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Redox profiling of preovulatory follicular fluid in the donkey is species-specific, and contributes to modulate sperm function

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The follicular fluid (FF) is crucial for providing oocytes with an ideal environment that promotes their development and maturation. Not only does this fluid supply nutrients and hormones, but also other components that protect both follicular cells and the oocyte itself from potential harmful factors, such as those inducing oxidative stress (OS). The FF has also been suggested to have beneficial effects on sperm when they reach the oviduct. The objectives of this study were to describe the presence and activity levels of redox biomarkers in the preovulatory follicular fluid (PFF), and to analyze the impact of PFF on the motility, viability and reactive oxygen species (ROS) levels of donkey sperm. For this purpose, ten PFF samples obtained from Catalan jennies and nine ejaculates collected from Catalan donkeys were used. Redox biomarkers –including enzymatic and non-enzymatic antioxidants, and oxidative biomarkers– were analyzed in jennies' PFF. After collection, each semen sample was split into two aliquots of equal volume. The two aliquots were centrifuged to remove the seminal plasma; one pellet was resuspended in Tris Buffered Medium (TBM), and the other was resuspended in TBM supplemented with 20% PFF. Motility parameters (CASA) and other semen quality biomarkers (flow cytometry) were assessed after 0, 60 and 120 min of incubation at 38 °C. Exposure of donkey sperm to PFF reduced intracellular ROS levels and helped maintain sperm motility and viability. These findings suggest that, in the oviduct, the PFF components protect donkey sperm from oxidative stress. Furthermore, knowing better the composition of donkey PFF in terms of antioxidant biomarkers may be used to improve the formulation of media for oocyte maturation and fertilization in this and other species.

Keywords Donkey, Follicular fluid, Sperm, Antioxidants, Oxidative stress

The follicle is the fundamental, structural and functional unit of the ovary. As the follicles grow and develop into antral follicles, the follicular fluid (FF) fills the follicular cavity[1,2]. The FF originates from the transudate of the theca and granulosa cells in the growing follicular antrum[3]. It has several essential functions: provides an optimal microenvironment for follicular maturation, supplies the nutrients necessary for oocyte development[4], facilitates communication between somatic and germ cells[5], protects follicular cells from oxidative damage[6], and promotes oocyte extrusion during ovulation[7]. Both the composition and amount of the fluid depend on the species, the stage of follicular development[6], the age of the animal[8], and the day of the estrus cycle[9].

Among the components found in the FF are steroid hormones, polysaccharides, proteins, reactive oxygen species (ROS), metabolites, and antioxidants[5]. It has been demonstrated that these FF components may affect oocyte quality, early embryonic development, and pregnancy outcomes[10–12]. For this reason, the FF has been

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used to supplement the in vitro maturation (IVM) medium in various species, including donkeys[13], where the FF enrichment was found to support the maturation of oocytes obtained through Ovum Pick Up (OPU), and the development of embryos obtained through Intracytoplasmic Sperm Injection (ICSI) and in vitro culture (IVC)[13].

The quality of oocytes is closely related to the microenvironment where they develop, and it particularly relies on oxidative stress (OS). Excessive levels of ROS negatively affect the development and quality of the oocyte, thereby reducing fertility[2,14–16]. Previous studies in mice, pigs, cattle, sheep, and humans have shown that supplementing the IVM medium with specific antioxidants positively impacts the outcomes of assisted reproductive techniques (ARTs), such as higher maturation rates, reduced proportion of abnormal oocytes, increased oocyte quality, and enhanced blastocyst cryotolerance (see[2] for review). Accordingly, maintaining the balance between oxidants and antioxidants in the FF is crucial for the performance of ARTs[2,14–18].

Upon ovulation, the FF fills the oviduct and comes into contact with sperm, supporting their proper functioning during fertilization[19] and maintaining the integrity of their chromatin by mitigating ROS and oxidative stress, one of the leading causes of sperm DNA damage[19,20]. In the particular case of donkeys, after mating (whether natural mating or artificial insemination), an intense physiological endometrial reaction occurs[21], leading to a significant migration of polymorphonuclear neutrophils (PMN) that move rapidly toward the endometrium to counteract the inflammatory response generated by the presence of semen[22–24]. In addition to eliminating infectious agents through phagocytosis, these PMNs can also degranulate/destroy their DNA, release bactericidal molecules (such as histones and enzymes), and form neutrophil extracellular traps (NETs), which are found in the extracellular space and create a unique type of cell death called NETosis[22,25,26]. This process generates large amounts of ROS, which are added to the ROS produced by sperm metabolism. The responsible for controlling these high ROS levels and their possible harmful effects on sperm is the seminal plasma (SP) through its antioxidant molecules[22]. On the other hand, as waves of sperm transport from the uterus start immediately after seminal deposition thanks to myometrium contractions[27,28], and a brief contact of SP with sperm in the uterus is assumed, the FF could play an important role in controlling ROS in sperm when these cells reach the oviduct. Moreover, some ART protocols include semen centrifugation and removal of seminal plasma, which can increase the susceptibility of sperm to the damage caused by ROS, leading to oxidative stress and decreasing semen fertility[29,30].

Considering the impact of the FF on oocytes and sperm, and the need for a redox balance in the oviductal environment, the first objective of this study was to analyze the presence and activity levels of redox biomarkers in the PFF, including (i) enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and paraoxonase type 1 (PON-1)); (ii) non-enzymatic antioxidants measured in terms of total thiols, cupric-reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), and Trolox equivalent antioxidant capacity (TEAC); and (iii) oxidative biomarkers, such as total oxidant status (TOS), peroxide activity (POX-Act), derivatives of reactive oxygen metabolites (d-ROMs), advanced oxidation protein products (AOPP), and the oxidative stress index (OSI). The second objective was to address whether the PFF influences sperm viability and motility, and regulates oxidative stress in donkey sperm, as evaluated in terms of total ROS production and superoxide levels after 0, 60, and 120 min of incubation at 38 °C in a base medium (TBM) with and without the PFF.

Results

Enzymatic and non-enzymatic antioxidants in donkey PFF

The activity levels of enzymatic antioxidants SOD, CAT, GPX and PON-1 detected in the preovulatory follicular fluid of jennies are provided in Table 1.

Table 2 shows the results of the activity levels of non-enzymatic antioxidants measured in terms of total thiols, CUPRAC, FRAP, and TEAC, evaluated in the preovulatory follicular fluid of jennies.

Oxidant status of donkey PFF

The results of oxidant biomarkers TOS, POX-Act, AOPP, and d-ROMs, as well as the OSI evaluated in the preovulatory follicular fluid of jennies are displayed in Table 3.

Total oxidant status (TOS), peroxide-activity (POX-Act), derivatives of reactive oxygen metabolites (d-ROMs), advanced oxidation protein products (AOPP), and oxidative stress index (OSI). Data are presented as mean ± standard error of the mean (SEM) and range of antioxidant activity levels in 10 different preovulatory follicles from jennies.

Enzymatic antioxidants	Mean ± SEM	Range (min–max)
SOD (IU/mL)	0.49 ± 0.02	0.31–0.58
GPX (IU/L)	267.43 ± 45.05	79.60–550.80
PON-1 (IU/L)	1.57 ± 0.08	1.24–2.10
CAT (IU/mL)	0.30 ± 0.02	0.23–0.46

Table 1. Activity levels of enzymatic antioxidants evaluated in donkey PFF. Superoxide dismutase (SOD), glutathione peroxidase (GPX), paraoxonase type 1 (PON-1), catalase (CAT). Data are presented as mean ± standard error of the mean (SEM) and range of antioxidant activity levels in 10 different preovulatory follicles from jennies.

Non-enzymatic antioxidants	Mean ± SEM	Range (min–max)
CUPRAC (mmol/L)	0.48 ± 0.01	0.45–0.56
FRAP (mmol/L)	0.34 ± 0.01	0.29–0.42
TEAC (mmol/L)	0.63 ± 0.02	0.57–0.77
Thiol (µmol/L)	303.18 ± 10.04	275.30–383.20

Table 2. Activity levels of non-enzymatic antioxidants assessed in donkey PFF. Cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), and total thiols (Thiol). Data are presented as mean ± standard error of the mean (SEM) and range of antioxidant activity levels in 10 different preovulatory follicles from jennies.

Oxidant biomarkers	Mean ± SEM	Range (min–max)
TOS (µmol/L)	3.45 ± 0.97	0.59–8.20
POX-Act (µmol/L)	203.14 ± 24.65	85.80–318.90
d-ROMs (U.Carr)	110.74 ± 6.54	84.0–138.50
AOPP (µmol/L)	63.36 ± 6.80	37.90–103.70
OSI (arbitrary unit)	5.29 ± 1.43	0.95–12.72

Table 3. Oxidant biomarkers evaluated in donkey PFF.

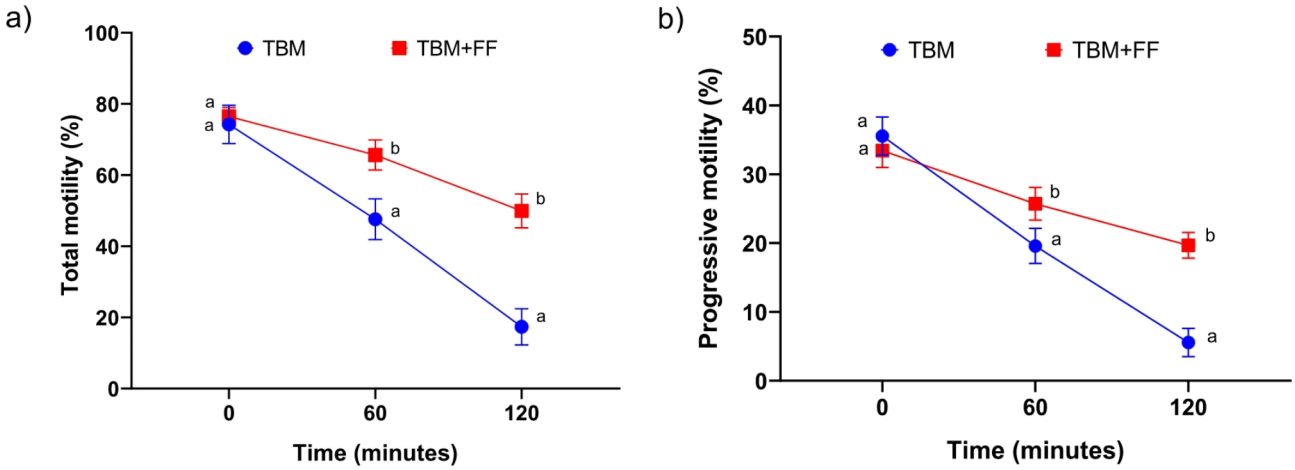

Fig. 1. Percentages of total motile **(a)** and progressively motile sperm **(b)** measured in samples resuspended in TBM medium (TBM; blue line) and resuspended in TBM supplemented with 20% preovulatory follicular fluid (TBM + FF; red line) at 0 min, and after 60 min and 120 min of incubation at 38 °C. Different superscript letters (**a**–**b**) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. Data are presented as mean ± standard error of the mean (SEM) of nine independent replicates.

Incubation of donkey sperm with PFF increases their motility

As Fig. 1 shows, supplementing TBM with 20% PFF (TBM + FF) significantly increased ($P < 0.05$) the percentage of total motile sperm compared to the control (TBM) after 60 min (mean ± SEM, TBM + FF: 65.65 ± 4.23% vs. TBM: 47.59 ± 5.73%) and 120 min (TBM + FF: 49.94 ± 4.76% vs. TBM: 17.33 ± 5.09%) of incubation. Similar results were observed for the percentage of progressively motile sperm (Fig. 1b), which was again greater ($P < 0.05$) in samples supplemented with 20% PFF than in the control after 60 min (TBM + FF: 25.70 ± 2.38 vs. TBM: 19.59 ± 2.54) and 120 min of incubation (TBM + FF: 18.52 ± 1.19 vs. TBM: 5.54 ± 2.04).

Regarding sperm kinetics (Table 4), incubating sperm with 20% PFF for 120 min significantly ($P < 0.05$) increased VCL (TBM + FF: 64.93 ± 3.62 vs. TBM: 44.87 ± 4.05), VSL (TBM + FF: 36.80 ± 3.17 vs. TBM: 22.76 ± 3.24), VAP (TBM + FF: 46.55 ± 3.84 vs. TBM: 28.08 ± 3.03), and WOB (TBM + FF: 70.13 ± 2.76 vs. TBM: 62.30 ± 2.50).

Incubation of donkey sperm with PFF better preserves their viability

As shown in Fig. 2, the percentage of sperm with an intact plasma membrane ($SYBR14^+/PI^-$; viable sperm) after 120 min of incubation at 38 °C was significantly ($P \leq 0.05$) higher in the TBM medium supplemented with 20% PFF (TBM + FF; 20.34 ± 2.15) than in its non-supplemented counterpart (TBM; 9.79 ± 1.64).

Time (min)	Treatment	Kinetic parameters (mean ± SEM)							
		VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
0	TBM	102.43 ± 4.60[a]	59.73 ± 4.57[a]	76.06 ± 4.67[a]	57.25 ± 3.39[a]	76.43 ± 2.33[a]	73.17 ± 2.47[a]	3.27 ± 0.12[a]	7.41 ± 0.20[a]
	TBM + FF	89.22 ± 4.05[a]	54.01 ± 4.13[a]	69.11 ± 4.27[a]	59.80 ± 3.52[a]	76.93 ± 2.33[a]	76.41 ± 2.57[a]	2.85 ± 0.15[a]	7.01 ± 0.22[a]
60	TBM	70.93 ± 6.13[a]	43.07 ± 4.43[a]	53.31 ± 5.23[a]	58.09 ± 2.42[a]	78.79 ± 2.35[a]	72.80 ± 1.78[a]	2.92 ± 0.16[a]	6.77 ± 0.31[a]
	TBM + FF	72.71 ± 3.95[a]	42.00 ± 3.74[a]	53.83 ± 3.86[a]	56.76 ± 2.96[a]	77.04 ± 2.83[a]	72.99 ± 1.93[a]	3.04 ± 0.12[a]	6.78 ± 0.29[a]
120	TBM	44.87 ± 4.05[a]	22.76 ± 3.24[a]	28.08 ± 3.03[a]	49.22 ± 3.49[a]	78.71 ± 3.52[a]	62,30 ± 2.50[a]	2.85 ± 0.19[a]	6.93 ± 0.85[a]
	TBM + FF	64.93 ± 3.62[b]	36.80 ± 3.17[b]	46.55 ± 3.84[b]	54.79 ± 2.52[a]	77.29 ± 2.04[a]	70.13 ± 2.76[b]	2.86 ± 0.24[a]	6.84 ± 1.50[a]

Table 4. Kinetic parameters assessed in sperm resuspended in TBM, and sperm resuspended in TBM supplemented with 20% PFF (TBM + FF) at 0 min, after 60 min and 120 min of incubation at 38 °C. 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). The different superscript letters (a-b) indicate significant differences ($P \leq 0.05$) between TBM and TBM + FF at a given time point. TBM: sperm resuspended in TBM; TBM + FF: sperm resuspended in TBM supplemented with 20% of preovulatory follicular fluid. Data are shown as mean ± standard error of the mean (SEM) of nine independent replicates.

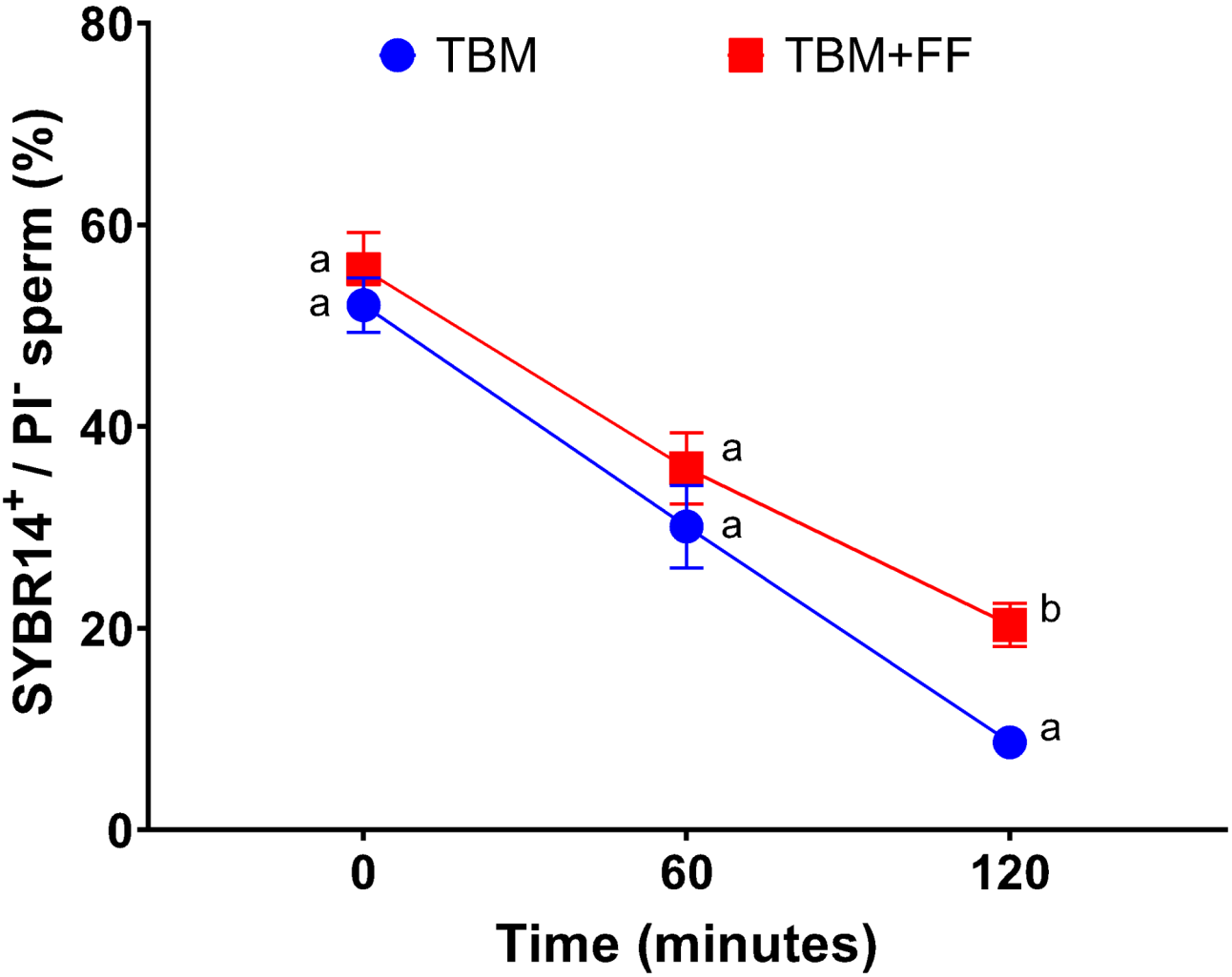

Fig. 2. Percentages of sperm with an intact plasma membrane (viable sperm) in samples resuspended in TBM medium (TBM; blue line) and resuspended with TBM supplemented with 20% preovulatory follicular fluid (TBM + FF; red line) at 0 min, and after 60 min and 120 min of incubation at 38 °C. Different superscript letters (a-b) indicate significant differences ($P \leq 0.05$) between treatments at the same time point. Data are presented as mean ± standard error of the mean (SEM) of nine independent replicates.

Incubation of donkey sperm with PFF decreases intracellular ROS levels

Incubation of sperm with TBM supplemented with 20% PFF for 120 min reduced the intracellular levels of total ROS (TBM + FF: 77474.22 ± 13329.88 vs. TBM: 145493.97 ± 16145.81; $P < 0.05$; Fig. 3a). In the case of intracellular O_2^- levels (Fig. 3b), sperm incubated with TBM supplemented with 20% PFF for 60 min and 120 min at 38 °C also exhibited significantly ($P < 0.05$) lower levels of superoxides compared to TBM (60 min, TBM + FF: 27545.79 ± 9019.24 vs. TBM: 61077.28 ± 16899.11; 120 min, TBM + FF: 40455.47 ± 7730.17 vs. 76255.85 ± 9972.43).

The PFF does not affect the mitochondrial activity of donkey sperm

Figure 4 Mitochondrial membrane potential. Ratio between JC-1 aggregates ($JC\text{-}1_{agg}$) and JC-1 monomers ($JC\text{-}1_{mon}$) in viable sperm treated with TBM and with TBM + FF (TBM supplemented with 20% preovulatory follicular fluid) at 0 min, and after 60 and 120 min of incubation at 38 °C. Data are presented as mean ± SEM of nine independent replicates.

Discussion

The present sought to investigate, for the first time, the presence and activity levels of antioxidant (enzymatic and non-enzymatic) and oxidant biomarkers in donkey PFF, and how this fluid may modulate sperm function.

Regarding enzymatic antioxidants, the activity levels of SOD in donkey PFF observed in this study (mean value of 0.49 IU/mL) were lower than those reported in cattle[31] and swine[32]. In these two species, it was found that the smaller the follicle, the higher the levels of SOD (mean values in cattle: 51.1, 29.9, and 14.8 IU/mL for small, medium, and large follicles, respectively; mean values in swine: 16.5, 12.0 and 10.5 IU/mL for small, medium, and large follicles, respectively). The SOD activity observed in donkey PFF was also lower than that of buffaloes (mean value: 16 IU/mL)[33] and humans (mean value: 1.76 IU/mL), where women suffering from the polycystic ovary syndrome (PCOS) exhibited slightly lower values (mean value 1.60 IU/mL)[34].

In the case of GPX, the activity levels in donkey PFF (mean value 267.43 IU/L) were much higher than those in pig PFF, where small follicles were found to have higher values than medium and large follicles (mean values of 2.9, 1.7 and 1.6 IU/mL for small, medium and large follicles respectively)[32]. On the contrary, the GPX activity levels in donkey PFF were lower than those in women (mean values: 418 and 472 IU/L in the group with non-superior quality and superior quality embryos, respectively)[35]. The GPX activity levels in bovine PFF (with mean values of 265, 265 and 274 IU/L in small, medium and large follicles, respectively) were similar to those observed in donkeys[36].

With respect to CAT, the activity levels in donkey PFF (mean value: 0.30 IU/mL) were lower than those reported in the PFF of humans. In effect, the activity levels of this enzymatic antioxidant in the PFF of women suffering from PCOS and those not having this condition were higher than in donkey PFF (mean values: 5.53 IU/mL and 8.94 IU/mL, respectively)[34]. Also, women with fertility issues were seen to display greater values of CAT activity (mean values: 6.3 IU/mL vs. 5.6 IU/mL in fertile women), and a negative correlation with ART outcomes[37]. Observations in the PFF of other species, such as buffaloes (mean values of 8 IU/mL during and 3.5 IU/mL outside the reproductive season)[33] and pigs (mean values 58.9 IU/mL, 61.8 IU/mL, and 61.9 IU/mL, respectively)[32], also brought greater activity values of CAT compared to donkeys.

Regarding PON-1 activity levels in donkey PFF (mean value: 1.57 IU/L) were higher than those detected in sheep (mean value: 148.8 IU/mL)[38] and the dominant follicles of cows (mean value: 47.2 IU/mL)[39].

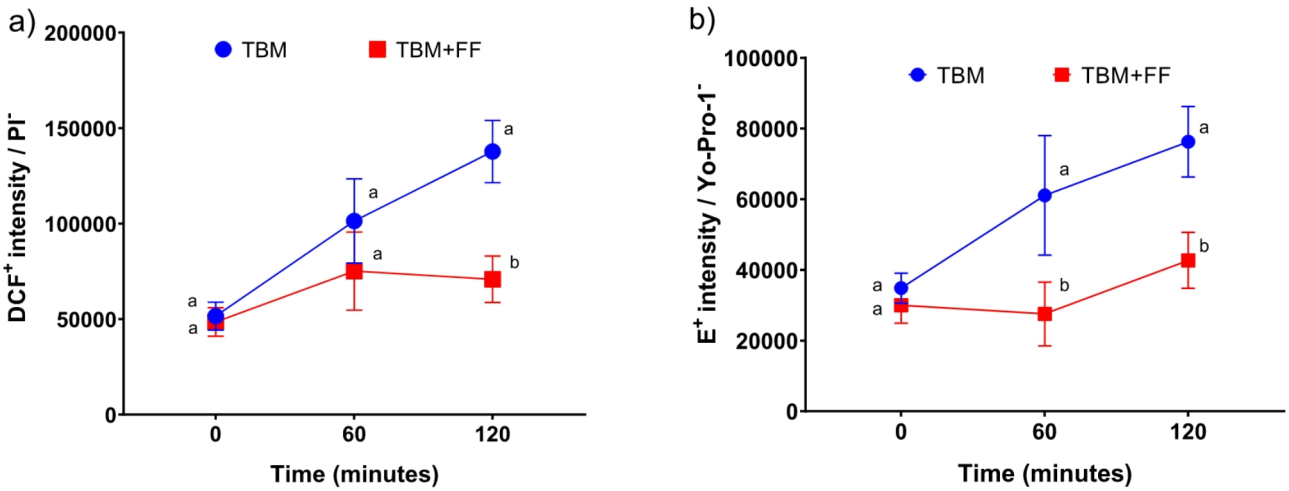

Fig. 3. Intracellular levels of ROS. **(a)** Total ROS, DCF^+ intensity in viable sperm (PI^-) and **(b)** superoxides (O_2^-), E^+ intensity in viable sperm (Yo-Pro-1^-) in samples resuspended in TBM (TBM; blue line) and TBM supplemented with 20% preovulatory follicular fluid (TBM + FF; red line) at 0 min, and after 60 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. Data are shown as mean ± SEM of nine independent replicates.

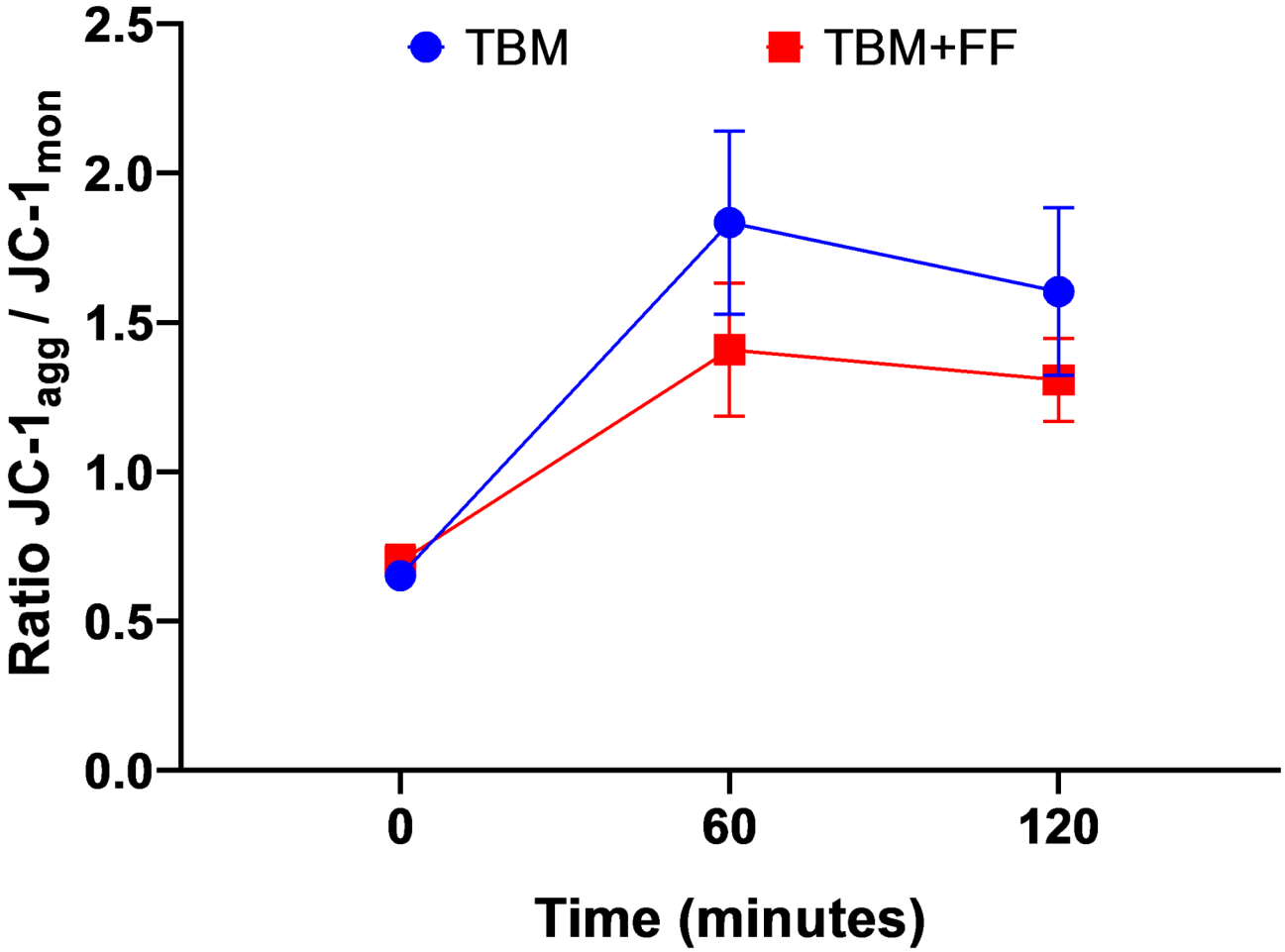

Fig. 4. shows the ratio between JC-1_{agg} and JC-1_{mon}, which provides a measurement of mitochondrial membrane potential. No significant differences in the mitochondrial membrane potential were found between treatments at any incubation time ($P > 0.05$).

Non-enzymatic antioxidant activity is often expressed as total antioxidant capacity (TAC). The analysis of TAC using the TEAC method revealed mean activity levels of 0.63 mmol/L in donkey PFF. These levels were lower than those reported in cattle by Gupta et al.[9] for small, medium, and large follicles, respectively, with differences between small and large follicles (mean values: 0.97, 1.16, 1.17 mmol Trolox equivalent/L). Furthermore, the values observed herein in donkey PFF were also lower than in humans, where two studies reported range values of 1.00-1.07 mmol/L[40] and 1.08 and 1.13 mmol/L[41]. On the other hand, FRAP levels were also observed to be lower in the FF of donkeys (mean value: 0.34 mmol/L) than in that of humans (control group mean value of 0.63 mmol/L)[42]. In the case of pigs, the FRAP levels of the fluid from small follicles (0.455 mmol/L) but not those from medium and large follicles (0.372 mmol/L and 0.388 mmol/L, respectively) were found to be higher than in donkeys[32].

As for TAC analysis based on CUPRAC, the levels obtained in this study (0.48 mmol/L) were also lower than those reported in humans by Appasamy et al.[43] (mean value in the control group of 0.75–0.8 mmol/L) and Nishihara et al.[44] (control group: mean value of 1.14 mmol/L). Moreover, total thiol levels in donkey FF (mean value: 303.18 µmol/L) were lower than those reported by Erdoğan et al.[45] in the FF of women with a diminished ovarian reserve (mean value: 448.14 µmol/L) and in that of women diagnosed with mild to moderate infertility (mean value: 481.18 µmol/L). Additionally, the levels found in donkey PFF were slightly lower than those detected in a study of women with and without PCOS (mean values: 323.8 and 352.0 µmol/L, respectively)[46].

As far as oxidative biomarkers are concerned, the TOS values observed in donkey PFF (3.45 µmol/L) were lower than those reported in women (from 14.9 to 20.8 µmol/L across different groups) by Topdagi et al.[47] and Ilhan et al.[41] in a study comparing women with a diminished ovarian reserve with a control group (mean values 7.07 and 6.77 µmol/L, respectively). Furthermore, the d-ROMs levels in donkey PFF were lower than those detected in a study of women with endometriosis (mean value: 456.6 U.CARR), PCOS (mean value: 418.4 U.CARR), tubal factor infertility (mean value: 392 U.CARR), and their healthy controls (mean value: 302.6 U.CARR)[48]. These levels were also lower than those reported in a study by Nakagawa et al.[49] involving women with endometrioma (mean value 328.7 U.CARR) and a control group (mean value 414.9 U.CARR), the latter value being consistent with the figure of 408.9 U.CARR found by Terao et al.[50]. In contrast, the concentrations

of d-ROMs in donkey PFF (mean value: 110.74 U.CARR) were higher than those reported in horse FF for small, medium, and large follicles (mean values 57.67 U.CARR, 63.05 U.CARR and 42.09 U.CARR, respectively)[51]. In cattle, the levels of d-ROMs in small follicles (mean value 106.43 U.CARR) were similar to those found in this study, but lower in medium (mean value of 98.94 U.CARR) and large follicles (mean value of 72.57 U.CARR)[36].

The AOPP levels in donkey PFF (mean value: 63.36 µmol/L) were again lower than those reported in humans in women with PCOS and women without that condition by two studies (mean values, PCOS: 112.1 µmol/L-135.3 µmol/L; control: 100.3-135.1 µmol/L)[52,53]. In contrast, the AOPP levels in donkey PFF were slightly higher than those found in a study in women with endometriosis compared to the control group (mean values of 51.5 vs. 41.8 µmol/L)[54]. Regarding OSI, while the values in donkey PFF (mean value: 5.29 AU) were lower than those reported by Ozturk et al.[55] in infertile women undergoing in vitro fertilization (mean values of 12.7 AU in pregnant women, and 13.5 AU in those non-pregnant), they were similar to those of women FF suffering from reproductive pathologies associated with PCOS (mean values of 3.99–7.31 AU) but higher than those of the corresponding control group (mean value: 0.89 AU)[56].

The observed differences in enzymatic and non-enzymatic antioxidant activity levels, as well as oxidative biomarkers, between the PFF of jennies and that of other species could be attributed to two particularities: (1) the FF analyzed in this study was obtained only from preovulatory follicles of clinically healthy animals, and (2) the volume of fluid produced by preovulatory donkey follicles was larger than that of other species, including the horse. Taking these reasons of caution into account, it becomes apparent that the composition of the FF, at least in terms of enzymatic and non-enzymatic antioxidant activity levels as well as oxidative biomarkers, is species-specific. This variability should be considered when designing media for in vitro oocyte maturation and fertilization across species, including the supplementation of these media with antioxidants, particularly in the case of donkeys, where not much research on the realm has been conducted.

Although the primary role of the FF is to promote the proper development of oocytes by modulating their maturation[57], cell communication[5], and protection against oxidative damage[6], it has also been suggested to affect sperm function during capacitation and fertilization[19]. While SP appears to control ROS and sperm oxidative damage (caused by sperm metabolism and NETs generation, etc.) within the uterine environment after semen deposition[22], the FF has been suggested to protect sperm from oxidative stress, thus maintaining the integrity of their DNA, when they reach the oviduct and come into contact with this fluid[19,20]. Because of the protective role of SP and FF on sperm, it is reasonable - in the context of this study - to compare the antioxidant activity of these two fluids in the donkey (i.e., jackass' SP and jenny's FF). Regarding enzymatic antioxidants, PON-1 levels were found to be higher in the PFF than in the SP[58] (mean values: 1.56 IU/mL vs. 0.25 IU/L, respectively). As for the other enzymatic antioxidants, GPX levels in PFF were lower (mean value: 267.43 IU/L) than those detected in SP by Papas et al.[59] (mean value of 845.4 IU/L) and Papas et al.[60] (mean values of 700 IU/L in good freezability ejaculates (GFE) and 200 IU/L in the poor freezability ones (PFE)). CAT levels observed in PFF (mean value: 0.30 IU/mL) were also lower than those detected in SP by Papas et al.[59] (mean value: 9.09 IU/mL) and Papas et al.[60] (mean levels of 9 IU/mL in the GFE group and 6 IU/mL in the PFE group). Yet, the most relevant difference was found in SOD levels, much lower in the PFF (mean value: 0.49 IU/mL) than in the SP, as reported by Catalan et al.[58] (mean value: 2168.80 IU/mL), Papas et al.[59] (mean value 1707.70 IU/mL) and Papas et al.[60] (with mean levels of 2500 IU/mL in the GFE group and 1250 IU/mL in the PFE). In the case of non-enzymatic antioxidants, only total thiol levels were found to be higher in the PFF than in the SP[58] (mean values: 303.18 µmol/L and 85.55 µmol/L, respectively). In the rest of the analyzed non-enzymatic antioxidants, all levels were lower in the PFF (mean values: CUPRAC 0.48 mmol/L; FRAP 0.34 mmol/L, and TEAC 0.63 mmol/L) than in the SP (mean values: CUPRAC 1.67 mmol/L; FRAP 1.71 mmol/L, and TEAC 2.30 mmol/L)[58]. These differences in composition could be related to the role of each of these fluids (SP and PFF) in the control of ROS. Indeed, in the case of SP, scavenging the high levels of ROS generated in the jenny's uterus post-breeding due to the intense endometrial reaction[21] and the formation of neutrophil extracellular traps (NETs)[22] could explain the greater levels of antioxidants in this fluid. In contrast, the lower levels of antioxidants in the FF would support that there is less ROS formation when sperm reach the oviduct. In the oviductal environment, however, the antioxidants of the FF could play a major role for sperm function after ovulation[19].

The potential influence of donkey PFF on the control of ROS in sperm within the oviduct was interrogated in the second experiment. For this purpose, sperm were incubated with TBM + PFF at 38 °C for 120 min. Compared to the control (TBM), incubating sperm with PFF reduced the levels of total ROS (at 60 min) and O_2^- (at 60 and 120 min), suggesting that the PFF components with antioxidant properties (enzymatic and non-enzymatic) protect sperm from oxidative stress in the oviduct. These findings were consistent with the motility results observed, as sperm incubated with PFF showed better motility than the control over time (at 60 and 120 min). These results differ from those reported in mice[61], rats[61], humans[61,62] and frozen-thawed cattle spermatozoa[63], where decreased motility was observed. Despite this, our results are similar to those obtained in sheep[64], buffaloes[65], and, curiously, to other studies in human sperm[66–69], where incubation with FF was found to improve sperm motility parameters. These studies tested the beneficial effects of FF on sperm motility using different doses. Whereas Abu-Musa et al.[66] and Mendoza and Tesarik[67] used 20% FF, Getpook and Wirotkarun[68] reported that the optimal proportion of FF to supplement sperm samples was around 20–50%, and Fabbri et al.[69] tested to supplement with 100% FF. Also, in humans, the beneficial effect of FF on sperm motility was found to be greater when sourced from mature (preovulatory) follicles compared to when it came from intermediate or immature follicles[70,71].

The positive effect of incubating sperm with FF was also observed in sperm viability, which was in agreement with Hasan et al.[72], who observed that frozen-thawed sperm viability improved when incubated with FF extracellular vesicles. These findings would also align with the findings of Kumar et al.[65], who reported an improvement in the viability of frozen-thawed buffalo sperm when the FF was used as an additive to the freezing medium. A possible explanation for the greater motility and viability of sperm in samples incubated with FF

could be that certain components of this fluid, such as chelating molecules, protect sperm from the activity of DNases[19], proteins and antioxidants[72,73]. These antioxidants would help regulate ROS levels and mitigate the main adverse effects of oxidative stress on sperm, such as DNA fragmentation, membrane lipid peroxidation, motility decline and depletion of ATP levels[74]. In spite of this, and even though previous studies demonstrated that excessive levels of ROS could have a detrimental effect on mitochondrial function in sperm[75,76], incubation of sperm with donkey PFF was not found to affect mitochondrial activity.

Conclusions

This study is the first to describe the levels of redox biomarkers in donkey PFF, including both enzymatic and non-enzymatic antioxidants, and oxidative biomarkers. The high degree of variability in these activity levels when compared with those reported in other species, suggested that they may be species-specific. Additionally, exposure of donkey sperm to PFF was found to reduce intracellular ROS levels and maintain their viability better, which would support that, in the oviductal environment, this fluid protects sperm from oxidative stress. The beneficial effects of donkey PFF on sperm quality and ROS scavenging observed in this study provide relevant information about the importance of PFF components, which should be considered to optimize the ART in this species. Furthermore, the description of redox biomarkers - especially antioxidants - in donkey PFF could be used to improve the formulation of media for in vitro oocyte maturation and fertilization in this and other species.

Methods

Reagents and suppliers

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

Animals and samples

Preovulatory 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, Genoa, Italy). Samples were obtained from follicles ≥ 40 mm in diameter, accompanied by uterine edema and without a corpus luteum in the ovaries, which is considered preovulatory for this species and breed, as described by Taberner et al.[77]. 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. The total volume of PFF extracted was approximately 30 mL per follicle. This volume was subsequently divided into 10-mL aliquots, which were stored at − 80 ºC until use.

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 (Minitüb GmbH, Tiefenbach, Germany) was utilized; this device was connected to a nylon mesh filter to remove the gel fraction. After removing this fraction, the semen volume of each sample was evaluated, and an aliquot was used to determine the sperm concentration (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 and heated 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 Sect. "Evaluation of sperm motility".), viability with a flow cytometer ($SYBR14^+/PI^-$; as detailed in Sect. "Flow cytometry analysis".), and morphology through eosin-nigrosine staining[78]. All samples met the standard quality thresholds (> 60% viable sperm and > 70% morphologically normal sperm). Each semen sample was then divided into two aliquots of equal volume and concentration (50 mL and 30 million sperm/mL).

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 protocols. This service functions under the approval of the Regional Government of Catalonia (Generalitat de Catalunya), Spain. The protocols and the methods developed in this study involving animals were approved by the ethical committee of the Autonomous University of Barcelona (Code: CEEAH 1424), and were carried out in accordance with the ARRIVE guidelines. All experiments were performed following the relevant guidelines and regulations.

Experimental design

Experiment 1: evaluation of the redox state of PFF

After collecting PFF samples and storing them at -80 °C, they were thawed on ice, and the activity levels of antioxidants (enzymatic and non-enzymatic) and oxidants were analyzed (see Sect. "Evaluation of the redox state of PFF").

Experiment 2: Evaluation of the impact of PFF on sperm quality parameters and ROS control in donkey sperm

After routine semen analysis and splitting each sample into two aliquots of equal volume and concentration (see Sect. "Semen samples"), the aliquots (in 50-mL tubes) were centrifuged, the supernatant was removed, and the pellet was resuspended in 20 mL of either Tris Buffered Medium (TBM: 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 $MgSO_4 \times 7\ H_2O$; aliquot 1), or TBM medium supplemented with 20% PFF (TBM + FF; aliquot 2). The amount of PFF added (i.e., 20%) was based on previous research conducted by Getpook and Wirotkarun[68]. Each of the resulting treatments was again split into three aliquots of 5 mL each, which were either evaluated immediately (0 min) or incubated for 60–120 min at 38 °C, 5% CO_2, 5% O_2 and a humidified atmosphere and then examined (see Sect. "Evaluation of the effects of PFF on donkey sperm"). At the corresponding time point (i.e., 0, 60, or 120 min), two 100 µL samples were taken from each aliquot. One was used to evaluate sperm motility with a computer-assisted sperm analysis (CASA) system, and the other was utilized to assess variables determined by flow cytometry.

Evaluation of the redox state of PFF

Evaluation of enzymatic antioxidants in PFF samples

The enzymatic antioxidants evaluated in jenny PFF were CAT, GPX, PON-1 and SOD[58]. The levels of activity of CAT, GPX and SOD were measured using commercially available kits, following the manufacturer's instructions (CAT: Sigma-Aldrich, St. Louis, MO, USA; GPX and SOD: Randox, Crumlin, United Kingdom). In brief, the assay for the antioxidant catalytic activity of CAT relied on the inhibition of color development in a urate assay that produces H_2O_2, wherein urate is present in excess. H_2O_2 production was quantified through Trinder reagent (4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulphonate) and horseradish peroxidase. The evaluation of GPX activity (Ransel kit) involved the oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) using cumene hydroperoxide and glutathione reductase. Finally, the SOD activity (Ransod kit) was determined by the generation of a red formazan dye produced through the interaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) with a superoxide radical supplied by xanthine and xanthine oxidase. The activity levels of PON-1 were assessed following the protocol described by Barranco et al.[79] and adapted to donkey PFF, measuring the hydrolysis of 4-nitrophenyl acetate into 4-nitrophenol. Whereas the determination of GPX, SOD and PON-1 activity was performed with an Olympus AU400 chemistry analyzer (Olympus Europe GmbH, Hamburg, Germany), that of CAT was measured using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA). The levels of GPX and PON-1 were expressed in IU/L, and those of SOD and CAT were expressed in IU/mL. Two technical replicates of each PFF sample were examined.

Evaluation of non-enzymatic antioxidants in PFF samples

The levels of non-enzymatic antioxidants were analyzed based on TEAC, CUPRAC, FRAP, and total thiol content following the protocols described by Li et al.[80] and adapted to donkey PFF in the authors' laboratory. The TEAC assay measures the sample's ability to reduce or inhibit the formation of oxidized products generated in the assay, such as the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^+$), a blue-green chromophore that decreases in intensity in the presence of antioxidants[81,82]. The CUPRAC assay evaluates the antioxidant capacity of a sample by measuring its ability to convert Cu^{2+} to Cu^{1+} in the presence of a chelating agent that forms stable colored complexes with Cu^{1+} [83,84]. Lastly, the FRAP assay operates on the principle of reducing the ferrictripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) at low pH through the action of antioxidants in the sample. The resulting blue-colored product (Fe^{2+-}TPTZ) undergoes changes in absorbance that are correlated to the antioxidant capacity of the sample[81,85]. An Olympus AU400 automated chemistry analyzer (Olympus Europe GmbH) was used for all the above determinations. The activity levels of TEAC, CUPRAC, and FRAP were expressed as mmol Trolox equivalent/L.

Evaluation of the oxidant status of PFF

The oxidant status of PFF samples was assessed by measuring the levels of TOS, POX-Act, AOPP, and d-ROMs. In addition, the oxidant/antioxidant ratio in PFF samples was determined using the Oxidative Stress Index (OSI). The determination of TOS was based on the method described by Erel[86], following a protocol adapted from donkey SP[58]. This test relies on the conversion of Fe^{2+} to Fe^{3+} in the presence of oxidants in an acidic medium, followed by the quantification of Fe^{3+} using xylenol orange. The assessment was conducted with an Olympus AU400 automated chemistry analyzer. TOS outcomes were expressed in terms of µmol H_2O_2 equivalent/L. The POX-Act assay was based on the determination of total peroxides through a peroxide-peroxidase reaction using tetramethylbenzidine as a chromogenic substrate[87]. This method was used and validated for human and canine sera and was adapted to donkey PFF; data were expressed as µmol/L. The AOPP determination was based on the presence of oxidized albumin and dityrosine-containing cross-linked proteins[88], previously measured in canine serum[89]. The protocol was adapted to donkey PFF and results were expressed in µmol/L. The d-ROM assay was based on the reaction of the sample with N, N,-diethyl-para-phenylenediamine (DEPPD) in an acidic medium, following a previously described method[90] and expressing the results in Carratelli Units (U.CARR). The OSI of PFF was calculated using the formula: OSI (arbitrary unit) = total oxidative status (TOS, µmol H_2O_2 equivalent/L) divided by TEAC (mmol Trolox equivalent/L)[91]. Two technical replicates of each PFF sample were examined.

In each test - non-enzymatic and enzymatic antioxidants, and oxidants of PFF -, the coefficient of variation was less than 10%.

Evaluation of the effects of PFF on donkey sperm

Evaluation of sperm motility

Sperm motility was evaluated using a CASA system (ISAS V1.0; Proiser S.L; Valencia, Spain). This system employs a high-resolution camera connected to a microscope that captures 25 frames per second (fps). For semen analysis, 3 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 with an Olympus BX41 microscope (Olympus, Tokyo, Japan) that had a stage at a controlled temperature of 38 °C. At least 1,000 sperm per sample were analyzed. 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%.

Flow cytometry analysis

The CytoFLEX flow cytometer from Beckman Coulter (Indianapolis, IN, USA) was used for this analysis. The side scatter and forward scatter channels were calibrated with 3-μm diameter flow control fluorospheres (Beckman Coulter). The evaluated parameters included sperm membrane integrity (SYBR14/propidium iodide [PI]), mitochondrial membrane potential (JC-1/[LIVE/DEAD far red]), and intracellular levels of total ROS (dichlorodihydrofluorescin diacetate [H_2DCFDA]/PI) and superoxides (dihydroethidium [HE]/Yo-Pro-1). Information on flow cytometry analyses is provided according to the MIFlowCyt guidelines[92].

Sperm were specifically selected from other particles/cells by using the Forward Scatter Detector (FSD), which analyzes the size of particles, and the Side Scatter Detector (SSD), which detects their roughness. Combining both detectors allowed for the discernment of sperm from other particles, such as debris and cell aggregates. All samples were excited with the blue laser (488 nm), except the ones labeled with the LIVE/DEAD fixable far red dead cell stain, which were also excited with the red laser (638 nm). The fluorescence emitted by SYBR14, Yo-Pro-1, DCF, 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 fixable far red fluorochrome was detected through the APC channel (bandpass 660/20 nm); and the fluorescence of PI and E was measured with the PC5.5 channel (bandpass 690/50 nm). For each sample, two technical replicates of at least 5000 sperm per replicate were evaluated.

Assessment of plasma membrane integrity

Plasma membrane integrity (viability) was determined with the LIVE/DEAD viability kit (Thermo Fisher Scientific; Waltham, MA, USA), following the protocol of Garner and Johnson[93], adapted to donkey sperm. In brief, semen samples were incubated with SYBR14 (final concentration: 31.5 nM) and PI (final concentration: 7.6 μM) at 38 °C in the dark for 10 min. Staining with the two fluorochromes resulted in four different populations: (i) viable sperm ($SYBR14^+/PI^-$); (ii) moribund sperm ($SYBR14^+/PI^+$); (iii) non-viable sperm ($SYBR14^-/PI^+$); and (iv) debris particles ($SYBR14^-/PI^-$). The percentage of debris particles ($SYBR14^-/PI^-$) was used to correct and recalculate the percentage of viable sperm in this and other staining protocols.

Assessment of intracellular ROS levels

A combination of H_2DCFDA and PI fluorochromes was used to assess the intracellular levels of total ROS[94]. Samples were incubated with H_2DCFDA (final concentration: 0.35 μM) at 38 °C for 20 min. The oxidation of H_2DCFDA (which is not fluorescent) to DCF (a highly fluorescent molecule that emits green fluorescence) indicates the presence of oxidative molecules. 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. For each sample, total ROS levels were expressed as the intensity of DCF^+ in viable sperm (PI^-).

For the analysis of intracellular superoxide (O_2^-) levels, 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[94]. The oxidation of HE to ethidium (E), which emits red fluorescence, indicates the presence of O_2^-. Four different sperm populations were distinguished: (i) non-viable sperm with low O_2^- levels (E^-/YO-PRO-1^+), (ii) viable sperm with low O_2^- levels (E^-/YO-PRO-1^-), (iii) non-viable sperm with high O_2^- levels (E^+/YO-PRO-1^+), and (iv) viable sperm with high O_2^- levels (E^+/YO-PRO-1^-). The fluorescence intensity of E^+ was measured in all sperm populations. For each sample, the intracellular levels of O_2^- were expressed as the intensity of E^+ in viable sperm (Yo-Pro-1^-).

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated after staining with JC-1 (final concentration: 750 nM) and the LIVE/DEAD fixable far red dead cell fluorochrome (Molecular Probes; Eugene, OR, United States), diluted at 1:8000 (v: v) in PBS, following the protocol of Garriga et al.[95] with minor modifications. Samples were stained at 38 °C in the dark for 30 min. When sperm have high mitochondrial membrane potential, JC-1 forms aggregates emitting orange fluorescence. On the contrary, low mitochondrial membrane potential maintains JC-1 molecules in the monomeric form, emitting green fluorescence. Four populations were determined: (i) viable sperm with high MMP; (ii) viable sperm with low MMP; (iii) non-viable sperm with high MMP; and (iv) non-viable sperm with low MMP. Mitochondrial membrane potential was expressed as the ratio of the fluorescence intensity of $JC\text{-}1_{agg}$ to $JC\text{-}1_{mon}$ in viable sperm.

Statistical analyses

Data were analyzed using IBM® SPSS® 27.0 statistical software for Windows (IBM Corp., Chicago, IL, USA). Before analysis, the normality of data was verified using the Shapiro-Wilk test, and the homogeneity of variances was checked using the Levene test.

A mixed linear model was run to evaluate the effects of the follicular fluid on sperm motility, intracellular ROS levels (total ROS and O_2^-), plasma membrane integrity (viability), and mitochondrial membrane potential. Pairwise comparisons were subsequently conducted using the Bonferroni test. The within-subject factor was the time of incubation (0 min, 60 min, and 120 min), the fixed between-subject factor was the treatment (TBM and TBM + FF), and the donkey was the random between-subject factor. All sperm parameters were considered dependent variables.

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

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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