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Original article

Supplementation of 17β-estradiol and progesterone in the co-culture medium of bovine oviductal epithelial cells and ovine spermatozoa reduces the sperm kinematics and capacitation

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ABSTRACT

This study investigated the effect that bovine oviductal epithelial cell (BOEC) and ovine spermatozoa co-culture exposed to different hormonal environments had on ram sperm function over the course of a 24-h incubation period. Ram cooled-stored spermatozoa were selected by swim-up and then co-cultured separately for 24 h at 38.5 °C under 5% CO_2 with either: (1) Fert-TALP medium (positive control [POSControl]), (2) Fert-TALP medium supplemented with 17β-estradiol (E2) and progesterone (P4) at concentrations similar to follicular phase (Follicular NEGControl), (3) Fert-TALP medium supplemented with E2 and P4 concentrations similar to luteal phase (Luteal NEGControl), (4) BOEC cultured in the same medium as that of the Follicular NEGControl group (Follicular BOEC group), or (5) BOEC cultured in the same medium as that of the Luteal NEGControl group (Luteal BOEC group). The sperm kinematics, capacitation status, and plasma membrane (PM) integrity were evaluated at different intervals. Sperm PM integrity was not affected ($P > 0.05$) by BOEC co-culture, regardless of the phase of the estrous cycle. After 4 h of incubation, the Luteal BOEC group presented lower ($P < 0.05$) progressive motility and total motility than the Luteal NEGControl group while the Follicular BOEC group showed lower ($P < 0.05$) velocimetric parameters and progressive motility than the Follicular NEGControl group. Throughout the incubation period, both BOEC co-culture groups showed a decrease ($P < 0.05$) in their capacitation rate in comparison to the POSControl group. Conversely, the Luteal BOEC group presented a higher ($P < 0.05$) non-capacitated rate than both the POSControl and Luteal NEGControl groups. In conclusion, BOEC co-culture with ovine spermatozoa at either the follicular or luteal phase decreases sperm kinematics and delays sperm capacitation.

1. Introduction

The oviduct of non-primates is an organ that provides the optimal microenvironment for the fertilization process [1]. After mating, millions of sperm are deposited in the female reproductive tract; however, only a fraction of the sperm reach the oviduct. Of those, many bind to the oviductal epithelial cells (OEC) and subsequently form a sperm reservoir. The interaction between sperm and the OEC plays an important role in ensuring the sperm remains viable until ovulation occurs [1]. Previous studies have concluded that the attachment of sperm to the OEC has a positive effect on sperm motility [2], enhances sperm viability [3], and delays premature capacitation [4]. Despite the positive correlation between spermatozoa-OEC binding and sperm function, there remains a lack of in vitro co-culture studies that have adequately replicated in vivo oviductal microenvironment conditions during the estrous cycle to investigate the physiology of sperm function and the capacitation process.

The oviductal epithelium consists of ciliated cells, which are involved in gametes and embryo transport, and secretory cells, which participate in protein secretion in the oviductal fluid [5,6]. During the estrous cycle, the oviduct epithelium undergoes physiological changes that are predominantly regulated by the 17β-estradiol (E2) and progesterone (P4) ovarian steroids. In the presence of E2, the ciliogenesis of bovine oviductal epithelium is stimulated. In contrast, P4 suppresses ciliogenesis leading to a deciliation process [7]. Furthermore, stimulation with these steroids promotes alterations in OEC transcriptome and, consequently, in the secreted product [8–10]. During the follicular phase—locally within the oviduct—the E2 concentration is high, and

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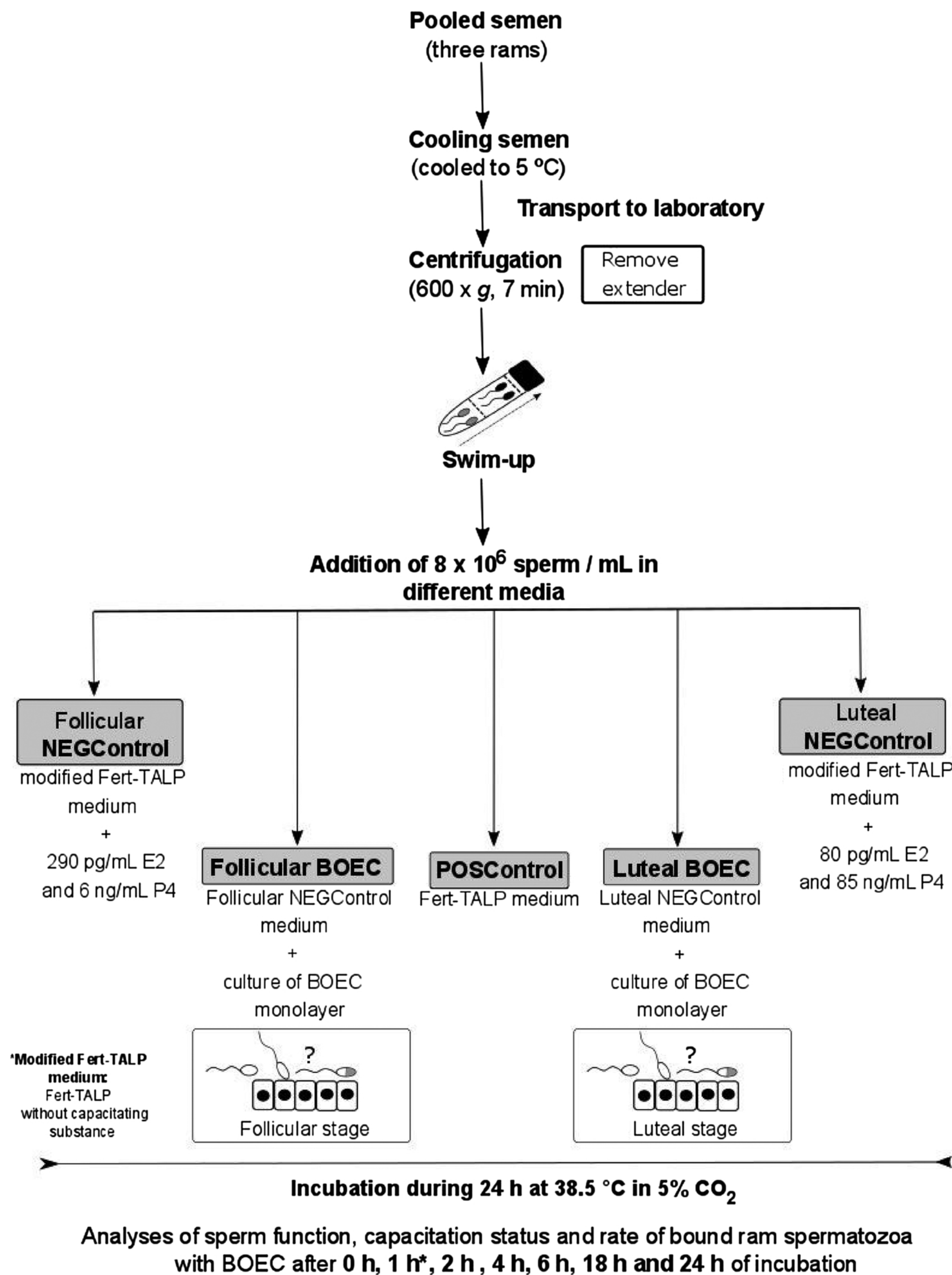

Fig. 1. Experimental design of the study. Effect of bovine oviductal epithelial cell (BOEC) on ram sperm function and capacitation status during incubation of 24 h at 38.5 °C in 5% CO_2.

P4 is low. By contrast, in the luteal phase, the opposite occurs [11]. As such, it is possible that throughout the estrous cycle, hormonal stimulation acts in oviductal epithelium altering this microenvironment and, therefore, modulating sperm function in different ways.

Steroid hormones also have dualistic effects on sperm function. Studies have ascertained that P4 is involved in sperm chemoattraction, the stimulation of capacitation and acrosome reaction processes, and the binding and fusion of sperm-zona pellucida [12,13]. Conversely, E2 suppresses the acrosome reaction and hyperactivation motility process induced by P4 [14]. However, the potential impact that dynamic changes in E2 and P4 can have on the regulation of sperm function during the estrous cycle is not clearly understood.

The majority of existing studies that have evaluated the sperm function in sperm-OEC co-culture have employed a homologous system [2,3,15,16]. However, heterologous systems have been successfully used as an equivalent to homologous systems in different species [17–19], including ovine species. For example, in one study, ram spermatozoa were able to attach to both sheep and hamster OEC [20]. Furthermore, a heterologous system could represent a viable alternative to a homologous system if the OEC cannot be routinely collected due to a lack of suitable animal samples (wild species and endangered species) and/or inaccessibility to a slaughterhouse. Therefore, the co-culture between ram spermatozoa and BOEC may be considered a feasible model for the investigation of sperm-OEC interactions.

In summary, there is still a lack of literature on the basic physiology of sperm function in the oviduct during the estrous cycle. Given previous indications that steroid hormones modify oviductal epithelium in different phases of the estrous cycle, we hypothesized that variations in

P4 and E2 concentrations alter the profile of the proteins secreted in vitro and subsequently have different impacts on the BOEC modulating sperm function and capacitation process during the estrous cycle. Thus, the objective of the current study was to evaluate the effect that the presence and direct contact of BOEC previously treated with E2 and P4 concentrations similar to the follicular and luteal phase of estrous cycle has on ram sperm function over the course of a 24-h incubation period.

2. Material and methods

2.1. Ethics

This research was approved by the Animal Care Committee of Universidade Federal Fluminense (protocol approval: 879/2016). In addition, this manuscript follows the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines.

2.2. Reagents

Unless specified otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Experimental design

The aim of this experiment was to evaluate the effect that sperm-BOEC co-culture supplemented with E2 and P4 at concentrations similar to either the follicular or luteal phase had on ovine sperm function and capacitation status over a 24-h duration. After sperm selection (swim-up), the sperm were diluted in a final concentration of 8×10^6 sperm / mL in different media: (1) POSControl: Fert-TALP medium; (2) Follicular NEGControl: Fert-TALP medium without capacitating agents, supplemented with E2 and P4 at concentrations similar to the follicular phase in bovine oviductal fluid (bOF); (3) Luteal NEGControl: Fert-TALP medium without capacitating agents, supplemented with E2 and P4 at concentrations similar to the luteal phase in bOF; (4) Follicular BOEC: BOEC cultured in the same medium of Follicular NEGControl group; and (5) Luteal BOEC: BOEC cultured in the same medium of Luteal NEGControl group. A separate culture well was set up for assessment at each time point, and the four-well culture dishes containing selected sperm suspended in 500 μL of fertilization medium in each experimental group were incubated for 24 h at 38.5 °C in 5% CO_2. The parameters of sperm kinematics and sperm PM integrity were evaluated after 0 h, 2 h, 4 h, 6 h, 18 h, and 24 h. Sperm capacitation status and the rate of ram sperm bound to BOEC were evaluated after 0 h, 1 h, 2 h, 4 h, 6 h, 18 h, and 24 h (Fig. 1). In the BOEC co-culture groups (either the Follicular or Luteal BOEC group), the sperm kinematics and sperm capacitation status of BOEC-binding sperms and freely swimming sperms (unbound sperms) were assessed during 24 h of co-incubation. At each sampling time, the medium containing unbound sperms were removed and replaced with BOEC co-culture medium. Then, BOEC-binding sperms were sampled by positioning the tip of the pipette at the bottom of the wells. Five replicates were performed.

2.4. Media

2.4.1. BOEC culture medium

BOEC were cultured in tissue culture medium-199 (TCM-199) supplemented with 10% of heat-treated fetal calf serum (FCS) (Gibco, Invitrogen Corp., Carlsbad, USA) and 1% of antibiotic/antimycotic solution (ATB/ATM). The culture medium was filtered with a 0.22 μm filter and stored at 4 °C.

2.4.2. Sperm-BOEC co-culture medium

BOEC co-culture was carried out in a modified Fert-TALP medium. This medium consisted of Fert-TALP without capacitating agents (caffeine, heparin, penicillamine, hypotaurine, and epinephrine). 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%), 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2$, 10 mM Hepes, 1 mg/mL bovine serum albumin (BSA; fraction V), 0.45 mM sodium pyruvate, and [1x] ATB/ATM. The co-culture medium was filtered with a 0.22 μm filter and stored at 4 °C.

2.4.3. Positive control group medium

The positive control (POScontrol) group medium consisted of 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 $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2$, 10 mM Hepes, 6 mg/mL BSA (fatty acid free), 0.45 mM sodium pyruvate, [1x] ATB/ATM, 5 IU/mL heparin, 1.47 mM hypotaurine, 29.4 mM penicillamine, and 0.14 mM epinephrine. The positive control medium was filtered with a 0.22 μm filter and stored at 4 °C.

2.4.4. Negative control groups media

The media of the negative control (NEGControl) group consisted of modified Fert-TALP medium, identical to that described per the BOEC co-culture medium. The negative control media were filtered with a 0.22 μm filter and stored at 4 °C.

2.5. Hormonal treatments

The in vivo oviductal microenvironment conditions were in vitro replicated as closely as possible by adding concentrations of E2 and P4 at the follicular (E2 = 290 pg/mL; P4 = 6 ng/mL) phase or the luteal (E2 = 80 pg/mL; P4 = 85 ng/mL) phase as per the measurements performed on the bovine oviductal fluid [11]. BOEC monolayers were pretreated for 24 h before the experiment with exogenous E2 (Sigma – Aldrich Corp.; E2758) and P4 (Sigma – Aldrich Corp.; P8783) according to the experimental group. On the day of the experiment, the BOEC culture medium was removed and replaced with BOEC co-culture medium supplemented with E2 and P4 at concentrations aligned with the estrous cycle phase (follicular or luteal) of the respective experimental group.

2.6. BOEC culture

The oviducts and ovaries of cows and/or heifers at random phases of estrous cycle were obtained at a local slaughterhouse, transported to the laboratory on ice within 1 h *post-mortem,* and processed immediately as per the following process. Once in the laboratory, the oviducts (n = 20) were separated from their attached ovaries and quickly washed: once with a 70% ethanol solution (Jand Química, São Paulo, Brazil) and twice with Dulbecco's phosphate-buffered saline (Nutricell, Campinas, Brazil). Then, the oviducts were transferred on ice to Petri dishes and any surrounding tissues were removed. BOECs were isolated by mechanical scraping of the oviduct with a sterile glass slide as previously described [21]. BOECs were then washed three times for 5 min by sedimentation with 5 mL of HEPES Buffered TCM-199 supplemented with BSA (fraction V; 3 mg/mL) and 0.25% of ATB/ATM. The resulting cellular pellet was diluted 100 times (dilution factor: 1/100) in TCM-199 supplemented with 10% of FCS (Gibco, Invitrogen Corp., Carlsbad, USA) and 1% of ATB/ATM. Following that, 5 mL of the cell suspension (final concentration: 2×10^5 cells/mL) was seeded into tissue culture flasks (25 cm^2; Corning, New York, USA) and placed in a humidified atmosphere 5% CO_2 at 38.5 °C. The culture medium was renewed after 48 h and subsequently half-renewed every 48 h until cell confluence (7–8 days). When cell confluence was 100% (Fig. 2), monolayers were trypsinized in a cocktail solution consisting of 0.4 mg/mL of collagenase type I-A, 0.4 mg/mL trypsin-EDTA, and 0.24 mg/mL of DNase (Invitrogen Corp., Carlsbad, USA). Then, the BOECs were frozen in order to avoid the lack of reproducibility between replicates

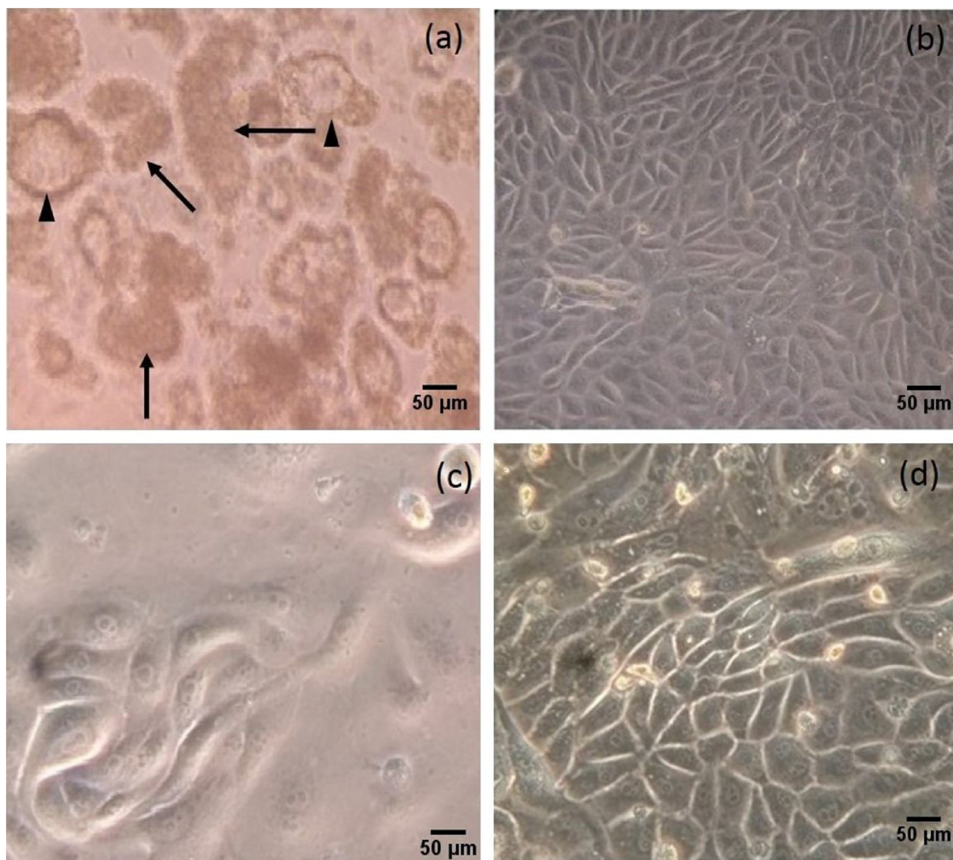

Fig. 2. Light micrographs of bovine oviductal epithelial cells (BOEC) in monolayer culture. (a) BOEC primary line after 2 days of culture presenting cell aggregates (arrows) capable of forming vesicles (arrowheads) that float in the culture medium. (b) Confluent monolayer of BOEC primary line after 7 days of culture showing a typical epithelial morphology with highly packed polygonal cells. (c) BOEC frozen/thawed after 2 days of culture showing a non-covered area. (d) Confluent monolayer of BOEC frozen/thawed after 7 days of culture.

that occur in studies that use different primary cultures [22]. The BOECs were frozen in a freezing medium (TCM-199 supplemented with 10% of dimethyl sulfoxide [DMSO], 20% of FCS and 0.5% of ATB/ATM) according to the previously described protocol [23] with some modifications. First, BOECs were cooled at 4 °C for 1 h, maintained at −80 °C for 24 h, and then stored in liquid nitrogen (−196 °C). After thawing, cells (final concentration: 1×10^5 cells/mL) were cultured until 100% of confluence (5–7 days) into individual wells of four-well plates (Nunc, Roskildle, Denmark) in a humidified atmosphere 5% CO_2 at 38.5 °C and subsequently used for sperm co-culture. The same BOEC-frozen/thawed line was used for each replicate of the experiment.

2.7. Semen collection and cooling process

Prior to the experiment, the extra-gonadal reserve was exhausted by daily semen collection for four consecutive days followed by two days of sexual rest [24]. In each replicate, fresh semen from three adult rams with proven fertility was collected using an artificial vagina. Semen was pooled with the objective of eliminating the individual factor as a variable of the analysis [25]. After measuring the volume of the semen, a sperm sample was immediately evaluated for motility, vigor, and concentration. Subsequently, the pooled semen was diluted with the OPTIXcell® extender (IMV Technologies, l'Aigle, France) to obtain 600×10^6 sptz/mL [26]. The diluted semen was packaged in 0.25 mL straws (IMV Technologies, l'Aigle, France), sealed, and then progressively cooled to 5 °C at a rate of −0.25 °C/min (TK3000® equipment; TK Equipamentos, Uberaba, Brazil) over the course of approximately 1.5 h. After cooling, straws were transported to the laboratory in a semen transport box (Botutainer®; Botupharma, Botucatu, Brazil) within 2 h. This semen transport thermobox maintained the temperature of the box at 5 °C for up to 36 h. In the laboratory, the straws were rewarmed to 37 °C and centrifuged (600 × *g*, 7 min) to flush out the extender [27].

2.8. Sperm selection (Swim-up technique)

The sperm selection procedure was performed using a method previously described by our group [28], with some modifications: 200 µL of cooled-stored sperm was carefully placed in the bottom of a 15 mL tube that contained 1 mL of modified Fert-TALP. The tube was positioned at a 45° angle and incubated for 45 min at 38.5 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant containing selected sperm was centrifuged (300 × *g*, 8 min). The sperm concentration of the pellet was estimated using a Neubauer counting chamber following dilution of the sperm sample in formaldehyde buffered saline (1:400).

2.9. Rate of ram sperm bound to BOEC

At each interval of incubation, medium with unbound spermatozoa was removed with a pipette, and the sperm concentration of the solution was calculated. The rate of spermatozoa attached to BOEC was estimated at different intervals of incubation (1, 2, 4, 6, 18 and 24 h) by subtracting the number of unbound spermatozoa from the total number of spermatozoa added to each well (i.e., 4×10^6 sperm) [29]. The rate of ram sperm bound to BOEC was estimated by dividing the number of spermatozoa attached to BOEC by 8×10^4.

2.10. Sperm evaluation

2.10.1. Sperm kinematics

Computer-assisted sperm analysis (CASA) was used to assess sperm kinematics. This was achieved using a computer with an SCA® system (Sperm Class Analyzer Microptic, Version 3.2.0, Spain) connected to a phase contrast and epifluorescence microscope (Nikon TM H5505, Eclipse 50i, Japan) equipped with a Basler Ace ACA780-75GC digital camera. The analysis was performed in phase contrast and to a

Table 1
Sperm kinematics of ram spermatozoa receiving different treatments during long-time incubation (Mean ± SEM).

Treatments*	Parameters									
	Incubation time	Total motility (%)	Progressive motility (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	LIN (%)	STR (%)	ALH (μm)	BCF (Hz)
	0 h‡	75.5 ± 4.3	27.4 ± 4.1	74.8 ± 11.7	89.6 ± 11.5	57.0 ± 9.4	61.8 ± 4.4	75.5 ± 2.6	2.6 ± 0.2	6.7 ± 0.3
POSControl	2 h	35.4 ± 7.0 ^{ab}	14.6 ± 6.0 ^{ab}	55.8 ± 8.3	63.3 ± 7.4	49.5 ± 8.7	75.8 ± 6.2	85.2 ± 3.9	1.4 ± 0.1	6.1 ± 0.4
Follicular NEGControl		40.0 ± 4.9 ^{a}	22.1 ± 4.2 ^{a}	70.3 ± 10.4	78.1 ± 10.1	64.3 ± 9.6	81.5 ± 2.1	91.3 ± 1.0	1.6 ± 0.0	6.5 ± 0.3
Luteal NEGControl		41.5 ± 3.6 ^{a}	21.9 ± 4.6 ^{a}	68.9 ± 11.2	75.5 ± 10.4	63.5 ± 11.0	81.6 ± 4.7	91.3 ± 1.5	1.6 ± 0.1	7.2 ± 0.2
Follicular BOEC		24.3 ± 5.0 ^{ab}	10.5 ± 3.8 ^{ab}	64.0 ± 13.3	71.4 ± 12.5	58.7 ± 12.8	76.7 ± 8.1	89.1 ± 3.3	1.4 ± 0.2	6.2 ± 0.8
Luteal BOEC		20.4 ± 5.3 ^{b}	5.6 ± 1.5 ^{b}	49.1 ± 5.5	57.8 ± 4.8	41.1 ± 5.7	69.7 ± 4.9	82.5 ± 3.3	1.4 ± 0.2	3.9 ± 0.5
POSControl	4 h	23.4 ± 4.1 ^{ab}	9.9 ± 3.2 ^{ab}	55.1 ± 6.1 ^{ab}	62.9 ± 5.5 ^{ab}	48.9 ± 6.1 ^{ab}	76.7 ± 3.7	88.0 ± 1.6	1.4 ± 0.0	5.7 ± 0.4 ^{ab}
Follicular NEGControl		36.8 ± 4.9 ^{ab}	18.0 ± 3.4 ^{a}	66.6 ± 2.9 ^{a}	73.5 ± 3.3 ^{a}	61.0 ± 2.7 ^{a}	83.0 ± 1.5	91.5 ± 1.2	1.5 ± 0.0	7.1 ± 0.1 ^{a}
Luteal NEGControl		40.8 ± 6.2 ^{a}	19.6 ± 4.8 ^{a}	63.9 ± 5.5 ^{a}	71.3 ± 5.9 ^{a}	57.5 ± 4.6 ^{a}	80.8 ± 1.7	90.3 ± 1.6	1.6 ± 0.1	7.3 ± 0.3 ^{a}
Follicular BOEC		25.5 ± 8.5 ^{ab}	6.7 ± 3.1 ^{b}	34.5 ± 12.9 ^{b}	43.1 ± 12.8 ^{b}	30.5 ± 12.0 ^{b}	55.2 ± 14.2	76.3 ± 10.9	1.2 ± 0.5	3.7 ± 1.5 ^{b}
Luteal BOEC		21.0 ± 3.4 ^{b}	5.2 ± 1.9 ^{b}	42.7 ± 7.3 ^{ab}	52.9 ± 3.7 ^{ab}	37.6 ± 8.2 ^{ab}	68.2 ± 13.5	81.4 ± 10.3	1.1 ± 0.2	4.5 ± 1.0 ^{ab}
POSControl	6 h	28.6 ± 6.3	11.6 ± 4.5	44.6 ± 5.9	51.0 ± 5.6	40.2 ± 5.7	78.2 ± 2.5	90.1 ± 1.2	1.3 ± 0.1	6.6 ± 0.1
Follicular NEGControl		31.2 ± 5.3	12.9 ± 2.6	49.4 ± 6.8	56.2 ± 5.9	45.6 ± 6.6	79.6 ± 3.8	91.9 ± 1.4	1.4 ± 0.1	7.0 ± 0.2
Luteal NEGControl		26.1 ± 2.4	11.2 ± 2.3	51.6 ± 8.0	58.2 ± 7.2	48.3 ± 7.9	81.4 ± 3.3	93.1 ± 0.9	1.2 ± 0.1	6.6 ± 0.4
Follicular BOEC		22.5 ± 5.2	5.3 ± 3.9	30.5 ± 13.1	39.6 ± 12.6	27.0 ± 12.8	51.8 ± 13.6	75.9 ± 8.5	0.9 ± 0.3	4.1 ± 1.1
Luteal BOEC		23.3 ± 6.2	6.2 ± 3.8	28.6 ± 9.3	37.6 ± 8.7	25.6 ± 9.0	59.7 ± 9.8	85.8 ± 3.8	0.9 ± 0.3	4.6 ± 1.4
POSControl	18 h	22.0 ± 1.8	5.1 ± 1.1	28.0 ± 3.8	33.8 ± 3.9	24.2 ± 3.4 ^{a}	70.9 ± 1.5 ^{a}	86.3 ± 0.5 ^{a}	1.2 ± 0.1 ^{a}	5.9 ± 0.4 ^{a}
Follicular NEGControl		20.4 ± 3.3	5.6 ± 2.3	26.5 ± 5.4	34.0 ± 4.8	23.7 ± 5.4 ^{a}	66.4 ± 6.4 ^{a}	88.0 ± 2.3 ^{a}	1.1 ± 0.1 ^{ab}	5.7 ± 0.9 ^{ab}
Luteal NEGControl		25.3 ± 6.5	8.8 ± 4.5	29.3 ± 8.6	36.4 ± 7.8	26.7 ± 8.3 ^{a}	65.1 ± 10.7 ^{a}	88.0 ± 2.8 ^{a}	1.1 ± 0.2 ^{ab}	5.6 ± 1.3 ^{abc}
Follicular BOEC		15.6 ± 4.7	1.4 ± 0.8	14.0 ± 3.7	23.8 ± 3.4	10.9 ± 3.6 ^{ab}	41.2 ± 8.9 ^{ab}	72.8 ± 6.1 ^{ab}	0.5 ± 0.1 ^{ab}	2.4 ± 0.5 ^{bc}
Luteal BOEC		15.1 ± 2.9	1.5 ± 1.1	13.2 ± 5.3	22.9 ± 4.5	9.7 ± 5.0 ^{b}	33.0 ± 13.6 ^{b}	55.7 ± 12.2 ^{b}	0.4 ± 0.3 ^{b}	2.3 ± 1.4 ^{c}
POSControl	24 h	14.8 ± 1.4	1.1 ± 0.4	15.5 ± 2.8	21.3 ± 2.4	12.9 ± 2.7	57.4 ± 7.6 ^{a}	80.2 ± 4.6	0.6 ± 0.2	2.9 ± 1.0
Follicular NEGControl		11.5 ± 1.7	0.7 ± 0.6	8.0 ± 3.0	18.2 ± 2.2	5.8 ± 3.0	27.2 ± 10.5 ^{b}	59.9 ± 10.1	0.3 ± 0.2	1.6 ± 1.1
Luteal NEGControl		17.8 ± 6.8	4.4 ± 4.1	13.2 ± 6.8	22.6 ± 5.9	11.0 ± 6.7	35.6 ± 12.7 ^{ab}	67.7 ± 9.1	0.4 ± 0.2	2.5 ± 1.2
Follicular BOEC		16.0 ± 2.1	2.1 ± 0.4	18.0 ± 4.3	25.5 ± 4.4	15.0 ± 4.1	53.8 ± 8.2 ^{ab}	79.8 ± 4.8	0.7 ± 0.2	3.3 ± 0.7
Luteal BOEC		12.6 ± 2.1	1.4 ± 0.5	15.2 ± 4.2	24.5 ± 4.2	11.5 ± 3.4	42.1 ± 8.9 ^{ab}	68.8 ± 10.6	0.6 ± 0.3	3.0 ± 1.2

Within a column, mean of values in the same incubation time followed by lower-case letters differed among them by Bonferroni test.

* Positive control (POScontrol): Fert-TALP medium; Follicular Negative control (Follicular NEGControl): Fert-TALP without capacitating substances + 290 pg/mL of E2 and 6 ng/mL of P4; Luteal Negative control (Luteal NEGControl): Fert-TALP without capacitating substances + 80 pg/mL of E2 and 85 ng/mL of P4; Follicular BOEC: Follicular NEGControl + cultive of bovine oviductal epithelial cells and Luteal BOEC: Luteal NEGControl + cultive of bovine oviductal epithelial cells.

‡ Incubation time (0 h): moment after sperm selection and before the addition of different media.

Raw data are shown but statistical comparisons were calculated after arc sine or logarithmic transformations.

Sperm motility parameters were evaluated by CASA system.

VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; LIN: linearity (ratio VSL/VCL); STR: straightness (ratio VSL/VAP); ALH: amplitude of lateral head displacement; BCF: beat/cross frequency.

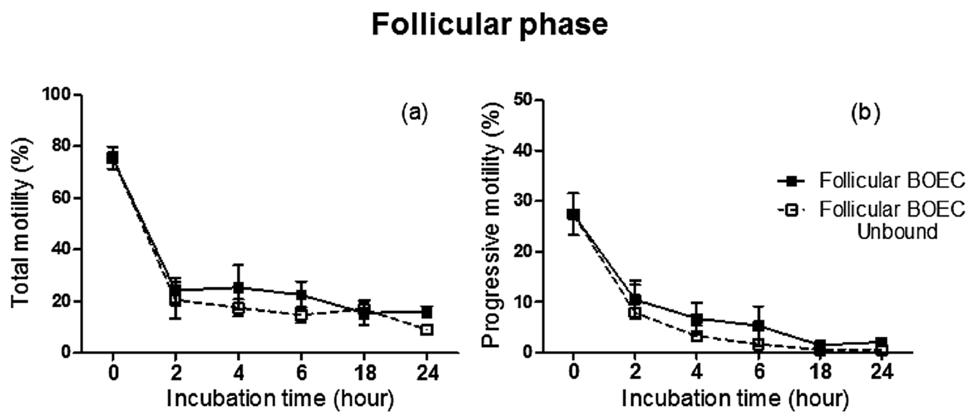

Fig. 3. Sperm motility of bound (Follicular BOEC) and unbound (Follicular BOEC Unbound) ram spermatozoa in co-culture with bovine oviductal epithelial cells at the follicular phase [total motility (a) and progressive motility (b)] during incubation of 24 h at 38.5 °C in 5% CO_2. Analyses were performed 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. Incubation time (0 h): moment after sperm selection and before the addition of different media.

magnifying power of x 100. 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 fields, including at least 200 cells, were analyzed [30]. Software settings were adjusted for ram sperm. The standard parameter settings were as follows: 25 frames/s, 18–60 μm^2 for sperm head area and curvilinear velocity (VCL) < 10 μm/s to classify spermatozoa as immotile. Sperm cells presenting straightness (STR) above 80% were featured as progressive. The variables analyzed were: total motility (TM; %), progressive motility (%), VCL (μm/s), straight-line velocity (VSL; μm/s), average path velocity (VAP; μm/s), straightness (STR: VSL/VAP; %), linearity (LIN: VSL/ VCL; %), amplitude of lateral head displacement (ALH; μm), and beat/cross frequency (BCF; Hz).

2.10.2. Sperm PM integrity

The parameter for this factor were determined according to the process described in [31] with slight modifications consisting of acridine orange (Sigma – Aldrich Corp.; A9231) [dilution factor: 1(acridine orange):9999 (distilled water), v/v] and 0.5 mg/mL propidium iodide probes. Acridine orange is a PM permeable probe selective to nucleic acids that emits green fluorescence. Propidium iodide is a non-PM permeable probe that binds to nucleic acids and subsequently emits red fluorescence. The test was performed using the CASA system connected to a microscope under epifluorescence illumination equipped with the appropriate filter sets (465–495 nm excitation and 515–555 nm emission) using x100 magnification. The analyzed slides included a minimum of 300 spermatozoa per slide [32].

2.10.3. Sperm capacitation status

Sperm capacitation status was assessed using chlortetracycline (CTC) staining as previously described [33] with some modifications [28]. Every day, a 0.75 mM CTC solution (pH 7.8) was prepared 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 and, after a few seconds, 1 μL of 4% paraformaldehyde was added [34]. A drop of 0.22 M 1,4-diaza-bicyclo (2,2,2) octane (DABCO) was also added to reduce the fading of the CTC fluorescence. The slides were covered with 24 × 24 mm coverslips and stored at 4 °C overnight in the dark. CTC patterns were evaluated using a microscope (Nikon Eclipse Ci[5]) under epifluorescence illumination, at 1000 x magnification with oil immersion. Cells were observed with a blue-violet 2 A (BV-2 A) filter with 400–440 nm excitation and 470 nm emission fluorescence. Two hundred spermatozoa per slide were classified according to the pre-defined criteria suggested in [35] into either uniform bright fluorescence over the whole head (non-capacitated cells, F pattern), fluorescence-free band in the post-acrosomal region (capacitated cells, B pattern), and full fluorescence over the whole head for a thin, bright band of fluorescence along the equatorial region (acrosome-reacted cells, AR pattern) [36].

2.11. Statistical analysis

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

3. Results

3.1. General aspects of BOEC morphology during culture

After 48 h of seeding in primary culture, active ciliary movement was observed under phase contrast microscopy. However, on Day 7 of culture, the monolayer achieved 100% confluence and the resulting monolayer presented loss of beating cilia (free cilia) from adhering cells. Cells showed similar morphology of epithelial cells presenting highly packed polygonal cells (Fig. 2).

3.2. Effect of treatment, time and their interaction on sperm parameters during incubation of 24 h

Two-way repeated measures ANOVA was performed to determine how a response was affected by two factors (treatment and incubation time) and to evaluate the interaction between these factors. The interaction of treatment × time was considered significant ($P < 0.05$) in some of the sperm parameters evaluated (VSL, LIN, STR, and BCF). When treatment and incubation time did not interact with each other ($P > 0.05$), the time effect was extremely significant ($P < 0.0001$) in all sperm parameters and the treatment had a significant ($P < 0.05$) effect on progressive motility, rates of capacitated, acrosome-reacted and non-capacitated sperm.

3.3. Effect of BOEC at either the follicular or luteal phase of co-culture on sperm kinematics during a 24-h incubation period

The kinematic parameters of sperm obtained during incubation with different media are shown in Table 1. Co-culturing BOEC with ram sperm, regardless of the phase of the estrous cycle, affected most of the sperm kinematics throughout the 24 h of incubation. Two hours after the start of co-culture, the proportion of progressive motility and total motility decreased ($P < 0.05$) more in the BOEC Luteal group

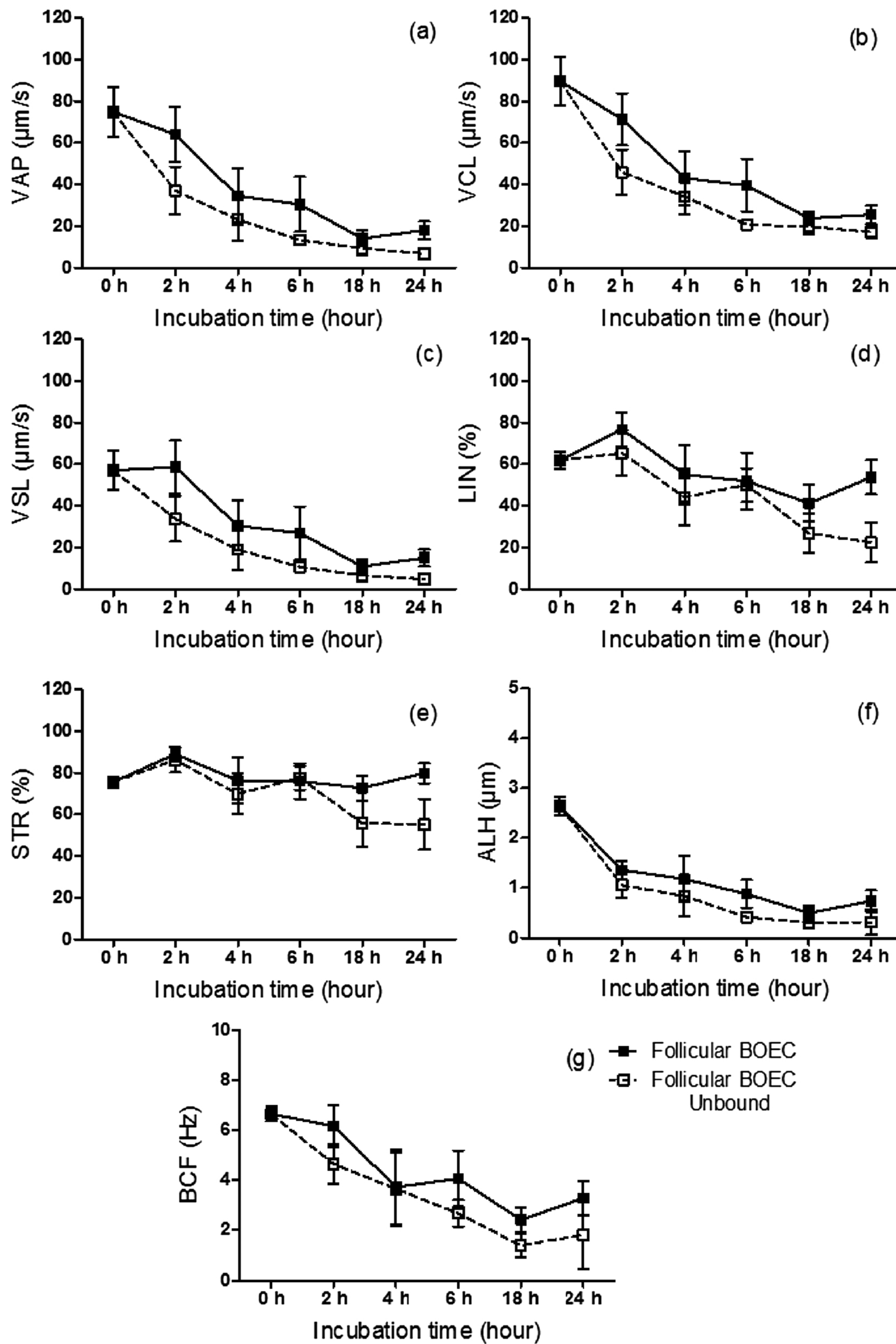

Fig. 4. Sperm kinematics of bound (Follicular BOEC) and unbound (Follicular BOEC Unbound) ram spermatozoa in co-culture with bovine oviductal epithelial cells at the follicular phase [VAP (a; average path velocity), VCL (b; curvilinear velocity), VSL (c; straight-line velocity), LIN (d; linearity (ratio 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 38.5 °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. Incubation time (0 h): moment after sperm selection and before the addition of different media.

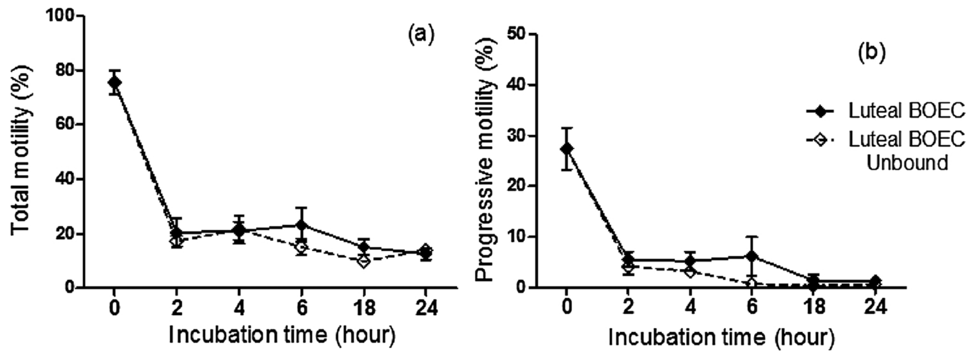

Fig. 5. Sperm motility of bound (Luteal BOEC) and unbound (Luteal BOEC Unbound) ram spermatozoa in co-culture with bovine oviductal epithelial cells at the luteal phase [total motility (a) and progressive motility (b)] during incubation of 24 h at 38.5 °C in 5% CO_2. Analyses were performed after 2 h, 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. Incubation time (0 h): moment after sperm selection and before the addition of different media.

compared with the Follicular and Luteal NEGControl groups (spermatozoa in the culture medium without cells). At 4 h of incubation, co-culture of BOEC at follicular phase reduced ($P < 0.05$) some of the kinematic parameters of sperm (VAP, VCL, VSL, BCF, and progressive motility) evaluated by the CASA system in comparison with the Luteal and Follicular NEGControl groups. At 6 h, the kinematic parameters of sperm evaluated by the CASA system were not affected ($P > 0.05$) by BOEC co-culture during either the follicular or luteal phases. However, the presence and interaction of BOEC in the luteal hormonal environment with ram sperm (BOEC Luteal group) continued promoting a reduction ($P < 0.05$) in some kinematic parameters of sperm (VSL, LIN, and STR) compared with the controls groups (POSControl, Luteal and Follicular NEGControl groups).

3.3.1. Ram spermatozoa bound to BOEC vs. Unbound ram spermatozoa

Data are shown in Figs. 3–6. The binding of ram sperm to BOEC in a hormonal environment similar to the follicular or luteal phase did not affect ($P > 0.05$) the kinematic parameters of sperm during the 24 h of incubation.

3.4. Effect of BOEC at either the follicular or luteal phase of co-culture on capacitation status and sperm PM integrity during a 24-h incubation period

The capacitation status and PM integrity during incubation with different media are presented in Fig. 7. The interaction between ram sperm and BOEC, regardless of the phase of the estrous cycle, decreased the sperm capacitation process during the 24-h incubation period. After 1 h of incubation, the proportion of capacitated spermatozoa was higher ($P < 0.05$) in the POSControl group than it was in the Follicular BOEC group but not the other groups. At 4 h of incubation, the proportion of capacitated spermatozoa was lower ($P < 0.05$) in the co-culture BOEC groups (Follicular and Luteal BOEC groups) than it was in the POSControl group. Correspondingly, the proportion of non-capacitated spermatozoa was higher ($P < 0.05$) in the Luteal BOEC group than the respective negative control group (NEGControl group). From 6 h to 24 h, the proportion of capacitated spermatozoa continued to be higher ($P < 0.05$) in the POSControl group in comparison to the Luteal BOEC group. On the other hand, the proportion of non-capacitated spermatozoa was higher ($P < 0.05$) in the Luteal BOEC group compared with the POSControl group. Co-culture of BOEC, regardless of the phase of the estrous cycle, had no effect ($P > 0.05$) on sperm PM integrity at all incubation intervals.

3.4.1. Ram spermatozoa bound to BOEC vs. Unbound ram spermatozoa

The capacitation status of the population of ram spermatozoa bound to BOEC in comparison to the population of unbound ram spermatozoa is shown in Fig. 8. The interaction between ram sperm and BOEC, regardless of the phase of the estrous cycle, had no effect on capacitation status at all incubation intervals (except at 2 h). At 2 h, the population of ram spermatozoa bound to BOEC in a hormonal environment similar to the luteal phase exhibited a lower ($P < 0.05$) proportion of capacitated spermatozoa in comparison to the population of unbound ram spermatozoa.

3.5. Rate of ram sperm bound to BOEC at follicular or luteal phase during a 24-h incubation period

The phase of the estrous cycle did not affect ($P > 0.05$) the ability of ram spermatozoa binding with BOEC during all incubation intervals (Fig. 9). Also, no differences ($P > 0.05$) were observed in the rate of ram sperm bound to BOEC throughout the incubation time in either follicular or luteal hormonal milieu (Follicular BOEC and Luteal BOEC groups).

4. Discussion

In addition to maintaining the fertilization ability of sperm, the oviduct reservoir also plays a key role in sperm selection, ensuring that only a competent sperm population is present at the time of ovulation [37]. This study investigated the effects of co-culture between ram sperm and BOEC pretreated with E2 and P4 concentrations similar to the follicular phase or the luteal phase on in vitro sperm capacitation and general sperm function during a prolonged period (18–24 h) of incubation. The main findings of this in vitro study are: (1) ram sperm co-incubation with BOEC, regardless of the phase of the estrous cycle, decreases the kinematic parameters of sperm until at least 4 h of incubation; (2) co-incubation with BOEC, regardless of the phase of the estrous cycle, delayed the in vitro ram sperm capacitation, indicating that a heterologous interaction could play a role in the capacitation process; (3) direct contact between ram sperm and BOEC, regardless of the phase of the estrous cycle, had no impact on the selection of ram spermatozoa with high motility.

Previous research has found that co-culture between OEC and sperm maintains sperm motility in several species [2,3,38,39]. However, other studies report that this interaction has the opposite effect on sperm motility [37,40]. Our findings indicate that the direct contact between ram sperm and BOEC in the presence of a hormonal concentration similar to either the follicular phase or the luteal phase promotes a negative effect on the kinematic parameters of sperm. These results are in agreement with those previously reported [40], which also observed a reduction in progressive motility in a homologous BOEC co-culture system. Indeed, attachment between spermatozoa and OEC is capable of suppressing motility and maintaining sperm quiescent to prevent a reduction in the ATP level and the reduction of the energy, ensuring adequate sperm energetic metabolism to fertilization. Conversely, when ovulation occurs and the ovulation-associated signal is released, spermatozoa detach from the OEC and acquire a “hyperactivated pattern” [41].

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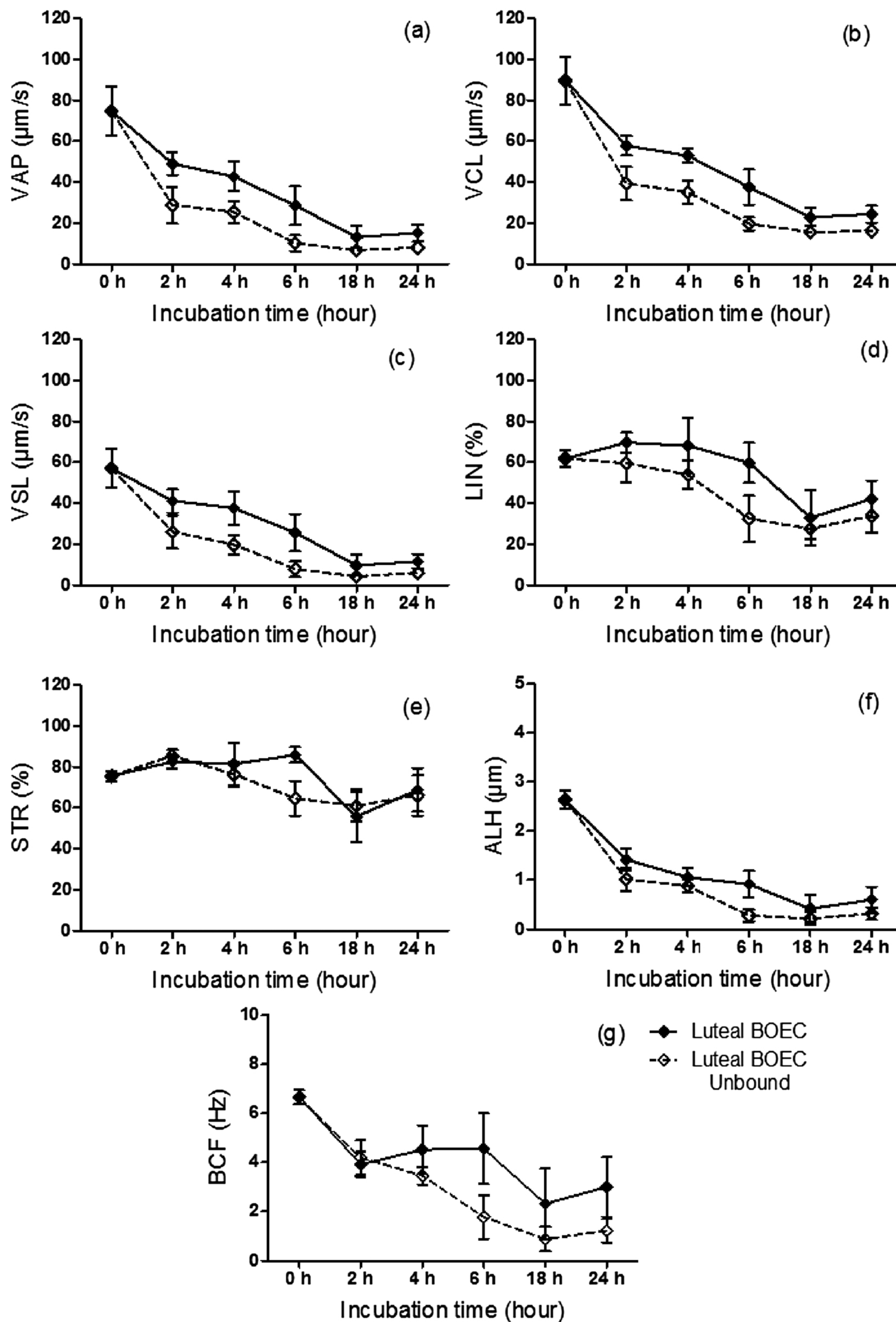

Fig. 6. Sperm kinematics of bound (Luteal BOEC) and unbound (Luteal BOEC Unbound) ram spermatozoa in co-culture with bovine oviductal epithelial cells at the luteal phase [VAP (a; average path velocity), VCL (b; curvilinear velocity), VSL (c; straight-line velocity), LIN (d; linearity (ratio VSL/VCL)), 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 38.5 °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. Incubation time (0 h): moment after sperm selection and before the addition of different media.

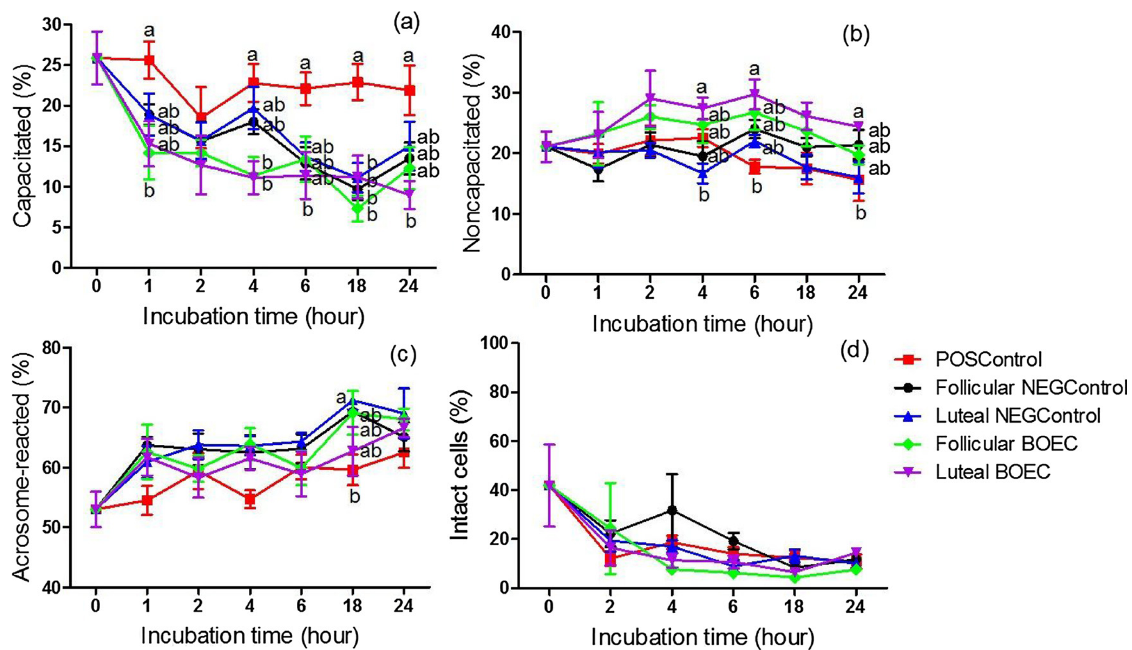

Fig. 7. Effect of bovine oviductal epithelial cells either at the follicular (Follicular BOEC) phase or the luteal (Luteal BOEC) phase on ram sperm capacitation status and sperm plasma membrane integrity [rate of capacitated sperm (a), rate of acrosome-reacted sperm (b), rate of non-capacitated sperm (c) and rate of intact cells (d)] during incubation of 24 h at 38.5 °C in 5% CO_2. Analyses were performed after 1 h (except sperm plasma membrane integrity), 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 to the Bonferroni test. Incubation time (0 h): moment after sperm selection and before the addition of different media.

During long-term co-incubation, the interaction between sperm and OEC seemed to slow down the process of membrane destabilization, promoting a beneficial effect on sperm viability [18]. The positive effect of the interaction between sperm and OEC in terms of sperm viability has been described in several others species [2,3,18,42]; however, in the present study, the heterologous OEC system appeared to have no impact on ram sperm viability. Our findings are in agreement with the results reported earlier [20], which also noted that neither the heterologous or homologous OEC system had a direct influence on ram sperm viability during 24-h period of incubation. These authors suggested that sperm viability could be regulated by oviductal products (proteins, glycoproteins, amino acids, and/or sugars) secreted into their surrounding medium as opposed to being the result of the direct contact with OEC. In this study, we used a monolayer model, which has a flat surface, to assess sperm-oviduct interactions. During sperm transport through the oviduct, sperm bind to ciliated cells on isthmus and the interaction prolongs sperm survival [1]. Thus, we believe that the presence of cilia in the oviductal epithelial cells has a positive effect on sperm viability.

Our results indicate that the co-culture between ram spermatozoa and BOEC, regardless of the phase of the estrous cycle, decreased sperm capacitation rate. These data support the view that sperm contact with OEC prolongs the functional lifespan of sperm through selection of uncapacitated sperm. This selection prevents the premature capacitation process, ensuring that it does not incur undesirable spermatozoa death before ovulation [43]. Our results are in agreement with those reported earlier in several species, the findings of which indicated that sperm interaction with OEC delays and decelerates capacitation, keeping cytosolic calcium concentration at the basal level [3,4,18,44].

In the present study, we observed that 1 h of co-incubation (ram sperm with BOEC) was sufficient to achieve~ 50% of spermatozoa bound to BOEC. This rate did not reduce throughout the incubation period, indicating that ram sperm quickly bound to BOEC and remained attached after a long period of incubation regardless of the influence of steroids and hormones. Recently, Lamy et al. [21] suggested that P4 induces the release of bound spermatozoa, and E2 (in concentrations above 100 pg/mL) inhibits the releasing effect of P4 on bound sperm. Although the finding was not significant, the addition of E2 in the concentration of 1 pg/mL appeared to inhibit the releasing effect of P4. In our study, we mimicked the oviductal environment during the estrous cycle by adding E2 and P4 to BOEC co-culture medium at the following concentrations: 290 pg/mL E2 and 6 ng/mL P4 (follicular phase) and 80 pg/mL E2 and 85 ng/mL P4 (luteal phase). Therefore, it is possible that the duality of effects between E2 and P4 was presented in both phases of the estrous cycle and the releasing effect was inhibited by the presence of E2.

In an attempt to recreate oviductal epithelium during estrous cycle in vivo, a previous in vitro study [45] reported changes in the morphological (including in ultrastructural level) and functional (gene expression) aspects of porcine OEC during diestrus (P4-domination) and estrus (E2-domination) phases. In contrast, our study showed that BOEC previously treated with E2 and P4 at concentrations similar to the follicular phase and the luteal phase did not have a different effect on ram sperm function and the capacitation process. This finding is in agreement with the findings of previous research [2], which also reported that the use of a homologous co-culture system did not have an estrous-cycle dependent effect. Therefore, our results suggest that the phase of the estrous cycle does not influence the modulation of sperm function and capacitation process in a heterologous OEC co-culture system. One possible explanation for this finding is that estrous cycle-dependent changes in OECs are time-dependent of hormonal stimulation [45] and also reliant on a precise definition of estrous cycle phase [5]. In our study, BOEC received a previous hormonal stimulation during 24 h, while in the study performed by [45], hormonal supplementation to mimic the estrous cycle phases lasted days (diestrus: 10 days and estrus: 2.5 days). Additionally, to the best of our knowledge, this is the first study of its kind to evaluate the effect the estrous cycle has on sperm function, simulating estrous cycle phases through the addition of steroids based on the hormonal levels present in the oviductal fluid. Therefore, the hormonal concentrations incorporated in our study were different from the concentrations used in other studies that were based on physiological blood hormone levels [5,45].

Fig. 8. Sperm capacitation status of bound and unbound ram spermatozoa in co-culture with bovine oviductal epithelial cells either at the follicular (Follicular BOEC) phase or the luteal (Luteal BOEC) phase [rate of capacitated sperm at the follicular phase (a), rate of capacitated sperm at the luteal phase (b), rate of acrosome-reacted sperm at the follicular phase (c), rate of acrosome-reacted sperm at the luteal phase (d) rate of non-capacitated sperm at the follicular phase (e) and rate of non-capacitated sperm at the luteal phase (f)] during incubation of 24 h at 38.5 °C in 5% CO_2. Analyses were performed after 1 h, 2 h, 4 h, 6 h, 18 h, and 24 h of incubation. Data are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$) among treatments according to the Bonferroni test. Incubation time (0 h): moment after sperm selection and before the addition of different media. At each sampling time, the medium containing unbound sperms were removed and replaced with BOEC co-culture medium. Then, BOEC-binding sperms were sampled by positioning the pipette tip at the bottom of the wells.

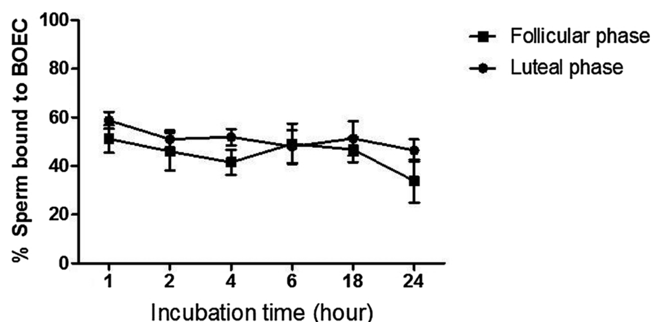

Fig. 9. Rate of ram sperm bound to BOEC either at the follicular phase or the luteal phase during incubation of 24 h at 38.5 °C in 5% CO_2. Analyses were performed after 1 h, 2 h, 4 h, 6 h, 18 h, and 24 h of incubation. Data are expressed as mean ± SEM.

Although co-culture between ram cooled-stored spermatozoa and BOEC previously treated with E2 and P4 at concentrations similar to the follicular phase or the luteal phase did not affect ram sperm PM integrity, it was able to modulate sperm capacitation and promote a negative effect on sperm motility. The interaction between ram sperm and BOEC reduced sperm capacitation, regardless of the phase of the estrous cycle, promoting a delay in sperm capacitation, which prolongs the lifespan of spermatozoa.

Declaration of interest

The authors have no conflict of interest to declare.

Author contributions

VAPA helped to conceive and design the experiment, acquire the data, analyze and interpret the data and wrote the original draft of the manuscript. JMGS-F and RITPB helped to conceive and design the experiment and critically revised the manuscript. FZB contributed with funding acquisition and critically revised the manuscript. LRC, GMB, CVS and LCC helped to acquire the data. All authors approved the final version of the manuscript.

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