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Binding Regulation of Porcine Spermatozoa to Oviductal Vesicles In Vitro

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ABSTRACT: In vivo, en route to the site of fertilization, mammalian spermatozoa bind to oviduct epithelial cells (OECs). This binding may favor sperm survival and capacitation, but little is known about the regulation of detachment of sperm from the oviduct in vivo. Therefore, we studied sperm-oviduct interaction in vitro using vesicles formed from OECs in primary culture. Porcine oviducts were collected from gilts at the slaughterhouse. OECs were obtained by compressing the oviduct and culturing them in TCM-199 medium with 10% fetal calf serum for 48 hours. For the first experiment, to test the hypothesis that progesterone (P_4) and estradiol (E_2) affect sperm-OEC binding, OECs were pretreated for 48 hours with 100 ng/mL of P_4 or E_2. In the second experiment, porcine follicular fluid (pFF, 5%), caffeine (1 μM), calcium ionophore A23187 (1 μM), and DMSO (0.01%) were added to the incubation medium to provide insights on the mechanisms of sperm release from the oviduct. For both experiments, 50×10^6 sperm were coincubated with 50 μL of OEC vesicles in 1 mL of incubation medium for up to 24 hours at 37°C and 5% CO_2. After an initial 30 minutes of coincubation, the vesicles settled and a sample of the supernatant was removed to evaluate sperm release and acrosomal status; subsequent samples were removed after 2, 4, and 24 hours of coincubation. To evaluate the effect of the different treatments on sperm integrity, the acrosome status of the spermatozoa was determined using fluorescein-labeled *Pisum sativum* agglutinin staining. Experiment 1 showed that P_4 pretreatment of OECs interferes with sperm binding compared with pretreatment with E_2 or controls ($P < .05$). In experiment 2, coincubation in the presence of A23187 increased sperm detachment compared with pretreatment with DMSO, pFF, caffeine, or controls ($P < .05$). For each experiment, the treatments did not affect the percentage of acrosome-reacted sperm compared with controls ($P < .05$).

Key words: Sperm, oviduct, progesterone, ionophore, acrosome reaction, pig.

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Oviduct epithelial cells (OECs) are believed to play a role in the survival (Smith and Yanagimachi, 1990; Raychoudhury and Suarez, 1991) and capacitation (Pollard et al, 1991) of bound or unbound sperm in vivo. Before fertilization, porcine spermatozoa are stored mainly in the isthmus by binding to OECs (Hunter, 1984; Raychoudhury and Suarez, 1991; Suarez, 1998). Sperm release in the oviduct has a major effect on fertilization because the number that reach the oocytes is crucial (Hunter and Nichol, 1983; Hunter, 1984, 1990; Mburu et al, 1997). A synchronized releasing process is necessary to permit sperm redistribution in the oviduct and favor fertilization while also limiting the number of spermatozoa around the oocytes to avoid high polyspermy rates (Hunter, 1993, 1996; Yanagimachi, 1994). The mechanisms involved in sperm release from OECs, however, are still unknown.

Based on in vivo observations, certain hypotheses have been proposed. It is known that when ovulation takes place, high concentrations of steroid hormones and several types of prostaglandins are present in the oviductal fluid (Hunter, 1988). Therefore, it is believed that these substances could be the signal to release the spermatozoa from the OECs. Estradiol (E_2) and progesterone (P_4) are among the steroid hormones known to affect OECs (Hunter, 1972, 1983, 1988; Raychoudhury and Suarez, 1991). Modifications in steroid concentrations provoke changes in the contractile activity of the oviduct; P_4 increases the electromyography activity and E_2 has the opposite effect (Rousseau and Ménézo, 1991).

Our laboratory is interested in understanding sperm-oviduct interactions in the pig. In this study, vesicles from OECs were coincubated with spermatozoa. OEC vesicles are a physiologically more-responsive model for the in vivo oviduct than are OEC monolayers. To test the hypothesis that P_4 and E_2 effect OEC-sperm interactions and sperm release in vitro, cells were preincubated with these hormones before coincubation with spermatozoa. Then, coincubation was carried out in the presence of substances potentially involved in the acquisition of sperm fertility,

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namely caffeine (Vandevoort et al, 1994), calcium ionophore (Fraser, 1982), and porcine follicular fluid (pFF; Lenz et al, 1982), to better understand the regulation of sperm release observed in the oviduct. Because these treatments are known to facilitate capacitation or the acrosome reaction, sperm release could be mediated by the sperm cells themselves.

Materials and Methods

Preparation of Oviductal Epithelial Cells

Porcine oviducts were collected from prepubertal gilts at the slaughterhouse, transported to the laboratory on ice, and dissected free from the ovaries and other tissues. Oviducts were then washed 3 times in saline and both ends were cut after each wash. Then they were washed once in Hanks medium (containing 5% fetal calf serum [FCS; Immunocorp, Montréal, Que, Canada] and 1% antibiotic-antimycotic) and the OECs were obtained by compressing the oviduct from the isthmus toward the infundibulum with a glass slide. The OECs were transferred to a 50-mL conical tube containing 15 mL Hanks medium and washed twice. About 100 μL of cells were transferred to 50-mL tissue culture flasks containing 10 mL of TCM-199 medium (supplemented with 10% FCS, 0.2 mM pyruvate, and 50 μg/mL gentamicin). OECs were initially cultured at 38.5°C and 5% CO_2 for 48 hours.

Preparation of Spermatozoa

Fresh sperm were obtained from semen collected from fertile boars by the gloved-hand method at the Centre d'insémination porcine du Québec (St-Lambert, Que, Canada) and diluted with Beltsville Thawing Solution (BTS; Clarke and Johnson, 1987). Semen was transported to the laboratory at room temperature (20°C) and then stored at 16°C for 48 hours. About 1 mL of semen was added to 1 mL of Dulbecco phosphate buffered saline (dPBS) supplemented with 0.1% bovine serum albumin (BSA; Sigma, Oakville, Ont, Canada) at 38.5°C. This preparation was layered onto a two-step Percoll gradient 65% and 70% as described by Mattioli et al (1989) with some modifications. After centrifugation at 2000 × *g* for 15 minutes (22°C), the sperm pellet was washed twice in 5 mL of dPBS + BSA medium and centrifuged for 10 minutes at 250 × *g*. The sperm pellet was then resuspended in either TCM-199 + 10% FCS or mTBM medium (mTBM recipe; Abeydeera and Day, 1997) for incubation. The mTBM medium is used for porcine in vitro fertilization, and therefore the experiment was conducted with these 2 media for optimization of the study.

Coincubation of Spermatozoa and OECs

After 48 hours of culture, the OECs were washed with the different coincubation media (either TCM-199 or mTBM). About 50 μL of cells were resuspended in 1 mL of incubation medium with 50 × 10^6 fresh spermatozoa obtained after Percoll treatment. In all experiments, sperm were coincubated with the vesicles for 30 minutes at 37°C and 5% CO_2 in air. During the incubation, the medium containing the vesicles and sperm was shaken gently to prevent the cells from settling. After 30 minutes of sperm-OEC coincubation for the first experiment and 30 minutes after the addition of substances known to affect sperm (calcium ionophore, caffeine, pFF) in the second experiment, the vesicles had settled and a supernatant aliquot was removed. The quantity and the motility of spermatozoa were counted with the hemocytometer, and this time corresponded to 0 hours. To continue the coincubation, 1 mL of coincubation medium was added to the vesicles and subsequent supernatant samples were removed after 2, 4, and 24 hours of incubation to evaluate the quantity of spermatozoa released from the vesicles over time and to assess acrosome reactions.

Evaluation of the Acrosome Reaction

The proportion of acrosome-reacted sperm was determined using fluorescein-labeled *Pisum sativum* agglutinin (PSA-FITC; Sigma, Oakville, Ont, Canada; Tardif et al, 1999). In brief, 15 μL of sperm suspension were smeared onto a slide. After drying, the cells were fixed and permeabilized in absolute ethanol for 1 minute. The slides were then kept at −20°C until staining. To stain, the smears were covered with 15 μL PSA-FITC (100 μg/mL; Sigma) and placed in the dark at room temperature for 20 minutes in a moist environment. The slides were rinsed and mounted with glycerolized water (10%) and coverslips were placed over them. Two hundred sperm were scored and classified as either acrosome-intact or acrosome-reacted with a Nikon microscope equipped with fluorescent optics (B2-A filter, 400×, excitation ~450–490 nm).

Experimental Design

In the first experiment (n = 4), OECs were initially cocultured for 48 hours with either 100 ng/mL of P_4 or E_2, after which sperm were added (as described). Hormones were diluted with ethanol. The coincubation media were either TCM-199 + 10% FCS or mTBM. About 50 × 10^6 sperm cells were coincubated with 50 μL of vesicles in 1 mL of coincubation medium.

For the second experiment (n = 4), factors that potentially induce capacitation or OEC release were added to the coincubation medium 30 minutes after the sperm were added to the OEC:pFF (5%), caffeine (1 μM), calcium ionophore A23187 (1 μM; Molecular Probes Inc, Eugene, Ore), and DMSO (0.01%). For this experiment, the coincubation medium was mTBM. About 50 × 10^6 sperm cells were coincubated with 50 μL of vesicles in 1 mL of coincubation medium.

Statistical Design

The difference between the percentage of sperm bound or not bound to vesicles or acrosome-intact versus acrosome-reacted sperm from 4 replicate trials were carried out by one-way analysis of variance followed by the least significant differences test when main effects were significant. A probability of less than 0.05 was considered statistically significant.

Results

Experiment 1

With the P_4 treatment, more spermatozoa were released from OEC vesicles during coincubation in either mTBM

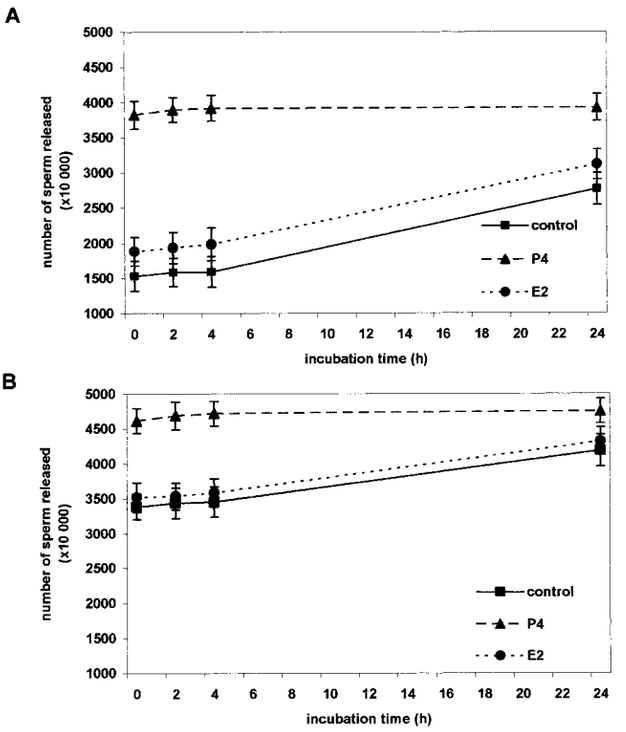

Figure 1. Effect of P_4 and E_2 on the detachment of spermatozoa from OEC vesicles during coincubation. OECs were cultured for 48 hours with 100 ng/mL of P_4 or E_2 prior to sperm addition. Coincubation medium was TCM-199 + 10% FCS **(A)** or mTBM **(B)**. The P_4 treatment caused a greater release of sperm compared with E_2 treatment or controls in both media ($P < .05$); the latter pair did not differ ($P > .05$). Data are means ± SEM.

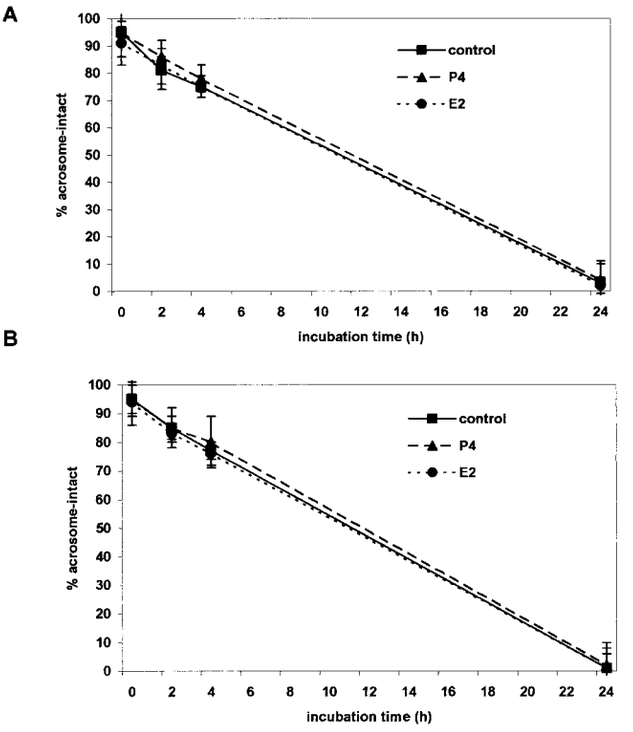

Figure 2. The influence of P_4 and E_2 on sperm integrity during coincubation with OEC vesicles. The acrosome reaction was determined by PSA-FITC coloration. OECs were previously cultured for 48 hours with 100 ng/mL of P_4 or E_2 prior to sperm addition. The coincubation medium was TCM-199 + 10% FCS **(A)** or mTBM **(B)**. There were no significant differences among the treatments ($P > .05$). Data are means ± SEM.

or TCM-199 in comparison with E_2 treatment or controls for all times ($P < .05$; Figure 1). No significant difference in sperm release was observed between E_2 and controls ($P > .05$). Sperm incubated with OEC vesicles demonstrated levels of acrosome reactions (Figure 2) and motility (Figure 3) that were not significantly different among the 3 treatments (P_4, E_2, and controls, $P > .05$).

Experiment 2

The sperm-OEC vesicle binding observed during coincubation among the control, DMSO, caffeine, and pFF treatments was not significantly different ($P > .05$). However, A23187 treatment resulted in more sperm released compared with the other treatments ($P < .05$; Figure 4). Levels of acrosome-reacted sperm (Figure 5) and motility (Figure 6) were not significantly different between any of the treatments ($P > .05$).

Discussion

It has been established for diverse mammalian species that the initial ratio between spermatozoa and oocytes at the ampullary-isthmic junction is close to unity (Hunter, 1993, 1996; Yanagimachi, 1994). In a polyovular species, such as the pig, there would clearly need to be a quantitative relationship between the number of eggs shed at ovulation and the initial number of spermatozoa released from the isthmus reservoir (Hunter, 1998). From the results of the first experiment, it can be concluded that P_4 pretreatment interferes with sperm binding compared with E_2 pretreatment or controls ($P < .05$). Some studies demonstrate that steroids have an effect on the regulation of tubal activity (Hunter, 1988), such that E_2 and P_4 affect the oviduct, the isthmus in particular. Estrogen tends to block tubal motility, whereas P_4 acts conversely (Hunter, 1988, 1991; Rousseau and Ménézo, 1991). This phenomenon could also have an effect on the release of spermatozoa from the OECs. In vivo, the concentrations of the tissue receptors for E_2 and P_4 in the porcine oviduct at different stages of the estrous cycle have been investigated, and the concentrations of P_4 receptors in the isthmus and ampulla increase from behavioral estrus onward (Stanchev et al, 1985). The E_2 receptors are more abundant in the ampulla compared with the isthmus. In pigs, for instance, the release of spermatozoa from the oviductal epithelium is believed to be mediated by the high level

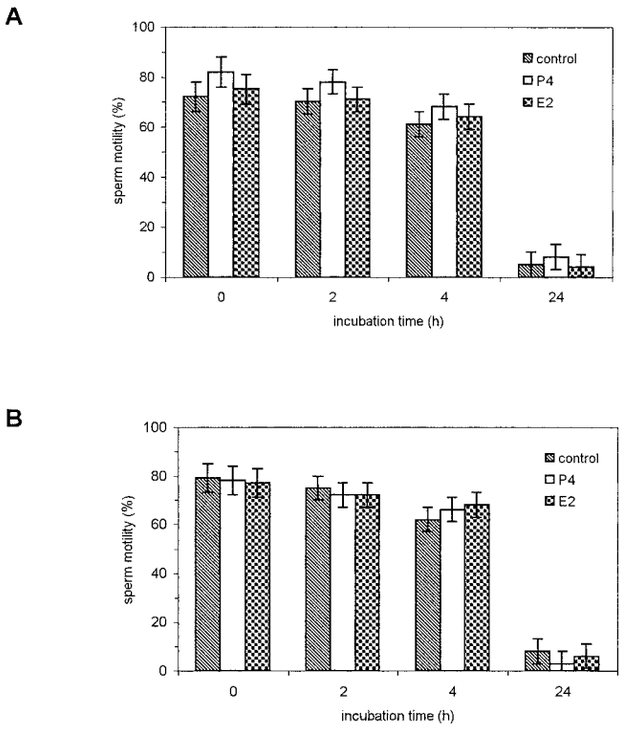

Figure 3. Effect of P_4 and E_2 on sperm motility during coincubation with OEC vesicles. OECs were cultured for 48 hours with 100 ng/mL of P_4 or E_2 prior to sperm addition. Coincubation medium was TCM-199 + 10% FCS **(A)** or mTBM **(B)**. There were no significant differences among the treatments ($P > .05$). Data are means ± SEM.

of steroids present locally through an anatomical structure that connects the ovarian vein with the oviductal artery. This creates a countercurrent mechanism that delivers large amounts of steroid hormones drained from the ruptured follicle to the isthmic wall (Gandolfi, 1995; Hunter et al, 1999). Hunter and coauthors (1999) have demonstrated in vivo a local involvement of preovulatory P_4 in the control of sperm release from the reservoir in the

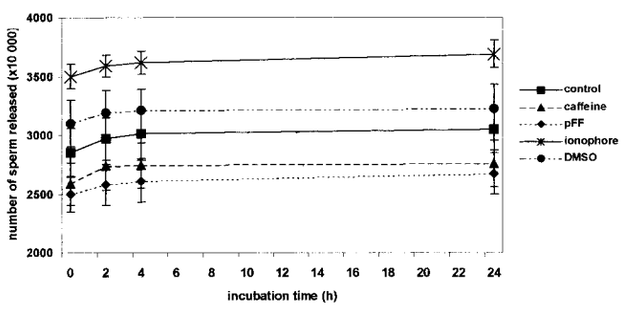

Figure 4. Effect of caffeine (1 μM), pFF (5%), ionophore (1 μM), and DMSO (0.01%) on the detachment of spermatozoa from OEC vesicles during coincubation. The substances were added to the coincubation medium, mTBM, 30 minutes after the beginning of sperm-OEC coincubation. The ionophore caused significantly more sperm to detach compared with pFF, caffeine, DMSO, or controls ($P < .05$); the latter treatments were not different ($P > .05$). Data are means ± SEM.

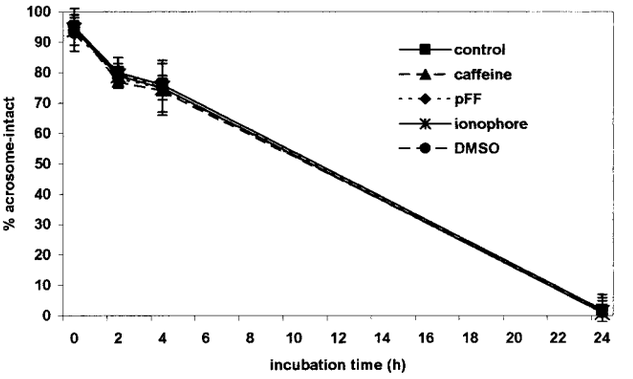

Figure 5. The influence of caffeine (1 μM), pFF (5%), ionophore (1 μM), and DMSO (0.01%) on sperm integrity during coincubation with OEC vesicles. The acrosome reaction was determined by PSA-FITC coloration. The substances were added to the coincubation medium, mTBM, 30 minutes after the beginning of sperm-OEC coincubation. There were no significant differences among the treatments ($P > .05$). Data are means ± SEM.

caudal isthmus. A local transfer of follicular hormones, such as P_4, from the ovarian vein to the ovarian and uterotubal arteries is proposed as a means of regulating oviduct function (Hunter, 1983). This P_4 effect could be modulated by an influx of Ca^{2+} ions into a discrete population of sperm bound to the OECs, thereby promoting a controlled activation and release (Hunter et al, 1999).

Barboni and coauthors (1995) demonstrated that P_4 improves the fertilizing ability of boar semen essentially by facilitating the process of capacitation. In stallion (Cheng et al, 1998) and human spermatozoa (Meizel and Turner, 1991), the existence of receptors that promote capacitation and the acrosome reaction in response to P_4 have been discovered on the plasma membrane of the sperm. The induction of the capacitation and the acrosome re-

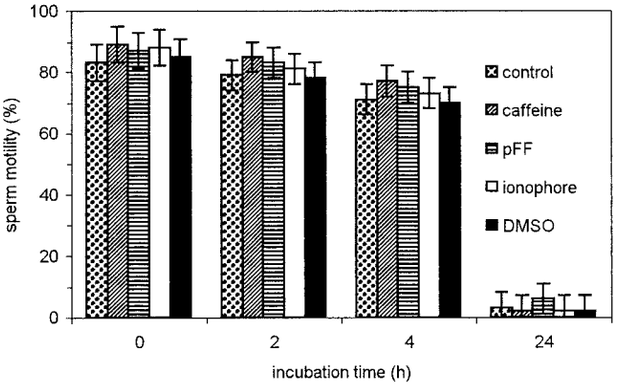

Figure 6. The influence of caffeine (1 μM), pFF (5%), ionophore (1 μM), and DMSO (0.01%) on sperm motility during coincubation with OEC vesicles. The substances were added to the coincubation medium, mTBM, 30 minutes after the beginning of sperm-OEC coincubation. There were no significant differences among the treatments ($P > .05$). Data are means ± SEM.

action has also been observed in porcine sperm but without evidence of these receptors (Melendrez et al, 1994). Progesterone can initiate the acrosome reaction of human (Osman et al, 1989), hamster (Meizel et al, 1990), and stallion sperm in vitro (Meyers et al, 1995). Melendrez and coauthors (1994) also reported that P_4 (1 μg/mL and 75 ng/mL) initiates a rapid and physiological acrosome reaction in porcine sperm. These observations could explain the large quantity of spermatozoa released with the P_4 treatment (Figure 1). However, our results with PSA-FITC staining did not show more acrosome-reacted spermatozoa with P_4 treatment compared with E_2 pretreatment and controls (Figure 2), and the results are the same for sperm motility (Figure 3). Although there is a clear increase of acrosome-reacted spermatozoa with time in culture, this is also true with sperm not incubated with cells (results not shown). Also, sperm incubated with P_4 only (100 ng/mL) do not show more acrosome-reacted spermatozoa compared with E_2 and controls (results not shown). This could indicate that P_4 acts mainly on OECs, but it does not rule out other actions of P_4 on the sperm not related to the acrosome reaction.

When substances potentially involved in the acquisition of sperm fertility were added to the coincubation medium, there were no significant differences in the acrosome reaction rates and sperm motility among all treatments, but the calcium ionophore A23187 provoked more sperm detachment compared with DMSO, pFF, caffeine, and controls ($P < .05$; Figure 4). Follicular fluid has been shown in vitro to possess the ability to promote sperm capacitation and the acrosome reaction in a number of species. It contains compounds such as proteoglycans that possess acrosome reaction–inducing ability (Lenz et al, 1982). The fact that pFF does not provoke a significant detachment of the sperm may be due to the low concentration of P_4 found in the follicle fluid compared with the concentration in the tubal fluid in the pig (Hunter, 1988). The pFF used in this experiment was obtained from follicles from ovaries of prepubertal gilts. It is possible that more mature follicles or a larger quantity of follicular fluid might have resulted in different results. Used in different species during in vitro fertilization, caffeine is presumed to increase or produce the same effect as increased intracellular levels of cyclic adenosine monophosphate (Vandevoort et al, 1994). Earlier experiments have shown that an exposure of sperm to ionophore A23187 significantly stimulated fertilization in vitro, a result consistent with promotion of capacitation-related changes in the sperm (Fraser, 1982). The ionophore A23187 promotes rises in intracellular levels of Ca^{2+}. All these substances have no effect on sperm integrity (Figure 5). However, the marked sperm release caused by A23187 treatment is not the result of an increase of the acrosome reaction.

The evaluation of the acrosome-reacted sperm shows that P_4 and ionophore actions are not caused by a mechanical disruption of the bound sperm following the loss of the acrosome. It may be interesting to evaluate the importance of the other components of the tubal fluid on their capacity to provoke sperm detachment, such as prostaglandins. In some species, the signal that stimulates the ascent of a selected population of male gametes to the fertilization site is believed to be prostaglandins of the F series secreted by the corona cells surrounding the ovulated oocytes (Gandolfi, 1995). The release is obtained by stimulation of the contraction of the smooth muscle layer of the isthmus wall, causing the detachment of some sperm. Prostaglandin E_2 acts as a relaxant (Ortego-Moreno, 1995), however, it is difficult to study prostaglandin effects in vitro because they accumulate in the culture medium in contrast to the rapid clearance in vivo (Fortier et al, 1988).

In conclusion, our results obtained in vitro are in agreement with the in vivo observations regarding the role of progesterone (Hunter, 1999) in sperm release and provide further insights as the possible mechanism (calcium increase) involved in progesterone action.

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