Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency

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Objective: To determine optimal conditions for the inclusion of oviductal fluid (OF) in IVF protocols.
Design: Experimental prospective study.
Setting: Mammalian reproduction research laboratory.
Animal(s): Oviducts and ovaries from porcine females were collected at a slaughterhouse. A total of 30 oviducts and 1,285 oocytes were used. Boar-ejaculated spermatozoa were also used.
Intervention(s): In vitro–matured porcine oocytes were preincubated with OF collected from animals before or after ovulation and later fertilized in vitro. Zona pellucida digestion time in oocytes after preincubation in OF was assessed. Concentrations of E_2 and P_4 in OF were measured. IVF was performed, including within the culture media the E_2 and P_4 concentrations found in the preovulatory OF. The effect of preovulatory OF on IVF efficiency was compared between fresh and frozen-thawed spermatozoa.
Main Outcome Measure(s): E_2 and P_4 concentrations in OF; penetration and monospermy rates; number of spermatozoa within the ooplasm and on the zona pellucida after IVF under different experimental conditions; zona pellucida resistance to protease digestion.
Result(s): Preincubation of oocytes in OF collected before ovulation enhances IVF efficiency in the pig compared with OF collected after ovulation (29.58 $\pm$ 3.84 vs. 11.03 $\pm$ 2.69). When frozen-thawed spermatozoa are used for the IVF of these OF-treated oocytes, their fertilization ability increases compared with fresh semen. OF collected before and after ovulation shows significantly different concentrations of E_2 (99.00 $\pm$ 8.72 vs. $<$10 pg/mL) and P_4 (2.53 $\pm$ 0.66 vs. 12.27 $\pm$ 2.33 ng/mL), respectively. Addition of E_2 and P_4 at concentrations similar to those in the OF before ovulation partially simulates the effect of the fluid on IVF outcome.
Conclusion(s): Preincubation of oocytes in OF collected before ovulation is a suitable protocol for increasing the efficiency of IVF with fresh semen in the pig model and could be a useful tool to increase the fertilization ability of frozen-thawed spermatozoa in other species. E_2 concentrations in preovulatory OF are higher than those reported in blood serum at the same phase of the estrous cycle. (Fertil Steril® 2014;102:1762–8. ©2014 by American Society for Reproductive Medicine.)
Key Words: Oviductal fluid, in vitro fertilization, frozen-thawed spermatozoa, steroids

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Despite the widespread use of intracytoplasmic sperm injection (ICSI) as the technique of choice for the fertilization of oocytes in human infertility clinics, conventional IVF represents a more physiological tool, where the penetrating spermatozoa are naturally selected and compete with many other sperm cells to reach the female gamete. Moreover, it has been suggested that the utilization of IVF in conjunction with in vitro maturation (IVM) treatment

(avoiding the risks associated with ovarian stimulation with gonadotropins) is a suitable, more cost-effective and less invasive method for creating more viable embryos with improved implantation and pregnancy potential than ICSI (1). These reasons support the interest of studies addressed to increase the efficiency of IVF methods in humans.

However, the introduction of novel protocols in human IVF requires their prior validation in animal models, and the pig is one species with considerable advantages for this purpose (2). Recently, the preincubation of oocytes in oviductal fluid (OF) was identified as a good method for increasing the efficiency of IVF in pigs (3), but, before transferring this biological observation into laboratory protocols, it is necessary to define the specific conditions required to turn this finding into an efficient and reliable tool.

Indeed, in the pig model and in other mammalian species (4), the contact of oocytes with OF before fertilization seems to be a key step in modifying the zona pellucida (ZP), by binding specific components from the fluid and increasing the possibilities of successful fertilization (3); however, two issues still remain to be clarified. One is the lack of precise criteria for the selection of OF batches with the desired effect or, alternatively, for the addition in the culture media of specific oviductal components capable of partially reproducing the effect of the OF (5). The second is the lack of information about the effects of the OF on the IVF assays when the spermatozoa come from frozen-thawed straws. The aim of the present study is to clarify these two issues.

Thus, despite previous data indicating that OF collected around the time of ovulation was most effective at inducing modifications in the ZP (specifically increasing the resistance of the ZP to protease digestion) and increasing the success of IVF (3), there are no data showing the effect on IVF of different batches of OF collected either some hours before or after ovulation.

It is known that hormone variations during the estrous cycle determine biochemical and physiological changes in the female reproductive tract, including modifications in OF composition (6). The best-known example is oviductal specific glycoprotein (OVGP1), which is secreted by the epithelial cells at estrous or during the mid-late follicular phase of the cycle, whose oviductal level depends on the E_2 concentration in blood circulation (7). In fact, this protein is considered to be the main factor, together with heparin-like glycosaminoglycans (GAGs), responsible for the increased efficiency in IVF results when oocytes are preincubated in OF before adding the spermatozoa in the culture dish (3).

Although E_2 in blood serum starts to decrease approximately 2 days before ovulation in sows (8), it is not known whether this drop is reflected immediately or later in the E_2 concentrations in OF or how this change might affect OVGP1 or other protein concentrations. In fact, there are a number of studies indicating a maximum secretion of OVGP1 at the late follicular phase (9, 10). This would correspond with the time at which the E_2 levels in serum are lower (11), but, to our knowledge, no data are available in the literature indicating the E_2 and P_4 concentrations in OF at every specific time of the estrous cycle. From these observations, the first two hypotheses proposed in the present study are [1] that OF collected before and after ovulation would differentially affect the IVF results and [2] that steroid concentrations in OF collected at these specific time points vary in a different way from those concentrations already described in blood serum.

Apart from the effect of E_2 on regulating the secretion of OVGP1 and other components by the oviductal epithelial cells, it is also possible that steroid concentrations may induce changes directly in the spermatozoa or oocyte that could affect their interaction because both gametes have shown steroid receptors on their membranes (12–15). Then, once the concentrations of both steroids in OF have been measured, it would be important to test the effect of their inclusion in IVF media on the efficiency of the process. The third hypothesis of this study was therefore that the addition of steroids to IVF media at concentrations similar to those found in the most effective OF would reproduce the beneficial effect.

Frozen-thawed spermatozoa are a source of male gametes with clear advantages in specific clinical situations, and any new protocol in IVF should be tested with such samples. Using fresh spermatozoa, it was shown that fertilization of porcine oocytes preincubated in periovulatory OF was more efficient than in the absence of OF (3). However, no data are available about a similar effect using frozen-thawed sperm cells. The last hypothesis to be tested in this study was that the preincubation of oocytes in OF enhances fertilization results when cryopreserved spermatozoa are used.

MATERIALS AND METHODS

This study was developed following institutional approval from the Bioethics Committee of the University of Murcia, and it was performed in accordance with the Animal Welfare regulations of that institution.

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A.

Collection of OF and Assessment of E_2 and P_4 Concentrations

Porcine oviducts (n = 30) were transported on ice from the slaughterhouse to the laboratory. They were classified as described elsewhere (16), and only those at the preovulatory (ovaries with follicles of 8–9 mm diameter and corpora albicans from previous cycle) or postovulatory (recent corpora hemorrhagica, small growing follicles, and presence or absence of corpora albicans) phases of the estrous cycle were used. Oviducts were dissected on ice and washed twice in saline solution. The fluid was collected by aspiration with an automatic pipette using a tip for a maximum 200-μL volume. The samples were centrifuged at 7,000 *g* for 10 minutes at 4°C to remove cellular debris, and the supernatant was stored at −80°C.

For hormone determinations, a total volume of 400 μL of OF from each phase of the estrous cycle (pre- and postovulatory) was assessed by a chemiluminiscent microparticle immunoassay (CMIA; Architect, Abbott). Three different batches of 400 μL of OF collected from animals before and after ovulation were assayed by CMIA.

Oocyte Collection and IVM

Ovaries from gilts were transported to the laboratory in saline containing 100 μg/mL kanamycin sulfate at 38°C and washed once in 0.04% cetrimide solution (w/v) and twice in saline within 30 minutes of slaughter.

Cumulus-oocyte complexes (COCs) were collected from antral follicles (3–6 mm diameter), washed twice with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1 mg/mL polyvinyl alcohol (PVA) and 0.005 mg/mL phenol red and twice more in maturation medium previously equilibrated for a minimum of 3 hours at 38.5°C under 5% CO_2 in air. Maturation medium was NCSU-37 (17) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 mg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG; Foligon; Intervet International BV), 10 IU/mL hCG (Veterin Corion; Divasa Farmavic), and 10% porcine follicular fluid (v/v). Only COCs with complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 μL maturation medium for 22 hours at 38.5°C under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional period of 20–22 hours (18).

IVF

The fertilization medium was TALP (19). COCs cultured for a total of 44 hours in maturation medium were stripped of cumulus cells by pipetting and washed 3 times with TALP medium, and groups of 50–55 oocytes were transferred into each well of a 4-well multidish containing 250 μL IVF medium previously equilibrated at 38.5°C under 5% CO_2. A sperm-rich fraction of semen from a mature, fertility-tested boar was collected by the gloved-hand method, immediately transported to the laboratory, and diluted at 1:8 v/v in Beltsville thawing solution (20). The same boar was used for the preparation of frozen-thawed samples according to the straw-freezing procedure described by Thilmant (21). Aliquots of the semen samples (0.5 mL) were centrifuged (740 *g*, 30 minutes) through a discontinuous Percoll (GE Healthcare) gradient (45 and 90 % v/v), and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 minutes at 740 *g*. Finally, the pellet was diluted in TALP, and 250 μL of this suspension was added to the wells containing the oocytes, giving a final concentration of 25,000 cells/mL.

The sperm and oocytes were cultured at 38.5°C under 5% CO_2 in air. At 18–20 hours post-insemination, putative zygotes were washed 10 times by aspiration through an automatic pipette and evaluated after fixation and staining (22). Penetration, monospermy, number of spermatozoa penetrating each oocyte, number of pronuclei and sperm decondensed heads inside each oocyte, and number of spermatozoa bound to ZP were assessed in each oocyte. Efficiency represents the final putative zygotes (with two pronuclei) in each group per 100 penetrated oocytes.

To test the first hypothesis of the study, groups of 50–55 IVM oocytes were preincubated for 30 minutes with preovulatory or postovulatory samples of OFs (one oocyte per microliter of OF) and in vitro fertilized in TALP medium supplemented with 1% preovulatory OF. A control group of oocytes without preincubation in OF was also included. To test the second hypothesis, the experimental group consisted of oocytes fertilized in the presence of E_2 and P_4 added to the IVF medium at the concentrations assessed in the preovulatory OF. An additional experiment, adding separately E_2 or P_4 to the experimental groups, and including a positive control ($E_2 + P_4$ group) to compare the data with those in the previous experiment, was also performed.

Assessment of ZP Solubility

After removing the cumulus cells, the IVM porcine oocytes were incubated in OF (one oocyte per microliter of fluid) and covered with mineral oil for 30 minutes at 38.5°C under 5% CO_2 in air. A control group of oocytes incubated in DPBS under the same conditions was used in all the experiments. After incubation in the OF, the oocytes were washed quickly in DPBS and transferred into drops of 50 μL of 0.5% (w/v pronase solution in DPBS). ZPs were continuously observed for dissolution under an inverted microscope equipped with a warm plate at 38.5°C. The dissolution time of the ZP of each oocyte was registered as the time between the placement of the oocytes in the pronase solution and the time when the zona was no longer visible at ×200 magnification. This time was referred to as "ZP digestion time" (3).

ZP resistance to protease digestion was assessed using the same experimental groups (control, preovulatory fluid– and postovulatory fluid–treated oocytes; n = 82) as those used in the IVF experiment.

Statistical Analysis

Data are presented as the mean ± SEM. The variables in all of the experiments were analyzed by one-way analysis of variance (ANOVA; steroid concentrations, ZP digestion time, and IVF parameters). When ANOVA revealed a significant effect, values were compared by the Tukey post hoc test. $P < .05$ was considered statistically significant.

RESULTS

Preincubation of Oocytes in Preovulatory OF Results in Higher IVF Efficiency than Preincubation in Postovulatory OF

Although the preincubation in preovulatory OF decreased the percentage of penetration by around 13% (Table 1), the final efficiency of the process was significantly higher with this fluid since the mean number of sperm bound to each ZP and the mean number of sperm penetrating each oocyte were lower ($P < .05$) and the percentage of monospermy was higher ($P < .05$) than in the control or postovulatory OF-treated group (Table 1).

Preincubation of Oocytes in Preovulatory OF Results in Higher Resistance of ZP to Proteolysis than Preincubation in Postovulatory OF

The results confirmed that the ZP from oocytes incubated in preovulatory OF was more resistant ($P < .05$) to enzymatic

TABLE 1

Effect of the pre- and postovulatory OF on IVF efficiency with fresh semen.

Group	n	Penetration (%)	Monospermy (%)	Sperm/oocyte	ZP binding[a]	Efficiency (%)
Control	140	100.00 ± 0.00[b]	3.23 ± 2.06[b]	8.00 ± 0.63[b]	95.38 ± 7.87[b]	3.23 ± 2.06[b]
Preovulatory	155	87.32 ± 2.80[c]	33.87 ± 4.26[c]	2.40 ± 0.12[c]	8.08 ± 0.38[c]	29.58 ± 3.84[c]
Postovulatory	150	95.59 ± 1.76[b,c]	11.54 ± 2.81[b]	3.57 ± 0.20[c]	13.05 ± 0.63[c]	11.03 ± 2.69[b]

Note: The superscripts b and c in the same column denote significant differences ($P<.05$).
[a] ZP binding is the mean number of sperm attached to ZP for each oocyte.

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digestion (42.70 ± 3.51 minutes) than the ZP from oocytes incubated in postovulatory OF (11.43 ± 1.92 minutes) and that ZP from both groups of fluids was more resistant ($P<.05$) than control oocytes (1.55 ± 1.20 minutes).

Steroids Concentrations in the Preovulatory OF are Different from Those in Postovulatory OFs

The E_2 concentration in the preovulatory samples was 99.00 ± 8.72 pg/mL, whereas in the postovulatory fluid this concentration dropped below the threshold of detection for the technique (<10 pg/mL). Regarding P_4, the concentration in the preovulatory fluid was significantly lower than in the postovulatory fluid (2.53 ± 0.66 vs. 12.27 ± 2.33 ng/mL, respectively; $P=.016$).

Addition of Steroids at the Concentration Found in the Preovulatory OF Modifies IVF Results

When the IVF experiment was run after adding 100 pg/mL of E_2 and 2.5 ng/mL of P_4 to the fertilization medium of one group of oocytes, and the results were compared with a control group in the absence of steroids, the penetration percentages were not different among groups (Table 2), although a trend was noted for a higher monospermy and efficiency in the presence of steroids ($P=.093$ and $=.071$, respectively). In agreement with this trend, the mean number of spermatozoa per oocyte and the mean number of spermatozoa bound to the ZP were lower in the group with added steroids in the IVF media than in the control (Table 2). In the additional experiment to corroborate these data (Supplemental Table 1), it was observed that the main effect was due to the action of P_4 in the absence of E_2, since the differences in penetration, monospermy, mean number of spermatozoa per oocyte, and mean number of spermatozoa bound to the ZP were mainly observed in this P_4-treated group ($P<.05$ for all the parameters assessed).

Preincubation of Oocytes in Preovulatory OF Enhances Penetration of Frozen-thawed Spermatozoa

The use of frozen-thawed spermatozoa for IVF (after preincubation of oocytes in preovulatory OF and the addition of 1% OF to the fertilization medium) showed a significant increase in the ability of the spermatozoa to penetrate the oocyte, thus rendering a high level of polyspermy and decreasing the final efficiency of the system (Table 3). In contrast, the same treatment, when fresh semen was used, resulted, as in experiment 1, in a significant increase of the IVF efficiency by enhancing the monospermy and decreasing the mean number of sperm per oocyte and the ZP binding (Table 3). This experiment was repeated 3 times.

DISCUSSION

The use of oviductal or uterine fluid as supplements in the culture media is not a reality today but remains a challenge for the future. Similar to blood serum, whose fractions are purified, stored, and used in different human therapies, the possibility of making available batches of biological fluids or their compounds, embryo tested and pathogen free, for enhancing IVF efficiency is envisioned, although further research is necessary before establishing the legal rules for its use. Thus, advances in the study of the composition and effect of these fluids are necessary in animal models.

What is inferred from the present data is that OF samples collected at close proximity in time, such as those at the preovulatory and the postovulatory phases of the cycle, separated by just some hours (8), differentially affect the IVF results. It seems clear that the porcine oocytes incubated in preovulatory OF, which would not be their natural environment under physiological conditions at fertilization time, are more resistant to penetration (owing to their harder ZP) than oocytes incubated in the postovulatory OF, the fluid

TABLE 2

Effect of the addition of steroids (E_2 + P_4), at the concentration found in preovulatory OF (100 pg/mL E_2 and 2.5 ng/mL P_4), on the IVF results.

Group	n	Penetration (%)	Monospermy (%)	Sperm/oocyte	ZP binding[a]	Efficiency (%)
Control	145	88.97 ± 2.61	14.73 ± 3.13	8.16 ± 0.52[b]	28.25 ± 3.39[b]	13.10 ± 2.81
E_2 + P_4	142	92.25 ± 2.25	22.90 ± 3.68	5.23 ± 0.37[c]	17.33 ± 1.87[c]	21.13 ± 3.43

Note: The superscripts b and c in the same column denote significant differences ($P<.05$).
[a] ZP binding is the mean number of sperm attached to ZP for each oocyte.

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TABLE 3

Effect of the cryopreservation of spermatozoa on the IVF results after preincubation of oocytes in preovulatory OF.

Sperm	OF	N	Penetration (%)	Monospermy (%)	Sperm/oocyte	ZP binding	Efficiency (%)
Fresh	−	126	$93.65 \pm 2.18^{a,b}$	$12.50 \pm 3.25^{a,b}$	3.58 ± 0.19^{a}	14.02 ± 0.95^{a}	$11.90 \pm 3.11^{a,b}$
	+	110	86.36 ± 3.29^{a}	36.84 ± 4.98^{c}	2.19 ± 0.12^{b}	3.77 ± 0.26^{b}	31.82 ± 4.46^{c}
Frozen	−	133	90.98 ± 2.49^{a}	$23.97 \pm 3.90^{b,c}$	3.07 ± 0.18^{a}	8.93 ± 0.89^{c}	$21.80 \pm 3.59^{b,c}$
	+	102	100.00 ± 0.00^{b}	2.94 ± 1.68^{a}	4.68 ± 0.22^{c}	$6.27 \pm 0.42^{b,c}$	2.94 ± 1.68^{a}

Note: The superscripts a, b, and c in the same column denote significant differences ($P < .05$).

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they would find after being shed from the follicles and reaching the oviductal ampulla in vivo. This is in agreement with the present results on ZP resistance to proteolysis, where again the preovulatory OF made the ZPs harder to digest than the postovulatory OF, in theory owing to the combined action of OVGP1 and GAGs (3). Thus, in the pig species, the use of the first type of fluid would represent an advantage because polyspermy is a well-known problem occurring during IVF (6, 23, 24) and any method contributing to reduce it would increase the efficiency of the process, as is the case in the present study. However, from the human point of view, the incubation of oocytes in a postovulatory OF would be likely to improve the final percentage of successful fertilizations because, as is shown, the number of spermatozoa penetrating the oocytes was higher for oocytes under this treatment and polyspermy is not such a serious problem in human IVF. Then, if human fluids were available in the future (e.g., through biobanks with samples from donors), the use of the postovulatory samples, which in turn are the ones that physiologically match the corresponding phase of the cycle during fertilization, would be recommended once their efficiency is demonstrated in the corresponding trials. In fact, although there are no data in humans about IVF results after treating the oocytes with OF, a beneficial effect of human OF on sperm motility and survival has been demonstrated previously (25).

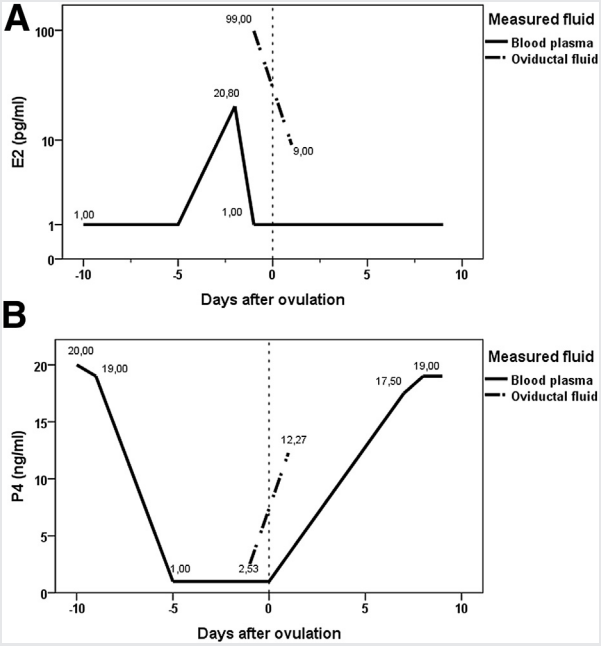
Data about concentration of steroids in blood serum or in follicular fluid of different species indicate seminal differences between both biological fluids. In human follicles containing mature oocytes, E_2 concentration ranged from 368.0 (26) to ~896.74 ng/mL (27) and P_4 was around 5.4 ng/mL (26). In contrast, E_2 levels from blood serum in patients were ~3.02 ng/mL before hCG administration and 4.05 ng/mL after hCG administration (28), which means a tenfold lower concentration compared with the follicular levels. Serum P_4 can reach up to 16 ng/mL during the luteal phase but is around 0.26–1.12 ng/mL during the follicular phase (29, 30), which, compared with the 5.4 ng/mL found in the follicles, represents a 4- to 20-fold lower concentration.

In the pig, the situation is similar, because the maximum E_2 levels in serum (2.5 days before ovulation) were around 22 pg/mL (8), 55.3 pg/mL (31), or 36.8 pmol/L (~9.8 pg/mL) (32), depending on the accuracy of the procedures. Maximum P_4 (at day 8 postovulation) was around 19 ng/mL (8) or 76.4 nmol/L (~25.9 ng/mL) (32). However, maximum steroid concentrations in follicular fluid were 848 ng/mL for E_2 (31), which represents a huge difference compared with serum, and 612 ng/mL for P_4, which is a 30-fold difference compared with serum.

In contrast, to our knowledge, data about steroid concentrations in porcine or human OF are not available. With the development of suitable protocols to collect human OF by minilaparotomy in the late 1980s and early 1990s, a number of proteins were identified (33–35), but no reference to steroids was found. From the results in the present study, it can be assumed that they are also higher than in blood serum. Moreover, from our data it seems clear that the drastic drop in E_2 concentration described in the pig serum from day −2.5 to day 0 (8) is delayed in the OF, where the values remain high before ovulation as occurs in the follicular fluid. While the origin of the high E_2 concentrations in the follicle is well known, as the follicular cells are their main source, the situation for OF needs further explanation. It was shown years ago that a countercurrent exchange between the ovarian vein and the uterotubal artery occurs in pigs (36), being most effective around the time of ovulation (37), and it has been proposed that hormones of low molecular weight (~300 Da) such as steroids and prostaglandins entering the ovarian vein can reach the oviduct locally by countercurrent transfer to the oviduct branch of the ovarian artery (6). In this manner, hormones secreted by a preovulatory follicle could reach the arterioles of the ipsilateral oviduct arcade and, finally, reach the oviductal lumen by selective transudation (38). If that were the situation, the drop in the E_2 concentrations, occurring in the transition between the periovulatory and the postovulatory stages, would be reflected in the OF 24–48 hours later than in serum, as our data seem to indicate and as is represented in Figure 1.

This specific concentrations of steroids in the OF would induce corresponding responses in gene expression of the oviductal tissues, but they could also have other types of response. Rapid or nongenomic actions of steroids, occurring at the cell surface, have been discovered over the past decades and are conserved from plants to humans (39). In the present study, and using steroid concentrations found in the oviduct at the preovulatory phase, it is shown that IVF parameters are affected. Bearing in mind that the addition of the steroids was at the beginning of the period of sperm-oocyte coincubation, the effect observed 18 hours later can be attributed either to a genomic or to a nongenomic mechanism. The genomic mechanism would involve binding to nuclear receptors, the activation or repression of gene transcription (or the interaction of the nuclear steroid-receptor complex with transcription

FIGURE 1



Comparative diagram of steroid concentrations in blood serum (data from Noguchi et al. 2010) and OF (data from the present study) of sows around the ovulation time. (**A**) E_2 and (**B**) P_4.

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factors), and the modulation of the subsequent protein expression. By contrast, the nongenomic possibility would be the consequence of a direct action of the E_2 and P_4 present in the OF on the gametes, since it is already known that steroid actions occurring at the cell surface provoke many fast nongenomic effects (39). As indicated, both gametes seem able to respond to steroids that bind to their membrane receptors (15, 40), although at this point the specific mechanisms responsible for the observed effect are unknown.

In the present study, the inclusion of E_2 and P_4 in the fertilization medium resulted in a significant decrease in the mean number of spermatozoa inside the oocyte and bound to the ZP. This is interpreted as a trend toward improving the monospermy rate and hence the final efficiency of the system, similar to the effect produced by the addition of preovulatory OF. Again, in the pig model, this could be a suitable tool for improving the IVF results by reducing polyspermy, but in the human model the addition of steroids at the concentration found in the postovulatory fluid would probably be a better option. Indeed, studies in the mouse have also demonstrated that E_2 stimulates capacitation and acrosome reaction and that the addition to the IVF medium of E_2 increases the fertilizing ability (41), as we corroborated in the present study.

Finally, a key result from the present study is the fact that frozen-thawed spermatozoa do not respond like their fresh counterparts to the presence of OF or its components bound to the ZP. It might be thought that a more proteolysis-resistant ZP would be harder for frozen-thawed spermatozoa to cross, which, in theory, might have poorer motility and physical strength. However, it is also known that cryopreservation produces a series of changes in the sperm membranes leading to a capacitation-like status and that OF components protect these cryopreserved sperm cells, support their viability and motility, and enhance protein phosphorylation (42–44). Previous studies have already shown that the percentage of phosphorylation in cryopreserved boar spermatozoa was greater when they were treated with preovulatory OF (43), an effect that would be in agreement with a higher fertilization ability, as seen in the present study. The higher level of penetration and polyspermy with frozen-thawed spermatozoa compared with the fresh samples of the same boar, in the presence of the same batch of preovulatory OF, lead us to conclude that the use of this type of OF would be beneficial for increasing the IVF efficiency with low-quality (fresh or frozen-thawed) semen samples, as is the case in many couples attending infertility clinics. Additional studies and efforts should be made before having OF samples from donors available for their use in different assisted reproductive technologies.

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SUPPLEMENTAL TABLE 1

Effect of the separated addition of E_2 (100 pg/mL) and/or P_4 (2.5 ng/mL) at the concentration found in preovulatory OF on the IVF results.

Group	n	Penetration (%)	Monospermy (%)	Sperm/oocyte	ZP binding[a]
Control	54	100.00 ± 00.00[b]	7.41 ± 3 60[b,c]	6.31 ± 0.43[b]	7.80 ± 0.63[b]
E_2	51	100.00 ± 00.00[b]	3.92 ± 2.74[b]	6.31 ± 0.60[b]	7.15 ± 1.01[b]
P_4	46	65.22 ± 7.10[c]	23.33 ± 7.85[c]	2.70 ± 0.40[c]	4.05 ± 0.50[c]
$E_2 + P_4$	50	96.00 ± 2.80[b]	20.83 ± 5.92[b,c]	3.77 ± 0.40[c]	7.25 ± 0.94[b]

Note: The superscripts b and c in the same column denote significant differences ($P < .05$).
[a] ZP binding is the mean number of sperm attached to ZP for each oocyte.

Ballester. Oviduct fluid and steroids effect in IVF. Fertil Steril 2014.