Principles of mammalian fertilization

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The principles of mammalian fertilization are defined in relation to studies of the intracytoplasmic injection of spermatozoa into human oocytes (ICSI). Sections are included on the production of mature oocytes and mature spermatozoa, sperm–oocyte interaction, and formation of the pronucleate oocyte. Current concepts in gametogenesis are discussed, including spermiogenesis, oogenesis, the biology and penetration of the zona pellucida including capacitation and the acrosome reaction, gamete fusion, the block to polyspermy and growth of the pronucleus. A concluding section relates the normal events of fertilization to those occurring after ICSI and discusses various types of anomalies found in the oocyte after ICSI.

Key words: acrosome reaction/capacitation/fertilization/gametogenesis/ICSI

Fertilization is a complex process of molecular events involving matured haploid male and female gametes and their mutual recognition and fusion to establish the genotype of a new individual. Fully grown, matured oocytes and normally differentiated haploid spermatozoa are the prerequisites for the success of the fertilization process. The topic has been recently reviewed in detail (Edwards and Brody, 1995), and the present text provides a brief and up-to-date summary of the major factors involved.

Production of the mature oocyte

Oocyte maturation is defined as the reinitiation and completion of the first meiotic division, subsequent progression to metaphase II and the completion of nuclear and cytoplasmic processes which are essential for fertilization and early embryo development. Oocytes are arrested in prophase I of meiosis during the fetal period, and this nucleated stage persists until maturation begins (Figure 1). During this prolonged period, the oocyte enlarges and synthesizes RNA and protein as the follicle grows. The oocyte and follicle become sensitive to the actions of gonadotrophins, and the follicle stimulating hormone (FSH) and luteinizing hormone (LH) surges in mid-cycle, or the external application of human chorionic gonadotrophin (HCG), initiate the onset of maturation in antral follicles and oocytes.

Completion of the first meiotic division takes place when oocytes have undergone extensive growth in cellular interaction with the granulosa and theca cells. The oocyte undergoes asymmetric cytokinesis and extrudes the first polar body containing a haploid chromosome complement. Immediately after the first meiotic division is completed, the second meiotic division is initiated, and oocytes arrest in metaphase II until fertilization occurs and activates the mature oocyte. Mechanisms of oocyte maturation are still under investigation. In-vitro models gave insight into the importance of substances affecting oocyte maturation and its inhibition such as cAMP, calcium, cell cycle proteins, growth factors, gonadotrophin-releasing hormone (GnRH), gonadotrophins, purines and steroids (Figure 2).

One of the major events of structural changes during oocyte maturation is germinal vesicle breakdown (GVB), indicating the reinitiation of meiosis and the disappearance of the germinal vesicle

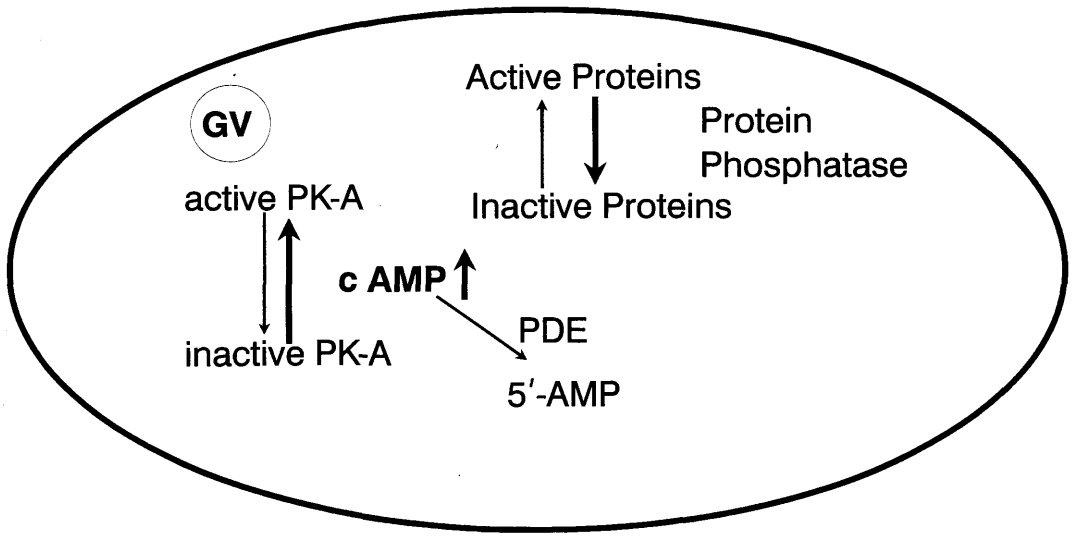

Figure 1. Maintenance of meiotic arrest. GV=germinal vesicle.

(Edwards and Brody, 1995). These structural changes of the oocytes nucleus are initiated *in vivo* by the LH surge. GVB begins with undulations of the nuclear envelope which continues for ~1–2 h (Van Blerkom, 1989). These undulations may correlate with the onset of chromosome condensation and the detachment of chromosomes from their site-specific partners in the nuclear membrane. The membranes of the nuclear envelope disappear and their rudiments are assumed to be involved in formation of pronuclear membranes after fertilization has occurred. Chromosome condensation and spindle formation are subsequent steps on the schedule of oocyte maturation.

Following GVB, the nuclear envelope and its fibrillar lining of laminins, decondense as chromosomes retract from the centre of the nucleus towards the undulating membranes as condensation occurs. Chiasmata terminalize (i.e. move to the ends of the chromosomes) and chromatin begins to condense. When condensation is complete, the emergent chromosomes become arranged in the centre of the oocyte as the metaphase spindle forms. During GVB and chromosome condensation, kinetochores and the microtubule system organize the formation of the spindle. The spindle apparatus is formed and moves to the periphery of the oocyte where the barrel-shaped spindle becomes surrounded by mitochondria, vacuoles and granules. Metaphase I lasts for a few hours, then leads to anaphase I, when chromosomal bivalents move towards the opposite ends of the spindle and the whole spindle rotates through 90°. During telophase I, the first polar body is extruded. Homologous chromosomes seperate and one half of them are extruded with cytoplasmic material such as mitochondria, ribosomes and cortical granules into the first polar body. The oocyte now moves rapidly to metaphase II. The spindle contains α- and γ-tubulin, with γ-tubulin being confined the anastral spindles in the mature human oocyte (Van Blerkonm *et al.*, 1995; George *et al.*, 1996). Progressive maturation beyond metaphase II depends on the activation of the oocyte at fertilization or through parthenogenetic stimulation.

The maturation cascade is regulated by a variety of substances mediated by intercellular communication of the cumulus and corona cells enclosing the oocyte and by ooplasmic factors. LH apparently induces GVB indirectly via granulosa cells, perhaps by inactivating the transfer of maturation arresting factors to the ooplasm (Dekel, 1988). Another hypothesis proposes that LH induces GVB by signals generated in the granulosa cells and transmitted by gap junctions (Downs *et al.*, 1988). These structures consists of proteins called connexons which act as channels permitting rapid passage of small molecules. LH activity also

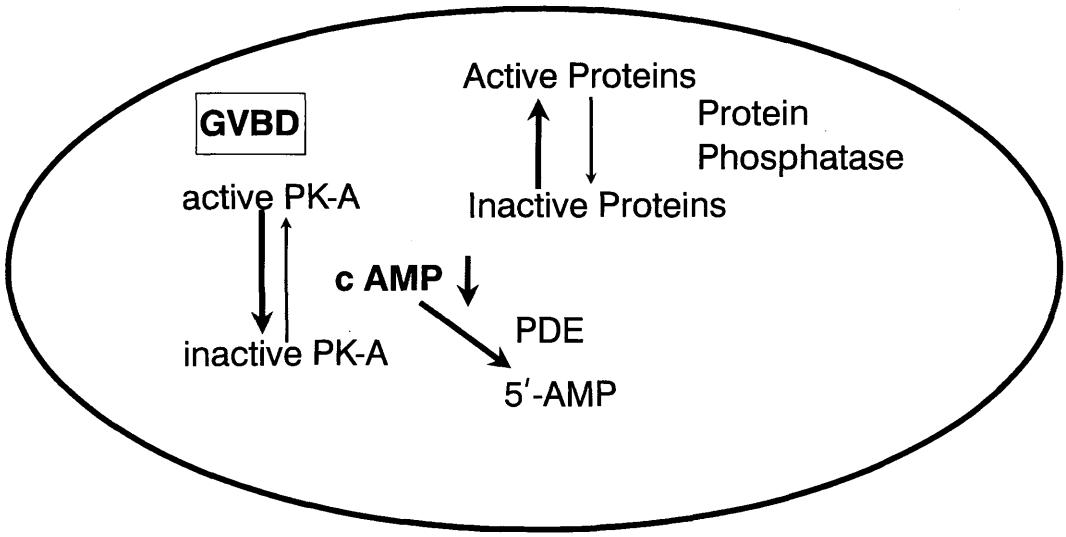

Figure 2. Resumption of meiotic maturation. GVBD=germinal vesicle breakdown.

increases the concentration of free inositol 1,4,5-trisphosphate (IP_3) and calcium in the granulosa cell (Homa *et al.*, 1991). Both substances are transmitted via gap junctions into the oocyte. IP_3 induces the mobilization of calcium stores within the oocytes. Free calcium activates cAMP phosphodiesterase which may decrease the cAMP concentration below the threshold needed to maintain meiotic arrest. cAMP activates a dependent protein kinase (PK-A), which phosphorylates proteins necessary for GVB. Continuous phosphorylation of these proteins maintains meiotic arrest, and their dephosphorylation induces a resumption of meiosis. The activation of phosphatidyl inositol and calcium is apparently associated with action of angiotensin II, perhaps regulated in turn by transforming growth factor-β (TGFβ).

Maturation-promoting factor (MPF) must be activated to induce nuclear envelope breakdown, chromosome condensation and formation of the spindle apparatus. MPF is a protein kinase consisting of two components, cyclin B and p 34, and the kinase activity of its subunit p 34 has to be activated by subunit cyclin B. Another oncogene, $pp39^{mos}$ encodes for mos protein (pp39mos) which may have a role in triggering cyclin B activation in some vertebrates. Mos is better known for its function in inducing meiotic arrest in metaphase II oocytes. It is actually the cytostatic factor which induces meiotic arrest (Sagata *et al.*, 1989) by its action in the inactivation of MPF. Both c-mos protein and cyclin Bl are expressed in specific patterns in human metaphase II oocytes and embryos (Heikenheimo *et al.*, 1995). The mRNA for c-mos is confined to oocytes and absent from granulosa cells, and high levels of cyclin B1 are found in oocytes; these maternal pools are degraded post-fertilization by the time of the 8-cell embryonic stage. In human oocytes, the 10^4–10^5 copies of c-mos mRNA were amplified by reverse transcriptase–polymerase chain reaction (RT–PCR) to obtain a 357 bp fragment of mos protein (Pal *et al.*, 1994). Knock-out studies on $pp39^{mos}$ have shown how metaphase II oocytes do not arrest in the normal fashion, and instead the oocyte proceeds parthenogenetically to anaphase and telophase I and to first polar body extrusion (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). The injection of anti-sense c-mos nucleotides also disturbs the arrest of oocytes at metaphase II (O'Keefe *et al.*,1989).

Production of the mature spermatozoon

Spermatogenesis and spermiogenesis are distinct processes. The progression from spermatogonia to mature spermatozoa requires ~60–70 days, with at least three mitotic and two meiotic divisions during

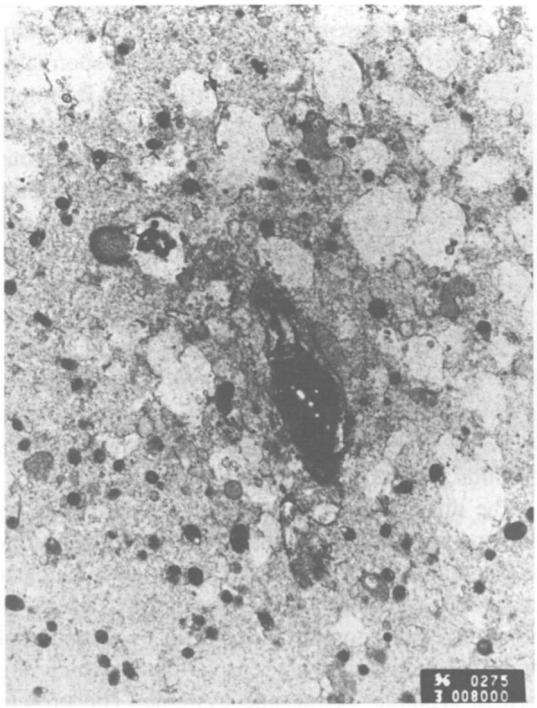

Figure 3. Undecondensed sperm head after intracytoplasmic sperm injection (ICSI).

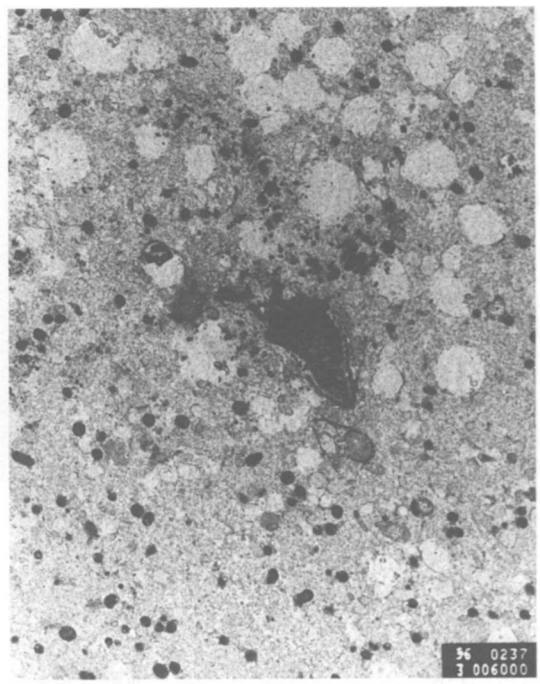

Figure 4. Partially decondensed sperm head after intracytoplasmic sperm injection (ICSI).

spermatogenesis. Spermatogenesis begins at puberty, continues throughout adult life and takes place inside the seminiferous tubules that are surrounded by a nutritive interstitial compartment consisting of Leydig cells, blood and lymphatic vessels. The tubular compartment consists of the spermatogenic cell line and supporting Sertoli cell.

Outside the tubules, Leydig cells are the prominent cell type of the interstitial compartment, besides macrophages. These cells display a high metabolic activity. Their ultrastructure shows a typical arrangement of tubules, smooth endoplasmic reticulum, mitochondria, lipid inclusions, a Golgi apparatus and rod-shaped crystals of Reinke (de Kretser and Kerr, 1988). In response to LH, mediated through LH receptors, Leydig cells are stimulated to synthesize androgens, in particular testosterone, cAMP, the aromatase system, corticotrophin-releasing factor (CFR) and endorphins. Stimulation of the EGF receptor contributes to the onset of testosterone synthesis. Leydig cells transport compounds, such as pregnancy-associated plasma protein (PAPP)-A from plasma to the tubules (Schindler *et al.*, 1986) and are active in secretion of paracrine factors, for example to induce interleukin-1α production in the Sertoli cell.

Sertoli cells are interconnected by tight junctions, and are mainly under control of FSH. Their ultrastructural elements are endoplasmatic reticula with extensive cisternae, few ribosomes, dense bodies, crystalloid inclusions, an extensive Golgi network and lysosome-like bodies. Sertoli cells provide a variety of functions to serve as a support system to spermatogenesis and protect the inner compartment, the tubules, against the surrounding environment. They remove residual material and cellular debris accumulated during spermatogenesis by phagocytosis. Sertoli cells express receptors for androgens, oestrogens, insulin and insulin-like growth factor (IGF). They respond to trophic stimuli and are able to produce many hormones under the influence of gonadotrophins. Sertoli cells produce growth factors such as acidic fibroblast growth factor (FGF), somatomedin C, IGF II, testibumin, inhibin, TGF-α and β1. Interleukin-lα regulates the testosterone production of Leydig cells. Two of the major important secretory

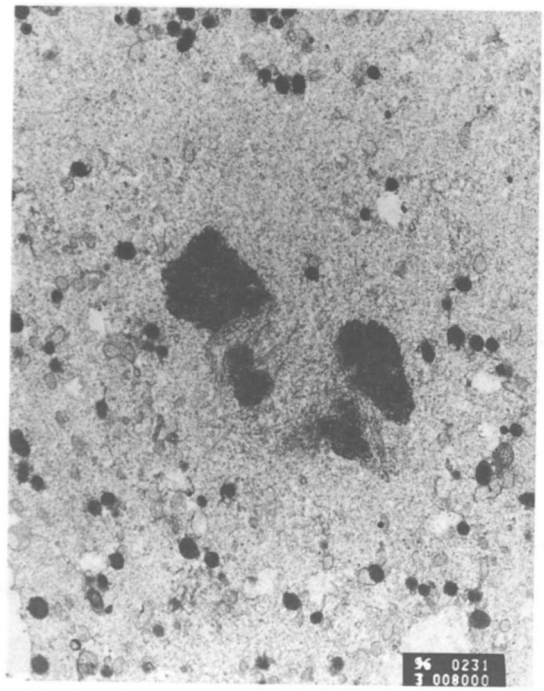

Figure 5. Maternal chromatin at the periphery of the oocyte at the beginning of spindle formation.

products of Sertoli cells are the androgen-binding protein (ABP) and the testosterone–oestradiol-binding globulin (TEBG), similar to the sex hormone-binding protein in peripheral blood, which are responsible for transport and localization of androgens within the tubule. This protein has been detected in all germ cells, in the latest stages of spermiogenesis it is mainly located in the cytoplasm of spermatids (Gerard *et al.*, 1994). Other substances that are transported to the germ cells are copper and iron, plasminogen activator, sulphated glycoprotein-l, glycoprotein-2 and β_2 microglobulin. Sertoli cells are assumed to be mainly involved in the regulation of spermatogonial multiplication or feedback actions between proliferating spermatogonia.

Spermatogenesis starts with the activation of type A stem spermatogonia from their resting phase in the adult gonad (Edwards and Brody, 1995). These cells undergo mitotic divisions to reach intermediate type B spermatogonia and then primary spermatocytes. The mitotic phase ends as primary spermatocytes form and enter the preleptotene stage. The beginning of meiosis is signalled as a final DNA synthesis occurs in dividing germ cells. The onset of meiosis in primary spermatocytes might be controlled by MPF, cdc 2 protein and cyclins. During the first meiotic division, synaptonemal complexes form as homologous chromosomes pair into bivalents, and chiasmata involving specific nucleotide sequences arise between homologous chromosomes to initiate crossing-over and gene recombination. Cells are quadrupled during the following two meiotic divisions of primary and secondary spermatocytes. The second meiotic division involved centromere division, and the separation of chroamtids into secondary spermatocytes. Chromosome numbers are thus reduced to haploid in spermatids.

The differentiation of secondary spermatocytes into spermatids and spermatozoa during spermiogenesis starts when meiosis is completed. Nuclear chromatin structure of the spermatids has to be reorganized. Histones are replaced by transitional proteins and more basic protamines, which are rich in arginine, serine and cysteine. The protein replacement coincides with compaction of the chromatin and the repression of transcriptional activity in the nucleus. Acrosomal granules from the Golgi complex build the acrosomal cap and the equatorial segment. The midpiece, rich in a helix of mitochondria overlying the flagellum and the major source of ATP, is formed in secondary spermatocytes and round spermatids. Selenium as well as zinc is essential as a protector against oxidative damage, and it is associated with polypeptides in the outer mitochondrial membranes. The flagellum arises from the centriole lying close to the base of the nucleus and extends to the distal tip of the tail. Its axoneme consists of two central microtubules, surrounded by nine outer microtubules, composed of two doublets. The outer doublets have two dynein arms. Their ATPase generates forces that regulate the sliding motions between adjacent outer doublets as flagellar beat begins.

Sperm–oocyte interaction

Transport of spermatozoa through the female reproductive tract occurs very rapidly since they take only 15 min to reach the abdominal cavity. After insemination, many acrosome-intact spermatozoa reach the mature oocyte within 1 h. This transport

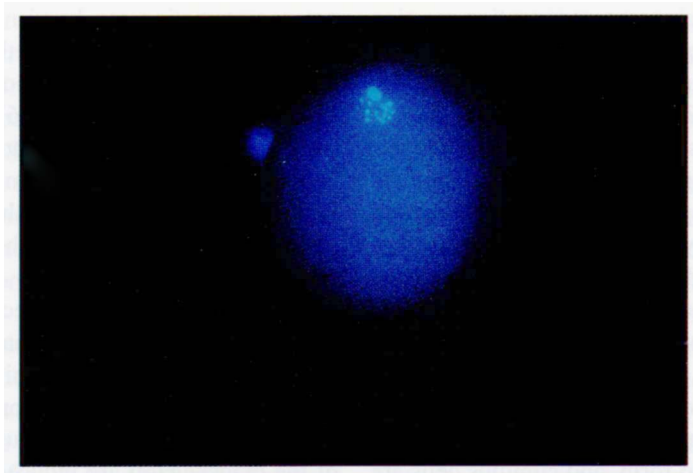

Figure 6. Failed intracytoplasmic sperm injection (ICSI): location of maternal chromatin and partially decondensed sperm chromatin 48 h after injection (Hoechst 33342 staining).

seems to be largely independent of the individual motility of spermatozoa. Chemotaxis does not attract spermatozoa to oocytes.

Ampullary spermatozoa are a highly selected population with a great facility to penetrate oocytes. To aquire fertilization ability, spermatozoa must undergo certain modifications in the female genital tract. This requires three steps: capacitation, hyperactivation and acrosome reaction (Mortimer, 1997). The first step of sperm maturation is capacitation. Capacitation is time-related and takes ~5–6 h in men. It is calcium-dependent, involves activation of adenosine triphosphatase (ATPase), and the redistribution of mannose receptors, glycoproteins and glycolipids on the sperm surface with subsequent changes in the properies of the membranes. Sialylglycoconjugates are involved during capacitation *in vitro*, and their distribution shifts from a general to a particular localization in the postacrosomal region (Focarelli *et al.*, 1995). Mannose ligand receptors also display similar changes in distribution, subsequent to the loss of cholesterol from the membrane during capacitation (Benoff *et al.*, 1993). The changes in membrane cholesterol/phospholipid ratio may be associated with modifications ocurring in membrane potential and in an increasing sensitivity and permeability to calcium ions (Bird and Houghton, 1991). Angiotensin converting enzyme (ACE) is released during capacitation and is assumed to participate in the acrosome reaction as the membranes show an increasing sensitivity and permeability to calcium ions (Foresta *et al.*, 1991; Bird and Houghton, 1991). The acquisition of hyperactivated motility is also a consequence of capacitation, and results in enhanced lateral head displacement, reduced linearity, beat frequency and flagellar curvature (Fraser and Ahuja, 1988; Pacey *et al.*, 1997). Factors in human prostatic secretions and seminal plasma related to thyrotrophin-releasing hormone (TRH), and called fertilization promoting peptides, may also stimulate capacitation and fertilization. Such compounds may exert their effects via cAMP or IP_3 (Green *et al.*, 1996a,b).

The spermatozoon initially binds to the zona pellucida, a semi-permeable investment permeable to molecules up to 170 kDa (Afzalpurkar *et al.*, 1997). Three sulphated zona proteins had been identified, ZP1, ZP2 and ZP3, each regulated by their corresponding genes and each with differing molecular weights. Human zona proteins have molecular masses of ~100, 75 and 60–70 for ZP1, ZP2 and ZP3 respectively (Shabanowitz and O'Rand, 1988). ZP2 and ZP3 form dimers which are linked by ZP1. The primary and most important receptor for spermatozoa on the zona pellucida seems to be ZP3, and its ability to induce the acrosome reaction in spermatozoa resides in *O*-linked oligosaccharides in its carboxyl regions (Litscher and Wassarman, 1996). ZP2 may bind

spermatozoa after their acrosome reaction reaction has occurred (Morales *et al.*, 1989). ZP1 may have a role in sperm binding in some species. There are $\sim 1 \times 10^9$ copies of ZP3 in the zona and the *O*-linked carbohydrates seem to be responsible for binding. Acrosome-reacted spermatozoa remain bound to ZP3 during zona penetration, although many are apparently released from it after the reaction is completed. The release of spermatozoa may be due to the modifications in the zona proteins which occur after sperm binding. Gene knock-out studies in mice show that homozygous transgenic animals nullisomic for ZP3 are infertile (Liu *et al.*, 1996; Rankin *et al.*, 1996). Indeed, the zona pellucida fails to form, and cumulus cells are disorganized in these animals, although ZP1 and ZP2 form at the oocyte surface. Maintenance of binding is supported by action of ZP2, which serves as a secondary sperm receptor, but perhaps only for acrosome-reacted spermatozoa.

The third step is the acrosome reaction. It is believed to begin at the time of sperm binding to the zona pellucida although it may be induced *in vivo* by cumulus cells and follicular fluid (Tesarik, 1985). The acrosome reaction in spermatozoa that achieve fertilization is believed by many investigators to occur as spermatozoa bind to ZP3 on the zona pellucida (Yeung *et al.*, 1997). Others suggest that zona can also be initiated by acrosome-reacted spermatozoa (Morales *et al.*, 1989).

The acrosome reaction involves fusion of outer and inner membranes of acrosomal membranes (reviewed by Brucker and Lilford, 1995). It is rapidly triggered by zona-receptor interaction and involves calcium uptake which induces changes in membrane potentials, modifications in pH, swelling and the release of acrosomal enzymes. This increase of intracellular calcium can be achieved *in vitro* by exposing spermatozoa to calcium ionophores or phosphodiesterase inhibitors. Calmodulin, located in the outer acrosomal membrane mediates the action of calcium. Calcium initiates phosphoinositide breakdown and protein kinase C activity. Activated protein kinase leads to the stimulation of protein phosphorylation. Acrosome reactions are also initiated by solubilized zonae pellucidae, and depend on the action of protein kinases A, C and G (Bielfeld *et al.*, 1994). The acrosome reaction can be visualized in individual spermatozoa. A variety of methods can be used to stain or mark the outer and exposed inner acrosomal membranes (Amin *et al.*, 1996). These include the use of chlortetracycline, mannosylated compounds and quinacrine. Quinacrine may detect the onset of the reaction, while the other two compounds react when the acrosome cap has been shed (Amin *et al.*, 1996).

The acrosome reaction is accompanied by modifications in the sperm plasma membrane, which exposes receptors for zona binding and possibly factors exposed on the equatorial segment in preparation for sperm–oocyte fusion. Modifications of the cytoskeleton are induced via the actions of calcium and calmodulin. In all, 14 or more proteins are apparently phosphorylated during the acrosome reaction, and large amounts of acrosin and proacrosin are released. Adenosine contributes to the activation of kinases and cAMP, and of G-protein receptors; acrosin may also be involved in activating adenylate cyclase and phospholipases A_2 and C. Inhibition of signal transduction occurs at the level of G protein (Wassarman, 1990). Acrosin and its precursor proacrosin are also assumed to participate directly in the sperm–zona binding process supporting the binding of spermatozoa tightly to the zona. Sperm–zona binding is a receptor-mediated process of recognition and attachment. The receptors are glycoproteins carrying saccharides such as fucose, galactose, aminosugars and mannose (Ahuja, 1985; Macek and Shur, 1988). Specific agents involved in binding include β1,4-galactosyl-transferase, zona receptor kinase and sp56 (Litscher and Wassarman, 1996). Sperm surface adhesins such as PH-30 may also be involved in the binding process.

Bound, acrosome-reacted spermatozoa attached tangentially to the zona pellucida may pass through it via their own motility. Contrary to many other species, human spermatozoa enter the zona in a straight line, implying that any zona lysins must be located in the perforatorium which is formed of perinuclear material stabilized by disulphide bonds and located at the tip of the sperm head. The inner acrosomal membrane and perforatorium thus form an effective cutting edge, while the posterior equatorial acrosomal segment is stable.

Acrosomal lytic enzymes are probably active during sperm penetration into the zona pellucida.

Capacitation and the acrosome reaction last for ~2 h, and sperm passage through the zona may require another h. Spermatozoa pass through the zona pellucida into the perivitelline space. Fusion then occurs between the sperm plasma membrane in the region of the equatorial segment and the oolemma. The fusion site on the equatorial segment may be excluded from the acrosome reaction (Chen and Sathananthan, 1986), and the plasma membrane is still intact on the anterior part of the post-acrosomal sheath and the equatorial segment. The sperm tail can be seen still beating outside the zona pellucida after its head and midpiece have entered the perivitelline space and the equatorial segment has made an initial contact with the oolemma. The sperm head fuses with microvilli on the oocyte vitelline membrane. After fusion has occurred, the microvilli retract and draw most or all of the spermatozoon into the ooplasm by means of filaments associated with subcortical layers of actin and myosin. The spermatozoon becomes immotile as it is incorporated into the oocyte, and all of the spermatozoon, including the sperm tail, is drawn subsequently into the ooplasm.

Fusion of gametes could involve several factors acting in union or separately. Fibronectin (Fusi and Bronson, 1992a), integrins (Blobel *et al.*, 1992; Bronson and Fusi, 1992b) and two distinct peptides, α and β, which are components of the adhesion protein PH-30, all seem to be involved. There are considerable areas of homology between integrins, PH-30 and EGF. These compounds are located especially in the post-equatorial segment and in the plasma membrane of the spermatozoon, and disintegrins may be present on the oolemma (Klentzeris *et al.*, 1995; Campbell *et al.*, 1995). The Asp-Gly-Arg (RGD) adhesion peptide typical of integrins seems to be involved in fusion. RGD and PH-30 have both EGF-like repeats and similar amino acid sequences. Fertilization promoting peptides may also enhance sperm–oocyte binding (Green *et al.*, 1996,a,b).

A series of susequent stages after membrane fusion include activation of the oocyte, exocytosis of the cortical granules to form the block to polyspermy and so prevent further sperm entry, and the activation of metaphase II leading to the formation of the second polar body and the appearance of pronuclei within the oocyte. Debate has recently been intense about the nature of the of the activating stimulus. A protein may be released from the fertilizing spermatozoon into the ooplasm within a very brief time after sperm–oocyte fusion, via a small channel formed during this interval between the ooplasm and spermplasm (Swann, 1993). A soluble protein has been activated from spermatozoa, and shown to be active in spiking, to increases the concentration of free calcium and activate the IP_3 system. This protein has oscillin-like properties, and may be released from the spermatozoon to initiate calcium spiking; it may act independently of IP_3 (Swann, 1996). This mechanism of oocyte activation could be very important factor in the success of ICSI, permitting the oocyte to be activated despite the lack of any sperm fusion of membrane hyperpolarization.

Calcium is released in spikes from oocyte stores soon after sperm entry, and activates resumption of meiosis and metaphase II, promotes the exocytosis of cortical granules and the extrusion of the second polar body (Ducibella, 1992). Within the mature oocyte, bundles of microfilaments lining the inner surface of the oolemma may assist in localising the cortical granules and facitating their exocytosis; they may also control the premature release of these granules. The oocyte may gain the ability to release its cortical granules and to respond to signals invoking calcium release only a few hours before fertilization occurs, perhaps just before it reaches metaphase II. Cortical granule release may begin in the region where sperm–oocyte fusion has occurred (Whitaker and Swann, 1993).

Calcium ions are released in newly-fertilized oocytes in a defined sequence of repetitive oscillations over a period of 24 h. These oscillations can be inhibited by use of calcium chelators which block the exocytosis of cortical granules, arrests the oocyte at metaphase and thus prevents the further fertilization process. At least two distinct calcium channels may be involved in spiking, each with specific receptors influencing the release of distinct stores of calcium within the ooplasm. One channel may be sensitive to IP_3 and the other to

ryanodone. It is possible that IP_3 channels have a distinct distribution within the oocyte, those near the oolemma controlling sperm entry, and the deeper channels controlling spiking (Tesarik *et al.*, 1995; Tesarik, 1996; Berridge, 1993, 1996). Calcium spiking induced by thimerosal, which acts via IP_3, occurred more frequently in metaphase II human oocytes than in metaphase-I oocytes, indicating that major changes occurred in calcium responses during oocyte maturation (Herbert *et al.*, 1995). Activation of the phosphoinositide pathway is linked to the activation of G-protein in somatic cells, but there is no certain evidence that this transduction system is involved in the fertilization process. While membrane hyperpolarization supports a monotonic outward K current, calcium is released by binding of IP_3 to its receptor. Calcium spiking also stimulates MPF and ultimately the onset of DNA synthesis in the fertilized oocyte.

After gamete fusion the cortical reaction takes place to form the block to polyspermy. The zona reaction occurs as cortical granules lying just below the vitelline surface fuse with the oolemma. These lysosome-like granules release hydrolytic enzymes, proteases and peroxidases into the perivitelline space and onto the inner surface of the zona pellucida. The oocyte surface increases as cortical granules fuse with it, characterized by an elongation of ist microvilli (Wassarman, 1987). This process takes ~15 min. Modifications of sperm receptors, mainly ZP1 and ZP2 are also induced. The desensitization of the zona for sperm binding and induction of acrosome reaction involves transformation of ZP3 to ZP3f. This process is called zona hardening. Another calcium-dependent process occurring immediately after sperm binding to the zona is the synthesis of calpain, which degrade mos protein or cytostatic factor to terminate the metaphase II arrest.

Formation of the pronucleate egg

Soon after activation of the oocyte through sperm entry the second polar body is extruded (Payne *et al.*, 1997). The sperm head enlarges in the ooplasm and chromosome decondensation takes a few hours by reduction of disulphide bonds between protamines by action of gluthathione. Histones generated by the oocyte replace the protamines. Decondensation of the sperm head begins 1 h after zona penetration. Male pronucleus formation occurs simultaneously with disappearance of the nuclear membrane, decondensation of the chromosomes and reformation of the pronuclear membrane from oocyte endoplasmic reticulum which is supported by action of growth factors. This coincides with decondensation of the maternal chromatin and the formation of the female pronucleus.

The pronuclei expand and move together in the ooplasm. The migration of the expanding sperm head and male pronucleus to the maternal perinuclear region is both rapid and displays distinct linear movement characteristics (Van Blerkom *et al.*, 1995). This migration may be associated with unusual configurations of the sperm tail, perhaps even some continued motility. The distribution of chromatin in the pronuclei is polarized and associated with the position of the sperm centrosome. Two pronuclei are usually a sign of normal fertilization, with one male and one female pronucleus. Some eggs have a single pronucleus, and may be gynogenetic or androgenetic; others with only one obvious pronucleus may actually be diploid because the second pronucleus has been overlooked or has formed later than normal. Eggs with a single pronucleus after in-vitro fertilization (IVF) mostly develop as diploid embryos with a Y chromosome, indicating they are normal and can be used for transfer, whereas those arising after ICSI are haploid and lack a Y chromosome (Sultan *et al.*, 1995). Three pronucei signify the occurrence of dispermy, or of digyny throught the incorporation of the second polar body within the oocyte. Attempts are being made to excise the second male pronucleus, in order to restore diploidy in the fertilized egg, a procedure that is successful in mouse eggs (Feng and Gordon, 1996). Many of the operated eggs develop to blastocysts, and some into normal mouse offspring. Nevertheless, there must be no doubt that the origins of each of the three pronuclei have been correctly identified. The consequences could be serious if, for example, the single female pronucleus was mistakenly removed from a dispermic tripronucleate egg, for the remaining two male pronuclei could encode for androgenetic development and the possible formation of hydatdiform moles.

Many of these normal events of fertilization and activation of the egg have been by-passed by the introduction of ICSI (Figures 3–6). One of the challenges of research today is to interpret the nature of oocyte activation in relation to the procedures of ICSI. Perhaps oscillins or some other compounds are released from the spermatozoon to activate the egg during ICSI in a manner that produces a virtually normal activation of the oocyte. It is not clear what happens to the acrosome or the cortical granules, or how the second polar body is extruded and if membrane hyperpolarization is involved. There are nevertheless indications of anomalies in the activation process after ICSI. More eggs than expected after IVF appear to be digynic, and others have a single pronucleus. Anomalies have also been found in a moderate number of eggs on the nature of pronucleus formation. In the eggs of rhesus monkeys, the presence of the acrosome in the ooplasm is sometimes associated with heterogeneous and temporary form of deconsation of sperm chromatin, together with other potential anomalies (Sutovsky *et al.*, 1996). Nevertheless, ICSI has opened a new light on the study of human fertilization, and on the alleviation of male infertility. It has also permitted the use of epididymal and testicular spermatozoa for fertilization in cases of severe male infertility, with fertilization rates only slightly lower than with the use of ejaculated spermatozoa (Palermo *et al.*, 1995; Silber *et al.*, 1996; Tournaye *et al.*, 1996). Spermatids can replace spermatozoa for use in ICSI, and several full-term pregnancies have been achieved (Edwards *et al.*, 1993; Tesarik *et al.*, 1995, 1996a,b) It is even possible that fertilization might soon be achieved through the transfer of a nucleus and a centriole. Tubulin was nucleated and a sperm aster was formed after a human centriole was transplanted into mature oocytes (Van Blerkom and Davis, 1995), and pronuclear transplants are being made into human oocytes (Azambuja *et al.*, 1996). Cytoplasmic transfers are also being made between oocytes in different stages of maturation (Levron *et al.*, 1996).

The pronuclear stage in fertilized human eggs lasts for almost 24 h, and consists of well-defined G1, S and G2 phases. The two pronuclei enlarge and move towards one another, but never fuse. The sperm centriole divides in the ooplasm to form the centrosomes of the first cleavage spindle (Sathananthan *et al.*, 1996). Male and female chromosomes condense, associate with the microtubules and move to the equator of the egg to form of the spindle of the first cleavage division. These events occur normally in the vast majority of human eggs fertilized *in vitro*. Recently, various novel forms of anomaly arising during these stages have illustrated new forms of infertility arising after IVF and ICSI, and possibly after natural conception. An inability of the centrosome to function normally leads to developmental arrest just before syngamy begins (Asch *et al.*, 1995; Van Blerkom *et al.*, 1995). It may fail to divide or the products of its division may migrate abnormally to the opposite poles of the first cleavage spindle; it may synthesize the incorrect form of tubulin and microtubules in the spindle apparatus. In normally-developing eggs, syngamy initiates the combination of the paternally- and maternally-inherited chromosomes as the embryo inherites its new genome. The first cleavage then proceeds normally, and initiates the beginning of a new cell cycle at prophase.

Conclusion: how does ICSI relate to the normal events of fertilization?

One of the most common questions is raised in many meetings is 'How did ICSI ever succeed in face of the complex events in normal fertilization?' It is a good question. Clearly, all the events normally associated with spermatozoon entering into the ooplasma are by-passed and seriously modified after ICSI. The normal events usually occurring after sperm entry are also compromised after ICSI, perhaps to a lesser extent but nevertheless decisively.

Concerning the events before and during sperm entry, questions arise about membrane depolarization, cortical granule extrusion, calcium utilization and extrusion of the second polar body. Do calcium transients induced by ICSI cause by a modified form membrane fusion between the spermatozoon and, for example, the endoplasmic reticulum? Are these transients normal? Recent observations regarding calcium oscillations occurring during ICSI showed different patterns from those of

normal fertilization. The question to be solved is whether different calcium-sensitive organelles such as mitochondria, secretory granules and endoplasmic reticulum are differently involved. Similar questions arise about oscillin, another substance that triggers calcium oscillations. Oscillin is concentrated in the intracellular part of of the equatorial segment of spermatozoa, i.e. the region that first fuses with the oocyte membrane during normal fertilization. Can low oscillin concentrations cause failed oocyte activation after insertion of the spermatozoon during ICSI? What happens to the cortical granules: are they discharged and are the zona proteins modified in a manner resembling that occurring after normal fertilization? How does the extrusion of the second polar body occur? After ICSI, many fertilized eggs are unipronucleate or digynic pronucleate eggs, whereas such anomalous forms are rare after normal fertilization.

Concerning events after sperm entry, questions must arise about the phagocytosis of the external acrosomal membrane, which is normally destroyed before fertilization during the acrosome reaction. There could well be problems in the normal protamine–histone transition and the decondensation of the male pronucleus, the destruction of the outer acrosomal membrane and the rapid onset of pronucleus formation after ICSI (Figures 3 and 4). No doubt some of these disparities in the formation of male and female pronuclei are due to a failure to insert the spermatozoon fully into the ooplasma. There could well be other causes.

Perhaps the success of ICSI has been due to the ability of human oocytes to avoid a series of near-disasters which are overcome by the regulatory processes occurring in gametes and eggs at fertilization. A succession of slight deformaties have been noted but, overall, the repair processes in the oocyte have apparently proved to be adequate to correct the anomalies.

Acknowledgements

Figures 3, 4 and 6 are by Prof. Dr W.Kühnel, Director of the Institute of Anatomy, Medical University Lübeck, Germany, to whom we express our gratitude for his significant contribution to our work.

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