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Societat Catalana de BIOLOGIA

ACTES DE LA XVIII JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

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BIOLOGIA DE LA REPRODUCCIÓ

ACTES DE LA XVIII JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

Editors

Judit Castillo, Meritxell Jodar i Rafael Oliva

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

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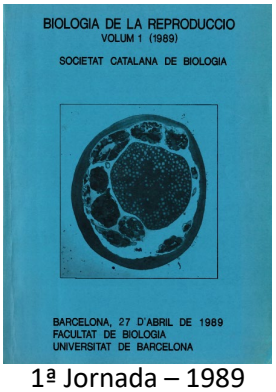
PRESENTACIÓ

Aquest volum recull les comunicacions presentades a la XVIII Jornada de Biologia de la Reproducció celebrades el 24 de maig de 2023 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



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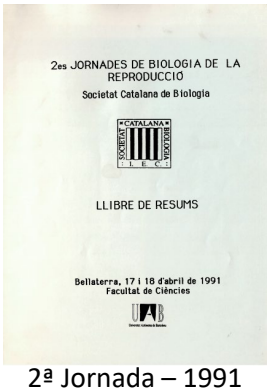

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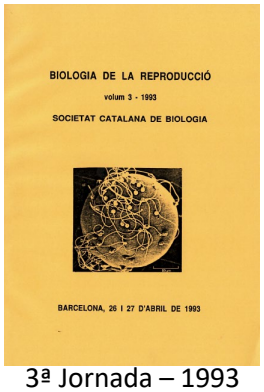

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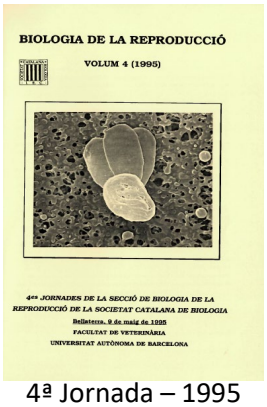

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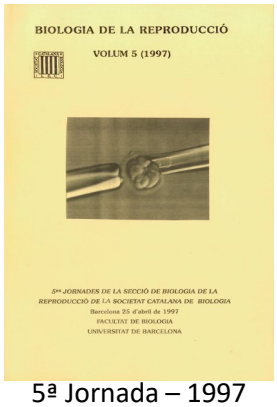

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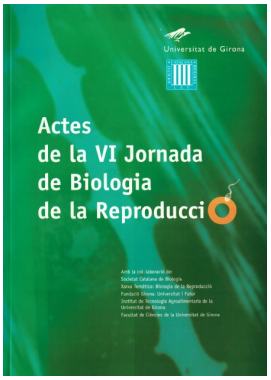

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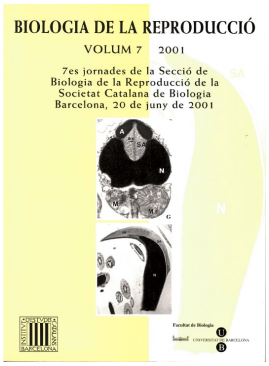

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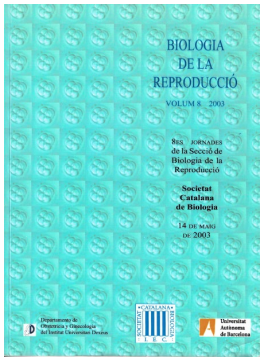

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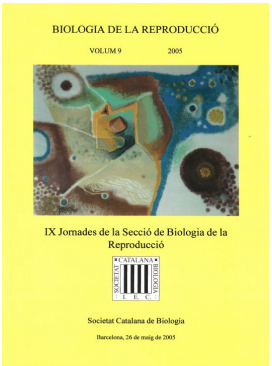

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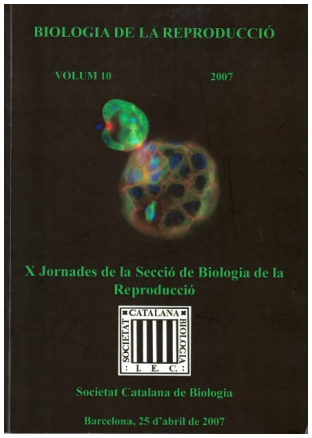

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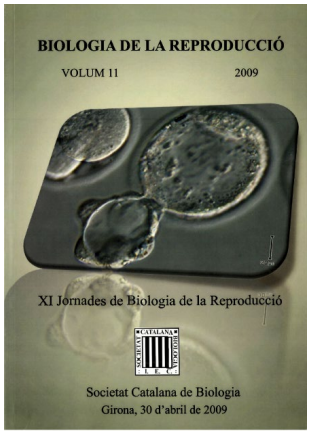

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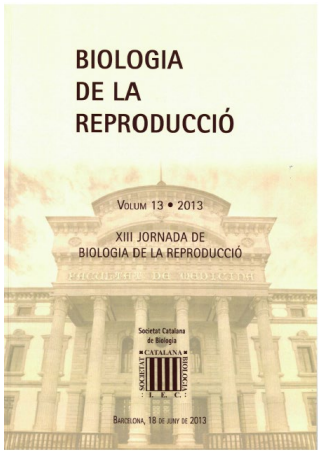

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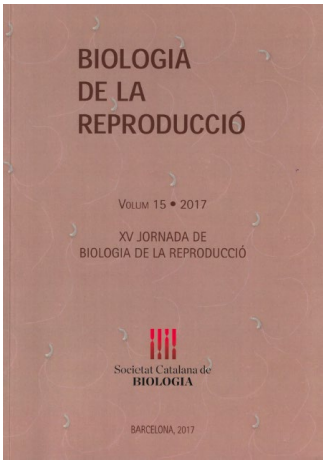

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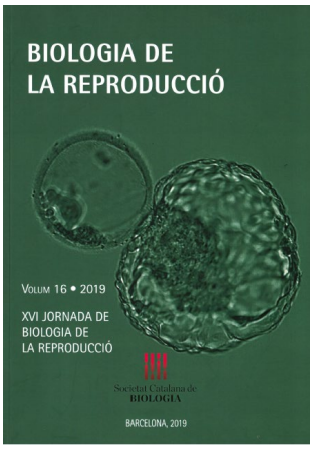

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NOTA BIOGRÀFICA
MERCÈ DURFORT I COLL (La Suze-sur-Sarthe, 4 d'abril de 1943 – Barcelona, 7 d'abril de 2022).

Enric Ribes[1]

[1]Departament de Biologia Cel·lular, Fisiologia i Immunologia. Facultat de Biologia. Universitat de Barcelona. Av. Diagonal, 643. 08028 Barcelona.
*Corresponding author: eribes@ub.edu

"Veure la ciència amb l'òptica de l'artista i l'art amb la de la vida"

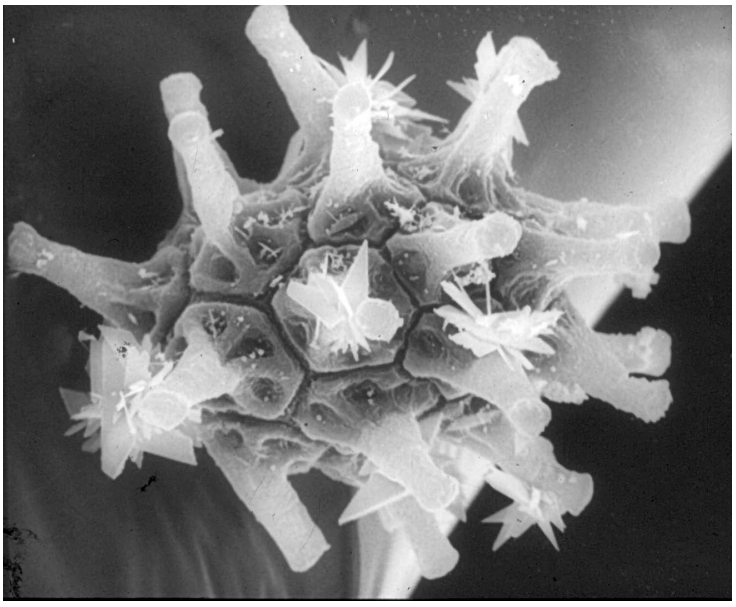
Aquesta reflexió de Nietzsche va acompanyar la vida de Mercè Durfort.

Mercè Durfort, va estudiar Ciències Biològiques a la Universitat de Barcelona (UB), l'any 1973 va obtenir el títol de doctora i l'any 1986 el de catedràtica de Biologia Cel·lular a la Facultat de Biologia de la UB.

Va exercir diferents càrrecs de gestió i de representació acadèmica, essent cap de departament en diversos períodes. L'any 2013 fou anomenada catedràtica emèrita de Biologia Cel·lular, aquest mateix any va tenir lloc un acte d'homenatge amb motiu de la seva jubilació (1), que va ser presidit pel rector de la UB, Dídac Ramírez, durant aquest els conferenciants recordaren el compromís de Mercè Durfort amb la institució universitària, destacant aspectes de la seva personalitat, com ara la generositat, el carisma, la intel·ligència emocional i la capacitat de treball. Va ser una gran professora que transmetre als seus alumnes els valors científics i la passió per la biologia, com s'ha demostrat en les manifestacions d'elogi i de simpatia que ha rebut al llarg dels anys tan dels estudiants com dels companys de professió.

Durfort fou una experta en biologia de la reproducció d'invertebrats, en estudis histopatològics de mol·luscs i de crustacis, en processos de bioacumulació en òrgans digestius de bivalves marins, i en la determinació de protozous paràsits, en especial els microsporidis. Va ser la primera científica catalana en

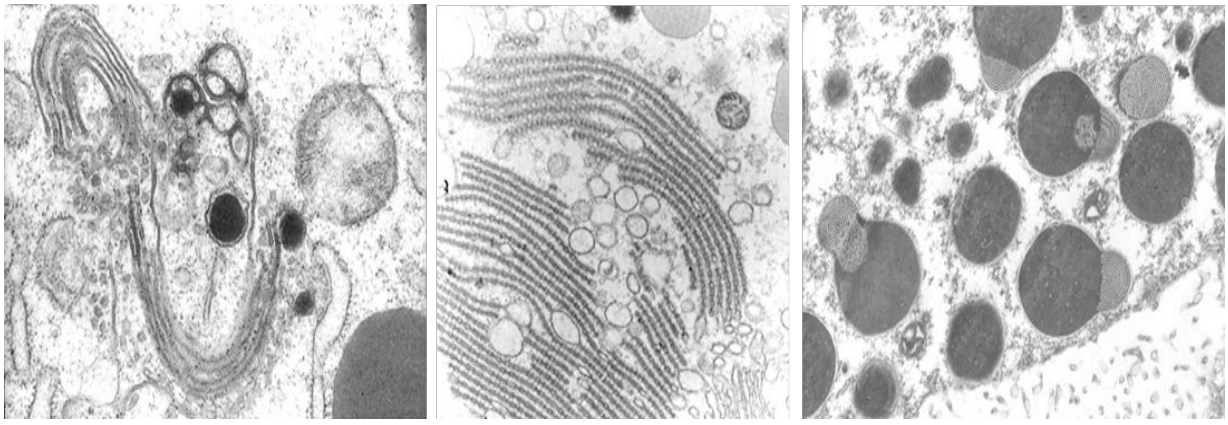
dedicar-se als estudis ultraestructurals cel·lulars, autora d'una tesi doctoral sobre l'ultraestructura de la gònada femenina de Mytilus *edulis*, essent també la primera en utilitzar el microscopi electrònic de transmissió Philips TEM 200, primer microscopi electrònic que es va instal·lar en la UB.



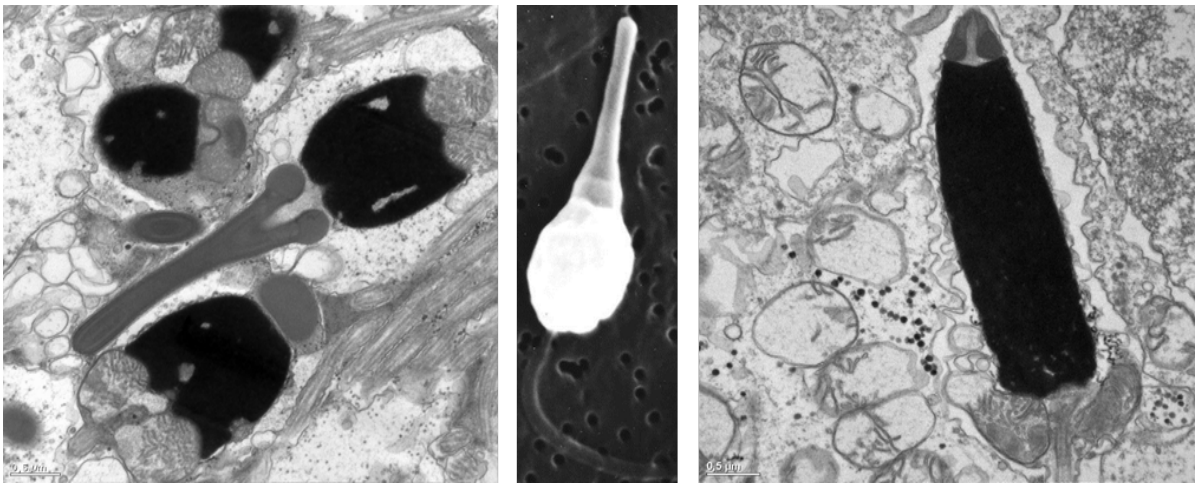
Imatge que mostra un oòcit de Trachydermon cinereus embolcallat per cèl·lules fol·liculars. Electromicrografia obtinguda amb un microscopi electrònic de rastreig. M.Durfort.

En el camp de la biologia de la reproducció, va centrar-se en l'estudi ultraestructural de la oogènesi de mol·lusc i de crustacis, mostrant un especial interès en el procés de formació de les plaquetes vitel·lines i en els orgànuls i estructures cel·lulars implicats en la seva síntesi, també per la morfologia de les cèl·lules fol·liculars i les seves funcions, aquests estudis els va fer en *Mytilus edulis, Trachydermon cinereus i Murex elenencis*.

Estudià l'espermatogènesi de diferents espècies de mol·luscs, com les de *Mytilus galloprovincialis, Crassostrea gigas, Donax trunculus i Murex brandaris*, En els darrers anys va mostrar interès en l'estudi de la gònada del cargol poma, *Pomacea canaliculata* espècie invasora que es troba majoritàriament al delta de l'Ebre.



Electromicrografies de estructures cel·lulars implicades en la formació de les plaquetes vitel·lines en els mol·luscs. Microscopi electrònic de transmissió (TEM). M. Durfort.



Electromicrografies d'espermatozoides i espermàtides de mol·luscs. TEM. M. Durfort

Va promoure la redacció d'un vocabulari de Biologia de la Reproducció[2] finançat per la secció de Ciències Biològiques de l'Institut d'Estudis Catalans on van participar experts en la matèria.
S'ha d'esmentar la seva participació en la constitució de la Secció de Biologia de la Reproducció de la Societat Catalana de Biologia, assolint la condició de membre fundadora, participant activament en les reunions i en les Jornades científiques organitzades per aquesta secció.

El seu compromís amb la llengua catalana, va comportar que fos presidenta de la Comissió de Dinamització Lingüística de la Facultat de Biologia de la UB que té com a finalitat fer una sèrie de vocabularis de terminologia científica dins de l'àmbit de la Biologia. Cal mencionar la seva participació en la traducció al català del llibre de Bruce Alberts *et al*., *Molecular biology of the cell*. 1a. ed. New York, Garland, 1983, un dels llibres de text més utilitzat de biologia cel·lular i molecular.

El seu interès per la història de la ciència i per la vida de rellevants científics la va portar a fer conferències amb menció especial a les que tractaven de la vida i obra de Santiago Ramon y Cajal[3, 4], a qui tenia una gran admiració, i a la del biòleg català Jaume Pujiola i Dilmé[5], també va fer ressenyes de la història dels estudis de biologia a la UB.
Va participar en la creació de la "*Galeria de científics catalans*"[6] que conté una recopilació de semblances de reconeguts científics.
Seguint amb aquesta inquietud per la història de la ciència, després de jubilar-se, va col·laborar en l'assignatura *Evolució del Pensament Biològic* que s'imparteix a la Facultat de Biologia.
Inicià la *Col·lecció d'Instruments Científics de la Facultat de Biologia*[7], amb microscopis i altres instruments científics obsolets de l'antic Departament de Morfologia Microscòpica, de l'antic Servei de Microscòpia Electrònica de la UB, de l'Institut Biològic de Sarrià, donats quan es va clausurar el 1985 i d'altres departaments de les Facultats de Biologia i de Medicina.
L'any 1993 ingressà a la Reial Acadèmia de Ciències i Arts de Barcelona, essent la primera dona en fer-ho, l'any 2000 va ser membre corresponent de la Reial Acadèmia de Medicina de Catalunya i membre corresponent de la Reial Acadèmia de Doctors de Espanya l'any 2007. Durant el període 1981-85 fou vicepresidenta de la Societat Catalana de Biologia i des de 1989 membre numerari de l'Institut d'Estudis Catalans (IEC), assolint la presidència de la Secció de Ciències Biològiques del 1993 al 2000.
Al 2002 va rebre la medalla Narcís Monturiol al mèrit científic i tecnològic i al 2004 la Creu de Sant Jordi per la seva activitat com a professora i científica.
Era sòcia honorària de la Institució Catalana d'Història Natural (2005) i de la Societat Catalana de Biologia (2014), filials de l'IEC, i membre d'honor de la Càtedra Santiago Ramón y Cajal de la Universitat de Ciències Mèdiques de l'Havana, a Cuba (2014).

Primer microscopi electrònic de transmissió que va comprar la Universitat de Barcelona, un Philips MET 200, que inicialment (1965-1966) va estar instal·lat i funcionant al Servei de Microscòpia Electrònica, a l'Edifici Històric de la UB. Aquest microscopi al va utilitzar M. Durfort per obtenir les electromicrografies de la seva tesi doctoral i d'altres publicacions.

El dia 15 de novembre de 2022, després de l' acte de homenatge a M. Durfort a l' aula magna Ramon Parés de la Facultat de Biologia organitzat per la Reial Acadèmia de Ciències i Arts de Barcelona i per la Facultat de Biologia de la Universitat de Barcelona[8], es va procedir a anomenar edifici Mercè Durfort a l'aulari de la Facultat, en reconeixement a la seva dedicació acadèmica.

REFERÈNCIES

(1) Acte d'homenatge a la catedràtica Mercè Durfort i Coll. Aula Magna de la Facultat de Biologia de la Universitat de Barcelona, octubre de 2023. *Notícies. Actualitat*, Universitat de Barcelona.
(2) Durfort, M. (2011) Vocabulari de biologia de la reproducció. Català – Castellà – Anglès. *Secció de Ciències Biològiques*. Institut d'Estudis Catalans, Barcelona.
(3) Durfort, M. (2007). La etapa barcelonesa de Santiago Ramon y Cajal, *Quark*, 39, 66-74.
(4) Fons bibliogràfic Santiago Ramon y Cajal. *Centre de Recursos per l'Aprenentatge i la Investigació*, CRAI, Universitat de Barcelona.
(5) Durfort, M. (1984). L'institut Biològic de Sarrià i la Institució Catalana d'Història Natural, *Butlletí de la Institució Catalana d'Història Natural*, 50; 1984. Barcelona.
(6) Galeria de Científics Catalans. Projecte de la Secció de Ciències Biològiques de l'Institut d'Estudis Catalans.
(7) Col·lecció d'Instruments Científics de la Facultat de Biologia. *Cultura i Patrimoni, Fons i Col·leccions* de la Universitat de Barcelona.
(8) Homenatge a la Dra. Mercè Durfort, 15 de novembre de 2022. Aula Magna Ramon Parés, Facultat de Biologia, Universitat de Barcelona. Acte organitzat per la Reial Acadèmia de Ciències i Arts de Barcelona.

La relació de treballs científics de Mercè Durfort es pot consultar entrant a Connecting research and researchers (ORCID https://orcid.org/0000-0003-3640-9580).

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

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EFFICACY OF OOCYTE FOLLICULAR ASPIRATION (OPU) TO OBTAIN EMBRYOS BY INTRACITOPLASMATIC SPERM INJECTION (ICSI) IN THE MARE AND DONKEY

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[2]Servei de Reproducció Equina, Departament de Medicina i Cirurgia Animal, Facultat de Veterinària, Universtitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Espanya.

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Abstract

The production of embryos in vitro through Ovum Pick Up (OPU) and intracytoplasmic sperm injection (ICSI) are the techniques currently used to produce embryos in vitro in the horse, due to the fact that in vitro fertilization (IVF) so far it offers sub-optimal results. However, although the use of OPU-ICSI can produce commercially acceptable results in the horse and is a technique that is becoming increasingly popular in the equestrian industry, there are no positive results reported so far in the case of the donkey. The aim of this study is to evaluate the efficiency of OPU- ICSI first procedures in horses performed at "Servei de reproducció equina de la Universitat Autònoma de Barcelona" and to compare it with the results obtained in donkeys as a way of improving the conservetion of the Catalan Donkey breed We performed 18 OPUs in mare and 8 in donkeys, from where we obtained oocytes that were maturated. From those matured we performed ICSI and finally assessed blastocyst formation. The results obtained in mares and jennies respectively were 64.08± 30.06% and 73.13±15.66%. of occyte collection, 68.51±22.92% and 65.31±22.40% of maturation, 78.25± 16.94 % and 62.26±33.14% for zigot clavage and 27.80±16.87% and 2.50±7.07% for blastocist/injected oocyte. o significant differences were observed in collection (P= 0.7414), oocyte maturation (P= 0.7705), cleavage (P=0.202129), but there is significant differences in the final result of blastocyst/injected oocytes (P=0.0018) In conclusion, the overall results in this study are comparable to those reported elsewhere and demonstrate that OPU can be done in donkeys as performed in mares but ICSI in jennies is still not as efficient as in horses. More research is needed in order to improve this results, taking specially emphasis on ICSI in donkeys. Furthermore, it is the first report of a donkey blastocyst obtained by ICSI, which was only accomplished when oocytes were maturated with preovulatory follicular fluid (PFF).

Resum

La producció in vitro d'embrions a través de aspiració fol•licular (OPU) i la injecció intracitoplasmàtica d'espermatozoides (ICSI) és l'única tècnica in-vitro utilitzada perquè la fecundació in vitro (IVF) segueix sent impossible, pel que la combinació de OPU-ICSI pot conduir a resultats comparables o fins i tot millors que la transferència convencional d'embrions. Malgrat la gran popularitat en les eugues encara no hi ha resultats presents per a burres. L'objectiu d'aquest estudi és avaluar l'eficàcia dels primers procediments OPU- ICSI en els cavalls que es realitzen a la "Servei de reproducció equina de la Universitat Autònoma de Barcelona" i comparar-los amb els resultats obtinguts en els rucs com a eina de millorar per a la conservació de la raça de Ruc Català. Es van realitzar 18 OPUs en euga i 8 en rucs, d'on es va obtenir oòcits que van ser madurats. A partir dels madurs es va realitzar ICSI i finalment avaluar si hi havia fecundació i formació de blastòcits. Els resultats obtinguts en les eugues i burres respectivament van ser 64.08± 30.06% i 73.13.15.66% en la obtenció d'occits, 68.51±22.92% i 65.31.22.40% en la maduració, 78.25. 16.94% i 62.26.33.14% per a la multiplicació del zigot i 27,80.16.87% i 2,50.7.07% per a blastocists/oòcit injectat. Es van observar o diferències significatives en la col·lecció (P= 0.7414), la

maduració oòcitaria (P= 0.7705), la multiplicació (P=0.202129), però hi ha diferències significatives en el resultat final de percentatge de oòcits blastòcits/injectes (P=0.0018). En conclusió, els resultats generals d'aquest estudi són comparables als reportats en altres llocs i demostren que l'OPU es pot fer en rucs tal com es realitza en eugues, però l'ICSI en burres encara no és tan eficient com en cavalls. Es necessària major recerca per millorar aquests resultats, posant especial èmfasi en la ICSI en els rucs. A més, aquest es el primer informe d'un blastocist de ruc obtingut per ICSI, que només es va aconseguir quan els oòcits van ser madurats amb PFF.

INTRODUCTION

In vitro embryo production via ovum pick up (OPU) and intracytoplasmatic sperm injection (ICSI) is a breeding technique that has become very popular during last years, mainly because *in vitro* fecundation (IVF) does not work in horses yet (Leemans et al., 2016) and therefore it is the newest way to maximize reproduction efficiency and genetic preservation (Squires, 2020). OPU-ICSI programs are an effective means of obtaining foals from mares that do not provide embryos for embryo transfer and/or are unable to successfully complete any pregnancy due to chronic endometritis, cervical defects, age, damage to the reproductive tract or any other problem that induces poor fertility (Herrera, 2018; Morris, 2018). Moreover, ICSI is also indicated for producing embryos from stallions with poor semen quality or quantity that would not fertilize any oocyte by their selves due to age, genetic problems, etc. (Roels et al., 2018). Other use of this technique is to optimize frozen semen from death stallions, by dilution of standard straw of frozen semen that can be sectioned into multiple ICSI doses (Rader et al., 2016). It is also a reproductive technique to consider for competition mares as it can be done at any time of the year and does not need many days of training rest. However, it is more expensive than conventional embryo transfer (Salamone et al., 2017; Rader et al., 2016). Although OPU-ICSI is getting more and more popular in equine breeders, there is anything reported about it in donkeys. The few studies focused in OPU in donkeys report the chemical activation of oocytes followed by IVF and vitrification of early embryos (Abdoon et al., 2018; Deleuze et al., 2018). At this point it is relevant to mention that despite the great improve in donkey semen cryopreservation, the use of thawed semen for artificial insemination produces low results of pregnancy in jennies (Álvarez et al., 2019; Canisso et al., 2011; Miró et al., 2020; Oliveira et al., 2012; Vidament et al., 2009).

After first demonstration of successful pregnancy from ICSI horse embryo (Galli et al., 2007) several results have been published to date, but many of them are controversial. In recent years, oocyte collection has been reported from 50-60% (Cuervo-Arango et al., 2019b; Rader et al., 2016; Claes et al., 2022; Barbacini et al., 2009), whereas oocyte maturation varies from 59-66% (Claes et al., 2022; Rader et al., 2016). The more successful rates of blastocyst per injected oocyte were 21.2% (Cuervo-Arango et al., 2019b) and 23.0% (Rader et al., 2016). However, the most common is from 10.0-17.6% (Meyers et al., 2019; Barbacini et al., 2009.), and in some individual cases it was up to 46.7% (Cuervo-Arango et al., 2019b). Furthermore, when talking about overall results per OPU session it has never been described more than 1.7 blastocysts/ OPU (Cuervo-Arango et al., 2019b). Results of OPU implemented in jennies yields in more variable recovery percentages from 34-76% (Deleuze et al., 2018).

The objective of this study was to evaluate and compare the efficacy of the OPU-ICSI procedures in horses and donkeys carried out at the “Servei de reproducción equina de la Universitat Autònoma de Barcelona”.

MATERIAL AND METHODS

Population. Seven mares of different breeds and five Catalan breed jennies were used in this study. The population of mares studied ranged from one with good genetics without any known fertility problems time to others that had subfertility results in past breeding seasons. In the case of jennies, they had not presented previous reproductive problems. In some animals, more than one OPU session was performed.

OPU. The oocyte recovery was done by aspiration of the immature follicles of > 10 mm. Oocytes were collected by transvaginal ultrasound guided follicular aspiration technique. The mares and jennies were

placed in palpation stocks and sedated using Detomidine hydrochloride (0.01mg/kg IV for mares, 0.02 mg/kg for jennies) and Butorphanol tartrate (0.02mg/kg IV for jennies, 0.03 mg/kg for jennies). They were also medicated with Butilscopolamine (0.15 mg/kg IV for mares, 0.12 mg/kg for jennie), Flunixin Meglumine (1.1mg/kg) and with a prophylactic antibiotic protocol consisting in Gentamicin (6.6 mg/kg IV) some minutes before the process. The tail was tied up, the rectum was evacuated, and the urinary bladder was proved and emptied of urine. The perineum and vulva were scrubbed three times with neutral soap.

A total of 26 OPU sessions were done with an ultrasound transducer with a section probe (E3123, MyLabGamma, Esaote®, Genova, Italy) placed in a holder with a follicular aspiration double lumen needle of 12G x 25" attached to a double vacuum pump (Minitüb, Tiefenbach, Germany). The follicle was flushed 5-10 times with a commercial recovery medium (Equiflush®, Minitüb, Tiefenbach, Germany) supplemented with Heparin (500UI/ml Heparinasodica®, ROVI, Madrid, Spain) prewarmed at 37ºC (Cuervo-Arango et al., 2019). All the fluid obtained was maintained at 37ºC into the vacuum equipment (Cuervo-Arango et al., 2019b). Once the aspiration of all oocytes was completed the collected fluid was transported to the laboratory for the oocyte searching process. The collected fluid was filtered through a sterile 70 mm embryo filter (EmCon®; IMV Technologies, L'Aigle, France), and the filtered content was evaluated under a stereomicroscope (SMZ800 N, Nikon Corporation, Tokyo, Japan) to identify the oocytes. The oocytes were placed in a 2 mL embryo holding media (Minitube, Bayern, Germany) and transported to the ICSI laboratory.

Oocyte maturation. Oocyte *in vitro* maturation is performed for 36-38 hours in 100 μL frops of bicarbonate-buffered Tissue Culture Medium (TCM-199 1,150-059, Thermo Fisher Scientific,Waltham, USA) supplemented with 10% v/v fetal bovine serum [FBS (10091148 8, Thermo Fisher Scien-tific, Waltham, USA)] and 1μL/mL Insulin-Transferrin-Selenium (51300044, Thermo Fisher Scientific, Waltham, USA), 1 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768), 10μg/mL follicle stimulating hormone (Folltropin-V, Vetoquinol Especiali-dades Veterinarias, Madrid, Spain), and 25μg/ml gentamycin (15710064, Thermo Fisher Scientific, Waltham, USA) under mineraloil (ART-4008PA, Origio Oil for Tissue Culture, Cooper Surgical Fertility Companies, Målov, Denmark) in 5% CO2 in humidified air at 38.2 ºC. For donkey oocytes a 10% of donkey preovulatory follicular fluid (PFF) was used instead of FBS in 24 oocytes.

Management of oocytes. After the maturation period, the dish containing the oocytes was removed from the incubator, and the oocytes were evaluated using a dissection microscope. The cumulus cells were mechanically removed in G-MOPSTM medium (10.130, Vitrolife, Goteborg, Sweden) to assess successfully maturation into MII (Hinrichs, 2010; Rodríguez et al., 2019; Metcalf et al., 2020). The oocytes with intact cytoplasmatic membrane without any visible polar body were classified as immature. Only those oocytes where the first polar body was observed, like oocyte B in figure 1, were placed back into maturation droplets and into the incubator to wait for ICSI.

ICSI. It is a micromanipulation technique that consists of an injection of a single spermatozoon into the cytoplasm of a mature oocyte. It involves the use of an inverted microscope (Nikon Eclipse Ti2-A, Nikon Corporation, Tokyo, Japan) and a micromanipulation system (Transferman 4r, Eppendorf Ibèrica S.L.U, SanSebastián de los Reyes, Spain). Frozen sperm was thawed by submerging in 1mL of G-MOPS at 37ºC was used in all conditions. ICSI was performed using a 7 μm glass sharp micropipette (IC-50-30, Origio, CooperSurgicalFertility Companies, Målov, Denmak).

The oocyte is subjected by the holding pipette, placing the polar body in 6-12 o'clock site and the zona pellucida of the oocyte is removed using Piezzo Drill pulses. Before injection, the pipette is used for the immobilization of a single motile spermatozoon with normal morphology. The sperm is aspirated with the pipette and both are inserted into the oocyte cytoplasm by breaking first the oolemma as in figure 2. (Brom-de-Luna et al., 2021; Smits et al., 2012)

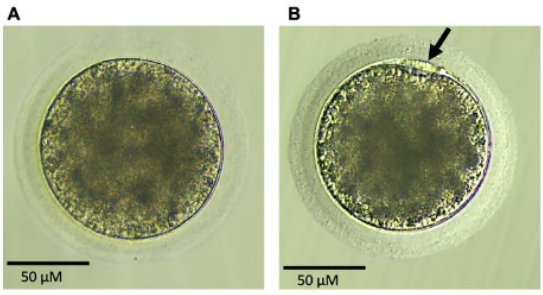

Figure 1: Immature oocyte (A) and mature oocyte Miró)

Figure 2. Donkey egg immediately before ICSI (image courtesy by Jordi Miró)

Embryo development. The future zygotes were cultured in a global medium (LGGG, LifeGlobal, Guilford, CT) with 10% v/v FBS for up to 11 day). At day 5 the media was renewed, and the cleavage was assessed. Embryos were often evaluated from day 7 to day 11, removing them from the incubator for a microscopic evaluation. However, in some cases, embryos were assessed from time-lapse images as in figure 3. In these cases, presumptive ICSI zygotes were cultured using 16-microwell Primo Vision microwell culture dish (Vitrolife, Goteborg, Sweden) under 50 mL of culture media drop covered with mineral oil (ART-4008PA, Origio CooperSurgicalFertility Companies, Målov, Denmak). To control the development of the embryo, images were captured every 5 minutes starting 5-20 min after ICSI using the Primo Vision Time-Lapse System. The image of each zygote was assessed evaluating the mitotic process, capturing images of each division until reaching the blastocyte stage. Blastocyst were vitrified for future transference into a reception mare or jenny or for future studies.

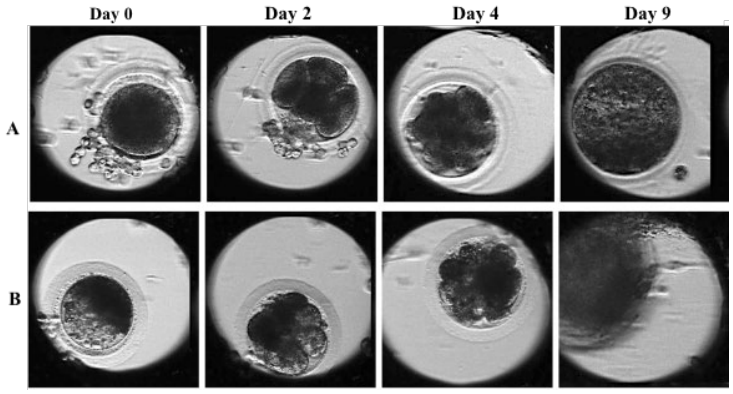

Figure 3. Time-lapse images of the development of donkey (A) and horse (B) embryos (image courtesy by Jordi Miró)

Statistical analyses. Statistical package R, using R-commander was used to analyze the results obtained in this work. Shapiro-Wilk and Levene tests were conducted to assess data distribution and homogeneity of variances respectively. Following this, Student-t test was performed for all data comparisons except for oocyte recuperation rate blastocyst/injection oocyte as the first data in mares and the second in jennies do not follow a normal distribution. A Wilcoxon-Mann-Whitney test was performed in these two cases.

RESULTS AND DISCUSSION

OPU

Follicular aspiration results in mares are present in table 1. Results of 50 to 60% of picking up oocyte have been previously reported the last (Cuervo-Arango et al., 2019b; Claes et al., 2022; Rader et al., 2016; Barbacini et al., 2009). Our results raised up of 64.08% success in horse and 73.13% in donkeys, validating our technique.

Table 1. Oocyte recuperation by follicular aspiration results in mares and jennies.

Specie	No. OPU	No. follicles aspirates	No. oocytes recovered	Oocyte collection efficiency (%)
Mare (n=8)	16	287	197	64.08± 30.06
Jennies (n=5)	8	65	45	73.13 ±15.66

No: number
Data are shown as mean ± SD
Oocyte collection efficiency was calculated as the no. oocytes recovered per no. of aspirated follicles.

In the present report, the mean number of oocytes recovered from jennies was 5.625± 2.00, which is higher than the last study reported (Deleuze et al., 2018), but less than in mares, not only on average (10.98) but on the maximum obtained individually (9 and 19 respectively). However, no significant differences were observed in collection (P= 0.7414).

When talking about individual results, in our study, from one mare there was not any oocyte obtained by OPU, perhaps because of an intrinsic reproductive problem. On the other hand, we can aspect huge variability between animals, but also in the same mare or jennie.

ICSI

Regarding the production of horse embryos ICSI results vary greatly between studies, from 10-23% of average blastocysts/ oocyte injected have been previously described (Meyers et al., 2019; Barbacini et al., 2009; Rader et al., 2016; Cuervo-Arango et al., 2019b). Moreover, in individual cases it has been reported a 46,7% of success (Cuervo-Arango et al., 2019b) They also described more than 1,67 blastocyst/OPU, the grater results published by the moment. As present in table 2, our study leads in a 27.80% ±16.87 %of blastocysts/injected oocyte in horses, an average greater than any mentioned before. However, some poor individual results are present, having 5 ICSI with less than 10%. It is important to point out that the percentage of cleavage is 78.25%± 16.94% in mares and 62.26%±33.14% in jennies. Few studies have produced results that definitively describes the embryo media requirements during this development. However, when comparing with other research (Brom-de-Luna et al., 2021; Cuervo-Arango et al., 2019b), this may indicate that the percentage achieved on mares with the present culture system is near the maximum possible considering de knowledge of this moment. Some modifications of oocyte handling and maturation systems are promising (Galli et al., 2018; Meyers et al., 2019). We suggest that culture of embryos is an important limitation and further research is required.

Table 2. ICSI results in mares and jennies

Specie	Maturation rate (%)	Cleaveage rate (%)	No. Blastocyst	Blastocyst/injected oocyte (%)
Mare (n=8)	68.51±22.92	78.25± 16.94	32 ± 1.26	27.80±16.87*
Jennies (n=5)	65.31 ± 22.40	62.26±33.14	1 ±0.35	2.50 ± 7.07*

No: number
Maturation rate was calculated as the no. of MII oocytes out of the total no. of oocytes collected. Cleavage rate was defined as the ratio of no. cleaved zygotes by the no. oocytes injected oocytes.
The subscript (*) means significant differences (P≤ 0.05) between results in mares and jennies.
Data are shown as mean ± SD

Similar oocyte maturation results are present in our study between the two species. However, after ICSI, the results between mares and jennies started to differ more significantly with a percentage of cleavage of only 78.25% in horses compared with 62.26% in jennies and a final efficacy result of 27.80%±16.87% and 2.50% ±7.07 respectively. No significant differences were observed in collection (P= 0.7414), oocyte maturation (P= 0.7705), cleavage (P=0.202129), but there is significant differences in the final result of blastocyst/injected oocytes (P=0.00208). This suggest that ICSI in donkeys might not be as efficient as in

horse. It has been proposed that when evaluating embryo development by time-lapse imaging, donkey *in vitro* embryos have a similar cell division patter compared to the horse (Flores Bragulat et al., 2023) that means that probably improper *in vitro* culture conditions and/or incomplete donkey oocyte activation after ICSI by donkey sperm are the main conditioning to lead in poor developmental rates on donkey ICSI embryos. Thus, the investigation of effects of the media on the sperm and the knowledge of which components can affect them negatively could improve greatly the results, not even in jennies but also in horse invitro production of blastocysts.

CONCLUSIONS

In conclusion, the overall results of this study are comparable to those reported in other studies in the case of the horse. It is important to note that this is the first time that obtaining a blastocyst by means of OPU-ICSI in the donkey has been reported. While these findings suggest that this technique provides an opportunity to breed valuable equine specimens and endangered breeds, more ICSI cycles are needed to confirm this trend. OPU horse protocols and oocyte incubation for maturation can be extrapolated to donkeys. However, the embryonic development potential after ICSI in donkeys is not as efficient as in mares. The preovulatory follicular fluid supports in vitro maturation and embryonic development of donkeys, only with this supplementation was the formation of blastocysts achieved. The possibility of collecting oocytes from live donkeys and producing donkey embryos in vitro makes a significant contribution to the genetic preservation of species of interest, such as the Catalan donkey.

Overall, the OPU-ICSI procedure is a potential breeding technique in equine species. As the current results are still suboptimal and the cost of the procedure is high, more research is needed to increase the number of blastocysts obtained in horses and especially donkeys.

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EXPLORING THE CORRELATIONS BETWEEN SPERM PARAMETERS, TYPES OF SPERM DNA FRAGMENTATION AND PROTAMINE ALTERATIONS

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Abstract

Fertility evaluation for men is currently limited to examining alterations in sperm count, motility, or morphology. Recently, sperm DNA fragmentation (SDF) has emerged as a new potential diagnostic tool for male infertility. SDF assesses the integrity of paternal genome and high levels of DNA fragmentation have been associated with a decreased male fertility. Some studies suggest that single-strand breaks (SSBs) and double-strand breaks (DSBs) have different implications for reproduction, being DSB the ones with a higher negative impact on male fertility. High levels of oxidative stress in semen have been proposed to increase SDF, although the tight condensation of sperm DNA by protamines might protect paternal DNA from this damage. Some authors correlate an abnormal sperm chromatin compaction to an increased SDF, but the relationship between protamine alterations and different types of SDF still requires more research. This study investigates the association between types of SDF, oxidative stress and alterations in sperm chromatin compaction using a selected population of idiopathic infertile men. SDF was measured using comet assay, and the samples were stratified based on the presence of high levels of DSBs, SSBs, or both. Oxidative stress was measured by MiOXYS. Finally, protamines were extracted and subjected to acid electrophoresis to measure the P1/P2 ratio and protamine/DNA content. Increased levels of sperm oxidative stress have a direct association with both types of SDF. However, no significant differences were observed between protamine levels and SDF. Thus, further research is necessary to fully elucidate the mechanisms underlying sperm chromatin structural abnormalities and their impact on SFD and male infertility.

Resum

Actualment, l'avaluació de la fertilitat dels homes es limita a examinar les alteracions en el recompte, la motilitat o la morfologia dels espermatozoides. En els últims anys, la fragmentació de l'ADN espermàtic (SDF, de l'anglès *sperm DNA fragmentation*) ha sorgit com una nova eina potencial de diagnòstic per a la infertilitat masculina. La SDF avalua la integritat del genoma patern i s'han associat nivells elevats de fragmentació de l'ADN amb una disminució de la fertilitat masculina. Alguns estudis suggereixen que els trencaments de cadena simple (SSB, de l'anglès *single strand breaks*) i els trencaments de doble cadena (DSB, de l'anglès *double strand breaks*) tenen implicacions diferents per a la reproducció, sent els DSB els que tenen un major impacte negatiu en la fertilitat masculina. S'ha proposat que nivells elevats d'estrès oxidatiu en el semen augmenten el SDF, tot i que la gran condensació de l'ADN dels espermatozoides per part de les protamines podria protegir l'ADN patern d'aquest dany. Alguns autors correlacionen una compactació anormal de la cromatina dels espermatozoides amb un SDF augmentat, però la relació entre les alteracions de les protamines i els diferents tipus de SDF necessita més estudi. Aquest projecte

investiga l'associació entre tipus de SDF, l'estrès oxidatiu i les alteracions en la compactació de la cromatina espermàtica, utilitzant una població seleccionada d'homes amb infertilitat idiopàtica. La SDF es va mesurar mitjançant l'assaig Comet i les mostres es van dividir en funció de la presència d'alts nivells de trencaments DSB, SSB o ambdós. L'estrès oxidatiu es va mesurar mitjançant MiOXYS. Finalment, es van extreure protamines i es van sotmetre a electroforesi àcida per mesurar la relació P1/P2 i el contingut de protamina/ADN. L'augment dels nivells d'estrès oxidatiu dels espermatozoides té una associació directa amb ambdós tipus de SDF. Tanmateix, no es van observar diferències significatives entre nivells de protamines i SDF. Per tant, calen més investigacions per dilucidar completament els mecanismes subjacents a les anomalies estructurals de la cromatina espermàtica i el seu impacte en la SFD i la infertilitat masculina.

INTRODUCTION

Overall, the male factor substantially contributes to approximately 50% of all infertility cases (Leslie et al., 2023). The evaluation of male infertility is very limited, as it is primarily based on the seminogram, which is able to reveal gross deficiencies in sperm count, motility, or morphology, thus resolving obvious cases of male infertility (Kumar & Singh, 2015). In approximately 55% of male infertility cases is not possible to determine the causes. Beyond basic sperm parameters, other molecular factors such as sperm DNA and chromatin integrities might have a detrimental impact on normal fertilization, embryo development, and success of assisted reproductive techniques (ART) (Agarwal et al., 2020).

Sperm cells are highly specialized delivery vehicles for paternal chromatin cargo composed of DNA and its associated proteins. In the nucleus, the most abundant sperm nuclear proteins are protamines, which pack approximately 85-95% of human sperm DNA, whereas the rest of the DNA remain associated to histones (Balhorn, 2007; de la Iglesia et al., 2023). Protamines are small and extremely basic specific sperm proteins (with 50-70 % of arginines residues in the sequence) that increase the level of DNA packaging, forming highly condensed toroidal and rod-like structures (Castillo et al., 2015; de la Iglesia et al., 2023). This is essential for the protection of the paternal genetic message from nucleases and contributes in obtaining the required hydrodynamic shape for mature sperm functionality (Oliva & Castillo, 2011). In humans, there are two types of protamines, protamine 1 (P1) and protamine 2 family (P2). P1 is translated as mature protein whereas P2 is translated as an immature protein (precursor P2; pre-P2) that after proteolytic processing generates the mature forms of P2 (HP2, HP3, and HP4), differentiated by the few amino acids at the N-terminal end (Atshan et al., 2020; Oliva, 2006). These two types of protamines are usually found in a 1:1 ratio (0.8-1.2) (Balhorn, 2007; Lettieri et al., 2020; Oliva, 2006). P1/P2 ratio alterations have been correlated with high levels of sperm DNA fragmentation (SDF), altered seminal parameters, and low success rate of ART (Agarwal, Majzoub, et al., 2020; Muratori et al., 2015). Furthermore, alterations in pre-P2 levels are also correlated with a lower fertility and higher levels of SDF (Atshan et al., 2020; de la Iglesia et al., 2023; Jodar et al., 2011; Okada, 2022; Wu & Chu, 2008).

DNA damage leading to SDF can occur during spermatogenesis and/or during transport through the reproductive tract, affecting one or both strands of the DNA helix and resulting in single- (SSBs) or double- (DSBs) strand DNA breaks (Agarwal, Barbăroșie, et al., 2020; Wallach et al., 2010). Extrinsic factors such as exposure to environmental pollutants, certain chemotherapeutics, heat, and smoking, as well as intrinsic factors like defective maturation, abortive apoptosis, and oxidative stress (OS), can cause DNA impairment (Agarwal et al., 2020; Muratori et al., 2015). Moreover, there seems to be a close relationship between abnormal sperm chromatin protamination, OS and SDF, although the causal relationship remains unclear. Recent studies have shown that different types of breaks in sperm DNA (such as SSBs and DSBs) can result in varying effects on reproductive outcomes. SSBs breaks can occur at multiple break points throughout the genome and are commonly associated with OS. These breaks have been known to contribute to a lack of clinical pregnancy or lead to a longer conception time (Ribas-Maynou & Benet, 2019). DDBs can result from the failure of DNA repair during meiosis and can cause an increased risk of miscarriage, lower embryo quality, and higher risks of implantation failure during

ICSI cycles (Ribas-Maynou & Benet, 2019). Therefore, understanding the pathophysiology of the different types of sperm DNA damage and their impact on male fertility is essential to provide possible screening tools, treatments and advice for men who are facing infertility issues. Additionally, the evaluation of the different types of SSBs and DSBs can be useful in determining an individual's predisposition to specific complications, thus improving the accuracy of diagnosis and reproductive treatment options.

The aim of this study is to investigate potential differences in seminal parameters, semen reduction-oxidative potential and protamine content among different groups if patients with varying grades and types of SDF.

MATERIAL AND METHODS

Sample collection and processing. Semen samples (n=165) were obtained with prior consent from men who attended the Andrology unitof the Hospital Clínic de Barcelona to undergo semen analysis for fertility purposes. Ejaculates were collected after 3-5 days of sexual abstinence and a seminogram was routinely performed evaluating sperm concentration, motility, vitality and morphology, according to the guidelines of the World Health Organization (World Health Organization, 2021). In addition, semen reduction-oxidation potential (sORP) was measured by galvanostatic system MiOXSYS and normalized by sperm concentration (sORP/C).

DSBs and SSBs evaluation using Comet assay. Comet assay is a methodology that allows distinguishing between SSB and DSB. This assay is based on sperm inclusion in an agarose gel, followed by a nuclear decompaction, and an electrophoresis under alkaline (main detection of SSBs) or neutral (main detection of DSBs) conditions (Ribas-Maynou et al., 2012; Simon & Carrell, 2013). DNA fragments (comet tail) migrate more than the whole intact chromatin (comet head), thus forming a shape similar to a comet and being able to discriminate between positive (fragmented) and negative (non-fragmented) cells [Figura1A]. Individual comet-shaped spermatozoa were stained and visualized using fluorescence microscopy, and the percentage of fragmented cells was quantified.

Protamine extraction and quantification. Protamine extraction was performed following the protocols standardized by our research group (Soler-Ventura et al., 2018) in some samples of each group of study (high DSBs and SSBs (n = 6), high DSBs (n = 5), high SSBs (n = 6), and low DSBs and SSBs (n = 5)) with at least 3 million spermatozoa (Mz) available after SDF evaluation by comet. Once the protamine extract was obtained, P1 and P2 were quantified by acid-urea polyacrylamide gel electrophoresis in conjunction with increasing quantities of a standard of human protamines. In addition, the DNA pellet obtained after protamine extraction was used for DNA quantification through 0.5 M perchloric acid hydrolysis (90°C for 20 min). The absorbance at 260 nm was determined using a NanoDrop spectrophotometer in order to normalize the amount of protamines with the amount of DNA for each sample.

Statistical analysis. All the data were analyzed using GraphPad Prism version 7.00. A histogram graph was plotted to visualize the frequency distribution of percentage of cell with SSB or DSB in our population (n = 165) [Figure 1]. The population was stratified into three categories based on the proportion of fragmented cells: high (> 36.69% SSBs; > 33.12% DSBs), medium (between 20.94-36.69% SSBs; between 21.84 – 33.12 DSBs), and low (< 20.94% SSBs; < 21.84% DSBs). The thresholds were determined via the terciles method, according to relevant literature (Ribas-Maynou et al., 2013; Simon & Carrell, 2013). For this study, the samples were selected and stratified based on the presence of high levels of double-strand breaks (DSBs), single-strand breaks (SSBs), or both, resulting in four categories: high DSBs and SSBs (n = 32), high DSBs (n = 7), high SSBs (n = 8), and low DSBs and SSBs (n = 25). The parameters examined included SDF, sperm concentration, sperm motility, sperm vitality, sperm

morphology, sORP/c, P1/P2 ratio, and protamine (P1, P2, and P1 + P2)/DNA content of the native semen, with mean ± SD values presented in Table 1 and 2. P-value <0.05 was considered statistically significant. The data were analyzed using ANOVA or Kruskal-Wallis test. Post-hoc analysis was conducted using Dunn's, Holm-Sidak's, and Tukey's multiple comparison tests to identify significant differences between groups.

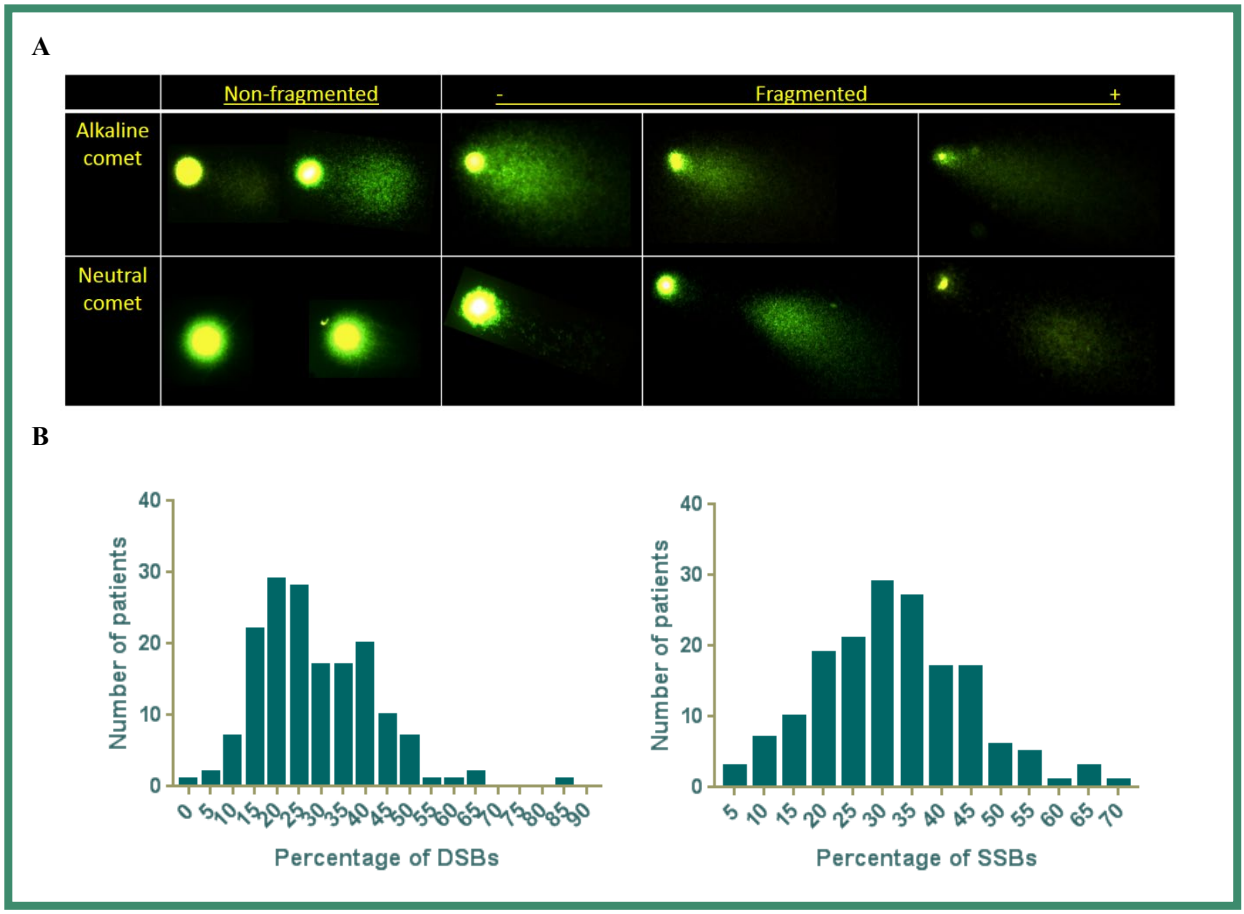

Figure 1. A) Summary table of the different comet-shaped sperm cells observed in the neutral and alkaline comet assays, ranked in order of increasing DNA fragmentation. B) Study population frequency distribution (n=165) represented as a histogram graphic according to the percentage of sperm with SSBs and DSBs.

RESULTS AND DISCUSSION

Association between sperm vitality, semen oxidative stress (sORP/C), and DNA fragmentation levels

Among classic semen parameters and oxidative stress-related parameters, vitality (P = 0.002), concentration (P = 0.043), and sORP/C levels (P = 0.0102) differed significantly between various groups of study [Table 1]. Subsequent multi-comparison test revealed significantly lower vitality (P = 0.0016) and higher sORP/C levels (P = 0.005) in samples featuring high SDF levels, for both DSBs and SSBs, compared to those characterized by low SDF [Table 1, Figure 2]. These findings imply a potential link between oxidative stress and SDF, since sORP/C is an indicator of redox state of semen, and it is known that dead spermatozoa are high ROS (Reactive Oxygen Species) producers.

Table 1. Correlation between DNA fragmentation and seminal and oxidative stress-related parameters. Results showed as mean ± SD. NS: Non-significant.

	sORP/C (mV/Mz/mL)	Concentration (Mz/mL)	Progressive Motility	Immotile sperm	Morphology	Vitality
1. High fragmentation (DSBs and SSBs)	9.16 ± 13.08	40.3 ± 48.9	16.75 ± 11.95	74.86 ± 20.64	4.1 ± 20.64	53.92 ± 13.88
2. High DSBs	3.53 ± 3.915	32.03 ± 35.93	27.27 ± 13.66	68.88 ± 14.67	6.857 ± 14.67	56.82 ± 21.2
3. High SSBs	2.127 ± 0.989	17.83 ± 11.62	20.28 ± 15.62	76.88 ± 15.91	2.714 ± 15.91	53.61 ± 10.45
4. Low fragmentation (DSBs and SSBs)	1.71 ± 2.802	66.04 ± 55.00	33.1 ± 27.17	61.95 ± 28.65	5.292 ± 28.65	68.81 ± 13.55
P value (ANOVA or Kruskal-Wallis test)	0.0102	0.0430	NS	NS	NS	0.0020
P value (Dunn's and Holm-Sidak's multiple comparisons test)	0.0050 1 vs 4	NS	NS	NS	NS	0.0016 1 vs 4

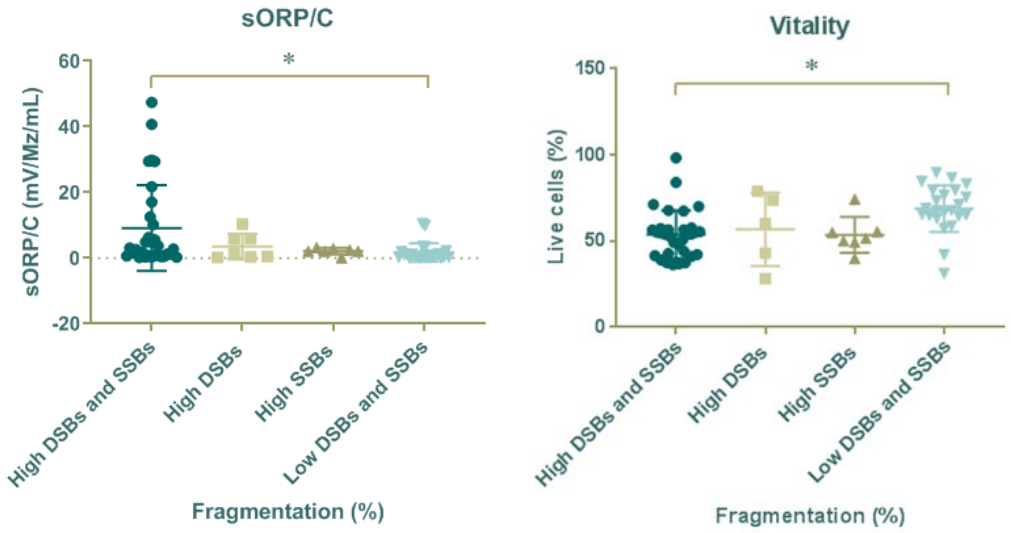

Figure 2. Graphical representation of semen oxidative stress levels evaluated sORP/C and sperm vitality illustrating the marked differences (*) observed between high levels of fragmentation of both DSBs and SSBs versus low levels of fragmentation of both DSBs and SSBs.

The complex relationship between sperm chromatin structure and DNA fragmentation

The analysis of the data showed no significant difference between protamination state and DNA fragmentation type [Table 2], suggesting that higher DNA damage and the type of breaks may not always happen together with alterations on P1/DNA, P2/DNA, P1+P2/DNA, or P1/P2 ratios. Our findings suggest that the complexity of sperm chromatin structure may be influenced by different protamine proteoforms and post-translational modifications, such as phosphorylated protamines. Further research is needed to fully understand the mechanisms underlying sperm chromatin structural abnormalities and their impact on male infertility.

Table 2. Protamine levels according to the presence and type of DNA fragmentation. Results showed as mean ± SD. NS: Non-significant.

	P1/P2	P1/DNA	P2/DNA	P1+P2/DNA
High fragmentation (DSBs and SSBs)	1.18600 ± 0.30290	0.00050 ± 0.00032	0.00135 ± 0.00234	0.00097 ± 0.00072
High DSBs	1.27200 ± 0.40480	0.00021 ± 2.507e-005	0.00016 ± 5.111e-005	0.00038 ± 6.711e-005
High SSBs	1.21600 ± 0.15110	0.00010 ± 2.837e-005	8.255e-005 ± 2.514e-005	0.00018 ± 5.342e-005
Low fragmentation (DSBs and SSBs)	1.05100 ± 0.25650	0.00043 ± 0.00020	0.00041 ± 0.00017	0.00084 ± 0.00035
P value (ANOVA or Kruskal-Wallis test)	NS	NS	NS	NS

CONCLUSIONS

It can be concluded that low sperm viability and increased levels of semen oxidative stress measured as sORP/C have an impact on both types of SDF, SSB and DSB. However, no significant differences were found on protamination state between the different types of SDF. Further research is necessary to fully understand the mechanisms underlying sperm chromatin structural abnormalities and their impact on male infertility. Overall, the study underscores the importance of studying sperm quality beyond conventional semen analysis, particularly in cases of unexplained infertility, to better comprehend the role of sperm in the generation of a new healthy individual.

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COMPARATIVE STUDY ON THE STATUS OF DNA DAMAGE OF SPERM SELECTED BY DENSITY GRADIENT CENTRIFUGATION AND MICROFLUIDIC DEVICES

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Abstract

Male infertility is a complex disease affecting 17% of couples at reproductive age. To overcome this medical issue, an increasing number of couples choose to rely on assisted reproductive technologies (ARTs). Sperm selection methods represent a crucial factor for the outcome of these procedures. In the last few years, microfluidic devices have been developed as a promising alternative to traditional sperm selection methodologies as density gradient centrifugation, which is currently the most utilized technique for clinical purposes, although it might affect sperm DNA integrity. Microfluidic devices have been shown to select sperm populations with good motility and morphology, but the knowledge on the effect at the molecular layer, such as the level of DNA damage, is still scarce. In this work, we carried out a comparative study on the status of DNA fragmentation of sperm selected by density gradient centrifugation and microfluidic devices, in comparison with native semen, by conducting alkaline and neutral comet assays and image analysis with CometAnalyser software. The results suggest that microfluidic devices allow the selection of sperm with good semen parameters and a better status of DNA damage in comparison to density gradient centrifugation.

Resum

La infertilitat masculina és una malaltia complexa que afecta el 17% de les parelles en edat reproductiva. Per superar aquest problema mèdic, un nombre creixent de parelles opten per confiar en les tecnologies de reproducció assistida (ART). Els mètodes de selecció d'espermatozoides representen un factor crucial per al resultat d'aquests procediments. En els últims anys s'han desenvolupat dispositius microfluídics com a alternativa prometedora de les tècniques tradicionals de selecció d'espermatozoides com la centrifugació en gradient de densitat, actualment la tècnica més utilitzada amb finalitats clíniques, tot i que podria afectar la integritat del DNA espermàtic. S'ha demostrat que els dispositius microfluídics seleccionen poblacions d'espermatozoides amb bona motilitat i morfologia, però no hi ha prou coneixement sobre els efectes a nivell molecular, com és el nivell de dany al DNA. En aquest treball hem realitzat un estudi comparatiu sobre l'estat de la integritat del DNA dels espermatozoides seleccionats per centrifugació de gradient de densitat i per dispositius microfluídics, en comparació amb l'estat inicial del semen, realitzant l'assaig cometa i anàlisi d'imatges amb el software CometAnalyzer. Els resultats suggereixen que els dispositius microfluídics permeten seleccionar espermatozoides amb bons paràmetres de semen i un millor estat de dany a l'ADN, en comparació amb la centrifugació en gradient de densitat.

INTRODUCTION

It is estimated that 17.5% of the population of reproductive age worldwide is affected by infertility (WHO, 2023), a complex condition in which male contribution is found in half of the cases (Agarwal et al., 2019). Male infertility is generally studied by semen analysis; however, this type of screening is often not sufficient to explain the complexity of the disease (Agarwal et al., 2019). Indeed, 60% of male infertility cases remain classified as idiopathic or with an unknown cause (De Jonge & Barratt, 2019; Corsini et al., 2022), suggesting that the investigation of the etiology of this condition should not be limited to the seminogram, but it should include the evaluation of molecular characteristics of sperm. Despite this lack of knowledge, the number of infertile couples using assisted reproductive technologies (ARTs) is increasing (De Geyter et al., 2018). Considering that these procedures are costly, invasive, time-consuming and have low success rates, further attention should be paid to the quality of the male gamete (Villani et al., 2021; Ribas-Maynou et al., 2022). In addition, the sperm selected for ARTs is of great concern for the health of the future offspring because the current selection methods bypass the natural barriers present in the female reproductive tract, with the possibility that fragmented DNA and genomic defects are transferred to the embryo (Leung et al., 2022). Therefore, it is crucial to develop reliable diagnostic tools for male infertility and to improve the current sperm selection techniques that are performed prior to ART.

Nowadays, the most utilized sperm selection method for clinical purposes is density gradient centrifugation (DGC) (Leung et al., 2022). With this technique, the ejaculate is placed on the top of a continuous or discontinuous density gradient and then centrifuged. The spermatozoa are selected based on their morphology and motility and are recovered at the bottom of the tube (Castillo et al., 2011; Baldini et al., 2021). This method is sometimes coupled with the swim-up, a technique based on the ability of sperm to swim to a culture medium (WHO, 2021). However, it has been suggested that DGC can damage the sperm genetic material, and this can have a negative impact on ART success (Twigg, 1998; Olatunji & More, 2022). In the last few years, new technologies based on microfluidics have been developed to overcome the limitations of the current approaches to sperm selection. Microfluidic devices mimic the female reproductive tract and allow the selection of sperm through the movement of fluids inside micrometer-sized channels (Vaughan & Sakkas, 2019). Although these devices select a sperm population with good motility and morphology, little is known about the molecular characteristics of the selected sperm, especially about the quality of sperm chromatin.

The integrity and the compaction of sperm chromatin are critical for ART success, embryo quality and the health of the future offspring (Azpiazu et al., 2014; de la Iglesia et al., 2022). Several studies have demonstrated that DNA fragmentation of the male gamete has a negative impact on the quality of semen and on the ART outcome (Simon et al., 2011; Ribas-Maynou et al., 2012; Simon et al., 2011; Simon, Emery & Carrell, 2017). There are two types of DNA breaks, single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs), both considered to have different consequences on reproductive health. SSBs has been associated to increased conception time and lack of clinical pregnancy, while DSBs seems to be linked with a higher risk of miscarriage and recurrent pregnancy loss (RPL), low embryo quality and a higher risk of implantation failure in ICSI cycles (Ribas-Maynou & Benet, 2019). One of the most reproducible and sensitive methods to detect SSBs in the DNA of individual sperm is the alkaline comet assay (Simon & Carrell, 2013), while a variant of it, the neutral comet assay, allows the detection of DSBs (Ribas-Maynou et al., 2012). These two methods are based on microgel electrophoresis in which agarose-embedded cells are lysed, subjected to an electric field and then visualized with fluorescent staining. When cells exhibit DNA damage, they appear as “comets” with a head consisting of undamaged genetic material and a tail consisting of nucleic acid fragments, since these fragments migrate faster than the intact DNA (Ribas-Maynou et al., 2012). Furthermore, thanks to commercial and open-source software’s for image analysis, it is possible to obtain a deeper description of the DNA damage revealed by the comet assay, through the extraction of several intensity and morphological features of the comet pictures (Beleon et al., 2022).

The aim of this study is to characterize sperm populations selected with DGC and microfluidic devices in terms of DNA integrity, by using comet assay and software analysis of comet images and comparing with the native semen.

MATERIAL AND METHODS

Semen samples collection. Semen samples from 12 normozoospermic patients with unexplained infertility attending the assisted reproductive unit of the Clinic Barcelona Hospital were collected under prior informed consent and after 3-5 days of sexual abstinence.

Processing of the semen samples and sperm selection methods. The surplus of the semen sample not used for ART or clinical evaluation purposes was subjected to selection by discontinuous density gradient centrifugation (40-80%) using Puresperm® (NidaCon) and by ZyMōt Multi 850 µl® microfluidic device, following the respective manufacturer's instructions. In addition, an aliquot of neat semen was kept aside for each semen sample. Different semen populations (Neat, Post-gradient and Post-ZyMōt) were assessed for seminal parameters such as sperm concentration, motility, morphology and vitality, following the WHO guidelines (WHO 2021). Finally, samples were cryopreserved in liquid nitrogen, as indicated by the manufacturer of the Cryosperm® cryoprotectant, for further use.

Evaluation of sperm DNA fragmentation with the alkaline and neutral comet assay. The alkaline and neutral comet assays were performed on the different cryopreserved sperm populations, based on Simon L. and Carrell D. (2013) and Ribas-Maynou, J., et al (2012) protocols, respectively. Cells were stained with SYBR Gold and observed under the fluorescent microscope. Then, the percentage of fragmented cells was determined in each population out of a total of at least 100 cells.

Analysis of comet assay images. To conduct a preliminary deeper analysis of the state of DNA fragmentation of each sperm selected population, the comet assay data of 5 patients were evaluated. Around 10 fragmented cells were randomly selected from each sperm population, captured under the fluorescent microscope and then analysed with CometAnalyser software (v 1.0) (Beleon et al., 2022). The features “Tail Percent DNA” and “Tail Extent Moment” were used to evaluate the entity of DNA damage. “Tail Percent DNA” is a measure of the proportion of the total DNA that is present in the comet tail, while “Tail Extent Moment” is a value that considers both the amount of DNA in the comet tail and the tail length.

Statistical analysis. Data analysis was conducted using the software GraphPad Prism 9. Differences in seminal parameters, % of cells with SSBs and DSBs, “Tail Percent DNA” and “Tail Extent Moment” between the sperm populations were analysed using Repeated-measures one-way ANOVA corrected by Tukey’s multiple comparisons test. The results were considered statistically significant for p-values < 0.05.

RESULTS

Semen parameters of neat semen in comparison to sperm subjected to different selection methods

The sperm population selected by ZyMōt Multi 850 µl® microfluidic device showed improved progressive motility, total motility, vitality and morphology compared to both neat semen and the sperm selected by 40-80% DGC (p-value < 0.05).

In contrast, the semen parameters of sperm processed with 40-80% DGC were not significantly different from the ones of neat semen [Table 1].

Table 1. *Comparison of semen parameters of different sperm populations. Data are expressed as mean ± standard deviation.*

	Progressive motility	Total motility	Vitality	Morphology
1. Neat semen	64.25 ± 15.88	63.37 ± 27.67	70.30 ± 14.52	7.14 ± 4.40
2. Post 40-80% DGC	71.69 ± 19.48	77.29 ± 16.94	70.43 ± 11.42	7.91 ± 4.55
3. Post-ZyMōt	94.71 ± 4.72	95.40 ± 4.55	88.96 ± 9.06	9.58 ± 5.11
P-value (ANOVA test)	<0.0001	<0.0001	0.0006	0.0102
P-value (Tukey's multiple comparison test)	<0.0001 1 vs 3 0.0026 2 vs 3	0.0001 1 vs 3 0.0041 2 vs 3	0.0090 1 vs 3 0.0073 2 vs 3	0.0004 1 vs 3 0.0184 2 vs 3

Comparison of sperm DNA fragmentation of neat semen and sperm selected with different methods

Sperm selected by ZyMōt Multi 850 µl® microfluidic device exhibited a reduction in the percentage of cells with DNA damage (both in the alkaline and neutral comet assay) when compared to neat semen and sperm selected by 40-80% DGC. The percentage of spermatozoa with DSBs was higher in the sperm selected with 40-80% DGC in comparison to neat semen, while no significant differences in the percentage of cells with SSBs were observed between these two sperm populations [Table 2].

Table 2. *Comparison of the percentage of sperm cells with DNA damage in different sperm populations. Data are expressed as mean ± standard deviation.*

	DNA damage	
	SSBs (Alkaline Comet assay)	**DSBs (Neutral Comet assay)**
1. Neat semen	19.77 ± 8.79	13.49 ± 6.33
2. Post 40-80% DGC	23.07 ± 7.89	24.02 ± 12.49
3. Post-ZyMōt	6.76 ± 4.70	4.48 ± 4.43
P-value (ANOVA test)	<0.0001	<0.0001
P-value (Tukey's multiple comparison test)	<0.0001 1 vs 3 0.0003 2 vs 3	0.0179 1 vs 2 0.0003 1 vs 3 0.0001 2 vs 3

Comparison of the features of DNA fragmentation of neat semen and sperm selected with different methods

The software analysis of the comet images of the different sperm populations [Figure 1] revealed a significant reduction in the alkaline "Tail Percent DNA" feature in the cells with fragmented DNA of the Post-ZyMōt population, in comparison to the ones of neat semen. While the ANOVA test detected significant differences among the three sperm populations in the alkaline "Tail Extent Moment" feature of the cells with damaged DNA, Tukey's test did not show any significant difference in the two-by-two comparisons between populations. On the other hand, no significant differences in the features "Tail Percent DNA" and "Tail Extent Moment" for the neutral comet assay were observed [Table 3].

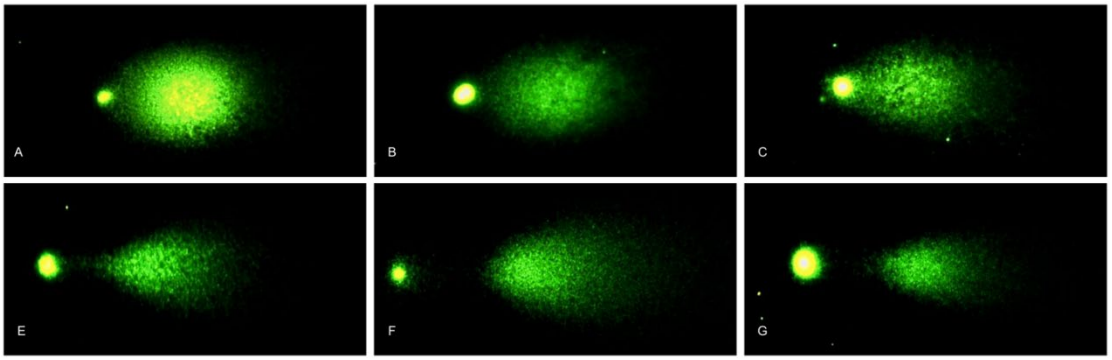

Figure 1. *Examples of cells with DNA damage detected by alkaline (first row) and neutral (second row) comet assay which were captured under the fluorescent microscope in Neat (A/E), Post 40-80% DGC (B/F) and Post-ZyMōt (C/G) sperm populations from the same patient.*

Table 3. *Comparison of the alkaline and neutral comet image features of different sperm populations analysed with CometAnalyser software. "Tail Percent DNA" is expressed in percentage, while "Tail Extent Moment" is expressed in arbitrary units. Data are presented as mean ± standard deviation. NS: non-significant.*

	Alkaline comet features		**Neutral comet features**	
	Tail Percent DNA	**Tail Extent Moment**	**Tail Percent DNA**	**Tail Extent Moment**
1. Neat semen	88.27 ± 2.36	479.41 ± 70.84	54.81 ± 13.73	496.54 ± 75.19
2. Post 40-80% DGC	84.80 ± 5.27	472.47 ± 131.56	48.55 ± 12.33	466.62 ± 132.29
3. Post-ZyMōt	79.24 ± 3.96	347.62 ± 65.78	44.23 ± 8.64	365.38 ± 89.50
P-value (ANOVA test)	0.0259	0.0218	NS	NS
P-value (Tukey's multiple comparison test)	0.0303 1 vs 3	NS	-	-

DISCUSSION

The data obtained from the semen analysis and the comet assay of the different sperm populations (Neat semen, Post-gradient and Post-ZyMōt) suggest that the microfluidic device allows the selection of sperm with good semen parameters and low levels of DNA fragmentation, similarly to what has been suggested by other authors (Quinn et al., 2018). On the other hand, the 40-80% DGC did not seem to select semen with statistically significant improved motility, vitality, and morphology. This appears to be different from what is reported in literature (de Mateo et al., 2011; Quinn et al., 2018; Ali et al., 2022) and it might be influenced by the lower number of patients considered in the current analysis. In addition, this selection method seemed to increase DSBs in the selected population in comparison to neat semen, most probably due to a detrimental effect of the centrifugation steps needed in this procedure. This result is consistent with other works (Twigg, 1998; Muratori et al., 2016; Olatunji & More, 2022), even though in literature there are some studies demonstrating comparable levels of DNA damage in neat semen and in semen after DGC (Malvezzi et al., 2014; Quinn et el., 2018). The preliminary software analysis of the comet assay images suggests that the sperm cells with SSBs in the Post-ZyMōt population present a lower amount of fragmented DNA (expressed by the feature "Tail Percent DNA"), compared to the ones of neat semen. Significant differences were found in the comparison of the feature "Tail Extent Moment" between the cells with SSBs of the different sperm populations, but these were not observed when only comparing the Post-ZyMōt and the neat semen populations. Although there is a trend of lower values of "Tail Percent DNA" and "Tail Extent Moment" in the spermatozoa with DSBs and selected with microfluidic devices, no significant differences were found in the comparisons to the other sperm populations. This suggests

that the entity of DSBs in the cells with DNA damage, evaluated in terms of proportion and size of the fragmented DNA, is comparable between the different sperm populations. However, all the results should be validated with further investigations with an increased number of samples.

CONCLUSION

The results of this study suggest that ZyMōt Multi 850 µl® microfluidic device represents a useful alternative to DGC for sperm selection previous ART, since it improves the quality and the status of DNA damage of the selected sperm, in comparison to neat semen and the traditional method based on density gradient centrifugation. Further studies with a higher number of patients are necessary to confirm these findings and to deeper explore whether there are differences in the characteristics of DNA fragmentation between the sperm cells presenting a compromised genetic material after the different selection methods.

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

(Ordenats per aparició en el programa de la Jornada)

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THE PERPLEXING GERM LINE: WHY TO TURN A CHROMOSOME OFF AND ON AGAIN

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The mammalian germline is characterized by extensive epigenetic reprogramming during development into eggs and sperm. Specifically, the epigenome requires resetting before parental marks can be established and transmitted to the next generation. In the female germline, X-chromosome inactivation and reactivation are among the most prominent epigenetic reprogramming events, yet little is known about their kinetics and biological function.

In our previous work (Severino et al., *EMBO J* 2022), we have investigated X-inactivation and reactivation dynamics using an *in vitro* system of mouse primordial germ cell-like cell (PGCLC) differentiation. We found that X-inactivation in PGCLCs is moderate compared to somatic cells, and characterized by many genes escaping X-inactivation. Subsequently we observed step-wise X-reactivation, which is mostly completed during meiotic prophase I. Importantly, we found that PGCLCs, which failed X-inactivation or reactivated too rapidly displayed impaired meiotic potential.

To follow-up this study, we now wanted to test, if X-dosage control is a direct functional requirement or rather a diagnostic mark for proper germ cell differentiation. Therefore, we have analyzed PGCLC differentiation in cells deficient for *Xist*, the master regulator of X-inactivation. *Xist*-KO cells undergo massive cell death and X-chromosome loss upon pluripotency exit, which constitutes a major bottleneck in their development towards germ cells. However, cells, which have cleared this selection event successfully, show a similar capacity to enter meiosis and oogenesis as wildtype cells, which have undergone X-inactivation successfully, despite differences in gene expression. In summary, active and inactive X-chromosome states present stage-specific constraints that are crucial for the normal development of female germ cells towards meiosis and oogenesis.

Reference: Severino, J. *et al.* Controlled X-chromosome dynamics defines meiotic potential of female mouse in vitro germ cells. *EMBO J.* **41**, e109457 (2022), https://doi.org/10.15252/embj.2021109457

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INTERROGATING THE IMPORTANCE OF X-CHROMOSOME INACTIVATION AND REACTIVATION FOR MEIOTIC POTENTIAL

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X-chromosome dosage compensation is achieved by X-chromosome inactivation, a process where one X chromosome is epigenetically silenced in female mammals in order to attain X-dosage parity with male cells. Subsequently, the silenced X chromosome uniquely reactivates during development of the germline, before the cells ultimately initiate meiosis. Using a dual X-chromosome reporter mouse embryonic cell line, we observed a subpopulation of primordial germ cells with two active X chromosomes, rather than the expected single active X, which were found to exhibit abnormal behavior and a low efficiency of meiotic entry. This data suggested that X-chromosome kinetics may have an important role in correct female germline development. In order to functionally test this, we developed a knockout model of Xist, a long non-coding RNA that mediates X-chromosome inactivation. Our results demonstrate that the majority of cells cannot maintain two active X chromosomes after differentiation, resulting in X-chromosome loss or cell death. However, primordial germ cells that survive this bottleneck event while maintaining two active X chromosomes can progress efficiently to meiosis prophase I, indicating that X-chromosome inactivation and reactivation may not be strictly required for female germ cell development. These findings provide insight into the prerequisites of X-chromosome epigenetic states for proper meiotic entry in female germ cells.

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THE ROLE OF SIRT7 IN MALE MEIOSIS AND REPRODUCTIVE AGING

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Sirtuins are NAD^+-dependent deacetylases that play major roles in genome integrity maintenance, cell metabolism, cell senescence, and aging. Sirtuins are paramount regulators of fertility in males and females, being key in different stages of gametogenesis. In this line, we previously described that SIRT7 deficiency in mouse females results in chromosome synapsis defects, a decreased ovarian reserve, and an overall age-dependent decline in fertility. Interestingly, our most recent data also point to a relevant role for SIRT7 in male gametogenesis during aging. $Sirt7^{-/-}$ males develop premature subfertility, evidenced by fewer offspring, histological defects in the testis, and a reduced testicular volume, as they age. Despite sperm counts being normal, comet assays in H2O2-challenged sperm revealed higher levels of DNA damage in the absence of SIRT7, suggestive of a diminished gamete quality. SIRT7 levels in mouse testes dramatically decrease in aged samples pointing to a key role in fertility maintenance with aging. Additionally, analysis of publicly available data indicated that SIRT7 is highly expressed in spermatogonia and spermatocytes, suggesting an important role in early meiosis. Accordingly, we observed a partial arrest at the leptotene-zygotene stage in $Sirt7^{-/-}$ aged mice, indicative of a defect in meiotic progression. SIRT7 is an epigenetic enzyme whose main histone mark target is H3K36ac. As expected, SIRT7 deficient spermatocytes presented a drastic increase in H3K36ac levels at all stages. This mark, however, was deregulated regardless of age, suggesting that its functional relevance may not be related to the observed aging phenotype. Further analysis of other epigenetic marks revealed an age-dependent downregulation of H3K9me3 in $Sirt7^{-/-}$ testes. Since, H3K9me3 is a chromatin mark related to heterochromatin and gene repression, this finding suggests that gene expression could be altered in SIRT7 deficient spermatogenic cells. Altogether, our findings point to SIRT7 as an essential factor for the preservation of reproductive potential throughout the aging process. Future experiments will provide deeper insights into the precise molecular mechanisms underlying SIRT7 control of gametogenesis and reproductive aging.

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MEIOTIC 3D CHROMATIN DYNAMICS IN THE MARSUPIAL GERM LINE

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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. Due to their key basal position in the mammalian evolutionary tree, marsupials offer a unique opportunity to explore previously uncharacterized meiotic features, from sex chromosome pairing strategies to chromosome occupancy within the nucleus. Here, we combine cytological analysis, fluorescence activated cell sorting and *in situ* chromosome conformation capture sequencing (Hi-C) to study the meiotic 3D chromatin dynamics in the Australian marsupial tammar wallaby. Ours results show that sex chromosomes form the so-called dense plate following different sex chromosome pairing strategies when compared to other marsupials, which correlates with differential sex chromosomes architecture and specific transcriptional patterns. Moreover, the spatial folding of chromosomes in meiotic (primary spermatocytes) and post-meiotic (round spermatids) cells showed different patterns of compartmentalisation in the tammar wallaby when compared to eutherian mammals (i.e., mouse). Overall, our results provide new insights into the regulation of chromatin in the germ line.

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DISENTANGLING THE EFFECT OF ROBERTSONIAN FUSIONS AND PRDM9 ALLELIC BACKGROUND ON MEIOTIC RECOMBINATION

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Spermatogenesis is a tightly regulated process that controls the transmission of genetic information through generations. Meiotic recombination is a key molecular event that generates genetic variability and ensures the proper segregation of homologous chromosomes. The recombination landscape can be affected by several factors such as structural variants (i.e., Rb fusions - balanced fusions between acrocentric chromosomes-), genetic factors (*Prdm9* allele), and mechanistic factors (chromatin state, interference between crossovers (COs), among others). Here, we take advantage of wild mice populations from the Barcelona Robertsonian System (BRbS) to evaluate the effect of the high variability of *Prdm9* in mice populations and the presence of Rb fusions on meiotic recombination. To this aim, we combined the cytological analysis of COs by immunofluorescence with the analysis of inferred recombination rates based on linkage disequilibrium using single nucleotide polymorphism data. Cytological results show that recombination decreases in spermatocytes of Rb mice, and it does so when the *Prdm9* alleles are in homozygosis. This reduction is related to mechanistic problems and increased COs interference, respectively. Levels of *in situ* recombination correlate with historical recombination per population: However, we also detected that the homozygosity of *Prdm9* allelic variants is a major determinant of the recombination levels, whereas Rb fusions have limited effects. When analysing the distribution of recombination events, they tend to accumulate at distal chromosomal regions, although the displacement from the centromere is more pronounced in Rb mice. This results in the redistribution of meiotic recombination across the genome due to the presence of chromosomal fusions. Taking it all together, our results provide a direct insight into the effect of the presence of Rb chromosomes and *Prdm9* homozygosis on the recombination.

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HOMOLOGOUS CHROMOSOMES PAIRING PRECEDING THE MEIOSIS ONSET IN MICE SPERMATOGENESIS

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During meiosis, pairing, synapsis and recombination of homologous chromosomes are key processes in the production of genetically diverse haploid gametes with respect to the parental genome. The study of different model organisms has shown that the molecular mechanisms and timing of synapsis and recombination are conserved. However, the knowledge regarding pairing is limited.

The objective of this study is to characterize the timing of the homologous chromosomes pairing during mice spermatogenesis. To do so we have applied a protocol consisting on the following steps: 1) testicular tissue enzymatic disaggregation; 2) immunolabeling of undifferentiated spermatogonia (GFRα1), differentiated spermatogonia (cKIT) and primary spermatocyte (SYCP3); 3) cell-populations isolation using Fluorescence-Activated Cell Sorting; 4) paring analysis using Fluorescence *In-situ* Hybridization; 5) image capture by confocal microscopy; 6) image analysis using ImageJ/Fiji and 7) statistical analysis.

With the use of this procedure, we have selected a GFRα1+ spermatogonia population, representing a 0,7% of the testicular cell suspension. Pureness assessment was carried on by fluorescence microscopy and shown a 100% of cells positive for GFRα1 with a conserved DAPI pattern with multiple chromocenters. On the other hand, cKIT+ population represents an 8,97% of the testicular cell suspension. The pureness assessment was carried on by a γH2AX and SYCP3 immunolabeling. 96% of the cells were negative for both markers, meaning that these cells have not entered meiosis. In addition, 100% of negative cells for γH2AX and SYCP3 shown the same DAPI pattern as the GFRα1+ population.

Concerning homologous chromosomes pairing analysis, the study of differentiated spermatogonia (cKIT+) has been initiated. Image analysis from chromosome 5 indicates that both homologs occupy a single territory at 38% of the analysed cells. This value is double the value obtained for somatic cells (19,47%), suggesting that chromosome movements that gathers homologous chromosomes during pairing begins before the onset of meiosis.

APARELLAMENT DELS CROMOSOMES HOMÒLEGS ABANS DE L'INICI DE LA MEIOSI EN L'ESPERMATOGÈNESI DE RATOLÍ

Durant la meiosi, els processos d'aparellament, sinapsi i recombinació dels cromosomes homòlegs son essencials per la producció de gàmetes haploides i genèticament diversos respecte el genoma parental. Mentre que els estudis en diferents espècies model han posat de manifest que els mecanismes moleculars i la temporatilitat de la sinapsi i la recombinació estan àmpliament conservats, el coneixement en el cas de l'aparellament és limitat.

L'objectiu d'aquest treball és realitzar una caracterització de la temporalitat de l'aparellament dels cromosomes homòlegs durant l'espermatogènesi de ratolí. Amb aquesta finalitat hem aplicat un protocol d'anàlisi que consta dels següents passos: 1) disgregació enzimàtica del teixit testicular; 2) immunodetecció d'espermatogonis no diferenciats (GFRα1), d'espermatogonis diferenciats (cKIT) i d'espermatòcits primaris (SYCP3); 3) obtenció de les poblacions mitjançant selecció cel·lular activada per fluorescència; 4) anàlisi de l'aparellament mitjançant hibridació *in-situ* fluorescent; 5)

captura d'imatges amb microscòpia confocal; 6) anàlisi de les imatges mitjançant ImageJ/Fiji i 7) anàlisi estadística.

Mitjançant aquest procediment, hem seleccionat una població d'espermatogonis GFRα1+ que representa el 0,70% de la suspensió de cèl·lules testiculars. La comprovació de la puresa d'aquesta població s'ha realitzat mitjançant microsòpia de fluorecència i va mostrar un 100% de cèl·lules amb marcatge positiu per GFRα1 amb un patró de DAPI conservat amb múltiples cromocentres. D'altra banda, la població cel·lular cKIT+ va representar un 8,97% del total de la suspensió cel·lular. La verificació de la puresa es va realitzar mitjançant una immunodetecció de γH2AX i SYCP3 i es va observar que un 96% de les cèl·lules eren negatives per ambdós marcadors, es a dir, eren cèl·lules que no havien iniciat la meiosi. A més, el 100% de les cèl·lules negatives per γH2AX i SYCP3 presentaven el mateix patró de DAPI que la població GFRα1+.

Pel que fa a l'anàlisi de l'aparellament dels cromosomes homòlegs, s'ha iniciat l'estudi a la població d'espermatogonis diferenciats (cKIT+). L'anàlisi de les imatges corresponents a la detecció del cromosoma 5 indiquen que ambdós homòlegs ocupen un sol territori al 38% de les cèl·lules analitzades. Aquest valor dobla el percentatge observat en cèl·lules somàtiques (19,47%), la qual cosa suggereix que els moviments cromosòmics que aproximen els cromosomes homòlegs durant l'aparellament comencen abans de l'inici de la meiosi.

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FAST EVOLUTION OF piRNA CLUSTERS IN MOUSE

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Piwi-interacting RNAs (piRNAs) are small non-coding RNAs responsible for the silencing of transposable elements in the germline of most animals. They are produced from long single-stranded transcripts that derive from discrete genomic loci called piRNA clusters. piRNAs and piRNA clusters are highly diverged between species showing almost no evidence of selection constraint. Considering their fast turnover, we wondered how the expression of piRNA clusters evolves in short evolutionary time scales. To address this, we focused on differences in postnatal piRNA expression in different inbred strains of mice and closely related murine species. We found significant differences in piRNA clusters within and across species. Within Mus musculus, we found that clusters with polymorphic endogenous retroviruses were overrepresented among those with highly variable piRNA cluster expression. Comparing closely related murine species that shared a common ancestor six million years ago, we found high divergence in the expression of piRNA clusters. We considered the effect of features previously associated with piRNA-producing protein-coding genes in the turnover of piRNA clusters. Although genes with long untranslated regions (UTR) and long first exons are significantly associated with piRNA production, we found no evidence that changes in UTR or exon length lead to changes in piRNA production between murine species. We found that the best predictor of piRNA cluster expression conservation is piRNA cluster expression level. Taken together our results reveal that young endogenous retroviruses are potent drivers of piRNA cluster gains and that piRNA abundance is linked to piRNA evolution.

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GONADAL DEVELOPMENT, GENE EXPRESSION PROFILE AND SEX REVERSAL MECHANISM IN SPOTTED SCAT, *SCATOPHAGUS ARGUS*

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The spotted scat (*Scatophagus argus*) is an economically important marine fish with an XX/XY sex-determining system and exhibits sexual growth dimorphism favoring females. We have previously developed genetic sex identification markers from *dmrt1* and *dmrt3⊿-Y* genes, both of which are exclusive to XY-males. Meanwhile, little is known about the development, patterns of gene expression in the early gonads, and sex reversibility status. Herein, we employed morphological and molecular means to fill in the above research gaps. Germ cells are an essential indicator of the undifferentiated gonads, and were found to significantly increase in female from 3 months post-hatch. Prior to males, females experienced sexual differentiation as evidenced by the primary oocyte and spermatocyte proliferations at 3.5 and 4 mph, respectively. Gonadal transcriptomics revealed a total of 37575 genes, of which 17656 were co-expressed, 18847 and 18728 were expressed in differentiating testes and ovaries, respectively. A total of 4410 DEGs were identified between the testes and ovaries. Genes attributable to male pathways such as *gsdf, amh, and cyp17a1* were significantly expressed in testes, while female-related genes *foxl2, cyp19a1a, 42sp50,* and *sox3,* were expressed considerably in ovaries. We also found a first incidence of a transient intersex associated with testis-ova (ectopic primary oocytes; Ecto-PO) in some male individuals reared in a controlled environment. These males with Ecto-PO are between 6-12 mph which degenerates during sexual maturation. The Ecto-PO in testes did not affect the expression patterns of male and female sex-related genes by qPCR. In addition, the administration of Estradiol (E_2) could reverse 2-3 mph XY-male to phenotypic female (XY-female) while Methyltestosterone (MT), and Letrozole (Le) did not. Whiles the gonads of E_2-treated fish were normal, that of MT and Le males were morphologically defective, and bulky in size, respectively. This study explicitly presents valuable information for research development on differentiation and sex control in spotted scat. The study also opens new research chapters to ascertain 1) the mystery behind the Ecto-PO, 2) the fertility status of XY-female, 3) the possibility of mono-sex production.

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DNA METHYLATION DURING EARLY DEVELOPMENT IN DIPLOID AND TRIPLOID EUROPEAN SEA BASS

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Triploidy can be induced by retention of the 2nd polar body by temperature or pressure shocks. Induced triploidy is used in the aquaculture of some fish and mollusks to increase growth and avoid the problems associated with sexual maturation, since induced triploids, particularly females, are sterile. Most research on the consequences of induced triploidy has been focused on survival, growth, and reproductive physiology. However, less is known on the epigenetic involvement and the limited literature reveals contrasting results. Thus, no differences were detected between diploid and triploid brown trout (*Salmo trutta*). In triploid oysters (*Crassostrea gigas*), different levels of DNA methylation were observed related to their fertility status. In allotriploid cyprinids, DNA methylation was involved in dosage compensation, resulting in similar gene expression levels than in diploids. Although recently disrupted gene expression was found in allotriploid hybrid grouper during the middle gastrula stage, no information is available on the effects of induced triploidy on DNA methylation patterns during early development in fish, which was the goal of this study. The European sea bass (*Dicentrarchus labrax*), was used as a model. Triploidization was induced by a cold shock of eggs, at 5 min post fertilization (mpf), at 0°C for 10 min. Three stages of development (90% epiboly, 30 mpf; hatching, 92.5 mpf; mouth opening, 5 days post hatching, dph) and two distinct families were analyzed to account for biological variation. Reduced representation bisulfite sequencing (RRBS) was used to quantify DNA methylation. Samples were processed in two different pools. Quality control analysis revealed a batch effect, as different number of CpGs were covered, depending on which pool the samples belonged to. In order to get with this issue, stringent parameters were used for the statistics, only considering that CpGs covered in all samples, correcting for over dispersion and accounting for covariate, when Differentially Methylated Cytosines (DMCs) were quantified. From a preliminary analysis, 379 DMCs (369 hyper- and 10 hypo-methylated) were identified by comparing triploid against diploid fish, regardless of the stage of development or the family. The genomic localization of these samples and the genes and functions affected will be discussed.

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PROTEOMIC PROFILING OF TELEOST SPERMIOGENESIS REVEALS THE ROLE OF CYTOPLASMIC TRANSLATION DURING SPERMATOZOA MATURATION

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In teleost fishes, the molecular mechanisms involved in the transformation of haploid germ cells (HGC) into mature and functional spermatozoa (spermiogenesis) are largely unknown. Here, this process was investigated in the gilthead seabream (*Sparus aurata*), a fish that does not condense chromatin with protamines, by determining the changes in the whole proteome of HGC and intratesticular (immature) and ejaculated (mature) spermatozoa (SPZ_{IT} and SPZ_{EJ}, respectively) using LC-MS/MS-based quantitative proteomics. The HGC and SPZ_{IT} were isolated by fluorescence-activated cell sorting, whereas SPZ_{EJ} were collected by manual stripping of naturally spermiating males. Proteomic analysis identified 539 proteins across HGC, SPZ_{IT} and SPZ_{EJ}, which could be clustered into four different protein dynamic profiles from HCG to SPZ_{IT} and SPZ_{EJ}, from which two clusters comprised proteins more accumulated in SPZ_{IT}, and the two remaining contained proteins higher expressed in SPZ_{EJ}. Gene ontology analysis revealed enrichment of categories associated with transcription, translation, and protein synthesis, folding and turnover, in SPZ_{IT}, while the majority of proteins significantly accumulated in SPZ_{EJ} were related to the formation of the cytoskeleton and flagella and cell movement. To investigate further whether transcriptional and/or translational events occur during the maturation of seabream spermatozoa, an additional comparative proteomic profiling of the nuclear fraction of immature spermatozoa collected from the efferent duct (SPZ_{ED}) and SPZ_{EJ} was conducted. This second analysis identified 224 proteins differentially accumulated between SPZ_{ED} and SPZ_{EJ} and confirmed that most of the proteins enriched in SPZ_{ED} were associated with RNA processing and cytoplasmic translation, suggesting that translational mechanisms occur in maturing spermatozoa. This hypothesis was tested and corroborated by *in vitro* translation experiments in which isolated SPZ_{ED} were incubated with lysine transfer RNA labeled with the fluorophore BODIPY. However, we found that some histone lysine methyltransferases, as well as specific epigenetic modifications of histone H3 (H3K14ac, H3k4me3 and H3K79me2) known to be related to transcriptional activation in vertebrates, were also more accumulated in SPZ_{ED}. These data indicate that seabream post-meiotic spermatozoa are translationally active and suggest that they may also be transcriptionally competent. Such late-stage transcriptional and translational mechanisms might be essential for the expression of genes and epigenetic marks required for the acquisition of full sperm function.

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TWO LUTEINIZING HORMONE RECEPTORS REGULATE FLATFISH SPERMIOGENESIS

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During the semi-cystic spermatogenesis of the marine flatfish Senegalese sole (*Solea senegalensis*), ligand-activation of the luteinizing hormone receptor ba (Lhcgrba) in mature spermatids released into the lumen of the seminiferous tubules promotes spermatozoa differentiation. To understand the regulation of this pathway in germ cells, we investigated the transcriptomic changes between immature and mature spermatids (i.e. attached to Sertoli cells or free in the lumen, respectively) , isolated by fluorescence-activated single cell sorting, using RNA-seq. Amongst the mRNAs upregulated in mature spermatids we identified a novel duplicated paralog of the Lhcgr, that we termed Lhcgrbb. The cloning and phylogenetic analysis of Lhcgrbb confirms that the coexistence of two Lhcgrs is conserved in many teleosts. Functional assays in HEK293T mammalian cells showed that while the Lhcgrba is specifically activated by rLh, the Lhcgrbb is also able to respond to high doses of rFsh. *In situ* hybridization confirmed redundant mRNA expression of the *lhcgrba* and *-bb* in the somatic Leydig cells, as well as in immature and mature spermatids. However, both RNA-seq and qRT-PCR indicated the upregulation of *lhcgrbb* transcripts in mature spermatids, while those of *lhcgrba* remain unchanged during spermatid maturation. immunostaining using paralog-specific antibodies revealed the sequential protein expression of each Lhcgr in spermatids, the Lhcgrba being expressed in early maturing spermatids whereas the Lhcgrbb was detected only in elongating spermatids and differentiated spermatozoa Interestingly, bioinformatic analyses of the putative gene promoters of *lhcgrba* and *-bb* predicted the presence of common and paralog-specific potential binding sites for transcription factors, some of which were found to be upregulated in mature spermatids by RNA-seq. In addition, transcriptomic analysis indicated the regulation of several hormonal pathways and eukaryotic translation initiation factors during spermatid maturationthat could be involved in the specific regulation of each Lhcgr during germ cell development. Altogether, our results therefore suggest the presence of two distinct Lhcgrs in sole spermatids which could be differentially regulated to control the differentiation of haploid germ cells into spermatozoa.

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CRYOPRESERVATION OF BULL SPERM INCREASES SINGLE-STRANDED DNA BREAKS, MAINLY IN TOROID LINKER REGIONS

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Cryopreservation is a routinely used method to preserve sperm in cattle. This procedure is, however, well known to cause major harm to sperm cells, affecting their function and survival. Regarding sperm chromatin, the integrity of Toroid Linker Regions (TLR) has been suggested to be critical for the capacity of sperm to generate viable embryos. The present study, therefore, aimed to address whether cryopreservation increases single- and double-stranded DNA breaks in bovine sperm, and which chromatin regions are mostly affected by this damage. For this purpose, sperm samples from 12 bulls were evaluated before and after freeze-thawing to determine: i) the incidence of single- and double-stranded DNA breaks (SSB and DSB) utilizing the Comet assay (OTM parameter), and ii) the size of the fragments generated by these DNA breaks through pulsed-field gel electrophoresis (PFGE). The Comet assay revealed an increase of SSB in frozen-thawed sperm compared to fresh sperm (9.087 ± 1.386 vs. 4.942 ± 1.009; $P < 0.0001$), whereas no significant changes in DSB were observed (1.372 ± 0.201 vs. 1.337 ± 0.295; $P > 0.05$). On the other hand, PFGE revealed that most DNA breaks (45.869 % ± 4.700 % of the total DNA damage) were associated to fragments ranging between 15 and 145 Kb, which would be compatible to the size of one to three toroidal structures. In conclusion, this study demonstrated, for the first time, that the cryopreservation- induced DNA damage in bovine sperm occurs in TLR and that it affects one rather than the two DNA strands.

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HARNESSING EXOSOMES FOR REPRODUCTIVE BIOLOGY: DELIVERY OF MOLECULAR CARGO TO MOUSE EMBRYOS

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Extracellular vesicles (EVs) are membrane-contained particles that play critical roles in cell-cell communication, are secreted by a variety of cell types, and are involved in physiological reproductive processes. EVs are increasingly being used as drug delivery vehicles as they can be synthesised in vitro, loaded with exogenous cargo, and delivered to recipient cells while being protected from extracellular degradation or modification. The aim of this study was to investigate the putative use of exosomes as a non-invasive delivery tool to mammalian embryos. Exosomes were produced in vitro from human embryonic kidney 293 (HEK293T) cells and loaded with fluorescent and genomic cargoes. Murine embryos were exposed to 1×109 mL-1 of naked exosomes, 2×109 mL-1 of naked exosomes, 1×109 mL-1 of BODIPY-labelled exosomes, or to 2×109 mL-1 of DNA-loaded exosomes for 48 hours via in vitro co-incubation or microinjection. The exposure of murine embryos to naked exosomes, BODIPY-labelled exosomes, or DNA-loaded exosomes had no detrimental impact on blastocyst quality, the expression levels of pluripotency genes, or DNA integrity. After 48-hours of in vitro culture, there was no significant difference ($p > 0.05$) in survival rate between non-exosome exposed embryos (controls) and exosome-exposed embryos, and more than 75% of exosome-exposed embryos had reached the blastocyst stage. There was no significant difference ($p > 0.05$) in the expression levels of Cdx2 and Nanog in embryos cultured with naked exosomes or BODIPY-labelled exosomes when compared to non-exosome exposed embryos. The DNA fragmentation index of exosome-exposed embryos (18.2%) did not differ significantly ($p > 0.05$) from non-exposed embryos (21.9%). In addition, PCR analysis demonstrated that DNA-loaded exosomes were successfully internalized by embryos after both direct microinjection and in vitro co-culture. Overall, our findings suggest that HEK293T-derived exosomes can be loaded with cargoes and successfully delivered to and taken up by murine embryos without compromising their development or quality.

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ROBUST CLASSIFICATION OF MAMMALIAN EMBRYOS AND OOCYTES BASED ON LABEL-FREE HYPERSPECTRAL IMAGING AND ARTIFICIAL INTELLIGENCE

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Metabolic assessment of oocyte and embryo is crucial for evaluating their developmental competence. However, current methods to measure oocyte and embryo metabolism are either invasive, slow, or based on surrogates. In this study, we developed an innovative non-invasive Hyper-Spectral (HS) imaging method to measure intrinsic metabolic signals of mammalian oocytes and embryos. By using the correct light excitation, the concentration of auto-fluorescent intracellular metabolites can be quantified using HS imaging, encoding rich metabolic information from multiple metabolites (6+) simultaneously. Then, a cutting-edge Artificial Intelligence (AI) pipeline was designed to transform the data and predict the oocytes and embryos quality under different metabolic conditions. More specifically, we used a Support Vector Machine (SVM) algorithm under the Akaike Information Criterion (AIC) model, and cross validation was performed using 80%/20%. For completeness, we used a 5-fold cross-validation repeated 50 times.
A total of 96 mouse embryos and 178 oocytes were analyzed for the study. Embryo cohorts included control, glucose-starved, pyruvate/lactate-starved, and glucose/pyruvate/lactate-starved embryos. Using brightfield images, human graders correctly classified a lower percentage of the samples compared to the AI algorithm, with an area under the curve (AUC) of 51% in the human graders compared to 93.7% of the AI algorithm. Oocyte cohorts included oocytes obtained from young female mice analyzed either immediately after collection or after overnight culture (in-vitro aged), and from old female mice. We could separate the oocyte classes with an average AUC of 96.2% and make a statistical correlation with their blastulation efficiency after fertilization with an 82.2% AUC.
We also segmented images of individual mouse oocytes and embryos by combining two features of the FAD+ metabolite: mitochondrial confinement and a specific spectrum range. Using this segmentation method, we were able to extract HS enriched signals coming from mitochondria and measure three parameters - average intensity, intensity variance, and occupancy - to evaluate their potential in improving our algorithm.
Our HS imaging method offers a 4D real-time image of mammalian embryo metabolism during development in a label-free fashion. This safe and robust method could be translated in the IVF clinic as a tool to score oocyte and embryo potential.

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COVID-19 COMPROMISES SPERMATOGENESIS IN MAN

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The SARS-CoV-2 virus has infected over 760 million people worldwide in the last three years and caused almost 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. 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. Moreover, UTF1-positive spermatogonia presented more DNA damage than control cells, suggesting that COVID-19 could compromise spermatogenesis even after recovery. So, we conducted a study to examine the impact of SARS-CoV-2 infection on the testes of a small group of male individuals who had recovered from the virus infection. These patients 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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EFFECT OF SARS-CoV-2 INFECTION ON SPERM PARAMETERS: A NORTH-EAST SPANISH MULTICENTRIC RETROSPECTIVE STUDY

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Different studies with small sample size suggested that sperm production might be altered during and after SARS-CoV-2 infection. In the current study, we conducted a retrospective study with a larger number of samples comparing semen quality in men who attended our assisted reproduction units before and after SARS-CoV-2 infection, in order to decipher the impact of SARS-CoV-2 on sperm parameters. To carry out this project, sperm parameters from 191 infertile men who underwent semen analysis for fertility purposes during 2018 and 2019 (previous to the initiation of the COVID-19 pandemic) and between 2020 and 2023 (after a validated positive test of SARS-CoV-2), were compared before and after the infection. As a control group, we included 593 infertile patients with two seminograms at different time points before COVID-19 pandemic (2017-2019). Semen volume is the only parameter being significantly reduced after SARS-CoV-2 infection ($p<0.001$) by T-test on paired samples. Although semen volume is also significantly different between the two seminograms analysed in the control population, linear general model suggests that SARS-CoV2 infection has a detrimental effect on semen volume values ($p =0.02$). Furthermore, analysing differences according to spermatogenic cycle after infection and infecting strain, alteration in semen volume seems to be only significantly lower during the first spermatogenic cycle after infection ($p=0.003$) and more pronounced in subjects infected with the EU1-Omicron SARS-CoV-2 strain ($p=0.03$). Additionally, we detected alterations in progressive motility in patients infected with the alpha SARS-CoV-2 strain ($p=0.01$). In conclusion, SARS-CoV-2 might have some small effect on semen volume and sperm motility in infertile men according to the infective strain, although pre-infection values can be recovered over one spermatogenic cycle after infection.

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IS TELOMERE LENGTH A BIOMARKER OF SPERM QUALITY? A SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES

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Importance: Telomeres, are essential for the integrity of chromosome ends during cell division and their involvement in different processes linked to aging has been established. These chromosome components are involved in spermatogenesis and seem to play an important role in fertilization and embryo development. Telomere length is shortened with each cell division. Recently, short sperm telomere length (TL) has been proposed as a potential biomarker of male infertility. Objective: To conduct a systematic review and meta-analysis (SRMA) of studies exploring the association between sperm and/or leukocyte telomere length with sperm quality parameters and different infertility conditions. Data sources: A Medline literature search was conducted via PUBMED and Cochrane Library databases until May 2022. Study selection and synthesis: Eligible studies included were prospective cohort, cross-sectional and case-control studies and telomere length in sperm and/or leukocytes cells was defined as the exposure. Main outcomes: Semen quality parameters or infertility conditions (e.g., oligozoospermia, asthenozoospermia, teratozoospermia, or other spermatogenic impairment combinations) were defined as the outcomes. Results: Twenty-four observational studies were included. In the qualitative analysis, a high heterogeneity was observed between studies regarding the associations between TL and semen parameters in different normozoospermic/fertile and oligozoospermic/infertile populations. In the meta-analysis, sperm and leukocyte TL was shorter in infertile compared to fertile individuals (mean difference, MD; 95% confidence interval, CI) (-1.43; -1.66 to -1.21; P-value<0.001, and -1.67; -2.02 to -1.31; P-value<0.001, respectively). Moreover, in terms of sperm TL, these differences were also significant between individuals with a normal seminogram and individuals with low quantity of spermatozoa in the ejaculate (-0.97; -1.32, -0.61; P-value<0.001). Conclusion and relevance: The current systematic review and meta-analysis suggest the potential role of sperm or leukocyte TL as a reliable biomarker of semen quality which may help distinguish between infertility conditions beyond the routine semen analysis.

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DEVELOPMENT OF A NEW AUTOMATIC WORKFLOW FOR THE ANALYSIS OF SPERM FLUORESCENCE IN SITU HYBRIDIZATION SIGNALS BASED ON IMAGEJ/FIJI PLUGINS

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Fluorescence in situ hybridization (FISH) is a highly demanding cytogenetic method for checking for aneuploidies in sperm nuclei from infertile patients, and hence, to attribute a genetic cause in some cases of male infertility. Nevertheless, some technical characteristics compromise its routine application in some laboratories (i.e. analysis of large numbers of cells, time-consuming, skilled personnel required for the analysis).
The automation of the process could be an alternative to overcome these limitations. Nevertheless, systems developed so far exhibit important limitations that hamper its clinical implementation: 1) Raw automatic results greatly differ from the manual ones, 2) Automatic systems not only identify spermatozoa so other cells types potentially present in the ejaculate can be included in the analysis, 3) Time required for the automatic and the manual analysis is similar, 4) Computerized systems are costly to implement regarding the acquisition of hardware and software.
To solve these limitations, we have developed a new computerized workflow for the analysis of human sperm aneuploidies and diploidies. Sperm images from triple-color FISH hybridizations using centromeric DNA probes for chromosomes X, Y and 18 were captured using a epifluorescent microscope equipped with MetaMorph® that allows automatic image acquisitions. Image analyses were done using two different ImageJ/Fiji plugins: one allows identifying spermatozoa while discarding any other cell types (according to its size and circularity), and the other one determines their chromosome content (considering signal size, intensity, and distance, as in the manual analysis). The specificity (true negative rate) and sensitivity (true positive rate) of the system were determined by comparing the manual and automatic results obtained after the analysis of six slides from six different semen samples.
The specificity and sensitivity of the automated method in identifying sperm cells were 74.55%±10.51 and 52.42%±23.06, respectively. Concerning the identification of the FISH signals, high values of specificity and sensitivity were observed in all samples 96.44%±5.16 and 92.04%±7.76, respectively. Importantly, the automatic method drastically reduced the time frame required for the analysis.
This new automated FISH scoring workflow is a highly reliable method for the evaluation of FISH signals, although there is still room for improvement in the identification of sperm cells.

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MEIOSIS AND MATURATION OF HUMAN OOCYTES ARE INFLUENCED BY MITOCHONDRIAL METABOLISM

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Recent studies in human found that poor quality oocytes have compromised mitochondrial function, while mice studies suggest that successful meiotic maturation and metabolic competence are functionally linked. However, this relationship has not been clearly established in human. Metabolic dynamics can be visualized by indirect measurements through mitochondrial staining and quantified directly using non-invasive FluorescenceLifetimeImagingMicroscopy (FLIM). This study aims to characterize oocyte metabolism in maturing GVs and to establish the role of mitochondrial metabolism for successful meiosis in human.
A total of 264 immature GVs were included in the study. GVs were matured in vitro, and maturation was determined by polar body extrusion. Oocytes were evaluated by FLIM (n=123) and immunofluorescence (IF) analysis (n=77). Furthermore, loss of function studies (n=64) were performed by treating GVs with 1μM Trifluoromethoxy-carbonylcyanide-phenylhydrazone (FCCP) for 30minutes. The proteins Dihydrolipoamide-S-Acetyltransferase (D-LAT) and Translocase-of-outer-mitochondrial-membrane (TOMM20) were analysed in oocytes by IF (Arbitrary-Units, AU) to assess mitochondrial activity and localization, respectively. Fluorescence mean intensities were quantified with ImageJ and compared by t-test; maturation rates were compared by Chi-squared-test. FLIM comprehensive metabolism measurements were taken at GV stage. FLIM parameters were evaluated individually and combined into Redox ratio (intensity NADH /FAD+) throughout the entire GV-MII transition.
Mitochondrial staining showed a uniform localization of mitochondria in the ooplasm (TOMM20) and a subcortical localization of active organelles (D-LAT). These patterns were confirmed by NADH visualization with FLIM. FLIM revealed changes in metabolism during GV-MII transition (NADH average intensity increase of 11.7%, FAD+ increase of 16.6% p=3.965e-05). Further, FLIM imaging also revealed that GVs with a higher metabolism were more likely to complete meiosis and reach MII stage (Redox ratio 2e+00±0.17 in GVs matured to MII, 1e+00±0.18 in non-matured, p=1.054e-06). To establish the functional relationship between higher metabolic levels and a successful progression through meiosis, we lowered the metabolism of GVs by FCCP treatment, and noted a concomitant drop in maturation rates of treated GVs compared to untreated ones (39.5% vs 86.3%, respectively, p=0.00001)
Our findings provide further evidence of a functional link between oocyte metabolism and impaired meiosis, which may contribute to characterize the mechanisms needed to obtain high quality oocytes in human.
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CONSIDERATIONS FOR INTEGRATING ARTIFICIAL INTELLIGENCE IN REPRODUCTIVE BIOLOGY

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Artificial Intelligence (AI) algorithms have become ubiquitous in our lives, and the field of biosciences is no exception. In recent years, we have seen a growing number of publications in scientific journals and conferences on different AI-based applications in our field. However, the gap between publications and integration into clinical practice is being filled by commercial companies. As biologists who work in different types of centres but want to incorporate this technology into daily practice, we need to be sufficiently critical and clear about which criteria to follow when evaluating a new AI system, whether developed in-house or commercialized by another company. The most important factor in deciding whether to implement AI technology in our laboratories is that any new addition must improve results or have sufficient guarantees that it will do so, and in case it does not, it must offer some advantage such as facilitating work or improving safety, as long as it does not worsen results. It would also be necessary to evaluate several factors such as the user interface, scalability, technical support, update plan, cost, ethical aspects, and the experimental design on which the AI predictive model is based. It is important to understand the process of creating predictive algorithms (Machine Learning Lifecycle) as well as to know which metrics should be interpreted to better understand the expectations of predictive models before deciding to incorporate them in routine practice. Like any technology used in the laboratory, it would be pertinent to carry out external and internal quality control to determine if its application is justified and therefore improves procedures performed exclusively by human operators.

Els algoritmes d'intel•ligència artificial (IA) han esdevingut omnipresents a les nostres vides, i el camp de les biociències no n'és cap excepció. En els darrers anys, hem vist un creixent nombre de publicacions en revistes científiques i conferències sobre diferents aplicacions basades en intel•ligència artificial en el nostre camp. No obstant, el forat entre les publicacions i la integració a la pràctica clínica està sent ocupat per les companyies comercials i nosaltres, com a biòlegs que treballem en diferents tipus de centres però que volem incorporar aquesta tecnologia a la pràctica diària, hem de ser suficientment crítics i tenir clar quins criteris hem de seguir a l'hora de valorar un nou sistema d'intel•ligència artificial, ja sigui desenvolupat in-house o comercialitzat per una altra empresa. El més important per decidir si implementar tecnologia IA als nostres laboratoris és que qualsevol nova incorporació ha de millorar els resultats o tenir-ne prou garanties que ho farà, i en cas de no fer-ho, ha d'oferir alguna avantatge com facilitar la feina o millorar la seguretat, sempre i quan no empitjorin els resultats. Caldria valorar també tota una sèrie de factors com la interfície d'usuari, l'escalabilitat, el suport tècnic, el pla d'actualització, el cost, aspectes ètics i el disseny experimental en què se sustenta el model predictiu d'intel•ligència artificial. És important entendre com és el procés de creació d'algoritmes predictius (Machine Learning Lifecycle), així com saber quines mètriques s'haurien de saber interpretar per conèixer bé les expectatives dels models predictius abans de decidir incorporar-los rutinàriament. Com qualsevol tecnologia d'ús al laboratori, seria pertinent la realització de control de qualitat extern i intern per determinar si la seva aplicació estigués justificada, i per tant, millorés els procediments realitzats exclusivament per operadors humans.

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SkQ1 TREATMENT PRESERVES OVARIAN RESERVE IN MICE.

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In mammals, oocyte development and maturation are critical processes for female fertility. Lifestyle and diet habits seem to affect these processes significantly, resulting in differences in fertility capability among populations. Nevertheless, the genetic mechanisms regulating the ovarian reserve and the female meiotic cells surveillance are just beginning to be described. Natural aging and exogenous factors, such as alcohol consumption, have been linked to a diminished ovarian reserve in humans. Specifically, since ethanol causes oxidative stress, which has been associated with a decline in the quality of aging oocytes, we wondered if moderate alcohol consumption could damage the ovarian reserve in mice and if treatment with a mitochondrial-targeted antioxidant could revert it. To address this hypothesis, we administered, through the drinking water, the mitochondria-targeted antioxidant, SkQ1, to young C57BL/6 female mice for 14 weeks. To evaluate SKQ1 efficacy, we administered ethanol, an inductor of ROS linked to a diminished ovarian reserve in humans and co-administered SkQ1 and ethanol. Our preliminary findings reveal a significant reduction in primordial follicles in mice exposed to ethanol, suggesting that ethanol consumption diminishes the ovarian reserve. In addition, the SkQ1 treatment could revert these effects on the ovarian reserve and even increase the number of primordial follicles to control levels. To test the effects of the SkQ1 treatment in natural aging, we administered SkQ1 dissolved in DMSO into the drinking water of young C57BL/6 female mice for 14 weeks. Untreated mice lost around 45% of their primordial follicles, contrary to the SkQ1-treated animals, where the loss was just 22%. So, SkQ1 consumption rescued approximately 40% of the primordial follicles loss during 14 weeks. Based on these findings, we propose that the daily consumption of ethanol, even in small doses, could significantly affect the fertility status of mammalian females. However, an SkQ1 treatment could counterbalance the ethanol effects. More importantly, SkQ1 treatment can revert the natural aging effects and highly preserve the mammalian ovarian reserve.

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IMPACT OF POLYCHLORINATED DIBENZO-P-DIOXINS (PCDDS) AND POLYCHLORINATED DIBENZOFURANS (PCDFS) ON HUMAN SEMEN QUALITY.

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Introduction: The foods we eat and how we produce them could be a determinant of population health and the development of different chronic diseases such as obesity, diabetes and infertility. In particular, the incidence of infertility has increased considerably in the last decade. It currently affects about 15% of the world's population and male factors are responsible for 40-50% of cases. Several modifiable factors have been linked to impaired sperm quality, including diet type and dietary exposure to potential endocrine disruptors. It has been hypothesized that dietary exposure to polychlorinated dibenzo-p-dioxins (PCDDs) and to polychlorinated dibenzofurans (PCDFs), recognized as endocrine disruptors (EDs), could play an important role in occurrence of infertility and other chronic diseases. It is known that more than 90% of total PCDD/Fs exposure come from dietary sources. However, studies assessing the relationship between the dietary exposure to PCDD/Fs and human sperm quality are limited. Objective: To cross-sectionally assess the associations between dietary intake of PCDD/Fs and sperm quality parameters including: pH and semen volume, total sperm count, sperm concentration, vitality, progressive, non-progressive and total motility, immotility, and morphology in an adult male population. Methods: The dietary exposure to PCDD/Fs was estimated in 191 participants aged 18-40 years from the LED-FERTYL study, using the most updated levels in food (expressed as toxic equivalents-TEQ) and a 143-item validated food-frequency questionnaire. Associations between tertiles of PCDD/Fs dietary intake (in pgTEQ/week) and sperm quality parameters were assessed using lineal and logistic regression models adjusted by confounders. Results: Our results reveled that the highest tertile of PCDD/Fs dietary intake (T3) was related with higher levels of body mass index (BMI) (p=0.041) and higher consumption of fish (p=<0.001), red meat and derivates (p=<0.001). In addition, Participants in T3 showed an increase (β-coefficient [confidence interval]) in the percentage of abnormalities of the sperm head (6.02 % [1.24;10.78]; P-trend= 0.031). No other significant association was found in T3 but the tendency was in the expected direction for other morphological parameters and for semen volume, sperm concentration, and total sperm count. Conclusion: Higher dietary intake of PCDD/Fs was associated with sperm head abnormalities and higher levels in BMI. These preliminary analyses already point out major findings that should be studied further.

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STUDY OF NANOPLASTIC TOXIC EFFECTS ON MAMMALIAN SPERM FUNCTIONALITY

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Around 70% of the plastic produced around the world remains scattered around the planet as plastic waste. Over time, plastic breaks down into tiny fragments, creating massive amounts of particles with different shapes, sizes, and chemical composition. These polluting particles have been found in a wide spectrum of animal species, including humans, and it has been proved they can enter the body through different pathways including ingestion or inhalation.

It has been described that the continued and involuntary exposure to these particles represents a health threat to living organisms, including the risk of developing infertility. In rodent models, some studies suggest that the exposition to plastics induces a decrease in ovarian reserve capacity, reduced oocyte quality and poor sperm quality and quantity. However, the data are still scarce and preliminary, especially in humans.

To shed more light on the potential adverse effects of nanoplastics (NPs) in the male reproductive function, we have designed a study to determine the harmful effects of an *in vitro* exposure to NPs on ejaculated human spermatozoa and epididymal mouse spermatozoa.

Sperm cells have been incubated with polystyrene nanospheres (100 nm diameter) for a time frame of 24h. A fraction of the spermatozoa was obtained at different time intervals and the following sperm functional parameters were analysed: motility, vitality (Trypan Blue staining), membrane integrity (HOS test), oxidative stress (CellROX™ Green), acrosome integrity (FluoAcro kit) and apoptosis (Annexin V staining). Additionally, spermatozoa were incubated with dyed polystyrene nanospheres (Dragon Green) and the plasma membrane stain CellMask™ (Deep Red). Subsequently, slides were prepared and visualized in a confocal microscope to assess whether these nanospheres were adhered or internalized into the cells.

Preliminary results suggest that polystyrene nanospheres adhere to spermatozoa, a circumstance that could potentially hamper the functional parameters analysed. Final results will be disclosed and discussed in the symposium.

ESTUDI DELS EFECTES TÒXICS DELS NANOPLÀSTICS SOBRE LA FUNCIONALITAT ESPERMÀTICA DE MAMÍFERS

Al voltant del 70% del plàstic produït arreu del món roman dispers pel planeta com a residus plàstics. Al llarg del temps, aquests s'acaben convertint en petits fragments, creant grans quantitats de partícules amb diverses formes, mides i composicions químiques. Aquestes partícules contaminants s'han trobat en un ampli espectre d'espècies animals, també en humans, i s'ha demostrat que poden entrar al cos a través de diferents vies, com poden ser la ingestió o la inhalació.

S'ha descrit que l'exposició continuada i involuntària a aquestes partícules representa una amenaça per a la salut dels organismes vius, i que pot fer augmentar el risc de desenvolupar infertilitat. En models de rosegadors, alguns estudis suggereixen que l'exposició als plàstics indueix la disminució de la reserva ovàrica, la reducció de la qualitat dels oòcits, i baixa qualitat i quantitat de l'esperma. No obstant això, les dades encara són escasses i preliminars, especialment en humans.

Per dilucidar els possibles efectes adversos dels nanoplàstics (NPs) sobre la funció reproductiva

masculina, hem dissenyat un estudi per determinar els efectes nocius d'una exposició in vitro a NPs en espermatozoides humans ejaculats i espermatozoides de ratolí epididimals.

Amb aquesta finalitat, s'han incubat els espermatozoides amb nanosferes de poliestirè (100 nm de diàmetre) durant un període de temps de 24h. A determinats intervals de temps s'han obtingut fraccions de la mostra i s'han analitzat els següents paràmetres funcionals espermàtics: motilitat, vitalitat (tinció amb Blau de Tripà), integritat de membrana (HOS test), estrès oxidatiu (CellROX™ Green), integritat de l'acrosoma (FluoAcro kit) i apoptosi (tinció amb Annexina V). Per altra banda, una altra fracció d'espermatozoides s'ha incubat amb nanosferes de poliestirè marcades amb fluorescència (Dragon Green) i amb el colorant de membrana CellMask™ (Deep Red). Posteriorment s'han preparat extensions i s'ha avaluat si aquestes nanosferes s'havien adherit o interioritzat a les cèl·lules mitjançant la seva visualització en un microscopi confocal.

Els resultats preliminars suggereixen que les nanosferes de poliestirè són capaces d'adherir-se als espermatozoides, fet que podria comprometre els paràmetres funcionals espermàtics analitzats. Els resultats finals es discutiran al simposi.

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EFFECTS OF IN VITRO EXPOSURE TO NANOPLASTICS DURING MOUSE OOCYTE MATURATION

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Plastic pollution is a growing environmental concern, as global increase in plastic production together with its non-biodegradability and poor waste management have led to massive accumulation of plastics in both terrestrial and aquatic environments. Plastic fragments smaller than 5 mm (microplastics, MPLs) and 1 μm (nanoplastics, NPLs) have been recently found in all environmental compartments, and in several human tissues and fluids, and classified as emergent pollutants due to their potential risks for human health. Among these risks, studies in laboratory rodents have reported adverse effects on the reproductive system of both females and males. However, the impact of MPLs and NPLs exposure on gametes, particularly on female gametes, remains largely unknown.
The present study aims to investigate the effects of NPLs on mouse oocyte maturation in vitro and on the quality of the matured oocytes obtained. Immature oocytes were collected from the ovaries of CD-1 female mice (6-8 weeks old), 48 h after administration of 7.5 IU Pregnant Mare Serum Gonadotrophin. Ovaries were isolated in Hepes-buffered M199 medium containing 10% Fetal Bovine Serum (FBS), and follicles were punctured to release the oocytes. Cumulus-enclosed oocytes and non-enclosed oocytes were separately cultured in drops of M199 medium supplemented with 10% FBS, 0.1 IU Follicle Stimulating Hormone and 10 ng/ml Epidermal Growth Factor (37ºC, 5% CO2). In the exposed groups, plain or fluorescent polystyrene microspheres of 100 nm were added to the maturation medium (100 μg/ml). After 18 h of culture, cumulus cells were removed and oocyte maturation rates were determined. Oocytes exposed to fluorescent NPLs were fixed in 4% paraformaldehyde, stained with 1.5 U/ml Texas Red-phalloidin, and examined under a confocal microscope to assess NPLs internalization. Oocytes exposed to plain NPLs were stained either with 5 μM CellROX, to measure reactive oxygen species levels, or with 20 nM TMRE, to label active mitochondria. Next, stained oocytes were fixed in 4% paraformaldehyde and processed for immunofluorescence detection of α-tubulin. Results of in vitro maturation rates, NPLs internalization, oxidative stress, mitochondrial activity, spindle organization and chromosome alignment in control and exposed oocytes will be presented.

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GENOTOXIC EFFECTS OF NANOPLASTICS IN HUMAN PLACENTA JEG-3 CELLS

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Micro and nanoplastics (MNPs) are one of the main pollutants in the environment nowadays. Their ubiquitous distribution entails high availability for being uptaken by living organisms, which raises serious concerns about their potential harmful effects on ecosystems and human health (SAPEA 2019).
Since a person might accumulate several thousand MPs in their body over their lifetime (Nor et al, 2021), the consequences of MPs exposure and accumulation on human and fetal development is of relevant interest. In laboratory animals, maternal transfer of MNPs to the developing fetus during gestation has been observed after MNPs animals' exposure via ingestion (Cary et al., 2023) and inhalation (Fournier et al., 2020). Of relevance, the very few published studies on intergenerational effects indicate an increase in embryo resorption rates (Hu et al., 2021) and a decrease in the number of live births per dam (Park et al., 2020) after maternal exposure. Regarding human health, recently, MNPs have been detected in both sides of human placenta, maternal and fetal (Regusa et al., 2022; Zhu et al., 2023). Given that MNPs can bypass the placenta in laboratory animals, it is imperative to study the consequences of exposure to MNPs in human placental cells to predict possible adverse health effects during fetal human development.
Our study aims to evaluate the possible genotoxic effects that NPs may have on human JEG-3 choriocarcinoma cells, focusing mainly on the generation of DNA lesions. JEG-3 cells will be exposed during 24 and 48h to 100 μg/ml of 100 nm nanoplastic particles of different composition: polystyrene carboxylated □plain and/or fluorescent□ and polyethylene terephthalate. The possible genotoxicity of the NPs will be evaluated in two ways. On the one hand, the induction of DNA double-strand breaks will be evaluated by immunofluorescence of □H2AX and 53BP1 in JEG-3 nuclei exposed to NPs. On the other hand, the ability to correctly segregate the genetic material will be evaluated, analyzing the presence of micronuclei, nuclear buds and nucleoplasmic bridges in binucleated cells obtained from JEG-3 cells exposed to NPs.

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IMPACT OF NANOPLASTICS ON THE ZEBRAFISH GERM LINE

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Pollution from nanoplastics (NPs) is a raising environmental concern whose impacts on biodiversity and human health are far from being understood. In the aquatic environment most species base their reproduction on external fertilization. Yet, the effect of NPs on reproduction is barely known. Gametogenesis is a tightly regulated process by which gametes (oocytes and spermatozoa) are produced by the consecution of two meiotic divisions. Here, we explore the consequences of NPs exposure in both female (oogenesis) and male (spermatogenesis) zebrafish germ line. To do so, we evaluated the effects of a short-term (96 h) exposure of zebrafish to differently surfaced charged engineered polystyrene NPs and mechanically broken-down high-density polyethylene (HDPE) NPs. We show that, in males, NPs induced an unusual histological distribution and clustering of germ cells within the testis. The histological effect of NPs resulted in an increase sperm clustering when compared to the control group. Moreover, in females we observed an alteration in cell stages frequencies during oogenesis, reflecting oocytes' maturation and growth alterations. Overall, our results show that acute exposure to NPs had an effect at the histological level, in both males and females, most probably compromising reproductive fitness.

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IN VITRO EFFECT OF MANGANESE AND MAGNESIUM IONS ON DNA FRAGMENTATION

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Sperm DNA fragmentation is one of the major affectations of chromatin leading to male infertility. In vitro incubation of murine epididymal or vas deferens sperm with Mn2+ causes DNA breaks in toroid-linker regions (TLRs) through a mechanism known as Sperm Chromatin Fragmentation (SCF) and thought to involve the participation of topoisomerases and/or DNAses. This work aimed to elucidate if SCF also occurs in ejaculated sperm by assessing: i) the dose and incubation time response to sperm DNA fragmentation induced by MnCa and MgCa, and ii) the size of the resulting DNA fragments. For this purpose, Comet assay and pulsed-field gel electrophoresis (PFGE) analysis were performed to ejaculate samples previously treated with MnCa and MgCa in a concentration range between 0.1 mM to 50 mM and from 2 to 60 min at 37 ºC. The Comet assay revealed an active role of MnCa in causing DNA breaks, whose impact varied between concentrations and incubation times ($P < 0.001$), while a minor effect of MgCa on DNA integrity was observed. Incubations with either MnCa or MgCa showed no differences between permeabilized and non-permeabilized sperm, supporting the implication of an intracellular mechanism. Furthermore, PFGE revealed a higher incidence of DNA fragments ranging between 33 Kb and 194 Kb ($P < 0.05$). In summary, this study demonstrated that both MnCa and MgCa are capable of activating SCF in fully condensed and non-permeabilized sperm, following a dose-dependent response and via a specific intracellular pathway.

RELACIÓ D'AUTORS

ANNOTACIONS DE LA XVIII JORNADA DE BIOLOGIA DE LA REPRODUCCIÓ

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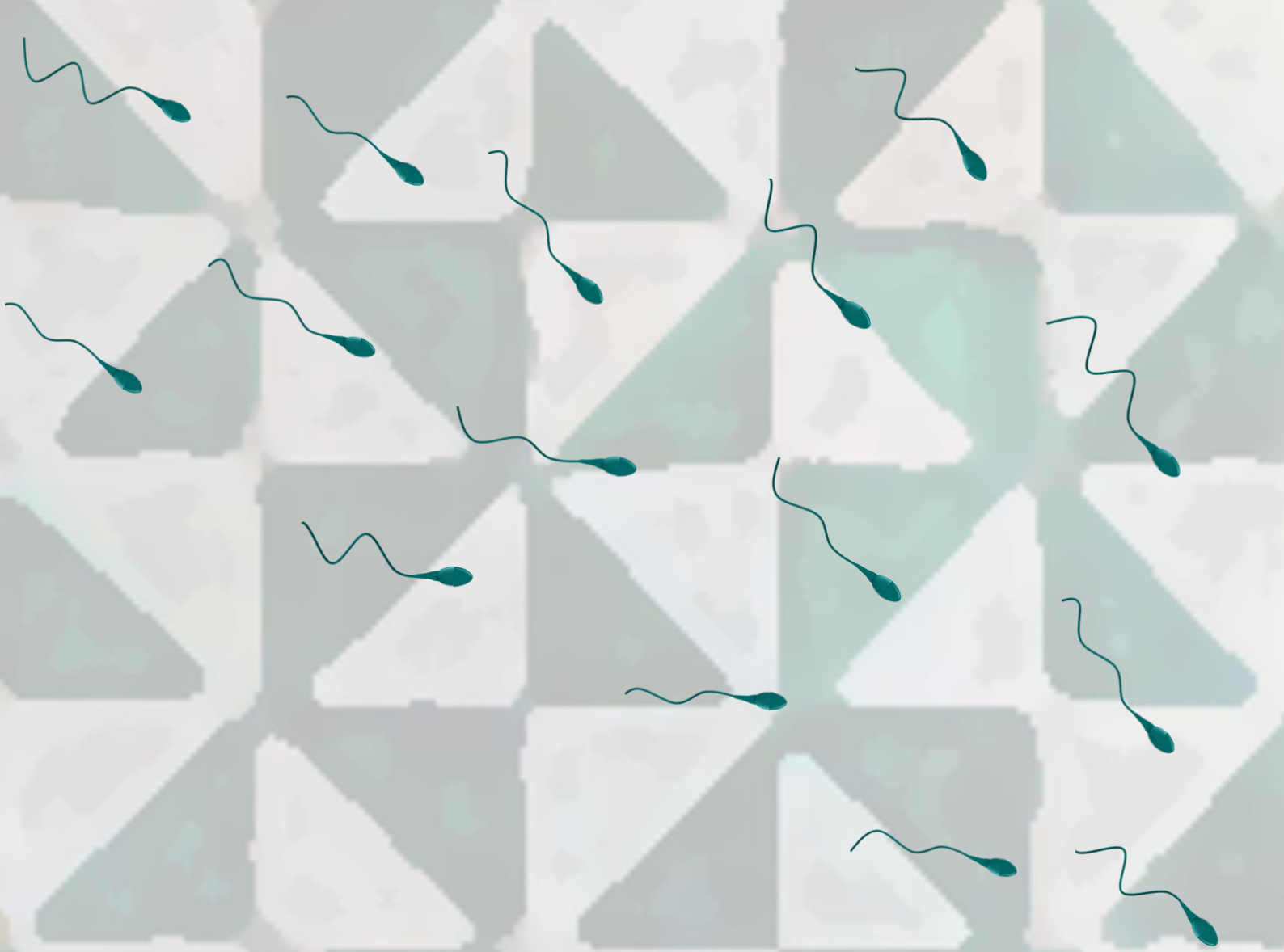

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