—Mini Review—

Extracytoplasmic Markers of Human Oocyte Quality

Thomas Ebner[1]

[1]*Landes- Frauen- und Kinderklinik, IVF-Unit, Krankenhausstr. 26-30, A-4020 Linz, Austria*

Abstract: *Within growing follicles oocytes gradually acquire developmental competence to further support early embryonic development. This process is intrinsically linked to folliculogenesis. Thus, the fate of the germ cell depends on the health of the developing follicle, e.g. its vascularization, oxygen content and cumulus cell characteristics. Consequently, gametes of varying quality are harvested after follicular puncture. This review focuses on extracytoplasmic anomalies that should clearly be distinguished from intracytoplasmic ones. In detail, all relevant dysmorphisms related to the zona pellucida and perivitelline space will be discussed. Interestingly, some correlation can be found between extracytoplasmic dysmorphisms and fertilization and preimplantation development. Since genetic constitution is not affected by anomalies of the outer shell, it is postulated that it occurs in the late stages of maturation.*
Key words: *Oocyte quality, Perivitelline, Space, Zona pellucida*

Introduction

Although there is evidence that accidental maturation and ovulation of germ cells of reduced developmental potential does occur in controlled ovarian hyperstimulation (COH) [1], the role of oocyte morphology in the decision-making process for the selection of an embryo or blastocyst for transfer is still controversial [1–5]. However, it is well accepted that the developmental fate of an oocyte reflects the follicular milieu because the viability of the individual gamete (and the corresponding embryo) is strongly correlated to optimal maturational processes in the ovary.

Within growing follicles oocytes gradually acquire

developmental competence to further support early embryonic development. This process is intrinsically linked to folliculogenesis. Thus, the fate of the germ cell depends on the health of the developing follicle, e.g. its vascularization, oxygen content and cumulus cell characteristics [6, 7]. In more detail, suboptimal gametes may be derived from follicles with reduced blood supply. If vascularization in ovaries is underdeveloped, some follicles will be exposed to hypoxia which in turn causes a change in energy metabolism by switching from oxidative phosphorylation to glycolysis. As a consequence, ATP production in the affected follicle may decrease dramatically. If oxygen saturation falls below a certain threshold, it has been reported [8] that gametes with a reduced internal cytoplasmic pH and ATP content may arise. A lack of metabolites may interfere with synchronization of oocyte maturational processes, e.g. coordination between nuclear and cytoplasmic maturation [5].

Due to these intrinsic problems numerous morphological anomalies may develop in human oocytes and only half of all MII gametes show a good morphology which, by definition, requires a clear, moderately granulate cytoplasm, a small perivitelline space (PVS), an intact first polar body, and a colorless zona pellucida [2, 9]. The following review exclusively focuses on extracytoplasmic anomalies of the oocyte including all dysmorphisms related to the zona pellucida and perivitelline space.

Zona Pellucida

As a result of the mutual dependence between somatic cells (e.g. cumulus cells) and the egg it is likely that any disturbance negatively affecting the follicle will have a comparable impact on the oocyte itself. Amongst the conceivable changes in oocyte performance it is possible that the secretion and patterning of the zona pellucida from the secondary

follicle onwards could be altered or interrupted [10, 11]. This could either result in dysmorphisms that can be seen under a light-microscopice, or in more subtle changes of the three-dimensional structure of the zona.

Consistency of the zona pellucida

It has been suggested previously that in ICSI the zona pellucida and/or oolemma display various reactions to the penetrating glass pipette [12]. Data from zona free ICSI indicate that it is mostly the zona pellucida that contributes to these various reactions [13]. Three distinct types of zona responses can be observed. Most of the injected oocytes show normal breakage, as assessed by formation of an invagination rupturing at the approximate center of the oocyte. A second type of response, sudden breakage of the zona/oolemma without creation of a funnel may be observed. This phenomenon is correlated with decreased rates of survival and fertilization [12, 14]. The third type of response is called "difficult breakage" and is characterized by delayed penetration. Difficult and sudden breakage patterns may serve as markers of an impaired zona pellucida and this is supported by recent data suggesting that MII- oocytes can show varying zona elasticities as demonstrated by micro tactile sensors [15].

Palermo *et al.* [12] described a correlation between different breakage patterns of the oolemma/zona and peripheral estradiol levels. In detail, they were able to show that oocytes with sudden breakage of the outer shell were found in patients with low levels of estradiol at the time of ovulation induction compared with the other two oolemma response patterns (P = 0.01).

Complete absence of the zona pellucida

Definitely the most severe form of impaired growth of the zona pellucida is its complete absence. Normally, up to four zona proteins [16, 17] contribute to the three-dimensional matrix of the outer protective shell. Filaments are constructed of repeating zona protein (ZP) 2 and 3 units which are cross-linked by ZP 1 [18], thus contributing to the structural integrity of the zona pellucida. Experiments on mice lacking the ZP1 gene showed that secreting only ZP2 and ZP3 results in a thinner, more loosely organized zona pellucida [19]. On the other hand, targeted disruption of ZP 2 and 3 led to complete absence of the acellular coat resulting in infertility [20].

Theoretically, these data could also be relevant to the human model. Defects in gene expression could cause the failure to create a glycoprotein coat, even though the ovum itself shows intact corona cells [21]. In such rare cases the ova fail to fertilize in conventional IVF. In ICSI, there is a considerable risk of exposing the gametes to mechanical stress (e.g. lysis) during the denudation process. Stanger *et al.* [21] successfully showed that in patients with zona-free eggs, pregnancies can be achieved by simply leaving the coronal cell layer attached, as it acts as a supporting structure keeping the oocyte in shape during injection.

A completely different etiology can be seen if the zona pellucida is present *a priori* but is lost during *in vitro* manipulation. Most probably this phenomenon is caused by a spontaneous breach in the zona matrix through which the gamete escapes leaving an empty zona behind. Case reports show that this type of zona-free oocyte is fertilizable and can proceed to the blastocyst stage [13, 22]. Since the shaping function of the zona is not present, such zona-free embryos tend to have a more elongated shape and a delayed development.

Variation in thickness of the zona pellucida

From conventional IVF, it is known that thicker zonae (e.g. > 21 μm) are associated with lower fertilization rates [23]. This observation has been linked to patient and stimulation parameters [24]. In ICSI, however, a thicker zona neither interferes with subsequent fertilization nor with implantation since assisted hatching can be utilized.

Recently, it has been shown that the multilaminar structure of the zona pellucida can also be analyzed quantitatively using polarized light microscopy [25]. Though considerable variation exists in the thickness of zona layers around individual eggs (Fig. 1) and between members of a cohort it is evident that the inner zona layer is the most dominant part of the zona [11, 25]. Thus, thinning in this area would cause the most evident effect on overall thickness. However, it has been shown that the mean difference in thickness between zonae from conception cycles (11.3 ± 1.4 μm) and failed ones (9.4 ± 1.7 μm) was around 1 μm [11], a value being beyond the limit of provability of most systems designed for measuring cells. In addition, the thickness of the inner zona layer seems to be associated with blastocyst formation [26]. In detail, 10–12 nm inner layers performed better (53.1%) than 8–10 nm ones (25.0%).

Since the inner layer of the zona is highly ordered it can clearly be depicted using polarized light. It has been reported that the birefringence of the inner zona is directly proportional to its thickness [11, 25, 26].

Shen *et al.* [11] found an almost 30% higher mean

Fig. 1.   Oocyte displaying zona pellucida thickness variations.
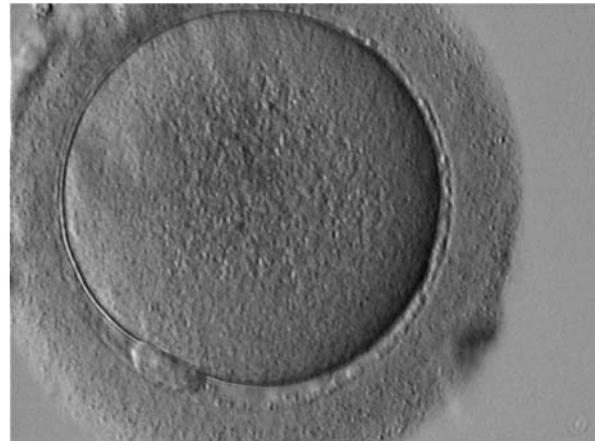
Fig. 2.   Discolored oocyte with a rather thick zona pellucida (25 μm).

light retardance in conception cycles, compared to non-conception cycles, indicating that some stimulated cycles yield oocytes of reduced quality. There are two retrospective studies which have suggested a relationship exists between zona birefringence (inner layer) and preimplantation development. Montag *et al.* [27] noted a higher rate of good quality embryos on day 3 (but not on day 2) in an oocyte group with high zona birefringence (41.7%) as compared to a cohort with low birefringence (24.4%). An Indian group [26], observed a difference in progression to the blastocyst stage. If the zona inner layer retardance was > 3 nm, the blastulation rate was 60.9% as compared to 14.1% if retardance was lower than 2 nm.

Discoloration

Irrespective of the actual thickness of the protective shell COH sometimes generates gametes showing a zona pellucida that appears dark or brownish under a light-microscope. Mostly, the egg itself is affected. In the literature, it is reported that the presence of discolored zonae seems to be a frequent phenomenon, e.g. 9.5% to 25.7% [2, 3, 28, 29].

It has to be kept in mind that it is not completely clear that dark or brown zonae/oocytes occur for the same reasons. Recently, a group from Canada termed the oocytes "brown eggs" (Fig. 2) if they were dark with a thick zona pellucida, and had a rather small perivitelline space (sometimes filled with debris), and granular cytoplasm [30]. Esfandiari *et al.* [30] prospectively compared the outcome of brown gametes with that of gametes of normal appearance. Although the zona in discolored eggs was thicker than that of control

gametes, in conventional IVF the fertilization rate was similar (63% vs. 58%). The same was true for the fertilization after ICSI (70% vs. 75%), embryo quality (44% vs. 43% grade A), implantation rate (7.6% vs. 8.0%), and clinical pregnancy rate (25.0% vs. 24.4%). However, it has to be mentioned that because of the thick zonae, brown oocytes were subjected to laser-assisted hatching significantly more often than the control group.

Shape anomalies

Even if the thickness or color of the zona pellucida are inconspicuous it is not automatic that the shape of the gametes is spherical. Indeed, there is evidence that ova showing extreme forms of shape anomaly exist [31]. Such ova have been shown to be fertilizable and may lead to the birth of a healthy baby. However, a major problem with reports of these cases is that the degree of the shape anomaly was not quantified, and rather more imprecise descriptions have been given (e.g. cucumber shaped).

Recently our group successfully measured ovoid oocytes [32] and calculated a roundness index (RI, length divided by width) which was initially used to quantify inner cell masses of blastocysts [33]. Actually, two indices were determined to assess whether the whole oocyte was affected (showing an ovoid ooplasm and zona) or only the zona pellucida was of ovoid shape (with the ooplasm being perfectly round). Special care was taken to detect splitting of the innermost zona layer that might keep ooplasms in round shape (while zona is ovoid). The latter dysmorphism was shown to be associated with implantation failure [11].
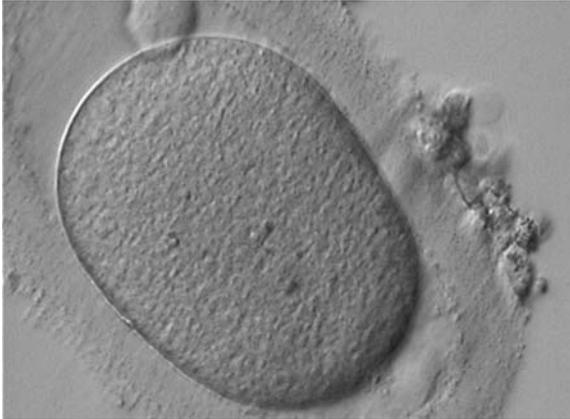
Fig. 3. Mature gamete of ovoid shape. Roundness index (length by width) is 1.52.

In a prospective one-year study [32], 18% of all ICSI cycles showed at least one elongated egg (Fig. 3). In the affected patients a total of 17% of all MII- oocytes was found to be ovoid, a percentage that decreased to 4% if all patients were taken into consideration. Out of 137 ovoid eggs, ooplasm deformation was involved in approximately 60% with a mean RI of 1.16 ± 0.14. The mean RI of the corresponding zona was 1.29 ± 0.14. Fifty-five gametes were considered round (RI between 1 and 1.03); however, their ZP were found to be ovoid (RI of 1.19 ± 0.09). Splitting of the inner zona layer was observed in 14% of all ovoid ova.

The degree of shape anomaly was neither correlated to fertilization nor embryo quality which is in line with previous data [31]. Interestingly, two types of cleavage pattern were observed on day 2. Either ovoid gametes cleaved normally like a tetrahedron (a crosswise arrangement of four cells with three blastomeres lying side-by-side) or, if the ovoid zona failed to exert its shaping function, resulting in a rather flat array of four blastomeres which was rather unusual. In detail, 61% ovoid embryos showed regular cleavage whereas the remaining 4-cell embryos showed irregularities. The roundness index of the zona pellucida ($P < 0.01$), but not of the ooplasm, was associated with the cleavage pattern. Since the abnormal pattern reduces the number of cell-to-cell contact points from 6 to 5 or 4, compaction and blastulation of the corresponding embryos may be delayed [32, 34].

Two possible mechanisms may account for the occurrence of ovoid oocytes. Firstly, mechanical stress during oocyte puncture and/or denudation processes could deform the egg. This unwanted occurrence would create ovoid gametes with both ooplasm and zona being affected. In these artificially damaged gametes a tendency towards recovery within a day has been suggested [32]. Thus, for the vast majority of ovoid ova it can be assumed that the deformation is a pre-existing anomaly generated during maturation within the follicle.

Perivitteline Space

It can be summarized that embryo/blastocysts from ovoid gametes may show different cleavage patterns. This is probably related to the dimension of the perivitteline space offering blastomeres enough room to move within ovoid zona. However, even if the shape is spherical extension of the PVS may differ.

Size of the perivitelline space
The size of the PVS is closely related to the maturational phase of the oocyte. While in prophase I (GV stage) expansion of PVS is minimal, however PVS begins to increase after the resumption of meiosis. In detail, at metaphase I, PVS can clearly be detected and after completion of maturation (metaphase II) its full size is reached.

Several authors have noted that 33% to 47% of all ova show a large PVS [35, 36]. In oocytes with a larger PVS (but without cytoplasmic anomalies) a lower fertilization rate was observed (67%) as compared to gametes with a normal (85%) PVS [35]. In addition, embryo quality was negatively affected by the size of the PVS [35]. This is more or less in line with the results of an Italian group [36] which showed that a large PVS is detrimental to fertilization and zygote morphology. Interestingly, patient parameters such as female age and indication did not seem to influence PVS performance [35] but the ratio of estradiol to testosterone (and to progesterone) did [37].

Data from *in vitro* matured oocytes indicate that a large PVS may be ascribed to over-mature eggs [38]. In other words, such eggs have shrunk in relation to the zona pellucida presenting a large gap between them. A large PVS would also occur if a larger portion of cytoplasm is extruded together with the haploid chromosomal set during first polar body formation. This would result in a large first polar body and a large PVS (Fig. 4).

First polar body morphology
For a long time it was thought that first polar body (1Pb) extrusion marks the completion of nuclear maturation ending in a metaphase II oocyte. Recently,
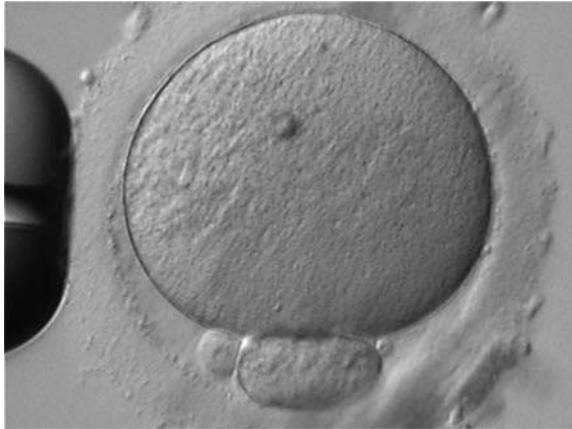
Fig. 4. Metaphase II oocyte showing a large and fragmented first polar body resulting in a large perivitteline space.

using polarized light microscopy it has been demonstrated that some oocytes showing a 1Pb were actually in telophase I and not —as expected— in metaphase II [39–41]. Otsuki *et al.* [42], recently, found a chromosome aggregation phase (gere phase) which occurred not only from germinal vesicle breakdown to metaphase I, but also from telophase I to metaphase II. Consequently, if ICSI is performed, although the chromosomes are unaligned, it may result in failed fertilization or three pronuclear zygotes due to abnormal chromosomal segregation.

The impact of 1Pb morphology on outcome is still a matter of debate. Though some 1Pbs in humans remain intact for more than 20 h after ovulation, they generally have a shorter life time [43]. Taking this time-dependency into consideration we may hypothesize that 1Pb morphology provides adequate information on the actual postovulatory age of the corresponding egg.

Ebner *et al.* [29] refined the scoring system of Xia [35] and tried to focus exclusively on the status of the 1Pb. Ova showing an intact 1Pb gave rise to higher rates of implantation and pregnancy [44], probably due to an increase in blastocyst formation [45]. However, these data are controversial [29, 36, 40, 45–48] as indicated in Table 1.

Apparently, the benefit of selecting oocytes according to the morphology of the 1Pb is somewhat reduced with increasing time span between ovulation induction and ICSI, since studies with different schedules could not find a relationship between constitution of the 1Pb and subsequent ICSI outcome [40, 47, 48]. In these data sets the percentage of eggs showing a fragmented polar body was also higher [35, 40, 47, 48] than that reported in the work of Ebner *et al.* [29] in which polar bodies were scored 2 h after collection (Table 1). This interesting since it has been found that of all intact 1Pb, 13% were already fragmented at a second inspection 3 h later [48].

Data from Hungary [47] suggest that a large 1Pb is the worst case. When large 1Pbs were extruded, embryos with multinucleated blastomeres were significantly ($P < 0.001$) more frequent (26.7%) than in all other 1Pb classes (ca. 8%). It has been postulated that the extrusion of an abnormally large 1Pb is due to dislocation of the meiotic spindle [49]. This would in part explain the observed impact on fertilization and embryo development [29, 36, 40].

Debris in the perivitelline space

Sometimes it is difficult to distinguish between heavily fragmented 1Pbs and debris within the PVS. The origin of the latter dysmorphism is unclear. Two hypotheses have been proposed both of which could explain the presence of granula in the PVS. One hypothesis is derived from ultrastructural data indicating the presence of an extracellular matrix comprised of granules and filaments in the space between oolemma and zona. This matrix appears to be identical to that found between cumulus cells and the corona radiata [50, 51].

The second hypothesis is based on the existence of coronal cell processes passing the zona pellucida and

Table 1. Possible correlation between first polar body (1Pb) morphology and fertilization rate

Authors	% fragmented 1Pb	2Pn fragmented 1Pb	2Pn intact 1Pb	Hours post egg retrieval
Ebner *et al.* [29]	20.8	112/159 (70.4)[a]	155/174 (89.1)[a]	2–3
Xia [35]	39.0	128/180 (71.1)	217/281 (77.2)	3–4
De Santis *et al.* [40]	30.7	161/251 (64.1)	202/340 (59.4)	3–5
Fancsovits *et al.* [45]	46.5	1088/1576 (69.0)	468/714 (65.6)	3–8
Ciotti *et al.* [46]	25.3	116/151 (76.8)	333/445 (74.8)	2–9

Values in parentheses are percentages. 2Pn: two pronuclei. [a]$P < 0.05$.

reaching the egg early in maturation (GV stage). It hypothesizes that after withdrawal of these processes some remnants remain within the PVS [52].

The findings of Hassan-Ali *et al.* [53] support the latter theory since they found a close relationship between the frequency of PVS granularity and maturation. In detail, they never detected debris in prophase I eggs, but found debris in 4% of the metaphase I and in 34.3% of metaphase II gametes. They were also able to show that the presence of PVS granules was gonadotropin dose-dependent. If less than 30 ampoules were used to stimulate the patient, 17.4% of the eggs were positive for this anomaly compared to 45.4% in high-dose patients (> 45 ampoules). Fertilization rate, cleavage rate and embryo quality were found to be unaffected by the presence of coarse granules in the PVS [53, 54]; however, rates of implantation and pregnancy seem to be influenced [54], since transfer of embryos derived from PVS granula free oocytes gave a 5% higher implantation rate and a 21% higher pregnancy rate.

Conclusion

To conclude, a variety of extracytoplasmic anomalies exist which in part negatively influence fertilization (consistency and thickness of the zona, 1Pb decay and debris within the PVS), blastulation (thickness of zona, 1Pb fragmentation), and pregnancy (thickness of zona, 1Pb morphology, debris in PVS). Characteristics of the zona pellucida and the PVS are most probably associated with the health of the developing follicle, e.g. its vacularization and oxygen content. Any disturbance during growth might severely alter oocyte morphology resulting in a pool of gametes with different prognoses. However, it must not be forgotten that information linking dysmorphisms with genetic disorders is scarce.

It is obvious that some anomalies, e.g. so-called giant eggs [55, 56] with an almost double-sized diameter, show a diploid chromosomal set, which contributes to digynic triploidy. In an interesting approach, Yakin *et al.* [57] checked the aneuploidy rate of 248 embryos. Embryos developing from oocytes with normal morphology showed a 58.1% euploidy rate. This ratio was comparable to that for oocytes showing extracytoplasmic (discoloration, PVS size) anomalies (53.2%) or shape anomalies (57.4%). Cytoplasmic dysmorphisms showed a lower rate of euploid cells (40.0%) but the difference was not significant.

Other authors [58] analyzed embryos genetically according to their polar body classes. No correlation was observed between polar body shape and genetic constitution; however, the only polar body group bearing a theoretical risk of chromosomal disorder, considering the larger volume of ooplasma in large polar bodies, was not analyzed.

According to a common hypothesis [59], the vast majority of extracytoplasmic anomalies occur late in maturation since they are associated with fertilization and developmental failure rather than with aneuploidy (e.g. giant eggs).

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