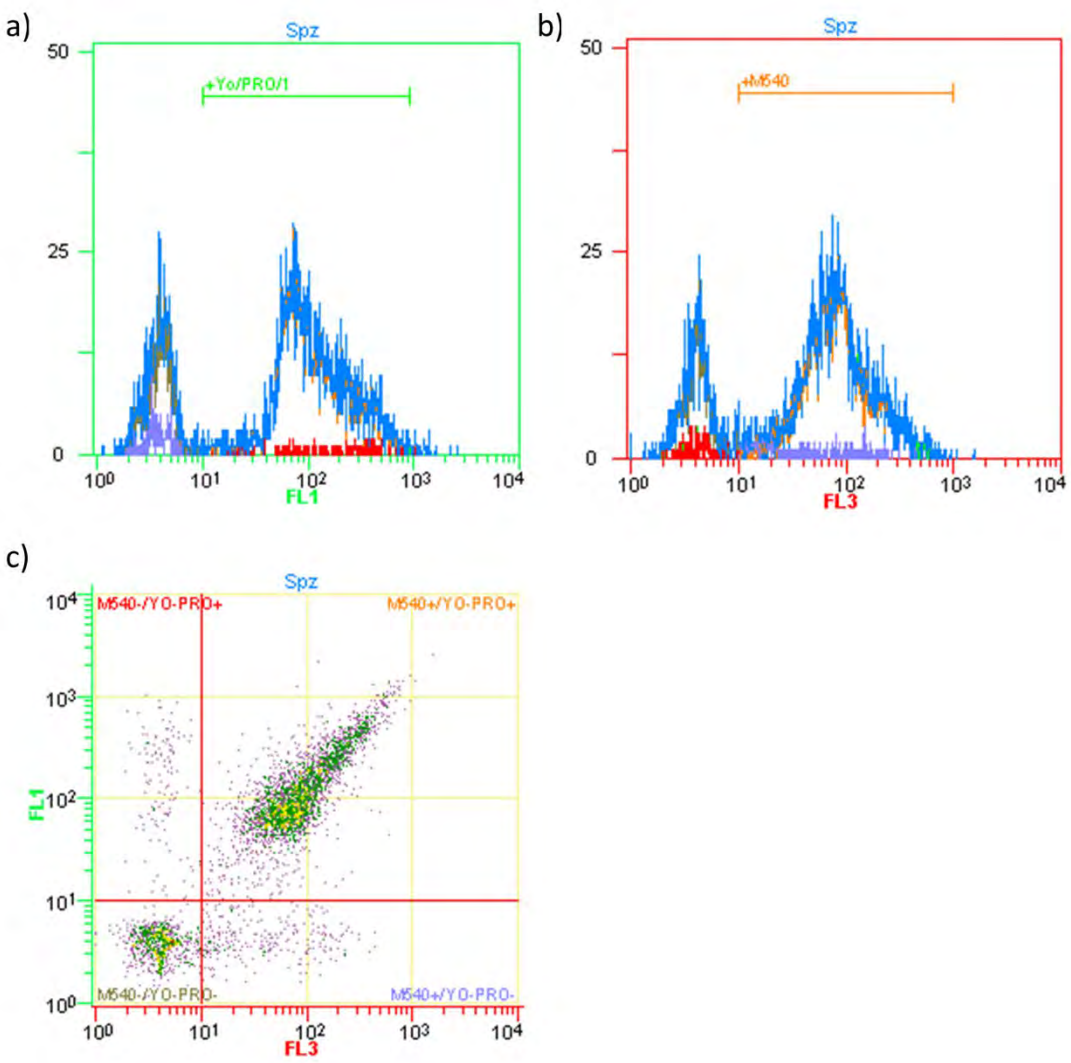

Figure S5: Representative flow cytometry YO-PRO (**a**) and M540 (**b**) histograms of sperm samples after 300 min of incubation in capacitation medium. **(c)** Dot plots of M540 and YO-PRO staining showing four distinct populations: M540^{-}/YO-PRO^{+}, M540^{+}/YO-PRO^{-}, M540^{-}/YO-PRO^{-}, and M540^{+}/YO-PRO^{+}.

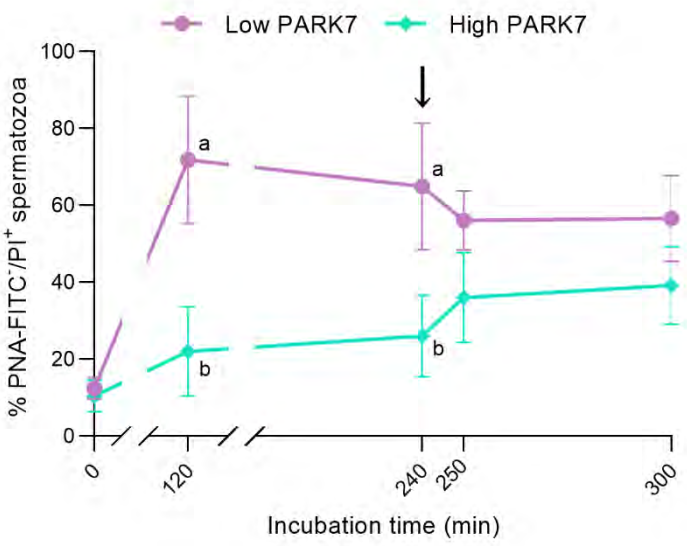

Figure S6: Percentage of non-viable spermatozoa with an acrosome that could not be fully intact (PNA-FITC$^+$/PI$^+$) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples with high or low PARK7 levels at 0 min of incubation.

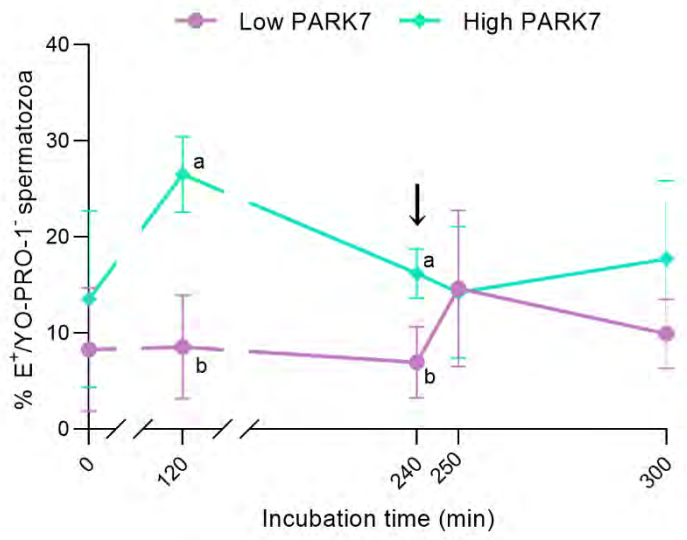

Figure S7: Percentage of viable sperm with high intracellular O_2^- levels (E$^+$/YO-PRO-1$^-$) during in vitro capacitation and progesterone-induced acrosome exocytosis (300 min). Black arrow indicates the time at which progesterone was added at a final concentration of 10 μg/mL (240 min). Data are shown as mean ± SD. Different letters indicate significant differences ($p < 0.05$) between sperm samples with high or low PARK7 levels at 0 min of incubation.

PAPER III

The presence of seminal plasma during liquid storage of pig spermatozoa at 17°c modulates their ability to elicit in vitro capacitation and trigger acrosomal exocytosis.

Ana Paula Pinoti Pavaneli, Sandra Recuero, Bruna Resende Chaves, Estela Garcia-Bonavila, Marc Llavanera, Elisabeth Pinart, Sergi Bonet, André Furugen Cesar De Andrade & Marc Yeste

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Article

The Presence of Seminal Plasma during Liquid Storage of Pig Spermatozoa at 17 °C Modulates Their Ability to Elicit In Vitro Capacitation and Trigger Acrosomal Exocytosis

Ana Paula Pinoti Pavaneli [1,2,3], Sandra Recuero [1,2], Bruna Resende Chaves [1,2,4], Estela Garcia-Bonavila [1,2], Marc Llavanera [1,2], Elisabeth Pinart [1,2], Sergi Bonet [1,2], André Furugen Cesar De Andrade [3] and Marc Yeste [1,2,*]

1 Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain; anapavaneli@usp.br (A.P.P.P.); sandra.recuero@udg.edu (S.R.); brunarufla@gmail.com (B.R.C.); estela.garcia@udg.edu (E.G.-B.); marc.llavanera@udg.edu (M.L.); elisabeth.pinart@udg.edu (E.P.); sergi.bonet@udg.edu (S.B.)
2 Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, E-17003 Girona, Spain
3 Laboratory of Andrology and Technology of Swine Embryos, Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, BR-13635-900 Pirassununga, Brazil; andrefc@usp.br
4 Department of Veterinary Medicine, Federal University of Lavras, BR-37200-000 Lavras, Brazil
* Correspondence: marc.yeste@udg.edu; Tel.: +34-972-418-514

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Abstract: Although seminal plasma is essential to maintain sperm integrity and function, it is diluted/removed prior to liquid storage and cryopreservation in most mammalian species. This study sought to evaluate, using the pig as a model, whether storing semen in the presence of seminal plasma affects the sperm ability to elicit in vitro capacitation and acrosomal exocytosis. Upon collection, seminal plasma was separated from sperm samples, which were diluted in a commercial extender, added with seminal plasma (15% or 30%), and stored at 17 °C for 48 or 72 h. Sperm cells were subsequently exposed to capacitating medium for 4 h, and then added with progesterone to induce acrosomal exocytosis. Sperm motility, acrosome integrity, membrane lipid disorder, intracellular Ca^{2+} levels, mitochondrial activity, and tyrosine phosphorylation levels of glycogen synthase kinase-3 (GSK3)α/β were determined after 0, 2, and 4 h of incubation, and after 5, 30, and 60 min of progesterone addition. Results showed that storing sperm at 17 °C with 15% or 30% seminal plasma led to reduced percentages of viable spermatozoa exhibiting an exocytosed acrosome, mitochondrial membrane potential, intracellular Ca^{2+} levels stained by Fluo3, and tyrosine phosphorylation levels of GSK3α/β after in vitro capacitation and progesterone-induced acrosomal exocytosis. Therefore, the direct contact between spermatozoa and seminal plasma during liquid storage at 17 °C modulated their ability to elicit in vitro capacitation and undergo acrosomal exocytosis, via signal transduction pathways involving Ca^{2+} and Tyr phosphorylation of GSK3α/β. Further research is required to address whether such a modulating effect has any impact upon sperm fertilizing ability.

Keywords: spermatozoa; seminal plasma; in vitro capacitation; acrosomal exocytosis

1. Introduction

Seminal plasma is composed of a mixture of secretions produced by the accessory glands of the male reproductive tract, and contains a variety of constituents, such as proteins, lipids, steroid hormones,

and ions [1]. Seminal plasma also contains extracellular vesicles (exosomes and microvesicles) [2,3] that infiltrate into the sperm membrane, thereby maintaining plasma membrane integrity [4]. These vesicles, which are also involved in the immune-related gene regulation in the uterus [5], are known to contain a repertoire of mRNA and small non-coding RNAs (microRNAs, miRNA; piwi-interacting RNAs, piRNA) that have been related to spermatogenesis and embryogenesis [6–8]. The mixture of spermatozoa with seminal plasma upon ejaculation underpins the idea that it is essential for these cells. In effect, despite not being well understood, the role of seminal plasma as a modulator of sperm function has become increasingly apparent in the last years. For this reason, its regulatory function on specific sperm parameters, such as motility and membrane permeability/fluidity, makes its use attractive to assisted reproductive technology [9].

In the case of cryopreservation protocols, removing extender and seminal plasma is a required step to increase sperm concentration and cryotolerance [10]. Related to this and considering the loss of the natural protection to spermatozoa provided by seminal plasma, previous studies have demonstrated that the reestablishment of that contact through post-thaw addition of seminal plasma can be beneficial for frozen-thawed spermatozoa, as it increases their motility, survival, and fertilizing ability [11–13]. Moreover, while other studies have investigated whether the presence of seminal plasma during liquid storage (15–20 °C) affects pig sperm function and survival, their results seem to be less consistent. Indeed, whilst some authors reported that removal of seminal plasma prior to liquid storage is a harmful practice, as it decreases sperm motility, viability, and acrosome integrity [14,15], others found that its elimination might be beneficial for sperm survival and in vivo fertilizing ability [16].

During capacitation, the process through which sperm cells acquire their ability to fertilize an oocyte, there are a series of changes that affect sperm motility, plasma membrane and acrosome integrity, membrane lipid disorder, mitochondrial activity, and Ca^{2+} homeostasis, and involve the phosphorylation of certain proteins [17–20]. Although seminal plasma proteins have been reported to inhibit sperm capacitation [21], the controversial results about to which extent the presence of seminal plasma is important to maintain sperm function during liquid storage [16] make it necessary to elucidate whether this prolonged interaction can affect the ability of mammalian sperm to elicit in vitro capacitation and trigger acrosomal exocytosis. Furthermore, mounting evidence in other species, like the sheep, indicates that not only is the overall sperm motility affected during in vitro capacitation but also the proportions of motile sperm subpopulations [22,23]. Therefore, whether storing pig sperm at 17 °C in the presence of seminal plasma tweaks the dynamics of these sperm subpopulations during in vitro capacitation also warrants further research.

Against this background, the present study aimed to evaluate how the presence of seminal plasma during liquid storage at 17 °C influences the ability of pig sperm to elicit in vitro capacitation and trigger acrosomal exocytosis. With this purpose, seminal plasma was separated from sperm upon collection. Thereafter, sperm samples were diluted in a commercial extender, supplemented with 15% or 30% seminal plasma, and stored at 17 °C for 48 h or 72 h. Control samples did not contain seminal plasma. After liquid storage, sperm samples were exposed to capacitating medium for 4 h; at this time point, acrosomal exocytosis was induced through the addition of progesterone. The following parameters were evaluated: plasma membrane and acrosome integrity, membrane lipid disorder, mitochondrial membrane potential, intracellular Ca^{2+} levels, and sperm motility and motile sperm subpopulations. Furthermore, tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) isoforms (α, β) was measured through immunoblotting, as this protein has been shown to be involved in the control of sperm motility, capacitation, and acrosome reaction in the pig [24] and other species [25,26].

2. Results

2.1. Sperm Motility

2.1.1. Total and Progressive Sperm Motility

Although total and progressive sperm motility decreased significantly ($p < 0.05$) throughout incubation time, there was no effect ($p > 0.05$) of the presence of seminal plasma on any of these parameters when samples were stored at 17 °C for either 48 h or 72 h (Figure 1a,b).

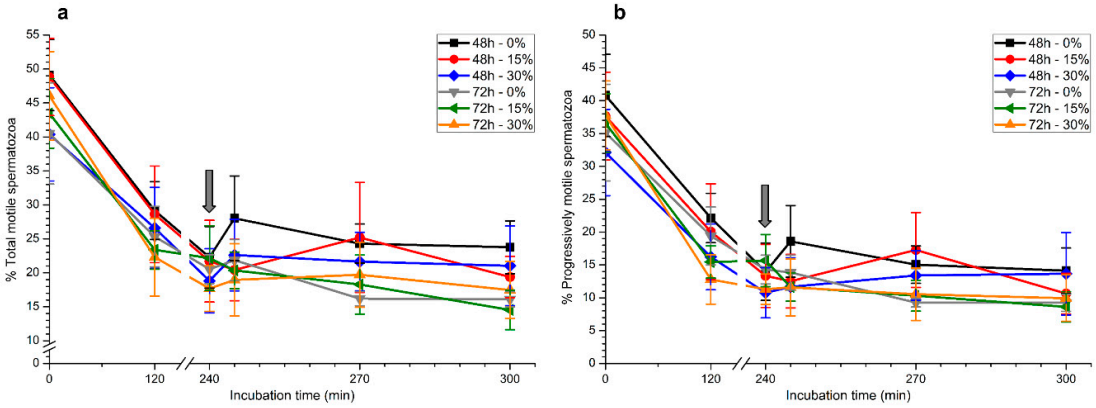

Figure 1. Percentages of total (**a**) and progressively motile spermatozoa (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). No significant differences ($p > 0.05$) between treatments were observed. Data are shown as mean ± standard error of the mean (SEM) for seven independent experiments.

2.1.2. Motile Sperm Subpopulations

To determine the number of motile sperm subpopulations and evaluate their changes throughout in vitro capacitation and progesterone-induced acrosomal exocytosis, we first ran a principal component analysis (PCA) with all kinematic parameters; two extracted components that accounted for 80.20% of the total variance were identified (Table 1).

Table 1. Principal component analyses (PCA) based on individual kinematic parameters of all sperm cells evaluated by computer-assisted sperm analysis (CASA) system. As a result, two PCA components were obtained.

Principal Component	Variance	Parameter	a_{ij}^2
Component 1	58.04%	LIN	0.94
		WOB	0.81
		STR	0.79
		VSL	0.62
		VAP	0.48
		BCF	0.44
		MAD	0.28
Component 2	22.16%	ALH	0.88
		VCL	0.78
		DANCE	0.93
Total	80.20%		

The loading factor (a_{ij}^2) represents the highest association between a given sperm kinematic parameter and the corresponding principal component. LIN, linearity; WOB, wobble; STR, straightness; VSL, straight-line velocity; VAP, average path velocity; BCF, beat-cross frequency; MAD, mean angular displacement; ALH, amplitude of lateral head displacement; VCL, curvilinear velocity; DANCE, VCL × ALH.

The first principal component was mainly related to straight-line velocity, linearity, and straightness (VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; WOB, wobble; STR, straightness; BCF, beat-cross frequency; and MAD, mean angular displacement), whereas the second one was highly related to curvilinear velocity and the amplitude of head displacement (VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; and DANCE, VCL × ALH). After clustering spermatozoa utilizing the regression scores obtained in the PCA, we found three motile sperm subpopulations (Table 2). Spermatozoa belonging to subpopulations 1 and 2 (SP1 and SP2) were faster spermatozoa

than those belonging to subpopulation 3 (SP3). In addition, whereas LIN, STR, and WOB were the highest in SP1, VCL and ALH reached their maximum in SP2. In contrast, VSL, VAP, and BCF values were similar between SP1 and SP2. The highest DANCE value was found in SP2, whereas the lowest MAD value was found in SP1. With the exception of MAD, all the other parameters were much lower in SP3.

Table 2. Descriptive statistics (mean ± SEM for seven experiments) of the three motile sperm subpopulations identified in this study.

	SP1	SP2	SP3
N	10,254	7458	3078
VCL (μm/s)	63.27 ± 0.27	92.87 ± 0.41	4.52 ± 0.19
VSL (μm/s)	49.91 ± 0.25	42.84 ± 0.44	2.86 ± 0.05
VAP (μm/s)	56.27 ± 0.27	61.39 ± 0.43	2.33 ± 0.10
LIN (%)	75.60 ± 0.15	41.49 ± 0.31	3.54 ± 0.16
STR (%)	86.47 ± 0.12	60.92 ± 0.33	6.91 ± 0.32
WOB (%)	86.94 ± 0.10	62.78 ± 0.25	8.76 ± 0.37
ALH (μm)	1.90 ± 0.01	3.57 ± 0.01	0.26 ± 0.01
BCF (Hz)	8.08 ± 0.03	7.79 ± 0.04	0.56 ± 0.03
DANCE ($\mu m^2/s$)	129.28 ± 0.79	357.99 ± 2.72	7.60 ± 0.35
MAD (°)	64.78 ± 0.39	107.85 ± 0.50	90.35 ± 0.73

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; (VAP/VCL); LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; DANCE (VCL × ALH); MAD, mean angular displacement.

The aforementioned motile sperm subpopulations were monitored throughout in vitro capacitation and progesterone-induced acrosomal exocytosis (Figure 2). Sperm samples stored with 30% seminal plasma for 48 h showed a marked decrease in SP1 (Figure 2a). The addition of progesterone led to a significant ($p < 0.05$) reduction in SP1 (5 min), the lowest value being observed in those samples that had been stored with 30% seminal plasma for 48 h. No significant differences between treatments were observed after 30 and 60 min of progesterone addition.

With regard to SP2, spermatozoa stored without seminal plasma for 72 h showed the highest value at 0 h (Figure 2b). In spite of this, no significant differences between treatments were observed at the other time points. Finally, proportions of spermatozoa belonging to SP3 did not differ between treatments, storage, or incubation times (Figure 2c).

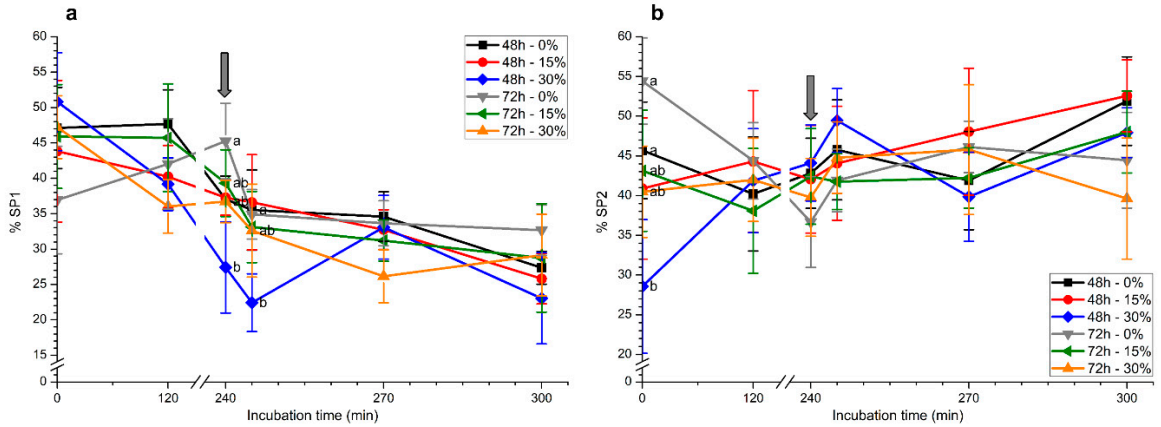

Figure 2. *Cont.*

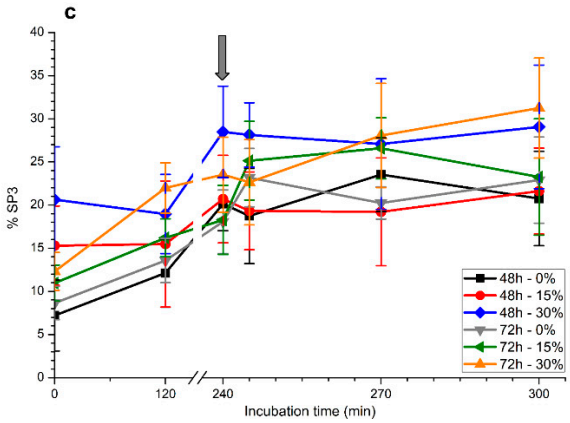

Figure 2. Percentages of motile sperm populations ((**a**) SP1, **(b)** SP2, and (**c**) SP3) during in vitro capacitation and progesterone-induced acrosomal exocytosis after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

2.2. Plasma Membrane and Acrosome Integrity

As expected, percentages of viable spermatozoa with an intact acrosome (PNA-FITC$^+$/EthD-1$^-$), which were significantly ($p < 0.05$) higher in samples stored for 48 h than in those stored for 72 h at the beginning of the experiment, decreased ($p < 0.05$) over the incubation period (Figure 3a).

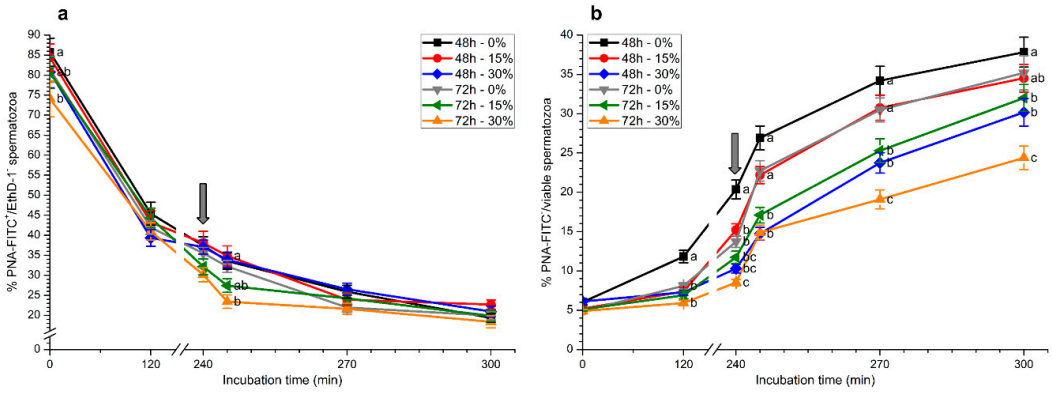

Figure 3. Percentages of viable spermatozoa with an intact acrosome (PNA-FITC$^+$/EthD-1$^-$; (**a**)) and with an exocytosed acrosome (PNA$^-$) in relation to total viable spermatozoa (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

Whereas percentages of viable spermatozoa with an intact acrosome after 5 min of adding progesterone (245 min) were significantly ($p < 0.05$) lower in samples stored with 30% seminal plasma than in the other treatments, no significant differences ($p > 0.05$) in the other time points were observed (Figure 3a).

Percentages of viable spermatozoa exhibiting an exocytosed acrosome (PNA-FITC$^-$/viable sperm) increased throughout incubation time and were significantly ($p < 0.05$) higher in samples stored without seminal plasma for 48 h than in the other treatments at 120 min and 240 min (Figure 3b). Previous storage with seminal plasma, especially in the treatment with 30% seminal plasma and 72 h storage,

showed significantly ($p < 0.05$) lower percentages of viable spermatozoa exhibiting an exocytosed acrosome and a reduced response to the addition of progesterone (Figure 3b).

2.3. Membrane Lipid Disorder

As expected, percentages of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$) significantly ($p < 0.05$) decreased along incubation with capacitating medium (Figure 4a). At 120 min, percentages of viable spermatozoa with low membrane lipid disorder were significantly ($p < 0.05$) higher in samples stored without seminal plasma for 72 h than in those stored with 15% or 30% seminal plasma for 48 h. No significant differences were observed between treatments at the other incubation time times (240 min, 245 min, 270 min, and 300 min; Figure 4a).

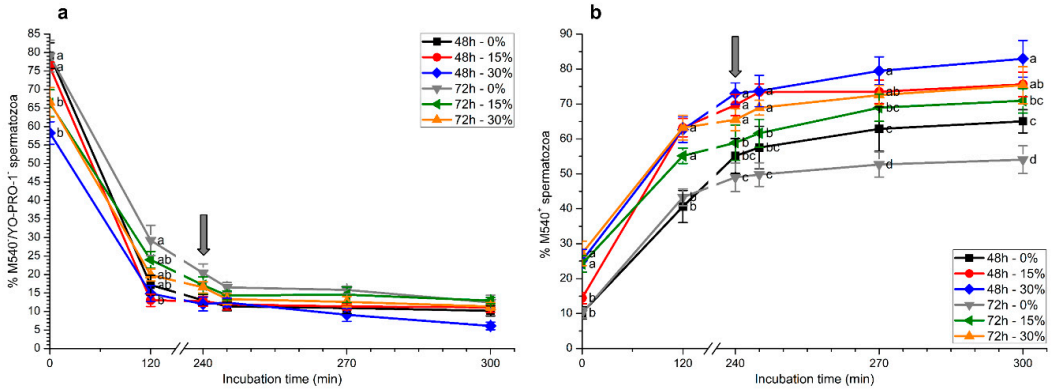

Figure 4. Percentages of viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$; (**a**)) and of $M540^+$ spermatozoa (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

Percentages of spermatozoa with a positive M540 signal ($M540^+$) showed a time-dependent increase ($p < 0.05$) during in vitro capacitation (Figure 4b). At 0 h, percentages of $M540^+$ spermatozoa in samples that were stored with seminal plasma (15 or 30%) for 72 h and those stored with seminal plasma at 30% for 48 h were significantly ($p < 0.05$) higher than in those stored without seminal plasma. These significant differences ($p < 0.05$) were maintained throughout in vitro capacitation and after progesterone addition. At the end of the experiment, samples that were stored with seminal plasma at 30% for 48 h and 72 h showed significantly ($p < 0.05$) higher proportions of $M540^+$ spermatozoa than those stored without seminal plasma. In addition, in the absence of seminal plasma, the percentages of $M540^+$ spermatozoa were significantly higher ($p < 0.05$) in samples stored for 48 h than in those stored for 72 h (Figure 4b).

2.4. Intracellular Calcium Levels

Percentages of $Fluo3^+$ spermatozoa ($Fluo3^+$) increased progressively throughout incubation in capacitating medium (Figure 5a). At 2 h and 4 h, percentages of $Fluo3^+$ spermatozoa were significantly ($p < 0.05$) higher in samples stored without seminal plasma, irrespective of the time of storage (48 h or 72 h). In addition, percentages of $Fluo3^+$ spermatozoa at 2 h in samples stored with 30% seminal plasma for 72 h were significantly ($p < 0.05$) lower than in the other treatments. After 5 and 30 min of progesterone addition, no significant differences between treatments were observed. However, at the end of the experiment, percentages of $Fluo3^+$ spermatozoa were significantly ($p < 0.05$) higher in samples stored without seminal plasma for 48 h than in those stored with or without seminal plasma for 72 h.

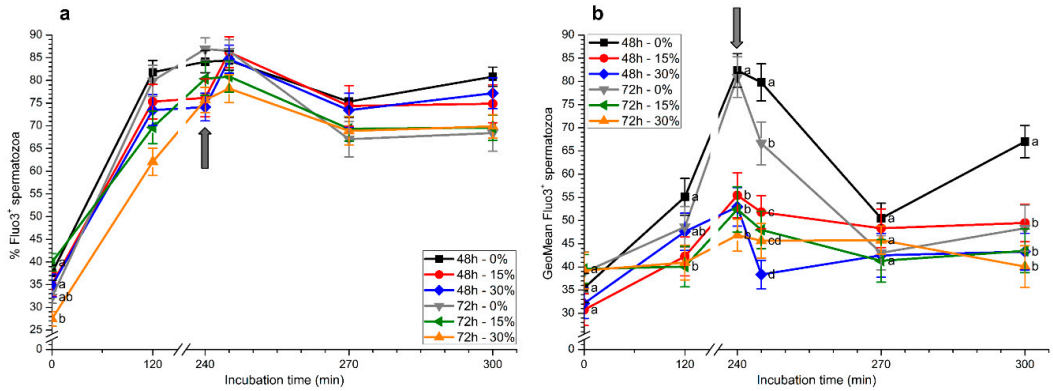

Figure 5. Percentages (**a**) and geometric mean intensity (**b**) of Fluo3$^+$ spermatozoa during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

With regard to Fluo3$^+$ intensity, maximum values of this parameter were observed after 4 h of incubation in samples stored for 48 h or 72 h in the absence of seminal plasma, in a similar fashion to that observed for the percentages of Fluo3$^+$ sperm (Figure 5b). Subsequent to progesterone addition (5 min after IVAE), significantly ($p < 0.05$) higher values were found in samples stored without seminal plasma for 48 h and 72 h. In contrast, samples stored with 30% seminal plasma for 48 h and 72 h showed the lowest values of this parameter. Furthermore, whereas all sperm samples displayed similar values 30 min after progesterone addition, the Fluo3$^+$ intensity was significantly ($p < 0.05$) higher in samples stored in the absence of seminal plasma for 48 h than in the other treatments (Figure 5b).

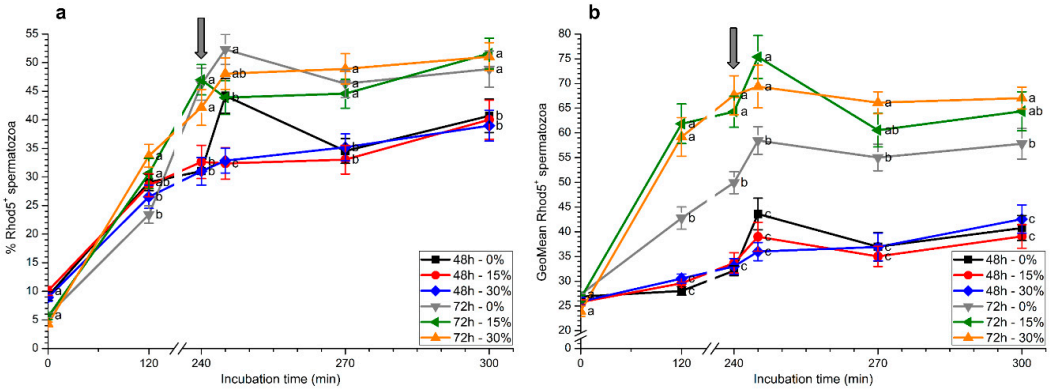

Figure 6. Percentages (**a**) and geometric mean intensity (**b**) of Rhod5$^+$ spermatozoa during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

Percentages of Rhod5$^+$ spermatozoa increased progressively during in vitro capacitation and, after 2 h of incubation, they were significantly ($p < 0.05$) higher in samples stored in the presence of seminal plasma (15% or 30%) for 72 h than in the others (Figure 6a). At 4 h, percentages of Rhod5$^+$ spermatozoa in samples stored for 72 h were significantly ($p < 0.05$) higher than those stored for 48 h, regardless of whether seminal plasma was present or absent. However, 5 min after progesterone addition, samples stored for 48 h without seminal plasma and those stored with and without seminal

plasma for 72 h showed significantly ($p < 0.05$) higher percentages of Rhod5$^+$ spermatozoa than those stored for 48 h with 15% and 30% seminal plasma. After 30 min and 60 min of progesterone addition, significantly ($p < 0.05$) higher percentages of Rhod5$^+$ spermatozoa were observed in samples stored for 72 h than in those stored for 48 h, irrespective of the presence of seminal plasma (Figure 6a).

Rhod5$^+$ fluorescence intensity was significantly ($p < 0.05$) higher in samples stored for 72 h than in those stored for 48 h, with and without seminal plasma, throughout all the incubation period (Figure 6b). In the case of samples stored for 72 h, values observed when spermatozoa were kept with seminal plasma (15% or 30%) were significantly ($p < 0.05$) higher than when they were stored without this fluid. The subsequent addition of progesterone (5 min after progesterone addition) induced an increase in this parameter for all samples, and spermatozoa stored with seminal plasma for 72 h again showed significantly ($p < 0.05$) higher values for this parameter. At the end of the experiment, Rhod5$^+$ fluorescence intensity was significantly ($p < 0.05$) higher in spermatozoa stored with 30% seminal plasma for 72 h than in the other treatments (Figure 6b).

2.5. Mitochondrial Membrane Potential

Samples stored with 30% seminal plasma for 48 h or 72 h showed reduced percentages of spermatozoa with high mitochondrial membrane potential (MMP) at 0 h ($p < 0.05$; Figure 7a). These differences were maintained throughout the entirety of the experimental period. Samples stored without seminal plasma for 72 h showed significantly ($p < 0.05$) higher percentages of spermatozoa with high MMP than those stored with 15% or 30% seminal plasma. After 5 min of progesterone addition, samples stored without seminal plasma again showed significantly ($p < 0.05$) higher percentages of spermatozoa with high MMP. At the end of the experiment, only samples stored with 15% or 30% seminal plasma for 48 h and those stored with 30% seminal plasma for 72 h exhibited significantly ($p < 0.05$) lower percentages of spermatozoa with high MMP than those stored without seminal plasma (Figure 7a).

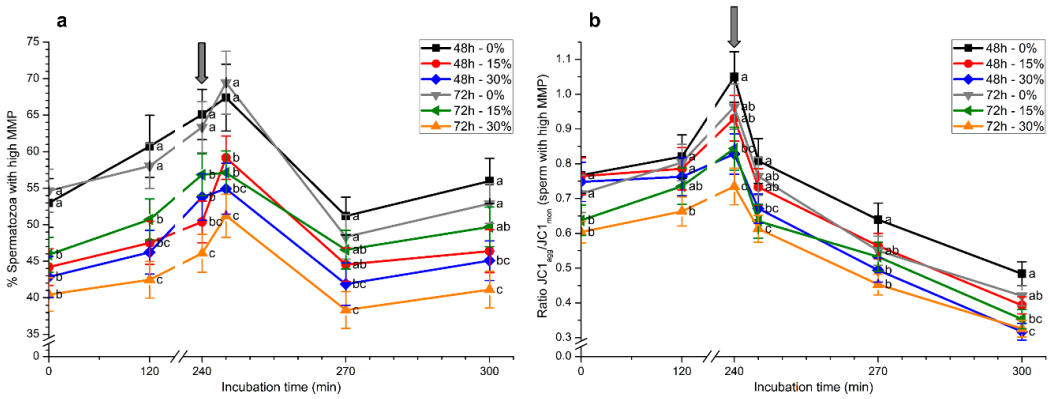

Figure 7. Percentages of spermatozoa with high mitochondrial membrane potential (MMP; (**a**)) and their $JC1_{agg}/JC1_{mon}$ ratios (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 µg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Data are shown as mean ± SEM for seven independent experiments.

Looking at the $JC1_{agg}/JC1_{mon}$ ratios in the sperm population with high MMP, significantly ($p < 0.05$) higher values were observed in samples stored without seminal plasma for 72 h compared to those stored with 30% seminal plasma for 48 h (Figure 7b). After 5 min of progesterone addition and until the end of the experiment, $JC1_{agg}/JC1_{mon}$ ratios in the sperm population with high MMP were significantly ($p < 0.05$) higher in samples stored without seminal plasma than in those stored with 30% seminal plasma, irrespective of the time of storage (48 h or 72 h).

2.6. Tyrosine Phosphorylation Levels of GSK3α/β

Similar results were obtained for tyrosine phosphorylation levels of GSK3α/β, regardless of whether blots were normalized against α-tubulin or total GSK3α/β (Figures 8 and 9). In spite of this, blots obtained using the former loading control were cleaner and the signal stronger than those observed with the latter.

In the absence of seminal plasma, tyrosine phosphorylation levels of GSK3α in spermatozoa at 0 h were significantly ($p < 0.05$) higher in samples stored for 72 h than in those stored for 48 h (Figures 8a and 9a). Tyrosine phosphorylation levels of GSK3α from 2 h and until the end of the experimental period were significantly ($p < 0.05$) higher in samples stored without than in those stored with seminal plasma, irrespective of the time of storage. Whereas tyrosine phosphorylation levels of GSK3α augmented along incubation, reaching maximum values after 5 min of progesterone addition, the extent of that increase was lower when samples were stored for 48 h or 72 h in the presence of 30% seminal plasma. In addition, tyrosine phosphorylation levels of GSK3α were the highest in spermatozoa stored without plasma for 72 h. Remarkably, samples stored in the presence of 30% seminal plasma showed the lowest tyrosine phosphorylation levels of GSK3α, especially at 270 min and 300 min of incubation (Figures 8a and 9a).

Samples stored for 72 h, with and without seminal plasma, had significantly ($p < 0.05$) higher tyrosine phosphorylation levels of GSK3β than those stored for 48 h. These differences, however, were observed in blots normalized against α-tubulin (Figure 8b), but not in those normalized against total GSK3α/β (Figure 9b).

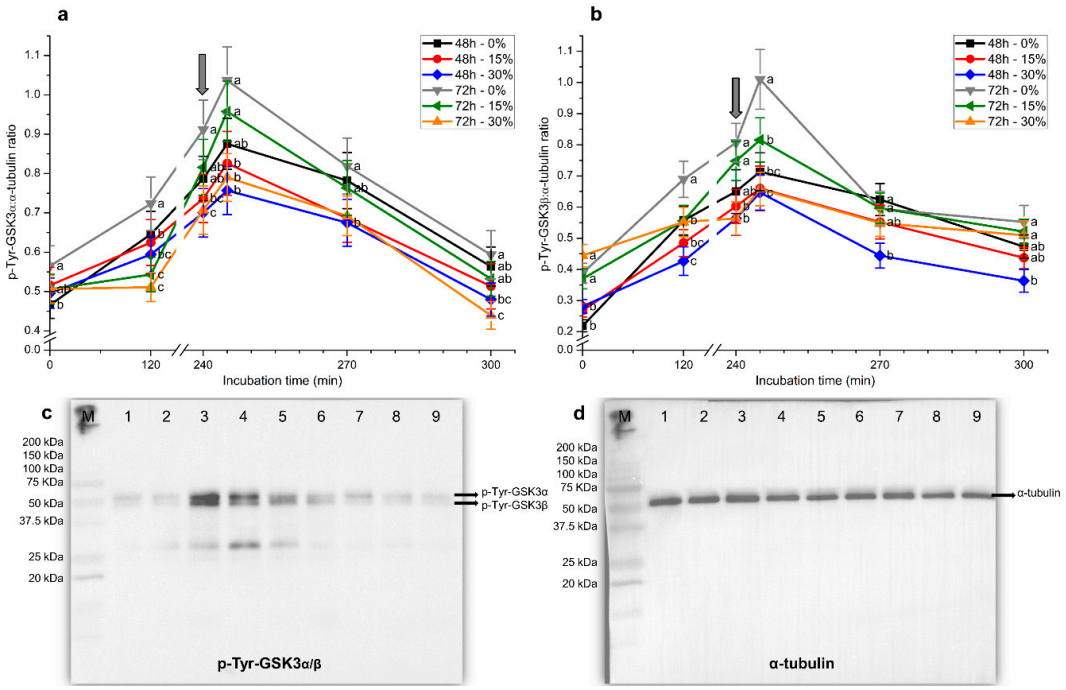

Figure 8. Relative tyrosine phosphorylation levels (using α-tubulin as a loading control) for glycogen synthase kinase-3 (GSK3)α (**a**) and GSK3β (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 μg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Results are shown as mean ± SEM for seven separate experiments. Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Representative blots for p-Tyr-GSK3α/β (**c**) and α-tubulin (**d**). Lanes: (M) protein ladder; (1) 72 h, 0% SP, 0 min; (2) 72 h, 0% SP, 120 min; (3) 72 h, 0% SP, 240 min; (4) 72 h, 0% SP, 245 min; (5) 72 h, 0% SP, 270 min; (6) 72 h, 0% SP, 300 min; (7) 72 h, 15% SP, 0 min; (8) 72 h, 15% SP, 120 min; (9) 72 h, 15% SP, 240 min.

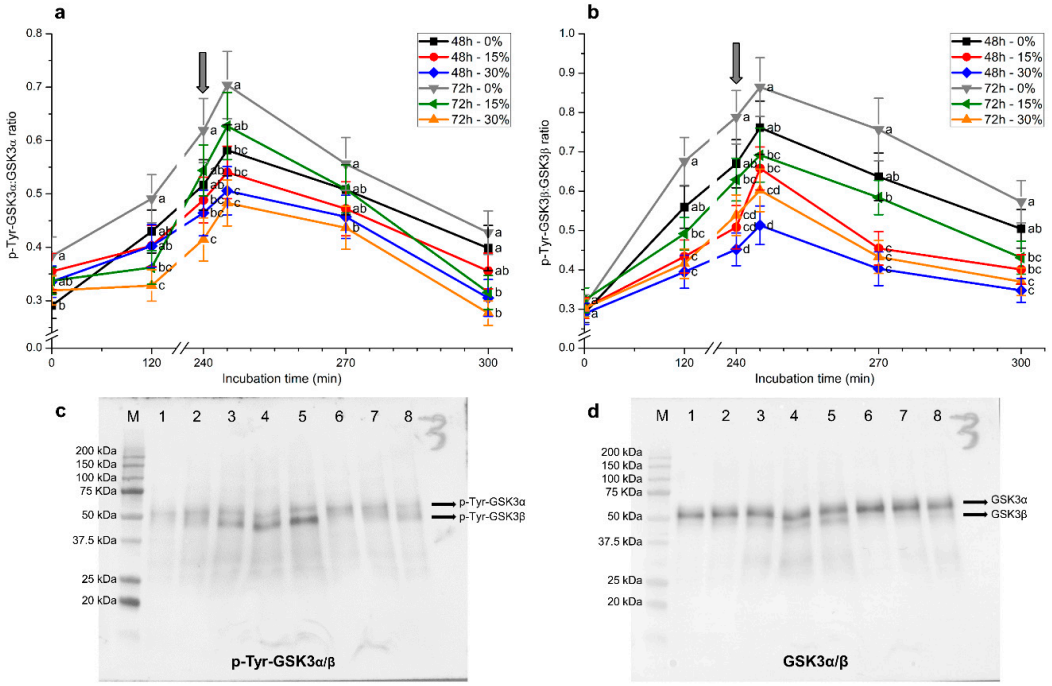

Figure 9. Relative tyrosine phosphorylation levels (using total GSK3α/β as a loading control) for GSK3α (**a**) and GSK3β (**b**) during in vitro capacitation and progesterone-induced acrosomal exocytosis (300 min) after previous storage of spermatozoa at 17 °C with different concentrations of seminal plasma (0%, 15%, and 30%) for 48 h or 72 h. Grey arrow indicates the time at which 10 μg/mL progesterone was added to induce acrosomal exocytosis (i.e., 240 min). Results are shown as mean ± SEM for seven separate experiments. Different letters mean significant ($p < 0.05$) differences between treatments at a given time point. Representative blots for p-Tyr-GSK3α/β (**c**) and α-tubulin (**d**). Lanes: (M) protein ladder; (1) 72 h, 0% SP, 0 min; (2) 72 h, 0% SP, 120 min; (3) 72 h, 0% SP, 240 min; (4) 72 h, 0% SP, 245 min; (5) 72 h, 0% SP, 270 min; (6) 72 h, 0% SP, 300 min; (7) 72 h, 15% SP, 0 min; (8) 72 h, 15% SP, 120 min.

In a similar fashion to that observed for p-Tyr-GSK3α levels, incubation of spermatozoa with capacitating medium led to an increase in p-Tyr-GSK3β levels, regardless of whether blots were normalized against α-tubulin or total GSK3α/β. These levels reached a peak after 5 min of progesterone addition and were significantly ($p < 0.05$) higher in sperm stored with seminal plasma for 72 h than in the other treatments. Again, the higher the concentration of seminal plasma with which sperm were stored, the lower the level of p-Tyr-GSK3β (Figures 8b and 9b). At the end of the experiment, samples stored with 30% seminal plasma for 48 h showed the lowest tyrosine phosphorylation levels of GSK3β.

3. Discussion

Despite the recent insights into the biochemistry of seminal plasma along with on the biological performance of its components, the mechanisms through which it modulates sperm function still remain largely unknown [1–8]. In this context, the current study aimed to investigate whether storing pig semen at 17 °C with seminal plasma affects the sperm ability to elicit in vitro capacitation and trigger acrosomal reaction, and which intracellular mechanisms could be involved.

Our results showed that the presence of seminal plasma during liquid storage of pig semen reduced the percentage of viable spermatozoa with an exocytosed acrosome, so that the longer the time of storage (72 h) and the higher the seminal plasma concentration (30%), the lower the sperm response to the induction of acrosomal exocytosis by progesterone. In addition, this previous storage with seminal plasma also affected intracellular calcium levels and membrane lipid disorder. This effect was observed as soon as sperm were separated from this fluid and were incubated in a capacitating medium (i.e., 0 h). Related to this, it is worth remembering that seminal plasma proteins are known to

be modulators of sperm capacitation [27]. Amongst these proteins, heparin-binding spermadhesins have been associated with the stabilization of sperm membrane by covering the cell surface, thereby preventing early capacitation and acrosomal exocytosis [21]. Therefore, our results suggest that, in addition to the inhibition of early membrane changes during cooling, proteins and other constituents present in the seminal plasma, such as extracellular vesicles [4], maintain sperm membrane integrity and affect the ability to elicit in vitro capacitation and trigger acrosomal exocytosis.

The current work also sought to elucidate the intracellular mechanisms that underlie the effects of the presence of seminal plasma during liquid storage of pig semen at 17 °C. It is widely known that Ca^{2+}, which was evaluated in this study by two separate fluorochromes, is involved in capacitation, hyperactivation, and acrosomal reaction [18,28,29]. Related to this, we observed that the intracellular mechanisms that regulate Ca^{2+} stores were modulated by the presence of seminal plasma and the time of storage. However, the impact on these stores relied upon whether they were located in the head (which are mainly stained by Rhod5) or in the mid-piece (which are mainly stained by Fluo3) [20]. On the one hand, spermatozoa stored in the presence of seminal plasma accumulated less Ca^{2+} (stained by Fluo3) in the mid-piece throughout in vitro capacitation, which could be related to the lower mitochondrial membrane potential observed in these treatments [30,31]. Related to this, it is worth mentioning that previous studies have demonstrated that in vitro sperm capacitation is associated with an increase in mitochondrial membrane potential [19,32] and Ca^{2+} levels in the sperm mid-piece [20]. Therefore, one could suggest that storing pig semen at 17 °C in the presence of seminal plasma affects the sperm ability to elicit in vitro capacitation and trigger acrosomal exocytosis through modulating mitochondrial function and altering Ca^{2+} storage in this compartment.

With regard to Ca^{2+} storage in the sperm head, there was an increase in the percentage of $Rhod5^{+}$ spermatozoa in samples stored for 72 h, with and without seminal plasma, which suggests that the permeability of sperm head membrane to Ca^{2+} increases with a longer period of storage. This would match with the reduced percentage of viable spermatozoa with an intact acrosome observed after 245 min of incubation in samples stored with 30% seminal plasma for 72 h. However, while the presence of seminal plasma during liquid storage led to an increase in the geometric mean intensity of $Rhod5^{+}$, this did not appear to be related to a higher sperm ability to undergo acrosome reaction following progesterone addition [33]. Whilst further studies are required to elucidate the separate Ca^{2+} channels that are involved in the different response of mid-piece and head stores, our results suggest that, in agreement with previous studies [20], the dynamics of these two stores differ and are reliant upon the time of storage and the presence of seminal plasma.

Previous studies have shown that high mitochondrial membrane potential is correlated with sperm motility [34–36] and fertilizing ability [37]. Although no significant differences between the presence and absence of seminal plasma were found in total and progressive sperm motility, we observed that storing pig semen at 17 °C with seminal plasma affected the structure of sperm motile subpopulations. In effect, sperm stored without seminal plasma exhibited higher percentages of SP1 (progressively motile and fastest spermatozoa) during in vitro capacitation and shortly after progesterone addition. These findings could be related to the higher mitochondrial membrane potential and accumulation of Ca^{2+} in the mitochondria observed in the absence of seminal plasma. Moreover, although no differences between treatments were observed with regard to SP2 and SP3, the percentages of spermatozoa belonging to SP2 tended to increase over the incubation time in samples stored with 30% seminal plasma. This increase was concomitant with a decrease in the percentages of spermatozoa belonging to SP1. In this study, SP2 was considered, in agreement with previous studies [38,39], as the hyperactivated sperm subpopulation. Therefore, one could suggest that storage of spermatozoa at 17 °C with seminal plasma could increase their ability to hyperactivate and increase membrane lipid disorder. This hypothesis would match with the higher membrane lipid disorder ($M540^{+}$) observed in samples stored with 30% seminal plasma for 48 h. Hyperactivation is an essential step for sperm fertilizing ability, characterized by highly asymmetrical waveforms and an increase in the amplitude of flagellar bends [28]. Although changes in sperm motility patterns are known to depend on intracellular Ca^{2+} levels [28,29,40–42], our results

indicate that seminal plasma affects head and mid-piece Ca^{2+} stores in a different manner. This could be related to the relevance of mitochondrial regulation during in vitro capacitation, as well as to the changes in the sperm head membrane prior to acrosomal exocytosis. Remarkably, while previous storage with seminal plasma appeared to increase the percentages of hyperactivated spermatozoa, they did not augment those of viable spermatozoa with an exocytosed acrosome. These findings would match with those reported in rams, since spermatozoa from this species are able to elicit in vitro capacitation without hyperactivation and its seminal plasma proteins induce hyperactivation while maintaining sperm in a decapacitated state [23].

Kinases and phosphatases take part in the molecular pathways that regulate, among other cell events, sperm motility and capacitation [43]. A crucial kinase in spermatozoa is glycogen synthase kinase-3 (GSK3), present in two isoforms (GSK3α and GSK3β) and ubiquitously expressed in mammalian tissues [44]. This protein is known to be related with sperm motility [24,45,46], sperm capacitation [24], acrosomal reaction [25], and fertilizing ability [26]. Serine and tyrosine phosphorylation of GSK3α and GSK3β has been established as a crucial regulatory mechanism for this protein. While phosphorylation at Ser21 or Ser9 (for α or β isoforms, respectively) is associated with GSK3-inhibition [47,48], tyrosine phosphorylation at Tyr279 or Tyr216 (for α or β isoforms, respectively) appears to increase its kinase activity [49,50]. However, some authors have suggested that the latter relationship is not as strict as the former, as pharmacological inhibition of GSK3 activity is not always correlated with reduced tyrosine phosphorylation [51]. While most works aiming at addressing role of GSK3 isoforms in mammalian spermatozoa have used serine phosphorylation as a method to determine its kinase activity [24,26,46], the involvement of tyrosine phosphorylation has been less studied. Herein, we investigated, for the first time, the changes of tyrosine phosphorylation of GSK3α and GSK3β that occur during in vitro capacitation and progesterone-induced acrosomal exocytosis. We used two different loading controls to normalize the intensity of the p-Tyr-GSK3α/β antibody (α-tubulin and total GSK3α/β); even though the results from the two approaches considerably coincided, signals were weaker and blots less clean when total GSK3α/β was used as the loading control. However, the fact that these two approaches were taken and agreed make the results more robust. Remarkably, we found that there was an increase of tyrosine phosphorylation in the two GSK3 isoforms when sperm were incubated under in vitro capacitating conditions, and that this increment was higher when samples were previously stored for a longer period (i.e., 72 h) without seminal plasma.

On the other hand, we observed that the presence of seminal plasma during liquid storage appeared to mitigate the capacitation-induced increase in tyrosine phosphorylation levels of GSK3α/β, which could be related to the reduced sperm ability to elicit in vitro capacitation [24]. Furthermore, storing sperm without seminal plasma for 72 h also increased tyrosine phosphorylation of GSK3β after progesterone addition. Since inhibition of GSK3β activity is related to a reduced sperm ability to undergo the acrosomal exocytosis induced by progesterone [25], our data suggest that storing pig sperm at 17 °C in the presence of seminal plasma for a longer period drops the kinase activity of GSK3β through a reduced phosphorylation of tyrosine residues, which results in a decreased percentage of spermatozoa that undergo acrosomal exocytosis.

Another interesting finding of our study was the lack of relationship between tyrosine phosphorylation of GSK3α/β and sperm motility. Previous studies conducted in other species reported that inhibition of GSK3α and GSK3β is required for activating sperm motility [24,46]. In this way, considering that tyrosine phosphorylation is related to an increase in the kinase activity of this protein [49,50], one would have expected more apparent differences between storage times and the presence/absence of seminal plasma with regard to sperm motility. However, the differences in tyrosine phosphorylation levels of GSK3α and GSK3β observed under different storage conditions had no impact on sperm motility parameters. While these results are in contrast with Vijayaraghavan et al. [45] who, working in bull spermatozoa, showed a positive correlation between tyrosine phosphorylation of GSK3 and sperm motility, the fact that our data were retrieved under capacitating conditions might explain these differences.

4. Materials and Methods

4.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany), and Panreac (Barcelona, Spain). All fluorochromes were provided by Thermo Fisher Scientific (Molecular Probes, Eugene, OR, USA) and were diluted with dimethyl sulfoxide (Sigma-Aldrich). Antibodies were acquired from Merck Millipore (Darmstadt, Germany), Agilent Technologies (Santa Clara, CA, USA), MyBioSource (San Diego, CA, USA), Cell Signaling (Danvers, MA, USA), and Dako (Glostrup, Denmark).

4.2. Semen Samples

Semen samples from seven boars were collected through the gloved-hand method by the technical staff of a local farm (Semen Cardona, S.L., Cardona, Spain). Handling of boars was performed in accordance with the EU Directive 2010/63/EU for animal experiments and the Animal Welfare Law issued by the Regional Government of Catalonia (Generalitat de Catalunya, Spain). However, the authors of this study did not manipulate any animal, as all samples were directly provided by the farm. Therefore, no specific ethical approval was required.

Upon collection, sperm-rich fraction were centrifuged at 2400× *g* and 17 °C for 5 min. The resulting supernatants (seminal plasma) were collected and stored at −20 °C. Sperm pellets were resuspended in a commercial extender (Duragen; Magapor, Ejea de los Caballeros, Spain) at a final concentration of 3×10^7 spermatozoa/mL and distributed into aliquots of 50 mL, which were cooled down to 17 °C. Seminal plasma and three sperm aliquots were transported to the laboratory within 2 h post-collection, at −20 °C and at 17 °C, respectively.

4.3. Treatments and Semen Storage Procedures

For each replicate, the three 50 mL sperm aliquots were pooled and split into six fractions. Seminal plasma was thawed and added as follows: (1) control treatment (25 mL diluted semen with no seminal plasma), (2) 15% seminal plasma (21.25 mL diluted semen + 3.75 mL seminal plasma), and (3) 30% seminal plasma (17.5 mL diluted semen + 7.5 mL seminal plasma). Sperm concentration was adjusted to 1×10^7 spermatozoa/mL. Each treatment was prepared in duplicate for evaluation after 48 h and 72 h of storage at 17 °C.

4.4. In Vitro Capacitation and Progesterone-Induced Acrosomal Exocytosis

Following liquid storage at 17 °C for 48 h or 72 h, samples were centrifuged at 600× *g* and 17 °C for 10 min and the resulting pellets were resuspended in 25 mL of capacitating medium (CM). This medium was composed of 20 mM HEPES (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 125 mM NaCl, 3.1 mM KCl, 5 mM glucose, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4{\cdot}7H_2O$, 4.5 mM $CaCl_2$, 15 mM $NaHCO_3$, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, and 5 mg/mL of bovine serum albumin (BSA); the pH was adjusted to 7.4. Spermatozoa were incubated at 38.5 °C and 5% CO_2 for 4 h (Heracell 150; Heraeus Instruments GmbH, Osterode, Germany), as described by Ramió-Lluch et al. [32]. In vitro capacitated spermatozoa were evaluated at 0, 2, and 4 h. After 4 h of incubation, progesterone (final concentration: 10 µg/mL) was added to induce acrosomal exocytosis [52,53]. Spermatozoa were further incubated under the same conditions and evaluated after 5, 30, and 60 min of progesterone addition. At each relevant time point, separate aliquots were taken for motility and flow cytometry analyses, and for protein extraction. In the latter case, aliquots were centrifuged at 2400× *g* and 17 °C for 5 min and supernatants were discarded. Pellets were stored at −80 °C until protein extraction.

4.5. Computer-Assisted Sperm Analysis

Sperm motility was evaluated using a computer-assisted sperm analysis (CASA; Integrated Sperm Analysis System, ISAS, V1.0; Proiser, Valencia, Spain). Briefly, samples were warmed at 38 °C for

15 min and a droplet of 5 µL was placed onto a previously warmed (38 °C) Makler chamber (Sefi Medical Instruments, Haifa, Isarel). A minimum of 200 spermatozoa were analyzed per sample and three separate fields were taken; two technical replicates were evaluated. This CASA system is based upon the analysis of 25 consecutive, digitalized photographic images (each image is captured every 40 ms) per field at a magnification of 100× (negative phase-contrast field, Olympus BX41 microscope; Olympus Europe GmbH, Hamburg, Germany). In addition to the mean values of total (TMOT, %) and progressive motility (PMOT, %), the following individual kinematic parameters were evaluated: straight linear velocity (VSL, µm/s), which represents the average velocity measured in a straight line from the beginning to the end of a given sperm track; curvilinear velocity (VCL, µm/s), which is the average velocity measured over the actual point-to-point track followed by the cell; average path velocity (VAP, µm/s), which corresponds to the average velocity of the smoothest cell pathway; linearity (LIN, %), which is provided by the quotient of VSL/VCL; straightness (STR, %), which results from dividing VSL by VAP; wobble coefficient (WOB, %), the ratio of VAP/VCL; amplitude of lateral head displacement (ALH, µm); beat cross frequency (BCF, Hz), which is the frequency at which the sperm cell head crosses the average pathway; the combination of the lateral and forward movement of the head (DANCE, $\mu m^2/s$), which results from multiplying VCL with ALH; and the mean angular displacement (MAD, degrees) [23,54]. A sperm cell was defined as being motile when VAP ≥ 10 µm/s and progressively motile when STR ≥ 45%.

Individual kinematic parameters were used to determine the number and characteristics of motile sperm subpopulations and to evaluate whether their structure changed in response to the presence of seminal plasma during liquid storage at 17 °C, and throughout in vitro capacitation and progesterone-induced acrosomal exocytosis.

4.6. Flow Cytometry

Plasma and acrosome integrity, membrane lipid disorder, intracellular calcium levels, and mitochondrial membrane potential were evaluated by flow cytometry. These analyses were conducted using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA), after excitation through an argon ion laser (488 nm) set at a power of 22 mW. Cell diameter/volume (i.e., electronic volume, EV) was measured using the Coulter principle for volume assessment, as this system has forward scatter (FSC) replaced by electronic volume (EV). Before spermatozoa were stained with fluorochromes, we adjusted concentration to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL [55]. Samples were assessed at a sheath flow rate of 4.17 µL/min with 10,000 cells being acquired per analysis. Three independent replicates were evaluated. To capture fluorochrome signals, we used three different optical filters: FL1 (green fluorescence)—Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width 505–545 nm; FL2 (orange fluorescence)—DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm; FL3 (red fluorescence)—LP filter: 670 nm, detection width: 655–685 nm. Debris (particle diameter < 7 µm) and aggregates (particle diameter > 12 µm) were excluded from the analysis by gating the particles on the basis of EV/side scatter (SSC) plots. Data obtained from flow cytometry analyses were corrected following the protocol described by Petrunkina et al. [56].

4.6.1. Acrosome Integrity

Acrosome integrity was evaluated through co-staining with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocyanate (FITC) and ethidium homodimer (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1), as described by Rocco et al. [57]. First, spermatozoa were incubated with EthD-1 (final concentration: 2.5 µg/mL) at 37.5 °C for 5 min in the dark. Following this, samples were centrifuged at 2000× *g* and 16 °C for 30 s and then resuspended with PBS supplemented with 4 mg/mL BSA. Thereafter, samples were centrifuged at 2000× *g* and 16 °C for 30 s, and subsequently fixed and permeabilized by adding 100 µL ice-cold methanol (100%) for 30 s. Methanol was removed by centrifugation at 2000× *g* and 16 °C for 30 s, and pellets were resuspended with 250 µL PBS. Finally, samples were stained with PNA-FITC (final concentration: 2.5 µM) at 25 °C for 15 min in the dark, washed twice with PBS at 2000× *g* for 30 s, and resuspended in PBS.

When stained samples were evaluated with the flow cytometer, four sperm populations were identified [58]: (i) viable sperm with an intact acrosome (PNA-FITC$^+$/EthD-1$^-$), (ii) viable sperm with an exocytosed acrosome (PNA-FITC$^-$/EthD-1$^-$), (iii) non-viable sperm with an intact acrosome (PNA-FITC$^+$/EthD-1$^+$), and (iv) non-viable sperm with an exocytosed acrosome (PNA-FITC$^-$/EthD-1$^+$). Fluorescence of EthD-1 was detected through FL3, and PNA-FITC fluorescence through FL1. Results are expressed as the percentage of viable sperm with an exocytosed acrosome (PNA-FITC$^-$) in relation to the total viable sperm population (EthD-1$^-$).

4.6.2. Membrane Lipid Disorder

Membrane lipid disorder was assessed using the co-staining protocol for merocyanine-540 (M540) and YO-PRO-1, as described by Harrison et al. [59]. Sperm samples collected at each time point were incubated with M540 (final concentration: 2.6 µM) and YO-PRO-1 (final concentration: 25 nM) at 38 °C in the dark for 10 min. Fluorescence emitted by M540 and YO-PRO-1 was detected through FL3 and FL1, respectively. Sperm cells stained with M540 corresponded to those exhibiting high membrane lipid disorder, whereas those stained with YO-PRO-1 indicated the occurrence of early changes in their membrane permeability. Four populations were identified: (i) spermatozoa with no changes in membrane permeability and low membrane lipid disorder (M540$^-$/YO-PRO-1$^-$), (ii) spermatozoa with no changes in membrane permeability and high membrane lipid disorder (M540$^+$/YO-PRO-1$^-$), (iii) spermatozoa with changes in membrane permeability and low membrane lipid disorder (M540$^-$/YO-PRO-1$^+$), and (iv) spermatozoa with changes in membrane permeability and high membrane lipid disorder (M540$^+$/YO-PRO-1$^+$).

4.6.3. Intracellular Calcium Levels

Intracellular calcium levels were evaluated with two different markers (Fluo3 and Rhod5). Fluo3 staining was performed following the protocol described by Harrison et al. [60] and modified by Kadirvel et al. [61], whereas Rhod5 staining was performed following the protocol described by Yeste et al. [20]. Whereas Fluo3 has more affinity for the Ca^{2+} stored in the mid-piece, Rhod5 preferentially stains that stored in the sperm head [20].

For Fluo3, spermatozoa were incubated at 38 °C in the dark for 10 min with Fluo3-AM (final concentration: 1 µM) and PI (final concentration: 12 µM), which were detected through FL1 and FL3, respectively. A total of four sperm populations could be identified: (i) viable spermatozoa with low levels of intracellular calcium (Fluo3$^-$/PI$^-$), (ii) viable spermatozoa with high levels of intracellular calcium (Fluo3$^+$/PI$^-$), (iii) non-viable spermatozoa with low levels of intracellular calcium (Fluo3$^-$/PI$^+$), and (iv) non-viable spermatozoa with high levels of intracellular calcium (Fluo3$^+$/PI$^+$). Fluo3 spill over into the FL3 channel (2.45%) and PI spill-over into the FL1 channel (28.72%) were compensated, and the geometric mean of Fluo3 intensity was recorded for all sperm populations.

On the other hand, spermatozoa were incubated at 38 °C for 10 min in the dark with Rhod5-N (final concentration: 5 µM) and YO-PRO-1 (final concentration: 25 nM). Filters used to detect the fluorescence from Rhod5 and YO-PRO-1 were FL3 and FL1, respectively. A total of four sperm populations were identified: (i) viable spermatozoa with low levels of intracellular calcium (Rhod5$^-$/YO-PRO-1$^-$), (ii) viable spermatozoa with high levels of intracellular calcium (Rhod5$^+$/YO-PRO-1$^-$), (iii) non-viable spermatozoa with low levels of intracellular calcium (Rhod5$^-$/YO-PRO-1$^+$), and (iv) non-viable spermatozoa with high levels of intracellular calcium (Rhod5$^+$/YO-PRO-1$^+$). Fluorescence from Rhod5 was compensated into the FL1 channel (3.16%), and the geometric mean of Rhod5 intensity was recorded for all sperm populations.

4.6.4. Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was determined using JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) fluorochrome, following the protocol described by Garner and Johnson [62]. With this purpose, sperm samples were incubated with JC1 (final concentration:

0.3 μM) at 38 °C in the dark for 30 min, and two sperm populations were distinguished: (i) spermatozoa with high mitochondrial membrane potential, and (ii) spermatozoa with low mitochondrial membrane potential. When MMP was high, JC1 inside mitochondria formed orange aggregates that were detected through FL2. At low MMP, JC1 remained as monomers and emitted green fluorescence that was detected through FL1 [63]. Geometric intensities of $JC1_{mon}$ (FL1) and $JC1_{agg}$ (FL2) were recorded and the ratio between $JC1_{agg}$ and $JC1_{mon}$ ($JC1_{agg}/JC1_{mon}$) was also calculated for each sperm population. FL1 spill-over into the FL2 channel was compensated (51.70%).

4.7. Tyrosine Phosphorylation Levels of GSK3α/β

Sperm pellets stored at −80 °C were thawed and resuspended in 400 μL ice-cold lysis buffer and maintained at 4 °C for 30 min under constant agitation. The lysis buffer was made up of 2% SDS, 1% Triton-X-100, 8 M Urea, 2 mM dithiothreitol (DDT), 0.5% Tween 20, and 50 mM Tris-HCl; the pH was adjusted to 7.4. On the day of use, the lysis buffer was added with 1% commercial protease inhibitor cocktail (Sigma-Aldrich), 1% phenylmethanesulfonyl fluoride (PMSF), and 0.15% sodium orthovanadate. Following this, samples were homogenized by sonication (50% amplitude; 10 long-lasting pulses; Bandelin Sonopuls HD 2070; Bandelin Electronic GmbH and Co., Heinrichstrasse, Berlin), and then centrifuged at 10,000× *g* and 4 °C for 15 min. Supernatants were carefully collected and total protein was quantified in triplicate with a detergent-compatible protein assay (DC Protein Assay; BioRad, Hercules, CA, USA). Standard curves were made with different concentrations of BSA (Quick Start Bovine Serum Albumin Standard; Bio-Rad).

A total of 10 μg of total protein was mixed with 2× Laemmli sample buffer and incubated at 90 °C for 5 min. Protein samples were subsequently loaded onto gradient commercial SDS-PAGE gels (Mini-Protean TGX Stain-Free gels; percentage acrylamide in the separating gel: 8–16%), together with a molecular weight marker (Precision Plus Protein All Blue Standards, Bio-Rad). Each gel was run at 20 mA under an initial voltage of 80 V and final voltage of 120–150 V through an electrophoresis system (Mini-PROTEAN Tetra Cell, Bio-Rad). Thereafter, proteins bands were transferred onto polyvinylidene fluoride membranes (PVDF; Immobilon-P; Merck Millipore) through a transfer system (Mini-Trans Blot Cell; Bio-Rad) at 240 mA for 2 h. Membranes were subsequently incubated at 4 °C overnight, and under constant agitation with a blocking solution consisting of 5% (*v*/*v*) BSA diluted in Tris-buffered saline containing Tween 20 (1 × TBS-Tween20). Following this, membranes were incubated with a primary anti-phospho-GSK3α/β (Tyr279/Tyr216) (ref. 05-413; Merck Millipore) antibody diluted 1:5000 (*v*/*v*) in blocking solution, under agitation and at room temperature for 1 h. After we washed the membranes three times (5 min per wash) with a solution made up of 10 mM Tris, 150 mM NaCl, and 0.05% Tween20 (pH = 7.3), they were incubated with a secondary anti-mouse polyclonal antibody conjugated with horseradish peroxidase (HRP; ref. P0260; Agilent Technologies) diluted 1:10,000 (*v*/*v*) in blocking solution, at room temperature for 1 h under constant agitation. Membranes were washed 5 times (5 min per wash) in the case of membranes that were further incubated with an anti-α-tubulin antibody and 10 times in that of membranes that were further incubated with anti-GSK3α plus anti-GSK3β antibodies. Reactive bands were subsequently visualized using a chemilumimnescent substrate (Immobilon Western Detection Reagents; Merck Millipore). Two reactive bands with different molecular weights corresponding to α (≈51 kDa) and β (≈47 kDa) GSK3 isoforms [24] were identified. No blocking peptide was available for this primary antibody, and thus no peptide blocking assay could be run. However, this primary antibody was raised against the human GSK3, whose sequence homology with regard to the pig is, according to Protein BLAST (Basic Local Alignment Search Tool) database (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA), 96% (GSK3α) and 99% (GSK3β). Images were acquired with Genesys software (Synoptics Limited, Cambridge, United Kingdom).

Anti-α-tubulin (ref. MABT205, Merck Millipore), anti-GSK3α (ref. 9338; Cell Signaling), and anti-GSK3β (ref. MBS8207657; MyBioSource) antibodies were separately used as internal standards to normalize the intensity of p-Tyr-GSK3α/β-bands [64]. In brief, membranes were stripped using

a buffer made up of 1.5% glycine, 0.1% SDS, and 1% Tween20, and the pH was adjusted to 2.2. Membranes were incubated with an anti-α-tubulin antibody diluted at 1:100,000 (*v*/*v*) or anti-GSK3α plus anti-GSK3β antibodies diluted at 1:5000 (*v*/*v*) in blocking solution and then washed three times (5 min per wash) in the case of anti-α-tubulin and five times in that of anti-GSK3α plus anti-GSK3β antibodies. Subsequently, membranes were incubated with a secondary anti-mouse polyclonal antibody conjugated with horseradish peroxidase (HRP; ref. P0260; Agilent Technologies) diluted at 1:150,000 (*v*/*v*) in the case of the anti-α-tubulin antibody and at 1:10,000 (*v*/*v*) in the case of anti-GSK3α/β antibodies. After washing membranes 5 times in the case of anti-α-tubulin antibody and 10 times in that of anti-GSK3α plus anti-GSK3β antibodies, we used a chemilumimnescent substrate (Immobilon Western Detection Reagents; Merck Millipore) to visualize the bands corresponding to α-tubulin or GSK3α plus GSK3β [62]. Images were taken with the Genesys software (Synoptics Limited, Cambridge, United Kingdom).

Quantification of protein bands in blot images was performed with Quantity One 1-D Analysis Software (Bio-Rad). Values were expressed as the total signal intensity corresponding to pixel intensity units (density, mm^2) present inside the boundary of the band. In addition, a background signal was excluded and the lowest intensity of a pixel was considered as zero [65]. Ratios between each p-Tyr-GSK3-band (i.e., α or β) and α-tubulin, or GSK3α/GSK3β (total) bands were calculated per lane. Although signals were clearer and blots cleaner when α-tubulin was used as an internal standard, differences between treatments with regard to these ratios were quite similar.

4.8. Statistical Analyses

Data were analyzed with a statistical package (IBM SPSS 25.0 for Windows; IBM Corp.; Chicago, IL, USA). First of all, normality (Shapiro–Wilk test) and homogeneity of variances (Levene test) were checked. Following this, the effects of treatment (0%, 15%, and 30% seminal plasma) and storage time (48 and 72 h) on sperm motility parameters, acrosome integrity, membrane lipid disorder, mitochondrial membrane potential, intracellular Ca^{2+} levels, and tyrosine phosphorylation of GSK3α/β (normalized against α-tubulin and total GSK3α/β) during in vitro capacitation and progesterone-induced acrosomal exocytosis were tested with a linear mixed model followed by a Sidak test for pair-wise comparisons. Treatment (presence/absence of seminal plasma) and storage conditions were the inter-subject factors, and the incubation time during in vitro capacitation and progesterone-induced acrosomal exocytosis was the intra-subject factor. All sperm parameters were considered as dependent variables.

Sperm subpopulations were set according to the procedure described by Luna et al. [23] and Estrada et al. [66] with minor modifications. First, the individual CASA parameters obtained for each sperm cell (VSL, VCL, VAP, LIN, STR, WOB, ALH, BCF, MAD, and DANCE) were used as independent variables in a principal component analysis (PCA). These kinematic parameters were sorted into separate PCA components and the obtained data matrix was rotated using the Varimax procedure with Kaiser normalization. As a result, regression scores for each PCA component were calculated per spermatozoon. On the basis of these regression scores, a two-step cluster analysis was run (log likelihood distance and Schwarz's Bayesian criterion). This analysis identified up to three motile sperm subpopulations, and the percentages of spermatozoa belonging to each subpopulation (SP1, SP2, or SP3) were calculated. Again, a linear mixed model followed by post-hoc Sidak test was run to determine the effects of treatment (presence/absence of seminal plasma), storage time (48 and 72 h), and incubation (in vitro capacitation and progesterone-induced acrosomal exocytosis) on the percentages of SP1, SP2, and SP3 spermatozoa.

The level of significance was set at $p \leq 0.05$ for all analysis, and data are shown as mean ± standard error of the mean (SEM).

5. Conclusions

Our results indicate that the presence of seminal plasma during liquid storage of pig semen at 17 °C modulated the sperm ability to elicit in vitro capacitation and trigger the acrosomal exocytosis induced

by progesterone. In addition, this previous contact for 72 h led to decreased tyrosine phosphorylation of GSK3α/β, which appeared to be related to reduced mitochondrial membrane potential and ability to undergo acrosomal exocytosis. However, because the effects of the presence of seminal plasma during liquid storage 17 °C were less consistent in other sperm parameters, especially in the case of intracellular Ca^{2+} levels, further studies looking into in vitro fertilization (IVF) and artificial insemination outcomes are necessary to address whether it may be a beneficial practice for pig breeding.

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Abbreviations

ALH	amplitude of lateral head displacement
BCF	beat-cross frequency
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
CM	capacitating medium
DANCE	forward movement of the head
EV	electronic volume
FSC	forward scatter
GSK3a	glycogen synthase kinase-3 isoform α
GSK3ß	glycogen synthase kinase-3 isoform β
ISAS	integrated sperm analysis system
LIN	linearity
M540	merocyanine-540
MAD	mean angular displacement
PCA	principal component analysis
PI	propidium iodide
PMOT	progressive motility
SEM	standard error of the mean
SP	motile subpopulation
SRF	sperm-rich fraction
SSC	side scatter
STR	straightness
TMOT	total motility
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight linear velocity
WOB	wobble coefficient

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PAPER IV

Mating to intact, but not vasectomized, males elicits changes in the endometrial transcriptome: insights from the bovine model

Sandra Recuero, José María Sánchez, Yentel Mateo-Otero, Sandra Bagés-Arnal, Michael McDonald, Susanta K Behura, Thomas E Spencer, David A Kenny, Marc Yeste, Pat Lonergan & Beatriz Fernandez-Fuertes

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Mating to Intact, but Not Vasectomized, Males Elicits Changes in the Endometrial Transcriptome: Insights From the Bovine Model

*Sandra Recuero[1], José María Sánchez[2], Yentel Mateo-Otero[1], Sandra Bagés-Arnal[2], Michael McDonald[2], Susanta K. Behura[3], Thomas E. Spencer[3], David A. Kenny[4], Marc Yeste[1], Pat Lonergan[2] and Beatriz Fernandez-Fuertes[1]**

[1] Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain, [2] School of Agriculture and Food Science, University College Dublin, Dublin, Ireland, [3] Division of Animal Sciences, University of Missouri, Columbia, MO, United States, [4] Animal and Bioscience Research Centre, Teagasc Grange, Meath, Ireland

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Tomer Avidor-Reiss, The University of Toledo, United States

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Susan S. Suarez, Cornell University, United States
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***Correspondence:**
Beatriz Fernandez-Fuertes
beatriz.fernandez@udg.edu

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An appropriate female reproductive environment is essential for pregnancy success. In several species, including mice, pigs and horses, seminal plasma (SP) components have been shown to modulate this environment, leading to increased embryo viability and implantation. Due to the characteristics of mating in the aforementioned species, SP comes into direct contact with the uterus. However, it is questionable whether any SP reaches the uterus in species that ejaculate inside the vagina, such as humans and cattle. Hence, we hypothesized that sperm, perhaps acting as a vehicle for SP factors, play a more important role in the modulation of the maternal uterine environment in these species. In addition, changes elicited by SP and/or sperm may originate in the vagina and propagate to more distal regions of the female reproductive tract. To test these hypotheses, a bovine model in which heifers were mated to intact or vasectomized bulls or were left unmated was used. RNA-sequencing of endometrial samples collected 24 h after mating with a vasectomized bull did not reveal any differentially expressed genes (DEGs) in comparison with control samples. However, the endometrium of heifers mated with intact bulls exhibited 24 DEGs when compared to heifers mated with vasectomized bulls, and 22 DEGs when compared to unmated control heifers. The expression of a set of cytokines (*IL6*, *IL1A*, *IL8,* and *TNFA*) and candidate genes identified in the endometrial RNA-sequencing (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB,* and *CEBPD*) were assessed by RT-qPCR in the vagina and oviductal ampulla. No differences in expression of these genes were observed between treatments in any region. However, mating to both intact and vasectomized bulls induced an increase in *IL1A* and *TNFA* expression in the vagina compared to the oviduct. These data indicate that sperm, but not secretions from the accessory glands alone, induce modest changes in endometrial gene expression after natural mating in cattle. However, it is not clear whether this effect is triggered by inherent sperm proteins or SP proteins bound to sperm surface at the time of ejaculation.

Keywords: seminal plasma, cattle, endometrium, transcriptome, maternal environment

INTRODUCTION

Embryonic loss is a major contributor to pregnancy failure in livestock species and humans, ranging from 20 to 40% (Macklon et al., 2002; Wiltbank et al., 2016). Most of these losses occur before implantation, highlighting the importance of this period that encompasses such critical events as the first embryonic cleavage divisions; embryonic genome activation; blastocyst formation and hatching; conceptus development; and the preparation of the endometrium to interact with the embryonic trophectoderm (Diskin and Morris, 2008; Niakan et al., 2012; Sandra et al., 2017). Many factors are involved in implantation failure, but in mice and pigs there is growing evidence of a role for the maternal immune system and its regulation by seminal plasma (SP) (Gangnuss et al., 2004; O'Leary et al., 2004, 2006; Song et al., 2016; Glynn et al., 2017).

Seminal plasma is a complex fluid resulting from the secretions of the testes, epididymides and accessory sex glands (in the bull: ampullae, seminal vesicles, prostate and bulbourethral glands). Although it is difficult to accurately calculate the precise contribution of each organ and gland to the final composition of this fluid, vasectomy in the bull by removal of a portion of each vas deferens and therefore removing the contribution of the epididymides, does not appear to significantly affect SP volume (Alexander et al., 1971), indicating a more prominent role of the accessory sex glands. However, vasectomy does lead to a reduction in amino acids in the bull ejaculate (Alexander et al., 1971), and to slight modifications in the proteome of human SP (Batruch et al., 2011). Traditionally, SP has been viewed as a mere vehicle for sperm that nourishes and supports these cells in the female reproductive tract. However, mounting evidence demonstrates an emerging role for SP components in the modulation of the endometrial and oviductal environment, which results in improved fertility and embryo survival and development (reviewed in Bromfeld, 2016; Morgan and Watkins, 2020). Exposure to SP in mice (Schjenken et al., 2015; Song et al., 2016; Glynn et al., 2017), as well as in pigs (O'Leary et al., 2004, 2006) and mares (Tunon et al., 2000; Palm et al., 2008; Fedorka et al., 2017), induces the expression of several endometrial cytokines, leading to leukocyte recruitment to the uterus. This migration of immune cells was thought to solely serve the purpose of clearing microorganisms and excess sperm (Pandya and Cohen, 1985; Thompson et al., 1992). However, it is now thought that the endometrial cytokine and chemokine cascade induced by SP is important to facilitate maternal tolerance toward paternal antigens (reviewed in Robertson, 2007). Indeed, in mice, mating drives the expansion of $CD4^+CD25^+$ T regulatory cells (Robertson et al., 2009; Shima et al., 2015), which can suppress or modulate the immune response of other cells (Sakaguchi et al., 2001). The increase in $CD4^+CD25^+$ is not observed when females are mated to vasectomized or seminal-vesicle-excised males, suggesting that this expansion is driven by secretions from the male accessory glands (Robertson et al., 2009). This effect likely explains why mating increases maternal tolerance toward paternal major histocompatibility complex (MHC) antigens (Robertson et al., 2009), which improves the ability of the semi-allogenic embryo to implant and develop normally in this species (Bromfield et al., 2014; Watkins et al., 2018). In addition to modifying the uterine environment, transcervical infusion of SP in pigs has been shown to modulate ovarian function by increasing corpora lutea (CL) weight and progesterone synthesis (O'Leary et al., 2006), which is essential for creating an appropriate uterine environment for the developing embryo. Moreover, in horses, a pivotal role of SP in protecting spermatozoa from neutrophil phagocytosis in the uterus has been suggested, improving fertility in this environment (Troedsson et al., 2002; Alghamdi et al., 2004).

Due to characteristics of mating in rodents, pigs and horses, SP reaches the uterus and can therefore interact directly with the endometrium (Hunter, 1981; Dean et al., 2011). It is not clear, however, whether any SP reaches the uterus in species that ejaculate intravaginally and in which the volume of the ejaculate is relatively low, such as cattle or humans. It is possible that in those species, SP has an indirect effect on the endometrial environment and/or that sperm act as vehicles for the transport of SP components to more distal regions of the reproductive tract. In this sense, the bovine model could be more appropriate than rodents or pigs in understanding the regulatory properties of SP in the maternal environment of women.

In vitro studies in humans have demonstrated the potential of SP to induce expression of cytokines and chemokines in vaginal, cervical and endometrial epithelial cell cultures (Gutsche et al., 2003; Sharkey et al., 2007, 2012a; Remes Lenicov et al., 2012). While gene expression changes and leukocyte recruitment have been described in the human cervix after unprotected, but not condom-protected, coitus (Sharkey et al., 2012b), there is currently no evidence of SP-induced changes in the endometrium *in vivo*.

In cattle, the expression of several inflammatory mediators (such as colony-stimulating factor 2 – *CSF2*, interleukins 1B, 6, 17A and 8 - *IL1B*, *IL6*, *IL17A*, *IL8*; Prostaglandin-endoperoxide synthase 2 – *PTGS2*, and transforming growth factor beta 1 – *TGF-B1*) in uterine horns ipsi- and contralateral to the CL was modified after SP infusion into the uterus, in the absence or presence of sperm (Ibrahim et al., 2019). Despite this, uterine infusion of SP at the time of artificial insemination (AI) does not increase pregnancy rate in heifers or cows (Odhiambo et al., 2009; Ortiz et al., 2019). As mentioned above, it is questionable whether SP reaches the uterus during mating in cattle, so results obtained from the infusion of SP into the uterus may not be representative of physiological conditions. Indeed, recently it has been shown that infusion of SP into the vagina, but not into the uterus, modifies endometrial levels of epidermal growth factor (Badrakh et al., 2020), which highlights the importance of considering the ejaculate deposition site in natural conception in these studies. We recently reported a modest increase in conceptus length in embryos that developed from Day 7 to Day 14 in the uterus of heifers mated to a vasectomized bull in comparison to unmated heifers (Mateo-Otero et al., 2020). However, exposure of heifers to vasectomized bulls prior to AI failed to increase pregnancy rates (Pfeiffer et al., 2012). In addition, although bulls that had their seminal vesicles resected exhibited reduced semen volume, there was no apparent effect on their subsequent fertility (Shah et al., 1968). Together with recent work from our group, demonstrating

a deleterious effect of bull SP on endometrial RNA integrity due to the presence of a seminal RNase (Fernandez-fuertes et al., 2019), the literature seems to suggest that SP does not play a significant role in pregnancy establishment in cattle.

Based on these data, we hypothesized that in species that ejaculate inside the vagina, changes in the female reproductive environment begin in this region and then propagate to more distal regions, such as the uterus and/or the oviduct. Also, because of the lack of direct contact with the seminal fluid, sperm probably play a more important role in the modulation of the uterine environment in these species. In order to test these hypotheses, RNA-sequencing analysis of endometrial samples was carried out following natural mating of heifers with vasectomized (whose ejaculate lack sperm and epididymal and testicular fluid) or intact (that ejaculate sperm and SP) bulls. In addition, the expression of a set of interesting candidate genes was assessed in the vagina and oviductal ampulla, with the aim of determining the effects of sperm and accessory gland secretions from the most proximal region of the female reproductive tract (vagina) to the distal region where gamete interaction takes place (oviductal ampulla).

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were sourced from Sigma-Aldrich (Arklow, Ireland).

Animals

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College Dublin and licensed by the Health Products Regulatory Authority (HPRA), Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes). For the duration of the study, all animals were housed in groups of 10–15, independent of treatment, and managed identically in terms of feeding and husbandry routines.

Vasectomy was carried out by removing approximately 5 cm of both vasa deferentia. This procedure took place 5–6 months prior to the trial. Vasectomized bulls underwent semen evaluation to confirm the lack of sperm and all ran as teasers for oestrus detection with 25 cows/heifers each during the breeding season preceding the study. Intact bulls underwent a breeding soundness evaluation prior to the study. Neither intact and vasectomized bulls had access to females for at least 5 months before the trial, nor during it (apart from the controlled mating to the experimental heifers).

Experimental Design

Estrous cycles of crossbreed beef heifers (Angus and Holstein-Friesian cross; n = 28) were synchronized using an 8-day intravaginal device (PRID® Delta, 1.55 g progesterone, Ceva Santé Animale, Libourne, France), together with a 2 mL intramuscular injection of a synthetic gonadotrophin releasing hormone (Ovarelin®, equivalent to 100 μg Gonadorelin, Ceva Santé Animale) administered on the day of PRID insertion. One day prior to PRID removal, all heifers received a 5 mL intramuscular injection of prostaglandin F2 alpha (Enzaprost®, equivalent to 25 mg of Dinoprost, Ceva Santé Animale) to induce luteolysis. Only heifers observed in standing estrus were used (n = 22). Heifers were blocked by weight and randomly allocated to one of three treatments (**Figure 1**): (1) mated to an intact bull (n = 7), (2) mated to a vasectomized bull (n = 8), or (3) left unmated (control; n = 7). Between 0 to 6 h after estrus detection, heifers were separated from the group and placed in a pen (one at a time) with one of three vasectomized Holstein Friesian bulls, or one of two intact Holstein Friesian bulls (**Supplementary Table 1**). Once the bull mounted and intromission was confirmed, the heifers were returned to the group. Bulls were allowed to mate no more than twice per day and the experiment was carried out over three consecutive days.

Tissue Collection

All heifers were slaughtered in a commercial abattoir 24 h (±6 h) after mating and their reproductive tracts were recovered. The ovaries were examined to determine the site of the preovulatory or freshly ovulated follicle (**Supplementary Figure 1**). Endometrial tissue samples were obtained from intercaruncular areas of the base of uterine horn ipsilateral to the preovulatory or freshly ovulated follicle. In cattle, the uterine glands are located in the intercaruncular areas of the endometrium, while the caruncular areas are aglandular. These glands are responsible for the secretion of the histotroph, which will nurture the developing embryo, and drive the maternal signals of implantation (Kelleher et al., 2019; Spencer et al., 2019). In addition, epithelial sections of anterior vagina and whole sections of the ampulla of the ipsilateral oviduct were obtained. Immediately after tissue collection, each sample was snap frozen in liquid nitrogen and stored at −80°C.

Experiment 1: Seminal Plasma Effects on the Endometrial Transcriptome

RNA Extraction

For total mRNA extraction, samples were first homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, United States) using a steel bead and the Qiagen tissue lyzer (2 × 120 s at maximum speed). On-column RNA purification was performed using the Qiagen RNeasy kit (Qiagen, Crawley, Sussex, United Kingdom) per the manufacturer's instructions. The quantity of RNA was determined using the Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Dublin, Ireland). Prior to endometrial RNA sequencing analysis, the RNA quality was assessed by the Agilent Bioanalyzer (Agilent Technologies, Cork, Ireland). Only samples that exhibited a minimum RNA integrity number (RIN) of 8 were used in this experiment (n = 6 heifers in each experimental group).

RNA Sequencing Analysis

RNA library preparation and sequencing were performed by the University of Missouri DNA Core Facility as described previously by Moraes et al. (2018). The raw sequences (fastq) were subjected

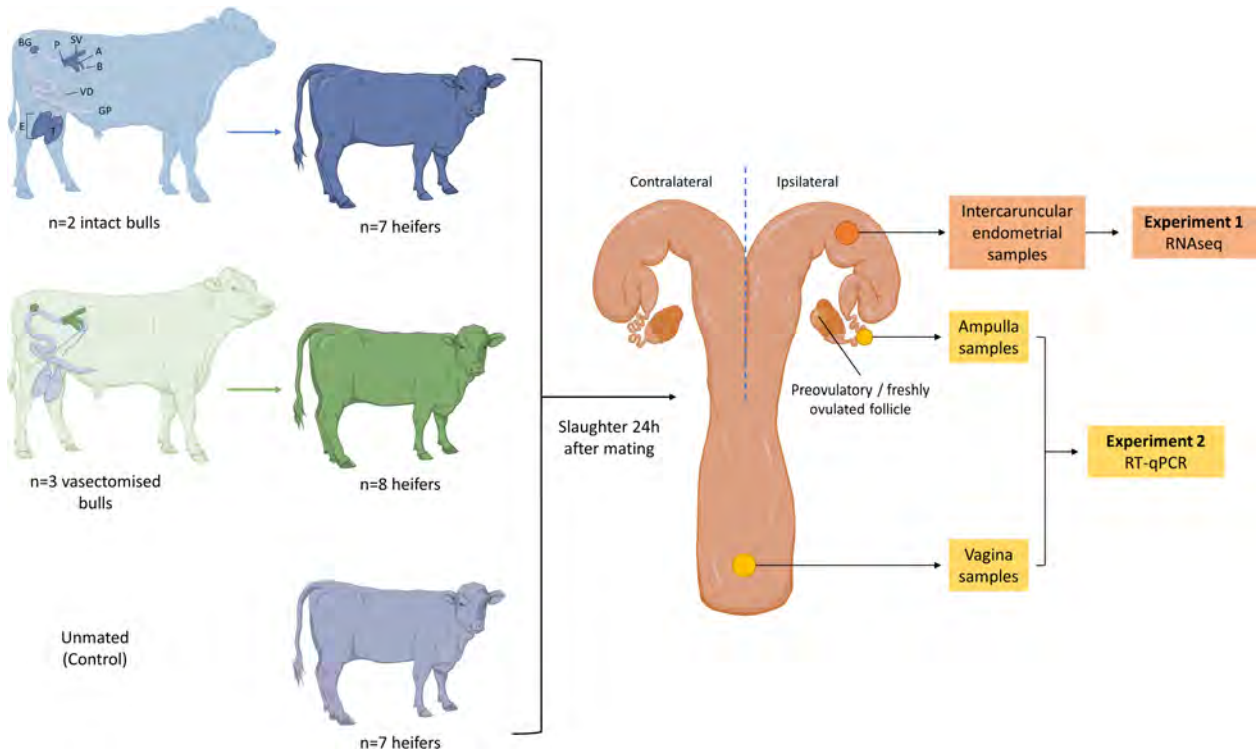

FIGURE 1 | Summary of the experimental design. Only heifers observed in standing estrus were mated with (1) an intact bull (blue; $n = 7$), (2) a vasectomized bull (green; $n = 8$) or (3) left unmated (gray; $n = 7$). A schematic representation of the male reproductive tract is shown, and the structures that contribute to the ejaculate in each case are highlighted (T, testis; E, epididymis; GP, glans penis; VD, vas deferens; B, bladder; A, ampulla; SV, seminal vesicle; P, prostate; BG, bulbourethral gland). Heifers were slaughtered 24 h after mating and their reproductive tracts were recovered. Endometrial samples were obtained from the base of the ipsilateral uterine horn to perform RNA sequencing (Experiment 1). Samples from ampulla and vagina were obtained to assess the expression of a set of genes (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB*, *CEBPD*, *IL1A*, *IL6*, *TNFA,* and *IL8*) by RT-qPCR (Experiment 2).

to quality trimming control using fqtrim[1]. Then, the quality reads were mapped to the bovine reference genome UMD3.1 using Hisat2 mapper (Kim et al., 2015). Read counts mapping to each gene were determined from the binary alignment map files of the samples using FeatureCounts (Liao et al., 2014). Differential expression analysis between different sample groups was performed using edgeR robust (Zhou et al., 2014).

For the annotated DEGs, the gene ontology analysis was performed using PANTHER[2].

Experiment 2: Seminal Plasma Effects on Gene Expression in the Vagina and Oviduct

RNA Extraction and cDNA Synthesis

Total mRNA extraction was carried out as described above. For each sample, cDNA was prepared from approximately 100 ng of total mRNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The cDNA obtained was diluted using RNase- and DNase-free water in a final concentration of 5 ng/mL and in a total volume of 100 μL. The diluted cDNA samples were stored at −20°C for subsequent analysis. As some samples were lost during the management of the experiment, the number of samples per group varies for the vagina region: $n = 6$ heifers in the control group, $n = 5$ in the intact group and $n = 7$ in the vasectomized group. For the ampulla region, there were $n = 7$ individuals in each experimental group (control, intact, and vasectomized). In addition, the RNA quality was assessed by the Agilent Bioanalyzer (Agilent Technologies, Cork, Ireland) (RIN = 7.81 ± 0.29; mean ± standard error of the mean, SEM).

Genes of Interest Selection for Expression Analysis

In order to determine SP effects on gene expression in vagina and oviduct regions, a set of genes (*IL1A*, *IL6*, *TNFA,* and *IL8*) were selected based on the literature. These genes were inflammatory mediators expression of which was reported to be modified by SP exposure in cattle and other species (O'Leary et al., 2004; Sharkey et al., 2012b; Schjenken et al., 2015; Introini et al., 2017; Ibrahim et al., 2019). Moreover, expression of some genes (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB,* and *CEBPD*) found differentially expressed in RNA-sequencing analysis of endometrium samples was also interrogated. The selection of these genes as interesting targets was based on literature searching (Wedel and Lömsziegler-Heitbrock, 1995; Blom et al., 2004;

[1]https://ccb.jhu.edu/software/fqtrim/
[2]pantherdb.org

Hannan et al., 2004; Tapia et al., 2008; Hayashi et al., 2017; Neupane et al., 2017; Pinto De Melo et al., 2017; Tríbulo et al., 2018) and GO term analysis.

Quantitative Real-Time PCR Analysis

All primers were designed using Primer Blast software[3] (**Supplementary Table 2**). Briefly, RT-qPCR assays were performed per duplicate in a total volume of 20 μL, containing 10 μL FastStart Universal SYBR Green Master (Roche Diagnostics Ltd., West Sussex, United Kingdom), 1.2 μL forward and reverse primer mix (300 nM final concentration), 5.6 μL nuclease-free water and 2 μL cDNA template on the ABI Prism 7500 Real-Time PCR System (Life Technologies). A total of 40 cycles were performed with the following thermo-cycling conditions for each cycle: 50°C for 2 min, 95°C for 10 min followed by 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s. The melt curve was also included to ensure specificity of amplification. The specificity of all targets was confirmed by the presence of a single sharp peak in the melt curve. A total of eight potential reference genes [Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*), Actin Cytoplasmic 1 (*ACTB*), 60S Ribosomal Protein L18 (*RPL18*), Peptidyl-Prolyl *Cis-Trans* Isomerase A (*PPIA*), 14-3-3 Protein Zeta/Delta (*YWHAZ*), RING Finger Protein 11 (*RNF11*), Histone H3.3 (*H3F3A*), Succinate Dehydrogenase Complex Subunit A Flavoprotein Variant (*SDHA*)] were analyzed using the geNorm function with the qbase + package (Biogazelle, Zwijnaarde, Belgium) to identify the best reference genes. Due to the high variability between samples, a total of four reference genes were selected: *RNF11, H3F3A, YWHAZ,* and *GADPH*, which were the most stably expressed (average geNorm $M \leq 0.5$).

Primer efficiency was carried out for the genes of interest, and RT-qPCR of 1:4 dilutions of a cDNA mix from a representative pool of samples were analyzed. The presence of a single sharp peak in the melt curve as well as the standard curve was used to confirm primer specificity. The threshold cycle (Ct) for each sample was automatically calculated using the default settings within the SDS software (SDS 1.4, ABI). In order to obtain the relative expression values of the genes of interest, $2^{-\Delta\Delta CT}$ method was used (Livak and Schmittgen, 2001). For each individual, the expression of the genes of interest was firstly normalized to the average of housekeeping genes previously selected (*RNF11, H3F3A, YWHAZ,* and *GADPH*) with the following formula: $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{(RNF11+H3F3A+YWHAZ+GADPH)/4}$. The values of $\Delta\Delta Ct$ were calculated normalizing the results to the mean across all individuals, including both tissue regions (vagina and ampulla), per each gene of interest. The subsequent statistical analysis was performed using ΔCt values whereas the results are represented as $2^{-\Delta\Delta CT}$.

Results expressed as ΔCT were analyzed with IBM SPSS 25.0 for Windows (Armonk; New York, NY, United States). Data were checked for normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene test) to confirm that parametric assumptions were fulfilled. When these premises were not, data (x) were linearly transformed using the square root ($\sqrt{x}$) and arcsine of the square root (arcsin $\sqrt{x}$). Thereafter, data (transformed or not depending on the case) were analyzed by a two-way ANOVA followed by a Sidak *post hoc* test for pair-wise comparisons. The expression of five genes (*CX3CL1*, *PLA2G10*, *TNFA*, *IL6,* and *CXCL8*), even after linear transformation, did not match parametric assumptions. For this reason, Scheirer–Ray–Hare and Mann–Whitney tests were used as non-parametric alternatives. In all cases, the significance level was established at $P \leq 0.05$.

[3]https://www.ncbi.nlm.nih.gov/tools/primer-blast/

RESULTS

Ovary Status

At the time of sample collection (24 ± 6 h after mating), a total of 13 heifers had a freshly ovulated follicle on their ovary, while the remaining animals exhibited a pre-ovulatory follicle (see **Supplementary Table 1** and **Supplementary Figure 1**). In the control group, three animals had ovulated and four exhibited a pre-ovulatory follicle. Regarding the heifers mated with vasectomized bulls, fresh ovulation was found in six animals and only two presented a pre-ovulatory follicle. While in the intact group, four ovulations and three preovulatory follicles were observed. The proportion of animals that had a fresh ovulation was balanced across treatments for subsequent gene expression analysis.

Effects of Seminal Plasma on the Endometrial Transcriptome

Sequencing of endometrial samples of heifers recovered 24 h after mating to intact bulls revealed a total of 22 differentially expressed genes (DEGs) compared with contemporary unmated animals (**Table 1** and **Supplementary File 1**). Of those DEGs, 12 were up-regulated and 10 down-regulated [False discovery rate (FDR) < 0.05]. Some of the genes that exhibited the lowest expression (logFC < -2) were serine protease 2 (*PRSS2*), complement C9 (*C9*), oxytocin/neurophysin I prepropeptide (*OXT*), a novel gene encoding for carbonic anhydrase 1 (ENSBTAG00000036116) and an uncharacterized novel gene (ENSBTAG00000050072). On the other hand, the genes with greater transcript abundance (logFC > 2) levels were coiled-coil domain containing 196 (*CCDC196*), solute carrier family 24 member 2 (*SLC24A2*), UDP glucuronosyltransferase family 2-member A1 complex locus (*UGT2A1*) and interferon gamma inducible protein 47 (*IFI47*). In contrast, the endometrium of heifers exposed only to SP (by mating with a vasectomized bull) did not exhibit DEGs compared with the control group. Comparison of endometrial transcriptomes of intact and vasectomized groups revealed a total of 24 DEGs, 18 up-regulated and 6 down-regulated (**Table 2** and **Supplementary File 1**) (FDR < 0.05). Amongst these, MHC, class II, DQ beta (*BOLA-DQB*), GSG1 like (*GSG1L*), potassium voltage-gated channel subfamily E regulatory subunit 1 (*KCNE1*) and the novel gene previously mentioned (ENSBTAG00000050072) were those that displayed lower logFC values (logFC < −2). In contrast, higher levels of expression (logFC > 2) were exhibited by interleukin

17F (*IL17F*), complement component 4 binding protein alpha (*C4BPA*), the aforementioned *IFI47* and *UGT2A1*, and a novel gene (ENSBTAG00000052851) which has been predicted to code for a protein containing an Ig-like domain.

Three DEGs (*UGT2A1*, *IFI47* and the novel gene ENSBTAG00000050072) were found to be common of DEGs detected between the intact group and the control and those detected when comparing the intact and vasectomized groups.

Gene Ontology (GO) Terms of DEGs

For the annotated genes in each comparison, the GO terms are shown in **Figure 2**. For the molecular function category, the main represented GO term was "catalytic activity" in the intact group compared with control or vasectomized samples (**Figure 2A**). "Cellular process" and "metabolic process" were the most represented terms for the biological process category in intact samples compared with the control (**Figure 2B**). Compared with the vasectomized group in the same category, in addition to "cellular process," "response to stimulus" was the most represented term (**Figure 2B**). In regard to pathway category, comparing the intact group with the control, all the terms represented were related to vascular regulation (**Figure 2D**). In contrast, compared with the vasectomized bull treatment group, the represented terms referred to immunity modulation and amino acid biosynthesis (**Figure 2D**). "Hydrolase" and "receptor" were the most represented protein class terms among the differentially regulated genes in the endometrium exposed to sperm and SP compared with the control samples (**Figure 2C**). On the other hand, compared with vasectomized samples, the most represented protein class terms were "cytoskeletal protein" and "immunity protein" (**Figure 2C**).

Effects of Seminal Plasma on Gene Expression in the Vagina and Oviduct

Based on the results obtained from the endometrial RNA-sequencing, we were interested in studying whether gene expression changes are more dramatic at the site of semen deposition (the vagina), and whether these changes can propagate to more distal regions (the oviductal ampulla). Thus, six DEGs from the sequencing analysis were selected (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB,* and *CEBPD*) based on evidence of their reproductive function found in the literature (Wedel and Lömsziegler-Heitbrock, 1995; Blom et al., 2004; Hannan et al., 2004; Tapia et al., 2008; Hayashi et al., 2017; Neupane et al., 2017; Pinto De Melo et al., 2017; Tríbulo et al., 2018), as well as their GO terms. In addition to these, *IL6*, *IL1A*, *TNFA,* and *IL8* expression was also assessed, as these are genes that have been observed to be regulated by SP in several species (O'Leary et al., 2004; Sharkey et al., 2012b; Schjenken et al., 2015; Introini et al., 2017; Ibrahim et al., 2019).

Differences in relative expression of *CEBPD* ($P < 0.01$; **Figure 3F**) and *IL8* ($P < 0.05$ in the control group and $P < 0.01$ in the intact and vasectomized groups; **Figure 3J**) between regions were observed in all groups, being up-regulated in the vagina in comparison with the ampulla, whereas *CX3CL1* was down-regulated in the vagina compared with the ampulla, only in the control group ($P < 0.01$; **Figure 3B**). In addition, *TNFA* ($P < 0.05$ in the intact group and $P < 0.01$ in the vasectomized group; **Figure 3I**) and *IL1A* ($P < 0.05$; **Figure 3H**) were up-regulated in the vagina compared with the ampulla in heifers that had been mated to an intact or a vasectomized bull, but not in unmated heifers. The remaining genes did not exhibit region-specific changes (**Figures 3A**,**C–E**,**G**). Conversely, when relative abundance of these genes was compared between treatment groups, no differences were detected ($P > 0.05$). It is also important to note that for many genes, especially those related to inflammation, there was considerable variability between animals.

DISCUSSION

The main findings of this study are: (1) mating to an intact bull induces subtle changes in the endometrial transcriptome; however, (2) these transcriptomic changes are not observed in heifers mated to vasectomized bulls; (3) expression of *PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB*, *CEBPD*, *IL1A*, *IL6*, *TNFA,* and *IL8* in the vagina and ampulla did not differ between treatments; and (4) *TNFA* and *IL1A* exhibited regional differences between vagina and ampulla of heifers mated to intact or vasectomized bulls.

Seminal plasma is a complex fluid the composition of which is determined by the size, storage capacity, and secretory output of different organs of the male reproductive tract, which in the bull include: testes, epididymides, ampullae of the vasa deferentia, seminal vesicles, prostate and bulbourethral glands. After completion of spermatogenesis, sperm enter the epididymis bathed in fluid produced by the rete testis, which will be absorbed in its majority by the epididymal epithelium (Amann et al., 1974). However, secreted factors of epididymal and/or testicular origin are found in the ejaculate. This is evidenced by studies comparing ejaculates before and after vasectomy, which show lower concentration of amino acids in bulls (Alexander et al., 1971), and proteins in humans (Batruch et al., 2011), after the procedure. Despite this, vasectomy does not lead to a decrease in bull ejaculate volume (Alexander et al., 1971), indicating a more prominent role of the male accessory glands in the production of SP components. It is challenging to precisely calculate the contribution of each gland to the final fluid volume and composition (Seidel and Foote, 1970); however, vesiculectomy (excision of the seminal vesicles) in the bull leads to a more than 50% decrease in ejaculate volume, together with a reduction in total protein and ion concentrations (chloride, potassium, calcium, sodium), lower sperm motility, viability and morphology, and increased pH (Faulkner et al., 1968; Alexander et al., 1971).

Growing evidence exists for a role of SP in the modulation of cellular and molecular events in the maternal tract of several species during early pregnancy (Gangnuss et al., 2004; O'Leary et al., 2004, 2006; Bromfeld, 2016; Song et al., 2016; Glynn et al., 2017; Morgan and Watkins, 2020). However, most of the *in vivo* evidence comes from pigs and mice, species in which SP reaches the uterus (Gangnuss et al., 2004; O'Leary et al., 2004, 2006;

TABLE 1 | List of differentially expressed genes (FDR < 0.05) in endometrial samples of heifers mated with intact bulls compared with unmated heifers.

Ensembl acc. number	Gene name	Gene description	logFC
ENSBTAG00000014234	*CCDC196*	Coiled-coil domain containing 196	8.63
ENSBTAG00000043972	*SLC24A2*	Solute carrier family 24 member 2	4.15
ENSBTAG00000004040	*UGT2A1*	UDP glucuronosyltransferase family 2 member A1 complex locus	3.11
ENSBTAG00000003529	*ASAH2*	*N*-acylsphingosine amidohydrolase 2	2.82
ENSBTAG00000015727	*IFI47*	Interferon gamma inducible protein 47	2.62
ENSBTAG00000049426	*STARD2*	Phosphatidylcholine transfer protein	1.72
ENSBTAG00000037929	*ADAM28*	ADAM-like, decysin 1	1.42
ENSBTAG00000019636	*SCARA5*	Scavenger receptor class A member 5	1.06
ENSBTAG00000017722	*F5*	Coagulation factor V	0.99
ENSBTAG00000019625	*EHHADH*	Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase	0.83
ENSBTAG00000008735	*VASH1*	Vasoinhibin 1	0.77
ENSBTAG00000001728	*IGSF10*	Immunoglobulin superfamily member 10	0.72
ENSBTAG00000046307	*CEBPD*	CCAAT enhancer binding protein delta	−0.86
ENSBTAG00000011079	*C18H19orf48*	Chromosome 18 C19orf48 homolog	−0.92
ENSBTAG00000007101	*F3*	Coagulation factor III, tissue factor	−1.04
ENSBTAG00000051812	*CA1L*	Carbonic anhydrase 1-like	−2.45
ENSBTAG00000008026	*OXT*	Oxytocin/neurophysin I prepropeptide	−2.76
ENSBTAG00000039446	*PI3L*	Elafin-like	−2.78
ENSBTAG00000036116	*CA1*	Carbonic anhydrase 1	−3.39
ENSBTAG00000016149	*C9*	Complement C9	−4.08
ENSBTAG00000050072		Novel gene	−4.99
ENSBTAG00000021565	*PRSS2*	Serine protease 2	−6.05

FDR, false discovery rate; logFC, logarithm of fold change.

TABLE 2 | List of differentially expressed genes (FDR < 0.05) in endometrial samples of heifers mated with intact bulls compared with heifers mated with vasectomized bulls.

Ensembl acc. number	Gene name	Gene description	logFC
ENSBTAG00000052851		Novel gene	4.57
ENSBTAG00000016835	*IL17F*	Interleukin 17F	4.50
ENSBTAG00000032884	*TNP2*	Transition protein 2	3.78
ENSBTAG00000019132	*DMP1*	Dentin matrix acidic phosphoprotein 1	3.34
ENSBTAG00000015727	*IFI47*	Interferon gamma inducible protein 47	3.18
ENSBTAG00000009876	*C4BPA*	Complement component 4 binding protein alpha	2.78
ENSBTAG00000004040	*UGT2A1*	UDP glucuronosyltransferase family 2 member A1 complex locus	2.47
ENSBTAG00000037539		Vascular cell adhesion molecule 1-like	1.81
ENSBTAG00000021764	*GLRB*	Glycine receptor beta	1.62
ENSBTAG00000002214	*TAT*	Tyrosine aminotransferase	1.38
ENSBTAG00000026779	*LYZ*	Lysozyme	1.32
ENSBTAG00000000601	*COL11A2*	Collagen type XI alpha 2 chain	1.14
ENSBTAG00000019588	*BLA-DQB*	MHC class II antigen	1.11
ENSBTAG00000034338	*C15H11orf88*	Chromosome 15 C11orf88 homolog	1.10
ENSBTAG00000021526	*RPRM*	Reprimo, TP53 dependent G2 arrest mediator homolog	1.05
ENSBTAG00000033429	*FAM229B*	Family with sequence similarity 229 member B	0.94
ENSBTAG00000024869	*CX3CL1*	C-X3-C motif chemokine ligand 1	0.78
ENSBTAG00000021522	*PLA2G10*	Group 10 secretory phospholipase A2	0.72
ENSBTAG00000012703	*GLO1*	Glyoxalase I	−0.49
ENSBTAG00000008147	*MICAL1*	Microtubule associated monooxygenase, calponin and LIM domain containing 1	−0.50
ENSBTAG00000001150	*KCNE1*	Potassium voltage-gated channel subfamily E regulatory subunit 1	−2.51
ENSBTAG00000004607	*GSG1L*	GSG1 like	−4.61
ENSBTAG00000050072		Novel gene	−4.94
ENSBTAG00000021077	*BOLA-DQB*	Major histocompatibility complex, class II, DQ beta	−5.05

FDR, false discovery rate; logFC: logarithm of fold change.

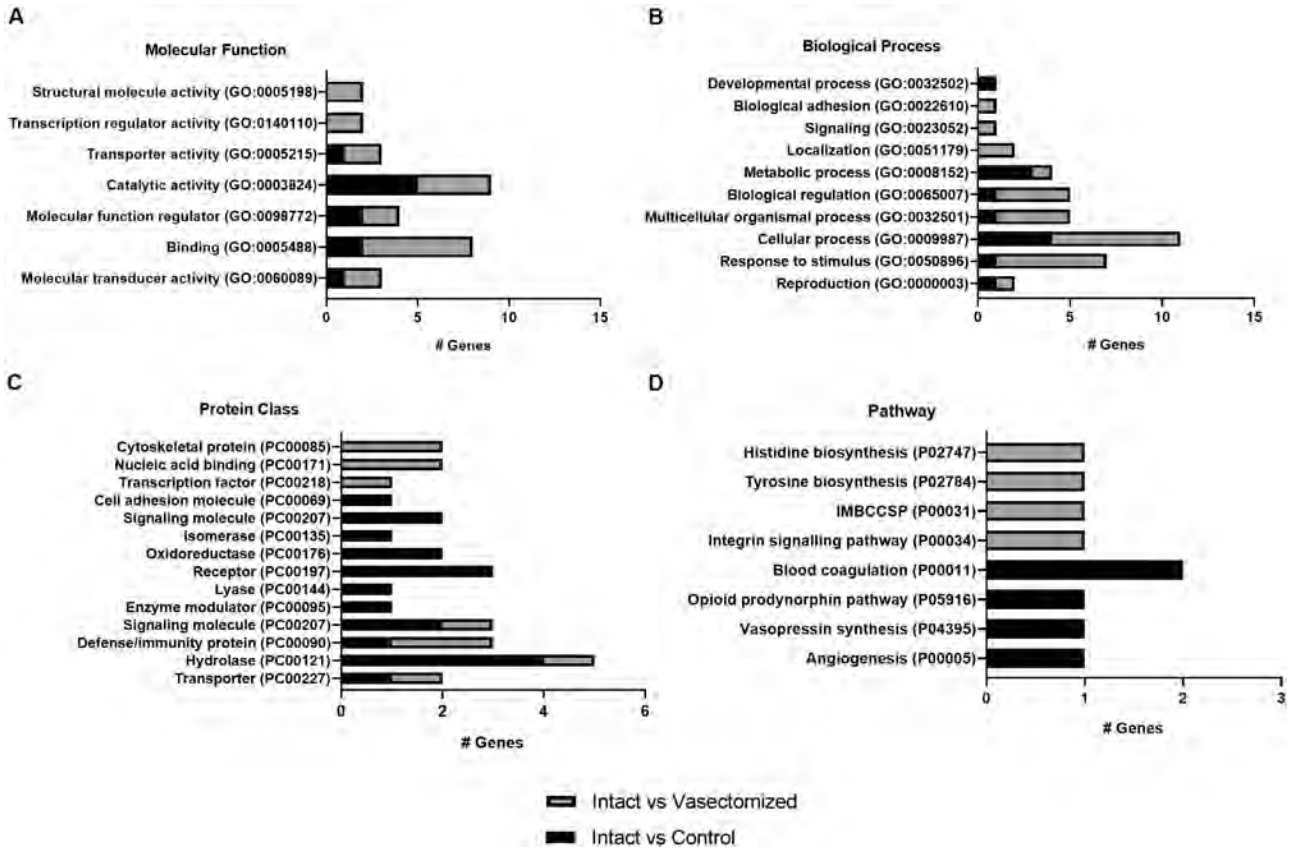

FIGURE 2 | (A–D) Representation of GO terms for annotated DEGs in each comparison. The main categories: **(A)** molecular function, **(B)** biological process, **(C)** protein class, and **(D)** pathway were represented. IMBCCSP, inflammation mediated by chemokine and cytokine signaling pathway.

Bromfield et al., 2014; Song et al., 2016; Glynn et al., 2017). Because of the relatively low volume of ejaculate in men (an average of 3.7 mL, Cooper et al., 2009) and bulls (around 5 mL), and the fact that the ejaculate is deposited in the vagina, it is questionable whether any SP reaches the uterus. Therefore, it is not clear whether this fluid has a critical role in the modulation of the uterine environment in these species. At the time of ejaculation, however, sperm come into contact with SP, leading to proteins binding tightly to the sperm plasma membrane (Pini et al., 2016). For example, seminal vesicle-derived Binder of Sperm Proteins (BSP) −1, −3, and −5 (previously called PDC-109 or BSP-A1/A2, BSP-A3 and BSP-30 kDa respectively), which make up approximately 50% of total protein in SP (Nauc and Manjunath, 2000), bind to sperm and play important roles during capacitation (Manjunath and Thérien, 2002) and formation of the sperm oviductal reservoir (Gwathmey et al., 2003, 2006). Thus, it is possible that in intravaginal ejaculators, sperm can act as a vehicle of SP proteins that interact with the reproductive epithelium to induce changes in the maternal environment. Indeed, bull sperm has been shown to interact with endometrial cells and induce a proinflammatory response *in vitro* (Elweza et al., 2018; Ezz et al., 2019).

To address this lack of basic knowledge, this study aimed to determine the effects of bovine SP and sperm exposure during natural mating on the endometrial transcriptome. Additionally, gene expression changes were assessed at the site of semen deposition (vagina) and the distal region where gamete interaction takes place (the oviductal ampulla) to determine whether SP-induced changes can propagate throughout the female reproductive tract.

In the present study, heifers were mated between 0 and 6 h after seen in standing estrus. Average time from estrus onset to ovulation is 27 h (Walker et al., 1996; Valenza et al., 2012; Randi et al., 2018). Thus, some animals had ovulated by the time of sample collection (24 ± 6 h after mating) whereas the rest exhibited a pre-ovulatory follicle. Ovulation and estrous cycle are orchestrated by an accurate hormonal regulation, and under this regulation, the endometrium experiments functional and morphological changes (Arai et al., 2013). In order to avoid any possible confounding factors due to ovulation having occurred or not, the number of ovulated and non-ovulated heifers that were analyzed by RNA-sequencing and RT-qPCR was balanced between treatments.

Strikingly, when heifers were exposed to SP in the absence of sperm and testicular and epididymal secretions (i.e., mated to a vasectomized bull) the endometrial RNA-sequencing analysis did not reveal any DEGs in comparison with samples from unmated animals. Conversely, the endometrial transcriptome of heifers

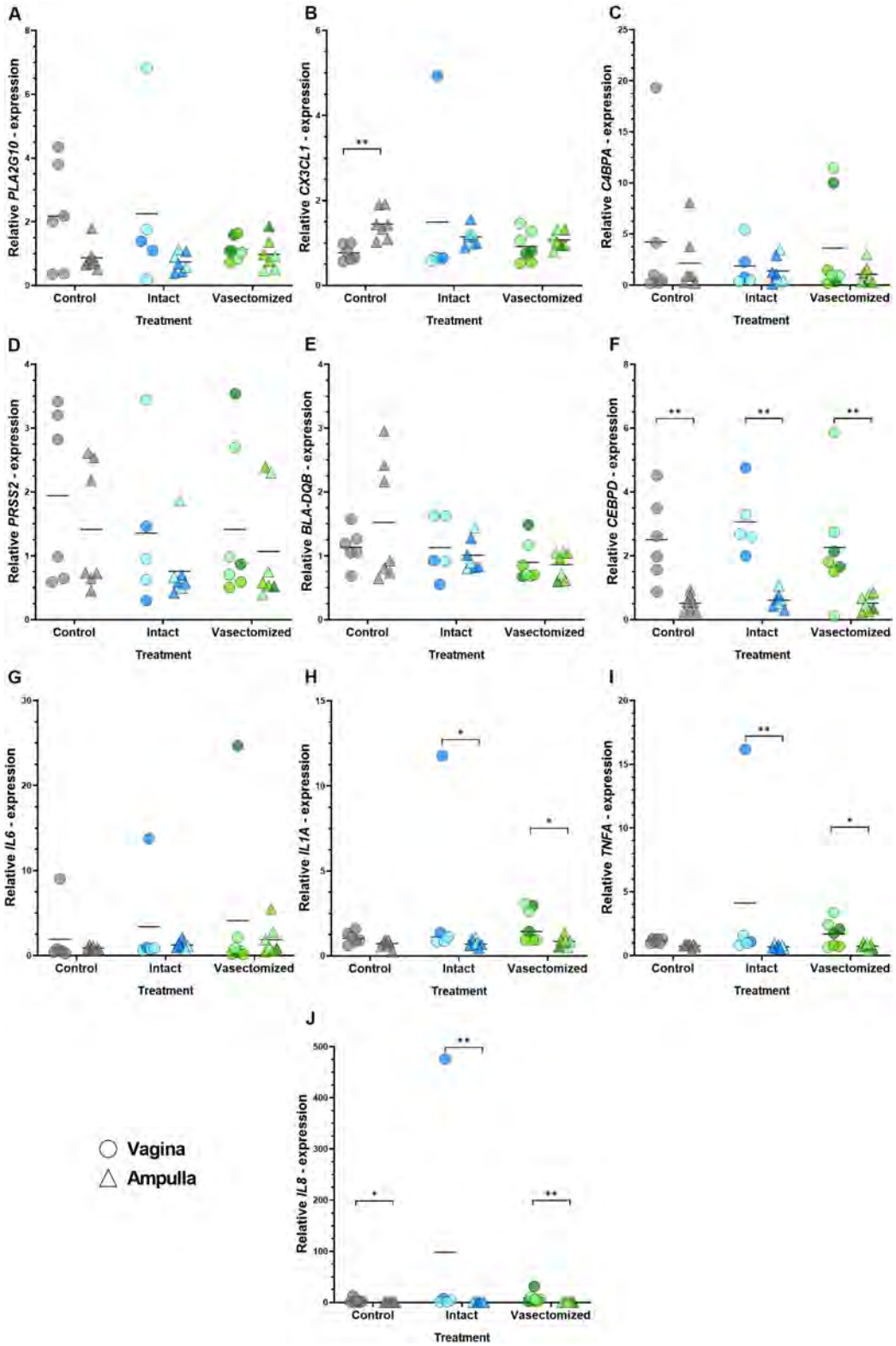

FIGURE 3 | (A–J) Relative expression values of all the genes assessed: **(A)** *PLA2G10*, **(B)** *CX3CL1*, **(C)** *C4BPA*, **(D)** *PRSS2,* **(E)** *BLA-DQB*, **(F)** *CEBPD*, **(G)** *IL6*, **(H)** *IL1A*, **(I)** *TNFA*, **(J)** *IL8* in the vagina (circles) and oviductal ampulla (triangle) in the different experimental groups (control, intact or vasectomized). Each circle/triangle refers to an individual heifer. Different color shades within groups correspond to the bull that was mated to that particular heifer, blue for intact group (n = 2 bulls) and green for vasectomized group (n = 3). Bars represent the mean of relative expression values and asterisks indicate significant (*$P < 0.05$, **$P < 0.01$) differences.

mated to intact bulls differed from the control and vasectomized groups, exhibiting differential regulation of a small number of genes that may play a role in bovine fertility. Although, these results could be due to SP reaching the uterus in both treatments, but only testicular of epididymal factors inducing a response; it is more likely that, in cattle, SP does not reach the uterus in the 24 h following natural mating, at least in the absence of a vehicle, such as sperm.

When compared with control endometrial samples, endometrium obtained from heifers mated to an intact bull exhibited up-regulation of 12 genes and down-regulation of 10 genes. Amongst these genes regulated by mating, some have been shown to participate in tissue remodeling, an important step preparing endometrium to embryo implantation. The gene coding for scavenger receptor class A, member 5 (*SCARA5)*, which participates in innate immunity (Jiang et al., 2006) was up-regulated. This gene has also been observed to be up-regulated in the endometrium of cows at day 20 of pregnancy (Mansouri-Attia et al., 2009), and has been proposed to play a role in the regulation of histotroph secretion and tissue remodeling, two critical processes for embryo implantation (Vitorino Carvalho et al., 2019). Additionally, serine protease 2 (*PRSS2*), which also participates in tissue remodeling by type 1 collagen degradation, was down-regulated by exposure to SP and sperm at mating. Interestingly, this gene is up-regulated in the endometrium of repeat breeder cows, those that are cycling normally and without clinical abnormalities but that fail to conceive after at least two successive inseminations (Hayashi et al., 2017). Mating to an intact bull also affected genes involved in cell proliferation, such as CCAAT enhancer binding protein delta (*CEBPD*), which was found to be down-regulated in the endometrium of heifers mated with intact bulls. This gene belongs to the C/EBP leucine-zipper transcription factor family involved in fat and hematopoietic progenitor cells differentiation (Wedel and Lömsziegler-Heitbrock, 1995). Another member of this family, CEBPB, has been identified as a regulator of proliferative events during decidualization in mice (Mantena et al., 2006). In regard to modulation of innate immunity, the gene coding for component 9 of complement system (*C9*) was down-regulated in the endometrium of heifers mated with intact bulls. The C9 component participates in the final steps of the complement cascade, in the formation of membrane attack complex (MAC), which mediates the formation of channels in the target cell membrane, leading to cell lysis and death (Janeway et al., 2001). Despite its importance, the function of the complement system in the context of reproduction is not well known. For instance, complement regulatory proteins have been found in bull sperm surface, such as CD59 (Byrne et al., 2012), which prevents the formation of MAC (Janeway et al., 2001). These complement regulatory proteins were also identified in human and mouse sperm and they have been proposed to play a role protecting sperm in the female tract (Harris et al., 2006). Moreover, mating with an intact bull resulted in a down-regulation of endometrial oxytocin (*OXT*). In cattle, high levels of OXT have been reported to impair embryo survival by promoting uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which induces luteolysis and consequently a drop in progesterone (Lemaster et al., 1999). Although endometrial OXT production has been observed also in mares (Bae and Watson, 2003), it is not known the locally effect of the OXT secreted by the endometrium during early pregnancy in cattle.

When comparing endometrial samples from heifers mated to intact bulls to those from heifers mated to vasectomized bulls, 18 genes were found to be up-regulated and four down-regulated. Amongst the up-regulated genes, literature on *CX3CL1*, *VCAM1-like*, *C4BPA*, *PLA2G10*, *IFI47*, *IL17F* and *BLA-DQB* suggest different roles of these genes in early pregnancy in different species. The chemokine CX3CL1 (C-X3-C motif chemokine ligand 1) has been identified as a potential bovine embryokine (Tríbulo et al., 2018). In addition, CX3CL1 induces recruitment of leukocytes during early pregnancy (Hannan et al., 2004), and also promotes trophoblast migration in women (Hannan et al., 2006). With importance for implantation, VCAM1 has been shown to be involved in the adhesion of the bovine conceptus to the endometrium (Bai et al., 2014). Further, in women with unexplained infertility the endometrial expression of *VCAM1* at the peri-implantation stage was significantly lower than control women (Konac et al., 2009). Additionally, the mRNA levels of complement component 4-binding protein alpha (*C4BPA*), a key inhibitor of the complement system (Blom et al., 2004), were increased during the implantation window in women (Tapia et al., 2011), but decreased in women which suffered repeated implantation failure and unexplained recurrent spontaneous abortion (Lee et al., 2007; Tapia et al., 2008). Although cattle and human implantation differs significantly, in both the time at which it takes place (around day 9 in humans and starting at day 21 in cattle) and the structure of the placenta (hemochorial in humans and epitheliochorial in bovine), it is likely that the up-regulation of *C4BPA* and *VCAM1* induced by exposure to SP and sperm during mating regulates peri-implantation events in cattle. On the other hand, group 10 secretory phospholipase A2 (*PLA2G10*), which was up-regulated in intact group samples, belongs to phospholipase A2 enzyme family, which is known to participate in inflammatory processes and to catalyze the release of arachidonic acid from phospholipids, needed to prostaglandin production (reviewed in Capper and Marshall, 2001). In addition, this gene has been associated with fertility in beef cattle (Neupane et al., 2017), and it was found highly down-regulated in the uterus of cows with negative energy balance (Wathes et al., 2009) which typically have lower pregnancy rates.

From an immunological point of view, the expression of interferon gamma inducible protein 47 (*IFI47*) was also found to be up-regulated in the endometrium of heifers mated with intact bulls in comparison with those mated to vasectomized bulls. Although its function in the uterus has not been defined, *IFI47* mRNA was more abundant in the endometrium of high fertility heifers compared to heifers classified as infertile (Minten et al., 2013). In addition, *IFI47* was up-regulated in the endometrium of heifers 13 days after embryo transfer (Spencer et al., 2013), probably under stimulation of interferon-tau secreted by the conceptus. Another immune-related gene up-regulated by mating with intact bulls is interleukin 17 F (*IL17F*), a cytokine produced by T helper 17 lymphocytes (reviewed in Iwakura et al., 2011). A similar response in expression of *IL17A*

was observed by Ibrahim et al. (2019). Both IL17A and IL17F share biological functions; indeed, both are highly homologous, can bind to the same receptor and moreover, can be secreted as heterodimers or homodimers (reviewed in Iwakura et al., 2011). Ibrahim et al. (2019) observed increased levels of *IL17A* after exposing endometrial cell cultures to SP and sperm or sperm alone, but not SP in the absence of sperm. Also, up-regulation of *IL17A* took place in *vivo*, after uterine infusion of semen (a combination of SP and sperm) but not SP alone (Ibrahim et al., 2019). Together with the data obtained in the present study, this suggests that regulation of *IL17* expression in the bovine endometrium is mediated by sperm action, not SP. This is interesting to note as, in mice, mating to a vasectomized male induces a similar increase in endometrial *IL17A* expression than mating with an intact male (Song et al., 2016). This gives weight to our hypothesis that in species that ejaculate intravaginally, sperm play a more important role than SP in regulation of the female reproductive environment. Moreover, the endometrial up-regulation of *IL17A* in bovine explants has been shown to be exclusively regulated by elongated Day 15 conceptuses, but not by interferon-tau, which is the main signal of pregnancy recognition in cattle, suggesting a role in the embryo – endometrium crosstalk during early pregnancy in cattle (Sánchez et al., 2019). During pregnancy, the maternal immune system must tolerate the presence of an embryo that expresses paternal antigens. The increase in endometrial *IL17* expression observed after mating in the aforementioned studies is reflective of an increase in the population of T helper 17 lymphocytes (Song et al., 2016), which probably participate in the establishment of this maternal tolerance toward paternal antigens. Another mechanism of maternal tolerance driven by male factors seems to be the regulation of the MHC. In the present study, mating to an intact bull elicited down-regulation of Bovine Lymphocyte antigen (referred to as MHC in other species) class II, DQ beta (*BOLA-DQB*), consistent with its down-regulation in pregnant heifers after natural breeding (Dickinson et al., 2018). Conversely, another MHC class II member, *BLA-DQB* was up-regulated in endometrial samples of heifers mated with intact bulls. Indeed, a list of genes of MHC class II family members, including *BLA-DQB* and *BOLA-DQB*, have been associated with reproductive performance in cattle (Pinto De Melo et al., 2017).

The lack of a response to mating with a vasectomized bull, together with the discovery of genes that were regulated by mating to intact bulls, led to the analysis of tissues that have direct contact with seminal fluid (i.e., the vagina). The ejaculation site during natural conception is an important factor to take into consideration since a recent study has shown that SP infusion into the vagina, but not into the uterus, could influence the levels of endometrial epidermal growth factor (Badrakh et al., 2020), which has been associated with fertility restoration in repeat breeder cows (Katagiri and Takahashi, 2006). In this sense, we were also interested in studying how far into the reproductive tract these changes could be observed, so gene expression analysis in the oviductal ampulla was also undertaken. Based on the sequencing results, genes identified as possible key regulators of uterine environment and pregnancy success (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB,* and *CEBPD*) were selected. In addition, the expression of inflammatory mediators regulated by SP components in other species (*IL1A*, *IL6*, *TNFA,* and *IL8*; O'Leary et al., 2004; Sharkey et al., 2012b; Schjenken et al., 2015; Introini et al., 2017; Ibrahim et al., 2019) was also analyzed. However, no difference between treatments was observed in the expression of any gene in the vagina or the oviduct. These results are not consistent with data in other species: in the mouse, *Il6* was reduced in the oviduct of females mated to males that had undergone a vesiculectomy with or without a vasectomy (Bromfield et al., 2014); while in the pig, natural mating induces up-regulation of *CEBPD* in the ampulla (Alvarez-Rodriguez et al., 2019); finally, in human cervix, an increase in *IL1A*, *IL6* and *IL8* is observed after coitus (Sharkey et al., 2012b). It is important to highlight, however, that the heifers in the present study had been estrous synchronized (both mated and control animals) with an intravaginal device that was removed 48 h prior to sample collection. Although all the animals were managed under the same conditions, the resulting manipulation could have had an impact on the inflammatory status of the vagina. Indeed, a higher dispersion within group is observed in the expression of inflammatory genes in the vagina, than in the ampulla region. This is especially noticeable in the intact group, were one heifer exhibits very high expression of *CX3CL1, IL6, IL1A, TNFA*, and *IL8*. Despite the lack of treatment effect, some genes were shown to be differentially expressed between tissues. A higher expression of *CX3CL1* was found in the ampulla compared with the vagina in control samples. In women, CXCL3 is present throughout the oviduct and, interestingly, its receptor was found in ejaculated sperm (Zhang et al., 2004). Conversely, *CEBPD* and *IL8* were found up-regulated in the vagina samples compared with ampulla in all treatments. On the one hand, the expression of *CEBPD* is a crucial factor during inflammatory acute-phase response, under regulation of a range of cytokines and other inflammatory agents (reviewed in Ramji and Foka, 2002) and, on the other hand, IL8 is well known to be a potent neutrophil chemoattractant (Leonard and Yoshimura, 1990). Therefore, a higher basal expression of both genes in the vagina compared with the ampulla might be expected since this tissue has contact with the outside and, thus, is more prone to environmental/external contaminants.

Interestingly, *IL1A* and *TNFA* were more highly expressed in vagina than in the ampulla tissue of heifers mated either to intact or vasectomized bulls, while control heifers did not exhibit this region-specific difference, suggesting a modulatory role induced by mating. In human exposure of ectocervical explants to SP resulted in the increase of *IL1A* and *TNFA* expression levels (Introini et al., 2017). Further, unprotected vaginal coitus, but not condom protected, induced the expression of *IL1A* in women (Sharkey et al., 2012b). However, in our study, the mechanical stimulus of mating cannot be ruled out.

CONCLUSION

The lack of changes in the endometrial transcriptome and in the expression of selected genes in the vagina and oviduct after mating to a vasectomized male do not support a role of SP (in

the absence of sperm nor testicular and epididymal secretions) in regulating early pregnancy and uterine environment in cattle. Rather, the subtle changes in the transcriptome of the endometrium and the vagina seem to be elicited by sperm. These data indicate that, in species that ejaculate intravaginally, sperm play a more critical role in the modulation of the female environment. This is most apparent when looking at regulation of *IL17*, which is driven by SP in mice (Song et al., 2016) and by sperm in cattle. However, further research is needed to elucidate the role of inherent sperm proteins or SP proteins that attach to sperm at ejaculation.

DATA AVAILABILITY STATEMENT

Gene expression data are publicly available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.s7h44j14r).

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Ethics Committee of University College Dublin, Ireland and licensed by the Health Products Regulatory Authority (HPRA), Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes).

AUTHOR CONTRIBUTIONS

SR carried out the laboratory work, analyzed the results, and wrote the draft. YM-O contributed to the laboratory work and the analysis of results. BF-F, JS, SB-A, MM, and DK carried out the animal work, including handling of bulls and heifers, estrus detection, mating and sample collection. SB and TS performed the RNA library preparation and sequencing analysis. MY performed the statistical analysis of the data. BF-F created the illustrations for **Figure 1**. PL, JS, MY, and BF-F contributed to the critical revision of the manuscript. BF-F, PL, and JS designed the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00547/full#supplementary-material

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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.

Supplementary Material

Supplementary Data

Supplementary Table 1: Relation of heifers and bulls used in the study. The time passed since mating (in the case of intact or vasectomized treatments) or standing estrus (in the case of control animals) is indicated, together with the ovarian structures observed at recovery time.

Heifer	Treatment	Bull	Time from mating/estrus to slaughter	Ovarian structures	Used in RNA-sequencing analysis
Ctr_1	Control	N/A	24 h	Fresh ovulation	✓
Ctr_2	Control	N/A	24 h	Fresh ovulation	✓
Ctr_3	Control	N/A	22 h	Preovulatory follicle	✓
Ctr_4	Control	N/A	23 h	Preovulatory follicle	✓
Ctr_5	Control	N/A	29 h	Preovulatory follicle	✓
Ctr_6	Control	N/A	30 h	Fresh ovulation	✓
Ctr_7	Control	N/A	24 h	Preovulatory follicle	-
Intact_1	Intact	I1	30 h	Fresh ovulation	✓
Intact_2	Intact	I2	24 h	Fresh ovulation	✓
Intact_3	Intact	I1	28 h	Fresh ovulation	✓
Intact_4	Intact	I2	29 h	Fresh ovulation	✓

Intact_5	Intact	I1	29 h	Preovulatory follicle	✓
Intact_6	Intact	I2	28 h	Preovulatory follicle	✓
Intact_7	Intact	I2	24 h	Preovulatory follicle	-
Vasec_1	Vasect	V1	30 h	Fresh ovulation	✓
Vasec_2	Vasect	V2	21 h	Preovulatory follicle	✓
Vasec_3	Vasect	V3	29 h	Fresh ovulation	✓
Vasec_4	Vasect	V2	24 h	Preovulatory follicle	✓
Vasec_5	Vasect	V2	23 h	Fresh ovulation	✓
Vasec_6	Vasect	V1	23 h	Fresh ovulation	✓
Vasec_7	Vasect	V1	23 h	Fresh ovulation	-
Vasec_8	Vasect	V3	24 h	Fresh ovulation	-

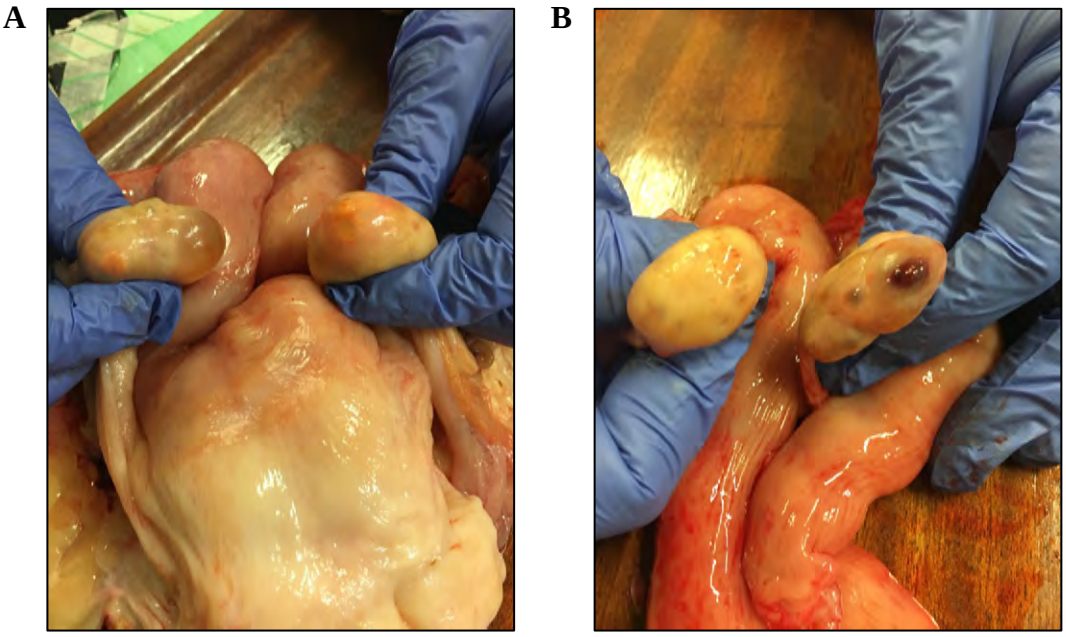
Supplementary Table 2: Gene symbol, forward and reverse primer sequences, amplicon size and accession number for each gene assessed by RT-qPCR.

Gene Symbol	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)	Size (bp)	Accession Number (Bos taurus)
GAPDH	TTCTACTGGCGCTGCCAAGG	GATCCACAACAGACACGTTGGG	107	NM_001034034.2

ACTB	CAGCAGATGTGGATCAGCAAGC	AACGCAGCTAACAGTCCGCC	91	NM_173979.3
RPL19	GAAAGGCAGGCATATGGGTA	TCATCCTCCTCATCCAGGTT	86	NM_001040516.1
PPIA	CATACAGGTCCTGGCATCTTGTCC	CACGTGCTTGCCATCCAACC	108	NM_178320.2
YWHAZ	TGAAGCCATTGCTGAACTTG	TCTCCTTGGGTATCCGATGT	114	NM_174814.2
RNF11	TCCGGGAGTGTGTGATCTGTATGAT	GCAGGAGGGGCACGTGAAGG	131	NM_001077953.1
H3F3A	CATGGCTCGTACAAAGCAGA	ACCAGGCCTGTAACGATGAG	136	NM_001014389.2
SDHA	ACTTCACCGTTGATGGCAATAA	CGCAGAAATCGCATCTGAAA	59	NM_174178.2
PLA2G10	GTGTCAAGTGTGAACCAACGG	GTTGTACTCTGCTCGGGCTA	95	XM_003587818.5
CX3CL1	AATGACCCAGAAGATTCCCGAG	TTCAGGCTACATGACAGCTCC	88	XM_015475803.2
C4BPA	TGGGCAAGTGATAGTTAAGACAGAT	AATGGTTGGAGGAGGTTCACAC	181	NM_174252.3
PRSS2	ATCCGCCACCCCAAGTACAG	GGGTAGTTGACGCCACTGCT	185	NM_174690.1
BLA-DQB	AGTACGTGCGGTTCGACAG	TAGGTTCCACTCGCCGCT	186	NM_001034668.3
CEBPD	TTCAGCGCCTACATCGACTC	GTTGAAGAGGTCGGCGAAGA	81	NM_174267.2
IL6	GCGCATGGTCGACAAAATCT	AAATCGCCTGATTGAACCCAGA	158	NM_173923.2
IL1A	GGTCCATACCTGACGGCTACTA	CAGGCATCTCCTTTAGCAAGACG	178	NM_174092.1

TNFA	TGGTTCAAACACTCAGGTCCTCT	TACGAGTCCCACCACCGGA	106	NM_173966.3
IL8	TGAGGACATGTGGAAGCACTTTA	TCCCATTTCTCCAAATTCATGCAC	73	NM_173925.2

Supplementary Figure 1: Representative pictures of the ovaries of heifers slaughtered 24 h (±6 h) after mating. **A)** Left ovary containing a pre-ovulatory follicle; regressing corpus luteum on the right ovary. **B)** Right ovary containing a freshly ovulated follicle.



Discussion

Semen is composed by a cell fraction (sperm) and a fluid portion (seminal plasma) that are mixed cells upon ejaculation (Rodríguez-Martínez et al., 2011). When they leave the testis, sperm are fully differentiated cells that present a head, composed of the acrosome and the nucleus, and a tail, containing the mitochondrial sheath and the molecular architecture necessary for sperm motility (Briz & Fàbrega, 2013). In addition, upon completing spermatogenesis, sperm become transcriptionally silent cells, so that their functions rely upon the existing borne proteins with their post-translational modifications (Maciel et al., 2019); this sperm protein content is modified throughout the epididymal transit. Besides this, the different fluids that sperm encounter during their passage throughout the male and female reproductive tracts (i.e., epididymal fluid, seminal plasma, and uterine and oviductal fluids) are also crucial for the modulation of their function and physiology (Gadella, 2017). Therefore, the main objective of this Dissertation was to investigate the role of sperm and seminal plasma proteins regulating the events that take place after ejaculation and during the sperm transit along the female tract.

Once they exit the testis, sperm undergo two different maturational processes. On the one hand, during the transit through the different regions of the epididymis, the sperm surface is remodelled through the removal and adsorption of sperm-coating proteins (Dacheux et al., 2005). Furthermore, upon ejaculation, sperm are unable to fertilize the oocyte and acquire that ability during the passage along the female tract in a process known as capacitation (Austin, 1951, 1952; Chang, 1951). After fast transport through the body and the uterine horns, sperm reach the utero-tubal junction and the lower region of the oviduct, also known as isthmus. Before ovulation, sperm bind to oviductal epithelial cells, forming a reservoir (Rodriguez-Martinez, 2007). Around the time of ovulation, sperm are released and exposed to oviductal fluid, which induces a variety of biochemical changes that ultimately give them the capacity to interact with and pass through oocyte vestments, and fuse with the oocyte membrane (Rodriguez-Martinez et al., 2001); this process is known as capacitation. These changes include alterations in the architecture of plasma (Harrison et al., 1996; van Gestel et al., 2005) and acrosome membranes (Tsai et al., 2007), changes in mitochondrial activity (Ramió-Lluch et al., 2011), an increase in intracellular pH

and calcium levels (Visconti et al., 2011; Yeste et al., 2015), generation of reactive oxygen species (ROS) (Betarelli et al., 2018), and phosphorylation of certain proteins (Flesch et al., 1999; Tardif et al., 2001). Only capacitated sperm can trigger the acrosome reaction, which involves the release of the acrosome content, allowing sperm cells to penetrate the zona pellucida and fertilise the oocyte (Flesch & Gadella, 2000). In this context, *in vitro* fertilisation relies upon the induction of sperm capacitation, which can be accomplished by incubating sperm in a defined medium mimicking the oviductal environment (Salicioni et al., 2007; Visconti, 2009).

Although in mice it is well established that calcium-activated potassium channels play a pivotal role in the accomplishment of sperm capacitation (De La Vega-Beltran et al., 2012; Santi et al., 2010), their role during this process remains to be elucidated in other species, such as the pig. Therefore, the first study of this Dissertation sought to determine the role of calcium-activated potassium channels during *in vitro* capacitation and acrosome reaction in pig sperm. To this end, two different inhibitors were used, quinine and paxilline. Quinine is a general inhibitor of potassium channels, including voltage-gated potassium channels, calcium-activated potassium channels, and tandem pore domain potassium channels (Grinstein & Foskett, 1990; Kuriyama et al., 1995; Mancilla & Rojas, 1990). On the other hand, paxilline (PAX) is a specific inhibitor of calcium-activated potassium channels (Sanchez & McManus, 1996).

Incubation of sperm in capacitation medium entails an increase in membrane lipid disorder (Harrison et al., 1996; van Gestel et al., 2005), as well as a decrease in membrane integrity and, therefore, sperm viability (Harrison, 1996; Lechniak et al., 2002). Despite all samples exhibiting this trend, incubation with the highest concentration of quinine (1 mM) resulted in higher plasma membrane integrity compared to the other treatments, whereas incubation with 1 mM PAX led to the lowest percentages of sperm with an intact plasma membrane. These data suggest that quinine treatment prevent sperm from eliciting *in vitro* capacitation. Contrary to what would have been expected, however, the highest percentages of viable sperm with high membrane lipid disorder were observed after incubation with 1 mM quinine, and the lowest after incubation with 1 mM PAX. Similarly, the inhibition of SLO1 channels, which are

also calcium-activated channels, prevents pig sperm from eliciting capacitation without altering the lipid architecture of plasma membrane (Yeste et al., 2019). Because, herein, quinine exerted this effect on the lipid architecture of the membrane, which was not observed after the specific inhibition of calcium-activated channels by PAX, it is reasonable to posit that potassium channels other than the calcium-activated ones are involved in the membrane lipid disorder increase. It is also worth mentioning that, in pig sperm, exposure to quinine prevents regulation of cell volume (Petrunkina et al., 2001). Shrinking or swelling impacts directly on structural elements of the sperm cell, such as cytoskeleton and plasma membrane, significantly affecting cell function (Lang et al., 1998). In this sense, a failure in the regulation of sperm volume, due to potassium channel inhibition through quinine, may induce a disturbance in plasma membrane, affecting its architecture and increasing its lipid disorder. Further research is, however, needed to understand the precise relationship between potassium channels and membrane lipid architecture.

Hyperactivation of sperm motility is one of the best characterised events during capacitation (Suarez & Ho, 2003). In the first study, after the induction of acrosome exocytosis, an increase in both total and progressive motility was observed, with the exception of samples incubated with quinine and 1 mM PAX. The highest concentration of quinine induced a drastic decrease in total and progressive motility from the beginning of the experiment. Inhibition of potassium channels by quinine has been reported to induce swelling in pig and human sperm, as a result of their inability to regulate cell volume (Petrunkina et al., 2001; Yeung & Cooper, 2001). It is known that sperm swelling induce alterations in kinematic parameters, leading to failure in migration and penetration of cervical mucus (Yeung & Cooper, 2001). It is important to highlight that samples treated with 0.1 mM quinine and 1 mM PAX also exhibited a decrease in total and progressive motility, although not to the same extent as the highest concentration of quinine did. Taken collectively, these data strongly suggest that modulation of sperm motility during capacitation involves voltage-gated and tandem pore domain potassium channels, in addition to the calcium-activated potassium ones.

Mitochondrial activity is essential to maintain appropriate energy levels for sperm motility (Agnihotri et al., 2016; Mukai & Okuno, 2004; Piomboni et al.,

2012). Mitochondrial membrane potential (MMP) directly reflects the generation of chemical energy in terms of the ATP needed to sustain sperm motility, especially in capacitated sperm (Agnihotri et al., 2016). It is well established that mitochondrial activity increases progressively during *in vitro* sperm capacitation and acrosome exocytosis, reaching a peak once the latter is achieved (Paventi et al., 2015; Ramió-Lluch et al., 2011). In the first study of this Dissertation, control samples were observed to increase their MMP during *in vitro* capacitation, also reaching a maximum level immediately after the induction of the acrosome reaction. In contrast, samples incubated with quinine or PAX showed lower MMP, the higher the concentration of these inhibitors the lower the percentages of sperm with high MMP and the $JC1_{agg}/JC1_{mon}$ ratios. To the best of our knowledge, there are no studies regarding the contribution of calcium-activated potassium channels to the modulation of mitochondrial activity during pig sperm capacitation. Considering, however, that PAX did not induce an effect as considerable as quinine did, it is reasonable to surmise that not only calcium-activated channels, but also the voltage-gated and tandem pore domain potassium ones play an instrumental function in the regulation of mitochondrial activity during *in vitro* capacitation of pig sperm. In regard to this, it is worth mentioning that quinine has been reported to inhibit mitochondrial ATP-regulated potassium channels in bovine myocardium cells (Bednarczyk et al., 2004), which might also contribute to explain the different response between quinine and PAX blockage.

Modulation of intracellular calcium levels is crucial for mammalian sperm function, including capacitation, acrosome exocytosis, motility hyperactivation and chemotaxis (Correia et al., 2015; Publicover et al., 2007). In mammalian sperm, intracellular calcium is stored in two different deposits. Whilst the head reservoir has been proposed to be related to acrosome exocytosis, calcium stored in sperm neck and midpiece has been suggested to be involved in mitochondria energy production and sperm motility (Costello et al., 2009; Yeste et al., 2015). Using two different fluorochromes (Fluo3 and Rhod5), these two calcium stores were analysed in this first study. Fluo3 marks both head and midpiece deposits, and Rhod5 specifically stains the calcium stored in the head (Takahashi et al., 1999; Yeste et al., 2015). In a dose-dependent manner, PAX

and quinine blockage resulted in significantly lower intracellular calcium in both deposits, suggesting that potassium channels, especially the calcium-activated ones, are involved in the regulation of intracellular calcium levels during pig sperm capacitation. Progesterone is known to induce acrosome exocytosis, rising intracellular calcium levels in both sperm head and midpiece deposits (Wu et al., 2006). In agreement to this, our results showed an increase in calcium influx five minutes after progesterone addition; notwithstanding, this rise was not as high as that seen in the control when samples were incubated with the highest concentrations of PAX or quinine. Remarkably, the decrease in intracellular calcium induced by PAX or quinine blockage was more apparent in the case of Fluo3 staining. These data emphasise the importance of potassium conductance to trigger calcium influx in the flagellum during *in vitro* capacitation and subsequent acrosome exocytosis. Moreover, CatSper channels, which are calcium voltage-gated and pH-sensitive channels, have been shown to be crucial in the regulation of sperm motility during *in vitro* capacitation of pig sperm (Vicente-Carrillo et al., 2017). As mentioned above, in addition to the general inhibition of potassium channels with quinine, blockage of calcium-activated potassium channels with PAX also resulted in a reduction of sperm motility. Thus, it is reasonable to hypothesise that, in porcine species, calcium-activated potassium channels could modulate calcium influx via regulating CatSper channels, which supports the notion that conductance of potassium and calcium ions are closely related.

Generation of ROS is also a characteristic event of sperm capacitation, which are needed in moderate levels for sperm to achieve the capacitated status (Betarelli et al., 2018; Boerke et al., 2013). An excessive ROS generation, however, may negatively impact sperm physiology and fertility. In effect, sustained oxidative stress involves a decrease in sperm motility, inability of sperm to interact with the oocyte, protein damage, DNA fragmentation, membrane lipid peroxidation, and even cell death (Tvrdá et al., 2011). A proper balance between ROS levels and antioxidant activity is thus crucial to avoid the detrimental effects induced by oxidative stress. For this reason, any new insight into the proteins involved in the sperm response to oxidative deserves our attention. In this context, the second study of this Dissertation investigated the role of Parkinson

disease protein 7 (PARK7), a multifunctional protein associated to oxidative stress response (Sharma et al., 2013; Sun et al., 2014). While the precise function of this protein in mammalian sperm is not fully understood, PARK7 has been positively correlated to membrane integrity, motility and SOD activity in human sperm (An et al., 2011; Favareto et al., 2010). Despite this, there are no studies regarding the relevance of PARK7 for mammalian sperm capacitation and acrosome reaction. Considering this, we interrogated if PARK7 is related to the sperm ability to undergo *in vitro* capacitation and trigger the acrosomal exocytosis induced by progesterone in porcine species. In addition, changes in the localisation of this protein were also analysed as well as whether its relative content in fresh sperm was related to their ability to withstand in *vitro* capacitation.

In human sperm, PARK7 has been reported to be present in the posterior part of the head, the midpiece and the flagellum (Yoshida et al., 2003). Herein, PARK7 was observed to localise to the post-acrosomal region and flagellum of pig sperm, a localisation pattern that is maintained during *in vitro* capacitation and acrosome exocytosis. In contrast, a decrease in PARK7 content was observed during incubation of sperm in capacitation medium, which was also reported by Choi et al. (2008). On the other hand, two different groups of sperm samples could be distinguished considering the relative content of PARK7 in fresh sperm at the beginning of the experiment (low and high PARK7 relative levels). Considering this, indicative cell parameters associated to sperm capacitation were analysed comparing the two groups in order to determine the relevance of PARK7 levels in the accomplishment of *in vitro* capacitation.

Concerning motility and kinematic parameters analysis, a positive correlation between PARK7 levels and sperm motility has been reported in human species (An et al., 2011). In our study, however, no differences in total and progressive sperm motility were observed between the two PARK7 level. Conversely, tendencies and differences in some kinematic parameters, including curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), lateral head displacement (ALH), and straightness (STR), were observed. Taking this into consideration, it is plausible to hypothesise that PARK7 is involved in the regulation of sperm motility during capacitation. Notwithstanding, further studies

with a higher number of individuals are needed to better stablish the role of PARK7 in the modulation of pig sperm motility.

As stated above, a decrease in membrane integrity, namely sperm viability, is expected during *in vitro* capacitation (Harrison, 1996; Lechniak et al., 2002). Whereas samples with high relative levels of PARK7 experimented a progressive decline in membrane integrity, those with a low content of this protein showed a drastic decrease. Moreover, the percentage of non-viable sperm with high lipid membrane disorder was significantly higher in the sperm population with low relative PARK7 levels. Due to its polyunsaturated fatty acid composition, the plasma membrane of mammalian sperm, particularly in pigs, is especially sensitive to oxidative stress (Cerolini et al., 2000). Based on our results and the observation that PARK7 is positively correlated to membrane integrity (Favareto et al., 2010), one could suggest that this protein preserves that integrity thanks to its antioxidant activity. It is widely understood that, although moderate levels of ROS are needed for sperm to achieve the capacitated status (Betarelli et al., 2018; Boerke et al., 2013), excessive amounts have detrimental effects on sperm cells, so an appropriate balance of ROS levels and antioxidant activity is essential during sperm capacitation (Bansal & Bilaspuri, 2010). In fact, the capacitation process can be modulated by some antioxidant molecules. For instance, paraoxonase 1 (PON-1), an enzyme with antioxidant activity, has been reported to prevent sperm from spontaneous acrosome exocytosis (Efrat et al., 2019). Similarly, glutathione peroxidase 6 (GPX6) removes ROS excess, protecting sperm from premature capacitation. In the present study, we observed that sperm samples with higher relative levels of PARK7 exhibited higher superoxide levels, as well as higher viability. It is worth mentioning that PARK7 levels in human sperm have been reported to be positively correlated to SOD activity, which catalyses the dismutation of superoxide molecules into hydrogen peroxide and oxygen (An et al., 2011). No differences, however, were observed regarding peroxide levels, so PARK7 content does not seem to be related with SOD activity during *in vitro* capacitation of pig sperm. Taken together, it can be posited that PARK7 may increase the resilience of sperm to the ROS produced during capacitation, which would result in a better maintenance of sperm viability.

As mentioned earlier in this Section, semen is composed by sperm (cell portion) and SP (fluid portion). This fluid contains a great variety of components that are involved in sperm protection and modulation of physiological processes such as capacitation, including enzymes or molecules with antioxidant properties, proteins (Juyena & Stelletta, 2012) and extracellular vesicles containing mRNAs and small non-coding RNAs (Barranco et al., 2019; Salas-Huetos et al., 2020). For this reason, in the last years, the role of SP as a modulator of sperm function has become an interesting focus of study with the aim to develop new biotechnological tools in the assisted reproduction field (Caballero et al., 2008, 2012). Despite this role, removal or extensive dilution of SP is a previous step in sperm preservation, both liquid-storage and cryopreservation, thus eliminating the natural protection provided by this SP. In fact, the presence of SP during sperm preservation has been reported to have detrimental effects for sperm cells (Höfner et al., 2020; Okazaki et al., 2009), as prolonged exposure of sperm to SP represents a non-physiological situation (Hunter, 1981). Whilst the supplementation of thawing media with SP seems to ameliorate the damage induced by cryopreservation procedures (Recuero et al., 2019), whether the presence of SP prior to liquid storage affects sperm survival and fertilising capacity is not clear. Furthermore, whereas some studies reported that the removal of SP prior to liquid storage might have advantageous effects on *in vivo* sperm fertilising ability (Pavaneli et al., 2019), other authors observed that the absence of SP could have a negative impact on the quality of liquid-stored semen (Chutia et al., 2014; Leal et al., 2018).

Although it is known that some SP proteins can inhibit sperm capacitation (Vadnais & Roberts, 2010), whether a prolonged contact of sperm with SP during liquid storage affects the ability of sperm to achieve the capacitated status and undergo acrosome reaction has not been investigated. In sheep, exposure to SP affects sperm motility and the structure of motile subpopulations during *in vitro* capacitation (Luna et al., 2017; Maree & van der Horst, 2013). Considering this and the conflicting evidence regarding sperm survival mentioned above, it is of special interest to evaluate how exposure to SP during liquid storage (17°C) affects the ability of sperm to elicit *in vitro* capacitation and trigger progesterone-induced acrosome exocytosis, which was the aim of the third study. The

mechanisms that regulate intracellular calcium levels in the different stores (sperm midpiece and head) were observed to be affected by the presence of SP and the time of storage. In effect, on the one hand, an increase in calcium levels in the sperm head deposit was observed in samples stored for 72 h, regardless of the presence of SP, suggesting that the permeability of sperm head to calcium increases with storage time. This could be associated to the reduction in the percentages of viable sperm with an intact acrosome observed after 245 min of incubation in samples stored with 30% SP for 72 h. While the treatments with SP and longer storage time resulted in an increase in the geometric mean intensity of Rhod5, a fluorochrome that mainly stains the calcium stored in the head (Yeste et al., 2015), this did not seem to be related to a higher sperm ability to trigger the acrosome exocytosis induced by progesterone. On the other hand, the presence of SP during liquid storage resulted in lower levels of calcium in the midpiece deposit, assessed by Fluo3 staining. As there was a concomitant reduction in the mitochondrial membrane potential in sperm stored in the presence of SP, these two events could be related (Gunter et al., 2004; Gürler et al., 2015). In fact, both calcium levels in the midpiece store (Yeste et al., 2015) and mitochondrial membrane potential (Ramió-Lluch et al., 2011, 2014) are expected to increase during sperm capacitation. It, therefore, looks that exposure of sperm to SP during liquid-storage could affect their ability to reach the capacitated status by inducing alterations in calcium levels in the midpiece store, and mitochondrial activity. Moreover, these results agree with those obtained by Yeste et al., (2015) and suggest that not only are the molecular mechanisms regulating calcium storage in the two deposits different, but they are also affected by the presence of SP and the time of storage at 17 ºC.

Regarding plasma and acrosomal membrane integrity, both the presence of SP and time of storage resulted in a lower percentage of viable sperm with an exocytosed acrosome. Indeed, the capacity of sperm to trigger the acrosome reaction diminished with longer storage times and higher concentrations of SP. The effects of SP-exposure on membrane lipid architecture were observed as soon as SP was removed, and sperm cells were incubated in capacitation medium. It is well known that SP contains proteins that can act as modulators of sperm capacitation (Rodríguez-Martínez et al., 2011). In effect, it has been

proposed that heparin-binding proteins, which cover sperm surface and stabilise plasma membrane, may act preventing sperm from premature capacitation and acrosome reaction (Vadnais & Roberts, 2010). Considering the results reported herein, other proteins or constituents of SP, including extracellular vesicles (Du et al., 2016), could be involved in maintaining the integrity of plasma membrane during liquid storage, thereby affecting the sperm ability to undergo *in vitro* capacitation and subsequent acrosome exocytosis.

Glycogen synthase kinase-3 (GSK3) is known to be involved in the molecular pathways that regulate sperm motility (Aparicio et al., 2007; Somanath et al., 2004; Vijayaraghavan et al., 2000), capacitation (Aparicio et al., 2007), and acrosome reaction (Reid et al., 2015). This crucial kinase is present in two isoforms, GSK3α and GSK3β, and its activity is regulated via phosphorylation of tyrosine and serine residues. Whereas the inhibition of GSK3α or GSK3β is associated with the phosphorylation at Ser21 or Ser9 residues, respectively (Frame et al., 2001; Stambolic & Woodgett, 1994), phosphorylation at Tyr279 (GSK3α) or Tyr216 (GSK3β) residues has been reported to increase its kinase activity (Pérez et al., 2003; Sayas et al., 2002). In this third study, we evaluated, for the first time, the changes in tyrosine phosphorylation that both GSK3 isoforms undergo during sperm capacitation and acrosome exocytosis. As expected, an increase in tyrosine phosphorylation in both GSK3α and GSK3β was observed during incubation in capacitation medium, reaching maximum levels after progesterone addition. Remarkably, while the extent of this increase was higher in samples stored for 72 h without SP, the lowest levels of tyrosine phosphorylation were observed in samples exposed to SP during liquid storage. It has been reported that inhibition of GSK3 hinders sperm from undergoing the acrosome exocytosis induced by progesterone (Reid et al., 2015). Along these lines, a lower percentage of sperm with reacted acrosomes after progesterone exposure was seen when storage time was longer and concentration of SP was higher. Taken collectively, our data suggest that the presence of SP during liquid-storage could reduce tyrosine phosphorylation of GSK3, diminishing its kinase activity; this would lead to a reduction in the sperm ability to trigger the acrosome reaction. As pointed out earlier, GSK3 activity has been related to the modulation of sperm motility in mammalian species, including pigs and bulls (Aparicio et al.,

2007; Somanath et al., 2004). In cattle, stimulation of sperm motility has been positively associated to an increase in tyrosine phosphorylation of GSK3 (Vijayaraghavan et al., 2000). Strikingly, despite changes in tyrosine phosphorylation, we did not observe significant effects of SP presence/absence or storage time on total and progressive motility. These different results may be explained by the fact that our study was conducted under capacitation conditions, in contrast to that of Vijayaraghavan et al. (2000).

Modulation of sperm motility is known to be related to calcium levels (Costello et al., 2009; Yeste et al., 2015) and high mitochondrial membrane potential (Gallon et al., 2006; Paoli et al., 2011), two parameters that were found to be altered by the presence of SP and storage time. Despite the lack of differences in total and progressive motility, changes in sperm motile subpopulations were observed. In effect, samples stored for 72 h in the absence of SP exhibited higher percentages of progressively motile and fastest sperm during *in vitro* capacitation, and shortly after the addition of progesterone. Samples stored in the absence of SP also exhibited higher accumulation of calcium in the midpiece region and higher mitochondrial membrane potential, which could underlie the increase in the progressively motile and fastest sperm subpopulation. On the other hand, the percentage of sperm belonging to the hyperactivated subpopulation (Pavaneli et al., 2017; Schmidt & Kamp, 2004) tended to increase during incubation time in samples stored in the presence of 30% SP; this was concomitant with a decrease in the percentage of progressively motile and fastest sperm. Hyperactivation is characterised by vigorous and high amplitude flagellar beating, and is essential for sperm to acquire fertilising capacity (Olson et al., 2011). Changes in sperm motility patterns rely upon intracellular calcium levels (Harayama, 2013; Ho & Suarez, 2003; Olson et al., 2010, 2011), albeit we observed that SP differentially affected calcium accumulation in head and midpiece stores. These findings could be related to changes in the sperm head membrane prior to acrosome exocytosis and to the regulation of mitochondrial activity during capacitation. Moreover, whereas the presence of SP during liquid-storage tended to increase the percentage of hyperactivated sperm, an increase in the percentage of viable sperm with a reacted acrosomes was not observed. These results would be in agreement with

those reported in rams, in which SP proteins induce hyperactivation while maintaining sperm in a decapacitated state, and sperm are able to elicit *in vitro* capacitation without hyperactivation (Luna et al., 2017).

As explained in more detail in the introduction section, SP performs two essential functions. On the one hand, it modulates sperm function, e.g., protecting the cell against oxidative stress and delaying capacitation (Caballero et al., 2008; Juyena & Stelletta, 2012), as it has also been reported in the third study. On the other hand, mounting evidence supports that SP has an effect on the female reproductive tract (Bromfield, 2016). Proteins are major components of SP, and can be found suspended in the fluid (Caballero et al., 2012), enclosed in extracellular vesicles (Yáñez-Mó et al., 2015), or attached to the sperm surface (Pini et al., 2016). Evidence of maternal tract modulation by SP comes from mice and pigs, in which ejaculate is deposited nearly or directly in the uterine lumen (Dean et al., 2011; Hunter, 1981); this supports that SP can interact directly with endometrial cells. In species that ejaculate intravaginally, such as humans or cattle, however, whether anatomical barriers (i.e., passage through cervix) modify the ability of SP and its components to interact with the endometrium is yet to be elucidated. In this context, it is reasonable to suggest that sperm cells, acting as vehicles for SP proteins adhered to their surface, could be the main paternal factor regulating the maternal environment in species with intravaginal ejaculation. In order to test this hypothesis, the fourth study was conducted with the aim to investigate whether sperm or SP elicit transcriptomic changes in the endometrium during natural mating, using the cattle as an animal model with intravaginal ejaculation. The RNA-sequencing analysis of endometrial samples was performed following natural mating of heifers with an intact bull (which ejaculates sperm and SP) or a vasectomised bull (which only ejaculates SP), and in unmated females (control animals). In order to determine whether SP or sperm effects are propagated along the female tract, not only were gene expression changes evaluated at the ejaculation deposition site (vagina), but also in the distal region where fertilisation takes place (oviductal ampulla).

Surprisingly, the comparison of the endometrial transcriptomes of unmated heifers and those mated to vasectomised bulls revealed no differentially expressed gene (DEG). In contrast, when heifers were mated to intact bulls, 22

(12 up- and 10 down-regulated) and 24 (18 up- and four down-regulated) endometrial genes were identified to be differentially expressed, compared to unmated animals and heifers mated to vasectomised bulls, respectively. These findings suggest that, in cattle, SP does not reach the uterus 24 h after natural mating, at least in the absence of a vehicle, such as sperm. Considering our data and the existing literature, some of the genes identified have been purported to potentially play a role in fertility. Amongst these, when comparing the control (unmated) with samples from heifers mated to intact bulls, some DEGs were found to be involved in tissue remodelling, which is an important step in the preparation of the endometrium for embryo implantation. For example, we found serine protease 2 (*PRSS2*), which is associated with degradation of type-1 collagen, down-regulated in response to mating to intact bulls. Interestingly, *PRSS2* has been reported to be up-regulated in endometrial tissue of repeat breeder cows, which are animals with no apparent anatomical abnormalities or infections that exhibit a variety of reproductive disturbances in a consistent pattern over three or more consecutive heat cycles (Hayashi et al., 2017). Gene encoding for scavenger receptor class A, member 5 (*SCARA5*), which was found to be up-regulated, is known to participate in immune defence (Jiang et al., 2006). The study of Mansouri-Attia et al. (2009) reported the up-regulation of this gene in the bovine endometrium during pregnancy. The SCARA5 has also been posited to be involved in tissue remodelling and histotroph secretion, both crucial for proper embryo development and implantation (Vitorino Carvalho et al., 2019). Regarding genes related to immune modulation, we found component 9 of complement system (*C9*) down-regulated in the endometrium of heifers mated to intact bulls. During the final steps of complement cascade, C9 participates in the constitution of the membrane attack complex (MAC), specifically mediating the formation of channels to induce lysis and death of targeted cells (Janeway et al., 2001). In the context of reproduction, the precise function of the complement system is not well stablished; however, some proteins involved in its regulation have been found in mouse and human sperm, which suggests that they could play a function in the protection of sperm in the female genital tract (Harris et al., 2006). Similarly, complement regulatory protein CD59, which is known to act impeding the formation of MAC (Janeway et al., 2001), has been identified in the surface of bovine sperm (Byrne et al., 2012). Moreover, mating to intact bulls

resulted in the down-regulation of the gene coding for CCAAT enhancer binding protein delta (*CEBPD*), which is a transcription factor implicated in cell proliferation (Wedel & Lömsziegler-Heitbrock, 1995). Although there is no evidence associating CEBPD with fertility or reproduction, CEBPB, which belongs to the same family of transcription factors, is noted to regulate proliferative events during decidualisation in mice (Mantena et al., 2006).

As aforementioned, comparison of endometrial transcriptomes of heifers mated to intact bulls and heifers mated to vasectomised bulls resulted in 18 up-regulated genes. Amongst these, *C4BPA, PLA2G10, CX3CL1, BLA-DQB,* and *IL17F* are immune-related genes and could play a role during early pregnancy in mammals. In humans, the complement component 4-binding protein alpha (C4BPA), a crucial inhibitor of the complement system (Blom et al., 2004), increases during the peri-implantation period (Tapia et al., 2011). In contrast, significant lower levels of C4BPA are detected in women suffering from unexplained, repeated spontaneous abortions and recurrent implantation failure (Lee et al., 2007; Tapia et al., 2008). While implantation time and placentation differ significantly between humans and cattle, it is reasonable to suggest in the latter that alterations in the expression of *C4BPA* in response to sperm and SP exposure could be involved in the regulation of peri-implantation events. Moreover, Group 10 secretory phospholipase A2 (PLA2G10) is a mediator of inflammatory processes involved in the synthesis pathway of prostaglandin (Capper & Marshall, 2001). In cattle, this protein has been defined as fertility-associated (Neupane et al., 2017) and, interestingly, the expression of its encoding gene (*PLA2G10*) has been found to be significantly reduced in the uterus of negative energy balance cows, which usually present lower pregnancy rates (Wathes et al., 2009). Another gene modified by mating to an intact bull that seems to play an important role modulating early maternal-embryo communication events is the C-X3-C motif chemokine ligand 1 (*CX3CL1*). In humans, this chemokine has been shown to mediate leukocyte infiltration into the endometrial epithelium during early pregnancy (Hannan et al., 2004), as well as to stimulate trophoblast migration (Hannan et al., 2006). In addition, Tríbulo et al. (2018) reported that CX3CL1 could potentially function as an embryokine in cattle.

Exposure of the female reproductive tract to sperm and SP during natural mating also resulted in alterations in the expression of immune-related genes that might drive immune tolerance during early pregnancy in cattle, such as Bovine Lymphocyte antigen (also known as MHC) class II, DQ beta (*BOLA-DQB*). In our study, mating to an intact bull resulted in the down-regulation of *BOLA-DQB*, similar to that observed in the study of (Dickinson et al., 2018), in which *BOLA-DQB* was also found down-regulated in pregnant heifers after natural breeding. In contrast, *BLA-DQB*, which also belongs to the MHC class II family, was observed to be up-regulated in endometrial samples after mating to an intact bull. Several genes belonging to the MHC class II family, including *BOLA-DQB* and *BLA-DQB*, have been significantly associated to reproductive traits in cattle (Melo et al., 2017), so they represent an interesting target for the study of processes driving paternal-antigen immune tolerance during early pregnancy. In this context, alterations in the expression of interleukin 17 F (*IL17F*), which was found to be up-regulated after mating to intact bulls, could also be related to immune tolerance regulation. This cytokine is produced by T helper 17 lymphocytes and shares high homology and biological functions with IL17A (Iwakura et al., 2011). Exposure to sperm, regardless of the presence of SP, but not to SP in the absence of sperm, is known to induce the up-regulation of *IL17A* in both *in vitro* (endometrial cell culture) and *in vivo* (uterine infusion) conditions (Ibrahim et al., 2019). Taken collectively, sperm, but not SP, would mediate changes in *IL17* expression in the bovine endometrium. It is worth mentioning that in mice, up-regulation of *IL17A* has been observed after mating to a vasectomised male (Song et al., 2016). Thus, whereas IL17 expression is mediated by SP in mice, it is modulated by sperm in cattle, reinforcing the hypothesis that sperm, rather than SP, have a modulatory role of the female tract environment in species with intravaginal ejaculation, in contrast to intrauterine depositors. In addition, an increase in the population of T helper 17 lymphocytes has been reported to occur together with an increment in the endometrial expression of *IL17* (Song et al., 2016), which supports that this protein is probably related to the establishment of immune tolerance toward paternal antigens.

Considering the detection of genes regulated by mating to an intact bull, together with the lack of effects observed after mating to a vasectomised bull, the

analysis of tissues in which ejaculate is deposited (i.e., the vagina) was also undertaken. A recent study conducted in bovine species showed a variation of EGF levels in the endometrium in response to SP infusion into the vagina, but not into the uterus (Badrakh et al., 2020), highlighting the relevance of the ejaculate deposition site during natural mating. In addition, in order to evaluate the extent these changes could be propagated into the reproductive tract, gene expression analysis of the oviductal ampulla was also performed. Based on the literature, genes identified in the RNA-sequencing analysis as candidates to be key regulators of uterine environment and early pregnancy events (*PLA2G10*, *CX3CL1*, *C4BPA*, *PRSS2*, *BLA-DQB*, and *CEBPD*) were selected to be also analysed in these two regions. Additionally, the expression of cytokines [IL1A, IL6, TNFA, and IL8 (Ibrahim et al., 2019; Introini et al., 2017; O'Leary et al., 2004; Schjenken et al., 2015; Sharkey et al., 2012)], which are known to be modulated by SP exposure in other species, was also assessed.

Whereas in pigs, natural mating induces up-regulation of *CEBPD* in the ampulla region after natural mating (Alvarez-Rodriguez et al., 2019), in mice, the expression of *Il6* decreases in the oviduct after mating to males that have undergone a vesiculectomy with or without a vasectomy (Bromfield et al., 2014). Furthermore, coitus is known to induce an increase in *IL1A*, *IL6* and *IL8* expression in the human cervix (Sharkey et al., 2012). Surprisingly, in contrast to this evidence, we did not observe in the expression of any gene in the vagina or the oviduct differences between the three animal groups. It is worth mentioning that the oestrous cycle of the animals used in the present study was synchronised with an intravaginal device that was removed 48 h before sample collection. The resulting manipulation, therefore, could have had an impact on the inflammatory status of the vagina, albeit all the heifers were managed under the same conditions. In fact, a higher variation in gene expression data was observed in the vagina compared to the ampulla region.

Despite the lack of treatment effect, some genes displayed different expression between tissues. In control samples, the expression of *CX3CL1* was higher in the ampulla than in the vagina. It is noteworthy to mention that CXCL3 has been detected throughout the oviduct and its receptor has been identified in human ejaculated sperm (Zhang et al., 2004). On the other hand, in all

treatments, higher levels of *CEBPD* and *IL8* were found in the vagina compared to the ampulla. Considering that both genes are related with inflammatory processes (Leonard & Yoshimura, 1990; Ramji & Foka, 2002) their higher basal expression in the vagina could be expected, because this organ is in direct contact with the outside and, thus, it is highly exposed to external pathogens. Finally, while mating either to intact or vasectomised bulls resulted in an increase in the expression of *IL1A* and *TNFA* in the vagina compared to the ampulla, such increase was not observed in control heifers. In a similar fashion, exposure to SP has been reported to induce an increment in the expression of *IL1A* and *TNFA* in human cervical explants (Introini et al., 2017). Interestingly, an increase in *IL1A* expression occurs after unprotected vaginal coitus, but not condom protected (Sharkey et al., 2012), thus demonstrating that the effects observed are induced by semen rather than by the mechanical stimulus of the coitus. Nevertheless, in our study, the mechanical stimulus of mating cannot be ruled out. While all these findings support that sperm rather than SP induce transcriptomic changes in the female reproductive tract when the male ejaculates into the vagina, further studies to address whether these changes are elicited by sperm or by SP proteins adhered onto sperm surface are needed.

Conclusions

1. Inhibition of potassium channels reduces the sperm ability to elicit in vitro capacitation by decreasing mitochondrial activity and sperm motility. In addition, calcium influx also decreases, thus preventing sperm from triggering acrosomal exocytosis.

2. In addition to calcium-activated potassium channels, other such as the voltage-gated, tandem-pore domain, and mitochondrial ATP-regulated ones are likely to be involved in the modulation of *in vitro* capacitation of pig sperm.

3. Sperm with higher relative content of PARK7 present a better resilience to ROS levels, displaying higher viability and exhibiting a more progressive *in vitro* capacitation process.

4. Prolonged exposure of sperm to seminal plasma at 17°C alters *in vitro* capacitation by reducing mitochondrial activity, tyrosine phosphorylation of GSK3, and the sperm capacity to trigger progesterone-induced acrosomal exocytosis.

5. During natural mating, seminal plasma, at least in the absence of a vehicle such as sperm, does not play a role in modulating the endometrial environment in cattle.

6. Sperm-borne proteins (intrinsic sperm proteins or those acquired from seminal plasma) induce moderate changes in the transcriptome of the endometrium and the vagina of cattle.

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