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The Human Pronuclear Ovum: Fine Structure of Monospermic and Polyspermic Fertilization In Vitro

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The fine structure of pronuclear ova (monospermy and polyspermy) and one-cell embryos has been investigated in our IVF programme. Sixteen oocytes were collected at laparoscopy after appropriate hormonal stimulation and were matured and fertilized in vitro by methods that have given rise to normal pregnancies.

Pronuclear ova showing monospermic fertilization had two vesicular pronuclei surrounded by aggregations of cellular organelles. The male pronucleus was closely associated with a sperm axoneme, while the female pronucleus was dismantling its envelope and condensing its chromatin ahead of its counterpart in late pronuclear ova. Each pronucleus had dispersed chromatin, dense compact nucleoli, and intranuclear annulate lamellae. Smooth endoplasmic reticulum, annulate lamellae, Golgi complexes, and mitochondria formed a conspicuous part of the perinuclear ooplasm. The one-cell embryos were either in syngamy or in the process of undergoing first cleavage. Positive evidence of cortical granule release and second polar bodies were detected in the perivitelline space. A block to polyspermy seemed to operate at the level of the inner zona.

Dispermic and polyspermic ova had 3–16 pronuclei resembling those of monospermic ova and had sperm tails in the ooplasm. Sperm were also seen penetrating the inner zona and were occasionally found in the perivitelline space. Incomplete cortical granule release and early signs of cytoplasmic fragmentation were noted in polyspermic ova.

Both normal and abnormal features of these ova are reported and compared with pronuclear structure in vivo and in vitro.

Key words: in vitro fertilization, pronuclear ova, one-cell embryo, monospermy, polyspermy, syngamy

INTRODUCTION

The pronuclear ovum is the stage at which monospermic fertilization is confirmed in an IVF programme [Trounson et al, 1982]. This is usually done about 16 h postinsemination, after the cumulus cells around the ovum are removed by dissection or gentle pipetting. It is also the stage when polyspermy can be identified by the presence of multiple pronuclei, before the embryo is allowed to develop further in fresh embryo culture medium. Although polyspermy is lethal in man, such ova are

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known to undergo apparently normal cleavage up to the morula stage [Van Blerkom et al, 1984] and may be inadvertently used for embryo transfer (ET).

One-cell embryos at syngamy have been successfuly used to produce normal pregnancies after ET [Trounson, 1983]. This stage, at which maternal and paternal chromosomes intermingle, is also used to determine chromosomal aberrations that may occur at fertilization [Edwards, 1980].

The early events associated with pronuclear formation have been reported elsewhere [Lopata et al, 1980]. This study was undertaken to confirm fertilization in zygotes obtained in our in vitro fertilization (IVF) programme and to assess their normality. The ultrastructural organization of pronuclear ova (both monospermic and polyspermic) and of the ovum at syngamy, obtained by IVF methods that produced normal pregnancies in our clinics [Mohr and Trounson, 1984], are reported in this paper.

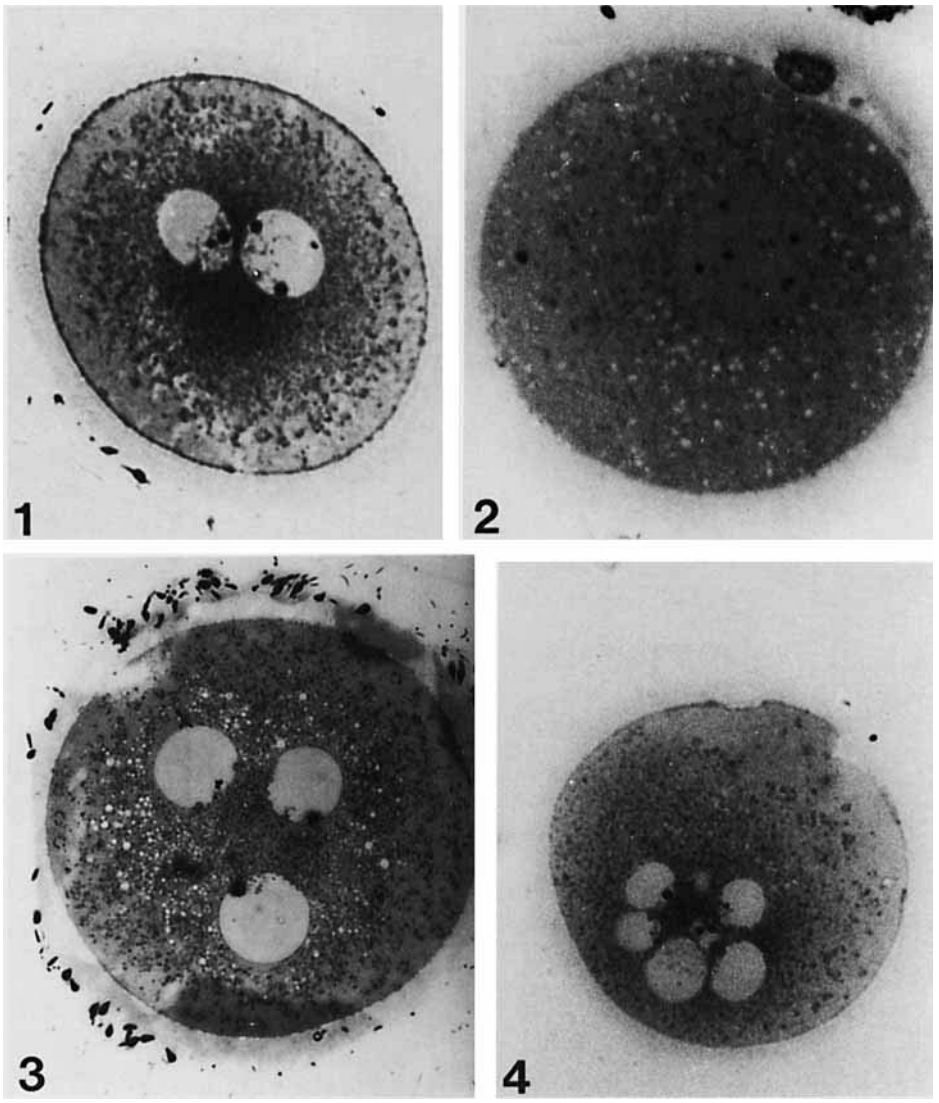
MATERIALS AND METHODS

Six pronuclear ova, eight polyspermic ova, and four single-cell embryos were examined by electron microscopy (Table 1).

The methods used for obtaining pronuclear ova and embryos have already been described [Trounson et al, 1982]. Briefly, the patients involved in this study had predominantly tubal infertility and were stimulated with clomiphene citrate or human menopausal gonadotropin or both, followed by the administration of human chorionic gonadotropin in cases where there was no spontaneous luteinizing hormone (LH) surge [Mohr and Trounson, 1984]. Preovulatory oocytes were recovered at laparoscopy and cultured 3–8 h before insemination to enable completion of maturation [Sathanathan and Trounson, 1982a; Trounson et al, 1982]. Semen was obtained from the patients' husbands by masturbation and the sperm were washed, centrifuged, and layered to harvest motile sperm. About 10,000 to 50,000 motile sperm were added to 1 ml of culture medium containing the oocyte [Mahadevan and Baker, 1984]. Four different culture media—Earl's, Ham's F10, Whitten's, and Whittingham's T6—were used in this study, and there was no significant difference between these media for fertilization, embryo cleavage, or pregnancy rate [Mohr and Trounson, 1984]. The fertilized ova were examined by light microscopy 12–18 h after insemination for the presence and number of pronuclei, and allowed to develop further in fresh embryo culture medium (17–72 h) before fixation. The fertilized ova and embryos were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) and stored 1–8 weeks in this fixative in a cold room. They were postfixed in 1% aqueous osmium tetroxide (30 min), dehydrated rapidly in ethyl alcohol followed by acetone, and then embedded in Araldite [Sathananthan and Trounson, 1982a,b]. Alternate series of thick (1 μm) and thin ($\sim$70 nm) sections were cut using glass and diamond knives, respectively. Thick sections were stained with toluidine blue for light microscopy. Thin sections were stained with alcoholic uranyl acetate and Reynold's lead citrate and examined with a Philips 301 electron microscope.

RESULTS

The general organization of pronuclear ova (monospermic or polyspermic) and the fertilized ovum in syngamy, revealed by light microscopy, is shown in figures



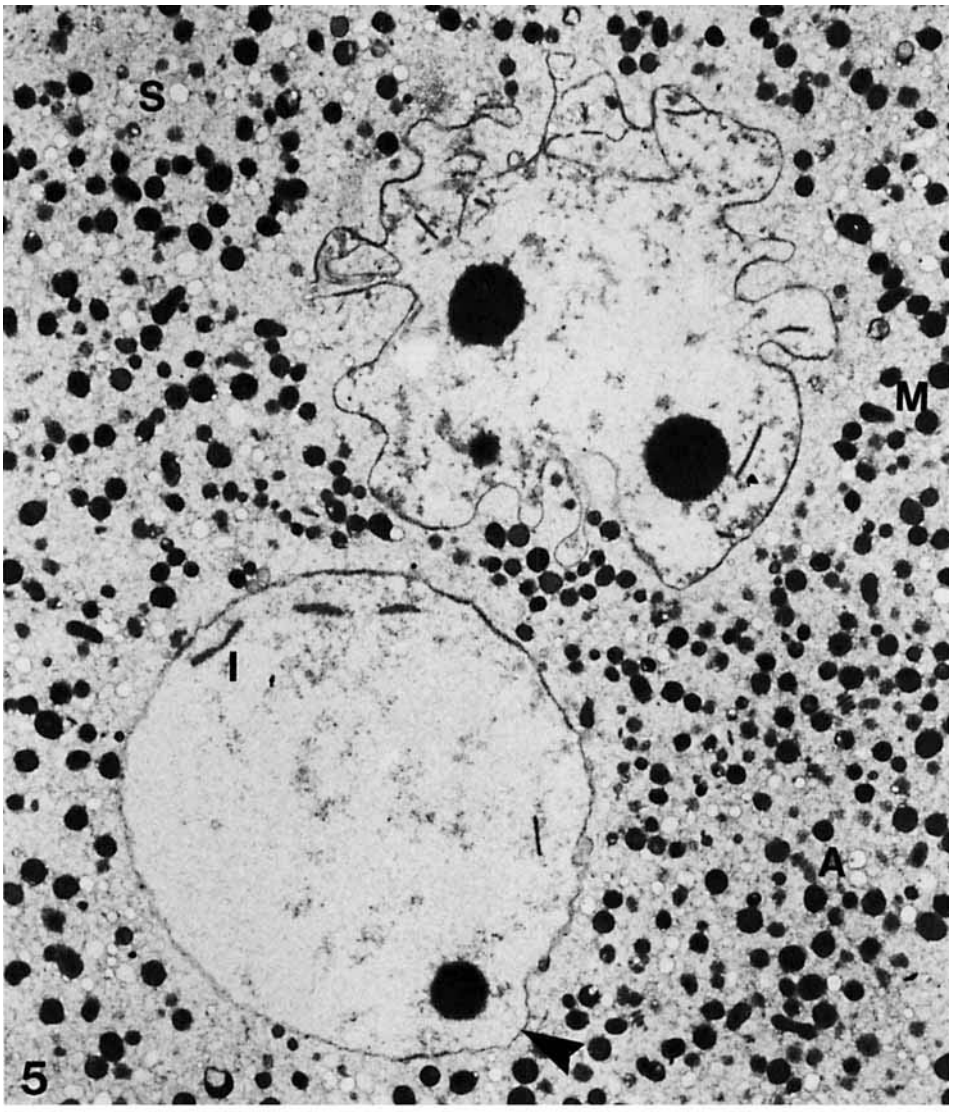
Figs. 1–4. Light micrographs of pronuclear ova and syngamy.

Fig. 1. Pronuclear ovum (monospermy) showing two vesicular pronuclei, almost equal in size, associated in the central ooplasm. Note aggregation of cellular organelles around pronuclei containing dense nucleoli. ×290 (17 h postinsemination).

Fig. 2. Fertilized ovum (1-cell embryo) in syngamy just prior to cleavage. The chromosomes, some associated in pairs, are located in an agranular zone in the central ooplasm. Clear vacuoles have already appeared in the ooplasm and a polar body is visible in the perivitelline space. ×460 (32 h postinsemination).

Fig. 3. Dispermic ovum with three pronuclei containing dense nucleoli. Numerous sperm are seen at the surface of the zona. This section has been restained. ×440 (48 h postinsemination).

Fig. 4. Polyspermic ovum sectioned through a "nest" of several pronuclei. Note accumulation of organelles around pronuclei and incomplete abstriction of the second polar body. ×230 (60 h postinsemination).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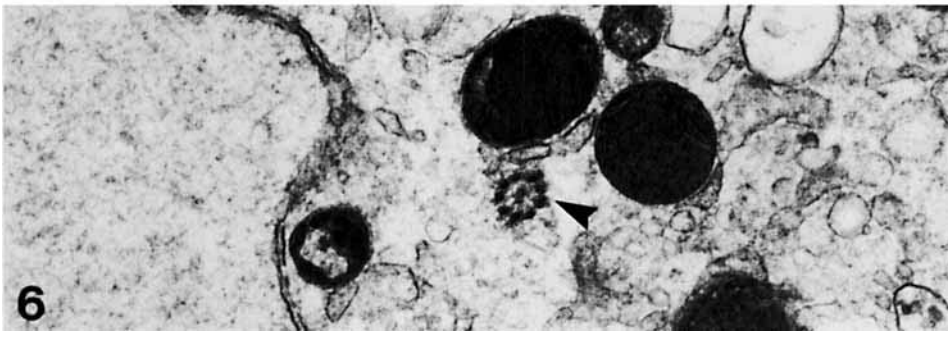
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1–4. Each ovum was surrounded by a zona pellucida, outside which were occasional clumps of cumulus cells that had retracted most of their processes from the surface of the oocyte. The oolemma extended into a few microvilli and marked pinocytotic activity was evident at the surface. Both acrosome reacted and non-reacted sperm were found between cumulus cells and at the surface of the zona, and some were phagocytosed by cumulus cells. Reacting or reacted sperm were often seen penetrating the outer zona.

Most ova had a nucleated second polar body (PB_2), in addition to the first (PB_1), located in the perivitelline space (PVS). Evidence of cortical granule (CG) release was observed in all ova, and their products were frequently seen in the PVS or interacting with the zona. At least two ova in each category examined revealed remnants of sperm axonemes in the ooplasm. These features considered collectively confirm that the ova were fertilized in vitro.

Monospermy

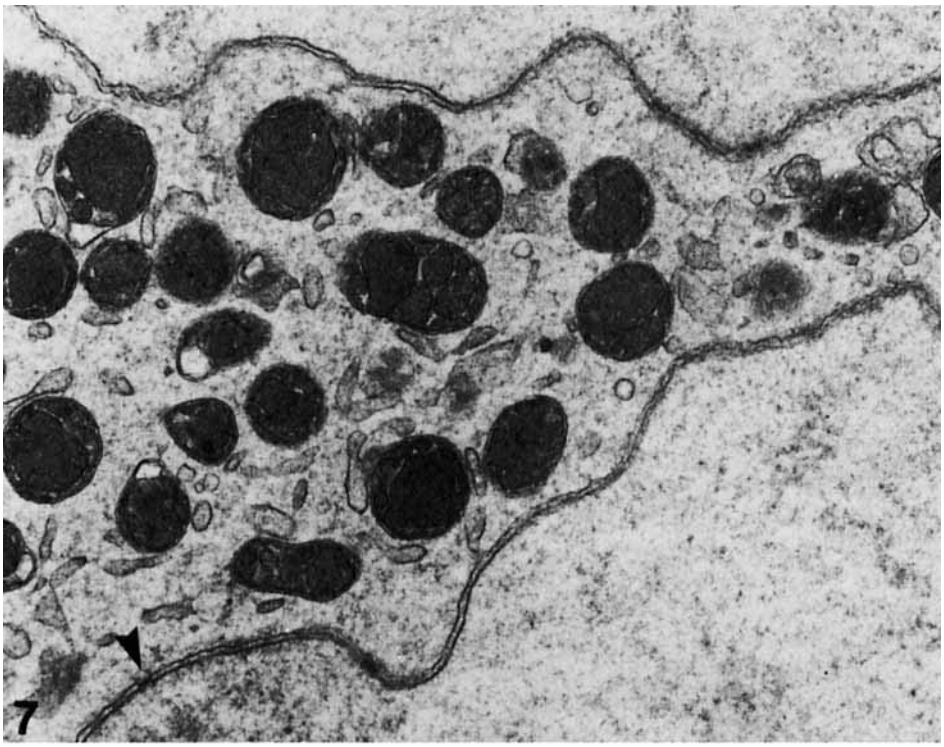
Most pronuclear ova showing monospermic fertilization had two well-defined vesicular pronuclei, male (MPN) and female (FPN), usually associated in the central ooplasm (Figs. 1,5). Both pronuclei were identical in structure; almost equal in size; and had up to eight dense compact nucleoli, dispersed chromatin, and isolated intranuclear annulate lamellae. There was often a distinct interpronuclear zone (Fig. 7) with mitochondria and elements of the smooth endoplasmic reticulum (SER). This zone may be very narrow and devoid of organelles, but interdigitation or fusion of the nuclear envelopes was never observed. The nuclear envelopes may show well-defined pores (Figs. 7,8) and somewhat dense, oval bodies arising from the inner nuclear membrane, located within the envelope (Fig. 8). Specks of heterochromatin may be associated with nucleoli, which were dense and predominantly fibrillar in appearance. Nucleoli may sometimes be closely associated with the nuclear envelope (Fig. 13). A sperm tail axoneme was seen opposite a pronucleus in one ovum (Fig. 6), while the other pronucleus was already dismantling its envelope (Fig. 12), which presented a quadrilaminar structure.

The cytoplasm of pronuclear ova consists of the usual organelles found in unfertilized oocytes and early embryos. These include mitochondria, SER, annulate lamellae (AL), Golgi membranes, and lysosomal inclusions. Mitochondria had dense stromae and few arc-like, reticulate, or transverse cristae (Fig. 7). The SER consisted of larger vesicular elements and smaller irregular profiles, often associated with mitochondria (Fig. 9). Ribosomes were rarely seen. AL were abundant and composed of stacks of parallel cisternae, closely resembling the nuclear envelope in fine structure (Figs. 9,10). These were sometimes continuous with elements of SER. Many

Figs. 5–16. Electron micrographs of pronuclear ova and 1-cell embryos.

Fig. 5. Late pronuclear ovum (monospermy) showing the two pronuclei associated in the central ooplasm. The MPN is still vesicular and had a sperm axoneme opposite the protuberance (arrow) in serial sections. The FPN has already begun to dismantle its envelope and condense its chromatin, ahead of the MPN. Both pronuclei have dense compact nucleoli and intranuclear annulate lamellae (I). Note abundance of cytoplasmic organelles around pronuclei. M, mitochondria; S, vesicular SER; A, annulate lamellae. ×6,300 (24 h postinsemination).

Fig. 6 Serial section of pronuclear ovum in Figure 5 showing the sperm axoneme opposite the protuberance of the MPN. The "9 + 2"organization of microtubules is apparent in the axoneme (arrow). M, mitochondria; S, vesicular SER. ×32,130 (24 h postinsemination).



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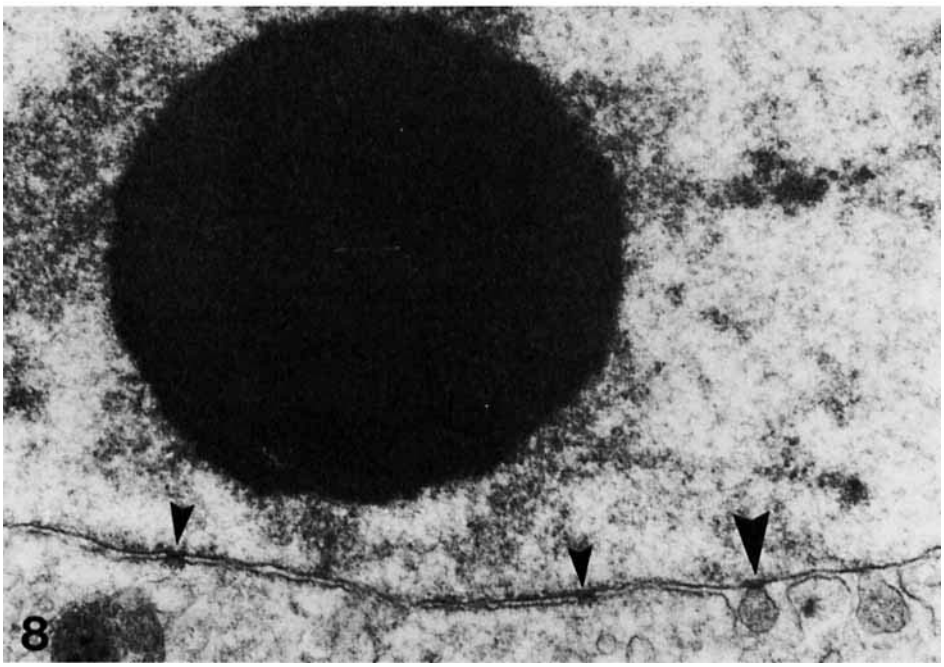

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hypertrophic Golgi complexes composed of tightly packed cisternae, vesicles, and clear vacuoles were evident (Fig. 11) and were frequently extensive. An elaborate system of SER, AL, and Golgi membranes were found in the ooplasm in close association with pronuclei. Primary lysosomes were identified (Fig. 15) as small membrane-bound vesicles with dense cores [Trounson and Sathananthan, 1984]. Multivesicular bodies, secondary lysosomes and residual bodies were heterogenous in overall appearance and constituted the other lysosomal inclusions.

Polyspermy

Ova with multiple pronuclei were observed in about 2% of oocytes fertilized in vitro [Mahadevan and Trounson, 1984]. Three ova examined were dispermic and had three pronuclei each (Fig. 3), while five were polyspermic and had up to 16 supernumerary pronuclei forming nests in the ooplasm (Fig. 4). All these ova resembled monospermic ova in their general organization, and their pronuclei were identical in structure to normal pronuclei (Fig. 14). However, delayed CG release and massive explosions of CGs were occasionally observed. Multiple sperm tails were identified within polyspermic ova and sperm heads had not fully decondensed their chromatin in some ova. Sperm were often seen penetrating the inner zona as well and were occasionally found in the PVS.

Polyspermic ova may show early signs of peripheral cytoplasmic fragmentation or incomplete abstriction of the second polar body (Fig. 4). Partial incorporation of chromatin into pronuclei and formation of smaller nuclei around scattered masses of chromatin were also encountered. The latter may contain nucleoli and may be attached to pronuclei. These are aberrant features often associated with polyspermy.

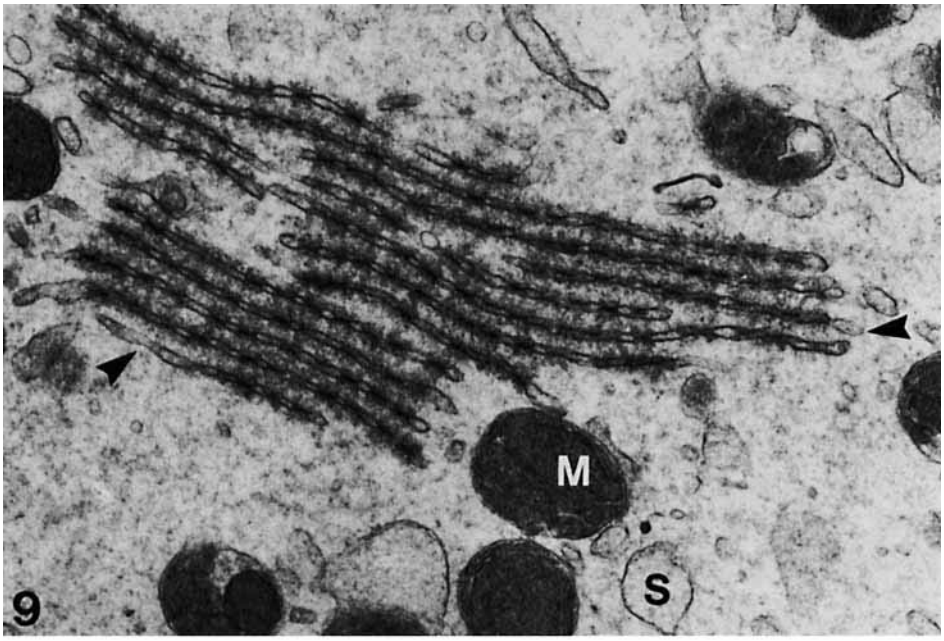
TABLE 1. Zygotes Examined by Electron Microscopy

Embryo	No. examined	Time in culture after insemination (h)[a]
Pronuclear ova (monospermy)	6	17–28
Pronuclear ova (polyspermy)	8[b]	48–72
One cell embryos (syngamy)	4[c]	32–60

[a]Ova kept longer in culture (36–72 h) showed progressive dilation of SER and Golgi membranes and increased density of mitochondria.
[b]Three ova were dispermic; the rest were polyspermic.
[c]One embryo showed delayed nuclear envelope breakdown of one pronucleus.

Fig. 7. Interpronuclear zone between two pronuclei of a monospermic ovum. This is occupied by mitochondria with dense stromae and few cristae, associated with smaller elements of SER. The pronuclear envelopes are composed of two membranes and have pores (arrow). ×32,130 (17 h postinsemination).

Fig. 8. Dense compact nucleolus in a pronucleus of a monospermic ovum. It is composed of fine fibrils and is associated with chromatin. The nuclear envelope has pores (small arrows) and two pear-shaped bodies arising from the inner membrane, where a pore-like structure is visible (large arrow). ×32,130 (17 h postinsemination).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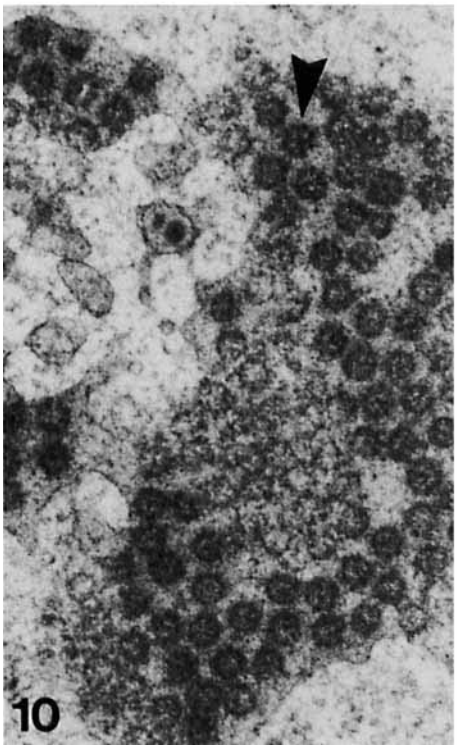
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Syngamy

The ovum in syngamy, at the onset of cleavage, had parental chromosomes organized in an organelle-free spindle zone in the central region of the ooplasm (Fig. 2). When the first cleavage was in progress, most chromosomes were aligned on the equatorial plate (meta-anaphase) of the spindle (Fig. 15) and attached to spindle microtubules via kinetochores. Centrioles and asters were absent and the spindle organization was similar to those of maturing oocytes. Evidence of possible non-disjunction of chromosomes in this embryo, arrested during cleavage, was observed.

DISCUSSION

Our ultrastructural observations agree with those of Zamboni et al [1966] for a pronuclear ovum obtained in vivo and of Soupart and Strong [1974, 1975], who described monospermic fertilization of oocytes in vitro, recovered from excised ovaries or ovarian follicles during elective surgery, without prior stimulation. Soupart and Strong [1975] also reported the fine structure of zona-free polyspermic oocytes fertilized in vitro, which basically agrees with our observations on polyspermic ova with intact zonae.

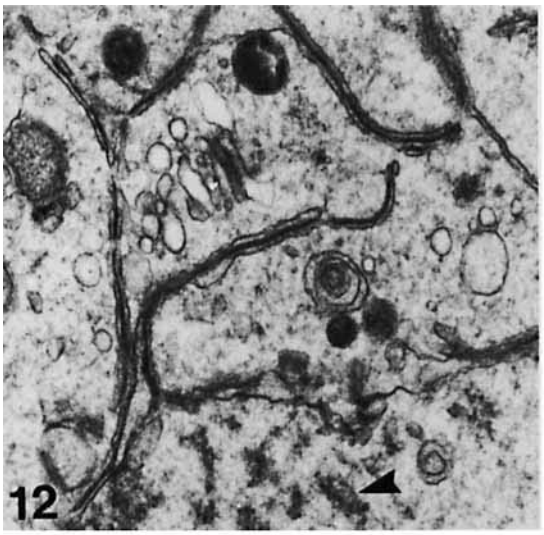
The identification of remnants of sperm axonemes in pronuclear ova (both monospermic and polyspermic) and in one-cell embryos, considered together with other criteria of fertilization, namely, evidence of PB_2 and CG release into the PVS, is proof that the eggs were fertilized in vitro and not the result of spontaneous or parthenogenetic activation. Unfertilized oocytes are known to develop multiple nuclei resembling pronuclei [Zamboni et al, 1972; Soupart and Strong, 1974]. These are apparently formed around scattered masses of chromatin, disconnected from spindle microtubules during meiosis. Nuclei were also detected in PB_1 of ageing oocytes, and these could well be mistaken for PB_2 when viewed with the light microscope. These aberrant oocytes and polar bodies, however, contain intact CGs.

The close proximity of the sperm axoneme to the MPN, also observed by previous workers, and the condensation of chromatin in the FPN ahead of the MPN in late pronuclear ova are useful criteria in their respective identification. The latter phenomenon has been reported in the mouse [see Edwards, 1980]. Chromosomal studies of polyspermic ova have confirmed male and female karyotypes in human pronuclei [Rudak et al, 1984]. Although sperm tails may not be so easily discernible with the light microscope, expanding sperm heads and developing pronuclei can be identified. Fully developed pronuclei should contain multiple nucleoli in contrast to large vacuoles that may occasionally appear in the ooplasm and confuse identification.

Fig. 9. Juxtanuclear stack of cytoplasmic annulate lamellae of a pronuclear ovum showing numerous pore-complexes lined by dense material (arrow). Note continuity of lamellae with small elements of the SER (arrows). S, vesicular SER; M, mitochondria. ×32,130 (32 h postinsemination).

Fig. 10. Tangential section of annulate lamellae of a pronuclear ovum. Each pore-complex consists of an annulus (arrow) organized of subunits arranged in 8-part radial symmetry. ×49,140 (32 h postinsemination).

Fig. 11. Well developed Golgi complex of a pronuclear ovum consisting of smooth-surfaced cisternae associated with small vesicles and large clear vacuoles. The vesicles appear to be budded off from the ends of cisternae while vacuoles are inflated segments of cisternae. The complex has a convex forming face and a concave secretory face. ×39,600 (30 h postinsemination).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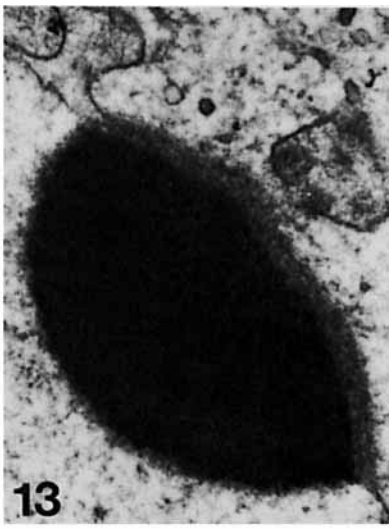
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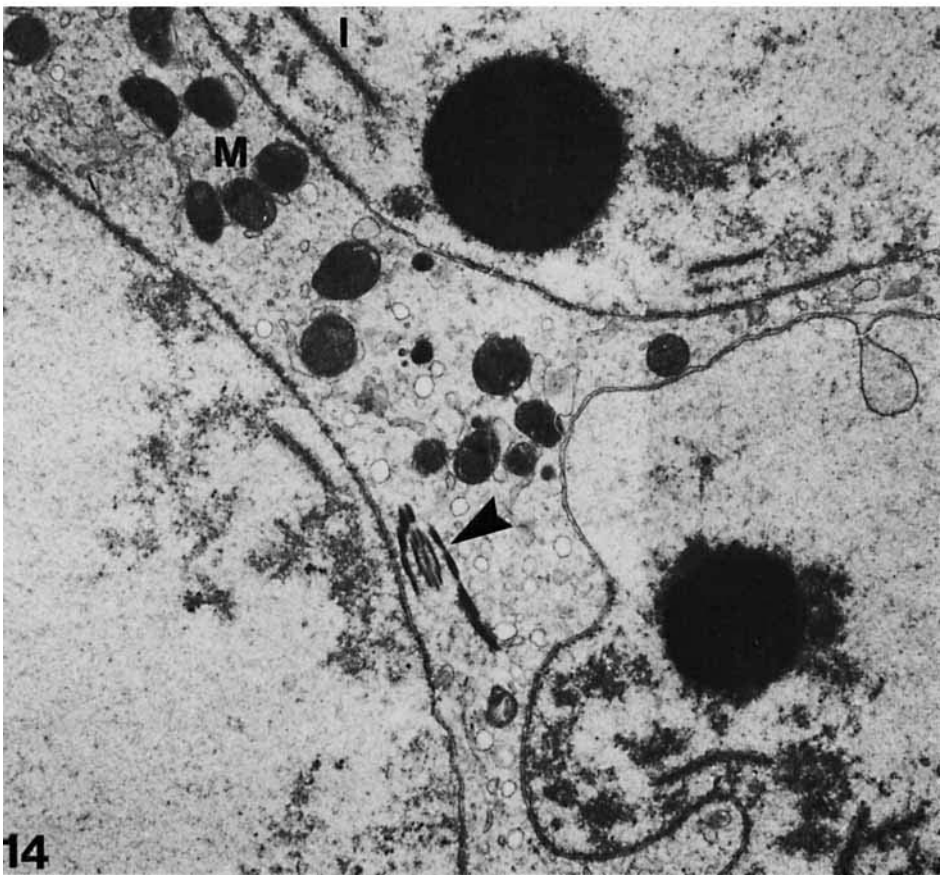
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The most significant feature of the pronuclear ovum is the extensive development of SER, Golgi complexes, and AL associated with mitochondria and the pronuclei themselves. This indicates high metabolic activity of the egg after fertilization. Small and large elements of the SER, AL, and also the nuclear envelope were occasionally continuous, and they may well belong to the same reticular system. These organelles, particularly AL, also predominate in polyspermic ova and in multinucleated blastomeres of early embryos [Sathananthan et al, 1982b; Trounson and Sathananthan, 1984], and their incidence is probably related to a high turnover of nuclear envelope material. Hypertrophic Golgi membranes are very likely linked to secretory activity where the secretion is evidently packaged into clear vesicles for exocytosis. The nature of this secretion is not known, but Golgi membranes were involved in formation of CGs and lysosomes in maturing oocytes [Sathananthan et al, 1984]. The abundance of mitochondria could be energy related [Soupart and Strong, 1975], particularly in association with the SER and nuclear envelope formation or may be due to their multiplication prior to cleavage [Zamboni et al, 1966].

The causes of polyspermy in vitro were mainly attributed to the immaturity of the oocyte at the time of sperm penetration [Sathananthan and Trounson, 1982a,b]. Polyspermic ova show evidence of delayed CG release and consequently a delayed zona reaction, which results in an ineffective or partial block to polyspermy at the level of the inner zona [Sathananthan et al, 1982a]. The presence of supernumerary sperm in the inner zona and occasionally the PVS is evidence of this suboptimal block to polyspermy. Polyspermy may be due to immaturitiy [Sathananthan and Trounson, 1982b], ageing of oocytes in culture, an inherent zona defect, or a breach in the zona caused during egg collection. The latter may be avoided by gentle aspiration at laparoscopy and careful handling of oocytes especially during the removal of cumulus cells. Wolf et al [1984] have reported that polyspermic fertilization is directly related to the concentration of motile sperm used to inseminate human ova. However, in our own IVF studies we have found that insemination with excessive number of sperm and large numbers bound to the surface of the oocyte does not seem to increase the incidence of polyspermy [Mahadevan and Trounson, 1984].

Polyspermic ova that continue to develop in culture may cleave normally, undergo partial fragmentation sooner or later, or develop into embryos with multinucleated blastomeres [Sathananthan et al, 1982b; Trounson and Sathananthan, 1984]. Embryos with fragments have given rise to normal pregnancies [Mohr and Trounson, 1984]. Multipronuclear ova may apparently cleave normally up to about the morula stage and may show multiple nuclei in about 25% of their blastomeres [Van Blerkom et al, 1984]. Hence, it is important to detect polyspermy as early as possible, preferably at the pronuclear stage prior to replacement in the uterus. The multiple

Fig. 12. Dismantling segment of FPN envelope of the ovum in Figure 5. The quadrilaminar structure of the envelope is evident in association with vesicular elements. The chromatin (arrow) is condensing. ×31,600 (24 h postinsemination).

Fig. 13. Hemispherical nucleolus of a pronuclear ovum closely associated with the nuclear envelope. The membranes of the envelope are masked by dense material and appear to be fused. ×31,600 (32 h postinsemination).

Fig. 14. Polyspermic ovum showing three pronuclei of a "nest" of several and a sperm tail in the ooplasm (arrow). Each pronucleus has dense nucleoli and chromatin which has condensed more in the nucleus with the undulated membrane (compare with FPN, Fig. 5). I, intranuclear annulate lamellae; M, mitochondria. ×13,860 (60 h postinsemination).

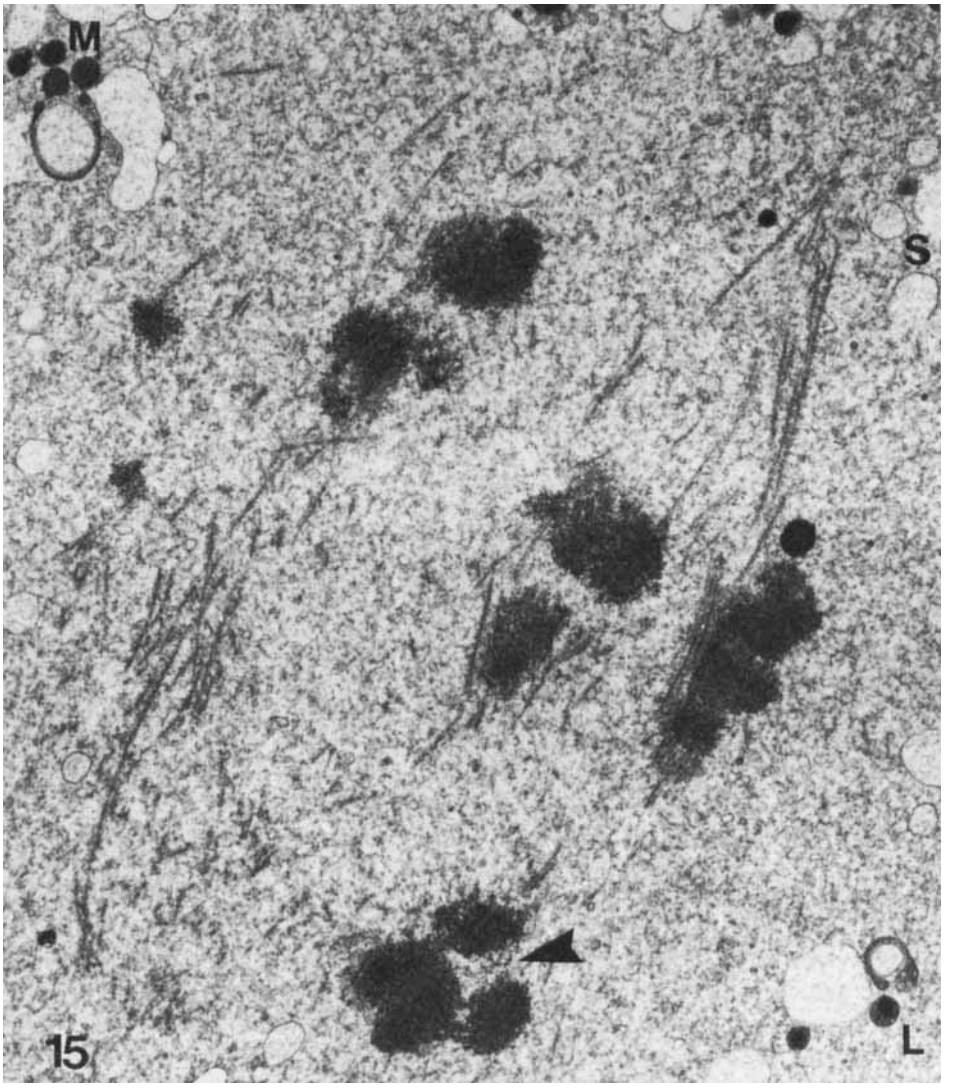

Fig. 15. One-cell embryo arrested at meta-anaphase of the first cleavage division. The spindle consists of microtubules, somewhat disorganized, ending abruptly in the ooplasm at either pole. Chromatids seem to be separating in two chromosomes while one set of chromosomes (arrow) does not appear to have separated (non-disjunction?). M, mitochondria; S, vesicular SER; L, primary lysosome. ×13,860 (40 h postinsemination).

MPN may be passively displaced to one or both blastomeres during the first cleavage division as in mouse ova [Witkowska, 1981], and this might explain the origin of multinucleated blastomeres at least in some of the human embryos. Fortunately, polyspermy is thought to be lethal in man, and such embryos may be aborted if transferred inadvertently. Wolf and Yu [1981] have shown that polyspermic mouse eggs are capable of disposing supernumerary sperm soon after fertilization. Karyotype analyses of some human embryos originally having three pronuclei have shown them

to be diploid (R. Angell and A. Trounson, unpublished data) indicating that a similar phenomenom may occur in the human.

Syngamy has been demonstrated in the human [Edwards, 1980] and is regarded as the culmination of fertilization. The one-cell embryo, usually developed 18–26 h after fertilization, is a rather difficult stage to assess in the IVF laboratory as it does not show nuclei, nor is it cleaving, and its chromosomes are not easily visible by light microscopy. It, however, should have at least two polar bodies in the PVS. Oocytes that do not show pronuclei should not be discarded, as they may be in syngamy and should be left to develop for a further 30–40 h after insemination. Chromosome staining techniques are still invaluable in determining the karyotype but are invasive, as they involve fixation of the embryo.

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REFERENCES

Edwards RG (1980): "Conception in the Human Female." London: Academic, chapter 8, pp 573–635.

Lopata A, Sathananthan AH, McBain JC, Johnsotn WIH, Spiers AL (1980): The ultrastructure of preovulatory human egg fertilized in vitro. Fertil Steril 33:12–20.

Mahadevan M, Baker G (1984): Assessment and preparation of semen for in vitro fertilization. In Wood C, Trounson A (eds): "Clinical In Vitro Fertilization." Berlin: Springer-Verlag, chapter 7, pp 83–97.

Mahadevan MM, Trounson AO (1984): The influence of seminal characteristics on the success rate of human in vitro fertilization. Fertil Steril 42:400–405.

Mohr L, Trounson A (1984): In vitro fertilization and embryo growth. In Wood C, Trounson A (eds): "Clinical In Vitro Fertilization." Berlin: Springer-Verlag, chapter 8, pp 99–115.

Rudak E, Dor J, Mashiach S. Nebel L, Goldman B (1984): Chromosome analysis of multipronuclear human oocytes fertilized in vitro. Fertil Steril 41:538–545.

Sathananthan AH, Trounson AO (1982a): Ultrastructural observations on cortical granules in human follicular oocytes cultured in vitro. Gamete Res 5:191–198.

Sathananthan AH, Trounson AO (1982b): Ultrastructure of cortical granule release and zona interaction in monospermic and polyspermic human ova fertilized in vitro. Gamete Res 6:225–234.

Sathananthan AH, Trounson AO, Wood C, Leeton J (1982a): Ultrastructural observations on the penetration of sperm into the zona pellucida of the human egg in vitro. J. Androl 3:356–364.

Sathananthan AH, Wood C, Leeton J (1982b): Ultrastructural evaluation of 8–16 cell human embryos developed in vitro. Micron 13:193–203.

Sathananthan AH, Ng SC, Chia CM, Law HY, Edirisinghe RW and Ratnam SS (1984): The origin and distribution of cortical granules in human oocytes with reference to Golgi, nucleolar and microfilament activity. In Edwards RG (ed): "In Vitro Fertilization and Embryo Transfer." New York Academy of Sciences 442:251–264.

Soupart P, Strong PA (1974): Ultrastructural observations on human oocytes fertilized in vitro. Fertil Steril 25:11–44.

Soupart P, Strong PA (1975): Ultrastructural observations on polyspermic penetration of zona pellucida-free human oocytes inseminated in vitro. Fertil Steril 26:523–537.

Trounson A (1983): Factors controlling normal embryo development and implantation of human oocytes fertilized in vitro. In Beier HM, Lindner HR (eds): "Fertilization of the Human Egg In Vitro." Berlin: Springer-Verlag, pp 315–322.

Trounson AO, Sathananthan AH (1984): The application of electron microscopy in the evaluation of 2–4 cell human embryos cultured in vitro for embryo transfer. J In Vitro Fert Embryo Transfer 1:153–165.

Trounson AO, Mohr LR, Wood C, Leeton JF (1982): Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. J Reprod Fertil 64:285–294.

Van Blerkom J, Henry GP, Porreco RP (1984): Preimplantation human embryonic development from polynuclear eggs after in vitro fertilization. Fertil Steril 41:686–696.

Witkowska A (1981): Pronuclear development and the first cleavage division in polyspermic mouse eggs. J Reprod Fertil 62:493–498.

Wolf DP, Yu SF (1981): Polyspermic mouse eggs can dispose of supernumerary sperm. Dev Biol 82:203–210.

Wolf DP, Byrd W, Dandekar P, Quigley MM (1984): Sperm concentration and the fertilization of human eggs in vitro. Biol Reprod 31:837–848.

Zamboni L, Mishell DR Jr, Bell JH, Baca M (1966): Fine structure of the human ovum in the pronuclear stage. J Cell Biol 30:579–600.

Zamboni L, Thompson RS, Moore Smith D (1972): Fine morphology of human oocyte maturation in vitro. Biol Reprod 7:425–457.