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The follicular fluid regulates sperm capacitation and the acrosome reaction in the donkey while alleviating oxidative stress

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ABSTRACT

The follicular fluid (FF) is a complex and dynamic biological medium that surrounds the oocytes during their development and growth. This fluid provides the necessary nutrients to both the oocyte and the surrounding cells, enabling the proper maturation of the oocyte. Additionally, the FF is involved in key sperm function processes, such as capacitation, which occurs when sperm reach the oviduct and are exposed to this fluid. Nevertheless, how this happens in donkeys has not been investigated. For this reason, the present study aimed to evaluate the influence of the FF on sperm capacitation in the donkey. Preovulatory FF was obtained from five different Catalan jennies (two follicles per animal), and nine ejaculates from fertile Catalan donkeys were collected. Sperm were resuspended in four different treatments: non-capacitating medium (TBM), non-capacitating medium supplemented with 20 % FF (TBM+FF), capacitating medium (TCM), and capacitating medium supplemented with 20 % FF (TCM+FF). Samples were incubated at 38 °C for 120 min, and at 60 min, progesterone was added to induce the acrosome reaction. After 0, 60, 70, and 120 min of incubation, sperm motility, membrane lipid disorder, acrosome integrity, mitochondrial membrane potential (MMP), and intracellular concentrations of calcium, reactive oxygen species (ROS) and superoxides were evaluated. These results suggest that the preovulatory FF promotes sperm capacitation in donkeys, as it increases intracellular calcium, membrane lipid disorder, and acrosome exocytosis, while maintaining sperm motility and reducing ROS concentration. Although the results are not entirely conclusive and further tests are needed, this study supports that the FF modulates sperm capacitation and alleviates the oxidative stress associated with this process.

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1. Introduction

Assisted Reproductive Technologies (ART) are revolutionising the equine industry by improving reproductive efficiency, maximising the genetic potential of individual animals, and offering valuable opportunities to produce offspring from specimens that have difficulty in reproducing, conceiving, or carrying a pregnancy to term (Hinrichs, 2018; Orsolini et al., 2021). In recent years, scientific interest has thus focused on the study and development of techniques, protocols, and procedures aimed at understanding and improving reproductive performance and ART outcomes (Cabeza and Gambini, 2023). Among these techniques, *in vitro* embryo production (IVP) has emerged as an essential tool, as it allows increasing the number of offspring that a female can produce throughout her reproductive life (Cuervo-Arango et al., 2019).

For decades, conventional *in vitro* fertilisation (IVF) has been widely used in both humans and animals; however, its application in equids has proven to be particularly challenging (Felix et al., 2022; Leemans et al., 2016a). In these species, after oocyte retrieval through ultrasound-guided follicular aspiration (OPU) and subsequent *in vitro* maturation (IVM), oocyte insemination is usually performed via intracytoplasmic sperm injection (ICSI) (Catalán et al., 2022), as traditional IVF has not produced successful results (Choi and Hinrichs, 2017; Galli et al., 2007). Nevertheless, recent studies have shown promising fertilisation rates using IVF in horses (Felix et al., 2022), which are phylogenetically close to donkeys but present significant reproductive disparities. Not only do these differences relate to sperm cells, including their motility and morphology, and how they interact with the female endometrium (Miró and Papas, 2018), but also to the composition of their seminal plasma (Catalán et al., 2023) and follicular fluid (Catalán et al., 2022).

While techniques such as OPU-ICSI have been developed and applied with commercially acceptable results in horses, the first report of a donkey embryo produced via OPU-ICSI was published only once (Flores Bragulat et al., 2023). It is important to note that the implementation of these techniques requires laboratories with costly equipment and highly trained personnel, so they are currently only available at specialised centres (Catalán et al., 2022). For this reason, there is a significant interest in developing and implementing conventional IVF in the donkey, yet a major obstacle is the lack of information on sperm capacitation in this species (Felix et al., 2022; Leemans et al., 2016a).

Capacitation is a biological process involving modifications in sperm as they travel through the female reproductive tract, enabling them to acquire the ability to fertilise an oocyte (Rathi et al., 2001; Visconti et al., 2011). This process involves a complex cascade of signalling mechanisms, including biochemical, biophysical, and functional changes in the sperm plasma membrane, such as increased membrane fluidity, cholesterol efflux, redox regulation, increased intracellular calcium, and tyrosine phosphorylation of proteins (Gervasi and Visconti, 2016; Hasan et al., 2021; Naz and Rajesh, 2004). Capacitation leads to a shift in the motility pattern known as hyperactivation and renders sperm competent to undergo the acrosome reaction, a physiological secretory event essential for fertilisation (Molina et al., 2018; Vigil et al., 2011; Visconti et al., 2011).

Mounting evidence suggests that, in the horse, the failure of IVF is due to the presence of not fully capacitated sperm, which prevents them from penetrating the zona pellucida and entering the oocyte (Maitan et al., 2022; Tremoleda et al., 2004). It is believed that in the donkey, like in the horse, this occurs because current capacitation or fertilisation media lack one or more oviductal factors necessary for proper capacitation (see ref. Leemans et al. 2016a for review). *In vivo*, the oviduct and its secretions provide a finely regulated microenvironment for the interaction of gametes. Several capacitation-related events are induced to regulate the release of sperm from the oviductal epithelium (Leemans et al., 2016a, 2016b, 2016c; Pérez-Cerezales et al., 2018; Suarez, 2008), and most studies indicate that exposure to oviductal secretions elicits sperm capacitation *in vivo* (Leemans et al., 2016a, 2016b; Pérez-Cerezales et al., 2018). It is therefore essential to thoroughly understand how donkey sperm become activated and acquire fertilising capacity within the oviductal microenvironment to identify the missing capacitation triggers in the currently used media (Catalán et al., 2025; Leemans et al., 2016a).

Within the oviduct, sperm are in contact not only with the fluids originating from the oviductal epithelium but also with those released alongside the oocyte, such as the follicular fluid (FF) (Hansen et al., 1991). The FF provides the microenvironment that supports oocyte maturation and, upon ovulation, is released together with the oocyte to the oviduct, where it can interact with sperm, potentially influencing fertilisation (Bartolomé et al., 2021; Getpook and Wirotkarun, 2007). This fluid contains nutrients, proteins, lipids, hormones, cytokines, and extracellular vesicles that, apart from being essential for oocyte development, they may directly influence sperm function (Bartolomé et al., 2021; Sysoeva et al., 2021). The components of the FF could, therefore, have the potential to modulate sperm capacitation, hyperactivation, and the acrosome reaction (Hasan et al., 2021; Jeon et al., 2001; Sysoeva et al., 2021).

Given the characteristics of the FF, there is particular interest in studying how this fluid may affect sperm function and, in particular, *in vitro* capacitation in donkeys, as the mechanisms of capacitation in this species are not yet fully understood. Thus, the present study aimed to address whether the FF modulates sperm capacitation. For this purpose, the response of donkey sperm after exposure to capacitating and non-capacitating media with or without 20 % FF was evaluated.

2. Materials and methods

2.1. Reagents and suppliers

Unless otherwise stated, all chemicals and reagents were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.2. Animals and samples

All donkeys used in this study (male and female) were fed a diet of mixed hay and basic concentrate, had *ad libitum* access to water, and were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), which operates under strict animal health and welfare regulations. This service functions under the approval of the Regional Government of Catalonia (Generalitat de Catalunya), Spain. Animal health status was good, as set in the guidelines established by Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016; thus, jackasses and jennies were free from equine infectious anaemia, contagious equine metritis, and equine viral arteritis. All protocols and procedures were carried out in accordance with the ARRIVE guidelines and all other applicable regulations for the use of animals in research (Law 5/1995, Generalitat de Catalunya; Royal Decree 53/2013, Spain; and Directive 2010/63/EU of the European Parliament and of the Council). This study was reviewed by the Ethics Committee of the Autonomous University of Barcelona (Code: CEEAH 1424), which declared it exempt from the requirement of obtaining ethics committee authorisation according to Article 1.5 of Directive 2010/63/EU.

2.2.1. Follicular fluid

Follicular fluid samples were obtained from 10 preovulatory follicles of five different Catalan jennies (two follicles per jenny) aged between 3 and 12 years. Before the extraction of these fluids, the reproductive tract, ovaries, and uterus of each jenny were examined via ultrasound (Esaote, MyLab™Gamma, Genova, Italy). The fluid was extracted from follicles $\geq$ 40 mm in diameter, accompanied by uterine oedema and in the absence of a corpus luteum in the ovaries, which is indicative of a preovulatory follicle in this species and breed as described by Taberner et al. (2008). The aspiration process consisted of an ultrasound-guided puncture through the flank, using a 20-mL syringe attached to a 20-g hypodermic needle, combined with the displacement of the ovary towards the flank by rectal palpation. Approximately 30 mL of fluid volume per follicle was extracted. This volume was subsequently divided into 5-mL aliquots, which were stored at −80 °C. Before use, the necessary FF aliquots were thawed and pooled into a single tube.

2.2.2. Semen samples

This study included nine ejaculates from three different Catalan donkeys (three ejaculates per donkey), aged between 4 and 11 years, and with proven fertility. To collect the semen, a Hannover artificial vagina (Minitube GmbH, Tiefenbach, Germany) was employed. This device was connected to a nylon mesh filter to remove the gel fraction. After that, the ejaculate volume was measured, and an aliquot was taken to determine the sperm concentration using a Neubauer chamber (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, Germany). Each ejaculate was then diluted (1:5; v:v) in INRA 96 extender (IMV Technologies, L'Aigle, France) previously warmed at 38 °C. Subsequently, a routine semen quality analysis was performed, evaluating sperm motility with a computer-assisted semen analysis (CASA) system (as detailed in Section 2.4.1), viability with a flow cytometer ($SYBR14^+/PI^-$; as detailed in Section 2.4.2), and morphology through eosin-nigrosine staining (Bamba, 1988). All samples met the standard quality thresholds (>60 % total motile sperm, >60 % viable sperm, and >70 % morphologically normal sperm). Each semen sample was then divided into four aliquots of equal volume and concentration (50 mL and 30 million sperm/mL).

2.3. Experimental design

After routine semen analysis and splitting each sample into four parts of equal volume and concentration, the aliquots (in 50-mL tubes) were centrifuged, and the supernatant was discarded. Subsequently, the pellet from each of the four tubes was resuspended in TBM (non-capacitating control medium; 20 mM HEPES, 96 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 21.6 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM $Na_2HPO_4 \times H_2O$, and 0.4 mM $MgSO_4 \times 7\ H_2O$), TBM supplemented with 20 % preovulatory FF (TBM+FF), TCM (capacitating control medium; 20 mM HEPES, 96 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 21.6 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4 \times 7\ H_2O$, 4.5 mM $CaCl_2{\cdot}2\ H_2O$, 3 mg/mL bovine serum albumin (BSA) and 10 mM sodium bicarbonate), or TCM supplemented with 20 % preovulatory FF (TCM+FF). Each of the resulting treatments was again split into four aliquots of 5 mL each, which were either evaluated immediately (0 min) or incubated for 60 min, 70 min, or 120 min at 38 °C, 5 % CO_2 and 5 % O_2 in a humidified atmosphere. After 60 min of incubation, progesterone (final concentration: 10 μM) was added to all treatments. At the corresponding time point (i.e., 0, 60, 70, or 120 min), 200 μL was taken from each aliquot. One hundred μL was used for motility assessment through a computer-assisted sperm analysis (CASA) system, and the remaining 100 μL was intended for flow cytometry analysis.

2.4. Evaluation of sperm motility

Sperm motility was evaluated using a CASA system (ISAS V1.0; Proiser S.L; Valencia, Spain). This system utilises a high-resolution camera connected to a microscope, capturing 25 frames per second (fps). Three μL of each sample was loaded into a Leja-20 chamber preheated to 38 °C. Samples were examined under a negative phase-contrast objective at 10 × magnification using an Olympus BX41 microscope (Olympus, Tokyo, Japan) equipped with a stage maintained at a controlled temperature of 38 °C. At least 1000 sperm cells were counted, analysing a minimum of five fields per sample. Each evaluation recorded the percentages of total motility (% TM) and progressive motility (% PM), along with kinetic parameters including straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), percentage of straightness (STR, %), percentage of linearity (LIN, %), percentage of oscillation (WOB, %), frequency of head displacement (BCF, Hz), and mean amplitude of lateral head displacement (ALH, μm). The CASA configuration adhered to the recommendations provided for equine sperm, which included the following settings: frames/s: 25 images

captured per second; connectivity: 6; particle area: > 4 and < 75 μm^2; and the minimum number of images to calculate ALH: 10. The cutoff value for motile sperm was VAP ≥ 10 μm/s, and for progressively motile sperm was STR ≥ 75 %.

2.5. Flow cytometry analysis

A CytoFLEX flow cytometer from Beckman Coulter (Indianapolis, IN, USA) was employed. Forward scatter (FSD) and side scatter (SSD) detectors were used to discriminate cells from debris. The following parameters were evaluated: acrosome integrity (*Arachis hypogaea* (peanut) conjugated with FITC [PNA-FITC]/[LIVE/DEAD] violet); membrane lipid disorder (merocyanine [M540]/Yo-Pro-1); intracellular concentrations of total ROS (dichlorodihyodrofluorescin diacetate [H_2DCFDA]/propidium iodide [PI]), superoxides (dihydroethidium [HE]/Yo-Pro-1), and Ca^{2+} (Fluo4-AM/PI); and mitochondrial membrane potential (JC-1/[LIVE/DEAD far red]). Information on flow cytometry analyses is provided according to the MIFlowCyt guidelines (Lee et al., 2008).

All samples were excited with the blue laser (488 nm), except LIVE/DEAD violet and LIVE/DEAD far-red fluorochromes, which were excited with ultraviolet (405 nm) and red lasers (638 nm), respectively. The fluorescence emitted by LIVE/DEAD violet was detected with the PB450 channel (450/45). The fluorescence emitted by SYBR14, Yo-Pro-1, M540, PNA-FITC, Fluo4, 2',7'-dichlorofluorescein (DCF; resulting from the oxidation and de-esterification of H_2DCFDA), and JC-1 monomers was collected through the FITC channel (bandpass 524/40 nm); the fluorescence emitted by JC-1 aggregates was detected via the PE channel (bandpass 585/42 nm); the fluorescence emitted by the LIVE/DEAD far-red fluorochrome was detected through the APC channel (bandpass 660/20 nm); and the fluorescence from PI and ethidium (E; resulting from the oxidation of HE) was measured with the PC5.5 channel (bandpass 690/50 nm). For each sample and analysis, two replicates of at least 10,000 sperm cells were evaluated.

2.5.1. Assessment of acrosome integrity

Acrosome integrity was evaluated following the protocol of Ritagliati et al. (2018) with minor modifications. Two hundred μL of each sperm sample was incubated with 1 μL LIVE/DEAD (LD) violet working solution (Thermo Fisher Scientific, Waltham, MA, USA) at 38 °C for 30 min in the dark, and then centrifuged at 660 g for 5 min. Samples were subsequently resuspended in a fixation solution (4 % paraformaldehyde; Thermofisher, Kandel, Germany) containing 0.1 % Triton X-100 and 0.3 % Tween-20, and incubated in a rotating shaker at room temperature for 30 min. Samples were centrifuged again at 800 g for 5 min, and pellets were resuspended in 200 μL PBS. The PNA-FITC fluorochrome (final concentration: 1.17 μM) was immediately added to resuspended samples, which were then incubated at 38 °C in the dark for 15 min. After incubation, samples were centrifuged at 8000 × g for 5 min; supernatants were discarded and pellets were resuspended in 150 μL PBS. Four sperm populations were identified in dot-plots: (1) viable sperm with an intact acrosome (PNA-FITC$^+$/LD$^-$), (2) viable sperm with an exocytosed acrosome (PNA-FITC$^-$/LD$^-$), (3) non-viable sperm with an intact acrosome (PNA-FITC$^+$/LD$^+$), and (4) non-viable sperm with an exocytosed acrosome (PNA-FITC$^-$/LD$^+$). Results are expressed as the percentage of viable sperm with either an intact (PNA-FITC$^+$/LD$^-$) or an exocytosed acrosome (PNA-FITC$^-$/LD$^-$).

2.5.2. Membrane lipid disorder (M540/Yo-Pro-1)

To assess membrane lipid disorder, sperm were co-stained with M540 and Yo-Pro-1, following the protocol of Rathi et al. (2001) with some modifications. Briefly, samples were stained with M540 (final concentration: 2.5 μM) and Yo-Pro-1 (final concentration: 25 nM) at 38 °C in the dark for 10 min. This protocol is based on the properties of M540, a hydrophobic fluorochrome that can intercalate within the plasma membrane. As the increase in membrane fluidity leads to a rise in M540 uptake, this fluorochrome is considered a reliable marker for plasma membrane destabilisation. Yo-Pro-1 is a vital stain that only labels sperm with increased membrane permeability. Four populations were identified: (i) viable sperm with low membrane lipid disorder (M540$^-$/Yo-Pro-1$^-$); (ii) viable sperm with high membrane lipid disorder (M540$^+$/Yo-Pro-1$^-$); (iii) non-viable sperm with low membrane lipid disorder (M540$^-$/Yo-Pro-1$^+$); and (iv) non-viable sperm with high membrane lipid disorder (M540$^+$/Yo-Pro-1$^+$). Results are expressed as the percentage of viable sperm with high (M540$^+$/Yo-Pro-1$^-$) membrane lipid disorder.

2.5.3. Intracellular Ca^{2+}

For the assessment of intracellular Ca^{2+} concentration, double staining with Fluo4-AM and PI was performed, following the protocol of Harrison et al. (1993) with some minor modifications. Fluo4-AM penetrates the cell and emits fluorescence after de-esterification and binding to Ca^{2+}; thus, the greater the fluorescence of Fluo4, the greater the intracellular concentration of Ca^{2+}. In brief, samples were stained with both Fluo4-AM (final concentration: 1.17 μM) and PI (final concentration: 5.6 μM) at 38 °C in the dark for 10 min. The Fluo4$^+$ intensity in viable sperm (PI$^-$) was used as a measure of intracellular Ca^{2+} concentration.

2.5.4. Intracellular ROS

A combination of H_2DCFDA and PI fluorochromes was used to assess the intracellular concentration of total ROS (Guthrie and Welch, 2006). Samples were incubated with H_2DCFDA (final concentration: 0.35 μM) at 38 °C for 20 min. H_2DCFDA is a non-fluorescent agent that can enter the sperm cell and react with ROS, thus converting into 2',7'-dichlorofluorescein (DCF), a green-fluorescent molecule. After the incubation period, PI (final concentration: 6 μM) was added to differentiate between viable and non-viable sperm, and samples were incubated for an additional 5-min period. For each sample, total ROS concentrations were expressed as the intensity of DCF in viable sperm (PI$^-$).

For the analysis of intracellular superoxide (O_2^-) concentration, samples were incubated with HE (final concentration: 5 μM) and Yo-Pro-1 (final concentration: 31.25 nM) at 38 °C for 30 min in the absence of light, as described by Guthrie and Welch (2006). HE permeates the sperm plasma membrane and is oxidised into ethidium (E), which emits red fluorescence, by O_2^-. Four different sperm

populations were distinguished: (i) non-viable sperm with low O_2^- concentrations (E^-/Yo-Pro-1^+), (ii) viable sperm with low O_2^- concentrations (E^-/Yo-Pro-1^-), (iii) non-viable sperm with high O_2^- concentrations (E^+/Yo-Pro-1^+), and (iv) viable sperm with high O_2^- concentrations (E^+/Yo-Pro-1^-). Results are expressed as the percentage of viable sperm with high superoxide concentrations (E^+/Yo-Pro-1^-) and the fluorescence intensity of E in the population of viable sperm (Yo-Pro-1^-).

2.5.5. Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated after staining with JC-1 (final concentration: 750 nM) and the LIVE/DEAD fixable far-red fluorochrome (Molecular Probes; Eugene, OR, United States), diluted at 1:8000 (v:v) in PBS, following the protocol of Garriga et al. (2024) with minor modifications. Samples were stained at 38 °C in the dark for 30 min. When sperm have a high mitochondrial membrane potential, JC-1 forms aggregates that emit orange fluorescence. On the contrary, low mitochondrial membrane potential maintains JC-1 molecules in the monomeric form, emitting green fluorescence. Mitochondrial membrane potential was expressed as the ratio between the fluorescence intensity of $JC\text{-}1_{agg}$ and $JC\text{-}1_{mon}$ in viable sperm.

2.6. Statistical analyses

Data were analysed using IBM® SPSS® 29.0 statistical package for Windows (IBM Corp., Chicago, IL, USA). Before analysis, the normality of the data was verified using the Shapiro-Wilk test, and the homogeneity of variances was checked with the Levene test.

A linear mixed model was used to evaluate the effects of treatments on sperm motility, membrane lipid disorder, acrosome integrity, mitochondrial membrane potential, and intracellular concentrations of calcium, total ROS and O_2^-. Pairwise comparisons were subsequently made using the post-hoc Sidak test. The within-subjects factor was the time of incubation (0 min, 60 min, 70 min, and 120 min), the fixed between-subjects factor was the treatment (TBM, TBM+FF, TCM or TCM+FF), and the random between-subjects factor was the donkey. All sperm parameters were considered dependent variables.

The significance level was set at $P \leq 0.05$, and all results are presented as the mean ± standard deviation (SD).

3. Results

3.1. Incubation of sperm with capacitating media or non-capacitating medium supplemented with FF increases sperm motility

As shown in Fig. 1a, total motility was greater ($P \leq 0.05$) in sperm incubated in TBM+FF, TCM, and TCM+FF than in those incubated in TBM, after 70 and 120 min at 38 °C. This was also observed in the percentages of progressively motile sperm (Fig. 1b), whose values were higher ($P \leq 0.05$) in sperm treated with TBM+FF, TCM, and TCM+FF than in those incubated in TBM, after 70 and 120 min of incubation at 38 °C.

Regarding kinetic parameters, as shown in Table 1, significant differences were observed between treatments at the same time point, with capacitating media (with or without FF) and non-capacitating media supplemented with FF showing higher values than the non-capacitating control (TBM). After 70 min of incubation at 38 °C, VCL values were higher ($P \leq 0.05$) in TCM and TCM+FF than in TBM and TBM+FF, and WOB was higher ($P \leq 0.05$) in TBM+FF than in TBM. At 120 min, VCL was higher ($P \leq 0.05$) in TBM+FF, TCM, and TCM+FF than in TBM. For VSL, the values observed in TBM+FF and TCM were higher ($P \leq 0.05$) than in TBM. Moreover, VAP and ALH were also higher ($P \leq 0.05$) in TBM+FF, TCM, and TCM+FF than in TBM.

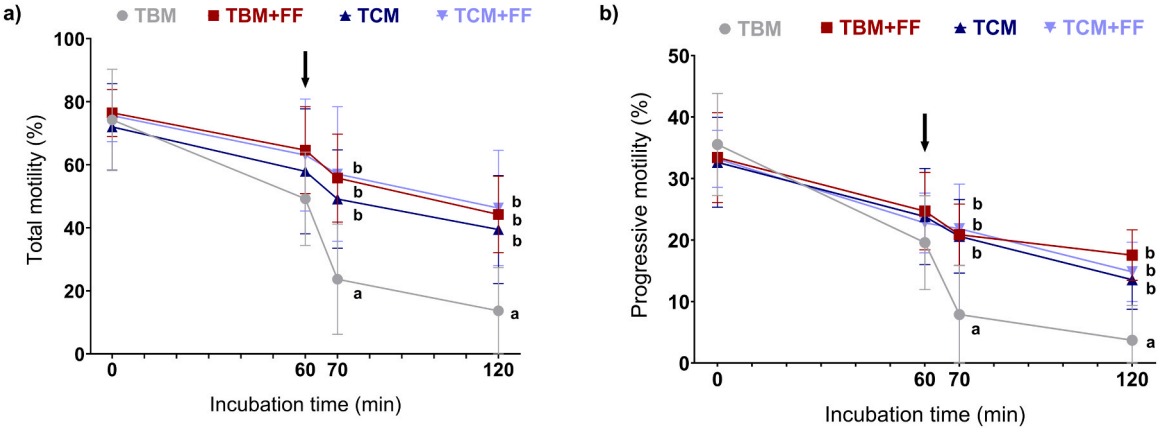

Fig. 1. Sperm motility. Percentages of **(a)** total motile and **(b)** progressively motile donkey sperm incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 0 min, 60 min, 70 min, and 120 min of incubation. Different superscript letters (a, b) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

Table 1
Kinetic variables of donkey sperm in the different treatments (TBM, TBM+FF, TCM, and TCM+FF) after 0, 60, 70, and 120 min of incubation at 38 °C.

Time (min)	Treatments	Sperm kinetic variables (mean ± SD)							
		VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
0	TBM	102.43 ± 13.79[a]	59.73 ± 13.73[a]	76.06 ± 14.00[a]	57.25 ± 10.18[a]	76.43 ± 6.99[a]	73.17 ± 7.41[a]	2.96 ± 0.19[a]	7.41 ± 0.61[a]
	TBM+FF	89.22 ± 12.145[a]	54.01 ± 12.39[a]	69.11 ± 12.82[a]	59.80 ± 10.57[a]	76.93 ± 6.99[a]	76.41 ± 7.71[a]	2.85 ± 0.45[a]	7.01 ± 0.66[a]
	TCM	91.50 ± 9.13[a]	56.15 ± 11.97[a]	70.89 ± 13.18[a]	60.15 ± 9.65[a]	77.69 ± 5.96[a]	76.14 ± 8.58[a]	2.91 ± 0.44[a]	6.94 ± 0.59[a]
	TCM+FF	87.46 ± 10.67[a]	53.74 ± 12.35[a]	68.03 ± 11.30[a]	59.74 ± 10.57[a]	76.40 ± 6.38[a]	76.36 ± 8.53[a]	2.74 ± 0.45[a]	6.67 ± 0.59[a]
60	TBM	70.92 ± 18.40[a]	43.07 ± 13.28[a]	53.31 ± 15.69[a]	58.09 ± 7.27[a]	78.79 ± 7.04[a]	72.80 ± 5.34[a]	2.92 ± 0.49[a]	6.77 ± 0.94[a]
	TBM+FF	72.71 ± 11.86[a]	41.99 ± 11.21[a]	53.84 ± 11.57[a]	56.76 ± 8.87[a]	77.04 ± 8.49[a]	72.99 ± 5.78[a]	3.04 ± 0.37[a]	6.78 ± 0.88[a]
	TCM	74.62 ± 15.43[a]	39.96 ± 9.87[a]	52.31 ± 11.27[a]	52.69 ± 6.37[a]	74.73 ± 5.03[a]	69.48 ± 5.48[a]	3.07 ± 0.52[a]	6.80 ± 0.66[a]
	TCM+FF	72.78 ± 10.67[a]	37.76 ± 8.61[a]	50.15 ± 10.08[a]	51.60 ± 6.63[a]	74.09 ± 6.38[a]	68.85 ± 6.26[a]	3.10 ± 0.52[a]	6.71 ± 0.99[a]
70	TBM	47.77 ± 15.96[a]	25.02 ± 12.31[a]	30.57 ± 13.47[a]	49.39 ± 9.06[a]	78.46 ± 8.47[a]	62.21 ± 8.12[a]	2.69 ± 0.78[a]	6.98 ± 2.70[a]
	TBM+FF	62.00 ± 9.08[a]	36.88 ± 8.76[a]	44.62 ± 8.36[a]	58.48 ± 7.29[a]	80.74 ± 4.21[a]	71.61 ± 5.54[b]	2.92 ± 0.35[a]	7.10 ± 0.89[a]
	TCM	64.68 ± 12.54[b]	36.40 ± 10.17[a]	44.04 ± 10.25[a]	55.27 ± 7.08[a]	80.87 ± 5.07[a]	67.66 ± 5.79[a]	3.21 ± 0.34[a]	8.12 ± 1.55[a]
	TCM+FF	63.4 ± 14.08[a]	35.12 ± 10.39[a]	44.08 ± 11.55[a]	54.19 ± 5.83[a]	77.75 ± 4.62[a]	68.81 ± 5.31[a]	2.98 ± 0.39[a]	7.24 ± 1.50[a]
120	TBM	39.40 ± 8.57[a]	18.60 ± 5.97[a]	23.80 ± 4.92[a]	46.76 ± 9.16[a]	76.67 ± 9.79[a]	60.89 ± 6.82[a]	2.79 ± 0.52[a]	6.98 ± 2.42[a]
	TBM+FF	62.76 ± 8.64[b]	33.84 ± 8.22[b]	43.30 ± 10.63[b]	52.25 ± 7.96[a]	76.52 ± 5.27[a]	67.51 ± 9.28[a]	3.50 ± 0.43[b]	6.76 ± 1.49[a]
	TCM	61.48 ± 12.90[b]	32.49 ± 9.45[b]	39.93 ± 11.80[b]	51.41 ± 5.84[a]	79.85 ± 6.68[a]	63.98 ± 7.35[a]	3.66 ± 0.35[b]	7.58 ± 1.90[a]
	TCM+FF	59.02 ± 10.94[b]	30.18 ± 6.29[a]	38.99 ± 9.14[b]	50.24 ± 5.00[a]	75.99 ± 5.65[a]	65.58 ± 7.03[a]	3.46 ± 0.20[b]	6.97 ± 1.37[a]

Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR), wobble coefficient (WOB), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF). Different superscript letters (a, b) indicate significant differences ($P \leq 0.05$) between the treatments within a single time point. Data are shown as mean ± standard deviation (SD) from nine separate experiments.

3.2. Treating sperm with capacitating media or non-capacitating medium supplemented with FF increases intracellular calcium concentration

The percentage of viable sperm with high intracellular Ca^{2+} concentration ($Fluo4^{+}/PI^{-}$; Fig. 2a) was higher ($P \leq 0.05$) in TBM+FF, TCM, and TCM+FF treatments than in TBM, after 60, 70, and 120 min of incubation. Additionally, this percentage was higher ($P \leq 0.05$) in TCM than in TBM+FF at 60, 70 and 120 min, and than in TCM+FF at 60 and 70 min. On the other hand, the intracellular calcium concentrations measured in viable sperm as Fluo4 fluorescence intensity (Fig. 2b) were higher ($P \leq 0.05$) in TCM than in TBM after 60, 70, and 120 min of incubation, and in TCM+FF than in TBM at 120 min.

3.3. Supplementing non-capacitating medium with FF has the same effect on membrane lipid disorder as its capacitating counterpart

When analysing the results of plasma membrane lipid disorder (Fig. 3), a greater percentage ($P \leq 0.05$) of viable sperm with high membrane lipid disorder was observed in TBM+FF, TCM, and TCM+FF treatments compared to the TBM treatment at 60, 70, and 120 min of incubation. Indeed, supplementing TBM with FF resulted in percentages of viable sperm with high membrane lipid disorder similar to those of TCM, regardless of whether this capacitation medium contained FF.

3.4. Adding FF to the capacitating medium increases the percentage of viable sperm that trigger acrosomal exocytosis in response to progesterone

Regarding acrosome integrity (Fig. 4), a higher percentage ($P \leq 0.05$) of viable sperm with an exocytosed acrosome (Fig. 4a) was observed in the treatments supplemented with FF (TBM+FF and TCM+FF) compared to non-supplemented media (TBM and TCM) after 60 and 70 min of incubation at 38 °C. Furthermore, at 120 min, percentages of viable sperm with an exocytosed acrosome were significantly higher in media supplemented with FF (TCM+FF and TBM+FF) than in non-supplemented media. Furthermore, the percentage of viable sperm with an exocytosed acrosome was higher ($P \leq 0.05$) in capacitating (TCM) than in non-capacitating (TBM) media. Related to these data, the percentage of viable sperm with an intact acrosome was lower ($P \leq 0.05$) in the capacitating medium supplemented with FF (TCM+FF; Fig. 4b) than in the non-capacitated, non-supplemented one (TBM) after 120 min of incubation at 38 °C.

3.5. Sperm incubated in non-capacitating media exhibit greater mitochondrial membrane potential (MMP) than those incubated in capacitating media, but only at 60 min

Fig. 5 shows the ratio between $JC\text{-}1_{agg}$ and $JC\text{-}1_{mon}$ in viable sperm, which serves as a measure of mitochondrial membrane potential. At 60 min, sperm incubated in non-capacitating, non-supplemented medium (TBM) exhibited greater mitochondrial membrane potential than those incubated in capacitating media (TCM and TCM+FF). No differences between treatments were observed at any other time points.

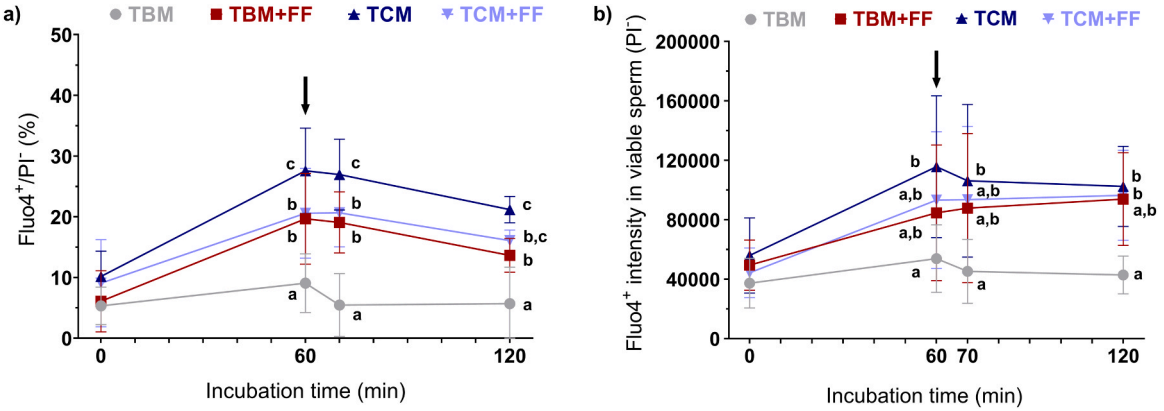

Fig. 2. Intracellular Ca^{2+} concentrations. **(a)** Percentage of viable sperm with high intracellular Ca^{2+} concentration ($Fluo4^{+}/PI^{-}$), and **(b)** fluorescence intensity of Fluo4 in viable sperm (PI^{-}), measured in donkey sperm incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 60 min, 70 min and 120 min of incubation at 38 °C. Different superscript letters (a–c) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

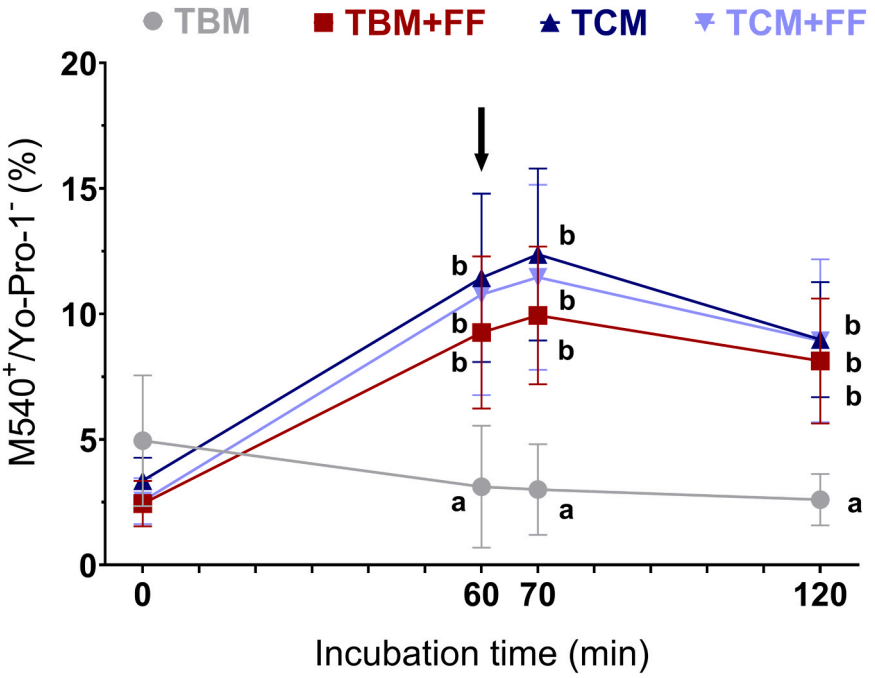

Fig. 3. Plasma membrane lipid disorder. Percentages of viable sperm (Yo-Pro-1$^-$) with high plasma membrane lipid disorder (M540$^+$) measured in donkey sperm incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 60 min, 70 min, and 120 min of incubation at 38 °C. Different superscript letters (a, b) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

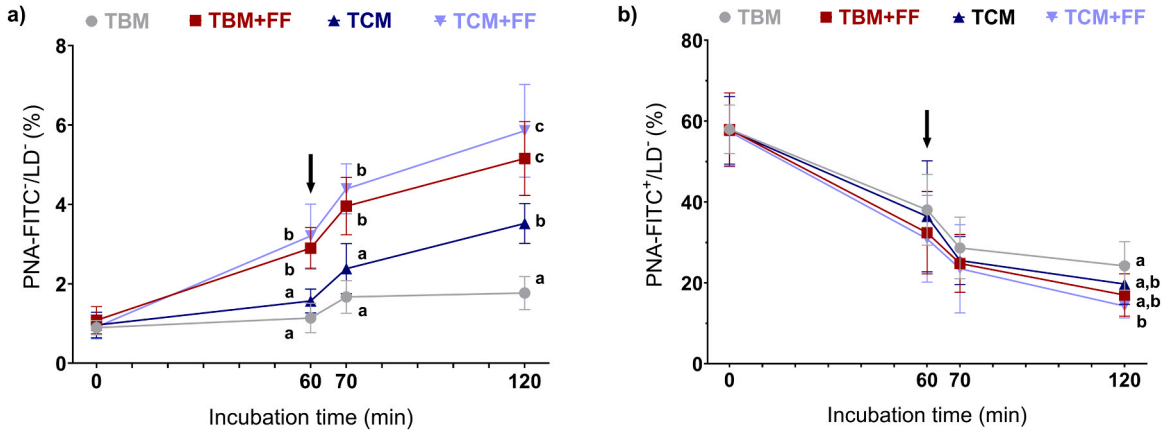

Fig. 4. Acrosomal integrity. Percentages of viable donkey sperm with **(a)** an exocytosed acrosome (PNA-FITC$^+$/LD$^-$) and **(b)** with an intact acrosome (PNA-FITC$^-$/LD$^-$) measured in samples incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 60 min, 70 min, and 120 min of incubation at 38 °C. Different superscript letters (a–c) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

3.6. The presence of FF mitigates the rise in total ROS and superoxide concentrations, regardless of whether sperm are incubated in capacitating or non-capacitating media

As depicted in Fig. 6a, intracellular concentrations of ROS in viable sperm were higher ($P \leq 0.05$) in the non-capacitating, non-supplemented medium (TBM) than in media (capacitating and non-capacitating) supplemented with the follicular fluid (TBM+FF and

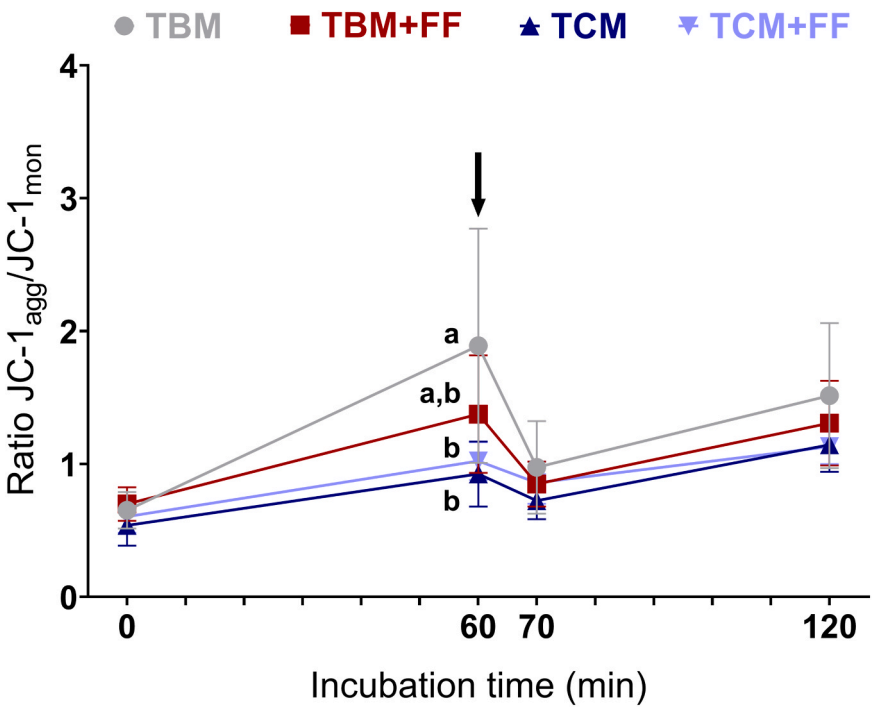

Fig. 5. Mitochondrial membrane potential. Ratio between JC-1 aggregates ($JC\text{-}1_{agg}$) and JC-1 monomers ($JC\text{-}1_{mon}$) in viable donkey sperm measured in samples incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 60 min, 70 min, and 120 min of incubation at 38 °C. Different superscript letters (a, b) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

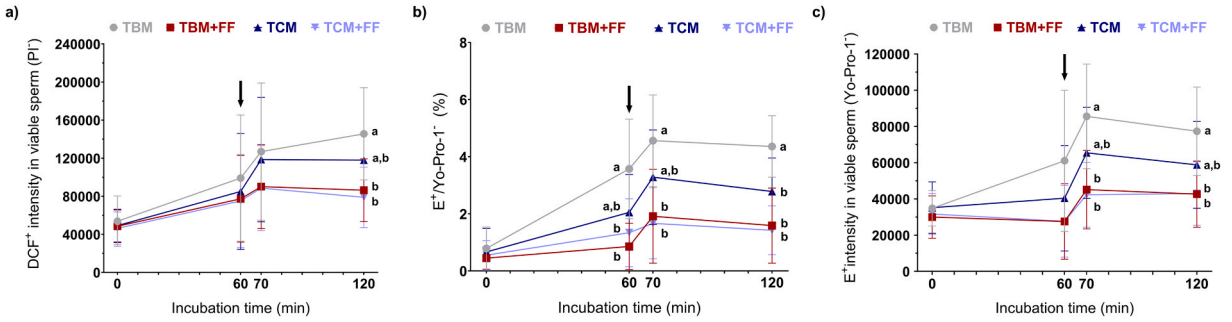

Fig. 6. Intracellular concentrations of ROS. **(a)** Total ROS as DCF^{+} intensity in viable sperm (PI^{-}), **(b)** percentages of viable sperm with high concentration of superoxides ($E^{+}/Yo\text{-}Pro\text{-}1^{-}$), and **(c)** superoxide concentration (O_2^{-}) as E^{+} intensity in viable sperm ($Yo\text{-}Pro\text{-}1^{-}$), measured in donkey samples incubated in non-capacitating medium (TBM; grey line), non-capacitating medium supplemented with 20 % follicular fluid (TBM+FF; burgundy line), capacitating medium (TCM; blue line), and capacitating medium supplemented with 20 % follicular fluid (TCM+FF; lilac line) at 0 min, and after 60 min, 70 min, and 120 min of incubation at 38 °C. Different superscript letters (a, b) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. The arrow indicates the addition of progesterone (60 min). Data are presented as mean ± standard deviation (SD) of nine independent replicates.

TCM+FF) after 120 min of incubation at 38 °C.

Similar results were observed for superoxides, as the percentage of viable sperm with a high concentration of superoxide in the non-capacitating, non-supplemented medium (TBM) was higher ($P \leq 0.05$) than in media (capacitating and non-capacitating) supplemented with the follicular fluid (TBM+FF and TCM+FF) (Fig. 6b), after 60 and 70 min of incubation at 38 °C. At 120 min, the percentage of viable sperm with a high concentration of superoxide was higher in TBM than in the other media. In agreement with these results, the relative amount of superoxides in viable sperm, as measured by E-fluorescence in viable sperm (Fig. 6c), increased in all treatments throughout incubation. Yet, the presence of the FF in capacitating (TCM+FF) and non-capacitating media (TBM+FF) mitigated that increase, as the E-intensity in viable sperm was higher ($P \leq 0.05$) in non-supplemented media (TBM and TCM) than in

their supplemented counterparts (TBM+FF and TCM+FF) after 70 and 120 min of incubation at 38 °C.

4. Discussion

Although fertility rates following artificial insemination (AI) with fresh and chilled semen are similar in the donkey and the horse, the outcomes of AI with frozen-thawed donkey sperm are very poor, limiting its use (de Oliveira et al., 2016). The development of other reproductive biotechnologies, such as IVP, is therefore of great interest for this species (Catalán et al., 2022). Moreover, the cryopreservation of IVP embryos would enable the conservation of male and female genetics, thus allowing for the restoration of breeds (Douet et al., 2017). In equids, however, there is no standardised, effective protocol for conventional IVF, with the lack of information on sperm capacitation being one of the main challenges.

As one of the periovulatory events consists of the encounter of sperm with the follicular fluid in the oviduct, the potential influence of the latter on the former has been investigated in other species (Esmaeilpour et al., 2014; Fabbri et al., 1998; Getpook and Wirotkarun, 2007; Leemans et al., 2015; Wang et al., 2001). These works have demonstrated that the FF may regulate functions such as sperm capacitation, acrosome reaction, chemotaxis, and sperm motility, which is consistent with the results obtained in the present study. Here, the impact of the FF on parameters related to sperm capacitation and functionality in the donkey was investigated, revealing a beneficial effect of incubating the male gametes with this fluid. Under our experimental conditions, not only did the FF enhance sperm motility but also promoted capacitation even in the absence of a specific capacitating medium, as the results of the TBM+FF treatment evidenced. In addition, compared to the capacitating medium (TCM), the FF attenuated the increase in the production of capacitation-associated ROS, without compromising this process. This finding suggests that the FF may modulate the oxidative status of sperm, enabling effective capacitation with controlled ROS levels, which is consistent with previous studies indicating that a certain concentration of ROS is necessary for sperm capacitation (Aitken, 2017; de Lamirande and Cagnon, 1993; Sánchez et al., 2010).

The higher values of sperm motility parameters – both total and progressive motility, as well as certain kinematic parameters – observed in the capacitating medium (TCM) and capacitating and non-capacitating media supplemented with 20 % FF (TBM+FF and TCM+FF) compared to the non-capacitating medium (TBM) suggest that the sperm motility pattern was influenced by both capacitating conditions and the FF presence, in addition to maintaining motility during incubation. The outcomes observed in the TCM treatment are in line with previous studies on sperm capacitation in species such as pigs (García Herreros et al., 2005) and cattle (Küçük et al., 2020), which may be attributed to the presence of calcium chloride ($CaCl_2$) and sodium bicarbonate ($NaHCO_3$) in this medium, compounds known to elicit capacitation (Harrison and Gadella, 2005; Popkiss et al., 2022). The effects of the capacitating medium would also be explained by components such as inorganic salts, carbohydrates (glucose, lactate, and pyruvate), amino acids, and proteins like BSA, which acts as a carrier for cholesterol during capacitation and has been demonstrated to support cell function and survival (Grasa et al., 2006; Molina et al., 2018; Travis et al., 2001).

The increase in sperm motility variables observed in the treatments containing the FF was similar to that reported in donkey (Catalán et al., 2025), human (Abu-Musa et al., 2001; Fabbri et al., 1998; Getpook and Wirotkarun, 2007; Mendoza and Tesarik, 1990) and sheep sperm incubated with the FF (El-Shahat et al., 2018). These beneficial effects could be attributed to specific FF components (e.g., hormones) that promote capacitation (Esmaeilpour et al., 2014; Fabbri et al., 1998; Getpook and Wirotkarun, 2007; Leemans et al., 2015; Wang et al., 2001), as well as to the presence of proteins, antioxidants (Hasan et al., 2021; Lee et al., 1992), and molecules with chelating activity, which protect sperm from the action of DNases (Bartolomé et al., 2021). The antioxidants would help regulate ROS concentration and mitigate the main adverse effects of oxidative stress in sperm cells (Catalán et al., 2025), which include DNA fragmentation, membrane lipid peroxidation, decreased motility, and ATP depletion (Catalán et al., 2024). Remarkably, the presence of FF in both capacitating and non-capacitating media alleviated the increase in total ROS and superoxides observed in their non-supplemented counterparts, along with improved maintenance of sperm motility. These findings are consistent with those of Catalán et al. (2025) in donkey semen. In contrast, sperm incubated in the non-capacitating medium exhibited increased intracellular ROS concentrations after 60 min of incubation, particularly superoxides, together with a simultaneous rise in mitochondrial activity and a decrease in sperm motility after the addition of progesterone (at 70 min). These observations could be explained, at least in part, by an uncontrolled increase in mitochondrial activity leading to ROS overproduction, potentially causing oxidative damage and impairing sperm functionality. Altogether, these findings support the hypothesis that the FF exerts a protective effect on sperm, possibly by modulating mitochondrial activity and oxidative status, which would allow in vitro capacitation conditions that better mimic what *occurs in vivo* within the oviduct.

Regarding the analysis of other parameters indicative of sperm capacitation, although our results are preliminary, they are very promising. The presence of the FF, even in the medium that, in principle, was non-capacitating (TBM), was found to induce *in vitro* capacitation. In particular, intracellular Ca^{2+} concentrations were higher in samples incubated in media supplemented with 20 % FF (TBM+FF and TCM+FF) and in the capacitating medium (TCM) than in those treated with the non-capacitating medium (TBM). Similarly, previous reports in humans (Brown et al., 2017; Fukui et al., 1995; Jeschke et al., 2021) and horses (Leemans et al., 2015) demonstrated an increase in intracellular Ca^{2+} concentration in sperm incubated with the FF or with specific components of this fluid. The increment in intracellular Ca^{2+} is a key event in the signalling cascade leading to capacitation, as it acts as an essential second messenger that triggers hyperactivation of motility and facilitates the acrosome reaction (Publicover et al., 2007; Yang et al., 2024). In other species, the FF has been reported to contain factors such as progesterone (which activates CatSper channels in sperm), molecules from extracellular vesicles, prostaglandins, and other modulators that induce a Ca^{2+} influx into the sperm cell, enhancing its responsiveness to physiological stimuli (Brown et al., 2017; Hasan et al., 2021; Jeschke et al., 2021; Suarez and Ho, 2003). Consistent with these findings, the percentage of viable sperm with high membrane lipid disorder followed a pattern similar to that of intracellular

Ca^{2+} concentration, as it was higher in non-capacitating and capacitating media supplemented with the FF (TBM+FF and TCM+FF) and in the non-supplemented capacitating medium (TCM) than in the non-supplemented, non-capacitating medium (TBM). The lipid disorder in the plasma membrane, detected using fluorescent probes such as merocyanine 540, reflects a redistribution of membrane lipids and a partial loss of cholesterol, which are characteristic features of capacitated sperm (Gadella and Harrison, 2002). These findings are consistent with those reported by Hamdi et al. (2010), who demonstrated that human FF and the high-density lipoproteins (HDL) it contains promote cholesterol efflux from the sperm plasma membrane and induce hyperactivation, processes associated with early stages of capacitation and changes in plasma membrane fluidity.

The higher percentage of viable sperm with a non-intact acrosome (pointing to acrosomal exocytosis) observed in capacitating and non-capacitating media supplemented with the FF (TCM+FF and TBM+FF) compared to their non-supplemented counterparts (TCM and TBM treatments) would indicate that this fluid also promotes the acrosome reaction. While these results may not be conclusive in suggesting that the FF can induce a strong acrosome reaction, this could be partly due to the short incubation time used. The results of this study are consistent with previous findings in horses (Cheng et al., 1998), humans (Yao et al., 2000), sheep (El-Shahat et al., 2018), hamsters (Yanagimachi, 1969), and pigs (Funahashi and Day, 1993; Hansen et al., 1991), which showed that sperm incubated with preovulatory FF (Cheng et al., 1998; Hansen et al., 1991; Yanagimachi, 1969; Yao et al., 2000) or FF from immature follicles (El-Shahat et al., 2018; Funahashi and Day, 1993) undergo the acrosome reaction, and that this reaction is dose- and time-dependent. Furthermore, our data, which revealed that the percentage of viable sperm with an intact acrosome was lower in the capacitation medium supplemented with 20 % FF (TCM + FF) than in the non-supplemented, non-capacitating medium (TBM) - indicating greater induction of the acrosome reaction in the former - are consistent with the findings of Rathi et al. (2006), who observed a lower percentage of viable sperm with an intact acrosome in the presence of 25 % or 50 % FF. On the other hand, a recently study in cattle demonstrated that extracellular vesicles derived from the fluid of small, medium, and large follicles modulate capacitation and the acrosome reaction in bovine sperm (Hasan et al., 2021). This reinforces the hypothesis that the FF components are involved in late capacitation events (Visconti, 2009) and the acrosome reaction.

While this study demonstrated a beneficial effect of the FF on the *in vitro* capacitation of donkey sperm, a limitation to consider is the low number of animals used. In addition, evidence from *in vivo* studies in other species suggests that the FF components are not solely responsible for inducing these processes (reviewed in Suarez and Ho, 2003). The FF may be involved in facilitating, stimulating, or initiating certain changes associated with capacitation. Still, its action is likely to be complemented by the oviductal environment through cellular and hormonal signals, as well as interactions with the epithelium and oviductal fluid (Coy et al., 2012; Mahé et al., 2021; Martinez et al., 2020).

5. Conclusion

The results of this study show that, in donkeys, the FF exerts a positive effect on motility and other variables indicative of sperm capacitation, such as increased intracellular Ca^{2+}, higher membrane lipid disorder, and acrosomal reaction. Moreover, the FF was found to mitigate the increase in ROS concentrations observed in non-supplemented capacitating and non-capacitating media, suggesting an antioxidant or protective effect that may contribute to the maintenance of sperm functionality. These findings highlight the importance of the FF components during the capacitation of donkey sperm, and also suggest their consideration as factors that could optimise capacitation and IVF protocols in this species. Yet, further research testing the effects of FF-incubated sperm on conventional IVF outcomes is needed.

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CRediT authorship contribution statement

Lorena Padilla: Methodology, Investigation. **Jaime Catalán:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marc Yeste:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Jordi Miró:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Jesús Martínez-Hernández:** Investigation, Data curation. **Carolina Maside:** Investigation.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Marc Yeste is an editor of Animal Reproduction Science, but was blinded from the peer review process for this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.108016.

Availability of data and material

The datasets prepared and analysed during the current study are available as Supplementary File 1.

References

Abu-Musa, A., Khalil, A., Hannoun, A., Sakhel, K., Takahashi, K., 2001. Effect of human follicular fluid on sperm survival. Clin. Exp. Obstet. Gynecol. 28, 40–42.

Aitken, R.J., 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol. Reprod. Dev. 84, 1039–1052. https://doi.org/10.1002/MRD.22871.

Bamba, K., 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology 29, 1245–1251. https://doi.org/10.1016/0093-691X(88)90004-0.

Bartolomé, J., Romeo, S.C., Dorado-Silva, M., García de la Vega, C., López, C., Sánchez-Martín, P., Johnston, S., Gosálvez, J., 2021. DNase activity in human seminal plasma and follicular fluid and its inhibition by follicular fluid chelating agents. Reprod. Biomed. Online 43, 1079–1086. https://doi.org/10.1016/j.rbmo.2021.09.015.

Brown, S.G., Costello, S., Kelly, M.C., Ramalingam, M., Drew, E., Publicover, S.J., Barratt, C.L.R., Da Silva, S.M., 2017. Complex CatSper-dependent and independent [Ca2+] I signalling in human spermatozoa induced by follicular fluid. Hum. Reprod. 32, 1995–2006. https://doi.org/10.1093/humrep/dex269.

Cabeza, J.P., Gambini, A., 2023. Advancements and challenges in in vitro reproductive technologies for the conservation of equine species. Theriogenol. Wild 2, 100036. https://doi.org/10.1016/j.therwi.2023.100036.

Catalán, J., Martínez-Rodero, I., Yánez-Ortiz, I., Mateo-Otero, Y., Bragulat, A.F., Nolis, P., Carluccio, A., Yeste, M., Miró, J., 2022. Metabolic profiling of preovulatory follicular fluid in jennies. Res. Vet. Sci. 153, 127–136. https://doi.org/10.1016/J.RVSC.2022.10.026.

Catalán, J., Yánez-Ortiz, I., Martínez-Rodero, I., Mateo-Otero, Y., Nolis, P., Yeste, M., Miró, J., 2023. Comparison of the metabolite profile of donkey and horse seminal plasma and its relationship with sperm viability and motility. Res. Vet. Sci. 165, 105046. https://doi.org/10.1016/j.rvsc.2023.105046.

Catalán, J., Yánez-Ortiz, I., Torres-Garrido, M., Ribas-Maynou, J., Llavanera, M., Barranco, I., Yeste, M., Miró, J., 2024. Impact of seminal plasma antioxidants on DNA fragmentation and lipid peroxidation of Frozen–Thawed horse sperm. Antioxidants 13, 322. https://doi.org/10.3390/antiox13030322.

Catalán, J., Padilla, L., Maside, C., Martínez-Hernández, J., Yánez-Ortiz, I., Tvarijonaviciute, A., Barranco, I., Bonet, S., Miró, J., Yeste, M., 2025. Redox profiling of preovulatory follicular fluid in the donkey is species-specific, and contributes to modulate sperm function. Sci. Rep. 15, 6522. https://doi.org/10.1038/s41598-025-91422-0.

Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Tremoleda, J.L., Bevers, M.M., Colenbrander, B., 1998. Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances in vitro binding to the zona pellucida. Int. J. Androl. 21, 57–66. https://doi.org/10.1046/j.1365-2605.1998.00096.x.

Choi, Y.-H., Hinrichs, K., 2017. Vitrification of in vitro-produced and in vivo-recovered equine blastocysts in a clinical program. Theriogenology 87, 48–54. https://doi.org/10.1016/j.theriogenology.2016.08.005.

Coy, P., García-Vázquez, F.A., Visconti, P.E., Avilés, M., 2012. Roles of the oviduct in mammalian fertilization. Reproduction 144, 649–660. https://doi.org/10.1530/REP-12-0279.

Cuervo-Arango, J., Claes, A.N., Stout, T.A., 2019. A retrospective comparison of the efficiency of different assisted reproductive techniques in the horse, emphasizing the impact of maternal age. Theriogenology 132, 36–44. https://doi.org/10.1016/j.theriogenology.2019.04.010.

Douet, C., Parodi, O., Martino, N.A., Lacalandra, G.M., Nicassio, M., Reigner, F., Deleuze, S., Dell'Aquila, M.E., Goudet, G., 2017. Exposure to follicular fluid during oocyte maturation and oviductal fluid during post-maturation does not improve in vitro embryo production in the horse. Zygote 25, 612–630. https://doi.org/10.1017/S096719941700048X.

El-Shahat, K.H., Taysser, M.I., Badr, M.R., Zaki, K.A., 2018. Effect of oviduct and follicular fluids on ram sperm capacitation and acrosome reaction in vitro. Int. J. Vet. Sci. Med. 6, S57–S62. https://doi.org/10.1016/j.ijvsm.2017.12.002.

Esmaeilpour, T., Zarei, M.R., Bahmanpour, S., Aliabadi, E., Hosseini, A., Jaberipour, M., 2014. Effect of follicular fluid and platelet-activating factor on lactate dehydrogenase c expression in human asthenozoospermic samples. Iran. J. Med. Sci. 39, 20–28.

Fabbri, R., Porcu, E., Lenzi, A., Gandini, L., Marsella, T., Flamigni, C., 1998. Follicular fluid and human granulosa cell cultures: influence on sperm kinetic parameters, hyperactivation, and acrosome reaction. Fertil. Steril. 69, 112–117. https://doi.org/10.1016/S0015-0282(97)00421-4.

Felix, M.R., Turner, R.M., Dobbie, T., Hinrichs, K., 2022. Successful in vitro fertilization in the horse: production of blastocysts and birth of foals after prolonged sperm incubation for capacitation. Biol. Reprod. 107, 1551–1564. https://doi.org/10.1093/biolre/ioac172.

Flores Bragulat, A.P., Ortiz, I., Catalán, J., Dorado, J., Hidalgo, M., Losinno, L., Dordas-Perpinyà, M., Yánez-Ortiz, I., Martínez-Rodero, I., Miró, J., Gambini, A., 2023. Time-lapse imaging and developmental competence of donkey eggs after ICSI: effect of preovulatory follicular fluid during oocyte in vitro maturation. Theriogenology 195, 199–208. https://doi.org/10.1016/j.theriogenology.2022.10.030.

Fukui, K., Takeda, Y., Yano, J., Kaura, R., Kitagawa, H., Suginami, H., Matsuura, S., Oka, K., 1995. The effect of follicular fluid on intracellular free calcium levels in human spermatozoa. J. Obstet. Gynaecol. 21, 43–49. https://doi.org/10.1111/j.1447-0756.1995.tb00896.x.

Funahashi, H., Day, B.N., 1993. Effects of follicular fluid at fertilization in vitro on sperm penetration in pig oocytes. J. Reprod. Fertil. 99, 97–103. https://doi.org/10.1530/jrf.0.0990097.

Gadella, B.M., Harrison, R.A.P., 2002. Capacitation induces cyclic adenosine 3′,5′-Monophosphate-Dependent, but Apoptosis-Unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol. Reprod. 67, 340–350. https://doi.org/10.1095/biolreprod67.1.340.

Galli, C., Colleoni, S., Duchi, R., Lagutina, I., Lazzari, G., 2007. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim. Reprod. Sci. 98, 39–55.

García Herreros, M., Aparicio, I.M., Núñez, I., García-Marín, L.J., Gil, M.C., Peña Vega, F.J., 2005. Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. Theriogenology 63, 795–805. https://doi.org/10.1016/j.theriogenology.2004.05.003.

Garriga, F., Martínez-Hernández, J., Gener-Velasco, N., Rodríguez-Gil, J.E., Yeste, M., 2024. Voltage-dependent anion channels are involved in the maintenance of pig sperm quality during liquid preservation. Theriogenology 224, 26–33. https://doi.org/10.1016/j.theriogenology.2024.05.003.

Gervasi, M.G., Visconti, P.E., 2016. Chang's meaning of capacitation: a molecular perspective. Mol. Reprod. Dev. 83, 860–874. https://doi.org/10.1002/mrd.22663.

Getpook, C., Wirotkarun, S., 2007. Sperm motility stimulation and preservation with various concentrations of follicular fluid. J. Assist. Reprod. Genet. 24, 425–428. https://doi.org/10.1007/s10815-007-9145-6.

Grasa, P., Cebrián-Pérez, J.Á., Muiño-Blanco, T., 2006. Signal transduction mechanisms involved in in vitro ram sperm capacitation. Reproduction 132, 721–732. https://doi.org/10.1530/rep.1.00770.

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

Hamdi, S.M., Vieitez, G., Jaspard, B., Barbaras, R., Perret, B., Mieusset, R., Parinaud, J., Collet, X., 2010. Effects of human follicular fluid and high-density lipoproteins on early spermatozoa hyperactivation and cholesterol efflux. J. Lipid Res. 51, 1363–1369. https://doi.org/10.1194/jlr.M000679.
Hansen, C., Srikandakumar, A., Downey, B.R., 1991. Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction. Mol. Reprod. Dev. 30, 148–153. https://doi.org/10.1002/mrd.1080300211.
Harrison, R., Gadella, B.M., 2005. Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology 63, 342–351. https://doi.org/10.1016/j.theriogenology.2004.09.016.
Harrison, R.A.P., Mairet, B., Miller, N.G.A., 1993. Flow cytometric studies of bicarbonate-mediated Ca2+ influx in boar sperm populations. Mol. Reprod. Dev. 35, 197–208. https://doi.org/10.1002/mrd.1080350214.
Hasan, M.M., Reshi, Q.U.A., Lättekivi, F., Viil, J., Godakumara, K., Dissanayake, K., Andronowska, A., Jaakma, Ü., Fazeli, A., 2021. Bovine follicular fluid derived extracellular vesicles modulate the viability, capacitation and acrosome reaction of bull spermatozoa. Biology 10, 1154. https://doi.org/10.3390/biology10111154.
Hinrichs, K., 2018. Assisted reproductive techniques in mares. Reprod. Domest. Anim. 53, 4–13. https://doi.org/10.1111/rda.13259.
Jeon, B.G., Moon, J.S., Kim, K.C., Lee, H.J., Choe, S.Y., Rho, G.J., 2001. Follicular fluid enhances sperm attraction and its motility in human. J. Assist. Reprod. Genet. 18, 407–412. https://doi.org/10.1023/A:1016674302652.
Jeschke, J.K., Biagioni, C., Schierling, T., Wagner, I.V., Börgel, F., Schepmann, D., Schüring, A., Kulle, A.E., Holterhus, P.M., von Wolff, M., Wünsch, B., Nordhoff, V., Strünker, T., Brenker, C., 2021. The action of reproductive fluids and contained steroids, prostaglandins, and Zn2+ on CatSper Ca2+ channels in human sperm. Front. Cell. Dev. Biol. 9, 699554. https://doi.org/10.3389/fcell.2021.699554.
Küçük, N., Lopes, J.S., Soriano-Úbeda, C., Hidalgo, C.O., Romar, R., Gadea, J., 2020. Effect of oviductal fluid on bull sperm functionality and fertility under non-capacitating and capacitating incubation conditions. Theriogenology 158, 406–415. https://doi.org/10.1016/j.theriogenology.2020.09.035.
de Lamirande, E., Cagnon, C., 1993. Human sperm hyperactivation and capacitation as parts of an oxidative process. Free. Radic. Biol. Med. 14, 157–166. https://doi.org/10.1016/0891-5849(93)90006-G.
Lee, J.A., Spidlen, J., Boyce, K., Cai, J., Crosbie, N., Dalphin, M., Furlong, J., Gasparetto, M., Goldberg, M., Goralczyk, E.M., Hyun, B., Jansen, K., Kollmann, T., Kong, M., Leif, R., McWeeney, S., Moloshok, T.D., Moore, W., Nolan, G., Nolan, J., Nikolich-Zugich, J., Parrish, D., Purcell, B., Qian, Y., Selvaraj, B., Smith, C., Tchuvatkina, O., Wertheimer, A., Wilkinson, P., Wilson, C., Wood, J., Zigon, R., Scheuermann, R.H., Brinkman, R.R., 2008. MIFlowCyt: the minimum information about a flow cytometry experiment. Cytom. Part A 73A, 926–930. https://doi.org/10.1002/cyto.a.20623.
Lee, S.-L., Kuo, Y.-M., Kao, C.-C., Hong, C.-Y., Wei, Y.-H., 1992. Purification of a sperm motility stimulator from porcine follicular fluid. Comp. Biochem. Physiol. B 101, 591–594. https://doi.org/10.1016/0305-0491(92)90344-Q.
Leemans, B., Gadella, B.M., Stout, T.A.E., Nelis, H., Hoogewijs, M., Van Soom, A., 2015. An alkaline follicular fluid fraction induces capacitation and limited release of oviduct epithelium-bound stallion sperm. Reproduction 150, 193–208. https://doi.org/10.1530/REP-15-0178.
Leemans, B., Gadella, B.M., Stout, T.A.E., Sostaric, E., De Schauwer, C., Nelis, H., Hoogewijs, M., Van Soom, A., 2016c. Combined albumin and bicarbonate induces head-to-head sperm agglutination which physically prevents equine sperm-oviduct binding. Reproduction 151, 313–330. https://doi.org/10.1530/REP-15-0471.
Leemans, B., Gadella, B.M., Stout, T.A.E., De Schauwer, C., Nelis, H., Hoogewijs, M., Van Soom, A., 2016b. The role of oviductal cells in activating stallion spermatozoa. J. Equine Vet. Sci. 43, S49–S55. https://doi.org/10.1016/j.jevs.2016.06.003.
Leemans, B., Gadella, B.M., Stout, T.A.E., De Schauwer, C., Nelis, H., Hoogewijs, M., Van Soom, A., 2016a. Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/fertilization. Reproduction 152, R233–R245. https://doi.org/10.1530/REP-16-0420.
Mahé, C., Zlotkowska, A.M., Reynaud, K., Tsikis, G., Mermillod, P., Druart, X., Schoen, J., Saint-Dizier, M., 2021. Sperm migration, selection, survival, and fertilizing ability in the mammalian oviduct. Biol. Reprod. 105, 317–331. https://doi.org/10.1093/biolre/ioab105.
Maitan, P., Bromfield, E.G., Stout, T.A.E., Gadella, B.M., Leemans, B., 2022. A stallion spermatozoon's journey through the mare's genital tract: in vivo and in vitro aspects of sperm capacitation. Anim. Reprod. Sci. 246, 106848. https://doi.org/10.1016/j.anireprosci.2021.106848.
Martinez, C.A., Alvarez-Rodriguez, M., Wright, D., Rodriguez-Martinez, H., 2020. Does the pre-ovulatory pig oviduct rule sperm capacitation in vivo mediating transcriptomics of catsper channels? Int. J. Mol. Sci. 21, 1840. https://doi.org/10.3390/ijms21051840.
Mendoza, C., Tesarik, J., 1990. Effect of follicular fluid on sperm movement characteristics. Fertil. Steril. 54, 1135–1139. https://doi.org/10.1016/S0015-0282(16)54017-5.
Miró, J., Papas, M., 2018. Post–Artificial insemination endometrial inflammation and its control in donkeys. J. Equine Vet. Sci. 65, 38–43. https://doi.org/10.1016/J.JEVS.2017.11.007.
Molina, L.C.P., Luque, G.M., Balestrini, P.A., Marín-Briggiler, C.I., Romarowski, A., Buffone, M.G., 2018. Molecular basis of human sperm capacitation. Front. Cell. Dev. Biol. 6, 72. https://doi.org/10.3389/fcell.2018.00072.
Naz, R.K., Rajesh, P.B., 2004. Role of tyrosine phosphorylation in sperm capacitation/acrosome reaction. Reprod. Biol. Endocrinol. 2, 75. https://doi.org/10.1186/1477-7827-2-75.
de Oliveira, J.V., de Luna Freire Oliveira, P.V., e Oña, C.M.M., Guasti, P.N., Monteiro, G.A., da Silva, Y.F.R.S., de Mello Papa, P., Alvarenga, M.A., Junior, J.A.D., Papa, F.O., 2016. Strategies to improve the fertility of fresh and frozen donkey semen. Theriogenology 85, 1267–1273. https://doi.org/10.1016/j.theriogenology.2015.12.010.
Orsolini, M.F., Meyers, S.A., Dini, P., 2021. An update on semen physiology, technologies, and selection techniques for the advancement of in vitro equine embryo production: section II. Animals 11, 3319. https://doi.org/10.3390/ani11113319.
Pérez-Cerezales, S., Ramos-Ibeas, P., Acuna, O.S., Avilés, M., Coy, P., Rizos, D., Gutiérrez-Adán, A., 2018. The oviduct: from sperm selection to the epigenetic landscape of the embryo. Biol. Reprod. 98, 262–276. https://doi.org/10.1093/biolre/iox173.
Popkiss, S., Horta, F., Vollenhoven, B., Green, M.P., Zander-Fox, D., 2022. Calcium chloride dihydrate supplementation at ICSI improves fertilization and pregnancy rates in patients with previous low fertilization: a retrospective paired treatment cycle study. J. Assist. Reprod. Genet. 39, 1055–1064. https://doi.org/10.1007/s10815-022-02407-1.
Publicover, S., Harper, C.V., Barratt, C., 2007. [Ca2+] I signalling in sperm — making the most of what you've got. Nat. Cell. Biol. 9, 235–242. https://doi.org/10.1038/ncb0307-235.
Rathi, R., Colenbrander, B., Bevers, M.M., Gadella, B.M., 2001. Evaluation of in vitro capacitation of stallion spermatozoa. Biol. Reprod. 65, 462–470. https://doi.org/10.1095/biolreprod65.2.462.
Rathi, R., Samper, J.C., Brault, L.S., Albrecht, B.A., Estrada, A., 2006. The effect of mare follicular fluid on the release of stallion spermatozoa from oviductal cells in vitro. Anim. Reprod. Sci. 94, 322–325. https://doi.org/10.1016/j.anireprosci.2006.04.024.
Ritagliati, C., Luque, G.M., Stival, C., Baro Graf, C., Buffone, M.G., Krapf, D., 2018. Lysine acetylation modulates mouse sperm capacitation. Sci. Rep. 8, 13334. https://doi.org/10.1038/s41598-018-31557-5.
Sánchez, R., Sepúlveda, C., Risopatrón, J., Villegas, J., Giojalas, L.C., 2010. Human sperm chemotaxis depends on critical levels of reactive oxygen species. Fertil. Steril. 93, 150–153. https://doi.org/10.1016/j.fertnstert.2008.09.049.
Suarez, S.S., 2008. Regulation of sperm storage and movement in the mammalian oviduct. Int. J. Dev. Biol. 52, 455–462. https://doi.org/10.1387/ijdb.072527ss.
Suarez, S.S., Ho, H.C., 2003. Hyperactivated motility in sperm. Reprod. Domest. Anim. 38, 119–124. https://doi.org/10.1046/j.1439-0531.2003.00397.x.
Sysoeva, A.P., Makarova, N.P., Silachev, D.N., Lobanova, N.N., Shevtsova, Y.A., Bragina, E.E., Kalinina, E.A., Sukhikh, G.T., 2021. Influence of extracellular vesicles of the follicular fluid on morphofunctional characteristics of human sperm. Bull. Exp. Biol. Med. 172, 254–262. https://doi.org/10.1007/s10517-021-05372-4.
Taberner, E., Medrano, A., Peña, A., Rigau, T., Miró, J., 2008. Oestrus cycle characteristics and prediction of ovulation in catalonian jennies. Theriogenology 70, 1489–1497. https://doi.org/10.1016/J.THERIOGENOLOGY.2008.06.096.
Travis, A.J., Jorgez, C.J., Merdiushev, T., Jones, B.H., Dess, D.M., Diaz-Cueto, L., Storey, B.T., Kopf, G.S., Moss, S.B., 2001. Functional relationships between Capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. J. Biol. Chem. 276, 7630–7636. https://doi.org/10.1074/jbc.M006217200.

Tremoleda, J.L., Stout, T.A.E., Gadella, B.M., Colenbrander, B., 2004. 286 spermoocyte interaction during in vitro fertilization in the horse. Reprod. Fertil. Dev. 16, 263. https://doi.org/10.1071/RDv16n1Ab286.

Vigil, P., Orellana, R.F., Cortés, M.E., 2011. Modulation of spermatozoon acrosome reaction. Biol. Res. 44, 151–159. https://doi.org/10.4067/S0716-97602011000200007.

Visconti, P.E., 2009. Understanding the molecular basis of sperm capacitation through kinase design. Proc. Natl. Acad. Sci. U. S. A. 106, 667–668. https://doi.org/10.1073/pnas.0811895106.

Visconti, P.E., Krapf, D., De La Vega-Beltrán, J.L., Acevedo, J.J., Darszon, A., 2011. Ion channels, phosphorylation and mammalian sperm capacitation. Asian J. Androl. 13, 395–405. https://doi.org/10.1038/aja.2010.69.

Wang, Y., Storeng, R., Dale, P.O., Abyholm, T., Tanbo, T., 2001. Effects of follicular fluid and steroid hormones on chemotaxis and motility of human spermatozoa in vitro. Gynecol. Endocrinol. 15, 286–292. https://doi.org/10.1080/gye.15.4.286.292.

Yanagimachi, R., 1969. In vitro capacitation of hamster spermatozoa by follicular fluid. J. Reprod. Fertil. 18, 275–286. https://doi.org/10.1530/jrf.0.0180275.

Yang, Y., Yang, L., Han, X., Wu, K., Mei, G., Wu, B., Cheng, Y., 2024. The regulation role of calcium channels in mammalian sperm function: a narrative review with a focus on humans and mice. PeerJ 12, 1–28. https://doi.org/10.7717/peerj.18429.

Yao, Y. qing, Ho, P. chung, Yeung, W.S.B., 2000. Effects of human follicular fluid on the capacitation and motility of human spermatozoa. Fertil. Steril. 73, 680–686. https://doi.org/10.1016/S0015-0282(99)00637-8.