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ARTICLE

Peritoneal fluid modifies the response of human spermatozoa to follicular fluid

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Abstract The aim of this study was to elucidate the mechanism involved in the acrosome reaction (AR) induced by follicular fluid (FF) in spermatozoa previously exposed to peritoneal fluid (PF). The influence of progesterone was also investigated. Semen samples were from 18 normozoospermic donors. PF samples were from 13 women with unexplained infertility and from a woman treated with synthetic progestagen. FF samples were collected from six women undergoing IVF/embryo transfer and pooled. Motile spermatozoa were capacitated overnight and a kinetic and inhibition study on the FF-induced AR was performed. Spermatozoa pretreated with PF were challenged with either FF or progesterone. The ability of progesterone- and progestagen-supplemented PF to induce AR was analysed. Enzyme-digested PF was also tested. Pre-incubation with PF for 60 min completely prevented the FF-induced AR; spermatozoa treated with PF were unable to respond to FF or progesterone and this effect was not reversible. Progesterone- and progestagen-supplemented PF stimulated the AR relative to controls. Enzyme-digested PF did not have an inhibitory capacity. These data strongly suggest that there are one or more inhibitory proteins in PF that interact with spermatozoa so as to prevent access of progesterone to its receptor and thus inhibit the occurrence of the AR.

KEYWORDS: acrosome reaction, capacitation, follicular fluid, human spermatozoa, peritoneal fluid, progesterone

Introduction

In mammals, the oviduct provides a stage for gamete transport and storage, fertilization and early embryo development. If ovulation has not occurred, spermatozoa may reside in the oviduct (considered as a reservoir) for several hours or even a few days awaiting oocyte arrival (for a review see Holt and Fazeli, 2010; Suarez and Pacey, 2006). During this stage, spermatozoa are exposed to several biological fluids secreted by the upper female genital tract which modulate (by as-yet unknown mechanisms) the acquisition of sperm fertilizing capacity (Barratt and Cooke, 1991). The peritoneal fluid (PF) environment is one that hosts the processes of ovulation, gamete transport or survival, spermatozoon–ovum interaction, early embryonic development and implantation. The cellular and acellular

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components of this dynamic fluid are in a constantly interactive state, being influenced by the physiological events and pelvic disease processes (Syrop and Halme, 1987). Since in humans there are no selective barriers separating the Fallopian tubes and the peritoneal cavity, it is expected that PF will be present in the fertilization milieu (Harper, 1988). Although changes in PF volume, cell concentration and hormones have been characterized during the normal menstrual cycle (Maathuis et al., 1978), little is known about the effect of this fluid on sperm function. However, direct intraperitoneal insemination of spermatozoa combined with ovulation induction is a well-known approach in assisted reproduction in patients with cervical patency (Forrler et al., 1986). Furthermore, a prospective, randomized trial comparing pregnancy rates of three insemination techniques (intraperitoneal insemination, Fallopian sperm perfusion and conventional intrauterine insemination) reported similar efficacy in the achievement of clinical pregnancy (Noci et al., 2007). It is accepted that PF sets up a stream of fluid towards the tube at ovulation, as has been demonstrated by following particles injected into the Douglas pouch and retrieving them at the uterine cavity (Pauerstein et al., 1975).

In order to investigate the effect of PF on sperm function, this study uses the fact previously reported that, *in vitro*, PF can support sperm survival and capacitation (Revelli et al., 1997). Because *in vivo*, spermatozoa might be first exposed to the PF and then to the follicular fluid (FF) in the vicinity of the oocyte, experiments were performed in which capacitated cells were sequentially incubated with PF followed by FF. When capacitated spermatozoa are exposed to a concentration of >20% (v/v) PF, they lose the ability to respond further to physiological stimuli for the acrosome reaction (AR) such as FF (Munuce et al., 2003).

It is possible that the PF, as part of the oviductal milieu, regulates the number of spermatozoa that undergo the AR, preventing a premature acrosomal loss. The aim of the present study was to elucidate the mechanism involved in the FF-induced AR in spermatozoa previously exposed to PF.

Materials and methods

The study was approved by the Institutional Review Board (resolution number 364/2011) of the School of Medicine of the University of Rosario. Written informed consent was obtained from each patient.

Initial semen sample processing and sperm capacitation

Semen samples were obtained from normozoospermic donors (n = 18) and collected by masturbation after 3–5 days of sexual abstinence. Written informed consent was obtained from each donor. Samples were allowed to liquefy and semen analysis was undertaken according to World Health Organization guidelines (WHO, 1999). Because each sample was used only once, the total number of samples used is the same as the number of experiments performed in this study.

Seminal plasma was removed by layering 1 ml semen on top of a discontinuous 90/70/50% Percoll gradient (Sigma Chemical, St Louis, MO, USA). Considering that only fully capacitated spermatozoa are able to respond to 20% FF (at least 8 h incubation is required; Calvo et al., 1989), overnight capacitation was selected in this study. The concentration of motile spermatozoa was adjusted to $2-8 \times 10^6$ cells/ml and capacitated for 20–22 h ('overnight') at 37°C in human tubal fluid (HTF; control; Gibco, BRL, Life Technologies, Grand Island, NY, USA) supplemented with 35 mg/ml bovine serum albumin (BSA fraction V; Sigma Chemical) in loosely capped 15-ml conical tubes under an atmosphere of CO_2/air (5:95). After each experiment, sperm viability was assessed by mixing one drop of sperm suspension with one drop of Eosin Y solution (0.5% in phosphate-buffered saline (PBS); Sigma) on a slide and examining 100 spermatozoa at ×400 magnification (WHO, 1999).

Recovery of peritoneal and follicular fluids

PF was collected from the Douglas pouch of patients scheduled for laparoscopy as part of the investigation for their cause of infertility. Collection was performed on cycle day 16.7 ± 0.6, as calculated from data of the preceding menstrual cycle. Only those patients classified as unexplained infertile were included in the study (mean age 25.5 ± 1.3 years). PF was aspirated into sterile plastic tubes and blood-free samples (microscopically assessed) were immediately transported to the laboratory (n = 13).

A pool of FF (n = 6) was obtained from women undergoing IVF. Multiple follicular developments were induced by injections of sequential human menopausal gonadotrophin and human chorionic gonadotrophin (Serono Laboratories, Argentina).

PF and the pool of FF were centrifuged (10 min, 600 ***g***) to remove cellular debris, filtered through a 0.22-μm membrane (Millipore, Bedford, MA, USA) and stored at −20°C until used (<12 months). The presence of antisperm antibodies in fluids was tested by the indirect Mar test (MarScreen; Fertility Technologies, Natick, MA, USA) and only antibody-free spermatozoa were used. Progesterone was measured in each PF sample (145.2 ± 68.5 ng/ml) and in the pool of FF (17 μg/ml) using an enzyme immunoassay (Enzymum test; Boehringer Manheim, Germany).

Progesterone solution

A 1 mg/ml stock solution of progesterone (M_r 314.47; Merck, Darmstadt, Germany) was prepared by dissolving the hormone in ethanol and was stored at 4°C until used. An aliquot of the stock solution was further diluted with HTF (1:10) and from this, aliquots were adjusted to the chosen concentration on the day of the experiment.

Kinetic and inhibition studies

The ability of capacitated spermatozoa to respond to 20% FF has been widely proposed as a measure of fertility potential (Calvo et al., 1989). A previous study (Munuce et al., 2003) showed that spermatozoa treated with ≤5% PF and then exposed to 20% FF had an increase in the number of reacted spermatozoa. However, ≥20% PF inhibited a further stimulus with FF. Considering that the present study's aim was to evaluate this inhibition, 20% PF was the selected concentration in all experiments. In order to determine whether PF inhibits

the occurrence of the FF-induced AR in a time-dependent manner, capacitated spermatozoa were exposed to 20% PF for 5, 15, 30 or 60 min and then incubated with 20% FF for an additional hour. Acrosomal status was then determined.

Progesterone as the active component in follicular fluid

Capacitated spermatozoa previously exposed to 20% PF or control medium for 60 min were exposed to 20% progesterone (adjusted to the same concentration as in FF, 17 μg/ml) or to 20% FF for 60 min. Acrosomal status was then determined.

Supplementation of peritoneal fluid with progesterone

Using the stock solution of progesterone, the concentration of progesterone in all PF was made equal to that in the pool of FF (17 μg/ml). The PF from a 37-year-old norethisterone-treated woman with a diagnostic of dysfunctional metrorrhagia was collected on day 18 and included as an in-vivo progestagen-supplemented PF (PF-T). Capacitated spermatozoa were exposed to 20% PF-P, PF-T, PF or control medium for 60 min. Acrosomal status was then determined.

Separation of peritoneal fluid

Capacitated spermatozoa, previously treated with PF as described above, were washed twice (5 min at 300 ***g***) with PBS at 37°C, resuspended in HTF plus albumin (35 mg/ml) and exposed to 20% progesterone solution for 60 min. Controls received the same volume of HTF or PF or were induced with progesterone with or without centrifugation. Acrosome status was then determined.

Enzymatic treatment of peritoneal fluid

A 2-ml aliquot of a pool of PF (n = 4) was digested with 5 U of proteinase K (Invitrogen Life Technologies, Carlsbad, California, USA) at 52°C for 4 h. Capacitated spermatozoa were exposed to 20% digested-PF or control medium for 60 min and to FF for another 60 min. Acrosomal status was then determined.

Evaluation of acrosome status

To determine the percentage of AR, spermatozoa were washed twice with 1 ml PBS by centrifugation at 3000 ***g*** for 10 min. Washed cells were left on a microscope slide until dry and fixed in cold methanol for 30 s. Permeabilized cells were stained for 30 min at room temperature with the fluorescent probe fluorescein isothiocyanate (FITC)-labelled *Pisum sativum* lectin (50 μg/ml; Sigma Chemical). Smears were examined at ×1000 magnification using an epifluorescence microscope (Olympus, Tokyo, Japan). Spermatozoa were considered reacted if staining was restricted to the equatorial segment. A total of 200 spermatozoa were counted in each experiment.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat program (GraphPad Software, San Diego, CA). Differences between treatments were determined by one-way ANOVA and the Tukey–Kramer multiple comparison post-test. Data were expressed as mean ± SEM. A P-value <0.05 was considered significant.

Results

Kinetic and inhibition studies

In order to determine the minimal time of exposure to PF required to observe inhibition, capacitated spermatozoa were exposed to 20% PF for several time intervals (5, 15, 30 or 60 min) and then induced with FF. The percentage of the AR was assessed. The results are presented in **Figure 1**. It was found that 60 min were required to significantly reduce ($P < 0.01$) the number of acrosome-reacted spermatozoa relative to the number induced in culture medium (FF) to values comparable with those in control medium. Incubation under capacitating conditions and the exposure of spermatozoa to PF did not affect sperm viability at any time (more than 80% of viable cells, data not shown).

Progesterone as the active component in follicular fluid

In order to determine whether progesterone present in FF is the active component responsible for AR induction, capacitated spermatozoa pretreated with PF for 60 min were challenged with either FF or progesterone at comparable concentrations. When spermatozoa were previously exposed to PF, no difference in stimulation with either FF or progesterone was observed, as judged by the fact that values remained similar to those in control medium and in PF (**Figure 2**). Therefore, in further experiments, either progesterone or FF was used. The potentially detrimental effect of ethanol used as

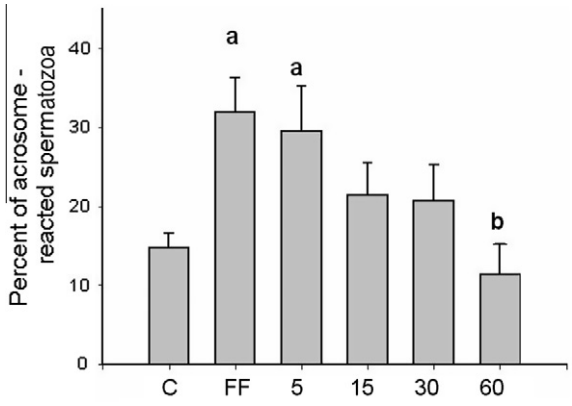

Figure 1 Kinetic and inhibition studies. Overnight-capacitated spermatozoa were exposed to 20% peritoneal fluid for 5, 15, 30 or 60 min and then to 20% follicular fluid. Aliquots incubated in human tubal fluid (C) or induced with 20% follicular fluid (FF) served as controls. The percentage of acrosome-reacted spermatozoa was determined by *Pisum sativum* agglutinin staining. The difference between C and FF represents a measure of the population of acrosome-inducible spermatozoa capacitated by FF. Results are mean ± SEM (n = 5). $^{a}P < 0.05$ with respect to C, 15 min and 30 min. $^{b}P < 0.01$ with respect to FF and 5 min.

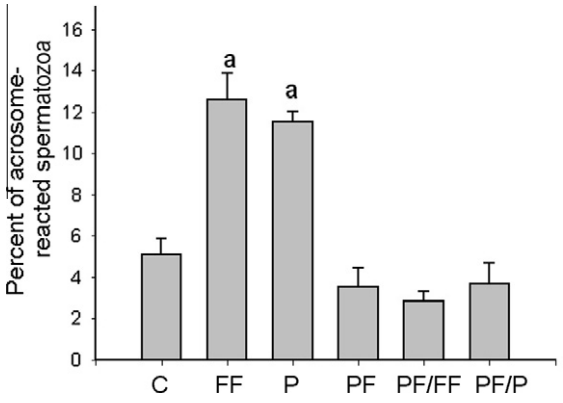

Figure 2 Effect of pretreatment with peritoneal fluid (PF) on the follicular fluid (FF)-induced and progesterone (P)-induced acrosome reaction. Overnight-capacitated spermatozoa were exposed to 20% PF for 60 min and then to 20% FF or progesterone for an additional hour. Aliquots incubated in human tubal fluid (C) or induced with FF or progesterone served as controls. The percentage of acrosome-reacted spermatozoa was determined by *Pisum sativum* agglutinin staining. Results are mean ± SEM ($n = 4$). $^{a}P < 0.001$ versus other conditions.

a solvent (<1%) was tested in parallel experiments; it was concluded that it had no toxic effect on the percentage of viable cells (data not shown).

Supplementation of peritoneal fluid with progesterone

A previous report showed evidence that the supplementation of PF with progesterone confers to the PF the ability to induce AR (Munuce et al., 2006). The present study compared the effect of supplementation of PF with 20% progesterone and norethisterone. In both groups, there was a significant increase in the percentage of AR ($P < 0.001$) relative to PF or control medium (**Figure 3**).

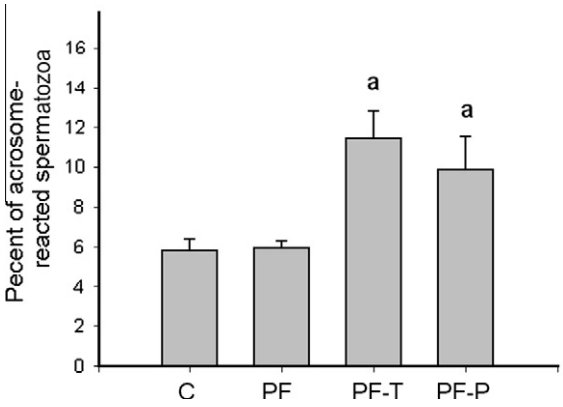

Figure 3 Effect of progesterone and progestagen-supplemented peritoneal fluid (PF) on sperm acrosome reaction. Overnight-capacitated spermatozoa were exposed to 20% progesterone-supplemented PF (PF-P) or norethisterone-treated PF (PF-T), peritoneal fluid (PF) or human tubal fluid (C) for 60 min. The percentage of acrosome-reacted spermatozoa was determined by *Pisum sativum* agglutinin staining. Results are mean ± SEM, $n = 3$. $^{a}P < 0.01$ versus other conditions.

Separation of peritoneal fluid

The sequential exposure of capacitated spermatozoa to PF inhibited the further ability to respond to the inducer (either FF or progesterone). The reversal of the mechanism of inhibition generated by incubation with PF was then investigated. Capacitated spermatozoa pretreated with PF for 60 min were centrifuged and resuspended in HTF plus albumin and then AR was induced with progesterone. The ability to respond to a further stimulus with progesterone is not restored, since values remained similar to those for nonstimulated spermatozoa in PF or control medium (**Figure 4**). No detrimental effect of centrifugation itself was observed since the percentages of acrosome-reacted cells in the washing and the nonwashing groups were comparable.

Enzyme treatment of peritoneal fluid

To determine whether the inhibitory activity of PF is due to protein, PF was enzymically digested. Capacitated spermatozoa were exposed either to 20% protein-free PF (PF-D) or to control medium and then induced with FF. The percentages of acrosome-reacted spermatozoa in both groups were comparable (**Figure 5**).

Discussion

A previous paper showed that when capacitated spermatozoa are exposed to ≥20% PF, there is a reduction in the rate of spermatozoa that undergo FF-induced AR (Munuce et al., 2003). The present study further investigated this mechanism of inhibition and provides evidence that 60 min are required to significantly reduce sperm response. Previously, Revelli et al. (1997) showed that the presence of PF in the

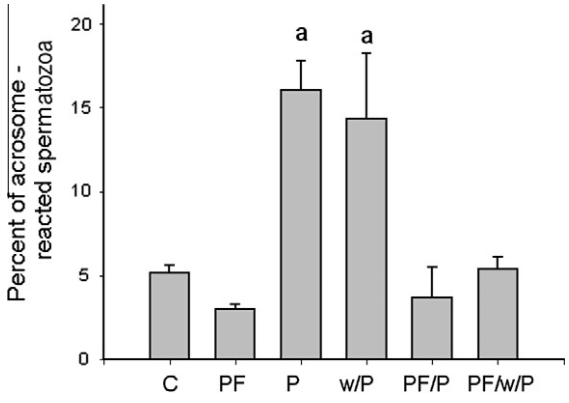

Figure 4 Separation of peritoneal fluid (PF). Overnight-capacitated spermatozoa were exposed to 20% PF for 60 min, washed with phosphate-buffered saline (5 min at 300 **g**), resuspended in human tubal fluid (HTF) plus albumin and then exposed to progesterone (P) for an additional hour (PF/w/P). Aliquots incubated in HTF (C), PF, exposed to PF and stimulated with progesterone (PF/P) or washed and induced with progesterone (w/P) served as controls. The percentage of acrosome-reacted spermatozoa was determined by *Pisum sativum* agglutinin staining. Results are mean ± SEM ($n = 3$). $^{a}P < 0.05$ versus other conditions.

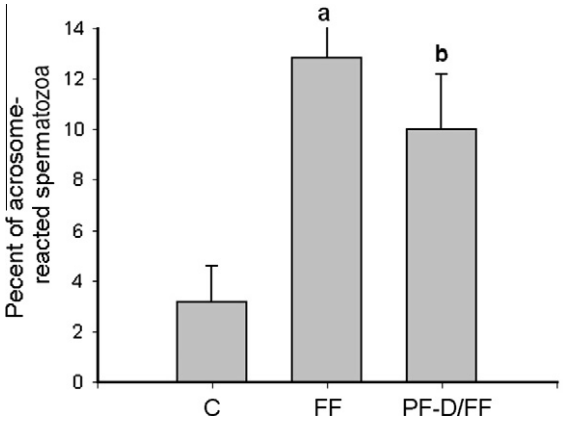

Figure 5 Enzymic treatment of peritoneal fluid (PF). Capacitated spermatozoa were exposed to 20% digested peritoneal fluid (PF-D) for 60 min and to follicular fluid (FF) for another 60 min. Aliquots incubated in human tubal fluid (C) or induced with 20% follicular fluid (FF) served as controls. The percentage of acrosome-reacted spermatozoa was determined by *Pisum sativum* agglutinin staining. Results are mean ± SEM ($n = 3$). $^{a}P < 0.01$ with respect to C. $^{b}P < 0.05$ with respect to C.

medium decreased the ability of spermatozoa to respond to a further AR stimulant. However, in those experiments, noncapacitated spermatozoa were directly mixed with PF/FF (1:1) and incubated for several hours to achieve capacitation before AR evaluation. Considering that only fully capacitated spermatozoa are able to respond to FF (at least 8 h incubation is required; Calvo et al., 1989), the lower response in FF-induced AR observed by Revelli et al. (1997) could be explained by an insufficient cohort of fully capacitated spermatozoa instead of an inhibitory effect of PF itself. In the current study, the experimental design was slightly different and took into account that in-vivo sperm capacitation occurs during transit through the Fallopian tubes and toward the ampullary environment where, depending on the ovulatory stage, different proportions of PF and FF could be present. Thus, this study sequentially exposed capacitated spermatozoa first to PF and then to FF, and AR was assessed as a marker of fertilizing potential. By measuring the set of signals, which led to phosphorylation of tyrosine proteins, it has been previously observed that there is no direct effect of PF on the occurrence of capacitation itself (Munuce et al., 2003). The present study thus reinforces the idea that in the presence of PF the inhibition of FF-induced AR is related to the process of exocytosis of acrosomal content and is not the result of a 'decapacitation' activity.

Progesterone concentration within the human reproductive genital tract throughout the time period that encompasses sperm capacitation and fertilization is unknown. It is widely accepted that progesterone is secreted by the cumulus matrix at a concentration of 1–10 μg/ml and is present in the periovulatory FF at a concentration of up to 20 μg/ml (Osman et al., 1989; Saaranen et al., 1993). After follicular rupture, progesterone in PF rises from 0.4–10 ng/ml to 30–350 ng/ml (Zorn et al., 1982).

Progesterone stimulates several sperm functions, including hyperactivation, chemotaxis towards the oocyte and physiological AR (Kaupp et al., 2008). It is well known that progesterone is the principal active component present in FF which, via a non-genomic membrane receptor (Morales et al., 1992; Osman et al., 1989), induces calcium influx, tyrosine phosphorylation of sperm proteins and other signalling cascades that end in the occurrence of the AR (Baldi et al., 2009; Blackmore et al., 1990, 1991). Recently, it has been described that CatSper, a pH-dependent Ca^{2+} channel of the sperm flagellum, serves as the non-genomic progesterone receptor of human spermatozoa (Lishko et al., 2011).

In view of these findings, it is possible to speculate that the action of PF on the FF-induced AR described here could be associated with an effect on the steroid molecule itself. This study offers evidence that when capacitated spermatozoa are first exposed to PF and then challenged with progesterone or FF, the reduction in the number of reacted cells is comparable. Two alternative explanations are possible: (i) PF component(s) bind to progesterone and render it inactive; and (ii) PF component(s) block the progesterone receptor on the sperm surface and thus prevent a further stimulatory effect.

Progesterone concentration in PF is 100-fold lower than in FF, which explains the incapacity of PF to stimulate the AR itself (Munuce et al., 2006). However, when capacitated spermatozoa are stimulated with PF supplemented with progesterone (PF-P or PF-T), there is an increase in the percentage of acrosome-reacted spermatozoa relative to those incubated with progesterone alone. Although it has been shown that norethisterone shows a weak agonist effect on the sperm non-genomic receptor (Blackmore et al., 1991), the present study observed a 95% increase in the rate of AR in spermatozoa exposed to PF-T. On one hand, progesterone has been shown to increase calcium influx (which leads to the AR) in less than 60 s (Blackmore et al., 1990). On the other hand, at least 60 min are required for PF to inhibit the FF-induced AR. The present experiments show that when PF and progesterone are added together, a clear stimulatory effect on AR is observed. A similar competition occurs when capacitated spermatozoa are exposed simultaneously to progesterone and the antiprogestin RU486, which binds with high affinity to the progesterone receptor: AR is significantly reduced but not abolished (Jang and Yi, 2002; Yang et al., 1994). It can be speculated that, under competitive conditions, the affinity of progesterone to reach sperm receptors seems to be higher than that of the blocking factor(s) in PF. A direct effect of PF on the progesterone molecule itself should be discarded.

In addition, these data show that, even after the removal of PF by centrifugation and resuspension of spermatozoa in HTF plus albumin, the response to progesterone was not restored, suggesting that the inhibitor fraction remains on the sperm surface. Stronger procedures, such as ionic force or pH gradients, could be used; however, sperm integrity and thus its ability to respond to progesterone could be affected. In these experiments, the percentage of progesterone-induced AR in washed spermatozoa was comparable to that in noncentrifuged spermatozoa, from which is concluded that sperm functionality was unaffected by washing. It is possible that, *in vivo*, a specific process displaces attached PF molecules from spermatozoa with fertilizing potential (maybe upon their passage through the cumulus). This study

shows that proteinase treatment abolishes the inhibitory activity present in PF and thus suggests that this factor could be a protein. However, further experiments will be necessary to elucidate the chemical composition of the inhibitory fraction. This study reinforces the idea that some PF component could block spermatozoa in a time-dependent manner and prevent a further response to progesterone.

In vivo, most spermatozoa have intact acrosomes and only a few undergo spontaneous AR in the oviduct (Yanagimachi, 1988). Although fertility potential is related to the ability of spermatozoa to respond to progesterone *in vitro* (Krausz et al., 1995, 1996; Oehninger et al., 1994), a premature stimulation of the sperm AR may not be beneficial for fertilization since the zona-binding capacity of spermatozoa is reduced after AR (Liu and Baker, 1992). However, a recent study has observed that mouse spermatozoa could begin the AR before reaching the zona (Jin et al., 2011). There is evidence that the progesterone receptor and the D-mannose-binding site (or zona pellucida receptor) seem to be topographically related on the sperm surface (Benoff et al., 1995). A former study described that, in the presence of PF, there is also a reduction in the number of detected of D-mannose-binding sites and the number of spermatozoa bound to the zona pellucida (Munuce et al., 2003). The possibility that some components in PF block both the D-mannose-binding site and the progesterone receptor in order to prevent a premature loss of zona affinity should be considered. Recently, it has been shown that glycodelin F is present in the FF and secreted by oviductal cells (Yeung et al., 2006, 2009). Both secretions produce suppression of the progesterone-induced AR (with no effect on spontaneous AR) and an inhibition in the zona binding by blocking the D-mannose receptor (Chiu et al., 2003a,b, 2004; Munuce et al., 2009; Zhu et al., 1994). The present study presents evidence that the mechanism involved in the inhibition of FF-induced AR by PF is time dependent, is abolished by enzymic digestion of PF, is not easy to remove and seems to involve a loss in sperm response to progesterone. Considering that PF, like FF, is an ovarian exudate which contributes to the fertilization milieu (Koninckx et al., 1980), the presence of such glycodelins in PF should be investigated.

As a practical matter, this study explains why intraperitoneal insemination results in fertilization rates comparable to those of other insemination techniques (Noci et al., 2007). Intraperitoneal insemination is still an interesting strategy to pursue the treatment of patients in which cervical patency is present.

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