Fertilization outcome could be regulated by binding of oviductal plasminogen to oocytes and by releasing of plasminogen activators during interplay between gametes

Irene Mondéjar, Ph.D., Luis A. Grullón, Ph.D., Francisco A. García-Vázquez, Ph.D., Raquel Romar, Ph.D., and Pilar Coy, Ph.D.

Department of Physiology, Faculty of Veterinary, University of Murcia, Murcia, Spain

Objective: To detect plasminogen and plasminogen activators (PA) in oviduct and oocytes and to clarify the role of the plasminogen/plasmin system on mammalian fertilization.
Design: Experimental prospective study.
Setting: Mammalian reproduction research laboratory.
Animal(s): Oviducts and ovaries from porcine and bovine females were collected at slaughterhouse. A total of 52 oviducts and 2,292 oocytes were used. Boar and bull ejaculated spermatozoa were also used.
Intervention(s): Plasminogen concentration in oviductal fluid (OF) through the cycle was measured. Immunolocalization of plasminogen and PAs in oocytes was carried out before and after fertilization. Porcine and bovine oocytes were in vitro fertilized, with plasminogen and plasmin added to the culture medium at different concentrations.
Main Outcome Measure(s): Plasminogen concentration in OF. Plasminogen and PAs immunolocalization in oocytes. Penetration and monospermy rates, number of spermatozoa in the ooplasma and on the zona pellucida (ZP) after IVF.
Result(s): Oviductal fluid contains about 92 μg/mL of plasminogen. The mature oocyte shows immunoreactivity toward plasminogen and toward PAs on its oolemma and ZP. After fertilization, plasminogen and PAs immunolabeling decreases in the oocyte, suggesting its conversion into plasmin. When exogenous plasminogen is added to the IVF medium, sperm entry into the oocyte is hampered, suggesting that the role of plasminogen activation during fertilization is to reduce the number of (or to select) penetrating spermatozoa.
Conclusion(s): The plasminogen/plasmin system is activated during gamete interaction and regulates the sperm entry into the oocyte. (Fertil Steril® 2012;97:453–61. ©2012 by American Society for Reproductive Medicine.)
Key Words: Plasminogen, IVF, oocyte activation, sperm-zona pellucida binding, polyspermy

The plasminogen/plasmin system, better known as the fibrinolytic system, is involved in a variety of physiological processes, such as blood clot lysis (1) and tumor growth and invasion (2). Briefly, the zymogen plasminogen is converted into the serine protease plasmin by the tissue plasminogen activator (t-PA) or the uroquinase plasminogen activator (u-PA). Plasmin degrades the extracellular matrix of most tissues and cleaves a variety of proteins. The system is regulated by plasminogen activator inhibitors (PAI-1, PAI-2, PAI-3) and by α-antiplasmin (3, 4).

Some of the elements of the system, such as t-PA, u-PA, or PAI-1 have been identified in human and boar sperm membrane (5), seminal plasma of human and various animal species (6), bovine follicular fluid (FF) (7), human endometrium (8), and the porcine and bovine oviduct (9, 10). The corresponding roles of the system in sperm motility and acrosome reaction (11), ovulation (12), or implantation (13) have been thus described, and its dysfunction has been related with subfertility in humans (14, 15). However, the presence of plasminogen itself in the oviduct and the precise localization of plasminogen, t-PA, and u-PA in the oocyte is almost unknown. In addition, the explanation for the presence of PAI-1, t-PA, or u-PA in the oocytes (12, 16) and oviduct (9, 10) and its possible role in fertilization has not yet been clarified.

Reverse transcriptase-polymerase chain reaction (RT-PCR) has shown high levels of u-PA messenger RNA

Received June 24, 2011; revised November 20, 2011; accepted November 23, 2011; published online December 16, 2011.
I.M. has nothing to disclose. L.A.G. has nothing to disclose. F.A.G.-V. has nothing to disclose. R.R. has nothing to disclose. P.C. has nothing to disclose.
Supported by Spanish Ministry of Science and Innovation and Fonds Européen de Développement Régional, Grant AGL2009-12512-C02-01.
Reprint requests: Pilar Coy, Ph.D., Department of Physiology, Faculty of Veterinary, University of Murcia, Campus de Espinardo, Murcia 30071, Spain (E-mail: pcoy@um.es).

Fertility and Sterility® Vol. 97, No. 2, February 2012 0015-0282/$36.00

doi:10.1016/j.fertnstert.2011.11.032

(mRNA) in oviduct just before ovulation with a significant decrease just after ovulation in cattle (10). In pigs, mRNA for PAI-1 has been also seen in the oviductal tissue (9) and plasminogen activator (PA) activity has been detected both in oviductal tissue and oviductal fluid (OF) of hamster (17). Bovine oocytes show u-PA activity (18). Mouse, rat, and Rhesus monkey oocytes show t-PA activity (12, 16), and the presence of t-PA in cortical granules of rat and cow oocytes has been also proposed (19, 20). Altogether, these studies suggest that the plasminogen/plasmin system may have a role during gamete interaction in the oviduct, but the results so far are controversial. Although an increased level of fertilization when plasminogen is added to the culture medium was first proposed in mouse (21), its participation in the regulation of polyspermy, hampering the sperm entry into the oocyte has also been shown in rats (19), as well as a lack of effect in pig and cattle (22, 23). The main objective of the present study was to clarify this controversy.

We hypothesize that the presence of the different components of the plasminogen/plasmin system in oocytes, spermatozoa, and the oviductal environment is related to its activation during gamete interaction and its participation in the regulation of sperm entry into the oocyte. If a specific role of the plasminogen/plasmin system in fertilization was demonstrated, then the relationship between the lack of plasminogen in IVF media and implantation failures of embryos produced in vitro should be investigated. To test our hypothesis, an animal model showing high levels of polyspermy at IVF (24), such as the pig, and an animal model with polyspermy levels at IVF similar to that found in humans (25), such as the cow, were used.

MATERIALS AND METHODS

This study was developed after institutional approval from the Bioethical Committee in the University of Murcia, and it was performed in accordance with the Animal Welfare regulations of that institution.

Oviductal Fluid Collection and Assessment of Plasminogen Concentration

Genital tracts from 14- to 20-month-old heifers (Charolais, Limousine, and Simmental) and 1- to 2-year-old sows (Landracex, Large White) were obtained at the abattoir and transported to the laboratory on ice. Classification of oviducts and OF collection are described in the Supplemental Material (available online). Plasminogen in OF was assayed using S-2251 (Chromogenix), a chromogenic substrate for determination of plasmin and streptokinase-activated plasminogen (26). A detailed description of the assay is also included in the Supplemental Material.

Oocyte Collection and In Vitro Maturation

Cow cumulus–oocyte complexes (COCs) were collected by aspiration from nonatretic follicles (diameter, 2–6 mm) of ovaries of animals aged 12–18 months obtained from the slaughterhouse. For the pig model, ovaries from 6- to 7-month-old animals, weighing 80–100 kg were transported to the laboratory within 30 minutes of slaughter. Cumulus–oocyte complexes were collected from antral follicles (diameter, 3–6 mm). A detailed description of in vitro maturation procedures is included in the Supplemental Material.

Immunocytochemical Detection of Plasminogen in Bovine and Porcine Oocytes

Porcine and bovine oocytes (N = 10 per group) with and without zona pellucida (ZP) were processed for immunolabeling with an antiplasminogen polyclonal antibody (BP750, polyclonal-antibody Acris). A secondary antibody, anti-rabbit IgG produced in goat (1:400) and conjugated to TRITC (tetramethyl rhodamine isothiocyanate), was used to reveal the signal. After treatment with antibodies, samples were visualized using a TCS NT confocal microscope (Leica) equipped with helium-neon laser for excitation of TRITC. Relative quantification of fluorescence intensity (Fig. 1) was performed with the free software ImageJ (National Institutes of Health) as explained in the Supplemental Material.

Immunocytochemical Detection of t-PA and u-PA in Bovine and Porcine Oocytes

Bovine and porcine oocytes (N = 10 per group) with and without ZP were processed for immunolabeling with an anti-t-PA (AP02244SU-N, Acris) or anti-u-PA (AP02255SU-S Acris) primary polyclonal antibody. A secondary antibody, anti-goat IgG produced in rabbit, and a tertiary antibody, anti-rabbit IgG produced in goat conjugated to TRITC, were used to reveal the signal. After treatment with antibodies, samples were visualized as previously explained for plasminogen. Relative fluorescence intensity in Figures 2 and 3 was quantified as explained in the Supplemental Material.

In Vitro Fertilization

Methods for IVF are given in the Supplemental Material. Sperm concentration in cow IVF was 10^6 spermatozoa/mL, whereas sperm concentration for pig IVF was 10^5 cells/mL. Evaluation of results was performed after fixation and staining of the putative zygotes (see Supplemental Material).

Statistical Analysis

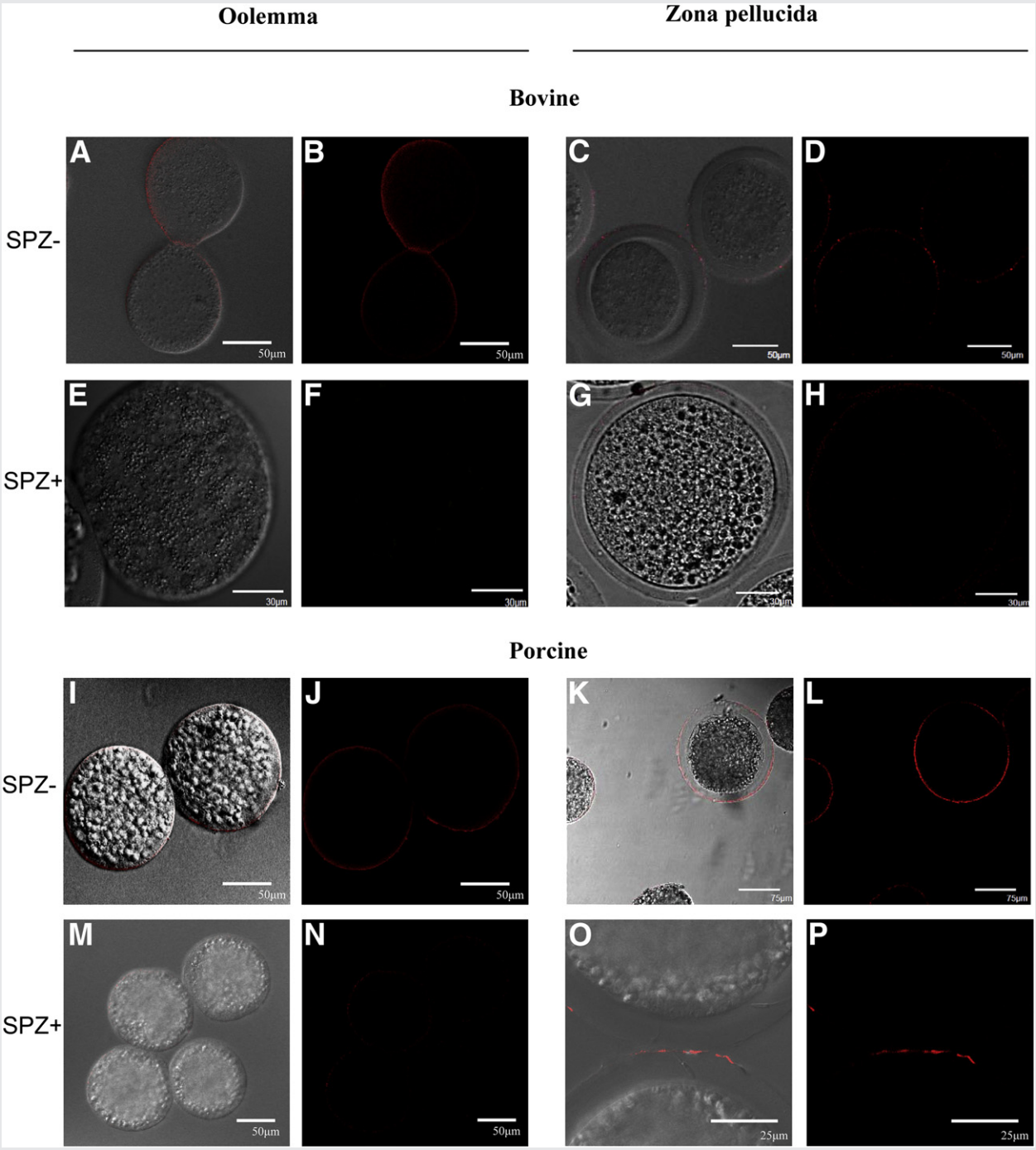
Data are presented as the mean ± SEM and all percentages were modeled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way analysis of variance (ANOVA) (percentage of oocyte penetration, number of sperm cells per penetrated oocyte, percentage of monospermy, number of sperm bound to the ZP). When ANOVA revealed a significant effect, values were compared by the Tukey test. A P value $< .05$ was taken to denote statistical significance.

RESULTS

Detection and Quantification of Plasminogen in OF

Plasminogen, and therefore plasmin activity, was detected in all the bovine and porcine samples of OF at a steady

FIGURE 1

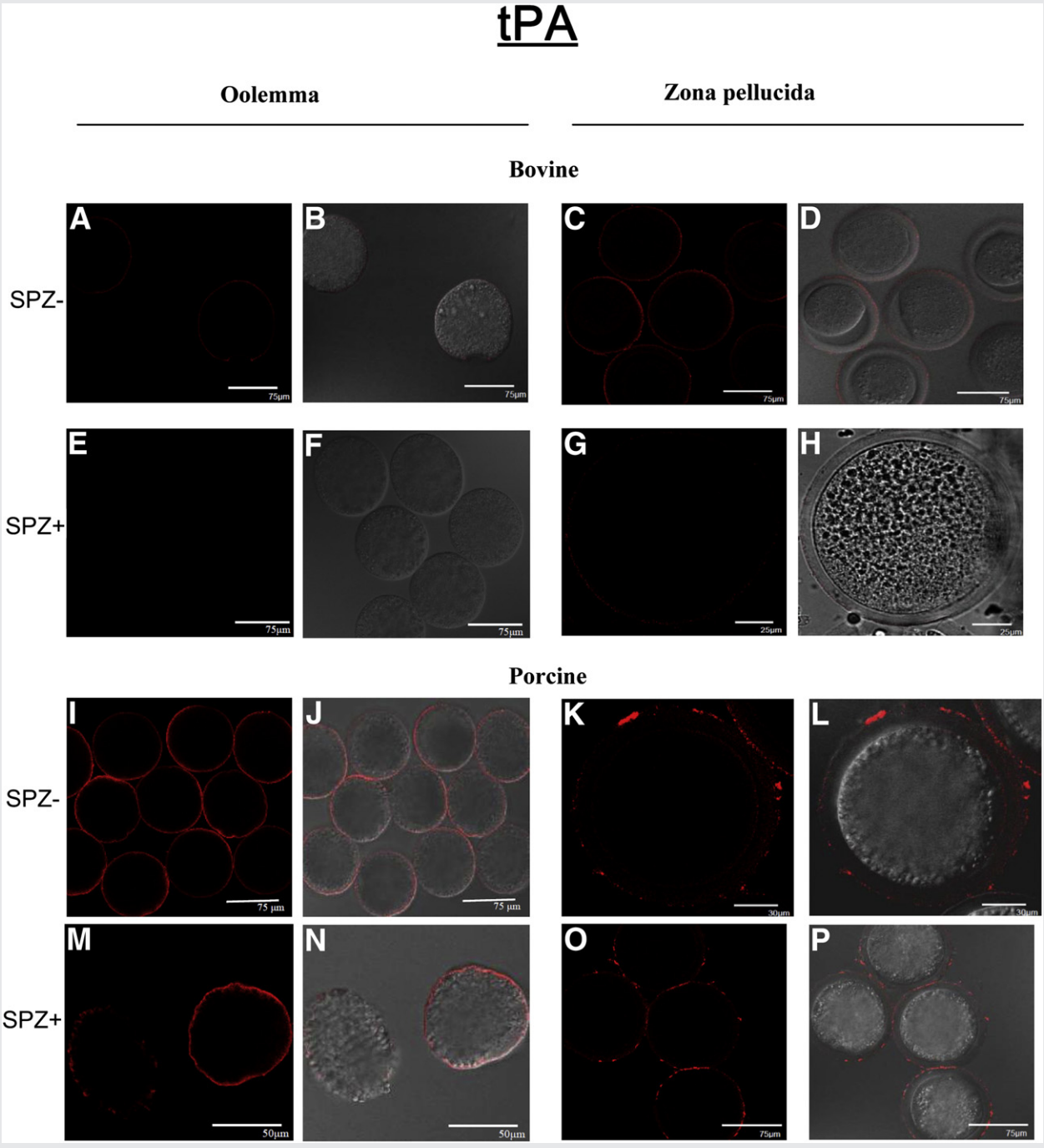


In vitro matured (SPZ−) and fertilized (SPZ+) (A, B, E, F) bovine and (I, J, M, N) porcine oocytes incubated with a primary antiplasminogen antibody and a secondary antibody conjugated to tetramethyl rhodamine isothiocyanate. Zona pelucida (ZP) was removed by pronase in some oocytes to better observe the antiplasminogen labeling at (C, D, G, H) oolemma and (K, L, O, P) ZP levels. Two pictures (transmitted and fluorescence light) are shown for each sample.

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.

concentration throughout the estrus cycle, with a mean ± SEM value of 93.15 ± 0.40 μg/mL, 92.59 ± 0.12 μg/mL, 92.78 ± 0.22 μg/mL, and 92.36 ± 0.15 μg/mL, respectively, for early follicular, late follicular, early luteal, and late luteal phase in pigs, and 92.46 ± 0.08 μg/mL, 92.75 ± 0.35 μg/mL, 92.52 ± 0.12 μg/mL, and 92.41 ± 0.17 μg/mL for the same phases in cow. For this reason, IVF experiments were conducted at concentrations near this quasi-physiological value.

FIGURE 2



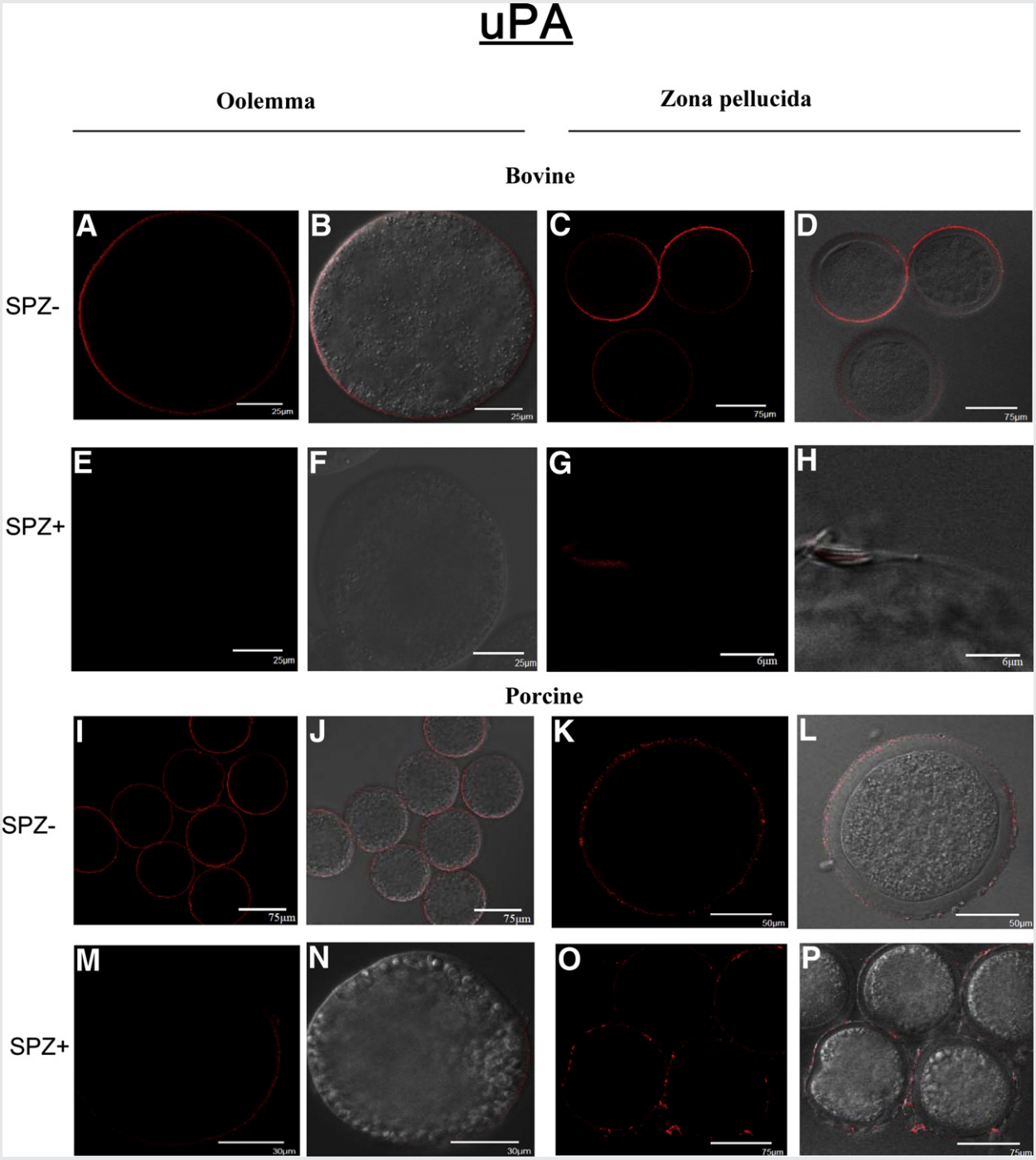
In vitro matured (SPZ−) and fertilized (SPZ+) (A-H) bovine and (I-P) porcine oocytes incubated with a primary anti-t-PA antibody, a secondary antibody anti-goat IgG produced in rabbit, and a tertiary antibody, anti-rabbit IgG produced in goat conjugated to tetramethyl rhodamine isothiocyanate. Zona pelucida (ZP) was removed by pronase in some oocytes to better observe the anti-t-PA labeling at oolemma and ZP levels. Two pictures (transmitted and fluorescence light) are shown for each sample.

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.

Immunolocalization of Plasminogen in Oocytes Before and After Fertilization

For bovine in vitro-matured oocytes, labeling with antiplasminogen and TRITC indicated the presence of plasminogen in the periphery of the ooplasm (Fig. 1A and B, with a relative fluorescence intensity [FI] of 4,827), and in the ZP (Fig. 1C and D, with a FI of 4,547). However, after fertilization, the signal was hardly visible in the oolemma (Fig. 1E and F, FI of

FIGURE 3



In vitro matured (SPZ−) and fertilized (SPZ+) (A-H) bovine and (I-P) porcine oocytes incubated with a primary anti-u-PA antibody, a secondary antibody anti-goat IgG produced in rabbit, and a tertiary antibody, anti-rabbit IgG produced in goat conjugated to tetramethyl rhodamine isothiocyanate. Zona pelucida (ZP) was removed by pronase in some oocytes to better observe the anti-u-PA labeling at oolemma and ZP levels. Two pictures (transmitted and fluorescence light) are shown for each sample.

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.

1,313), and showed lower intensity in the ZP (Fig. 1G and H, FI of 3,633).

For the porcine oocytes, immunoreactivity toward plasminogen was also detected around the oolemma (Fig. 1I and J, FI of 7,065) and it was especially strong in the ZP (Fig. 1K and L, FI of 15,652). After fertilization, the signal was less visible in the oolemma (Fig. 1M and N, FI of 4,742) and remained visible in the ZP, but only at the site of sperm binding (Fig. 1O and P, FI of 12,781).

Immunolocalization of t-PA and u-PA in Oocytes Before and After Fertilization

Both PAs, t-PA and u-PA, were immunolocalized surrounding the oolemma and ZP of in vitro matured bovine and porcine oocytes (Figs. 2 and 3). In the bovine oocytes, the immunoreactivity was clearly visible in the oolemma before IVF (relative FI of 5,341 in Fig. 2A and B) and completely disappeared after IVF (Fig. 2E and F). For the ZP, the immunoreactivity before IVF was higher (FI of 14,646 in Fig. 2C and D) than after IVF (FI of 4,075; Fig. 2G and H). Similar results were found in pig. Immunoreactivity in the oolemma was higher before IVF (FI of 10,184 in Fig. 2I and J) than after IVF (FI of 7,819 in Fig. 2M and N, oocyte at the left). Figure 2M and N shows a nonfertilized oocyte (right) and a fertilized oocyte (left), where not only the FI but also the discontinuous distribution of the labeling indicates a change in the pattern of t-PA immunolocalization. In the ZP, no differences were found before and after fertilization (Fig. 2K, 2L, 2O, and 2P), but it could be clearly observed that the pattern of fluorescence distribution was different. The areas labeled in the fertilized oocytes corresponded to sperm-ZP binding sites (Fig. 2O and P).

Regarding u-PA, the results were similar. The bovine oolemma showed a clear immunoreactivity before IVF (FI of 9,443 in Fig. 3A and B), which completely disappeared after IVF (Fig. 3E and F). The immunolabeling of the ZP was like a circle surrounding it before IVF (Fig. 3C and D), but it was restricted to the spermatozoa-ZP binding sites once fertilization took place (Fig. 3G and H). In the pig, in the oolemma immunoreactivity was higher before IVF (FI of 11,220 in Fig. 3I and J) than after IVF (FI of 6,664 in Fig. 3M and N). Again, the ZP labeling was circular-shaped before IVF (Fig. 3K and L) and adopted a pattern related to the sperm binding sites after IVF (Fig. 3O and P).

Effect of Plasminogen Added to the Culture Medium on IVF Outcome

Knowing that plasminogen was present in the OF and bound to oocytes, this experiment was designed to determine the effect of plasminogen added into the culture medium on the IVF outcome and whether the effect was dose dependent. For this purpose, bovine (N = 899) and porcine (N = 767) oocytes were in vitro fertilized in conventional IVF medium (control group) and in IVF medium supplemented with 150 μg/mL, 75 μg/mL, or 37.5 μg/mL of plasminogen. Three to four replicates were performed for each experiment.

In both species, the addition of 150 μg/mL plasminogen to the IVF medium had a significant effect on most parameters assessed (Table 1A). Overall, there was a decrease of penetration rate, average number of sperm penetrated per oocyte, and average number of spermatozoa bound to the ZP. This resulted in an increase in the percentage of monospermy.

When the dose of plasminogen added to the IVF medium was half (75 μg/mL) or 25% (37.5 μg/mL) than that used in the previous experiment (150 μg/mL), the results indicated differences between groups. The trend was the same as that observed in the previous experiment, showing lower rates of penetration and average number of sperm per oocyte in plasminogen groups than in the control group. As the plasminogen concentration increased, the penetration rate decreased (Table 1B). The average number of spermatozoa bound to the ZP also decreased by adding plasminogen and the percentage of monospermy increased. The effect was clearly dose-dependent for monospermy, sperm/oocyte, and sperm/ZP parameters in pig (Table 1B).

Effect of Plasmin (75 μg/mL) Added to the Culture Medium on IVF Outcome

This experiment was designed to determine whether the addition of 100% activated plasminogen (i.e. plasmin) to the IVF medium produced an effect on IVF outcome similar to that found for plasminogen. If so, this could be a first indication that in the previous experiments the plasminogen in the IVF medium had been activated into plasmin by PAs released from the gametes. For this purpose, bovine and porcine oocytes (N = 506) were in vitro fertilized in IVF medium without (control) or with 75 μg/mL of plasmin. Three replicates were performed with 40–50 oocytes per group.

In the bovine species, when plasmin (active form of plasminogen) was added to the fertilization medium there was a decrease in the percentage of penetration (Supplemental Table 1, available online), as observed in the previous experiments with the addition of plasminogen. However, unlike in these same experiments, no significant differences in other parameters evaluated were observed (Supplemental Table 1). In pigs, the results were similar to

TABLE 1A

Effect of plasminogen (PLG), added at concentration of 150 μg/mL, on IVF parameters in bovine and porcine species.

Treatment	No.	Penetration (%)	Monospermy (%)	Sperm/oocyte (n)	Sperm/ZP (n)
Bovine oocytes					
Control	225	94.22 ± 1.56[a]	71.70 ± 3.10[a]	1.40 ± 0.05[a]	2.14 ± 0.32[a]
PLG (150)	212	24.53 ± 3.18[b]	93.88 ± 3.46[b]	1.06 ± 0.03[b]	0.96 ± 0.27[b]
Porcine oocytes					
Control	177	94.35 ± 1.74[a]	19.16 ± 3.06[a]	3.94 ± 0.22[a]	23.58 ± 1.84[a]
PLG (150)	199	43.72 ± 3.53[b]	50.57 ± 5.39[b]	1.79 ± 0.12[b]	6.51 ± 0.82[b]

Note: ZP = zona pelucida.
[a,b] In the same column for the same species denote significant differences ($P<.05$).

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.

TABLE 1B

Effect of plasminogen (PLG), added at concentrations of 37.5 μg/mL and 75 μg/mL, on IVF parameters in bovine and porcine species.

Treatment	No.	Penetration (%)	Monospermy (%)	Sperm/oocyte (n)	Sperm/ZP (n)
Bovine oocytes					
Control	156	93.59 ± 1.97[a]	82.88 ± 3.13[a]	1.27 ± 0.05[a]	2.81 ± 0.26[a]
PLG (37.5)	154	60.39 ± 3.95[b]	91.40 ± 2.92[a,b]	1.12 ± 0.04[a,b]	1.56 ± 0.14[b]
PLG (75)	152	37.50 ± 3.94[c]	100.0[b]	1.0 ± 0.0[b]	0.99 ± 0.10[b]
Porcine oocytes					
Control	132	94.70 ± 1.96[a]	0[a]	21.54 ± 0.45[a]	84.73 ± 1.59[a]
PLG (37.5)	131	85.50 ± 3.10[a,b]	16.96 ± 3.56[a]	10.18 ± 0.53[b]	36.10 ± 1.89[b]
PLG (75)	128	73.44 ± 3.91[b]	39.36 ± 5.07[b]	2.33 ± 0.16[c]	16.85 ± 0.83[c]

Note: ZP = zona pelucida.
[a,b,c] In the same column for the same species denote significant differences ($P < .05$).

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.

those obtained with plasminogen in previous experiments, but the increase was particularly marked for monospermy, assuming a difference of 63% compared with the control group, as in this group all penetrated oocytes were polyspermic (Supplemental Table 1). This was due to higher average number of sperm binding to the ZP and penetrating each oocyte in the control group in this experiment, compared with the plasmin-treated group.

DISCUSSION

Our working hypothesis, as already mentioned, assumed that the plasminogen/plasmin system was involved in fertilization. To our knowledge, our data show and quantify for the first time the presence of plasminogen in the OF of any mammal. In addition, for the first time we localize plasminogen activators immunoreactivity to porcine or bovine oocytes. Because plasminogen is a zymogen, with no proteolytic activity, the demonstration of an effect after the addition of plasminogen to the IVF medium would suggest its conversion into plasmin by PAs present in gametes. Our results were consistent with the proposed hypothesis, as both PAs, t-PA and u-PA, were immunolocalized at ZP and oolemma levels and their signal decreased after fertilization. Furthermore, addition of plasminogen reduced the number of spermatozoa binding to the ZP and entering the oocyte, thus increasing the percentage of monospermy. Depending on the experiment, this reduction in penetration reached up to 70% of oocytes (bovine species) and 50% of oocytes (porcine species).

The first deduction to be gained from the obtained data suggests that the plasminogen present in OF and absent from IVF media may have a role in the control of polyspermy, or at least in the regulation of sperm penetration of oocytes under physiological conditions, which until the present had not been taken into account in IVF systems. The OF is partially composed of plasma transudate and plasminogen presence in this fluid, coming from plasma, was not an unexpected result. Concentration of plasminogen in human plasma is 120.2 μg/mL, varying from 77.0–168.0 μg/mL (27). Therefore our data for porcine and bovine OF are within this range. The absence of significant fluctuations through the estrous cycle indicates that the plasminogen concentration of OF is out of the control of gonadotropins. This is logical, considering that plasminogen is mainly synthesized by the liver, although other tissues synthesize plasminogen to a lesser extent (28). Our hypothesis is that the plasminogen level in OF remains constant and outside the control of gonadotropins, whereas proteolitic activity of the system through the estrous cycle would be controlled by changes in PA and PAI activity. In fact, there have been reported changes in the synthesis or activity of PAs and/or PAIs during the estrous cycle in pig (29) and hamster (17).

Plasminogen receptors are abundant on cell surfaces (37,000 sites/platelet, $>10^7$ sites/endothelial cell) (30) with lysine, lysine analogues, and peptides with carboxyl-terminal lysines residues exposed, and oocytes should not be an exception. Huarte et al. (21) first suggested that gametes and cumulus cells from mouse can bind plasminogen on their cell surface. They used ^{125}I-plasminogen, and cell-associated radioactivity in ligand-binding assays was determined in a gamma counter. Our results not only show that porcine and bovine oocytes bind plasminogen, but that the specific localization is restricted to the oolemma and ZP. In addition, the decrease in plasminogen and PAs immunoreactivity of the oocytes after fertilization, together with the results about IVF outcome, strongly suggest plasminogen activation into plasmin.

Colocalization of PAs and plasminogen on cell surfaces serves to increase the local concentrations of reactants and to enhance plasminogen activation (4), and the majority of the cells that bind plasminogen also express cell-surface receptors for the PAs (t-PA, u-PA) (31) supporting the possibility of an activation of plasminogen by the gametes. Results from the present study show that both PAs, t-PA and u-PA, are immunolocalized to the oolemma and ZP of bovine and porcine matured oocytes. After fertilization, the presence of the labeling decreases or disappears. These data strongly suggest that plasminogen is activated on the oocyte oolemma and ZP during interaction with spermatozoa and converts into plasmin. This hypothesis is coherent with the wide localization of the plasminogen/plasmin system in different protein matrices and tissues and its participation in a number of biological events (4). In the mouse, Huarte et al. (32) demonstrated high levels of t-PA mRNA in primary oocytes by Northern blot, with the signal disappearing in fertilized oocytes. Various studies demonstrate the expression of t-PA mRNA in cumulus cells (33, 34). Independently of the t-PA origin (either synthesized by the oocyte or by the cumulus

cells), it seems clear in the present studies that the interaction with the sperm is responsible for the decrease in the signal, which is related to plasminogen activation. As indicated, most cell receptors for t-PA bind plasminogen, with those favoring the plasmin formation focused on the cell surface, and this could also be the case in the oocyte. Supporting this idea, it is known that integrins $\alpha_m\beta_2$ and $\alpha_5\beta_1$ are plasminogen receptors that interact with Glu-plasminogen deposited in the extracellular matrix, and play a role in cellular adhesion in blood neutrophils, U937 monocytoid cells, and human embryonic kidney cells (35). Because integrin $\alpha_6\beta_1$ is present in the oocyte membrane and participates in human and mouse gamete fusion (36), the parallels between both models should be considered.

The u-PA is synthesized by cumulus cells (37) and its activity has also been detected in in vitro matured bovine oocytes (38). Because u-PA receptors can bind integrins (39) and it has been suggested that oolemma-anchored integrins are receptors for sperm ADAM proteins, it would plausible that sperm binding to oolemma mediated by integrins triggers u-PA binding to its receptor, finally activating plasminogen. This last possibility should be further investigated.

Previous studies have shown that plasminogen in the IVF medium increases penetration rate in mouse (21), decreases penetration rate in rat (19), has no effect on penetration rate in cow (23) or pig (22), or even induces a slight increase in penetration rate in the pig with doses 10 times lower than those used in the present study (40). Our results, tested in two species, either with the zymogen (plasminogen) or the active enzyme (plasmin), at concentrations similar to those found in the oviduct, consistently produced a reduction in sperm penetration. We therefore agree with the proposal by Zhang et al. (19) that t-PA is released during oocyte activation and converts plasminogen into plasmin, which, in rats, would bring about the proteolysis of ZP and the subsequent hardening after fertilization. In the pig or cow, as demonstrated previously, the effect of the released plasmin should be different as ZP hardening is a prefertilization event (24).

Supporting the suggestions of Zhang et al. (19), Rekkas et al. (20) identified t-PA by aspirating peri-oolemma content from bovine oocytes and proposed, like Zhang et al. (19), that the activator is present in the cortical granules. However, as suggested by Hoodbhoy and Talbot (41), the detection of t-PA in the peri-oolemma content does not necessarily mean that this activator comes from cortical granules. The possibility that t-PA was secreted by the oocyte, independently of cortical granule exocytosis, in a manner similar to that of calreticulin (42), should be tested.

As already mentioned, the activators responsible for plasmin generation in the present experiments must come from the gametes because no OF was added to the IVF medium. Cannon and Menino (43) suggested that spermatozoa could activate plasminogen of oviductal origin, which is bound to their cellular surface by sperm u-PA anchored to the u-PA-receptor, resulting in localized proteolysis (plasmin mediated) of ZP during sperm penetration.

Finally, our results also suggest that differences in polyspermy levels between bovine and porcine species could be related to the differences in the release of plasminogen and PAs from the ZP and oolemma. As it was observed in the immunolabeling of oocytes with antiplasminogen, anti-t-PA and anti-u-PA antibodies, porcine oocytes seemed to release plasminogen and PAs after IVF in a lower proportion than bovine oocytes. Consequently, if the activation of plasminogen in the oocytes is a mechanism contributing to the reduction in polyspermy during fertilization, these differences could partially explain the different polyspermy levels between bovine and porcine oocytes (24).

In conclusion, plasminogen binds to mature oocytes and, after gamete interaction, is partially released from the oolemma and ZP. Plasminogen activators are present at the ZP and oolemma level in both bovine and porcine matured oocytes and their immunolocalization decreases after IVF. The addition of plasminogen to the IVF medium reduces sperm penetration and polyspermy levels. Because addition of plasmin produces a similar effect to plasminogen, it is proposed that PAs are released from gametes during their interaction and the newly generated plasmin in the medium contributes to the reduction in the number of sperm penetrating the oocyte. This role of the plasminogen/plasmin system during fertilization should be considered to determine the specific concentrations necessary to reach a regulation of polyspermy in in vitro systems close to that observed during physiological fertilization in mammals.

Acknowledgments: The authors thank Dave Ginsberg, Vice President of Operations from Molecular Innovations, for his technical support with the use of plasminogen/plasmin reagents. We also thank Ruth Tunn for her help with the English spelling and Fara Saez for the image analysis. The authors also thank Juan A. Carvajal, Dario Abril, and Soledad Rodríguez for their generous technical assistance; Dalland SA farm and Breeders Association of "Asturiana de los Valles" for providing the boar and bull semen samples, respectively; and the staff of the slaughterhouse El Pozo and Orihuela SA for supplying the ovaries and oviducts.

REFERENCES

1. Collen D, Lijnen H. Molecular basis of fibrinolysis, as relevant for thrombolytic therapy. Thromb Haemost 1995;74:167–71.
2. Andreasen P, Egelund R, Petersen H. The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 2000;57:25–40.
3. Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. Cell Mol Life Sci 2011;68:785–801.
4. Castellino FJ, Ploplis VA. Structure and function of the plasminogen/plasmin system. Thromb Haemost 2005;93:647–54.
5. Smokovitis A, Kokolis N, Taitzoglou I, Rekkas C. Plasminogen activator: the identification of an additional proteinase at the outer acrosomal membrane of human and boar spermatozoa. Int J Fertil 1992;37:308–14.
6. Smokovitis A, Kokolis N, Alexopoulos C, Alexaki E, Eleftheriou E. Plasminogen activator activity, plasminogen activator inhibition and plasmin inhibition in spermatozoa and seminal plasma of man and various animal species, Effect of plasmin on sperm motility. Fibrinolysis 1987;1:253–7.
7. Beers WH. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 1975;6:379–86.
8. Bruse C, Bergqvist A, Carlström K, Fianu-Jonasson A, Lecander I, Astedt B. Fibrinolytic factors in endometriotic tissue, endometrium, peritoneal fluid, and plasma from women with endometriosis and in endometrium and peritoneal fluid from healthy women. Fertil Steril 1998;70:821–6.

9. Kouba AJ, Alvarez IM, Buhi WC. Identification and localization of plasminogen activator inhibitor-1 within the porcine oviduct. Biol Reprod 2000;62:501–10.
10. Gabler C, Killian GJ, Einspanier R. Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. Reproduction 2001;122:121–30.
11. Hong C, Chiang B, Huang J, Wu P. Two plasminogen activators, streptokinase and urokinase, stimulate human sperm motility. Andrologia 1985;17:317–20.
12. Liu Y. Plasminogen activator/plasminogen activator inhibitors in ovarian physiology. Front Biosci 2004;9:3356–73.
13. Menino AJ, Hogan A, Schultz G, Novak S, Dixon W, Foxcroft G. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. Dev Genet 1997;21:68–74.
14. Ebisch I, Thomas C, Wetzels A, Willemsen W, Sweep F, Steegers-Theunissen R. Review of the role of the plasminogen activator system and vascular endothelial growth factor in subfertility. Fertil Steril 2008;90:2340–50.
15. Ebisch I, Steegers-Theunissen R, Sweep F, Zielhuis G, Geurts-Moespot A, Thomas C. Possible role of the plasminogen activation system in human subfertility. Fertil Steril 2007;87:619–26.
16. Huarte J, Belin D, Vassalli J. Plasminogen activator in mouse and rat oocytes: induction during meiotic maturation. Cell 1985;43:551–8.
17. Jimenez Diaz M, Giunta S, Valz-Gianinet J, Pereyra-Alfonso S, Flores V, Miceli D. Proteases with plasminogen activator activity in hamster oviduct. Mol Reprod Dev 2000;55:47–54.
18. Park K, Choi S, Song X, Funahashi H, Niwa K. Production of plasminogen activators (PAs) in bovine cumulus-oocyte complexes during maturation in vitro: effects of epidermal growth factor on production of PAs in oocytes and cumulus cells. Biol Reprod 1999;61:298–304.
19. Zhang X, Rutledge J, Khamsi F, Armstrong D. Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction. Mol Reprod Dev 1992;32:28–32.
20. Rekkas CA, Besenfelder U, Havlicek V, Vainas E, Brem G. Plasminogen activator activity in cortical granules of bovine oocytes during in vitro maturation. Theriogenology 2002;57:1897–905.
21. Huarte J, Vassalli JD, Belin D, Sakkas D. Involvement of the plasminogen activator/plasmin proteolytic cascade in fertilization. Dev Biol 1993;157:539–46.
22. Kim TS, Sa SJ, Shin MY, Jang DM, Kwon SH, Kwon EH, et al. Stimulation of plasminogen activator activity by free radicals in boar spermatozoa. Anim Reprod Sci 2009;114:228–37.
23. Papanikolaou T, Amiridis G, Dimitriadis I, Vainas E, Rekkas C. Effect of plasmin, plasminogen activators and a plasmin inhibitor on bovine in vitro embryo production. Reprod Fertil Dev 2008;20:320–7.
24. Coy P, Cánovas S, Mondéjar I, Saavedra M, Romar R, Grullón L, et al. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. Proc Natl Acad Sci U S A 2008;105:15809–14.
25. Coy P, Avilés M. What controls polyspermy in mammals, the oviduct or the oocyte? Biol Rev Camb Philos Soc 2010;85:593–605.
26. Friberger P, Knös M, Gustavsson S, Aurell L, Claeson G. Methods for determination of plasmin, antiplasmin and plasminogen by means of substrate S-2251. Haemostasis 1978;7:138–45.
27. Leipnitz G, Miyashita C, Heiden M, von Blohn G, Kohler M, Wenzel E. Reference values and variability of plasminogen in healthy blood donors and its relation to parameters of the fibrinolytic system. Haemostasis 1988;18(Suppl 1):61–8.
28. Zhang L, Seiffert D, Fowler BJ, Jenkins GR, Thinnes TC, Loskutoff DJ, et al. Plasminogen has a broad extrahepatic distribution. Thromb Haemost 2002;87:493–501.
29. Tsantarliotou M, Zervos I, Vatzias G, Billinis C, Taitzoglou I, Kokolis N. Plasminogen activator activity in the porcine oviduct during the oestrous cycle. Theriogenology 2005;64:1007–15.
30. Miles LA, Dahlberg CM, Plow EF. The cell-binding domains of plasminogen and their function in plasma. J Biol Chem 1988;263:11928–34.
31. Pluskota E, Soloviev DA, Bdeir K, Cines DB, Plow EF. Integrin alphaMbeta2 orchestrates and accelerates plasminogen activation and fibrinolysis by neutrophils. J Biol Chem 2004;279:18063–72.
32. Huarte J, Belin D, Vassalli A, Strickland S, Vassalli JD. Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. Genes Dev 1987;1:1201–11.
33. Ny T, Liu YX, Ohlsson M, Jones PB, Hsueh AJ. Regulation of tissue-type plasminogen activator activity and messenger RNA levels by gonadotropin-releasing hormone in cultured rat granulosa cells and cumulus-oocyte complexes. J Biol Chem 1987;262:11790–3.
34. O'Connell M, Canipari R, Strickland S. Hormonal regulation of tissue plasminogen activator secretion and mRNA levels in rat granulosa cells. J Biol Chem 1987;262:2339–44.
35. Lishko VK, Novokhatny VV, Yakubenko VP, Skomorovska-Prokvolit HV, Ugarova TP. Characterization of plasminogen as an adhesive ligand for integrins alphaMbeta2 (Mac-1) and alpha5beta1 (VLA-5). Blood 2004;104:719–26.
36. Ziyyat A, Rubinstein E, Monier-Gavelle F, Barraud V, Kulski O, Prenant M, et al. CD9 controls the formation of clusters that contain tetraspanins and the integrin alpha 6 beta 1, which are involved in human and mouse gamete fusion. J Cell Sci 2006;119:416–24.
37. D'Alessandris C, Canipari R, Di Giacomo M, Epifano O, Camaioni A, Siracusa G, et al. Control of mouse cumulus cell-oocyte complex integrity before and after ovulation: plasminogen activator synthesis and matrix degradation. Endocrinology 2001;142:3033–40.
38. Park KW, Choi SH, Song XX, Funahashi H, Niwa K. Production of plasminogen activators (PAs) in bovine cumulus-oocyte complexes during maturation in vitro: effects of epidermal growth factor on production of PAs in oocytes and cumulus cells. Biol Reprod 1999;61:298–304.
39. Irigoyen J, Muñoz-Cánoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: biology and regulation. Cell Mol Life Sci 1999;56:104–32.
40. Sa S, Rhee H, Cheong H, Yang B, Park C. Effects of plasmin on sperm-oocyte interactions during in vitro fertilization in the pig. Anim Reprod Sci 2006;95:273–82.
41. Hoodbhoy T, Talbot P. Mammalian cortical granules: contents, fate, and function. Mol Reprod Dev 1994;39:439–48.
42. Tutuncu L, Stein P, Ord T, Jorgez C, Williams C. Calreticulin on the mouse egg surface mediates transmembrane signaling linked to cell cycle resumption. Dev Biol 2004;270:246–60.
43. Cannon MJ, Menino AR Jr. Changes in the bovine zona pellucida induced by plasmin or embryonic plasminogen activator. Mol Reprod Dev 1998;51:330–8.

SUPPLEMENTAL MATERIALS AND METHODS

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma–Aldrich Química SA.

Oviductal Fluid Collection and Assessment of Plasminogen Concentration

Genital tracts from 14 20-month-old heifers (Charolais, Limousine, and Simmental) and 1- to 2-year-old sows (Landracex, Large White) were obtained at the abattoir and transported to the laboratory on ice. Once in the laboratory, the stage of the estrous cycle (early follicular, late follicular, early luteal, and late luteal) was assessed on the basis of ovary morphology (1, 2). Once classified, 27 porcine and 25 bovine oviducts were separated from the tracts and quickly washed once with 0.4% vol/vol cetrimide solution, twice in saline, and transferred to Petri dishes on ice and dissected. Once dissected, the oviductal fluid (OF) from the whole oviduct was collected by aspiration with an automatic pipette, as previously described (1, 2). The OF was centrifuged (7,000 × *g*, 10 minutes, 4°C) to remove cellular debris and the supernatant immediately stored at −80°C until use. Plasminogen was assayed using S-2251 (Chromogenix), a chromogenic substrate for determination of plasmin and streptokinase-activated plasminogen (3). For the assay, OF samples were thawed and diluted 1:40 (vol/vol) in Tris-NaCl buffer (pH 7.4). Following manufacturer's instructions, 20 μL of diluted samples were incubated (37°C, 10 minutes) with 10 μL (5 U) of streptokinase. Finally, 70 μL of chromogenic substrate diluted in Tris-NaCl buffer (final concentration 3 mM) were added and absorbance immediately recorded at 405 nm on a spectrophotometer (FLUOstar Galaxy, BMG Lab Technologies) for 180 minutes. A blank in each sample was run without adding streptokinase (20 μL diluted OF + 80 μL substrate buffer). Positive control was prepared with 3.75 U of plasmin (20 μL plasmin + 80 μL substrate buffer), negative control with 100 μg/mL plasminogen (BPLG, Molecular Innovations; 20 μL plasminogen + 80 μL substrate buffer), and calibration curve with standard solutions of plasminogen from 0–250 μg/mL (20 μL solution + 10 μL streptokinase + 70 μL substrate buffer). Samples, blanks, controls, and standards were run concurrently and in duplicate. Three OF samples for each phase and species were analyzed. A calibration curve was produced by plotting the absorbance of the standards versus their concentration, and the plasminogen concentration of the OF calculated.

Oocyte Collection and In Vitro Maturation

Cow. The medium used for in vitro maturation of cow oocytes was TCM-199, supplemented in our laboratory, as described previously (4). Cow cumulus–oocyte complexes (COCs) were collected by aspiration from nonatretic follicles (diameter, 2–6 mm) of ovaries of 12- to 18-month-old animals obtained from the slaughterhouse. The COCs were then washed twice in TCM-199 with Hank's salts, 10.0 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2% fetal bovine serum, 2.0 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, and once in maturation medium previously equilibrated for 5 hours at 38.5°C under 5% CO_2 in air. Groups of 45–55 COCs were cultured in 500 μL maturation medium for 24 hours at 38.5°C under 5% CO_2 in air.

Pig. The medium used for in vitro maturation of pig oocytes was NCSU-37, prepared in our laboratory, as described previously (4). Within 30 minutes of slaughter, ovaries from 6- to 7-month-old animals weighing 80–100 kg were transported to the laboratory in saline containing 100 μg/mL kanamycin sulfate at 38°C, washed once in 0.04% cetrimide solution and twice in saline. The COCs were collected from antral follicles (diameter, 3–6 mm), washed twice with Dulbecco's phosphate-buffered saline (PBS) supplemented with 1 mg/mL polyvinyl alcohol and 0.005 mg/mL red phenol, and twice more in maturation medium previously equilibrated for a minimum of 3 hours at 38.5°C under 5% CO_2 in air. Only COCs with complete and dense cumulus oophorus were used for the experiments. Groups of 45–55 COCs were cultured in 500 μL maturation medium for 22 hours at 38.5°C under 5% CO_2 in air. After culture, COCs were washed twice in fresh maturation medium without dibutyryl cyclic adenosine 3′:5′ monophosphate (cAMP), equine chorionic gonadotropin (or PMSG), and hCG and cultured for an additional 20–22 hours (5).

Immunocytochemical Detection of Plasminogen in Bovine and Porcine Oocytes

Ca^{2+} and Mg^{2+} free PBS was used for this procedure. After in vitro maturation or IVF, the COCs or putative zygotes (N = 10 per group) were mechanically denuded in PBS. For oolemma observation, the zona pelucida (ZP) was removed using a rapid wash in 3% pronase in PBS. After two washes to remove traces of pronase, the oocytes were fixed in paraformaldehyde 2% (wt/vol) in PBS for 30 minutes at room temperature, washed twice for 15 minutes in PBS supplemented with 1% glycine, and an additional three times in PBS. After permeabilization for 10 minutes with Triton X-100 at 0.1% (vol/vol) in PBS they were again washed twice and incubated for 1 hour in a humidified chamber protected from light in droplets containing the primary antiplasminogen antibody (BP750, polyclonal-antibody Acris) diluted 1:100 (vol/vol) in PBS supplemented with 1% bovine serum albumin (BSA). After primary antibody incubation, the oocytes were washed three times in PBS and incubated again for 1 hour with secondary antibody, anti-rabbit IgG produced in goat (1:50) and conjugated to tetramethyl rhodamine isothiocyanate (TRITC). Controls were performed with anti-rabbit IgG conjugated to TRITC to discard its nonspecific binding to in vitro matured oocytes and in vitro fertilized oocytes.

This was the standard protocol followed in our study (ZP with pronase digestion and permeabilization with Triton X-100), although preliminary tests were carried out in some oocytes with ZP digested in acid Tyrode's solution and with nonpermeabilized oocytes. No differences were found compared to our standard protocol.

After treatment with antibodies, oocytes were washed in PBS and placed on a slide with mounting medium (Slow Fade Antifade Kit; Invitrogen). The samples were stored at 4°C in the dark and assessed within a maximum of 2 weeks. All samples were visualized in a TCS NT confocal microscope (Leica)

equipped with a helium-neon laser for excitation of rhodamine (TRITC). The images were obtained using an objective PL APO Leica UV ×40 1.32NA and recorded digitally at the equatorial region of the oocyte.

Controls for specific reactivity of antibodies were carried out by incubating oocytes only with the secondary anti-rabbit IgG-TRITC antibody. The evaluation showed that in the absence of primary antibody, this antibody was not bound to bovine or porcine oocytes, either fertilized or not.

Immunocytochemical Detection of t-PA and u-PA in Bovine and Porcine Oocytes

Protocols were the same described for immunocytochemical detection of plasminogen but the primary antibodies consisted of anti-t-PA (AP02244SU-N, Acris) or anti-u-PA (AP02255SU-S Acris) diluted 1:100 (vol/vol) in PBS supplemented with 1% BSA. After primary antibody incubation, the oocytes were washed three times in PBS and incubated again for 1 hour with a secondary antibody anti-goat IgG produced in rabbit (1:400). Thereafter, the oocytes were washed again three times and incubated with a tertiary antibody anti-rabbit IgG produced in goat (1:50) conjugated to TRITC. Samples were processed and visualized as described for plasminogen using a TCS NT confocal microscope (Leica).

Controls for antibody specificity were carried out by incubating oocytes with the secondary and tertiary antibodies only as well as with secondary and tertiary antibodies together. This evaluation showed that in the absence of primary antibody, these antibodies were not bound to bovine or porcine oocytes either fertilized or not.

Image Processing and Analysis

Confocal images were treated with the deconvolution software Huygens (SVI), and a quantification of fluorescence in Figures 1–3 (see original article) was carried out using the freeware ImageJ (National Institutes of Health). We worked on the stacks of images using a binary mask that covered the surface marked. The binary mask always covered the same range of pixel gray level for all treatments and time (from 1–255 gray level). The evaluation of each of these binary masks returns a value of mean gray level, which gives us a parameter to assess the intensity of fluorescence.

In Vitro Fertilization

Cow. The IVF medium for the bovine species consisted of IVF-TALP as previously described by Parrish et al. (6). At the end of the maturation period, 45–55 COCs were transferred to wells containing 500 μL of IVF-TALP medium. A volume of 25 μL penicillamine-epinephrine-hypotaurine (6) was added to each well 30 minutes before insemination. Spermatozoa were obtained by centrifugation of two 0.5-mL straws of frozen-thawed semen on a 45:90 discontinuous Percoll (Pharmacia) gradient for 10 minutes at 900 × *g*. The pellet was resuspended in 10 mL sperm-TALP medium (6) and washed again for 8 minutes at 300 × *g*. The final pellet was resuspended in 500 μL of IVF-TALP, sperm concentration adjusted, and cells added at a final concentration of 10^6 spermatozoa/mL to the IVF-TALP wells containing the COCs.

Pig. Medium used for pig IVF was essentially the same as that used by Rath et al. (7). The COCs cultured for a total of 44 hours in maturation medium were washed three times with TALP medium and groups of 45–55 oocytes were transferred into each well of a four-well multidish containing 250 μL of IVF medium previously equilibrated at 38.5°C under 5% CO_2. A sperm-rich fraction of semen from a mature, fertility-tested boar was collected by the gloved hand method and immediately transported to the laboratory diluted at 1:8 in Beltsville thawing solution (8). Aliquots of the semen samples (0.5 mL) were centrifuged (700 × *g*, 30 minutes) through a discontinuous Percoll (Pharmacia) gradient (45% and 90% vol/vol) and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 minutes at 100 × *g*. Finally, the pellet was diluted in TALP and 250 μL of this suspension were added to the wells containing the oocytes, giving a final concentration of 10^5 cells/mL.

Hoechst Staining

At 18–20 hours after insemination putative zygotes were washed in PBS to remove excess attached sperm, fixed for 30 minutes (0.5% glutaraldehyde in PBS), stained for 15 minutes (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/mL polyvinylpyrrolidone, and mounted on glass slides. Oocytes were examined under an epifluorescence microscope at ×200 and ×400 magnification. Penetration, number of spermatozoa per oocyte, and number of sperm bound to the ZP (in penetrated oocytes) were assessed in each oocyte.

REFERENCES

1. Carrasco LC, Romar R, Aviles M, Gadea J, Coy P. Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. Reproduction 2008;136:833–42.
2. Carrasco LC, Coy P, Aviles M, Gadea J, Romar R. Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle. Reprod Fert Dev 2008;20:808–17.
3. Friberger P, Knös M, Gustavsson S, Aurell L, Claeson G. Methods for determination of plasmin, antiplasmin and plasminogen by means of substrate S-2251. Haemostasis 1978;7:138–45.
4. Coy P, Grullon L, Canovas S, Romar R, Matas C, Aviles M. Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. Reproduction 2008;135:19–27.
5. Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod 1997;57:49–53.
6. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986;25:591–600.
7. Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL, Johnson LA. In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci 1999;77:3346–52.
8. Pursel V, Johnson L. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. J Anim Sci 1975;40:99–102.

SUPPLEMENTAL TABLE 1

Effect of plasmin (75 μg/mL) on IVF parameters in bovine and porcine species.

Treatment	No.	Penetration (%)	Monospermy (%)	Sperm/oocyte (n)	Sperm/ZP (n)
Bovine oocytes					
Control	136	94.85 ± 1.90[a]	84.5 ± 3.19	1.24 ± 0.05	2.57 ± 0.26
Plasmin	137	72.99 ± 3.8[b]	87.0 ± 3.38	1.15 ± 0.04	2.08 ± 0.17
Porcine oocytes					
Control	117	94.02 ± 2.20[a]	0[a]	21.11 ± 0.48[a]	86.03 ± 1.67[a]
Plasmin	116	61.21 ± 4.54[b]	63.38 ± 5.75[b]	1.61 ± 0.11[b]	5.19 ± 0.34[b]

Note: ZP = zona pelucida.
[a,b] In the same column for the same species denote significant differences ($P < .05$).

Mondéjar. Role of plasminogen during fertilization. Fertil Steril 2012.