

Cytochemical and biochemical evidences for a complex tridimensional structure of the hamster zona pellucida

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Summary. Zona pellucida (ZP) is an extracellular matrix that surrounds eggs and pre-implantation embryos and is required for *in vivo* fertility. A key event in successful fertilization is sperm binding to the surface of the ZP. It has been previously described that the hamster sperm binds mainly the outer region of the ZP which corresponds to the porous region in contact with the cumulus cells. Using ultrastructural cytochemistry approaches with an antibody developed against porcine ZP, this study shows that the pig ZP shares epitopes with some rodent species like hamster, rat and mouse. In the hamster, these epitopes are located mainly in the outer region of the ZP of preovulatory and ovulated oocytes. By means of biochemical approaches it was demonstrated that 1) the antibody is specific for the native hamster ZP3, 2) four different bands with a molecular weight of 67, 60, 48 and 38 kDa after N-linked deglycosylation suggesting that the hamster ZP is formed by four proteins, and 3) the different composition observed in the outer region of the hamster ZP could be due to a specific supramolecular structure that makes some epitopes accessible for the antibodies. In summary, this study provides evidence that the different composition observed in the different regions of the ZP is mediated by a different organization of the components of the ZP produced during the oocyte maturation. This different organization could be responsible for the different sperm binding affinity observed for sperm to the outer region versus the inner region of the ZP.

Key words: Zona pellucida, Cytochemistry, Hamster, Heterogeneity, Composition

Introduction

The zona pellucida (ZP) is a relatively thick extracellular coat that surrounds mammalian oocytes. The ZP contains sperm ligands that mediate sperm-egg interaction as a prelude to fertilization; it also induces the acrosome reaction and participates in the block to polyspermy following fertilization of eggs (Yanagimachi, 1994; Wassarman, 2002; Litscher and Wassarman, 2007). Wassarman et al. (1996) suggested that the ZP is formed by filaments composed by dimers of ZP2 and ZP3. These filaments are linked by dimers of ZP1 glycoprotein (Green, 1997; Wassarman et al., 2001). However, recent molecular biology approaches, using knock-out mice, provide new information about the ZP and suggest a reconsideration of some aspects of the previously described model (Dean, 2004). Thus, it was suggested that the ZP contains filaments composed by ZP3/ZP2 dimers but also by ZP3/ZP1 dimers (Rankin et al., 2003; Dean, 2004).

Previous morphological studies have demonstrated that the ZP shows a complex histological structure. Thus, scanning electron microscopy revealed a different structure of the ZP when comparing the region facing the oocyte and the region facing the cumulus cells, called inner and outer region of the ZP, respectively (Familiari et al., 1992, 2006; Phillips and Shalgi, 1980). Other studies combining transmission electron microscopy and lectin cytochemical techniques showed evidence for a heterogeneous carbohydrate composition of inner and outer regions of the ZP in different species

(Avilés et al., 1996, 1997b, 1999, 2000a,b; Jiménez-Movilla et al., 2004). The use of the Polscope microscopy showed that the ZP of hamster and human oocytes is organized in three different layers; however, the significance of this finding is not yet determined (Keefe et al., 1997; Pelletier et al., 2004). The morphological complexity of the ZP could be the basis of previous physiological evidence showing substantial differences in the binding of sperm to the inner and outer regions of the ZP of different species such as hamster (Phillips and Shalgi, 1980; Ahuja and Bolwell, 1983; Yazawa et al., 1995), pig (Fazeli et al., 1997) and human (Burkman et al., 1988).

In the present study, the structure and composition of the hamster ZP was investigated by means of biochemical and ultrastructural cytochemistry approaches. A specific polyclonal antibody developed against ZP isolated from porcine ovaries was used. This study showed that the pig ZP shared epitopes with other rodent species including hamster, rat and mouse. A differential distribution over the thickness of the ZP was described in hamster, mouse and rat. In hamster, these epitopes were located mainly in the outer region of the ZP of preovulatory and ovulated oocytes. However, the distribution is throughout the ZP in more immature oocytes.

Lectin blot analysis of the hamster ZP isolated from ovaries demonstrated the presence of two broad bands. The 56 kDa band was specifically recognized by the anti-pig ZP antibody by Western blot. However, four specific bands were detected after the remove of N-linked oligosaccharides. These data strongly suggest the presence of four different ZP glycoproteins in the hamster ZP. This new protein has an apparent mobility of 56 kDa and co-migrates with the hamster ZP3.

Material and methods

Reagents

WGA-peroxidase (HRP), HRP-conjugated goat anti-rat IgG, N-acetyl-glucosamine, DNase I, soy bean trypsin inhibitor, PVDF membrane, hyaluronidase from bovine testes and bovine serum albumin were obtained from Sigma (Madrid, Spain). Prestained molecular-mass standards were from Gibco laboratories. Protein A-colloidal gold conjugate (15 nm) was from the Department of Cell Biology (Utrecht University, Utrecht, the Netherlands). Protein A-agarose and HRP conjugated rabbit anti-goat IgG antibody were from Santa Cruz Biotechnology. A polyclonal antibody developed against total pig ZP purified by heat solubilization from pig ovarian oocytes was used (Barber et al. 2001). M1.4 (rat IgG2a), IE-3 (rat IgG) and IE-10 (rat IgG) monoclonal antibodies specific to mouse ZP1, ZP2 and ZP3 were kindly provided by Dr Jurrien Dean (NIH, USA) (East and Dean, 1984; East et al., 1985; Rankin et al., 1998).

Cytochemical analysis

Collection of ovaries and oocytes from hamster, mice and rats

Adult female CD1 mice (n=3), Wistar rats (n=3) and hamsters (*Mesocricetus auratus*) (n=3) were stimulated to superovulate by an intraperitoneal injection (i.p.) of 5 IU, 15 IU and 25 IU of PMSG respectively followed 48 hours later by an i.p. injection of 5 IU, 15 IU and 25 IU of hCG. Females were killed by CO₂ inhalation 17 h after injection with hCG. Ovaries and oviducts were removed and placed in the well of a glass dish containing 0.1 M cacodylate buffer (pH 7.4). To collect postovulatory oocyte-cumulus complexes, the ampullary region of the oviduct was identified and torn open with fine steel tweezers under a dissecting microscope. Ovaries and oocytes were fixed with 2% glutaraldehyde in cacodylate buffer pH 7.4 as previously described (Avilés et al., 1994) for electron microscopy.

Collection of pig ovaries

Ovaries from prepubertal gilts were transported to the laboratory within 30 min after slaughter in saline solution (0.9% (w/v) NaCl) containing 100 mg ml⁻¹ kanamycin at 37°C. For ultrastructural study, the pig ovaries were fixed for 2 hours at 4°C by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4).

Tissue processing

For preparation of thin sections, ovaries and oocyte cumulus complexes were processed for embedding in Lowicryl medium as previously described (Avilés et al., 1994, 1996, 1997a,b) and LR White resin according to routine procedures (Newman, 1999). Thin sections were obtained with an ultramicrotome (Microm International GmbH) and mounted on formvar coated nickel grids.

Lectin- and immuno-cytochemistry at the ultrastructural level

Grids were incubated with the lectin WGA-HRP as previously described (Jiménez-Movilla et al., 2004). For immunocytochemistry, grids were first incubated with 1% BSA in PBS for 10 min. Then, the grids were incubated for 1 hour with a drop of polyclonal anti-pig ZP antibody (1:1000) diluted in PBS supplemented with 1% BSA. After three washes in PBS of 5 min each, grids were incubated for 1 hour with gold labeled protein-A (15 nm) (1:60). After washing with the corresponding buffer and bidistilled water, the grids were counterstained with uranyl acetate and lead citrate.

Controls

Controls were carried out by the substitution of the

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primary antibody by the preimmune serum. For lectin cytochemistry two different controls were performed: 1) substitution of conjugated and unconjugated molecules by the corresponding buffer and 2) preincubation of the WGA-HRP lectin with the corresponding hapten-sugar inhibitor, N-acetyl-glucosamine, at 0.2 M.

Quantitative analysis

In the present study, the ZP was divided into two zones of approximately equal thickness: inner and outer zones. The inner zone is located close to the oocyte itself while the outer zone faces the cumulus cells and mediates sperm binding. These two regions show different morphological and cytochemical characteristics. For quantitative analysis, at least five randomly selected areas of ZP of each oocyte were photographed on the electron microscope at 4,500 magnification. Fifteen to twenty fields (squares of 2.5 μm side) of the different regions of the ZP from each oocyte were used for the quantitative evaluation. Density of labelling was assessed with a computer-assisted image analyser model Q500MC from Leica (Barcelona, Spain). The density of labelling was determined by automatic counting of the gold particles enclosed in manually delineated areas. A statistical comparison was performed for lectin and antibody labelling in the two regions of the ZP previously described. Labelling densities were compared using analysis of variance and Student's t-test with $p < 0.05$ performed with a SPSS v10.

Biochemical analysis

Purification of zona pellucida glycoproteins

Hamster and mouse. The ovaries from hamster ($n=16$) and mouse ($n=22$) were trimmed using small scissors under a dissecting microscope to remove fat and connective tissue. The ovaries were homogenized with a Polytron at a setting of 4 for 5 seconds in 3 ml of homogenization buffer (25 mM triethanolamine HCl, 150 mM NaCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 8.5) supplied with 12 mg of soybean trypsin inhibitor, 4 mg of bovine testicular hyaluronidase and 1% NP40. The homogenate was placed on ice for 1 min and then homogenized twice as above. 0.4 ml of 0.1 g/ml deoxycholic acid solution was added to the homogenate and was placed on ice for 10 min. The homogenate was further homogenized by 10 strokes in a glass homogenizer and subsequently centrifuged at 4°C (5 min, 10000xg). The supernatant was discarded and the pellet was washed first with TEA buffer (25 mM triethanolamine HCl, 150 mM NaCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 8.5) and second, with high salt PBS buffer (10 mM PBS plus 1 M NaCl, pH 7.0). The sample was finally resuspended in 1 ml of 10 mM phosphate buffer and heat solubilized at 65°C for 45 min. Finally, the sample was centrifuged

again at room temperature (5 min, 10000xg) and the supernatant which contained the heat solubilized ZP was stored at -80°C.

Electroelution

Purification of the upper and 56 kDa bands of hamster ZP was carried out by electroelution from SDS-PAGE gels. Electroelution was accomplished with a Model 422 Electro-Eluter (Bio-Rad Laboratories) at 8-10 mA/glass tube constant current for 4h at room temperature using an elution buffer of 25 mM Tris, 192 mM Glycine and 0.1% SDS.

Fig. Oocytes surrounded by four or more layers of cumulus cells were obtained from pig ovarian follicles (3-8 mm diameter) as previously described (Coy et al., 2002). Isolated ZP were obtained by passing the cumulus free oocytes through a glass pipette of approximately 80 μm diameter.

Immunoprecipitation

Hamster ZP glycoproteins were diluted in 100 μl of PBS buffer and precleared by incubation with 20 μl of prewashed protein A-agarose for 1 h at 4°C. The precleared ZP was incubated for 4h at 4°C in the presence of 5 μl of anti-pig ZP antibody. Immunoprecipitation was then performed by adding 20 μl of protein A-agarose for 1 h at 4°C with continuous shaking. After centrifugation (5 min, 7000xg) the pellet was washed four times in PBS buffer and resuspended in reducing SDS-PAGE loading buffer (Laemmli, 1970). After boiling (5 min) and centrifugation (5 min, 15000xg), the proteins in the supernatants were separated by SDS-PAGE.

PNGase F Digestion

The electroeluted 56 kDa band and the heat solubilized hamster ZP were heated at 95°C for 5 min prior to incubation at 37°C overnight in the presence of 6 UI of PNGase F (EC 3.5.1.52, Roche) in 50 mM phosphate buffer, pH 7.0, containing 10 mM EDTA and 0.1% SDS. Control samples were treated under identical conditions, except for the omission of the glycosidase.

SDS-PAGE and Western-blotting

Purified ZP obtained as described above were dissolved in sample buffer in reducing (5% β -mercaptoethanol (vol/vol)) and non-reducing conditions. After boiling for 5 min, samples were separated by 12% polyacrylamide SDS-PAGE (Laemmli, 1970). In brief, 4% stacking and 12% separating gels were used and run using 25 mM Tris/0.2 M glycine buffers, pH 8.6, containing 0.1% SDS for 1.5h at 150 V at room temperature. For western blots, the SDS-PAGE slab gels

were equilibrated for 30 min in 25 mM Tris, 192 mM glycine in 20% (vol/vol) methanol, and proteins were electrotransferred to PVDF membrane at 100 V for 1h.

Lectin-immunoblotting

After appropriate washings and blocking in 50 mM Tris buffer, pH 7.4, 150 mM NaCl and 0.1% Tween 20 (TBST) supplemented with 2% BSA, the membrane was incubated with anti-mouse ZP1, ZP2 and ZP3 (1:1000) monoclonal antibodies and anti-pig ZP (1:1000) polyclonal antibody. After washing in TBST and for detection the monoclonal and polyclonal antibodies, the membranes were incubated with HRP-conjugated goat anti-rat IgG (1:500) and rabbit anti-goat IgG antibody, respectively. For lectin-blots, the membranes were incubated with WGA-HRP (0.5 μ g/ml). After washing, visualization was accomplished using the ECL method following the manufacturer's instructions (Amersham).

Results

Cytochemical analysis of the zona pellucida

Ovary

The immunolabeling obtained with the anti-pig ZP was specifically located at the ZP of the different

follicles from rat, mouse, pig and hamster ovary. At ultrastructural level, it was observed that the immunolabeling was homogeneously distributed over the entire thickness of the ZP of the different follicles in pig (Fig. 1a), mouse (Fig. 1b) and rat ovary. A quantitative analysis was performed in the pig antral ovarian follicles. No statistical differences were observed between the inner and outer regions of the ZP (100.96 ± 2.2 versus 103.9 ± 2.4 respectively). However, a different immunolabeling pattern was observed in the hamster ovary. Small ovarian follicles (Fig. 2a) showed a uniform labelling over the entire ZP (Fig. 2b); however, the immunolabeling observed in the large ovarian follicles was heterogeneously distributed. Thus, the immunolabeling is much higher in the region of the ZP in contact with the follicular cells (outer region) (Fig.

Table 1. Immunolabeling densities (gold particles/ μm^2) in the ZP of tubal oocyte-cumulus complexes of different species¹.

Species	Inner region	Outer region
Hamster*	7.68 \pm 0.96	29.3 \pm 0.91
Mouse*	23.59 \pm 0.72	16.38 \pm 0.76
Rat*	32.67 \pm 1.27	24.49 \pm 1.3

¹Labeling densities were expressed as Mean \pm Standard Error (SE).
*Statistically significant differences between inner and outer zone of ZP in unfertilized eggs ($P < 0.05$).

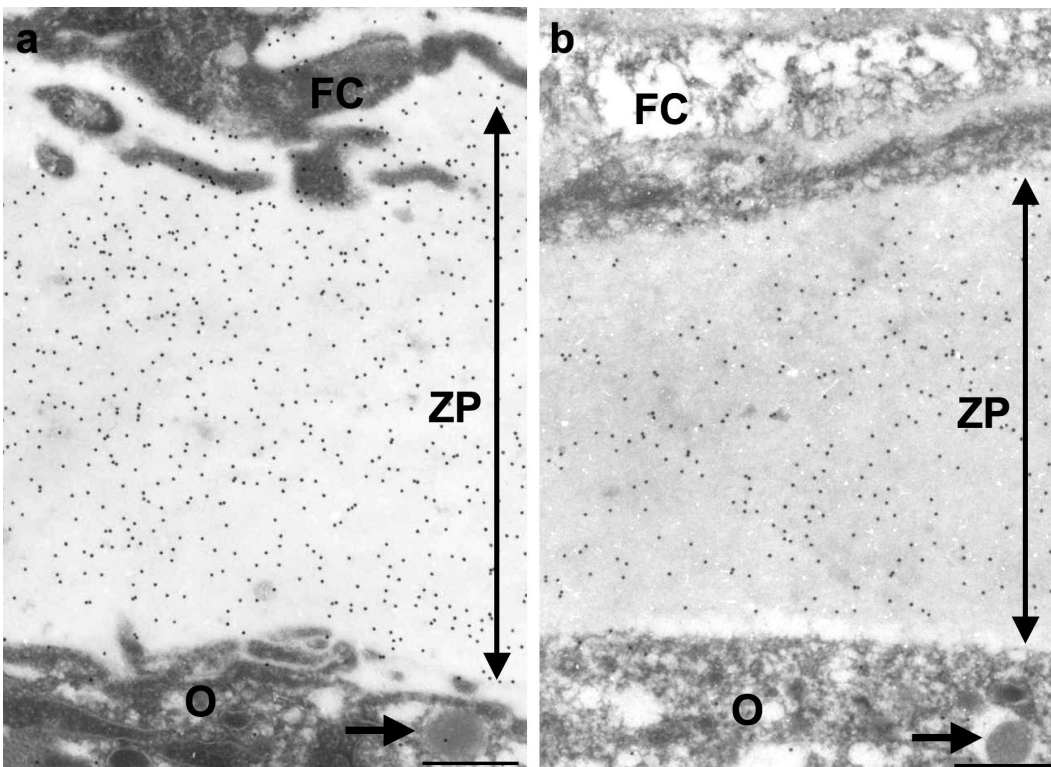


Fig. 1. Electron microscope immunolabelling of pig and mouse ovary stained with anti-pig ZP antibody. **a.** Pig antral ovarian follicle. **b.** Mouse antral ovarian follicle. The immunolabeling is distributed throughout the ZP in both oocytes. Note an unreactive cortical granule (arrow) in the ooplasm. FC: follicular cell, O: oocyte, ZP: zona pellucida. Bars: 0.5 μm .

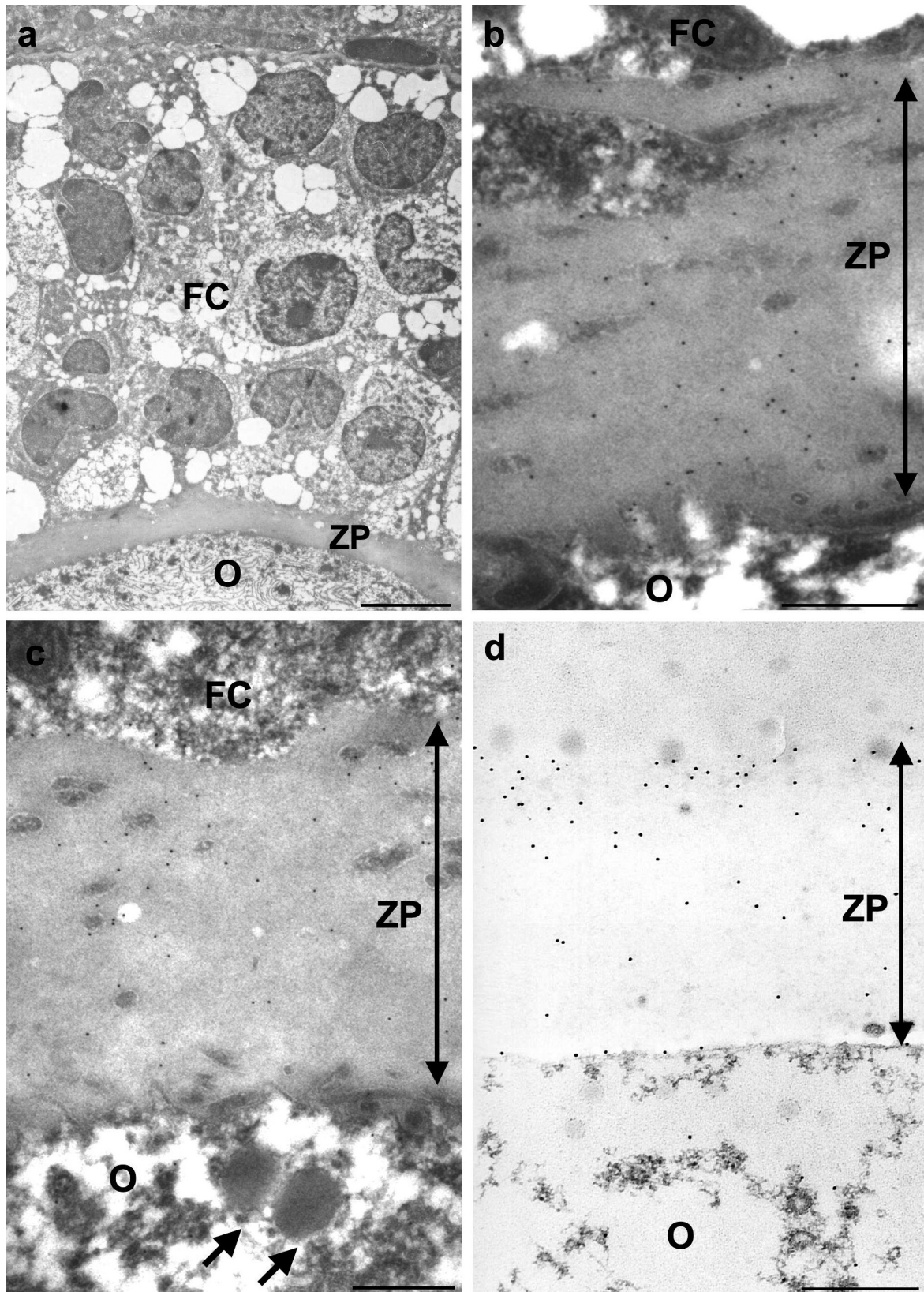


Fig. 2. Electron microscope immunolabeling of hamster ovary and postovulatory oocyte stained with anti-pig ZP. **a.** Multilaminar primary follicle. **b.** At higher magnification the ZP. The immunolabeling is homogeneously distributed throughout the ZP. **c.** Antral ovarian follicle. The immunolabeling is mainly located in the outer region of the ZP. Note the absence of labelling over the cortical granules (arrows). **d.** Postovulatory oocyte. Only the outer region of the ZP is mainly immunolabeled. FC: follicular cell, O: oocyte, ZP: zona pellucida. Bars: a, 10 μm ; b-d, 0.5 μm .

2c).

Tubal oocyte-cumulus complexes

The heterogeneous pattern observed in the antral ovarian follicles of the hamster was also observed in tubal oocytes (Fig. 2d). However, mouse and rat tubal oocytes showed a labelling over the entire thickness of the ZP. A quantitative analysis was performed in the tubal oocytes of the three rodent species (Table 1). It was observed that there is statistical significant difference in the labeling density between the inner and the outer region of the ZP in the three different species. However, this quantitative analysis demonstrates that the immunolabeling in mouse and rat was higher in the inner region of the ZP when compared with the outer zone. On the contrary, in the hamster, the immunolabeling observed was higher in the outer region of the ZP.

Biochemical analysis of the zona pellucida

To further characterize the specificity of the polyclonal anti-pig ZP antibodies used in this study, SDS-PAGE and western blot analyses were performed to identify the ZP glycoproteins recognized by this antibody using pig, mouse and hamster ZP. Pig ZP proteins migrate as a single broad band of apparent molecular mass of 48-80 kDa as described (Yurewicz et al., 1983) and that was specifically recognized by the anti-pig ZP antibody in its entire thickness (Fig. 3a). Under non-reducing conditions, the mouse ZP glycoproteins are separated in three different bands of apparent molecular masses of 180-200, 120-140 and 83 kDa that correspond to ZP1, ZP2 and ZP3 respectively

as previously described (Bleil and Wassarman, 1980; Shimizu et al., 1983). The three different glycoproteins are specifically recognized by the anti-pig ZP antibody (Fig. 3b, lane 2). A similar result was obtained using the WGA lectin (Fig. 3b, lane 1) and the monoclonal antibodies specific to mouse ZP1, ZP2 and ZP3 (data not shown). The isolated hamster ZP glycoproteins were separated by SDS-PAGE under reducing conditions. Lectin blot analysis using WGA demonstrated the presence of two broad bands. The upper band showed a molecular weight over 90 kDa and the lower band showed an average molecular weight of 56 kDa (Fig. 3c, lane 1). These two bands have been previously described to correspond with ZP1 and ZP2 (upper band) and with ZP3 (lower band) (Moller et al., 1990). Only the lower band of 56 kDa was specifically immunolabelled with the anti-pig ZP (Fig. 3c, lane 2). To investigate if the specific cross-reactivity showed by the anti-pig ZP antibodies is dependent on the carbohydrate residues or protein backbone a partial deglycosylation procedure was performed. After the specific deglycosylation with PNGase F, four bands with an apparent molecular weight of 67, 60, 48 and 38 kDa respectively were detected (Fig. 4a). The 48 and 38 kDa bands were detected when the purified 56 kDa band was deglycosylated using the N-glycosidase F enzyme (Fig. 4b).

Immunoprecipitation analysis of the hamster zona pellucida

Purified hamster ZP glycoproteins were immunoprecipitated using the anti-pig ZP antibody and protein A-agarose beads. Immunoprecipitation of the total hamster ZP using the anti-pig ZP demonstrates the

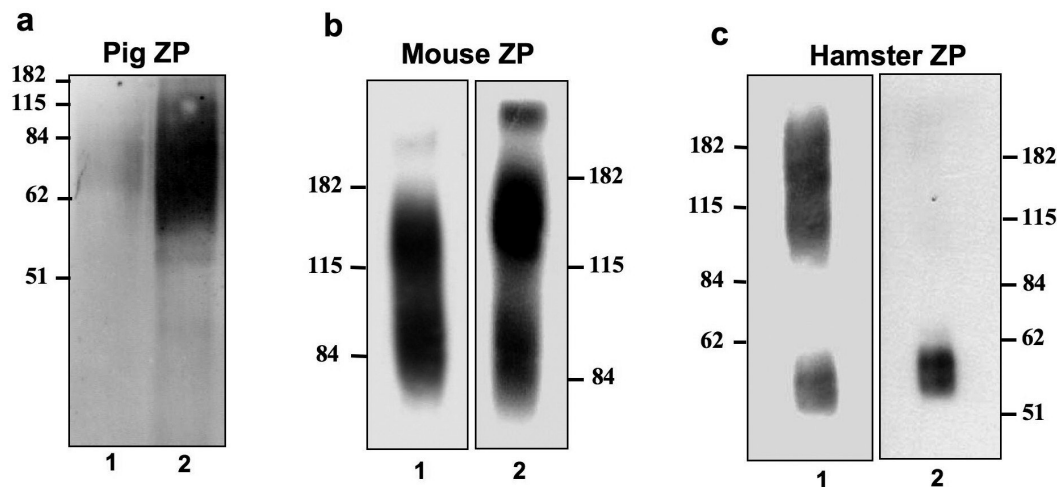


Fig. 3. Solubilized ZP from pig, mouse and hamster were probed with anti-pig ZP antibody and WGA lectin. **a.** Isolated pig ZP was probed with anti-pig ZP antibody under reducing conditions. Lane 1, 3 isolated ZPs. Lane 2, 10 isolated ZPs. Note that pig ZP migrate as a unique broad band that was specifically recognized by the antibody. **b.** Heat solubilised mouse ZP was separated by SDS-PAGE under non-reducing conditions and transferred to PVDF membrane. Lane 1, the blot was incubated with WGA lectin. The lectin showed

affinity for ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (83 kDa). Lane 2, the blot was incubated with anti-pig ZP. The antibody was reactive at ZP1, ZP2 and ZP3. **c.** Purified heat solubilised hamster ZP by reducing SDS-PAGE and western blot. Lane 1, heat solubilised hamster ZP was characterized with the lectin WGA. The lectin showed affinity for upper band (over 90 kDa) and 56 kDa band. Lane 2, heat solubilised hamster ZP was characterized with anti-pig ZP antibody. The antibody shows affinity for the 56 kDa band but no immunoreactivity was observed for upper band. The films were exposed for 15-30 seconds.

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presence of the 56 kDa band in the corresponding pellet containing the epitopes recognized by the anti-pig ZP antibodies. No immunolabeling was detected in the

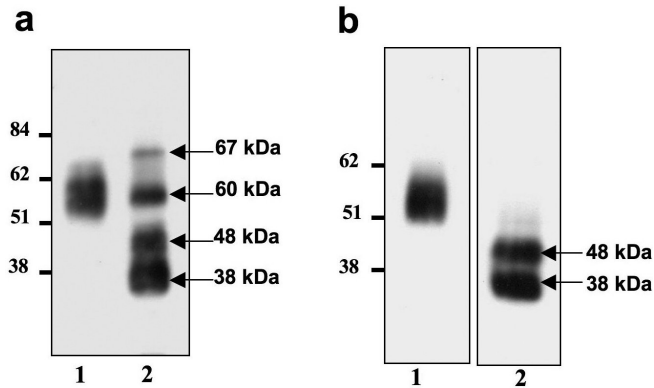


Fig. 4. Digestion of hamster ZP with PNGase-F. The samples were separated by SDS-PAGE under reducing conditions and the blot was incubated with anti-pig ZP antibody. **a.** Heat solubilized hamster ZP was digested with PNGase F. Lane 1, hamster ZP was treated under identical conditions, except for the omission of the glycosidase. Lane 2, solubilised hamster ZP was heated at 95°C for 5 min prior to incubation at 37°C overnight in the presence of 6 units of PNGase F. The blot showed that the antibody had affinity for the 56 kDa band, and when the N-glycans were deleted, four bands become reactive. We identify ZP1 (60 kDa), ZP2 (67 kDa), ZP3 (38 kDa) and ZP4 (48 kDa) based on molecular weight. **b.** The electroeluted 56 kDa band was digested with PNGase F. Lane 1, no treatment. Lane 2, electroeluted 56 kDa band was digested with PNGase F. The unique broad 56 kDa band appears as two different bands after the enzymatic digestion. The upper band corresponds to ZP4 and the lower one to ZP3 according to the apparent molecular weight. The films were exposed during 15-30 seconds.

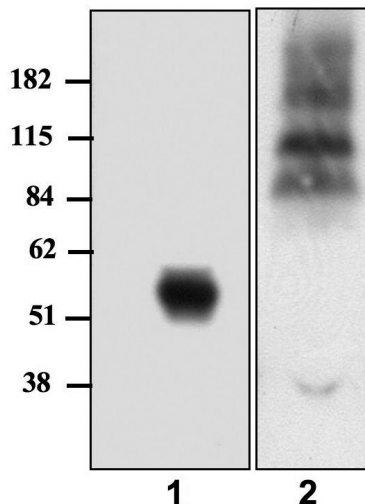


Fig. 5. Heat solubilized hamster ZP immunoprecipitated with anti-pig ZP. Lane 1, Pellet. Lane 2, Supernatant. The samples were detected with WGA lectin by means of western blot. The 56 kDa band was precipitate totally with the antibody. The films were exposed during 15-30 seconds.

supernatant using the anti-pig ZP antibody. The PVDF membranes were tested using WGA lectin (Fig. 5). Only a broad band corresponding to the hamster ZP3 was detected in the immunoprecipitated fraction; however, in the supernatant a major band corresponding to ZP1 and ZP2 glycoproteins was detected, thus indicating that no hamster ZP3 is contained in the supernatant fraction.

Discussion

Heterogeneity of the zona pellucida

It was previously described that hamster sperm binds mainly to the outer region of the ZP, which corresponds with the porous region in contact with the cumulus cells (Phillips and Shalgi, 1980; Ahuja and Bolwell, 1983). In these species, it was also observed that the outer surface of the ZP has a strong ability to induce the sperm acrosome reaction; however the inner surface of the ZP does not have this ability (Yazawa et al., 1995). Similar results have also been reported in human and pig ZP (Burkman et al., 1988; Fazeli et al., 1997). However, the cause of this fact remains unresolved. Since the carbohydrate residues are directly implicated in the sperm binding process, a different carbohydrate composition of the glycoproteins present in the outer and inner region of the ZP might be responsible for this process. A heterogeneous carbohydrate composition of the ZP was observed in different species using lectin cytochemistry (Avilés et al., 1996, 2000b; Raz et al., 1996; Jiménez-Movilla et al., 2004). The carbohydrate composition and distribution of the hamster ZP has been established using lectin cytochemistry (Roux and Kan, 1991; El-Mestrah and Kan, 2001). In these studies, it was reported that most carbohydrates are homogeneously distributed or mainly located in the inner region of the ZP. However, It was not previously described any carbohydrate residues mainly located in the outer region of the ZP. In this study, we have demonstrated that an epitope present in the pig ZP and shared by mouse, rat and hamster ZP is mainly located in the outer region of the hamster ZP. This is the first report demonstrating a difference between the inner and outer region of the hamster ZP that could be related with the different sperm binding properties showed by the inner and outer regions of the ZP.

In order to investigate how this heterogeneous distribution of the epitopes is generated and taking into consideration that the ZP is synthesized during the folliculogenesis, a cytochemical analysis of the ZP of different ovarian follicles was performed. The cytochemical analysis of the distribution of this epitope during the folliculogenesis demonstrated that it was expressed throughout the thickness of the ZP early during the folliculogenesis (multilaminar primary follicles). The ZP of large ovarian follicles (antral ovarian follicles) showed these epitopes in the outer region of the ZP; and almost no immunoreactivity was detected in the inner region of the ZP. The use of

epitopes-tagged ZP in combination with laser scanning confocal microscopy (Qi et al., 2002) and previous electron microscopical studies (Haddad and Nagai, 1977; Avilés et al., 1999, 2000a,b) suggested that the ZP is formed by the addition of newly synthesized ZP to the innermost region of the ZP. According to these studies, our cytochemical results could be interpreted by a selective oocyte expression of the epitopes detected by the anti-pig ZP during the early stages of the folliculogenesis. Western blot analysis demonstrated that the antibody used is specific for the 56 kDa band previously described as hamster ZP3 (Kinloch et al., 1990; Moller et al., 1990). A direct interpretation of these results suggests that the hamster ZP3 in the mature oocyte is located in the outer region of the ZP. This interpretation is contradictory with the established model of ZP synthesis and formation in mammals, especially in the mouse species. Thus, ZP glycoproteins are synthesized and secreted by growing oocytes (Bleil and Wassarman, 1980; Epifano et al., 1995). The distribution of the different ZP glycoproteins in the entire thickness of the ZP has been previously demonstrated by ultrastructural cytochemistry (Avilés et al. 1997b; El-Mestrah et al., 2002). Therefore, a more logical interpretation would be that these epitopes are not accessible to the antibodies in the inner region of the ZP of more mature ovarian follicles. To address this point an immunoprecipitation and biochemical analysis was performed to investigate whether two different ZP glycoproteins of 56 kDa exist on the basis of the reactivity to the anti-pig ZP antibody. The immunoprecipitation analyses showed that all ZP glycoproteins contained in the 56 kDa band present epitopes for the anti-pig ZP and consequently these glycoproteins were precipitated. These results, together with the cytochemical analysis described above, strongly support the hypothesis that some epitopes present in the ZP glycoproteins of the 56 kDa band are not accessible to the antibody in the native ZP; however, these epitopes are detected in the ZP treated for SDS-PAGE and Western-blot analyses. A similar result showing differences between the cytochemical and biochemical analysis of the egg envelope of *Discoglossus pictus* (Amphibia) was previously described (Caputo et al., 2001). The difference in the reactivity or accessibility of the antibody in the native ZP is probably due to a specific tridimensional organization or structure of the ZP. The ZP extracellular matrix changes continuously and thus the properties are also modified during the folliculogenesis (zona maturation) and after fertilization (zona reaction) (Oehninger et al., 1991; Avilés et al., 2000a,b; Sun, 2003; Dean, 2004; Nara et al., 2006). These changes could be produced by the participation of oocyte and granulosa cells. Thus, the oocyte can produce changes in the oligosaccharides composition of the ZP glycoproteins (Avilés et al., 1999, 2000a). Other processes, like the previously described precocious exocytosis of cortical granules, could be involved in this modification (Ducibella et al., 1988). These examples

and others could be responsible for the changes in the ZP (biochemical properties, tridimensional structure, etc). However, the molecular mechanism controlling the different organization of ZP glycoproteins remains to be clarified.

Evidence for the presence of four glycoproteins: ZP1, ZP2, ZP3 and ZP4

It has been accepted until recently that the ZP is formed by three glycoproteins. However, two recent proteomic studies have demonstrated that the human and rat ZP are formed by four glycoproteins ZP1, ZP2, ZP3 and ZP4 (Lefièvre et al., 2004; Boja et al., 2003, 2005; Hoobhoy et al., 2005). The difficulty in determining the composition of the ZP in different animal models was due mainly to: 1) the small amount of material available, and 2) the heterogeneity of glycosylation of the different ZP glycoproteins. This latter reason is responsible for the appearance of a broad band when the ZP glycoproteins are separated by SDS-PAGE. Thus, the identification of the different pig ZP glycoproteins was performed after deglycosylation (Yonezawa and Nakano, 2003). In this study, four different bands were identified by an anti-pig ZP antibody after the removal of the N-linked oligosaccharides. Immunocytochemical approaches demonstrated that this antibody recognizes mice, hamster and rat ZP. Western blot analysis of the mouse ZP using specific antibodies against the mouse ZP1, ZP2 and ZP3 corroborated this finding. Moreover, the isolation of the mRNA codifying for the hamster ZP1 (accession number EU003563), ZP2 (accession number AAW66610), ZP3 (accession number M63629) and ZP4 (accession number DQ838550) glycoprotein support that hamster ZP is formed by four glycoproteins.

Table 2. The amino acid sequence identities between pig, hamster and mouse zona pellucida proteins.

	PIG		
	ZP2	ZP3	ZP4
Hamster			
ZP1	32%	20%	47%
ZP2	54%	17%	33%
ZP3	19%	66%	NSS
ZP4	36%	22%	60%
Mouse			
ZP1	34%	27%	50%
ZP2	55%	NSS	33%
ZP3	NSS	67%	NSS

The identities between the proteins were estimated using the BLAST 2 Sequences, NCBI and this information is based on information available in public databases. Accession numbers: pig ZP2, NM_213848; pig ZP3, NM_213893; pig ZP4, NM_214045; hamster ZP1, EU003563; hamster ZP2, AY876920; hamster ZP3, M63629; hamster ZP4, DQ838550; mouse ZP1, NM_009580; mouse ZP2, NM_011775; mouse ZP3, NM_011776. NSS: No significant similarity.

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Future proteomic analysis and the production of antibodies against specific peptides of the different ZP glycoproteins are necessary to confirm the presence of these proteins, the molecular weight and their distribution in the native ZP.

Similarities between the ZP of different species

Previous reports have demonstrated that the ZP of different species show similar antigenic properties based on the observed cross-reactivity of the antibodies against the solubilized ZP from pig ovaries (Isojima et al., 1984; Maresh and Dunbar, 1987; Takai et al., 1987; Barber et al., 2001; Carino et al., 2002). In this study, we report that mouse, rat and hamster ZP share some antigenic sites with the porcine ZP. The cross-reactivity detected between the different species and the pig ZP is probably caused by the presence of similar carbohydrate and/or similar protein sequence. It is known that ZP proteins are glycosylated, and, it is possible that the common antigenic properties observed were generated by a similar carbohydrate composition of the ZP from different species. This was previously and thoroughly analyzed by lectin cytochemical studies and also by biochemical and biophysical analysis (Avilés et al., 1996, 1997; Easton et al., 2000; Nakano and Yonezawa, 2001; Dell et al., 2003; Jiménez-Movilla et al., 2004). However, the cross-reactivity observed between both the mouse and hamster ZP with the pig ZP may also be due to the described homology of the ZP proteins (Table 2). We consider that this is the main reason because the anti-pig ZP antibody used in this study has a strong affinity to partial deglycosylated ZP. Thus, the amino acid homology observed between hamster and pig ZP3 is approximately 66% (Table 2) making reasonable the observed cross-reactivity. In relation to the mouse, ZP2 and ZP3 of mouse and ZP2, ZP3 of pig shared a homology of 55% and 67% respectively. ZP1 glycoprotein is not present in the pig ZP. However, ZP1 glycoprotein has been reported to be present in the mouse ZP. The anti-pig ZP antibody used in this study has affinity for the mouse ZP1 and for a 67 kDa band that probably corresponds to hamster ZP1 according to the deduced protein sequence (accession number EU003563). This result is probably due to the 50% and 47% homology observed between the mouse, hamster ZP1 and pig ZP4, respectively. Hamster ZP2 gene was cloned (accession number AAW66610) and shared a 54% homology with the pig ZP2. However, despite the similar homology observed among the hamster, mouse and pig ZP2, cross-reactivity was observed only for the mouse, but not for the hamster ZP2 contained in the upper band of 90-182 kDa molecular weight (Moller et al., 1990). This difference could be due to different posttranslational modifications, e.g. glycosylation, which could prevent the binding of the antibody by steric hindrance. However, a band with a molecular weight of 48 kDa was specifically detected after deglycosylation of the hamster ZP. This molecular weight is very similar

to that expected for the mature secreted protein sequence that is formed by the removal of the signal peptide and cleavage at the furin consensus site as described for other ZP glycoproteins (Boja et al., 2003, 2005; Zhao et al., 2004;). A similar finding was observed for the hamster ZP4. The predicted hamster ZP4 protein sequence (accession number DQ838550) shares 60% homology with the pig ZP4 (ZPB). The estimated molecular weight of the mature secreted protein is very similar to the 48 kDa band detected after deglycosylation of the total hamster ZP and 56 kDa band.

In summary, this study demonstrated that a change is produced in the structure of the hamster ZP during the folliculogenesis that could be responsible for a differential exposure of some ZP determinants. This structural change could be related to the differential sperm binding affinity observed in the thickness of the ZP. Another major finding of this study was that the polyclonal antibody produced against the pig ZP cross-reacts specifically with the native hamster ZP glycoprotein with an average molecular weight of 56 kDa. However, the deglycosylation treatment reveals that the antibody reacts with four different bands, suggesting that the hamster ZP is formed by four different glycoproteins as in human and rat.

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