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Combination of oviduct fluid and heparin to improve monospermic zygotes production during porcine *in vitro* fertilization

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ABSTRACT

In vivo, the oviduct provides appropriate microenvironment conditions for monospermic fertilization and early embryo development. In addition, glycosaminoglycans such as heparin are present in the oviduct and have been shown to modulate the activity of oviduct-secreted proteins on the regulation of sperms parameters. Thus, the present study was designed to evaluate the effect of porcine oocytes exposure to oviduct fluid (OF) before *in vitro* fertilization (IVF; incubation of oocytes in OF for 30 minutes before IVF), during IVF (supplementation of IVF medium with 10% OF), and during IVF in combination with heparin (10% OF + 10-µg/mL heparin) on IVF parameters. Regardless of sperm concentration used (0.5, 1.5, or 4.5×10^5 cells/mL), exposure of oocytes to OF led to an increased ($P < 0.05$) monospermy rate, without alteration ($P > 0.05$) of the penetration rate in comparison with the control group. This resulted in a general increase ($P < 0.05$) in the final output of the IVF system in terms of zygotes with two pronuclei in OF-exposed groups: $56 \pm 9\%$ (OF before) and $60 \pm 7\%$ (10% OF during IVF), compared with control ($21 \pm 8\%$), when IVF was performed with 4.5×10^5 cells/mL. The combination of 10% OF with heparin during IVF induced a decrease ($P < 0.05$) of the penetration rate, with no effect ($P > 0.05$) on the monospermy rate in comparison with 10% OF alone. This resulted in a general reduction ($P < 0.05$) in the final output of the IVF system (%), which was $33 \pm 6\%$ and $52 \pm 8\%$, for 10% OF + heparin and 10% OF, respectively. In conclusion, the OF, used in porcine IVF, exerted a beneficial effect on oocytes by reducing the incidence of polyspermy without decreasing the penetration rate. However, the association of the OF with heparin reduced the efficiency of monospermic zygotes' production.
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1. Introduction

Penetration of the oocyte cytoplasm by more than one spermatozoon, called polyspermy, is a pathologic condition in placental mammals, usually causing early death of the embryo. Polyspermy is a common problem of *in vitro* fertilization (IVF) in cows [1], sheeps [2], goats [3], humans [4], and pigs [5], although its prevalence under natural conditions is moderate. Particularly in pigs, this defect affects approximately 50% of *in vitro* fertilized oocytes [6], whereas *in vivo* the prevalence is less than 5% [7]. Consequently, the *in vitro* production of porcine embryos for genetic improvement or recovery of endangered breeds is hampered, as well as for advanced biotechnologies such as transgenesis and genome editing [5,8].

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In vivo, two groups of mechanisms have traditionally been proposed as contributing to the block to polyspermy in mammals: mechanisms on the basis of the oviduct, avoiding the mass arrival of spermatozoa in the vicinity of the oocyte, and oocyte-based mechanisms, including alterations in membrane and zona pellucida (ZP) in reaction to sperm fertilization [9]. In the temporal sequence of events, the first major mechanism to prevent polyspermy in pigs occurs when male gametes crossing the uterotubal junction [10,11]. Once in the oviduct, the epithelial cells in the caudal region of the isthmus can bind the sperms in humans, sheeps, cows, and pigs [12–14], decreasing their movements and prolonging their survival [15,16]. Sperms selection and binding avoid a massive, simultaneous arrival of male gametes to the proximity of the oocyte [9].

In contact with spermatozoa in the ampulla region of the oviduct, the mammalian oocyte prevents polyspermy by a process known as cortical reaction. This process consists on a structure modification of the ZP and oocyte plasma membrane [17–20] after fertilization. Cortical granules (CG) exocytosis, which is initiated by calcium oscillations during sperm penetration [21], induces the fusion of the CG to the ooplasma and the exocytosis of their content into the perivitelline space which modifies the oocyte plasma membrane and the ZP, rendering the oocyte refractory to additional sperm binding and penetration [22].

Normally, the number of spermatozoa around the oocyte during IVF is higher than under *in vivo* conditions [11], which has been associated with high incidence of polyspermy. However, in pigs, decreasing the sperm concentration during IVF induces a parallel reduction in penetration frequency, decreasing polyspermy but not avoiding it [23,24]. On the other hand, delay in CG exocytosis in *in vitro* matured oocytes was also proposed as being responsible for a high degree of polyspermy [25]. However, Wang et al. [26] reported that the time of cortical reaction is similar between *in vivo* and *in vitro* matured oocytes under appropriate culture conditions.

Sathananthan et al. [27] and Van Der Ven et al. [28] suggested that the final maturation of the oocyte in the oviductal environment is a necessary step for successful fertilization and embryo development. Indeed, many studies support a functional role of the oviduct and its secretions (which are rich in estrus-associated glycoproteins) during fertilization, regulating processes such as sperm–ZP binding, the establishment of species-specific ZP barriers, and early embryonic development [29–32]. Glycosaminoglycans (GAG) such as heparin are present in the oviduct and have been shown to modulate the activity of oviduct-secreted proteins on the regulation of spermatozoa parameters. Coy et al. [6] have shown that incubation of oocytes for 30 minutes before IVF in oviduct fluid (OF) decreases the incidence of polyspermy and that heparin is responsible for the stability of the ZP resistance to digestion with pronase. Thus, the present study was designed to examine the effect of porcine oocytes exposure to OF before or during IVF and the association of heparin with OF in the occurrence of polyspermy and *in vitro* embryo production rates.

2. Material and methods

Chemicals were purchased from the Sigma Chemical Co. (Saint Louis, MO, USA) unless otherwise indicated.

2.1. Oviducts classification and OF recovery

Genital tracts from sows were obtained at a local slaughterhouse and transported to the laboratory on ice. The estrous cycle stage of the females was assessed in the laboratory, on the basis of ovarian morphology on both ovaries from each female. Oviducts from sows were classified as early follicular, late follicular, early luteal, or late luteal phase, according to the criteria defined by Hafez and Hafez [33]. Both oviducts coming from the same genital tract, classified as late follicular phase, were used. Tracts with ovaries not clearly matching these criteria, polycystic, and from pregnant females were discarded. After classification, sow oviducts were quickly washed once with 70% ethanol solution and two times with Dulbecco's PBS and transferred to Petri dishes on ice and dissected free of surrounding tissues. The oviducts flushed with 500 μL of PBS were introduced into the ampulla tip for washing the lumen. Making a manual ascendant pressure from the ampulla to the isthmus, the liquid was recovered. This procedure was repeated in 10 oviducts. The same fluid recovered in the first oviduct was reused to wash the next one. The oviductal flush was centrifuged at $7000 \times g$ for 10 minutes at 4 °C to remove cellular debris. Then, the supernatant was immediately stored at −20 °C until use.

2.2. In vitro maturation

Ovaries were collected from slaughtered peripubertal gilts and transported to the laboratory within 2 hours in 0.9% NaCl at 30 °C. Cumulus oocyte complexes (COC) were aspirated from antral follicles (3–6 mm in diameter) with an 18-gauge short needle connected to a Falcon tube under controlled vacuum (30 mm Hg). Then, COC were recovered under a stereomicroscope. Immature COC with a compact cumulus cell mass were washed three times in 25-mM Hepes-buffered tissue culture medium 199 (TCM199) with Earle's salts supplemented with 4-μg/mL gentamicin (G1272) and 1-mg/mL BSA (A9647) and washed once in maturation medium. Groups of 50 COC were transferred into 4-well plates (Nunc Roskilde, Denmark); each well containing 500 μL of maturation medium and cultured for 44 hours at 38.8 °C in an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium consisted of TCM199 with Earle's salts (M4530) supplemented with 10 ng/mL of epidermal growth factor (E4127), 400 ng/mL of follicle-stimulating hormone (PRIMUFOL, Rhône Mérieux, Lyon, France), 570-μM cysteamine (M9768), and 10% fetal calf serum (F2442; [34]).

2.3. Preparation of spermatozoa

Straws of frozen semen were prepared from a pool of three Large White boar ejaculates [35]. After thawing in a

water bath at 37 °C for 30 seconds, sperm from one straw were washed in Beltsville thawing solution (Landata, France) by centrifugation at 100 × *g* for 10 minutes at room temperature. Motile spermatozoa were obtained by centrifugation of the pellet on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous gradient (2 mL of 45% over 2 mL of 90%) for 30 minutes at 700 × *g*. Cells collected at the bottom of the 90% fraction were washed in fertilization medium by centrifugation at 100 × *g* for 10 minutes. The sperm pellet was then resuspended to give a concentration of 2×10^8 cells/mL.

2.4. In vitro fertilization

After the maturation period, oocytes were denuded by vortexing 2.0 minutes in 2 mL of TCM199 hepes then washed three times in the same medium and once in fertilization medium before being transferred in groups of 50 oocytes into 4-well plates. Each well contained 250 μL of fertilization medium. The fertilization medium consisted in modified Tris-buffered medium (1), with 113.1-mM NaCl, 3-mM KCl, 10-mM $CaCl_2$, 20-mM Tris, 11-mM glucose, 5-mM sodium pyruvate, 1-mM caffeine, and 0.1% BSA EFAF [34]. Aliquot of sperm suspension was added to each fertilization well to obtain their final concentrations: 0.5, 1.5, or 4.5×10^5 cells/mL. Fertilization was performed at 38.8 °C, 100% humidity in an atmosphere of 5% CO_2 in air.

2.5. In vitro development

Embryo development took place in modified synthetic oviduct fluid (SOF) medium under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.8 °C [36]. The SOF medium contained 107.7-mM NaCl, 7.16-mM KCl, 1.19-mM KH_2PO4, 1.71-mM $CaC1_2$, 0.49-mM $MgCl_2$, 25.07-mM $NaHCO_3$, 3.3-mM Na lactate, 0.3-mM Na pyruvate, 1-mM glutamine, 3% essential (B-6766) and 1% nonessential (M-7145) amino acids, and 0.3% BSA fraction V [34]. After 20 hours of fertilization, sperm were removed by gentle vortexing of the putative zygotes. They were then washed three times in TCM199 Hepes and once in SOF medium before being transferred in groups of 20 into oil overlaid 25-μL droplets of SOF medium, supplemented with 10% fetal calf serum. On Day 8, all expanded blastocysts were transferred into washing plates, washed to remove the mineral oil, fixed, and stained with Hoechst to count their total cell number. Cell counting was conducted under an epifluorescence microscope.

2.6. Assessment of fertilization

After fertilization, five putative zygotes from each group in each replicate were randomly collected and placed on a slide, air-dried, and fixed in absolute ethanol for 24 hours. They then were stained with Hoechst 33342 (10 μg/mL in 2.3% sodium citrate) and visualized with an epifluorescence microscope for analysis of total number of spermatozoa bound to the ZP. Another group of 15 presumptive zygotes per replicate were fixed (alcohol-chloroform-acetic acid, 80:10:10; v/v), stained with 1% (w/v) lacmoid, and examined at × 400 magnification for evidence of sperm penetration and pronuclear formation under a phase contrast microscope.

2.7. Experimental design

In experiment 1 (Fig. 1), the effect of oocyte incubation with porcine OF before IVF or the addition of OF during IVF, on IVF and *in vitro* development (IVD) results, were investigated. After *in vitro* maturation (IVM), COC were randomly allocated to one of three groups: (1) control—IVF in Tris-buffered medium (TBM); (2) OF 30′ before—incubation of oocytes in pure OF for 30 minutes, followed by three washings in TBM medium, then transfer into fertilization well; and (3) 10% OF—IVF in TBM medium supplemented with 10% OF. For each replicate, approximately 450 oocytes were equally allocated into one of the three treatments. For IVF, oocytes from each experimental group were allocated into three wells (around 50 oocytes per well) and co-cultured with either 0.5, 1.5, or 4.5×10^5 sperm cells/mL (totaling nine groups) for a period of 20 hours. After IVF, samples of 20 presumptive zygotes from each group were fixed and stained for analysis of number of spermatozoa bound to ZP, number of pronuclei per oocyte, and IVF efficiency (percentage of monospermic from total inseminated). The remaining presumptive zygotes were transferred to IVD, where embryo cleavage and blastocyst formation were evaluated: on Days 2 and 7 after fertilization, respectively. On Day 8, all blastocysts were fixed for counting their total cell number. Five replicates were performed.

In experiment 2 (Fig. 2), the effect of the association of OF with heparin (10 μg/mL; Calbiochem 375 095) on IVF parameters was evaluated. After IVM, COC were denuded and randomly allocated to one of the four treatments based on TBM IVF medium: (1) TBM + 10% OF + heparin (n = 150); (2) TBM + 10% OF (n = 136); (3) TBM + heparin (n = 146), and (4) TBM alone, control (n = 145). Co-culture with spermatozoa (4.5×10^5 cells/mL) was performed for 20 hours. Presumptive zygotes from each group were fixed and stained for analysis of number of spermatozoa bound to ZP, number of pronuclei per oocyte, and IVF efficiency (percentage of monospermic zygotes from total oocytes inseminated). IVD parameters were not evaluated in this experiment. Five replicates were performed.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean. Data for all rates were modeled according to the binomial model of parameters. The rate of oocyte penetration, number of sperm cells per penetrated oocyte, male pronucleus formation, monospermy rate, and total numbers of spermatozoa bound to the ZP were tested for normality using the Kolmogorov–Smirnov test. The parameters were compared using one-way ANOVA followed by Tukey's test. Differences were considered statistically different at $P < 0.05$. The parameters of embryonic development, cleavage, and blastocyst rates were compared by chi-square test.

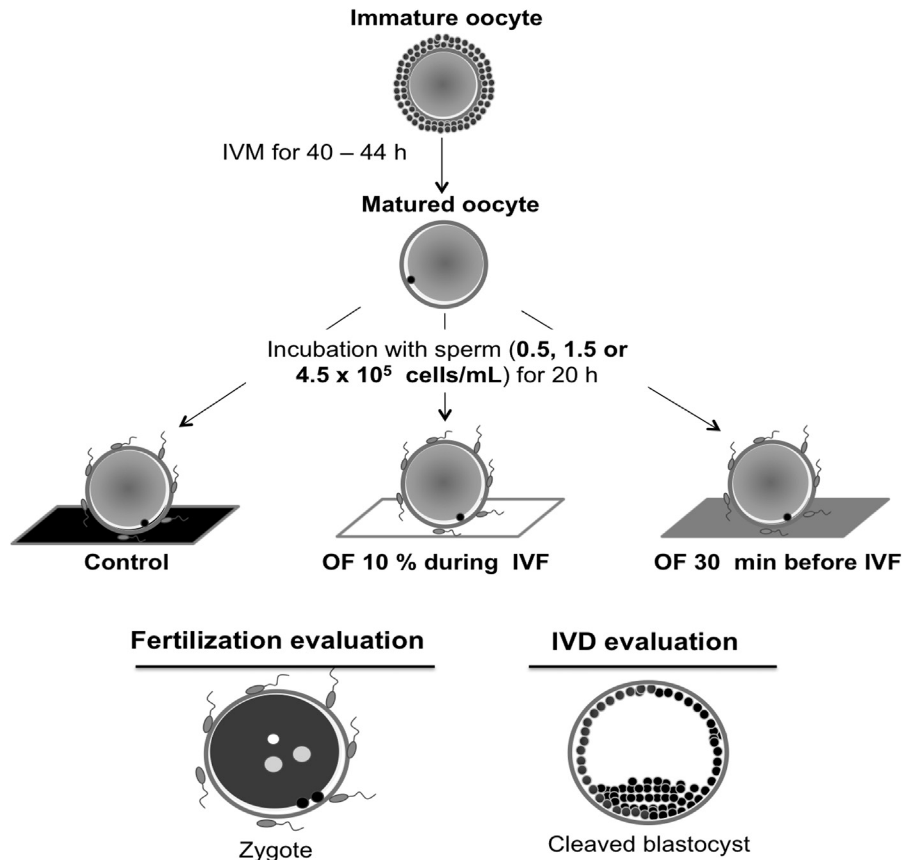

Fig. 1. Experimental design of experiment 1. Effect of oviduct fluid before or during IVF on the incidence of fertilization, polyspermy, and development rate of porcine oocytes. IVF, *in vitro* fertilization.

3. Results

3.1. Experiment 1. Effect of oocytes incubation with OF before and during IVF on IVF and IVD results

Regardless of sperm concentration, incubation of oocytes with OF both before and during IVF led to increased ($P < 0.05$) monospermy rate (Fig. 3B), with no effect ($P > 0.05$) on penetration rate (Fig. 3A) compared with the control group. This resulted in an increased ($P < 0.05$) efficiency of the IVF system in terms of production of normally fertilized zygotes from total oocytes: OF-exposed groups: 56 ± 9% (10% OF during IVF) and 60 ± 7% (OF 30 minutes before), compared with control (21 ± 8%), when using sperm concentration of 4.5×10^5 cells/mL (Fig. 3C). In both OF-exposed groups, the number of penetrated spermatozoa per oocyte (Fig. 3D) and the number of spermatozoa bound to the ZP (Fig. 3E) were lower ($P < 0.05$) in comparison with the control group. However, the number of spermatozoa bound to the ZP increased ($P < 0.05$) with higher sperm concentration in all groups, and this was much more pronounced in the control group.

The developmental rates and number of cells in blastocysts are listed in Table 1. The cleavage rate was not significantly affected by sperm concentration or exposure to OF. However, the blastocyst formation rate was lower ($P < 0.05$) in the control group when compared with the OF-exposed groups in sperm concentration of 1.5 and 4.5×10^5 cells/mL. When using the concentration of 0.5×10^5, this difference was not observed. The blastocyst production in the OF-exposed groups was lower when using a sperm concentration of 0.5×10^5 compared with 1.5×10^5 and 4.5×10^5 cells/mL. The total cell number per blastocyst was similar in all the groups.

3.2. Experiment 2. Effect of OF and heparin on IVF results

The IVF results of groups exposed or not to OF and/or heparin are shown in Figure 4. The combination of heparin with 10% OF decreased ($P < 0.05$) the penetration rate in comparison with the other groups (Fig. 4A). However, the monospermy rate in 10% OF + heparin was similar to 10% OF group, and both were higher than the control and heparin groups (Fig. 4B). This resulted in a reduction ($P < 0.05$) of the production of normally fertilized zygotes with OF and heparin compared with OF alone (33 ± 6 and 52 ± 8, for 10% OF + heparin and 10% OF, respectively, Fig. 4C). In OF-exposed groups, the number of pronuclei per oocyte (Fig. 4D) and the number of spermatozoa bound to the ZP (Fig. 4E) decreased in comparison with control and heparin groups.

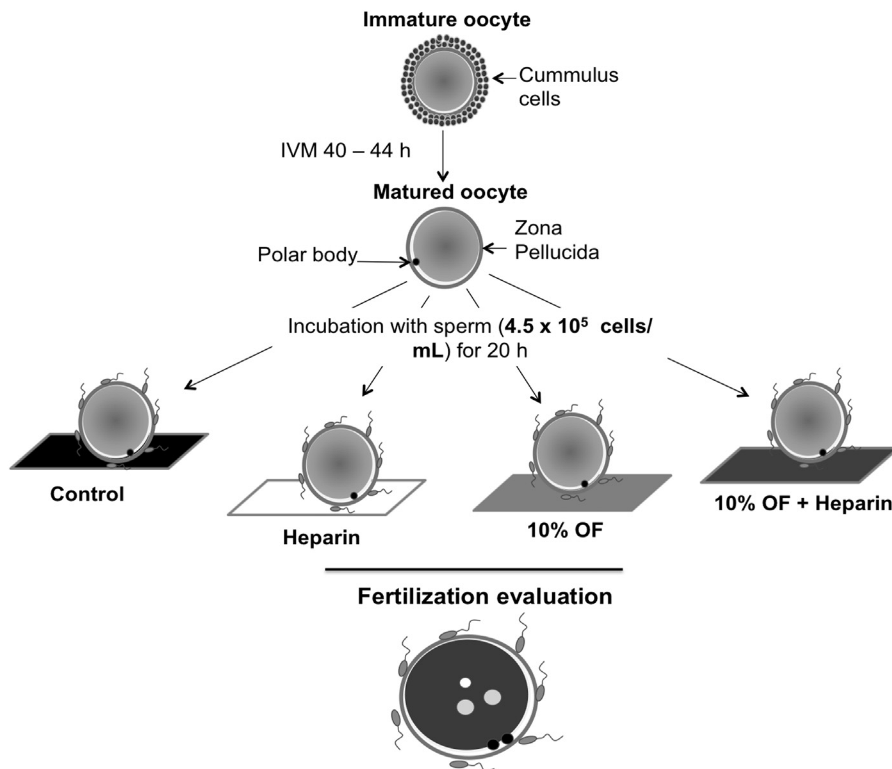

Fig. 2. Experimental design of experiment 2. Effect of oviduct fluid and heparin during IVF on the incidence of fertilization and polyspermy. IVF, *in vitro* fertilization.

4. Discussion

The results of this study showed that incubation of IVM oocytes with OF before or during IVF decreased the number of sperm-bound ZP and the incidence of polyspermy, regardless of sperm concentration. These data support the hypothesis of the presence of important factors in OF that contribute to the regulation of fertilization. Several proteins in the oviductal fluid can bind to the ZP, modifying both its proteins and carbohydrates composition [37]. Indeed, it has been reported that oviduct-specific glycoprotein (OVGP1), osteopontin, lipocalin-type prostaglandin D synthase, and lactoferrin can associate with the ZP in different species [38]. OVGP1 is the most studied ZP-associated protein and its role in fertilization seems to be related with the ZP hardening and the control of the number of spermatozoa bound to the ZP [37].

In the case of ZP hardening, a series of experiments with oocytes from nine species and oviductal fluids from five species indicated that the short incubation of the oocyte with oviductal fluid produces a clear change in ZP resistance to enzyme digestion [39]. It was suggested that OVGP could be masking the sites where proteinases act by steric hindrance and therefore prolonging the digestion time needed by the proteinase to hydrolyze the ZP. On the other hand, according to Coy et al. [6], a possible role of the ZP-anchored OVGP is to mask the sperm/ZP binding sites. However, both mechanisms can lead to a reduction of the number of sperms bound to ZP and, therefore, to a reduction of polyspermy. This is in agreement with a maturation of the ZP in the oviductal environment, leading to increased proteinase resistance and, in turn, to polyspermy limitation, as proposed before [40], especially in porcine species.

The analysis of our results when the sperm concentration of 0.5×10^5 cells/mL was used suggests the existence of an additional mechanism to polyspermy control exerted by OF. Even with no differences in the sperm number bound to the ZP, a significant increase in the production of monospermic zygote was observed compared with the control group. These data do not support the hypothesis proposed by Coy et al. [6]. For these authors, sperm-recognizing labels coming from OF can be incorporated in the ZP, helping the selection of specific subpopulations of capacitated spermatozoa (i.e., those whose receptors had high affinity for specific labels), even though the final number of spermatozoa around the oocyte at the fertilization time is reduced *in vivo*. It is interesting to note that the presence of OF before or during IVF allows to increase the sperm concentration without affecting the monospermy rate. This maybe of interest when using boars of unknown fertility for IVP, allowing to secure the penetration rate by using relatively high sperm concentrations without affecting monospermy.

Ballester et al. [41] reported that preincubation of oocytes in pure preovulatory OF-improved monospermic zygotes production when fresh semen was used for IVF, whereas the same treatment increased polyspermy after

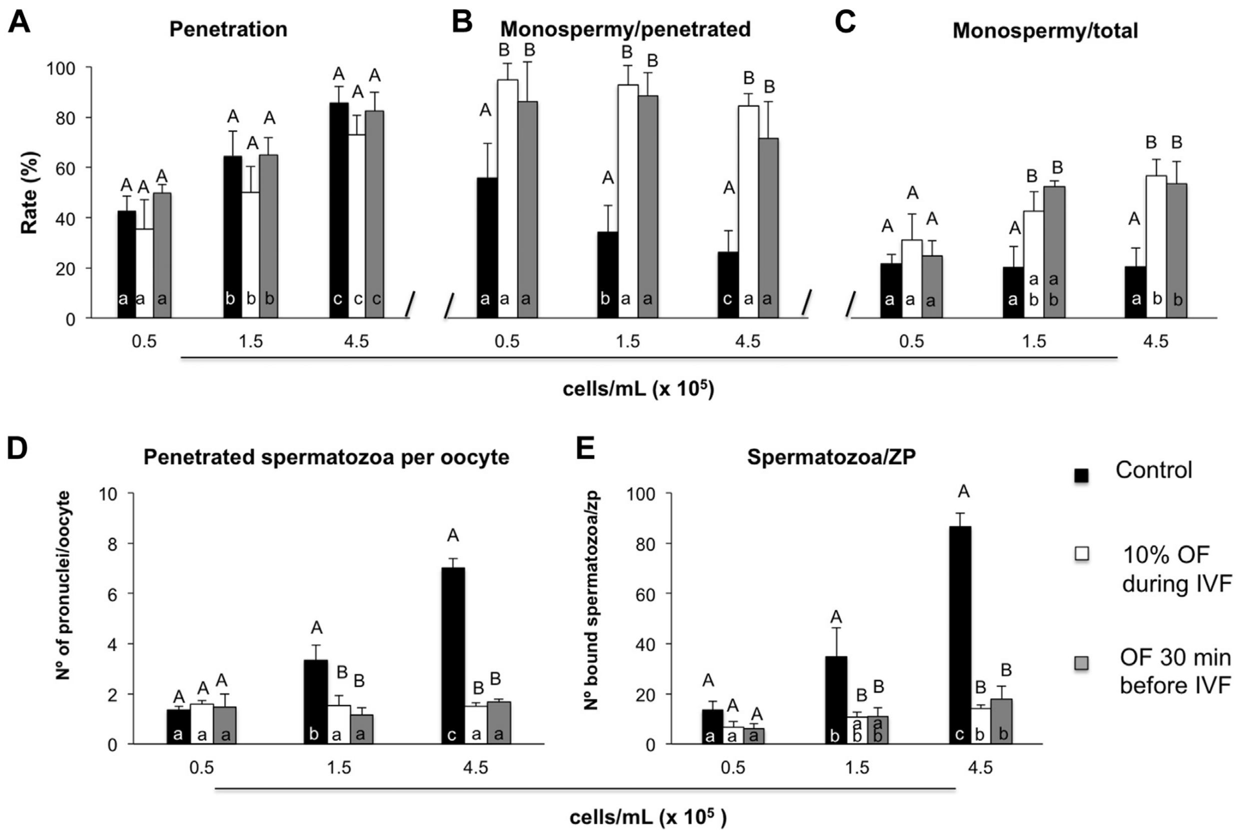

Fig. 3. Effect of the presence of oviduct fluid (OF) for 30 min before IVF or during IVF on fertilization results (penetration [A] and monospermy [B] rate, IVF efficiency [C] number of spermatozoa per oocyte [E] and number of bound spermatozoa per ZP [D]) of porcine oocytes. Each bar represents mean ± SEM. For each group, approximately 100 oocytes were analyzed. Different letters indicate significant differences ($P > 0.05$) between (a,b,c) or within (A,B) sperm concentration treatments. IVF, *in vitro* fertilization; SEM, standard error of the mean; ZP, zona pellucida.

IVF with frozen/thawed spermatozoa. The differences of these previous observations with our own results may be explained by the use of pure OF in Ballester's experiments, although we were using only 10% of oviduct flush in the present experiments.

Heparin and probably other sulfated GAG present in the oviduct are important regulators of oviductal (prefertilization) ZP modification. Previous studies predicted that the binding of GAG, which modifies ZP solubility and consequently makes it more resistant to sperm penetration, stabilizes the ZP network together with OVGP1 and other OF components [6]. This theory could partially explain the results on the strongest and most consistent effect of heparin in combination with OF compared with OF

Table 1
Effect of oviduct fluid during (10% OF) or before (OF 30′ Before) IVF on the development rate of porcine oocytes fertilized with three different sperm concentrations.

Sperms/mL ($\times 10^5$)	Treatment	n	Cleavage (%)	Blastocysts/COC (%)	Blast/cleavage (%)	Cells in blastocyst (mean)
0.5	Control[c]	137	$45.7 \pm 10.8^{a,A}$	$5.8 \pm 2.3^{a,A}$	$13.3 \pm 5.9^{a,A}$	$44.0 \pm 5.2^{a,A}$
	10% OF[d]	138	$39.8 \pm 4.0^{a,A}$	$6.5 \pm 1.4^{a,A}$	$16.8 \pm 5.6^{a,A}$	$46.5 \pm 5.0^{a,A}$
	OF 30′ before[e]	137	$35.7 \pm 3.8^{a,A}$	$5.1 \pm 2.8^{a,A}$	$14.4 \pm 7.4^{a,A}$	$43.5 \pm 6.7^{a,A}$
1.5	Control[c]	140	$43.6 \pm 4.9^{a,A}$	$5.0 \pm 1.4^{a,A}$	$11.7 \pm 3.9^{a,A}$	$46.8 \pm 4.1^{a,A}$
	10% OF[d]	139	$56.2 \pm 12.9^{a,A}$	$13.7 \pm 3.6^{b,B}$	$25.4 \pm 9.4^{a,A}$	$56.8 \pm 8.4^{a,A}$
	OF 30′ before[e]	141	$63.9 \pm 6.6^{a,A}$	$12.8 \pm 3.8^{b,B}$	$19.8 \pm 4.3^{a,A}$	$47.0 \pm 3.8^{a,A}$
4.5	Control[c]	138	$39.1 \pm 6.4^{a,A}$	$3.8 \pm 1.8^{a,A}$	$9.6 \pm 4.6^{a,A}$	$45.5 \pm 7.7^{a,A}$
	10% OF[d]	141	$56.0 \pm 5.8^{a,A}$	$11.3 \pm 3.2^{b,B}$	$20.5 \pm 7.0^{a,A}$	$54.0 \pm 8.6^{a,A}$
	OF 30′ before[e]	138	$55.2 \pm 9.8^{a,A}$	$10.2 \pm 3.8^{b,A,B}$	$19.2 \pm 9.0^{a,A}$	$57.8 \pm 7.5^{a,A}$

Uppercase letters are the comparison between IVF treatments, and lowercase letters are the comparison between different sperm concentrations. Within a column, values with different superscripts differ significantly ($P > 0.05$) by chi-square test.
Abbreviations: COC, cumulus oocyte complexes; IVF, *in vitro* fertilization; OF, oviduct fluid; n, number of oocytes.
[c] Control—IVF in Tris-buffered medium (TBM).
[d] 10% OF—IVF in TBM medium supplemented with 10% OF.
[e] OF 30′ before—incubation of oocytes in OF for 30 min, followed by three washings in TBM medium, then transference to fertilization well.

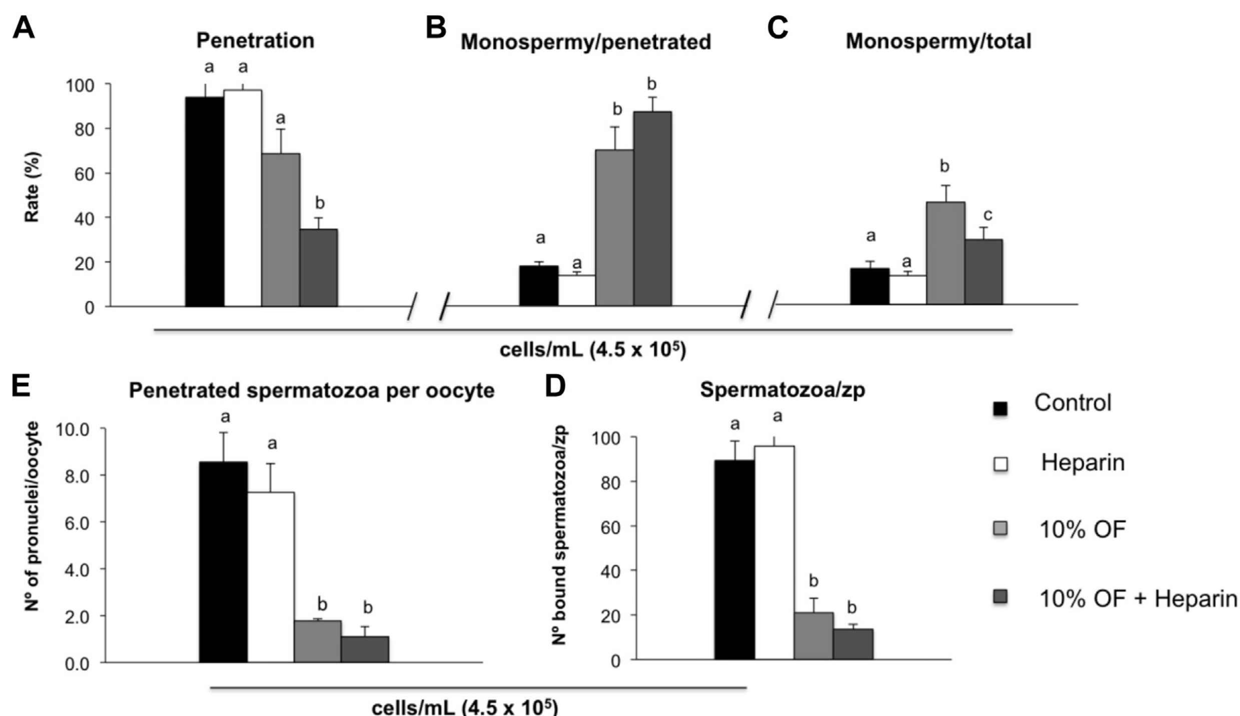

Fig. 4. Effect of the presence of oviduct fluid (OF) and heparin alone or in association during porcine IVF on fertilization results (penetration [A] and monospermy [B] rate, IVF efficiency [C] number of spermatozoa per oocyte [E] and number of bound spermatozoa per ZP [D]). Each bar represents mean ± SEM. For each group, approximately 100 oocytes were analyzed. Different letters indicate significant differences ($P > 0.05$). (i) IVF media + 10% OF + heparin ($n = 150$); (ii) IVF media + 10% OF ($n = 136$); (iii) IVF media + heparin ($n = 146$); and (iv) IVF media, control ($n = 145$). IVF, *in vitro* fertilization; SEM, standard error of the mean.

on ZP resistance to proteinases. However, our results have found that this combination is less effective for zygote production, by reducing drastically the penetration rate, resulting in low zygote production rate although the monospermy rate was increased. Although heparin is used as a capacitating agent in other species, heparin alone had no affect on penetration and polyspermy rates in pigs, at least at the sperm concentration tested.

An issue that confounds the problem of polyspermic fertilization in porcine IVP systems is that polyspermic embryos are able to develop to the blastocyst stage. Indeed, Han et al. [42] reported that porcine IVP zygotes containing three or four pronuclei developed to the blastocyst stage at the same rate as those containing two normal pronuclei. Depending on the location of the pronuclei, polyploid zygotes form triploid, aneuploid, or mixoploid embryos, of which only the latter develop to term [43]. Nevertheless, in the present study, a reduction in blastocyst production was observed in the control group compared with OF-exposed groups when IVF was performed with 1.5 and 4.5×10^5 cells/mL. Both sperm concentrations resulted in significant increased polyspermy in the control group compared with OF-exposed groups, suggesting an adversely effect of high rate of pronuclei formation in polyspermic embryos developing to the blastocyst stage.

4.1. Conclusion

Regardless of the spermatic concentration used in this study, exposure of oocytes to pure OF before IVF or diluted (10%) OF during IVF improved the efficiency of production of monospermic zygotes. Under our experimental conditions, the most effective combination for having a higher monospermic zygote production in IVF is the use of sperm concentration of 4.5×10^5 cells/mL and oocyte exposure to OF either before or during IVF. Furthermore, we have found that although heparin alone had no effect on IVF results, it reduced the penetration rate in the presence of OF, resulting in higher monospermy rate but lower efficiency of the monospermic zygotes production. These findings will contribute to the development of new approaches to improve the production of monospermic porcine zygotes by IVF.

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