Surface morphology of the zona pellucida surrounding human blastocysts obtained after *in vitro* fertilization*

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Summary. Human zona pellucida (ZP) is maintained up to the blastocyst stage prior to hatching. In *in vitro* fertilized (IVF) embryos, it eventually acts as a morphodynamic interface between the cultured embryo and its microenvironment. Ultrastructural data on the ZP of IVF blastocysts are scarce in humans. We employed correlated phase contrast microscopy (PCM) and scanning electron microscopy (SEM) to study retrospectively the ultrastructural morphology of the ZP outer surface of 20 IVF human blastocysts from 16 Japanese patients (28–44 years of age, average 36.7 ± 4.2) with a history of infertility. Blastocysts were derived from conventional *in vitro* fertilization (cIVF) (n = 10) and from intracytoplasmic sperm injection (ICSI) (n = 10). Both cIVF and ICSI groups included "clear blastocysts" (n = 5) and "dark blastocysts" (n = 5). By PCM, the clear blastocysts exhibited a regular, round-shaped contour and consisted of clear and voluminous cells. By SEM, they displayed a spongy ZP with numerous fenestrations formed by networked filaments. By PCM, dark

blastocysts appeared irregularly shaped and often collapsed, and comprised dark cells and debris. By SEM, their ZP were smooth with remnants of compact fenestrations. In conclusion, viable blastocysts presented a normal ZP outer surface ultrastructure, whereas unhealthy blastocysts showed an altered ZP outer surface, comparable to that of immature/atretic oocytes. Such alterations could reflect sub-optimal culture conditions and/or could be related to blastocyst degenerative processes. The blastocyst ZP surface ultrastructure was unaffected by the fertilization technique (cIVF or ICSI). These data suggest that blastocyst survival *in vitro* is related to ZP ultrastructure maintenance.

Introduction

The zona pellucida (ZP), a special extracellular matrix that surrounds the oocyte and the early embryo, is multifunctional in mammals, including humans. From ovulation until blastocyst hatching shortly before implantation, the ZP undergoes structural and biochemical changes and serves in several crucial events. The ZP protects the oocyte, regulates oocyte-sperm interactions in a species-specific manner, promotes acrosome reaction, and impedes polyspermy. After fertilization, the ZP avoids blastomere disgregation, facilitates embryo passage through the oviduct, and prevents premature attachment of the embryo to oviductal and endometrial surfaces (Nichols and Gardner, 1989; Wassarman *et al.*, 1999; Denker, 2000; Herrler and Beier, 2000; Motta *et al.*, 2003). Further, the ZP is able to protect the embryo against pathogenic agents (bacteria, viruses), toxins, and phagocytes and modulates exchanges of nutrients and embryo-maternal early signaling (Nichols and Gardner, 1989; Denker, 2000; Herrler and Beier, 2000).

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In recent years, interest has been increasing in the structural characteristics as well as the chemo-physical properties of embryo ZP during assisted reproduction technologies (ART). It has been observed that some adverse conditions arising from a prolonged embryonic culture might negatively influence ZP properties (thickness, hardness, density, elasticity), leading to a hatching failure of about three-quarters of embryos produced during *in vitro* fertilization (IVF) (Latterie, 1997). This may be offset through micro-assisted embryo manipulation, including assisted hatching, ZP thinning, or complete zona removal (Cohen *et al.*, 1990; Antinori *et al.*, 1996; Urman *et al.*, 2002; Veeck and Zaninovic, 2003), but the effectiveness of such techniques remains controversial (Veeck and Zaninovic, 2003). In addition, as reported in bovines, the ZP of IVF blastocysts is thinner (Abe *et al.*, 1999) and more fragile than that of the blastocysts developed *in vivo*, as seen following exposure to cryoprotectant and cryopreservation or enzymatic digestion (Fair *et al.*, 2001).

Although several ultrastructural studies were carried out on IVF human pronuclear eggs as well as on two- to eight-cell embryos and their vestments (Motta *et al.*, 1988, 1995; Familiari *et al.*, 1989, 1992; Nottola *et al.*, 1991, 2001; Sathananthan *et al.*, 1993; Makabe *et al.*, 1998, 2001), the ultrastructural morphology of *in vitro* grown human blastocysts and their vestments has not been thoroughly investigated to the present (Lopata *et al.*, 1982; Van Blerkom *et al.*, 1984; Lindenberg *et al.*, 1986; Sathananthan

et al., 1990; Ménéz *et al.*, 1999; Makabe *et al.*, 2001; Veeck and Zaninovic, 2003). Further, comprehensive studies on the blastocyst ZP ultrastructure are lacking in humans. Thus, very little is known about the response of the blastocyst ZP ultrastructure to prolonged extra-corporeal management. In the last years, scanning electron microscopy (SEM) and correlated ultrastructural procedures represented the technique of choice to investigate the ZP ultrastructural morphology of oocytes, pronuclear eggs, and early embryos in several mammals, including humans (Familiari *et al.*, 2001; Motta *et al.*, 2003). These studies, mainly performed during ART protocols, contributed toward understanding the functional relationship of the ZP ultrastructure with crucial processes such as oocyte maturation, oocyte-sperm interactions, fertilization, and early embryo development (Sundstrom, 1982; Tsuiki *et al.*, 1986; Familiari *et al.*, 1988, 1989, 1992, 2001; Motta *et al.*, 1988, 1995, 2003; Nikas *et al.*, 1994).

In the present study we performed a retrospective analysis of data concerning the structural and ultrastructural characteristics of the human blastocyst ZP after prolonged culture, as evaluated by phase contrast microscopy (PCM) and SEM. The aim of the study was to correlate the ZP ultrastructure — with special regard to its outer surface — to human blastocyst viability *in vitro*. The ultrastructure of the blastocyst ZP outer surface was also compared in two different ART protocols: conventional IVF (cIVF) and intracytoplasmic sperm injection (ICSI).

Fig. 1. Clear cIVF early blastocyst. This blastocyst shows a regular, rounded contour. A well-preserved ZP encircles it. Voluminous cells with a clear cytoplasm form this blastocyst. An evident blastocoel cavity is still not recognizable. Numerous spermatozoa (S) adhere to the ZP. PCM: $\times 150$

Fig. 2. Clear ICSI full blastocyst. This blastocyst shows a regular, rounded contour. A well-preserved ZP encircles it. Voluminous clear cells form this blastocyst. They appear organized in an inner cell mass (ICM) and an outer cell mass (OCM). B: blastocoel cavity. Asterisk: cellular debris. PCM: $\times 135$

Fig. 3. Clear cIVF early blastocyst. Panoramic view of the blastocyst shown in Figure 1. The blastocyst is spherical in shape. It presents clustered spermatozoa (S) and remnants of corona cells (CC) on its surface. SEM: $\times 1,100$

Fig. 4. Clear ICSI full blastocyst. Panoramic view of the blastocyst shown in Figure 2. This blastocyst appears irregularly spherical. SEM: $\times 1,000$

Fig. 5. Clear cIVF early blastocyst. ZP outer surface of the blastocyst of Figure 1. A spermatozoon (S) appears partially enclosed in the ZP matrix. The ZP appears melted in the direct proximity of the penetrating sperm. **Inset:** High magnification of Figure 5: remnants of the ZP microfilamentous texture around the penetrating sperm are seen. SEM: $\times 12,500$; inset: $\times 52,000$

Fig. 6. Clear ICSI full blastocyst. ZP outer surface of the blastocyst of Figure 2. The ZP is characterized by a spongy texture with expanded and flattened fenestrations (F). **Inset:** Detail of a fenestration on the ZP outer surface of the blastocyst shown in Figure 2. Note the microfilamentous texture of such a fenestration. SEM: $\times 13,000$; inset: $\times 50,000$

Fig. 1 – 6. Legends on the opposite page.

Materials and Methods

The retrospective analysis was performed on a collection of micrographs of blastocysts provided by the Department of Obstetrics and Gynaecology, Toho University (Tokyo) from March 1996 to November 1997.

The present study was carried out according to the European Community and Japan guidelines and concluded before the law of *February 19, 2004 n.40, G.U. February 24, 2004 n.45* came into force in Italy.

Patients, in vitro fertilization techniques, and blastocyst culture

Sixteen Japanese patients from twenty-eight to forty-four years of age (average: 36.7 ± 4.2), who underwent ART procedures for infertility problems, were stimulated with gonadotropin-releasing hormone agonist and follicle-stimulating hormone *plus* human menopausal gonadotropin (long protocol) to induce ovulation. Oocytes were fertilized using cIVF and ICSI techniques. Embryos were cultured up to the blastocyst stage (6 days) in Quinn's Advantage HEPES medium. All the patients gave their informed consent for this study.

Phase contrast microscopy and scanning electron microscopy: sample processing and observation

The blastocysts were fixed in 1.5% glutaraldehyde in 0.1 M PBS for at least 48 h. The samples were examined and photographed by PCM (Olympus, Melville, NY, USA) at the time of fixation and then processed for SEM evaluation as follows (Murakami, 1974; Nottola *et al.*, 1991): 1) rinsed in 0.1 M PBS, 2) post-fixed in osmium tetroxide (1%) in 0.1 M PBS for 20 min, 3) rinsed in 0.1 M PBS, 4) treated with tannic acid (1%) in distilled water for 30 min, 5) rinsed in 0.1 M PBS, 6) treated again with osmium tetroxide (1%) in 0.1 M PBS for 20 min, 7) rinsed in 0.1 M PBS, 8) placed in microporous specimen capsules (SIC Scientific Division, s.r.l., Rome, Italy), 9) dehydrated in an ascending series of graded ethylic alcohols, 10) critical-point dried in a CO₂ atmosphere (Balzers Union CPD020, Furstentum, Liechtenstein), and 11) mounted on aluminum stubs and coated with platinum (4nm in thickness) (Emitech K550 sputter coater, Ashford, Kent, UK). SEM observations were performed in a field emission scanning electron microscope (Hitachi S-4000, Danbury, CT, USA) operating at low voltage (5–10 kV).

The micrographs of 20 IVF blastocysts were analyzed in this study. Blastocysts were derived from cIVF ($n = 10$) and from ICSI ($n = 10$). Both cIVF and ICSI groups comprised

“clear blastocysts” ($n = 5$) and “dark blastocysts” ($n = 5$) as classified by means of PCM (Dokras *et al.*, 1993). The blastocysts were either surplus material or samples that regularly would not be implanted due to their retarded (or arrested) cleavage (for reference see: Dokras *et al.*, 1993; Gardner and Sakkas, 2003). Our samples were classified as: “early blastocysts”, “blastocysts” and “full blastocysts” according to Veeck and Zaninovic (2003).

Statistics

Measurement were taken on PCM micrographs and on SEM micrographs released at the same tilt angle and magnifications. Statistical analysis of the results was performed using Student's *t*-test (MedCalc3000© 1998–2004 Foundation Internet Service). A $P < 0.05$ was considered statistically significant.

Results

Clear cIVF and ICSI human blastocysts

By PCM, these blastocysts generally showed a regular, round-shaped contour and consisted of voluminous cells with a clear cytoplasm (Fig. 1, 2). An intact and very thin ZP encircled these blastocysts (Fig. 1, 2). The ZP average thickness for all samples was $7.8 \mu\text{m} \pm 0.7 \mu\text{m}$. Numerous spermatozoa, sometimes arranged in large clumps, were often found adhering to the cIVF blastocyst surface (Fig. 1). Those blastocysts classified as “blastocysts” and “full blastocysts” showed an outer cellular shell (the outer cell mass -OCM-), the blastocoel cavity, and an inner cell mass (ICM). The latter appeared as a voluminous cellular aggregate that protruded from one pole of the blastocyst inside the blastocoel cavity (Fig. 2). In the “early blastocysts”, where the blastocoel cavity is forming and numerous scattered cells still fill the blastocoel cavity, the ICM and the OCM were not clearly distinguishable (Fig. 1). Cellular debris was occasionally detected inside the blastocoel cavity of both cIVF and ICSI blastocysts (Fig. 2).

By SEM, at low magnification, clear cIVF and ICSI blastocysts displayed a spherical contour (Fig. 3, 4). Numerous spermatozoa, together with some remnants of cumulus-corona cells, were frequently found on the cIVF blastocyst surface (Fig. 3). The ZP outer surface of clear cIVF and ICSI blastocysts showed wide areas provided with a spongy-like texture due to the presence of large and flattened ZP fenestrations. In the whole population of clear blastocysts, the average maximum diameter of these fenestrations was $2.34 \mu\text{m} \pm 0.46 \mu\text{m}$ (Fig. 5,

5 inset, 6, 6 inset). These fenestrations were largest at the ZP outer surface and decreased in size centripetally (Fig. 6 inset). At higher magnifications, finely networked thin filaments formed these fenestrations (Fig. 6 inset). At low magnifications, some spermatozoa still appeared entrapped in the ZP fenestrations of the cIVF blastocysts. In the direct proximity of a sperm penetration site, the ZP outer surface occasionally showed a melted appearance (Fig. 5, 5 inset). However, at higher magnifications, the microfilamentous texture of the ZP fenestrations was still recognizable in these areas (Fig. 5 inset).

Dark cIVF and ICSI human blastocysts

By PCM, these blastocysts generally showed an irregular contour. Numerous cells with a dark and condensed cytoplasm and abundant cellular debris were found in these blastocysts. A thin, intact ZP entirely enveloped the blastocysts. The average ZP thickness for all samples was $7.7 \mu\text{m} \pm 0.6 \mu\text{m}$. The ZP sometimes showed focal points of disconnection from the embryonic cells. In the dark cIVF and ICSI blastocyst, the blastocoel cavity was often collapsed and the OCM and the ICM were hardly recognizable (Fig. 7).

By SEM, at low magnification, dark cIVF and ICSI blastocysts showed an irregular profile and often appeared collapsed (Fig. 8). In these blastocysts, the ZP outer surface showed wide areas with a smooth melted appearance (Fig. 9). A few small remnants of ZP fenestrations were sometimes recognized on the ZP outer surface (Fig. 9 inset). In the whole population of dark blastocysts, the

Fig. 7. Dark cIVF blastocyst. A blastocyst showing an irregular contour. An intact ZP encircles it. Cells with a dark cytoplasm and cellular debris constitute this blastocyst. The blastocoel cavity appears collapsed. PCM: $\times 135$

Fig. 8. Dark cIVF blastocyst. A panoramic view of the blastocyst of Figure 7. The blastocyst surface is irregularly shaped. SEM: $\times 1,200$

Fig. 9. Dark cIVF blastocyst. ZP outer surface of the blastocyst of Figure 7. Note the smooth appearance of the ZP. **Inset:** High magnification of a different area of the same blastocyst, where a remnant of a fenestration is visible (F). Note the compact texture of this fenestration. SEM: $\times 8,500$; inset: $\times 22,000$

average maximum diameter of ZP fenestrations was $1.21 \mu\text{m} \pm 0.47 \mu\text{m}$. At higher magnification, remnants of the ZP fenestrations showed a compact texture (Fig. 9 inset).

^{a, b} Values with these different superscripts within each size group are not significantly different ($P > 0.05$)

^{a', b'} Values with these different superscripts within each size group are significantly different ($P < 0.01$)

Discussion

In the present retrospective study we correlated the ultrastructure of the ZP outer surface to human blastocyst viability *in vitro* during different ART protocols (cIVF and ICSI).

For SEM study we selected both clear blastocysts and dark blastocysts as observed in PCM micrographs. Clear blastocysts, regular in shape and provided with a blastocoel cavity, are considered good quality embryos. Dark blastocysts that are provided with necrotic ICM and/or trophoctoderm and that appear often collapsed are considered poor quality embryos with a low developmental potential (Dokras *et al.*, 1993).

In this study we originally observed by SEM that the ZP outer surface microarchitecture is largely intact and well preserved in clear blastocysts produced by cIVF or ICSI. In fact, clear blastocysts showed a spongy-like microfilamentous pattern of the ZP outer surface similar to that of healthy oocytes, polypronuclear ova, and early developing human embryos (Motta *et al.*, 1988; Familiari *et al.*, 1989, 1992, 1998; Nottola *et al.*, 2001). Such a spongy-like pattern of the blastocyst ZP outer surface is lost during embryo degeneration in culture. In fact, dark blastocysts exhibited a smooth, melted fashion for their outer ZP and, where remnants of fenestrations were present, they appeared compact and significantly reduced in maximum diameter in comparison to those found on the outer ZP of clear blastocysts. Such a blastocyst ZP compaction, similar to that occurring on the surface of unhealthy oocytes, probably represents the ultrastructural correlate of a ZP alteration, which could ultimately affect blastocyst development. On the other hand, the smooth, melted fashion of the outer ZP could be the consequence — rather than the cause — of primary degenerative changes occurring in the blastocyst cells. Thus, our results indicate that the spongy and microfilamentous pattern of blastocyst ZP outer surface is related to human blastocyst viability *in vitro*. It could be hypothesized that blastocyst ZP fenestrations, which regulate nutrient penetration and early embryo

maternal signaling *in vivo*, could exert a similar function also *in vitro*, modulating exchanges between the embryo and the culture microenvironment (Nichols and Gardner, 1989; Denker, 2000; Herrler and Beier, 2000). It has also been demonstrated in IVF-produced bovine oocytes, early embryos and blastocysts that only the preservation of the microfilamentous texture of the ZP fenestrations prevents the penetration of pathogenic agents inside the embryonic cells during *in vitro* culture (Vanroose *et al.*, 2000).

We found that the cIVF blastocysts may show a local, smooth fashion of the ZP outer surface at the level of the sperm penetration site, where the microfilamentous texture is only visible at high magnification. We suggest that this ultrastructural feature of the ZP is physiologic (and not degenerative). It is likely caused by the lytic action of acrosomal enzymes of the spermatozoon that are released during the fertilization process (Vanroose *et al.*, 2000).

In the present study the blastocyst ZP thickness *in vitro* appeared by PCM to be significantly reduced when compared with the ZP measurements reported for polypronuclear ova or early embryos developed *in vitro* (Roux *et al.*, 1995; Garside *et al.*, 1997; our unpublished observations). In addition, we have not found in this study any significant difference in ZP thickness between clear and dark blastocysts. On the basis of our original ultrastructural observations, blastocyst ZP thinning does not seem to be caused by a loss of ZP material — as previously suggested (Schiewe *et al.*, 1995), but it is probably due to the pressure exerted by the actively dividing embryonic cells (which rapidly increase in number in a very small space) and by the mechanical stretching due to blastocoel expansion (Montag *et al.*, 2000). In fact, we found by SEM that the blastocyst ZP appears quite unchanged in comparison to that of embryos at earlier developmental stages (Motta *et al.*, 1988; Familiari *et al.*, 1998; Nottola *et al.*, 2001). The only exception is represented by the enlargement and flattening of the ZP fenestrations in clear blastocysts (likely related to the process of the blastocoel expansion) in comparison with the ZP fenestrations of pronuclear eggs or early embryos developed *in vitro*, which appear smaller in size and deeper (our unpublished observations). Thus, our ultrastructural observations seem to corroborate the hypothesis that ZP thinning offers evidence of blastocyst maturity and is crucial for correct blastocyst hatching (Montag *et al.*, 2000).

We did not observe a significant correlation between the ART protocol utilized to achieve fertilization (cIVF or ICSI) and the outer surface architecture of the blastocyst ZP (spongy-like or smooth). This seems of relevant interest, especially after several reports indicating the negative consequences of the ICSI procedure. It has been found (Aytoz *et al.*, 1999) that the miscarriage rate

after frozen/thawed ICSI embryo transfer is significantly higher than that observed after frozen/thawed IVF embryo transfer. The ICSI procedure, when compared with IVF, reduces the blastocyst formation rate *in vitro* (Jones *et al.*, 1998; Shoukir *et al.*, 1998; Griffiths *et al.*, 2000). This deficiency is probably a consequence of the post embryonic genome activation when the centrosomal or genetic defects in the spermatozoa of infertile men begin to exert their negative influence (Jones *et al.*, 1998; Shoukir *et al.*, 1998). Actually, whether or not the injection process itself is a causative factor is still an unresolved debate. In our previous SEM studies, we found an isolated unusual hole on the ZP of human ICSI pronuclear eggs and early embryos (4-cell stage), likely corresponding to the residual gap through which the sperm was introduced (Makabe *et al.*, 1998). As seen by SEM, the diameter of such holes on the ZP outer surface long after fertilization — at the time of segmentation — is much reduced in size, probably due to the retractile elasticity of the ZP material that remains “unaffected” after ICSI. In other previous studies by our research group, a wide smooth endoplasmic reticulum (SER) aggregate was occasionally found by transmission electron microscopy in one of the blastomeres of four- to six/seven-cell ICSI embryos (Makabe *et al.*, 2001; Nottola *et al.*, 2001). This feature, not detected in cIVF samples, could be considered a remnant of the microinjected sperm-associated tubular SER, as reported for human oocytes unfertilized after ICSI (Sousa and Tesarik, 1994), but its eventual consequences on embryo developmental potential have not been ruled out up to now (Makabe *et al.*, 2001).

Therefore, prolonged extracorporeal management did not affect the ZP outer surface ultrastructure in good quality blastocysts. However, degenerated blastocysts were provided with an altered ZP. We suggest that these ultrastructural modifications of the ZP outer surface could reflect sub-optimal culture conditions, representing the ultrastructural correlate of an impairment of an effective filtering action of the ZP that ultimately affects blastocyst development. ZP alterations could also be the consequence of degenerative processes that occur primarily in the blastocyst. In addition, on the basis of our results, the blastocyst ZP ultrastructure seems to be independent from the protocol utilized to achieve fertilization (cIVF or ICSI).

It is concluded that preservation of the ZP ultrastructure is related to human blastocyst viability *in vitro*.

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