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Original article

Texture of the zona pellucida of the mature pig oocyte. The mammalian egg envelope revisited*

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Abstract – The zona pellucida (ZP) of mature pig oocytes is believed to consist of a dense filamentous meshwork, less compact on the inner and outer faces. The uneven surface of the ZP is made of unordered and stretched fibrils surrounding deep funnels which are the openings of the radial canaliculi. The topography of the ZP surface may contribute to the initial interplay between male and female gametes. Using cytochemical techniques for transmission electron microscopy (TEM), such as tannic acid and ruthenium red treatments, we found that the ZP of pig oocytes was essentially made of bundles of fibrils distributed in concentric layers (except in the innermost and outer parts). A correlation appears between the dense structure of the core layer of the ZP and its texture: it is constituted of superposed layers of fibril bundles, whereas only a random meshwork is found in a very thin innermost and in the outer layer. The fascicular configuration may control the permeability of the ZP, giving its semi-rigidity and elasticity, and may facilitate sperm penetration. The liquid crystal-like design of the core layer of the ZP is similar to textures found in the the vitelline envelope (zona radiata) of other vertebrates and possibly of all the deuterostomes. Such texture is probably related to the unique ZP protein composition and to a coordinated synthesis.

vitelline envelope / ultrastructural cytochemistry / glycoproteins / liquid crystal-like texture / *Sus scrofa*

Abbreviations
PVS: perivitelline space; SEM: scanning electron microscopy; TEM: transmission electron microscopy; ZP: zona pellucida.

1. INTRODUCTION

The oocytes of animals, including vertebrates, are surrounded as a rule by an envelope called either the vitelline envelope, the chorion, the zona radiata or the zona pellucida [1]. The thickness of the mammalian zona pellucida (ZP) is species-specific, e.g.

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around 5 μm in the mouse and around 10 μm in the pig [2, 3]; the multiple roles of the ZP have been thoroughly reviewed [4, 5]. The site of ZP synthesis is the oocyte during its growth phase, at least in the mouse [6–8] and/or the follicular cells in other species [3, 9]. A unifying hypothesis, at least on the composition of these envelopes, has been offered by molecular biology: the constitutive proteins are homologous and all belong to the ZP protein family [3, 10–12]. Does a common composition induce a similar structure of egg envelopes? The answer was readily given for several groups of vertebrates by electron microscope observations: in fishes and amphibians, e.g., the main part of the envelopes appear to be made of fibril bundles or sheets distributed in superposed layers [1, 13, 14]. Similar observations are lacking for mammals where "it would be of great interest... to compare the high-resolution structures of vitelline envelope and ZP glycoproteins" [15]. The aim of the present work was to obtain preliminary results on the ZP substructure of the pig as a model for mammals. We therefore used cytochemical techniques on maturing and ovulated oocytes of two different breeds. In all cases, the main part of the pig ZP appeared to be built of superposed layers of fibril bundles.

2. MATERIALS AND METHODS

2.1. Collection of oocytes

We used the same animals as in a previous work [16]. Ten prepubertal 5–6 month old gilts of miniature pigs (a cross-breed of Minnesota and Göttingen strains) were stimulated by 1000 IU pregnant mare serum gonadotropin (PMSG) (Antex, Leo, Copenhagen) and 72 h later by 1500 IU human chorionic gonadotropin (hCG) (Praedyn, Spofa, Prague) to obtain synchronized maturation. Injection of hCG represented time 0 for subsequent in vivo or in vitro meiotic maturation. Moreover, untreated cyclic gilts of the Large White breed were monitored daily for the onset of oestrus with a boar. Unpenetrated oocytes were recovered by flushing the oviducts with phosphate buffered saline.

2.2. In vitro maturation

Oocyte-cumulus complexes (OCC) were mechanically isolated from excised ovaries of gilts at different times during treatment, after rupture of the follicular wall of the large preovulatory follicles. Some of the OCC were cultured in 0.1mL of the following medium under paraffin oil at 38 °C with 5% CO2 in air: 18 mL of 1.45% NaHCO3, 0.002% phenol red, 10 mL of 5.5% (w/v) glucose solution, 4 mg sodium pyruvate, 30 $mg \cdot mL^{-1}$ freeze-dried calf serum growth proteins (Usol, Prague), 50 IU penicillin and 5 $\mu g \cdot mL^{-1}$ streptomycin were added to 72 mL of isotonic TC 199 medium (Usol, Prague). During in vitro maturation, the oocytes had reached MI after one day of culture and were in MII after two days of culture.

2.3. Electron microscope morphological and cytochemical studies

Preparations for transmission electron microscopy (TEM) were made as described previously [16]. For the better preservation of glycoproteins and/or glycosaminoglycans, preovulatory oocytes, oocytes cultured in vitro and ovulated oocytes were treated during osmium tetroxide postfixation with 0.5% ruthenium red [17] or 1.0% tannic acid [18]. A series of ovulated oocytes were treated with 1% pronase (Calbiochem B grade) during 10 to 30 min at 38 °C before fixation as above and ruthenium red treatment.

3. RESULTS

We call structure, the general architecture (overall morphology, compactness, transverse canaliculi) of the ZP and texture, its submicroscopic constitution (filamentous meshwork organization).

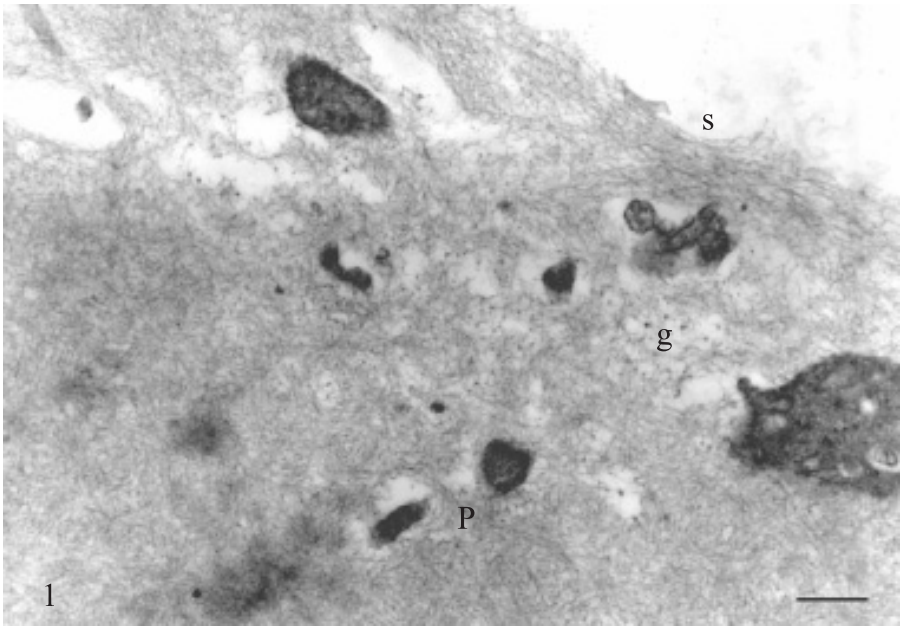

Figure 1. The outer part of the ZP of a pig oocyte collected 20 h after hCG and cultured in vitro until MII, shows a transition between inner fibril bundles and a porous surface (S) meshwork. Both layers are traversed by corona cell projections (P). Granules (g) contrasted by ruthenium red are found at the intersection of fibrils, mainly at the limit of the main and outer part of the ZP. Scale bar: 1 μm.

3.1. Structure

With standard techniques of TEM, the structure of the ZP of maturing and mature oocytes appeared as a dense fibrillar meshwork, less compact in the very thin inner and in the outer layer. The ZP was perforated by many funnel-like canaliculi containing cytoplasmic projections of the corona cells. When contrast was enhanced by use of tannic acid or ruthenium red, a fine random meshwork was visible in the outer (and a thin inner) layer of the ZP where electron dense granules were sometimes observed at fibril crossings (Fig. 1).

3.2. Texture

A different texture was observed in most of the thickness of the ZP: its fibrillar meshwork appeared not randomly arranged, but organized in closely apposed parallel bundles, themselves distributed in stratified layers (Figs. 1 and 2). A high magnification of the bundles is shown in Figure 3. Investigation on a limited number of samples showed that the diameter of the fibrils cut transversely varied from 6 to 14 nm after tannic acid or from 6 to 13 nm after ruthenium red treatment and the diameter of the bundles was 170 to 230–240 nm in both cases. The diameters were grouped around two values for fibrils, 6 and 9–12 nm and around 200 nm for the bundles. The distance from the centre to the centre of the bundles in a row was approximately 250 nm, which means that the bundles may be separated by a small space.

The bundle arrangement was observed in the ZP during in vitro maturation, after both full in vivo or in vitro maturation of miniature pig oocytes, and also in ovulated

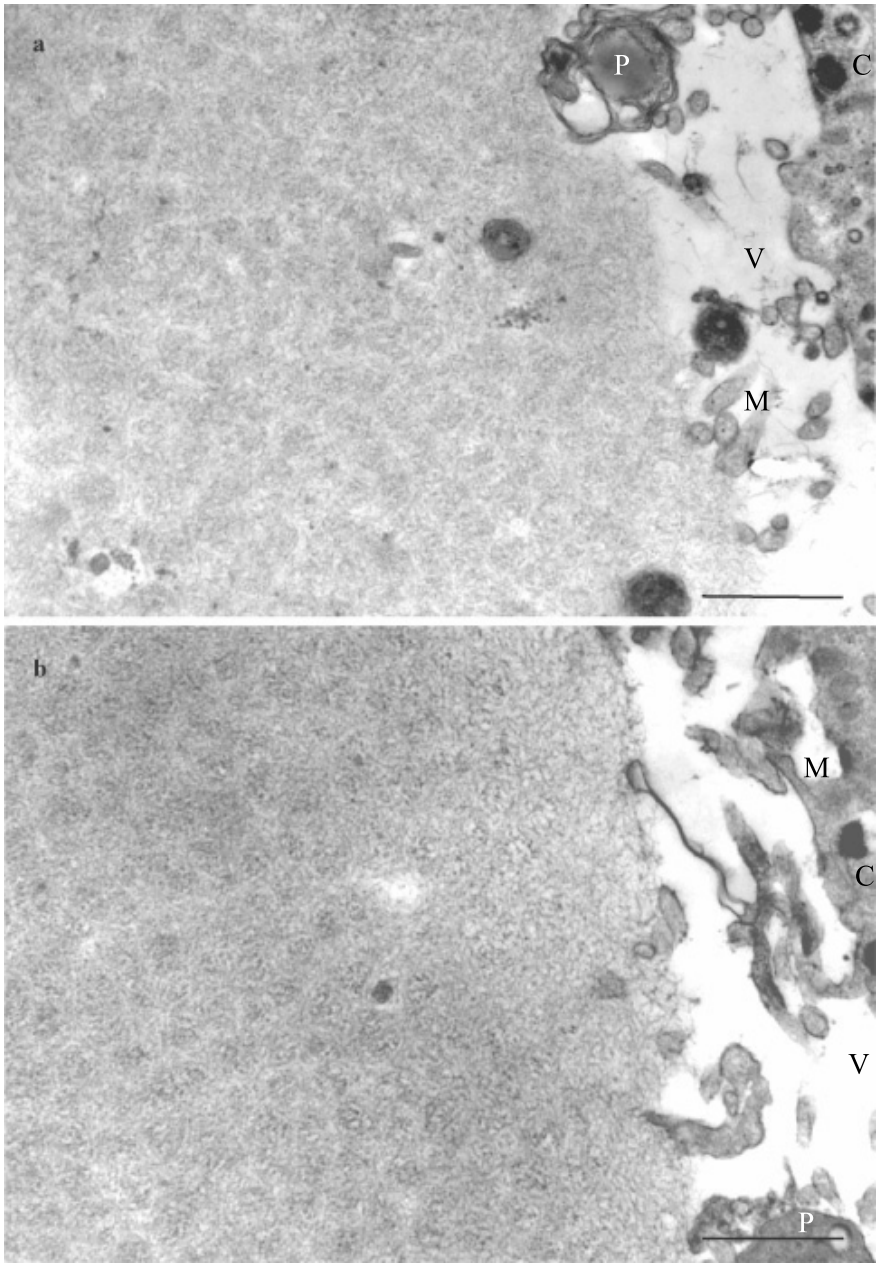

Figure 2. TEM of the ZP (central and inner part) of pig oocytes after tannic acid (**a**) and ruthenium red treatment (**b**). (**a**) Oocyte collected 20 h after hCG and cultured until MII. (**b**) Oocyte entirely matured in vitro. In both cases the ZP appears to be made of bundles of fine fibrils oriented parallel in cross section. Such regular texture is progressively less defined towards the inner face. Cytoplasmic structures in the PVS are corona cell projections (P) and oocyte microvilli (M). Cortical granules (C) are visible under the oolemma. Scale bar: 1 μm.

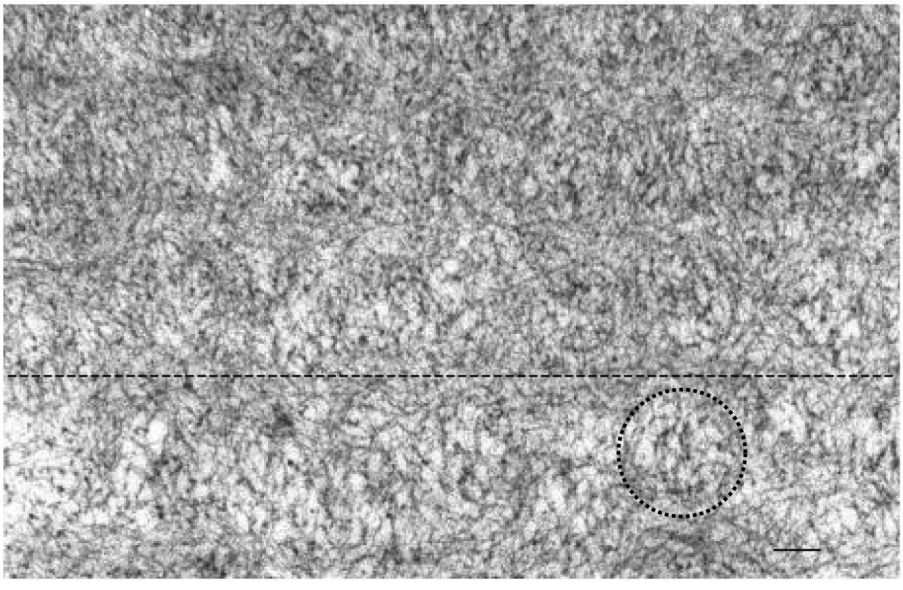

Figure 3. High magnification of a portion of the main part of the ZP of an oocyte matured in vitro until MII after ruthenium red treatment. The over-contrasted view shows the profile of the parallel fibril bundles in cross section. The contour of one bundle and the limit between two bundle layers were marked. Scale bar: 0.1 μm.

oocytes of the Large White breed. Such texture was even more evident when the ZP was partially digested by pronase in conditions giving complete lysis in around 30 min; in this case, the bundles appeared separated from each other before being digested (Fig. 4). A scheme representing the distribution of the parallel fibril bundles in superposed layers is given in Figure 5.

4. DISCUSSION

We will focus the discussion on the similarity of texture between the main part of the pig egg envelope and that of other vertebrates. In a second part of the discussion, we will analyse the relationship between texture and chemical composition and evaluate the implications of the dual texture of the ZP on its functions at the time of fertilization.

4.1. ZP Structure and texture

4.1.1. Structure

After in vivo or in vitro maturation, the ZP of pig oocytes showed the same structure as that observed in other eutherians, that is a dense fibrillar meshwork, becoming less compact on both faces or at least on the outer face [19–22]. The ZP is perforated by radial canaliculi containing the cytoplasmic processes of the corona cells and contacting the surface of the immature oocyte [23]. It should be stressed that these corona cell expansions are not at any time actively penetrating the ZP; the latter is built around these processes as well as around the short oocyte microvilli [24].

The trans-zona corona cell expansions form crater-like openings on the ZP surface, as illustrated by SEM observations of mature

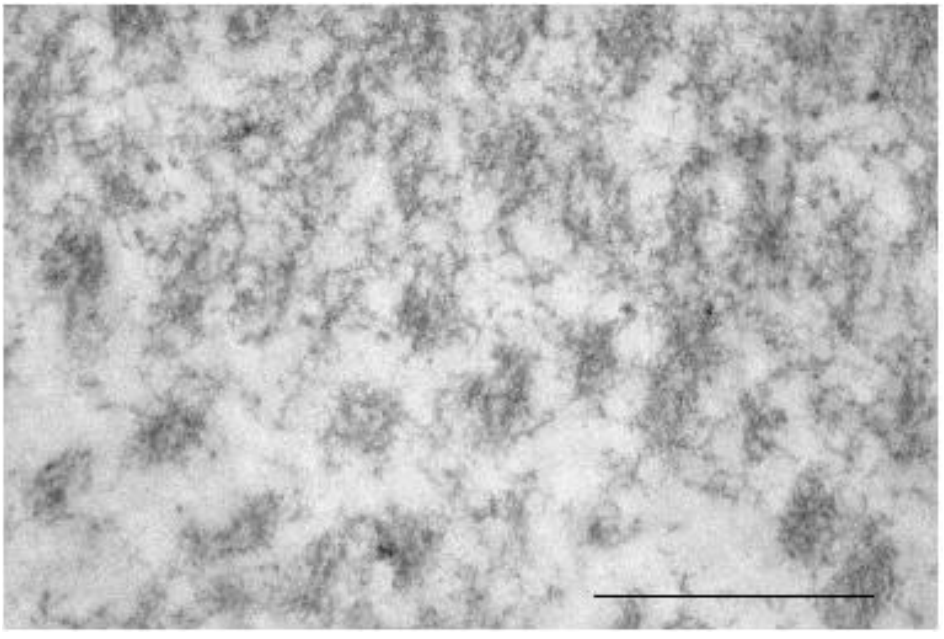

Figure 4. Progressive –from outside (bottom-left) to inside (top-right)– dissociation and lysis of the fibril bundles in the main part of the ZP of an ovulated oocyte after treatment with 1% pronase (25 min at 38 °C) before fixation and ruthenium red treatment. Scale bar: 1 μm.

Figure 5. Scheme of a few stratified layers of parallel fibril bundles in the core layer of the ZP. On a short length, the bundles appear parallel and rectilinear. The eventual small space between the bundles is neglected.

pig oocytes [25] and of oocytes of other species [26, 27]. A porous outer surface was also observed on the ZP of marsupial oocytes [28].

4.1.2. Texture

Following tannic acid and ruthenium red treatments, the observed diameter of the pig ZP fibrils (6 to 12 nm) was comparable to the dimensions of rat fibrils (4–7 nm) contrasted with tannic acid [29] and to extracted mouse fibrils, looking like "beads on a string", whose beads measured about 9 nm in diameter [22]. With both techniques, the two more frequent diameters observed for fibrils may correspond to the beads and the strings.

After use of ruthenium red, we occasionally observed granules at the fibril intersections in the superficial meshwork of the ZP which looked like the granules described at the onset of ZP deposition between the follicular cells and the oocyte [24]. They probably represent accumulations of the contrasting agents [26] rather than the structures of unknown nature described throughout the rabbit [30] and pig ZP [24].

Tannic acid and ruthenium red treatments showed that the core layer of the ZP was built of bundles of fibrils oriented in strata parallel to the oocyte surface. This corresponds to a cholesteric liquid crystal-like state found in many biological systems [31].The diameter of the bundles and their alinement were not rigorously uniform, organic structures being subject to variations and defects even present in the mineral crystals. How is it possible to interpret the distribution of bundles in three dimensions? The bundles may be arranged like the threads in a wool ball, forming with each other angles too small to be appreciated on a transverse section. The texture of fibril bundles did not change during maturation, all the more if the latter was incomplete in vitro, as we found it identical after ovulation. The formation of the ZP is probably achieved at the final stage of oocyte growth [32] and it is readily penetrable by sperm in germinal vesicle oocytes [33]. Although alterations in ZP thickness have been reported after ovulation [20], our results show that, if true, they do not affect the fasciculated configuration. However, changes in bundle association (compaction) were observed in the vitelline envelope of *Xenopus*, after ovulation [34], and modifications may also occur particularly in the outer layer. Differences in pig ZP between oocytes matured in vitro or in vivo may be attributed to an incomplete maturation in the first case and/or to extracellular matrix addition in the second case [20, 35].

The fascicular configuration of pig ZP was the same in miniature pigs and in the Large White breed. It was not an artefact due to in vitro maturation or to the treatment by tannic acid or ruthenium red, since we observed the same texture after in vivo maturation, even without the use of tannic acid or ruthenium red. However the contrast was very low in the latter case.

Superposed sheets or bundles of fibrils were observed in teleost inner zona radiata [36, 37], in *Xenopus laevis* [14] and *Discoglossus pictus* [13] egg envelopes. Moreover, in echinoderms, the oocyte envelope also looks threaded like a tennis ball framework [38]. So it is suggested that the egg vestment has evolved in the deuterostome phylum from a common model, which must be correlated with the coordinate deposition of its components [21, 39]. If the texture of pig ZP is similar to that of various vertebrates, it may be taken as a plausible model for other eutheriens.

The uniformity of ZP texture in vertebrates can be explained by a common composition actually limited to a few glycosylated proteins, such as in the pig [21]. These glycoproteins all belong to the ZP family and generally contain a common domain or "ZP module" [9]. There are three single-copy ZP genes in mammals ZPA, ZPB, ZPC [3] including marsupials [11]. Depending on the species, ZPA in the mouse [40] or ZPB in the pig [3, 41] would constitute sperm receptors. More generally, it appears that all egg envelope glycoproteins of vertebrates belong to the same family [10, 12, 21, 42].

In the mouse, the non covalently bound proteins ZP2 and ZP3 form filamentous polymers cross-linked by the third protein ZP1 [8]. Such macromolecular complexes may well give rise to the fibril bundles we observed. The ZP proteins are sensitive to proteases (including acrosin) inducing specific cleavages in ZP proteins, but not complete lysis, [9], to disulphide-bond cleaving agents and low pH (inducing changes in glycoprotein conformation, but not disruption of covalent bonds [43]). The use of pronase (in the present work) may explain the unveiling of the pig ZP texture by separating the fibril bundles. Solubilization with Li–3,5 di-iodosalicylate of pig ovarian oocytes resulted in the complete separation of the fibrils on the ZP surface and in the disclosure of fibril bundles in the interior [19]. The authors have overlooked the meaning of their observations which is presently revealed. Conversely we suspect that destabilization with saponin before fixation may disorganize the native texture of the ZP [20, 26]. Nevertheless "areas of major condensation",

probably bundles, are revealed this way in the ZP core layer of in vitro fertilized human ova [26].

4.2. Is the ZP texture correlated to its local chemical composition?

Our results show that the outer (and the thin inner) part of the ZP are not only less compact than the core layer as already known, but that they also have a different physical texture. Are the pig ZP layers of different chemical composition and/or cellular origin? Several, non exclusive either physical or chemical explanations can be given to the heterogeneity of the ZP.

The zona radiata of fishes shows several layers of different texture corresponding to the successive steps of deposition [1]. In a teleost, "the amorphous material of the inner zona radiata is secreted by the oocyte and assembles in a filament which then forms part of a highly ordered... layer" [37]. Similarly, the mode of deposition of the mammalian ZP proteins may differ from the onset to the end; the ZP first appears as disconnected patches of a fuzzy meshwork between the oocyte and follicle cells [24]. When becoming continuous, the ZP may gain a more regular texture. Moreover, at the end of the process, the first deposited and now peripheral meshwork may be stretched and/or disrupted during the increase of ZP thickness. On the inner side of the ZP, the building of bundles may be interrupted when the ZP protein synthesis is declining.

The texture heterogeneity may explain a difference in layer permeability, responsible for a pseudo chemical heterogeneity. Effectively, although the ZP is permeable to a large spectrum of components [23, 44], differential staining of the ZP layers was obtained by treatment *en bloc* of the oocytes with antibodies or lectins [19, 45, 46]. The diffusion of (immuno)cytochemical reactions must be easy in the spongy outer layer; it may be progressively hampered while the ZP is becoming a plugged filter or when reagents precipitate in the periphery [47, 48]. The radial canaliculi would not play any role in permeability if they are effectively closed after corona projections withdrawal. In vivo contamination of the ZP surfaces by the extracellular matrix during maturation [16, 44, 49] may also alter stainability [50].

The polymorphism of ZP proteins can result from many post-transcriptional modifications including Asn-(N-) and Ser/Thr-(O-) glycosylation and addition of disulphide-bonds and sialic acid groups [15]. The staining of ovarian sections with lectins for sugar distribution revealed heterogeneities in the ZP (layers of different affinities) of eutherians [50, 51] and marsupials [52]. So glycosylation may change, particularly at the onset and/or the end of ZP secretion, inducing stronger lectin binding in the outer and/or inner faces of the ZP.

Immunochemistry on thin sections using antibodies to ZP1, ZP2, ZP3 revealed a homogeneous protein distribution in the ZP of mouse ovarian oocytes, although local densities may correspond to fibril aggregates (bundles?) [6]. However, in other species, changes may occur in ZP protein gene expression during the course of synthesis and/or according to the site of synthesis, that is corona cells versus oocytes [3]. In pigs, although a contribution to ZP3 by follicular cells has been reported [53], transcripts of ZP1 were found only in growing oocytes [54], and no labeling was observed at the TEM level in follicular cells using three monoclonal antibodies to ZP proteins [55].

In clonclusion, the model of mammalian ZP we propose has at least two layers of different texture probably responsible for unequal permeability and contamination by secretions of the oocyte and of the cumulus oophorus. ZP heterogeneities may just result from the order of synthesis or from mechanical effects during deposition. Moreover they may also be due to a different chemical composition (changes in ZP proteins or in glycolysation).

4.3. Physiological properties of the Zp in mature oocytes inferred by its texture

4.3.1. Surface

The chaotic structure of the surface, and particularly the craters, when made empty by the loss of corona cells which begin to disperse 3 h after ovulation in the pig, may eventually be mechanical traps where spermatozoa start to penetrate the ZP tangentially (Fig. 2 in [56]). In this species, the supernumerary heads remain trapped almost parallel to the surface of the ZP in its non compacted easier to penetrate outer part [57]. SEM observations also indicate that sperm heads initiate penetration parallel to the surface of the hamster ZP [58, 59] and that the physical properties of the outer surface of the ZP may be important for human sperm adhesion [60]. An extreme case of physical interaction between mammalian gametes is given by a shrew, the sperm of which appears to bind to the ZP after the acrosomal reaction by barbs of the perforatorium [61]. Anyway, adhesion to- and onset of penetration of the ZP would probably be less easy if its surface was flat and smooth. The physical interactions between the sperm and ZP surface could be redundant with the elusive ligand-receptor system [62]. Both phenomena may explain why sperm do not bind efficiently to the inner face of the ZP when externalized [45, 63]. So the surface of the ZP appears to be functionally adapted by different means including its structure to sperm binding.

4.3.2. Core layer

The mechanical properties of the ZP were previously compared to those of a sulfated glycoprotein gel [21]. In fact, the texture of the main part of the ZP, made of several layers of fibril bundles (like a tire armature), can explain the well known envelope resistance and elasticity useful for micromanipulation.

The generally oblique (parabolic?) "penetration curve", is observed in the ZP of several species including the pig [64, 65]; it may result from the sliding of the flat and asymmetrical (spoon-like or hook-like) acrosome-reacted sperm head, due to a hyperactive movement of the flagellum [66], through the parallel fibril bundles. If the penetration is purely mechanical [67], the insinuation of the sperm head between the bundles may just slightly distort the compact material of the core layer in eutherians [68] as well as in marsupials [28]. Contribution of acrosomal enzymes remains to be clearly demonstrated. In didelphid marsupials however, the thin protease sensitive ZP would allow its perforation by acrosomal enzymes [67]. In teleost oocytes, it is well known that sperm penetrates via the micropyle [1]; in this case, the layers of fibril bundles are oriented at different angles as in plywood, a type of texture that would inhibit sperm edging; in mammals, the fibril bundles would be almost parallel, as discussed above. So in vertebrates, sperm penetration could depend on the physico-chemical and substructure variants of the vitelline envelope, although at least its main part has a common constitution of ZP proteins forming superposed fibril layers or bundles. In this context, unveiling the texture of the ZP in other mammalian species, particularly in those where sperm penetration is atypical, will be rewarding. It will also be interesting to consider how important the ZP texture is on the "zona reaction" after fertilization and on zona permeability, distension and shedding during early development.

5. CONCLUSION

Besides the known uniformity of composition, the present demonstration that the texture of the main part of a mammalian ZP is not an exception among that of vertebrates, is a new confirmation that "Vertebrate egg envelopes… are basically similar" [1]. It appears that the composition and substructure of the egg envelopes of vertebrates are very

well adapted to their functions, since they appear to have evolved little even after the divergence of the tetrapodes from fishes.

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