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Original article

Bovine oviductal fluid (bOF) collected in the follicular or luteal phase of the estrous cycle exerts similar effects on ram sperm kinematics and acrosome reactivity *in vitro*

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ABSTRACT

This study examined the effects of bovine oviductal fluid (bOF) obtained during the follicular or luteal phase of the estrous cycle on ram sperm kinematics, capacitation status and plasma membrane (PM) integrity at various time points during the 24-h incubation period. Fresh ram spermatozoa were selected using the swim-up technique and then incubated separately with either follicular phase (FbOF) or luteal phase (LbOF) bovine oviductal fluid added to Fert-TALP medium (positive control - POSControl) or in Fert-TALP medium without capacitating agents (negative control - NEGControl) at 38 °C under 5% CO_2. Incubation with FbOF or LbOF for 2 h and 4 h promoted an increase ($P < 0.05$) in most of the sperm motility parameters as compared with the NEGControl group, and bOF-induced changes in sperm kinematics were similar ($P > 0.05$) to those seen in the POSControl group. After 6 h of incubation, the stimulatory effect of FbOF or LbOF on ram sperm kinematics was no longer observed ($P > 0.05$). Sperm PM integrity was not affected ($P > 0.05$) by incubation in bOF-supplemented media or in absence of capacitating factors (NEGControl). Although neither FbOF nor LbOF had any effect on sperm capacitation rates, the proportion of acrosome-reacted spermatozoa was greater ($P < 0.05$) for bOF-containing media compared with the NEGControl group during the long incubation periods (18 h and 24 h). In conclusion, bOF from either follicular or luteal phase of the estrous cycle enhances ram sperm motility for up to 4 h and the rate of acrosome reaction after long (18–24 h) incubation periods without affecting sperm viability.

1. Introduction

Oviductal fluid contains several constituents (*e.g.* glucose, lactate, pyruvate and amino acids) derived from blood plasma and secreted by epithelial cells [1]. The presence of growth factors (*e.g.*, epidermal growth factor, transforming growth factor, granulocyte-macrophage colony stimulating factor, insulin growth factor), hormones (*e.g.*, oestradiol, oxytocin, progesterone, prostaglandins, relaxin), proteases, antioxidant protective agents, glycosidases and glycosyltransferases in the oviductal fluid have also been reported in human, bovine and porcine species [2]. There is a great deal of evidence to suggest that these components may influence different processes that occur in the oviduct, including final maturation of female and male gametes and fertilization [3].

In the oviductal isthmus, spermatozoa bind to epithelial cells and establish a sperm reservoir in which they are retained for ≥18 h [4]. During that period, the sperm is bathed by the oviductal fluid (OF) that can modulate sperm function and affect the capacitation process [5]. Earlier studies have shown that OF composition (ions, amino acids and energy substrates) varies between the follicular and luteal phase of the estrous cycle due mainly to variations in steroid hormone secretion and bioavailability. Estradiol stimulates secretory cells of the oviductal epithelium and progesterone antagonizes this effect [6,7]. Therefore, it is feasible that OF collected at various stages of the interovulatory interval exerts different effects on sperm function.

Several studies have evaluated the effects of conspecific OF on boar and bull sperm function and maturation [8–10]. However, there has been no study on the effects of heterospecific OF obtained at different

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phases of the estrous cycle on ram spermatozoa. Heterospecific OF (cow, pig, rabbit, sheep, goat and human) has similar effects on enzymatic ZP hardening to conspecific OF [11], suggesting that at least certain influences of OF on oocytes are not species-dependent. Since sperm capacitation can occur in the reproductive tract of a heterospecific female [12], oviductal secretions from the members of other species may modulate sperm function in a similar way to conspecific OF. Therefore, the animals of different species may provide a suitable and useful tool to investigate the effects of OF on sperm physiology when conspecific OF cannot be easily obtained due to a low number of animals or animal samples available (*e.g.*, wild animals and endangered species) and/or a lack of access to a slaughterhouse. We hypothesized that the effects of bOF on ram sperm motility and capacitation would be different for OF collected at the follicular or luteal stage of the estrous cycle. The present *in vitro* study evaluated the effect of bOF on ram sperm capacitation, motility and PM integrity over a long (18–24-h) incubation period.

2. Material and methods

2.1. General experimental design

Semen was collected from three adult rams with proven fertility using an artificial vagina filled with heated water (40–45 °C) to avoid cold shock. Polled semen was submitted to a centrifugation to remove seminal plasma and then to a sperm selection technique (swim-up). Following a swim-up procedure, sperm (8×10^6 sperm /mL) were incubated in different media: (1) positive control (POSControl): Fert-TALP medium; (2) negative control (NEGControl): Fert-TALP medium without capacitating agents (caffeine, heparin, penicillamine, hypotaurine and epinephrine); (3) bOF collected *ex vivo* from oviducts in the follicular phase (FbOF): NEGControl medium supplemented with 10% FbOF; and (4) bOF collected *ex vivo* from oviducts in the luteal phase (LbOF): NEGControl medium supplemented with 10% LbOF. Sperm were incubated at 38 °C in 5% CO_2 for 24 h. Sperm motility characteristics, PM integrity and capacitation status were evaluated after 0 h, 2 h, 4 h, 6 h, 18 h and 24 h of incubation (Fig. 1). Five replicates were performed.

2.2. Ethics

The Animal Care Committee at the Universidade Federal Fluminense approved of the present experimental design (protocol no.: 879/2016). All experimental procedures followed the guidelines of the Animal Research: Reporting of *In vivo* Experiments (ARRIVE).

2.3. Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) excluding Dulbecco's Phosphate Buffered Saline (DPBS), which was obtained from Nutricell (Campinas, SP, Brazil), and the 70% ethanol solution, which was obtained from Jand Química (São Paulo, SP, Brazil).

2.4. Oviduct classification and bOF collection

Reproductive tracts of cows were obtained at a local slaughterhouse and transported on ice to the laboratory within 1 h of collection. All reproductive tracts with apparent pathologies such as polycystic ovaries and hydrometra were discarded. In the laboratory, the reproductive tracts (n = 40) were classified based on the ovarian morphology as the follicular phase or the luteal phase tracts, as previously described [13], and all oviducts (n = 40) collected. Specifically, stages I (n = 4 tracts), II (n = 10 tracts) and III (n = 6 tracts) were considered the luteal phase and stage IV was regarded as the follicular phase. The stages were defined as follows: stage I (days 1–4 or post-ovulatory period; day 1 = ovulation) – corpus luteum (CL) (red) of 0.5–1.5 cm in diameter and absence of antral follicles > 10 mm; stage II (days 5–10; early-to-mid luteal phase) – point of follicle rupture completely covered, presence of CL of 1.6–2 cm in diameter and with visible peripheral vasculature and the red/brown apex protruding above the surface of the ovary, and presence of follicles > 10 mm; stage III (days 11–17; late luteal phase) – CL diameter of 1.6–2 cm and orange external/internal coloration; and stage IV (days 18–20; pre-ovulatory) – presence of at least one large antral follicle (> 10 mm) and of regressing CL with no surface vasculature. The oviducts separated from the tracts were quickly washed in 70% ethanol solution (once) and DPBS (twice). Then, the oviducts were transferred on ice to Petri dishes and dissected. After dissection, 1000 µL of PBS was injected into the ampulla to rinse the oviductal lumen; the liquid was recovered manually by gently squeezing the oviduct from the ampulla to the isthmus as previously described by our group [14]. The fluid was centrifuged at 6000 × *g* for 5 min at 4 °C to remove cellular debris and the supernatant was immediately frozen and stored at −20 °C until the use at a later date.

2.5. Semen collection and processing

Prior to the experiment, semen was collected from rams for four consecutive days (to deplete extra-gonadal pool of spermatozoa) followed by two days of sexual rest [15]. In each replicate, semen was collected from three Santa Ines adult rams (between 3 and 5 years) with proven fertility using an artificial vagina coupled to a pre-warmed plastic tube, and protected from the light with aluminum foil. Semen from all three rams was pooled to eliminate the individual variability factor from the analysis [16]. Semen (1 mL) was then diluted in 10 mL of Fert-TALP medium without capacitating agents (caffeine, heparin, penicillamine, hypotaurine and epinephrine), and centrifuged (800 × *g*, 10 min) to remove seminal plasma [17]; this was done to eliminate the potential influence of seminal plasma components on sperm function [18]. Sperm selection was performed using the swim-up technique. The procedure was similar to that described by our group [19], but with some modifications: 300 µL of fresh sperm were carefully placed at the bottom of a 15-mL tube containing 3 mL of modified Fert-TALP, held at a 45° angle and incubated for 45 min at 38 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was centrifuged (300 × *g* for 8 min) and sperm concentration in the resultant pellet was determined, following the dilution (1:400), in a Neubauer counting chamber.

2.6. Experimental groups and incubation of ram spermatozoa with or without bOF

Following completion of the swim-up technique, semen was allocated to one of the four experimental groups. In the POSControl group, semen was incubated in a Fert-TALP medium, which is commonly used for *in vitro* fertilization (IVF). This medium contained 114 mM NaCl, 3.1 mM KCl, 0.4 mM NaH_2PO_4, 10 mM sodium lactate (60%), 25 mM $NaHCO_3$, 10 µg/mL phenol red (0.5%), 1.4 mM caffeine, 2.0 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2$, 10 mM Hepes, 6 mg/mL BSA (fatty acid free), 0.45 mM sodium pyruvate, [1x] antibiotic/antimycotic solution (ATB/ATM), 5 IU/mL heparin, 1.47 mM hypotaurine, 29.4 mM penicillamine and 0.14 mM epinephrine. The NEGControl group utilized the same medium but without capacitating agents (caffeine, heparin, penicillamine, hypotaurine and epinephrine), containing 114 mM NaCl, 3.1 mM KCl, 0.4 mM NaH_2PO_4, 10 mM sodium lactate (60%), 25 mM $NaHCO_3$, 10 µg/mL phenol red (0.5%), 2.0 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2$, 10 mM Hepes, 1 mg/mL BSA (Fraction V), 0.45 mM sodium pyruvate and [1x] ATB/ATM. The two experimental groups utilized the medium used for the NEGControl group supplemented with 10% FbOF or 10% LbOF (vol/vol) (concentration used during IVF; [12]). Semen samples were diluted to a final concentration of 8×10^6 sperm /mL in all groups. Aliquots of spermatozoa were taken just after swim-up,

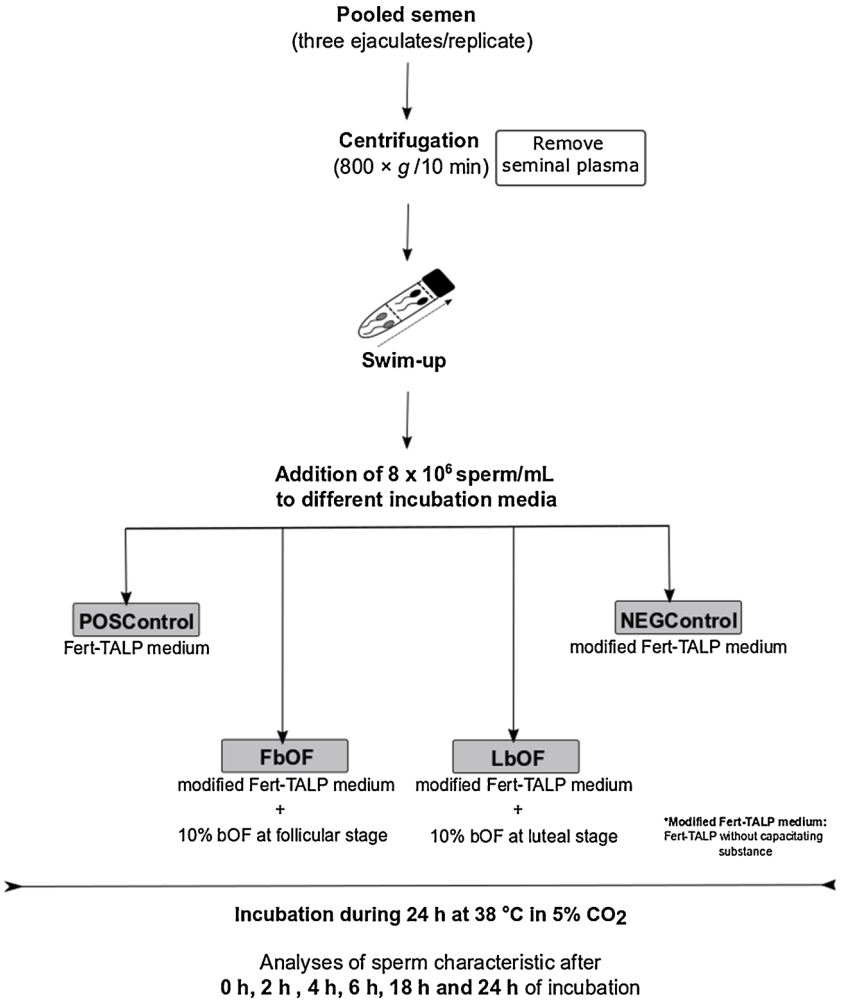

Fig. 1. Experimental design of the study. Effect of bovine oviductal fluid either at the follicular (FbOF) phase or the luteal (LbOF) on ram sperm function and capacitation status during incubation for 24 h at 38 °C in 5% CO_2.

before the addition of different media (0 h) and at various time points during incubation (2 h, 4 h, 6 h, 18 h and 24 h) for the assessment of sperm motility, PM integrity and capacitation status. In all replicates, a separate culture well was used for sample collection at each time point and the four-well culture dishes containing selected sperm suspended in 500 µL of sperm incubation medium were incubated for 24 h at 38 °C in 5% CO_2.

2.7. Sperm evaluation

2.7.1. Sperm motility

Computerized SCA® CASA system (Sperm Class Analyzer Microptic, version 3.2.0, Spain) connected to a contrast phase and epifluorescence microscope (Nikon TM H5505, Eclipse 50i, Japan) equipped with a Basler Ace ACA780-75GC digital camera were used to assess sperm motility. The analyses were performed at x100 image magnification. Aliquots of 10 µL were placed on a pre-warmed slide (37 °C) and covered with a 24 x 24 mm coverslip. For each evaluation, 10 microscopic view fields including at least 200 cells were analyzed [20]. Software settings were adjusted for ram sperm: 25 frames/s, 18–60 μm^2 for sperm head area and curvilinear velocity (VCL) < 10 µm/s to classify the spermatozoa as immotile. Sperm cells exhibiting straightness (STR) > 80% were identified as exhibiting progressive movement. The variables analyzed were: total motility (TM; %), progressive motility (%), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), average path velocity (VAP; µm/s), straightness (STR: VSL/VAPx100; %), linearity (LIN: VSL/VCLx100; %), amplitude of lateral head displacement (ALH; µm), and beat/cross frequency (BCF; Hz).

2.7.2. Sperm PM integrity

Sperm PM integrity was determined as previously described [21] with slight modifications including the addition of acridine orange [10.000 ×] and 0.5 mg/mL of propidium iodide probes. Acridine orange is a PM permeable probe selective to nucleic acids that emits green fluorescence and propidium iodide is a non-permeable probe that binds to nucleic acids and emits red fluorescence. The test was performed using the SCA® system connected to a fluorescent microscope equipped with the appropriate filter sets (465–495 nm excitation and 515–555 nm emission) at ×100 magnification. The analyzed samples included a minimum of 300 spermatozoa per slide [22].

2.7.3. Sperm capacitation status

Sperm capacitation status was assessed using chlortetracycline (CTC) staining as described elsewhere [18]. A 0.75 mM CTC solution (pH 7.8) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 mM l-cysteine. The sperm sample was mixed with an equal volume of CTC solution (10 µL) on a glass slide. A drop of 0.22 M 1,4-diaza-bicyclo (2,2,2) octane (DABCO) was added to prevent the fading of the CTC fluorescence. The slides were covered with 24 × 24 mm coverslips and CTC patterns were evaluated using a microscope (Nikon Eclipse Ci^5) under epifluorescence illumination and x1000 magnification with oil immersion. Cells were observed with a blue-violet 2A filter

(400–440 nm excitation and 470 nm emission fluorescence). Two hundred spermatozoa per slide were classified according to defined criteria [23]; the spermatozoa were categorized as non-capacitated cells (F pattern; uniform bright fluorescence of the whole head), capacitated cells (B pattern; fluorescence-free band in the post-acrosomal region) or acrosome-reacted cells (AR pattern; full fluorescence of the whole head with a thin, bright band of fluorescence along the equatorial region) [24].

2.8. Statistical analysis

Statistical analysis was performed using the Prism 5.01 software (GraphPad software, San Diego, CA, USA) and SAEG statistical software (SAEG 9.0; Viçosa, MG, Brazil). The results are presented as mean and standard error of the mean (SEM). All quantitative variables were subjected to a normality test (Lilliefors test) and homoscedasticity test (Bartlett test). Whenever necessary, the data were transformed (arc sine or logarithmic transformation) prior to a two-way repeated measures ANOVA (mixed model). The statistical model included the effects of media, incubation time and their interaction on sperm characteristics. When the ANOVA revealed a significant main effect, mean values were compared using the Bonferroni *post hoc* test. Differences with a *P*-value less than 0.05 were considered statistically significant.

3. Results

3.1. General outcomes of statistical comparisons

The results of two-way repeated measures ANOVA performed in the present study are presented in Supplemental Table 1 (Supplementary material). Treatment × Incubation Time interaction was significant ($P < 0.05$) for most sperm characteristics analyzed, indicating that the effects of media varied across the study period (*i.e.*, were affected by the duration of incubation period). Consequently, multiple comparisons among treatments were performed at each incubation period.

3.2. Effect of bOF on sperm motility characteristics

Sperm motility parameters recorded during incubation in different media are summarized in Figs. 2 and 3. After 2 h of incubation, the percentage of motile spermatozoa (total motility) was greater ($P < 0.05$) in the FbOF and LbOF groups compared with the NEGControl group (Fig. 2a). The percentage of sperm with progressive motility was greater ($P < 0.05$) in POSControl than in NEGControl after 2h and 4 h of incubation and it was greater ($P < 0.05$) in POSControl than in both bOF-supplemented groups after 18 h of incubation (Fig. 2b). The velocity parameters (VCL and VAP) were both greater ($P < 0.05$) in FbOF and LbOF than in NEGControl after 2h and 4 h of incubation but they were lower ($P < 0.05$) compared with those in NEGControl at 18-h time point (Fig. 3). ALH and BCF were lower ($P < 0.05$) in the NEGControl compared with all other groups after 2 h of incubation; in addition, BCF was greater ($P < 0.05$) in POSControl than in the remaining three experimental groups after 18 h of incubation (Fig. 3). STR was greater ($P < 0.05$) in POSControl than in NEGControl after 2 h and 6 h of incubation, it was greater ($P < 0.05$) than in FbOF after 24-h period, and it was greater ($P < 0.05$) than in all three remaining groups at 18 h (Fig. 3).

The parameters of progressive motility (VCL, VSL, VAP and LIN) did not vary ($P > 0.05$) among the POSControl, LbOF and FbOF groups at 2 h and 4 h of incubation. At 6 h of incubation, all sperm motility parameters except for STR and LIN were similar ($P > 0.05$) among all groups. After 18 h of incubation, no differences ($P > 0.05$) between the NEGControl and bOF groups were observed in relation to any kinematic parameter, regardless of the estrous cycle phase. However, the FbOF and LbOF groups showed lower values ($P < 0.05$) for these kinematic parameters compared with the POSControl group. After 24 h of incubation, no difference ($P > 0.05$) among the groups was observed in relation to most kinematic parameters.

3.3. Effect of bOF on sperm capacitation status and sperm PM integrity

Ram sperm capacitation status and PM integrity during incubation in different media are shown in Fig. 4. The addition of FbOF or LbOF resulted in a decreased ($P < 0.05$) proportion of capacitated spermatozoa compared with the NEGControl group after 4 h of incubation (Fig. 4a). After 18 h and 24 h of incubation, however, the percentage of capacitated sperm was greater ($P < 0.05$) in the NEGControl group compared with FbOF. At the same two observation time points (18 h and 24 h), the proportion of acrosome-reacted spermatozoa was significantly lower in NEGControl than in both bOF-supplemented groups and at 18 h it was also lower ($P < 0.05$) in NEGControl compared with POSControl (Fig. 4b). There were no shifts in the percentage of non-capacitated sperm during the entire incubation period in different media (Fig. 4c). Finally, the proportion of intact cells was lower ($P < 0.05$) in POSControl than in NEGControl after 4 h of incubation and it was lower ($P < 0.05$) in POSControl compared with NEGControl and FbOF after 6 h of incubation (Fig. 4d). Incubation with either FbOF or LbOF had no effect on sperm PM integrity. However, after 4 h and 6 h of incubation, the POSControl group had a lower ($P < 0.05$) percentage of intact spermatozoa compared with the NEGControl group.

In contrast, the proportion of acrosome-reacted spermatozoa was similar ($P > 0.05$) among the groups for up to 6 h of incubation. After a long incubation period (18–24 h), supplementation of bOF regardless of the phase of the estrous cycle phase (FbOF and LbOF groups) promoted an increase ($P < 0.05$) in the proportion of acrosome-reacted spermatozoa compared with the non-capacitated medium (NEGControl group). Furthermore, this supplementation caused a similar ($P > 0.05$) effect in the proportion of acrosome-reacted spermatozoa to the capacitated medium (POSControl group).

4. Discussion

The main objective of this study was to evaluate the effect of supplementing a non-capacitating medium with 10% bOF obtained in the follicular or the luteal phase of the estrous cycle on ram sperm capacitation and general function during a long-term incubation period.

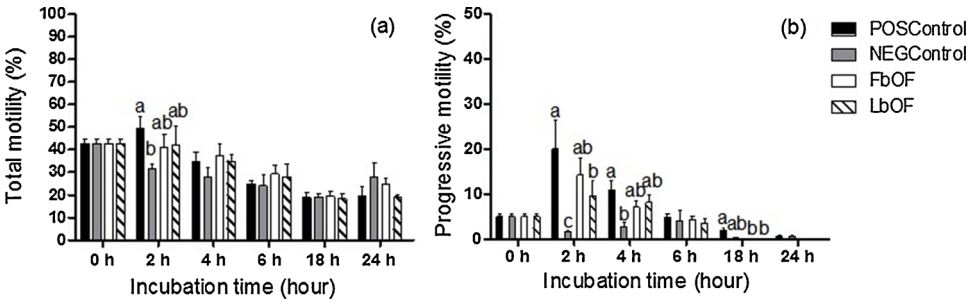

Fig. 2. Effect of bovine oviductal fluid either at the follicular (FbOF) phase or the luteal (LbOF) phase on ram sperm motility [total motility (a) and progressive motility (b)] during incubation of 24 h at 38 °C in 5% CO_2. Analyses were performed after 2 h, 4 h, 6 h, 18 h and 24 h of incubation and parameters were evaluated using a CASA system. Data are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$) among treatments (Bonferroni test). Incubation time (0 h): time after sperm selection and before the addition of different media.

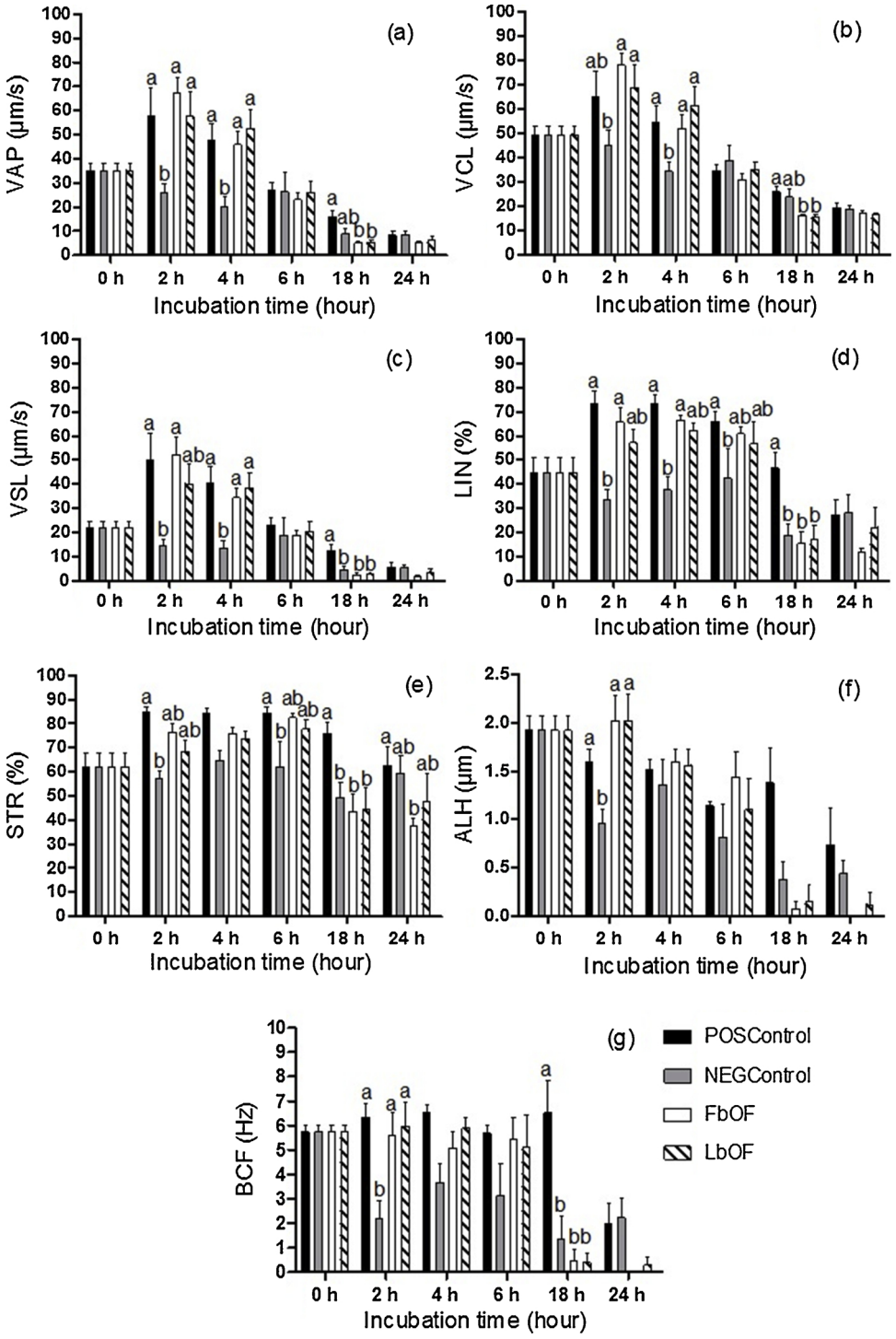

Fig. 3. Effect of bovine oviductal fluid either at the follicular (FbOF) phase or the luteal (LbOF) phase on ram sperm kinematics [VAP (a; average path velocity), VCL (b; curvilinear velocity), VSL (c; straight-line velocity), LIN (d; linearity (ratio VSL/VCL)), STR (e; straightness (ratio VSL/VAP)), ALH (f; amplitude of lateral head displacement) and BCF (g; beat/cross frequency)] during incubation of 24 h at 38 °C in 5% CO_2. Analyses were performed after 2 h, 4 h, 6 h, 18 h and 24 h of incubation and parameters were evaluated using a CASA system. Data are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$) among treatments (Bonferroni test). Incubation time (0 h): time after sperm selection and before the addition of different media.

Three main conclusions can be drawn from our study: i. beneficial influences of bOF supplementation were independent of the estrous cycle phase in which OF was collected; ii. heterospecific OF effectively modulated ram sperm motility and capacitation; and iii. although the addition of bOF did not significantly affect sperm PM integrity, it increased the proportion of acrosome-reacted rate after a long (≥18 h) incubation period.

A positive effect of 20% porcine OF from the pre-ovulatory period (proestrus and estrus) on porcine frozen-thawed sperm motility and a lack of similar effects with the use of OF obtained in the post-ovulatory phase (metestrus) have previously been described [24]. Grippo et al. [8] reported changes in sperm motility after incubation of freshly ejaculated bull semen with 40% luteal and non-luteal bOF but not with 20% luteal and non-luteal bOF. Interestingly, those earlier studies found no differences between the supplementation with luteal or non-luteal phase bOF on sperm kinematics [8], which is in complete agreement with our findings. The results of the present study indicate that 10% bOF obtained in the follicular or luteal phase of the estrous cycle and added to a non-capacitating medium has a positive effect on ram sperm motility.

In the present experiment, none of the characteristics of sperm progressive motility (VCL, VSL, VAP and LIN) varied among the POSControl, LbOF and FbOF groups after 2 h and 4 h of incubation, and after 6 h, all sperm motility parameters except for STR and LIN were

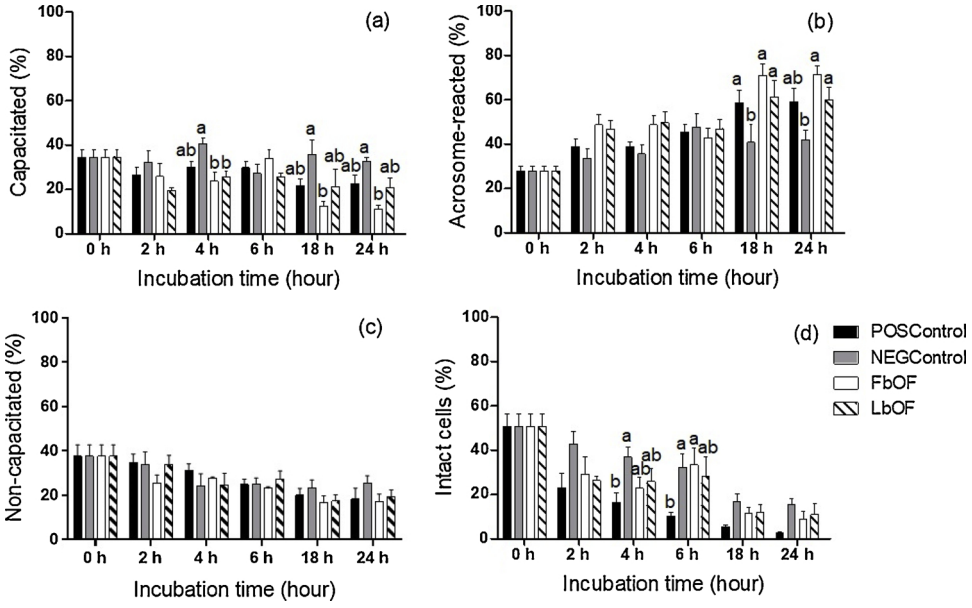

Fig. 4. Effect of bovine oviductal fluid either at the follicular (FbOF) phase or the luteal (LbOF) phase on the sperm capacitation status and plasma membrane integrity of ram spermatozoa [rate of capacitated sperm (a; B pattern), rate of acrosome-reacted sperm (b; AR pattern), rate of non-capacitated sperm (c) and rate of intact cells (d; F pattern)] during incubation of 24 h at 38 °C in 5% CO_2. Analyses were performed after 2 h, 4 h, 6 h, 18 h and 24 h of incubation. Data are expressed as mean ± SEM. Different letters indicate differences ($P < 0.05$) among treatments according (Bonferroni test). Incubation time (0 h): time after sperm selection and before the addition of different media. Non-capacitated cell: F pattern evaluated by CTC staining. Intact cell: sperm that presented plasma membrane integrity evaluated by acridine orange/propidium iodide staining.

similar among the four incubation conditions. Those observations agree with the results of previous studies [25,26] in which no effect of porcine isthmic OF on frozen/thawed boar sperm were observed after the short-term incubation (6 h). After 18 h of incubation, there were no apparent differences in sperm motility between the NEGControl and both bOF groups, but FbOF and LbOF treatments showed lower values ($P < 0.05$) for these motility parameters compared with the POSControl group. Clearly, the effects of bOF on sperm kinematics after an extended incubation period (18 h) were like those found in non-capacitating conditions but were less pronounced compared with those elicited by the capacitating media. After 24 h of incubation, there were no significant difference for most sperm motility parameters among the four groups.

Based on the comparison of sperm kinematics among bOF-supplemented groups and NEGControl, our results can be interpreted to suggest that the influence of bOF on ram sperm kinematics is restricted to the initial 4-h incubation period. Previous studies in pigs evaluated the effect of conspecific OF on sperm function during a short incubation period [9,10] but the effects of OF during a long incubation period have not been studied. The suppressive effects of isthmic non-luteal bOF on bull sperm motility were noted after 6 h of incubation [8]. Considering the *in vivo* situation, we hypothesized that OF components have a short-time positive effect (up to 4 h incubation) on sperm motility, while the attachment of spermatozoa to oviductal epithelial cells could be related to the promotion of a beneficial effect on sperm motility after a long incubation period (24 h) [27].

Several components (mainly GAGs) present in OF may affect sperm capacitation and acrosome reaction [28]. It has been demonstrated that the addition of porcine and bovine OF stimulates sperm capacitation in boars and bulls, respectively [10,29]. Our results indicate that bOF obtained at various stages of the estrous cycle results in a reduced ram sperm capacitation after 4 h of incubation. Several hours are necessary to induce capacitation of boar, bull, stallion and human spermatozoa *in vitro*. However, sperm capacitation in the ram typically occurs over 1–2 h [30]. The capacitation process is a pre-requisite for sperm to undergo the acrosome reaction [31]. Therefore, the reduction of *in vitro* ram sperm capacitation is expected after 4 h of incubation as at this time capacitated spermatozoa undergo acrosome reaction. We hypothesized that bOF could have a stimulatory effect in *in vitro* ram sperm capacitation during a short-term incubation (< 2 h). Human OF exerted a stimulatory effect on acrosome reaction although bOF had no effect on acrosome reaction in human sperm [32]. Interestingly, our results indicate that bOF can promote the acrosome reaction process in ram sperm suggesting that the stimulatory effect of OF on that process in ruminants can be both conspecific and heterospecific. It has been well established that oviductal microenvironment plays an important role in sperm selection [1]; therefore, it is attractive to suggest that OF acts to 'filter out' the pool of capacitated spermatozoa by inducing acrosome reaction.

In the present study, bOF had no significant effects on PM integrity of ram spermatozoa throughout the 24-h incubation period. This observation agrees with a previous study in cattle [8] in which bOF obtained in different phases of the estrous cycle phase and from different regions of the oviduct did not alter bull sperm viability during the 6-h incubation. However, there is substantial evidence to suggest that OF can maintain or enhance sperm viability due to the presence of oviductal proteins such as oviductin, GAGs, oviduct-specific glycoproteins and osteopontin [33]. It was demonstrated that 20 min of pre-exposure to porcine OF collected in the follicular phase of the estrous cycle increased boar sperm viability [9]. It is feasible that the effects of OF on sperm viability are very rapid (20 min or even less) or vary between different livestock species. More studies are needed to corroborate those differences.

The adverse effects of supplementation with heparin, pencillamine, hypotaurine and epinephrine on sperm PM has previously been reported [34]. Considering the negative effects of the traditional capacitating agents on sperm viability, the use of bOF may ameliorate sperm function and capacitation leading to improved fertilization rates. That would provide a new method to improve the outcome of *in vitro* fertilization (IVF) in sheep. Indeed, bOF could be an alternative to expensive synthetic additives. In an attempt to mimic microenvironment that *in vivo* fertilization occurs, strategies of supplementation of OF on IVF media have been used [35–37]. Moreover, a recent study by our group [13] has shown that conspecific OF exposure of porcine oocytes just before or during IVF decreases the incidence of polyspermy (*i.e.*, significantly increases the proportion of monospermic zygotes). Despite those beneficial effects of OF on fertilization, there are still no reports on the application of OF, either conspecific or heterospecific, in ovine *in vitro* embryo production systems.

In the present study we aimed to evaluate the effect of bOF collected at different stages of the estrous cycle on ram sperm parameters. The stage of the estrous cycle on which bovine reproductive tracts were dissected was based on the ovarian morphology, as previously described by [13]. This is an adequate method of estimating the phase of

the estrous cycle in cattle as the detection and apparence of corpora lutea (CL) are highly correlated with the actual day of the estrous cycle studied [13,39]. However, bOF chemical composition, including protein and steroid content, varies not only according to the stage of the estrous cycle but can also be affected by the presence or absence of CL and/or large (dominat) antral follicles in the inpsilateral ovary [38,39]. However, certain chemical constituents of bOF are regulated by the endocrine millieu during the specific stage of the interovulatory interval but not by side of ovulation. For example, bOF phospholipids playing a major role in the regulation of sperm capacitation but bOF lipid profiles are not affected the side of ovulation [40]. Systemic ovarian steroids hormones (progesterone and estradiol) appear to be main modulators of the secretory activity in the oviducts [6]. Nonetheless, future studies are necessary to evaluate the effects of bOF obtained from the oviducts ipsitaleteral and cotralateral to CL and large antral follicles.

In conclusion, the results of the present study indicate that *in vitro* exposure to heterospecific OF could enhance ram sperm kinematics for up to 4 h and optimize the ability of ram spermatozoa to undergo acrosome reaction, without affecting their viability OF, regardless the phase of the estrous cycle (follicular or luteal) during which the OF was obtained.

Author contributions

VAPA designed the present experiment as well as acquired, analyzed and interpreted the data, and wrote the original draft of the manuscript. JMGS-F and RITPB co-designed the experiment and critically revised the manuscript. FZB secured funding and critically revised the manuscript. PMB revised the english grammar of the manuscript and critically revised the manuscript. LRC, GMB and CVS helped to acquire the data. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.07.004.

References

[1] Pérez-Cerezales S, Ramos-Ibeas P, Acuña OS, Avilés M, Coy P, Rizos D, et al. The oviduct: from sperm selection to the epigenetic landscape of the embryo. Biol Reprod 2018;98:262–76. https://doi.org/10.1093/biolre/iox173.

[2] Aguilar J, Reyley M. The uterine tubal fluid: secretion, composition and biological effects. Anim Front 2015;5:25–31.

[3] Aviles M, Coy P, Rizos D. The oviduct: a key organ for the success of early reproductive events. Anim Reprod 2005;2:91–105. https://doi.org/10.2527/af.2015-0005.

[4] Hunter RH. Experimental studies of sperm transport in sheep, cows and pigs. Vet Rec 1985;116:188.

[5] Coy P, Garcia-Vazquez FA, Visconti PE, Aviles M. Roles of the oviduct in mammalian fertilization. Reproduction 2012;144:649–60. https://doi.org/10.1530/REP-12-0279.

[6] Hunter RHF. Components of oviduct physiology in eutherian mammals. Biol Rev Camb Philos Soc 2012;87:244–55. https://doi.org/10.1111/j.1469-185X.2011.00196.x.

[7] Hugentobler SA, Sreenan JM, Humpherson PG, Leese HJ, Diskin MG, Morris DG. Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. Reprod Fertil Dev 2010;22:684–94. https://doi.org/10.1071/rd09129.

[8] Grippo AA, Way AL, Killian GJ. Effect of bovine ampullary and isthmic oviductal fluid on motility, acrosome reaction and fertility of bull spermatozoa. J Reprod Fertil 1995;105:57–64. https://doi.org/10.1530/jrf.0.1050057.

[9] Coy P, Lloyd R, Romar R, Satake N, Matas C, Gadea J, et al. Effects of porcine preovulatory oviductal fluid on boar sperm function. Theriogenology 2010;74:632–42. https://doi.org/10.1016/j.theriogenology.2010.03.005.

[10] Kumaresan A, González R, Johannisson A, Berqvist A-S. Dynamic quantification of intracellular calcium and protein tyrosine phosphorylation in cryopreserved boar spermatozoa during short-time incubation with oviductal fluid. Theriogenology 2014;82:1145–53. https://doi.org/10.1016/j.theriogenology.2014.07.029.

[11] Mondéjar I, Avilés M, Coy P. The human is an exception to the evolutionarily-conserved phenomenon of pre-fertilization zona pellucida resistance to proteolysis induced by oviductal fluid. Hum Reprod Oxf Engl 2013;28:718–28. https://doi.org/10.1093/humrep/des423.

[12] Saling PM, Bedford JM. Absence of species specificity for mammalian sperm capacitation in vivo. J Reprod Fertil 1981;63:119–23.

[13] Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci 1980;63:155–60. https://doi.org/10.3168/jds.S0022-0302(80)82901-8.

[14] Batista RITP, Moro LN, Corbin E, Alminana C, Souza-Fabjan JMG, de Figueirêdo Freitas VJ, et al. Combination of oviduct fluid and heparin to improve monospermic zygotes production during porcine in vitro fertilization. Theriogenology 2016;86:495–502. https://doi.org/10.1016/j.theriogenology.2016.01.031.

[15] Kaya A, Aksoy M, Tekeli T. Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. Small Rumin Res 2002;44:153–8. https://doi.org/10.1016/S0921-4488(02)00051-2.

[16] Bucak MN, Tekin N. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. Small Rumin Res 2007;73:103–8. https://doi.org/10.1016/j.smallrumres.2006.12.001.

[17] Mata-Campuzano M, Soleilhavoup C, Tsikis G, Martinez-Pastor F, de Graaf SP, Druart X. Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration. Anim Reprod Sci 2015;162:31–6. https://doi.org/10.1016/j.anireprosci.2015.09.004.

[18] Maxwell W, de Graaf S, Ghaoui RE-H, Evans G. Seminal plasma effects on sperm handling and female fertility. Reprod Domest Rumin 2007;6:13–38. https://doi.org/10.5661/RDR-VI-13.

[19] Olivares CCS, de Souza-Fabjan JMG, da Fonseca JF, Balaro MFA, de Figueirêdo Freitas VJ, de Oliveira RV, et al. Comparison of different sperm selection techniques in ram frozen-thawed sperm. Acta Sci Vet 2017;45:1–11.

[20] Najafi A, Daghigh-Kia H, Dodaran HV, Mehdipour M, Alvarez-Rodriguez M. Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. Anim Reprod Sci 2017;177:35–41. https://doi.org/10.1016/j.anireprosci.2016.12.004.

[21] Yániz JL, Palacín I, Vicente-Fiel S, Gosalvez J, López-Fernández C, Santolaria P. Comparison of membrane-permeant fluorescent probes for sperm viability assessment in the ram. Reprod Domest Anim Zuchthyg 2013;48:598–603. https://doi.org/10.1111/rda.12132.

[22] Santolaria P, Vicente-Fiel S, Palacín I, Fantova E, Blasco ME, Silvestre MA, et al. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Anim Reprod Sci 2015;163:82–8. https://doi.org/10.1016/j.anireprosci.2015.10.001.

[23] Cormier N, Sirard M-A, Bailey JL. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. J Androl 1997;18:461–8. https://doi.org/10.1002/j.1939-4640.1997.tb01953.x.

[24] Fraser LR, Abeydeera LR, Niwa K. Ca(2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev 1995;40:233–41. https://doi.org/10.1002/mrd.1080400213.

[25] Kumaresan A, Johannisson A, Saravia F, Bergqvist AS. The effect of oviductal fluid on protein tyrosine phosphorylation in cryopreserved boar spermatozoa differs with the freezing method. Theriogenology 2012;77:588–99. https://doi.org/10.1016/j.theriogenology.2011.08.035.

[26] Kumaresan A, Johannisson A, Humblot P, Bergqvist A-S. Oviductal fluid modulates the dynamics of tyrosine phosphorylation in cryopreserved boar spermatozoa during capacitation. Mol Reprod Dev 2012;79:525–40. https://doi.org/10.1002/mrd.22058.

[27] Gutiérrez A, Garde J, García-Artiga C, Vázquez I. Ram spermatozoa cocultured with epithelial cell monolayers: an in vitro model for the study of capacitation and the acrosome reaction. Mol Reprod Dev 1993;36:338–45. https://doi.org/10.1002/mrd.1080360309.

[28] Miller D. Regulation of sperm function by oviduct fluid and the epithelium: insight into the role of glycans. Reprod Domest Anim 2015;50:31–9. https://doi.org/10.1111/rda.12570.

[29] Bergqvist A-S, Ballester J, Johannisson A, Hernandez M, Lundeheim N, Rodríguez-Martínez H. In vitro capacitation of bull spermatozoa by oviductal fluid and its components. Zygote 2006;14:259. https://doi.org/10.1017/S0967199406003777.
[30] Vadnais ML, Galantino-Homer HL, Althouse GC. Current concepts of molecular events during bovine and porcine spermatozoa capacitation. Arch Androl 2007;53:109–23. https://doi.org/10.1080/01485010701329386.
[31] Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 1998;59:1–6. https://doi.org/10.1095/biolreprod59.1.1.
[32] Jonge CJ, Barratt CL, Radwanska E, Cooke ID. The acrosome reaction-inducing effect of human follicular and oviductal fluid. J Androl 1993;14:359–65. https://doi.org/10.1002/j.1939-4640.1993.tb00400.x.
[33] Ghersevich S, Massa E, Zumoffen C. Oviductal secretion and gamete interaction. Reprod Camb Engl 2015;149:R1–14. https://doi.org/10.1530/REP-14-0145.
[34] Gonçalves FS, Barretto LSS, Arruda RP, Perri SHV, Mingoti GZ. Heparin and penicillamine–hypotaurine–epinephrine (PHE) solution during bovine in vitro fertilization procedures impair the quality of spermatozoa but improve normal oocyte fecundation and early embryonic development. Vitro Cell Dev Biol - Anim 2014;50:39–47. https://doi.org/10.1007/s11626-013-9675-4.
[35] Way AL, Schuler AM, Killian GJ. Influence of bovine ampullary and isthmic oviductal fluid on sperm–egg binding and fertilization in vitro. J Reprod Fertil 1997;109:95–101.
[36] Cebrian-Serrano A, Salvador I, García-Roselló E, Pericuesta E, Pérez-Cerezales S, Gutierrez-Adán A, et al. Effect of the bovine oviductal fluid on *in vitro* fertilization, development and gene expression of *in vitro*-produced bovine blastocysts: oviductal fluid on *in vitro* fertilized bovine blastocysts. Reprod Domest Anim 2013;48:331–8. https://doi.org/10.1111/j.1439-0531.2012.02157.x.
[37] Lloyd RE, Romar R, Matas C, Gutierrez-Adan A, Holt WV, Coy P. Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced in vitro. Reproduction 2009;137:679–87. https://doi.org/10.1530/REP-08-0405.
[38] Lamy J, Labas V, Harichaux G, Tsikis G, Mermillod P, Saint-Dizier M. Regulation of the bovine oviductal fluid proteome. Reproduction 2016;152:629–44. https://doi.org/10.1530/REP-16-0397.
[39] Lamy J, Liere P, Pianos A, Aprahamian F, Mermillod P, Saint-Dizier M. Steroid hormones in bovine oviductal fluid during the estrous cycle. Theriogenology 2016;86:1409–20. https://doi.org/10.1016/j.theriogenology.2016.04.086.
[40] Banliat C, Tomas D, Teixeira-Gomes AP, Uzbekova S, Guyonnet B, Labas V, et al. Stage-dependent changes in oviductal phospholipid profiles throughout the estrous cycle in cattle. Theriogenology 2019;135:65–72. https://doi.org/10.1016/j.theriogenology.2019.06.011.