Oviduct-specific glycoprotein and heparin modulate sperm–zona pellucida interaction during fertilization and contribute to the control of polyspermy

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Polyspermy is an important anomaly of fertilization in placental mammals, causing premature death of the embryo. It is especially frequent under *in vitro* conditions, complicating the successful generation of viable embryos. A block to polyspermy develops as a result of changes after sperm entry (i.e., cortical granule exocytosis). However, additional factors may play an important role in regulating polyspermy by acting on gametes before sperm–oocyte interaction. Most studies have used rodents as models, but ungulates may differ in mechanisms preventing polyspermy. We hypothesize that zona pellucida (ZP) changes during transit of the oocyte along the oviductal ampulla modulate the interaction with spermatozoa, contributing to the regulation of polyspermy. We report here that periovulatory oviductal fluid (OF) from sows and heifers increases (both, con- and heterospecifically) ZP resistance to digestion with pronase (a parameter commonly used to measure the block to polyspermy), changing from digestion times of ≈1 min (pig) or 2 min (cattle) to 45 min (pig) or several hours (cattle). Exposure of oocytes to OF increases monospermy after *in vitro* fertilization in both species, and in pigs, sperm–ZP binding decreases. The resistance of OF-exposed oocytes to pronase was abolished by exposure to heparin-depleted medium; in a medium with heparin it was not altered. Proteomic analysis of the content released in the heparin-depleted medium after removal of OF-exposed oocytes allowed the isolation and identification of oviduct-specific glycoprotein. Thus, an oviduct-specific glycoprotein–heparin protein complex seems to be responsible for ZP changes in the oviduct before fertilization, affecting sperm binding and contributing to the regulation of polyspermy.

sperm–oocyte interaction | oviductal fluid | ZP hardening

Polyspermy (the penetration of the egg cytoplasm by more than one spermatozoa) is a pathologic condition in placental mammals, usually causing early death of the embryo (1). Although the prevalence of polyspermy under natural conditions is moderate, in *in vitro* fertilization (IVF) systems polyspermy remains a major obstacle to successful development of viable embryos in different species, including humans (2). Mechanisms underlying the block to polyspermy in mammals have been partially uncovered and characterized, mainly with use of rodents as animal models and usually related to events occurring after sperm entry into the oocyte.

The entrance of the spermatozoon into the oocyte's cytoplasm induces the release of cortical granule contents, which modify the vitelline membrane, the zona pellucida (ZP), or both, rendering the oocyte refractory to additional sperm binding and penetration (3) and ending in changes in the mechanical properties and resistance to protease throughout the ZP (4). Yet assuming strong similarities in fertilization mechanisms among rodents and ungulates, observations in ovulated unfertilized porcine and bovine oocytes, showing that ZP resistance to pronase lasts from hours to days (5–8), contrast with the much shorter resistance (seconds to minutes) exhibited by ZP of mouse or hamster oviductal oocytes before fertilization (9, 10). Similarly, ZP from oocytes of follicular, preovulatory origin and from *in vitro*–matured (IVM) porcine and bovine oocytes before and even after fertilization (8, 11–13) show shorter digestion times (i.e., resistance to pronase). These observations prompted us to hypothesize whether the ZP in ungulates could be undergoing modifications during transit in the oviduct (before fertilization) that affect its resistance to pronase digestion and consequently its interaction with the sperm, and whether this may represent an additional mechanism to control polyspermy, different from the changes brought about by the cortical reaction. The rationale underlying this issue could support the view that spermatozoa penetrate the zona, at least in part, using physical thrust (14), and only those with the ability (or the force) to cross that protease-resistant ("hardened") ZP would be successful at fertilizing the oocyte. However, it cannot be ruled out that oviductal modifications of the ZP could also involve the coating of ZP sperm-binding sites, representing a different mechanism to select the fertilizing spermatozoon. In fact, previous observations have revealed differences between ovulated and antral follicular mouse oocytes in ZP sites for sperm (15). Whether an equivalent situation occurs in ungulates is unknown. According to Lyng and Shur (16), the newly ovulated mouse oocyte is surrounded by coating glycoproteins of oviductal epithelium origin, as was also shown by Oikawa *et al.* in hamster eggs (17). If such a sperm selection could be demonstrated, prefertilization resistance to pronase and selective sperm binding might be considered as two links in the same chain leading to final "oviductal maturation" of ZP, which in turn may represent one more way by which polyspermy is regulated.

Many studies support a functional role for the oviduct and its secretions (which are rich in estrus-associated glycoproteins) in fertilization, regulating processes such as sperm–ZP binding, the establishment of species-specific ZP barriers, and early embryonic development (1, 10, 13, 18–21). However, the molecular mechanisms underlying oviductal regulation are not fully understood, and few studies on this topic have been conducted with domestic ungulates as models. In an experimental design to demonstrate our hypothesis, the possible oviductal factor must fulfil two conditions: (*i*) reversibility (i.e., induction of ZP resistance to proteinases has to be "reversible" because proteolysis of the ZP is required for hatching of blastocysts in uterus), and (*ii*) localization in the oviductal fluid. Although previous attempts to prolong ZP resistance to proteinases (for hours at least) in pigs using oviductal secretions or soluble molecules have been unsuccessful (18, 22, 23),

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new methodologic approaches could be designed to explain how oviductal porcine and bovine oocytes become resistant to pronase.

The present work was designed to (*i*) search for a possible relationship between ZP resistance to proteinases induced by oviductal factors and sperm–oocyte interactions, and (*ii*) investigate the presence in the OF of any factor/s responsible for making ZP resistant to proteinases and, in turn, that may underlie modulation of the sperm–oocyte interaction.

Results

Oviductal Fluid Makes ZP Resistant to Pronase in Pig and Cow Oocytes. Porcine and bovine oocytes collected from oviducts resist proteolysis for at least 3–8 h (8). To determine whether this effect was due to soluble factors present in the oviductal fluid, samples from pigs (porcine oviductal fluid, pOF) and cows (bovine oviductal fluid, bOF) at different stages of the estrous cycle were collected to test their ability to increase ZP resistance to proteolysis. Oocytes from both species were matured *in vitro*, and ZP digestion time was assessed. Without exposure to OF, ZP was digested in 65.3 ± 9.5 s (≈1 min) in pigs and 124.2 ± 5.9 s (≈2 min) in the cow. On the other hand, after 30 min in OF, the digestion times increased in both species, with differences depending on the source of the fluid (Fig. 1). In pigs, the strongest effect ($P < 0.001$) was observed with OF obtained from adult females around ovulation time (sows at follicular phase; Fig. 1*A*), with ZP resistant to pronase digestion increasing to 2,866.83 ± 94.4 s (a 45-fold increase). Using OF from gilts at follicular phase, the digestion time was 509.08 ± 98.5 s (a 10-fold increase). In contrast, OF from either gilts or sows obtained during the luteal phase did not increase the ZP digestion time. Bovine oocytes incubated in bOF samples obtained from heifers older than 14 months, at the follicular phase of the estrous cycle, showed consistent resistance to ZP digestion, with mean values of 4,301.1 ± 441.7 s (34 times higher than control; Fig. 1*B*). Given the need of OF from ovulating sows for proteolysis tests and the difficulty of obtaining it from the slaughterhouse (commercial gilts are usually killed at approximately 6 months of age), an additional experiment was performed to test the effect of the OF from heifers on ZP resistance to pronase of porcine oocytes. Interestingly, pig oocytes became resistant to pronase by incubation with bOF, demonstrating cross-reactivity of bovine fluid with pig oocytes (Fig. 1*C*). Although differences between bOF batches were observed, ZP digestion times for pig IVM oocytes were always longer than 10,000 s (more than 2.7 h), approximately 166-fold greater than in control. These data revealed that OF contains soluble factors capable of inducing resistance to proteolysis in ZP; they exist in porcine and bovine OF during the follicular phase of the estrous cycle, and they lack species specificity. Moreover, this effect is independent of the presence or absence of cumulus cells [see supporting information (SI) Table S1], and it was not observed in mouse oocytes (Table S2).

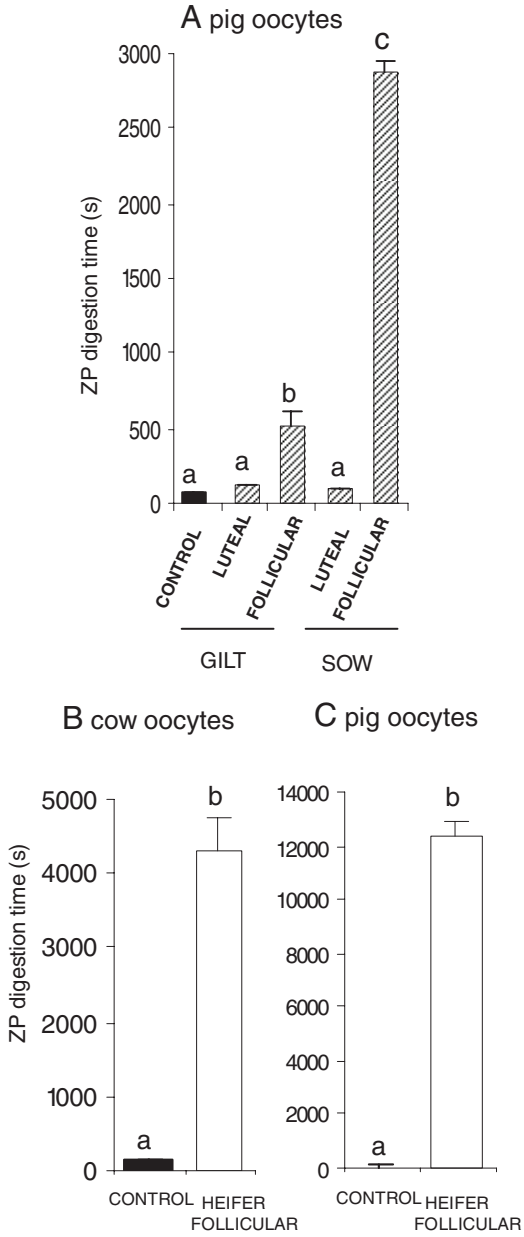

Fig. 1. Effect of different types of porcine (sow or gilt, follicular or luteal phase) and bovine (heifer at follicular phase) OF on the ZP resistance to protease digestion. The fluid from ovulating sows and from heifers at the follicular phase made the ZP highly resistant to digestion. Each bar represents the digestion time (mean ± SEM) of 30–45 IVM oocytes incubated in the fluid for 30 min and later transferred to a pronase solution (0.5% wt/vol in PBS). Experiments were carried out in triplicate. a, b, and c in each graphic indicate significant differences among groups ($P < 0.001$).

Oviductal Fluid Modulates Sperm–ZP Interaction and Monospermy. A comparison of the different IVF parameters between oocytes exposed to OF or not exposed was carried out in pigs and cows. In pigs, the results showed a 10-fold increase in the percentage of monospermy for the pOF-exposed oocytes (5.56 ± 3.8 vs. 50.0 ± 10.0; Fig. 2*A*), without changes in percentages of penetration (100 vs. 92.86 ± 4.9; Fig. 2*A*). Moreover, both the number of penetrated spermatozoa per oocyte (4.92 ± 0.5 vs. 1.73 ± 0.3; Fig. 2*B*) and the number of spermatozoa bound to the ZP (10.28 ± 0.8 vs. 6.61 ± 0.5; Fig. 2*C*) decreased in the pOF-exposed group. In contrast, incubation of bovine oocytes with homologous bOF led to a decrease in the percentage of penetration (58.1 ± 3.3 vs. 38.4 ± 3.3; Fig. 2*D*) yet increased the percentage of monospermy (80.8 ± 3.5 vs. 91.7 ± 3.0; Fig. 2*D*). The mean number of spermatozoa per oocyte remained invariable and low (Fig. 2*E*), as well as the number of spermatozoa bound to the ZP (Fig. 2*F*), which was expected in the cow because polyspermy in this species usually involves dispermic or trispermic penetration, in contrast to the frequent penetration of pig oocytes by 5–15 or even more spermatozoa. To test the lack of species specificity in the OF effect on IVF, porcine oocytes were incubated in bOF and *in vitro* fertilized under the same conditions. The results were similar to those obtained with the homologous fluid (Fig. S1). These results revealed that preincubation of oocytes with OF not only produced ZP resistance to proteolysis but also contributed to the reduction of polyspermy in both species.

ZP Modification Induced by OF Is Reversible. An experiment was designed to test the possible reversibility of the OF effect on pronase resistance of ZPs incubated in media used for porcine

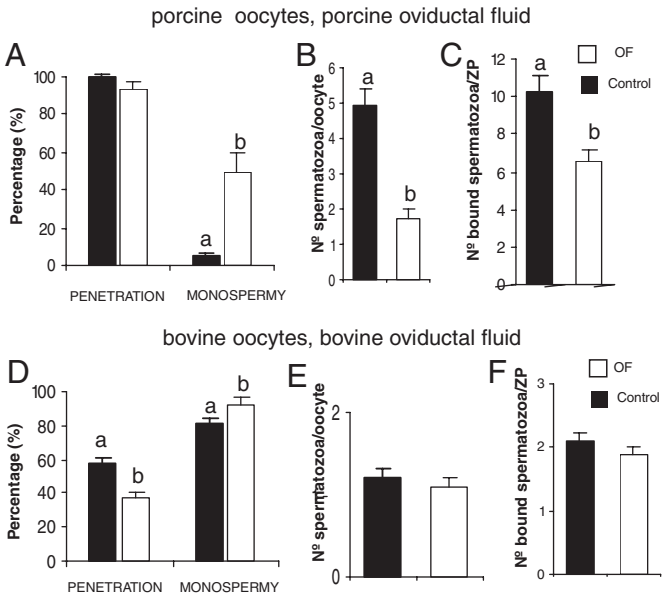

Fig. 2. Effect of incubation of IVM oocytes in oviductal fluid on IVF results. In porcine IVF the parameters penetration and monospermy rate (*A*), number of spermatozoa per oocyte (*B*), and number of bound spermatozoa per ZP (*C*) were evaluated. In bovine IVF, the same parameters were recorded (*D–F*). Each bar represents mean ± SEM for each parameter. Experiments were carried out in triplicate. Each replicate consisted of 10 oocytes for pOF and 40 oocytes for bOF. Different letters (a, b) in each graphic and parameter indicate significant differences ($P < 0.001$).

(TALPp) or bovine (TALPb) IVF. In the first set of experiments, pig and cow oocytes matured *in vitro* were exposed to bOF for 30 min and incubated in TALPp for various times (15–300 min), and the ZP digestion time was determined. The data showed that the digestion time was highest ($P < 0.01$) for pig oocytes after incubating in bOF (Fig. 3*A*), then decreased sharply after a 15-min incubation in TALPp and continued decreasing steadily when longer incubation times were tested. With bovine oocytes, a similar pattern was observed (Fig. 3*B*). Another experiment was carried out in the fertilization medium used for the bovine IVF (TALPb). Porcine and bovine IVM oocytes were incubated for 30 min in bOF and placed in TALPb for up to 240 min, and the ZP digestion time

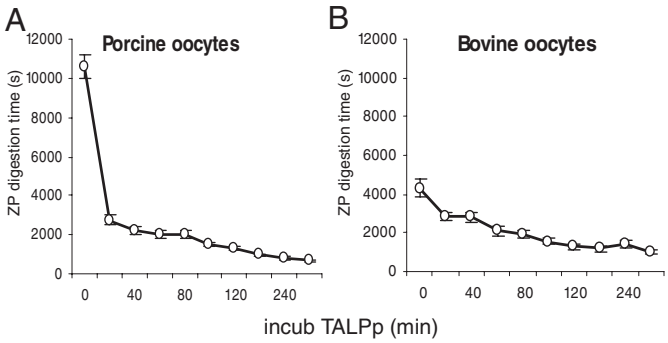

Fig. 3. Effect of bOF on resistance to pronase of ZP from oocytes incubated in TALP medium for different periods of time. Porcine (*A*) and bovine (*B*) IVM oocytes were incubated in bOF for 30 min and later incubated in TALPp medium for different times. The *x* axis indicates the time interval that the oocytes remained in TALPp after incubation in bOF and before assessing ZP resistance to proteases. The *y* axis indicates the time span between placement of the samples in pronase solution and complete dissolution of the ZP. Experiments were carried out in triplicate. Each replicate consisted of 20 oocytes for each time assayed. ($P < 0.01$).

Table 1. Resistance to protease induced by bOF in pig oocytes incubated in TALPb for different times

IVM porcine oocytes (treatment)	*n*	Nondigested ZP after 4 h, %	Nondigested ZP after 20 h, %
30 min in OFb (control)	38	44.74 ± 4.7a	7.89 ± 4.4a
bOF + 15 min TALPb	36	100b	63.89 ± 8.1b
bOF + 60 min TALPb	38	100b	68.42 ± 7.6b,c
bOF + 120 min TALPb	37	100b	72.97 ± 7.4b,c
bOF + 240 min TALPb	39	100b	89.74 ± 4.9c

Resistance to protease induced by bOF was not altered after 4 h in pronase solution, and more than 60% of the ZP remained undigested after 20 h in pronase solution. Each value represents the percentage of oocytes with nondigested ZP (mean ± SEM). Experiments were carried out in triplicate. Each replicate consisted of 12–14 oocytes. a, b, and c indicate significant differences among groups ($P < 0.01$).

was recorded in oocytes removed at different times and exposed to pronase. After 4 h in pronase, 100% of bovine oocytes kept the ZP intact (i.e., not digested). In pig, 44.7% ± 4.7% oocytes in the control group (30 min in bOF) lost their ZP after 4 h in pronase, leaving undigested all of the ZP from the groups incubated in TALPb for different times (Table 1). After 20 h of incubation in pronase, more than 60% of the pig oocytes for each group kept their ZPs intact, compared with 7.89% in the control group (Table 1). These results demonstrated that the maintenance of ZP resistance to pronase as the result of OF incubation depended on the IVF medium used for the subsequent oocyte incubation.

Heparin Is Responsible for the Stability in ZP Resistance to Pronase in TALPb Medium. Because differences between TALPb and TALPp media relate to calcium concentration and the presence or absence of heparin, the effect of these two components was tested. When TALPp medium was prepared with different amounts of calcium and the ZP digestion time was assessed, the results did not vary among groups, and the reversibility (in both porcine and bovine oocytes) was like that shown in Fig. 3. To test the effect of heparin in TALPb medium, IVM pig oocytes were incubated in bOF for 30 min and then incubated for various times in TALPb with and without heparin. In TALPb without heparin, the longer the incubation time, the lower the proportions of undigested ZPs at 4 h or 20 h of incubation in pronase solution (Fig. 4*A*). On the other hand, in TALPb with heparin, almost 100% of oocytes from each group after 4 h in pronase and 64%–93% of oocytes, depending on the group, after 20 h in pronase, kept their ZP unsolved (Fig. 4*B*).

Oviduct-Specific Glycoprotein Seems to Be Responsible for the Changes in the ZP. We examined whether the oviductal factor responsible for ZP changes resistance to proteolysis and sperm–oocyte interaction matched any of the previously described factors, such as proteinases (24, 25), a peroxidase (26), and a disulfide bond-forming reagent (27) that have been described as factors responsible for cortical granule effects. When pig IVM oocytes were incubated for 30 min in bOF with a mixture of proteinase inhibitors, the ZP did not dissolve even after 206 min in pronase solution, compared with 1 min in control oocytes. This result suggests very strongly that proteinases are not the factors responsible for OF effects on ZP. The results were similar with sodium azide, a strong peroxidase inhibitor, and with iodoacetamide, which prevents disulfide bond formation by blocking free cysteine amino acid residues. At this stage, evidence pointed to an oviduct-specific glycoprotein (OGP) as the possible candidate for the ZP resistance to pronase. OGP displays some of the expected features of this candidate: (*i*) OGP is regulated by estrogen and is present in the oviduct around the time of ovulation (21, 28), and this fits with the strong effect of the putative factor(s) at this stage of the estrous

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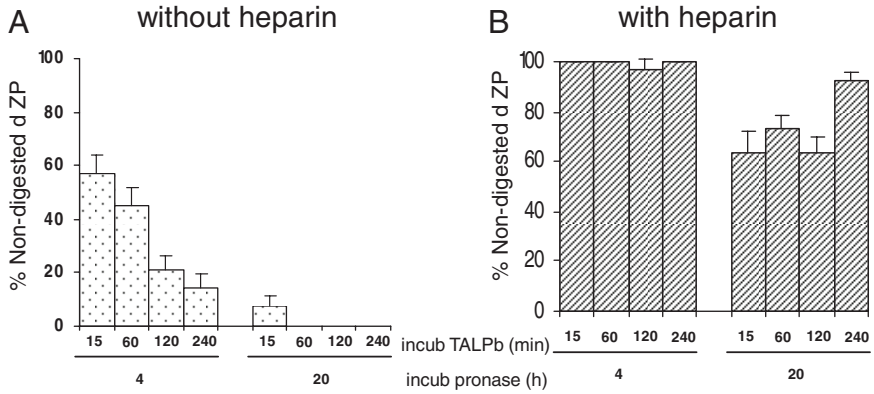
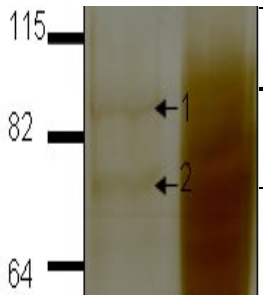


Fig. 4. Effect of heparin on the reversibility of the ZP resistance to proteases induced by bOF in porcine oocytes matured *in vitro*. Oocytes were preincubated in bOF for 30 min, transferred to TALPb medium without (*A*) or with (*B*) heparin and evaluated for resistance to proteases after 15, 60, 120, and 240 min of incubation. Each bar represents the percentage of nondigested ZP after 4 and 20 h in pronase solution (0.5% wt/vol in PBS), respectively, for each group. Experiments were carried out in triplicate. Each replicate consisted of 20 oocytes for each time assayed. ($P < 0.001$).

cycle, (*ii*) nucleotide and amino acid sequences of OGP are highly conserved between species (29) (Table S3), which agrees with the crossed action of porcine and bovine fluids, and (*iii*) OGP possesses heparin-binding consensus sequences (29) and heparin-binding sites (30), which would explain why heparin in the incubation medium stabilizes the hardening effect. To demonstrate the involvement of OGP in ZP modifications and its detachment in a medium lacking heparin, different experiments were performed. First, specific immunoreactivity to OGP was observed in the ZP of oocytes incubated in OF using a polyclonal antibody anti-OGP (Fig. S2). Second, changes in OGP immunoreactivity and their correspondence with ZP resistance to proteolysis in the absence or the presence of heparin during incubation were observed (Fig. S3 *a* and *b*). Third, a last experiment was designed to confirm the identity of OGP as the component unbound from the ZP after incubation in a medium without heparin. Isolated porcine ZPs and OF samples, with tested effect on ZP resistance to pronase, were incubated for 30 min and then transferred to a TALPp medium for 1 h. After removal from the medium, the fall in ZP resistance to pronase was confirmed. The medium (without ZPs) showed only two bands of ≈95 kDa and ≈75 kDa (Fig. 5), respectively, in SDS-PAGE. The identities of these bands were confirmed to be that of oviduct-specific glycoprotein [National Center for Biotechnology Information (NCBI) accession no. 2493675] by in-gel tryptic digestion and analysis with HPLC/ESI-Trap-MS/MS with a sequence coverage of 11% (protein score: 67.03; five peptide fragments) and 10% (protein score: 69.65; five peptide fragments) as retrieved from Agilent Spectrum Mill Workbench search.



Band No	NCBI Protein Accesion Number	Identified peptides
1	Oviduct-specific glycoprotein 2493675	(R)FVSSVIALLR(T) (R)THGFDGLDLFFLYPGLR(G) (R)WINDQYVPYAFK(G) (R)FTTMLSTFSNR(E) (K)LLMGLPTYGR(T)
2	Oviduct-specific glycoprotein 2493675	(R)LLLSAAVSGDPHVVQK(A) (K)ILYPEFNK(L) (K)TLLSIGGWNFGTVR(F) (K)LLMGLPTYGR(T) (R)FVSSVIALLR(T)

Fig. 5. Identification of OGP as the molecule unbound from ZP after incubation in oviductal fluid (30 min) and further incubation in TALPp medium (1 h). One hundred ZPs from IVM porcine oocytes were preincubated in bOF (1 ZP/0.5 μl OF) and later in TALPp medium. Lane1 shows the SDS-PAGE electrophoresis under reducing conditions of the TALPp medium. Lane 2 shows the lysate of 100 ZPs after being removed from the medium. The two bands observed in lane 1, of ≈95 and ≈75 kDa, were analyzed on an Agilent 1100 Series HPLC. Five peptides in the 95-kDa band and five peptides in the 75-kDa band corresponding to OGP were identified.

Discussion

The molecular mechanisms underlying gamete interactions in the oviductal environment are not fully understood. The present findings showing that the incubation in OF of porcine IVM oocytes makes the ZP resistant to proteolytic digestion, decreases sperm–ZP binding, and increases the incidence of monospermy, clearly indicates the presence of important factors in the oviduct regulating the fertilization process.

Previous studies have shown that oocytes from cow acquire ZP resistance when placed in sheep or rabbit oviducts (7). Hence, the oviductal-acquired resistance of the ZP seems to be a relatively common event, not species specific, as shown in sheep, rabbit, cow, and pig oviducts (8, 31). In mice or hamster, however, the ZP resistance to proteinases is not acquired in the oviduct (10, 32), but the postfertilization increased resistance of the ZP to pronase arises from the cortical reaction (3, 33, 34). Moreover, we have shown that mouse eggs exposed to bOF do not acquire resistance to proteinase and, intriguingly, mouse is an exception for the OGP association with the ZP, despite its evidence in the perivitelline space (21). These two issues suggest that in the mouse OGP is unrelated to ZP resistance to proteinases or to sperm–ZP binding, as has been recently described (15).

These observations have many practical implications. IVF is usually carried out with follicular oocytes, either preovulatory (as in humans), in which case no *in vitro* maturation is needed, or antral (pig, cow), for which *in vitro* maturation is required. In either case oocytes do not interact with oviductal secretions. A basic characteristic of pig and cow oocytes is that resistance to pronase as a consequence of fertilization (cortical granule exocytosis) does not happen (11, 12), as opposed to the situation in other models, such as the mouse. However, in the oviduct, resistance to proteolysis takes place before fertilization. The possible change in protein composition and barrier properties of ZP when incubated with OF components can facilitate the passage of specific sperm populations throughout the remodeled ZP network. This function of the oviduct secretions, the cooperation in the sperm selection and in the control of polyspermy, should be considered when IVF techniques are performed. But why have OF-induced ZP changes not been previously reported in *in vitro* experiments? From studies in which periovulatory OF was used in the pig model (13), the low increase in ZP resistance observed can be attributed to the reversibility of the effect found in the present study in absence of heparin and to the use of diluted OF in IVF medium.

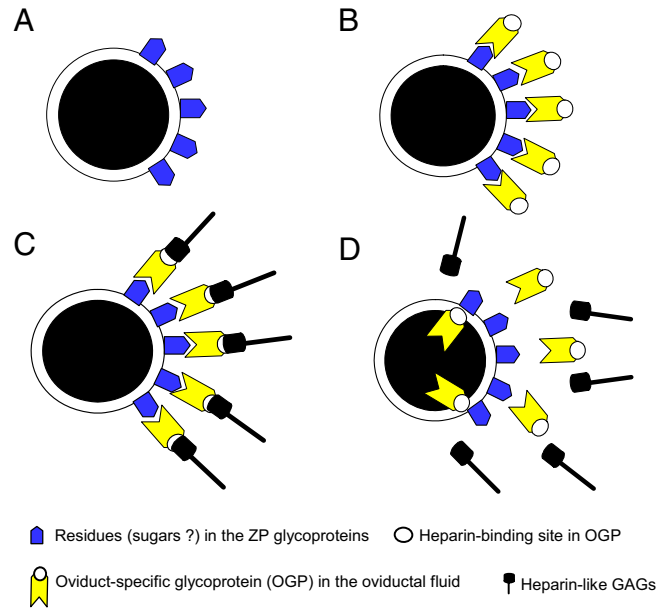


Fig. 6. Hypothesized mechanism by which oviductal proteins surround the oocyte in a ''shell'' that is responsible for the prefertilization ZP changes. (*A*) Oocyte in the preovulatory follicle. (*B*) In the oviduct, the residues (sugars?) in ZP-glycoproteins are recognized and bound by OGP. (*C*) Heparin-like GAGs in the oviduct fluid stabilize and reinforce the binding of OGP with residues in ZP-glycoprotein. (*D*) In the transit toward the uterus the system is destabilized and the OGP is partially unbound or internalized.

A model is proposed based on the present results suggesting binding of the oviduct factor to ZP of recently ovulated oocytes in the ampulla (Fig. 6 *A* and *B*) and, once fertilization and blocking of polyspermy have occurred, the factor is either partially and slowly removed from the zona during its transit toward the uterus (Fig. 6*D*) or incorporated through the perivitelline space to the plasma membrane, as has been shown in pigs for the OGP (35). The cumulus cells accompanying the oocyte from the follicle are physiologically removed shortly after ovulation, although their presence does not hamper the OGP binding to the ZP (36). From the present experiments, heparin [and probably other sulfated glycosaminoglycans (GAGs) in the oviduct] is revealed as an important regulator of oviductal (prefertilization) ZP modification. It is then plausible to predict that the ZP network that contains OGP and other elements and surrounds oocytes is stabilized by the binding of GAGs, which modifies ZP solubility and consequently makes it more resistant to sperm penetration (Fig. 6*C*). The higher content of GAGs in cow OF (37) than in pigs (38) could partially explain our results regarding the stronger and consistent effect of bovine compared with porcine OF on ZP resistance to proteinases.

We propose that OGP could associate with oviductal ZP of pig and cow, masking the sites where proteinases act by steric hindrance and therefore prolonging the digestion time needed by the proteinase to hydrolyze the ZP. This proposal agrees with Bedford's suggestion that "oviductal maturation of the ZP is necessary to acquire the insensitivity to proteinases and ability to select the entering spermatozoa" (14). Our results reported here in pig and cow lend strong support to Bedford's hypothesis and to our proposal. We suggest that sperm-recognizing labels coming from OF can be incorporated in the ZP and by this means select specific subpopulations of capacitated spermatozoa (i.e., those whose receptors had high affinity for particular labels), even though the final number of spermatozoa around the oocyte at the fertilization time is reduced *in vivo* (39). In support of this are the results showing OGP binding sites in cattle and hamster spermatozoa (40, 41). Another possible role of the ZP-anchored OGP is to mask the sperm's ZP binding sites. Both processes can lead to a reduction of the number of sperm bound to ZP and, therefore, polyspermy.

Concerning the homology between OGP in animal species, pig (NCBI accession no. 2493679) and cow (NCBI accession no. 2493675) OGP are 78% homologous on the basis of the amino acid sequence. In our study similar results were obtained for the effect of bovine and porcine OGP on pig ZP resistance to proteolysis, sperm–ZP binding, and monospermy. It would be of interest to examine whether OGP variants, differing in target and activity, can associate with ZP, perivitelline space, or vitelline or blastomere membrane (21). Similarly, it would be very interesting to study the possibility that the different glycosilation of OGP around the ovulation time, demonstrated in hamster (42), could affect the OGP function and be responsible for the differences observed in the activity of OF from follicular and luteal phases. These last facts might explain the different role that OGP plays on ZP in bovine and porcine species vs. that in the mouse model. In conclusion, we demonstrate here that the biochemical and biologic properties of the ZP are modified by oviductal secretions and, even more importantly, that this effect is reversible. This finding matches the *in vivo* situation in ungulates but not in the murine model. Moreover, we demonstrate the role of heparin-like GAGs and OGP as a complex working together in the development of these ZP modifications and, consequently, affecting the sperm–oocyte interaction. This is, in turn, a mechanism in the control of polyspermy in ungulates. This finding may contribute to the development of new approaches for improving current *IVF* techniques for basic, clinical, and commercial purposes.

Materials and Methods

Culture Media. Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química. The medium used for oocyte maturation in pig was NCSU-37 supplemented as described previously (8). The basic medium used for IVF in the pig, designated as TALPp medium, consists of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate.5H_2O, 0.5 mM $MgCl_2$.6H_2O, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mM Na-lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/ml BSA-FAF(A-6003), 1 mg/ml PVA, and 0.17 mM kanamycin sulfate. The *in vitro* maturation medium for cow was TCM-199 with Earle's salts, supplemented as described previously (8). The IVF medium for this species was designated as TALPb and consists of 114 mM NaCl, 3.2 mM KCl, 10 mM Na lactate, 0.5 mM $MgCl_2$ 6H_2O, 0.3 mM NaH_2PO_4 H_2O, 25 mM $NaHCO_3$, 0.2 mM Na-pyruvate, 2 mM $CaCl_2$.2H_2O, 6 mg/ml BSA-FAF(A-6003), 1.75 units/ml heparin, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Oocyte Collection and *in Vitro* Maturation. Methods for *in vitro* maturation in pig and cow were those described previously (8). Ovaries from Landrace × Large White gilts and Charolais, Limousine, and Simmental heifers were collected at the slaughterhouse.

Collection of Oviductal Fluid. Porcine or bovine oviducts were obtained at the slaughterhouse and transported in saline to the laboratory at room temperature. For cattle, all of the oviducts came from heifers 14–20 months old, and only those coming from animals at the late follicular phase were used. For pigs, oviducts were obtained from females whose age and stage of estrous cycle were known (gilt or sow, follicular or luteal phase). Oviducts were washed twice, transferred to Petri dishes placed on ice, and dissected. Oviductal fluid (bOF, pOF) was collected by aspiration with an automatic pipette using a tip for a maximum 200 ml volume and centrifuged at 7,000 × *g* for 10 min at 4°C to remove cellular debris. The supernatant was stored at −80°C until use.

Treatment of Pig and Cow IVM Oocytes with Oviductal Fluid. Preliminary experiments revealed that the effect of OF on ZP resistance to pronase was independent of the presence or absence of cumulus cells. Therefore, in all experiments the cumulus cells were removed to facilitate oocytes manipulation. After *in vitro* maturation and before IVF, porcine and bovine oocytes were incubated in undiluted OF (1 oocyte per microliter of fluid) in groups of 10–30 oocytes covered with mineral oil for 30 min at 38.5°C under 5% CO_2 in air. A control group of oocytes incubated in IVF medium under the same conditions was used in all experiments. Oocytes were washed three times and transferred to fresh IVF medium for insemination or assessed for ZP digestion time. To identify the factor responsible for the ZP resistance to proteinases in the OF, a mini-

proteinase inhibitor mixture (1 μl 10× solution/10 μl OF, Roche), sodium azide 1 mM (Merck 822335), or iodoacetamide 30 mM (Sigma A-3221) were added to the OF before introducing the oocytes, and the results were compared with those obtained in undiluted OF.

Assessment of ZP Solubility. The IVM oocytes from gilts or heifers were transferred into PBS and placed into 50 μl of 0.5% (wt/vol) pronase solution in PBS (12). ZPs were continuously observed for dissolution under an inverted microscope equipped with a warm plate at 37°C. The dissolution time of the zona of each oocyte was registered as the time between placement of samples in the pronase solution and time at which the zona was no longer visible at ×200 magnification. This time was referred to as ''ZP digestion time.''

***In Vitro* Fertilization.** Porcine and bovine IVF were carried out as described previously (8).

Hoechst Staining. Putative zygotes (22 h after insemination) were fixed for 30 min (0.5% glutaraldehyde in PBS), stained for 15 min (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/ml polyvinylpyrrolidone, and mounted on glass slides. They were examined under an epifluorescence microscope at ×200 and ×400 magnifications. Penetration, number of spermatozoa per oocyte, number of spermatozoa bound per ZP, and pronuclear formation were assessed in each putative zygote.

Oviductal-Specific Glycoprotein Identification. Oviducts from heifers at the late follicular phase of the estrous cycle (ovaries showing a predominant or preovulatory follicle) were collected at the slaughterhouse, dissected, and the fluid obtained as described above. Immature porcine cumulus–oocyte complexes were matured *in vitro* for 44 h in NCSU-37 medium. After the maturation period, the complexes were stripped of cumulus cells, washed in PBS, and quickly washed through purified water. Then, oocytes were lysed in a fresh water droplet by gentle pipetting using a narrow-bore glass pipette. Once lysed, empty ZPs were collected and washed for 30 min in PBS 1 M NaCl to remove possible attached cytoplasmic proteins. One hundred porcine ZPs were incubated for 30 min in bOF (1 ZP per 0.5 μl OF) that had been previously tested for its activity (i.e., capable of inducing ZP resistance to proteinases) and then transferred to modified TALPp (without BSA) for 1 h. The ZPs were removed from the medium and time of ZP digestion quantified, observing the expected decrease in the resistance to proteolysis. The medium without ZPs, in which the factor responsible for the resistance should be contained, was subjected to SDS-PAGE electrophoresis under reducing conditions. Two different bands (≈95 kDa and ≈75 kDa) were identified in the gel after the silver staining procedure. These bands were cut and processed for proteomic analysis, which was carried out on an HPLC/MS system consisting of an Agilent 1100 Series HPLC connected to an MSD Trap XCT Plus (Agilent Technologies) using an ESI (electrospray) interface.

Statistical Analysis. Data are presented as mean ± SEM, and all percentages were modeled according to the binomial model of variables and arcsine transformation to achieve normal distribution. The variables in all of the experiments were analyzed by one-way or two-way ANOVA (ZP digestion time, percentage of oocytes with nondigested ZP after 4 h, percentage of oocytes with nondigested ZP after 20 h, percentage of oocyte penetration, mean number of sperm cells per penetrated oocyte, mean number of sperm cells bound to each ZP, and percentage of monospermy). When ANOVAs revealed a significant effect, values were compared by the Tukey test. A P value ≤0.01 was taken to denote statistical significance.

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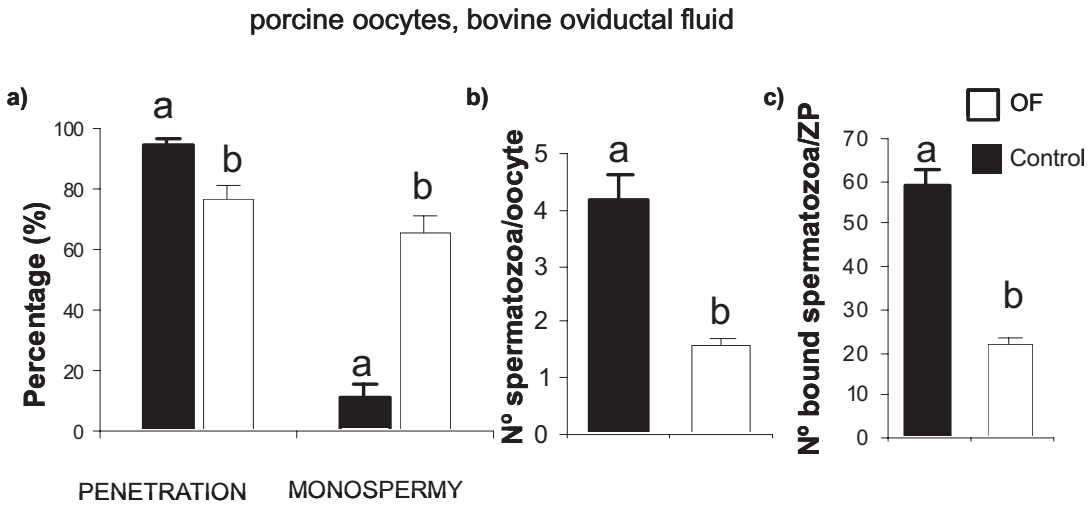
Supporting Information

Coy *et al.* 10.1073/pnas.0804422105

Supporting Information corrected 12/22/2008



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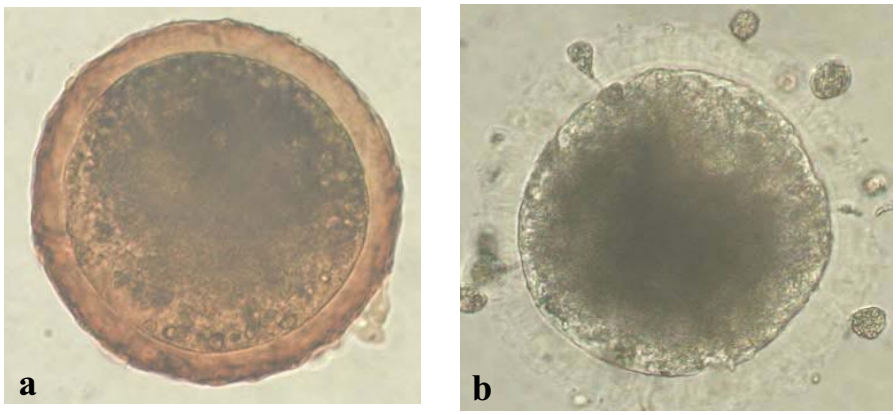

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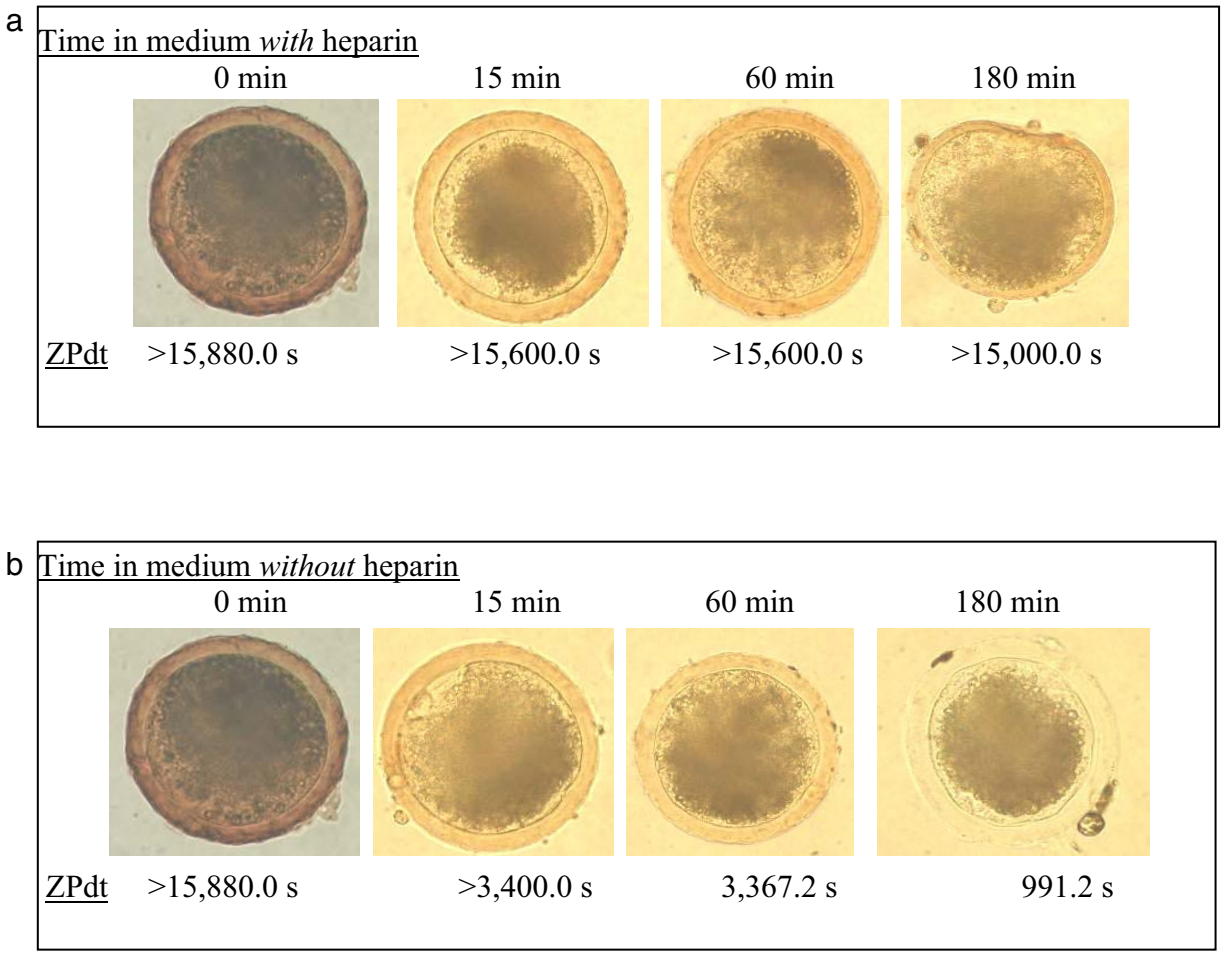

Fig. S3. Experiment to test changes in immunoreactivity in the absence or presence of heparin during incubation. Porcine IVM oocytes were incubated with OF for 30 min and then incubated for different periods in media with (*a*) and without (*b*) heparin. Subsamples of the oocytes were taken from both media at 0, 15, 60, and 180 min, and the presence of OGP by immunocytochemistry and pronase digestion time were assessed. The time that an oocyte was incubated in medium after being exposed to OF, and the time for ZP digestion with pronase (ZPdt), are given. As can be clearly seen in *b*, ZPdt decreased as the immunostaining detected by the antibody anti-OGP disappeared.

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Oocytes, *n*	With cumulus cells	Without cumulus cells
20	28,890.02 ± 1,135.60	30,241.12 ± 3,798.89

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P value		0.083

A sample of the follicular oocytes was incubated for 30 min in bOF before pronase assay.

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Canis familiaris	67%	66%
Homo sapiens	73%	73%
Macaca mulatta	74%	70%
Macaca radiata	70%	69%
Mesocricetus auratus (golden hamster)	66%	63%
Mus musculus	69%	70%
Oryctolagus cuniculus	74%	73%
Ovis aries	78%	94%
Pan troglodytes isoform 1	66%	63%
Pan troglodytes isoform 2	68%	66%
Papio anubis	74%	71%
Sus scrofa		78%

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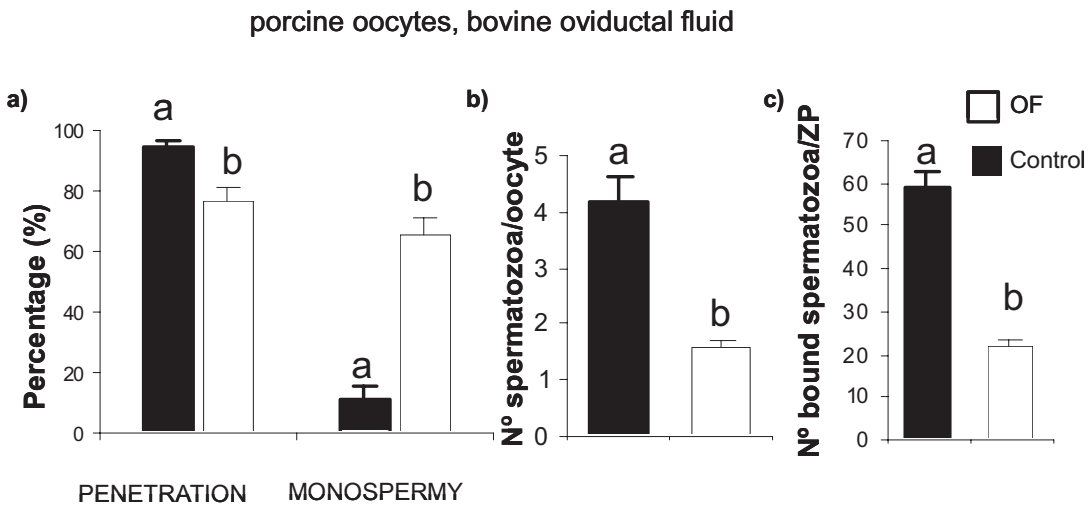

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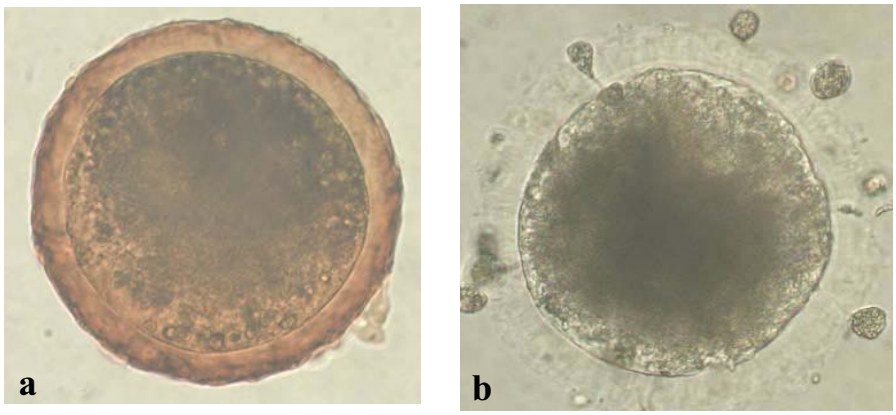

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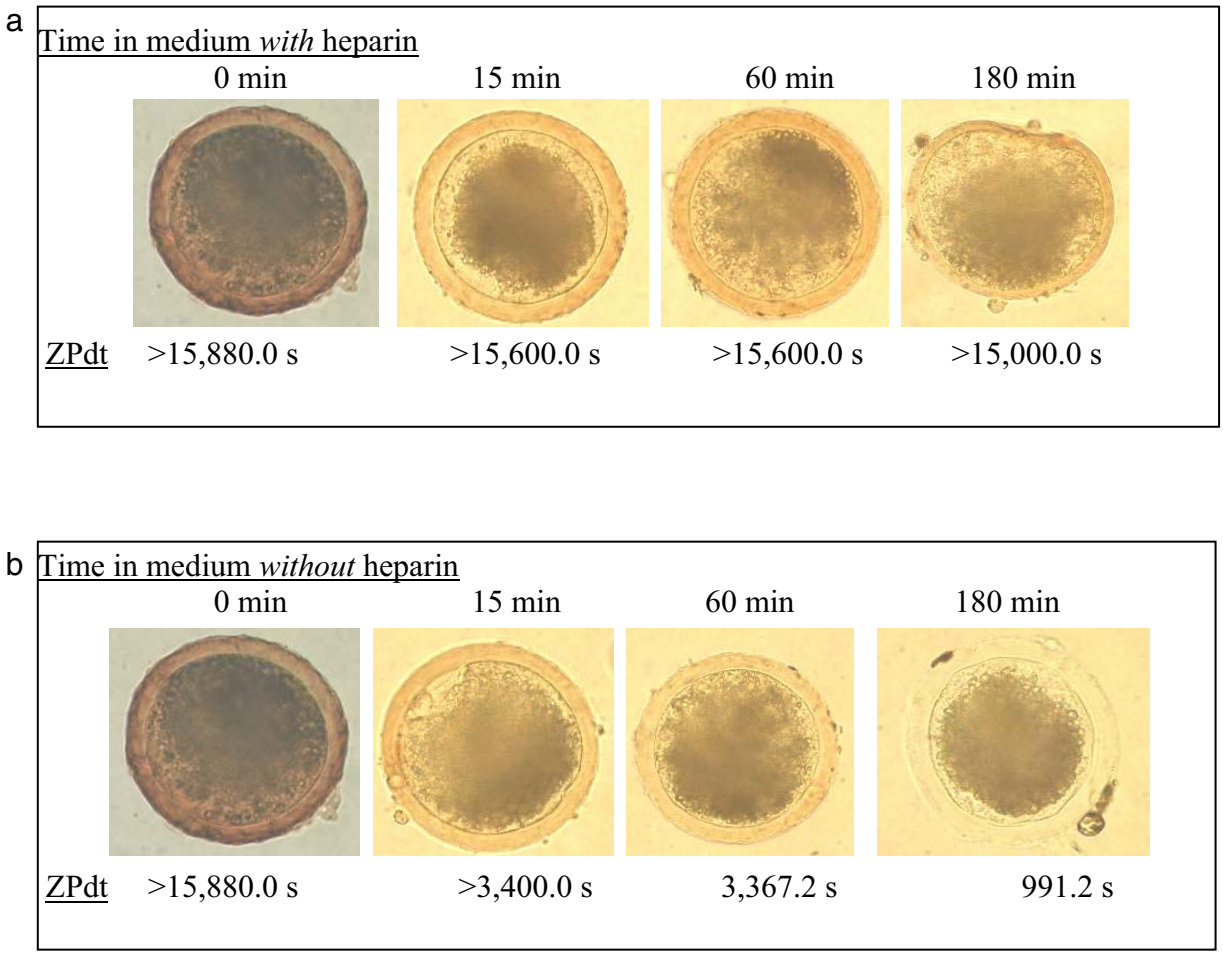

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