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ORIGINAL ARTICLE *Embryology*

Oocytes use the plasminogen-plasmin system to remove supernumerary spermatozoa

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BACKGROUND: The role of the plasminogen-plasmin (PLG-PLA) system in fertilization is unknown, although its dysfunction has been associated with subfertility in humans. We have recently detected and quantified plasminogen in the oviductal fluid of two mammals and showed a reduction in sperm penetration during IVF when plasminogen is present. The objective of this study was to describe the mechanism by which PLG-PLA system regulates sperm entry into the oocyte.

METHODS AND RESULTS: By combining biochemical, functional, electron microscopic, immunocytochemical and live cell imaging methods, we show here that (i) plasminogen is activated into the protease plasmin, by gamete interaction; (ii) urokinase-type and tissue-type plasminogen activators are present in oocytes, but they are not of cortical granule origin; (iii) sperm binding to oocytes triggers the releasing of plasminogen activators and (iv) the generated plasmin causes sperm detachment from the zona pellucida.

CONCLUSIONS: Our results describe a novel mechanism for the success or failure of fertilization in mammals, by which molecules present in the oviductal environment are activated by molecules originating within the gametes. We anticipate that therapeutic up- or down-regulation of this physiological mechanism may be used to help in conception or as a contraceptive tool. Since components of the PLG-PLA system are already available as drugs for heart attacks or cancer therapies, basic research on this novel function would be rapidly transferable for clinical application.

Key words: fertilization / plasminogen / plasmin / plasminogen activators / sperm–zona pellucida binding

Introduction

The oocyte's response to sperm contact in mammals is a stepwise process whose description was initiated in the early 1950s (Austin and Braden, 1953; Braden *et al.*, 1954) and completed some years later (Barros and Yanagimachi, 1971), with an accurate compendium of the information published in 1994, which is still valid today (Yanagimachi, 1994). Briefly, it is generally accepted that, after cumulus cell penetration, spermatozoa bind to the oocyte's extracellular coat [zona pellucida (ZP)] via surface receptors and establish the so-called primary binding, which is a relatively loose adhesion. The primary attached spermatozoon undergoes the acrosome reaction and establishes secondary binding (Bedford, 2004), being now able to penetrate the ZP, cross the perivitelline space, fuse with the oolemma, deliver the sperm-specific phospholipase C-zeta (Saunders *et al.*, 2002) and induce Ca^{+2} oscillations that activate the oocyte. Oocyte activation includes, as main events, the resumption of meiosis and the exocytosis of the cortical granules, specific oocyte vesicles lying beneath the oolemma. Cortical granule contents finally produce the ZP block to the entrance of additional spermatozoa (block to polyspermy) by inducing: (i) proteolytic cleavage of ZP2 (Moller and Wassarman, 1989), one of the three or four glycoproteins forming the ZP in mammals, named ZP1, ZP2, ZP3 and ZP4; (ii) increased resistance of the ZP to proteolytic digestion (Gulyas and Yuan, 1985) and (iii) enzymatic removal of glycan ligands in ZP3 for the sperm receptor activity (Miller *et al.*, 1993).

Recently, these three consequences of cortical granule exocytosis have been questioned by (i) evidence showing that transgenic mouse lines mutated to prevent cleavage of ZP2 ($Zp2^{Mut}$) can be fertilized at the same rate as normal controls and still display an effective post-fertilization block to polyspermy (Gahlay *et al.*, 2010); (ii) the observation that increased resistance of ZP to protease digestion ('hardening') is a pre-fertilization event in some mammals, depending on the oocyte's contact with oviductal fluid (Coy *et al.*, 2008a) and (iii) the

demonstration that spermatozoa can bind and fertilize oocytes from mouse lines lacking the specific glycans proposed as ligands on ZP ($Zp3^{Mut}$; Gahlay *et al.*, 2010) and that a deglycosylated ZP3 product induces inhibitory effects on sperm–ZP binding (Clark, 2011). At the same time, new evidence identifying the specific oligosaccharide involved in human sperm–ZP binding (Pang *et al.*, 2011) confirms the complexity of the biological mechanisms implicated in the process and emphasizes the need for using different models to reach general conclusions.

While the biochemical and transgenic approaches have provided consistent data to increase our understanding of the block to polyspermy, they have always been focused on molecules present either in the oocyte or in the spermatozoon. However, during gamete interaction, molecules present in the oviductal fluid, the physiological milieu surrounding gametes during their rendezvous, may also be playing an active role in the block to polyspermy, as is the case with the oviductal-specific glycoprotein (OVGP1; Coy *et al.*, 2008a), and this could also be the case if regulated protease activity in the oviductal fluid was identified.

The serine protease identified in the cortical granules of echinoderms (Haley and Wessel, 1999), activated at fertilization, and responsible for the protease-mediated reactions that contribute to the block to polyspermy, has its counterpart in the hamster (Cherr *et al.*, 1988) and in the mouse (Hoodbhoy and Talbot, 1994). In other mammals, the identity of this enzyme is unknown (Wong and Wessel, 2006) although serine-protease activity, mediated by the releasing of a tissue-type plasminogen activator (tPA) from cortical granules, has been proposed as a mechanism contributing to the regulation of sperm penetration into rat and bovine oocytes (Zhang *et al.*, 1992; Rekkas *et al.*, 2002). This tPA is part of the plasminogen-plasmin system (PLG-PLA). Plasminogen is a zymogen synthesized by the liver and other extrahepatic tissues (Zhang *et al.*, 2002) that is converted into the serine protease, plasmin, by tPA or by the uroquinase-type plasminogen activator (uPA). Plasmin, in turn, degrades the extracellular matrix of most of the tissues and cleaves a variety of proteins, and thus its role in blood clot lysis is the most well known (Schaller and Gerber, 2011).

The role of the PLG-PLA in fertilization has been largely discussed but never clarified. Most of the studies have been focused on the identification of their components and the study of its effect on the spermatozoa (Smokovitis *et al.*, 1987; Kim *et al.*, 2009; Zervos *et al.*, 2010) and few of them have studied the effect of the addition of plasminogen or plasmin into the fertilization medium on IVF outcomes. The results have been controversial: an increased level of fertilization when plasminogen was present in the culture medium was first proposed in mouse (Huarte *et al.*, 1993), whereas its participation in the regulation of polyspermy hampering sperm entry into the oocyte was also proposed in rats (Zhang *et al.*, 1992) as above mentioned.

Recently, the role of plasminogen in fertilization has been partially clarified. We have detected and quantified, for the first time, plasminogen in the oviductal fluid (Mondéjar *et al.*, 2012). By using plasminogen concentrations similar to those detected in the oviductal fluid, which are markedly different from those used in previous studies (Sa *et al.*, 2006), a reduction in the number of spermatozoa bound to the ZP and in the sperm penetration into oocytes was observed in the porcine and bovine species (Mondéjar *et al.*, 2012), either in the presence (cattle) or absence (pig) of cumulus cells. These data lead us to hypothesize that plasminogen binds to ZP and oolemma, as suggested by Roldán-Olarte *et al.* (2005), and becomes activated into plasmin during fertilization, and that this plasmin is responsible for the observed reduction in sperm penetration. Therefore, the main objectives of the present study were to test this hypothesis and to describe the mechanism by which the PLG-PLA system decreases polyspermy in the pig species.

Materials and Methods

In vitro maturation and fertilization

Procedures were basically those previously described (Coy *et al.*, 2008b). Cumulus–oocyte complexes (COCs) were collected from antral follicles (3–6 mm diameter) of ovaries in slaughtered 6–7-month-old animals, weighing 80–100 kg. Groups of 50–55 COCs were *in vitro* matured for 44 h in the NCSU-37 medium prepared in our laboratory. A sperm-rich fraction of semen from a mature, fertility-tested boar was selected through a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (45 and 90% v/v) and added to the IVF medium (TALP) containing the oocytes at a final concentration of 10^5 cells/ml. Plasminogen (BPLG, Molecular Innovations, USA) was added at a concentration of 100 μg/ml or not added (control) to the IVF dishes for the different experiments.

Enzymatic assays

The IVF medium was centrifuged to separate the putative zygotes and the supernatant and both fractions were later diluted 1:40 (v/v) in Tris–NaCl buffer (pH 7.4). Diluted samples were mixed with S-2251 chromogenic substrate (Chromogenix, Milan, Italy, Friberger *et al.*, 1978) diluted in Tris–NaCl buffer (final concentration 3 mM) and the absorbance was immediately recorded at 405 nm on a spectrophotometer (FLUOstar Galaxy, BMG Lab Technologies, Durham, USA). Negative controls were solutions containing exactly the same amount of buffer and chromogenic substrate S-2251 than the experimental groups but without gametes and with either plasminogen (negative control, 300 μg/ml) or plasmin (positive control, 10 μg/ml). Calibration curve was prepared with standard solutions of *p*-nitroaniline from 0 to 1.25 mM. Samples, controls and standards were run concurrently, in duplicate, during 120 min at 37°C. A calibration curve was produced by plotting the absorbance of the standards against their concentration during a period of 120 min. One unit of plasmin activity was defined as the amount of enzyme necessary to hydrolyse 1 mmol substrate per hour at 37°C. Samples from each group were analyzed in duplicate.

Live recording of effect of plasmin on sperm–ZP binding

After 2 h of oocyte and sperm incubation (see section *in vitro* maturation and fertilization under section Materials and methods), the oocytes were gently aspirated up and down using an automatic pipette set up to 200 μl volume until loosely attached spermatozoa were removed leaving only a few sperm strongly attached. The oocytes with the spermatozoa attached were placed on a Petri dish (each one in 4 μl PBS drop and a central drop containing plasmin) covered with mineral oil. The experiment was conducted on a heated plate at 200× magnification using an inverted microscope (Nikon Diaphot 300, Tokyo, Japan). The oocyte was immobilized using a holding pipette (Eppendorf Sterile VacuTips) by micromanipulator. Just after the immobilization, we tried to remove the spermatozoa using the end of an injection micropipette (Eppendorf Sterile VacuTips). Thereafter, the injection micropipette was filled with plasmin (2×10^{-5} μl from a 150 μg/ml solution). The plasmin was placed locally at the

sperm–ZP binding site and after 30 s we tried again to remove the same spermatozoa. The videoclips were recorded by digital camera attached to the microscope.

Sperm–ZP binding assay

A total of 160 oocytes were used for this assay. Oocytes were washed twice in PBS after IVM and passed repeatedly through a heat-narrowed Pasteur pipette with a pore diameter less than the size of the oocytes. Thus, the oocytes were broken and the ooplasm and empty ZP were released into the dishes. The ZPs were washed three times in PBS and transferred to fertilization medium where they were incubated with sperm following the protocols already explained for IVF. After removing the sperm not firmly attached by repeated pipetting, the ZPs with strongly bound sperm were stained with Hoechst (1% Hoechst 33342 in PBS). The number of sperm bound to each ZP was registered at different times. In the first group, sperm were allowed to be in contact with the ZP for 15 min in order to check the initial binding under control conditions. In a second group, sperm–ZP contact lasted 3 h, but half of the group were under control conditions and half of them were incubated with plasmin (75 μg/ml) added 30 min after the beginning of coincubation. In the third group, the sperm–ZP contact lasted 24 h and, again, half of the ZPs were incubated with sperm under control conditions and half of them with plasmin added 30 min after the beginning of coincubation. This experimental design was used to find out if plasmin impaired sperm–ZP binding or if the effect of plasmin was to detach the spermatozoa already attached.

Electron microscopy

In vitro matured and fertilized oocytes were fixed with 1% glutaraldehyde in cacodylate buffer, pH 7.4, for 2 h at 4°C. After extensive washing in cacodylate buffer, samples were dehydrated through a graded series of ethanol and processed for embedding in LR-White resin. For immunocytochemistry, a three step-method was used. All antibodies were diluted in PBS without calcium and magnesium supplemented with 1% (w/v) bovine serum albumin. Ultrathin sections were incubated with an anti-tPA polyclonal antibody from goat (1:400 v/v, AP02244SU-N, Acris antibodies, Herford, Germany) and anti-uPA polyclonal antibody from goat (1:200 v/v, AP02255SU-N, Acris antibodies), respectively. After that, samples were treated with polyclonal rabbit anti-goat IgG antibody (1:5000 v/v, G 5518, Sigma-Aldrich) and finally a protein A-colloidal gold conjugate (1:70 v/v; 15 nm) was used to detect signal. Ultrathin sections were counterstained with uranyl acetate followed by lead citrate and imaged in a Jeol JEL-1011 Electron Microscope.

Inmunoblots

Fractions recovered from the microaspiration experiment and ZP-free oocytes treated with pronase were separated by SDS-PAGE and transferred to PVDF membranes which were probed with polyclonal antibodies to tPA (1:2000 v/v), uPA (1:1000 v/v) or actin-HRP conjugate (1:1.000 v/v, Abcam, Cambrige, UK). To detect tPA and uPA signal, a rabbit anti-goat IgG-HRP conjugate antibody was used (1:10000 v/v, AP106P, Millipore). Blots were visualized by chemiluminescence (Typhoon 9410 Variable Mode Imager, GE Healthcare) and antibody signals were quantified by Image Quant TL v2005 software (GE Health Care, Piscatawat, NJ, USA).

Live cell imaging in confocal microscope

Polyclonal antibodies anti-tPA and anti-uPA were employed as markers for live confocal imaging. Cumulus cell-free oocytes were washed in the TALP medium and ZPs were removed quickly in 3% pronase solution. After washing, they were incubated in the primary antibody (1:15) for 1 h, three times washed and incubated in the secondary antibody rabbit anti-goat IgG-FITC conjugate (1:15 v/v, F0250, DAKO) for 1 h. After another three washes, they were visualized using a Leica TCS-NT/SP confocal microscope with CO_2 supply and a warm plate. FITC and Hoechst fluorochromes were excited using an argon laser at 488 and 364 nm, respectively. For each oocyte, 25–35 2D images (4 μm width, from the top of the oocyte to the bottom) were collected. The 3D image reconstruction was made by Imaris Scientific 3D software. For groups with sperm added, the time 0 pictures were taken 2 min before the addition of sperm.

Statistical analysis

Data were analyzed by ANOVA. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison *post hoc* test (Tukey). Differences were considered statistically significant at $P < 0.05$.

Results

Gamete interaction activates plasminogen into plasmin

We determined plasmin activity in the IVF medium where porcine gametes were interacting and in both the oocyte and spermatozoa. The assay revealed the activity of this enzyme (Fig. 1A–C), both in the gametes (gametes after interaction for 24 h, pellets) and in the medium (medium where gametes had interacted for 24 h, supernatant), when plasminogen was added. When no plasminogen was added (−PLG), plasmin activity was low but was nevertheless present, probably due to plasminogen of follicular origin bound to the oocytes. Since the control without gametes revealed no plasmin activity (Fig. 1A), the source of plasminogen activators in this experiment should be in the gametes. When gametes were analyzed separately, it was confirmed that sperm and oocytes could generate plasmin in the presence of plasminogen (Fig. 1D–F), but at a lower level than during their interaction. The source of plasminogen *in vivo* is the oviductal fluid; so, this result suggests that in the oviduct, plasmin is acting during physiological fertilization.

Plasmin detaches sperm bound to ZP

The consequences of plasmin activity could be related to a reduction in the number of spermatozoa being able to attach the ZP or to the detachment of the previously attached spermatozoa. To test these two possibilities, an experiment for the direct observation of the plasmin effect and a sperm–ZP binding assay were designed. Since plasmin can be an enzyme of rapid action at 37°C, we tried to determine, by real-time observation, whether plasmin can detach spermatozoa attached to the ZP a few seconds after its addition (Fig. 2A). Excess spermatozoa attached to the ZP of *in vitro* fertilized oocytes (loosely bound) were removed by pipetting and an attempt to detach the remaining attached sperm was done by using a microinjection pipette (Eppendorf Sterile VacuTips). The movies recorded show that the removal of spermatozoa bound to the ZP was not possible (Supplementary data, Movie S1A). Plasmin (2×10^{-5} μl from a 150 μg/ml solution) was spread near the sperm–ZP binding sites in the same oocytes and, after 30 s, an attempt to remove the attached spermatozoa was done. The videoclips recorded show that the spermatozoa were easily removed from the ZP with the

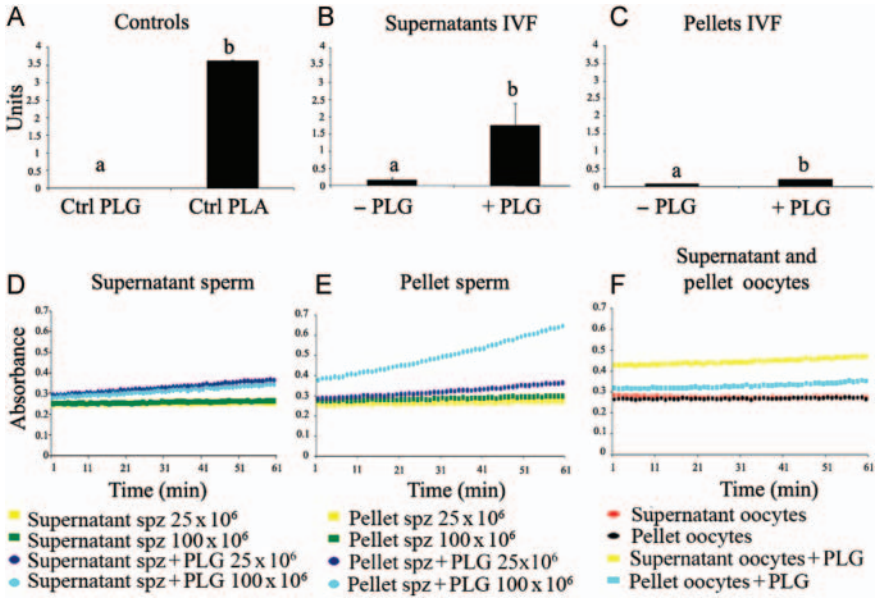

Figure 1 Plasminogen is activated into plasmin during gamete interaction. (**A**) Plasmin (PLA) activity (units) in negative (PLG, 300 μg/ml) and positive controls (PLA 10 μg/ml) used in the experiments. $P < 0.001$. (**B** and **C**) PLA activity in fertilization droplets collected 24 h after gamete interaction in the absence (−) or presence (+) of plasminogen (PLG). Supernatant (medium where gametes interacted for 24 h) contained the medium ($P = 0.044$) and pellets (gametes after interaction for 24 h) contained the gametes ($P = 0.006$). (**D** and **E**) Representation of PLA activity in one experiment for spermatozoa lysate (supernatant and pellet) obtained from 25 and 100 × 10^6 cells immediately after addition or not of PLG. PLA activity in pellets was 0.10, 0.11, 0.28 and 0.77 units, respectively, for 25 × 10^6, 100 × 10^6, 25 × 10^6 + PLG and 100 × 10^6 + PLG; and 0.07, 0.09, 0.28 and 0.22 units in the supernatants. (**F**) Representation of PLA activity in one experiment from oocytes lysate (supernatant and pellet) obtained from 200 cells immediately after addition or not of PLG. PLA activity reached 0.08, 0.03, 0.17 and 0.21 units, respectively, for supernatant, pellet, supernatant + PLG and pellet + PLG. Data are given as mean ± s.e.m. of three to five experiments if not otherwise stated.

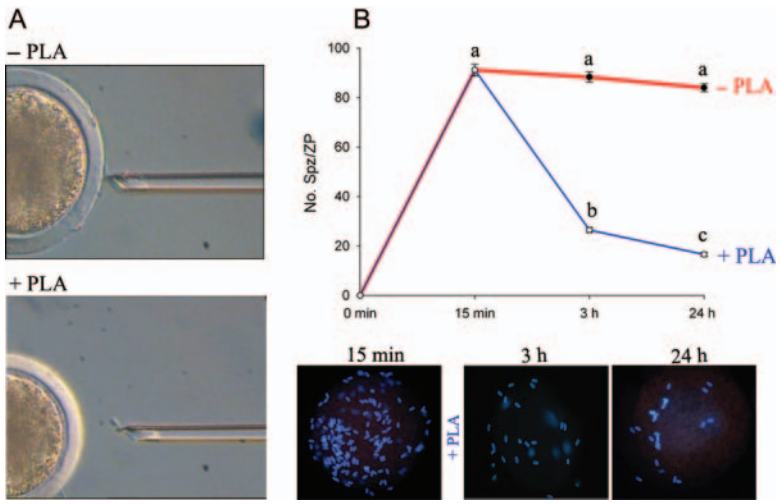

Figure 2 Plasmin induces sperm detaching from the ZP. (**A**) Pictures representative of the real-time observation (Supplementary data, Movie S1A) showing that spermatozoa attached to the ZP cannot be removed with the microinjection pipette (−PLA), but they are easily released from their ZP attachment when plasmin is added (+PLA; Supplementary data, Movie S1B). (**B**) Number of spermatozoa attached to the ZP after 15 min of gamete coincubation and after 3 and 24 h in the presence (+PLA) or absence (−PLA) of plasmin. $P < 0.001$. Representative pictures of stained spermatozoa attached to the ZPs at different times. A decrease in the number of sperm attached to the zona was observed in the presence of plasmin. Data are given as mean ± s.e.m. $n = 120$ ZPs per group.

microinjection pipette (Supplementary data, Movie S1B). In the same way, the sperm–ZP binding assay revealed that the number of spermatozoa attached to the ZP after 15 min of contact and assessed 3 h later, decreased if plasmin was present, but remained steady in the absence of plasmin (Fig. 2B).

Plasminogen activators are localized in the oolemma and ZP of oocytes, but are decreased in the presence of sperm

When the sperm are present in the IVF medium, immunolabeling for plasminogen activators in oocytes decreases. Once the effect of the PLG-PLA system had been established, a series of additional experiments were designed to search for the mechanism responsible for the plasminogen activation during the gamete interaction. The proteolytic activity of mature mouse gametes in their close environment and their ability to bind plasminogen was demonstrated some years ago (Huarte *et al.*, 1993). However, the precise localization of plasminogen activators in the oocytes was not described. By the immunolocalization of tPA and uPA in electron microscopy, we saw that both activators were present in the oolemma and in the ZP of oocytes (Fig. 3A) but were not associated with the cortical granules. When the oocytes were fertilized *in vitro* with spermatozoa, the immunolabeling decreased significantly (Fig. 3B), suggesting their reaction with the plasminogen bound to oocytes and the subsequent generation of plasmin.

To test whether the decreased immunoreactivity was an effect of the sperm contact both at ZP and oolemma level and not an effect of oocyte washing by the IVF medium, two groups of oocytes were incubated for 4 h in the IVF medium in the presence or absence of spermatozoa. By aspirating the oocyte content with the microinjection pipette (Supplementary data, Movie S2), two fractions, corresponding to ZPs and ooplasms, were obtained and analyzed by SDS-PAGE and western blot. The results revealed significant decreases in tPA and uPA in fertilized oocytes compared with their non-fertilized counterparts (Fig. 3C and D), thus suggesting an active role of the spermatozoa in the releasing of activators.

Sperm binding to oolemma triggers the releasing of plasminogen activators

In order to visualize the oocyte's reaction to sperm contact in real time at the oolemma level, we introduced tPA and uPA immunolabeled *in vitro* matured oocytes into an incubation chamber adapted for use with confocal microscopy with a CO_2 supply and a warm plate. The direct observation of these oocytes in the confocal microscope and the corresponding reconstructions of the three-dimensional images showed that oocytes in the IVF medium retained the immunolabeling for both tPA and uPA after 18 min. However, when we added spermatozoa to the dish, it was observed that as more spermatozoa bound to the ooplasm, less immunolabeling remained (Fig. 4).

Since in this experiment we removed the ZP by pronase digestion, and pronase could have digested also the plasminogen activators bound to the oolemma, we ran an additional western blot analysis to test whether tPA and uPA immunolabeling persisted in oocytes after short pronase treatment, both before and after IVF (Supplementary data, Fig. S3). The results were similar to those obtained by microaspiration of the cell contents, although the signal in both groups was proportionally lower, probably due to the action of pronase.

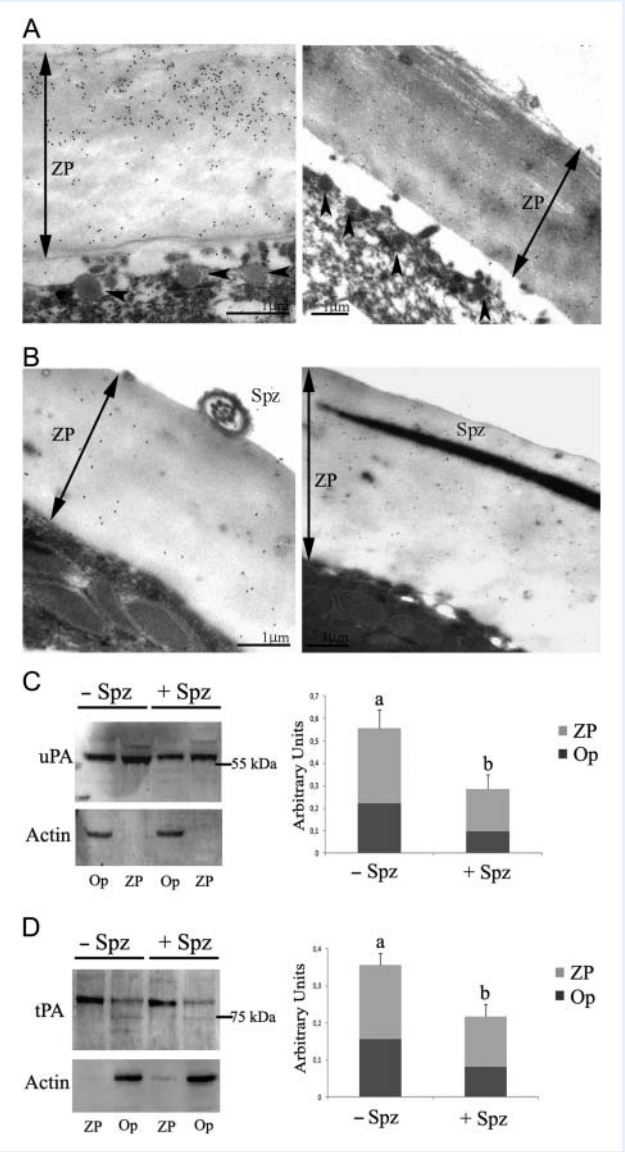

Figure 3 uPA and tPA labeling decrease in the ZP and ooplasm after oocyte-sperm coincubation. (**A**) *In vitro* matured oocytes immunostained with tPA (left panel) and uPA (right panel) antibodies showed specific labelling on the ZP. Note that cortical granules (arrows) beneath the ooplasm are unlabelled. (**B**) tPA and uPA labelling decreased in *in vitro* fertilized oocytes. Spz indicates tails or heads from the sperm. (**C** and **D**) Ooplasm (Op) and zona pellucida (ZP) separated after oocyte content aspiration (supplementary data, Movie S2) were analyzed by western blot with uPA and tPA antibodies and actin was used for loading normalization. Average data from three experiments were quantified by image analysis. Immunolabeling decreased in oocytes incubated with sperm both in uPA ($P = 0.041$) and tPA ($P = 0.024$).

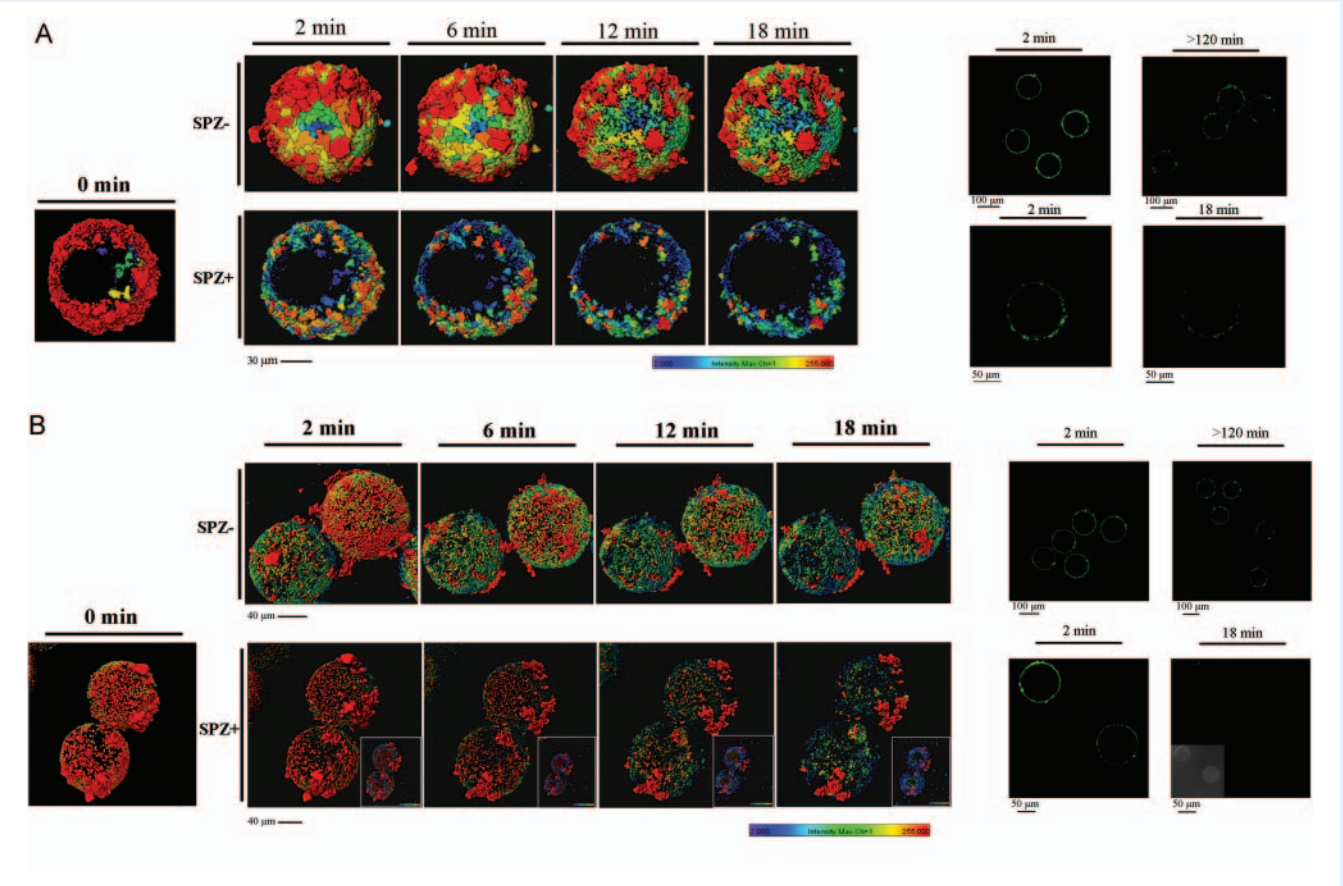

Figure 4 Sperm–oocyte contact induces plasminogen activator (uPA/tPA) release from the oocyte plasma membrane. Three-dimensional reconstructions (25–35 2D images) showing high (red color) and low (green color) fluorescence intensities in ZP-free *in vitro* matured (SPZ−) and fertilized (SPZ+) oocytes incubated with uPA (**A**) and tPA (**B**) antibodies. The fluorescence signal in the SPZ+ groups started decreasing considerably just 2 min after the sperm–ooplasm contact and during the incubation time (6–18 min) in comparison with the SPZ− groups. For the tPA SPZ+ group, the sperm were stained with Hoechst (inset) to identify the duration of sperm contact with the ooplasm. Equatorial sections (right panels) showed that after 120 min of culture, the fluorescence remained in SPZ− oocytes but it disappeared by 18 min in SPZ+ oocytes.

Discussion

Our data suggest that the oocyte, once the spermatozoon contacts it, releases plasminogen activators and displays plasmin activity both at the ZP and at the oolemma levels. This activity would remove some of the spermatozoa previously attached to the ZP contributing to regulate polyspermy or participating in other functions independent of cortical granules. Hence, despite previous suggestions indicating the possible presence of plasminogen activators inside cortical granules (Zhang, *et al.*, 1992, Rekkas, *et al.*, 2002), this was never demonstrated. Zhang *et al.* (1992) showed that tPA was released by activated oocytes but they did not detect its localization inside the ooplasm, and Rekkas *et al.* (2002) detected tPA after micro-aspiration of the peri-oolemma content in cow oocytes. Both results are compatible with a location of tPA in the oocyte's peri-oolemma, as we demonstrate for the first time in our electron microscopy pictures in the present manuscript (Fig. 3, panel A): cortical granules were un-reactive to specific antibodies against tPA (left) and uPA (right), but both plasminogen activators were present in the peri-oolemma content. These results agree with Hoodbhoy and Talbot's statement: (Hoodbhoy and Talbot, 1994): 'it seems unlikely that tPA is packaged into the CG' and we suggest, as Liu did (Liu, 2011), that tPA could be secreted by the oocyte, independently of cortical granule exocytosis, in a manner similar to that of calreticulin (Tutuncu *et al.*, 2004). Having said that, a possible role of CG secretions on the release of plasminogen activators from the ZP cannot be discarded: the observed decrease in the labeling of ZP for tPA and uPA after fertilization needs to be further clarified because the effect was not restricted to the sperm–ZP binding sites but it was observed in the whole ZP. We hypothesize that either factors of CG origin or the proteolytic action of plasmin itself on the ZP could be involved in the decrease of PAs at this level.

A schematic representation of our proposed mechanism is found in Fig. 5. The mechanism is compatible with all the other forms to control polyspermy already proposed, including the action of proteases from the cortical granules origin (Moller and Wassarman, 1989), which could work more efficiently on the inner portion of the ZP.

Differently from mouse, 'considerable numbers of accessory spermatozoa may accumulate within the outer portion of the ZP of fertilized

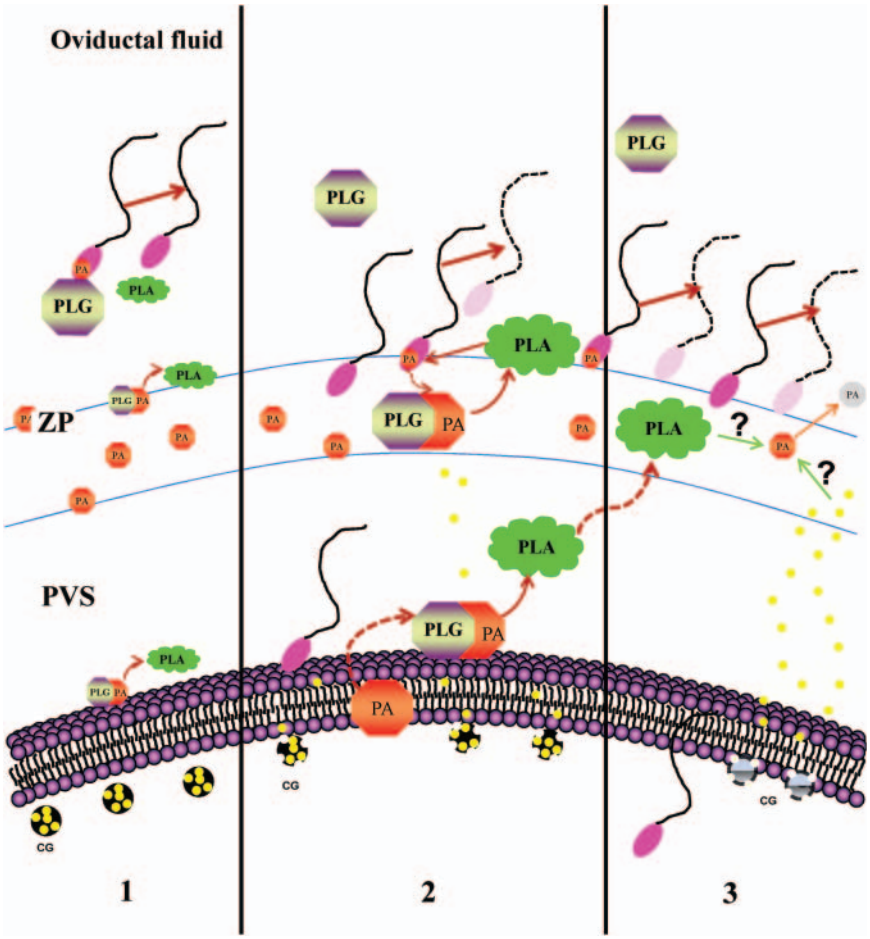

Figure 5 Schematic representation of the proposed model for the role of the fibrinolytic system during fertilization. (1) In the oocyte, plasminogen (PLG) and plasminogen activators (PAs) are present in the oolemma and in the ZP. Discrete PLG activation could be participating in the remodeling of ZP or in the maintaining of the perivitelline space (PVS) viscosity by the proteolytic action of plasmin (PLA). In the oviductal fluid, some spermatozoa could be also generating PLA by interacting with PLG. (2) When the spermatozoon contacts the ZP, generation of PLA is increased by participation of PLG and PA bound to the ZP. The same situation occurs at the oolemma level. (3) Plasmin generated breaks sperm–ZP binding. The role of cortical granule (CG) content or plasmin in the decrease of PAs at the ZP level is also suggested.

eggs in various species of domestic animals' (Hunter, 2003) and it is plausible that under *in vivo* conditions, the PLG-PLA system plays a different role depending on the species, to avoid the penetration of these attached sperm. In fact, we show here that the PLG-PLA system does not remove the spermatozoa loosely attached to the ZP but breaks or avoids their tight binding and, consequently, their penetration into the oocyte. Thus, it is possible to see zygotes after IVF with numerous spermatozoa attached to the ZP both in control and plasminogen-treated oocytes but, after passing them through a narrow pipette, more spermatozoa are easily detached if plasminogen has been present in the IVF medium than if plasminogen was absent. Apart from the possible role in the regulation of polyspermy, this could be important during the transport of the zygote through the isthmus, when contact with the epithelium would permit the removal of the excess spermatozoa attached to the zona and the establishment of appropriate signals between the mother and the incipient embryo by direct contact of ZP and oviductal cells. This could also be the mechanism involved in the sperm displacement of the ZP by additional sperm recently described by Gahlay *et al.* (2010).

Proteolytic degradation of the extracellular matrix by the protease plasmin is a universal mechanism that determines health and disease. Under physiological conditions, plasmin-induced proteolysis is not only involved in the resolution of blood clots (Collen, 1987) but also in cell migration and the remodeling of organs. Reduced activity of the system can be compensated by the administration of plasminogen activators, i.e. for heart-attack patients (Hatcher and Starr, 2011). On the contrary, exacerbated activity of the system is involved in tumor progression (Andreasen *et al.*, 2000). Within the reproductive function, reduced fertility was also shown in mutant mice with a deficiency in plasminogen activators (Carmeliet *et al.*, 1994). Dissecting the mechanism by which the system participates in fertilization, as we have shown here, may help to develop new contraceptive

strategies, by increasing the activity of the PLG-PLA system, or may contribute towards the treatment of subfertility (Ebisch *et al.*, 2007), by reducing the activity of some of their components.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

R.R. and L.G. performed enzymatic assays; F.A.G. and P.C. prepared gametes and performed movies. F.A.G., I.M. and P.C. performed confocal live cell experiments; M.J.M. carried out western blot and electron microscopy studies. P.C., R.R., F.A.G. and M.J.M. were involved in study design and data analysis. P.C. conceived the project and wrote the paper. All authors discussed the results and commented on the manuscript.

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Conflict of interest

None declared.

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