Universitat de Girona

RELEVANCE OF SPERM CHROMATIN PROTAMINATION, CONDENSATION AND DNA INTEGRITY FOR SPERM FUNCTION AND FERTILITY: THE ROLE OF TOROID LINKER REGIONS

Estel Viñolas Vergés

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DOCTORAL THESIS

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Doctoral programme in Technology

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

Universitat de Girona

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DECLARE:

That the Thesis entitled "**Relevance of sperm chromatin protamination, condensation and DNA integrity for sperm function and fertility: the role of toroid linker regions**", submitted by Miss. Estel Viñolas Vergés, has been completed under our supervision and meets the requirements for obtaining the degree of Doctor of Philosophy from the University of Girona.

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

Signatures

Girona, 29th June 2023

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"The important thing is not to stop questioning.

Curiosity has its own reason for existing"

Albert Einstein.

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Em sento molt afortunada de que formeu part de la meva vida.

LIST OF ABBREVIATIONS

8-OHdG	8-OH-2′-deoxyguanosine
-TX	Non-permeabilised
+TX	Permeabilised
ANOVA	One-way analysis of variance
AOPPs	Advanced oxidation protein products
ATP	Adenosine triphosphate
AU	Arbitrary units
BER	Base excision repair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CASA	Computer-Assisted Semen Analysis
CMA_3	Chromomycin A_3
CO_2	Carbon dioxide
COC	Cumulus-oocyte complex
CUPRAC	Cupric reducing antioxidant capacity
DBB	Dibromobimane
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
E^+	Ethidium
H_2DCFDA	2′7′-dichlorodihydrofuorescein
HCO_3^+	Bicarbonate

HDS	High DNA Stainability
HE	Hydroethidine
ICSI	Intracytoplasmic sperm injection
IP_3	Inositol-1,4,5-triphosphate
IQR	Interquartile range
IVF	*In vitro* fecundation
IZUMO1	Izumo sperm-oocyte fusion 1 protein
JUNO	Folate receptor 4 protein
LMP	Low melting point
MAR	Matrix attachment regions
Mg^{2+}	Magnesium
Mn^{2+}	Manganese
NRR	Non-return rate
NOX5	NADPH Oxidase 5
O_2^-	Superoxide anion
OGG	Oxoguanine glycosylase
OSi	Oxidative stress index
OTM	Olive tail moment
P1	Protamine 1
P2	Protamine 2
PAGE	Polyacrylamide acid-urea gel electrophoresis
PBS	Phosphate-buffered saline
PFGE	Pulsed-field Gel Electrophoresis
PI	Propidium Iodide
PKA	Protein kinase A

PLCζ	Phospholipase C zeta
PSRF	Post sperm-rich fraction
R	Correlation coefficient
ROS	Reactive oxygen species
sACY	Soluble adenylyl cyclase
SCD	Sperm chromatin dispersion test
SD	Standard deviation
SCF	Sperm chromatin fragmentation
SCSA	Sperm chromatin structure assay
SP	Seminal Plasma
SSB	Single-strand breaks
SRF	Sperm-rich fraction
SRF-P1	10 mL of the sperm-rich fraction
SRF-P2	Rest of the sperm-rich fraction
STR	Straightness index
TLR	Toroid linker regions
TOPIIβ	Topoisomerase IIb
TP	Transition proteins
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VAP	Average path velocity
ZP3	Zona pellucida sperm-binding protein 3

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GENERAL CONTENTS

ABSTRACT

Male fertilising capacity has traditionally been predicted by the assessment of sperm concentration, motility and morphology. During the last few decades, however, research has been particularly focused on the analysis of sperm chromatin, in terms of protamination, condensation and DNA integrity, as a valuable approach to evaluate male fertility. During spermiogenesis nuclear histones are replaced by protamines, resulting in a tightly condensed structure that is stabilised through intra- and inter-protamine disulphide bonds formed during epididymal maturation. This highly condensed organisation protects the paternal genetic information from damaging agents during the transit of sperm through both male and female genital tracts. Poor protamination after spermiogenesis may result in an elevated number of sperm cells with immature chromatin, which is featured by a higher incidence of histone-packed DNA regions; these regions have been described to be more susceptible to DNA damage than those properly protaminated. Sperm DNA fragmentation is defined by the incidence of single- and double-strand breaks caused by oxidative stress and enzymatic activity, respectively. Sperm DNA fragmentation has been associated to poor sperm quality and impaired fertility outcomes both *in vitro* and *in vivo*. Because of the relationship between DNA and chromatin integrity, it is reasonable to hypothesise that abnormal chromatin protamination and condensation can also affect sperm DNA negatively, which could ultimately cause male subfertility or infertility. In the light of the aforementioned, the present Doctoral Thesis aimed to investigate the relationship of sperm chromatin protamination and condensation, and DNA integrity with sperm function and fertilising ability. For this purpose, experiments included in Chapter I sought to describe how sperm chromatin from bovine sperm is altered during incubation at 38 °C, and whether such changes are related to their quality and fertilising capacity. To address the effects on sperm chromatin, protamination was determined

with CMA_3 staining, condensation was examined using the sperm chromatin dispersion test, and DNA integrity was evaluated through neutral and alkaline Comet assays for double-strand and global DNA damage, respectively. Chromatin protamination of frozen-thawed bovine sperm was observed to decrease upon incubation at 38 °C, whereas chromatin condensation was not affected under the same conditions. Furthermore, both global damage and double-strand DNA breaks were found to increase upon incubation of frozen-thawed bovine sperm at 38 °C. Regarding sperm quality, which was evaluated by flow cytometry and computer assisted sperm analysis (CASA), post-thawing incubation at 38 °C reduced sperm viability, motility, and the percentage of sperm with high intracellular ROS levels, whereas intracellular superoxide and calcium levels raised. Besides, fertility rates were found to correlate positively with total motility and percentages of sperm with high intracellular ROS levels. On the other hand, experiments conducted in Chapter II aimed to investigate whether chromatin protamination, condensation and DNA integrity of sperm vary between the separate fractions of the pig ejaculate (SRF-P1, SRF-P2 and PSRF). As results from CMA_3 and DBB tests indicated, sperm from SRF-P1 and SRF-P2 exhibited greater chromatin condensation. Moreover, an increased imbalance between oxidative stress and antioxidant capacity was noticed in the SRF-P1, which was correlated to sperm concentration. Finally, Chapter III was conducted to explore if sperm chromatin fragmentation (SCF) can be activated in ejaculated sperm *in vitro,* and to elucidate whether the DNA breaks generated by this mechanism are mainly located in the toroid linker regions. For this purpose, ejaculated sperm were incubated with different concentrations of Mn^{2+} and Mg^{2+} ions for distinct incubation times at 37 °C. Data from neutral and alkaline Comet assays confirmed that sperm DNA breaks can be induced *in vitro* in pig ejaculated sperm through the incubation with

intracellular divalent ions in a dose-dependent manner, with Mn^{2+} being more effective than Mg^{2+}. In addition, these incubations resulted in DNA fragments similar in size to toroidal structures (33 to 194 Kb). Finally, while the induction of SCF impaired sperm motility and induced sperm agglutination, it had no effect on *in vitro* fertilisation outcomes.

RESUM

Tradicionalment, la capacitat fecundant masculina s'ha determinat mitjançant l'avaluació de la concentració, motilitat i morfologia dels espermatozoides. No obstant això, durant les últimes dècades, les investigacions s'han centrat especialment en l'anàlisi del grau de protaminació i condensació de la cromatina espermàtica, i de la integritat de l'ADN, com a paràmetres complementaris per a l'avaluació de la fertilitat masculina. Durant l'espermiogènesi la major part d'histones són substituïdes per protamines, donant lloc a una estructura molt compacta que s'estabilitza mitjançant enllaços disulfur intra- i inter-protamines formats durant la maduració epididimària. Aquesta organització altament condensada protegeix la informació genètica paterna enfront d'agents perjudicials durant el trànsit dels espermatozoides pels tractes genitals masculí i femení. Una protaminació deficient durant l'espermiogènesi pot resultar en un major nombre de cèl·lules espermàtiques immadures, que presenten una incidència més elevada de regions d'ADN compactades per histones. S'ha descrit que aquests regions menys protaminades són més susceptibles de patir dany a l'ADN que aquelles que estan correctament protaminades. La fragmentació de l'ADN espermàtic es defineix per la incidència de trencaments de cadena simple i doble causades per l'estrès oxidatiu i l'activitat enzimàtica, respectivament. Aquesta fragmentació s'ha associat amb una baixa qualitat de l'esperma i una fertilitat menor, tant *in vitro* com *in vivo*. Tenint en compte la relació entre la integritat de la cromatina i l'ADN, hom pot suggerir que les anomalies en la protaminació i condensació de la cromatina també poden afectar negativament la funcionalitat espermàtica, la qual cosa podria causar subfertilitat o infertilitat masculina. En aquest context, aquesta Tesi Doctoral va tenir com a objectiu investigar la relació de la cromatina espermàtica, en termes de protaminació, condensació i integritat de l'ADN, amb la funcionalitat espermàtica i la capacitat fecundant dels

espermatozoides. Per això, els experiments inclosos en el Capítol I es van dissenyar per analitzar els canvis que pateix la cromatina de l'espermatozoide boví descongelat durant la seva incubació a 38 °C, i per determinar si aquests canvis estan relacionats amb la qualitat i fertilitat de l'espermatozoide. Per tal de testar els efectes sobre la cromatina espermàtica, la protaminació es va examinar mitjançant el test CMA_3, la condensació es va determinar amb el test de dispersió de la cromatina espermàtica i la integritat de l'ADN (fragmentació de cadena doble i global de l'ADN) es va avaluar mitjançant els assajos Comet neutre i alcalí. Els resultats van mostrar que la protaminació de la cromatina espermàtica dels espermatozoides de toro descongelats disminuïa després de la seva incubació a 38 °C, mentre que la condensació de la cromatina espermàtica no es veia alterada sota les mateixes condicions. A més, tant la fragmentació global com la de doble cadena de l'ADN augmentaven després de la incubació a 38 °C dels espermatozoides descongelats. Pel que fa a la qualitat espermàtica, avaluada mitjançant citometria de flux i el sistema d'anàlisi espermàtica automatitzat (CASA), la incubació a 38 °C reduïa la viabilitat i la motilitat espermàtiques, així com el percentatge d'espermatozoides amb alts nivells intracel·lulars de ROS, mentre que augmentava els nivells de superòxids i calci intracel·lulars. A més, es va observar que les taxes de fertilitat es correlacionaven amb la motilitat total i el percentatge d'espermatozoides amb alts nivells de ROS intracel·lular. Paral·lelament, els experiments realitzats en el Capítol II tenien com a objectiu investigar si la protaminació i la condensació de la cromatina, i la integritat de l'ADN dels espermatozoides, varien entre les diferents fraccions de l'ejaculació porcina (SRF-P1, SRF-P2 i PSRF). Els resultats dels assajos CMA_3 i DBB van mostrar que els espermatozoides procedents de les fraccions SRF-P1 i SRF-P2 presentaven una major condensació de la cromatina espermàtica. A més, la SRF-P1 presentava un major desequilibri

entre l'estrès oxidatiu i la capacitat antioxidant, la qual cosa es va veure que estava correlacionada amb la concentració d'espermatozoides. Finalment, el Capítol III es va dur a terme per explorar si la fragmentació de la cromatina espermàtica (SCF) pot activar-se en espermatozoides ejaculats *in vitro* i per dilucidar si es produeixen trencaments d'ADN a les regions que uneixen els toroides (TLR). Amb aquest objectiu, es van incubar espermatozoides ejaculats amb diferents concentracions de Mn^{2+} i Mg^{2+} durant diferents temps d'incubació a 37 °C. Els resultats dels assajos Comet neutre i alcalí van confirmar que es poden induir trencaments de l'ADN espermàtic *in vitro* en els espermatozoides ejaculats de porcí mitjançant la incubació amb ions divalents intracel·lulars de forma dosi-dependent, i que el Mn^{2+} és més efectiu que el Mg^{2+}. Addicionalment, aquesta incubació va donar lloc a fragments d'ADN de mida similar a les estructures toroidals (33 to 194 Kb). Finalment, es va examinar l'impacte de la fragmentació de l'ADN produïda per la SCF, i es va veure que la inducció d'aquest mecanisme comprometia la motilitat espermàtica i induïa l'aglutinació dels espermatozoides. Tanmateix, aquesta SCF induïda pel Mn^{2+} en els espermatozoides ejaculats no va influir en els resultats de la fecundació *in vitro*.

RESUMEN

Tradicionalmente, la capacidad fecundante masculina se ha determinado mediante la evaluación de la concentración, motilidad y morfología de los espermatozoides. Sin embargo, durante las últimas décadas, la investigación se ha centrado especialmente en el análisis del grado de protaminización y condensación de la cromatina espermática, y de la integridad del ADN, como parámetros complementarios para la evaluación de la fertilidad masculina. Durante la espermiogénesis la mayoría de les histonas son sustituidas por protaminas, dando lugar a una estructura muy compacta que se estabiliza mediante enlaces disulfuro intra- e inter-protaminas formados durante la maduración espermática. Esta organización altamente condensada protege la información genética paterna frente a agentes dañinos durante el tránsito de los espermatozoides por los tractos genitales masculino y femenino. Una protaminación deficiente durante la espermiogénesis puede resultar en un mayor número de células espermáticas inmaduras con una mayor incidencia de regiones de ADN compactadas por histonas, las cuales se ha descrito que son más susceptibles de sufrir daño en el ADN que las regiones correctamente protaminadas. La fragmentación del ADN espermático se define por la incidencia de roturas de cadena simple y doble causadas por el estrés oxidativo y la actividad enzimática, respectivamente. Dichas roturas se han asociado con una baja calidad del esperma y una menor fertilidad, tanto *in vitro* como *in vivo*. Teniendo en cuenta la relación entre la integridad de la cromatina y el ADN, se puede sugerir que las anomalías en la protaminación y condensación de la cromatina también pueden afectar negativamente la funcionalidad espermática, lo que podría causar subfertilidad o infertilidad masculina. En este contexto, esta Tesis Doctoral tuvo como objetivo investigar la relación de la cromatina espermática, en términos de protaminación, condensación e integridad del ADN, con la funcionalidad espermática y la capacidad

fecundante de los espermatozoides. Para ello, los experimentos incluidos en el Capítulo I se diseñaron para analizar los cambios que sufre la cromatina del espermatozoide bovino descongelado durante su incubación a 38 °C, y si estos cambios están relacionados con la calidad y fertilidad del espermatozoide. Para evaluar los efectos sobre la cromatina espermática, la protaminación se determinó mediante el test CMA_3, la condensación se examinó con el test de dispersión de la cromatina espermática y la integridad del ADN (fragmentación de cadena doble y global del ADN) se evaluó mediante los ensayos Comet neutro y alcalino. Los resultados mostraron que la protaminación de la cromatina espermática de los espermatozoides de toro descongelados disminuía tras su incubación a 38 °C, mientras que la condensación de la cromatina espermática no se veía alterada en las mismas condiciones. Además, tanto la fragmentación global cuanto la de doble cadena del ADN aumentaban tras la incubación de espermatozoides de toro descongelados a 38 °C. En lo que respecta a la calidad espermática, evaluada mediante citometría de flujo y el sistema de análisis espermático automatizado (CASA), la incubación a 38 °C posterior a la descongelación reducía la viabilidad y la motilidad espermáticas, así como el porcentaje de espermatozoides con altos niveles intracelulares de ROS, mientras que aumentaban los niveles de superóxidos y calcio intracelulares. Además, se observó que las tasas de fertilidad se correlacionaban con la motilidad total y el porcentaje de espermatozoides con altos niveles de ROS intracelular. Paralelamente, los experimentos realizados en el Capítulo II tenían como objetivo investigar si la protaminación y la condensación de la cromatina, y la integridad del ADN de los espermatozoides varían entre las distintas fracciones del eyaculado porcino (SRF-P1, SRF-P2 y PSRF). Los resultados de los ensayos CMA_3 y DBB mostraron que los espermatozoides procedentes de las fracciones SRF-P1 y la SRF-P2 presentaban una mayor condensación de la

cromatina espermática. Además, la SRF-P1 presentaba un mayor desequilibrio entre el estrés oxidativo y la capacidad antioxidante, lo cual se vio que estaba correlacionado con la concentración de espermatozoides. Por último, el Capítulo III se llevó a cabo para explorar si la fragmentación de la cromatina espermática (SCF) puede activarse en espermatozoides eyaculados *in vitro* y para dilucidar si se producen roturas de ADN en las regiones que unen los toroides (TLR). Con este objetivo, se incubaron espermatozoides eyaculados con distintas concentraciones de Mn^{2+} y Mg^{2+} durante diferentes tiempos de incubación a 37 °C. Los resultados de los ensayos Comet neutro y alcalino confirmaron que se pueden inducir roturas del ADN espermático *in vitro* en los espermatozoides eyaculados de porcino mediante la incubación con iones divalentes intracelulares de forma dosis-dependiente, y que el Mn^{2+} es más efectivo que el Mg^{2+}. Además, de esta incubación resultaron fragmentos de ADN de tamaño similar a las estructuras toroidales (33 a 194 Kb). Finalmente, se examinó el impacto de la fragmentación del ADN producida por SCF, y se vio que la inducción de la SCF comprometía la motilidad espermática e inducía la aglutinación de los espermatozoides. A pesar de ello, esta SCF inducida por el Mn^{2+} en los espermatozoides eyaculados no influyó en los resultados de la fecundación *in vitro*.

INTRODUCTION

1. Mammalian ejaculation

1.1. Semen and components

Semen is the liquid suspension released during ejaculation. It is composed by sperm cells and the seminal plasma (SP), which is made up of secretions from the male accessory glands and provides sperm with a suitable medium to support their function upon ejaculation and, in mammals, during the transport through the female reproductive tract towards the oocyte (Garner & Hafez, 2000; Sancho & Vilagran, 2013).

1.1.1. Sperm

Sperm cells are the cellular component of the ejaculate. The evolution provided sperm cells with an optimised anatomy that enables them to reach and fertilise the oocyte, achieving an effective propagation of the paternal genetic information. Yet, before a spermatozoon becomes competent to fertilise an oocyte, it needs to undergo a myriad of processes, both in the male and female reproductive tracts. The processes through which a germinal stem cell evolves to a mature spermatozoon with fertilising capacity include spermatogenesis, maturation, capacitation and acrosome reaction, and take place in the testicular seminiferous epithelium, in the epididymis and in the oviduct (fallopian tube), respectively.

Sperm are small, elongated cells that can be morphologically and functionally divided into two main regions, the head and the tail, which are connected by a region designated as connecting piece or neck (Alberts et al., 2008; Briz & Fàbrega, 2013). The sperm head is composed by a haploid nucleus containing a highly condensed and transcriptionally inactive DNA

that minimises the cell volume (Alberts et al., 2008). Together with the nucleus, a membrane-bound vesicle known as acrosome is found in the head of most mammalian sperm. The acrosome is an important specialised secretory vesicle located in the apical region of the sperm head and covers approximately 80 % of its volume. It carries hydrolytic enzymes, mainly acrosin and hyaluronidase, which are involved in the acrosome reaction, a crucial step that allows sperm penetration through the zona pellucida, enabling the further fusion with the oocyte plasma membrane (Garner & Hafez, 2000). The sperm tail is composed by a flagellum, whose central axoneme emerges from the basal region of the head. The axoneme, composed of two central microtubules surrounded by nine pairs of microtubules, is enclosed by nine outer dense fibres that restrict the flexibility of the flagellum and protects it from the forces resulting from its movement (Alberts et al., 2008). The sperm tail can be further subdivided into three different regions: the mid, principal, and end pieces. In the midpiece, the layer of dense fibres is surrounded by mitochondria, which are crucial organelles carrying out oxidative phosphorylation – necessary to produce ATP – and are involved in the regulation of sperm capacitation and hyperactivated movement (Alberts et al., 2008; Garner & Hafez, 2000). The longest segment of the sperm tail is the principal piece, which is attached to the midpiece by the Jensen's ring. More specifically, the Jensen's ring separates the layer of mitochondria of the midpiece from the principal piece fibrous cover (Lehti & Sironen, 2017). Finally, the end piece is the shorter segment of the sperm tail, and only contains a disorganised axoneme (Briz & Fàbrega, 2013).

1.1.2. Seminal plasma

The seminal plasma is the liquid portion of semen, and is composed by secretions from the testis, epididymis, and mostly, from the male accessory sex glands (seminal vesicles, prostate and bulbourethral glands) (Bromfield, 2014; Garner & Hafez, 2000). Seminal plasma joins sperm during ejaculation in order to create the appropriate medium to carry, protect and nourish ejaculated sperm (Gilany et al., 2015; Perez-Patiño et al., 2016). Besides this well-established function of SP, emerging evidence supports that it plays an essential role in regulating sperm fertilising ability (Bromfield, 2016; Locatello et al., 2013; Rodríguez-Martínez et al., 2011, 2021). In addition, SP interacts with the female reproductive tract, developing an important mission in the modulation of the immune system response, which has been shown to be required for an appropriate embryo development, implantation and pregnancy (Bromfield, 2016; O'Leary et al., 2004; Robertson, 2007; Rodríguez-Martínez et al., 2021; Schjenken & Robertson, 2014)

The SP is composed by inorganic compounds, proteins and metabolites (Bromfield, 2014; Garner & Hafez, 2000), whose concentration has been reported to vary across species, mainly due to the differences in the presence and dimensions of the accessory glands. Moreover, the composition of SP has also been described to present inter-individual variations and even variations between ejaculates from the same individual (Strzeżek et al., 2005; Valverde et al., 2016). The protein fraction is the most abundant SP component. Seminal plasma proteins are also able to interact with sperm during the epididymal transit, and during/after ejaculation, promoting membrane changes associated to sperm capacitation (Rodríguez-Martínez et al., 2011). On the other hand, inorganic compounds such as sodium, potassium, calcium and magnesium, are also present in the SP (Cragle et al., 1958), and have been reported to exert a

further impact on sperm function, once ejaculated, in relation to their concentration (Hamamah & Gatti, 1998). Metabolites present in the SP, which include carbohydrates, lipids, amino acids and hormones, are known to be involved in several physiological processes in sperm, such as metabolism and motility (Bieniek et al., 2016). In this regard, while phospholipids and cholesterol are known to be important for membrane structure and sperm function, carbohydrates are directly involved in the energy production process (Juyena & Stelletta, 2012). Finally, hormones like oestradiol and progesterone have been reported to be present in the SP, but their precise function is yet to be defined (Juyena & Stelletta, 2012).

1.2. Ejaculation

Ejaculation is the physiological process involving the ejection of sperm and SP outside the male body. As mentioned above, sperm are produced in the seminiferous tubules of the testicle (Alberts et al., 2008; Gilbert, 2000b; Parrish et al., 2017). Once sperm cells are completely differentiated at the seminiferous tubules, they are pushed through the *rete testis* and *vas efferens* to finally reach the epididymis (Alberts et al., 2008; Carlson, 2019). During its transit through the epididymis (divided into caput, corpus and cauda), sperm undergo a functional maturation, acquiring motility and higher nuclear condensation, and withstanding changes in the composition of their plasma membrane. This process ends when they reach the cauda epididymis, where sperm are stored awaiting ejaculation. Upon ejaculation, sperm migrate through *vas deferens* to the pelvic urethra, where they mix with secretions from seminal vesicles, the prostate, and bulbourethral glands to constitute the final ejaculate (Bonet et al., 2013; Carlson, 2019; Gilbert, 2000b), which is deposited in the female reproductive tract (Suarez & Pacey, 2006).

Ejaculates of mammalian species, such as humans, pigs and horses, are expelled into two main fractions after the pre-sperm one: the sperm-rich fraction (SRF) and the post sperm-rich fraction (PSRF) (Eliasson & Lindholmer, 1972; Rodríguez-Martínez et al., 2021). As mentioned above, ejaculate volume and composition have been found to vary across species, among individuals and among ejaculates from the same individual (Mann & Lutwak-Mann, 1981). Also, intra-ejaculate differences in terms of sperm and SP composition have also been reported (Hebles et al., 2015; Kareskoski et al., 2011; Rodriguez-Martinez et al., 2021), each ejaculate fraction being different from the other. The collection of split ejaculates is a method that has been attempted in humans in order to obtain the semen fraction containing the sperm with the highest quality to be used for fertilisation (Amelar & Hotchkiss, 1965; de la Torre et al., 2017). Collection of human ejaculate fractions, however, exhibits a high degree of variability, probably due to the fact that harvesting split fractions highly relies on the ability of the patient to fraction their ejaculates. To overcome this limitation, one may consider animal models like pigs, as semen collection methods are highly standardised because of their commercial production for artificial insemination purposes.

Ejaculate fractions differ in sperm concentration and SP volume, origin and composition (Amelar & Hotchkiss, 1965; de la Torre et al., 2017; Einarsson, 1971; Kareskoski et al., 2011a; 2011b). Whereas the SRF has the major contribution in sperm cells to the final ejaculate (80-90 %) and contains a small volume of SP, the PSRF is the largest in volume and only contains a small proportion of sperm (10-20 %) (Mann & Lutwak-Mann, 1981). These changes between fractions are known to be due to the differential origin of the fluid, the SP from the SRF being mainly composed by epididymal and prostate secretions (Einarsson, 1971; Eliasson & Lindholmer, 1972; Rodríguez-Martínez et al., 2009) and that from the PSRF

being mainly formed by secretions from seminal vesicles and bulbourethral glands (Eliasson & Lindholmer, 1972; Rodríguez-Martínez et al., 2009). Thus, different studies have evidenced variations between fractions in terms of SP components, such as proteins (Björndahl & Kvist, 2003; Kareskoski et al., 2011b; Perez-Patiño et al., 2016; Rodríguez-Martínez et al., 2011), antioxidants (Barranco et al., 2015; Kowalczyk, 2022a; Pahune, 2013) and metabolites (Arienti et al., 1999; Clegg & Foote, 1973; Mateo-Otero et al., 2020; Valsa et al., 2012). As a result of these differences in sperm and SP composition between ejaculate fractions, distinct sperm quality outcomes in terms of viability, motility and DNA integrity have been reported for each fraction (de la Torre et al., 2017; Dziekońska et al., 2017; Hebles et al., 2015; Kumar et al., 2011). In effect, several studies have shown that sperm contained in the SRF exhibit the highest motility (Dziekońska et al., 2017; Hebles et al., 2015; Santolaria et al., 2016; Valsa et al., 2012), membrane stability (Peña, 2003; Sellés et al., 2001) and DNA integrity (de la Torre et al., 2017; Hebles et al., 2015; Kumar et al., 2011), concluding that the SRF presents the greatest sperm quality and, therefore, the highest fertility potential (Barranco et al., 2015; Lindholmer, 1973; Sokol et al., 2009).

2. Sperm formation

2.1. Spermatogenesis

Spermatogenesis is a complex biological process through which a type B spermatogonium is divided into four fully differentiated sperm. Spermatogenesis occurs in the epithelium of the seminiferous tubules of the male testis, starting at puberty and throughout the remainder of the

male life, and can be subdivided in three processes: first, the proliferation and differentiation of spermatogonia; second, meiosis; and third, cell differentiation or spermiogenesis (Clermont et al., 1993; Sharma & Agarwal, 2011).

During foetal development, primordial germ cells migrate to the epithelium of the seminiferous cords, the precursors of the seminiferous tubules, and start to proliferate and differentiate to lead to type A spermatogonia (2n). These stem cells are constantly renewed by mitotic divisions and, once puberty is reached, they start to differentiate into type B spermatogonia (2n), while maintaining the type A lineage. Type B spermatogonia enter meiosis, deriving to primary spermatocytes, which undergo two meiotic divisions, the first one resulting in two secondary spermatocytes (n) and the second one resulting in four round spermatids (n), two per secondary spermatocyte (Gilbert, 2000b; Sharma & Agarwal, 2011). Among all the molecular processes occurring during meiosis, it is important to mention that during prophase I of the first meiotic division, homologous chromosomes interchange genetic material, providing genetic variability in the resulting daughter cells. This process is initiated by the recognition of the telomeres of the homologous chromosomes forming the bouquet, followed by a complete pairing of the homologous chromosomes and the generation of double-strand DNA breaks by the SPO11 nuclease, which enables the production of crossovers and the further resolution of chiasmata (Lin & Matzuk, 2014). After the second meiotic division, immature round spermatids begin a cell differentiation process, known as spermiogenesis, which mainly comprises cytoplasmic and morphological changes turning the cell from large round-shaped to small elongated spermatids, and changes in the chromatin configuration (Hermo et al., 2010a; Leblond & Clermont, 1952; Lin & Troyer, 2014). Regarding cytoplasmic changes, the acrosome formation from the Golgi

apparatus provides sperm with the ability to penetrate oocyte vestments during fertilisation (Hermo et al., 2010). Furthermore, the formation of the sperm tail starts with the polarisation of centrioles (Clermont et al., 1993; Leblond & Clermont, 1952), the spermatid cytoplasm is reduced and mitochondria are confined to the midpiece (Lin & Troyer, 2014). Finally, it is during spermiogenesis that most histones of the nuclear chromatin are replaced by protamines, leading to a highly-condensed state. Due to the relevance of these chromatin changes, they will be approached separately in Section 2.1.1. After completion of cell differentiation, testicular sperm are released from the seminiferous epithelium to the lumen of the seminiferous tubule during a process known as spermiation.

At the end of spermatogenesis, sperm are highly differentiated cells with no motility or fertilising ability, features that are acquired after their journey along the epididymis, where sperm undergo the maturation process (Cornwall, 2008; Gervasi & Visconti, 2017; Sullivan & Mieusset, 2016).

2.1.1. Sperm chromatin remodelling

During mammalian spermatogenesis, an important chromatin reorganisation occurs leading to severe changes in the chromatin condensation status, turning from a histone-based chromatin structure to a structure mostly composed of protamines (*Figure 1*) (Braun, 2001; Dadoune, 2003; Miller et al., 2010; Steger, 1999), which are the most abundant chromatin component in mature sperm (Oliva, 2006). In general, these chromatin changes occur mostly during spermiogenesis in a stepwise manner, first replacing some of the canonical histones by testis-specific histone variants, then replacing most histones by transition proteins (TP) and, finally, replacing these transition proteins by protamines

(Braun, 2001; Steger, 1999). Yet, the presence of transition proteins has only been described in some species, such as humans, sheep, mice and rats (Gosálvez et al., 2011). Histone-to-protamine replacement starts thanks to the activity of DNA topoisomerase II, which produces transient DNA breaks to alleviate the torsional stress resulting from supercoiling and whose function is facilitated by the high degree of histone hyperacetylation (Laberge & Boissonneault, 2005; Marcon & Boissonneault, 2004; S. McPherson & Longo, 1993a; 1993b). Initially, some canonical histones are replaced by testis-specific histone variants (Meistrich et al., 2003; Miller et al., 2010; Sharma & Agarwal, 2011). Then, in the species where transition proteins are involved, histones are replaced by transition nuclear proteins (TP1 and TP2), which are basic proteins that represent up to the 90 % of chromatin proteins during the histone-to-protamine replacement process (Meistrich et al., 2003). TP1 has been reported to contribute to histone displacement and, together with TP2, leads chromatin to achieve a greater condensation status (Boissonneault, 2002; Brewer et al., 2002; Lévesque et al., 1998). Finally, TPs are subsequently replaced by protamines, which leads to the highest degree of chromatin condensation (Braun, 2001). Even though most mammalian species present an unique type of protamine in sperm (protamine 1), mice, horses and humans exhibit similar amounts of two types of protamines, protamine 1 and protamine 2 (Balhorn et al., 1988; D. T. Carrell et al., 2007; Mengual et al., 2003). Not only has the abnormal proportion of protamine 1 and protamine 2 been linked to infertility (Francis et al., 2014; Nanassy et al., 2011; Torregrosa et al., 2006), but it has also been reported to negatively impact *in vitro* fertilisation outcomes and impair early embryo development (Aoki et al., 2006). Compared to histones, protamines are characterised by their high amounts of arginine residues, which are basic and contain amino groups ($-NH_3^+$) that neutralise the negative DNA phosphate groups ($-PO_4^{3-}$). This

results in a net positive charge that leads to a strong binding of these proteins to the major groove of the double helix of DNA (Björndahl et al., 2010; Oliva & Castillo, 2011). In addition, protamines contain a significant number of cysteine residues (between five and ten, depending on the species) that promote the formation of multiple inter- and intra-protamine disulphide bonds, essential to stabilise the highly condensed chromatin in mature sperm (Balhorn et al., 1988; Carrell et al., 2007; Ward, 2017). Furthermore, zinc bridges have been reported to be present in inter-protamine binding, contributing to the stability of the structure (Björndahl et al., 2010). All these features confer a high degree of DNA packaging, with a chromatin that is six to ten times more condensed than mitotic chromosomes, and a nuclear volume that is 44 times smaller than that of mouse liver cells (Ward & Coffey, 1991). This high degree of packaging of the genetic information allows a better protection of the paternal DNA during the sperm journey along the male and female reproductive tracts towards the oocyte. In spite of this, between 2 and 15 % of histones remain in certain regions of the mature sperm genome, being potentially involved in the epigenetic regulation of the first embryo stages (Braun, 2001; D. T. Carrell et al., 2007; Miller et al., 2010; Oliva, 2006). In this regard, several studies have reported that abnormally high levels of histones in mature sperm are correlated with male subfertility and infertility (Francis et al., 2014; Hamad, 2019).

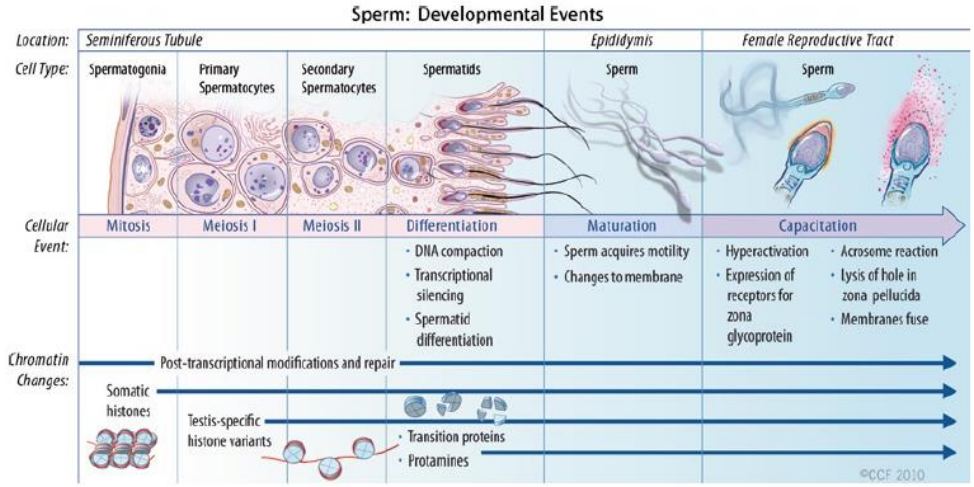

Figure 1. Cellular and chromatin changes during the life of a sperm cell, from its formation and testicular release, to epididymal maturation and journey through the female reproductive tract. Retrieved from Sharma & Agarwal (2011).

Condensation of sperm DNA with protamines promotes a very specific chromatin organisation, only found in sperm cells. This organisation is formed by the condensation of chromatin loop domains of 25 to 50 kilobases into toroidal structures, which are the basic packaging structure of sperm chromatin (Brewer et al., 1999; Miller et al., 2010). These toroidal structures become attached to each other and to the nuclear matrix thanks to nuclease-sensitive regions named Toroid Linker Regions (TLR), which are supposed to contain matrix attachment regions (MAR) (Aoki & Carrell, 2003; Singh, 1997; Wykes & Krawetz, 2003) (*Figure 2*). Particularities of this model that are supported by scientific evidence are: a) the presence of histones instead of protamines in large segments of DNA (Hammoud et al., 2009; Wykes & Krawetz, 2003); b) the formation of toroids by the attachment of protamines to the DNA, which results in structures highly resilient to nuclease digestion (Brewer et al., 1999; Hud et al., 1993; Sotolongo et al., 2003, 2005); and c) the organisation of protamine-DNA toroids in loops (Sotolongo et al., 2003) that are attached

to the nuclear matrix (Kramer & Krawetz, 1996; Ward et al., 1989). Despite advances in the recent years, some aspects are still theoretical and need to be experimentally proven, including the stacked disposition of toroids, the presence of histones rather than protamines in TLRs, and the specific localisation of TLRs in sperm chromatin (Ribas-Maynou et al., 2022b).

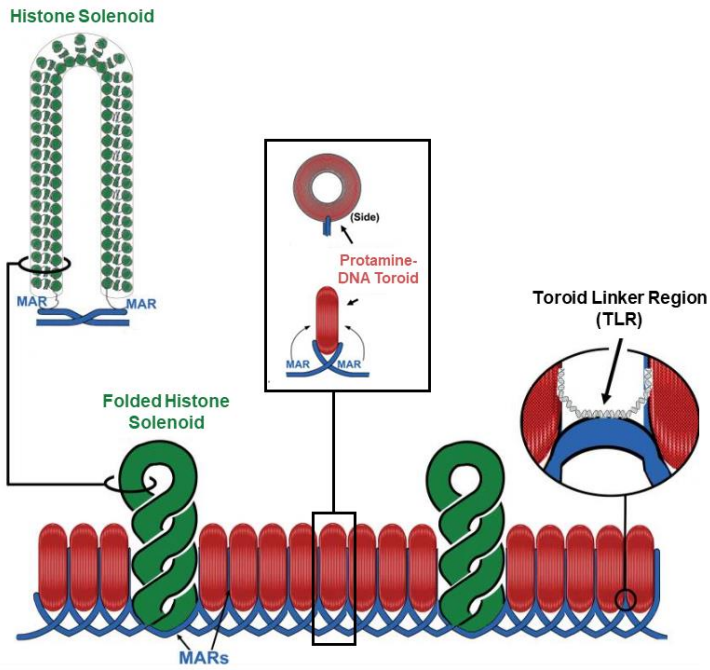

Figure 2. Organisation of sperm chromatin in protamine toroids (red) and histone-packed regions (green). Protamine-toroids are linked to DNA by matrix attachment regions (MAR, Blue) and to each other by toroid linker regions (TLR). Modified from Ward (1993).

2.2. Sperm physiology

2.2.1. Epididymal maturation

After spermatogenesis, sperm undergo a maturation process during their transit through the epididymis. Amongst other changes, most disulphide bonds between cysteine residues of protamines (Baker et al., 2015) are

formed and sperm acquire progressive motility (Cornwall, 2008; Gervasi & Visconti, 2017; Sullivan & Mieusset, 2016).

Epididymal maturation events are dependent on the surrounding environment, highly influenced by the region-specific secretory and adsorptive activities of epididymal epithelial cells (Browne et al., 2016; Zhou et al., 2018). The composition of the epididymal fluid is controlled by these adsorptive and secretion activities, and includes proteins, enzymes, ions, hormones, miRNAs and small organic molecules of the epithelial cells, which can be found free or contained in extracellular vesicles (epididymosomes) (Machtinger et al., 2016). The composition of the epididymal fluid differs between epididymal regions (Brooks, 1981; Da Silva et al., 2006; Turner et al., 1979), which has been linked to the particular functions of each region. In this regard, the composition in the caput and corpus regions has been described to be appropriate for morphological and physiological changes of sperm, whereas that of the cauda has been related to sperm survival during storage in the cauda before ejaculation (Browne et al., 2016; Zhou et al., 2018).

The most important modifications during epididymal maturation affect the lipids and proteins of the plasma membrane, which contributes to reshaping the sperm surface (Dacheux & Voglmayr, 1983; Scott et al., 1967). These changes include the proteolytic removal of sperm surface proteins, as well as their relocalisation, changes in the molecular weight, and modification of their glycosyl units by glycosylation or deglycosylation. These modifications are essential for sperm to undergo post-ejaculatory events like capacitation, acrosome reaction and fusion with the oocyte membrane (Dacheux & Dacheux, 2014). In addition, the specific set of proteins acquired during the epididymal maturation, together with the increased intracellular cAMP levels, are essential for the acquirement of sperm motility, which includes the initiation of the flagellar movement

followed by its coordination into a wave-shaped whipping motion that enables mature sperm to advance progressively (Dacheux & Dacheux, 2014).

Finally, regarding sperm chromatin, during the sperm transit through the epididymis, protamines undergo the oxidation of thiol groups forming inter- and intra-protamines disulphide bonds (Baker et al., 2015; Shalgi et al., 1989). Moreover, the developing sperm cells can internalise proteins and miRNAs, which are released in an epididymal region-specific manner to the intraluminal fluid both free and/or contained in epididymosomes, and play an active role in sperm maturation. These proteins are involved in: i) the regulation of sperm membrane calcium channels, important for proper sperm capacitation (i.e., CRISP1 and LCN6); ii) the posttranslational modification of sperm proteins, which provide the control of protein activity in sperm, as sperm cells are transcriptionally silent (i.e., RNAse10, INPP5b and SPINK13); iii) changes of the sperm membrane lipid composition (i.e., CES5A and SERPINA16); and iv) cell-to-cell interactions essential to recognise and fertilise the oocyte (i.e., DCXR and SOB2) (Björkgren & Sipilä, 2019; Zhou et al., 2018). Besides, the miRNAs present in the intraluminal fluid (free and/or contained in epididymosomes) have been reported to regulate the gene expression of the epithelial cells from the differentiated epididymal segments (Belleannée et al., 2013; Browne et al., 2018; Twenter et al., 2017).

2.2.2. Sperm capacitation and acrosome reaction

Capacitation is the gradual process that sperm undergo during their journey along the female reproductive tract to become fertilising competent. This process involves several physiological and biochemical modifications, including changes in the motility pattern and plasma

membrane architecture, the activation of several signalling pathways, and the acquisition of the ability to trigger the acrosome reaction (Signorelli et al., 2012; Visconti, 2009; Zaneveld et al., 1991; Zigo et al., 2020). As a result, capacitated sperm develop the potential to bind and penetrate the zona pellucida and the oolemma, to further activate the fertilised oocyte (Puga Molina et al., 2018; Yanagimachi, 1994; Zaneveld et al., 1991).

Capacitation events are initially blocked when sperm are mixed with SP, as the latter contains decapacitating factors. Two types of events are distinguished in sperm capacitation: the fast/early, which occur upon ejaculation – when sperm are deposited in the female tract – and include motility activation; and the slow/late, which take place in the female oviduct and include motility hyperactivation. Both in the fast/early and slow/late events, bicarbonate (HCO_3^-) plays a main role (Ickowicz et al., 2012; Jin & Yang, 2017; Puga Molina et al., 2018). When sperm is ejaculated and enter the female reproductive tract, the increased concentration of HCO_3^- present in the SP stimulates the activation of the sperm-specific, soluble Adenylyl Cyclase (sACY). As a result of sACY activity, cyclic adenosine monophosphate (cAMP) levels are increased, promoting the activation of the Protein Kinase A (PKA) pathway (Ickowicz et al., 2012; Jin & Yang, 2017), which stimulates the phosphorylation of several substrates, leading to sperm motility activation (Visconti, 2009). The initial activation of the cAMP/PKA pathway induces other capacitation-associated events, including the increase of intracellular pH (Wang et al., 2003, 2007; Zeng et al., 1996), the hyperpolarisation of the plasma membrane (Demarco et al., 2003), the removal of cholesterol from the plasma membrane (Gadella & Harrison, 2002; Harrison & Miller, 2000) and the increase of Ca^{2+} permeability via specific calcium channels (Ickowicz et al., 2012; Signorelli et al., 2012), thus promoting the release of sperm from the oviductal reservoir and initiating late/slow capacitation events. The simultaneous

HCO_3^- and Ca^{2+} influx contributes to the phosphorylation of serine-threonine protein residues by PKA, finally increasing protein tyrosine phosphorylation (Ickowicz et al., 2012; Visconti, 2009). Overall, sperm that complete capacitation display hyperactivated motility and have the ability to elicit the acrosome reaction (Visconti, 2009).

As explained above, capacitated sperm in the upper isthmus of the oviduct are prepared to trigger the acrosome reaction, which is likely to be induced by progesterone and other factors released by cumulus cells and/or present in the follicular fluid (Bray et al., 2002; Buffone, Hirohashi, et al., 2014; La Spina et al., 2016; Shi, 1997; Torres-Fuentes et al., 2015). Over the years, the induction of acrosome reaction was attributed to the initial contact between hyperactivated sperm and the zona pellucida (Yanagimachi & Phillips, 1984; Yeste, 2013), but recent investigations support the potential implication of progesterone and other factors released by the cumulus cells to the oviductal fluid. Thus, acrosome reaction would be triggered in the upper isthmus of the oviduct before sperm interact with oocyte vestments (Buffone, Wertheimer, et al., 2014; La Spina et al., 2016; Yanagimachi & Phillips, 1984; Yeste et al., 2017). The acrosome reaction is a Ca^{2+}-dependent process that involves the fusion of the sperm plasma membrane with the outer acrosome membrane, releasing the acrosomal content, which enzymatically degrades oocyte vestments and contributes sperm to reach the oolemma (Ickowicz et al., 2012; Yanagimachi, 2011; Zaneveld et al., 1991). In mammals, mature oocytes are surrounded by the zona pellucida that, in turn, is covered by several cumulus cell layers which synthesise hyaluronic acid. This implies that sperm need to pass through cumulus layers and then through the zona pellucida. In this itinerary, sperm appear to rely upon both the mechanical properties of hyperactivated motility resulting from capacitation (Quill et al., 2003) and the release of the acrosome enzymatic content, which

digests the extracellular matrix surrounding cumulus cells and allows sperm penetration to reach the zona pellucida. Once there, sperm galactosyl transferase binds to ZP3 protein in a species-specific process and penetrates the zona pellucida. Finally, sperm and oocyte plasma membranes bind thanks to IZUMO1 and JUNO proteins from sperm and oocyte, respectively (Bianchi et al., 2014; Gadella & Evans, 2011; Stein et al., 2004).

2.3. Sperm implications in fertilisation

Upon fertilisation, sperm-specific proteins such as phospholipase C zeta (PLCζ) activate the oocyte via a yet unidentified mechanism (Saunders et al., 2002). Phospholipase C zeta hydrolyses phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol-1,4,5-triphosphate, which interacts with its receptor in the endoplasmic reticulum thus initiating Ca^{2+} oscillations that promote several processes related to embryo progression. These include polyspermy blocking, metaphase II resumption and pronuclear formation, amongst others (Gilbert, 2000a; Liu, 2011).

Also after fertilisation, while the oocyte is completing the second meiotic division (anaphase II and telophase II), sperm chromatin undergoes a second remodelling of its structure and composition (McLay & Clarke, 2003). First, the disulphide bonds of paternal protamines are reduced, which leads to chromatin decondensation and activates protamine replacement by oocyte-provided histones through an ATP-dependent process. After histone assembly, paternal chromatin becomes again organised into nucleosomes. It is during this stage that methylation of paternal DNA starts to decrease actively due to demethylases, whereas the maternal pronucleus does not exhibit differences in the degree of

methylation (Dean et al., 2001; Ivanova et al., 2020; Oswald et al., 2000; Santos et al., 2002; Smith et al., 2014).

Mounting evidence supports oocyte cytoplasm as the main player governing sperm chromatin remodelling after fertilisation (McLay & Clarke, 2003; Zafar et al., 2021). In effect, whether proper paternal chromatin remodelling occurs depends on the stage of the oocyte, as earlier experiments showed that if oocytes are fertilised at prophase I, sperm chromatin does not undergo morphological changes and remains strongly condensed (Clarke & Masui, 1986; Maeda et al., 1998; Usui & Yanagimachi, 1976). In contrast, when metaphase II oocytes are fertilised, sperm chromatin is quickly dispersed to replace protamines with histones (Clarke & Masui, 1986; McLay & Clarke, 1997). Based on these findings, the remodelling of paternal chromatin seems to largely rely upon the meiotic maturation stage of the oocyte. Furthermore, the extensive decondensation of the paternal pronucleus also requires the oocyte to complete the M-phase and enter the interphase. This progression in the cell cycle, which is also governed by the Ca^{2+} oscillations triggered by PLCζ (Ducibella et al., 2002; Ducibella & Fissore, 2008; Saunders et al., 2002), entails the oocyte cytoplasm changes required for paternal chromatin decondensation.

3. Alterations in sperm chromatin

Conventional sperm quality parameters include concentration, motility and morphology, and their evaluation is aimed at predicting the male fertilising capacity. During the last decades, nevertheless, special emphasis on sperm DNA integrity has been made, as affectation of this biomolecule

may result in fertilisation failure, poor embryo development, pregnancy loss and developmental defects in the offspring (Huang et al., 2005; Morris, 2002; Virro et al., 2004). In addition, other sperm chromatin alterations such as poor protamination and poor condensation are now considered. The evaluation of these variables provides a comprehensive picture of the sperm chromatin status, and also contributes to determine the male fertilising capacity better.

3.1. Types of chromatin alterations

3.1.1. Protamine alterations

During spermiogenesis, nuclear histones are replaced by protamines, which results in the organisation of sperm DNA into a tightly packed structure that helps the preservation of paternal genome integrity during the journey of sperm cells through both male and female genital tracts and until they interact with the oocyte (Balhorn, 2007; Oliva, 2006). In mammals, an appropriate protamination plays a crucial role in sperm chromatin conformation (Gill-Sharma et al., 2011; Miller et al., 2010; Ward, 2010); therefore, insufficient protamination during spermiogenesis may result in an increased number of sperm cells with immature chromatin, which exhibit a higher incidence of histone-packed DNA regions (Oliva, 2006). As these regions have been shown to be more susceptible to DNA damage than those properly protaminated, abnormal chromatin protamination can negatively impair sperm function and cause male subfertility or infertility (Oliva, 2006; Sakkas, 1999; Sharma et al., 2004; Souza et al., 2018). Indeed, chromatin deprotamination has been described to be higher in non-viable than in viable sperm in cattle (Llavanera et al., 2021), and correlated with lower sperm concentration,

decreased motility and altered morphology in humans (Manochantr et al., 2012). Furthermore, insufficient chromatin protamination is more frequent in infertile than in fertile men (Jumeau et al., 2022; Manochantr et al., 2012).

The ratio between protamines and histones in sperm chromatin has also been associated with male fertility in different studies (Hamad, 2019; Zhang et al., 2006; Zini et al., 2007), as sperm from infertile men exhibit a lower protamine:histone ratio compared to their fertile counterparts (Zini et al., 2007). In other species like cattle, sperm protamine content has been positively correlated with *in vivo* fertility as increased protamine levels are exhibited by bulls with high fertility (Dogan et al., 2015; Souza et al., 2018). Thus, low sperm chromatin protamination has been linked to reduced male fertility. In a similar line of evidence, pre-pubertal bulls and rat males have been found to present a high percentage of sperm with protamination deficiency compared to reproductive mature individuals (Kipper et al., 2017; Zubkova et al., 2005), which suggests that sperm protamination depends on the male reproductive maturity. Sperm deprotamination, in addition to being associated to lower fertilisation, is also linked to reduced sperm quality. Chromatin protamination could thus be regarded as a feature of immature sperm, as its degree is lower in infertile men with abnormal sperm function parameters such as motility and morphology (Iranpour et al., 2000).

It is known that humans, mice and horses exhibit a similar content of two types of protamines, protamine 1 (P1) and protamine 2 (P2) (Balhorn et al., 1988; Bower et al., 1987; Gusse et al., 1986; Mengual et al., 2003). Several studies reported that an appropriate ratio between P1 and P2 in sperm chromatin is necessary to maintain male fertility, as an increased P1/P2 ratio is associated to men infertility (Balhorn et al., 1988; Mengual et al., 2003; Torregrosa et al., 2006). This P1/P2 imbalance has been related with a reduction of the P2 content (Belokopytova et al., 1993; de Yebra et

al., 1993, 1998), which has been reported to cause DNA damage and impair embryo development *in vitro* (Cho et al., 2003; Lolis et al., 1996; Nasr-Esfahani et al., 2005). In this regard, P1/P2 ratio in sperm cells is considered as a predictive indicator of male fertility and *in vitro* embryo development.

3.1.2. Chromatin decondensation

Sperm chromatin condensation involves the stabilisation of sperm protamines through intra- and inter-protamine disulphide bonds, which become fully oxidised during epididymal transit. This protamine-mediated stabilisation and condensation of sperm DNA protects genetic information from damaging agents, during the journey of sperm along the male and female reproductive tracts. While maintaining a condensed chromatin is very important over that transit, its decondensation is essential after gamete fusion for the formation of the male pronucleus (McLay & Clarke, 2003; Mudrak et al., 2009). Thus, changes in chromatin condensation before fertilisation, which can be caused by different factors such as zinc or protamine deficiencies (Aoki et al., 2006; Balhorn et al., 1988; Kvist et al., 1988), might have a detrimental effect on sperm fertility.

Although chromatin condensation and protamination are different features of sperm chromatin, the scientific literature usually confounds these terms and relates the assessment of protamine content to chromatin condensation. In this regard, a recent study in human healthy patients showed that while protamination evaluated using the CMA_3 test is related to the fertilising ability of sperm cells, chromatin condensation evaluated through the dibromobimane test is associated to higher blastocyst development (Ribas-Maynou et al., 2023).

3.1.3. DNA fragmentation

Sperm DNA fragmentation has been extensively studied during the last decades as a complementary parameter of sperm fertilisation potential, as it is related to the reproductive success (Simon et al., 2010). DNA damage may result from intrinsic or extrinsic effector mechanisms, and can take place at either testicular level or post-testicular stages (Sakkas & Alvarez, 2010).

Sperm DNA fragmentation is characterised by the presence of single- and double-strand breaks in the genome. Single-strand breaks (SSB) are mostly caused by oxidative stress, as reactive oxygen species can oxidise 8-OH-guanine into 8-OH-2′-deoxyguanosine (8-OHdG). 8-OH-2′-deoxyguanosine is then excised by oxoguanine glycosylase (OGG), the first enzyme of the base excision repair mechanism (BER), forming an abasic site (Barzilai & Yamamoto, 2004; De Iuliis et al., 2009; Santiso et al., 2010). On the other hand, double-strand breaks (DSB) are mainly associated with a defective enzymatic activity during spermatogenesis and spermiogenesis (Ribas-Maynou et al., 2012b). As SSB can occur in both protamine- and histone- condensed regions, they can be found in both toroidal and TLR regions. In contrast, DSB have been suggested to be preferentially located at the TLR regions, due to their accessibility (Ribas-Maynou et al., 2012b).

Sperm DNA fragmentation has been related to semen quality and fertility outcomes both *in vitro* and *in vivo*. In terms of sperm quality, a high incidence of DNA fragmentation is concomitant with a poor semen quality (Lopes et al., 1998), as low sperm concentration, viability and progressive motility are observed together with a greater percentage of sperm with fragmented DNA (Le et al., 2019; Mateo-Otero et al., 2022; Muriel et al., 2006; Sivanarayana et al., 2014). Moreover, sperm malformations, such as

elongated heads or the lack of lateral symmetry, have also been associated with DNA damage (Dariš et al., 2010; Le et al., 2019; Utsuno et al., 2013). Besides, high DNA fragmentation has been observed to be linked to *in vitro* and *in vivo* fertility rates (Bichara et al., 2019; Bungum et al., 2011; Dogan et al., 2015; Souza et al., 2018). Regarding natural conception, studies in humans, horses and pigs reported the relationship of sperm DNA fragmentation with the lack of clinical pregnancy and the increase of conception time (Boe-Hansen et al., 2008; Didion et al., 2009; Evenson, 1999; Giwercman et al., 2010; Kenney et al., 1995; Ribas-Maynou, et al., 2012a). On the other hand, there exist inconsistent results regarding the relationship between sperm DNA damage and fertility outcomes when using assisted reproductive techniques. In IVF treatments, sperm DNA fragmentation has been reported to exert a negative impact on fertilisation, implantation and pregnancy rates; impair embryo quality; and reduce live-birth rate (Evenson & Wixon, 2006; Henkel et al., 2004; Niu et al., 2011; Ribas-Maynou et al., 2021; Simon et al., 2017; Zhang, 2008; Zhang et al., 2015). Conversely, opposite results were found regarding the effects of sperm DNA fragmentation on embryo quality, implantation and pregnancy rates following ICSI (Agarwal et al., 2020; Casanovas et al., 2019; Evenson & Wixon, 2006; Ribas-Maynou et al., 2021; Simon et al., 2017; Zhang et al., 2015; Zini et al., 2005).

3.2. Aetiology of sperm DNA breaks

The loss of sperm chromatin integrity can be attributed to either intrinsic or extrinsic factors (Delbes et al., 2010; Hekmatdoost et al., 2009). Intrinsic factors include pathophysiological conditions that may occur during spermatogenesis, spermiogenesis, epididymal maturation or storage in the cauda epididymis. These circumstances include an imbalance between

reactive oxygen species levels and the antioxidant capacity, the activation of enzymatic mechanisms and apoptosis-like events. Extrinsic factors, such as high temperature, infections, radiations, environmental toxins or even nutrition (González-Marín et al., 2012; Panner Selvam et al., 2021), can also induce DNA breaks, and may be combined with the intrinsic ones.

3.2.1. Oxidative stress

Oxidative stress results from the disrupted balance between the production of reactive oxygen species (ROS) and the activity of intracellular and extracellular antioxidants (Sikka, 2001). It has been described as one of the main factors generating genotoxic defects such as DNA breaks (Aitken & Krausz, 2001), thus negatively affecting sperm function and fertilising ability. For this reason, oxidative stress has been associated with infertility (Agarwal et al., 2007). Oxidative stress can ensue from either endogenous production or an exogenous source. In mammalian sperm cells, one of the main endogenous sources of ROS is the activity of plasma membrane NADPH oxidase NOX5 (Ghanbari et al., 2018; Keshtgar & Ghani, 2022; Musset et al., 2012). Besides, oxidative phosphorylation in the mitochondria, which is used to produce ATP together with glycolysis (Dutta et al., 2020; Holland et al., 1982) is also involved in ROS generation(Koppers et al., 2008). It should be noted, however, that sustained levels of oxygen radicals are physiologically necessary to maintain the sperm cell function along maturation, capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion (Agarwal et al., 2003; Du Plessis et al., 2015). Specifically, this active role of ROS is mainly carried out through the regulation of the redox status of thiol groups. This action stimulates adenylyl cyclase by increasing intracellular cAMP concentration which, in turn, activates PKA, thereby triggering

various specific downstream cell signalling pathways resulting in the hyperactivation of sperm motility and a complete capacitated status (Betarelli et al., 2018; Du Plessis et al., 2015; Dutta et al., 2020; Griveau & Lannou, 1997; Thompson et al., 2013). Also, nitric oxide and hydrogen peroxide are compounds involved in certain processes such as capacitation and acrosome reaction (Aitken & De Iuliis, 2010; Marzec-Wróblewska et al., 2012). Moreover, ROS levels are needed to trigger the acrosome reaction (de Lamirande & Gagnon, 1993) and enhance the sperm ability to fertilise the oocyte (Kodama et al., 1996).

Despite playing an essential role in maintaining cell function, excessive ROS generation may produce oxidative stress as it disrupts the balance between oxidative activity and antioxidant capacity; this has a negatively impact upon sperm physiology and fertility (Agarwal et al., 2003; Garrido et al., 2004; Sikka, 2001; Zini et al., 1993). Related with this, it is worth keeping in mind that intracellular and extracellular antioxidants scavenge the excess of ROS and thus contribute to maintain redox homeostasis and, consequently, sperm function and fertilising ability (Barratt et al., 2017; de Lamirande, 1997; Kowalczyk, 2022; Showell et al., 2014; Smits et al., 2018). Yet, when the excess of ROS cannot be mitigated by antioxidant activity, they may interact with - and damage - other components of the cell, such as membrane phospholipids and DNA (Agarwal et al., 2003; Aitken & Krausz, 2001; Kodama et al., 1996). Elevated amounts of ROS thus increase lipid peroxidation levels, resulting in the destabilisation of plasma and acrosome membranes (Agarwal et al., 2003; Aitken & Krausz, 2001; Kodama et al., 1996; Padron et al., 1997). In terms of DNA damage, oxidative stress has been reported to induce histone modifications, alterations in DNA methylation and DNA fragmentation (Kreuz & Fischle, 2016), specifically causing SSB in both protamine- and histone-condensed regions (Agarwal et al., 2007; Aitken & De Iuliis, 2010;

Enciso et al., 2009; Ribas-Maynou et al., 2014). Furthermore, ROS levels in semen have been shown to negatively correlate with normal sperm function and underlie in/subfertility (Agarwal et al., 2007; Aitken et al., 1998). It is thus essential maintaining proper ROS homeostasis to prevent sperm cell damage.

3.2.2 Enzymatic mechanisms

Activity of nucleases has been detected in both sperm cells and seminal fluid from different species such as mice and humans (Fernandez-Encinas et al., 2016; Maione et al., 1997; Mann & Lutwak-Mann, 1981; Sotolongo et al., 2005). The activation of an endogenous nuclease in mature sperm cells has been suggested as a natural defence against the intrusion of exogenous molecules such as exogenous DNA (Maione et al., 1997). In addition, activity of intracellular and extracellular nucleases in mouse epididymal and vas deferens sperm has been observed to induce sperm chromatin fragmentation both *in vitro* (Yamauchi, Shaman, & Ward, 2007) and *in vivo* (Ribas-Maynou et al., 2022a). Moreover, nuclease activity has been reported to differ between the epididymis and ejaculated sperm harvested from the female uterus (Yamauchi et al., 2007a). Such a nuclease activity in sperm and surrounding fluids appears to increase through the epididymis and upon ejaculation, as it is low in the corpus, slightly higher in the cauda, moderate in the vas deferens, and the highest in the post-ejaculated fraction (Yamauchi et al., 2007a). This strong nuclease activity of ejaculated sperm could account for their increased susceptibility to DNA damage under stress, which could be part of a mechanism to positively select the most resilient (i.e., best) sperm (Yamauchi et al., 2007a).

It is known that nuclease activity may generate chromatin damage at the toroid linker regions, which have been shown to be accessible to

enzymes and, therefore, nuclease-sensitive (Ward, 2010). Noticeably, poorly protaminated and histone-condensed chromatin may be more accessible to endogenous and exogenous nucleases, and thus exhibit a higher susceptibility to DNA damage (Sakkas & Alvarez, 2010). This is also associated to a reduction of sperm fertilising capacity.

Other enzymatic activity that could lead to DNA damage was observed in experiments that used Mn^{2+} and Ca^{2+} to induce sperm chromatin fragmentation via an intracellular topoisomerase, known as topoisomerase IIβ (TOPIIβ) (Shaman et al., 2006; Yamauchi, Shaman, & Ward, 2007). This topoisomerase could be activated *in vitro* to produce DNA breaks in the toroid linker regions through a sperm chromatin fragmentation mechanism (Sotolongo et al., 2005), causing a delay in DNA replication at the first stages of embryo development and an impaired development to blastocyst (Gawecka et al., 2015). In these experiments, TOPIIβ was seen to act together with an exonuclease present in the vas deferens fluid, which could cleave the DNA into small fragments (Shaman et al., 2006; Yamauchi, Shaman, & Ward, 2007). A decade after, additional experiments demonstrated that the DSB produced through that topoisomerase during the epididymal stage occurred in DNA regions attached to the nuclear matrix, thus facilitating the further repair of these DNA breaks by the fertilised oocyte before the formation of the male pronucleus after fertilisation (Ribas-Maynou et al., 2014).

3.2.3. Abortive apoptosis

Apoptosis is a cell process that entails its programmed death. This process also occurs in germ cells during spermatogenesis, regulating sperm overproduction and ensuring normal proliferation (Sinha Hikim & Swerdloff, 1999). In the case of mature/ejaculated sperm, however,

apoptotic pathways appear to be truncated, so that one cannot establish the existence of a proper apoptotic process, but an apoptosis-like one that also causes DNA breaks (Aitken & De Iuliis, 2010).

During spermatogenesis, Sertoli cells are responsible for maintaining a normal proliferation of germ cells, as they remove abnormal meiotic or post-meiotic cells; this works like a quality control of sperm production. One of the possible key mediators of germ cell apoptosis in the testis is the Fas/FasL system (Lee et al., 1997), which is based on the expression of the Fas protein in the membrane of the cells that must be removed, and the presence of its ligand, the FasL protein, in the membrane of Sertoli cells (Pentikäinen et al., 1999). Binding of FasL to Fas initiates the apoptosis process, which ends up with the phagocytosis of apoptotic germ cells by Sertoli cells (Eguchi et al., 2002; Lee et al., 1997). The relevance of this pathway was confirmed by the observed correlation between Fas expression and germ cell degeneration during meiotic and post-meiotic phases of spermatogenesis in humans (Francavilla, 2002).

Besides, several authors identified other apoptotic markers such as BCL2 and TP53 in ejaculated sperm exhibiting different alterations (Barroso et al., 2000; Oldereid et al., 2001; Sakkas et al., 2002). As aforementioned, while apoptotic markers can be found in ejaculated sperm, one should be cautious when speaking about apoptosis in these cells and rather designate this event as apoptosis-like, abortive apoptosis or spermptosis (Aitken & De Iuliis, 2010; Martín Muñoz et al., 2018). Be that as it may, this process is known to be initiated by oxidative stress, which triggers a caspase cascade that subsequently induces the activation of an endonuclease. This endonuclease then cleaves the sperm DNA into fragments (Muratori et al., 2015; Sakkas, 1999; Sakkas et al., 2004).

3.3. Sperm chromatin and DNA integrity assays

3.3.1. Sperm chromatin protamination

During routine semen analysis, the chromatin protamination status is not usually evaluated, even though there is evidence of its important role in male fertility (Llavanera et al., 2021; Ribas-Maynou et al., 2020a; Ribas-Maynou et al., 2023). Among the methods used in research to determine the amount of protamines present in sperm chromatin, the most commonly used are Chromomycin A_3 and Aniline Blue staining (Dutta et al., 2021; Ni et al., 2016; Tanga et al., 2021). The amount of protamines with respect to histones can also be semi-quantitatively evaluated through Western blot (Hamilton et al., 2019; Mengual et al., 2003).

Chromomycin A_3 (CMA_3)

Chromomycin A_3 (CMA_3) is a fluorescent dye that specifically binds to guanine-cytosine sequences in the DNA. As it competes with protamines for the same binding sites in the DNA, high levels of CMA_3 fluorescence are indicative of low levels of protamination in sperm. The detection of protamine-deficient DNA thus provides an estimation of the number of sperm with poor chromatin protamination (Bianchi et al., 1993; Lolis et al., 1996; Manicardi et al., 1995; Srivastava & Megha Pande, 2017). Positive CMA_3 staining has been shown to be related to morphologically abnormal and immotile sperm (Bianchi et al., 1996; Nijs et al., 2009), leading to male infertility. Also, poor chromatin protamination has been associated to sperm decondensation failure after fertilisation using IVF and ICSI procedures, resulting in low fertilisation rates (Esterhuizen et al., 2000; Llavanera et al., 2021; Lolis et al., 1996; Nasr-Esfahani et al., 2008; Sakkas

et al., 1996, 1998). Thus, evaluation of chromatin protamination with CMA_3 may predict the IVF success (Esterhuizenet al., 2000).

Acidic Aniline Blue Staining

The degree of chromatin protamination can be assessed with acidic aniline blue staining. This dye reveals differences in the composition of basic amino acids from sperm nuclear proteins, by discriminating between lysine-rich histones and arginine/cysteine-rich protamines (Hofmann & Hilscher, 1991). Staining of sperm cells with aniline blue indicates the presence of lysine in nuclear proteins. Because histones, but not protamines, are rich in lysine, strong aniline blue staining indicates low protamination (Hammadeh et al., 2001; Hofmann & Hilscher, 1991). After this procedure, the percentage of sperm exhibiting abnormal chromatin protamination can be calculated as the ratio between the number of blue-stained sperm and the total number of sperm analysed (Srivastava & Megha Pande, 2017). Chromatin protamination results obtained through this technique seem to be a good predictor of human male infertility and IVF outcomes, despite some works having not observed an association with ICSI outcomes (Auger et al., 1990; Hammadeh et al., 2001, 2009).

Acetic acid-urea polyacrylamide gel electrophoresis

Polyacrylamide acid-urea gel electrophoresis (PAGE) is a technique used to distinguish similar proteins based on differences in their molecular weight and effective charge (acid or basic) determined by their charge-to-mass ratio (Hamilton et al., 2021). Thus, proteins that are more acidic and have phosphorylated threonine or serine side chains or acetylated lysine side chains exhibit a different electrophoretic mobility compared to their

basic counterparts. The PAGE method has been used to evaluate the relative amounts of histones, protamines and transition proteins, and their posttranslational variants. In particular, it is useful to easily separate protamines from histones, but also to distinguish acetylated histones or the different protamine forms (protamine 1 and protamine 2) (de Yebra & Oliva, 1993; Hamilton et al., 2019; Liu et al., 2013; Panyim & Chalkley, 1969). Following PAGE, the different proteins and its variants can be identified and quantified by Western blot (Hamilton et al., 2019; Hazzalin & Mahadevan, 2017).

3.3.2. Sperm chromatin condensation

As previously explained in Section *2.1.1.* about *Sperm chromatin remodelling*, binding of protamines to DNA drives condensation of sperm chromatin into toroidal structures that are stabilised thanks to disulphide bonds. The assessment of protamine oxidation-reduction status in sperm chromatin thus estimates the degree of condensation (Brewer et al., 2003).

The analysis of chromatin condensation through the determination of oxidation-reduction status of disulphide bridges can be assessed with dibromobimane (DBB) (Ribas-Maynou et al., 2023). This technique is based on the capacity of DBB to bind the free thiol groups (-SH) resulting from the reduction (i.e., disruption) of disulphide bonds. Since mammalian sperm protamine 1 contain between five and ten cysteine residues (Gosálvez et al., 2011), the major amount of thiol groups in these cells is due to protamine disulphide bridges. As a result of the covalent bond between DBB and a free thiol, DBB emits fluorescence with a peak at 477 nm. In contrast, if protamine disulphide bridges are oxidised (i.e., formed), DBB is not able to react with free thiols and, therefore, no fluorescence is emitted. Thus, the fluorescence emitted by DBB is negatively correlated to

chromatin condensation, as the higher the proportion of free thiols - and thus the lower the number of the disulphide bridges formed -, the lower the sperm chromatin condensation (Ribas-Maynou et al., 2023).

3.3.3. Sperm DNA fragmentation

Sperm chromatin structure assay (SCSA)

The sperm chromatin structure assay (SCSA) is a flow-cytometry based test that evaluates the susceptibility of sperm DNA to denaturation, a feature that is highly associated with DNA fragmentation (Bungum et al., 2011; Evenson, 2016). This procedure involves the treatment of the sample with an acid solution to unwind the DNA strands, which occurs preferentially at the sites where DNA breaks are present. After staining with acridine orange, a fluorescent dye, the fluorescence intensity is measured with a flow cytometer and the percentage of cells with a denatured DNA can be calculated.

The metachromatic properties of acridine orange, which emits green fluorescence (510-515 nm) when interspersed as a monomer into double-strand DNA, and red fluorescence (630 nm) as a result of its binding to single-strand DNA in an aggregate manner (Evenson et al., 2007) is the basis of the detection mechanism, as DNA containing breaks is more susceptible to denaturate. This differential emission allows the detection of sperm with a high incidence of DNA breaks. In this sense, sperm with low DNA fragmentation emit green fluorescence and sperm with highly fragmented DNA emit mostly red fluorescence. The main outcome of SCSA is the DNA fragmentation index (DFI), which reflects the percentage of sperm with fragmented DNA and can be calculated by the ratio between red and the sum of red and green fluorescence (Evenson,

2013, 2016; Shaman et al., 2006). In addition, a sperm subpopulation with high chromatin immaturity, named High DNA Stainability (HDS), can be detected through this method. This subpopulation has been described to correspond to those cells that have a better access to all chromatin due to a poor interchange of histones by protamines (Evenson, 2016).

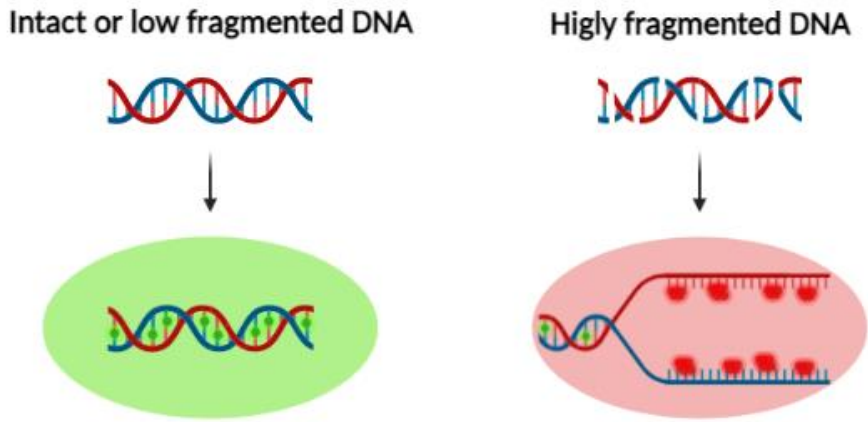

Figure 3. Schematic representation of the sperm chromatin structure assay (SCSA). Acridine orange emits green or red fluorescence when binds to the DNA as a monomer (intact or low fragmented DNA) or aggregate (fragmented DNA), respectively. *Figure created with BioRender*.

Sperm Chromatin Dispersion Test (SCD)

The sperm chromatin dispersion test (SCD) relies on the fact that damaged sperm present greater DNA decondensation when sperm are treated with an acidic solution and the nuclear proteins are removed. In most mammals, the nucleus of sperm with non-fragmented DNA exhibit small and compact dispersion halos, and the presence of DNA breaks results in an extended halo dispersion, usually not visible due to the large dispersion of chromatin (Fernández et al., 2003, 2011). In men, however, dispersion halos are absent when sperm DNA is fragmented, whereas the nuclei of non-fragmented DNA exhibit a visible dispersion halo. The resulting halos can

be evaluated by optical or fluorescence microscopy, and the percentage of sperm with non-dispersed and dispersed nuclei can be determined. Thus, the SCD test is a simple, fast, and reproducible test to evaluate sperm DNA fragmentation (Fernández et al., 2003, 2011).

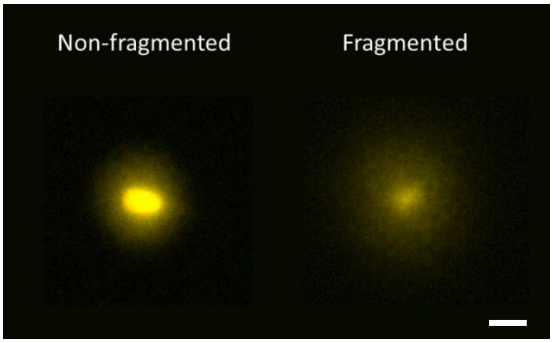

Figure 4. Sperm chromatin dispersion test (SCD) evaluation. In most mammals but not in humans, sperm with non-fragmented DNA exhibit small and compact dispersion halos, while the presence of DNA fragmentation results in an extended halo dispersion (usually not visible). Scale bar: 10 µm.

Single-Cell Gel electrophoresis assay (Comet)

The Comet assay is a commonly used assay in somatic cells for toxicology studies and was adapted to sperm cells by Singh (1988) (Singh et al., 1988). The Comet assay is a laborious method with a wide range of protocols, that mainly differ in the lysis step and electrophoresis times (Enciso et al., 2009; Simon et al., 2011; Singh et al., 1988; Villani et al., 2010). Briefly, sperm cells are first embedded in an agarose matrix and then exposed to a lysis buffer. Then, lysed sperm cells are subject to electrophoresis, in which the DNA presenting breaks migrate towards the anode, resulting in a "comet tail". This step of electrophoresis can be conducted in a neutral pH or alkaline pH solution, depending on the type of DNA breaks that are examined. Whereas electrophoresis conducted in a neutral pH solution leads to the detection of DSB (Neutral Comet) (Shaposhnikov et al., 2008),

the application of an alkaline pH leads to the detection of both SSB and DSB (Alkaline Comet) (Simon & Carrell, 2013). Results are observed by fluorescence microscopy after staining the sample. Next, sperm cells with fragmented DNA show a brightly fluorescent comet halo and comet tail, whose length and fluorescence intensity rely on the number of DNA breaks. Using a software, the fluorescence intensity of the Comet tail indicates the incidence of DNA breaks in each cell. In addition, using non-damaged reference controls, the percentage of cells with increased DNA fragmentation can be expressed (Simon & Carrell, 2013). Only, the combination of alkaline and neutral Comet allows the determination of global damage, SSB and DSB.

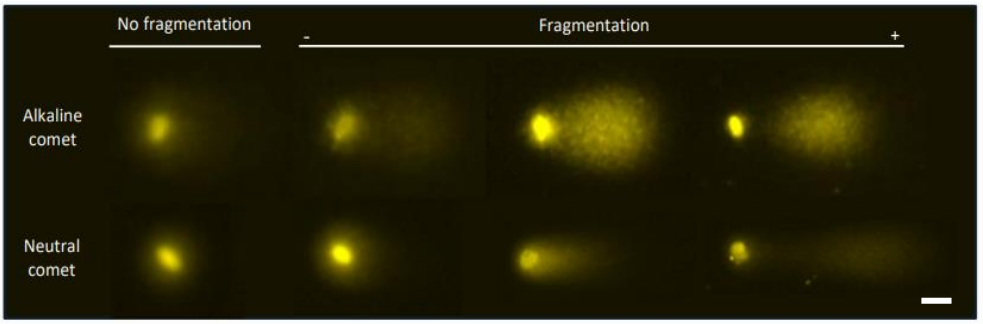

Figure 5. Non-fragmented and fragmented sperm in alkaline and neutral Comet assays. Scale bar: 10 µm.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) Assay

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay is based on the enzymatic incorporation of fluorochrome-conjugated oligonucleotides to free 3′ DNA strands by a terminal deoxynucleotidyl transferase (Sharma et al., 2016). Deoxyuridine triphosphates (dUTP), which are conjugated with a fluorochrome, emit fluorescence that can be assessed by either fluorescence microscopy or

flow cytometry. As the 3'OH DNA ends correspond to single- and double-strand DNA fragmentation, the global percentage of sperm with DNA breaks is obtained as a result (Gorczyca et al., 1993; Sharma et al., 2016).

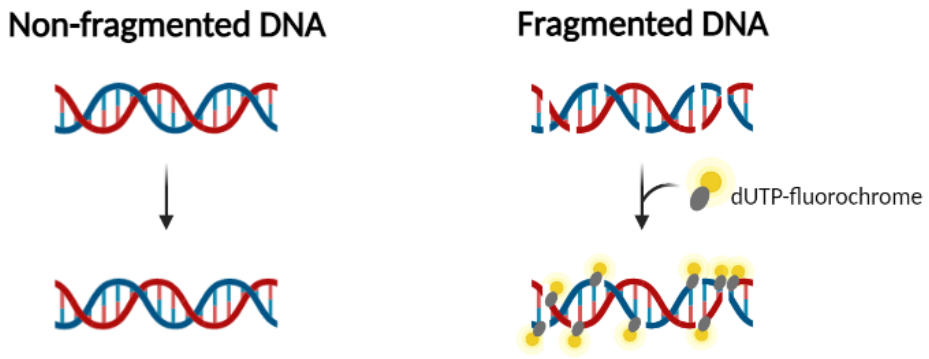

Figure 6. Schematic representation of the terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL). The dUTP-fluorochrome conjugate binds to free 3' DNA strands resulting from DNA breaks, and emits fluorescence. *Figure created with BioRender*.

OBJECTIVES

Against the background compiled in the previous section, the main aim of the present Doctoral Thesis was to elucidate the relevance of chromatin condensation and protamination, and DNA integrity for sperm physiology and fertility.

For this purpose, four specific objectives were set:

1. To determine how frozen-thawed bull sperm chromatin is modified over time after *in vitro* incubation at 38 °C.
2. To investigate the relationship of sperm chromatin condensation, protamination and DNA integrity with sperm function and fertilising ability.
3. To identify whether boar ejaculate fractions differ in the DNA integrity and chromatin condensation and protamination of their sperm.
4. To address whether the sperm chromatin fragmentation (SCF) mechanism can be activated *in vitro* in ejaculated sperm.
5. To evaluate the impact of the DNA fragmentation generated by induced SCF on sperm function and *in vitro* fertilising ability.

METHODS

1. Collection of bovine ejaculates

Ejaculates were collected from bulls housed at Cenero AI centre in Gijón, Asturias (Spain), complying with all European Union regulations for animal husbandry, under standard feeding and housing conditions, to produce commercially available cryopreserved sperm straws. The collection of bull ejaculates was performed through an artificial vagina, with an internal temperature of 45 °C at weekly intervals for five weeks.

2. Cryopreservation and thawing of bovine semen

After collection, ejaculates with 2–8 mL of volume, $> 10^9$ sperm/mL and > 85 % of total motile sperm were subject to cryopreservation. Ejaculates were cryopreserved following the standard protocol described by Muiño et al. (2008) (Muiño et al., 2008). First, sperm concentration was adjusted to 92×10^6 sperm/mL using a commercial extender (Bioxcell; IMV Technologies L'Aigle, France) at 22 °C. Subsequently, ejaculate samples were cooled at a rate of − 0.2 °C/min to a final temperature of 4 °C, and equilibrated at this temperature for 3 h. Samples were subsequently packaged into 0.25-mL straws and cryopreserved using a controlled-rate freezer (Digit-cool; IMV Technologies), with the following cooling rates: 5 °C/min from 4 °C to − 10 °C; 40 °C/min from − 10 °C to − 100 °C; and 20 °C/min from − 100 °C to − 140 °C. Finally, straws were stored in liquid nitrogen until use.

Thawing of frozen samples was performed through immersion of straws at 38 °C for 20 s in a water bath and incubated at the same temperature for up to 4 h.

3. Collection of porcine ejaculates

Pig ejaculate samples were obtained from an AI centre (AIM Ibérica) located in Calasparra (Murcia, Spain), which follows the current Spanish (registration number: ES300130640127, August 2006) and European (registration number: ES13RS04P, July 2012) regulations for commercialisation of porcine semen, and for animal health and welfare. Ejaculates were obtained from healthy and sexually mature boars (aged 18-36 months) from different breeds (Large White and Pietrain). All boars were housed in individual pens with controlled temperature (15-25 °C) and 16 h of light per day. Moreover, animals were fed a commercial diet according to the nutritional requirements of adult boars (Chiba, 2009) and had *ad libitum* access to water.

Upon ejaculation, sperm concentration, motility and morphology were assessed. All ejaculate samples included in the study fulfilled the standard thresholds for the preparation of semen AI-doses, which include a concentration of at least 200 x 10^6 sperm/mL, a minimum of 70 % of motile sperm, and more than 75 % of sperm with normal morphology.

4. Sperm quality evaluation

4.1. Sperm motility

Sperm motility was assessed using a computer assisted sperm analysis system (CASA) (Integrates Sperm Analysis System, ISAS V1.0; Proiser S.L.; Valencia, Spain) coupled to an Olympus BX41 microscope (Olympus; Tokyo, Japan) equipped with a negative field phase contrast (Olympus 10×0.30 PLAN objective, Olympus). Sperm samples were first incubated at 38 °C for 10 min, and 3 µL of porcine or bovine semen were then placed

into a pre-warmed 20 µm Leja chamber slide (Leja Products BV; Nieuw-Vennep, The Netherlands). Two technical replicates were examined, evaluating 1,000 sperm per replicate at 100× magnification. Percentages of total and progressive motility were recorded for each sample. Sperm were considered motile when their average path velocity (VAP) was ≥ 10 µm/s, and progressively motile when their straightness index (STR) was ≥ 70 % in the case of bovine sperm, or ≥ 45 % in the case of porcine sperm. The following kinematic parameters were also recorded: velocity (fast, medium or slow); curvilinear velocity (VCL, µm/s); straight-line velocity (VSL, µm/s); average path velocity (VAP, µm/s); linearity (LIN = VSL/VCL×100, %); straightness (STR = VSL/VAP×100, %); oscillation (WOB = VAP/VCL×100, %); lateral head displacement (ALH, µm); and frequency of head displacement (BCF, Hz).

4.2. Sperm concentration

Sperm concentration (sperm/mL) was determined using a Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel) under an Olympus BX41 microscope (Olympus; Tokyo, Japan). First, samples were diluted in PBS when sperm concentration was very high and individual cells could not be distinguished. Once adjusted to 35 - 50 × 10^6 sperm/mL (Johnson et al., 1996), 5 µL of each sample was placed onto the Makler chamber. Then, under the microscope, the number of sperm contained in the squares of three rows was recorded and the average of the three counts was calculated. After evaluating three replicates, the average cell count obtained corresponded to sperm concentration (× 10^6 sperm/mL).

4.3. Evaluation of sperm with flow cytometry

Flow cytometry parameters described below (viability, and intracellular levels of total ROS, superoxides and calcium) were analysed using a CytoFLEX Flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with red, blue and violet lasers (637, 488 and 405 nm). After adjusting sperm concentration, the corresponding protocol was applied for each parameter, two replicates were examined evaluating 10,000 sperm per replicate (flow rate was between 10 µL/s and 60 µL/s). The analysis of the flow cytometry dot-plots was conducted through the CytExpert Software (Beckman Coulter; Fullerton, CA, USA) in order to obtain the different populations and/or the fluorescence intensity. The mean fluorescence intensity peak (arbitrary units) was exported to Microsoft Excel (Microsoft, Redmond, WA, USA). The device was calibrated daily using the CytoFLEX Daily QC Fluorospheres (Beckman Coulter; Fullerton, CA, USA), as recommended by the manufacturer.

4.3.1. Sperm viability

Sperm viability was determined using SYBR-14 combined with Propidium Iodide (PI). While SYBR-14 is able to penetrate the plasma membrane of viable and non-viable sperm, PI is incapable to penetrate an intact membrane. Thus, viable sperm become stained by SYBR-14$^+$ (green fluorescence), whereas non-viable sperm are labelled by both SYBR-14$^+$ and PI$^+$, or only by PI$^+$ (red fluorescence). Regarding the protocol, sperm concentration was first adjusted to 1×10^6 sperm/mL using pre-warmed and filtered PBS, and sperm were then incubated for 15 min at 38 °C in the dark with SYBR-14 (TermoFisher, Waltham, MA, USA) at a final concentration of 32 nM, and PI (TermoFisher, Waltham, MA, USA) at a final concentration of 7.5 µM. Once stained, samples were excited using the 488 nm laser; the

fluorescence emitted (Arbitrary Units) by SYBR-14 was detected with the FITC channel (525/40) and that emitted by PI was detected through the PC5.5 channel (690/50). Staining with SYBR-14 and PI resulted in three sperm populations: i) viable sperm (SYBR-14$^+$/PI$^-$); ii) non-viable sperm (SYBR-14$^-$/PI$^+$); and iii) moribund sperm, which were also considered as non-viable (SYBR14$^+$/PI$^+$). Percentages of viable (SYBR-14$^+$/PI$^-$) and non-viable sperm (SYBR-14$^-$/PI$^+$ and SYBR-14$^+$/PI$^+$) were recorded.

4.3.2. Intracellular superoxide radicals

Intracellular superoxide levels (O_2^-) were determined using hydroethidine (HE), a compound that is oxidised into ethidium (E^+) in the presence of O_2^-. The fluorescence intensity emitted by E^+ therefore represents an indicator of superoxide levels. In the same protocol, sperm are co-stained with YO-PRO-1, which is only able to penetrate membrane-damaged cells, indicating the viability of sperm cells. Sperm concentration was first adjusted with pre-warmed filtered PBS to 1×10^6 sperm/mL. Then, samples were incubated in HE (final concentration of 5 µM) (TermoFisher, Waltham, MA, USA) and YO-PRO-1 (final concentration of 31.2 nM) (TermoFisher, Waltham, MA, USA) at 38 °C for 20 min in the dark, and subsequently analysed with the flow cytometer. Samples were excited at 488 nm, and the fluorescence emitted by E^+ was detected with the FITC channel (2.24 % compensation) and the one emitted by YO-PRO-1 was collected through the PE channel (7.5 % compensation). After analysis, four subpopulations could be identified: i) viable sperm with low levels of superoxides (E^-/YO-PRO-1$^-$); ii) viable sperm with high levels of superoxides (E^+/YO-PRO-1$^-$); non-viable sperm with low levels of superoxides (E^-/YO-PRO-1$^+$); and iv) non-viable sperm with high levels of superoxides (E^+/YO-PRO-1$^+$).

4.3.3. Intracellular reactive oxygen species

Total ROS levels in sperm were evaluated using 2′,7′-dichlorodihydrofuorescein (H_2DCFDA), a non-fluorescent probe that penetrates sperm plasma membrane. When high levels of ROS are present, H_2DCFDA is intracellularly oxidised and converted into 2′,7′-dichlorofluorescein (DCF^+), which is highly fluorescent. After sperm concentration was adjusted to 1×10^6 sperm/mL in PBS, sperm samples were co-stained with H_2DCFDA (TermoFisher, Waltham, MA, USA) at a final concentration of 100 µM, and PI (TermoFisher, Waltham, MA, USA) at a final concentration of 5.6 µM. Samples were incubated for 20 min at 38 °C in the dark, and subsequently analysed with the flow cytometer. H_2DCFDA and PI were excited with the 488-nm laser and the fluorescence emitted by each fluorochrome was detected through FITC (525/40) and PC5.5 (690/50) channels, respectively. Four subpopulations could be identified after analysis: i) viable sperm with low levels of ROS (DCF^-/PI^-); ii) viable sperm with high levels of ROS (DCF^+/PI^-); iii) non-viable sperm with low levels of ROS (DCF^-/PI^+); and iv) non-viable sperm with high levels of ROS (DCF^+/PI^+).

4.3.4. Intracellular calcium

Fluo3, a membrane-permeable dye that emits fluorescence when bound to Ca^{2+}, was used to measure intracellular calcium levels in sperm. After adjusting sperm concentration to 1×10^6 sperm/mL in PBS, all samples were co-incubated with Fluo3 (TermoFisher, Waltham, MA, USA) (final concentration of 1.17 µM) and PI (final concentration of 5.56 µM) for 10 min at 38 °C in the dark, and subsequently analysed using the flow cytometer. Samples were excited at 488 nm and the fluorescence from Fluo3 and PI was detected through FITC (525/40) and PC5.5 (690/50) channels,

respectively. Four subpopulations were identified in dot plots: i) viable sperm with low levels of Ca^{2+} ($Fluo3^{-}/PI^{-}$); ii) viable sperm with high levels of Ca^{2+} ($Fluo3^{+}/PI^{-}$); iii) non-viable sperm with low levels of Ca^{2+} ($Fluo3^{-}/PI^{+}$); and iv) non-viable sperm with high levels of Ca^{2+} ($Fluo3^{+}/PI^{+}$).

5. Evaluation of sperm agglutination

The degree of sperm agglutination was assessed following the protocol described by Harayama et al. (1994). At least 250 sperm cells per sample were counted under a phase-contrast microscope at 100× magnification. Each sperm cell was classified as either agglutinated or non-agglutinated. Finally, the percentage of agglutinated sperm was calculated as: (Number of sperm cells agglutinated / Number of total sperm cells) × 100.

6. Assessment of chromatin protamination and condensation

6.1. Analysis of chromatin protamination

Chromatin protamination was assessed using the chromomycin A_3 test (CMA_3). In the presence of Mg^{2+}, CMA_3 binds to the minor groove of the DNA, resulting in the emission of fluorescence with a peak in 590 nm when excited at 430 nm. In sperm, CMA_3 competes with protamines for a similar DNA binding site, thus limiting its binding only to non-protaminated regions. In brief, a CMA_3 (Sigma-Aldrich, St. Louis, MO, USA) stock solution at 0.5 mg/mL in ethanol was first prepared before staining. Thereafter, sperm samples were diluted 1:1 (v:v) in 2× McIlvine buffer (60 mM citric acid, 280 mM Na_2HPO_4 and 20 mM $MgCl_2$) to a final concentration of

20×10^6 sperm/mL. Diluted samples were subsequently stained with CMA_3 to a final concentration of 12.5 mg/mL, and incubated at room temperature for 20 minutes in the dark. Following the labelling step, samples were diluted 1:10 (v:v) in filtered PBS and analysed using the CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA), with the fluorescence gain previously set and calibrated by means of CytoFlex Daily QC Fluorospheres (Beckman Coulter, Fullerton, CA, USA). A negative control without CMA_3 was included for each sample in order to establish the threshold value for positive CMA_3 cells. Moreover, a positive control was prepared by incubating sperm with a saline solution (5 mM DTT + 1 M NaCl) for 8 min at room temperature before sample dilution and labelling steps.

Samples were excited with the violet laser (405 nm), and the fluorescence emitted (arbitrary units) was collected through the Violet 610 channel (610/20 band pass). The mean fluorescence intensity peak (arbitrary units) was exported to Microsoft Excel (Microsoft, Redmond, WA, USA). For each sample, at least 10,000 sperm were evaluated at a flow rate between 10 and 60 mL/s. The fluorescence intensity of CMA_3 from both sperm populations was recorded as an indicator of the chromatin protamination degree.

6.2. Analysis of chromatin condensation

6.2.1. Dibromobimane test

Sperm chromatin condensation was evaluated through the oxidation-reduction status of disulphide bridges present in protamines using dibromobimane (DBB). Dibromobimane is a cell-permeant compound that, when covalently binds the thiol pairs resulting from reduced

disulphide bonds and is excited at 394 nm, emits fluorescence at 490 nm. For this purpose, a stock solution of 5 mM DBB (Sigma-Aldrich, St. Louis, MO, USA) was first prepared in 100 % dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). Besides, sperm were diluted in PBS to reach a final concentration of 1×10^6 sperm/mL. Subsequently, samples were incubated in 20 µM DBB at room temperature for 20 min in the dark. Following this, the fluorescence intensity emitted was evaluated using a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA), with the fluorescence gain being previously calibrated by means of the CytoFlex Daily QC Fluorospheres (Beckman Coulter, Fullerton, CA, USA). Samples were excited with the violet laser (405 nm) and the fluorescence emitted was collected through the KO525 channel (525/40 band pass). A negative control without DBB was included for each sample in order to establish the basal fluorescence intensity. Dot plots were analysed through the CytoExpert Software (Beckman Coulter, Fullerton, CA, USA), and the mean fluorescence intensity peak (arbitrary units) in the KO525 channel was exported to Microsoft Excel (Microsoft, Redmond, WA, USA). For each sample, at least 10,000 sperm were evaluated at a flow rate between 10 and 60 mL/s. Positive (DBB^+) and negative (DBB^-) cells were identified on the basis of the negative control. In addition, the fluorescence intensity of DBB was recorded as an indicator of chromatin condensation status.

6.2.2. Neutral halo assay

The susceptibility of sperm chromatin to decondensation can be assessed by the sperm chromatin dispersion test (halo assay). Sperm samples were first diluted in PBS to a final concentration of 2×10^6 sperm/mL, while an aliquot of 1 % low melting point (LMP) agarose (Thermo Fisher Scientific; Waltham, MA, USA) was melted at 70 °C for 10 min and subsequently

cooled down to 37.5 °C for further 10 min. Then, samples were incubated at 37.5 °C and mixed 1:2 (v:v) with 1 % LMP agarose at the same temperature. Subsequently, a 6.5-µL drop of each mixture was poured onto an agarose-treated slide, covered with an 8-mm circular coverslip, and allowed to gel on a metal cold plate at 4 °C for 5 min. Following this, coverslips were gently removed and samples were incubated at room temperature with three lysis solutions: i) the first containing 0.8 M Tris-HCl, 0.8 M DTT and 1 % SDS (pH = 7.5) for 30 min; ii) the second containing 0.4 M Tris-HCl, 0.4 M DTT, 50 mM EDTA, 2 M NaCl and 1 % Tween20 (pH = 7.5) for 30 min; and iii) the third containing 0.4 M Tris-HCl, 0.4 M DTT, 50 mM EDTA, 2 M NaCl, 1 % Tween20 and 100 µg/mL proteinase K (pH = 7.5) for 180 min. After lysis steps, slides were washed in distilled water and dehydrated in an increasing ethanol series (70 %, 90 % and 100 %) for 2 min each. Finally, samples were led to dry horizontally. All samples were evaluated under a fluorescence microscope (Zeiss Imager Z1, Carl Zeiss AG, Oberkochen, Germany), and dispersed and non-dispersed halos were differentiated, recording the percentage of sperm with dispersed chromatin.

7. Determination of DNA integrity

7.1. Global damage and double-strand breaks

Sperm DNA damage was assessed using Single-Cell Gel Electrophoresis (Comet assay). The alkaline and neutral variants were used to evaluate the incidence of global DNA damage and of DSB in sperm, respectively. For this purpose, the protocol of Ribas-Maynou et al. (2021) was followed. Slides were treated in horizontal position throughout the following steps:

i) sample preparation and lysis; ii) electrophoresis and fixation; and iii) staining, imaging and analysis.

Sample preparation and lysis: For each sperm sample, two slides were prepared and treated following this protocol, one for the alkaline comet and the other for the neutral comet. First, sperm samples were diluted in PBS to a final concentration of 5×10^5 sperm/mL. Meanwhile, 1 % LMP agarose (Thermo Fisher Scientific; Waltham, MA, USA) was melted at 70 °C for 10 min and cooled down to 38 °C for further 10 min. Then, sperm samples were mixed with low melting point agarose at a ratio of 1:2 (v:v), and 6.5 µL of each mixture was placed onto an agarose pre-treated slide, covered with an 8-mm diameter round coverslip and allowed to gel for 5 min at 4 °C on the top of a metal plate. Following this, coverslips were gently removed and slides were incubated at room temperature in three lysis solutions: i) the first solution containing 0.8 M Tris-HCl, 0.8 M DTT and 1 % SDS (pH = 7.5), for 30 min; ii) the second one containing 0.4 M Tris-HCl, 0.4 M DTT, 50 Mm EDTA, 2 M NaCl and 1 % Tween20 (pH = 7.5), for 30 min; and iii) the third solution containing 0.4 M Tris-HCl, 0.4 M DTT, 50 mM EDTA, 2 M NaCl, 1 % Tween20 and 100 mg/µL Proteinase K (pH = 7.5), for 3 h. Finally, slides were washed in distilled water for 2 min.

Electrophoresis and fixation: This step was performed differentially for alkaline and neutral Comet slides. Alkaline Comet slides were first denatured through incubation in a cold alkaline solution (4 °C) containing 0.03 M NaOH and 1 M NaCl (pH = 13) for 5 min. Then, slides were electrophoresed in alkaline buffer (0.03 M NaOH; pH =13) at 1 V/cm for 4 min. In parallel, neutral Comet slides were directly electrophoresed in TBE buffer containing 0.445 M Tris-HCl, 0.445 M Boric acid and 0.01 M EDTA (pH = 8) at 1 V/cm for 12.5 min, and then washed in a 0.9 % NaCl solution for 2 min. After electrophoresis, both slides were neutralised in 0.4 M Tris-HCl (pH = 7.5) for 5 min, and subsequently dehydrated in 70 %, 90 % and

100 % ethanol for 2 min each. Thereafter, slides were dried in horizontal position at room temperature.

Staining, Imaging and Analysis: Comet slides were stained by immersion in 1× SYTOX orange (Invitrogen, Whaltham, MA, USA) in distilled water, for 15 min at room temperature. The prepared Comet samples were observed under an epifluorescence microscope (Zeiss Imager Z1, Carl Zeiss AG, Oberkochen, Germany) at 100× magnification, and at least 100 sperm cells per sample were captured using the Axiovision 4.6 software (Carl Zeiss AG, Oberkochen, Germany), adjusting the exposure time to avoid overexposure of Comet heads or tails.

Afterwards, microscope captures were used for the quantitative analysis of fluorescence intensity using the automatic function of the CometScore v2.0 software (RexHoover, http://rexhoover.com). A manual review of each Comet image analysis was performed to delete the overlapping comets or signals that did not correspond to comets, and to correct the automatic identification of comet heads and tails, if necessary. At least 50 correctly analysed comets were required to obtain an average quantification of sperm DNA breaks for each sample. The Olive Tail Moment (OTM), calculated in each sample as (Tail mean intensity – Head mean intensity) × (Tail intensity / 100) was used as the standard parameter.

7.2. Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field Gel Electrophoresis (PFGE) was used as a method to identify the size of DNA fragments, when DNA damage was present. First, sperm samples were centrifuged at 600 g for 5 min and the cellular fraction (pellet) was resuspended in pre-warmed PBS (37 °C) to a final sperm concentration of 400×10^6 sperm/mL. After that, samples were mixed 1:1

(v:v) with 2 % LMP agarose previously melted at 70 °C and tempered at 38 °C. Immediately after, around 90 µL of each sample was poured onto BioRad plug molds (Bio-Rad; Hercules, CA, USA) and cooled to 4 °C for 10 min. Subsequently, plugs were unmolded and incubated in 2 mL of lysis buffer containing 10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 20 mM DTT, 2 % SDS and 2 mg/mL proteinase K (pH = 8.0) for 60 min at 53 °C. After incubation, plugs were washed three times in TE buffer (10 Mm Tris-HCl, 0.1 mM EDTA; pH=8).

Half of each plug was cut-off and loaded into a well of a 1 % PFGE agarose gel (Pulsed-Field Certified Agarose BioRad; Hercules, CA, USA) along with a slice of an agarose-embedded Low Range PFG DNA Marker (New England Biolabs; Ipswich, MA, USA). The agarose gel was then placed in a Bio-Rad CHEF DRIII system with 0.5× TBE buffer (Tris-borate 50 mM, EDTA 0.1 mM) at 14 °C. Electrophoresis was conducted at 4 V/cm for 27.1 h with a rotation (angle) of 120° and a pulse change ramp from 6.7 to 33.7 seconds. Following electrophoresis, the gel was stained with ethidium bromide and imaged under ultraviolet light using the GelDoc System (BioRad; Hercules, CA, USA).

The intensity of the DNA smear in the gel was quantified using the Image Studio Lite (LI-COR Biosciences, Lincoln, NE, USA). The size of DNA fragments was determined using the DNA ladder, distinguishing: i) fragments smaller than a toroid (<33 Kb), ii) fragments ranging the size between one and multiple toroids (15 – 194 Kb), and iii) undamaged DNA (>194 Kb).

8. Determination of oxidant capacity and antioxidant activity of seminal plasma

8.1. Oxidant capacity

Advanced oxidation protein products (AOPPs), which are indicators of protein damage caused by oxidative stress, were determined following the procedure described by Witko-Sarsat (1996) adapted to pig SP (Barranco et al., 2021; Witko-Sarsat et al., 1996). Briefly, 10 µL of a SP sample was mixed with 160 µL of 0.074 M potassium iodide, and 25 µL of 50 % acetic acid, and then incubated at 37 °C for 40 s. The resulting change in absorbance was measured at 340 nm using an automated analyser (Olympus AU600 Automatic Chemistry Analyser). The assay was calibrated with chloramine-T (0–500 µM) and the results were expressed as µmol/L of chloramine-T equivalents. Two technical replicates were evaluated for each sample. Intra- and inter-assay coefficient variations were lower than 10 %, indicating good linearity in serial dilutions.

8.2. Antioxidant activity

The cupric reducing antioxidant capacity (CUPRAC) assay with bathocuproinedisulfonic acid disodium salt as chelating agent was used to determine the total antioxidant capacity of SP. This method was described by Campos et al. (2009) and adapted to pig SP by Li et al., (2018) (Campos et al., 2009; Li et al., 2018). First, 5 µL of SP was mixed with 195 µL of 0.25 mM bathocuproinedisulfonic acid disodium salt, and the absorbance was measured at 480 nm using an automated analyser (Olympus AU600 Automatic Chemistry Analyser, Olympus Europe GmbH, Hamburg, Germany). Next, 50 µL of 0.5 mM $CuSO_4$ was added, and the resulting

mixture was incubated at 37 °C for 4 min and 40 s. Then, the absorbance was measured again at 480 nm in the same automatic analyser. The difference between the two absorbance readings was used to calculate the antioxidant capacity of the SP sample. To calibrate this assay, 0 mM to 2 mM Trolox solutions were used, and the results were expressed as mmol/L of Trolox equivalents. Each SP sample was measured per duplicate and the intra- and inter-assay coefficient variations were lower than 10 %, indicating high linearity in serial dilutions.

9. *In Vitro* Fertilisation

Oocyte collection: Ovaries from pre-puberal gilts were obtained from a local abattoir (Frigorífics Costa Brava; Riudellots de la Selva, Girona) and transported to the laboratory in 0.9 % NaCl supplemented with 70 µg/mL kanamycin at 38 °C. Cumulus-oocyte complexes (COC) were collected from follicles through aspiration with insulin needles, and the ones with a complete, compact cumulus mass were selected under the stereomicroscope. These COCs were washed in Dulbecco's PBS (Gibco, ThermoFisher, Waltham, USA) supplemented with 4 mg/mL of BSA.

Oocyte maturation: For oocyte maturation, TCM-199 (Gibco, ThermoFisher, Waltham, USA) medium, supplemented with 0.57 mM cysteine, 0.1 % (w:v) polyvinyl alcohol, 10 ng/mL human epidermal growth factor, 75 µg/mL of penicillin-G potassium and 50 µg/mL of streptomycin sulphate was used. Groups of 40-50 COCs were transferred to a four-well multi-dish (Nunc, ThermoFisher; Waltham, USA) containing 500 µL of pre-equilibrated maturation medium supplemented with 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International B.V.; Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotropin

(hCG; Veterin Corion; Divasa Farmavic S.A.; Gurb, Barcelona, Spain). Oocytes were incubated for 20-22 h at 38 °C under a humidified atmosphere of 5 % CO_2 in air. After this incubation, oocytes were transferred to 500 µL of pre-equilibrated maturation medium without hormones.

IVF protocol: For the fertilisation protocol, matured oocytes were denuded in Dulbecco's PBS (Gibco, ThermoFisher) and placed in 50-µL drops of pre-equilibrated *in vitro* fertilisation medium (Tris-buffered medium modified from (Abeydeera & Day, 1997) containing 1 mM caffeine). Meanwhile, semen samples were adjusted to a final concentration of 1,000 sperm per oocyte in fertilisation medium, and oocytes and sperm were then co-incubated for 5 h at 38 °C under a humidified atmosphere containing 5 % CO_2 in air.

Embryo culture: After incubation, presumptive zygotes were washed and transferred (40 zygotes/well) to a four-well multi-dish containing 500 µL of NCSU23 medium (Peters et al., 2001), supplemented with 0.4 % BSA, 0.3 mM pyruvate and 4.5 mM lactate. Cleaved embryos were evaluated after two days, and the fertilisation rate was calculated. Then, embryos were changed to NCSU23 medium supplemented with 0.4 % BSA and 5.5 mM glucose. After culturing embryos for 5 days, they were evaluated and classified according to the Gardner's established criterion (Balaban & Gardner, 2013). Percentages of morulae, early blastocysts/blastocysts, hatching/hatched blastocysts and total embryos (sum of morulae, early blastocysts/blastocysts and hatching/hatched blastocysts) were calculated at Day 6 post-fertilisation. All incubations carried out during embryo culture were performed at 38.5 °C in a humidified atmosphere containing 5 % CO_2 in air.

10. Statistical analysis

Statistical analyses were conducted with IBM SPSS 27.0 for Windows (IBM Corp.; Armonk, NY, USA) and graphs were prepared using GraphPad Prism v8 (GraphPad Software, La Jolla, CA, USA). Normal distribution and homogeneity of variances were tested with Shapiro-Wilk and Levene tests, respectively. In all cases, parameters did not fit with parametric assumptions, and this was not remedied after linear transformation (arcsin $\sqrt{x}$, $\sqrt{x}$); therefore, non-parametric tests were used as an alternative. When related samples were assessed, differences between groups were evaluated through a repeated measures one-way ANOVA (Friedman test) followed by Dunn's post-hoc test for pair-wise comparisons. When independent groups were assessed, differences were evaluated using a one-way ANOVA (Kruskal-Wallis test), followed by Dunn' or Mann-Whitney post-hoc tests. For multiple comparisons accounting two factors, the Scheirer-Ray-Hare Test as an alternative to two-way ANOVA was run followed by the Wilcoxon test. Finally, correlations were examined with the Spearman coefficient. For all tests, the level of statistical significance was set at $P \leq 0.05$.

CHAPTER I

Post-thaw incubation of bovine sperm affects sperm chromatin and sperm physiology

1. Experimental design

The present set of experiments were aimed to evaluate the effects of post-thaw incubation at 38 °C upon sperm characteristics, including sperm chromatin and sperm physiology, in order to establish the sequence of events occurring during cell degradation and determine whether they can be useful to predict the fertilising capacity of a given sample. For this purpose, 25 sexually mature Holstein bulls housed at an Artificial Insemination Centre located in Cenero (Gijón, Spain) were involved. For each bull, three ejaculates collected at three different periods separated with at least five weeks were obtained and, just after thawing, they were pooled and evaluated after 0 h, 2 h and 4 h of incubation at 38 °C. This provided a dynamic evaluation of the sperm resilience to post-thawing incubation. This dynamic evaluation was expressed by the rates of variation [(final value – initial value) / time] between 0 and 2 h, 2 and 4 h, and 0 and 4 h, of all the assessed sperm chromatin and functionality parameters.

The first experiment sought to address the effects on sperm chromatin. Chromatin protamination was determined using the CMA_3 test, chromatin condensation was examined with the halo test, double-strand DNA damage was evaluated through the neutral Comet assay, and the global incidence of DNA breaks was assessed using the alkaline Comet assay.

The second experiment was aimed at investigating the effects on sperm physiological characteristics. Briefly, sperm motility was evaluated using a Computer-Assisted Sperm Analysis system, and sperm viability (SYBR-14/PI), intracellular levels of total ROS (H_2DCFDA), superoxides (HE) and intracellular Ca^{2+} (Fluo3) were examined with flow cytometry.

The third experiment was devised to determine whether the static evaluation at 0 h, 2 h and 4 h, or the dynamic evaluation (variation rates

between 0 h and 2 h and between 2 h and 4 h of incubation) were able to predict bull fertility, measured as non-return to oestrus rates after 90 days (NRR).

2. Results

2.1. Experiment 1: Evaluation of the resilience of bovine sperm chromatin to freeze-thawing

2.1.1. Sperm chromatin protamination decreases upon incubation of frozen-thawed bovine sperm at 38 ºC

The degree of chromatin protamination was evaluated after thawing through CMA_3 fluorescence intensity at 0 h, and after 2 h and 4 h of incubation at 38 °C. As shown in *Figure 7* and *Table 1*, a higher chromatin protamination degree was observed at 0 h compared to 2 h of incubation ($P < 0.001$) and 4 h of incubation ($P < 0.001$). Moreover, after 4 h of incubation, chromatin protamination decreased compared to 2 h ($P < 0.001$). These results suggest that the chromatin of frozen-thawed bovine sperm becomes deprotaminated upon incubation at 38 °C.

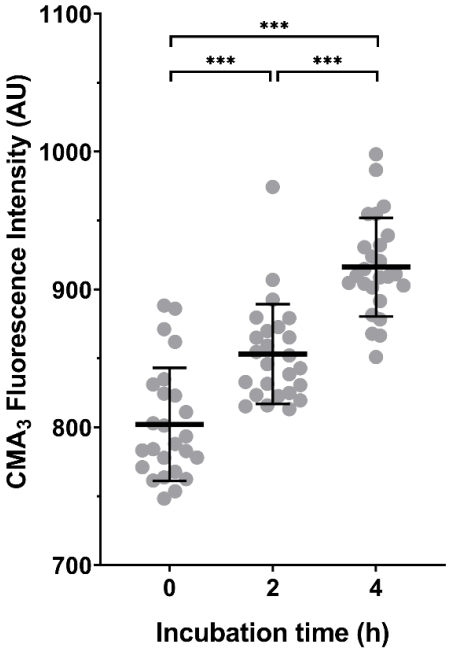

Figure 7. Effects of post-thawing incubation at 38 °C on chromatin protamination of bovine sperm. For each incubation-time, chromatin protamination values corresponding to each bull are depicted as dots, and the mean ± standard deviation (SD) are represented by lines. (***) indicates statistically significant differences with a $P < 0.001$. AU: Arbitrary units.

Table 1. Effects of post-thawing incubation time on sperm chromatin protamination. Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h of incubation ($P < 0.001$); and ([b]) statistical differences compared to 2 h of incubation ($P < 0.001$). AU: Arbitrary units.

	T = 0 h Mean ± SD	**T = 2 h** Mean ± SD	**T = 4 h** Mean ± SD
Sperm chromatin protamination (CMA_3 intensity, AU)	802.04 ± 41.03	853.12 ± 36.20 [a]	916.18 ± 35.83 [a,b]

Changes in chromatin protamination during post-thawing incubation at 38 °C were evaluated in two separate periods (*Table 2*): i) from 0 to 2 h of incubation; and ii) from 2 h to 4 h of incubation. Despite the differences in sperm chromatin protamination along the four hours of post-thawing incubation, no significant differences in the rate of protamination decrease were observed between the two periods of incubation (first period: 0 to 2 h; second period: 2 to 4 h) ($P > 0.05$).

Table 2. Rates of variation between the two periods for the degree of sperm chromatin protamination. Values are presented as mean ± standard deviation (SD). AU: Arbitrary units.

	T = 0 - 2 h Mean ± SD	**T = 2 - 4 h** Mean ± SD	**T = 0 - 4 h** Mean ± SD
Sperm chromatin protamination (CMA_3 intensity, AU)	25.54 ± 26.32	31.53 ± 24.66	28.54 ± 10.6

2.1.2. Sperm chromatin condensation is not impaired after a short incubation of frozen-thawed bovine sperm at 38 °C

Sperm chromatin condensation was evaluated immediately after thawing (0 h) and after 2 h and 4 h of incubation at 38 °C through the Halo test, which determines the ability of chromatin to decondense. Results, expressed as the percentage of sperm with highly decondensed chromatin, are shown in *Figure 8* and *Table 3*. Post-thawing incubation did not alter sperm chromatin condensation ($P > 0.05$), but there was a slight increase in the percentage of sperm with decondensed chromatin after 2 h (3.03 ± 1.06 % sperm with decondensed chromatin) and 4 h (3.53 ± 1.78 % sperm with decondensed chromatin) of incubation. Additionally, regarding DNA decondensation rate, no statistical differences were

observed between the first and the second periods of incubation ($P > 0.05$) (*Table 4).*

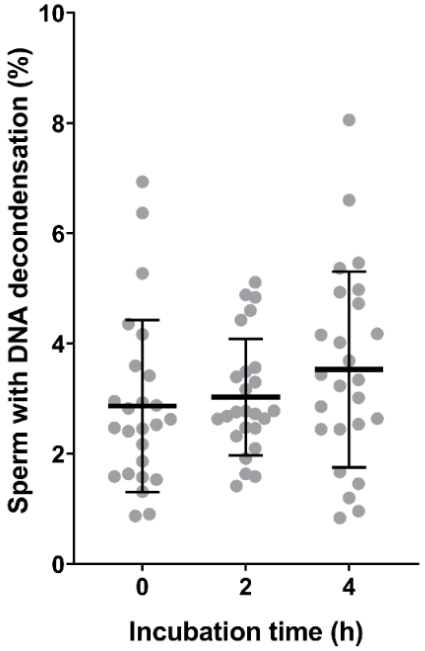

Figure 8. Effects of post-thawing incubation at 38 °C on sperm chromatin condensation. For each incubation time, the chromatin condensation values of each individual bull are presented by dots, and the mean ± standard deviation (SD) are represented by the lines. No significant differences between time points were observed.

Table 3. Effects of post-thawing incubation time on chromatin condensation. Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h of incubation ($P < 0.001$); and ([b]) statistical differences compared to 2 h of incubation ($P < 0.001$).

	T = 0 h Mean ± SD	**T = 2 h** Mean ± SD	**T = 4 h** Mean ± SD
Sperm chromatin decondensation (Halo test, %)	2.87 ± 1.56	3.03 ± 1.06	3.53 ± 1.78

Table 4. Rates of variation for sperm chromatin condensation between the two periods. Values are presented as mean ± standard deviation (SD).

	T = 0 - 2 h Mean ± SD	**T = 2 - 4 h** Mean ± SD	**T = 0 - 4 h** Mean ± SD
Sperm chromatin decondensation (Halo test, %)	0.08 ± 0.61	0.25 ± 0.84	0.17 ± 0.41

2.1.3. Both global damage and double-strand DNA breaks increase upon incubation of frozen-thawed bovine sperm at 38 ºC, but this increase is notably greater during the second period of incubation

The incidence of double-strand DNA breaks and global DNA damage (Olive Tail Moment, OTM) was determined through neutral and alkaline Comet assays, respectively, in frozen-thawed sperm at 0 h, and after 2 h and 4 h of incubation at 38 °C. Results obtained are shown in *Figure 9* and *Table 5*.

Regarding the incidence of global DNA damage (single- and double-strand DNA breaks) in sperm, a statistically significant increase was observed after 4 h of incubation, compared to 0 h and 2 h ($P < 0.001$) (*Figure 9A*). Also, a statistically significant increase in the incidence of double-strand DNA breaks was noticed between 0 h and 4 h of post-thawing incubation ($P = 0.024$) (*Figure 9B*).

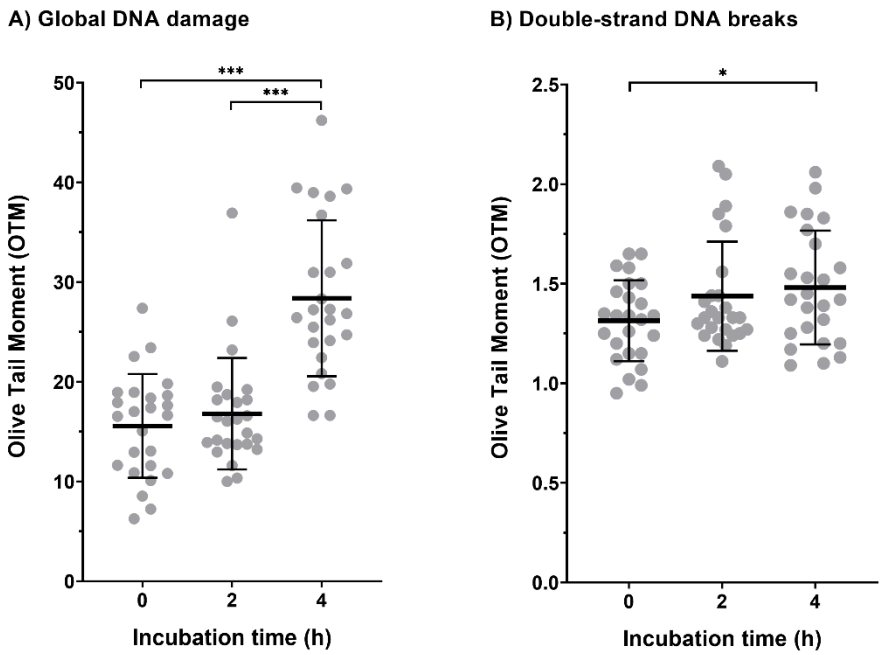

Figure 9. Effects of post-thawing incubation at 38 °C on (A) Global DNA damage (alkaline Comet) and (B) double-strand DNA breaks (neutral Comet). For each incubation time, the values for individual bulls are represented by dots, and the mean ± standard deviation are shown as lines. (*) indicates statistically significant differences with a $P < 0.05$; and (***) indicates statistically significant differences with a $P < 0.001$.

Table 5. Effects of post-thawing incubation time on the incidence of double-strand breaks and global DNA damage in bovine sperm. Values are presented as mean ± standard deviation (SD). ([a]) Statistically significant differences compared to 0 h of incubation ($P < 0.01$); ([b]) Statistically significant differences compared to 2 h of incubation ($P < 0.01$).

	T = 0 h Mean ± SD	**T = 2 h** Mean ± SD	**T = 4 h** Mean ± SD
Incidence of Global DNA damage (OTM)	15.57 ± 5.20	16.80 ± 5.59	28.38 ± 7.82 [a,b]
Incidence of double-strand DNA breaks (OTM)	1.31 ± 0.20	1.44 ± 0.27	1.48 ± 0.29 [a]

Although the incidence of double-strand DNA breaks in sperm was only found to significantly increase when 0 h and 4 h were compared, the extent of that increase was similar in the first (from 0 h to 2 h) and second periods of incubation (0.06 ± 0.16 AU/h and 0.02 ± 0.14 AU/h, respectively) (*Table 6*). Conversely, while post-thawing incubation also increased the incidence of global DNA damage, the extent of that increase was much greater in the second than in the first period (0.61 ± 1.65 AU/h and 5.79 ± 3.05 AU/h, respectively, $P < 0.001$) (*Table 6*).

Table 6. Rates of increase of global DNA damage and double-strand DNA breaks in frozen-thawed bovine sperm, between the two periods of incubation (0 h to 2 h and 2 h to 4 h). Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to the first period (0 to 2 h) ($P < 0.01$); and ([b]) statistical differences compared to the second period (2 to 4 h) ($P < 0.01$).

	T = 0 - 2 h Mean ± SD	**T = 2 - 4 h** Mean ± SD	**T = 0 - 4 h** Mean ± SD
Incidence of Global DNA damage (OTM)	0.61 ± 1.65	5.79 ± 3.05 [a]	3.20 ± 1.57 [a,b]
Incidence of double-strand DNA breaks (OTM)	0.06 ± 0.16	0.02 ± 0.14	0.04 ± 0.07

2.2. Experiment 2: Effects of post-thawing incubation on sperm quality

2.2.1. Sperm motility is one of the first parameters affected upon post-thawing incubation at 38 °C

As shown in *Figure 10A* and *Table 7*, total sperm motility decreased over the incubation period ($P \leq 0.001$), the lowest value being observed after 4 h of incubation. While this reduction of sperm motility was greater in the first (0 to 2 h; -8.51 ± 11.50 %/h) than in the second period (2 to 4 h; -22.39 ± 11.44 %/h), these values were not statistically significant ($P > 0.05$) (*Figure 10B* and *Table 8*).

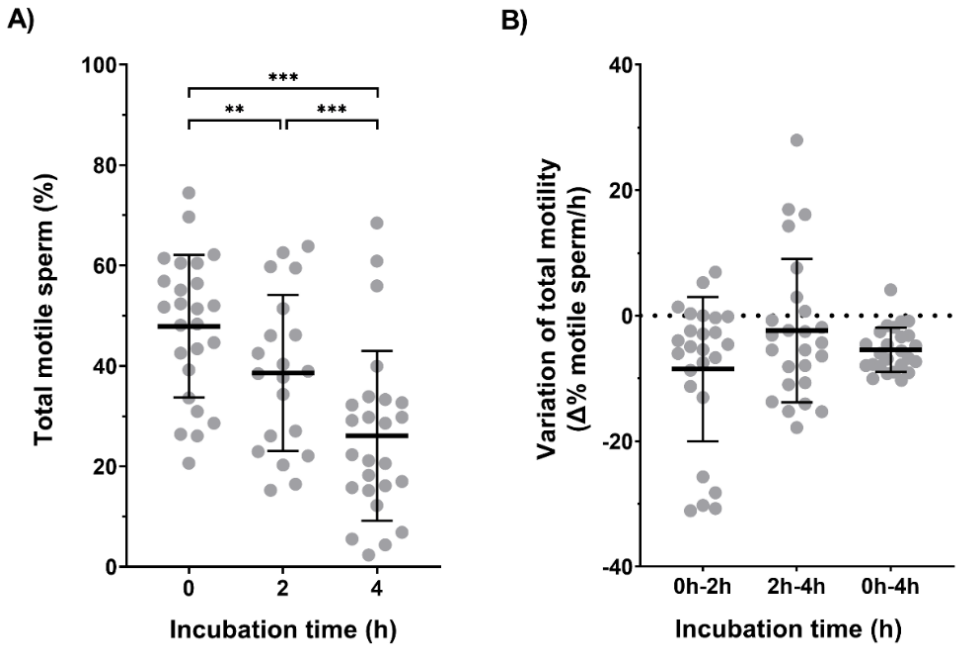

Figure 10. (A) Effects of post-thawing incubation at 38 °C on total sperm motility. (B) Variation of total sperm motility during the first (0 h – 2 h), second (2 h – 4 h) and entire periods of incubation (0 h – 4 h). Values for individual bulls are presented by dots, and lines represent the mean ± standard deviation. (**) Indicates statistically significant differences with a $P < 0.01$; and (***) indicates statistically significant differences with a $P < 0.001$.

Table 8. Rates of variation of total motility and sperm viability during the first (0 h – 2 h), second (2 h – 4 h) and entire periods of incubation (0 h – 4 h). Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h ($P < 0.05$); and ([b]) statistical differences compared to 2 h of incubation ($P < 0.05$).

	T = 0 - 2 h Mean ± SD	**T = 2 - 4 h** Mean ± SD	**T = 0 - 4 h** Mean ± SD
Total motility (%)	-8.51 ± 11.50	-7.13 ± 5.96	-5.45 ± 3.52
Viable sperm (SYBR-14$^+$/PI$^-$) (%)	-4.49 ± 1.79	-2.50 ± 3.39 [a]	-3.50 ± 1.99 [a,b]

Besides, progressive motility also showed a reduction as time advanced ($P < 0.001$) (*Table 7*) showing a rate of decrease of -4.70 ± 3.42 %/h and -1.89 ± 2.97 %/h for the first and second periods, respectively. For kinematic parameters, a similar trend was observed after 4 h of incubation, when all parameters (except medium velocity) suffered a reduction compared to 0 h post-thawing ($P < 0.05$) (*Table 7*).

Table 7. Effects of post-thawing incubation on total and progressive motility, and kinematic parameters. Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h ($P < 0.05$); and ([b]) statistical differences compared to 2 h ($P < 0.05$).

	T = 0 h			**T = 2 h**			**T = 4 h**		
	Mean	±	SD	Mean	±	SD	Mean	±	SD
Motility									
Total motility (%)	47.89	±	13.91	38.58	±	15.16 [a]	26.09	±	16.60 [a,b]
Progressive motility (%)	27.96	±	9.16	22.35	±	9.12 [a]	12.96	±	8.83 [a,b]
Kinematic parameters									
Fast velocity (%)	31.00	±	11.24	24.22	±	11.04 [a]	12.62	±	9.89 [a,b]
Medium velocity (%)	8.89	±	2,68	8.48	±	2.91	8.30	±	4.07
Slow velocity (%)	8.76	±	7.81	5.88	±	4.02 [a]	5.16	±	5.46 [a]
Curvilinear velocity (VCL) (μm/s)	81.97	±	15.97	73.22	±	12.90 [a]	51.96	±	13.70 [a,b]
Straight-line velocity (VSL) (μm/s)	43.68	±	12.80	37.63	±	10.34 [a]	22.39	±	10.17 [a,b]
Average path velocity (VAP) (μm/s)	50.10	±	12.83	43.68	±	10.36 [a]	28.42	±	9.97 [a,b]
Linearity (LIN) (%)	52.37	±	6.69	50.38	±	7.02	41.08	±	9.18 [a,b]
Straightness (STR) (%)	86.26	±	4.70	85.07	±	5.59	75.76	±	10.40 [a,b]
Oscillation (WOB) (%)	60.49	±	5.24	58.92	±	5.16	53.61	±	5.48 [a,b]
Lateral head displacement (ALH) (μm)	3.11	±	0.48	2.77	±	0.41 [a]	2.17	±	0.51 [a,b]
Frequency of head displacement (BCF) (Hz)	12.28	±	1.34	11.99	±	1.71	8.80	±	3.58 [a,b]
Viability									
Viable sperm (SYBR-14$^+$/PI$^-$) (%)	50.95	±	9.55	41.97	±	9.03 [a]	36.96	±	10.76 [a,b]

2.2.2. Sperm viability is reduced at a higher rate during the first period of incubation

Regarding viability (*Figure 11A, Table 7*), a statistically significant reduction of viable sperm was observed during post-thawing incubation ($P < 0.01$). Besides, the rate of the reduction of viable sperm was different between periods, with a faster decrease during the first (0 to 2 h; -4.49 ± 1.79 %/h) than during the second incubation period (2 to 4 h; -2.50 ± 3.39 %/h) ($P = 0.032$) (*Figure 11B* and *Table 8*).

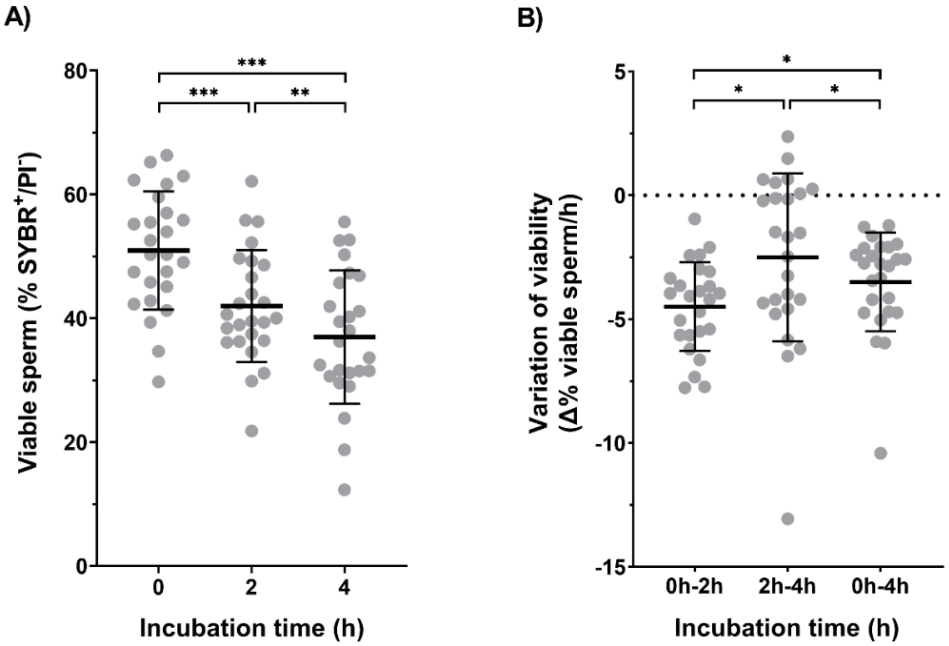

Figure 11. (A) Effects of post-thawing incubation at 38 °C on sperm viability. (B) Variation of sperm viability during the first (0 h – 2 h), second (2 h – 4 h) and the entire (0 h – 4 h) incubation period. Values for individual bulls are presented by dots, and the mean ± standard deviation are represented by lines. (*) Indicates statistically significant differences with a $P < 0.05$; (**) indicates statistically significant differences with a $P < 0.01$; and (***) indicates statistically significant differences with a $P < 0.001$.

2.2.3. Incubation of frozen-thawed sperm at 38 °C for 4 h decreases the percentage of sperm with high intracellular ROS levels

Incubation of frozen-thawed sperm for 4 h significantly reduced the percentage of sperm with high ROS levels (*Figure 12A* and *Table 9*; $P < 0.05$). When comparing the two periods of incubation, the rate of ROS decrease was similar (0 to 2 h: -1.08 ± 2.95 %/h; 2 to 4 h: -1.04 ± 1.47 %/h; $P > 0.05$) (*Figure 12B* and *Table 10*).

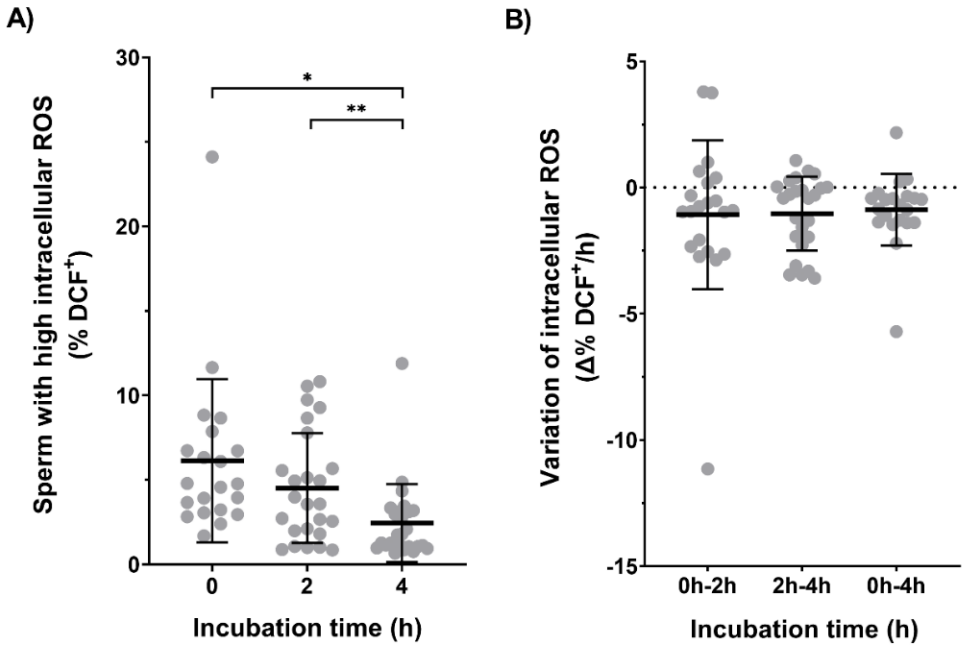

Figure 12. (A) Effects of post-thawing incubation at 38 °C on the percentage of sperm with high intracellular ROS. (B) Variation of the percentage of sperm with high intracellular ROS during the first (0 h – 2 h), second (2 h – 4 h) and entire incubation period (0 h – 4 h). Values for individual bulls are presented by dots, and the mean ± standard deviation are represented by lines. (*) Indicates statistically significant differences with a $P < 0.05$; and (**) indicates statistically significant differences with a $P < 0.01$.

2.2.4. The percentage of sperm with high intracellular superoxides increase at a higher rate during the first period of incubation

As shown in *Figure 13A* and *Table 9*, the percentage of sperm with high levels of intracellular superoxides (E^+) was significantly higher at 2 h ($P < 0.001$) and 4 h ($P = 0.011$) post-thawing, compared to 0 h. Comparing the rates of variation *(Figure 13B* and *Table 10*) between periods, the percentage of sperm with high intracellular superoxides increased at a greater rate during the first (3.16 ± 3.16 %/h) than the second period (0.76 ± 3.96 %/h) ($P < 0.01$).

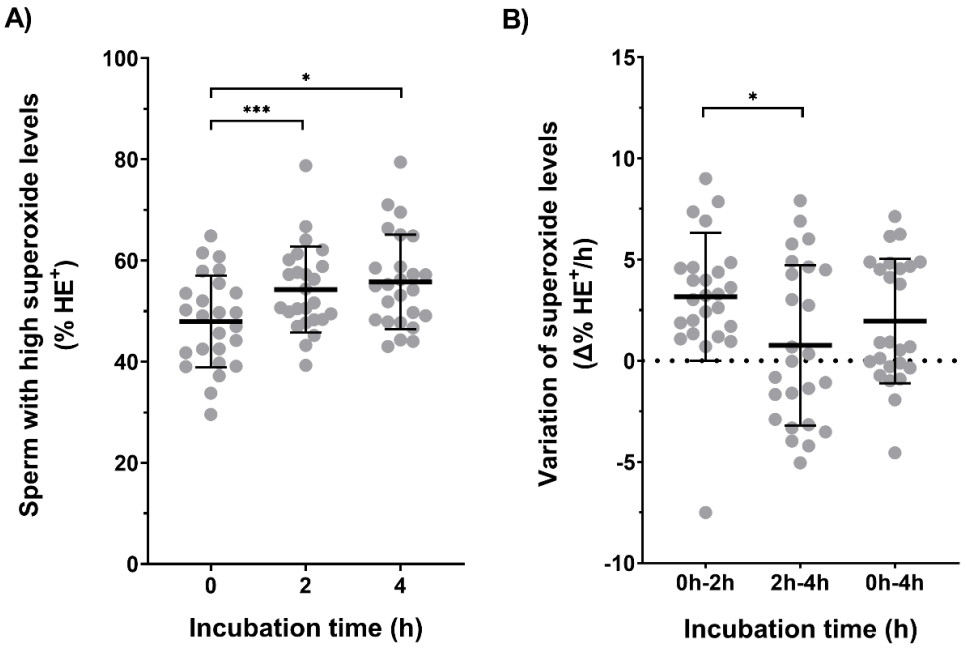

Figure 13. (A) Effects of post-thawing incubation at 38 °C on the percentages of sperm with high levels of superoxides. (B) Variation in the percentage of sperm with high superoxide levels during the first (0 h – 2 h), second (2 h – 4 h) and entire (0 h – 4 h) periods of incubation. Values for individual bulls are presented by dots, and the mean ± standard deviation (SD) are represented by lines. (*) Indicates statistically significant differences with a $P < 0.05$; and (***) indicates statistically significant differences with a $P < 0.001$.

2.2.5. Post-thawing incubation at 38 °C increases the percentage of sperm with high intracellular calcium

The percentage of sperm with high intracellular calcium levels ($Fluo3^+$) was found to significantly increase during post-thawing incubation, both between 0 h and 2 h ($P = 0.002$) and between 2 h and 4 h ($P < 0.001$) (*Figure 14A* and *Table 9*). As depicted in *Figure 14B* and *Table 10*, nevertheless, no statistically significant differences in variation rates were observed when the first and second incubation periods were compared (9.75 ± 11.57 %/h and 4.14 ± 4.89 %/h, respectively; $P > 0.05$).

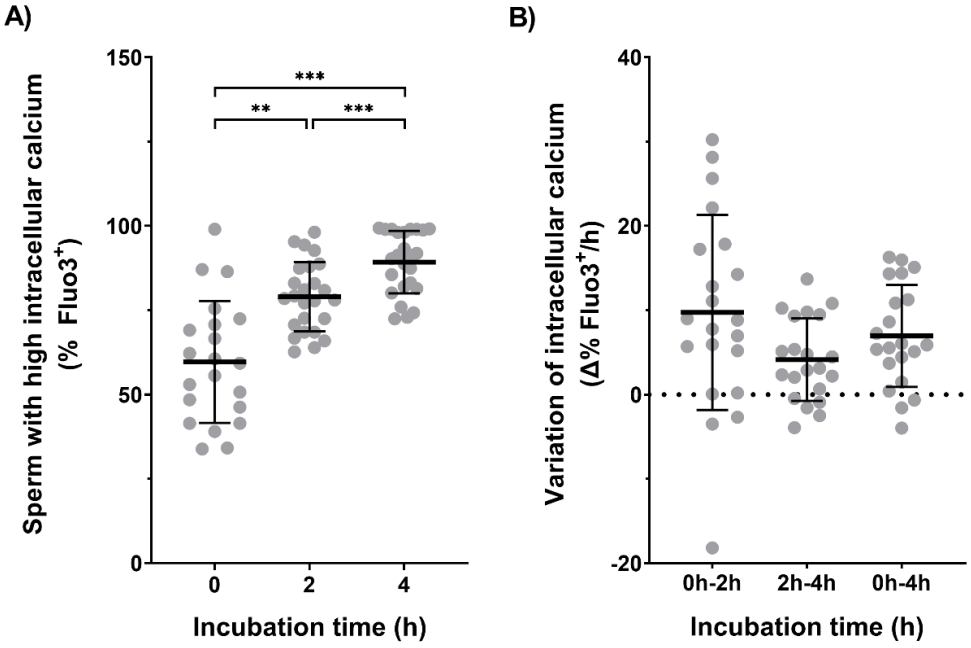

Figure 14. (A) Effects of post-thawing incubation at 38 °C on the percentages of sperm with high intracellular calcium levels. (B) Variation in the percentages of sperm with high intracellular calcium levels during the first (0 h – 2 h), second (2 h – 4 h) and the entire (0 h – 4 h) incubation period. Values for individual bulls are presented by dots, and the mean ± standard deviation are represented by lines. (**) indicates statistically significant differences with a $P < 0.01$; and (***) indicates statistically significant differences with a $P < 0.001$.

Table 9. Effects of post-thawing incubation at 38 °C on the percentages of sperm with high intracellular ROS, sperm with high superoxides and sperm with high calcium levels. Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h of incubation ($P < 0.05$); and ([b]) statistical differences compared to 2 h of incubation ($P < 0.05$).

	T = 0 h Mean ± SD	**T = 2 h** Mean ± SD	**T = 4 h** Mean ± SD
Sperm with high ROS (DCF^+) (%)	6.13 ± 4.83	4.51 ± 3.24	2.44 ± 2.31 [a,b]
Sperm with high superoxides (E^+) (%)	47.94 ± 9.09	54.26 ± 8.51 [a]	55.78 ± 9.33 [a]
Sperm with high calcium ($Fluo3^+$) (%)	59.66 ± 18.09	79.00 ± 10.28[a]	89.26 ± 9.27 [a,b]

Table 10. Rates of variation during the first (0 h – 2 h), second (2 h – 4 h) and entire (0 h – 4 h) incubation periods for the percentages of sperm with high intracellular ROS, sperm with high superoxides and sperm with high calcium levels. Values are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to 0 h of incubation ($P < 0.05$); and ([b]) statistical differences compared to 2 h of incubation ($P < 0.05$).

	T = 0 – 2 h Mean ± SD	**T = 2 – 4 h** Mean ± SD	**T = 0 – 4 h** Mean ± SD
Sperm with high ROS (DCF^+) (%)	-1.08 ± 2.95	-1.04 ± 1.47	-0.88 ± 1.42
Sperm with high superoxides (E^+) (%)	3.16 ± 3.16	0.76 ± 3.96 [a]	1.96 ± 3.07 [a,b]
Sperm with high calcium ($Fluo3^+$) (%)	9.75 ± 11.57	5.13 ± 5.26	6.94 ± 6.04

2.2.6. Correlations between chromatin parameters and sperm function variables

Correlations between variation rates of DNA damage (global and double-strand), sperm chromatin condensation and protamination, and sperm functionality parameters are shown in *Table 11*.

Regarding the first incubation period (0 h to 2 h), the rate of double-strand DNA breaks variation showed statistically significant positive correlations with the variation rates of global DNA damage and the percentage of sperm with decondensed chromatin (P = 0.020 and P = 0.016, respectively). Also, positive correlations between the rate of chromatin deprotamination (CMA_3^+) and the percentages of sperm with high superoxide and calcium levels (P = 0.020 and P = 0.034, respectively) were observed.

For the second period of incubation (2 h to 4 h), chromatin damage was observed to happen together with a detrimental effect on sperm function. The rate of increase observed in the percentage of sperm with high intracellular superoxides was found to be negatively correlated to both global DNA damage and chromatin protamination (P = 0.039 and P < 0.001, respectively). Moreover, the variation rate of viable sperm was observed to be correlated to chromatin protamination (P = 0.029). Besides, the incidence of global DNA damage and chromatin protamination were also found to be correlated (P = 0.001).

When considering the entire incubation period (0 h to 4 h), a positive correlation between the rate of variation of global DNA damage and chromatin decondensation (P = 0.032) was found, together with a negative correlation between chromatin deprotamination and the percentage of sperm with high intracellular ROS levels (P = 0.049).

Table 11. Correlations between the rates of variation of sperm DNA fragmentation, chromatin decondensation, chromatin deprotamination and sperm functionality parameters, separated by the period of incubation.

		Global DNA damage (OTM)	Double-strand DNA breaks (OTM)	Chromatin decondensation (%)	Chromatin deprotamination (AU)	Viability (%)	Total motility (%)	Sperm with high ROS (DCF^+)	Sperm with high superoxides (HE^+)	Sperm with high calcium ($F3^+$)
First incubation period (0 h – 2 h)										
Global DNA damage (OTM)	*Rs*	-	**0.463**	0.350	0.228	0.162	0.126	0.127	0.248	0.321
	P value		**0.020**	0.086	0.274	0.440	0.557	0.582	0.231	0.156
Double-strand DNA breaks (OTM)	*Rs*	-	-	**0.477**	0.179	0.022	0.153	-0.047	0.104	0.322
	P value			**0.016**	0.393	0.916	0.476	0.838	0.621	0.154
Chromatin decondensation (%)	*Rs*	-	-	-	0.028	0.204	0.299	0.329	0.152	0.256
	P value				0.895	0.328	0.156	0.146	0.467	0.263
Chromatin deprotamination (AU)	*Rs*	-	-	-	-	-0.188	0.121	-0.208	**0.598**	**0.464**
	P value					0.369	0.574	0.366	**0.002**	**0.034**
First incubation period (2 h – 4 h)										
Global DNA damage (OTM)	*Rs*	-	0.201	0.335	**0.607**	0.285	0.027	0.044	**-0.415**	0.078
	P value		0.337	0.101	**0.001**	0.167	0.910	0.835	**0.039**	0.709
Double-strand DNA breaks (OTM)	*Rs*	-	-	0.096	0.187	0.162	-0.391	0.169	-0.112	-0.304
	P value			0.647	0.372	0.438	0.089	0.421	0.594	0.139
Chromatin decondensation (%)	*Rs*	-	-	-	0.390	0.223	0.069	0.028	-0.128	0.049
	P value				0.054	0.284	0.772	0.893	0.541	0.815
Chromatin deprotamination (AU)	*Rs*	-	-	-	-	**0.437**	0.092	0.306	**-0.675**	-0.122
	P value					**0.029**	0.701	0.137	**< 0.001**	0.560

Table 11. (Continued).

		Global DNA damage (OTM)	Double-strand DNA breaks (OTM)	Chromatin decondensation (%)	Chromatin deprotamination (AU)	Viability (%)	Total motility (%)	Sperm with high ROS (DCF^+)	Sperm with high superoxides (HE^+)	Sperm with high calcium ($F3^+$)
Global incubation (0 h – 4 h)										
Global DNA damage (OTM)	*Rs*	-	0.167	**0.430**	0.125	0.032	-0.124	0.305	-0.242	0.030
	P value		0.424	**0.032**	0.550	0.878	0.555	0.179	0.245	0.898
Double-strand DNA breaks (OTM)	*Rs*	-	-	0.088	-0.332	0.073	-0.065	0.029	0.217	0.104
	P value			0.677	0.105	0.727	0.756	0.902	0.297	0.654
Chromatin decondensation (%)	*Rs*	-	-	-	0.002	0.048	0.241	0.166	-0.324	0.052
	P value				0.991	0.818	0.246	0.471	0.114	0.823
Chromatin deprotamination (AU)	*Rs*	-	-	-	-	-0.169	0.065	**-0.434**	-0.095	0.114
	P value					0.419	0.759	**0.049**	0.650	0.622

2.3. Experiment 3: Correlations between sperm quality evaluated after thawing and fertility outcomes

After assessing sperm quality, all static (i.e., measured at a specific time point) and dynamic parameters (i.e., variation between two time points) were tested for correlation with non-return rates (NRR) after artificial insemination. *Table 12* lists the parameters for which correlations were found to be significant.

Regarding static parameters, fertility rates were found to be correlated to total motility at 2 h *(Rs* = 0.512, *P* = 0.021) and the percentage of sperm with high intracellular ROS levels at 4 h (*Rs* = 0.553, *P* = 0.004). Conversely, no dynamic parameter correlated to non-return rates (*P* > 0.05).

Table 12. Statistically significant correlations (*P* < 0.05) of static and dynamic parameters and NRR.

	Rs	**95% Confidence interval**	***P value***
Static parameters			
T0 Progressive motility (%)	0.511	0.133 to 0.759	0.009
T0 Fast motility (%)	0.489	0.104 to 0.746	0.013
T2 Total motility (%)	0.512	0.076 to 0.784	0.021
T2 Progressive motility (%)	0.509	0.072 to 0.782	0.022
T2 Fast motility (%)	0.469	0.019 to 0.761	0.037
T4 Sperm with high ROS (% DCF^{+})	0.553	0.190 to 0.783	0.004
Dynamic parameters			
No statistically significant correlations			

CHAPTER II

Pig sperm-rich fraction exhibits higher sperm chromatin condensation, but does not differ in sperm DNA integrity

1. Experimental design

This Chapter sought to describe the chromatin condensation and protamination, and DNA integrity of sperm from the different ejaculate fractions. The study was conducted in the pig model, as the collection of different semen fractions in this species is highly standardised. Ejaculates from eight healthy, fertile and mature boars were collected in three separate fractions: the first 10 mL of the sperm rich fraction (SRP-P1), the rest of the sperm-rich fraction (SRF-P2) and the post sperm-rich fraction (PSRF). In the first experiment, an aliquot of each fraction per boar was taken to examine sperm chromatin protamination (Chromomycin A_3 test, CMA_3), sperm chromatin condensation (Dibromobimane test, DBB), the incidence of double-strand DNA breaks (neutral Comet assay) and global sperm DNA damage (alkaline Comet assay).

Based on the results of Experiment 1, a second experiment was performed to test whether sperm concentration and the oxidative stress index, which differ between fractions, were correlated. To assess that, six pools of four animals (n=24) were obtained by mixing the sperm samples from the animals involved in Experiment 1. For each pool, sperm concentration (sperm/mL), oxidant activity (AOPP method) and total antioxidant capacity (CUPRAC method) were evaluated. Finally, the oxidative stress index (OSi) was defined by dividing the relative amount of oxidant products by the total antioxidant capacity. The formula used was: OSi (µmol oxidants / µmol antioxidants) = AOPPs / CUPRAC.

2. Results

2.1. Experiment 1: Evaluation of sperm chromatin

2.1.1. Sperm chromatin protamination is similar between the ejaculate fractions.

Sperm chromatin protamination of ejaculate fractions, measured by CMA_3 fluorescence intensity, was assessed at 0 h (*Figure 15* and *Table 13*). No significant differences between fractions were observed ($P > 0.05$).

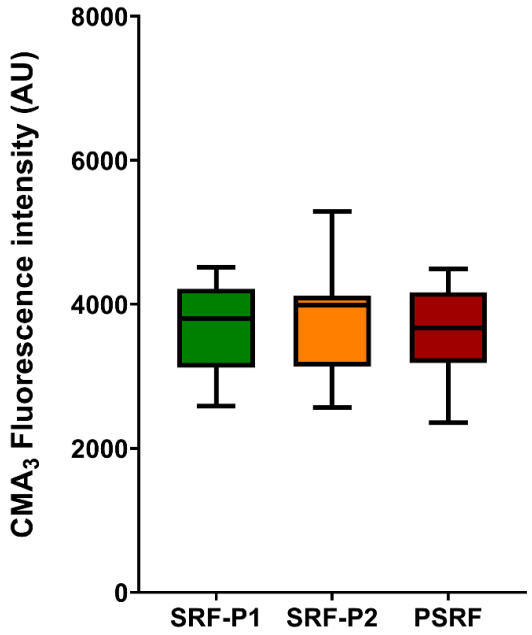

Figure 15. Box-whisker plots showing sperm chromatin protamination (CMA_3 fluorescence intensity, AU) in each ejaculate fraction. Each box encloses data between the 25th and 75th percentiles, whereas the whiskers extend from the minimum to the maximum values and the line indicates the median. AU: arbitrary units.

Table 13. Sperm chromatin protamination in each ejaculate fraction (CMA_3 fluorescence intensity, AU). Data are presented as mean ± standard deviation (SD). AU: arbitrary units.

CMA_3 fluorescence intensity (AU)		
SRF-P1 Mean ± SD	**SRF-P2** Mean ± SD	**PSRF** Mean ± SD
3679.50 ± 643.63	3829.58 ± 819.65	3623.23 ± 672.62

2.1.2. Sperm chromatin is more condensed in SRP-P1 and SRP-P2 than in PSRF

Dibromobimane, a reagent that reacts with free thiols and emits green fluorescence, was used to inversely evaluate chromatin condensation; indeed, the greater the fluorescence emitted by DBB, the lower the degree of chromatin condensation (and the higher the degree of chromatin decondensation). *Figure 16* and *Table 14* show the degree of chromatin condensation (DBB fluorescence intensity) exhibited by the sperm from SRP-P1, SRP-P2 and PSRF. Significantly lower chromatin condensation was detected in the sperm of the PSRF compared to the sperm of SRP-P1 (P = 0.018) and SRF-P2 (P = 0.004).

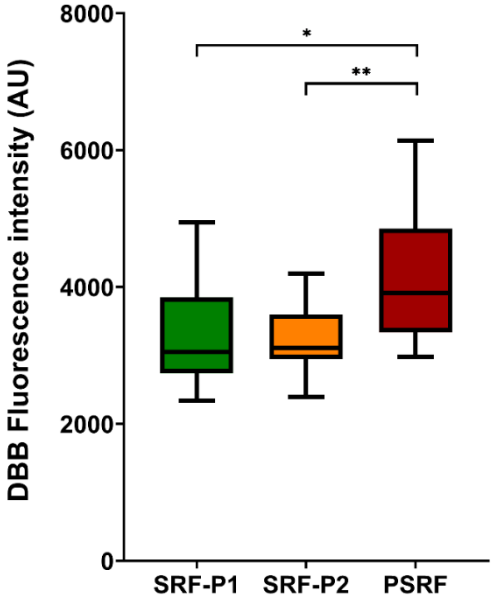

Figure 16. Box-whisker plots showing sperm chromatin condensation (DBB fluorescence intensity, AU). Each box encloses data between the 25th and 75th percentiles, whereas the whiskers extend from the minimum to the maximum values and the line indicates the median. (*) Indicates statistically significant differences with $P \leq 0.05$; and (**) indicates statistically significant differences with $P \leq 0.01$. AU: arbitrary units.

Table 14. Sperm chromatin condensation (DBB fluorescence intensity, AU) in each ejaculate fraction. Data are presented as mean ± standard deviation (SD). ([a]) Statistical differences compared to SRP-P1 ($P < 0.05$); and ([b]) statistical differences compared to SRF-P2 ($P < 0.05$). AU: arbitrary units.

DBB fluorescence intensity (AU)		
SRF-P1	**SRF-P2**	**PSRF**
Mean ± SD	Mean ± SD	Mean ± SD
3308.71 ± 835.20	3226.04 ± 537.89	4144.33 [a,b] ± 1025.65

2.1.3. Sperm from the different ejaculate fractions exhibit a similar incidence of DNA damage

The incidence of global DNA damage, which includes both single and double-strand DNA breaks, was evaluated in all ejaculate fractions using the alkaline Comet assay (*Figure 17B* and *Table 15*). No significant differences between fractions were observed ($P > 0.05$).

Moreover, the incidence of double-strand DNA breaks in sperm from all ejaculate fractions was determined using the neutral Comet assay (*Figure 17A* and *Table 15)*. In the same fashion to that observed for global DNA damage, the incidence of double-strand DNA breaks did not differ between ejaculate fractions ($P > 0.05$).

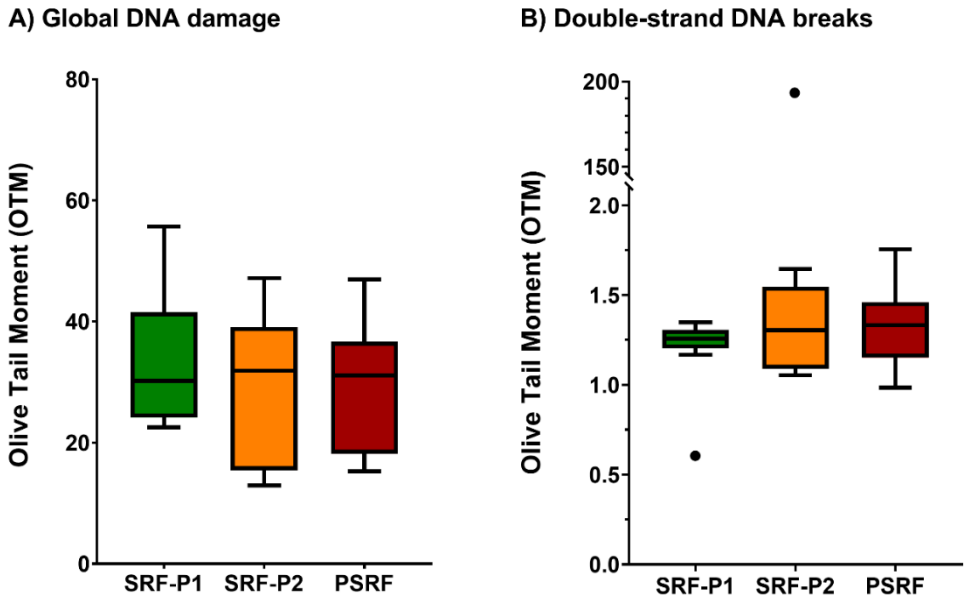

Figure 17. Box-whisker plots showing the incidence of (A) global DNA damage (Alkaline comet), and (B) double-strand DNA breaks (Neutral comet) in each ejaculate fraction. Each box encloses data between the 25th and 75th percentiles, whereas the whiskers extend from the minimum to the maximum values and the line indicates the median. Dots represent outlier values.

Table 15. Incidence of global DNA damage and double-strand DNA breaks. Data are presented as mean ± standard deviation (SD).

SRF-P1 Mean ± SD	**SRF-P2** Mean ± SD	**PSRF** Mean ± SD
Global DNA damage (OTM)		
33.85 ± 11.28	28.98 ± 12.79	29-16 ± 11.09
Double-strand fragmentation (OTM)		
1.25 ± 0.06	1.32 ± 0.22	1.33 ± 0.24

2.2. Experiment 2: The Oxidative Stress Index (OSi) is higher in the SRP-P1 than in SRP-P2 and PSRF, and is correlated to sperm concentration

Sperm concentration, oxidant activity, total antioxidant capacity and OSi of all fractions are depicted in *Table 16*. The SRP-P1 showed a higher sperm concentration and oxidant activity compared to SRP-P2 and PSRF ($P < 0.01$), and a lower total antioxidant capacity than the PSRF ($P < 0.01$). In contrast, the PSRF exhibited the lowest sperm concentration and oxidant activity ($P < 0.01$), and a higher antioxidant capacity than the SRP-P1 ($P < 0.01$). More importantly, the relationship between oxidant and antioxidant components, evaluated as OSi, was significantly higher in the SRP-P1 than in the SRP-P2 ($P = 0.002$), and higher in the SRP-P2 than in the PSRF ($P = 0.023$). Moreover, sperm concentration was found to be positively correlated to OSi (Rs = 0.973; $P < 0.001$) (*Figure 18*), so that it followed the same trend as OSi, the sperm concentration and OSi being the lowest in the PSRF, and the highest in the SRP-P1.

Table 16. Sperm concentration, oxidant activity (AOPP), total antioxidant capacity (CUPRAC) and oxidative stress index (OSi = AOPP / CUPRAC) in the different ejaculate-fractions (SRP-P1, SRP-P2 and PSRF). Data are presented as mean ± SD. ([a]) Statistical differences compared to SRP-P1 ($P < 0.05$); ([b]) statistical differences compared to SRP-P2 ($P < 0.05$); ([c]) Statistical differences compared to PSRF ($P < 0.05$).

	Concentration (spz/mL) Mean ± SD	**AOPP (µmol/L)** Mean ± SD	**CUPRAC (µmol/L)** Mean ± SD	**OSi (AOPP/CUPRAC)** Mean ± SD
SRF-P1	$1.34\times10^{9} \pm 1.88\times10^{8}$ [b,c]	78.90 ± 15.60 [b,c]	188.40 ± 15.42 [c]	0.42 ± 0.06 [b,c]
SRF-P2	$5.66\times10^{8} \pm 1.27\times10^{8}$ [a,c]	48.25 ± 11.85 [a,c]	224.23 ± 41.66	0.23 ± 0.09 [a,c]
PSRF	$1.32\times10^{8} \pm 3.90\times10^{7}$ [a,b]	20.15 ± 1.28 [a,b]	250.32 ± 9.58 [a]	0.08 ± 0.00 [a,b]

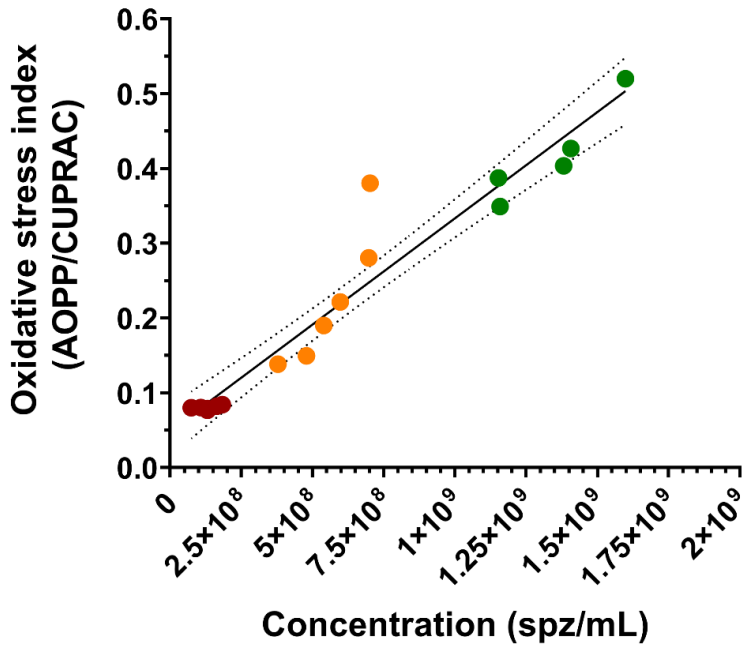

Figure 18. Spearman correlation between sperm count (sperm/mL) and the oxidative stress index in each ejaculate fraction (SRP-P1, SRP-P2 and PSRF). Each fraction is represented by a different colour: green for the sperm from SRP-P1, orange for the sperm from SRF-P2, and red for the sperm from PSRF.

CHAPTER III

An intracellular, non-oxidative mechanism activates sperm chromatin fragmentation in ejaculated sperm *in vitro*

1. Experimental design

In this Chapter, separate experiments were devised: (i) to investigate whether the SCF mechanism can be activated in ejaculated sperm; (ii) to examine whether Mg^{2+} ions can trigger SCF *in vitro*; (iii) to determine if the SCF activation entails other consequences for sperm function and survival; and (iv) to evaluate whether SCF causes an effect on embryo development. In order to address these aims, four experiments were designed, interrogating each of the previously mentioned aspects.

In the first experiment, the generation of DNA breaks in ejaculated sperm by Mn^{2+} and Mg^{2+} ions was evaluated. For this purpose, ejaculated sperm were incubated with these ions at different concentrations and for different times. In addition, permeabilised and non-permeabilised sperm samples were used to investigate whether the mechanism responsible for inducing DNA breaks is triggered outside the cell or involves the intracellular machinery. For this purpose, 50 mL of three semen samples from different boars (n=3) containing 33×10^6 sperm/mL were centrifuged at 600g for 10 min at room temperature. Then, for each sample, the supernatant was removed, and the pellet fraction was resuspended in TKB buffer (25 mM Tris-HCl, 150 mM KCl, pH=7.5). Subsequently, to permeabilise the sperm, samples were incubated with 0.25 % Triton X-100 for 10 min on ice. Following this, each sample (permeabilised and non-permeabilised) was split into two aliquots (for incubation with Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+}, respectively), and each aliquot into 10 tubes (five to assess the dose-response and five to evaluate the time-response). To evaluate the dose-response, samples were incubated with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} at 0 mM (Control), 0.1 mM, 1 mM, 5 mM and 50 mM (prepared with the appropriate volumes of $MnCl_2$, $MgCl_2$ and $CaCl_2$, all at 0.5 M) for 10 min at 37 °C. Furthermore, to evaluate the time-response, samples were

incubated with 10 mM Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} for 0 min (Control), 2 min, 10 min, 30 min or 60 min at 37 °C. After incubation, the incidence of single and double DNA breaks was determined through the Comet assay.

After confirming that incubation with Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} induces DNA breaks, pulsed-field gel electrophoresis (PFGE) was used in the second experiment to identify the size of the resulting DNA fragments in non-permeabilised ejaculated sperm. Briefly, three semen samples from different boars (n=3), containing 33×10^6 sperm/mL, were centrifuged at 600g and room temperature for 10 min and the pellet fraction was resuspended in TKB buffer (25 mM Tris-HCl, 150 mM KCl, pH=7.5). Samples were subsequently mixed 1:1 (v:v) with 1 % PFGE (Pulsed-Field Certified Agarose BioRad; Hercules, CA, USA), placed into moulds and allowed to solidify at 4 °C. Thereafter, the resulting plugs were incubated with 10 mM Mn^{2+}/Ca^{2+} or 10 mM Mg^{2+}/Ca^{2+} at 37 °C for 30 min, and with lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 20 mM DTT, 2 % SDS and 20 mg/mL proteinase K, pH = 8.0) at 53 °C for 1 h. Finally, samples were subject to PFGE. Negative controls were non-treated sperm in TKB buffer (25 mM Tris-HCl, 150 mM KCl, pH = 7.5) without Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+}, and were also incubated at 37 °C for 30 min.

For the third experiment, flow cytometry and Computer Assisted Sperm Analysis (CASA) were used to determine the impact of different Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} concentrations and incubation times on sperm function and survival. For all treatments, 50 mL of each sample (n=3, from three different boars) were centrifuged at 600g and room temperature for 10 min to remove the preservation medium. After centrifugation, the supernatant was removed and the pellet was resuspended in 50 mL of PBS, previously warmed at 37 °C. Then, samples were distributed in 1-mL aliquots according to the number of treatments and incubation times tested. Following this, the corresponding Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+}

treatments were added to sperm samples. To evaluate the dose and incubation time response, sperm samples were incubated with different concentrations of Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} (Control, 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM) for different incubation times (2 min, 10 min, 30 min and 60 min) at 37 °C. Once conducted, sperm viability and the percentages of sperm with high intracellular ROS and superoxide levels were assessed by flow cytometry. Also, sperm motility was assessed using CASA, and sperm agglutination was evaluated under a phase-contrast microscope.

In the fourth experiment of this Chapter, *in vitro* fertilisation was used to investigate how incubation of sperm with different doses of Mn^{2+}/Ca^{2+} affects their fertilising capacity. For this purpose, four pools made up of equal volumes of semen from four boars were treated with different concentrations of Mn^{2+}/Ca^{2+} before its use for *in vitro* fertilisation. To this end, 40 mL of each pool were centrifuged at 600g and room temperature for 10 min. After centrifugation, the supernatant was removed and the pellet was resuspended in 40 mL of PBS, previously tempered at 37 °C. Then, each sample was distributed in five aliquots of 3 mL each. Subsequently, the corresponding amount of Mn^{2+}/Ca^{2+} was added to each aliquot to obtain final concentrations of 0.1 mM, 1 mM, 5 mM, 10 mM, prior to incubating the samples at 37 °C for 10 min. The remaining aliquot was used as the untreated control. After incubation of sperm with Mn^{2+}/Ca^{2+}, IVF was carried out.

2. Results

2.1. Experiment 1: Sperm DNA breaks can be induced in ejaculated sperm through intracellular divalent ions, but Mn^{2+} is more effective than Mg^{2+}

Dose-response results for both Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} treatments in non-permeabilised and permeabilised sperm samples are shown in *Figure 19* and *Table 17*. On the one hand, Mn^{2+}/Ca^{2+} exhibited a dose-dependent effect on the induction of DNA breaks, both in non-permeabilised and permeabilised samples (*Figure 19A*). Specifically, significant differences were found among Mn^{2+}/Ca^{2+} treatments ($P < 0.05$), except between 5 mM and 50 mM in non-permeabilised sperm and between control and 0.1 mM in permeabilised samples ($P > 0.05$). In contrast, incubation with Mg^{2+}/Ca^{2+} showed no dose-dependent effect as statistical differences were only observed between the control and 5 mM ($P = 0.010$), and between 50 mM Mg^{2+}/Ca^{2+} and all the other treatments ($P < 0.05$), in both non-permeabilised and permeabilised samples ($P < 0.05$) (*Figure 19B*). Furthermore, no statistically significant differences were found between non-permeabilised and permeabilised samples after incubation with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} ($P > 0.05$) (*Figure 20*), evidencing that an intracellular component could be involved in triggering the SCF mechanism and thus generate DNA breaks.

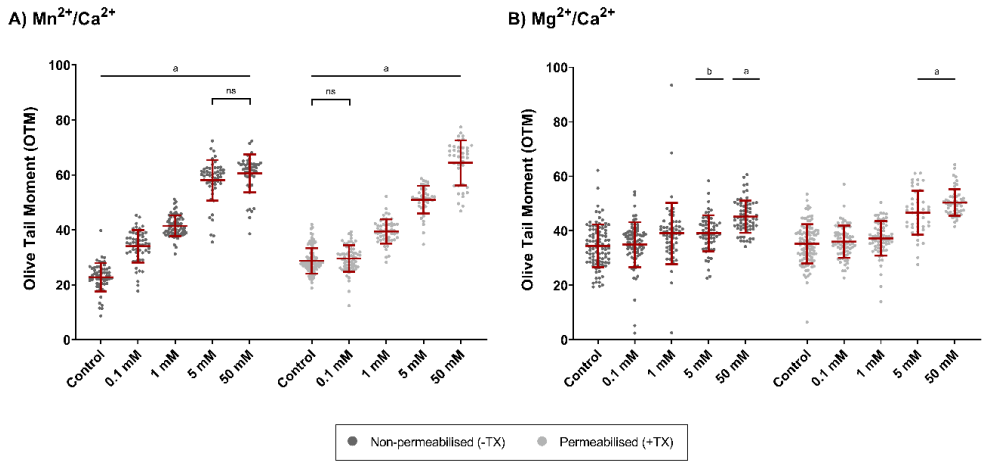

Figure 19. Comet assay Olive Tail Moment (OTM) after incubation with different concentrations (0 mM, 0.1 mM, 1 mM, 5 mM and 50 mM) of (A) Mn^{2+}/Ca^{2+} and (B) Mg^{2+}/Ca^{2+} in non-permeabilised (-TX) and permeabilised (+TX) samples. Data are shown as mean ± SD. ([a]) Statistical differences compared to all treatments within the same TX group ($P < 0.05$) unless indicated by *ns*. ([b]) Statistical differences compared to the control within the same TX group ($P < 0.05$).

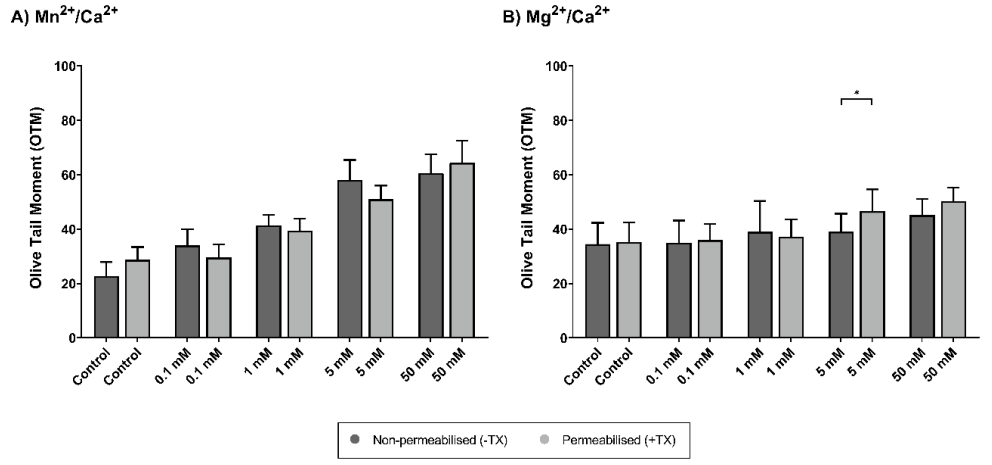

Figure 20. Comet assay Olive Tail Moment (OTM) obtained after incubation with different concentrations (0 mM, 0.1 mM, 1 mM, 5 mM and 50 mM) of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+} in non-permeabilised (-TX) and permeabilised (+TX) samples. Data are shown as mean ± SD. (*) Statistically significant differences ($P < 0.05$).

Table 17. Olive Tail Moment (OTM) (mean ± SD) values of the alkaline Comet after incubation of sperm with different concentrations of (A) Mn^{2+}/Ca^{2+} and (B) Mg^{2+}/Ca^{2+} (0 mM, 0.1 mM, 1 mM, 5 mM and 50 mM) in non-permeabilised (-TX) and permeabilised (+TX) samples. Data are shown as mean ± SD. ([a]) Statistical differences compared to all treatments within the same TX group ($P < 0.05$), unless indicated by *ns*. ([b]) Statistical differences compared to the control within the same TX group ($P < 0.05$).

	Olive Tail Moment					
	Non-permeabilised (- TX)			**Permeabilised (+ TX)**		
	Mean	±	SD	Mean	±	SD
(A) Mn^{2+}/Ca^{2+}						
Control (0 mM)	22.72	±	5.18 [a]	28.73	±	4.65 [a]
0.1 mM	34.03	±	5.88 [a]	29.55	±	4.84 [a]
1 mM	41.45	±	3.81 [a]	39.45	±	4.41 [a]
5 mM	58.07	±	7.38 [a]	51.01	±	5.02 [a]
50 mM	60.53	±	6.93 [a]	64.41	±	8.17 [a]
(B) Mg^{2+}/Ca^{2+}						
Control (0 mM)	34.48	±	7.86	35.20	±	7.20
0.1 mM	34.93	±	8.22	35.99	±	5.90
1 mM	39.06	±	11.26	37.21	±	6.32
5 mM	39.08	±	6.61 [b]	46.63	±	8.01 [a]
50 mM	45.18	±	5.94 [a]	50.35	±	4.89 [a]

On the other hand, there was no effect of incubation time on the activation of the SCF mechanism regardless of the permeabilisation status of sperm (*Figure 21; Table 18*). Incubations with 5 mM Mn^{2+}/Ca^{2+} for different times (2 min, 10 min, 30 min and 60 min) resulted in similar DNA damage ($P > 0.05$). In the case of Mg^{2+}, only the longest incubation (60 min) with Mg^{2+}/Ca^{2+} appeared to evoke SCF, as only this period was significantly different from the others (2 min, 10 min and 30 min) and from the control ($P < 0.05$).

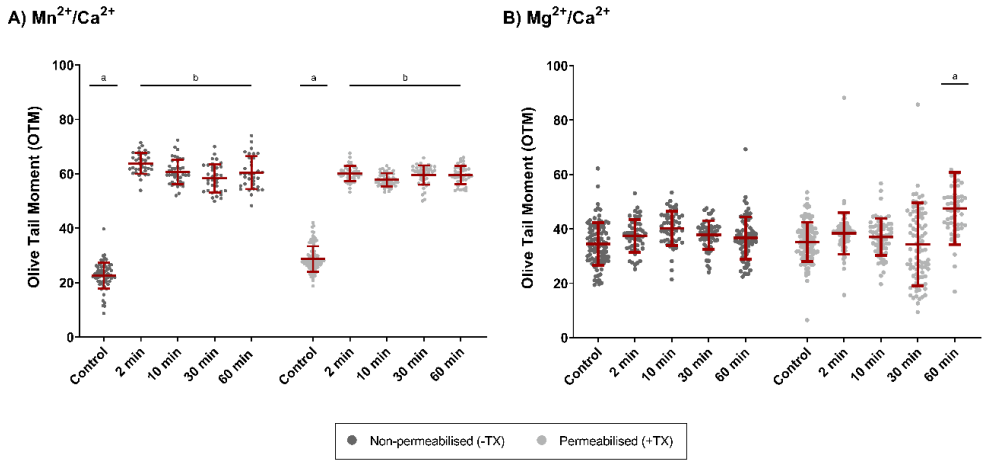

Figure 21. Comet assay Olive Tail Moment (OTM) obtained after incubation of sperm for different times (0 min, 2 min, 10 min, 30 min, 60 min) with 10 mM of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+} in non-permeabilised (-TX) and permeabilised (+TX) samples. Data are shown as mean ± SD. ([a]) Statistical differences compared to all treatments within the same TX group ($P < 0.05$). ([b]) Statistical differences compared to the control within the same TX group ($P < 0.05$).

Finally, while the effects of incubation time did not differ between non-permeabilised and permeabilised sperm in Mn^{2+}/Ca^{2+} treatments, the extent of DNA damage was higher in permeabilised than in non-permeabilised samples after incubation with Mg^{2+}/Ca^{2+} for 60 min ($P < 0.05$) (*Figure 22*).

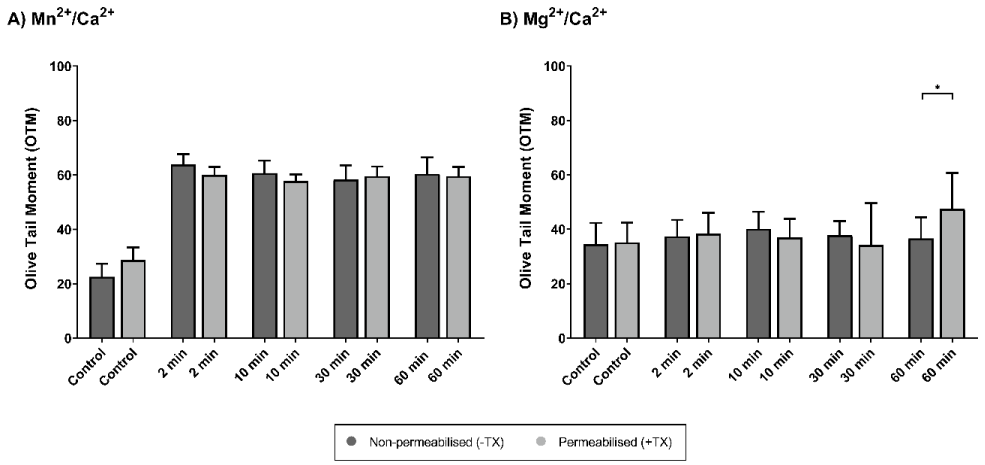

Figure 22. Comet assay Olive Tail Moment (OTM) obtained after incubation of sperm for different times (0 min, 2 min, 10 min, 30 min, 60 min) with 10 mM of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+} in non-permeabilised (-TX) and permeabilised (+TX) samples. Data are shown as mean ± SD. (*) Statistically significant differences ($P < 0.05$).

Table 18. Comet assay Olive Tail Moment (OTM) (mean ± SD) obtained after incubation of sperm for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min) with 10 mM of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+} in non-permeabilised (-TX) and permeabilised (+TX) samples. ([a]) Statistical differences compared to all treatments within the same TX group ($P < 0.05$). ([b]) Statistical differences compared to the control within the same TX group ($P < 0.05$).

	Olive Tail Moment					
	Non-permeabilised (- TX)			**Permeabilised (+ TX)**		
	Mean	±	SD	Mean	±	SD
(A) 10 mM Mn^{2+}/Ca^{2+}						
Control (0 min)	22.57	±	4.82 [a]	28.73	±	4.65 [a]
2 min	63.80	±	3.80 [b]	60.07	±	2.82 [b]
10 min	60.71	±	4.55 [b]	57.85	±	2.39 [b]
30 min	58.34	±	5.24 [b]	59.55	±	3.52 [b]
60 min	60.42	±	6.03 [b]	59.59	±	3.33 [b]

Table 18. (Continued).

	Olive Tail Moment					
	Non-permeabilised (- TX)			**Permeabilised (+ TX)**		
	Mean	±	SD	Mean	±	SD
(B) 10 mM Mg^{2+}/Ca^{2+}						
Control (0 min)	34.48	±	7.86	35.20	±	7.20
2 min	37.44	±	6.01	38.35	±	7.67
10 min	40.16	±	6.29	37.04	±	6.75
30 min	37.76	±	5.24	34.30	±	15.30
60 min	36.63	±	7.79	47.51	±	13.27 [a]

2.2. Experiment 2: Incubation with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} triggers the SCF mechanism in ejaculated sperm *in vitro*

After confirming that incubation with Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} induces DNA breaks, pulsed-field gel electrophoresis was used to determine the sizes of the fragments generated. Noticeably, incubation with Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} increased the amount of DNA fragments ranging between 33 Kb and 194 Kb ($P < 0.05$), a size that would be compatible with the DNA condensed into one to four toroids (*Figure 23*). This would suggest that Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} trigger SCF in ejaculated sperm *in vitro,* in a similar fashion to that observed before in epididymal and vas deferens mouse sperm *in vivo* (Ribas-Maynou et al., 2022a).

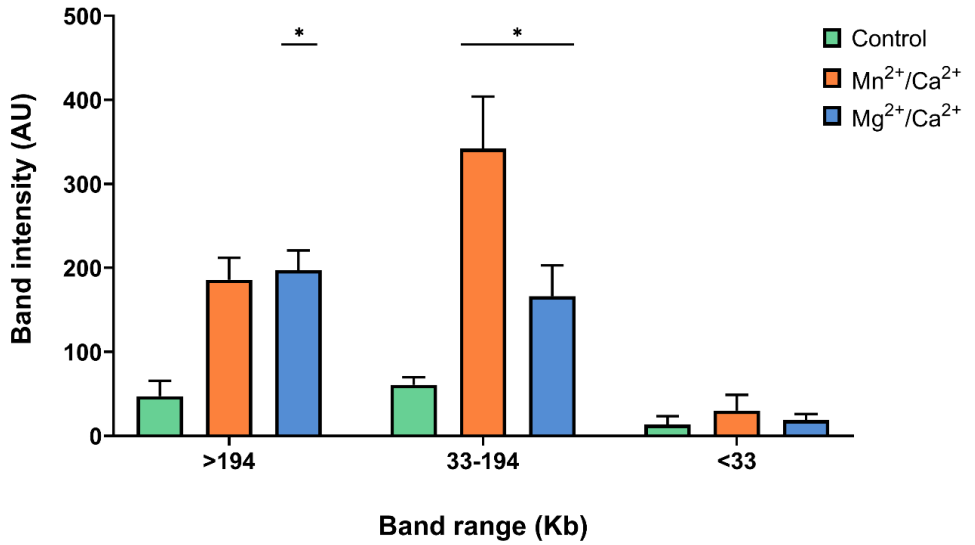

Figure 23. Intensity of bands after Pulsed Field Gel Electrophoresis (mean ± SD). Different DNA lengths were analysed: DNA fragments larger than 194 Kb, compatible to mostly intact DNA; DNA fragments ranging between 33 Kb and 194 Kb, compatible to the size of DNA packed into one to four toroids; and DNA fragments shorter than 33 Kb, compatible to DNA sizes smaller than one toroid. (*) Statistically significant differences ($P < 0.05$).

2.3. Experiment 3: The induction of SCF *in vitro* impairs sperm motility and induces sperm agglutination

In addition to sperm DNA integrity, the effects of Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} on sperm motility, agglutination, viability and levels of total ROS and superoxides were also examined.

2.3.1. Mn^{2+}/Ca^{2+} impairs sperm motility in a dose-dependent manner, whereas Mg^{2+}/Ca^{2+} only has an effect at high concentrations

Figure 24A (*Table 19*) shows that Mn^{2+}/Ca^{2+} caused a concentration-dependent decrease in sperm motility, with a noticeable reduction at high

concentrations ($P < 0.05$). Yet, incubation time did not have a significant effect on sperm motility. On the other hand, incubation with Mg^{2+}/Ca^{2+} also caused a drastic decrease on sperm motility, but only at the highest concentration (50 mM) ($P < 0.05$), while the other concentrations (Control, 0.1 mM, 1 mM, 5 mM, and 10 mM) did not have any effect ($P > 0.05$) (*Figure 24B, Table 20*).

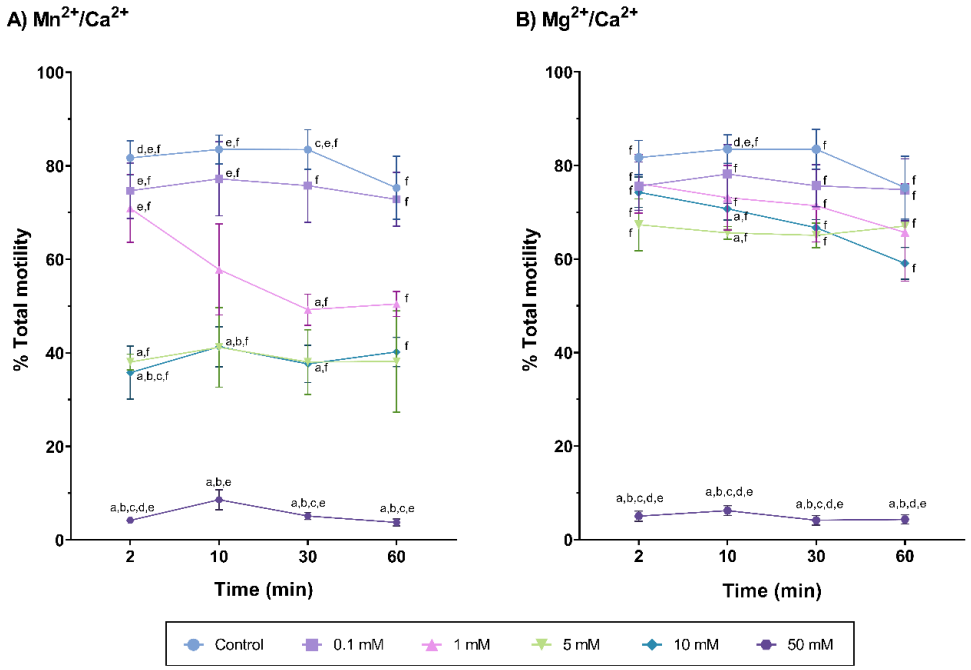

Figure 24. Percentages of motile sperm after incubation with different concentrations (0 mM [Control], 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM) of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+}, for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD. ([a]) Statistical differences compared to the control (0 mM); ([b]) Statistical differences compared to 0.1 mM treatment; ([c]) Statistical differences compared to 1 mM treatment; ([d]) Statistical differences compared to 5 mM treatment; ([e]) Statistical differences compared to 10 mM treatment; and ([f]) Statistical differences compared to 50 mM treatment.

2.3.2. Incubation with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} has no effect on sperm viability

The effects of incubation with Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} on sperm viability are depicted in *Figure 25* (*Table 19* and *Table 20*, respectively). Statistical analysis showed that neither Mn^{2+}/Ca^{2+} nor Mg^{2+}/Ca^{2+} had a significant impact on sperm viability ($P > 0.05$). Higher concentrations of Mn^{2+}/Ca^{2+} (50 mM) and Mg^{2+}/Ca^{2+} (10 and 50 mM) as well as longer incubation times (60 minutes) did, however, tend to decrease the percentage of viable sperm.

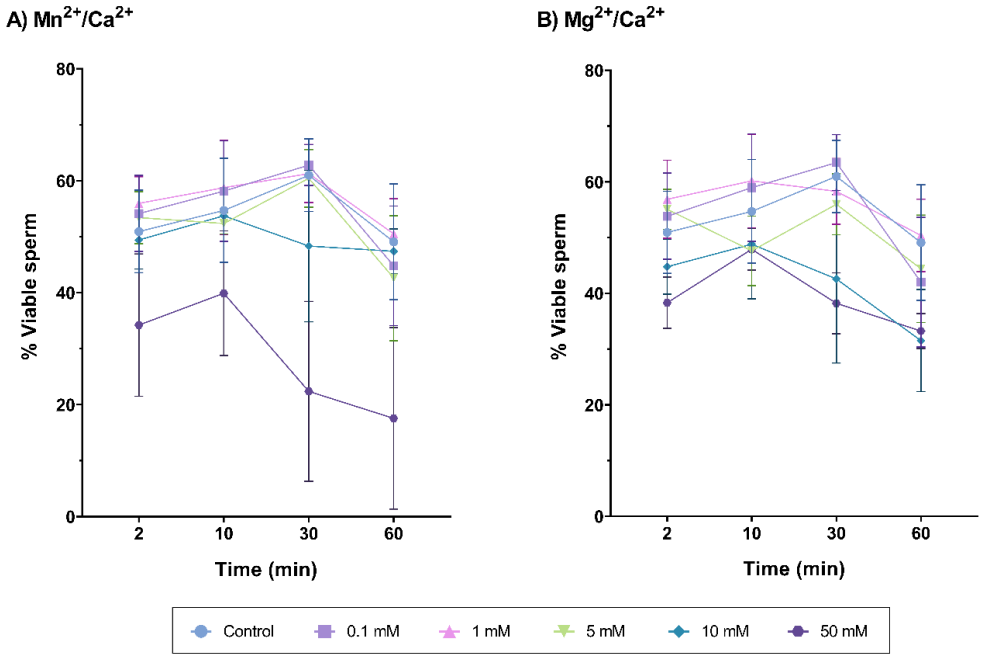

Figure 25. Percentages of viable sperm after incubation with different concentrations (0 mM [Control], 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM) of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+}, for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD.

2.3.3. SCF is not related to oxidative stress, as neither total ROS nor superoxide levels are altered following incubation of sperm with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+}

As shown in *Figure 26*, *Table 19* and *Table 20*, no significant increase in the percentages of sperm with high intracellular levels of total ROS or superoxides was observed after incubation with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} ($P > 0.05$). The fact that reactive oxygen species did not increase following the activation of SCF suggests that this mechanism does not rely upon oxidative stress.

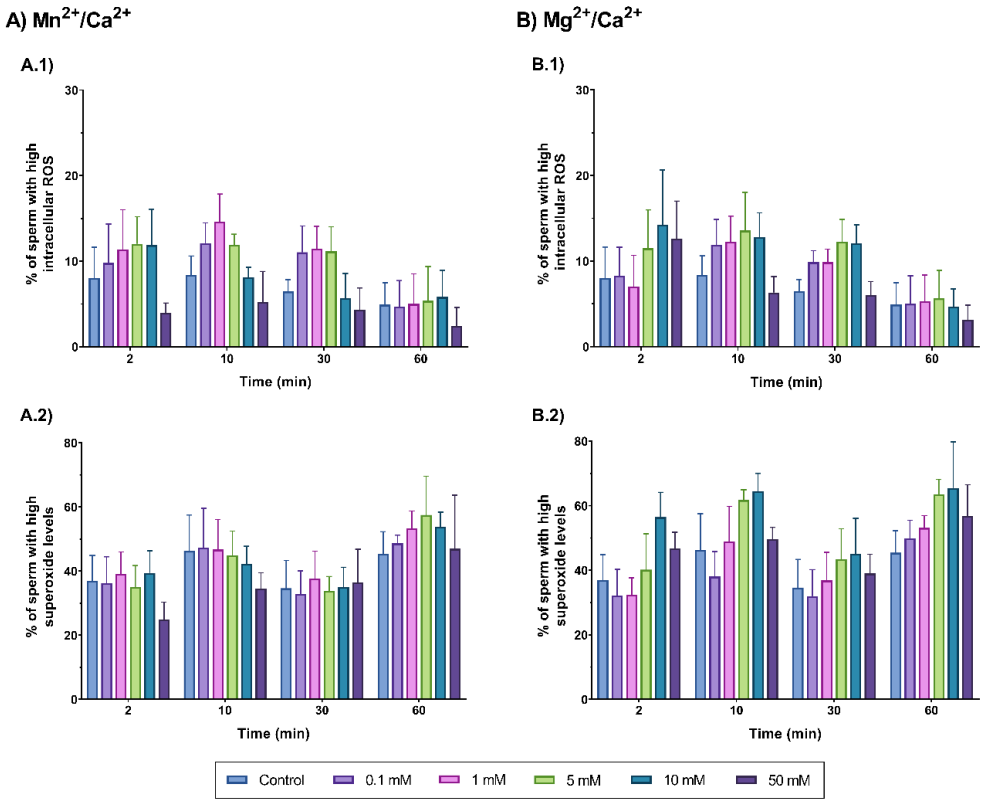

Figure 26. Percentages of sperm with high total ROS and superoxide levels after incubation with different concentrations (0 mM [Control], 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM) of Mn^{2+}/Ca^{2+} (A.1 and A.2) or Mg^{2+}/Ca^{2+} (B.1 and B.2), for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD.

2.3.4. Incubation with both Mn^{2+}/Ca^{2+} and Mg^{2+}/Ca^{2+} induces sperm agglutination

As presented in *Figure 27, Table 19* and *Table 20*, the percentage of agglutinated sperm increased in a dose-dependent manner after incubation with Mn^{2+}/Ca^{2+} or Mg^{2+}/Ca^{2+} ($P < 0.05$). In contrast, there was no influence from the incubation time ($P > 0.05$). A significant increase of sperm agglutination was observed at high concentrations of Mn^{2+}/Ca^{2+} (10 mM and 50 mM), with the percentage of agglutination being greater than 80 %.

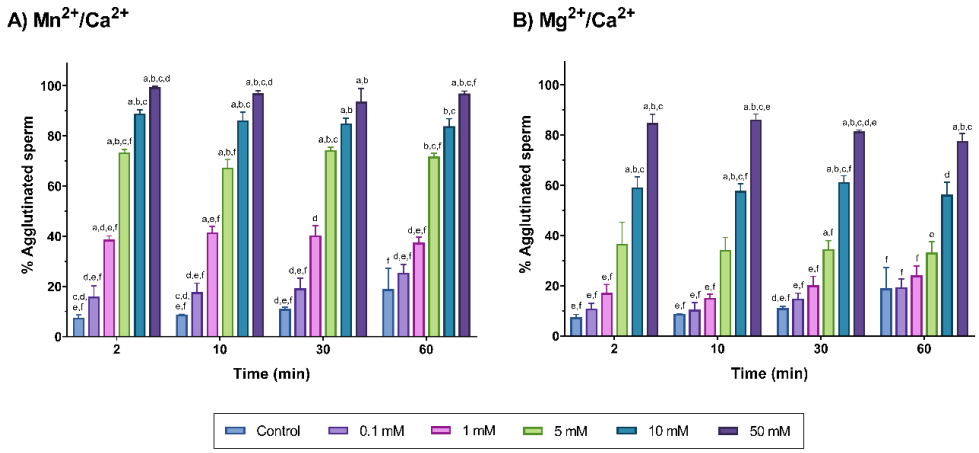

Figure 27. Percentages of agglutinated sperm after incubation with different concentrations (0 mM [Control], 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM) of (A) Mn^{2+}/Ca^{2+} or (B) Mg^{2+}/Ca^{2+}, for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD. ([a]) Statistical differences compared to the control (0 mM). ([b]) Statistical differences compared to 0.1 mM treatment. ([c]) Statistical differences compared to 1 mM treatment. ([d]) Statistical differences compared to 5 mM treatment. ([e]) Statistical differences compared to 10 mM treatment. ([f]) Statistical differences compared to 50 mM treatment.

Table 19. Percentages of motility, viability, sperm with high intracellular ROS, sperm with high intracellular superoxides, and agglutinated sperm after incubation with different concentrations of Mn^{2+}/Ca^{2+} (0 mM, 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM), for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD. ([a]) Statistical differences compared to the control (0 mM). ([b]) Statistical differences compared to 0.1 mM treatment. ([c]) Statistical differences compared to 1 mM treatment. ([d]) Statistical differences compared to 5 mM treatment. ([e]) Statistical differences compared to 10 mM treatment. ([f]) Statistical differences compared to 50 mM treatment.

	MOTILITY (%)	**VIABILITY (%)**	**TOTAL ROS (%)**	**SUPEROXIDES (%)**	**AGGLUTINATION (%)**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
2 min					
Control (0 mM)	81.69 ± 6.36 [d,e,f]	50.93 ± 12.74	8.01 ± 6.28	36.95 ± 13.67	7.48 ± 2.05 [c,d,e,f]
0.1 mM	74.66 ± 10.29 [e,f]	54.13 ± 11.77	9.80 ± 7.86	36.28 ± 14.19	15.97 ± 6.69 [d,e,f]
1 mM	70.91 ± 12.54 [e,f]	55.96 ± 8.27	11.35 ± 8.07	39.13 ± 11.86	38.70 ± 2.74 [a,d,e,f]
5 mM	38.06 ± 2.91 [a,f]	53.41 ± 8.02	12.03 ± 5.48	35.00 ± 11.59	73.42 ± 2.54 [a,b,c,f]
10 mM	35.78 ± 9.80 [a,b,c,f]	49.42 ± 9.00	11.88 ± 7.28	39.45 ± 11.88	88.88 ± 2.97 [a,b,c]
50 mM	4.19 ± 0.61 [a,b,c,d,e]	34.21 ± 22.00	3.95 ± 1.99	24.90 ± 9.42	99.34 ± 0.84 [a,b,c,d]
10 min					
Control (0 mM)	83.49 ± 5.29 [e,f]	54.70 ± 16.13	8.38 ± 3.84	46.28 ± 19.39	8.81 ± 1.44 [c,d,e,f]
0.1 mM	77.22 ± 13.69 [e,f]	58.15 ± 15.55	12.12 ± 4.09	47.35 ± 21.23	17.67 ± 5.73 [d,e,f]
1 mM	57.84 ± 16.84	58.77 ± 14.60	14.61 ± 5.59	46.70 ± 16.20	41.52 ± 4.67 [a,e,f]
5 mM	41.19 ± 14.74	52.37 ± 3.30	11.90 ± 2.14	44.87 ± 13.15	67.43 ± 5.41 [a,b,f]
10 mM	41.33 ± 7.41 [a,b,f]	53.74 ± 7.88	8.12 ± 1.99	42.28 ± 9.42	86.14 ± 5.37 [a,b,c]
50 mM	8.61 ± 3.72 [a,b,e]	39.89 ± 19.33	5.25 ± 6.15	34.59 ± 8.42	96.99 ± 1.95 [a,b,c,d]

Table 19. (Continued)

	MOTILITY (%)	VIABILITY (%)	TOTAL ROS (%)	SUPEROXIDES (%)	AGGLUTINATION (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
30 min					
Control (0 mM)	83.47 ± 7.34 [c,e,f]	60.98 ± 11.23	6.46 ± 2.40	34.65 ± 15.04	11.02 ± 1.43 [d,e,f]
0.1 mM	75.76 ± 13.61 [f]	62.79 ± 6.32	11.05 ± 5.29	32.91 ± 12.34	19.26 ± 7.07 [d,e,f]
1 mM	49.24 ± 5.77 [a,f]	61.28 ± 8.99	11.45 ± 4.58	37.68 ± 14.78	40.28 ± 6.25 [d]
5 mM	38.05 ± 11.98	60.43 ± 8.92	11.15 ± 4.97	33.77 ± 7.83	74.32 ± 2.76 [a,b,c]
10 mM	37.65 ± 6.89 [a,f]	48.34 ± 23.48	5.69 ± 4.98	35.01 ± 10.71	84.95 ± 3.83 [a,b]
50 mM	5.12 ± 1.29 [a,b,c,e]	22.36 ± 27.83	4.32 ± 4.43	36.42 ± 18.03	93.63 ± 8.18 [a,b]
60 min					
Control (0 mM)	75.29 ± 11.65 [f]	49.09 ± 17.94	4.91 ± 4.41	45.43 ± 11.86	19.00 ± 12.95 [f]
0.1 mM	72.83 ± 10.00 [f]	44.80 ± 18.50	4.68 ± 5.28	48.65 ± 4.46	25.43 ± 5.34 [d,e,f]
1 mM	50.46 ± 4.64 [f]	50.51 ± 10.83	5.01 ± 6.06	53.38 ± 9.25	37.47 ± 4.27 [d,e,f]
5 mM	38.17 ± 18.72	42.61 ± 19.33	5.37 ± 6.96	57.51 ± 20.88	71.71 ± 2.98 [b,c,f]
10 mM	40.20 ± 5.43 [f]	47.41 ± 6.94	5.82 ± 5.40	53.82 ± 7.85	83.80 ± 4.90 [b,c]
50 mM	3.75 ± 1.30 [a,b,c,e]	17.54 ± 28.07	2.44 ± 3.68	46.96 ± 28.95	96.87 ± 3.02 [a,b,c,f]

Table 20. Percentages of motility, viability, sperm with high intracellular ROS, sperm with high intracellular superoxides, and agglutinated sperm after incubation with different concentrations of Mg^{2+}/Ca^{2+} (0 mM, 0.1 mM, 1 mM, 5 mM, 10 mM and 50 mM), for different incubation times (0 min, 2 min, 10 min, 30 min, 60 min). Data are shown as mean ± SD. ([a]) Statistical differences compared to the control (0 mM). ([b]) Statistical differences compared to 0.1 mM treatment. ([c]) Statistical differences compared to 1 mM treatment. ([d]) Statistical differences compared to 5 mM treatment. ([e]) Statistical differences compared to 10 mM treatment. ([f]) Statistical differences compared to 50 mM treatment.

	MOTILITY (%)	**VIABILITY (%)**	**TOTAL ROS (%)**	**SUPEROXIDES (%)**	**AGGLUTINATION (%)**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
2 min					
Control (0 mM)	81.69 ± 6.36 [f]	50.93 ± 12.74	8.01 ± 6.28	36.95 ± 13.67	7.48 ± 2.05 [e,f]
0.1 mM	75.59 ± 8.97 [f]	53.82 ± 13.40	8.30 ± 5.73	32.18 ± 14.13	10.85 ± 3.35 [e,f]
1 mM	76.12 ± 10.89 [f]	56.87 ± 12.12	7.01 ± 6.31	32.42 ± 9.04	17.18 ± 5.10 [e,f]
5 mM	67.32 ± 9.64 [f]	55.09 ± 6.24	11.50 ± 7.77	40.16 ± 19.29	36.70 ± 19.72
10 mM	74.28 ± 5.60 [f]	44.76 ± 8.49	14.25 ± 11.05	56.46 ± 13.32	59.09 ± 6.82 [a,b,c]
50 mM	5.02 ± 1.89 [a,b,c,d,e]	38.31 ± 7.94	12.61 ± 7.60	46.77 ± 8.64	84.77 ± 5.56 [a,b,c]
10 min					
Control (0 mM)	83.49 ± 5.29 [d,e,f]	54.70 ± 16.13	8.38 ± 3.84	46.28 ± 19.39	8.81 ± 1.44 [e,f]
0.1 mM	78.16 ± 10.83 [f]	58.95 ± 16.71	10.90 ± 5.12	38.04 ± 13.45	10.41 ± 4.47 [e,f]
1 mM	73.11 ± 11.92 [f]	60.16 ± 14.58	12.23 ± 5.23	49.00 ± 18.74	15.11 ± 2.44 [e,f]
5 mM	65.59 ± 2.35 [a,f]	47.63 ± 10.77	13.56 ± 7.69	61.78 ± 5.44	34.24 ± 7.84
10 mM	70.74 ± 4.18 [a,f]	48.81 ± 16.93	12.80 ± 4.90	64.50 ± 9.59	57.82 ± 4.85. [a,b,c,f]
50 mM	6.19 ± 1.84 [a,b,c,d,e]	47.90 ± 6.50	6.34 ± 3.22	49.68 ± 6.26	86.15 ± 3.89 [a,b,c,e]

Table 20. (Continued)

	MOTILITY (%)	VIABILITY (%)	TOTAL ROS (%)	SUPEROXIDES (%)	AGGLUTINATION (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
30 min					
Control (0 mM)	83.47 ± 7.34 [f]	60.98 ± 11.23	6.46 ± 2.40	34.65 ± 15.04	11.02 ± 1.43 [d,e,f]
0.1 mM	75.67 ± 7.75 [f]	63.44 ± 8.67	9.87 ± 2.33	32.02 ± 14.21	14.80 ± 3.63 [e,f]
1 mM	71.39 ± 13.43 [f]	58.29 ± 10.18	9.85 ± 2.68	36.89 ± 15.05	20.17 ± 5.94 [e,f]
5 mM	65.06 ± 4.57 [f]	55.97 ± 9.46	12.26 ± 4.55	43.39 ± 16.35	34.56 ± 5.70 [a,f]
10 mM	66.69 ± 2.99 [f]	42.57 ± 26.12	12.07 ± 3.73	45.16 ± 18.95	61.23 ± 5.97 [a,b,c,f]
50 mM	4.14 ± 1.74 [a,b,c,d,e]	38.20 ± 9.45	6.04 ± 2.72	39.00 ± 10.36	81.44 ± 2.36 [a,b,c,d,e]
60 min					
Control (0 mM)	75.29 ± 11.65 [f]	49.09 ± 17.94	4.91 ± 4.41	45.43 ± 11.86	19.00 ± 12.95 [f]
0.1 mM	74.82 ± 11.46 [f]	42.00 ± 20.10	5.00 ± 5.68	49.88 ± 9.71	19.35 ± 5.11 [f]
1 mM	65.67 ± 17.99	50.38 ± 11.28	5.31 ± 5.31	53.14 ± 6.49	24.11 ± 6.06 [f]
5 mM	67.04 ± 0.97 [f]	44.39 ± 6.67	5.64 ± 5.67	63.49 ± 8.07	33.15 ± 6.93 [e]
10 mM	59.06 ± 5.92 [f]	31.52 ± 15.86	4.68 ± 3.55	65.53 ± 24.71	56.22 ± 11.57 [d]
50 mM	4.32 ± 1.71 [a,b,d,e]	33.24 ± 5.47	3.12 ± 2.97	56.88 ± 16.61	77.70 ± 7.48 [a,b,c]

2.4. Experiment 4: The SCF induced by Mn^{2+} in ejaculated sperm has no impact on fertilisation rates or embryo development

In vitro fertilisation outcomes, including fertilisation rate and the percentages of total embryos, morulae, blastocysts and hatching/hatched blastocysts, are shown in *Table 21*. Although these *in vitro* fertility parameters tended to be lower when sperm had been previously treated with 10 mM Mn^{2+}/Ca^{2+}, no statistically significant differences between treatments were observed in any of the variables evaluated ($P > 0.05$).

Table 21. Fertilisation rate at Day 2, and percentages of morulae, blastocysts, and hatched blastocysts at Day 6 post-fertilisation resulting from sperm previously treated with different concentrations of Mn^{2+}/Ca^{2+} (0 mM [Control], 0.1 mM, 1 mM, 5 mM, and 10 mM). Data are presented as mean ± standard deviation (SD).

	Fertilisation rate (%) Mean ± SD	**Embryos (%)** Mean ± SD	**Morulae (%)** Mean ± SD	**Blastocysts (%)** Mean ± SD	**Hatched (%)** Mean ± SD
Control	46.75 ± 15.15	60.54 ± 14.50	13.87 ± 6.31	13.44 ± 8.00	2.39 ± 1.48
0.1 mM	50.74 ± 10.05	65.85 ± 11.31	15.76 ± 3.84	9.08 ± 3.68	3.32 ± 3.23
1 mM	47.84 ± 11.44	58.83 ± 9.09	13.94 ± 8.24	14.98 ± 5.55	2.98 ± 2.30
5 mM	49.47 ± 6.23	64.91 ± 4.52	17.14 ± 8.69	13.71 ± 6.36	3.12 ± 4.83
10 mM	36.81 ± 11.32	55.87 ± 17.44	14.68 ± 8.39	9.21 ± 4.98	0.81 ± 0.99

DISCUSSION

The assessment of sperm concentration, motility and morphology have traditionally been used to predict the male fertilising capacity. Over the past few decades, however, the analysis of sperm chromatin status has become a valuable, complementary parameter for evaluating male fertility. In effect, different features of sperm chromatin (integrity, protamination and condensation) have been reported to be associated to several sperm quality parameters, such as motility and morphology among others (Irvine et al., 2000; Muratori et al., 2000; Sharma et al., 2004). Besides, not only has poor sperm DNA integrity been associated to impaired sperm quality but also to altered fertility outcomes, such as fertilisation failure, poor embryo development, pregnancy loss, and developmental defects in the offspring (Huang et al., 2005; Morris, 2002; Virro et al., 2004).

Against this background, the present Doctoral Thesis aimed to investigate the resilience of sperm chromatin, evaluated as chromatin protamination, condensation and DNA integrity, during incubation after ejaculation. Moreover, it also sought to address whether such a resilience as well as the possible modifications that sperm chromatin undergoes during the aforementioned incubation are linked to the impairment of sperm functionality and fertilising ability, as this may help elucidate the relevance of sperm chromatin integrity for early embryo development.

Experiments included in **Chapter I** were devised with the purpose of describing how sperm chromatin is altered, in terms of protamination, condensation and DNA fragmentation during incubation after ejaculation at 38 °C, and whether such changes are related to the bovine sperm quality decline observed during that period. In addition, the relationship between chromatin status and sperm fertility was assessed in order to determine if chromatin integrity can be used as a biomarker to predict pregnancy rates. On the other hand, although earlier research consistently showed that sperm quality and function differ between ejaculate fractions (de la Torre

et al., 2017; Dziekońska et al., 2017; Hebles et al., 2015; Valsa et al., 2012), no study previously investigated if chromatin protamination and condensation, and DNA integrity of sperm chromatin also vary when the sperm from the separate fractions are compared (SRF-P1, SRF-P2 and PSRF), nor whether the differences in the composition of seminal plasma have any impact on the resilience of sperm chromatin to incubation after ejaculation. Experiments conducted in **Chapter II**, therefore, aimed to compare chromatin protamination and condensation, and DNA integrity of sperm between fractions. Finally, **Chapter III** explored how sperm chromatin fragmentation (SCF) occurs in ejaculated sperm *in vitro*, using the pig as a model. The possibility that a mechanism similar to that described in mouse sperm from the epididymis and vas deferens (Ribas-Maynou et al., 2022a) also exists in ejaculated sperm could contribute to understand how sperm chromatin is organised, and to elucidate whether DNA breaks occur more often in toroid linker regions. In addition, the impact of the DNA fragmentation generated by the SCF mechanism on sperm function and *in vitro* fertilisation was also interrogated.

Sperm chromatin condensation and protamination

The evaluation of sperm chromatin in post-thawed bovine sperm conducted in Chapter I (Experiment I) showed that the degree of chromatin protamination decreases when sperm are incubated at 38 °C (*Figure 7*). Moreover, although a slight increase in chromatin decondensation could be seen (*Figure 8*), no differences in chromatin condensation were observed for the period assessed (4 h). The experiments conducted in Chapter II, which compared the different sperm fractions, demonstrated that the disulphide bridges between protamines presented a higher degree of oxidation in the sperm from the sperm-rich fraction (SRF-P1 and

SRF-P2), thus indicating that chromatin condensation is higher in these ejaculate fractions compared to PSRF (*Figure 16*). No changes were, however, observed in chromatin protamination degree between SRF and PSRF (*Figure 15*). Together, these results suggest that the organisation of chromatin in the ejaculated sperm of the distinct fractions only differs in terms of chromatin condensation.

The aforementioned results denote that even though chromatin protamination and condensation are usually treated as synonyms in multiple studies assessing sperm chromatin, the use of both chromomycin A_3 and dibromobimane allows differentiating these two features. First, chromatin protamination leads to the packaging of protamine-DNA into toroidal structures of approximately 50 Kb (Braun, 2001; Miller et al., 2010; Ward, 2010); these toroidal structures are stabilised by intraprotamine and interprotamine disulphide bridges between cysteine residues (Balhorn et al., 1991). Protamines contain between 5 and 10 cysteine residues that, during the transit of sperm cells through the epididymis, become mostly oxidised (Chapman & Michael, 2003), thus accounting for chromatin condensation (Hutchison et al., 2017). Upon fertilisation, the reduction of these disulphide bonds is known to be essential for paternal chromatin decondensation (Cheng et al., 2009; Jager et al., 1990; Perreault et al., 1984; Zirkin et al., 1989). The oxidation and reduction of disulphide bonds between and within protamines could, therefore, not only regulate the degree of condensation/decondensation after gamete fusion, but also along the transit of the developing and subsequently mature sperm cells through male and female reproductive tracts.

Furthermore, and to the best of the author's knowledge, Chapter II (Experiment 1) of this Doctoral Thesis evaluated, for the first time, chromatin protamination and condensation in the sperm of the different pig ejaculate fractions. Therein, sperm of the SRF-P1 and SRF-P2 were

found to exhibit greater condensation than the PSRF (*Figure 16*). This higher degree of chromatin condensation could provide the sperm from the SRF with a stronger protection against DNA damage, which would be related to the greater quality and fertility potential that was previously reported for such an ejaculate fraction (Rodríguez-Martínez et al., 2005; Saravia et al., 2009; Sebastián-Abad et al., 2021).

Sperm DNA integrity

Regarding the effects of incubation at physiological temperature on sperm DNA integrity, results obtained in frozen-thawed bovine sperm (Chapter I, Experiment 2) showed a slight increase in the incidence of double-strand DNA breaks, and a statistically significant increase of global DNA damage (single- and double-strand DNA breaks), especially after 4 h of incubation (*Figure 9*). This suggests that, in cattle, a greater incidence of SSB rather than of DSB occurs when sperm are incubated at 38 °C, in agreement with that observed before in pigs (Ribas-Maynou et al., 2021) and humans (Tímermans et al., 2020). Related to this, it is worth mentioning that SSB are thought to be mainly produced by oxidative stress in DNA regions condensed by either protamines or histones (Enciso et al., 2009; Simon & Carrell, 2013).

Results from Chapter I (Experiment 2) showed that chromatin decondensation was directly correlated to the rate of global DNA fragmentation after incubation for 4 h. This relationship between abnormal chromatin condensation and DNA fragmentation was previously reported in several studies, which revealed that poorly protaminated chromatin is strongly linked to high DNA fragmentation (Cho et al., 2003; Llavanera et al., 2021; Manochantr et al., 2012; Nasr-Esfahani et al., 2005). Poor chromatin protamination would thus appear to enhance the effect of

damaging agents producing DNA fragmentation (Aitken & De Iuliis, 2010). Earlier research concluded that sperm chromatin stability is closely associated to the amount of inter- and intra- protamine interactions (Balhorn et al., 1991; Ward & Coffey, 1991; Ward, 2017). Thus, protamine-deficient sperm exhibit a less stable chromatin structure, resulting in an increased susceptibility to DNA damage (Aoki, 2005; Love & Kenney, 1999; Ribas-Maynou et al., 2021). Data presented in Chapter I, together with findings from other investigations, indicate that not only are protamines involved in DNA protection, but also in preventing DNA damage. Moreover, the concomitant alteration of chromatin protamination and DNA integrity pointed out in Chapter I supports the two-step hypothesis proposed by Aitken and De Iuliis (2010) to explain the origin of DNA damage (Aitken & De Iuliis, 2010).

After observing that sperm DNA integrity was altered upon incubation at 37 °C, differences in the sperm of the distinct ejaculate fractions were posited due to differences in the content of their seminal plasma. In the experiments comparing ejaculate fractions (Chapter II), the greater chromatin condensation observed in SRF-P1 and SRF-P2 initially suggested that the sperm contained in these fractions would be more resilient to DNA damage, compared to those of the PSRF. Surprisingly, the incidence of both global DNA damage and double-strand DNA breaks was similar between ejaculate fractions (*Figure 17*). While this result could not seem to make sense at first glance, the further experiments conducted in Chapter II, assessing sperm concentration, reactive oxygen species and antioxidant capacity, could explain these findings. The oxidative stress index, assessed as the ratio between ROS levels and total antioxidant capacity, was determined in the sperm of all ejaculate fractions (Chapter II, Experiment 2). Thanks to these evaluations, the oxidative stress index was found to be positively and significantly correlated to sperm concentration,

indicating that the higher the sperm count, the greater the imbalance between ROS (oxidant activity) and antioxidant activity. As SRF-P1 and SRF-P2 fractions presented higher sperm count and higher oxidative stress (*Figure 18*), it could be suggested that ROS production in these fractions overwhelmed the antioxidant capacity of their respective SP. Strong evidence suggests that high levels of ROS mediate the occurrence of an increased frequency of DNA fragmentation in sperm from infertile men (Aitken & Krausz, 2001; Kodama et al., 1996; Lopes et al., 1998); in addition, a significant positive correlation between ROS and DNA fragmentation was previously reported (Barroso et al., 2000). In this sense, the increased chromatin condensation exhibited by the sperm from SRF-P1 and SRF-P2 would not be strong enough to protect their DNA from being damaged by the ROS to which they are exposed. Besides, chromatin of the sperm from the PSRF is less condensed and, therefore, more susceptible to DNA damage. A greater balance between ROS levels and antioxidant capacity is, however, exhibited by the PSRF, resulting in a reduced DNA exposure to the damaging effects of high ROS levels. Together, this may explain why no differences between ejaculate fractions are found in terms of DNA damage.

Accumulating evidence indicates that high ROS levels are associated to histone modifications, DNA base modifications, SSB and other alterations, such as changes in membrane phospholipids or oxidation of other proteins (Agarwal et al., 2007; Aitken & De Iuliis, 2010; Enciso et al., 2009; Ribas-Maynou et al., 2014). Elevated ROS levels have thus been described to exert a detrimental effect upon sperm quality and survival (Agarwal et al., 2003; Saleh et al., 2002). The specific structure of sperm chromatin, which is highly condensed, and the presence of antioxidants in seminal plasma are known to contribute to minimising the adverse impact of excessive amounts of ROS on sperm DNA integrity

(Bennetts & Aitken, 2005; Kowalczyk, 2022; Pahune et al., 2013). Yet, when ROS generation increases, there may be an imbalance between ROS levels and antioxidant protection, which leads to oxidative stress. Such an oxidative stress, either intrinsically or extrinsically produced, is one of the main causes of sperm DNA damage (Ribas-Maynou & Yeste, 2020). On the one hand, the major source of intrinsic ROS production is sperm metabolism, specifically the activity of mitochondria, which is essential for sperm motility, particularly during capacitation. These organelles produce ROS as a by-product during oxidative phosphorylation (Chianese & Pierantoni, 2021; Gallo et al., 2021). Intrinsic ROS production has also been attributed to NOX5, a calcium-dependent enzyme present in sperm plasma membrane, whose activation is essential for the sperm capacitation process (Bedard & Krause, 2007; Moreno-Irusta et al., 2020). On the other hand, increased levels of ROS can be produced by exogenous conditions including varicocele, the activation of the immune system by infections, alcohol consumption, the increase of testicular temperature, and the exposure to both ionising and non-ionising radiations and environmental pollution (Ribas-Maynou & Yeste, 2020).

Although oxidative stress has been proposed as a possible cause of DNA damage, other enzymatic mechanisms have been described to be potentially involved. It is known that the nuclease activity may generate DSB at the toroid linker regions (TLR), which have been shown to be accessible to enzymes thanks to the lack of protamines (Boaz et al., 2008; Sotolongo et al., 2005). As it is easier for endogenous and exogenous nucleases to access to poorly protaminated chromatin, they are also more likely to inflict DNA damage in sperm exhibiting deprotamined chromatin (Sakkas & Alvarez, 2010).

In vitro induction of SCF as an approach to investigate TLR

Several studies in humans and animal models have reported that sperm DNA integrity is strongly associated with embryo development outcomes (Mateo-Otero et al., 2022; Ribas-Maynou et al., 2021; Sedó et al., 2017; Simon et al., 2014; Zheng et al., 2018). Particularly, not only is the repercussion of SSB and DSBs different (Agarwal et al., 2020), but the latter mainly occurs in TLRs and is caused by enzymatic activity (Ribas-Maynou et al., 2012b). In humans, the incidence of DSB in TLRs has been linked to recurrent miscarriage, slower embryo kinetics and lower developmental rates to blastocyst (Casanovas et al., 2019; Ribas-Maynou et al., 2012b). Based on these studies, it can be hypothesised that the genetic information contained in TLRs plays an important role during the initial stages of embryonic development (Kumar et al., 2013; Ward, 2010; Yamauchi et al., 2007, 2011). Moreover, chromatin in TLRs is condensed with retained histones, which are the only sperm nucleoproteins that bear epigenetic signatures. These signatures reside in gamete-imprinted genes and can influence gene expression after fertilisation and during the initial stages of embryo development (Jung et al., 2017; Yoshida et al., 2018). In spite of this, whether regions condensed with histones contain genes or non-coding information is unclear, as some studies found an increased presence of histones in gene-rich regions (Yoshida et al., 2018), whereas others mainly identified histones in repetitive, intergenic regions (Samans et al., 2014).

In vitro activation of SCF was only described before in epididymal and vas deferens mouse sperm (Gawecka et al., 2013; Shaman et al., 2006; Sotolongo et al., 2005). Whether SCF is also triggered in ejaculated sperm was thus not previously investigated, so that Chapter III set the conditions to induce DNA breaks in the TLRs of ejaculated sperm. Inducing these DNA breaks was of interest as a previous step to determine the length of the

resulting DNA fragments, and to thus elucidate if fragmentation occurs more frequently in specific DNA regions such as TLRs. Besides, how the *in vitro* activated SCF affects sperm function and *in vitro* fertilisation outcomes was also investigated in Chapter III. In addition, through this approach, the genetic information contained in these regions may be investigated in the future, which may in turn provide the basis for confirming if TLRs have a crucial role for embryo development as previous research suggested. Incubation of ejaculated sperm with Mn^{2+} and Mg^{2+} ions resulted in DNA fragments similar in size to those corresponding to toroidal structures (33 to 194 Kb) (*Figure 23*). These observations demonstrated that the size of the fragments generated after the activation of SCF *in vitro*, a process through which sperm degrade their own DNA, was compatible with that of toroids (Boaz et al., 2008; Sotolongo et al., 2005).

In previous research, Mn^{2+} at a concentration of 10 mM, with or without Ca^{2+}, was found to trigger the SCF mechanism (Ribas-Maynou et al., 2014; Shaman et al., 2006; Yamauchi, Shaman, & Ward, 2007). How this mechanism relies upon Mn^{2+} concentrations was not, nevertheless, interrogated before, that is why a set of experiments tested the effects of different Mn^{2+}/Ca^{2+} concentrations. In this Doctoral Thesis, the effects of Mn^{2+}/Ca^{2+} were, for the first time, revealed to be dose-dependent, as greater concentrations of Mn^{2+}/Ca^{2+} lead to a higher incidence of sperm DNA breaks (*Figure 19*). Furthermore, the effect of Mn^{2+}/Ca^{2+} was fast and obvious as early as 2 min post-incubation, and a longer incubation period did not result in a higher incidence of sperm DNA breaks (*Figure 21*). Based on these findings, one could suggest that the activation of the SCF mechanism *in vitro* relies on the concentration of Mn^{2+}/Ca^{2+} but not on the incubation period. Besides, incubation with Mg^{2+}/Ca^{2+} was also seen to be able to activate SCF in ejaculated sperm, also cleaving sperm DNA at TLRs (*Figure 19*). The results, however, showed that the sensitivity to Mg^{2+}/Ca^{2+}

was lower compared to Mn^{2+}/Ca^{2+}, as lower concentrations of Mn^{2+}/Ca^{2+} were sufficient to activate SCF, indicating that the intracellular machinery involved could be ion-independent with differing affinities to the distinct divalent ions. Several nucleases with DNA cleavage activities have been described to require divalent cations to activate. In this regard, there exist DNAse I enzymes and endonucleases that require Mg^{2+} or Mn^{2+} as cofactors and other nucleases which are Ca^{2+}- and Mg^{2+}-dependent (Dupureur, 2008; Guéroult et al., 2010; Pan et al., 1998).

Establishing an *in vitro* SCF model in ejaculated sperm allowed determining whether the DNA damage generated at TLRs is caused by oxidative effectors. This aspect is important, as most studies consider that global DNA fragmentation is mainly caused by ROS (Muratori et al., 2020; Ozmen et al., 2007; Samplaski et al., 2015; Zandieh et al., 2018), despite the fact that other enzymatic-dependent mechanisms may also be involved (Ribas-Maynou, Nguyen, Valle, et al., 2022; Yamauchi et al., 2007a). In Chapter III (Experiment 3), incubation with either Mg^{2+}/Ca^{2+} or Mn^{2+}/Ca^{2+} was found to cause SCF without increasing ROS production, suggesting that a non-oxidative mechanism is involved in the *in vitro* generation of DNA breaks. This mechanism, which probably requires the activation of an enzymatic cascade, could be related to the intrinsic apoptotic-like pathway of sperm (Gorczyca et al., 1993). In this regard, an active endogenous nuclease was identified in vasectomised mice, which suggests that this enzyme promotes DNA cleavage *in vivo*, rendering sperm non-functional (Ribas-Maynou et al., 2022a). In addition to this, the participation of topoisomerase IIB has to be considered, as previous investigations indicated that this enzyme is located near to TLRs, and cleaves DNA into loop-sized fragments of 50 Kb (Boaz et al., 2008; Ribas-Maynou et al., 2014; Shaman et al., 2006; Sotolongo et al., 2005; Yamauchi et al., 2007b).

Triggering *in vitro* the mechanism described above to induce specific DNA breaks at TLRs allows starting the investigation of these regions through sequencing technologies. In this sense, it could be addressed whether these regions contain specific or repetitive sequences, as well as if they contain genes involved in specific biological pathways.

Relationship of sperm chromatin protamination and decondensation, and DNA integrity with sperm function

Sperm function is defined by different parameters, including motility, plasma membrane integrity, mitochondrial activity, and intracellular levels of ROS and Ca^{2+}. In this Doctoral Thesis, whether such variables are related to sperm chromatin protamination and condensation, and DNA integrity was investigated, in addition to the effects of incubation at 38 °C. It is worth mentioning that the impact of those parameters on sperm function differed between frozen-thawed and fresh sperm, as well as between different species.

First, and as expected, experiments conducted in Chapter I (Experiment 3) revealed that viability and motility of bovine sperm decreased over post-thawing incubation at 38 °C. Interestingly, despite sperm motility (total and progressive motility) being seen to be constantly reduced throughout the different incubation periods (0-2 h and 2-4 h), sperm viability was mostly affected in the first period of incubation. This effect could be due to the activity of the ROS generated during freezing and thawing (Pezo et al., 2021; Ugur et al., 2019), which would oxidise lipids from mitochondrial and plasma membranes. As a consequence, a rapid loss of mitochondrial membrane potential and plasma membrane integrity would occur, finally leading to the reduction of sperm motility and the induction of cell death (Aitken, 2020; Drevet & Aitken, 2019).

Moreover, data also showed that sperm motility and viability were altered before chromatin integrity alterations appeared, a fact that could be explained by the high protection that chromatin possesses thanks to its organisation into toroids.

Conventional sperm quality parameters have been related to DNA integrity in previous studies. Most of such studies indicated that sperm motility, concentration, viability and morphology are correlated to DNA integrity (Irvine et al., 2000; Muratori et al., 2000; Sharma et al., 2004). Although no correlations between DNA damage and sperm functionality parameters were identified in bovine sperm herein (Chapter I, Experiment 2), the reduction of DNA integrity in pig sperm caused by the *in vitro* activation of SCF using Mg^{2+}/Ca^{2+} and Mn^{2+}/Ca^{2+} treatments (Chapter III, Experiment 3) resulted in an impaired sperm motility and an increased agglutination in a concentration-dependent manner (*Figure 24* and *Figure 27*, respectively). As previously suggested, these effects could be related to the destabilisation of sperm membrane caused by oxidative stress (Kodama et al., 1996), notwithstanding the alteration of sperm viability was not observed in this Doctoral Thesis. Since sperm viability is assessed on the basis of plasma membrane integrity, further investigation is needed to understand why incubation with Mg^{2+}/Ca^{2+} or Mn^{2+}/Ca^{2+} leads to sperm agglutination, especially at high concentrations. In order to determine whether oxidative stress was responsible for the SCF mechanism and the concomitant alterations, intracellular ROS and superoxides levels were evaluated. Results showed that neither ROS nor superoxide levels showed an increase after SCF activation (*Figure 26*), dismissing the previously hypothesised involvement of an oxidative stress mechanism on the impairment of sperm function. These results would, therefore, support that the *in vitro* triggering of the SCF mechanism relies upon the activation of specific enzymes.

Impact of sperm chromatin protamination and condensation, and DNA integrity on *in vivo* and *in vitro* fertility outcomes

Previous works showed that impaired sperm quality parameters such as total motility or sperm morphology cause a reduction of fertility outcomes (Bongso et al., 1989; Carrell & Liu, 2001; Franken et al., 1999; Liu & Baker, 1992). The consequences of this reduction vary depending on the species, from male infertility in humans to subfertility and economic losses in the livestock breeding industry. In this regard, results presented in Chapter I (Experiment 3) showed that sperm motility correlated to artificial insemination outcomes, which is in agreement with other studies performed in different species that found an association between sperm fertility and both motility and morphology (Ibanescu et al., 2020; Kathiravan et al., 2011; Quintero-Moreno et al., 2003; Schulze & Waberski, 2022; Shulman et al., 1998; Yániz et al., 2015).

Mounting evidence in farm animals supports that DNA fragmentation negatively affects reproductive outcomes, reducing both *in vitro* and *in vivo* fertility (Boe-Hansen et al., 2008; Didion et al., 2009; Dogan et al., 2015; Kenney et al., 1995; Souza et al., 2018). In humans, however, the association between sperm DNA damage and fertility outcomes remains unclear, as there exist differences between IVF and ICSI. Several studies reported that sperm DNA damage is associated with clinical outcomes following IVF (Evenson & Wixon, 2006; Henkel et al., 2004; Niu et al., 2011; Ribas-Maynou et al., 2021b; Simon et al., 2017; Zhang, 2008; Zhang et al., 2015), but not after ICSI (Agarwal et al., 2020; Casanovas et al., 2019; Evenson & Wixon, 2006; Ribas-Maynou et al., 2021b; Simon et al., 2017; Zhang et al., 2015; Zini et al., 2005). This difference could potentially be explained by the technical differences

between these two techniques, as ICSI involves the selection of the sperm exhibiting progressive motility and good morphology, features that are known to be associated with DNA integrity (Aitken & De Iuliis, 2010). Thus, sperm with a potentially lower incidence of SSB are used, resulting in the lack of an effect of DNA damage on clinical outcomes after ICSI (Ribas-Maynou et al., 2021b). In this regard, Chapter I (Experiment 3) assessed, for the first time, the relationship between the incidence of global and DSB in sperm and artificial insemination outcomes in cattle, but no correlation was observed. In addition, *in vitro* SCF triggered by incubation with Mn^{2+} (Chapter III, Experiment 4) did not show any effect on *in vitro* fertilisation success in pigs. It is important to bear in mind that *in vitro* induction of SCF was found to specifically produce DNA breaks in TLRs, and that the DNA affected by these breaks remains attached to the nuclear matrix by a still unknown protein/factor (Ribas-Maynou et al., 2014), providing the embryo with an opportunity to repair paternal DNA.

The results obtained in Chapter I also showed that viable sperm with high ROS at 4 h post-thaw were correlated to non-return to oestrus rates after 90 days (NRR) (*Table 12*). As broadly discussed above, oxidative stress is one of the major effectors leading to damage not only to paternal genetic content through base modifications, but also to different components of the sperm cell, such as plasma membrane, acrosome and proteins (Aitken, 2020). Reactive oxygen species, however, when present in small amounts, are important components driving sperm capacitation (Aitken, 2017; Mostek et al., 2021). In cattle, previous studies suggested that highly imbalanced oxidative stress is directly related to lower fertility rates, both *in vivo* (Leite et al., 2022) and *in vitro* (Ribas-Maynou et al., 2020b).

Based on these observations, one of the main explanations for these findings could be the ability of early embryos to repair paternal DNA

damage by activating maternally driven mechanisms throughout embryonic development, which was first interrogated by Generoso et al. (1979) (Generoso et al., 1979). Since then, several investigations have been focused on describing how this process is initiated, which mechanisms are involved, and how it is regulated (Derijck et al., 2008; Fernández-Díez et al., 2016; González-Marín et al., 2012; Horta et al., 2020; Marchetti et al., 2007). Specifically, and as aforementioned, the breaks induced by the SCF mechanism are located in the TLRs, particularly in the DNA that remains attached to the nuclear matrix, thus providing a scaffold for DNA repair (Ribas-Maynou et al., 2014).

CONCLUSIONS

The main conclusions of this Doctoral Thesis are:

1. Sperm chromatin protamination and condensation, and DNA integrity are related to sperm functionality parameters such as sperm viability and motility.
2. Sperm chromatin protamination and condensation differ between ejaculate fractions, being greater in the SRF-P1 than in the PRSF, which would explain the higher sperm quality of the SRF-P1.
3. Sperm chromatin fragmentation can be induced *in vitro* in ejaculated sperm through incubation with intracellular divalent ions.
4. Sperm chromatin fragmentation is a non-oxidative mechanism that produces DNA breaks in toroid linker regions, decreasing sperm motility and increasing sperm agglutination.

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