

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, pre-

ovulatory 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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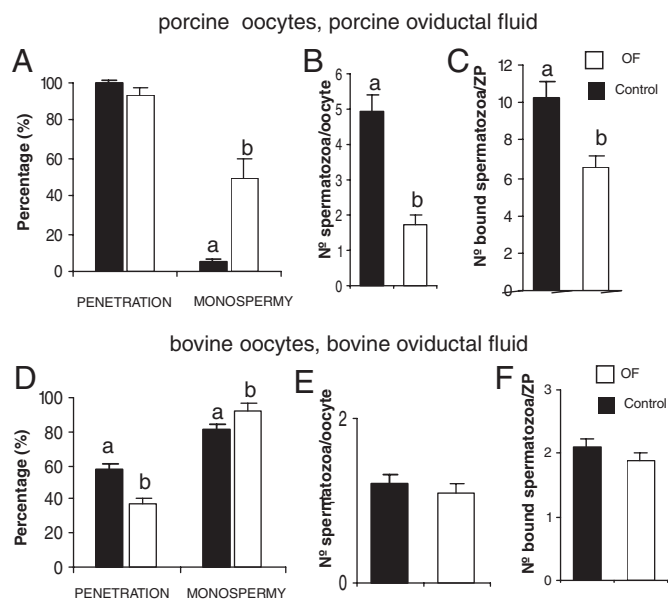


Fig. 2. Effect of incubation of IVM oocytes in oviductal fluid on IVF results. In porcine IVF the parameters penetration and monospermy rate (A), number of spermatozoa per oocyte (B), and number of bound spermatozoa per ZP (C) were evaluated. In bovine IVF, the same parameters were recorded (D–F). Each bar represents mean \pm 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. 3A), 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. 3B). 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

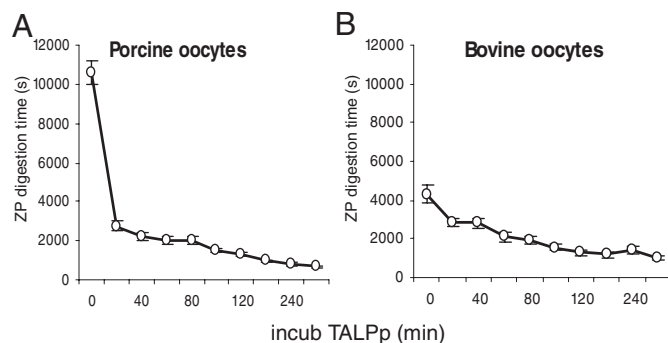


Fig. 3. Effect of bOF on resistance to pronase of ZP from oocytes incubated in TALPp medium for different periods of time. Porcine (A) and bovine (B) IVM oocytes were incubated in bOF for 30 min and later incubated in TALPp medium for different times. The x axis indicates the time interval that the oocytes 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 \pm 4.7a	7.89 \pm 4.4a
bOF + 15 min TALPb	36	100b	63.89 \pm 8.1b
bOF + 60 min TALPb	38	100b	68.42 \pm 7.6b,c
bOF + 120 min TALPb	37	100b	72.97 \pm 7.4b,c
bOF + 240 min TALPb	39	100b	89.74 \pm 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 \pm 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% \pm 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. 4A). 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. 4B).

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

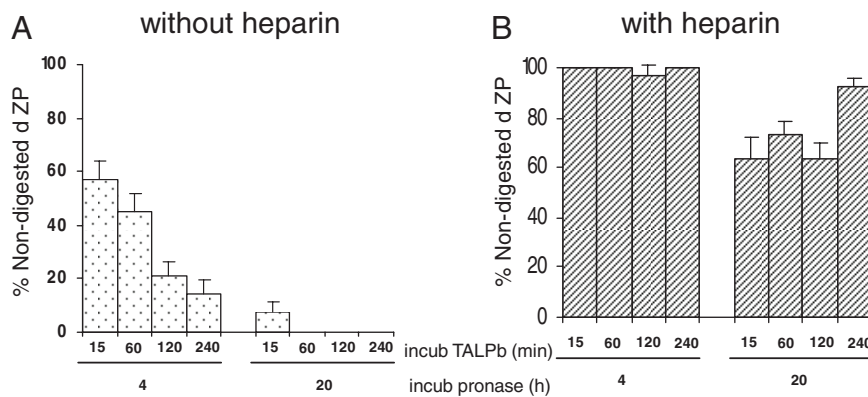


Fig. 4. Effect of heparin on the reversibility of the ZP resistance to proteases induced by bOF in porcine oocytes matured *in vitro*. Oocytes were preincubated in bOF for 30 min, transferred to TALPb medium without (A) or with (B) heparin and evaluated for resistance to proteases after 15, 60, 120, 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.

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 perioovulatory 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.

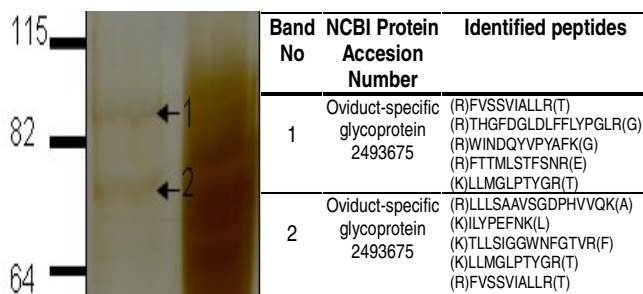


Fig. 5. Identification of OGP as the molecule unbound from ZP after incubation in oviductal fluid (30 min) and further incubation in TALPp medium (1 h). One hundred ZPs from IVM porcine oocytes were preincubated in bOF (1 ZP/0.5 μ l OF) and later in TALPp medium. Lane 1 shows the SDS-PAGE electrophoresis 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.

proteinase inhibitor mixture (1 μ l 10 \times solution/10 μ l 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 μ l 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 \times 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 \times 200 and \times 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 prevulatory 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 μ l 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 (\approx 95 kDa and \approx 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 \pm 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 \leq 0.01 was taken to denote statistical significance.

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