Oviduct fluid during IVF moderately modulates polyspermy in in vitro-produced goat embryos during the non-breeding season

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Revised

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Running title: Oviduct fluid and polyspermy in goat embryos

ABSTRACT

The present study determined i) the presence of proteins (oviduct-specific glycoprotein, OVGP1; heat shock protein-70A, HSPA1A; heat shock protein-A8, HSPA8; annexin A1, ANXA1; annexin A5, ANXA5; and myosin-9, MYH9) known to be involved in early reproduction in the oviduct fluid (OF) of anestrous goats; and ii) the functional effect of OF during IVF on polyspermy modulation and embryonic development. *In vitro*-matured oocytes were co-cultured with spermatozoa (1.0, 2.0, or 4.0 x 10^6 cells/mL) for 18 h in SOF medium supplemented with 5 µg/mL of heparin, 4 µg/mL gentamicin, and 10% estrus sheep serum (CTRL1, CTRL2, and CTRL4 groups) or the same medium plus 10% OF (OF1, OF2, and OF4 groups) obtained from anestrus goats. The analysis of OF by western blotting confirmed the presence of the six proteins tested for. The increase in sperm concentration had no effect ($P > 0.05$) on the penetration rate in any group; however, monospermy rate decreased as sperm concentration was increased in both OF and CTRL. Regardless of the concentration used, when data were pooled, OF supplementation improved ($P < 0.05$) monospermy and tended ($P = 0.057$) to enhance IVF efficiency. Additionally, IVF efficiency was higher ($P < 0.05$) in OF1 than in OF4 [60 ± 13 vs 37 ± 5%). The development capacity was not affected ($P > 0.05$) by the sperm concentration and OF treatment, and the average values were cleavage (72 ± 2.6%), blastocyst (37 ± 3.0%), blastocyst in relation to the cleaved (51 ± 4.8%), hatched (62 ± 1.2%), and number of cells per blastocyst (174 ± 1.8%). In conclusion, the six proteins analyzed are present in the OF of anestrous goats, and the supplementation of this OF during IVF may modulate the polyspermy incidence and enhance IVF efficiency, especially when 1x10^6 sperm per mL is used.

Keywords: *anestrus; caprine; IVP; monospermy; oviduct proteins.*

1. Introduction

In most mammalian, gamete transport, final sperm capacitation, fertilization, and early embryo development are reproductive events that occur in the oviduct lumen [1]. In this microenvironment, gametes and early embryos are immersed in the oviduct fluid (OF), which is a mixture of plasma exudate, epithelial cell secretion, and follicular fluid released during ovulation [2]. In addition to metabolic components such as glucose, lactate, pyruvate, and amino acids, OF contains a wide variety of proteins which contribute to the success of early reproductive events [3]. Extracellular vesicles (EVs), including microvesicles and exosomes, are present in OF and play a fundamental role in the dialogue of oviduct epithelial cells with gametes/embryos [4,5]. Analyses of transcripts from oviduct epithelial cells [6] and protein composition [7] suggest that the composition of OF is dynamic and changes according to the concentration of sex steroid hormones such as 17β-estradiol (E2) and progesterone (P4), during the estrous cycle.

In sheep expressing spontaneous estrus, 624 proteins were identified in the OF, among which 280 were found to be differentially expressed throughout the estrous cycle, with 64 (23%) being more abundant at estrus and 17 (6%) at luteal phase [8]. Among the proteins in abundance at estrus, the oviduct glycoprotein-1 (OVGP1) has a role during the fertilization and interacts with heat shock protein (HSP) family members, such as HSPA8 and HSPA1A, and in the zona pellucida (ZP) hardening of the oocyte [9]. HSPA8 sperm exposure enhanced the sperm survival in boars and bulls and improved monospermy in pigs [10]. Annexins (*i.e.*, ANXA1 and ANXA5) are oviduct sperm-binding proteins involved with the sperm reservoir formation. This mechanism keeps the sperm viable near the site of fertilization, controlling the number of sperm cells arriving around the oocyte, and may intrinsically reduce the risk of polyspermy [4,11]. Myosin-9 (MYH9) has been identified as an important sperm-interacting protein with a possible role in sperm capacitation around fertilization. It acts by forming

66 complexes with other proteins of the OF (*e.g.,* OVGP1) before interacting with the sperm
67 surface [12].

68 In IVF, oocyte polyspermic penetration is a common problem observed in several
69 species, including goats [13–16]. It leads to embryo development failure and consequently a
70 reduction in IVF efficiency. High rates of polyspermy have been observed in prepuberal
71 (~48%) [17] and adult goats (~34%) [13]. Studies of different species have already
72 demonstrated that IVF medium supplementations or gametes treatment with OF or EVs
73 extracted from OF could promote gametes viability, fertilization rate, and embryo production
74 [18–20]. One of the benefits of using OF in *in vitro* systems is that it enhances monospermic
75 penetrations, possibly through inducing ZP hardening [9,14,21]. An improvement of cleavage
76 and blastocyst rates was also reported using OVGP1 purified from OF in a goat IVF protocol
77 [20]. OF effects on gametes were observed when using heterologous co-incubation systems,
78 such as ram sperm/bovine OF [18] and oocytes from several species (cows, sheep, goats,
79 pigs, rats, rabbits, and humans) with OF from different species [22].

80 There is no information about the OF proteome in goats, and only OVGP1 has been
81 reported in the literature [20,23,24]. Proteome studies in bovine [25] and ovine [8] showed
82 some similarities of OF protein expression profile throughout the estrous cycle. The follicular
83 phase (late or pre-ovulatory) is related with high E2 concentration, which is the major
84 modulator of the OF proteins involved in the gamete interactions and fertilization [5,26,27].
85 In small ruminants under temperate climate conditions, the seasonal anestrus compromises
86 natural reproduction, although follicular waves and steroid production are maintained [28].
87 Thus, based on this evidence, a study that determines the protein profile and role of OF from
88 anestrous females seems sound. Therefore, the present study was designed to i) determine the
89 presence of proteins involved in the fertilization process (OVGP1, ANXA1, ANAX5,

HSPA8, HSPA1A, and MYH9) in the OF of goats in anestrus; and ii) assess the functional effect of this fluid during IVF on polyspermy and embryo development.

2. Materials and Methods

The study was performed at Unité de Physiologie de la Reproduction et des Comportements in Nouzilly, France (47º22'N and 00º41'E), during the non-breeding season (June to September) [29,30]. Chemicals were purchased from the Sigma Chemical Co. (Saint Louis, MO, USA), except where otherwise indicated.

2.1. *Experimental design*

The goat oviducts and ovaries used during the study were obtained at a local slaughterhouse. In June, the flushing containing OF was centrifuged, aliquoted, and stored at -80 ºC. From early July to early September, a sample was used to determine the presence of important proteins by western blotting [4]. Over six runs 1,576 COCs were used and submitted to *in vitro* maturation (IVM), followed by co-culture with three different sperm concentrations (1.0, 2.0, and 4.0 x 10^6 cells/mL) in IVF medium supplemented with 10% oviduct flushing, corresponding to ± 10% OF (OF1, OF2, and OF4) or without (CTRL1, CTRL2, and CTRL4). After IVM, the COCs were allocated to the following groups: CTRL1 (n=259), CTRL2 (n=270), and CTRL4 (n=254); and OF1 (n=261), OF2 (n=266), and OF4 (n=266) for the IVF. After IVF, a part of the presumptive zygotes (n=628) was denuded, fixed, stained, and used to evaluate polyspermy, while the others (n=914) were cultured until day 8 to evaluate development.

2.2. *OF recovery*

Genital tracts and the attached ovaries were transported to the laboratory in individual bottles (dry) in a thermos box at 30 ºC within 2–3 h of collection. The estrous cycle phase was assessed by ovarian morphology, according to Camp et al. [31]. The absence of corpus luteum (CL) and preovulatory follicles, characterizing acyclicity, was observed throughout the experiment. Consequently, all the oviducts and ovaries used were classified as being in seasonal anestrous. Oviducts were dissected free of surrounding tissues: A needle coupled to a 1 mL syringe was inserted into the infundibulum and 500 µL of IVF medium were slowly and carefully injected into the oviduct lumen. A manual descendent (from ampulla to isthmus) pressure was applied to collect the flushing. The oviductal flushing of the pair of oviducts from each female was recovered in a falcon tube of 15 mL and centrifuged at 300 × *g* for 15 min at room temperature to remove the cells. The supernatant was transferred into a new tube (FALCON white lid) and centrifuged at 12,000 × *g* for 15 min at 4 ºC to remove cellular debris and apoptotic bodies. Clarified OF was stored at -80 ºC until use. Before use, a pool of samples from three females was prepared, aliquoted, and frozen for use throughout the experiment.

2.3 *Western blotting*

Proteins were selected based on the previous identification of oviduct proteins with known reproductive roles in bovine [4,32] and porcine [14,19] species. Therefore, bovine and porcine OF were used as positive control in western blots (WB). For each species, an OF pool from three animals was used. OF samples were diluted in reducing Laemmli loading buffer (2x buffer; composition: 125 mM Tris, 20% glycerol, 4% sodium dodecyl sulfate, and 10% tris (2-carboxyethyl)phosphine), followed by vortexing, heating in a water bath at 95 ºC for 5 min and being centrifuged at 12,000 *g* for 5 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide using a 10% polyacrylamide gel containing 40 µg of proteins per lane

139 at 180 V for 45 min [33]. After separation, proteins were transferred onto nitrocellulose
140 membranes (Bio-Rad, 1704271) over 30 min up to 1.0 A and 25 V using semi-liquid
141 Transblot® Turbo™ System (Bio-Rad). After transfer, membranes were washed with distilled
142 water and blocked with Tris-buffered saline solution containing Tween 20 (0.5% w/v; TBST),
143 supplemented with lyophilized low-fat milk (5% w/v; TBST-milk) for 2 h at room
144 temperature. Then, membranes were incubated with primary antibodies overnight at 4 °C,
145 washed four times in TBST, and incubated with IRDye® Fluorescent secondary antibodies for
146 45 min at 37 °C in the dark, both under gentle agitation. The protein bands were revealed with
147 UV light exposure, and images were digitalized using Odyssey CLx Near-Infrared
148 Fluorescence Imaging System (LICOR), in triplicate. The primary antibodies used for
149 immunoblotting detection were anti-oviduct-specific glycoprotein (OVGP1; Santa Cruz
150 Biotechnology, sc-377267); anti-annexin A1 and A5 (ANXA1 and 5; Santa Cruz
151 Biotechnology); anti-heat-shock protein 70 (HSP1A1; Stressgen, SPA-810); anti-myosin
152 heavy chain 9 (MYH9-H40, Santa Cruz Biotechnology, sc-98978); and Anti-Hsc70 (HSPA8;
153 Abcam, ab65170). Secondary antibodies were IRDye® 800CW anti-Rabbit IgG and IRDye®
154 680RD anti-Mouse IgG (1:10000; LICOR).
155
156 *2.4. Cumulus-oocyte complexes (COCs) recovery*
157 Slaughterhouse ovaries from adult goats were collected and transported to the
158 laboratory in a thermos box containing saline solution (0.9% NaCl) at 30 ºC within 4 h of
159 collection. Ovaries were washed with fresh saline solution at 30 ºC and the stage of estrous
160 cycle was classified. Oocytes were aspirated from all visible follicles between 2 and 6 mm in
161 diameter with an 18-ga short bevel needle connected to a Falcon tube under controlled
162 vacuum (30 mm Hg). The collection tubes had been previously filled with 3 mL of HEPES-
163 buffered TCM 199 supplemented with 10 IU/mL heparin (Choay; Glaxo Wellcome

Production, Notre Dame de Bondeville, France), 4 µg/mL gentamicin (G1272), and 1 mg/mL BSA (A 9647).

2.5. In vitro maturation

COCs were isolated under stereomicroscope (Nikon Corporation, Japan) and screened by morphology. Only COCs surrounded by at least one complete layer of unexpanded cumulus cells and finely granulated oocyte cytoplasm were used for IVM; the rest were discarded [34]. COCs were washed four times in four-well petri dishes (Nunc, Roskilde, Denmark) containing 500 µL of TCM 199 (M4530) supplemented with gentamicin (4 µg/mL). Then, 40 to 50 COC groups were transferred into another four-well petri dish containing 500 µL of TCM199 supplemented with 10 ng/mL EGF and 100 µM cysteamine, and incubated for 22 h at 38.8 ºC in a humidified atmosphere of 5% CO_2 in air.

2.6. Semen preparation and in vitro fertilization

For each replicate used in the experiments, two straws of frozen semen (frozen at the same time) from the same batch and from the same two bucks were thawed (37 ºC for 30 s) and pooled. Motile sperm were separated by centrifugation (15 min at 700 × *g*) on 3 mL of Percoll (Pharmacia, Uppsala, Sweden), discontinuous density gradient (45/90%). The sperm pellet was washed by centrifugation (100 × *g* for 5 min) in 600 µL of IVF-Hepes medium that consisted of synthetic oviduct fluid [SOF; 107.7 mM NaCI, 7.16 mM KCl, 1.19 mM KH2PO4, 1.71 mM CaC12, 0.49 mM MgCl2, 25.07 mM NaHCO3, 3.3 mM Na lactate, 0.3 mM Na pyruvate, 1.3 µg/mL phenol red, 1 mM glutamine, 3% essential (M-6766), 1% nonessential (B-7145) amino acids, and 0.3% BSA fraction V; pH = 7.3; 280 m0sm] supplemented with 2.4 mg/mL Hepes (H3375), and 4 µg/mL gentamicin. The sperm pellet was resuspended in IVF medium (described below).

After maturation, the COCs were washed once in 500 µL of IVF medium and transferred into the fertilization well containing 250 µL of medium. Sperm suspension was diluted in IVF medium to reach final concentrations of 1.0, 2.0, or 4.0 × 10^6 cells/mL by adding 250 µL to IVF wells. The IVF medium consisted in SOF (already described) containing 10% of heat inactivated estrus sheep serum (ESS), 5 µg/mL heparin (Calbiochem 375095) and 4 µg/mL gentamicin (control groups; CTRL1, CTRL2 and CTRL4) or this same medium supplemented with 10% of OF (OF groups; OF1, OF2, and OF4). Sperm and COCs (40 to 50/well) were co-incubated for 18 h at 38.8 ºC in a humidified atmosphere of 5% CO_2 in air.

2.7. Assessment of fertilization

The presumptive zygotes reserved for evaluation of polyspermy (approximately 40% of the initial number of inseminated COCs) from each group were transferred within a 10 µL droplet onto a grease-free slide and dried at room temperature. They were then fixed in 100% ethanol for at least 3 h. After drying, structures were stained in 1 µg/mL Hoechst 33342 fluorochrome (stains all nuclei) in Vectashield mounting medium (Vector Labs, Burlingame, CA, USA), and overlaid with a cover slip sealed with nail varnish. Slides were stored at 4 ºC until they were examined using epifluorescence microscopy to evaluate fertilization. Zygotes with three or more pronuclei (or decondensing sperm heads) were considered polyspermic.

2.8. In vitro development (IVD)

After IVF, the zygotes were placed into 15 mL Falcon tubes containing 2 mL of washing medium (SOF; 2.4 mg/mL Hepes and 2 µL/mL BSA) and vortexed for 2 min to remove cumulus cells. The presumptive zygotes were recovered in 35 mm petri plates, washed four times in culture medium (SOF; supplemented with 3 mg/mL BSA), and reserved

for evaluation of polyspermy or transferred in groups of maximum 25 into four-well petri dishes containing 25 µL (1 µL/structure) drops of culture medium covered with 700 µL mineral oil. The presumptive zygotes were cultured for 8 days at 38.8 ºC in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. At 48 h post-insemination (PI), the drops were supplemented with 10% fetal calf serum (FCS). On day 8, all blastocysts were washed, spotted onto microscopy slides, fixed in ethanol 100% for 3 h, dried, and stained with Hoechst (1 µg/mL) to count their total cell number under epifluorescence microscope.

2.9. Statistical analyses

The efficiency of development was evaluated (1) as the percentage of cleaved embryos 2 days PI and the percentage of blastocysts at 8 days PI, expressed (2) on the basis of the number of oocytes entering into IVD or (3) on the basis of the number of cleaved embryos at day 2. The following end points were assessed: penetration rate (penetrated zygotes/total oocytes x 100); monospermy rate (monospermic/penetrated zygotes x 100); and IVF efficiency (monospermic zygotes/total oocytes x 100). Data were tested for normality using the Kolmogorov-Smirnov test. Variables were compared by one-way ANOVA followed by Tukey test. Differences were considered significant when $P < 0.05$ and tendency when $P < 0.10$. Data are shown in mean ± S.E.M.

3. Results

3.1. Western blotting

The analysis of candidate proteins confirmed the presence of the six proteins (OVGP1, ANXA1, ANXA5, HSPA1A, HSPA8, and MYH9) associated with reproductive functions in the OF obtained from goats in anestrus. The size of each protein observed in the goat sample

238 is compatible with the sizes observed in the bovine and swine samples used as positive
239 controls (Fig. 1).
240
241 *3.2. Effect of OF on IVF outcomes*
242 When comparing OF with CTRL at the same sperm concentration (OF1 vs. CTRL1,
243 OF2 vs. CTRL2, and OF4 vs. CTRL4, respectively), the fertilization parameters, such as
244 penetration rate (Fig. 2A, P = 0.32, P = 0.83, P = 0.62), monospermy rate (Fig. 2B, P = 0.14,
245 P = 0.08, P = 0.18), and IVF efficiency (Fig. 2C, P = 0.11, P = 0.55, P = 0.15), were similar.
246 The increase in sperm concentration had no effect on the penetration rate (Fig. 2A) either for
247 control (P = 0.82, CTRL1: 67 ± 11; CTRL2: 69 ± 16, and CTRL4: 72 ± 15%) or OF groups (P
248 = 0.74, OF1: 74 ± 13; OF2: 71 ± 14, and OF4: 67 ± 17%). Regardless of the concentration
249 used, when data were pooled, gametes exposure to OF during IVF improved (P = 0.04)
250 monospermy and tended (P = 0.06) to enhance IVF efficiency in terms of production of
251 normally fertilized zygotes from total oocytes (Fig. 2D). In addition, IVF efficiency was
252 greater (P = 0.002) in OF1 than OF4 [60 ± 13 vs 37 ± 5%), with no effect (P = 0.11) in the
253 control groups [CTRL1: 45 ± 16; CTRL2: 46 ± 13, and CTRL4: 31 ± 9%) (Fig. 2C). The
254 monospermy rate decreased as sperm concentration was increased, in both OF and CTRL
255 (Fig. 2B).
256
257 *3.3. Embryo development*
258 Gametes exposure to OF during IVF did not affect any parameter related to
259 developmental competence, inferred by rates of cleavage (P = 0.63, P = 0.61, P = 0.94),
260 blastocysts in relation to oocytes (P = 0.75, P = 0.85, P = 0.94), blastocysts in relation to
261 cleaved embryos (P = 0.26, P = 0.86, P = 0.92) and hatched blastocysts in relation to
262 blastocysts (P = 0.99, P = 0.83, P = 0.89), as well as the total number of cells per blastocyst (P

263 = 0.94, P = 0.83, P = 0.89, Table 1). When comparing OF1 to OF4, there was a tendency (P =
264 0.08) to favor OF1 in the blastocyst/cleaved oocytes parameter. When the data were pooled
265 regardless of the treatment, average cleavage was 72 ± 2.6%, blastocyst 37 ± 3.0%, blastocyst
266 in relation to cleaved 51 ± 4.8%, hatched 62 ± 1.2%, and total number of cells per blastocyst
267 174 ± 1.8. Goat blastocysts are shown in Fig. 3.
268
269 **4. Discussion**
270 To mimic physiological reproductive conditions *in vivo*, co-culture of
271 gametes/embryos with oviduct epithelial cells, EVs (microvesicles and exosomes), and
272 purified or integral OF proteins may represent the best models of *in vitro* systems [1].
273 Interestingly, in this study we demonstrated that the main proteins (OVGP1, ANXA1,
274 ANAX5, HSPA8, HSPA1A, and MYH9) involved in the fertilization process are present in
275 the OF of anestrus goats. However, a moderate effect only was observed on fertilization
276 parameters evaluated after the OF exposure of the gametes during IVF. Even though no
277 differences were found when treatments using the same sperm concentrations (OF1 vs.
278 CTRL1, OF2 vs. CTRL2, and OF4 vs. CTRL4) were compared, when data were pooled
279 regardless of concentrations, the OF exposure significantly improved monospermy and tended
280 to enhance IVF efficiency in terms of production of normally fertilized zygotes.
281 A comparison of the extreme concentrations within the same treatment (CTRL1 vs
282 CTRL4 and OF1 vs OF4) revealed that penetration rate was not affected; however, a
283 monospermy decrease was observed when 4 x 10^6 sperm/mL was used in both (CTRL4 and
284 OF4). These data partly diverge from those previously described in prepubertal goats [35],
285 where penetration rate was enhanced when 4 x 10^6 sperm/mL was used, but polyspermy was
286 also increased. For adult goats, the standard sperm concentration used for IVF is between 1
287 and 2 x 10^6 sperm/mL [13,36], but 4 x 10^6 sperm/mL has also been reported in the literature,

aiming to enhance oocyte penetration [20,37]. However, our data demonstrate that penetration was not affected by concentration and thus 1 x 10^6 sperm/mL could be recommended for adult goat IVF.

Importantly, IVF efficiency was greater in OF1 than in OF4. Conversely, no effect was observed on the control (CTRL1 vs CTRL4). We believe that the effect observed in OF groups could be due to an enhancement of sperm quality. In this way, we recently demonstrated that bovine OF was able to enhance ram sperm motility for up to 4 h as well as rate of acrosome reaction after long (18–24 h) incubation periods without affecting sperm viability [18]. In addition, there was a tendency to favor OF1 in the rate of blastocyst in relation to the cleaved structures. This parameter is often associated with the quality of the oocyte and its capacity to sustain embryonic events until the maternal to zygotic transition, which occurs at 8-16 cells in goats [38]. However, in the current study, the oocytes were pooled at the beginning of the experiment, before the treatments were applied, and it is unlikely that this affected this parameter in any way. One aspect that may certainly not be overlooked is the possibility that the development of the polyspermic embryos was blocked before they reached the blastocyst stage [39]. When data were pooled regardless of sperm concentration, the OF total average (OF1+OF2+OF4) promoted a greater monospermy rate than the CTRL total average and tended to increase IVF efficiency. Overall, these data reinforce our hypothesis that the association of 10% OF and 1.0 x 10^6 cells/mL sperm during IVF is the best combination to increase monospermy, obviously reducing polyspermy, improving IVF efficiency, and resulting in a lower number of embryos being blocked. Based on these data, we suggest that when OF is present, the IVF must be performed using a lower sperm concentration.

It is important to highlight that there were no significant differences in the comparison between treatments using the same sperm concentrations (OF1 vs. CTRL1, OF2 vs. CTRL2,

313 and OF4 vs. CTRL4). It should be emphasized that the OF was collected from anestrus goats.
314 Considering the high influence of the season on small ruminant reproductive systems due to
315 hormonal alterations [40], we suppose that this may have affected the overall protein
316 concentrations and their role in the polyspermy modulation. The OF depends on the
317 concentration of ovarian steroid hormones, which modulate the physiological and
318 reproductive events that occur in the oviduct lumen [31]. During the estrous cycle,
319 fluctuations of E2 and P4 concentrations induce changes in the epithelium of the oviduct and
320 secretory function [2,3], which alter the proteomic and metabolomic profile of OF [4,30] to
321 provide an optimal microenvironment for fertilization and early development. The practically
322 constant concentrations of E2 during anestrus [28] may have modified the profile of proteins
323 involved in the fertilization process, which were capable of exerting only a moderate control
324 of polyspermy in the present study.

325 According to previous studies, throughout the estrous cycle the OF proteins OVGP1,
326 HSPA8, HSPA1A, and MYH9 were most abundant at estrus in sheep showing a spontaneous
327 cycle [8]. OVGP1 and HSPA8 were also detected at luteal phase but in much lower quantities
328 than during estrus [8]. In bovine, at ipsilateral to ovulation side, ANXA1 and MYH9 are most
329 abundant in the pre- and post-ovulatory periods, respectively. OVGP1 is overabundant in both
330 pre- and post-ovulatory stages compared with mid-luteal, HSPA8 is more abundant in the pre-
331 ovulatory stage, and there is no significant fluctuation in HSPA1A during the estrous cycle
332 phases [25]. In heifers with induced estrus (synchronization by Ovsynch® protocol plus
333 buserelin application) HSPA8, OVGP1, MHY9, and ANXA1 were among the 20 most
334 abundant proteins [41].

335 OVGP1 has been proposed as the main player in monospermy regulation, and ZP
336 hardening could be the mechanism involved [7,13]. While in aquatic organisms a rapid
337 depolarization of the oocyte plasma membrane prevents polyspermy within a few seconds, a

similar mechanism is still controversial in mammals even though a primary block to polyspermy, before the release of cortical granules, is also suspected. In mice and humans, the oocyte Juno membrane protein has been shown to play a role in polyspermy regulation before the cortical granule reaction [42]. Shortly after fertilization, Juno is released in the perivitelline space and binds to the Izumo partner of additional sperms, preventing their fusion to the oocyte membrane [43]. Juno and Izumo orthologs are found in most mammalian species, but their role in domestic species polyspermy regulation is yet to be explored. However, it has been shown that anti-Izumo antibodies can reduce fertilization in pigs [44]. Nevertheless, the possible effect of OF on Juno-Izumo interactions needs to be investigated.

There is evidence in the literature that monospermy modulation in goats may be different to that in other species. Possibly, the OVGP1 has a concentration-dependent effect in goats, or it is only active in a short temporal window near ovulation, as occurs in swine [3]. Besides OVGP1, MYH9 participates in the gamete-oviduct interaction process; OVGP1 may bind to spermatozoa and oocytes through the partner MYH9 by a non-glycosylated N-terminal conserved region [45]. It has been proposed that monospermic fertilization may be enhanced by the functional effects of OVGP1 and MYH9 via EVs, binding to the ZP, perhaps through modification of its carbohydrate and protein composition [11]. Additionally, EVs could bind to spermatozoa by annexins (e.g., ANXA1 and ANXA5), preventing a massive spermatozoa arrival at the oocyte and fertilization by apoptotic spermatozoa, leading to improvements in IVF efficiency [11]. In addition, OVGP1 and ANXA1 are amongst the most abundant embryo-interacting proteins from OF in 4-6 cells and morula stage embryos [46].

HSPA8 increases sperm viability *in vitro* and has been associated with the maintenance of sperm survival around ovulation [10]. HSPA1A is an EVs marker, an exosomal-specific protein present in 89% of proteomic studies [4,47], which confirms the presence of EVs in goats' OF used in our study. A study demonstrated that at adult oviduct,

363 the percentage of HSPA1A positive cells tended to decrease during estrus and increase during
364 diestrus, and was associated with the rise of the percentage of estrogen receptor-positive cells.
365 HSPA1A is an oviduct protein that is modulated differentially between oviduct regions, being
366 more abundant in highly steroid-responsive regions, such as the infundibulum and ampulla.
367 Moreover, HSPA1A-positive cells were found to be more abundant during rats in early
368 pregnancy than in non-pregnant rats, suggesting they play a role in early embryo development
369 [48].

370 Data in the literature support our speculation that the moderate OF effect seen in our
371 study could be associated with the low steroid levels during the non-breeding season, which
372 decreased the concentration of some OF proteins. The constant level of E2, with rare
373 fluctuations in small ruminant females in anestrus [28,40], may indicate a moderate role of
374 OF in monospermy modulation. Therefore, future studies aiming to compare the IVF effect of
375 OF throughout the estrous cycle at breeding season might be of interest. In addition, the
376 characterization of the protein profile of OF at different stages of the estrous cycle may be
377 important for the identification of additional proteins that enhance the beneficial effect of OF
378 on monospermic fertilization. Another possibility would be to supplement goat IVF medium
379 with heterologous OF from a non-seasonal species (e.g., cows) as an alternative to reduce
380 polyspermy in goat IVF.

381 In conclusion, the main proteins (OVGP1, ANXA1, ANAX5, HSPA8, HSPA1A, and
382 MYH9) involved in the fertilization process are present in the OF of anestrus goats.
383 Moreover, the OF supplementation in IVF during gametes co-culture may moderately
384 modulate polyspermy incidence and enhance IVF efficiency, especially when 1.0×10^6 sperm
385 is used.

386

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Conflict of interest

None of the authors have any conflict of interest to declare.

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551 Table 1. Effect of adult anestrous goat oviduct fluid (OF) supplemented at IVF on *in vitro*
552 embryo production (IVP) system, considering different sperm concentrations at IVF. Mean
553 ± S.E.M.

Treatment	n	Cleavage (%)	Bl (%)[#]	Bl/cleaved (%)[#]	Hbl/total bl (%)[#]	Total cells (n)[#]
OF1[*]	148	65 ± 7.1	41 ± 4.7	65 ± 8.1	64 ± 3.7	174 ± 11.5
CTRL1[**]	154	71 ± 6.6	38 ± 6.5	54 ± 5.8	65 ± 8.6	175 ± 15.0
OF2[*]	155	77 ± 1.7	39 ± 5.8	50 ± 7.6	59 ± 7.9	181 ± 11.6
CTRL2[**]	156	72 ± 4.7	38 ± 4.3	52 ± 4.7	65 ± 8.7	177 ± 14.9
OF4[*]	152	75 ± 6.5	33 ± 3.5	44 ± 1.9	59 ± 8.5	168 ± 12.1
CTRL4[**]	149	74 ± 4.3	32 ± 5.7	43 ± 7.1	61 ± 13.9	170 ± 14.4
Total OF[***]	455	72 ± 3.3	38 ± 2.7	53 ± 4.2	61 ± 3.9	174 ± 3.8
Total CTRL[***]	459	72 ± 2.9	36 ± 3.1	50 ± 3.4	63 ± 5.8	174 ± 2.1

554 "n" represents the number of structures after IVF submitted to IVD

555 [A,B] differ within column between treatments (OF vs. CTRL) in the same sperm concentration

556 [a,b] differ within column among sperm concentrations (1 vs 2 vs 4) in the same treatment

557 [C,D] differ within column between the total averages (Total OF vs Total CTRL)

558 [*] OF1, OF2 and OF4: IVF medium supplemented with 10% of OF and sperm concentration used at
559 IVF was 1.0, 2.0, and 4.0 × 10^6 cell/mL

560 [**] CTRL1, CTRL2, and CTRL4: no supplementation with OF, and sperm concentration used at IVF
561 was 1.0, 2.0, and 4.0 × 10^6 cell/mL

562 [***] Total average for OF (OF1, OF2, and OF4) and CTRL (CTRL1, CTRL2, and CTRL4) groups

563 [#]Bl: blastocyst, Hbl: hatched blastocyst, and Total cells: number of blastomeres per blastocyst
564 (n=22/group in three runs).

565 **Figure captions**

566 **Fig. 1.** Presence of proteins related to monospermy modulation in oviduct fluid (OF), such as
567 annexin A1 (ANXA1), annexin A5 (ANXA5), anti-myosin heavy chain 9 (MYH9), anti-heat-
568 shock protein 70 (HSPA1A), heat shock cognate protein 70 (HSPA8), and oviduct-specific
569 glycoprotein (oviductin, OVGP1). Standard (St) and samples: bOF and sOF (bovine and
570 swine oviduct fluid, positive controls), and gOF (goat oviduct fluid). The gOF was collected
571 from goats in the anestrus season.

572

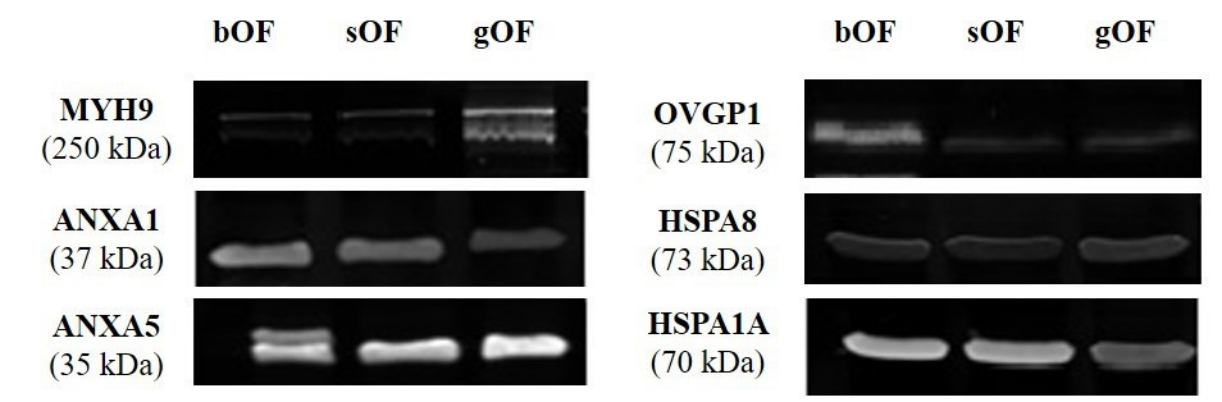
573 **Fig. 2.** Effect of goat oviduct fluid (OF) supplementation or not (Control: CTRL) at IVF with
574 three sperm concentrations: 1.0 x 10^6 (OF1 and CTRL1), 2.0 x 10^6 (OF2 and CTRL2), and
575 4.0 x 10^6 (OF4 and CTRL4) on the goat IVF parameters. (A,D) Penetration rate; (B,D)
576 monospermy/penetration rate; (C,D) IVF efficiency in terms of production of normally
577 fertilized zygotes from total oocytes; (E) normal monospermic zygote containing two
578 pronuclei; (F) polyspermic zygote containing three pronuclei plus a decondensed spermatozoa
579 head under Hoechst stain. (A-D): each bar represents mean ± SEM. For each group,
580 approximately 628 presumed zygotes were evaluated. ^{A,B} differ between treatments (OF vs.
581 CTRL) at the same sperm concentration (A-C); ^{a,b} differ among sperm concentrations (1 vs 2
582 vs 4) in the same treatment; ^{C,D} differ between the total averages (Total OF vs Total CTRL).

583

584 **Fig. 3.** (A) Goat embryos at day 7 of culture produced from oocytes recovered in the non-
585 breeding season (100 x); (B) Fresh blastocyst (400 x) and (C) Blastocyst stained with Hoechst
586 (400 x).

587

588


bOF
sOF
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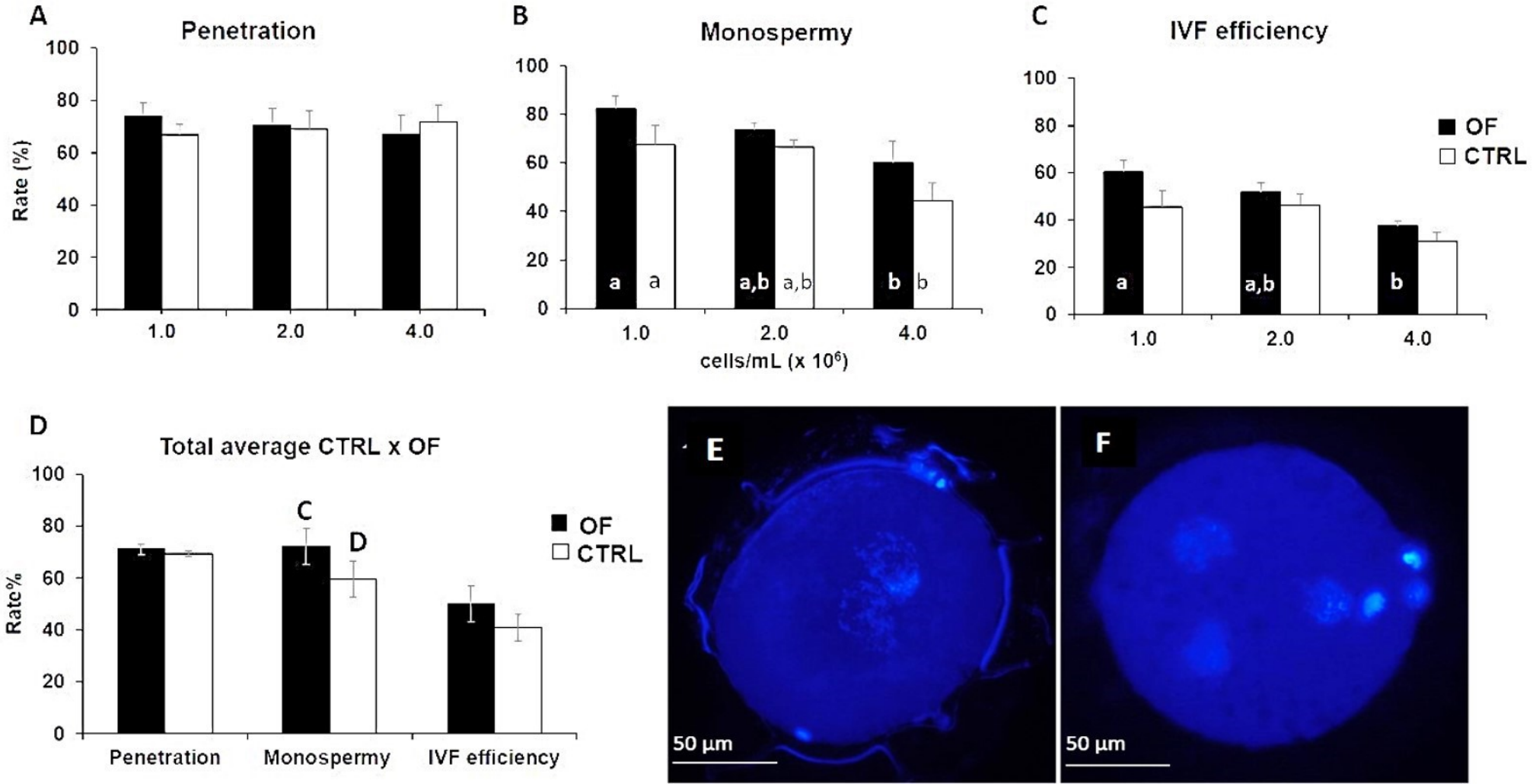
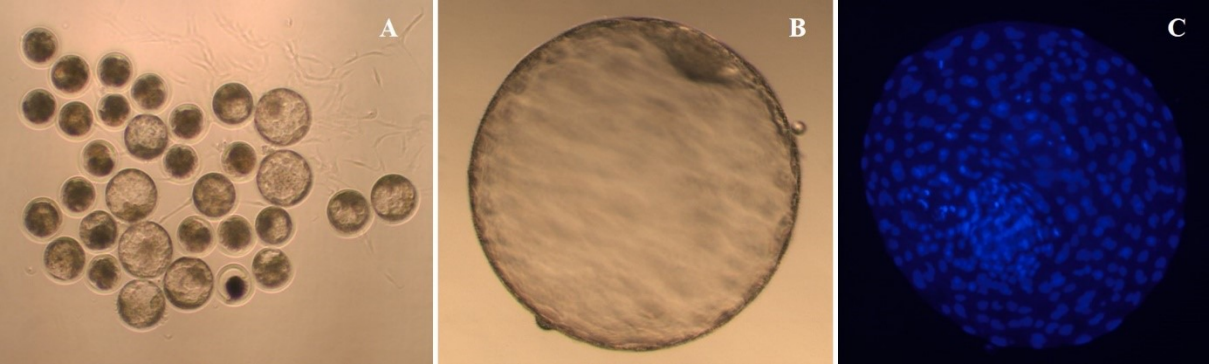
MYH9
(250 kDa)
ANXA1
(37 kDa)
ANXA5
(35 kDa)
bOF
sOF
gOF
OVGP1
(75 kDa)
HSPA8
(73 kDa)
HSPA1A
(70 kDa)


A
Penetration
Rate (%)
100
80
60
40
20
0
1.0
2.0
4.0
B
Monospermy
a
a
a,b
a,b
b
b
cells/mL (x 10⁶)
C
IVF efficiency
OF
CTRL
D
Total average CTRL x OF
Rate%
C
D
Penetration
Monospermy
IVF efficiency
E
50 µm
F
50 µm


A
B
C