

Structural changes of the zona pellucida during fertilization and embryo development

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1. ABSTRACT

The zona pellucida (ZP) is a unique extracellular coat surrounding the maturing oocyte, during ovulation, fertilization, and early embryo development. It is formed by three/four glycoproteins. Ultrastructural data obtained with transmission (TEM) and scanning electron microscopy (SEM) were compared with molecular data on the glycoproteins network from ovulation to blastocyst formation. Molecular models are quite different to the morphology obtained with TEM, which shows a microfibrillar architecture, or with SEM, which shows a spongy or smooth surface. The saponin-ruthenium red-osmium tetroxide-thiocarbohydrazide technique allows to show the ZP real microfilamentous structure and the related functional changes. These results support an ultrastructural *supramolecular model*, more similar and comparable to molecular models related with the glycoprotein network. A detailed mapping of single mammalian ZP proteins and their relationship within the supramolecular architecture of the zona matrix would clearly supply insights into the molecular basis of sperm-egg recognition. Differences in ZP glycoproteins among mammals do not affect structural morphology; further studies are needed to clarify the relationships between ultrastructural and molecular organizations.

2. INTRODUCTION

The mammalian zona pellucida (ZP) is a particular extracellular coat that surrounds the oocyte at ovulation and that plays an important role in the process of fertilization up to embryo implantation. Mammalian ZP mediates initial sperm-egg recognition, an essential step in fertilization, undergoing structural modification after fertilization and blocking polyspermy. Sperm-egg recognition is taxon-specific, particularly in humans, and is mediated by receptors for homologous sperm in the egg (1-13).

The ultrastructural and functional variation as well as the biochemical and molecular composition of the ZP have been extensively studied during the last 40 years in different mammalian species (14-27). A very important knowledge is that mammalian ZP is composed by a highly ordered filament structure which constituting glycoproteins bear remarkably similar structural features (28-29). The molecular composition of mammalian ZP is characterized by the presence of long and thin filaments that are visualized after solubilization of the ZP and negative contrast but that are not visible using conventional ultrastructural techniques such as transmission (TEM) and scanning (SEM) electron microscopy (18). In fact, the use

of these techniques shows a similar ZP morphology in almost all species observed although the molecular composition varies among different species (30).

Several studies have focused on the molecular mechanisms at the basis of mammalian fertilization. Nevertheless, due to difficulties related to direct studies performed in humans, the mouse is still considered a good model for studying human fertilization. In fact, human studies on ZP morphology generally correspond to invasive analysis performed mainly on oocytes failing fertilization or containing abnormalities (28). Recently, orientation-independent polarizing microscopy (Polscope) became a breakthrough in that it is a non-invasive analysis that can be applied to study paracrystalline structures such as the ZP (28).

With the aim to review and integrate ultrastructural and molecular data on the ZP during ovulation, fertilization, and preimplantation development, this work is devoted to analyze and compare the microanatomy and molecular organization of mammalian ZP, including human, and the possible relationship existent between these features and the functional properties of the ZP during the post-ovulatory period, fertilization, and preimplantation development with special reference to human reproduction.

3. ULTRASTRUCTURE OF THE ZP IN OOCYTES AT OVULATION

Concerning the origin of mammalian ZP, there is evidence that ZP glycoproteins are very likely synthesized by the oocyte and/or the follicle cells (31-33). However, the horse zona protein synthesis during *in vivo* maturation is completely overtaken by the cumulus cells (34). Zona layers are laid down in a temporal sequence, with the inner layer laid down last in mice (35). The formation of the ZP matrix involves a regulated proteolytic process mediated by a furin convertase family member (36).

There is considerable interspecies variation in the thickness of mammalian ZP (30). ZP thickness of human MII oocytes is about 15-20 μm , twice as thick as that of the mouse (4,8,28,37-38), and is inversely related to estrogen levels (39). The structural proteins of the inner portion of the zona are more densely packed than those of the outer portion, in relation to the stretching of the external portion of the matrix necessary to contain the oocyte enlargement, over 300-fold increase in cell volume (40).

3.1. Conventional electron microscopy

If observed by conventional TEM the mammalian ZP displays a homogeneous microgranular appearance throughout its thickness and is characterized by an irregular, rough outer margin. Fine filaments are seen that are more loosely arranged on the outer surface of the ZP, and large regions of the ZP interdigitate with surrounding cumulus oophorus cells (Figure 1) (30, 41). By conventional SEM, in turn, the mammalian ZP shows different structural patterns in its outer and inner surfaces. In mice, the outer surface is filled by numerous

fenestrations, giving it a somewhat spongy appearance whereas the inner surface is relatively smooth and compact (42-44). Regional differences have been also reported in the surface of hamster ZP (43-46). In a similar way, the ZP of canine, rabbit, bovine, equine and porcine oocytes appears as a fibrous and fenestrated network (33-34,47-52).

Whether the ultrastructure of the ZP relates to the maturational stage of the oocyte and/or to sperm binding and penetration is still matter of debate. The ZP of human oocytes approaching meiotic maturity undergoes maturational changes and becomes more susceptible to sperm penetration. In fact, spermatozoa are able to fertilize metaphase II but not metaphase I oocytes, ascribing this to differences in ZP ultrastructure (53). Whereas the ZP of metaphase I oocytes showed a compact and homogeneous aspect, the ZP of metaphase II oocytes appeared as a highly porous structure (53). A correlation between the morphology of the ZP surface and the degree of oocyte maturity has been described in mice (54) and humans (55-57).

In our studies performed with conventional SEM, the ZP structure in human oocytes shows two basically different patterns: the first one is a multilayered large network resembling a spongy structure, the second one which shows a compact and smooth surface (Figures 2, 3) (55-57). The former was usually seen in cultured mature oocytes whereas the latter was commonly seen in cultured oocytes belonging to immature and atretic groups. On the other hand, a greater number of penetrating spermatozoa was noted in oocytes having a spongy ZP (Figure 4) whereas few if any sperm appeared flattened against the surface of oocytes showing the more compact, smoother ZP (Figure 5). Nevertheless, the inner surface of the ZP, as seen by conventional SEM, showed a compact structure in all cases. We hypothesized that the condensation of the outer aspect of the ZP caused a disorientation of sperm-binding sites, which would result in reduced sperm binding and penetration capacity, thus leading to impairment of *in vitro* oocyte fertilizability.

Windt *et al.* (58) evaluated at the ultrastructural level recurrent and *in vitro* maturation-resistant metaphase I-arrested oocytes and they considered the "narrow and fibrous" appearance of the ZP as characteristic of the immature stage. These results were not further confirmed in which no significant structural changes were seen between immature oocytes and those matured *in vitro* for 48 h, even if at 24 h the ZP structures were more similar to those of *in vivo* matured oocytes (59). On the other hand, using traditional SEM observations, Magerkurth *et al.* (60) reported that human oocytes had an extremely heterogeneous morphology of the ZP surface, within and among patients. Furthermore, these authors described four different types of ZP morphology considering four categories of oocytes (mature, immature, fertilized and unfertilized oocytes), from a porous, net-like structure to a nearly smooth and compact surface. According to these authors, no correlation could be established between ZP type and oocyte maturity or fertilizability and, therefore,

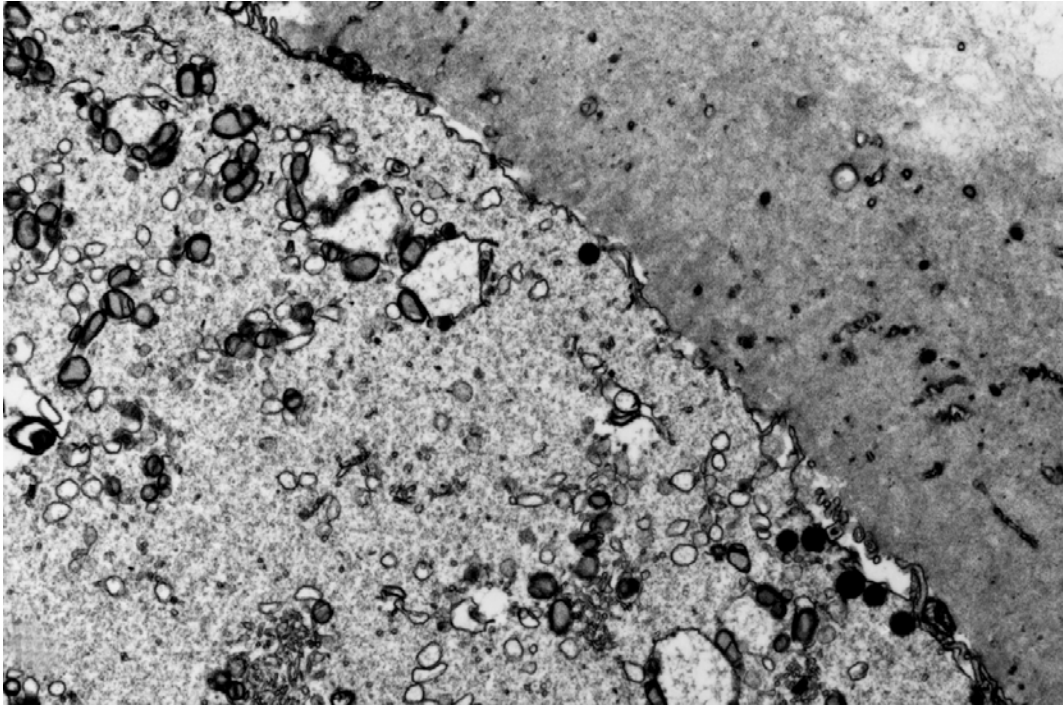


Figure 1. Mature oocyte. Zona pellucida displays a homogeneous microgranular appearance throughout its thickness and is characterized by an irregular, rough outer margin. TEM, 3800X. Reproduced with permission from (99).

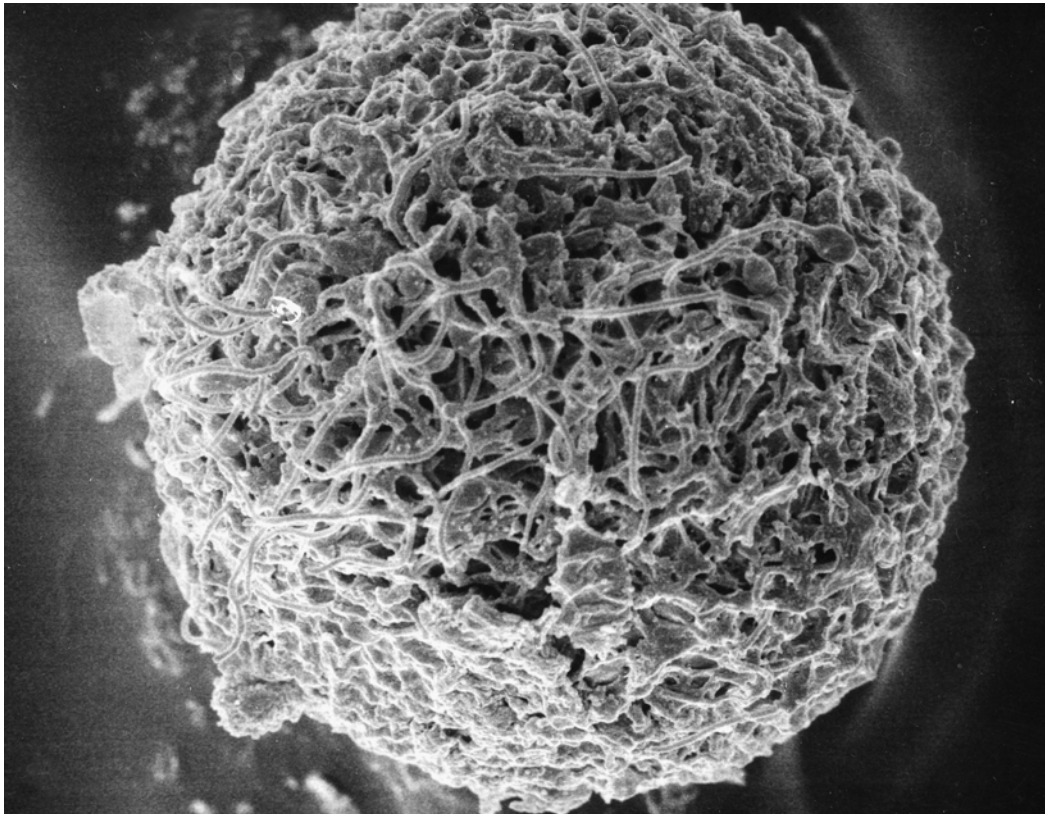


Figure 2. Mature oocyte, the outer surface of the zona pellucida looks spongy. SEM, 1200X.

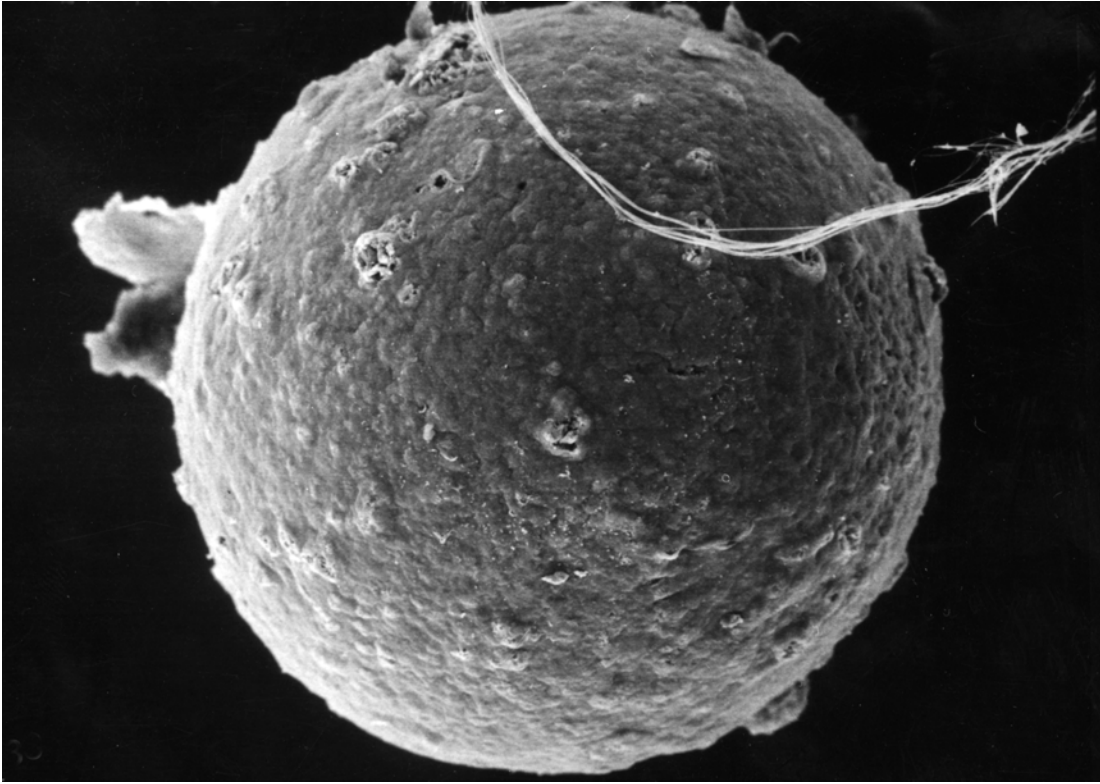


Figure 3. Atretic oocyte, the outer surface of the zona pellucida appears smooth and compact. SEM, 700X.

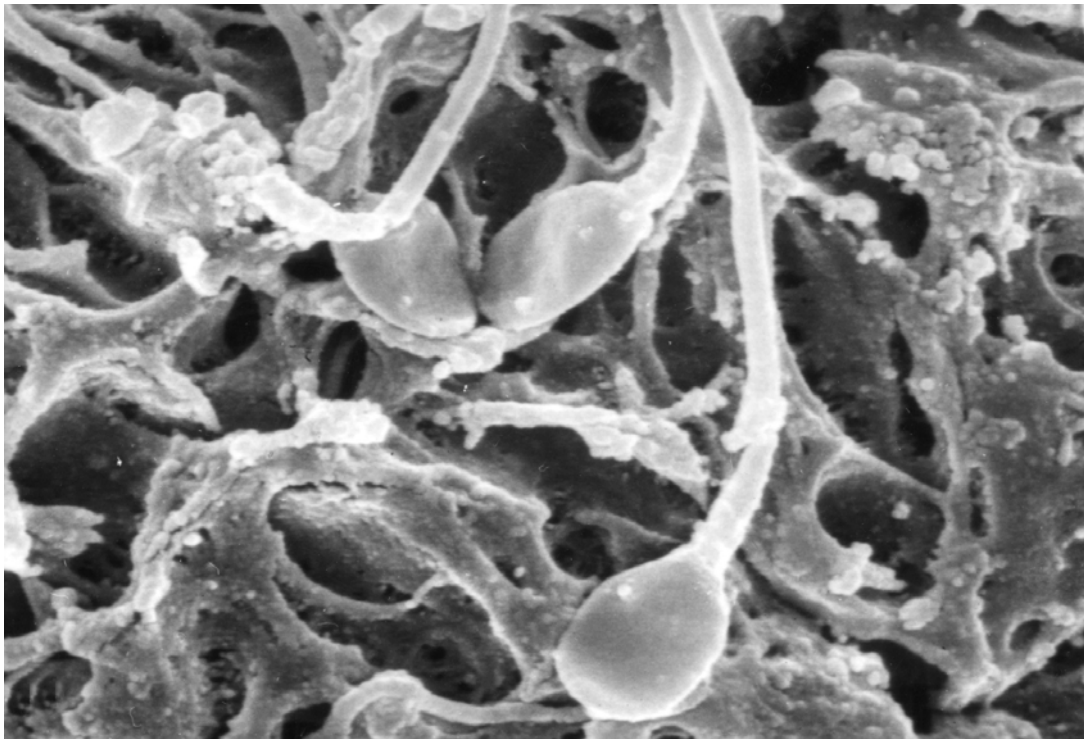


Figure 4. Oocyte with spongy zona and sperm tangentially attached to its surface. SEM, 3000X. Reproduced with permission from (55).



Figure 5. Oocyte with compact zona and sperm flattened on its surface. SEM, 3000X. Reproduced with permission from (55).

they suggested that the heterogeneous morphology of the ZP plays no important role in sperm-oocyte interaction.

Studies of Henkel *et al.* (61) on the influence of elevated pH levels on the morphofunctional features of human ZP corroborate a possible relationship between surface ZP morphology, i.e. spongy vs compact/smooth microarchitecture, and its sperm-binding capability. The percentage of binding increase as the structure becomes less tight. Acidic ZP solvents may significantly improve sperm binding to immature ZP in sperm-oocyte co-incubation if compared to control samples supplemented with basic ZP solvents which have exhibited sperm binding ranges corresponding with those reported for prophase and metaphase I oocytes (61). On the other hand, reduction or disappearance of cortical granules and consequent zona hardening are observed either in fertilized oocytes, as well as in unfertilized mature (frozen/thawed) and aged oocytes (61-62).

3.2. High Resolution SEM and TEM with saponin-ruthenium red-osmium tetroxide-thiocarbohydrazide (Sap-RR-Os-Tc)

It has been pointed out that the study of the ZP by means of classical SEM or TEM yields a microstructural ZP appearance which is not consistent with the molecular model in which ZP2 and ZP3 form long filaments anastomosed by means of ZP1 (mice) and ZP1/ZP4 (human) proteins (3). In fact, using these methods it is possible to see the surface of the ZP with small or large fenestrae or smooth appearance. The interpretation of the precise ZP structure seen with traditional SEM is difficult, because the ZP is much hydrated and thus shrinks considerably when dried, even if a critical-point drying apparatus is employed.

A true detailed description of the filaments contained within the multilayered lattice of the ZP was obtained by TEM and high resolution SEM using the Sap-RR-Os-Tc method that offers several advantages in terms of ZP stabilization and preservation of the morphology of its delicate filaments (57, 63). Moreover, RR acts as a staining and a stabilizing agent of structural glycoproteins and polyanionic carbohydrates by preventing their dissolution and/or alteration induced by aqueous fixatives, and may be also used together with tannic acid in order to improve contrast (33). Even if the use of cationic dyes such as RR results in a molecular collapse of polyanionic chains causing these to appear as condensed granules (64), the addition of saponin avoids the formation of globular artifacts (41, 63). Osmium-thiocarbohydrazide treatment increases the molecular weight and yields a very fine structure under the electron beam. Besides, it hardens and preserves the glycoprotein matrix filaments from the mechanical stress induced by dehydration and critical point drying and thus, reduces filaments packing and shrinkage.

This method allowed the observation of thin anastomosed, globule-bearing filaments, 0.1 to 0.4 μm in length and 10 to 14 nm in thickness, as seen by TEM, 22 to 28 nm thick as seen by SEM (difference in thickness is due to the chemical coating used in SEM sample preparation). The filaments observed by means of this particular technique, probably corresponded to ZP2/ZP3 filaments, and the intersections corresponded to ZP1/ZP4 bounds. The filament arrangement was remarkably different between the inner and outer surfaces of the ZP and among the various maturation stages of the oocytes studied (Figures 6, 7, 8, 9). The outer surface of mature oocyte mainly consisted of filaments arranged in a multilayered network that appears

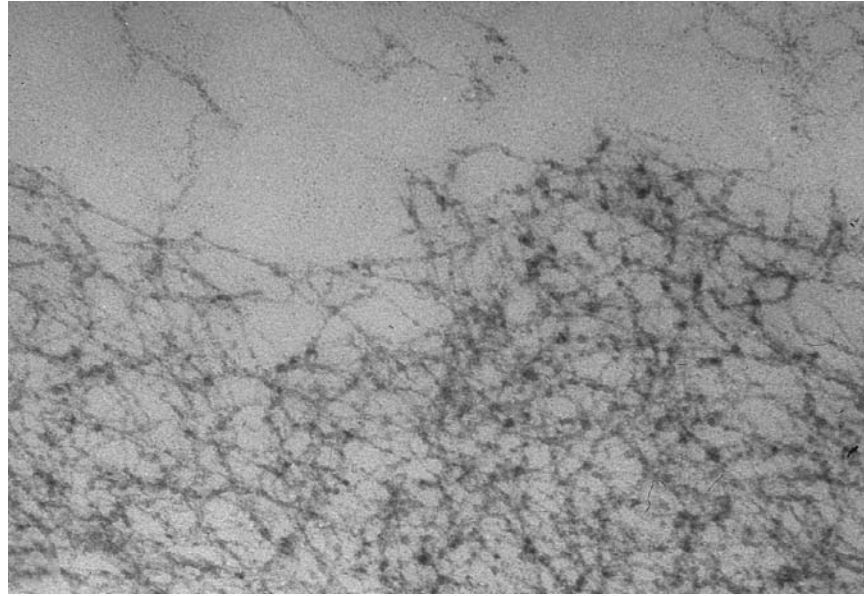


Figure 6. Mature oocyte with spongy zona. Filaments are arranged in a loosely-meshed network. Sap-RR-Os-Tc method. TEM, 27700X. Reproduced with permission from (41).

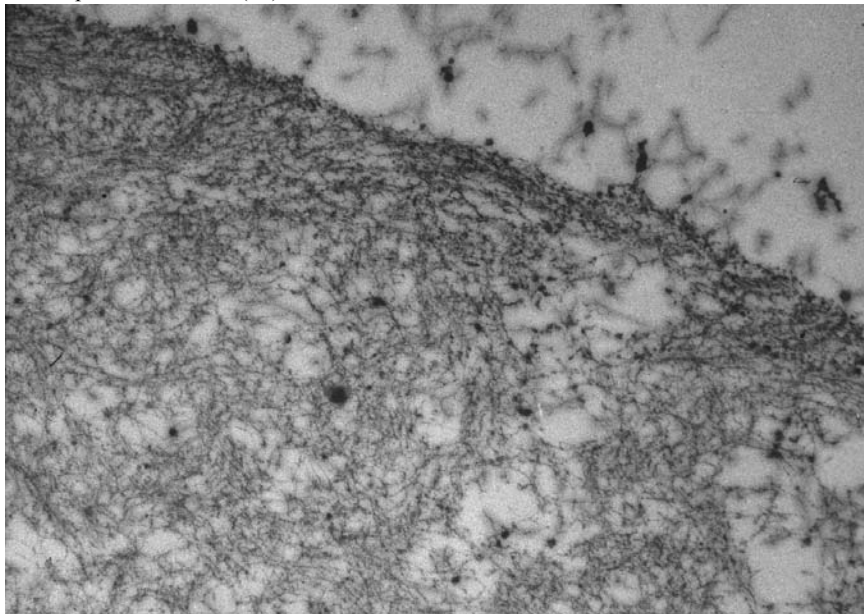


Figure 7. Atretic oocyte with compact zona. Filaments are arranged in a tightly-meshed network. Sap-RR-Os-Tc method. TEM, 27700X. Reproduced with permission from (41).

compact in the parts delimiting the fenestrations of the ZP, whereas it appears loose in correspondence with the fenestrations (Figures 8, 9). The latter seemed empty when observed with conventional SEM. These were, in turn, made up of a loose filament arrangement when observed with the Sap-RR-Os-Tc method. Immature and atretic oocytes displayed almost a tight meshed network of filaments. The inner surface of the ZP belonging to unfertilized oocytes at any stage was arranged in repetitive structures characterized by numerous short and straight filaments that anastomosed with each other, sometimes

forming small, rounded structures at the intersections (Figure 10) (1-2,41,57,65).

Further observations of our group on the microanatomy of the ZP surface of human oocytes (1-2,65) supported own previous results (41, 55). Following a comparative analysis of traditional SEM techniques (gold coating and conductive staining methods) and Sap-RR-Os-Tc method, the main aspect of the ZP surrounding human mature oocytes by traditional SEM consisted mostly (78,5%) in a porous, net-like structure. After the Sap-RR-

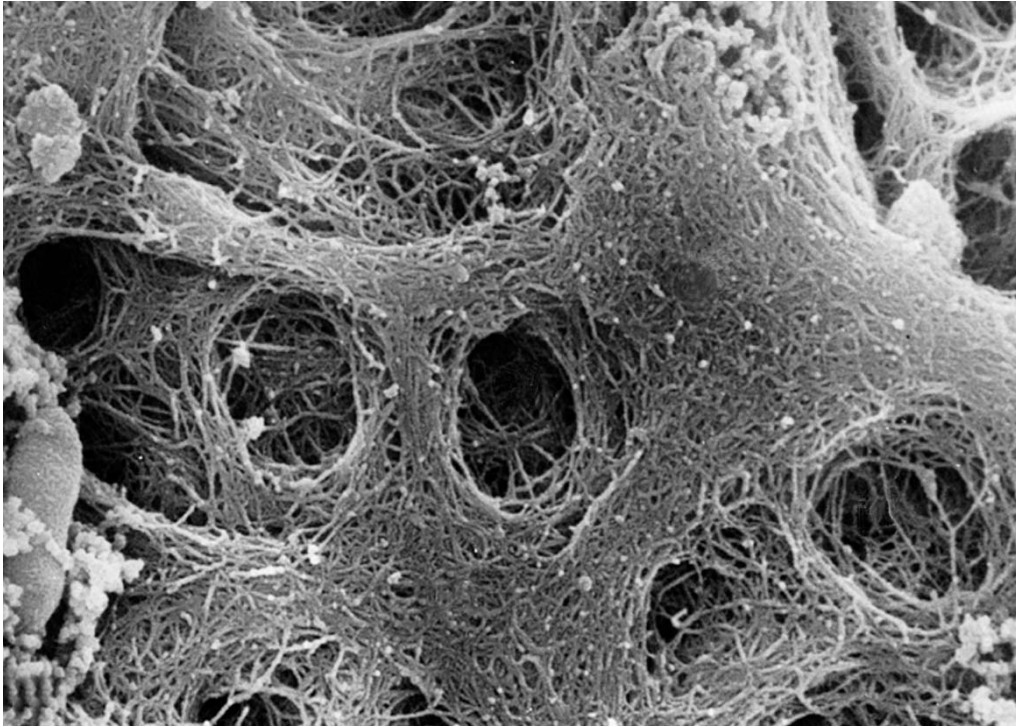


Figure 8. Mature oocyte showing the outer surface of the spongy zona pellucida. Filaments arranged differently within the branches than in between the branches. Sap-RR-Os-Tc method. SEM, 9000X. Reproduced with permission from. (41).

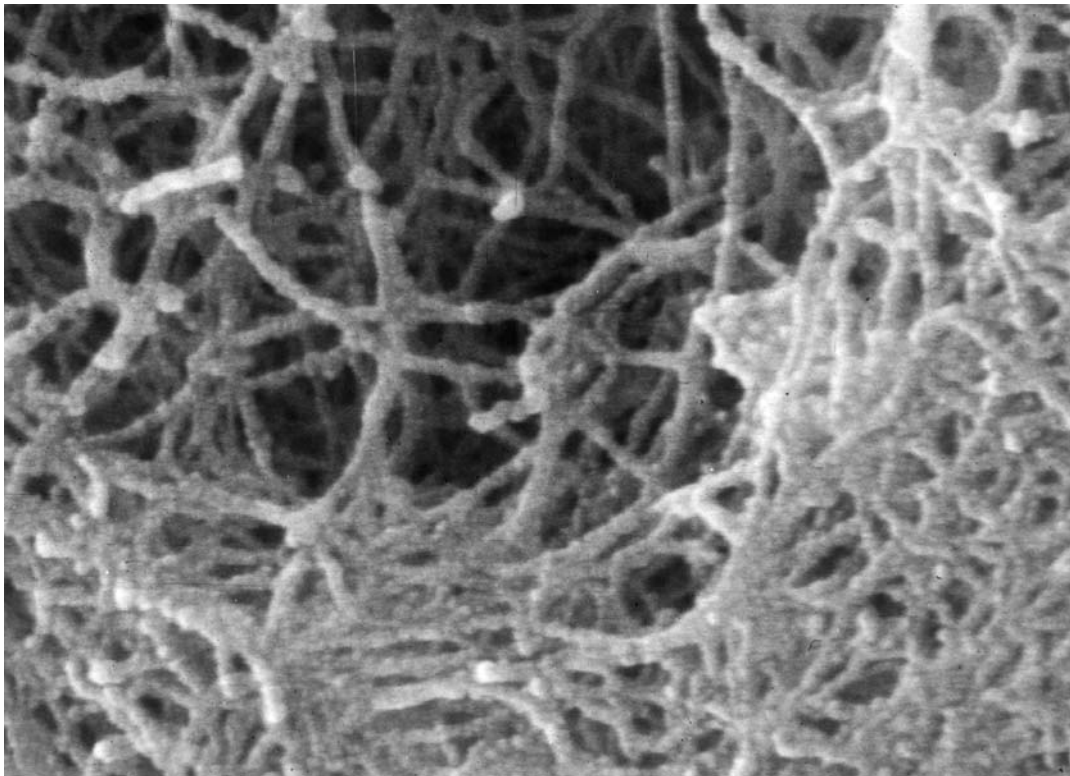


Figure 9. Mature oocyte. High magnification of the outer surface of the zona. There is a different arrangement of filaments appearing as globule-bearing structures. Sap-RR-Os-Tc method. SEM, 50000X.

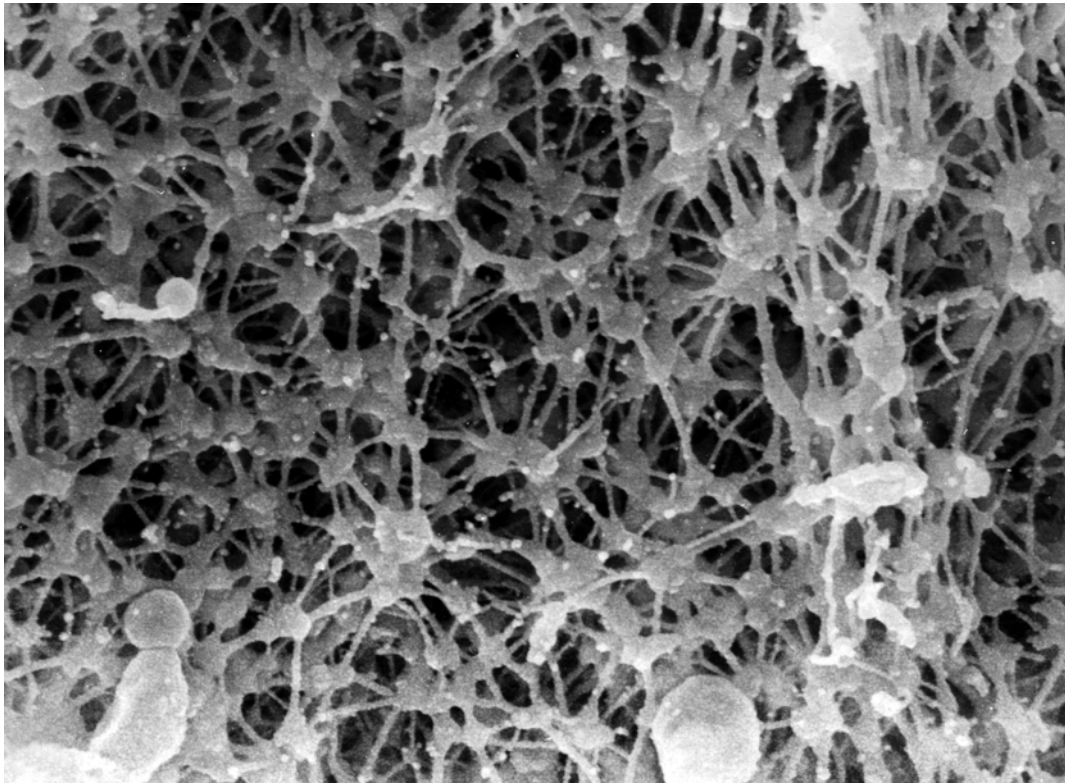


Figure 10. Mature oocyte showing the inner surface of the zona with regularly arranged filaments. Sap-RR-Os-Tc method. SEM, 22000X. Reproduced with permission from (41).

Os-Tc method, most human mature oocytes (86,1%) showed the ZP consisting of alternating tight and large meshed networks (65).

Therefore, the well standardized procedures, the stabilizing action of the conductive staining on the ZP material and the results obtained with the Sap-RR-Os-Tc method, strongly emphasize that ZP changes occurring in oocytes of various groups are not artifacts but genuine features, very likely related to their actual maturation status.

On the basis of these ultrastructural and functional data it is possible to hypothesize that oocyte maturation in humans is accompanied by three-dimensional modifications in the arrangement of ZP filaments observed *in situ*, which may be relevant in the processes of sperm binding, penetration and selection (1-2,41,65).

It has been difficult to assign sperm binding to specific ZP protein or carbohydrate determinant. This raises the possibility that supramolecular structures exert essential roles in sperm-egg recognition (4). Moreover, molecular support to our ultrastructural data arises from the so-called *supramolecular model* at the basis of egg-sperm recognition (4-5,66). As it is well known, the ZP consists of a paracrystalline meshwork composed of heterodimeric and cross-linked protein filaments (28). In particular, these filaments are formed by ZP2 and ZP3 proteins cross-linked

by ZP1 proteins. Rankin *et al.* (66) proposed a model in which the ZP is composed at least of ZP2 and ZP3 forming a three-dimensional matrix around ovulated eggs to which sperm will bind. Following fertilization and cortical granule exocytosis, the cleavage of ZP2 modifies the three-dimensional structure of the ZP matrix without loss of zona components, so that sperm can no longer bind (41,50,65,67). On the basis of the above evidence, it is rather logical to hypothesize that the three-dimensional network of ZP filaments is not randomly arranged but structured to accomplish fertilization of the mature oocyte.

4. ULTRASTRUCTURE OF FERTILIZATION: SPERM PENETRATION OF THE ZP

The ZP allows sperm penetration and subsequent fusion with the egg oolemma, and facilitates fertilization (68). It triggers the acrosome reaction so only acrosome-intact spermatozoa bind to the ZP (8,68). Human ZP is a strong inducer of hyperactivated motility among spermatozoa and this effect is stronger in sperm populations with normal morphology (69).

Fertilization rates strongly results from the ability of sperm to bind to the ZP. The zona binding is powerfully influenced by sperm morphology, in particular, both normal shape of the acrosome and nuclear chromatin (70-71). Results presented by Liu and Baker (72) showed that defective sperm-zona interaction is the major cause for low

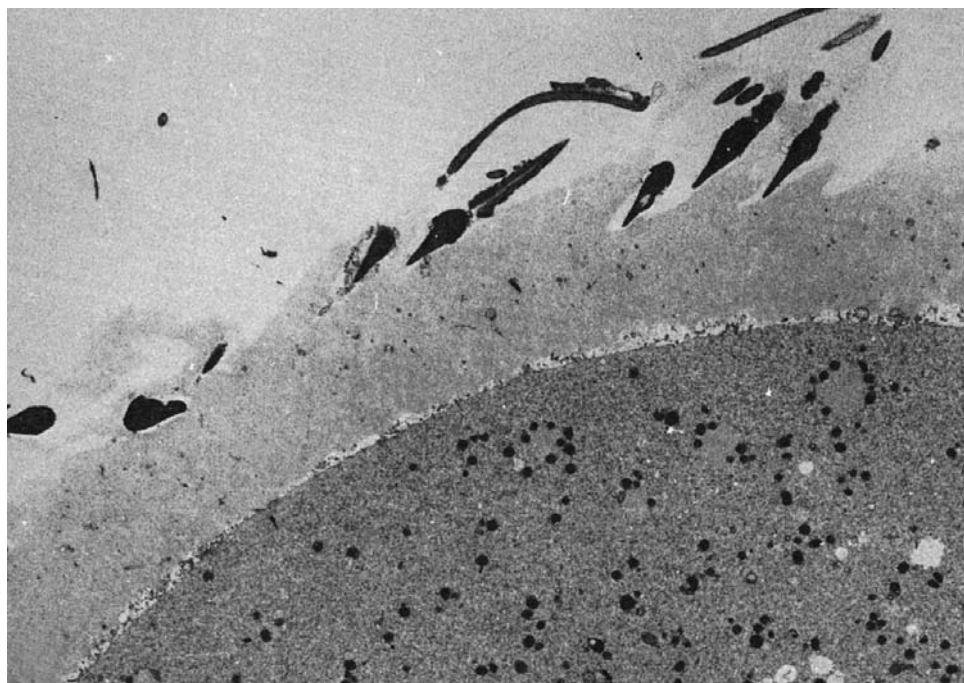


Figure 11. Numerous acrosome-reacted sperm approach and enter into the outer region of the zona pellucida of a polyspermic oocyte. TEM, 7500X. Reproduced with permission from (78).

fertilization rates with standard IVF. However, sperm with abnormal acrosomes can accomplish the acrosome reaction (73). In fertile men, only a very small proportion of motile sperm (average of 14%) is capable of binding to the ZP *in vitro* when unlimited ZP receptors are provided (74).

The physiological fertilization process involves binding of acrosome intact sperm to the surface of the ZP and the binding process stimulates the acrosome reaction to occur on the surface of the zona (8,75). ZP from fertilized human oocytes induces a voltage-dependent calcium influx and sperm acrosome reaction (76). These findings are corroborated by electron microscopy studies on human fertilization, in which it is clearly shown that sperm within the inner third of the zona are all completely acrosome-reacted, and that the zona material, during sperm penetration shows clear areas around sperm heads presumably representing digested pathways by acrosomal enzymes (73,77-78). Similar results arose from ultrastructural studies in mammalian fertilization (30). Sperm penetration of the ZP depends on acrosomal proteases acting enzymatically and on the oscillating thrust of the sperm (80). The latter transduces a mechanosensory signal that leads to the induction of sperm acrosome reaction as recently proposed in mice by Baibakov *et al.* (81).

The shape and the relative size of the acrosome region of the sperm head, as well as anterior symmetry of the sperm and width of the midpiece region at the junction with the head are important for sperm to be bound to the zona (70,82). According to Hoodbhoy *et al.* (4), it is

interesting to point out that there are substantial differences in size between human and mouse sperm head length (human's is half that of mice), ZP thickness (human's is about twice that of mice), and egg diameter (120 μm vs 80 μm , respectively). Whether these differences affect the three-dimensional molecular structures which are relevant for taxon-specific sperm-egg recognition (4), and consequently, egg penetration, remains to be elucidated.

At SEM and TEM analysis, most commonly, a flat and tangential attachment of the sperm head to the surface of the ZP appears, which is then followed by intrusion into the zona in precisely this horizontal position (Figures 4, 11). However, vertical binding with penetration by the tip of the head also occurs. In oocytes where large, cluster-like numbers of bound spermatozoa are visible, vertical binding and penetration is the most usual position (55-56,78-79,83). Regardless the means spermatozoa use to pass through the mammalian ZP, it has often been noted that the sharply defined penetration slit has a distinctly tangential trajectory (80). Moreover, Bedford suggested that the tangential trajectory of that oblique path ensures that it then closes as the ZP stretches and thins during expansion of the blastocyst, so preventing the development of a hole that could lead to trophoblast herniation, compromising normal hatching (80).

4.1. The perivitelline space (PVS)

The PVS is a space between the ZP and the oolemma that contains a hyaluronan-rich extracellular matrix (84). This space is relatively small in immature oocytes, whereas it becomes asymmetrical and enlarged around the first polar body after its extrusion, retaining this

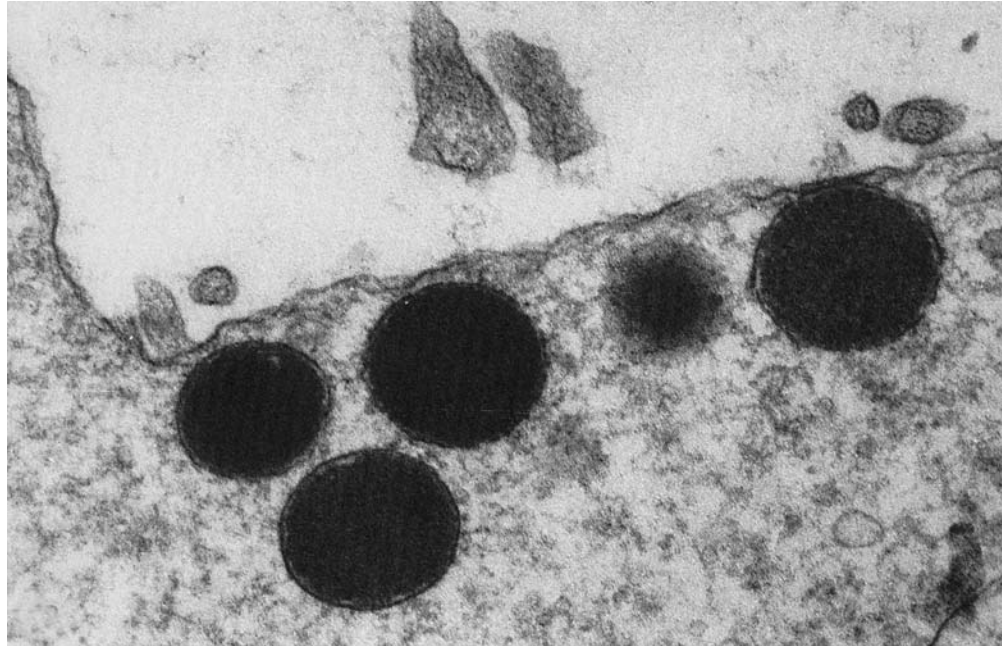


Figure 12. Mature unfertilized oocyte, cortical granules beneath the oolemma. TEM, 70000X. Reproduced with permission from (88).

appearance until fertilization. When observed with TEM in the presence of RR, this matrix is shown to be constituted by electron dense granules and filaments, both of which attach to the oolemma (85). Talbot and Dandekar hypothesize a role during fertilization in that hyaluronan, which can inhibit membrane fusion, must be negotiated by the fertilizing sperm (84).

5. ULTRASTRUCTURE OF FERTILIZATION: CORTICAL REACTION AND ZONA REACTION

Cortical granules beneath the oolemma release their contents into the PVS by exocytosis in response to inositol triphosphate-induced calcium rise, generated by the fusion of the sperm with the egg (86-87). This reaction is believed to take place in a few seconds right all around the egg, usually beginning at the point of sperm fusion. Once the granular contents of the cortical granules are released into the PVS they diffuse and interact with the ZP, resulting in the so-called zona reaction and in the block of polyspermy (Figures 12, 13) (79,88-89). However, their direct role in this process is still not fully understood, due to the scarce data about cortical granule composition and their effect on the ZP (10,87,90).

As commonly known, the zona reaction involves the interaction of cortical granules contents with the glycoproteins of the ZP establishing a primary block to polyspermy at the level of the ZP. The reaction is described as a chemical hardening process that prevents supernumerary sperm from entering the perivitelline space after fertilization. This process, at the ultrastructural level, is characterized by morphological changes that begin in the inner ZP and then are seen in the mid ZP portion, described as the presence of “blobs or striae of dense material”

(Figure 14) (78,88-89,91). Dandekar and Talbot (92) demonstrated the interaction of cortical granule exudates with the PVS matrix in fertilized human eggs, using the RR method. In particular, these authors showed that not all cortical granule components diffuse into the ZP, but much of the cortical granule exudates dehisce and is trapped within the perivitelline space forming a new coat around the fertilized oocyte. This material, called *cortical granule envelope*, is observed in all developmental stages, up to blastocyst. If observed with TEM, this envelope is made up of electron dense granules, distinct in size and texture from those found around unfertilized oocytes (85). Talbot and Dandekar proposed that it plays a role in blocking polyspermy in mammals, including humans (84).

When observed with both high resolution SEM and TEM using Sap-RR-Os-Tc (63), the inner part and surface of the human ZP in fertilized ova and embryos displayed numerous areas wherein filaments fuse together (Figures 15, 16) (41). On the contrary, the outer ZP evidenced the same pattern observed in human mature oocytes, suggesting that the cortical reaction induces modifications in the inner layer of the ZP consisting in regularly disposed areas of closely packed filaments (41). It is possible that this filament condensation could be related to the process of zona reaction, which plays a role in the mechanisms related to the block of polyspermy, together with the formation of the cortical nuclear envelope (84, 92).

Other morphological three-dimensional observations obtained in human (93) and porcine (59) fertilized oocytes using traditional SEM, showed that ZP had a compact surface. In particular, Nikas *et al.* postulated that the substances released by the oocyte rapidly diffuse through the inner pores of the ZP, exerting a lytic action on

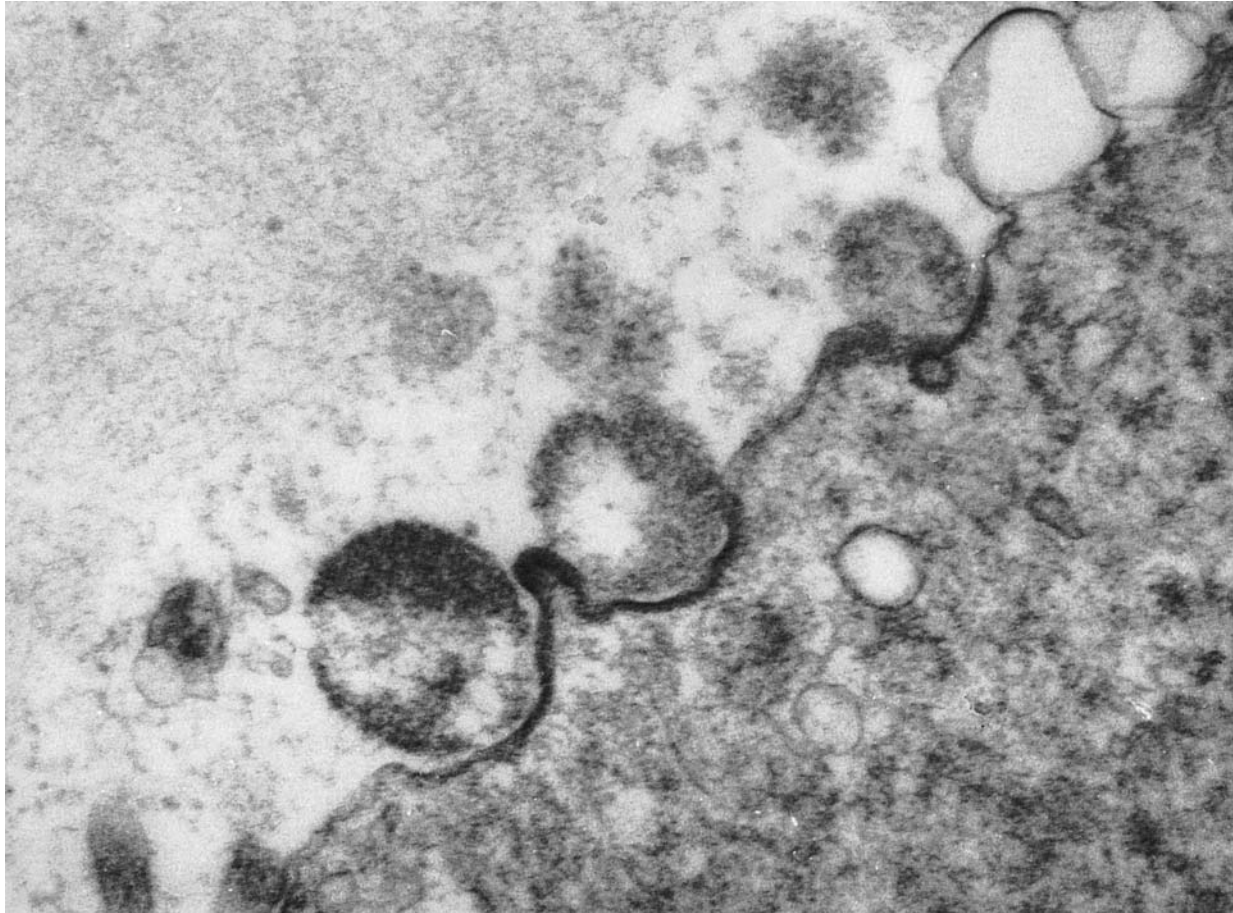


Figure 13. Mature fertilized oocyte, cortical reaction. Exocytosis of cortical granules into the perivitelline space wherein they interact with the inner portion of the zona. TEM, 70000X. Reproduced with permission from (89).

P structures and causing them to melt and obstruct the pores, thus becoming a barrier for further sperm penetration (93). These observations are in contrast with our studies on human and bovine ZP following fertilization and during cleavage. In fact, our studies demonstrated that the ultrastructural surface aspects of the ZP do not change after fertilization or during cleavage (2,41,55-56,94). The discrepancy is probably due to the different preparatory techniques employed. In particular, our observations were performed with both high resolution SEM and TEM observations, using Sap-RR-Tc as filament stabilizers. We showed changes in the stereo-configuration of ZP filaments; up until then other observations had described only the presence or absence of superficial pores of the ZP that exclusively represent the result of filament collapse during traditional SEM technical procedures (1-2,41,65).

Further interesting results were obtained by Funahashi *et al.* (50) in pig oocytes about zona reaction, and using TEM and the RR-Sap technique (63). The fine structure of the ZP and the ZP reaction at sperm penetration differs between pig oocytes fertilized *in vivo* and *in vitro*. The outer and inner pig ZP have different network organizations, probably due to insufficient final maturation of the ZP during *in vitro* maturation, lacking oviductal

glycoproteins (50). In particular, the outer area of the ZP always formed a concentrically arranged fibrillar network, whereas the inner area showed a much more compact, trabecule-like mesh. However, both areas, but particularly the outer network, were much more compacted after the zona reaction. Clear differences in the degree of fibrillar aggregation of the inner zona were also observed in *in vitro* and *in vivo* zygotes, being much higher in the latter (50). Therefore, it is also possible to propose that the zona reaction results from ultrastructural modifications that include ZP variations as well as cortical granule envelope formation. Nevertheless, it is possible to suppose that the zona reaction, detected mainly in *in vitro* studies, may be different in the *in vivo* situation.

Other data (95) are consistent with our observations concerning the three-dimensional compaction of ZP filaments in the inner layer of the ZP during the post-fertilization period (41,67). Sun *et al.* (95) investigated protein structure changes in mice ZP related to fertilization with a microrobotic system. Their experimental studies quantitatively describe a stiffness increase seen in the mouse ZP after fertilization, providing an understanding of ZP protein structure modification, related to an increase in the number of protein ZP1 cross-links with ZP2 and ZP3 at

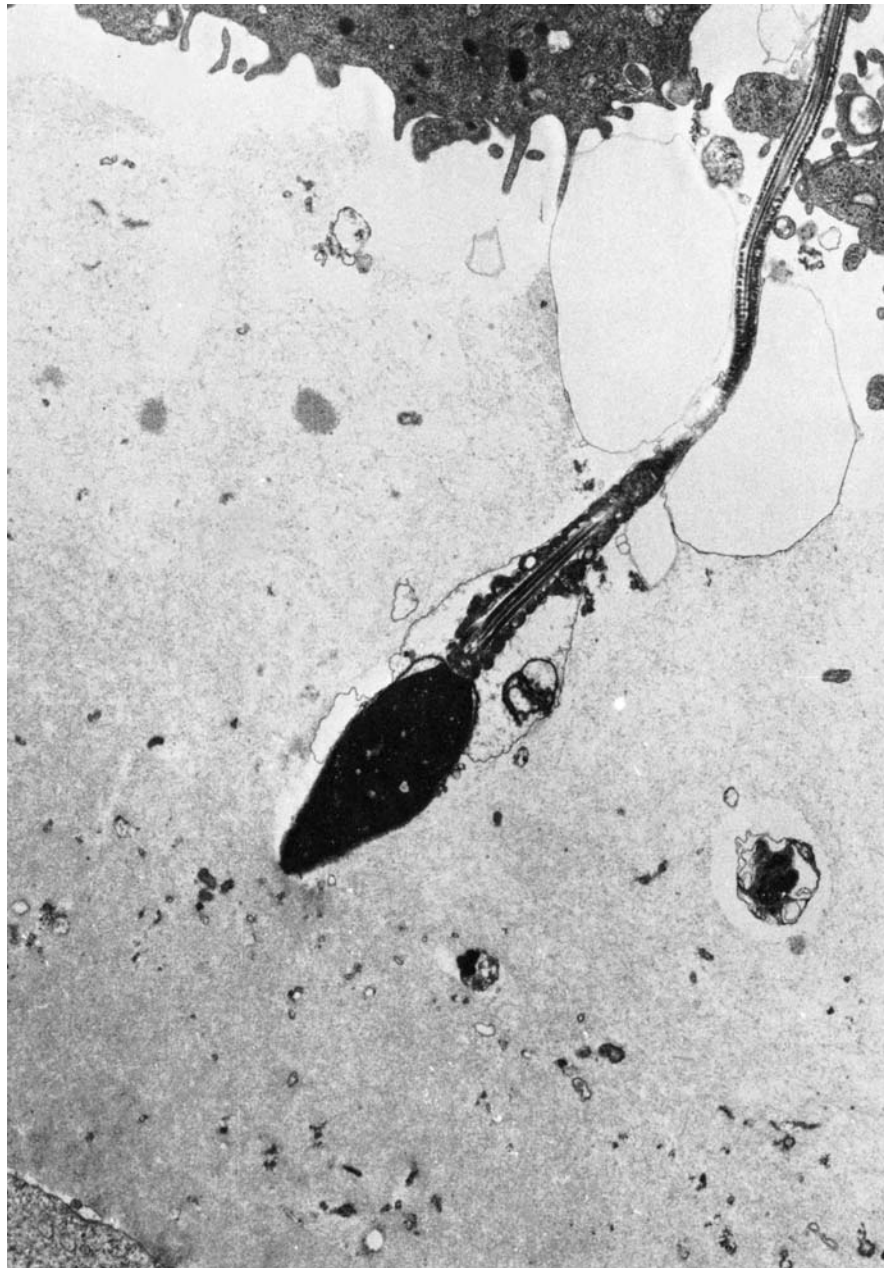


Figure 14. Polyspermy block. An acrosome-reacted sperm has penetrated about half the thickness of the zona pellucida, virtually blocked just outside the inner surface of the zona. TEM, 9100X. Reproduced with permission from (78).

the basis of ZP hardening and related to stiffness increase of the ZP.

Additional data on the ZP structure were obtained with the Polscope. Using this apparatus, Wang *et al.* (96) showed that ZP of normally fertilized human oocytes has a trilaminar structure, whereas in polyspermic oocytes ZP exhibited minute discontinuities in the inner zona layer (96). These observations provide a method to diagnose abnormality in the block of polyspermy in the inner layer of the ZP as ultrastructurally shown in our previous studies

(41,63). Wang *et al.* (96) supposed that discontinuities in the inner ZP layer might be related to polyspermy.

6. ULTRASTRUCTURE OF THE ZP SURROUNDING THE EMBRYO

The ZP has important functions during preimplantation development until the blastocyst stage, at which time the embryo escapes from the zona and implants in the endometrium (8). The presence of the ZP around the early embryo creates a sort of embryo encapsulation that

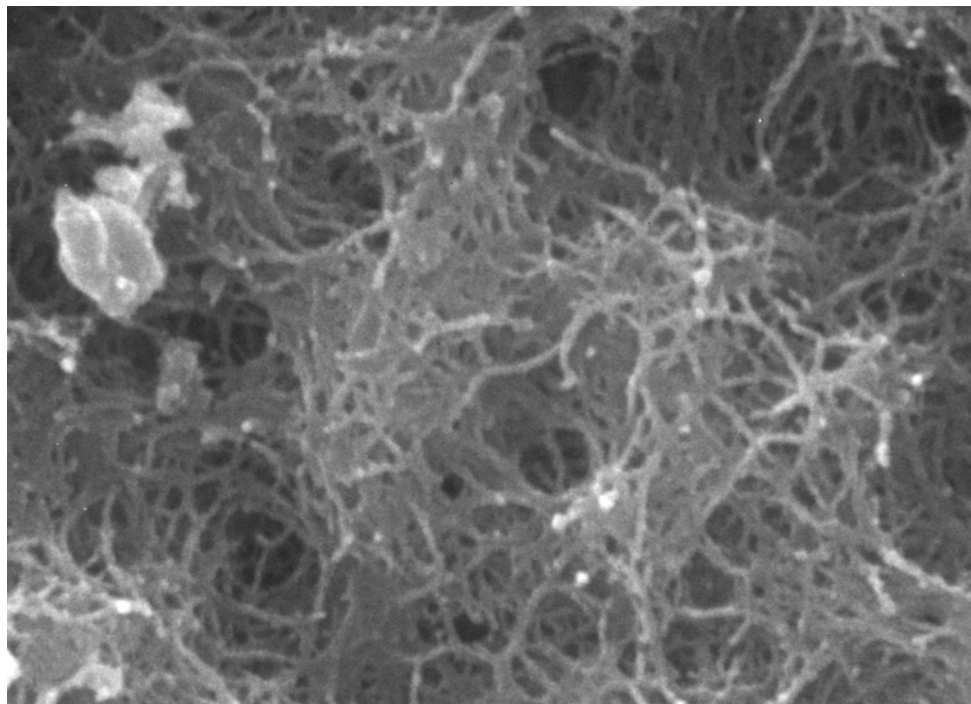


Figure 15. Fertilized ovum, inner portion of the zona pellucida. Note the presence of bulging areas of condensed, closely packed filaments. Sap-RR-Os-Tc method. SEM, 29800X.

likely helps to create a suitable microenvironment allowing autocrine stimulation of blastomeres by embryo-derived substances. It also ensures maximum contact between blastomeres prior to compaction, affords protection for the early embryo both during its transit down the oviduct and from the maternal immune system, and inhibits ectopic tubal implantation (8,68,97-98). Furthermore, the ZP is involved in embryo protection against pathogenic agents and also modulates embryo-maternal early signaling (97-98).

Garside *et al.* (99) used the inverted microscope in order to measure the average ZP thickness of *in vitro* cultured embryos. They concluded that this parameter decreases during *in vitro* culture ($17.7 \pm 0.14 \mu\text{m}$ on day 1; $16.3 \pm 0.14 \mu\text{m}$ on day 2 and $14.9 \pm 0.14 \mu\text{m}$ on day 3) and that these changes are more evident in good quality embryos. Pelletier *et al.* (100), in turn, have quantitatively characterized the ultrastructural arrangement of three different layers of ZP in living cleavage-stage embryos by means of the Polscope. All the three constituting layers (i.e. 1, 2, 3) of the ZP are thinner in embryos ($15.2 \pm 2.9 \mu\text{m}$) than in eggs. However, layers 1 and 2 contribute proportionally less with respect to layer 3 (the outer one) to the overall thinning process, thus indicating a possible stretching of the zona as the embryo increases in diameter (100). The ZP thickness of transferred embryos significantly correlates with the number of blastomeres, embryo grade and fragmentation. Therefore, it may have a clinical diagnostic value representing a potentially reliable parameter for embryo selection (99,101).

SEM studies in the ZP in human polypronuclear embryos (two-four blastomeres) displayed a spongy, fenestrated outer surface (Figure 17) (41,56). On the contrary, other morphological three-dimensional observations obtained in human (93) and porcine (59) embryos using traditional SEM showed that ZP had a compact outer surface.

Furthermore, SEM data of the ZP belonging to human blastocysts obtained throughout assisted reproduction protocols by Nottola *et al.* (102) showed that the ZP ultrastructure in healthy blastocysts was characterized by numerous large fenestrations, formed by interwoven filaments, rather similar to that of healthy mature oocytes and early embryos (Figure 18). In contrast to this, degenerated, dark and irregular blastocysts showed a more compact ZP, comparable to that of immature/atretic oocytes (41, 55-56). The ZP is increasingly stretched until it is almost invisible (103-104). Nevertheless, the characteristic hatching process observed *in vitro* that includes expansion of the blastocoele, distension and thinning of the ZP before rupture, may even correspond to artifacts (38). Normally, the blastocyst hatches *in vivo* on days 6 or 7 after fertilization, time at which it implants in the endometrium (105-106).

Although some controversy exists (8), the ZP is not an essential component of human embryogenesis nor pregnancy. In fact, its absence do not inhibit embryo development in that the cumulus corona cells provide enough support to maintain blastomere interaction, embryo viability and pregnancy (68).

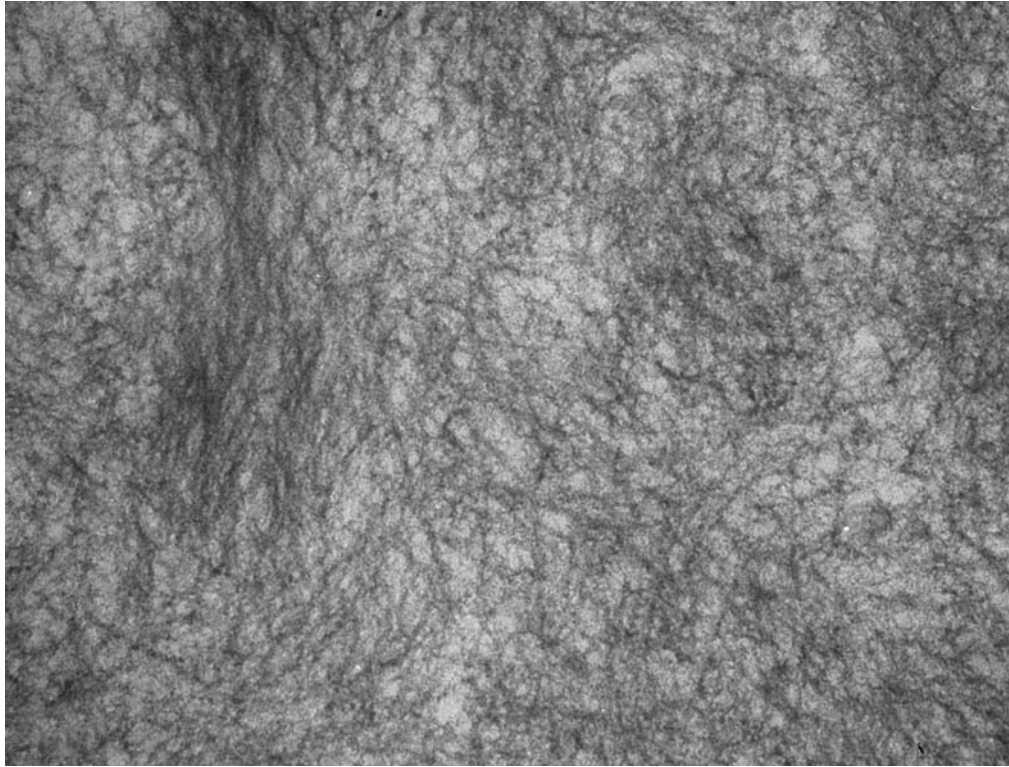


Figure 16. Four-cell polypronuclear embryo; inner surface of the zona. Within the network of filaments, areas of major condensation are present. Sap-RR-Os-Tc method. TEM, 27700X. Reproduced with permission from (41).

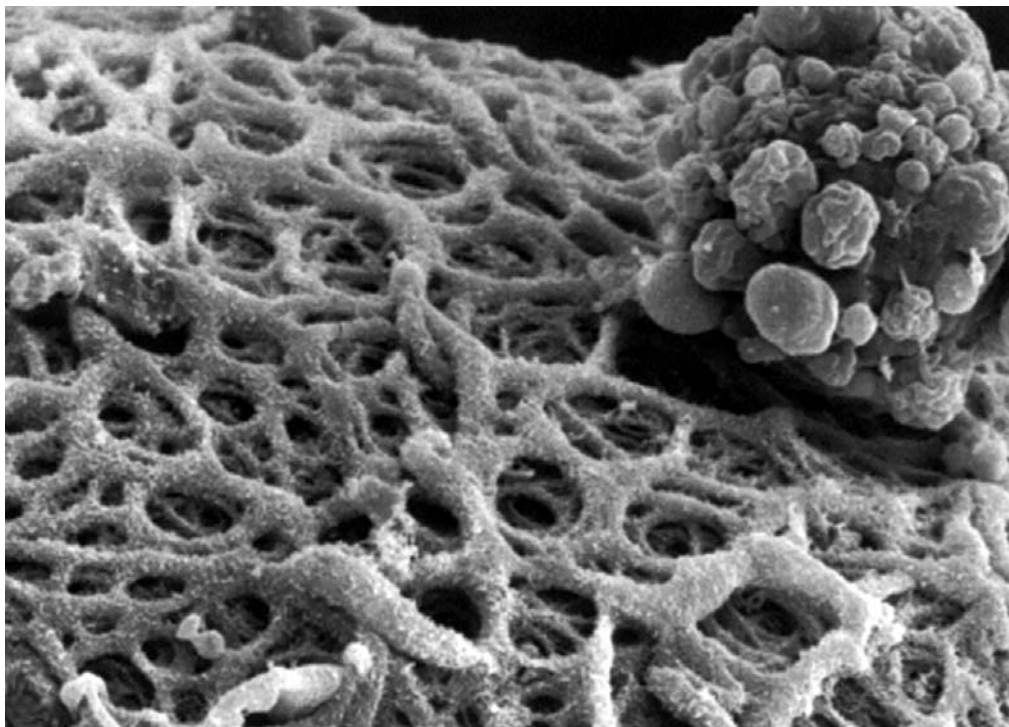


Figure 17. Two-cell polypronuclear embryo; outer surface of the zona showing a spongy surface. SEM, 1500X. Reproduced with permission from. (41).

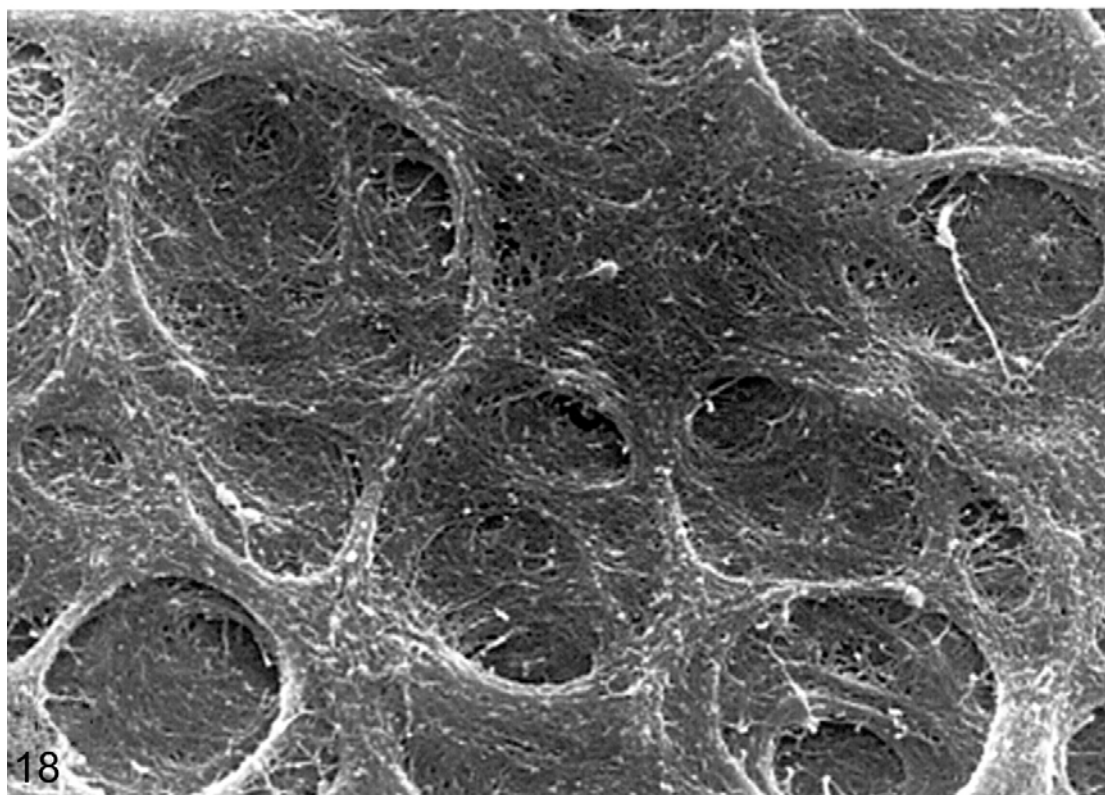


Figure 18. Full blastocyst (ICSI). The outer surface of the zona pellucida shows a spongy texture with wide and flattened fenestrations. SEM, 13000X. Reproduced with permission from (102).

7. MOLECULAR ORGANIZATION OF ZP GLYCOPROTEINS

Mice ZP has been the most studied one, it is composed of three glycoproteins named ZP1, ZP2 and ZP3 (18). This model is formed by repeating units of ZP2-ZP3 heterodimers arranged in filaments that are cross-linked by ZP1 homodimers (18), and has been accepted until now as a model for the ZP arrangement in higher vertebrates. Mice ZP1 was proposed as the linker molecule that acts as stabilizing element (18,107), ZP2 has been acknowledged as a secondary receptor for binding with acrosome-reacted spermatozoon (108-110), and ZP3 acts as the primary sperm receptor, which binds the intact sperm and induces the acrosome receptor (111).

Gene sequence information, currently available on a public database (112), shows that the human ZP is composed of four glycoproteins: ZP1, ZP2, ZP3, and ZP4/ZPB (113), the genetic loci of their four genes potentially encoding ZP proteins have been already identified among somatic chromosomes 11, 16, 7, and 1, respectively (112). The related glycoprotein levels were determined by mass spectrometry; ZP2, ZP3, ZP4 were present in the same amount whereas ZP1 was the least represented (113). Four ZP genes can be also found in the chimpanzee, macaque, rat, and chicken genomes, while 3 genes and one pseudogene are present in the mouse.

A hypothetical model of the zona containing four ZP proteins assumes that basic structural elements of the zona are conserved between species and that the relationship between ZP1 and ZP4 bespeaks a conserved function. Probably, ZP2/ZP3 filament structure is similar to that of the mouse but may contain different types of filament crosslinkers, such as ZP1 homodimers, ZP4 homodimers and ZP1/ZP4 heterodimers (25).

7.1. ZP glycoproteins and sperm binding and penetration

Sperm initially contact the ZP proteins by means of plasma membrane in the anterior head region. As the acrosome reaction progress, this membrane domain vesiculates and is sloughed. Sperm must remain attached to the ZP during the completion of this process, and after dispersal of acrosomal contents, it begins penetration into the ZP (3). Some evidence suggested that at this point adhesion may be mediated by binding sites on the sperm inner acrosomal membrane that interact with ZP2, the other unit of the ZP filament (109-110). Zona penetration further requires the participation of flagellar motility as well as sperm proteases that may assist penetration by proteolytic cleavage of ZP proteins (114).

Direct data on the function of human ZP proteins can be founded in Chakravarty *et al.* (115). They have shown that capacitated human sperm binds to recombinant

ZP3 and ZP4 to either acrosomal cap or equatorial region, whereas no binding of ZP2 was observed.

Rankin *et al.* (66) showed that “humanized” mouse zonae matrices expressing human ZP2 and ZP3 can bind mouse sperm but are unable to bind human sperm, possibly related to a requirement for species-specific glycosylation (116). As supposed by Conner *et al.* (117), this result may alternatively reflect human sperm having evolved to bind to a ZP consisting of four ZP proteins rather than three. It should be also considered that ZP4 itself is required for a direct interaction as part of the sperm receptor on the ZP likely other mammalian species in which the ZP4 is supposed to have sperm binding activity (118-120). Nevertheless, as shown by Hoodbhoy *et al.* (4), human sperm do not bind to rat ZP despite the presence of four homologous glycoproteins.

The human ZP reveals a variable glycosylation throughout its thickness, with marked differences between the outermost and innermost regions of this matrix. In a similar way, the canine ZP shows a regionalization of oligosaccharide chains within three concentric bands of the zona matrix, with a higher concentration in the inner portion (121). This heterogeneous carbohydrate composition could be responsible for the different sperm binding ability detected between the outer and inner regions of the ZP (122). Besides, human ZP glycoproteins express some carbohydrate sequences not previously detected in other species (122). Studies using Polscope (polarizing microscopy) confirmed the bilaminar, tripartite nature of the zona in human mature unfertilized oocytes and suggest that the inner layer appears as a marker for oocyte developmental potential and conception cycles (28).

At present, the burden of evidence indicates that ZP3 represents the sperm adhesion molecule of the ZP, related with a tenacious form of contact between sperm and ZP referred as binding. There is a wide agreement from studies in a number of species that tenacious adhesion is likely due to sperm interaction with a ZP3 glycan (3). Interestingly, considering that the ZP surrounding ovulated eggs, but not two-cell embryos, is permissive for sperm binding, two models have been considered for sperm-egg recognition (81): the “glycan release model” and the “zona scaffold” model. Much of the evidence supports the latter model (66,81) which, however, does not discriminate on a protein and/or a carbohydrate basis. According to Baibakov *et al.* (81), the ZP three-dimensional microarchitecture likely plays a crucial role in forming the cognate binding site.

7.2. ZP glycoproteins and the block of polyspermy

The molecular changes resulting from differential effects on various layers within the ZP remain to be fully established. A ZP block model involves modifications of specific ZP proteins that could account for the penetration of additional sperm binding of the ZP, undergoing acrosome reaction and penetration of the ZP (3).

After fertilization, ZP3 appear to undergo an important functional modification which is likely located in

the carbohydrate portion (123) involved in sperm binding and acrosome reaction induction. Other evidence suggested that ZP2 undergoes proteolytic cleavage after fertilization in mice (124) and probably humans (5). In human *in vitro* fertilization, ZP2 cleavage is observed in some inseminated eggs that fail to fertilize (125). It has been speculated that ZP2 proteolytic cleavage results in functionally important changes in the supramolecular structure of the ZP (4-5). Several proteins and N-acetylglucosaminidase activity, localized in intact cortical granules are related with ZP2 cleavage (126) and with the modification of ZP3 (127).

Fertilized human ZP retains the ability to bind spermatozoa and to induce acrosome reaction but cannot be penetrated by spermatozoa; probably post-fertilization architectural alterations could provoke the hardening of the zona making impossible the lytic action of acrosomal enzymes (76). Fertilization does not abolish the bioactivity of the ZP even though the biochemical status of ZP2 is modified, from an intact to a cleaved form (76).

7.3. ZP glycoproteins defects

Even though human ZP genes are currently under investigation by means of genomic and proteomic research, a pathology which is strictly linked to a precise defect in ZP genes has not been found up until now. Anyway, an association between sequence variations in human ZP genes and fertilization failure has been proved by Mannikko *et al.* (128). They have found several sequence variations of the four ZP human genes in the pool of infertile women studied. In the examined group ZP1 was the gene that showed the highest variability.

An interesting hypothesis on the molecular level mechanism of gamete interaction has been proposed by Castle (129). It involves the action of multiple low-affinity binding sites, and implies that a single nucleotide mutation in one or in several genes cannot cause the complete fertilization failure. This hypothesis has been corroborated by the finding that no single point mutation, observed in infertile women examined by Mannikko *et al.* (128), solely accounts for fertilization failure. Various sequence variations were instead found by the authors in the four human ZP genes, one in the regulatory region of ZP3 gene, another in one of the structures that binds to the equatorial segment and the post acrosomal sheath of the human sperm.

The multiple low-affinity binding sites theory implies that the interacting molecules on the sperm and on the egg have to possess a precise three-dimensional architecture, not only in the backbone polypeptide structure but also in the O-linked sugar moieties, to act correctly. Further molecular studies are needed in order to confirm this fascinating hypothesis in human, a more precise comprehension of the structure-function relationship as well as a better molecular characterization of human ZP genes/proteins will allow a better counseling for treatment in infertile couples.

Integrity in ZP glycoproteins is important, as shown in experiments using knock-out mice for the three

genes codifying ZP (130-131). In fact, defective zonae pellucidae in ZP2-null mice disrupt folliculogenesis, fertility, and development, whereas abnormal zona pellucida in mice lacking ZP1 results in early embryonic loss (130-131).

8. CONCLUDING REMARKS AND PERSPECTIVES

Numerous studies have been built up on the structure and properties of the ZP in various species at both the ultrastructural and molecular levels. However, it is still very difficult to compare ultrastructural data obtained with the use of standard TEM and SEM with molecular data related to the structural glycoproteins network. In fact, molecular models are quite different to the standard morphology of the ZP obtained with TEM (the microfibrillar architecture) or with standard SEM (spongy or smooth surface). A particular approach in the study of the ZP ultrastructure throughout the Sap-RR-Os-Tc technique, shows its real microfilamentous structure and the related functional changes during oocyte maturation and fertilization. This supports a morphological ultrastructural supramolecular model, more similar and comparable to molecular models related with the glycoprotein network. A detailed mapping of single human ZP proteins and their relationship within the supramolecular architecture of the zona matrix would clearly supply insights into the molecular basis of sperm-egg recognition. Nevertheless, some questions still remain to be clarified.

The first question concerns the ZP three-dimensional appearance observed with conventional SEM, that comprises both a spongy and a compact/smooth ZP aspect. Some authors affirm that there is no correlation between ZP morphological changes and the maturation stage of the oocyte, and that spongy or compact ZP aspects found in oocytes belonging to groups of different ages is a just a random, casual finding. On the contrary, there is evidence for a real correlation existing among ZP structure, function, and oocyte age (or maturation stage) as extensively reported in several species, including humans. In order to explain the three-dimensional morphological differences it should be pointed out, once more, that a glycoprotein matrix such as the ZP, due to its extremely hydration state, is very difficult to be preserved during ordinary electron microscopy procedures. In particular, strong solutions of ethanol or glutaraldehyde may cause shrinkage of the ZP gel/paracrystalline structure, thus generating relevant artifacts. Therefore, in order to correctly interpret and compare the morphology of ZP in oocytes submitted to different treatment, experimental design or timing, particular care should be given in treating ZP for SEM examination and standardized procedures; this will certainly reduce or even avoid ultrastructural artifacts. We thus emphasize the concept that a modern view of the ZP surface is a three-dimensional network of crossing filaments as seen with high resolution SEM. In particular, this method shows the spongy or compact appearance of the ZP which reflects different filament network arrangements, according to the particular oocyte maturation stage.

The second question concerns the possibility that ZP morphological changes that are related to the spongy or compact appearance with traditional SEM may correlate with sperm-binding and penetration capacity. As already reported, data from the current literature are not univocally oriented. An answer to this question could be found out just considering the role that the ZP plays in that structure and function develop side by side. ZP is the sperm-binding site and it has to be crossed by the sperm in order to achieve fertilization. Considering the question from an evolutionist point of view, it is reasonable to hypothesize a ZP structure model as a three-dimensional lattice, which can well accomplish those tasks. A wide texture may not only avoid the exposition of adequate sperm binding sites but may also favor an easier digestion with acrosomal enzymes. A tight texture, in turn, may hamper sperm binding and would render enzymatic digestion more difficult. Such a theoretical model has practical evidence that arises from ultrastructural studies that show the arrangement of ZP filaments and the related modifications according to the maturational stage of the oocyte and fertilization, but also when considering genetic and biochemical assays such as the supramolecular model at the basis of egg-sperm recognition.

Last question arises: is the mouse still a good model for human fertilization? As extensively shown, there are great similarities in the ultrastructural organization between mouse and human ZP, when seen with both SEM and TEM as well as in the microarrangement of the ZP filaments, and ZP morphological modifications related to development, aging and fertilization. On the other hand, there are great differences in the ZP glycoproteins, since mouse ZP is made up of three glycoproteins whereas human ZP is made up of four glycoproteins. At present, glycoprotein differences do not affect structural morphology and further studies are needed to clarify the relationships between the ultrastructural and molecular organization, even if the increased complexity of the ZP in humans and other species across the evolutionary tree now demands that we reconsider our reliance on the mouse model for understanding early fertilization events.

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Abbreviations: Os: osmium; PVS: perivitelline space; RR: ruthenium red; Sap: saponin; SEM: scanning electron microscopy; Tc: thiocarbohydrazide; TEM: transmission electron microscopy; ZP: zona pellucida

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