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Societat Catalana de BIOLOGIA

ACTES DE LA XIX JORNADA
DE BIOLOGIA DE LA REPRODUCCIÓ

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BIOLOGIA DE LA REPRODUCCIÓ

ACTES DE LA XIX JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

Editors

Meritxell Jodar, Judit Castillo, Rafael Oliva i Ignasi Roig

Amb la col·laboració de:

Institut d'Estudis Catalans
Unitat de Genètica, Departament de Biomedicina, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona
Biologia Cel·lular, Fisiologia i Immunologia, Unitat de Citologia i Histologia, Facultat de Biociències, Universitat Autònoma de Barcelona

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PRESENTACIÓ

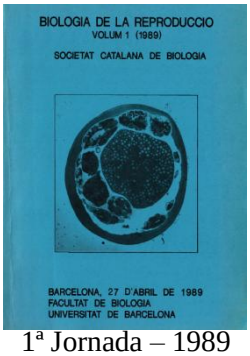
Aquest volum recull les comunicacions presentades a la XIX Jornada de Biologia de la Reproducció celebrades el 12 de juny de 2025 a l'Institut d'Estudis Catalans. L'objectiu d'aquesta jornada, que es va organitzar per primera vegada l'any 1989 i que s'organitza cada dos anys, és reunir els investigadors que treballen en el camp de la biologia de la reproducció, i difondre les diverses línies de treball, compartir coneixements i poder establir noves col·laboracions. La jornada representa també una excel·lent oportunitat per a la participació del investigadors més joves. Les comunicacions presentades en aquesta edició inclouen tant contribucions fonamentals com aplicades a la reproducció en una diversitat d'espècies. Voldríem agrair a l'Institut d'Estudis Catalans pels ajuts que han permès editar aquest llibre i cobrir la resta de despeses associades a la jornada.

ELS ORGANITZADORS

PRESENTATION

This volume collects the communications presented at the XIX Biology of Reproduction Symposium held on June 12, 2025, at the Institut d'Estudis Catalans. The objective of this symposium, which was first organized in 1989 and is organized every two years, is to bring together researchers working in the field of biology of reproduction, and to disseminate the various research lines, exchange knowledge and stimulate new collaborations. The symposium also represents an excellent opportunity for the participation of young researchers. The communications presented in this edition include both fundamental and applied contributions to reproduction in a diversity of species. We would like to thank the Institut d'Estudis Catalans for the economic support that has allowed us to publish this book, as well as to cover the remaining expenses associated with the symposium.

THE ORGANIZERS



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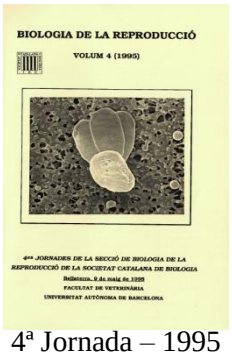

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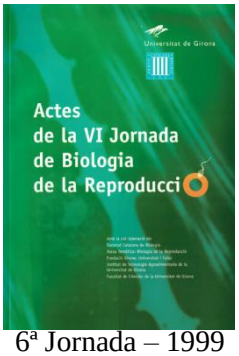

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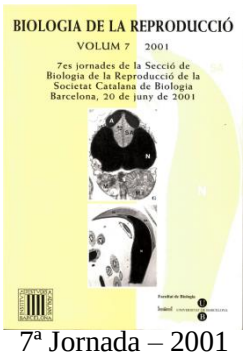

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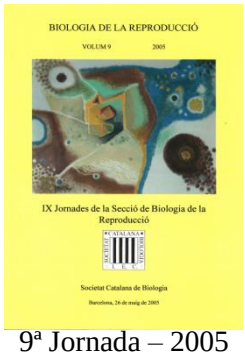

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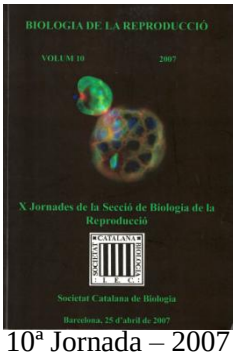

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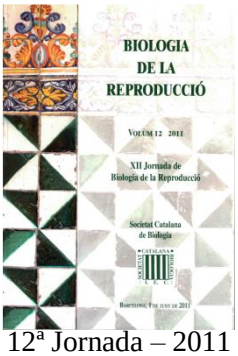

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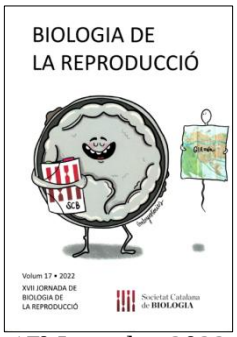

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ARTICLES CORRESPONENTS A LES COMUNICACIONS PRESENTADES A LA XIX JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

(Ordenats per aparició en el programa de la Jornada)

ARTICLES CORRESPONDING TO THE COMMUNICATIONS PRESENTED AT THE XIX BIOLOGY OF REPRODUCTION SYMPOSIUM

(*Ordered according to appearance in the Symposium program*)

Biologia de la Reproducció
Vol. 19, p. 13-18 (2025)

SPERM NUCLEAR BASIC PROTEINS (SNBPs): FROM INTRINSIC PROTEIN DISORDER TO LIQUID-LIQUID PHASE SEPARATION OF CHROMATIN

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Abstract

Commencing with a brief historical review from a personal perspective, I will analyze the evolution of the sperm nuclear basic proteins (SNBPs), emphasizing the pivotal role of histone H1 (H5) in this process. I will discuss the intrinsically disordered nature that is common to all these proteins (IDPs) and its profound implications for the crucial chromatin transitions during spermatogenesis. Moreover, I will describe how these transitions often involve liquid-liquid phase separation (LLPS), particularly by spinodal decomposition (SD).

Resum

Començant amb una breu revisió històrica des d'una perspectiva personal, analitzaré l'evolució de les proteïnes bàsiques nuclears espermàtiques (SNBP), posant èmfasi en el paper fonamental de la histona H1 (H5) en aquest procés. Discutiré la naturalesa intrínsecament desordenada que és comuna a totes aquestes proteïnes (IDP) i les seves profundes implicacions per a les transicions crucials de la cromatina durant l'espermatogènesi. A més, descriuré com aquestes transicions sovint impliquen la separació de fases líquid-líquid (LLPS), especialment per la descomposició espinodal (SD).

THE MUSSELS FROM "LA BOQUERIA"

My first encounter with chromatin, in particular sperm chromatin, took place by the middle of the summer of 1975 when I started working on my Ph. D. in Juan Antonio Subirana's lab. It involved the biochemical and biophysical (analytical ultracentrifuge, X-ray diffraction) characterization of the chromatin of the sperm of the blue mussel *Mytilus edulis*. The large amounts of protein needed at the time to perform those experiments required gathering large number of mussels (about 30 Kg) which I obtained from the Boqueria market in downtown Barcelona. I also used the surf clam *Spisula soliddisima* which was collected at the Marine Biological Laboratory (MBL) at Woods Hole (USA). During the time at Juan's lab and because of my Biology background I was very impressed by a paper published in that lab about the evolution of protamines and other basic proteins from the sperm of molluscs (Subirana, 1973) and became interested in this topic that I would pursue years later. Hence, biophysics, biochemistry and evolution of sperm chromatin became the pillars of my future career and it all started in Barcelona at a time when this already was a well-recognized world leading city in the topic of chromatin.

SNBP EVOLUTION

The 1973 paper by Subirana and co-authors (Subirana, 1973) postulated that the diversity in electrophoretic mobility and amino acid composition observed in the sperm basic proteins of Mollusca could be explained from a potential relation to a somatic histone precursor. Protamine-like intermediates like those observed in *M. edulis* may have led to arginine-rich protamines such as those found in the squid *Loligo paleaii*. However, the detailed molecular mechanism involved in the transitions was not

clear. Several years later, after I had left Subirana's lab, the structural and biochemical characterization of the large (455 amino acids) *Spisula*'s SNBP revealed that, similar to members of the histone H1 family, this protein had a trypsin resistant domain. Moreover, its amino acid sequence was extremely similar to that which is found in the core somatic histone H5. Such core corresponds to the winged helix domain (WHD) that is characteristic of all members of the histone H1 family (Figure 1A).

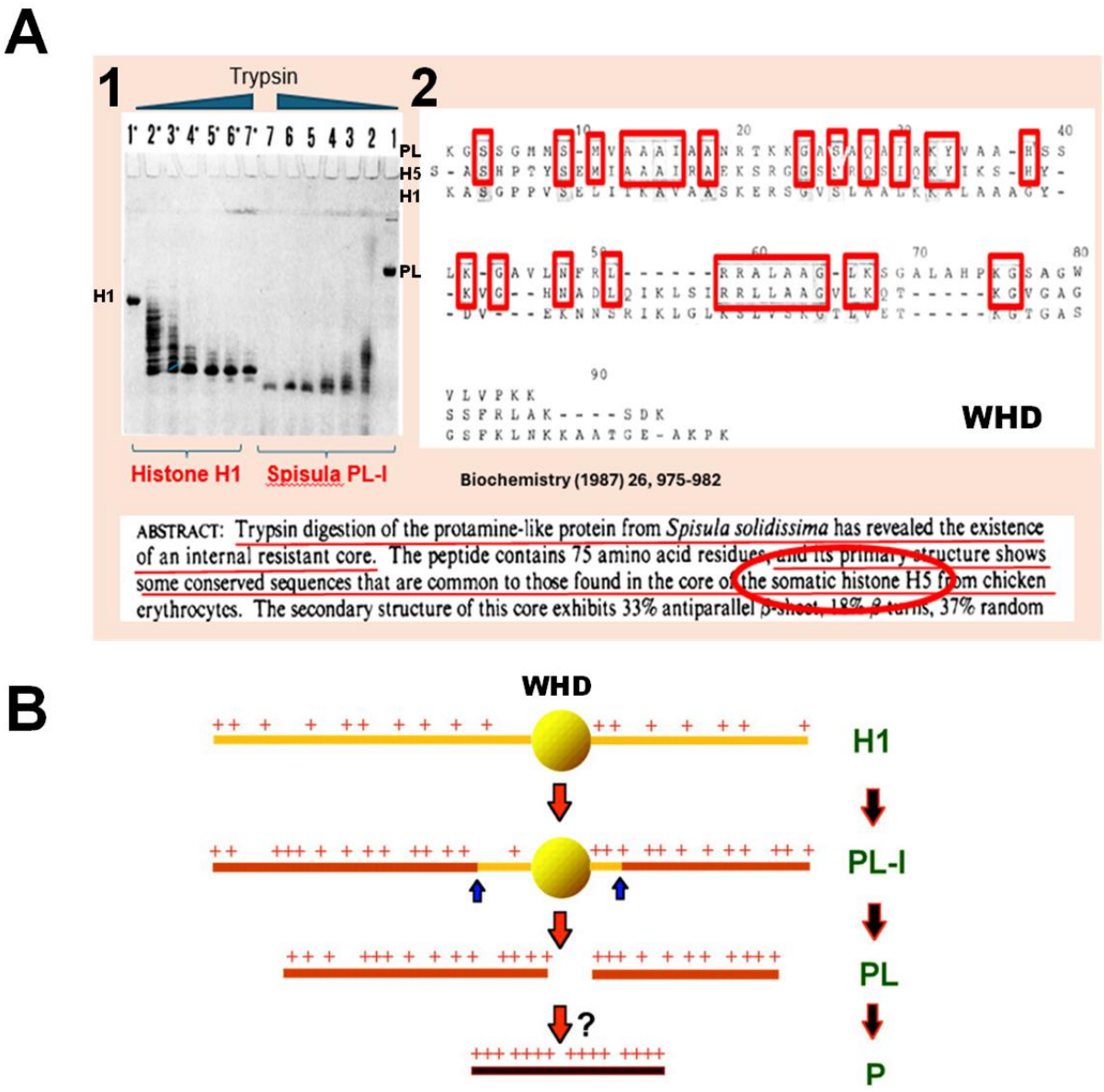

Figure 1. A-1) Comparative trypsin digestion of PL-I from *S. solidissima* (surf clam) and histone H1 from calf thymus. A-2) Amino acid sequence alignment of the trypsin resistant peptide of *S. Solidissim*, histone H5 from chicken erythrocyte and histone H1 (Ausió et al., 1987). B) Histone H1 hypothesis of the orgin of protamines. WHD: winged helix domain.

The relation of a sperm nuclear protein of a clam to a highly specialized somatic histone H5 from chicken erythrocytes was not initially obvious. However, histone H5 is a member of the replication independent histone H1.0 subfamily which is often found in terminally differentiated cells as in the case of both avian erythrocytes and sperm. This led to the proposal of the hypothesis that SNBPs are related to the histone H5 type within the larger H1 histone family (Ausió, 1999) shown in Figure 1B for which

my lab has gathered strong evidence with many publications on diverse organisms from different phylogenetic groups (Leyden et al., 2024).

SNBPs ARE INTRINSICALLY DISORDERED PROTEINS

Over the years, we have been able to show that SNBPs can be classified into three main groups: Histone (H), protamine-like (PL) and protamine (P) types (Ausió, 1999; Eirin-Lopez and Ausio, 2009). An important structural characteristic shared by all these proteins is their intrinsic disorder and relatively low levels of secondary and tertiary structure (Figure 2A). In native protein structure, intrinsically disordered regions coexist with folded domains and the interplay between the different domains may be critical for the protein's function as shown in the trinity proposal (Dunker et al., 2001) (Figure 2B). In fact, there is a continuum of folding between folded and random coil with different extents of transient and stable secondary and tertiary structure (Figure 2C) (Watson and Stott, 2019). Indeed, interaction of IDPs with their binding partners often results in secondary structure formation (Figure 2D) of which protamines provide a good example (Roque et al., 2012).

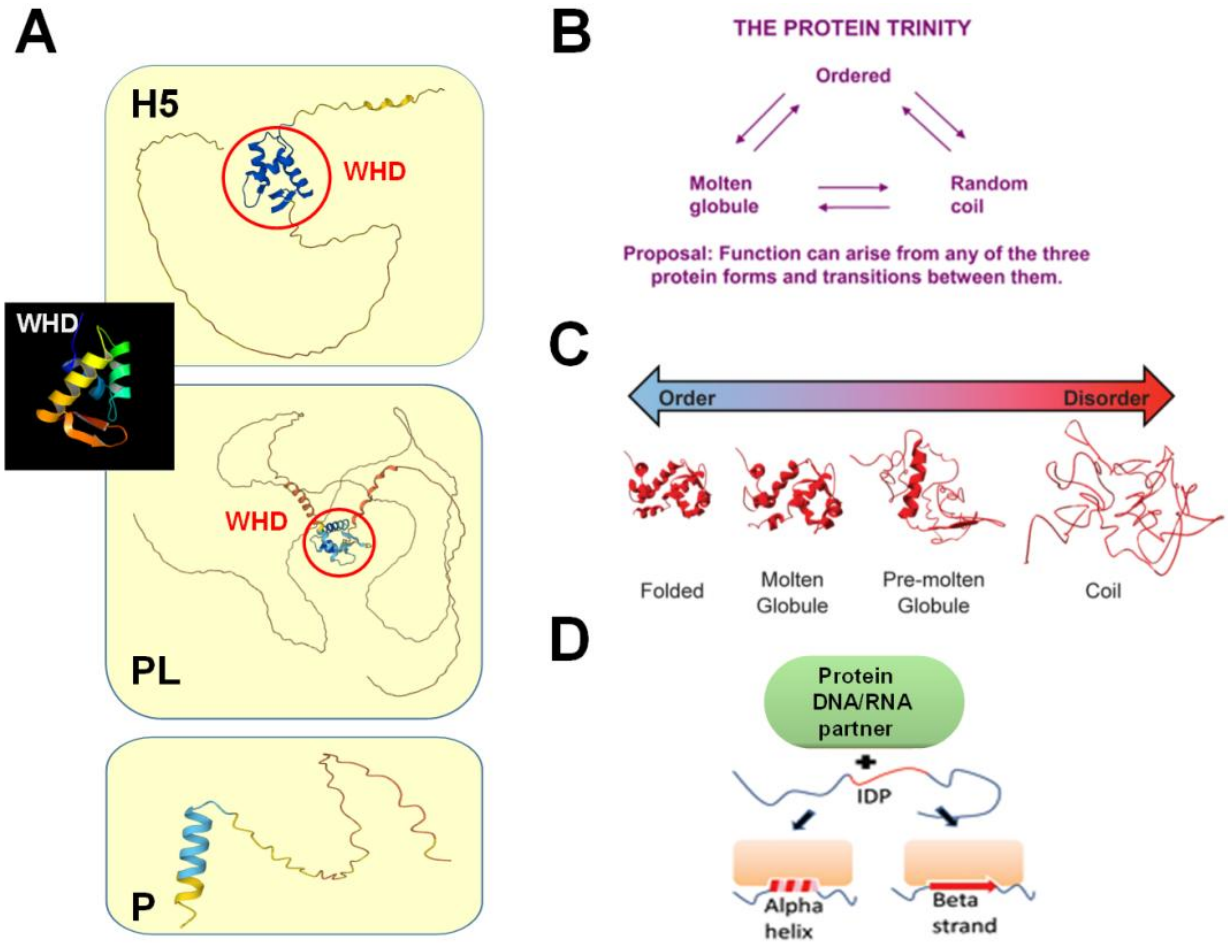

Figure 2. A) AlphaFold-generated structure of histone H5, *S.solidissima* (top), PL-I (middle) and *Loligo opalescens* squid protamine (P) (bottom). The three-dimensional organization of the WHD is also shown. B) The protein trinity proposal (Dunker et al., 2001). C) The continuum of protein disorder (Watson and Stott, 2019). D) IDPs can adopt structure upon interaction with binding partners.

THE MAIN TYPES OF SPERM CHROMATIN

The main H, PL and P types of SNBPs lead to three corresponding types of chromatin organization where the last two types possess higher levels of compaction (Figure 3A).

In some groups of organisms such as in fish and amphibians as well as in several groups of invertebrates and plants, the H, PL and P chromatin types are distributed (Leyden et al., 2024) in a sporadic but non-random distribution.

As shown in Figure 3B, sperm-chromatin compaction increases with the extent of protein disorder. The co-existence of different chromatin types in amphibia (*Rana catesbeiana*, frog, with almost 100% heterochomatinized nucleus) H type and (*Bufo bufo*, toad) P-type allowed us to estimate the extent of sperm nuclear compaction undergone in transitioning from histones to protamines to be about 2-fold (Figure 3B).

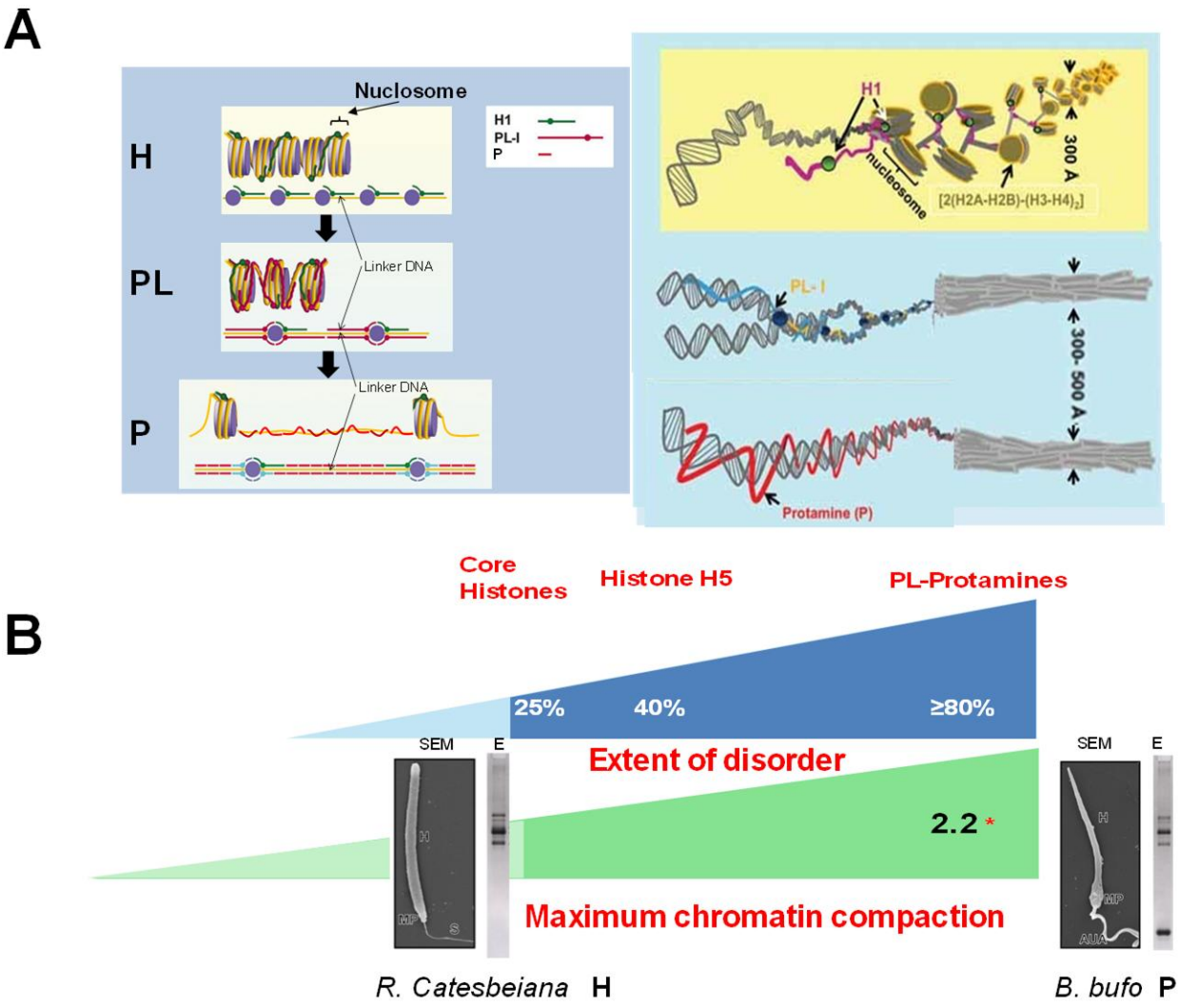

Figure 3. A) Two different depictions of the chromatin structures adopted upon interaction of the three main types of SNBPs with DNA. B) Chromatin disorder and chromatin compaction. The extent of compaction in the highly condensed chromatin in the nucleus of sperm of the toad (*Bufo bufo*) increases by 2 compared to that of the frog *Rana catesbeiana* (H-type). E: electrophoretic SNBP pattern; SEM: scanning electron microscopy.

SPERM CHROMATIN OFTEN UNDERGOES LLPS DURING SPERMATOGENESIS

Figure 4A shows one of the most unique chromatin organizations. The figure represents a snapshot of an extremely dynamic process of lamellar chromatin organization in the marine snail *Murex brandaris* (Harrison et al., 2005). Although a similar kind of organization had been described in the dogfish

Scylorhinus canicula as early as in 1978 (Gusse and Chevaillier, 1978), this unique arrangement was first attributed to SD in 2005 (Harrison et al., 2005). It represents one of the first descriptions of the involvement of LLPS in chromatin folding, a topic that in more recent years has pervaded the attention in the field.

Very simplistically, LLPS is a phase separation like that observed when two insoluble liquids like water and oil are mixed and the oil droplets separate from the aqueous phase. It involves a complex thermodynamic process (Figure 4B) (Kasinsky et al., 2012). In sperm chromatin, this is mediated by protein (protamine) precursor processing and post-translational modifications (PTMs) and is often facilitated by intrinsic protein disorder. This is a widespread process that has been described to occur during spermatogenesis of several groups of invertebrates, particularly in insects, vertebrates and plants (Leyden et al., 2024). Interestingly, the last paper of my lab focused on spider SNBPs showed that spermatogenesis in the genus *Steatoda* also involves SD. Moreover, this is mediated by a protamine that is a fragment of a precursor protein whose gene was previously annotated as a histone H5 like protein (Leyden et al., 2024). This finding nicely closes a cycle in this review that brings it back to the histone H1 (H5) proposal described at the beginning.

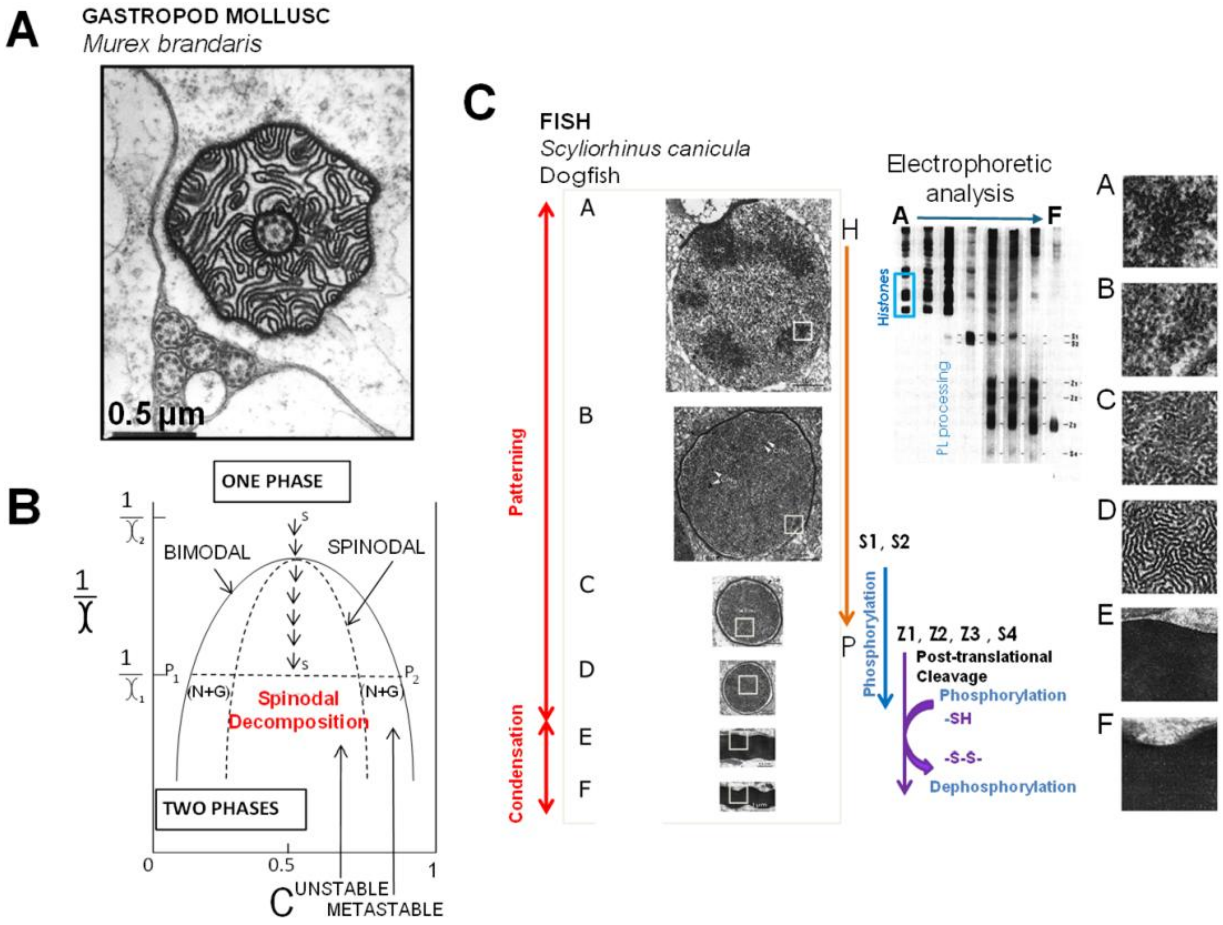

Figure 4. A) Transmission electron microscopy (TEM) micrograph of a transverse section of the nucleus depicting the lamellar chromatin organization in elongated spermatids of *Murex brandaris.* B) Phase diagram for spinodal decomposition (Kasinsky et al., 2012). χ: interaction parameter; P: phase; G: growth; N: nucleation; S; composition at $1/\chi_2$. C) TEM and electrophoretic analysis of the transitions undergone by chromatin and SNBPs in the dogfish *S. canicula* (Gusse and Chevaillier, 1978) . The TEMs A to F on the right-hand side correspond to magnified images from the white squares of those shown on the left side.

ACKNOWLEDGMENTS

The research published in this review would have not been possible without the help of many important collaborators in particular, Manel Chiva, Núria Saperas, Harold Kasinsky and José María Eirín-López. I am also very indebted to the Natural Sciences and Engineering Research Council of Canada (NSERC) for unconditionally supporting this research since 1991 to the present day.

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GENETIC FINDINGS IN AZOOSPERMIC PATIENTS WITH MEIOTIC ARREST

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Abstract

Non-obstructive azoospermia (NOA) is a major cause of male infertility, frequently linked to underlying genetic defects. We studied 44 azoospermic patients, classifying them according to testicular histology into pure (n=34) and mixed (n=10) phenotypes. Pure phenotypes included Sertoli cell-only phenotype (SCO), hypospermatogenesis, conserved spermatogenesis, germ cell arrest (GCA), and tubular shadows. Karyotyping, Y chromosome microdeletions analysis, *CFTR* gene variant screening and hormonal analyses were performed in all patients. Hormonal analysis showed that SCO patients had significantly elevated FSH and decreased Inhibin compared to other groups. Four patients with pure germ cell arrest underwent exome sequencing, which revealed likely pathogenic variants in meiosis-related genes (*TEX11*, *HFM1*) and the testis-specific histone gene *H2BC*1 in three individuals. These findings support the relevance of genetic screening in NOA, particularly in cases of meiotic arrest. In addition, linking specific genetic variants to germ cell arrest provide valuable insights into the molecular mechanisms that regulate spermatogenesis. This understanding will not only improve our knowledge of the underlying causes of male infertility but will also provide prognostic information to guide the recovery of viable sperm from the testis, potentially reducing the need for unnecessary biopsies.

Resum

L'azoospèrmia no obstructiva (NOA) és una causa principal d'infertilitat masculina, sovint relacionada amb defectes genètics subjacents. Hem estudiat 44 pacients azoospèrmics, classificats segons la histologia testicular en fenotips purs (n=34) i mixtos (n=10). Els fenotips purs inclouen: només cèl·lules de Sertoli (SCO), hipospermatogènesi, espermatogènesi conservada, aturada de cèl·lules germinals (GCA) i ombres tubulars. A tots els pacients se'ls van fer anàlisis de cariotip, microdelecions del cromosoma Y, variants del gen CFTR i estudis hormonals. L'anàlisi hormonal va mostrar que els pacients amb SCO presentaven nivells de FSH significativament elevats i nivells d'inhibina B disminuïts respecte a la resta de grups. Quatre pacients amb un fenotip pur d'aturada de cèl·lules germinals van ser sotmesos a seqüenciació de l'exoma, que va revelar variants probablement patogèniques en gens relacionats amb la meiosi (*TEX11*, *HFM1*) i en el gen de l'histona específica del testicle *H2B*C1 en tres d'ells. Aquests resultats recolzen la importància del cribratge genètic en la NOA, especialment en casos d'aturada meiótica. A més, la relació entre variants genètiques específiques i l'aturada de cèl·lules germinals aporta informació rellevant sobre els mecanismes moleculars que regulen l'espermatogènesi. Aquesta comprensió no només millora el coneixement de les causes de la infertilitat masculina, sinó que també proporciona valor pronòstic per orientar la recuperació de espermatozoides viables del testicle, reduint potencialment la necessitat de biòpsies innecessàries.

INTRODUCTION

Azoospermia, defined as the absence of spermatozoa in the ejaculate, accounts for approximately 3%-10% of male infertility cases. It is broadly classified into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). OA is caused by physical blockage in the male reproductive tract and is typically associated with preserved testicular function and normal spermatogenesis. Known causes of OA include acquired factors (e.g., vasectomy, iatrogenic injury) and congenital anomalies such as congenital bilateral absence of the vas deferens, often linked to pathogenic variants in the *CFTR* gene. In contrast, NOA accounts for around 60% of azoospermia cases and results from intrinsic testicular dysfunction. This includes primary testicular failure (elevated LH and FSH levels, small testes, affecting up 10% of infertile men), secondary testicular failure (congenital hypogonadotropic hypogonadism with low gonadotropin levels) and other causes such as toxic exposures, abnormal testicular development, cryptorchidism, varicocele, and idiopathic factors. Clinically, patients with NOA often present with small, soft, and atrophic testes, in contrast to OA patients, who typically have normal-sized testes (Wosnitze et al. 2014).

In rare cases of NOA, particularly those associated with hypogonadotropic hypogonadism, hormone replacement therapy may induce spermatogenesis. Additionally, in men with NOA and varicocele, varicocelectomy has shown some success. However, for most other cases of NOA, the only viable option for achieving pregnancy is intracytoplasmic sperm injection (ICSI) combined with surgical testicular sperm extraction (TESE), though the success rate remains low (Takeshima et al. 2024). Conventional clinical genetic testing such as conventional karyotyping and Y chromosome microdeletion analysis can identify underlying causes of NOA, thereby providing prognostic information regarding the probability of successful sperm retrieval after testicular biopsy. However, nowadays TESE is contraindicated only in NOA patients with a 46,XX and 47,XXY karyotypes or with microdeletions of AZFa and/or AZFb regions of the Y chromosome (Wosnitze et al. 2014; Wyrwoll et al. 2023). All these external factors, testicular problems, hormonal or genetic alterations can explain approximately 45% of the cases of NOA (Krausz et al. 2018). However, a significant proportion of cases remain unexplained. In the preceding decade, whole-exome sequencing (WES) has been utilised to identify novel genetic defects associated with spermatogenesis failure (Lillepea et al. 2024; Wyrwoll et al. 2024). The integration of WES into the clinical diagnosis of azoospermia, in conjunction with conventional genetic testing, has been demonstrated to enhance diagnostic efficiency.

Based on the testicular histology, NOA patients can be subclassified into several patterns including Sertoli-cell-only phenotype (SCO), tubular shadows, hypospermatogenesis, spermiation failure and germ cell arrest (Wyrwoll et al. 2024). The germ cell arrest pattern can be further divided into specific arrest stages such as spermatogonial arrest, meiotic arrest (MA), also known as spermatocyte arrest, and round spermatid arrest. Although complete idiopathic germ cell arrest is less common than other histological patterns, it is increasingly recognized as the phenotype most frequently associated with a clear monogenic aetiology. Recent studies suggest that many of these cases involve homozygous or hemizygous variants, following either an autosomal recessive or X-linked pattern of inheritance (Krausz et al. 2020).

Germ cell arrest, observed in over 150 knockout mouse models, has highlighted the critical role of numerous genes in murine male meiosis. However, to date, fewer than 30 genes have been linked to germ cell arrest in humans. Identification of genetic causes associated with germ cell arrest provide valuable insights into the molecular mechanisms that regulate spermatogenesis but also provide prognostic information to guide the recovery of viable sperm from the testis, potentially reducing the need for unnecessary biopsies. This study aims to identify genetic causes of idiopathic NOA characterized by germ cell arrest in our population.

MATERIAL AND METHODS

A total of 44 azoospermic patients treated at our hospital between 2018 and 2023 were identified through semen analysis and classified into five histological categories based on testicular biopsy findings: conserved spermatogenesis, hypospermatogenesis, MA, SCO and tubular shadows. All

patients included in the study provided informed consent and the study was approved by the ethical committee of the Hospital Clínic de Barcelona.

Whole blood samples were collected in sterile 5mL EDTA tubes for comprehensive analyses, including genetic testing (karyotyping, Y chromosome microdeletions analysis and *CFTR* gene variant screening) and hormonal profiling. DNA was extracted using the MagNA Pure 96 DNA and Viral NA Large Volume Kit following the manufacturer's guidelines in the extraction equipment a MagNA Pure96 Instrument (Roche Diagnostics). DNA purity and concentration were assessed using a NanoDrop spectrophotometer and samples were stored at –20ºC until further processing.

Whole-genome sequencing (WGS) was conducted on 4 patients with a pure phenotype of MA. Library preparation was performed using the SureSelect Human All Exon v8 kit (Agilent) followed by sequencing on the NextSeq2000 platform. Bioinformatics analysis was carried out using the coreBM-Germline_1.0.0 pipeline with the GRCh38/hg38 human genome reference. Detailed information regarding the software tools, versions, and pipeline code is available in the GitHub repository (https://github.com/CDB-coreBM/coreBM-Germline) and can be accessed upon request. Variant annotation and filtering were conducted using the Jnomics platform (https://www.jnomics.es/).

Variants classified as pathogenic or of uncertain clinical significance in any of the 146 genes associated with azoospermia were prioritized. Genetic variant classification followed the guidelines of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015), utilizing standard diagnostic databases such as ClinVar, gnomAD, and HGMD. Finally, selected variants were validated by PCR and/or Sanger sequencing according to standard protocols.

RESULTS AND DISCUSSION

In our cohort of 44 patients, 77.3% (n=34) presented pure testicular phenotypes, while 22.7% (n=10) showed combined phenotypes. Among the pure phenotypes, SCO was the most frequent (29.4%, n=10), followed by hypospermatogenesis (26.5%, n=9), conserved spermatogenesis (23.5%, n=8), germ cell arrest (11.7%, n=4), and tubular shadows (5.9%, n=2). Biallelic pathogenic variants in the *CFTR* gene were identified exclusively in 3 patients with conserved spermatogenesis. No karyotype alterations or Y chromosome microdeletions were detected among patients with pure phenotypes. Among those with combined phenotypes, three patients presented genetic alterations: one with maturation arrest plus hypospermatogenesis had a 47,XYY karyotype, while two patients with SCO plus hypospermatogenesis showed a Robertsonian translocation (45,XY,der(13;14)) and a Y chromosome microdeletion in the AZFc region, respectively.

Significant differences were observed in LH (luteinizing hormone), FSH (follicle-stimulating hormone) and inhibin hormone levels between patients with pure phenotypes (Table 1 and Figure 1). SCO patients exhibited the most different hormonal profile, with significantly elevated FSH compared to all other histological groups and markedly reduced Inhibin B, which was significantly lower than in both the conserved spermatogenesis and hypospermatogenesis groups. LH levels in SCO were also higher than in conserved spermatogenesis (Table 1 and Figure 1).

Table 1. Mean ± SD of FSH, LH and Inhibin B levels in azoospermic patients with pure phenotypes, according to testicular histology.

	FSH (U/L)	**LH (U/L)**	**Inhibin (ng/L)**
Conserved spermatogenesis	5,21 ± 3 ,64	2,82 ± 0,68	236,04 ± 112,86
Hypospermatogenesis	10,43 ± 8,90	5,02 ± 2,3	134,84± 90,88
Germ cell arrest	10,47 ± 5,01	6,67	110,15 ± 47,03
Sertoli cell only	25,09 ± 10,35	5,96 ± 2,12	17,48 ± 9,87

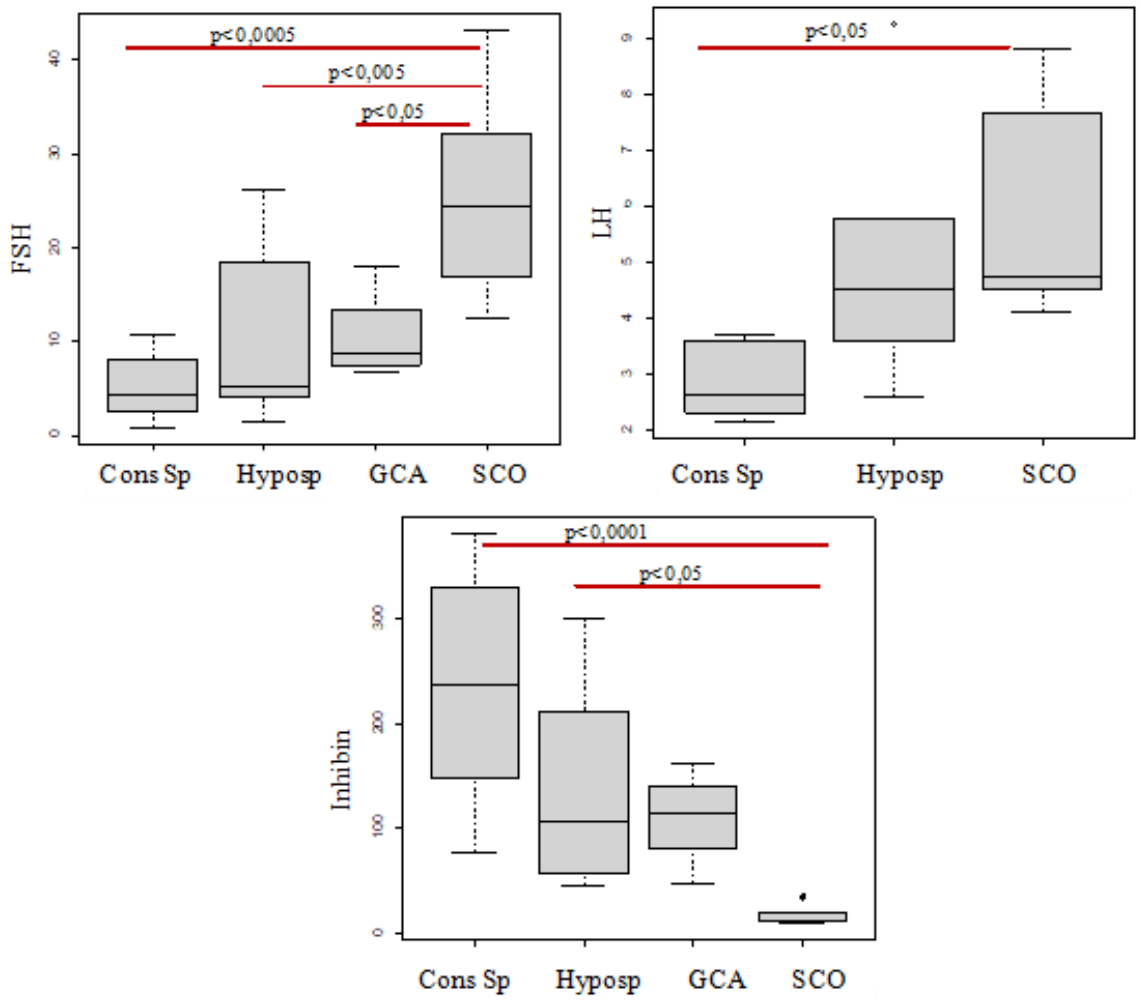

Figure 1. Boxplots of FSH, LH and Inhibin B levels in azoospermic patients with pure phenotypes, according to testicular histology *(Cons Sp – conserved spermatogenesis; Hypsp – Hypospermatogenesis; GCA – Germ cell arrest; SCO – Sertoli cell Only; FSH U/L, LH U/L, Inhibin ng/L)*

Four azoospermic patients with a pure germ cell arrest phenotype underwent whole-exome sequencing (**Table 2**). Likely pathogenic (LP) variants in a hemizygous or homozygous state—affecting meiosis-related genes (Testis expressed 11 -*TEX11* and Helicase for meiosis 1- *HFM1*) or a chromatin-remodelling gene (H2B Clustered Histone 1- *H2BC1*)—were identified in three of the four patients, which may be the cause of the observed germ cell arrest. In addition, runs of homozygosity (ROH) analysis revealed multiple segments > 1 Mb in patients GCA1 and GCA3, consistent with a degree of parental consanguinity.

The association of both *TEX11* and *HFM1* pathogenic variants with meiotic arrest is well established in humans and mice. *TEX11* encodes a known fertility factor involved in meiotic recombination and pathogenic variants including single mutations and deletions in this gene are estimated to account for 1-2% of all NOA diagnoses (Yatsenko et al. 2015). Similarly, *HFM1* encodes an evolutionarily conserved DNA helicase that is essential for crossover formation and completion of meiosis in germline cells. Biallelic pathogenic variants in *HFM1* have been associated with azoospermia but also with premature ovarian failure (Xie et al., 2022; Ke et al., 2023).

Table 2. Hormonal levels (FSH and Inhibin), histological evaluation and genetic variants identified in four azoospermic patients with pure germ cell arrest (GCA) phenotype.

	Hormone level		Histological evaluation		Genetic analysis		
	FSH (U/L)	Inhibin (ng/L)	Assessed tubules	Germ cell arrested type	Genetic variant	Pathogenicity	State (inheritance)
GCA1	8,42	118,3	150-170/ 80-90	Spermatocyte	*HFM1:*c.2452G>A p.(Glu818Lys)	LP (PM2, PP3)	Homozygote (AR)
GCA2	17,86	47,8	NA/58	Spermatogonia	*TEX14:*c.777G>A p.(Trp259Ter)	LP (PVS1, PM2)	Heterozygote (AR)
GCA3	8,92	161,9	50/70	Spermatocyte	*TEX11:* delX(70907753-70929997)	CNV LP	Hemizygote (XLR)
GCA4	6,68	112,6	100/50	Secondary spermatocyte	*H2BC1:* c.33_34insA p(Ser12IlefsTer6)	LP (PM2, PM3)	Homozygote (?)

Regarding *H2BC1*, this represents the first reported case of maturation arrest linked to biallelic pathogenic variants in the testis-specific histone H2BC1. H2BC1 is essential for the histone-to-protamine transition and genome packaging, particularly during the final conversion of nucleosomes into protamine-rich structures (Rusevski et al. 2022). A defect in H2BC1 may therefore underlie the observed arrest at the secondary spermatocyte stage. However, histone gene clusters encode proteins with overlapping, often redundant functions; when one variant is non-functional, other cluster members can typically compensate. Further studies are needed to determine whether H2BC1 performs a unique role that cannot be fulfilled by other histones, thus explaining this patient's specific phenotype.

In the case of patient GCA2, we identified only a single variant in the testis-expressed gene TEX14 (c.777G>A, p.(Trp259Ter)), which cannot fully account for the phenotype, as TEX14-related azoospermia follows an autosomal recessive inheritance pattern. Histological analysis of this patient revealed an arrest at the spermatogonial stage, indicating that meiosis fails to initiate. Consistent with this finding, the patient exhibited elevated FSH and reduced Inhibin B levels, similar to the hormonal profile of SCO phenotype. These observations suggest a block prior to meiotic entry. Other genetic studies—such as whole-genome sequencing and long-read technologies—could be used to identify potential structural variants or other genetic causes underlying this phenotype.

Overall, our findings highlight genetic causes are involved in meiotic arrest and support routine, comprehensive genetic screening in NOA patients—both to inform clinical management such as testicular sperm retrieval prognosis and to provide reproductive counselling to the couple. Future work should integrate multi-omics approaches and larger patient cohorts to uncover additional rare or structural variants, refine genotype–phenotype correlations, and ultimately improve diagnostic yield and therapeutic options for men with NOA.

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IMPACTS OF IN VITRO EXPOSURE TO NANOPLASTICS ON MOUSE SPERMATOZOA AND PREIMPLANTATION EMBRYOS

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Abstract

Plastic pollution is a growing concern due to the ubiquitous presence of microplastics (MPLs) and nanoplastics (NPLs) in the environment. Their accumulation in human tissues, especially in testes and ovaries, raises concerns about potential fertility disruption.

This study examined the effects of *in vitro* NPLs exposure on spermatozoa and preimplantation embryos in a mouse model. Epididymal sperm and *in vivo* fertilized zygotes were exposed to 100 nm polystyrene NPLs (100 µg/mL). Sperm motility, membrane integrity, acrosome reaction, reactive oxygen species (ROS) production and DNA fragmentation were measured at different time-points up to 24 h. In embryos with and without zona pellucida (ZP), development was monitored for up to 120 h. Blastocysts were processed for ROS quantification and blastocyst quality was assessed by inner cell mass (ICM), trophectoderm and total cell counts. Additionally, fluorescent NPLs of the same size and concentration were used to evaluate adhesion and internalisation in sperm and embryos.

Exposed sperm showed increased NPLs adhesion over time, initially localized to the acrosome and head, and progressively extending to the tail. Motility and membrane integrity were significantly reduced after 4 h, with consistently lower acrosome reaction. No significant increases in ROS production or DNA fragmentation were observed. In embryos, NPLs adhered to the ZP and internalisation increased over time, with intracellular clusters appearing only at 72 and 96 h. Overall development, ROS production, and blastocyst quality were unaffected. However, when the ZP was removed prior to NPLs exposure, NPLs internalisation and ROS production increased, impairing compaction and blastocyst formation. Blastocyst quality declined, with lower ICM counts and decreased cell ratios.

NPLs exposure negatively affects sperm function and impairs embryonic development in the absence of ZP protection, highlighting potential risks to fertility.

Resum

La contaminació per plàstics és una preocupació creixent per la presència de microplàstics (MPLs) i nanoplàstics (NPLs) al medi ambient. La seva acumulació en teixits humans, especialment en testicles i ovaris, genera preocupació sobre possibles alteracions en la fertilitat.

Aquest estudi analitza els efectes de l'exposició *in vitro* a NPLs sobre espermatozoides i embrions preimplantacionals en un model de ratolí. Es van exposar espermatozoides epididimals i zigots fecundats *in vivo* a NPLs de poliestirè de 100 nm (100 µg/mL). Es va analitzar la motilitat, la integritat de la membrana, la reacció acrosòmica, la producció d'espècies reactives de l'oxigen (ROS) i la fragmentació de l'ADN fins a 24 h postexposició. En embrions amb i sense zona pel·lúcida (ZP), es va controlar el desenvolupament fins a 120 h. Els blastocists resultants es van processar per a la quantificació de ROS i se'n va avaluar la qualitat mitjançant recomptes cel·lulars. A més, es van utilitzar NPL fluorescents de la mateixa mida i concentració per avaluar l'adhesió i la internalització en espermatozoides i embrions.

Els resultats mostren que els NPLs s'adhereixen progressivament als espermatozoides, inicialment per l'acrosoma i el cap, i posteriorment cap a la cua. Després de 4 h d'exposició, es va observar una reducció significativa de la motilitat i integritat de la membrana, així com una disminució en la reacció acrosòmica. No es van detectar increments de ROS ni dany a l'ADN. En embrions, els NPLs

s'adheriren a la ZP i s'internalitzen progressivament, formant agregats intracel·lulars a les 72–96 h. El desenvolupament, la qualitat dels blastocists i els nivells de ROS no es van veure afectats, llevat que la ZP s'eliminés abans de l'exposició. En aquest cas, l'exposició als NPLs va augmentar la producció de ROS, interferint amb la compactació i reduint la qualitat dels blastocists.
Els NPLs afecten negativament la funció espermàtica i el desenvolupament embrionari si no hi ha protecció de la ZP, destacant possibles riscos per a la fertilitat.

INTRODUCTION

Plastic production continues to rise, yet waste management remains inadequate, leading to accumulation in landfills and environmental leakage (Fan *et al.*, 2022). While large plastic debris poses environmental threats, recent focus has turned to microplastics (MPLs, <5 mm) and nanoplastics (NPLs, <1 □m) due to their enhanced mobility, persistence, and potential for biological interaction (Mitrano, Wick and Nowack, 2021).

MNPLs exposure takes place mainly through ingestion (Barboza *et al.*, 2018), but also via inhalation and dermal contact (Revel, Châtel and Mouneyrac, 2018). Consequently, MNPLs have been detected in the bloodstream and multiple organs, suggesting systemic distribution via circulation (Salvia *et al.*, 2023). NPLs can enter cells through endocytosis or passive membrane diffusion (Liu *et al.*, 2021), where they may accumulate in lysosomes or distribute among cellular organelles (Liu *et al.*, 2022).

A key concern regarding MNPLs exposure is its potential toxicity. MNPLs can directly interact with cell membranes or enter cells, potentially triggering oxidative stress through the production of reactive oxygen species (ROS) (Płuciennik *et al.*, 2024). ROS are crucial at controlled physiological levels for regulating sperm functions such as motility, capacitation, and the acrosome reaction. However, sperm cells are also particularly susceptible to excessive ROS due to their polyunsaturated fatty acid-rich membranes, with excessive ROS impairing the same functions (Henkel, 2011). Similarly, controlled ROS levels are critical for early embryonic development, with fluctuations necessary for key processes such as compaction and blastocyst formation. Although embryos possess protective mechanisms like apoptosis and autophagy, excessive ROS can cause mitochondrial and nuclear DNA damage, compromising development (You *et al.*, 2024).

Despite increasing concern, few studies have explored the effects of NPLs on gametes and embryos. This study aimed to examine the impact of *in vitro* exposure to NPLs on sperm functionality and the development and quality of preimplantation embryos in a mouse model.

MATERIAL AND METHODS

Animals. Outbred CD-1 and hybrid B6CBAF1 (C57BL/6J × CBA/J) mice were used (males: 8-12 weeks; females: 6-10 weeks). Animals were housed under standard conditions (12 h light/dark, 20-25°C, 50-70% humidity) with *ad libitum* access to food and water. All procedures were approved by the Animal Experimentation Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (4762UAB).

Nanoplastics. Carboxylated polystyrene nanospheres (100 nm; Polybead® Carboxylate Polystyrene Microspheres, Polysciences) were used at 100 µg/ml. For adhesion and internalization studies, fluorescent polystyrene NPLs (100 nm; Fluoresbrite® Yellow Green Carboxylate Polystyrene Microspheres, Polysciences) were used at 100 µg/ml for embryos and 35 µg/ml for sperm.

Collection of samples. Spermatozoa were retrieved from cauda epididymides in HTF medium. One-cell embryos were obtained from the oviducts of superovulated CD-1 females mated with B6CBAF1 males. Embryos were collected in H-KSOM medium and denuded with hyaluronidase. For some experiments, the zona pellucida was removed using 0.5% pronase. Embryos were cultured in KSOM drops under mineral oil at 37°C with 5% CO_2 for up to 120 h.

NPLs adhesion on spermatozoa. Adhesion of NPLs was evaluated in one replicate. At timepoints 0, 2, 4, 8, 12 and 24 h after exposure, a fraction of spermatozoa was analysed using an epifluorescence microscope (Olympus BX60). NPLs adhesion was evaluated based on their localization on the acrosome, head, midpiece and tail. For each time-point, 100 spermatozoa were analysed.

Sperm functionality analysis. To assess sperm functionality, several parameters were analysed including motility, membrane integrity, acrosome reaction, ROS production, and DNA fragmentation. Three replicates were performed for all experiments, each with a control and an NPLs-exposed group. For each time-point and each group, 100 spermatozoa were analysed, unless indicated otherwise.

- **Motility** was analysed at 0, 2, 4, 8, 12 and 24 h under a bright-field microscope (Olympus CH30). Sperm motility was classified into three distinct groups: progressive motility, non-progressive motility, and immotile.
- **Membrane integrity** was evaluated at 0, 2, 4, 8, 12 and 24 h using the hypoosmotic swelling (HOS) test and visualized under a bright-field microscope (Olympus CH30). Sperm were classified into two groups: reactive, meaning the plasma membrane is intact, allowing water to pass through and causing the swelling and curving of its tail; and non-reactive, meaning the plasma membrane is not functional thus sperm does not show any sperm tail swelling/curving reaction.
- **Acrosome reaction** capability was assessed at 0, 2, 4, 8, 12 and 24 h using the FluoAcro test (Microptic S. L.) and analysed using an epifluorescence microscope (Olympus BX60). Sperm were classified in two groups: reactive, corresponding to those which had undergone acrosome reaction and consequently did not retain the acrosome, and non-reactive, corresponding to sperm which had not undergone acrosome reaction and retained the acrosome.
- **ROS generation** was assessed at 0, 4, 12, and 24 h with each replicate including a non-exposed sample, a positive control (non-exposed sample treated with antimycin A), and a NPLs-exposed sample. Samples were stained with 4 µM DHE to detect ROS and with Hoechst for nuclear counterstaining. Flow cytometry (Leica Microsystems) was used to analyse at least 5000 events per sample, identifying sperm-like cells based on dual fluorescence. The threshold for ROS positivity was established using the positive control and applied to determine the percentage of ROS-positive cells in non-exposed and NPLs-exposed samples.
- **DNA fragmentation** in sperm was assessed using the TUNEL assay (Roche Diagnostics) at 0, 2, 12, and 24 h timepoints. Sperm suspensions were divided into four groups: NPLs-exposed, non-exposed control, positive control (DNase I-treated), and negative control (no TdT enzyme). After incubation, samples were fixed, permeabilized, and stained with the TUNEL reaction mixture. Propidium iodide was used as a counterstain. At least 5000 events were analysed by flow cytometry (Leica Microsystems), and the percentage of TUNEL-positive cells was quantified using CytExpert software.

NPLs adhesion and internalisation in embryos. Adhesion and internalisation of NPLs were evaluated in embryos with (ZP+) and without ZP (ZP-). Every 24 h, three embryos per group were fixed and stained with Texas Red phalloidin for actin and Hoechst 33258 for nuclei. Samples were analysed by confocal microscopy (Leica SP5).

Embryo development and quality analysis

- **Embryo development** was assessed in ZP+ and ZP- embryos after up to 120 h of culture (6 replicates per group). One-cell embryos were randomly divided into control (C; KSOM medium) and NPLs-exposed groups (NPLs; KSOM + NPLs) and the rates of embryos reaching the two-cell, morula and blastocyst stages were recorded.
- **ROS levels** were assessed in blastocysts at 96 h after culture of ZP+ and ZP- one-cell embryos (3 replicates per group). A subset of ZP+ control embryos was treated with antimycin A as a positive control. All groups were incubated with 10 µM DHE. Blastocysts were imaged using an inverted fluorescence microscope (Olympus IX71), and corrected total cell fluorescence (CTCF) was quantified using Fiji software. Fluorescence values were normalized to the positive control.
- **Blastocyst quality** was assessed by OCT-4 and CDX2 immunofluorescence detection (3 replicates for ZP+ and 5 replicates for ZP-). Embryos were imaged via epifluorescence microscopy, and the number of inner cell mass (ICM) and trophectoderm (TE) cells were counted in Fiji.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software, with a significance level set at $p < 0.05$. Two-way ANOVA was applied to assess differences in ROS levels and DNA fragmentation in spermatozoa. Fisher's exact test was used to compare embryo development rates and sperm functionality tests. The Mann-Whitney test was employed for comparing ROS levels in embryos and blastocyst cell counts and ratios.

RESULTS AND DISCUSSION

Results in spermatozoa

NPLs adhesion on spermatozoa progressed over time, particularly in the acrosomal and head regions where capacitation-related membrane changes occur (Figure 1). As capacitation reduces the negative surface charge, primarily due to the loss of sialic acid and changes in phospholipid composition (Ma *et al.*, 2012), the likelihood of carboxylate NPLs binding increases over time . This possibly explains the observed functional impairments *in vitro*. Sperm motility was notably reduced from 4 h of exposure onward, with declines in both progressive and non-progressive motility (Figure 2A). Membrane integrity also decreased at 4, 8, and 24 h post-exposure (Figure 2B), and the acrosome reaction was consistently lower in NPLs-exposed samples at all time points (Figure 2C).

Adhesion of NPLs may interfere with surface proteins such as ATPases, essential for sperm motility, as previously described in studies using gold nanoparticles (Taylor *et al.*, 2014). Additionally, impaired sperm motility, membrane integrity, and acrosome reaction have been similarly reported in human sperm exposed to 50 and 100 nm NPLs (Contino et al., 2023). Acrosome reaction inhibition may stem from NPLs-induced blocking of calcium channels or interference with calcium ionophores, both of which are essential for acrosome exocytosis. Damage to the sperm membrane may further prevent the necessary fusion between plasma and acrosome membranes.

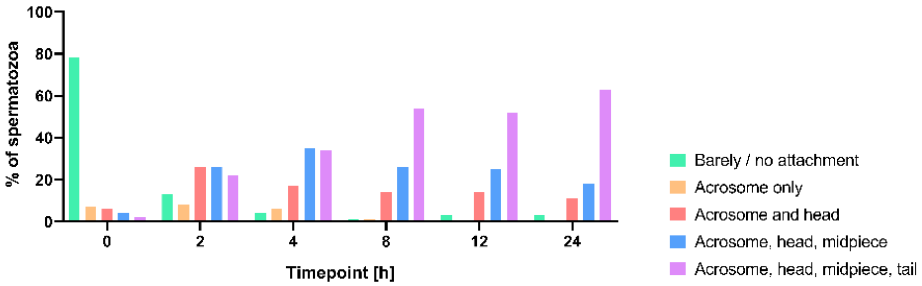

Figure 1: Adhesion of NPLs to the different regions of spermatozoa at different timepoints up to 24 h. NPLs display more adherence over time, with attachment reaching the tail as the observation period progresses.

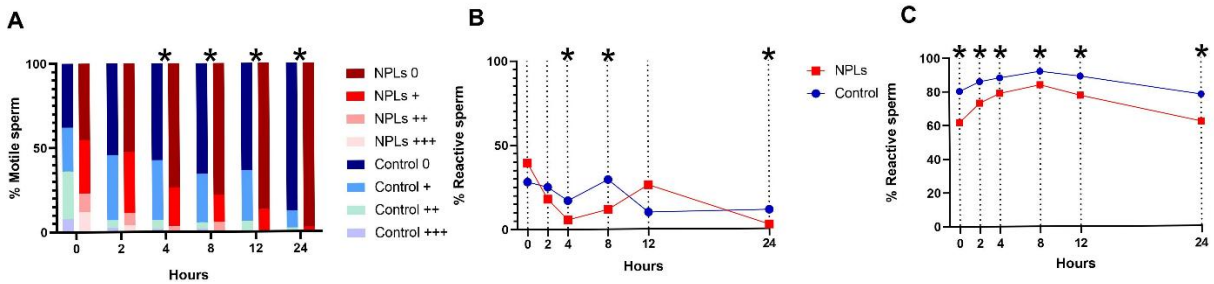

Figure 2: Sperm functionality results in control and NPLs-exposed spermatozoa along the different time points. (A) Percentage of different types of motilities indicated in different shades of blue (control) and red (exposed) with immotility (0), non-progressive motility (+) and progressive motility (++, +++); (B) Percentage of sperm with an intact membrane (HOS-reactive). (C) Percentage of sperm that underwent acrosome reaction (reactive). *p-value<0.05 using Fisher's exact test.

Regardless, our results indicate that NPLs exposure does not induce ROS production in spermatozoa at any of the measured time points (Figure 3A). Although sperm display high levels of endocytosis during spermatogenesis (Segretain *et al.*, 1992), mature sperm cells likely have limited abilities to internalise

NPLs, which explains the lack of uptake and consequently ROS production. Another study similarly observed adherence of polystyrene MPLs to bovine spermatozoa after 2 h of incubation but reported no internalisation or ROS induction (Grechi *et al.*, 2024). Given that NPL internalisation by mature spermatozoa is unlikely, ROS generation is not expected.

Additionally, no significant DNA fragmentation was detected across all assessed time points (Figure 3B). Oxidative stress is a major contributor to DNA damage in spermatozoa (Sanocka and Kurpisz, 2004). However, sperm chromatin is highly compacted due to the replacement of histones with protamines, providing substantial resistance to oxidative stress and structural damage (Agarwal and Said, 2003). Since our results showed no increase in ROS, no DNA fragmentation is expected.

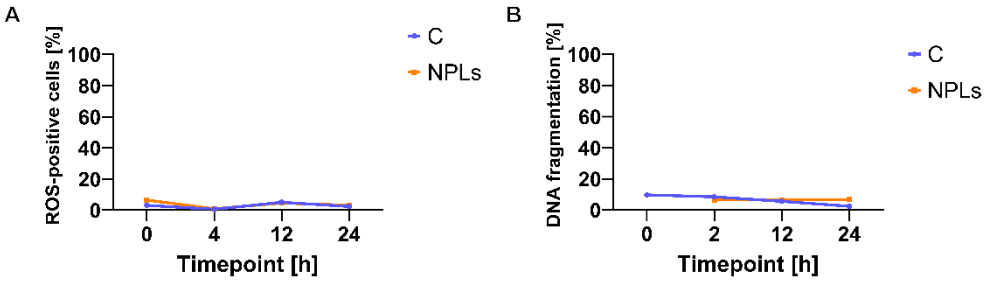

Figure 3: (A) Generation of ROS in spermatozoa. Percentage of ROS-positive spermatozoa at timepoints 0, 4, 12 and 24 h. (B) DNA fragmentation in spermatozoa. Percentage of DNA fragmentation at timepoints 0, 2, 12 and 24 h. Colors indicate groups: control (blue) and NPLs-exposed (orange). Data is represented as mean values from three replicates. No significant differences were detected using two-way ANOVA (p-value > 0.05).

These findings align with other results, where they observed DNA fragmentation in mature human spermatozoa only with 50 nm NPLs but not with 100 nm particles. Their study also reported ROS production and acrosomal damage with smaller NPLs, indicating a potential size-dependent effect. (Contino *et al.*, 2023)

Overall, while NPLs adhere progressively to sperm membranes and impair motility, membrane integrity, and acrosome reaction, they do not induce oxidative stress or DNA fragmentation under these *in vitro* conditions.

Results in embryos

In our study, NPLs quickly adhered to the ZP, but internalisation was only observed at late time points (from 72 h of exposure), emphasizing the modulatory role of the ZP in NPLs uptake. In embryos where the ZP was removed, internalisation of NPLs occurred as early as 24 h.

The mammalian ZP is composed of three glycoproteins in mice and four in humans (Litscher and Wassarman, 2020), with its matrix rich in neutral and acidic carbohydrates and proteins (Green, 1997). After fertilization, the ZP undergoes "zona hardening," which increases its mechanical strength and reduces enzymatic solubility, further limiting foreign particle penetration (Schiewe *et al.*, 1995; Sun *et al.*, 2003). Despite the protective function of the ZP, other studies have reported internalisation even in its presence. You et al. found that polymethylmethacrylate NPLs (25 nm; 100 µg/mL) rapidly entered mouse embryos, including the two-cell stage, with increasing uptake and ROS production over time that resulted in impaired development (You *et al.*, 2024). A separate study showed internalisation of 44 nm PS-NPLs into blastomeres (but not nuclei) of bovine embryos, impairing cleavage rates without affecting blastocyst quality (Barbato *et al.*, 2020). The larger size of NPLs used in our study could explain their limited internalisation in ZP+ embryos.

In our experiments, embryos with intact ZP showed no differences in ROS levels compared to controls following NPLs exposure (Figure 4A), whereas ZP- embryos exhibited significantly increased ROS levels (Figure 4B). ROS naturally occur in preimplantation embryos as by-products of ATP metabolism

and are essential for key developmental processes (Deluao *et al.*, 2022). However, excessive ROS can lead to DNA damage, embryo arrest, and abnormal development (You *et al.*, 2024).

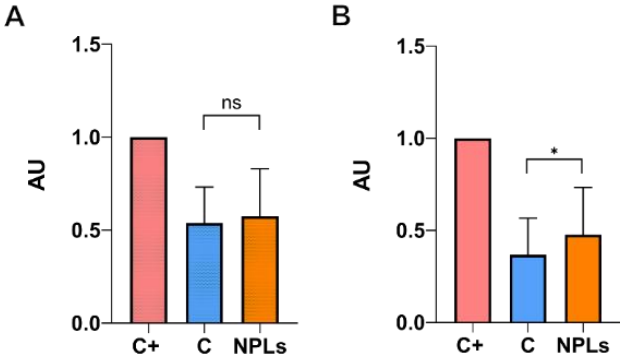

Figure 4: ROS generation in control and NPLs-exposed embryos. (A) with ZP (C+: n=18, C: n=58, NPLs: n=55) and (B) without ZP (C+: n=14, C: n=65, NPLs: n=62). Positive control (C+, red), control (C, blue), NPLs-exposed group (NPLs, orange). In embryos with ZP, ROS levels between groups are equivalent, whereas a significant increase is detected in embryos without ZP (Mann-Whitney test, p-value < 0.05). Data is represented as fluorescence intensity (CTCF mean ± SD) normalized to the positive control.

ZP+ embryos showed no developmental defects or blastocyst quality alterations (Figure 5A, Figure 6A and B), consistent with limited NPLs internalisation and lack of ROS generation. However, ZP- embryos exposed to NPLs displayed significant impairments in morula and blastocyst formation, reduced ICM cell numbers, and altered ratios, though total cell and TE numbers remained unaffected (Figure 5B, Figure 6C and D). These disruptions likely result from increased internalisation and ROS production following ZP removal.

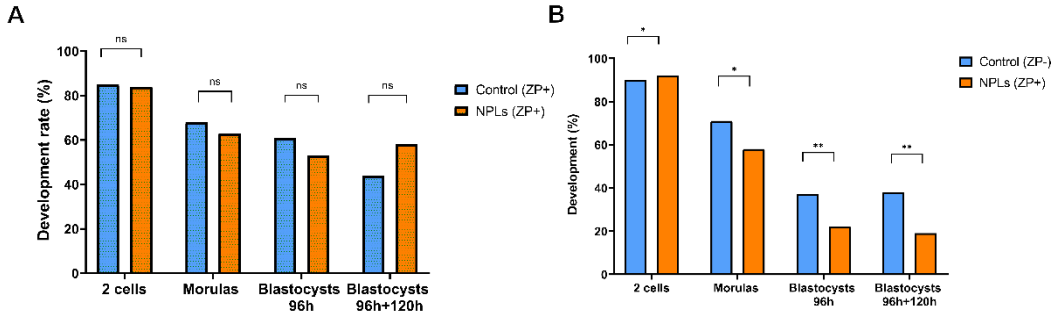

Figure 5: Embryonic development rates to the 2-cell, morula and blastocyst stages in control and NPLs-exposed embryos. (A) with ZP (C: n=168, NPLs: n=149) and (B) without ZP (C: n=183, NPLs: n=198). Control (C, blue), NPLs-exposed group (NPLs, orange). NPLs-exposed embryos without ZP show impaired morula and blastocysts development rates compared to the control group (Fisher's exact test, *p-value < 0.05, ** p-value < 0.01).

Our data reinforce the hypothesis that the ZP functions as a critical barrier that delays or prevents NPLs uptake and associated oxidative stress. In the absence of the ZP, embryos are more susceptible to early and extensive NPLs internalisation, leading to elevated ROS levels, impaired development, and reduced blastocyst quality. The observed reductions in ICM cell numbers and altered ratios suggest potential consequences for implantation success and embryonic viability.

In conclusion, our results indicate that sperm and embryos are vulnerable to NPLs exposure, although the zona pellucida acts as a crucial protective barrier. These findings emphasize the potential threats of plastic pollution to reproductive health and highlight the need for further research into the long-term consequences of MNPLs exposure on fertility and embryonic development.

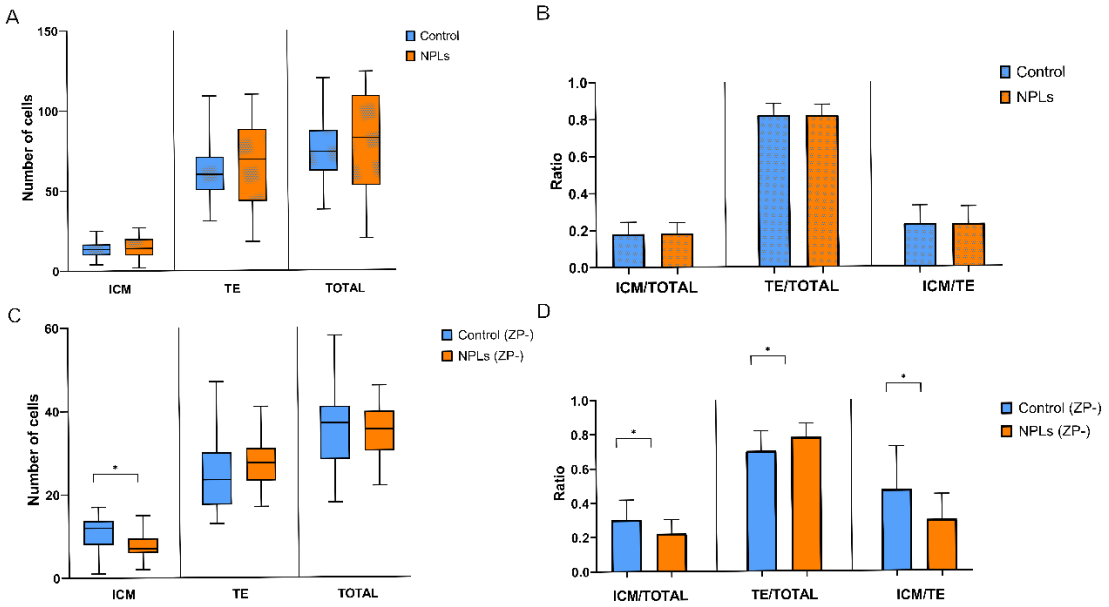

Figure 6: Blastocyst quality in control and NPLs-exposed embryos. (A, B) with ZP (C: n=32, NPLs: n=30) and (C, D) without ZP (C: n=28, NPLs: n=20). Control (C, blue), NPLs-exposed group (NPLs, orange). (A, C) Cell counts of inner cell mass (ICM), trophectoderm (TE) and total cells. (B, D) Ratios of ICM to total cell count (ICM/TOTAL), TE to total cell count (TE/TOTAL) and ICM to TE (ICM/TE). ZP+ showed no differences in cell counts or ratios. ZP- embryos exposed to NPLs showed reduced ICM counts and significant differences in all ratios (Mann-Whitney test, *p-value < 0.05, ** p-value < 0.01).

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AGE-RELATED EPIGENETIC CHANGES IN MALE GERM CELLS AND THEIR ASSOCIATION WITH NEUROPSYCHIATRIC DISORDERNS IN THE PROGENY

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Abstract

The growing trend of delayed fatherhood has brought increasing attention to the influence of paternal factors on offspring health, as well as the importance of the biological changes of reproductive aging. It is well established that advanced paternal age correlates with an increased risk of having a child with neuropsychiatric disorders, particularly autism spectrum disorder (ASD) and schizophrenia (SCZ). Recent evidence indicates that male epigenetic inheritance may have a causative role.

Male germline transmission relies on spermatogenesis which ensures the production of high-quality sperm. Epigenetic modifications play a crucial role in this process by regulating multiple aspects including gene expression and chromosome organization. Age-related epigenetic alterations have been identified in the sperm of older men, including global hypermethylation along with gene-specific hypomethylation in sites related to neural development and neuropsychiatric conditions. In addition, protamine levels are also disrupted with age, potentially affecting fertilization and early embryonic development. During murine germ cell development, the dynamics of histone post-translational modifications are also altered with age, affecting both active and repressive epigenetic marks, indicating a broad epigenetic network deregulation. Emerging epigenetic regulators have also been associated with sperm aging and progeny health. That is the case of tRNA-derived small RNA, which has been reported to be altered in aged sperm and have been linked to behavioural abnormalities in offspring, such as anxiety and autism-like traits. Furthermore, m6A RNA modification in aged sperm correlates with neuroinflammation in the offspring.

Multiple ASD mice models have confirmed that such epigenetic alterations are heritable through the paternal germline and may contribute to the development of neuropsychiatric disorders.

This review compiles the existing evidence on age-related epigenetic changes in male germ cells in both human and animal models, providing a comprehensive overview on the known mechanisms underlying advanced paternal age and the risk of neuropsychiatric disorders in the progeny. Further to this, it reveals areas for which no evidence has yet been reported, representing promising directions for future research.

Resum

La creixent tendència de posposar la paternitat ha posat en evidència la influència dels factors paterns en la salut de la descendència, així com la importància de les conseqüències biològiques de l'envelliment reproductiu. Està ben establert que l'edat paterna avançada es correlaciona amb un risc augmentat de tenir descendència amb desordres neuropsiquiàtrics, especialment trastorn de l'espectre autista (ASD) i esquizofrènia (SCZ). L'evidència recent indica que l'herència epigenètica paterna podria tenir un rol causal.

La transmissió de la línia germinal masculina depèn de l'espermatogènesi, que garanteix la producció d'espermatozoides d'alta qualitat. Les modificacions epigenètiques tenen un paper crucial en aquest procés mitjançat la regulació de múltiples aspectes, incloent-hi l'expressió gènica i l'organització dels

cromosomes. S'han identificat alteracions epigenètiques associades a l'edat en l'esperma d'homes d'edat avançada, incloent una hipermetilació global juntament amb una hipometilació regional en gens implicats en el desenvolupament neural i malalties neuropsiquiàtriques. Els nivells de protamines també es veuen alterats amb l'edat, fet que pot afectar potencialment la fecundació i el desenvolupament embrionari primerenc. Durant el desenvolupament de les cèl·lules germinals masculines murines, la dinàmica de les modificacions post-traduccionals de les histones s'altera amb l'edat, afectant tant marques epigenètiques activadores com repressores i suggerint una àmplia desregulació de les xarxes epigenètics. Els reguladors epigenètics emergents també s'han associat amb l'envelliment espermàtic i la salut de la progènie. És el cas dels petits RNA derivats de tRNA, els quals s'han reportat alterats en esperma envellit i han estat relacionats a anomalies conductuals en la descendència, com comportament ansiós i comportaments similars a l'autisme. D'altra banda, la modificació d'RNA m6A en esperma envellit es correlaciona amb neuroinflamació en la descendència.
Diversos models murins d'ASD han demostrat que aquestes alteracions epigenètiques són heretables a través de la línia germinal paterna i poden contribuir al desenvolupament de trastorns neuropsiquiàtrics.
Aquesta revisió recopila l'evidència existent sobre els canvis epigenètics en les cèl·lules germinals masculines relacionats amb l'edat, tant en humans com en models animals, i ofereix una visió global dels mecanismes coneguts subjacents a l'edat paterna avançada i el risc de trastorns neuropsiquiàtrics en la descendència. A més, posa de manifest aspectes pels quals no s'ha reportat evidència, representant direccions prometedores per a la recerca futura.

INTRODUCTION

Aging is a progressive decline in cellular and physiological functions driven by complex interactions between environmental factors and stress-response molecular events, being epigenetic alterations key hallmarks underlying the aging process (López-Otín et al., 2023). In particular, primary epigenetic alterations arise when the homeostasis of DNA methylation, post-translational modifications (PTMs) and/or non-coding RNAs (ncRNAs) is disrupted (Gibney & Nolan, 2010). While epigenetic aging has been extensively studied in somatic cells, little is known about the epigenetic changes occurring during germ cell aging. Thus, investigating epigenetic alterations derived from reproductive aging holds significant potential and represent an expanding area of research.

Spermatogenesis is a complex biological process that involves mitosis, meiosis, and spermiogenesis, resulting in the formation of mature sperm (de Kretser et al., 1998). Epigenetic regulation is chief for this process and emerging evidence highlights its critical role in subsequent embryonic development (Burton & Torres-Padilla, 2025).

Numerous population-based studies have identified advanced paternal age (APA) as a risk factor for the development of psychiatric disorders in the offspring, being the strongest association with ASD and SCZ (Buizer-Voskamp et al., 2011; de Kluiver et al., 2017; Krug et al., 2020). While there is not a vastly accepted definition of APA, a frequently used criteria is the father being more than 40 or 50 years-old at the time of conception (Toriello & Meck, 2008). A study conducted in the Dutch population (Buizer-Voskamp et al., 2011) determined that the risk of having a child with ASD was 3.3 times higher for fathers over 40 years of age compared to those under 20. Additionally, an odds ratio of 1.27 was also reported for the risk of fathering a child with SCZ among fathers older than 35 years. Nonetheless, the biological mechanisms underlying this association are not yet elucidated. Several hypotheses have been proposed, including epigenetic mechanisms related to male epigenetic inheritance (de Kluiver et al., 2017; Soubry et al., 2014). Remarkably, these findings highlight the relevance of the paternal factor in conception and subsequent offspring development.

ASD comprises a group of neurodevelopmental conditions characterized by a wide range of deficits in communication, social interaction, and behaviour (NIMH, 2022). SCZ, on the other hand, is a severe psychiatric disorder that affects an individual's thinking, emotional regulation, and communication (NIMH, 2024).

The aim of the present review is to compile the current evidence on age-related epigenetic alterations in male germ cells in both mice and humans, and examine the existing knowledge on how these changes may contribute to the development of ASD and SCZ in the progeny of fathers of advanced age.

MATERIAL AND METHODS

Scientific evidence searches. A literature review was conducted through PubMed and Google Scholar searches using the keywords "aging," "paternal age," "epigenetics," "autism,", "schizophrenia", "inheritance," "neurodevelopmental disorders," "ncRNA," "PTM," and "DNA methylation." Databases such as UniProt, SFARI, GWAS Catalog, Genome Browser, and miRDB were used to perform specific and more accurate searches.

RESULTS AND DISCUSSION

DNA methylation.

DNA methylation involves the addition of a methyl group to the C5 position of cytosine, thereby forming 5-methylcytosine (5-mC). Through the oxidation of 5-mC, 5-hydroxymethylcytocine (5-hmC) is formed. Both 5-mC and 5-hmC regulate gene expression by recruiting transcription factors or modulating their binding to DNA, with the former associated with heterochromatin (Jin et al., 2011) and the latter with euchromatin (López et al., 2017). DNA methylation is a dynamic process that includes both *de novo* methylation and demethylation. Notably, in the zygote, the paternal genome undergoes global demethylation —with the exception of imprinted regions— and re-methylation, representing some of the earliest epigenetic events occurring during early embryonic development (Moore et al., 2013). This process is fundamental, as it establishes tissue-specific gene expression profiles, playing a crucial role in development and genomic imprinting, hence its dysregulation contributes to a wide range of diseases (Jin et al., 2011).

Altered DNA methylation patterns have been observed in the sperm of men of advanced age, with genome-wide correlation studies reporting a global age-associated hypermethylation (Bernhardt et al., 2023; Jenkins et al., 2014), in contrast to the global age-related hypomethylation observed in somatic cells (López-Otín et al., 2023). Likewise, 5-hmC levels display a similar trend, showing a positive correlation between paternal age and sperm global 5-hmC levels and increasing by 5% per year approximately (Jenkins et al., 2013). Despite these observations, the role of 5-hmC in aged male germ cells remains unexplored and current evidence offers limited conclusions.

Regardless the global hypermethylation in aged sperm, there is a pronounced bias towards regional gene-associated hypomethylation, with only a limited number of loci showing significant hypermethylation. In this regard, hypomethylated regions tend to be located near transcription start sites and nucleosome retention sites, while hypermethylated regions are generally found in more distal gene-associated regions. Intriguingly, methylation differences between young and aged sperm donors are modest, suggesting that multiple changes of little size impact contribute to paternal age effects in the progeny, rather than single or few highly penetrant epimutations. Nevertheless, findings indicate that age exerts a stronger influence on methylation aberrations in sperm compared to somatic cells (Bernhardt et al., 2023; Jenkins et al., 2014).

In human aged sperm, gene ontology analysis of genes exhibiting age-related methylation changes display signatures related to neurodevelopment and neuron synapses, as well as association with bipolar disorder and SCZ disease (Bernhardt et al., 2023). Among the different hypomethylated genes identified is DRD4, which codifies the D(4) dopamine receptor and widely implicated in the pathology of SCZ and other neuropsychiatric disorders.

An important gene is BEGAIN, which has been found to be hypomethylated in APA across five different studies (Bernhardt et al., 2023). This protein is a guanylate kinase-associated protein that activates postsynaptic potential in addition to participate in learning and memory processes (Katano et al., 2023). BEGAIN bisulphite sequencing conducted on sperm samples and offspring peripheral blood, revealed a negative correlation between BEGAIN promoter methylation and the age of the sperm donor, with an equivalent correlation observed in foetal cord blood samples from male offspring, but surprisingly not females (Potabattula et al., 2023). Displaying a similar trend, BEGAIN promoter hypomethylation was observed in the same study in the peripheral blood of individuals with ASD. Together, these findings point to BEGAIN as a potential candidate for a male-inherited epigenetic mark contributor to the increased risk of ASD in the progeny. It is worth emphasizing, however, that while paternal age contributes to BEGAIN promoter hypomethylation, its effect is partially explained by genetic polymorphisms (Potabattula et al., 2023).

In addition to the aforementioned studies in humans, different ASD mouse models have also identified age-related changes in sperm DNA methylome and their association with progeny health. Age-associated differentially methylated regions (ageDMRs) in the sperm of aged sires were predominantly located at intergenic and intragenic regions of genes involved in neuronal pathways and brain development (Yoshizaki et al., 2021; Zhao et al., 2020). Additionally, transcription binding sequence analysis of these ageDMRs identified an enrichment for the consensus motif of REST/NRSF (Yoshizaki et al., 2021), a master regulator of the nervous system involved in neuronal differentiation, plasticity, and survival (Arizmendi-Izazaga et al., 2023). Remarkably, transcriptome analysis in the forebrains of APA-derived embryos revealed expression changes in REST/NRSF target genes, correlating with autistic-like behaviour after birth. Most importantly, treatment of young mice with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine resulted in autistic-like traits in the offspring, as observed in the progeny of aged mice, hence establishing a causal link between DNA methylation changes in aged sperm and impaired neurodevelopment (Yoshizaki et al., 2021).

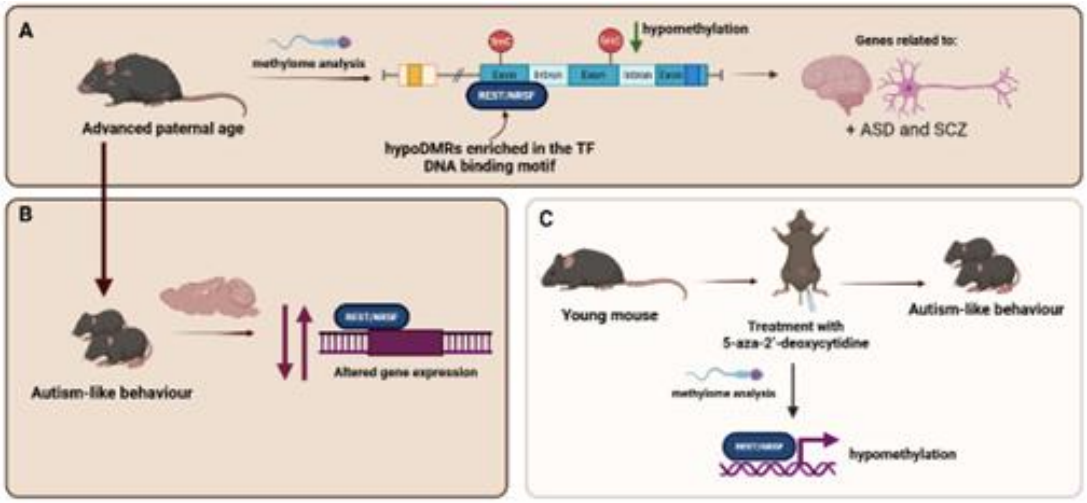

Figure 1. Age-related hypomethylation in male germ cells involving REST/NRSF contributes to anxiety-like behaviour in the offspring. A) Sperm from aged mice exhibit hypomethylation in REST/NRSF target genes linked to neural pathways. B) Their offspring display autism-like behaviours and altered forebrain gene expression. **C)** DNA methyltransferase inhibition with 5-aza-2'deoxycytidine in young mice induces comparable sperm hypomethylation and replicates the APA effects in the progeny (Yoshizaki et al., 2021).

Although the mechanisms by which paternal DNA methylation inheritance influences offspring health remain poorly understood, emerging evidence suggests that sperm DNA methylation regulates early epigenetic events following fertilization, shaping chromatin structure and gene expression programs in the developing embryo and potentially impacting the progeny health in the long term (Fanourgakis et al., 2025).

Protamines.

Protamines are arginine-rich proteins that replace histones during spermatogenesis, bind to sperm DNA and allow further DNA compaction within sperm head, event required for normal sperm function. In mammals, the primary protamines are protamine 1 (P1) and protamine 2 (P2), with the latter being only present in primates. The P1/P2 ratio is species-specific, critical for fertilization, and the disruption of its normal balance has been reported to negatively impact early embryonic development (Balhorn, 2007; Simon et al., 2011). In humans, alterations in P1/P2 ratio correlate with abnormal sperm morphology, reduced motility and fertilization rate and increased DNA fragmentation (Klutstein & Gonen, 2023). The sperm of men aged 51 to 81 years present lower P1 and P2 expression levels compared to men aged 20 to 32, indicating that aging is associated with a decline of major protamine members. More importantly, the P1/P2 mRNA ratio is also altered with age, with older individuals presenting a ratio of

0.63, in contrast to the 0.94 ratio observed in younger subjects (Paoli et al., 2019). Given that one of the primary functions of protamines is to preserve DNA integrity within the sperm head, such alterations may lead to impaired chromatin compaction and increased DNA fragmentation (García-Peiró et al., 2011).
Despite the negative impact of age-associated changes in protamines on sperm integrity and fertilization capacity (Simon et al., 2011), the exact implications of these alterations for disease transmission in the offspring remains still unknown.

Histone and RNA post-translational modifications.
The basic unit of chromatin is the nucleosome, composed of a histone octamer (two dimers of H2A-H2B and two dimers of H3-H4), which enables the packaging of DNA (Luger et al., 1997). Histones are proteins subject to covalent PTMs, constituting epigenetic marks that play a crucial role in genome homeostasis. There are multiple types of PTMs, the prevailing ones being acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation of lysine (K), arginine (R), serine (S), and threonine (T) residues (Ramazi et al., 2020). Each PTM exerts different effects, therefore their different combinations define the concept of the "epigenetic code", proposed in 1993 (Turner, 1993). The balance among different PTMs represents an essential regulatory element, hence the disruption of this homeostasis can lead to pathological conditions (Ramazi et al., 2020).
Changes in histone acetylation and methylation have been reported in the context of reproductive aging. Imaging analysis of histone PTM panel in male germ cells of aged mice compared to young controls, revealed reduced intensity of H3K9me3 and H3K4me2, along with increased levels of H3K27me2/3 through all spermatogenesis. Furthermore, H3K27ac levels were decreased at specific spermatogenetic stages (Tatehana et al., 2020). Whether these changes also occur during human spermatogenesis is currently unknown.
Considering that H3K9me3 and H3K27me2/3 are repressive epigenetics marks, whereas H3K4me2 is activating, these alterations likely impact euchromatic and heterochromatic domains and gene regulation. For instance, a decrease in H3K9me3, which constitutes pericentromeric heterochromatin, has been shown to impair gene repression and chromosomal stability (Burton & Torres-Padilla, 2025; Cheng et al., 2021). Meanwhile, H3K27 and H3K4 methylations are key players in early gene expression during embryonic development. Importantly, H3K4me in the paternal pronucleus is essential for minor zygotic genome activation (ZGA) (Aoshima et al., 2015) and evidence show that H3K4me3 aberrations in sperm are retained in the embryo and correlate with deregulated embryo gene expression, which is related to birth defects (Lismer et al., 2021). Nonetheless, the impact that age-related histone PTM alterations may have in the neuropsychiatric disorders of the offspring is yet to be uncovered.
N6-methyladenosine (m6A) is the most prevalent epigenetic modification in eukaryotic RNA and plays a critical role in regulating RNA splicing, translation, stability, translocation, and secondary structure. The YTHDC protein acts as an m6A reader by binding to m6A-containing transcripts and regulating RNA dynamics (Jiang et al., 2021). Recent evidence has demonstrated that YTHDC binds to m6A marks on *Nr4a2* and suppresses its translation. NR4A2 is a key transcriptional regulator required for the differentiation and maintenance of dopaminergic neurons during development (Flaig et al., 2005). Importantly, mutations in NR4A2 have been identified in disorders involving dopaminergic dysfunction, including SCZ (Buervenich et al., 2000; Le et al., 2003). In a mouse model of ASD using offspring of APA mice, analysis of hippocampal tissue and microglia revealed an increased neuroinflammation and microglia overactivation in addition to autistic-like behaviour compared to the progeny derived from young sires. Methylated RNA immunoprecipitation coupled to high-throughput sequencing (Me-RIP-seq) in paternal sperm and offspring hippocampus, identified m6A hypermethylation of *Nr4a2* mRNA, consistent with reduced NR4A2 protein levels. At the same time, YTHDC1 protein levels were significantly elevated in aged sperm. Notably, microglial ablation in APA progeny ameliorated the autism-like behaviours, while suppression of Ythdc1 attenuated neuroinflammation and improved cognitive defects (Mao et al., 2024). Whether the increased YTHDC1 levels in aged sperm are causally linked to ASD development in the offspring has not yet been demonstrated.

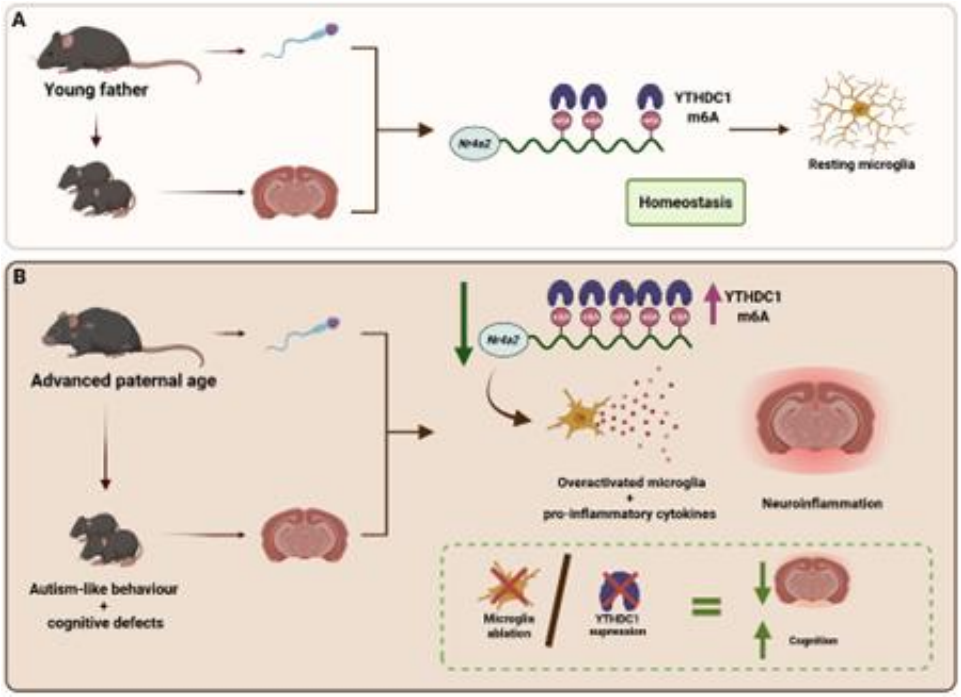

Figure 2. Epigenetic RNA modification mediates paternal age effects on offspring neural homeostasis. A) Normal *Nr4a2* m6A modification in young sires and preserved neural homeostasis in offspring B) Aged mice and their progeny display reduced NR4A2 protein levels due to increased m6A mRNA methylation and YTHDC1 expression, leading to neuroinflammation, overactivated microglia and autism-like behaviours in the progeny. Microglia ablation and Ythdc1 suppression mitigate these effects. (Mao et al., 2024)

Non-coding RNA.

ncRNAs originate from diverse genomic regions, including introns and exons of non-coding genes, as well as introns of protein-coding genes (Zhou et al., 2010). Among the various functional classes of ncRNAs, the following section focuses on those shown to be altered in the context of APA. First, microRNAs (miRNAs) are small RNA molecules of approximately 22 nucleotides in length that regulate gene expression at the post-transcriptional level, mainly by binding to complementary sequences on target messenger RNAs (mRNAs) and leading to either translational inhibition or mRNA degradation. They are involved in cellular processes such as development, differentiation, apoptosis, and proliferation. Similar to DNA methylation and histone PTMs, deregulation of miRNAs has been implicated in diseases including cancer, neurological disorders, and cardiovascular conditions (Bartel, 2004). Secondly, another type of ncRNA are tRNA-derived small RNAs (tsRNAs), which originate through specific cleavage events from transfer RNAs (tRNAs). tsRNAs play important roles in gene expression regulation by interacting with ribosomes, modulating target mRNAs, and even associating with stress factors and regulatory proteins. Altered expression of tsRNA has been observed in metabolic disorders, neurodegenerative diseases, and various cancers (Oberbauer & Schaefer, 2018).

Studies of seminal plasma from older men have identified several miRNAs with altered levels compared to young donors. Among those, miRNAs involved in aging and spermatogenesis, such as miR-146a, miR-371-3p, and miR-122a, were found to be downregulated (Paoli et al., 2019). Furthermore, two independent studies reported decreased levels of miR-29b in human sperm and serum samples from older individuals (Paoli et al., 2019; H. Zhang et al., 2015). miR-29b miRNA negatively regulates TCF4, a transcription factor involved in neuronal differentiation (Y. Zhang et al., 2021). Indeed, deletions and loss-of-function mutations in TCF4 cause Pitt-Hopkins Syndrome, a condition characterized by impaired intellectual development (Zweier et al., 2007). Additionally, recent studies have identified a single nucleotide variant within an intron of TCF4 that provides an increased risk (OR = 1.23) for developing SCZ (Stefansson et al., 2009). Nonetheless, no studies have explored the causal link between aged-related miR-29b deregulation, TCF4 expression and SCZ disease in the offspring.

Emerging evidence suggests that tsRNA profiles also play a role in paternal epigenetic inheritance, with altered tsRNA patterns reported in the sperm of aged mice (Guo et al., 2021). Sperm RNA sequencing comparing old versus young mice, revealed tsRNA deregulation in old individuals, affecting genes involved in axon guidance and cholinergic synapses. To determine whether tsRNAs could be responsible for transmitting these effects, tsRNAs were extracted from the sperm of aged males and microinjected into zygotes derived from young sires. The resulting offspring developed anxiety-like behaviours comparable to those observed in progeny born to aged males, establishing a causal link between altered sperm tsRNA profiles and progeny disease manifestation. Cortex and hippocampal tissue transcriptomic analysis from these mice revealed multiple deregulated genes, many of which were involved in aging and neurodevelopment pathways. When microinjecting tsRNA from aged male in two-cell embryos, when major ZGA occurs, and blastocysts, similar transcriptomic alterations were observed, reinforcing the connection between early gene expression changes and later offspring health outcomes. Further analysis of sperm RNA from older men revealed comparable results, supporting the consistency of the evidence and the translational relevance of these findings across species. (Guo et al., 2021).

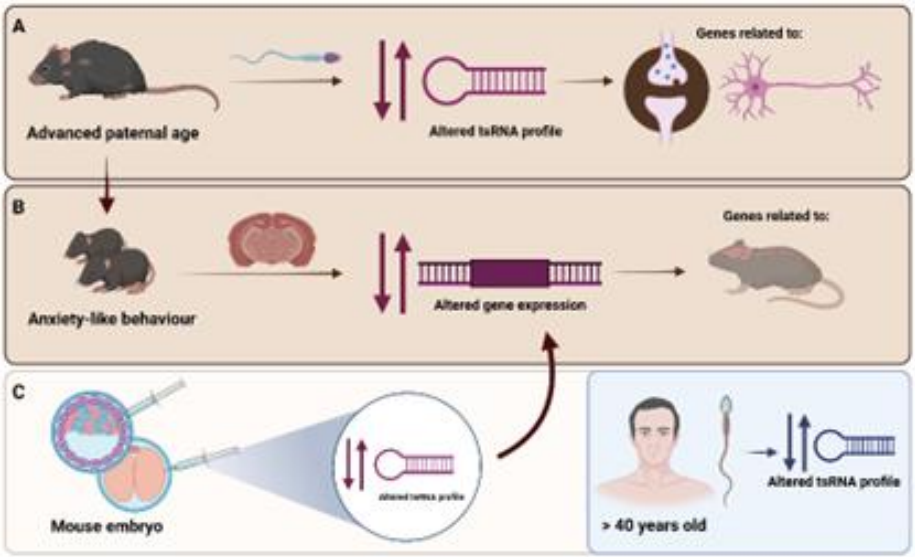

Figure 3. Altered sperm tsRNA due to advanced paternal age promotes anxiety-like behaviour in offspring. A) Aged mice show altered tsRNA targeting neurodevelopmental genes and B) their offspring display anxiety-like behaviour, along with central nervous system gene expression changes. C) Injecting age-altered tsRNA into embryos replicate these phenotypes. Similar tsRNA alterations are seen in sperm from older men (Guo et al., 2021).

CONCLUSIONS

The influence of paternal factors on offspring health has long been considered of low importance. However, the growing trend of delaying fatherhood has stressed the importance of understanding the biological consequences of reproductive aging.

While the association between paternal age and increased risk of neuropsychiatric disorders in the progeny, such as ASD and SCZ, is well-documented, the underlying mechanisms are only beginning to be understood. Recent findings suggest age-associated epigenetic modifications in male germ cells as key factors that may interfere with early embryonic and neural development, more specifically ageDMR, protamine ratio imbalances, changes in histone PTMs and ncRNA. Importantly, multiples studies using ASD mouse models demonstrate that these epigenetic alterations can be inherited and might be causal factors contributing to behavioural phenotypes of ASD.

Despite evidence seems to unravel the major mechanisms underlaying such complex diseases, it must be beard in mind the multifactorial and polygenic aetiology of neuropsychiatric disorders. Consequently, APA cannot function as a unique causal factor, but rather as one of several interacting components which collectively influence child developmental outcomes.

Although many questions remain unanswered, current knowledge provides a solid foundation for understanding how male germline aging may impact the next generation. These evidence not only extent our comprehension of the biological basis of neuropsychiatric disorders, but also opens promising new research lines to further explore the role of paternal age in reproductive aging.

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MOLECULAR EFFECTS OF SARS-CoV-2 INFECTION ON SPERMATOZOA ACCORDING TO COVID-19 SEVERITY.

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Abstract

SARS-CoV-2 infection has affected a large proportion of the population, raising some concerns about its potential impact on male reproduction and fertility. Our previously published case-control retrospective study has demonstrated significant reversible mild detrimental effects of COVID-19 disease on seminal parameters. Based on that, this study was designed to investigate the presence of SARS-CoV-2 RNA in semen samples from COVID-19 patients during the acute phase of the infection and to systematically evaluate potential alterations in seminal and hormonal parameters and sperm DNA fragmentation according to disease severity. Results revealed a clear disease severity-dependent pattern on semen quality and sperm DNA integrity. Notably, the absence of viral RNA in all semen samples suggested that the observed impairments are likely mediated through indirect mechanisms. These findings indicate that the adverse effects on male reproductive function may be transient and potentially reversible.

Resum

La infecció per SARS-CoV-2 ha afectat una gran proporció de la població i ha generat preocupacions sobre el seu possible impacte en la reproducció i la fertilitat masculina. El nostre estudi retrospectiu previ de casos i controls va mostrar que la infecció per SARS-CoV-2 resulta en un lleuger efecte advers significatiu i reversible en la qualitat dels paràmetres seminals. D'aquesta manera, hem dissenyat el present estudi per investigar la presència de RNA víric en mostres de semen de pacients amb COVID-19 durant la fase aguda d'infecció i avaluar sistemàticament les possibles alteracions en els paràmetres seminals i hormonals, així com en la fragmentació del DNA espermàtic, segons la gravetat de la malaltia. Els resultats obtinguts van revelar una afectació clara en la qualitat del semen i la integritat del DNA depenent de la simptomatologia. Cal destacar que, l'absència de RNA viral a totes les mostres de semen va suggerir que les alteracions observades probablement es troben mediades per mecanismes indirectes. Aquestes troballes indiquen que els efectes adversos sobre la funció reproductiva masculina poden ser transitoris i potencialment reversibles.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus infection, responsible for Coronavirus disease 2019 (COVID-19), is one of the most serious public health events in recent years. In addition to respiratory symptoms, some patients also showed delayed reactions or sequelae in extrapulmonary organs, including male reproductive system, potentially affecting fertility (Li et al. 2020; Anifandis et al. 2021; Borges et al. 2021).

The pathogenesis of infection by SARS-CoV-2 mainly involves Angiotensin Converting Enzyme 2 (ACE2) and Transmembrane Serine Protease 2 (TMPRSS2) (Hoffmann et al. 2020; Wrapp et al. 2020). Specifically, TMPRSS2 primes and cleaves the S1/S2 site on the SARS-CoV-2 spike protein. The S1 protein then binds to ACE2 to facilitate the entrance of the virus into the host cell (Hoffmann et al. 2020; Wrapp et al. 2020). Therefore, cells expressing high levels of ACE2 may be more susceptible to viral entry.

According to the Human Protein Atlas (Uhlén et al. 2015), within the male reproductive tract, the ACE2 receptor is mainly expressed in Sertoli cells, Leydig cells, epididymis and seminal vesicles, although it has also been detected in spermatogonia (Wang et al. 2020). In addition, TMPRSS2 is highly expressed in the epithelium of prostate and epididymis, but it is low expressed in testicular cells (Chen et al. 2010). This provides support for the possibility that the male reproductive system and fertility may be adversely affected by SARS-CoV-2 infection.

Although some studies have detected SARS-CoV-2 RNA in testicular tissue (Stein et al. 2022), there is still controversy over whether SARS-CoV-2 RNA exists in semen (Alvarez et al. 2023; Hallak et al. 2024) or viral particles in the testes (Ata et al. 2023). Several authors have suggested that possible testicular damage associated with COVID-19 disease may be a secondary effect of the immunological and inflammatory response induced by the infection (He et al. 2021). Inflammatory responses to infection can lead to the release of a large number of cytokines, including interleukin-6 (IL-6). Elevated IL-6 levels in critical patients have been closely linked to the serum SARS-CoV-2 viral load and may contribute to acute respiratory distress syndrome development (Antushevich 2020; Gusev et al. 2021).

Studies have shown that SARS-CoV-2 can have reversible adverse effects on male fertility, mainly manifested in decreased semen quality and altered hormone levels during COVID-19 disease (Gacci et al. 2021; Corona et al. 2022; Kumar et al. 2023; Lauritsen et al. 2023, Kadihasanoglu et al. 2021). In our recent retrospective study, we found reduced semen volume and impaired progressive motility during the first spermatogenic cycle after SARS-COV-2 infection and mainly in unvaccinated patients (Jodar et al 2024). However, there is no consensus on the potential changes in male fertility, as other studies have found no adverse effects on semen parameters after COVID-19 disease (Gul et al. 2021; Edimiris et al. 2023; Paoli et al. 2023).

The aim of this study is to measure the presence of SARS-CoV-2 RNA and elucidate potential differences in seminal parameters including sperm DNA fragmentation, hormone levels and an inflammation marker during the acute phase of the infection according to COVID-19 severity.

MATERIAL AND METHODS

Study design and participants recruitment. A prospective study of 87 infertile patients with a positive PCR test for SARS-CoV-2 was designed. All patients included in the study provided informed consent and the study was approved by the ethical committee of the Clínic Barcelona hospital. Semen and blood samples were collected from men who attended the Andrology unit of the the Clínic Barcelona hospital to undergo semen analysis during the phase of COVID-19 disease infection. Ejaculates were collected after 3-5 days of sexual abstinence and a seminogram was routinely performed evaluating sperm concentration, motility and morphology, according to the guidelines of the World Health Organization (World Health Organization, 2021). Samples were stratified in two groups considering the severity of the COVID-19 disease developed after viral infection according to symptoms: asymptomatic patients were classified as “mild” (n=65) and hospitalized patients were classified as “severe” (n=22).

Presence of SARS-CoV-2 RNA. RNA was isolated from semen in a semi-automated platform (MagnaPure Compact, Roche®) and the presence of SARS-CoV-2 RNA was tested using reverse transcription polymerase chain reaction (RT-PCR), targeting the viral E protein gene (Corman et al. 2020).

Evaluation of IL-6. Inflammation was evaluated by measuring IL-6 levels in seminal plasma employing an enzyme-linked immunosorbent assay (ELISA) kit specific to IL-6 at the Biochemistry and Molecular Genetics Service of the Clínic Barcelona hospital (KAP1261, DIAsource, Belgium).

Hormone levels. Blood samples were collected on the same day of ejaculates and hormonal levels related to male reproductive function, including follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone and inhibin (INHB) were assessed by immunoenzymatic assays commercially available at the Biochemistry and Molecular Genetics Service of the Clínic Barcelona hospital.

Double and single strand sperm DNA fragmentation evaluation by neutral and alkaline comet assay. Neutral and alkaline comet assay were performed to evaluate double strand breaks (DSB) and single strand breaks (SSB) in sperm DNA, respectively. The protocol was made following the two cited publications (Ribas-Maynou et al. 2012; Simon & Carrell, 2013). Under the action of the electric field, negatively charged DNA fragmentation moved fester than intact DNA toward the positive electrode, forming the tail of the comet. The gels were stained with SYBR Gold, a fluorescent dye specific for DNA. The percentage of sperms with fragmented DNA was quantified using fluorescence microscopy.

Statistical analyses. R software version 4.4.2 (http://www.r-project.org) was used for all data analyses and only p-values below 0.05 were considered statistically significant. Comparative Shapiro test was performed to evaluate if variables studied were normal distributed. Since the assessed variables were not normally distributed, non-parametric Wilcoxon test was performed.

RESULTS AND DISCUSSION

Absence of SARS-CoV-2 RNA in semen

SARS CoV-2 RNA was not detected in any of the semen samples assessed (17 severe and 10 mild). Our results are in accordance with other studies (Alvarez, 2023; Chaput, 2024; Hallak et al. 2024).

Alterations in seminal and hormonal parameters in severe COVID-19 patients during the acute phase of infection

The comprehensive comparative analysis revealed significant differential effects of COVID-19 severity on male reproductive parameters (**Table 1, Figure 1**). Specifically, patients who experienced severe COVID-19 disease demonstrated greater impairment in semen quality, including sperm concentration, total sperm count and progressive motility, compared to those with mild disease symptomatology. Furthermore, assessment of sperm DNA integrity through comet assay showed a significantly higher number of sperm cells with DNA fragmentation in severe cases. Evaluation of inflammation marker showed higher IL-6 levels in the severe group. In contrast, no differences were detected at hormonal level according to severity of the symptoms. These findings suggest an association between COVID-19 disease severity and the extent of reproductive dysfunction, potentially mediated through mechanisms like systemic inflammation.

CONCLUSION

In conclusion, the adverse effects of COVID-19 on the male reproductive system and fertility at the moment of infection have been confirmed and are related to the severity of the disease. However, these reproductive impairments are not mediated through direct cytopathic effects of SARS-CoV-2 on spermatozoa, but rather through secondary systemic mechanisms associated with the infection.

Table 1. Comparison of seminal and hormonal parameters and DNA fragmentation according to COVID-19 disease severity. Results showed as mean ± standard deviation. P-values <0.05 were considered significant. NS: no significance; IL-6: interleukin-6; FSH: follicle stimulating hormone; LH: luteinizing hormone; INHB: inhibin; SSB: single strand breaks; DSB: double strand breaks.

Variables	Mild (n=65)	Severe (n=22)	p-value
Volume (mL)	2.72 ± 1.17	2.20 ± 1.37	NS
Concentration (Mz/mL)	69.04 ± 80.43	33.16 ± 46.99	<0.01
Total Sperm Count (Mz)	178.04 ± 230.23	57.90 ± 94.97	<0.001
Progressive Motility (%)	39.83 ± 20.40	17.21 ± 13.54	<0.001
Normal Forms (%)	6.62 ± 6.23	7.20 ± 4.92	NS
IL-6 (pg/mL)	24.24 ± 42.15	36.78 ± 30.04	<0.05
FSH (U/L)	7.98 ± 7.11	8.74 ± 13.70	NS
LH (U/L)	4.68 ± 2.18	3.76 ± 1.44	NS
Testosterone (ng/dL)	429.52 ± 281.17	485.06 ± 208.66	NS
INHB (ng/L)	166.13 ± 78.22	173.92 ± 60.91	NS
SSB (%)	24.57 ± 10.96	44.07 ± 26.32	<0.01
DSB (%)	16.48 ± 8.76	36.35 ± 26.14	<0.01

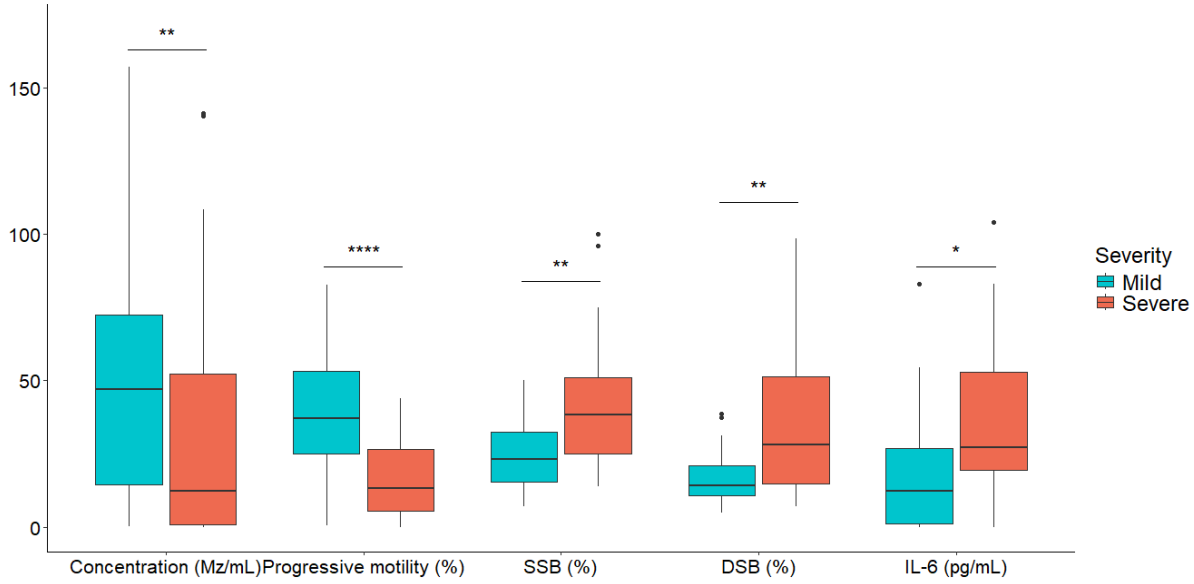

Figure 1. Selected parameters that showed significant differences according to COVID-19 severity at the moment of infection. P-values <0.05 were considered significant by Wilcox test. *p-value<0.05; **p-value<0.01; ****p-value<0.0001. SSB: single strand breaks; DSB: double strand breaks; IL-6: interleukin-6.

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CASE REPORT: IS THERE AN ASSOCIATION BETWEEN PATHOGENIC VARIANTS IN *BRCA2* AND SPERM DNA FRAGMENTATION? – IMPLICATIONS FOR MALE FERTILITY AND SPERM SELECTION STRATEGIES

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Abstract
BRCA2 plays a critical role in maintaining genomic stability during spermatogenesis by facilitating the repair of DNA double-strand breaks (DSBs) through homologous recombination. Pathogenic variants that impair BRCA2 function can compromise sperm DNA integrity, potentially affecting male fertility. We present a case of an infertile patient who was a carrier of a heterozygous pathogenic *BRCA2* variant. Despite undergoing two embryo transfers using healthy donor oocytes and presenting only a mild male factor, no gestation was achieved. A COMET assay revealed a high percentage of sperm with DNA fragmentation. To clinically address this issue, various strategies were considered, but frequent ejaculation was recommended due to its simplicity and immediate applicability. Following this intervention, sperm DNA integrity significantly improved, although some semen parameters showed a decline. These findings suggest that *BRCA2*-deficient sperm cells are not only less capable of repairing DSBs due to impaired homologous recombination but are also more sensitive to reactive oxygen species (ROS). Reducing sperm exposure time to oxidative stress in the epididymis through frequent ejaculation appears to enhance sperm DNA quality. Additionally, we investigated whether haploid sperm carrying the pathogenic *BRCA2* variant might be more susceptible to DNA damage than those without the variant. To explore this, we performed microfluidic selection to isolate sperm with intact DNA and analyzed the frequency of the pathogenic variant before and after selection. PCR and sequencing results showed no difference in variant frequency between the studied fractions. This likely reflects the well-documented phenomenon of gene product sharing across cytoplasmic bridges in the early stages of spermatid development, is consistent the expression pattern of *BRCA2* during spermatid development (high in spermatogonia and spermatocytes but low in early and late spermatids) and results in phenotypic equivalence among sperm cells regardless of whether they carry the pathogenic *BRCA2* variant.

Resum
BRCA2 exerceix un paper crític en el manteniment de l'estabilitat genòmica durant l'espermatogènesi, facilitant la reparació de les ruptures del DNA de doble cadena (DSBs) mitjançant la recombinació homòloga. Les variants patogèniques que afecten la funció de *BRCA2* poden comprometre la integritat del DNA espermàtic, potencialment afectant la fertilitat masculina. Presentem el cas d'un pacient infèrtil portador d'una variant patogènica de *BRCA2* en heterozigosi. Tot i haver-se sotmès a dues transferències embrionàries amb oòcits sans de donant i presentar sol un factor masculí lleu, no es va aconseguir cap gestació. Mitjançant l'assaig COMET, es va observar un alt percentatge de espermatozoides amb fragmentació del DNA espermàtic. Per abordar clínicament aquest problema, es

van considerar diverses estratègies, però, per la seva simplicitat i aplicabilitat immediata, es va recomanar l'ejaculació freqüent. Després d'aquesta intervenció, la integritat del DNA espermàtic va millorar significativament, tot i que alguns paràmetres seminals van mostrar un lleuger descens. Aquests resultats suggereixen que els espermatozoides amb dèficit de *BRCA2* no només tenen una capacitat reduïda per reparar les DSBs a causa d'una recombinació homòloga defectuosa, sinó que també són més sensibles a les espècies reactives de l'oxigen (ROS). Reduir el temps d'exposició dels espermatozoides a l'estrès oxidatiu de l'epidídim mitjançant ejaculacions freqüents sembla millorar la qualitat del DNA espermàtic. A més, vam investigar si els espermatozoides haploides que porten la variant patogènica de *BRCA2* podrien ser més susceptibles al dany del DNA que aquells sense la variant. Per explorar-ho, vam fer ús de microfluídics per seleccionar espermatozoides amb DNA intacte i vam analitzar la freqüència de la variant patogènica abans i després del mètode de selecció. Els resultats de PCR i seqüenciació no van mostrar diferències en la freqüència de la variant entre les fraccions estudiades. Això probablement reflecteix el fenomen ben documentat de l'intercanvi de productes gènics a través dels ponts citoplasmàtics en les primeres etapes del desenvolupament de les espermàtides, és consistent en el patró d'expressió de *BRCA2* durant el desenvolupament de les espermàtides (alt en espermatogònies i espermatòcits però baix en espermàtides primerenques i tardanes) i dóna lloc a una equivalència fenotípica entre els espermatozoides, independentment de si porten la variant patogènica en *BRCA2*.

INTRODUCTION

BRCA1 and BRCA2, known as tumor suppressors, are crucial for maintaining genomic stability and are closely linked to the risk of breast and ovarian cancer (Miki Y, 1994; Wooster R, 1995). BRCA1 is responsible for double strain breaks (DSBs) repair in somatic cells via homologous recombination (HR) (Royo et al., 2010). It also contributes to the inactivation of meiotic sex chromosomes (MSCI) in males and supports DSBs repair during meiotic recombination (Broering et al., 2014). Impairments in BRCA1-mediated meiotic DSBs repair can disrupt meiotic silencing, leading to programmed cell death of spermatocytes and the activation of harmful genes on the sex chromosomes (Royo et al., 2010). Similarly, BRCA2 plays a pivotal role in HR repairing DSBs (Moynahan et al., 2001; Holloman, 2013), which is essential for maintaining genomic stability during spermatogenesis. Loss of function pathogenic variants or deficiencies in BRCA2 compromise DNA repair, resulting in increased DNA damage, meiotic arrest, and infertility in males by negatively affecting sperm motility, concentration, and morphology (Kabartan et al., 2019).

Although research on fertility-related issues in male carriers of pathogenic variants in *BRCA* genes is limited, recent studies have demonstrated significant reproductive consequences in animal models. Heterozygous rats carrying a pathogenic variant in exon 11 of *BRCA2* exhibit increased DSBs and apoptosis in spermatogonia and spermatocytes, leading to accelerated testicular germ cell loss, reduced sperm quality, and early male reproductive dysfunction (Motooka et al., 2025). Similarly, *BRCA1* haploinsufficiency in male mice has been associated with comparable reproductive impairments (Stobezki et al., 2020). Furthermore, in zebrafish, a homozygous *BRCA2* genetic variant disrupts spermatogenesis, resulting in the presence of only spermatogonia and primary spermatocytes, indicating defective sperm cell development (Mensah et al., 2019; Shive et al., 2010). Further research is required to establish the negative impact of *BRCA* pathogenic variants on human male fertility.

MATERIALS AND METHODS

CASE REPORT

Patient Background

A male patient heterozygous for the pathogenic variant c.4404dupT p. (Asp1469Ter) in the *BRCA2* gene (NM_000059.4). This variant creates a premature stop codon, resulting in a prematurely truncated protein, which has a negative functional impact. As shown in Figure 1, the patient has a family history of cancer. His aunt, a carrier of the pathogenic variant, was diagnosed with breast cancer at the age of 60, while his grandmother developed breast cancer at 61 and ovarian cancer at 68. Regarding his

fertility history, although he has previous offspring, currently he presents multiple unsuccessful fertilization attempts: two embryo transferences with no gestation using healthy oocytes (with previous births) from oocyte donation.
With this patient background, sperm DNA fragmentation study was prescribed.

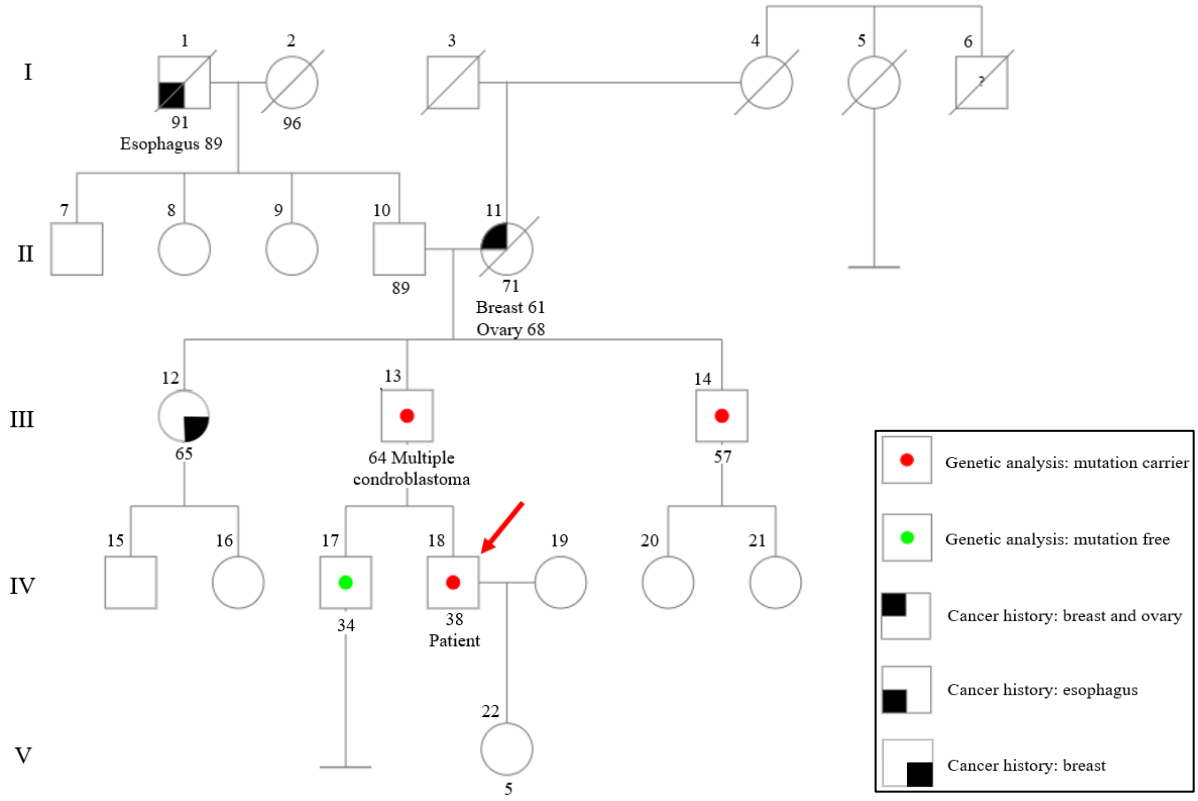

Figure 1: Pedigree of the patient showing family history of cancer. In the lower right corner is the legend of the scheme: Squares represent men and circles, women. Figures with red dot represent carriers of *BRCA2* pathogenic variant. Figures with green dot represent mutant free people. Each closed quarter of rectangle and quadrant indicates cancers in the figure: breast and ovary/ esophagus. Red flag points out the patient (nº18).

Semen Sample Collection

Semen samples from the patient were collected after 3-5 days of sexual abstinence with prior informed consent at the Andrology Unit of the Hospital Clínic de Barcelona. During the previous period to sample collection, no medication or any lifestyle modification was advised. Neat sample is obtained by preserving it in its fresh state, without any gradient or additional procedures applied.

Evaluation of DNA fragmentation

The Comet assay is a technique used to detect DNA damage, specifically single-strand breaks (SSBs) and double-strand breaks (DSBs), by analyzing the migration of DNA fragments (Simon & Carrell, 2013). Sperm cells from cryopreserved semen are embedded in agarose gels and subjected to lysis and to electrophoresis under alkaline (for SSBs) (Simon & Carrell, 2013) or neutral (for DSBs) (Ribas-Maynou et al., 2012) conditions. DNA fragments migrate further than intact chromatin, forming a "comet tail" that allows for the detection of fragmented DNA.
The process begins with embedding sperm in low melting point agarose on slides treated with normal melting point agarose to prevent detachment. Positive controls were prepared by treating samples with hydrogen peroxide. The samples undergo a three-phase lysis treatment, followed by incubation with DNase for neutral assays. Electrophoresis is conducted under alkaline or neutral pH conditions, depending on which type of breaks we want to detect. After electrophoresis, slides were treated and stained with SYBR Gold to visualize comet tails under a microscope. The extent of fragmentation was assessed by counting 60-120 sperm cells per gel, providing a measure of DNA damage.

Sperm separation by microfluidic device

Following the manufacturer's instructions, the neat semen from the patient was separated using microfluidic device (ZyMōt Multi (850 µL), ZyMōt Fertility, Inc.®) to isolate high-motility, low-DNA-fragmentation sperm using a membrane-based microfluidic approach that mimics natural sperm selection in the female reproductive tract. Semen samples were liquefied by incubating them for 20–30 minutes. Afterward, an 850 µL aliquot of the liquefied semen was drawn using a Luer-tip syringe. This aliquot was injected into the device's Inlet Port with slow and steady pressure to avoid bubble formation. The device's Outlet Port was primed with approximately 50 µL from a new syringe with 750 µL of media, followed by dropping the remaining (700 µL) on the upper membrane to ensure thorough coverage without tilting the device. The device was then incubated horizontally in a covered Petri dish at 37°C for maximum 30 minutes. Post-incubation, a new syringe was used to aspirate a maximum of 500 µL of sperm-containing fluid from the Outlet Port. This fraction was termed the selected sperm cells. Sperm cells remaining on the membrane from the device were collected and termed the non-selected sperm cells.

Sperm DNA extraction

The extraction of DNA was performed following a protocol adapted for sperm cells using the QIAmp DNA Mini Kit. In summary, sperm cells were added to a solution of 1M NaCl-0.5M EDTA and centrifuged. This step was repeated and the pellet was then resuspended in a solution of 500mM NaCl-10mM EDTA-100mM Tris-HCl (pH 8)-1% SDS-2% β-mercaptoethanol, along with 106 µL of proteinase K, and incubated at 55°C for 2 hours. After incubation, an additional 21.2 µL of proteinase K was added, followed by another 1-hour incubation at the same temperature, with occasional tube inversion. After incubation, a lysis solution containing guanidine hydrochloride and ethanol was added, and the mixture was vortexed until homogeneous. The sample was then transferred to a Qiamp Mini spin column and centrifuged at 6000g for 1 minute. After centrifugation, two washes were performed using the solutions provided in the kit, and the DNA was eluted from the column. The extracted DNA was stored long-term at -20°C in the elution buffer.

To assess the quantity and quality of the extracted genetic material, the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) was used.

Direct sequencing

Exon 11 from the *BRCA2* gene was amplified using standard polymerase chain reaction (PCR) techniques with the following primers (Forward: AAAAATATTAGTGTCGCCAAAGAG and Reverse: TGTATGAAAACCCAACAGAGTAGG). The PCR-amplified fragments were purified using ExoSAP-IT (USB, Cleveland, OH, USA) and directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and analyzed using a 3500 automated DNA sequencer (Applied Biosystems).

RESULTS

Analysis of neat sample

The seminogram from neat sample showed values close to the normal range stablished by the World Health Organization (WHO) in 2021, however the progressive motility revealed a mild masculine factor (asthenozoospermia) (Table 1). After two failed in vitro fertilization attempts and the seminogram results, DNA fragmentation assays were required for further investigation.

As observed in the neutral COMET assay, the patient's neat sperm exhibited a high percentage of spermatozoa with dsDNA fragmentation (Figure 2, A-C).

Strategies to reduce sperm DNA fragmentation

Based on the findings, various strategies were considered to mitigate sperm DNA fragmentation: (1) antioxidant supplementation has been shown to reduce reactive oxygen species (ROS) levels, thereby improving sperm quality (Bradley et al., 2016); (2) frequent ejaculation was shown to reduce sperm DNA damage by limiting sperm exposure to reactive oxygen species within the testicular ducts and epididymis. Consequently, it led to decreased DNA damage and an improved DNA fragmentation index, offering a potential strategy to enhance male fertility (Kaur et al., 2020); (3) microfluidic sperm selection enables the isolation of high-quality sperm with lower DNA fragmentation (Baldini et al., 2021a); (4) Testicular Sperm Extraction (TESE), a surgical biopsy procedure, allows for the retrieval of

testicular sperm, which are less exposed to oxidative stress and may present lower fragmentation rates, making it a viable option for IVF cycles in severe cases (Bradley et al., 2016). Given that antioxidant therapy involves a long-term approach (at least 3 months) and TESE was deemed too invasive, the frequent ejaculation protocol was selected as the first approach due to its non-invasive nature and simplicity.

Results indicated that most of the semen parameters analyzed in sperm obtained by frequent ejaculation were reduced compared to the neat sample (Table 1), however the post-intervention COMET assay revealed a reduction in percentage of sperm with dsDNA fragmentation (Figure 2, B-D).assay revealed a reduction in percentage of sperm with dsDNA fragmentation (Figure 2, B-D).

Table 1. Comparison of semen parameters of neat sample and sample after frequent ejaculation protocol with the minimum values stablished by the WHO (World Health Organization) in 2021. F.E.: Frequent ejaculations.

Semen parameters	Neat sample	Sample after F.E.	WHO 2021
Volume (mL)	5	2,4	1,4
Sperm concentration (x10^6/mL)	9,34	9,01	16
Total sperm number (x10^6/mL)	46,7	21,62	39
Total motility (%)	37,86	25	42
Progressive motility (%)	26,62	15	30
Normal morphology (%)	4	4	4

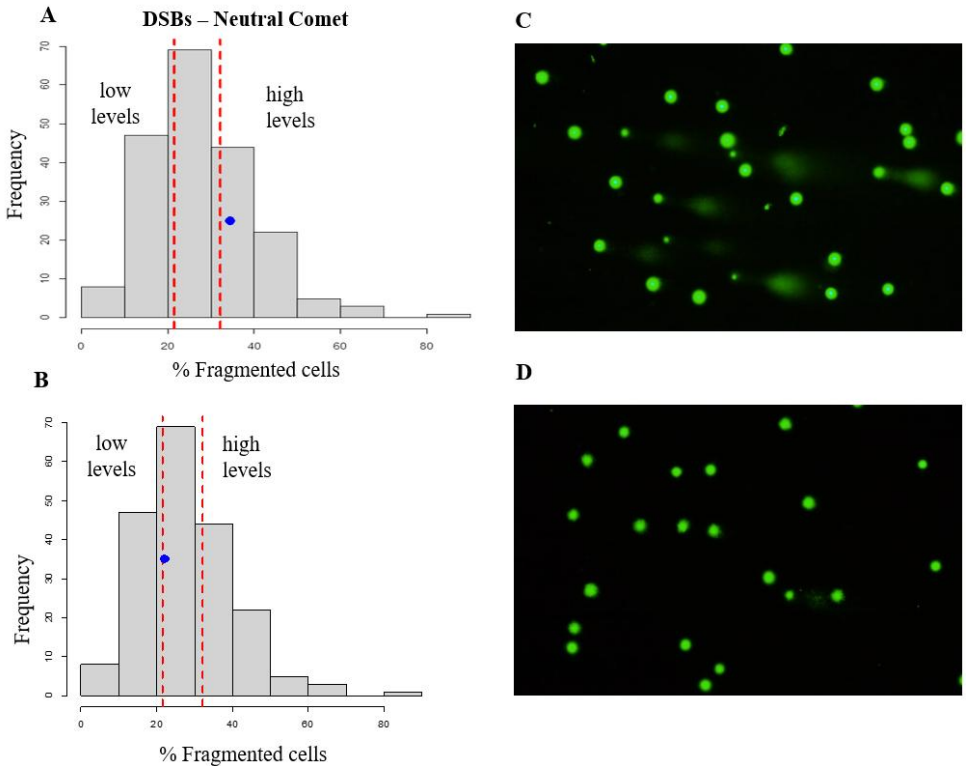

Figure 2: Histogram showing the percentage of double-strand (DSBs) fragmented cells and their frequency in neutral comet assay from ~200 studied patients with infertility issues. The blue dot represents the result obtained in the patient after 3-5 days abstinence (A) and after frequent ejaculation protocol (B). The red dashed lines mark the boundaries of what are considered high and low levels of fragmentation in each case. Representative image of neutral comet assay was captured under the fluorescent microscope from the patient after 3-5 days abstinence (C) and after frequent ejaculation protocol (D).

***BRCA2* pathogenic variant distribution in sperm cells before and after microfluidic selection**

During mammalian spermatogenesis germ cells are connected by cytoplasmic bridges before sperm are released into the lumen of the seminiferous tubules. This syncytial organization provides a mechanism by which genetically different sperm in a male can be made biochemically equivalent (Braun et al., 1989). However, there are some exceptions such as t haplotype in mice (Amaral & Herrmann, 2021). Since the patient is a heterozygous carrier of the c.4404dupT pathogenic variant in the *BRCA2* gene, we hypothesized that sperm with the pathogenic variant may be more susceptible to DNA damage than the sperm without the variant.

To assess our hypothesis, we performed sperm selection on frequent ejaculation collected sample using the microfluidic device ZyMōt. These devices mimic the natural microenvironment of the female reproductive tract (FRT) to select sperm with improved motility and morphology (reducing DNA fragmentation up to 70%, results from our group).

After applying this sorting method, we obtained three fractions: the neat sample (pre-selected sperm cells), the selected sperm cells (post-selection), and the non-selected sperm cells (post-selection). DNA was extracted from these fractions and analysed to check the pathogenic variant prevalence. However, PCR and sequencing results showed no differences between the fractions (data not shown).

DISCUSSION

Sperm DNA fragmentation arises from intrinsic factors like apoptosis, recombination errors, protamine imbalances, and oxidative stress, as well as extrinsic factors such as storage, handling, infections, and medications. While DNA damage repair occurs during spermatogenesis, mature sperm lack this capability due to the cessation of transcription after spermiogenesis. Consequently, the ability of oocytes and early embryos to repair sperm DNA damage plays a crucial role, making the impact of fragmentation dependent on both sperm chromatin integrity and oocyte repair capacity (González-Marín et al., 2012; Li et al., 2024).

The patient presented in this case report is heterozygous for *BRCA2* c.4404dupT and has a high percentage of sperm with dsDNA fragmentation. Given that *BRCA2* pathogenic variants impair DSB repair mechanisms during spermatogenesis, germ cells in affected individuals experience an accumulation of unrepaired breaks, ultimately leading to increased apoptosis and diminished sperm quality. In addition, it has been shown that BRCA1 and BRCA2 deficiencies increase cellular sensitivity to oxidative stress, as exposure to ROS like hydrogen peroxide induces oxidative DNA damage and DSBs. In the context of male fertility, this sensitivity may contribute to sperm DNA fragmentation, particularly when sperm undergo prolonged epididymal storage (Fridlich et al., 2016). Therefore, frequent ejaculation was suggested as an intervention to improve sperm DNA integrity by minimizing exposure to ROS, as shorter abstinence periods yield sperm with reduced fragmentation and better overall quality (Kaur et al., 2020). A study in rats shows that pathogenic *BRCA2* variants in heterozygosis induces accelerated age-dependent decline in sperm quality (Motooka et al., 2025b). Notably, the patient with a *BRCA2* pathogenic variant had successfully fathered a child before experiencing infertility, which may be explained by the gradual accumulation of DNA fragmentation over time.

Furthermore, given the patient's heterozygosity for the *BRCA2* c.4404dupT, we hypothesized that half of the sperm population, carrying the pathogenic variant, are more susceptible to DNA damage than sperm without the variant. Frequent ejaculation and microfluidic selection successfully enriched high-quality sperm. However, PCR and sequencing showed no difference in the pathogenic variant frequency among fractions, indicating the selection process did not discriminate against the pathogenic variant. This is likely due to cytoplasmic bridges between spermatids, which enable gene product sharing, homogenizing phenotypic traits (Braun et al., 1989). *BRCA2* is not an exception, as it follows this pattern, with transcript levels declining from early to late spermatids (Bhutani et al., 2021). Consequently, the selection of sperm based on phenotypic quality is not necessarily correlated with the absence of the mutant haplotype.

This study sets a precedent for the investigation of fertility in men carrying *BRCA* pathogenic variants. As demonstrated in this case, a patient experiencing failed fertilization attempts with a male factor contributing to infertility may have high DNA fragmentation, which can significantly compromise fertilization potential. This fragmentation can be easily assessed using a COMET assay, allowing for the identification of the most appropriate strategies to improve DNA integrity based on individual cases. In any case, further research is essential to determine whether all BRCA pathogenic variant

carriers exhibit increased DNA fragmentation and if so, whether it can be recovered with simple frequent ejaculation protocol.

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RESUMS CORRESPONENTS A LES COMUNICACIONS PRESENTADES A LA XIX JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

(Ordenats per aparició en el programa de la Jornada)

ABSTRACTS CORRESPONDING TO THE COMMUNICATIONS PRESENTED AT THE XIX BIOLOGY OF REPRODUCTION SYMPOSIUM

(*Ordered according to appearance in the Symposium program*)

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MEIOTIC 3D CHROMATIN DYNAMICS IN THE GERM LINE OF VERTEBRATES

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During mammalian spermatogenesis, genetically variable haploid gametes are generated in a tightly regulated process that includes homologous chromosome pairing and recombination, which are accompanied by the reshuffling of the three-dimensional (3D) chromatin architecture. Errors in these processes may lead to aneuploidy and infertility problems. Exploring the similarities and differences of chromatin folding across evolutionary lineages is central to developing an appreciation of both the dynamics of genome function and, ultimately, the effects on speciation. Yet, our understanding of the mechanisms regulating genome folding and gene expression during the generation of germ cells in divergent vertebrate species is incomplete. By combining cytological analysis, fluorescence activated cell sorting, in situ chromosome conformation capture sequencing (Hi-C) and single cell-RNAseq, we describe principles of 3D genome folding and expression in the germ line of different vertebrate species (eutherian, marsupials and reptiles). Our results show unique patterns on the diversity of the spatial folding of chromosomes in meiotic (primary spermatocytes) and post-meiotic cells (round spermatids). Importantly, our results show that both chromosome morphology and genome size strongly influence DNA loop size and inter-chromosomal interactions in meiocytes. Moreover, we explored the crosstalk between 3D genome remodelling and gene expression in the context of the meiotic sex chromosome inactivation (MSCI). We found that certain genomic regions in the tammar wallaby were permeable to MSCI, suggesting that silencing is not complete in marsupials. Overall, our results provide new insights into the regulation of chromatin in the germ line of vertebrates at an unprecedent level of resolution.

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ROLE OF SECISBP2 PROTEIN ON FEMALE AND MALE GAMETOGENESIS

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Gametogenesis is influenced by factors such as age, genetics, environment, and oxidative stress (OS). It is well known that, in physiological amounts, Reactive Oxidative Species (ROS) play an essential role in regulating multiple fertility processes. Despite being crucial for signaling pathways, when these molecules accumulate, OS occurs. Furthermore, meiosis is affected by OS. Selenoproteins are essential to regulate ROS levels and protect cells from damage in this context. Furthermore, at least 25 selenoproteins have been identified in the human proteome, with diverse functions, including regulating redox-sensitive biochemical pathways, preserving redox potential, guarding against oxidative damage to genetic material, proteins, and membranes, metabolizing thyroid hormones, controlling protein folding, and regulating gene expression. The biogenesis of the selenoproteins is highly mediated by the Selenocysteine Insertion Sequence Binding Protein 2 (SECISBP2). Consequently, previous studies have described that the absence of a copy of Secisbp2 causes a decrease in selenoprotein synthesis. However, our knowledge of this gene and its relationship with fertility remains limited. Nevertheless, Secisbp2 has been identified as a candidate gene for regulating the timing of natural menopause. In this regard, the present study aims to analyze the effects of the decrease in SECISBP2 levels on gametogenesis in Mus musculus. We will present the preliminary characterization of the gametogenic phenotype of mice with decreased expression of SECISBP2 and Secisbp2 conditional mutant mice with decreased expression of SECISBP2 limited to the germline to elucidate its role in ovarian aging and spermatogenesis.

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SINGLE-CELL MULTIOMIC PROFILING REVEALS TRANSCRIPTIONAL DYNAMICS AND REGULATORY TRAJECTORIES OF GERMLINE CELLS DURING PORCINE SPERMATOGENESIS

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Spermatogenesis is a complex developmental process occurring in the testis, involving multiple germline and somatic cell types, and ultimately resulting in the formation of spermatozoa. The function, morphology, and fertility of sperm cells are directly influenced by a series of tightly regulated molecular events that take place sequentially in each cell type throughout this process. Therefore, characterizing the transcriptomic and gene regulatory landscapes of the distinct cell populations involved in spermatogenesis at the genomic level is essential for gaining a comprehensive understanding of the molecular mechanisms underlying semen quality and male fertility.
We isolated nuclei from flash-frozen testicular tissue of four adult Large White boars using a citric acid buffer-based protocol, and performed single-cell multiome sequencing (ATAC + Gene Expression) to simultaneously capture transcriptomic profiles and chromatin accessibility. After preprocessing and quality control based on gene count, total counts, and mitochondrial read percentage, we identified 8,346 germline cells, in 11 clusters, representing all major male germline cells.
Here we present the results of reclustering germline cells to refine their characterization and identify gene markers using SEURAT with default parameters. We identified 14 cell clusters, therefore with minimal improvement in cluster refinement when compared with the original UMAP topography. These clusters corresponded to proliferating and differentiating spermatogonia, pre-leptotene, leptotene, pachytene and diplotene spermatocytes, as well as early and late spermatids. Cluster C11, consisting of late spermatids, had the largest number of cluster-specific genes (UMI ≥ 3 in that cluster and UMI <1 in any other cluster), with 250 genes, followed by clusters C6, C5 and C8, corresponding to different spermatocyte stages. Pseudotime analysis confirmed the expected order of spermatogenesis.

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SYCP3 AND SIRT7 ARE ESSENTIAL FOR PREMEIOTIC HOMOLOGOUS ASSOCIATION

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Homologous chromosome pairing is essential for meiotic synapsis and recombination. While synapsis and recombination are well characterized processes, the timing of initiation and the specific factors involved in the pairing process, remain unknown.

In this study, we examined the initiation of homologous chromosome pairing and explored the effect of SYCP3 and SIRT7 depletion, two key regulators of the meiotic synaptic process, in mice. To achieve this, we employed a combination of Fluorescence-Activated Cell Sorting (FACS) and Fluorescence In-Situ Hybridization (FISH) to isolate and characterize undifferentiated (GFRα1+) and differentiated (cKIT+) spermatogonia. Homologous chromosome association was evaluated using painting probes and contrasted with data obtained from somatic cells. Images were acquired through confocal microscopy and analysed using ImageJ/FIJI. Chromosomes were classified as paired when presenting a single fluorescence signal and unpaired when two distinct signals were observed.

In wild-type (WT) mice, homologous chromosome pairing was observed in 38.65% of undifferentiated spermatogonia, increasing to 42.06% in differentiated spermatogonia. Among differentiated spermatogonia, chromosomes 1 and 19 showed the highest pairing rates (47%; $p=0.0074$ and 49.19%; $p=0.0119$, respectively), whereas chromosomes 5 and 16 did not exhibit significant differences in pairing compared to somatic cells. To assess the impact of SYCP3 and SIRT7 depletion, we compared homologous pairing rates between differentiated spermatogonia in WT and mutants. In the absence of SYCP3, the pairing rate dropped significantly to 25.92% ($p=0.0045$). SIRT7 depletion produced a similar, though less pronounced reduction (30.78%; $p=0.0325$). Pairing levels in both mutants closely resembled those observed in WT somatic cells (29.74%; $p>0.05$).

Altogether, our results provide new insights into the molecular regulation of chromosome dynamics during early spermatogenesis. Here we demonstrate that the pairing process of homologous chromosomes begins asynchronously in differentiated spermatogonia and that SYCP3 and SIRT7 play a significant role in the process.

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DIVERGENT MEIOTIC DYNAMICS ACROSS MARSUPIALS WITH DIFFERENT SEX CHROMOSOME SYSTEMS

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During meiotic prophase I, homologous chromosomes undergo pairing, synapsis, and recombination in a tightly regulated process, ensuring the generation of genetically diverse haploid gametes. While this process displays highly conserved canonical features in mammals, notable differences arise among taxa. Regarding sex chromosomes, marsupial species typically possess sex chromosomes without homology, unlike eutherians, whose sex chromosomes share a pseudo-autosomal region (PAR). Yet, the implications of achiasmatic sex chromosomes during meiosis progression are still poorly understood. Here, we describe the meiotic dynamics of the XY_1Y_2 sex chromosome system in the swamp wallaby (*Wallabia bicolor*: family Macropodidae), which originated from the fusion of an autosome with the ancestral X chromosome. We also compare the regulatory mechanisms of meiosis in the swamp wallaby with those in three other Australian marsupial species with distinct sex chromosome systems: one with an XY_1Y_2 system, the greater bilby (*Macrotis lagotis*: family Thylacomyidae), and two with the ancestral XY system, the tammar wallaby (*Macropus eugenii:* family Macropodidae) and the fat-tailed dunnart (*Sminthopsis crassicaudata*: family Dasyuridae). For that, we conducted a cytological analysis of the meiotic prophase I, including the study of chromosome synapsis, centromere and telomere homeostasis, and meiotic sex chromosome inactivation (MSCI). Our results illustrate that marsupial species exhibit divergent dynamics of sex chromosomes during meiosis including distinct pairing and MSCI strategies. This highlights the intricate relationship between the meiotic behaviour of sex chromosomes and their implications for evolutionary reproduction.

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MEIOTIC RECOMBINATION PLASTICITY IN RESPONSE TO TEMPERATURE VARIATION IN AN ECTOTHERM SPECIES

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The ability to adapt to environmental changes is key for species' survival. Of the environmental stimuli that can influence chromatin regulation, temperature is the most common. Although it is known that organisms can respond to temperature by activating a common transcriptional programme, the effect on recombination is less explored. Previous studies in worms, flies and plants have shown that the frequency of recombination during meiotic prophase I can be affected when temperatures are extreme, however whether this effect is also conserved in vertebrates is not known at this stage. In this context, the study of the exposure to different temperatures in ectotherms vertebrates will allow us to understand the possible effect of environmental factors on recombination during the process of gamete formation, especially during meiotic prophase I when homologous chromosomes pair, synapse and recombine. Here, we studied the effect of temperature on recombination in the Guibé's ground gecko (*Paroedura guibeae*), an ectotherm species. We analyzed the formation of double stand breaks (DSBs) and crossovers (COs) by the immunolocalization of proteins involved in these processes. Moreover, we determined the frequency and chromosomal location of crossovers and the levels of double stand breaks formation comparing these individuals. We show the presence of hyper-crossover spermatocytes in individuals treated at both at high and low temperatures. Importantly, this significantly increase of crossovers was related to an increased level of meiotic double stand breaks formation. Overall, our results provide new insights into the effects of environment temperature fluctuations on meiotic recombination in ectotherm species.

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REPROGRAMMING OF EPIGENETIC MARKS DURING EARLY DEVELOPMENT IN THE EUROPEAN SEA BASS

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Epigenetic reprogramming refers to the process by which epigenetic marks, such as DNA methylation and histone modifications, are erased and re-established during specific stages of early development, enabling the formation of a new individual. This is well studied in mammals, where both paternal and maternal genomes undergo a global erasure of the marks and resetting. In contrast, the dynamics in fish remain not fully understood. For instance, in zebrafish only a few events of de- and remethylation occur in the maternal genome, to resemble sperm levels. More controversial is the situation in medaka, since both mammalian and zebrafish patterns have been proposed. Furthermore, in the self-fertilizing *Kryptolebias marmoratus*, epigenome reprogramming takes longer than in other vertebrates as demethylation is achieved at the end of gastrulation, whereas in the three-spined stickleback methylation levels are lower than in other species and passive demethylation of paternal genome takes place during the cleavage phase. The objective of the current study was to characterize epigenetic reprogramming during European sea bass early development. Preliminary results suggest that although unfertilized eggs still remain demethylated with respect to the sperm, the difference is less pronounced when compared to other species, resembling patterns observed in zebrafish and medaka. In the zygote, methylation levels are slightly lower than gametic levels and during the 2-cell stage a short demethylation wave drops levels below those of oocytes. By the 16-cell stage, remethylation is nearly complete, resulting in levels exceeding those of sperm, remaining stable throughout the 90% epiboly stage. Our study confirms, along with others mentioned above, that global erasure of methyl marks, like in mammals, is absent in some fish such as the sea bass. Species-specific differences of reprogramming in teleost needs to be further investigated to fully understand their evolutionary significance and to rule out potential biases stemming from distinct methylation assessment methods.

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THE LANDSCAPE OF circRNAs IN OBESE SPERMATOZOA

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In the last decades, obesity is becoming an alarming health concern for developed countries thus to reach epidemic dimensions. Several studies suggest that nutrition and lifestyle factors play an important role in negatively affecting male reproductive skills. Various mechanisms could contribute to obesity-induced male infertility, including hormonal alterations, genetic implications, oxidative stress, DNA fragmentation and blood-testis barrier disruption. In this scenario, the discovery of key players involved in the regulation of sperm quality in both physiological and pathological condition is a matter of great interest. CircRNAs can be precisely attributed to such functions. In detail, they constitute a class of stable, covalently closed non-coding RNAs, modulating sperm quality setting as well as nuclear remodeling dynamics.

Recently, we have profiled a circRNA cargo in obese spermatozoa by microarray strategy. The validation of selected differentially expressed circRNAs has enabled us to identify some circRNAs of interest trapped in networks functionally related to apoptosis pathways as well as to embryo development. Furthermore, their characterization in extracellular vesicles, important effectors in cell-to-cell communication, enhances the idea of their use as biomarkers of sperm quality.

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ROLE OF SIRTUINS IN SPERMATOGONIA MAINTENANCE DURING AGING AND CHEMOTHERAPY EXPOSURE

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Spermatogonia, the precursor cells that sustain spermatogenesis, are critical for maintaining male fertility throughout life. Environmental insults, such as chemotherapeutic agents, can compromise this cell population, potentially leading to infertility. The chemotherapy-induced infertility is particularly concerning in prepubertal male oncologic patients, for whom fertility preservation options remain experimental.

In this study, we investigated the role of the NAD^+-dependent enzyme SIRT7 in spermatogonia using a SIRT7 knockout mouse model. We observed that SIRT7 is essential for maintaining chromatin dynamics and genome stability in spermatogonia, supporting efficient spermatogenesis with age. Analysis of publicly available data revealed that expression levels of many sirtuin members are high in these cells, suggesting broader roles for sirtuins in this context. Using an ex vivo gonadal culture system, we cultured prepubertal mouse testes, which are mainly formed by spermatogonia. In this model, we demonstrated that sirtuin-activating compounds (STACs) exert a beneficial effect on spermatogonial biology. These findings suggest that STACs may be used as gonadoprotective agents, with the potential to preserve fertility in patients undergoing chemotherapy.

Collectively, our results highlight SIRT7's pivotal role in regulating genomic stability in spermatogonia and support further exploration of sirtuin-targeted strategies for protecting reproductive health in cancer patients.

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EFFECTS OF HIGH TEMPERATURE EXPOSURE ON THE PHENOTYPE OF EUROPEAN SEA BASS: A STUDY OVER THREE GENERATIONS

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There is increasing global evidence of natural sex reversal in species with temperature-sensitive sex determination. The uncertain outcome for these species and their ecosystems under rising temperatures due to global warming highlights the need for research in this field. However, studies rarely extend beyond one generation. Here, the transgenerational phenotypic effects of high-temperature exposure (TE) were investigated in three generations of European sea bass (*Dicentrarchus labrax*), a species with polygenic sex determination. Both genetic (i.e., the sex tendency: low for males, high for females) and environmental factors, particularly temperature, play a role in sex determination. Notably, elevated temperatures lead to a higher proportion of males in the population. In the present study, fish were exposed to either control (C) or elevated temperature (T) during the thermosensitive period over three generations (F0, F1 and F2). Males from each generation were selected to produce the next. The F2 involved eight different combinations of C and T treatments over the three generations including different forms of ancestral and developmental exposure. Fish were sampled at one year of age and the sex was visually determined (n=1465). Sex proportions, growth parameters, gonadal morphology and gonadal chemical elemental composition were then compared across groups. As expected, T exposure consistently increased male proportion in each generation. However, transgenerational effects varied: some groups showed expected masculinization, others cumulative masculinization and others even feminization. Fish with low sex tendency experienced cumulative masculinization, while those with high sex tendency underwent feminization when both the F1 and F2 were exposed to high temperatures. We hypothesize that ancestral heat exposure triggers a compensatory effect based on sex tendency, leading to these outcomes. Histological analysis revealed a delayed sexual development in TE groups in males (but not in females), enhanced by repeated ancestral TE, supporting a cumulative transgenerational temperature effect. X-ray energy dispersion spectrometry of gonadal composition (C, N, O, Na, P, S, Ca) showed clear sex-related differences but no TE effect within each sex. These findings highlight novel transgenerational temperature effects on fish sex ratios and gonadal morphology, enhancing our knowledge on sex determination mechanisms and climate change impacts on natural populations.

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SARS-COV-2 INFECTION REDUCES NUMBER OF SPERMATOGONIAL STEM CELLS AND DYSREGULATES THE TRANSCRIPTIONAL LANDSCAPE OF HUMAN TESTIS

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The SARS-CoV-2 virus has infected over 770 million people worldwide in the last five years and caused over 7 million deaths. SARS-CoV-2 uses the TMPRSS2 protease and ACE2 receptor to infect host cells. Even though it is mainly a respiratory disease, both proteins are expressed in many tissues, including several testicular cell types. Abnormal levels of sex hormones and a decrease in sperm quality have been observed in patients during and after recovery from COVID-19. Furthermore, severe damage caused by inflammation has been detected in the testis of infected men. In addition, SARS-CoV-2 has been found in the testis. Thus, our objective was to explore the potential impact of COVID-19 on the male reproductive system. First, we analyzed the morphology of testis sections from patients deceased by COVID-19 and compared them to control samples of similar ages. Overall, COVID-19 samples displayed various anomalies commonly associated with compromised spermatogenesis, such as vacuolization of Sertoli cells, detachment of the germinal epithelium, or thickening of the basal lamina. Next, we studied the presence of different relevant biomarkers of spermatogenic cells, DNA damage, and leukocytes in these samples. A higher fraction of T lymphocytes and macrophages were detected in the peritubular spaces of COVID-19 samples compared to controls, thus confirming the infiltration of immune cells in the peritubular tissue of the testis. In addition, the seminiferous tubules of COVID-19 samples showed fewer UTF1-positive spermatogonia, which represents the spermatogonial stem cell population from which all sperm cells derive, and these cells presented more DNA damage than control cells, suggesting that COVID-19 could compromise spermatogenesis even after recovery. Moreover, the analysis of a snRNAseq assay showed differentially expressed genes in testicular cells from COVID-19 samples compared to controls. A small group of male individuals who had recovered from the virus infection also showed a decrease in the number of UTF1-positive spermatogonia, which presented more DNA damage, compared to controls. Finally, viral RNA was found in a fraction of COVID-19 necropsies. Nonetheless, more studies are needed to understand the impact of COVID-19 in spermatogenesis, especially in those patients that have recovered from the infection.

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THE MANCHETTE, A MOLECULAR CORSET FOR SPERMATIDS

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During spermiogenesis, a temporal organelle called the manchette assembles and facilitates the cellular reorganization of round spermatids to sperm cells. The manchette primarily comprises microtubules (MTs), highly dynamic, hollow biopolymers involved in many fundamental cellular processes, including cell division and cellular motility. The manchette is relevant for our understanding of MT regulation and stabilization, as it maintains its structural integrity for several weeks and is used as a scaffold for the redistribution of protein cargo. Errors in spermiogenesis, and specifically in manchette function, can lead to misshaped sperm cells that lack motility or structural integrity and thus lie at the basis of unspecified male infertility. This significant health concern necessitates better understanding. Here, we used cryogenic-electron microscopy (cryo-EM) and tomography (cryo-ET) to study manchettes isolated from rat spermatids at a high resolution. We show the decoration of the MTs with dynein-dynactin complexes and actin filaments in the manchette. We further find factors regulating MT ends in the manchette and reveal a microtubule inner protein (MIP) regularly binding along the seam of manchette MTs. Our work provides important insights into the molecular and structural mechanisms of manchette stabilization and function during spermiogenesis.

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EFFECTS OF INHIBITING CALCIUM-ACTIVATED CHLORIDE CHANNELS ON SPERM CRYOTOLERANCE

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Cryopreservation is the most suitable method for storing sperm in the long term. Previous research has consistently shown that aquaporins, particularly aquaglyceroporins, play an essential role in the adaptation of sperm to osmotic changes, allowing the transport of permeable cryoprotectants such as glycerol. Yet, not only water balance but also that of ions is important for sperm cryotolerance. In that regard, the contribution of calcium-activated chloride channels (CaCCs) to sperm cryotolerance has not been addressed. Herein, and using the pig as a model, we tested the hypothesis that CaCCs are relevant for sperm resilience to freeze-thawing. Eight biological replicates were evaluated. Before starting the freezing protocol, CaCCs were inhibited with two different blockers: a general one (5-Nitro-2-(3-phenylpropylamino)benzoic acid; NPPB) and another targeting exclusively TMEM16A/B protein, a central component of calcium-activated chloride channels (3,5-Dibromo-4-hydroxyphenyl)(2-ethyl-3-benzofuranyl)methanone; benzobromarone, BBR). Three combined treatments were tested (100 µm NPPB+10 µM BBR, 100 µm NPPB+100 µM BBR, and 200 µm NPPB+100 µM BBR). Before cryopreservation and after 30 min and 240 min of thawing, sperm motility was evaluated with a computer-assisted sperm analysis (CASA) system, and viability, acrosome integrity, membrane lipid disorder, mitochondrial membrane potential, and intracellular levels of reactive oxygen species (ROS), superoxides and calcium were determined with flow cytometry. The effects of channel blocking on sperm cryopreservation were analysed with a linear mixed model followed by a post-hoc Sidak test. The level of significance was set at $P \leq 0.05$. Inhibition of CaCCs, particularly at the highest concentration of the two pharmacological agents (i.e., 200 µm NPPB+100 µM BBR), reduced total and progressive motility at 30 and 240 min post-thaw. A similar decline was observed in sperm viability, acrosome integrity, mitochondrial membrane potential, and the percentage of sperm with high membrane lipid disorder increased, particularly after 240 min of incubation at 38 °C ($P<0.01$). Conversely, no changes in ROS and superoxide levels were observed ($P>0.05$). These results support that CaCC channels regulate sperm cryotolerance, thus suggesting that the cell response to the oxidative, thermal, and osmotic stress related to freezing and thawing relies not only on water channels but also on chloride ions. Further research interrogating the molecular mechanisms underlying the effects of blocking CaCCs is warranted. Funding: Ministry of Science, Innovation and Universities, Spain (PID2020-113320RB-I00); AGAUR (2021-SGR-00900), ICREA, and University of Girona (IF-UdG scheme).

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ROLE OF BAX AND BAK DURING LIQUID PRESERVATION OF PIG SPERM

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Pig semen is commonly used for artificial insemination (AI) in pig production. Currently, liquid storage is the most used preservation method in the swine breeding industry; however, sperm quality is impaired during this preservation process, resulting in reduced fertility rates. In this context, the study of the molecular pathways involved in the regulation of cell lifespan is of high relevance. Multiple apoptotic signals culminate in mitochondrial outer membrane permeabilisation (MOMP), which leads to the activation of caspases and initiates caspase-independent mitochondrial dysfunction. BAX and BAK are multidomain pro-apoptotic essential effectors that regulate the intrinsic pathway of apoptosis, resulting in MOMP. In this study, three pools of three ejaculates each (n=9) were split into aliquots and added with different concentrations of MSN-125 (0.5 µM, 2.5 µM, 5 µM) and BH3-I (10 µM, 50 µM), which inhibit and activate apoptosis, respectively, by regulating BAX and BAK oligomerisation. Samples were then stored at 17 °C for four days and flow cytometry analyses were performed on days 0, 1 and 4 to assess membrane lipid disorder, mitochondrial membrane potential (MMP) and intracellular levels of activated caspases 3 and 7, Ca^{2+}, superoxide and total reactive oxygen species (ROS). Data were subsequently analysed with a mixed model followed by a post-hoc Sidak test. On the one hand, BH3-I, an apoptosis inducer, caused a drastic decrease in sperm viability and MMP, increased membrane lipid disorder and intracellular levels of total ROS and superoxides, and activated caspases 3 and 7 (P<0.05). On the other hand, incubation of samples with MSN-125, a BAX/BAK inhibitor, did not lead to significant changes in any of the assessed sperm function parameters. These results suggest that BAX and BAK might not be involved in cell senescence during sperm preservation at 17 °C. Further studies, including more replicates and analysis analyses to assess other sperm functional parameters, might help unravel to which extent apoptotic-like changes compromise sperm function during liquid preservation.

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COPPER CHLORIDE AND ZINC CHLORIDE EXERT AN ANTIMICROBIAL ACTIVITY WHILE MAINTAINING BOAR SPERM QUALITY IN SAMPLES PRESERVED AT 17ºC

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Bacterial contamination is a major issue in various fields, with the rise of multidrug-resistant bacteria becoming a global concern. While antibiotics are essential for bacterial control, their overuse has accelerated resistance, emphasizing the need for alternative antimicrobial strategies. In pigs, bacterial overgrowth poses a challenge to liquid state sperm storage, necessitating the continuous development of new bacterial control approaches. This study aims to evaluate the antimicrobial potential of different ions against Escherichia coli and Clostridium perfringens, while maintaining boar sperm quality at 17ºC. For that, we assessed sperm function in terms of motility, viability and oxidative stress, and determined the antimicrobial activity through the determination of the minimum inhibitory and minimum bactericidal concentrations in samples stored for 48h in increasing concentrations (100mM, 300mM, 500mM, 1mM and 10 mM) of silver sulfadiazine, aluminum chloride, zinc chloride or cooper chloride. Results showed that silver sulfadiazine exerted an antimicrobial effect for all concentrations tested, but it also negatively affected boar sperm quality ($P<0.05$). In contrast, aluminum chloride preserved sperm quality, but failed to inhibit bacterial growth under any tested conditions ($P>0.05$). Finally, copper and zinc chloride demonstrated promising antimicrobial potential combined with the preservation of sperm quality at concentrations of 1mM ($P<0.05$). Despite that, their inhibition of bacterial growth was not complete, thus suggesting that they could be used to reduce antibiotic concentrations. Further studies should assess the synergistic ability of these compounds when combined with antibiotics.

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BLOCKING CHLORIDE CHANNELS WITH 9-ANTHRACENEACARBOXYLIC ACID DURING IN VITRO CAPACITATION INDUCES SPERM MEMBRANE LIPID DISORDER EARLIER

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After their deposition in the female reproductive tract of mammals, sperm start to undergo a series of changes that are known as capacitation. Late capacitation events, the ones that are mimicked during in vitro capacitation, occur within the oviduct. While it is now understood that cations channels (Ca^{2+}, Na^{+}, K^{+}, H^{+}) , either voltage- or ligand-gated, play an essential role during sperm capacitation, whether anion channels, such as those of chloride (Cl^{-}), are involved has not been investigated. In this study, chloride channels (ClC) were blocked with different concentrations of 9-anthracenecarboxylic acid (9-AC), an inhibitor of these channels, particularly of ClC-1, whose presence was previously confirmed in pig sperm. Seven semen pools, each prepared with ejaculates from three separate boars, were in vitro capacitated in a standard medium (TCM, control) at 38.5 °C and 5% CO_2 for 120 min; progesterone (10 μg/mL) was then added and samples incubated for an additional 60-min period. Treatments consisted of TCM supplemented with 100 μM, 1 mM or 10 mM. At 0 min, and after 60, 120, 130 and 180 min of incubation at the aforementioned conditions, sperm motility was determined with a computer-assisted sperm analysis (CASA) system, and acrosome integrity, membrane lipid disorder, and intracellular levels of calcium, superoxides and total ROS were evaluated with a flow cytometer. A linear mixed model followed by a post-hoc Sidak's test for pair-wise comparisons was run to determine the effects of 9-AC treatments and incubation time. The level of significance was set at $P \leq 0.05$. Blocking ClC with 1 mM and 10 mM 9-AC significantly ($P<0.05$) increased membrane lipid disorder after 60 min of incubation, indicating that it could induce cholesterol efflux from the sperm plasmalemma earlier than the control. This effect was dose-dependent, with no impact at the concentration of 100 μM, and the most apparent at 10 mM. Conversely, neither motility nor acrosome integrity and the intracellular levels of calcium, superoxides and total ROS were altered when chloride channels were blocked with 9-AC. These data suggest that inhibiting these channels mainly affects the PKA-mediated downstream events related to membrane fluidity, but does not regulate motility or mitochondria, at least with the inhibitors and concentrations tested in this work. Yet, further research using other blocking agents for chloride channels is needed before their role during sperm capacitation can be addressed entirely. Besides, the molecular mechanisms underlying the ClC-modulation of plasma membrane lipid disorder warrant additional studies.

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THE NAKCC COTRANSPORTER IS INVOLVED IN THE REGULATION OF MAMMALIAN SPERM CAPACITATION

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Previous research has consistently demonstrated the involvement of ion channels (Ca^{2+}, Na^{+}, K^{+}, H^{+}) and exchangers (Na^{+}/Ca^{2+}, Na^{+}/K^{+}, Na^{+}/HCO_3^{-}) in the regulation of sperm capacitation, a process that occurs within the female reproductive and that is required in order for sperm to fertilise the oocyte. To the best of the authors' knowledge, however, the role of the Na^{+}/K^{+}-$2Cl^{-}$ cotransporter (NaKCC) has not been interrogated. Here, we induced in vitro capacitation of porcine sperm with a standard medium (TCM; control), and tested the effects of two NaKCC blockers, bumetanide (BMD) and furosemide (FSM), with the following treatments: 100 µM BMD + 100 µM FSM; 100 µM BMD + 1 mM FSM; 1 mM BMD + 100 µM FSM; and 1 mM BMD + 1 mM FSM. Samples were incubated at 38.5 ºC and 5% CO_2 for 120 min, and then added with 10 µg/mL progesterone to induce the acrosome reaction; samples were then incubated further for 60 min under the same conditions. Six independent experiments, each biological replicate consisting of a pool of three seminal samples from a different male, were conducted, and sperm were evaluated at 0, 60, 120, 130 and 180 min. At each time point, sperm motility was evaluated with a computer-assisted sperm analysis (CASA) system, and membrane lipid disorder, acrosome integrity, mitochondrial membrane potential, and intracellular levels of calcium, superoxides and total ROS were determined by flow cytometry. The effects of treatments throughout the incubation time were examined with a linear mixed model followed by a post-hoc Sidak's test, and the level of significance was set at $P \leq 0.05$. Inhibition of NKCC with 100 µM BMD + 1 mM FSM, 1 mM BMD + 100 µM FSM, and, particularly, 1 mM BMD + 1 mM FSM significantly ($P<0.05$) reduced total and progressive motility after 60, 120, 130 and 180 min of incubation, whereas the treatment containing 100 µM BMD + 100 µM FSM had no impact. Membrane lipid disorder was also found to be impaired by those treatments, notwithstanding this was only observed at 60 min. Furthermore, inhibition of NKCC with 1 mM BMD + 1 mM FSM significantly increased the intracellular levels of calcium and total ROS in viable sperm after 60 and 120 min of incubation, respectively, and increased the percentage of acrosome-damaged sperm before adding progesterone. These findings suggest that NKCC may be involved in the regulation of sperm capacitation, as their proper functioning is needed for a timely achievement of the capacitated status.

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CALCIUM TIGHTLY REGULATES SPERM FUNCTION AND METABOLISM DURING LIQUID PRESERVATION

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Recent studies suggested that calcium (Ca^{2+}) modulates the metabolism of poultry sperm cooled at 4 ºC; however, its role in the liquid preservation of mammalian semen has not been interrogated. Using the pig as an animal model, this study aims to evaluate the role of Ca^{2+} on sperm physiology and metabolism during liquid storage at 17 ºC, through the extracellular and intracellular chelation of this cation with EGTA and EGTA-AM, respectively. Samples (n =12) were incubated in four different media at 17 ºC for four days: (i) control (BTS without EDTA), and the same medium supplemented with (ii) 1 mM Ca^{2+}, (iii) 6.8 mM EGTA, and (iv) 20 µM EGTA-AM. Sperm quality parameters were evaluated daily by flow cytometry: sperm viability (SYBR-14), membrane lipid disorder (M540), acrosome integrity (PNA), intracellular Ca^{2+} (Fluo4), mitochondrial membrane potential (MMP; JC-1), total ROS (H_2DCFDA) and superoxide levels (HE). Sperm motility was evaluated using a CASA system. Data were analysed through a linear mixed model (significance at $P \leq 0.05$). Results showed that Ca^{2+} supplementation has a detrimental effect on plasma and acrosome membranes, whereas its chelation brings no effect. This may indicate the induction of Ca^{2+}-mediated capacitation-like changes in sperm preserved at 17 ºC. Analyses also confirmed that intracellular - but not extracellular - chelation reduces total and progressive motility, which is consistent with previous studies showing that Ca^{2+} is essential for motility. Moreover, while Ca^{2+} supplementation was found not to impact MMP, its intracellular chelation stimulated it and reduced total ROS, suggesting a blockage of mitochondrial respiration in the absence of Ca^{2+}. These findings suggest that Ca^{2+} influences the antioxidant system, increasing superoxide levels and reducing total ROS. In conclusion, the present study shows that, although Ca^{2+} has a detrimental effect on sperm viability and membrane integrity during liquid preservation, its presence is crucial for mitochondrial respiration, energy production and motility. These results open the door to investigate further the involvement of Ca^{2+} in the regulation of sperm metabolic pathways, as well as the development of novel preservation media to optimise sperm quality not only in porcine but also in other mammalian species.

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EFFECT OF MORPHOLOGICAL QUALITY ON IMPLANTATION OF HUMAN BLASTOCYSTS IN AN *IN VITRO* MODEL

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Implantation failure is one of the most common causes of unsuccessful pregnancies. In fact, about 30% of all conceptions are lost at the time of implantation. Therefore, studying the embryo implantation process *in vitro* is crucial to understanding the underlying mechanisms.

We developed an *in vitro* implantation model consisting of a collagen-based 2D or 3D matrix which allows the culture of post-implantation human embryos. Day 5 human blastocysts were thawed, graded based on Gardner system and transferred to the matrix. Embryos could be cultured in the matrix until up to day 12 of development. Immunostaining of epiblast marker OCT4, primitive endoderm marker GATA6 and actin staining revealed that embryos were able to maintain their 3D architecture, displaying a pro-amniotic cavity and a primitive yolk sac. Moreover, human embryos penetrated deeply into the 2D matrix, consistent with the interstitial implantation observed *in vivo*.

We studied the correlation between the morphological quality of the blastocyst and its implantation potential on our 2D matrix. First, embryos were classified into 5 categories based on their morphological grading (excellent, good, fair, bad and worst). Then, the area and the penetration of the embryo into the collagen matrix (depth) were measured at day 9. Results showed a positive correlation between embryo area and depth at day 9 (Spearman ρ=0.9018). Both parameters were significantly lower in worst quality embryos, and a general trend showing that higher quality embryos had higher area and implantation depth was observed. When analysing the inner cell mass (ICM) and trophectoderm (TE) quality individually, no significant differences were found in the implantation depth among embryos with ICM grade A, B or C, but embryos with TE grade C showed a significantly lower implantation depth at day 9 than embryos with TE grade A (p=0.0004), thus highlighting the importance of a good quality TE for proper embryo implantation.

These findings validate the use of our model to extend the preclinical studies of human embryo development beyond implantation. Thanks to this tool, we have been able to perform live imaging of embryo implantation, carry out mechanical studies or even test new culture media supplements.

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NO EFFECT OF MATERNAL AGE ON EMBRYO IMPLANTATION AND LIVE BIRTH RATES AFTER PREIMPLANTATION GENETIC TESTING FOR ANEUPLOIDIES: RETROSPECTIVE ANALYSIS OF 11,855 SINGLE BLASTOCYST TRANSFERS

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Age-related fertility decline is linked to increased chromosomal abnormalities and reduced oocyte yield. While preimplantation genetic testing for aneuploidy (PGT-A) helps mitigate maternal age effects by selecting euploid embryos, its ability to fully counteract age-related fertility decline remains unclear. Studies examining reproductive outcomes after euploid embryo transfer in older women have delivered mixed results, raising questions about the role of embryo quality and endometrial factors. This retrospective multicentre study analyzed 11,855 single euploid blastocyst transfers from autologous PGT-A cycles across seven IVF centers (January 2015 - October 2024). Primary outcomes included implantation (IR), clinical pregnancy (CPR), and live birth (LBR). Embryo quality was evaluated using a modified Gardner scoring system, classifying embryos as excellent, good or poor. Maternal age was categorized based on SART criteria: <35 (n=4,141), 35-37 (n=3,320), 38-40 (n=3,202), 41-42 (n=988), and >42 years of age (n=174). A multivariate mixed-effects logistic regression model analyzed maternal age impact on outcomes, adjusting for paternal age, cycle number, biopsy day, embryo quality (fixed effects), and center/provider (random effect). The interaction between maternal age and embryo quality on LBR was also modeled. In our cohort, mean maternal age (±SD) was 35.8 (±3.8) years. As anticipated, higher maternal age correlated with fewer embryos available for biopsy (Pearson's R^2=-0.26, p<0.01). Multivariate analysis showed no significant impact of maternal nor paternal age on LBR, IR or CPR (p>0.05). However, embryo quality (excellent vs. good, OR=0.55, [95%CI: 0.51-0.60]; poor, OR=0.29, [95%CI: 0.20-0.42], p<0.001) and biopsy day (day 5 vs. day 6, OR=0.86, [95%CI: 0.79-0.93]; day 7, OR=0.29, [95%CI: 0.22-0.39], p<0.001) were associated with reduced LBR. Older patients exhibited increased rates of poor-quality embryos (3.2%, 2.2%, 3%, 5.3%, and 9.2%, <35, 35-37, 38-40, 41-42, >42 years, respectively). No significant decline in LBR was observed with advancing maternal age after transferring excellent or good-quality embryos. However, women >42 years showed reduced LBR when transferring poor-quality embryos (OR=0.46, [95%CI: 0.21-1.01], p=0.053). Thus, advanced maternal age does not compromise clinical outcomes following euploid embryo transfer. The decline in success rates with age may primarily stem from reduced euploid embryo availability and lower likelihood of obtaining good-quality blastocysts suitable for biopsy.

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SKQ1 TREATMENT PREVENTS OVARIAN AGING IN MICE.

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In mammals, oocyte development and maturation are essential for female fertility. Female mammals are born with a finite number of follicles, known as the ovarian reserve, which decreases throughout their reproductive lifespan. In humans, this reserve is gradually depleted, leading to menopause around the age of natural cessation of reproduction. Previous data from our lab have shown that the age-related decline in primordial follicles is linked to the activation of the DNA damage response (DDR). Moreover, these studies highlighted the role of oxidative stress in the regulation of the age of natural menopause in women. Considering all the above, we aimed to evaluate the potential of antioxidant treatment to mitigate the effects of oxidative stress and preserve the ovarian reserve. Young female C57BL/6 mice were treated with various compounds, including ethanol, a known inducer of oxidative stress, as well as several antioxidant agents, such as SkQ1 (a mitochondria-targeted antioxidant). Our preliminary findings reveal a significant reduction in primordial follicles in mice exposed to ethanol, suggesting that daily ethanol consumption diminishes the ovarian reserve. However, co-administration of SkQ1 with ethanol appeared to reverse this effect. Interestingly, in the context of natural aging, an increased number of primordial follicles was detected in mice treated with SkQ1. To explore the underlying mechanisms, proteomics, metabolomics and lipidomics revealed alterations in metabolism and DNA related pathways. To summarize our findings, we conclude that daily ethanol intake significantly reduces the mouse ovarian reserve, resulting in the selective elimination of primordial follicles. However, treatment with SKQ1 appears to counteract the harmful effects of chronic ethanol consumption on murine ovarian reserve. Additionally, SkQ1 treatment protects the ovarian reserve from age-related decline. The pathways implicated in these changes appear to be related to mitochondrial function, metabolism, autophagy, and DNA-related processes. Based on these findings, we can confidently assert that oxidative stress plays a crucial role in limiting the ovarian reserve and regulating the aging process of the ovary.

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CLINICAL STUDY TO VERIFY THE EFFICACY OF AN ANTIOXIDANT IN THE IMPROVEMENT OF SEMEN QUALITY IN PATIENTS WITH IDIOPATHIC INFERTILITY.

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Recent studies have highlighted oxidative stress caused by environmental and pathological components as a potential factor of unexplained male infertility. Likewise, consumption of antioxidants to fight against oxidative damage has been booming within assisted reproduction patients. Nevertheless, knowledge about clinical assessment of oxidative stress and the effects of antioxidants on semen quality is insufficient. To fill this gap, we carried out an extended double-blind randomized and placebo-controlled clinical study to confirm the usefulness of dietary supplementation with the antioxidant TetraSOD® (a phytoplankton extract with high Superoxide Dismutase activity) for 3 months (one spermatogenic cycle) in the improvement of sperm quality including sperm DNA fragmentation. To achieve this objective, we gathered 66 Astheno/Oligo/Oligoasthenozoospermic patients attending the assisted reproduction clinic of the Clinic Barcelona hospital. From each patient two semen samples were collected, before (T0) and after (T3) 3 months of antioxidant/placebo supplementation. We evaluated socio-demographic data (BMI, toxic habits), hormonal parameters [Inhibin, Testosterone, Luteinizing hormone (LH), Follicle Stimulating Hormone and Sex Hormone Binding Globulin (SHBG)] and semen parameters (standard values), along with static oxidation-reduction potential (sORP/C) by MiOXSYS system and single and double strand sperm DNA fragmentation by alkaline and neutral comet assay, respectively. In general, we observed that patients receiving TetraSOD® showed a significant increase in blood LH levels after 3 months. However, we only detected a direct and significant impact on semen quality in patients with an altered sORP/C (increased sperm concentration and decreased double-stranded sperm DNA fragmentation), patients with asthenozoospermia (improved progressive sperm motility), or oligozoospermia (decreased double-stranded sperm DNA fragmentation). In contrast, no improvements were detected in patients who did not show any signs of oxidative stress. Overall, antioxidant consumption seems to improve semen quality in men with a compromised redox balance. It may be beneficial at a clinical level to check the oxidative status of infertile men before administering an antioxidant to select better the population that can really benefit from this kind of supplements.

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THE UTILITY OF SPERM DNA FRAGMENTATION AS A DIAGNOSTIC TOOL FOR MALE INFERTILITY AND ITS PREDICTIVE VALUE FOR ASSISTED REPRODUCTIVE TECHNOLOGY OUTCOMES.

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Male infertility management primarily relies on the assessment of seminal parameters. However, semen examination results often lack correlation with Assisted Reproductive Technology (ART) outcomes, highlighting the need for molecular-based determinations. This prospective study evaluates the potential of sperm DNA fragmentation (SDF) as a diagnostic tool for male infertility and as a predictive biomarker for ART success through validation of its association with seminal alterations and ART-related outcomes. Semen samples from 20 donors (proven fertility) and 46 infertile patients either with altered semen parameters (n=40) or unexplained infertility (normozoospermia; n=6), were collected. These samples were used for ART cycles according to the procedures established by the collaborating centres. A fraction of each semen sample was analysed for SDF through TUNEL assay. Positive cells were detected by flow cytometry and analysed using the Cytexpert software. Statistical analyses included SDF-level comparisons between patients and donors and correlation analyses of SDF with seminal parameters and ART-related outcomes. ROC analyses were applied to evaluate the diagnostic and predictive potential of SDF.The mean SDF was significantly lower ($p<0.01$) in donors (22.19±8.37) compared to all infertile patients (33.03±13.46) and the stratified groups according to seminal categories: asthenozoospermia (38.18±10.14) oligozoospermia (37.46±14.01), teratozoospermia (32.3±14.63) and normozoospermia (34.80±13.45). Negative correlations of SDF were observed with sperm count ($r=-0.4096$; $p<0.001$) and motility ($r=-0.5828$; $p<0.001$). Regarding the potential of SDF as a diagnostic tool, ROC analysis across all patients yielded an Area Under the Curve (AUC) of 0.7455 (p-value=0.0018). Regarding the potential of SDF as a predictive biomarker for ART success, significant SDF differences (23.36±8.766 vs 30.66±12.50; $p=0.0016$) were observed between infertile patients with a high and a low embryo quality rate. Moreover, ROC analysis showed an AUC=0.68 (p-value=0.0017) for embryo quality. No predictive value was observed for fertilization and blastulation rates. This study confirms the robustness of SDF assessment as a diagnostic biomarker of seminal alterations, and its predictive value as biomarker of embryo quality.

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EVALUATION OF CENTROMERE AND TELOMERE REGION-SPECIFIC SPERM DNA INTEGRITY

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Sperm DNA fragmentation (SDF) has emerged as a key biomarker of sperm quality, demonstrating an association with fertility and serving as a predictor of assisted reproductive outcomes. Whether specific genomic regions are more susceptible to or protected from DNA fragmentation due to their specific nuclear architecture remains unknown. The present study aimed to evaluate whether the incidence of double-stranded breaks (DSB) differs between centromeric and telomeric regions, compared to the whole sperm DNA. For this purpose, a single-cell gel electrophoresis (Comet) assay was combined with fluorescent in situ hybridization (FISH) to assess region-specific DSB in human sperm cells. An α-satellite pan-centromeric probe (Cambio, Cambridge, UK) was used to detect all centromeres, while a peptide nucleic acid (PNA)-conjugated telomeric probe (Panagene, South Korea) was used to identify telomeres. Fluorescence intensity of the pan-centromeric probe was quantified using ImageJ software, categorizing the analysis into non-fragmented or fragmented regions for each evaluated Comet. The number of telomeres located in regions with or without DNA fragmentation was analyzed using TFL-Telo v2 software. Total DNA damage was quantified using the %TailDNA parameter in CometScore software. Total incidence of DSBs in the 32 analyzed Comets was 18.0 ± 9.5% (95%CI: 15.0-22.0), the incidence of DSB in centromeric regions was 24.0 ± 9.3% (95%CI: 20.0-27.0), and the percentage of telomeres in regions with nearby DSBs was 27.0 ± 11.0 % (95%CI: 23.0-31.0). Statistically significant differences were observed between total DSB and centromeric DSBs (P=0.043), as well as between total DSB and telomeres with nearby DSB (P=0.005). Previous studies have shown that repetitive regions exhibit higher nucleosome retention. Our results suggest that the increased incidence of DSB observed in centromeres and telomeres could be related to increased accessibility of histone-associated regions. Future studies should investigate the clinical significance of the sperm chromatin integrity in these regions for mammalian reproduction.

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MOLECULAR EVALUATION OF SPERM QUALITY AND MATURITY AFTER SELECTION PROCEDURES IN COUPLES WITH UNEXPLAINED INFERTILITY

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Infertility is a complex condition affecting 1 in 6 couples of reproductive age. To address this medical issue, an increasing number of couples turn to assisted reproductive technologies (ARTs). A crucial factor influencing the success of these procedures is the sperm selection method. Recently, microfluidic devices have emerged as a promising alternative to discontinuous density gradient centrifugation (DGC), the most widely used clinical sperm selection technique. These devices select sperm with better motility and morphology, while avoiding the detrimental effects of centrifugation associated to DGC. However, little is known about the selection capacity at the molecular level. This study aimed to compare sperm quality and maturity at molecular level following selection by two distinct procedures before ART: a 40-80% DGC and a microfluidic device (ZyMōt™ Multi 850µL®). Sperm selection was performed on neat semen from normozoospermic infertile patients with no toxic habits, no genetic alterations and a BMI < 30Kg/m^2. Semen quality was evaluated through key sperm parameters, including motility, morphology, and vitality (n = 12); DNA integrity was assessed using both Alkaline and Neutral Comet Assay (n = 37) and proteomic profiling was conducted via LC-MS/MS (n=5). We confirmed that sperm selection by microfluidic devices significantly improved motility (p-value <0.0001), vitality (p-value < 0.0001) and morphology (p-value = 0.0012) compared to both DGC and native semen. Additionally, a significant lower amount of sperm with DNA fragmentation was selected with microfluidics devices (p-value <0.001) whereas 40-80% DGC significantly increase the number of cells with damaged DNA (p-value< 0.002). These results suggest the harmful effects of the centrifugation included in the process. Out of 4,348 identified proteins, 1,882 proteins showed significant differential abundance when comparing selected sperm populations to neat semen (p-value <0.05 and FC ≥ 1.5). Spermatozoa selected by the microfluidic device exhibited an enrichment in proteins associated with physiological functions essential for sperm viability and fertilization potential, such as energy production, mitochondrial functioning, structuring of the tail and midpiece of the sperm, redox balance maintenance, sperm capacitation and prevention of premature acrosomal reaction. In contrast, spermatozoa selected by using the conventional 40-80% DGC demonstrated a molecular composition similar to neat semen. These findings highlight the potential benefit of passive microfluidic sperm selection in enhancing seminal quality by selecting spermatozoa with distinct molecular profile that favors key physiological functions for fertilization. Further research is needed to determine its potential benefits in increasing success rates of ART outcomes.
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DEVELOPMENT OF AN *EX UTERO* EMBRYO CULTURE PLATFORM TO ADDRESS POST-IMPLANTATION DEVELOPMENT AND EARLY PLACENTATION

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Infertility is defined by the failure to conceive a child after 12 months or more of regular unprotected sexual intercourse and affects about 15% of reproductive-aged couples worldwide. One of the critical points for pregnancy progression is embryo implantation, since most unsuccessful conceptions are lost in this step and only one third of conceptions progress and lead to a live birth. This natural inefficiency of human implantation has not been significantly improved with the arrival of assisted reproductive techniques. Therefore, to overcome this roadblock in human reproduction, we need a deeper understanding of the events that control the formation and progression of the human conceptus during implantation. The current embryo culture technology patches different stages of embryo development, ranging from preimplantation stages to early organogenesis. But, so far, nobody has accomplished a continue culture from zygote all the way until organogenesis. The static culture conditions used in those systems, where the distribution of nutrients and gases depends only on passive diffusion, is one of the limiting factors. Here, we propose an embryonic culture platform integrated into a microfluidic device which allows us to apply a flow of media to the culture system. These features enable us to adapt both gases and nutrient supply to the developmental stage of the embryos, while also facilitating the removal of waste products. The device is fabricated by replica molding and consists of a culture platform with an integrated hydrogel layer on which the embryos can implant. Fabrication and sterilization protocols have been adapted to host mouse embryos. They have been cultured from blastocyst stage (day 4.5 after fertilization) to early-gastrulation stage after 4 days of culture in the device. Embryos have an implantation morphology similar to those shown in literature, and express Oct4, a marker of the embryonic epiblast, precursor to the future fetus, and Cdx2, a marker of the trophectoderm, the extraembryonic tissue precursor of the placenta. Altogether, we have engineered a microfluidic platform that will help us to study mouse and human embryo implantation under controlled culture conditions enabling us to study embryo development from zygote to post-gastrulation stages.

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ANOTACIONS DE LA XIX JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

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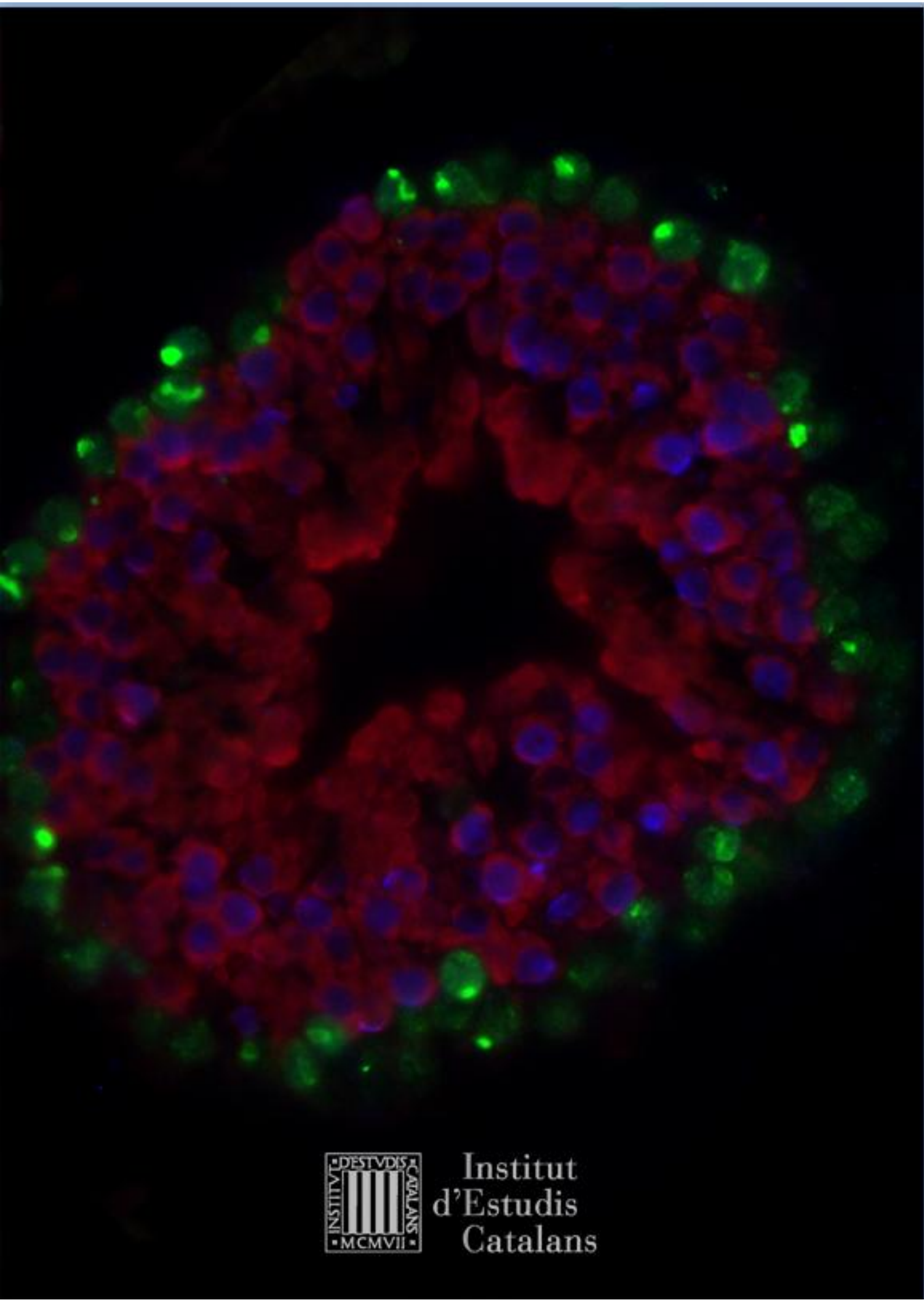

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