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Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow

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Abstract

One of the proposed mechanisms of polyspermy block is an increased resistance of the zona pellucida (ZP) to proteolytic digestion (ZP hardening) as a consequence of cortical granule exocytosis that occurs soon after fertilization. However, evidence is available that the zonae pellucidae of freshly ovulated pig and cow oocytes harden considerably before fertilization. It was thought that such pre-fertilization ZP hardening could be involved in the control of polyspermy, and its lack in the oocytes matured *in vitro* could be one of the reasons for the extremely high incidence of polyspermy in pig *in vitro* fertilization (IVF). To test this hypothesis, two different types of cross-linking reagents were employed and their effects on ZP hardening and IVF efficiency were examined. The sulfhydryl-reactive cross-linkers produced a slight hardening of ZP ($P<0.001$) of treated oocytes compared with control oocytes, and totally inhibited sperm penetration into pig oocytes after IVF. In the cow, sperm penetration into eggs was reduced to 10%. It is proposed that formation of disulfide bonds in ZP or blocking of SH groups in the oocyte plasma membrane proteins prevents sperm penetration. An amine-reactive cross-linker was then assayed and produced strong ZP hardening, increasing the incidence of monospermy in both pig and cow oocytes after fertilization. When the cross-linker concentration was optimized, a 45% improvement for pig IVF efficiency was reached. It is proposed that the observed physiological ZP hardening is a mechanism to control polyspermy, differentially affecting various mammalian species and can be imitated by the use of amine-reactive cross-linkers during IVF.

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Introduction

Fertilization is the union of a single sperm and an egg, an event that results in a diploid zygote. Successful embryonic development is enhanced when the zygote is protected from excess sperm and invasive micro-organisms or parasites. In most animals, a mechanical barrier against sperm and microorganisms is established in the egg's extracellular matrix (ZP, zona pellucida in mammals) by action of the egg's secretory vesicles, called cortical granules (CG), which contain structural proteins and/or enzymes (Wong & Wessel 2006).

The role of ZP in polyspermy block at fertilization is not completely understood (Bleil & Wassarman 1980, Schmell *et al*. 1983, Wassarman 1994, Dean 2004). In mice the fusion between a fertilizing sperm and the oocyte results in exocytosis of CG. Their contents modify (harden) the ZP to prevent penetration of excess spermatozoa. This is called 'zona hardening' (Kurasawa *et al*. 1989, Ducibella *et al*. 1990, Vincent *et al*. 1990, 1991). It is characterized by an increased resistance of the ZP to proteolytic digestion (Austin & Braden 1956, Gwatkin 1964, Barros & Yanagimachi 1971, Inoue & Wolf 1974, Gulyas & Yuan 1985), perhaps due to masking the sites of attack to proteolytic enzymes or cross-linking the ZP to prevent unfolding of proteins (Green 1997). Supporting the hypothesis of ZP cross-linking, it has been proposed that ZP proteins remain inter- and intramolecularly bound by means of disulfide bonds (Dean 2004).

Polyspermy is a common problem of *in vitro* fertilization (IVF) in the cow (Wang *et al*. 1997, Roh *et al*. 2002, Coy *et al*. 2005), sheep (Fukui *et al*. 1988, Slavik *et al*. 2005), goat (De Smedt *et al*. 1992, Mogas *et al*. 1997), human (van der Ven *et al*. 1985, Balakier 1993, Aoki *et al*. 2005), and in particular, pig (Coy & Romar 2002, Hao *et al*. 2006). Since the reported levels of polyspermy after IVF are higher than 50% of pig oocytes and as high as 25% of cow oocytes, these two species were chosen as models for this study. Both are particularly important livestock species with well-established *in vitro* maturation (IVM) systems and unlimited oocyte availability at slaughterhouses. Although the reasons for polyspermy at IVF are not clear, they do not seem to be related to failures in the exocytosis of CG if the oocyte maturation is complete. Incomplete or delayed ZP reaction could be a major cause of polyspermy

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in the pig (Wang *et al*. 1998*a*, 1998*b*, Funahashi *et al*. 2001, Coy *et al*. 2002).

Even though ZP hardening after fertilization is usually understood as a common event in mammals (Sun 2003), previous studies from our laboratory revealed that *in vitro* matured pig and cow oocytes did not undergo ZP hardening after IVF (Coy *et al*. 2002, 2005). This prompted us to enhance the post-fertilization zona hardening at IVF. However, we were surprised to find references reporting zona hardening of pig and cow oviductal oocytes before fertilization (Broermann *et al*. 1989, Katska *et al*. 1999, Kolbe & Holtz 2005). We hypothesize that, at least in the pig and cow, ZP hardening before fertilization is a plausible physiological mechanism to reduce polyspermy.

To test our hypothesis, two different groups of cross-linking reagents were employed to induce ZP hardening in *in vitro*-matured pig and cow oocytes before IVF. The first included BM(PEO)$_2$ (1,8-bis-maleimidodiethyleneglycol; BM2) and BM(PEO)$_3$ (1,11-bis-maleimidotriethyleneglycol; BM3), which are sulfhydryl-reactive cross-linkers forming stable thioether linkages among cysteines between pH 6.5 and 7.5, as has been proposed for ZP after IVF in cows (Iwamoto *et al*. 1999). The second group of reagents included DSP (dithiobis(succinimidyl propionate) or Lomant's reagent), which forms a covalent amide bond with α-amine groups present on N-termini of proteins and ε-amines on lysine, glutamine, arginine, or asparagine residues of proteins. This cross-linker was chosen because the number of primary amines in ZP proteins is higher that of cysteines, and it was thought that a strong hardening of ZP, similar to that observed in the oviductal oocytes before fertilization, could be obtained by this reagent. As the formation of glutamine–NH_2 bonds among ZP2 and ZP3 is a mechanism to form the outer layer of the fertilization envelope in carp (Chang *et al*. 2002), this approach seemed suitable to mimic our hypothesized pre-fertilization hardening and test its effect on the control of polyspermy.

Results

The time taken for 0.5% pronase to digest the ZP from oviductal oocytes from pig and cow (13691.9±1228.1 s and 31448.0±4856.0 s respectively) was longer than for follicular ones from pig and cow (96.8±4.2 and 234.7±11.4 s respectively).

Experiment 1: sulfhydryl-reactive cross-linkers BM2 and BM3 induce a slight hardening in pig and cow ZP

When pig IVM oocytes were exposed to BM2 or BM3 cross-linkers for 30 min, the ZP digestion time increased significantly with respect to the control IVM oocytes at the higher assayed concentration and also at the intermediate one for BM3 (Table 1). This increase was around 10–40 s.

When cow IVM oocytes were exposed to BM2 or BM3 cross-linkers for 30 min, the ZP digestion time increased significantly with respect to the control IVM oocytes at the higher and intermediate assayed concentration and also at the lower one for BM3 (Table 2).

Experiment 2: treatment of oocytes with sulfhydryl-reactive cross-linkers BM2 and BM3 totally (pig) or partially (cow) inhibits sperm penetration

BM2-treated pig oocytes were not penetrated after IVF (Table 3). When cow oocytes were employed, the percentage of penetration was very low (10.2%) as was the percentage of male pronuclear formation (55.6%); it was 100% in control group. When a lower BM2 concentration was used (0.06 mg/ml), the results did not show a significant increase in the percentages of penetration or male pronucleus formation (data not shown).

Experiment 3: the amine-reactive cross-linker DSP induces a strong hardening in pig and cow ZP

DSP cross-linker produced a significant increase in ZP hardening of pig and cow oocytes at any of the assayed concentrations compared with control (Fig. 1A and B). Differences among the three DSP concentrations were also significant; a 40-fold increase in pig ZP hardening and a 22-fold increase in cow ZP hardening were obtained with the highest DSP concentration (0.60 mg/ml) compared with control.

Table 1 Effect of different concentrations of BM2 and BM3 on pig zona pellucida (ZP) digestion time (seconds).

	Cross-linker concentration		
Cross-linker	0.06 mg/ml	0.30 mg/ml	0.60 mg/ml
Pig			
BM2	92.6±10.0[a] (*N*=83)	87.4±6.5[a] (*N*=86)	98.9±6.0[b,] * (*N*=91)
BM3	79.9±8.9[a] (*N*=68)	111.9±4.9[b,]* (*N*=72)	112.5±6.0[b,] * (*N*=71)
Control=69.0±3.9 (*N*=73)			

[a, b]Different letters in the same row indicate values statistically different ($P<0.001$). *Significantly different from control group.

Table 2 Effect of different concentrations of BM2 and BM3 on cow zona pellucida (ZP) digestion time (seconds).

	Cross-linker concentration		
Cross-linker	0.06 mg/ml	0.30 mg/ml	0.60 mg/ml
Cow			
BM2	107.9 ± 7.0^{a} ($N=60$)	$142.2 \pm 8.1^{b,*}$ ($N=59$)	$169.5 \pm 10.0^{b,*}$ ($N=60$)
BM3	$217.4 \pm 10.8^{a,*}$ ($N=70$)	$203.0 \pm 7.2^{a,*}$ ($N=70$)	$224.3 \pm 10.6^{a,*}$ ($N=70$)
Control = 93.5 ± 5.1 ($N=61$)			

^{a,b}Different letters in the same row indicate statistically different values ($P<0.001$). *Significantly different from control group.

Experiment 4: treatment of oocytes with the amine-reactive cross-linker DSP permits sperm penetration but reduces polyspermy

Treatment of pig oocytes with DSP before IVF produced a significant decrease in the percentage of penetration and in the mean number of spermatozoa per oocyte, with a significant increase in the percentage of monospermy (Table 4). That resulted in a general increase in the final output of the IVF system (percentage of penetrated oocytes being monospermic, fertilization efficiency), which was around 7.3% in the control group and 26.2% in the DSP group.

Treatment of cow oocytes with DSP before IVF also produced a significant decrease in the percentage of penetration, with a significant increase in the percentage of monospermy and no change in the mean number of spermatozoa per oocyte (Table 4). However, the final output of the IVF system (fertilization efficiency) decreased, being around 49.9% in the control group and 36.9% in the DSP group.

Experiment 5: treatment of oocytes with the amine-reactive cross-linker DSP at 0.30 mg/ml induces a tenfold increase in fertilization efficiency in the pig

Final output of the pig IVF (fertilization efficiency) was optimized when a concentration of 0.30 mg/ml DSP was used, increasing from 5.1 in control group to 47.6 in DSP-treated group (Fig. 2). Percentage of penetration after treatment with 0.06 or 0.30 mg/ml DSP was similar to that in the control group (Table 5), while percentage of monospermy was increased after treatment with 0.30 or 0.60 mg/ml DSP and mean number of sperm per oocyte also decreased (Table 5).

Discussion

For many years, it has been proposed that ZP hardening is a post-fertilization event contributing to polyspermy block (Austin & Braden 1956, Gwatkin 1964, Barros & Yanagimachi 1971, Inoue & Wolf 1974, Gulyas & Yuan 1985, Kurasawa *et al.* 1989, Ducibella *et al.* 1990, Vincent *et al.* 1990, 1991, Sun *et al.* 2003, Dean 2004, Lindsay & Hedrick 2004). However, most of the previous studies have been carried out in rodents. The situation may be different in the cow and the pig.

Zona hardening is usually assessed by determining the time necessary to digest ZP with pronase or chymotrypsin. In the mouse (Gulyas & Yuan 1985, DeMeestere *et al.* 1997), human (Schiewe *et al.* 1995), and cow (Iwamoto *et al.* 1999), ZP digestion time after IVF increases a few seconds or few minutes. Data from our laboratory, as well as those from others (Broermann *et al.* 1989, Katska *et al.* 1999, Kolbe & Holtz 2005), demonstrated that ZP of naturally ovulated oviductal pig and cow oocytes is resistant to enzyme digestion; it takes hours or even days. Since ZP of pig and cow oocytes from pre-ovulatory follicles are digested by enzymes in <4 min, ZP hardening must take place in the oviduct independently from fertilization. It is clear that IVM oocytes used by Iwamoto *et al.* (1999) and our group (Coy *et al.* 2002) in cow and pig are completely different from the oviductal *in vivo*-matured oocytes in term of ZP resistance to pronase digestion. Morphological differences in ZP of *in vitro* and *in vivo* matured and fertilized oocytes have been already described (Wang *et al.* 1998*b*, Funahashi *et al.* 2001), although they were never discussed in terms of ZP hardening and polyspermy block before sperm penetration.

Table 3 Effect of incubation of oocytes in BM2 (0.60 mg/ml) on pig and cow *in vitro* fertilization (IVF) results.

Species	Treatment	*N*	% PEN	% MON	SPZ/O	% MPN
Pig	Control	81	91.6 ± 3.0^{a}	9.0 ± 2.5	6.0 ± 0.3	97.3 ± 1.4
	BM2	87	0^{b}			
Cow	Control	78	60.3 ± 5.6^{a}	66.0 ± 7.0^{a}	1.3 ± 0.1^{a}	100^{a}
	BM2	88	10.2 ± 3.2^{b}	100^{b}	1.0^{b}	55.6 ± 17.6^{b}

^{a,b}Different superscripts in the same column for the same species indicate significant differences ($P<0.001$). PEN, penetration percentage from *N*; MON, monospermy percentage calculated from penetrated oocytes; SPZ/O, mean number of sperm per penetrated oocyte; MPN, percentage of male pronuclear formation.

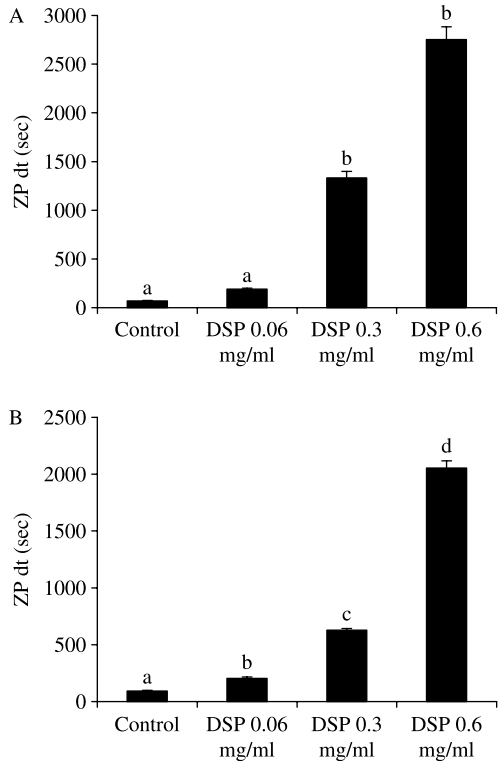

Figure 1 (A) Effect of DSP on pig zona pellucida digestion time (ZP dt). Different letters indicate significant differences ($P<0.001$). (B) Effect of DSP on cow zona pellucida digestion time (ZP dt). Different letters indicate significant differences ($P<0.001$).

Protein cross-linking agents have been used to determine near-neighbor relationships, analyze three-dimensional structures of proteins and complexes, prepare antibody–enzyme conjugates, immobilize molecules and conjugate haptens to carrier. Based on previous reports that S–S linkages among cysteines in ZP proteins could be responsible for ZP hardening in cow, mouse, or rat (Zhang *et al*. 1991, Iwamoto *et al*. 1999, Hoodbhoy & Dean 2004), we used two sulfhydryl-reactive cross-linkers (BM2 and BM3) to induce ZP hardening in IVM oocytes and to check its effectiveness in the control of polyspermy. We found a significant increase in the ZP digestion time, which, although it was far from the values observed in the oviductal oocytes, resulted in a very effective block to sperm penetration. These results would then match the theory of a post-fertilization slight hardening of ZP (from seconds to minutes) as a result of the formation of disulfide bonds (Iwamoto *et al*. 1999), making the ZP almost impenetrable for the sperm, but would not fit yet with our proposed hypothesis about the pre-fertilization hardening, as observed in oviductal oocytes, contributing to reducing polyspermy. However, this slight post-fertilization ZP hardening might not be a real mechanism for the reduction of polyspermy. Another possible explanation for our results could coincide with the recent finding suggesting that members of the protein disulfide isomerase family, present in the sperm head and catalyzing the thiol-disulfide exchange reaction, are necessary to sperm–egg fusion (Ellerman *et al*. 2006). Thereafter, the induced cross-linking of the sulfhydryl groups on the oocyte plasma membrane by BM2 and BM3 reagents in the present experiments could have avoided the sperm penetration by blocking the thiol groups from its surface. In any case, it seems clear that such mechanisms are not happening in the oviduct before fertilization (otherwise penetration would not be possible) or are responsible for the physiological ZP–sperm penetration permissible hardening observed in the oviductal oocytes.

The formation of glutamine–NH_2 bonds among ZP2 and ZP3 has been described as a mechanism to form the outer layer of fertilization envelope in carp (Chang *et al*. 2002). This approach seemed suitable to imitate our hypothesized pre-fertilization hardening as a mechanism to control polyspermy. Thereafter, the second series of experiments, with amine-reactive DSP cross-linker, was carried out and showed a completely new finding with crucial implications for IVF systems. DSP-treated oocytes acquired a moderately hard but functional ZP that continued to permit sperm penetration. This is similar to the physiological situation, where oviductal oocytes, with high-resistant ZP to pronase digestion, are penetrated most of the time by only one sperm.

By studying the ZP amino acid sequence, it seems clear that the number of bonds produced by the sulfhydryl-reactive cross-linkers must be lower than that produced by the amine-reactive cross-linker DSP because the total number of cysteine residues in the different ZP glycoproteins is lower than the number of amino acids containing primary amines. Moreover, the cysteines that could react with the BM cross-linkers are

Table 4 Effect of dithiobis(succinimidyl propionate) (DSP; 0.60 mg/ml) on pig and cow *in vitro* fertilization (IVF) results.

Species	Treatment	% PEN	% MON	SPZ/O	% MPN
Pig	Control ($N=149$)	97.9 ± 1.1^{a}	7.5 ± 2.2^{a}	12.7 ± 0.5^{a}	99.1 ± 0.7
	DSP ($N=149$)	71.1 ± 3.7^{b}	36.8 ± 4.7^{b}	2.8 ± 0.2^{b}	94.5 ± 3.0
Cow	Control ($N=74$)	91.9 ± 3.2^{a}	54.4 ± 6.1^{a}	1.68 ± 0.1	93.2 ± 3.2
	DSP ($N=64$)	57.8 ± 6.2^{b}	63.9 ± 8.1^{b}	1.58 ± 0.2	94.1 ± 3.9

^{a,b}Different superscripts in the same column within the same species indicates significant differences ($P<0.001$). PEN, penetrated oocytes from *N*; MON, monospermy percentage calculated from penetrated oocytes; SPZ/O, mean number of spermatozoa per penetrated oocyte; MPN, percentage of male pronuclear formation.

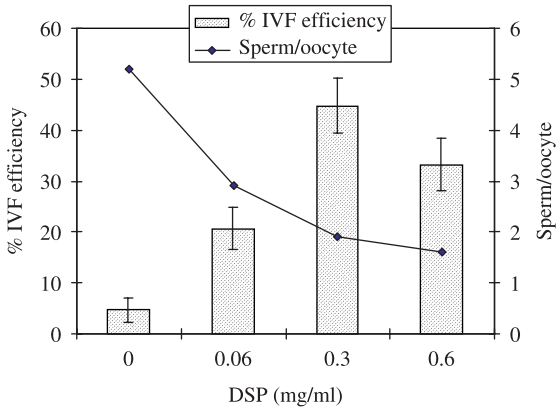

Figure 2 Final output of the IVF system (percentage of penetrated oocytes that were monospermic) after treating the oocytes with different concentrations of DSP.

approximately half of the total amount of cysteines in the ZP because these cysteines are already involved in the folding of the proteins and crosslinkages. This was previously described for mouse, rat, and human ZP proteins by means of mass spectrometry analysis (Boja *et al*. 2003, Zhao *et al*. 2004, Hoodbhoy *et al*. 2005) and, although the specific cysteines involved in cow and pig ZP disulphide bonds are not yet identified, the cysteines involved in these bonds, described previously in other species, are totally (or mainly) conserved in the bovine and porcine ones. Thus, this could explain the differences among the two types of cross-linkers in terms of ZP resistance to pronase digestion.

Regarding the differences in penetration, it seems clear that the cross-linkage produced by the amine-reactive reagent DSP (longer ZP digestion time, higher resistance to proteolytic digestion) permits more easily the passage of the spermatozoa through the ZP than the cross-linkage induced by the sulfhydryl-reactive reagents (shorter ZP digestion time, lower resistance to proteolytic digestion). Ultrastructural evidence for requisite ZP proteolysis does not exist in mammals, and mechanical penetration of an oocyte's ZP is possible by piercing through with a long narrow head or by pushing filaments aside using an oscillating motion (Wakayama *et al*. 1996, Wong & Wessel 2006). The sperm could then gain access to the egg membrane without severely altering the integrity or the mass of the ZP. Thus, higher resistance to proteolytic digestion of ZP does not have to mean a higher resistance to sperm penetration. Additionally, cross-linking proteins with NHS esters may result in a conformational change of the protein when the NHS ester cross-linker reacts with primary amines on the molecule's surface. As yet the specific residues involved in the sperm–ZP binding are not well described, and the tertiary structure of the ZP remains in controversy (Green 1997). Thus, the changes induced by DSP, even rendering the ZP more resistant to proteolytic digestion because the number of established bonds is high, could be compatible with a selective sperm penetration. In fact, the proposal of a kind of oviductal effect on the oocyte prior fertilization permitting that only a very 'strong' spermatozoon is able to penetrate oocytes has already been proposed (Okada *et al*. 1986), although related to a premature partial cortical granule exocytosis, which is different from this case. This would explain the higher levels of monospermy observed, whereas the changes induced by the sulfhydryl-reactive cross-linkers could severely mask the sperm-binding sites, or produce conformational changes in the ZP structure incompatible with the sperm penetration (Nara *et al*. 2006), or just avoid the sperm–oocyte fusion by blocking the thiol groups from the oocyte membrane as it has been explained above (Ellerman *et al*. 2006).

After treatment with DSP, the increase observed in the efficiency of the system is higher than that reported in other pig-IVF systems. The best results were obtained by Hao *et al*. (2006); using osteopontin to increase monospermy in pig they showed a difference in fertilization efficiency between control and treated groups of 13% (31.6 vs 44.6%). In our case, even using a high sperm concentration that produces a very high polyspermy level in the control group with a final fertilization efficiency of only 5.1%, the DSP-treated oocytes rendered a 47.6% of final efficiency, that is, a difference of 42.5%. Moreover, the male pronuclear formation in the system described in the present paper was always more than 95% for both control and DSP-treated groups, quite far from the levels reported by Hao *et al*. (2006), which varied from 40 to 65%.

Why this same improvement was not reached in cow oocytes is a new challenge to be investigated. Experiments to allow not only testing the effect of DSP concentrations in bovine IVF but also the effect of incubation time and other different cross-linkers in a

Table 5 Effect of different concentrations of dithiobis(succinimidyl propionate) (DSP) on pig *in vitro* fertilization (IVF) results.

Treatment	*N*	% PEN	% MON	SPZ/O	% MPN
Control	83	97.6±1.7[a]	5.2±2.5[a]	5.2±0.3[a]	97.5±1.7
DSP 0.06 mg/ml	94	91.5±2.9[a]	27.0±5[b]	2.9±0.9[b]	100±0.0
DSP 0.30 mg/ml	83	93.9±2.6[a]	50.7±5.8[c]	1.9±0.1[c]	97.4±1.8
DSP 0.60 mg/ml	78	60.3±5.6[b]	62.2±7.3[c]	1.6±0.1[c]	100±0.0

[a,b,c]Different superscripts in the same column indicates significant differences ($P<0.001$). PEN, penetrated oocytes from *N*; MON, monospermy percentage calculated from penetrated oocytes; SPZ/O, mean number of spermatozoa per penetrated oocyte; MPN, percentage of male pronuclear formation.

bovine-IVF system are currently being performed. On the other hand, it is possible that the block to polyspermy in the cow is more dependent on cortical granule exudates than in the pig, and this hypothesis is supported by the fact that polyspermy in cows after IVF is not as high as it is in pigs. As it has been proposed, selection would favor the most efficient mechanism of monospermy in each species (Wong & Wessel 2006).

What is evident from the results in our paper is that pre-fertilization ZP hardening must take a role in pig defense against polyspermy, and the physiological mechanism involved is currently under study. In marsupials, CG exocytosis does not provide a full block to polyspermy; instead, the CG-derived permanent block is supplemented by the application of a mucoid shell over the modified zona during its travel across secretory epithelium of the oviduct isthmus (Rodger & Bedford 1982, Selwood 1992). Specific experiments to investigate a similar role for pig oviduct are being carried out.

In conclusion, a relevant scientific and a technical finding can be gained from this study. The first one involves the existence of a mechanism preventing polyspermy by pre-fertilization ZP hardening, which could affect different mammal species in a different manner. The second one offers a new way to improve IVF efficiency in pig with high increases in the levels of monospermy.

Materials and Methods

Culture media

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

The medium used for pig oocyte maturation was NCSU-37 (Petters & Wells 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/ml insulin, 50 μM β-mercaptoethanol, 10 IU/ml equine chorionic gonadotropin (eCG; Foligon; Intervet International BV, Boxmeer, Holland), 10 IU/ml hCG (Veterin Corion; Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (v/v).

The basic medium used for pig IVF was essentially the same as that used by Rath *et al.* (1999). This medium, designated as TALP medium, consists of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate·$5H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 ml/l Na lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/ml BSA (A-9647), 1 mg/ml polyvinyl alcohol (PVA), and 0.17 mM kanamycin sulfate.

The IVM medium for cow oocytes was TCM-199 with Earle's salts, 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 0.2 mM sodium pyruvate, 50 mg/ml gentamicin, 10 IU/ml eCG (Foligon; Intervet International BV), and 10 IU/ml hCG (Veterin Corion Divasa Farmavic). The IVF medium for this species consisted of IVF-TALP as previously described by Parrish *et al.* (1986).

Oocyte collection and IVM

Pig

Within 30 min of slaughter, ovaries from pre-pubertal gilts 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. Cumulus–oocyte complexes (COCs) were collected from antral follicles (3–6 mm diameter), washed twice with Dulbecco's PBS supplemented with 1 mg/ml PVA and 0.005 mg/ml red phenol, and twice more in maturation medium previously equilibrated for a minimum of 3 h 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 50 COCs were cultured in 500 μl maturation medium for 22 h at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20–22 h (Funahashi *et al.* 1997).

Cow

Cow COCs were collected by aspiration from non-atretic follicles (2–6 mm in diameter) of ovaries from the slaughterhouse. COCs were then washed twice in TCM-199 with Hank's salts, 10.0 mM HEPES, 2% FBS, 2.0 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and once in maturation medium previously equilibrated for 5 h at 38.5 °C under 5% CO_2 in air. Groups of 30–40 oocytes were cultured in 500 μl maturation medium for 24 h at 38.5 °C under 5% CO_2 in air.

Treatment of pig and cow IVM oocytes with different cross-linkers

Three cross-linkers were used: $BM(PEO)_3$; (BM3; Pierce, Rockford, IL, USA); $BM(PEO)_2$, (BM2; Pierce), and di(*N*-succinimidyl) 3,3′-dithiodipropionate DSP, (DSP or Lomant's reagent; Fluka, Madrid, Spain). Cross-linkers were diluted in dimethylsulfoxide at a concentration of 25 mg/ml and stored frozen at −80 °C until used. Stock millimolar concentrations were 71 mM, 81 mM, and 62 mM respectively for BM3, BM2, and DSP.

After IVM and before IVF, pig and cow oocytes were gently stripped of cumulus cells by pipetting and incubated in IVF medium with any of the different cross-linkers at 0.06, 0.30, or 0.60 mg/ml for 30 min. A control group of oocytes incubated in IVF medium for 30 min without any cross-linker was used in all the experiments. Thereafter, the oocytes were washed three times and transferred to fresh IVF medium for insemination or just assessed for recording ZP digestion time.

In vitro *fertilization*

Pig

COCs cultured for a total of 44 h in maturation medium were washed three times with TALP medium and groups of 30–35 oocytes were transferred into each well of a 4-well

multidish containing 250 µl 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 (Pursel & Johnson 1975). Aliquots of the semen samples (0.5 ml) were centrifuged (700 ***g***, 30 min) through a discontinuous Percoll (Pharmacia) gradient (45 and 90% v/v) and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 min 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. At 4 h post-insemination (hpi), oocytes were washed twice with fresh TALP by gentle aspiration through a glass pipette and allowed to continue in culture, at 38.5 °C under 5% CO_2 till fixation. At 18–20 hpi, putative zygotes were stained with Hoechst 33342 and examined at 400× magnification for evidence of sperm penetration and pronuclear formation under an epifluorescence microscope.

Cow

At the end of the maturation period, 30–40 oocytes were transferred to wells containing 500 µl of IVF–TALP medium. A volume of 25 µl penicillamine–epinephrine–hypotaurine (PHE, Parrish *et al*. 1986) were added to each well ~30 min 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 min at 900 ***g***. The pellet was resuspended in 10 ml Sperm–TALP medium and washed again for 8 min at 300 ***g***. The final pellet was resuspended in 500 µl IVF–TALP, sperm concentration adjusted, and cells added at a final concentration of 10^6 spermatozoa/ml to the IVF–TALP wells containing the oocytes. Eighteen to twenty hours post-fertilization, putative zygotes were washed in PBS to remove excess of sperm attached, and fixed for evaluation. Fertilization efficiency was calculated as the percentage of penetrated oocytes being monospermic.

Hoechst staining

Putative zygotes were fixed for 15 min (0.5% glutaraldehyde in PBS), stained for 30 min (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 pronuclear formation were assessed in each oocyte.

Assessment of ZP solubility

The IVM oocytes from pigs or cows were transferred into PBS, washed by pipetting, and introduced into 50 µl of 0.5% (w/v) pronase solution in PBS (Coy *et al*. 2002). Zonae pellucidae 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 interval between placement of the samples in pronase solution and that when the zona was no longer visible at a magnification of 200× (Fig. 3). Pig or cow *in vivo*-matured oocytes collected from oviducts of recently ovulated females (3 and 7 oviducts respectively) or from pre-ovulatory follicles (22 from sows and 19 from cows) were also assessed for ZP digestion time with the same pronase solution used for the remaining experiments.

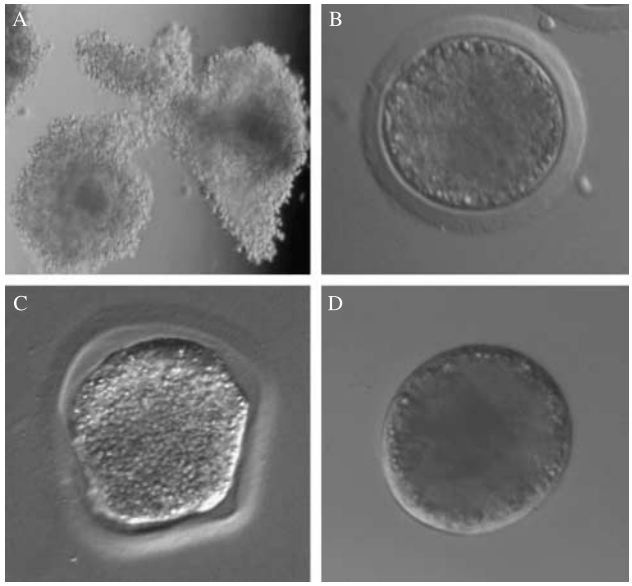

Figure 3 Digestion of ZP in pronase solution (0.5%). (A) Pig oocyte surrounded by cumulus cells. (B) Pig oocyte after denudation. (C) Beginning of ZP digestion by effect of pronase. (D) Pig oocyte where ZP is no longer visible, because it has been completely digested by pronase.

Experimental design

Experiment 1: effect of two different sulfhydryl-reactive cross-linkers (BM2 and BM3) on pig and cow ZP hardening

In order to know if any of the two selected sulfhydryl-reactive cross-linkers produced an increase in ZP digestion time, groups of 18–22 (pig) and 15–18 (cow) *in vitro* matured oocytes were incubated for 30 min with BM2 or BM3 at 0.06 (0.17 mM), 0.30 (0.85 mM), or 0.60 (1.7 mM) mg/ml. The ZP digestion time was assessed with pronase. Control IVM oocytes were also evaluated. This experiment was repeated four times.

Experiment 2: effect of BM2 on pig and cow IVF efficiency

A previous experiment with pig oocytes treated with BM3 was performed, but very poor penetration percentage was observed (data not shown) and it was decided to evaluate the effect of BM2. Oocytes previously treated with 0.60 mg/ml (1.7 mM) BM2, which produced a significant ZP hardening in experiment 1 for both species, were assayed for IVF. This experiment was performed separately with pig and cow oocytes and repeated four times for each species. Moreover, an additional experiment with BM2-treated cow oocytes was

carried out at 0.06 mg/ml to check the possibility of improving the low percentages of male pronuclear formation obtained with the former and higher 0.60 mg/ml concentration.

Experiment 3: effect of the amine-reactive cross-linker DSP on pig and cow ZP hardening

The amine-reactive cross-linker DSP was investigated to see whether it produced an increase in ZP digestion time. Groups of 18–22 (pig) and 15–18 (cow) *in vitro* matured oocytes were incubated for 30 min with DSP at 0.06 (0.15 mM), 0.30 (0.75 mM), or 0.60 (1.5 mM) mg/ml. ZP digestion time was assessed with pronase. Control IVM oocytes were also evaluated. This experiment was repeated thrice.

Experiment 4: effect of DSP on pig and cow IVF efficiency

Pig and cow oocytes previously treated with 0.60 mg/ml (1.5 mM) DSP, which resulted in positive induction of ZP hardening in experiment 3, were assayed for IVF. This experiment was performed separately with pig and cow oocytes and repeated four times for each species.

Experiment 5: effect of different concentrations of DSP on pig IVF efficiency

As results in experiment 4 demonstrated a significant increase in the final output of pig but not cow IVF, a final experiment was designed to search the optimal DSP concentration for pig IVF systems. So far, porcine IVM oocytes were incubated for 30 min with 0.06 (0.15 mM), 0.30 (0.75 mM), or 0.60 (1.5 mM) mg/ml DSP and then *in vitro* fertilized. This experiment was repeated three times.

Statistical analysis

Data are presented as the mean $\pm$ S.E.M. and all percentages were modeled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way ANOVA (ZP digestion time, percentage of oocyte penetration, number of sperm cells per penetrated oocyte, percentage of monospermy). When ANOVA revealed a significant effect, values were compared by the Tukey test. A value of $P<0.001$ was taken to denote statistical significance.

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