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What controls polyspermy in mammals, the oviduct or the oocyte?

Pilar Coy[1,*] and Manuel Avilés[2]

[1] *Department of Physiology, Faculty of Veterinary, University of Murcia, Spain*
[2] *Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Spain*

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ABSTRACT

A block to polyspermy is required for successful fertilisation and embryo survival in mammals. A higher incidence of polyspermy is observed during *in vitro* fertilisation (IVF) compared with the *in vivo* situation in several species. Two groups of mechanisms have traditionally been proposed as contributing to the block to polyspermy in mammals: oviduct-based mechanisms, avoiding a massive arrival of spermatozoa in the proximity of the oocyte, and egg-based mechanisms, including changes in the membrane and zona pellucida (ZP) in reaction to the fertilising sperm. Additionally, a mechanism has been described recently which involves modifications of the ZP in the oviduct before the oocyte interacts with spermatozoa, termed "pre-fertilisation zona pellucida hardening". This mechanism is mediated by the oviductal-specific glycoprotein (OVGP1) secreted by the oviductal epithelial cells around the time of ovulation, and is reinforced by heparin-like glycosaminoglycans (S-GAGs) present in oviductal fluid. Identification of the molecules contributing to the ZP modifications in the oviduct will improve our knowledge of the mechanisms of sperm-egg interaction and could help to increase the success of IVF systems in domestic animals and humans.

Key words: polyspermy, zona pellucida, *in vitro* fertilisation (IVF), oviduct-specific glycoprotein, glycosaminoglycans, farm mammals.

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I. INTRODUCTION

A block to polyspermy in mammals, a mechanism that ensures fertilisation by a single sperm, is required for successful fertilisation and embryo survival. Different regions in the female reproductive system, and the oviduct in particular, help the oocyte to protect itself from an excess of sperm. Upon sperm entry into the female tract, the selection process begins in the uterus; male gametes reaching the utero-tubal junction are further subjected to strict control in some mammalian species in order to pass through this barrier (Hunter, 2005; Suarez, 2007). Once in the oviduct, the epithelial cells in the caudal region of the isthmus can retain the sperm in human, sheep, cow and pig (Flechon & Hunter, 1981; Hunter, 1981; Hunter & Nichol, 1983; Hunter & Wilmut, 1984; Pacey *et al.*, 1995; Suarez, 1998) by binding; such bound sperm show decreased movement and

* Address for correspondence: (E-mail: pcoy@um.es)

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prolonged survival (Fazeli *et al.*, 2003; Suarez, 2008). This is a method of avoiding a massive, simultaneous arrival of male gametes in the proximity of the oocyte. In the temporal sequence of events it represents the first mechanism for the prevention of polyspermy (Yanagimachi, 1994); it is common to many terrestrial animals and herein is referred to as the "oviduct-based mechanism".

Upon first contact with the spermatozoon, mammalian eggs exhibit two different types of polyspermy-preventing reactions, grouped here as "egg-based mechanisms": (i) the so-called membrane block that, in non-mammalian species, is a rapid reaction involving a transient depolarisation of the egg plasma membrane potential (Gould-Somero, Jaffe & Holland, 1979), but in mammals is a slower and less well-characterised reaction probably involving Ca^{2+} signalling (Gardner & Evans, 2006), and (ii) the zona pellucida (ZP) block or "zona reaction" (vitelline envelope block in non-mammals), an extensively studied mechanism involving the exocytosis of the cortical granule (CG) contents from the cortex of the egg. The exocytosis of the CGs, a process known as the cortical reaction, is triggered by the Ca^{2+} oscillations induced by the fertilising sperm, and considered until now the principal mechanism responsible for the egg's block to polyspermy in most domestic mammals, with some exceptions such as the rabbit (Austin & Braden, 1953, 1956; Barros & Yanagimachi, 1971; Bleil & Wassarman, 1980*a*; Schmell, Gulyas & Hedrick, 1983; Wassarman, 1994; Yanagimachi, 1994; Menkhorst & Selwood, 2008). The present review will focus on the ZP block and will also describe the existence of additional ZP modifications by the oviductal secretions before the arrival of sperm. These modifications represent a pre-fertilisation mechanism contributing to the regulation of polyspermy in mammals. The identification of the molecules participating in such a mechanism could help refine assisted reproduction technologies (ARTs) by minimizing the differences between *in vitro* and *in vivo* fertilisation.

II. THE INCIDENCE OF POLYSPERMY IN MAMMALS UNDER *IN VITRO* CONDITIONS

Although the ZP block has been extensively studied in mammals, some classical concepts concerning this process will be revisited here in an attempt to shed light on the high incidence of polyspermy observed during *in vitro* fertilisation (IVF) compared with fertilisation *in vivo*. In humans, for example, the incidence of polyspermy during IVF ranges from less than 3% to over 30% (van der Ven *et al.*, 1985; Balakier, 1993; Aoki *et al.*, 2005; Kang & Rosenwaks, 2008) and this abnormality usually results in early embryonic death and/or spontaneous abortion (Jacobs *et al.*, 1978). Bearing in mind that a regular IVF cycle costs approximately 5,000€ (with high variation among countries), and that the number of IVF cycles [without intracytoplasmic sperm injection (ICSI)] performed every year is at least 132,000 in Europe (Andersen *et al.*, 2007), 25,000 in the USA (Wright *et al.*, 2008) and 191,000 worldwide (Adamson *et al.*, 2006), the economic importance of even a 1% reduction in the percentage of abnormal fertilisations due to polyspermy could be justified, with the emotional benefits probably being much higher.

The pig provides an example of a high incidence of polyspermy during IVF, with large numbers of spermatozoa (up to several decens in some experimental conditions) penetrating a single oocyte. Up to 65% polyspermy with *in vitro* matured oocytes and 28% in ovulated oocytes fertilised *in vitro* under the same conditions has been reported (Wang *et al.*, 1998*b*). A review (Abeydeera, 2001) reported differences in polyspermy ranging from 14% to 93% (with percentages of penetration ranging from 19 to 95%, respectively). Consequently, the *in vitro* production of porcine embryos for transgenesis, xenotransplantation, genetic improvement or recovery of endangered breeds is hampered (Wheeler & Walters, 2001; Coy & Romar, 2002; Funahashi, 2003). Thus, this species is a good model to examine further how the block to polyspermy operates.

The reported percentages of polyspermy in cattle range from 5% to 45 % for *in vitro* systems (Wang *et al.*, 1997*a*; Roh *et al.*, 2002; Coy *et al.*, 2005; Iwata *et al.*, 2008). In sheep and goats, polyspermy after IVF is also the main abnormality detected, affecting almost 20% of the inseminated oocytes (Fukui *et al.*, 1988; De Smedt *et al.*, 1992; Mogas *et al.*, 1997; Slavik *et al.*, 2005). Experimental studies in golden hamsters, mice, rabbits and rats also showed polyspermy in these species during IVF (Austin & Braden, 1953; Barros, Vliegenthart & Franklin, 1972; Fraser & Maudlin, 1979; Santalo *et al.*, 1992), but the reasons for such differences from the physiological situation are not fully understood. Thus, this review will examine previously proposed reasons for polyspermy during IVF in different mammals before describing recent experimental insights into the ZP block that could explain the increased frequency of polyspermy during IVF.

Under *in vitro* conditions in the golden hamster, Barros & Yanagimachi (1972) proposed that completion of the ZP and membrane blocks to polyspermy is not fast enough to prevent entry of the excess spermatozoa *in vitro* compared to the *in vivo* situation. This is analogous to a hypothetical failure of the oviduct-based mechanism *in vivo*. Such an explanation could apply to many species where IVF is performed, because the numbers of sperm employed are always higher than under *in vivo* conditions (Suarez, 2007). Usually, when the sperm concentration *in vitro* is reduced, polyspermy decreases to levels that do not present a major problem in humans, although it still exists (van der Ven *et al.*, 1985). In pigs, decreasing the sperm concentration during IVF induces a parallel reduction in penetration frequency, decreasing polyspermy but not eliminating it (Coy *et al.*, 1993; Abeydeera & Day, 1997).

In humans, it has been reported that oocyte maturation, particularly as far as CG migration and CG exocytosis are concerned (i.e. the egg-based mechanism of zona block), plays a pivotal role in decreasing the incidence of polyspermy (Sathananthan *et al.*, 1985; van der Ven

et al., 1985). However, the current use of *in vivo* matured oocytes recovered from stimulated follicles does not avoid the problem of polyspermy; dispermy is the most common anomaly of fertilisation in clinical IVF (Feng & Hershlag, 2003). Although intracytoplasmic injection of a single sperm (ICSI) is today a widespread option to avoid polyspermy ($>$50%, Adamson *et al.*, 2006), this technology bypasses key steps in the fertilisation process that could help select spermatozoa with a higher chance of producing a healthy embryo, as well as increasing the risk of aneuploidy and introducing sperm components into the oocyte that would not normally be present (Schultz & Williams, 2002). As described for human IVF, studies in our laboratory (P. Coy, unpublished observations) have shown that dispermy and trispermy are the most common forms of polyspermy during IVF in cattle.

Although delayed CG exocytosis in *in vitro* matured oocytes was proposed as responsible for a high degree of polyspermy (Wang, Hosoe & Shioya, 1997*b*.) further studies suggested that this is not the explanation because it has been demonstrated that the cortical reaction is similar in *in vivo* and *in vitro* matured oocytes under appropriate culture conditions (Wang *et al.*, 1998*b*).

III. THE ZONA BLOCK TO POLYSPERMY IN MAMMALS

There are hundreds of articles on post-fertilisation zona changes. The intention of the present review is simply to outline the main conclusions and to refer readers to other reviews published in recent years before focussing on the pre-fertilisation mechanism recently proposed.

Extensive studies have searched for the molecular cues involved in sperm-egg recognition, fusion, and triggering of CG exocytosis (reviewed by Hoodbhoy & Dean, 2004; Wassarman *et al.*, 2005; Gardner & Evans, 2006; Tsaadon *et al.*, 2006; Wong & Wessel, 2006). Briefly, it is generally accepted that the process of recognition and binding between spermatozoon and oocyte is mediated by the coupling of carbohydrates present in the ZP glycoproteins to specific (not fully characterised) proteins on the sperm membrane (Benoff, 1997; Dell *et al.*, 1999; Wassarman, Jovine & Litscher, 2001; Dean, 2004; Shur, Rodeheffer & Ensslin, 2004). Enhanced by progesterone synthesized in the cumulus oophorus (Hong *et al.*, 2004; Shi *et al.*, 2005), the initial contact between the spermatozoon and the ZP induces the acrosome reaction (AR), facilitating the exposure of moieties and binding to the secondary ZP receptor. After secondary binding, the sperm cell migrates through the ZP and crosses the small perivitelline space to contact the oolemma. Lastly, as a result of sperm-egg fusion, delivery of the sperm-borne sperm-specific phospholipase C-zeta (PLC-zeta) into the oocyte cytoplasm triggers Ca^{2+} signalling (Saunders *et al.*, 2002; Swann *et al.*, 2006). Ca^{2+} signalling is thought to be involved in driving the plasma membrane block to polyspermy (Gardner & Evans, 2006) and also leads to CG release which modifies the biochemical and biological properties of the ZP, establishing the zona block (zona reaction) (Bleil & Wassarman, 1980*a*; Bleil, Beall & Wassarman, 1981; Miller *et al.*, 1993; Green, 1997).

CG release is the physiological mechanism considered primarily responsible for avoiding an excess of sperm binding to, and crossing, the ZP in mammals (Dean, 2004; Hoodbhoy & Dean, 2004). Specific glycosidases and proteases present in the CG remove carbohydrates involved in sperm-ZP binding (Miller *et al.*, 1993), and cleave the ZP glycoproteins, finally causing its "hardening" and the prevention of sperm penetration (Gwatkin *et al.*, 1973; Gwatkin & Williams, 1974; Moller & Wassarman, 1989).

From studies in rodents, "zona hardening" is usually taken to mean the increased resistance of the ZP to the proteolytic digestion that takes place as a consequence of CG exocytosis after oocyte penetration by the spermatozoon (Austin & Braden, 1956; Gwatkin, 1964; Barros & Yanagimachi, 1971; Inoue & Wolf, 1974; Gulyas & Yuan, 1985).

These changes modify the actions of proteolytic enzymes in some animals, perhaps by masking the reaction sites or by cross-linking the ZP to prevent proteins from unfolding (Green, 1997). There is evidence, notably from mice, that the contents of the CG modify the ZP to prevent sperm penetration and to increase resistance to proteolysis (Kurasawa, Schultz & Kopf, 1989; Ducibella *et al.*, 1990; Vincent, Pickering & Johnson, 1990; Vincent *et al.*, 1991), but the situation in other mammals could be different. The reported timescale for ZP resistance to proteolysis after fertilisation varies widely among species (Table 1). While in mice and rats ZP resistance is significantly higher after fertilisation or artificial activation (Gulyas & Yuan, 1985; Zhang *et al.*, 1992; DeMeestere, Barlow & Leroy, 1997), there is no clear pattern in humans (Schiewe *et al.*, 1995; Manna *et al.*, 2001) and this increase does not take place in cattle (Iwamoto *et al.*, 1999; Coy *et al.*, 2005) or pigs (Coy *et al.*, 2002; Kolbe & Holtz, 2005). Here we use the term "ZP hardening" when both resistance to proteolysis and a decrease in sperm binding and sperm penetration are implied, whereas the term "ZP resistance to proteolysis" will be used when there is no relationship with sperm penetration.

At the molecular level, ZP hardening has been proposed to be a consequence of the selective proteolysis of ZP2 (one of the four glycoprotein families in the mammalian ZP) by enzymes in the CGs in, for example, pigs, cows, mice and humans (Moller & Wassarman, 1989; Hasegawa *et al.*, 1994; Noguchi *et al.*, 1994; Bauskin *et al.*, 1999). Cleavage in ZP2 probably initiates a series of conformational changes along a ZP protofilament, terminating in the acquisition of protease resistance throughout the ZP (Sun *et al.*, 2003; Dean, 2004; Lindsay & Hedrick, 2004). However, contrary to general belief, neither pig nor cow zygotes are resistant to pronase digestion under *in vitro* conditions (Coy *et al.*, 2002, 2005, Table 1), suggesting that mechanisms other than ZP2 cleavage could also be involved in the resistance of the zona to proteolytic digestion.

Table 1. Effect of fertilization (or chemical activation) on zona pellucida (ZP) resistance to proteolytic digestion ("post-fertilisation ZP hardening") in mammals

Species	**Protease used**	**Oocyte source**	**ZP digestion time pre-fertilization (min)**	**ZP digestion time post-fertilization (min)**	**Reference**
Mouse	Chymotrypsin	*In vivo* matured (oviduct) and fertilized	0.5–2.5 (120)	270 (120)	Gulyas &Yuan (1985)
Mouse	Chymotrypsin	*In vivo* matured (oviduct) and fertilized	40.7 (24)	65.3* (28)	DeMeestere *et al.* (1997)
Rat	Chymotrypsin	*In vivo* matured (oviduct) and chemically activated	1.2 ± 0.2 (90)	12.4 ± 0.8* (90) (after chemical activation)	Zhang *et al.* (1992)
Human	Alpha-chymotrypsin	*In vivo* matured (ovarian follicle) and *in vitro* fertilized	25.8 ± 0.6	32.2 ± 1.8*	Schiewe *et al.* (1995)
Human	Alpha-chymotrypsin	*In vivo* matured (ovarian follicle) and *in vitro* fertilized	24.1 ± 0.9–46.7 ± 2.0 (41)	45.3 ± 3.4 (18)	Manna *et al.* (2001)
Human	Pronase 0.5%	*In vivo* matured (ovarian follicle) and *in vitro* fertilized	1.62 ± 0.32 (15)	1.36 ± 0.07 (21)	P. Coy, R. Romar (unpublished data)
Cow	Pronase 0.1%	*In vitro* matured and fertilized	1.6 ± 0.15 (10)	1.7 ± 0.23 (10)	Iwamoto *et al.* (1999)
Cow	Mercaptoethanol and urea	*In vitro* matured and fertilized	11.3 ± 0.35 (10)	12.4 ± 0.36 (10)	Iwamoto *et al.* (1999)
Cow	Pronase 0.1%	*In vitro* matured and fertilized	3.25–3.76 (374)	3.01–3.78 (354)	Coy *et al.* (2005)
Pig	Pronase 0.1%	*In vitro* matured and fertilized	3.6 ± 0.4 (40)	2.7 ± 0.2* (40)	Coy *et al.* (2002)
Pig	Pronase 0.5%	*In vitro* matured and fertilized	1.8 ± 0.0 (60)	1.5 ± 0.1* (61)	Kolbe & Holtz (2005)

*Statistical differences ($P < 0.05$) between columns, when available, are indicated by **Values are means ± S.E.M. (N) or ranges. Number of oocytes (N) is indicated when available.

In recent studies using transgenic mice models, mouse oocytes with a humanized ZP containing human ZP2 or both ZP2 and ZP3 exhibited an effective block to polyspermy; however, no cleavage of the human ZP2 was apparent (Rankin *et al.*, 2003; Dean, 2004), again suggesting that other factors may contribute to ZP hardening.

After ZP2 cleavage, terminal N- and C- protein fragments remain bound intramolecularly by disulphide bonds (Hoodbhoy & Dean, 2004). Additionally, intramolecular disulphide bonds in ZP2 and intra- and intermolecular disulphide bonds in ZP4 are formed during fertilisation in the cow and pig (Iwamoto *et al.*, 1999; Topfer-Petersen *et al.*, 2008), probably making the ZP much less porous or accessible to proteolytic enzymes. It also has been observed that treatment of oocytes with a crosslinking reagent [Di-(N-succinimidyl)-3,3′-dithiodipropionate (DSP)] which forms bonds between the NH_2 groups of proteins increases ZP resistance to pronase digestion and to sperm penetration in the pig and cow (Coy *et al.*, 2008*b*; Cánovas *et al.*, 2009). For these reasons, the block to polyspermy and the hardening of the ZP have been associated with the formation of new bonds among proteins (Iwamoto *et al.*, 1999; Topfer-Petersen, Ekhlasi-Hundrieser & Tsolova, 2008) and with changes in the secondary structure of the zona proteins (Nara *et al.*, 2006) that take place at fertilisation and could modify the accessibility of the ZP receptors for spermatozoa and proteolytic enzymes.

Indeed, apart from ZP2 cleavage, reversible interactions between the CG enzyme N-acetylglucosaminidase in mice and its preferred oligosaccharides (Miller *et al.*, 1993) and steric modifications have been proposed as participating in ZP hardening and inhibition of gamete interactions in eutherians (Wong & Wessel, 2006). Diverse evidence supports the idea that specific sugar moieties in the ZP which probably participate in sperm-ZP binding are modified by glycosidases in the CGs (Avilés *et al.*, 1996,1997; Katsumata *et al.*, 1996; Raz Skutelsky & Shalgi, 1996; Velásquez *et al.*, 2007).

IV. PRE-FERTILISATION ZONA PELLUCIDA HARDENING

(1) A mechanism to reduce polyspermy

As mentioned above, the complete block to polyspermy commonly fails under IVF conditions employing fully mature oocytes and capacitated sperm from proven-fertility animals. It has often been suggested that final maturation of the oocyte, in addition to follicular maturation, in the oviductal environment is a necessary step for successful fertilisation and embryo development (Sathananthan *et al.*, 1985; van der Ven *et al.*, 1985). It was suggested two decades ago by Yang & Yanagimachi (1989), that "zonae of ovarian oocytes became like those of oviductal oocytes only when they were exposed to ampullary and/or isthmic fluids. The zona-altering factors in the oviductal fluid (oviductal glycoproteins), which are apparently integrated into the native zona, may act to enhance the various functions of the zona" (p 63). It is possible that the association of specific oviductal glycoproteins (OVGP1s) with the ZP and their localisation in the perivitelline space (Buhi, 2002) plays an important role in this process, which we term "oviductal zona maturation". This concept of oviductal zona maturation has been reinforced by recent findings suggesting incomplete secretion of ZP2 and reduced presentation of polysulphate structures by *in vitro* matured pig oocytes compared with oviductal (*in vivo* matured) oocytes (Topfer-Petersen *et al.*, 2008). However, the function and molecular relationship between OVGP1s or other oviductal components and the block to polyspermy has remained elusive until recently.

There are clear differences among animal species in the composition of the ZP which is formed by different glycoproteins in different mammals due to the progressive loss of zona pellucida genes in a pseudogenization process (Goudet *et al.*, 2008). Animals such as mouse, pig, cow and dog have three proteins whereas others, including hamster, rat and human have four (Bleil & Wassarman, 1980*b*; Lefievre *et al.*, 2004; Boja *et al.*, 2005; Hoodbhoy *et al.*, 2005; Izquierdo-Rico *et al.*, 2009). Moreover, in species with three ZP proteins, these proteins are not identical. In mice, the ZP proteins are ZP1, ZP2 and ZP3 whereas in pig, cow and dog they are ZP2, ZP3 and ZP4 (Goudet *et al.*, 2008). It is therefore possible to hypothesize that the role of the ZP in the fertilisation process could differ among rodents, humans and ungulates and that the concept of ZP hardening may be more subtle than previously thought.

Zhang, Rutledge & Armstrong (1991) described ZP resistance to chymotrypsin digestion in rats following *in vitro* maturation in a serum-free medium. An increase in the ZP resistance also occured during spontaneous maturation of murine oocytes in a defined medium (De Felici & Siracusa, 1982). Similar spontaneous zona resistance has been correlated with oocyte aging (Fukuda, Roudebush & Thatcher, 1992), premature CG exocytosis, and with the failure of oocytes to be fertilised *in vitro* (Gianfortoni & Gulyas, 1985; Downs, Schroeder & Eppig, 1986). Spontaneous resistance can be inhibited with the glycoprotein fetuin in rats (Schroeder *et al.*, 1990). This compound was therefore employed in horses with the aim of facilitating sperm penetration, since the hypothetical increase in zona resistance to proteolysis was considered responsible for the low fertilisation rate of *in-vitro*-matured equine oocytes (Dell'Aquila *et al.*, 1999). However, although the presence of fetuin reduced ZP resistance to proteolysis in a dose-dependent manner, it did not influence subsequent sperm penetration, indicating that the processes are similar but not identical in the different animal models.

These studies emphasize that data from rodents should not be extended to other mammals in the absence of a consistent body of research. There are numerous publications where ZP hardening is described as a post-fertilisation event, taking place as a consequence of CG exocytosis in every mammal, with the "dogma" stating that the first

spermatozoon that fuses with the oolemma triggers the release of the protease-active content of the CG, inducing ZP resistance to proteolysis and blocking polyspermy (Gulyas & Yuan, 1985; Iwamoto *et al.*, 1999; Hoyer *et al.*, 2001; Sun, 2003; Wong & Wessel, 2006). Consequently, pre-fertilisation resistance of the ZP may appear as an abnormality due to premature exocytosis of the CG because of oocyte aging, and would therefore reduce fertilisation, but is this true in mammals other than the mouse or rat?

From the available literature and from our own experiments (Table 2), we found intriguing data relating to ZP resistance to proteolysis in different farm animals. While the ZP digestion time was 2–3 min in *in-vitro*-matured bovine oocytes (Coy *et al.*, 2008*b*; Katska *et al.*, 1999) and 0–20 min in the oocytes obtained from preovulatory follicles (Coy *et al.*, 2008*b*, Katska *et al.*, 1989), retrieved oocytes from the oviduct required a 60–262-fold increase in the time for ZP digestion (Coy *et al.*, 2008*b*, Katska *et al.*, 1989). Similarly, when pig oocytes were employed by Coy *et al.*, (2008*b*), the ZP digestion time following *in vitro* maturation was 1 min and follicular oocytes took 1.5 min to be digested, whereas oviductal oocytes took 228 min, a 152-fold increase. Measurements were not affected by the presence or absence of cumulus cells (Coy *et al.*, 2008*a*). Broermann *et al.* (1989) reported that oviductal oocytes from pigs took more than 24 h to be digested with pronase solution under their experimental conditions and Kolbe & Holtz (2005) reported digestion times for unfertilised pig oocytes ranging from 1.4 min in follicular oocytes to 47.1 min in oviductal-derived oocytes. The increase in ZP resistance to digestion occurred in bovine oocytes placed in sheep or rabbit oviducts (Smorag & Katska, 1988; Katska *et al.*, 1999) and in pig oocytes placed in oviductal fluid from cows (Coy *et al.*, 2008a), indicating that this process is not species-specific. Moreover, the increase in resistance occurs in both mature and immature oocytes (Smorag & Katska, 1988). However, no differences were found in the ZP digestion time among follicular and oviductal mouse oocytes (Table 2).

The data in Table 2 suggest that oviductal oocytes have a more resistant ZP than follicular oocytes or those matured *in vitro*. We also observed that *in-vitro*-matured pig or cow oocytes incubated for 30 min in oviductal fluid (OF) showed a 211- and 33.5-fold increase, respectively, in their ZP resistance to pronase digestion (Coy *et al.*, 2008a). Similar experiments using 30% oviductal fluid (Kim *et al.*, 1996) reported an increase in ZP digestion time from 1.2 ± 0.3 to 6.4 ± 0.8 min (Mean ± S.E.M., $P < 0.05$). Light micrographs of the oocyte before and after contact with oviductal fluid indicate visible differences in ZP appearance, with unidentified components bound (Fig. 1A, B). One of these components bound to the ZP was identified as OVGP1 by immunocytochemistry (Fig. 1C).

These data indicate for pig and cow oocytes, and also for ewe and goat (I. Mondéjar, M. Avilés and P. Coy, unpublished data; Table 2) that the ZP becomes more resistant while they are in the oviduct or in contact with oviductal fluid (Broermann *et al.*, 1989; Katska *et al* 1999; Kolbe & Holtz, 2005; Coy *et al.*, 2008*a*, *b*). Experimental data also show that sperm penetration can occur through this resistant ZP, and that pre-fertilisation ZP resistance contributes to a reduction in polyspermy in pig oocytes (5.6% monospermy in control oocytes *versus* 50.0 % monospermy in oocytes incubated with oviductal fluid) and to a lesser extent in bovine oocytes (80.8% monospermy in controls *versus* 91.7% in oviductal-fluid-treated oocytes) (Coy *et al.*, 2008*a*).

Taken together, these observations lead us to hypothesize that oviductal-fluid-induced modification of the ZP, giving it resistance to proteolysis, is a mechanism for pre-fertilisation ZP hardening, fully independent of CG exocytosis. Pre-fertilisation ZP resistance to proteolysis and post-fertilisation modification of the ZP due to its CG content should be considered two independent events in ungulates, although both result in a decreased number of sperm penetrating the oocyte.

(2) Role of oviduct-specific glycoprotein (OVGP1) and sulphated glycosaminoglycans (S-GAGs)

Experiments were conducted to characterize the oviductal factor/s involved in the pre-fertilisation hardening. An effect of oviductal fluid in pigs was observed only with fluid samples collected in the very late follicular phase of the oestrous cycle (Coy *et al.*, 2008*a*). Oviduct-specific glycoprotein (OVGP1) was suggested as a possible candidate for inducing ZP resistance to pronase because OVGP1 is regulated by oestradiol levels and is present in the oviduct around the time of ovulation (Kan *et al.*, 1990; Buhi, 2002). The cross-reactivity of oviductal fluid between cows and pigs (Coy *et al.*, 2008a) also pointed to OVGP1 since identity in the amino acid sequences for this protein between pig and cow is 78% (Buhi *et al.*, 1996). The ZP acquired resistance to proteolysis was found to be reversible since the ZP of oocytes incubated for 30 min in oviductal fluid and later incubated in an IVF medium without heparin, exhibited significantly ($P < 0.001$) lower resistance after 15 min than the ZP of oocytes incubated in IVF medium with heparin (Fig. 2). Thereafter, heparin was shown to stabilize the acquired ZP resistance to proteolysis overcomimg the reversibility of the effect (Fig. 2). A role of OVGP1 again is consistent with these observations because it possesses heparin-binding consensus sequences (Buhi *et al.*, 1996) and heparin binding sites (Kouba *et al.*, 2000). OVGP1 was identified by proteomic analysis as becoming bound and later unbound from the ZP in IVF medium without heparin, and hence was likely responsible for the changes in the ZP that we termed pre-fertilisation hardening, i.e. making it resistant to proteolysis and to sperm penetration (Coy *et al.*, 2008*a*). The mean levels of sulphated glycosaminoglycans (S-GAGs) increase in pig oviductal fluid collected during the late follicular phase, that is pre-ovulatory oestrus (Tienthai *et al.*, 2000). Together, these data could explain why the ZP of oocytes in IVF medium with heparin (a highly sulphated glycosaminoglycan) becomes resistant to proteolysis for long periods of time, because the heparin (S-GAGs in the oviduct) would bind to OVGP1 via its

Table 2. Effect of oviductal factors on zona pellucida (ZP) resistance to proteolytic digestion in different mammals ("pre-fertilisation ZP hardening"). (OF: oviductal fluid; OVGP1: oviduct-specific glycoprotein)

Species	Protease used	Source of oocyte	ZP digestion time pre-treatment (min)	ZP digestion time post-treatment (min)	Reference
Mouse	Pronase 0.1%	*In vivo* matured (ovarian follicle)	2.9 ± 0.02 (12)	3.1 ± 0.1 (oviduct) (22) 2.8 ± 0.03 (after 30 min in OF) (16)	Coy *et al.* (2008*a*)
Pig	Pronase 0.1% + trypsin 0.1%	*In vivo* matured (ovarian follicle)	6 ± 0.6 (42)	1284 ±174* (after 30 min in oviduct) (29)	Broerman *et al.*(1989)
Pig	Pronase 0.1% + trypsin 0.1%	*In vivo* matured (oviductal oocytes)	1440 ± 0 (57)	1440 ± 0 (oviductal zygotes and embryos) (68)	Broerman *et al.* (1989)
Pig	Pronase 0.1%	*In vitro* matured	1.2 ± 0.3 (49)	6.4 ± 0.8* (after treatment with 30% oviductal fluid) (57)	Kim *et al.* (1996)
Pig	Pronase 0.1%	*In vitro* matured	2.18 ± 0.12 (57)	>120* (oviduct) (41)	Wang *et al.*(1998*b*)
Pig	Pronase 0.1%	*In vitro* matured	1–6 (30)	1–6 (after OVGP1 treatment) (30)	Kouba *et al.* (2000)
Pig	Pronase 0.5%	*In vivo* matured (ovarian follicle)	1.6 ± 0–07 (22)	228.18 ± 20.4* (oviduct) (27)	Coy *et al.* (2008*b*)
Pig	Pronase 0.5%	*In vitro* matured	1.08 ± 0.15 (40)	47.7 ± 1.5* (after 30 min in OF) (40)	Coy *et al.* (2008*a*)
Pig	Pronase 0.5%	*In vivo* matured (ovarian follicle)	1.4 ± 0 (28)	47.1 ± 7.3* (oviduct) (35)	Kolbe and & Holtz (2005)
Pig	Pronase 0.1%	*In vitro* matured	1.9 ± 0.15 (6)	3.4 ± 0.15* (after treatment with osteopontin) (6)	Hao *et al.* (2006)
Cow	Pronase 0.1%	Immature	0–10 (66)	120–720* (after 2 h in sheep or rabbit oviducts) (88)	Smorag & Katska (1988)
Cow	Pronase 0.1%	*In vivo* matured (ovarian follicle)	0–20 (17)	240–1440* (after 4–5 h in rabbit oviducts) (7)	Katska *et al.* (1989)
Cow	Pronase 0.1%	*In vivo* matured (ovarian follicle)	0–20 (17)	60–240* (oviduct) (13)	Katska *et al.* (1989)
Cow	Pronase 0.1%	*In vitro* matured	2.4 (25)	35.5* (after 40 min in oviducts) (20)	Katska *et al.* (1999)
Cow	Pronase 0.5%	*In vivo* matured (ovarian follicle)	3.9 ± 0.19 (19)	524.13 ± 50.9* (oviduct) (7)	Coy *et al.* (2008*b*)
Cow	Pronase 0.5%	*In vitro* matured	2.07 ± 0.1 (42)	71.7 ± 7.3* (after 30 min in OF) (40)	Coy *et al.* (2008*a*)
Ewe	Pronase 0.5%	*In vitro* matured	2.9 ± 0.02 (33)	223.01 ± 16.1* (after 30 min in OF) (43)	I. Mondéjar, M. Avilés & P. Coy, (unpublished data)
Goat	Pronase 0.5%	*In vitro* matured	6 ± 0.6 (16)	169.5 ± 13.26* (after 30 min in OF) (30)	I. Mondéjar, M. Avilés & P. Coy, (unpublished data)

*Statistical differences ($P < 0.05$) between columns are indicated by **Values are means ± S.E.M. (N).

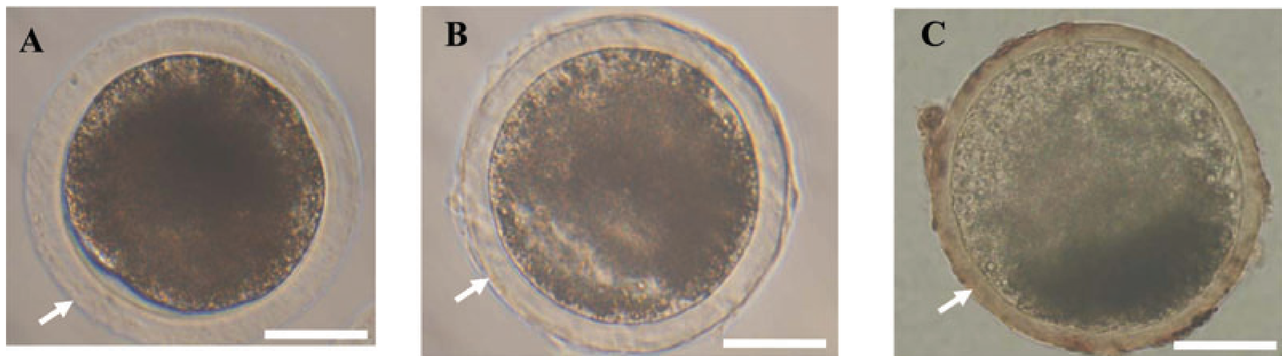

Fig. 1. Light micrographs showing changes in the zona pellucida (ZP, arrow) after incubation in oviductal fluid. (A) *In-vitro*-matured pig oocyte. (B) *In-vitro*-matured pig oocyte after 30 min incubation in pig oviductal fluid and three washes in phosphate-buffered saline to remove not firmly bound components or possible artefacts. Note the different appearance of the ZP, which looks less smooth and uniform after contact with oviductal fluid. (C) *In-vitro*-matured pig oocyte after 30 min incubation in pig oviductal fluid, three washes in phosphate-buffered saline and further processing by immunocytochemistry to detect oviduct-specific glycoprotein (OVGP1) [detailed methods and supporting information available in Coy *et al.*, 2008*a*]. The brown staining of the ZP indicates the presence of OVGP1. Scale bar, 50 μm.

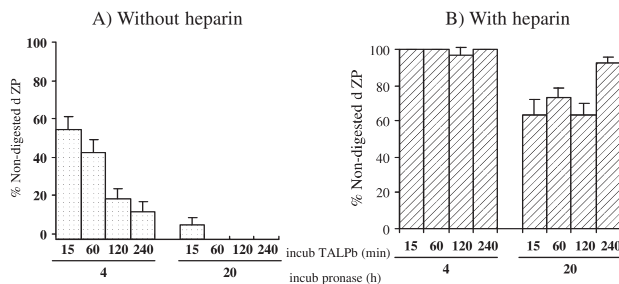

Fig. 2. Effect of heparin on the reversibility of porcine oocyte zona pellucida (ZP) resistance to proteases induced by incubation in bovine oviductal fluid (OF). *In vitro*-matured oocytes were preincubated in bovine OF for 30 min, transferred to bovine *in vitro* fertilisation (IVF) medium (TALPb) without (A) or with (B) heparin and evaluated for resistance to protease after various periods of incubation. Each bar represents the percentage (mean ± S.E.M.) of non-digested ZP after 4 or 20 h in pronase solution (0.5% w/v in phosphate-buffered saline), respectively, for each group. Experiments were carried out in triplicate. Each replicate consisted of 20 oocytes for each time assayed. Differences among every time group with or without heparin were significant ($P < 0.001$). From Coy *et al.* (2008*a*).

heparin binding site, and OVGP1 would also be bound to the ZP, stabilizing its resistance to proteolysis (Figs. 1C & 2). The proposed mechanism is represented in Fig. 3.

Observations in pigs and other species have shown that, under physiological conditions, sperm/egg ratios can be close to unity during early stages of fertilisation (Hunter, 1996). However, during an unspecified period of time, subsequent groups of sperm are released progressively from the isthmus to guarantee the fertilisation of all the eggs (Hunter, 1974). Resistance of the zona pellucida to sperm penetration, until the block to polyspermy is fully established by CG exocytosis, could be crucial during these first minutes or hours. Later, when such resistance becomes unnecessary because the membrane and/or zona blocks have been fully established, the numbers of accessory sperm attached to the zona pellucida (but not entering) increase dramatically (Hunter, 1974). This would agree with the mechanism proposed in Fig. 3, where a decrease in S-GAG concentration in the oviductal fluid could cause the OVGP1 to unbind from the ZP, and with observations that by the time the blastocyst is ready to leave the egg, the ZP has no resistance to proteolysis (Kolbe & Holtz, 2005). Thus, we propose that two mechanisms, zona pellucida hardening, mediated by the oviductal fluid, and the ZP block mediated by CG exocytosis work together to achieve successful fertilisation. In the pig, it could be necessary that the oocytes are in contact with oviductal fluid from the peri-ovulatory stage, as samples of oviductal fluid collected few hours before or after ovulation do not produce ZP hardening (Coy *et al.*, 2008*a*). This may explain the higher levels of polyspermy observed after delayed insemination in pigs (Hunter, 1967).

A mechanism to make the egg resistant to fertilisation may be thought counterproductive but penetration percentages of pig oocytes incubated in oviductal fluid are not lower than in controls, although there is a decrease in the case of bovine oocytes (Coy *et al.*, 2008*a*). The widespread occurrence of ZP resistance to proteolysis in ungulates (in pigs, cattle and now in sheep and goats: I. Mondéjar, M. Avilés & P. Coy, unpublished data), may mean that the changes in protein composition and properties of the ZP when incubated with oviductal fluid may have other additional functions, such as facilitation of sperm selection.

If this mechanism is widespread in ungulates, then why is not found in all species, such as the mouse, as OVGP1 is highly conserved? As indicated by Yong *et al.* (2002) few data exist to investigate the biological roles assigned to OVGP1. While a comparative analysis of OVGP1 amino acid sequences of different species indicates a high degree of similarity, especially in the N-terminal region of the protein (Verhage *et al.*, 1997; Buhi *et al.*, 1996), considerable divergence has been observed in the carboxy

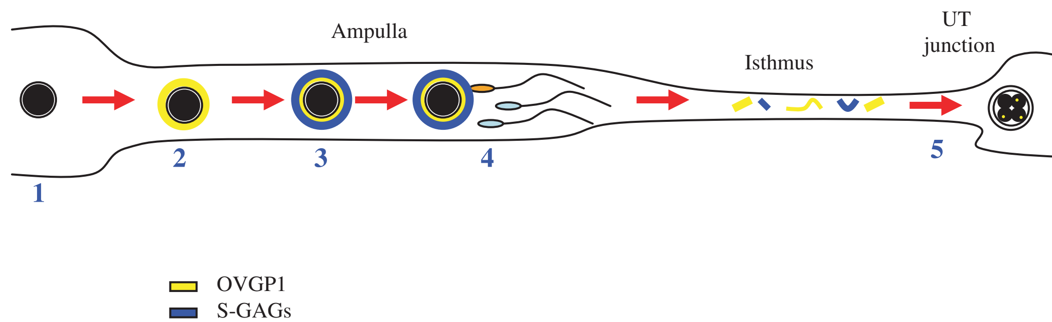

Fig. 3. Description of the proposed pre-fertilisation mechanism preventing polyspermy in ungulates. When the oocyte is shed into the ampulla (1) soon after ovulation, oviduct-specific glycoprotein (OVGP1) surrounds it in a "shell" that is responsible for the resistance of the zona pellucida (ZP) to proteolysis (2). Heparin-like glycosaminoglycans (S-GAGs) in the oviduct fluid stabilize and reinforce the binding of OVGP1 with the ZP (3), affecting its interaction with selected spermatozoa (4). During the transition of the fertilised oocyte to the uterus, the system is destabilized and the OVGP1 is partially unbound or absorbed into the egg (5).

terminal region, where the number of Ser/Thr-rich tandemly repeated motifs varies among species. Perhaps this could explain differences in the effects of OVGP1 among species. Moreover, alignment of different OVGP1s indicates that the region lost is not identical in all species. Positive Darwinian selection may promote the divergence of OVGP1 among mammals (Swanson *et al.*, 2001). Second, different biological roles for OVGP1 could be caused by different extents of glycosylation. In hamsters, the apparent molecular weight of OVGP1 ranges from 160 to 350 kDa (Malette & Bleau, 1993), indicating that carbohydrates are a major component. Most of the carbohydrates are present in O-linked chains but N-linked chains have also been detected (Malette & Bleau, 1993). Glycosylation differences detected over the oestrous cycle could be responsible for a different biological role of oviductal-secreted glycoproteins (McBride *et al.*, 2004; McBride Brockhausen, & Kan, 2005). Third, OVGP1 activity could be affected by ZP composition. As already mentioned, the number and composition of ZP glycoproteins differ among mammals. In the mouse, although OVGP1 is a component of oviductal fluid, its association with the ZP has not been demonstrated (Buhi, 2002). Mouse OVGP1 is very similar to hamster OVGP1 (Verhage *et al.*, 1997); however, hamster OVGP1 has an affinity for hamster ZP (Malette & Bleau, 1993). The species differ in the composition of the ZP (three proteins *versus* four proteins, respectively) and ZP4 is present in the hamster but not in the mouse ZP (Izquierdo-Rico *et al.*, 2009). Moreover, ZP2 and ZP3 are among the most divergent 10% of proteins in mammals (Monne *et al.*, 2008) and this could affect how OVGP1 functions in different species. Indeed, the relevance of the different ZP proteins to the functions of OVGP1 interaction could be a fruitful area of future research. The use of heterologous assays could provide valuable new information concerning OVGP1 functioning and binding to the ZP.

Attempts to demonstrate an effect of OVGP1 on pig ZP hardening and on the frequency of polyspermy during IVF have met with little success perhaps because the ability of OVGP1 to unbind from the ZP, and the role of heparin, were then unknown (Kouba *et al.*, 2000; McCauley *et al.*, 2003). No information is available in the human although some experimental data showed that baboon OVGP1 binds to the human ZP (O'Day-Bowman *et al.*, 1996).

V. CONCLUSIONS

(1) Oviductal secretions contribute to the regulation of polyspermy in different mammals by inducing a pre-fertilisation modification in the ZP. This modification consists of the binding of OVGP1 to render the ZP more resistant to protease digestion and to sperm penetration. ZP resistance to pronase digestion increases from 1–2 min in oocytes without contact with oviductal secretions to several hours or even days in oocytes incubated for 30 min in oviductal fluid (Coy *et al.*, 2008*a*). Following existing nomenclature, that uses the term "ZP hardening" to describe the post-fertilisation modifications in the ZP that increase its resistance to proteolysis and to sperm penetration, this additional mechanism can be termed "pre-fertilisation ZP hardening".

(2) Evidence now exists that demonstrates a role for S-GAGs in pre-fertilisation ZP hardening. When there are no S-GAGs in the fertilisation medium, resistance to proteolysis decreases (presumably because the OVGP1 becomes unbound from the ZP). Different levels of oviductal S-GAGs known to occur during the oestrous cycle (Parrish *et al.*, 1989; Tienthai *et al.*, 2000; Talevi & Gualtieri, 2001) may regulate ZP hardening allowing a return to the "non-resistant" form by the time of blastocyst hatching (Kolbe & Holtz, 2005).

(3) A universal characteristic of OVGP1s is their association with the ZP and presence in the perivitelline space of oocytes and embryos (Buhi, 2002). A variety of functional roles during fertilisation and early embryonic development has been proposed, but the specific mechanisms and functions of OVGP1 remain elusive (Buhi, 2002). Differences in OVGP1

length, amino acid sequence, and glycosylation as well as interspecific differences in the ZP glycoproteins present may explain the diversity of roles assigned to this oviductal glycoprotein. The use of heterologous assays could provide valuable information in determining the functions of OVGP1 during fertilisation and early embryo development.

(4) The presence of pre-fertilisation ZP hardening opens up a series of new questions on sperm-ZP interaction. The newly identified modified ZP of oviductal oocytes and binding between ZP glycoproteins and the complex OVGP1-S-GAGs will involve a matrix of contact for the spermatozoa different from that employed in current IVF systems. A recent study found that oocyte contact with oviductal secretions before fertilisation had a beneficial effect on embryo development, quality and gene expression (Lloyd *et al.*, 2009). These observations make it reasonable to propose future genomic and proteomic approaches to investigate further the already identified oviductal components (Seytanoglu *et al.*, 2008) that contribute to fertilisation and early embryo development. Although oviduct-derived glycoproteins are molecules which seem to fill the role of "missing factors" *in vitro* (Leese, 1988), proteins other than OVGP1 in oviductal fluid also associate with the zona pellucida, such as alpha-1 acid glycoprotein, which is thought to influence sperm-ZP binding (Kratz *et al.*, 2003), and the complement component C3, which works as an embryotrophic factor in the human (Lee *et al.*, 2003, 2004). The latter has been identified in the porcine oviduct mainly during oestrus (Buhi & Alvarez, 2003). Other molecules of interest are glycodelins (Chiu *et al.*, 2007; Yeung *et al.*, 2007), retinol-binding protein, haemoglobin beta chain, Ig kappa light chain and variable region, fibrinogen A-alpha-chain, fibrinogen beta chain precursor, (Georgiou *et al.*, 2007) different sulphated and non-sulphated glycosaminoglycans (Tienthai *et al.*, 2000; Bergqvist & Rodriguez-Martinez, 2006; Liberda *et al.*, 2006), and glycosidases (Carrasco *et al.*, 2008*a*,*b*). The inclusion of recombinant human albumin and hyaluronan in current human IVF media provides a precedent for the use of recombinant OVGP1, C3 or different S-GAGs in future culture media. This could be beneficial for ART programs in human clinics, for the animal embryo transfer industry and for scientists investigating the genetic regulation of embryonic development (Boice *et al.*, 1990).

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