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REPRODUCTIVE SCIENCE

The Ultrastructural Study of the Cytoskeleton of the Human Oocytes Subjected to Micromanipulation

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ABSTRACT

Objectives The objectives of the study are :- 1. To establish a technique for preparing the human oocytes for transmission electron microscopy 2. To develop an immunogold method for the localisation of cytoskeletal elements of the human oocytes 3. To study the ultrastructure of the human oocytes and the cytoskeleton, subjected to micromanipulation.

Design Experimental study.

Setting Nottingham University Research and Treatment Unit in Reproduction (NURTURE), Queen's medical centre, Nottingham University, Nottingham, UK.

Subjects and methods The oocytes, which underwent in vitro fertilization (IVF) or microassisted fertilization (MAF) e.g. Subzonal Injection of Sperms (SUZI), Direct Injection of Sperm to Cytoplasm of the Oocyte (DISCO), were collected and assigned to the control and study groups respectively at NUTURE, Queen's medical centre from 25th October to 24th December 1993.

Results The cytoskeleton (microtubules and microfilaments) has been examined by electron microscopy and immunocytochemistry. Due to their small size and number of the human oocytes, a new method for handling and preparation for transmission electron microscopy (TEM) has been developed. This method combined protein embedding with centrifugation to locate the specimens on the face of a Beem capsule mould. Therefore, it facilitated both the processing of human oocytes with minimal loss and rapid location of the specimens within the block of serial sectioning, staining and examination. The effects of microassisted (SUZI ; Subzonal Injection of Sperm, DISCO ; Direct Injection of Sperm to Cytoplasm of the Oocyte),

sucrose and sonic sword on the microfilamentous and microtubular systems of the human oocytes were studied.

Microfilaments of the human oocytes could be found at the core of microvilli and at the periphery of the cytocortex of all the groups studied by electron microscopy and electron microscopic immunocytochemistry. Most of the oocytes had a uniform microfilament distribution by light microscopic immunocytochemistry except those oocytes exposed to multiple risk factors (SUZI, sucrose, sonic sword) or the direct disturbance of the cytoskeleton system produced by DISCO.

Microtubular system of the human oocytes could be detected in all the groups by light microscopic immunocytochemistry. This showed that only two out of six oocytes from normal in vitro fertilization (IVF) (control group) and the manual microinjection of spermatozoa without sucrose and sonic sword had the normal barrel shape spindles, whilst the others had abnormal spindles. By TEM study, microtubules could be found in only one section which was cut through the chromosome and microtubule level transversely. The microtubular system could not be detected in any groups by electron microscopic immunocytochemistry. However, there was no difference in the character and distribution of other organelles in both control and study groups.

Conclusion Microassisted fertilization (SUZI, DISCO), sucrose, and sonic sword may be the risk factors of the human oocyte cytoskeleton abnormality, especially those exposed to combined risk factors or the direct disturbance of the cytoskeleton system produced by DISCO.

Key words : ultrastructure, human oocyte cytoskeleton

Although the modern era of the research in cytoskeleton is only about a decade old, the roots of the cytoskeleton concept can be traced back to the early days of the cell theory. The term "cytoskeleton" was introduced by John T. Needham more than 2,000 years ago. The cytoskeleton is the system of fibrillar structures in the cytoplasm of eukaryotic cells. The three main types of fibrils forming these structures are microtubules, microfilaments and intermediate filaments. This integrated system of molecules provides the cells with shape, internal spartial organization, motility, cell division, gamete fertilization, and possibly a means of communication with other cells and the environment.

With the advent of in vitro fertilization (IVF) and microassisted fertilization (MAF) as accepted clinical procedures in the modern reproduction, it becomes interesting to study the ultrastructure of cytoskeleton in the human oocytes and embryos in order to understand some kinds of abnormalities such as triploidy, aneuploidy and also growth differentiation of the embryo.

Materials and Methods

The stimulation protocols, (Clomid/HMG, HMG/FSH and Buserelin/HMG), were used to obtain multiple oocytes at Nottingham University Research and Treatment Unit in Reproduction (NURTURE), Department of Obstetrics and Gynaecology, Queen's Medical Centre Nottingham University from 25th October - 24th December 1993. Oocyte recovery from the follicles was achieved by a transvaginal ultrasound technique approximately 36 hours after the injection of human chorionic gonadotrophin (HCG). The

retrieved oocytes were transferred into microdrops of Earls' media containing 10% human serum under liquid paraffin oil in an atmosphere 5% CO_2 in air and maintained at 37° C for not less than 6 hours. The oocytes underwent in vitro fertilization (IVF), or microassisted fertilization (MAF) e.g. Subzonal Injection of Sperms (SUZI), Direct Injection of Sperm to Cytoplasm of the Oocyte (DISCO) (Embryology protocol manual, NURTURE, 1993). After 14-22 hours, the oocytes were examined for pronuclei formation. The mature unfertilized oocytes (aged 48 hours) were collected and assigned to the control and study groups.

Control Group :

Unfertilized nonmicroinjected human oocytes and fertilized abnormal nonmicroinjected human oocytes

Study Group :

Unfertilized microinjected human oocytes and fertilized abnormal microinjected human occytes

Group A = Subzonal injection of sperm (SUZI) group

Group A1 = Normal (manual) microinjected oocytes

Group A2 = Microinjected oocytes with sonic sword

Group A3 = Manual microinjected oocytes by using sucrose to create more perivitelline space

Group A4 = Microinjected oocytes with sonic sword by using sucrose to create more perivitelline space

Group B = Direct injection of sperm to cytoplasm of the oocyte (DISCO) group

Following examination, all the oocytes were then transferred with a heat-polished micropipette to drops of 2.5% glutaraldehyde in 0.1M phosphate buffer (GA ; pH 7.4) and fixed at 37° C for 45 minutes. The specimens were washed three times in phosphate buffer saline (PBS) and kept at 4° C for examination by electron microscopy and immunocytochemistry.

Electron Microscopic (EM) Study of the Oocytes

Five oocytes in each group were washed in 10% bovine serum albumin in Dulbecco's phosphate buffer (BSAD). Then, those in each group were transferred to a dust-free Beem capsule containing one drop of BSAD. The oocytes were left for 30 minutes to settle on the bottom of the capsule. Any specimens adhering to the sides of the capsule were gently dislodged under a dissecting microscope with the closed end of a heat-polished micropipette. When the specimens were situated on the bottom, the capsule was centrifuged horizontally for 15 minutes at 1,800g. Three drops of GA were dropped onto the surface of the BSAD and the capsule was centrifuged as described for an additional 60 minutes. The capsule was then filled with GA and refrigerated at 4°C overnight.

The following morning,GA was poured off. The capsule bisected longitudinally with a scalpel blade and the gel containing the occytes was removed. This specimen was transferred to a vial for post-fixation in 1% aqueous osmium tetroxide at room temperature for 2 hours, dehydration and infiltration. For embedding, the gel was carefully aligned in the bottom of a Beem capsule containing three drops of resin. The capsule was then filled with resin and polymerized at 60°C for 18 hours. 1 μm sections were cut with glass knives using a Reichert-Jung ultracut ultramicrotome and stained with 1% toluidine blue in borax.

Ultrathin sections (approximately 70nm) were obtained with Diatome diamond knives using LKB and Reichert ultramicrotomes. Alternate series of 1 μm and ultrathin sections were cut for routine examination. Thick sections were photographed with Leitz microscopes. Thin sections were mounted on uncoated copper grids, stained with alcoholic uranyl acetate and Reynoldís lead citrate, and examined with a Jeol JEM1200 EX electron microscope and photographed at original magnification ranging from x 5,000 to x 100,000.

Immunocytochemistry Study

Light Microscopic Level With Immunogold-Silver Staining

Two oocytes in each group were treated in 0.04% Triton X-100 in PBS for 30 minutes and then washed twice in PBS for 5 minutes. Oocytes were transferred in 50 mM ammonium chloride for 15 minutes and then washed twice again in PBS for 5 minutes. One oocyte in each group was then incubated in monoclonal mouse and alpha-actin (1 : 50) and the other was incubated in monoclonal mouse anti alpha-tubulin antisera (1 : 1000) for 60 minutes at room temperature. Following incubation, the oocytes were washed twice in PBS for 15 minutes and incubated in 5 nM immunogold conjugated goat antimouse immunoglobulin G (1gG) (1 : 50) for 30 minutes. They were then washed three times in distilled water for 3 minutes and incubated in silver enhancer for 10 minutes. The oocytes were washed again in distilled water for 3 minutes three times. Whole oocytes were mounted in Aqueous Mounting Media (Nustain). Qualitative evaluation of the morphology of the cytoskeleton was done with a Leitz microscope at a magnification of x 400. If the meiotic spindle was present, its morphology was considered normal when barrel-shaped and abnormal when reduced, elongated, or disrupted. For the methodology control, an oocyte from the control group was attained as detailed above, except that incubation in the primary antiserum was omitted. Photographs were taken on Fuji film using Leitz photographic installation.

EM-Level With Post-Embedding Immunogold Staining

Two ultrathin sections from one oocyte of each group were collected onto pre-cleaned uncoated nickel grids (200-300 mesh). Grids were put into drops of a saturated aqueous solution of sodium metaperiodate for 15 minutes at room temperature. Subsequently, the grids were washed in drops of water three times for 15 minutes and then put in drops of 1% Triton X-100 for 15 minutes. The grids were then put in drops of 1:5 swine serum for 1 hour. One grid from each oocyte was then incubated in drops of monoclonal mouse anti alpha-actin (1 : 50) and the other was incubated in monoclonal mouse and anti-tubulin (1 : 1,000) antisera overnight at 4° C. Following incubation, grids were thoroughly washed five times in 1% BSA in TRIS-buffered saline (TBS-BSA) for 15 minutes and then incubated for 60 minutes at 37°C with the immunogold conjugated goat and mouse IgG diluted 1 : 15 in TBS-BSA. After extensive washing in TBS-BSA, sections were postfixed for 10 minutes at room temperature in 2.5% glutaraldehyde in 0.1M PBS and then washed twice in distilled water for 15 minutes and allowed to dry in a dust free environment. For the methodology control, the ultrathin sections from one of the control oocytes was stained as detailed above, except that incubation in the primary antiserum was omitted. Grids were counterstained with Reynoldís lead citrate and uranyl acetate,

and examined with Jeol JEM 1200EX electron microscopes. The specimens were photographed at magnification ranging from x 5,000 to x 130,000.

Results

In order to assist in the interpretation of the results concerning changes in the cytoskeleton system of the human oocytes subjected to micro manipulation, it was considered necessary to document the basic ultrastructure of the oocytes in each group first. Therefore, the new method for handling and preparing the human oocytes for EM was done. The blocks of resin were cut into thick sections (1 μm) and stained with Toluidine blue before the ultrathin sections (approximately 70 nm) were obtained.

Toluidine blue is extremely useful when sections for EM are needed as they give a general idea of the orientation of the tissue and enable one to pinpoint areas of interest in the block face prior to further trimming of the block of ultramicrotomy. Toluidine blue stained slides will also allow the quality of fixation and embedding to be assessed. One μm resin sections of oocytes stained with toluidine blue are of considerable aid to the embryologist to assess the oocytes roughly at different levels (Fig.1).

Basic Ultrastructural Study by TEM

The human oocyte was surrounded by a fibrillar zona pellucida, outside which there were several layers of cumulus cells. The perivitelline space was found within the zona pellucida. The oolemma protruded into numerous microvilli during the early stages of maturation, which progressively decreased in number as maturation proceeded. Each microvillus had a core of microfilaments which could be found in all of control and study groups.

Microfilaments also formed a more or less continuous band beneath the oolemma of the immature oocyte. This band could be found only in the group A2 (Fig. 2).

Cortical granules(CG) were found in both control and study groups without any significant difference. They became progressively abundant as the oocyte completed maturation[1] and migrated to the surface beneath the oolemma. These granules, characteristic of all mammalian oocytes, were bound by well-defined membranes and contained extremely electron-dense cores. They were formed by hypertrophic Golgi complexes, initially, abundant in the cortical ooplasm.[2] The cortical reaction involved the exocytosis of the contents of cortical granules when the sperm fused with the oocyte at fertilization. A wave of cortical granule release is believed to be propagated circumferentially from the point of sperm entry, and the reaction is completed within a few minutes. The cortical granules burst open and their membranes fused with the overlying oolemma, releasing their contents into the perivitelline space, by a process of cell secretion (Fig. 3). The oolemma at the point of exocytosis was quite dense and appeared to be straitened (Fig. 3).

Golgi complexes were composed of elongated cisternae, vesicles, and vacuoles, there was no difference in distribution in both control and study groups.

Lysosomes existed in a variety of forms and were involved in intracellular digestion. They were acid phosphatase positive and heterogenous in overall appearance. Primary lysosomes were small vesicles bound by a limiting membrane and contained a dense core, often eccentric in position. Secondary lysosomes were larger and showed membranous and vesicular profiles associated with dense material. These were

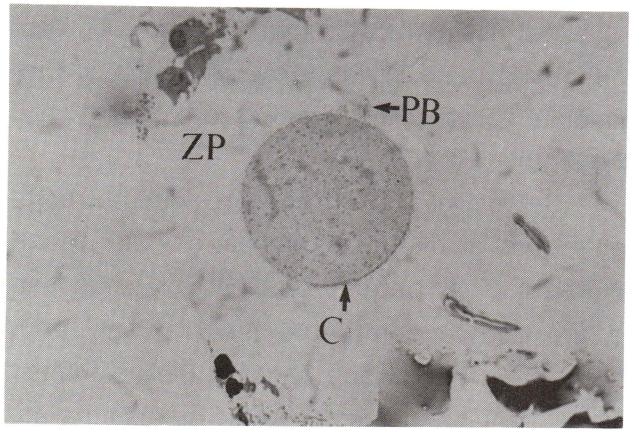


Fig. 1. The IVF mature oocyte of the control group x 400.

This light micrograph shows the zona pellucida (ZP) and one polar body (PB) in the perivitelline space. The ooplasm has an even distribution of organelles. Numerous cortical granules (CG) are organized in one discontinuous layer beneath the oolemma. The cumulus cells (CC) have been removed but there are some left.

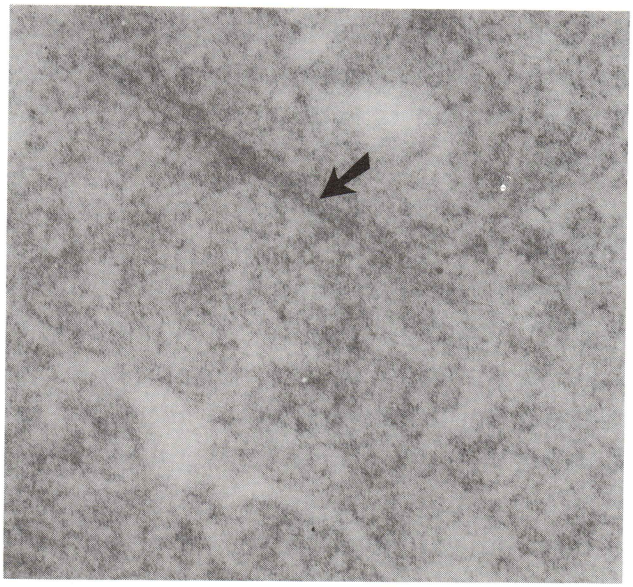


Fig. 2. The cytocortex of the human oocyte (group A2) TEM x 10,000.

This micrograph reveals the surface architecture of the junction of the polar body and the oocyte. The disrupted band of microfilaments (arrow) locates beneath the oolemma.

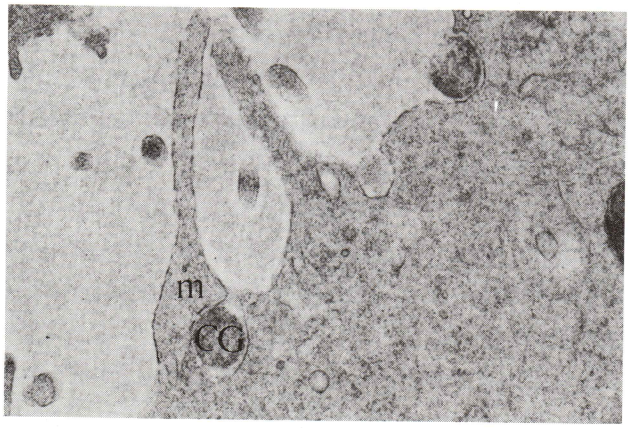


Fig. 3. The cortical granules (group A1) TEM x 30,000.

These cortical granules burst open and the membranes fuse with the overlying oolemma, releasing their contents into the perivitelline space. the oolemma at the point of exocytosis is quite dense and appears to be straitened.

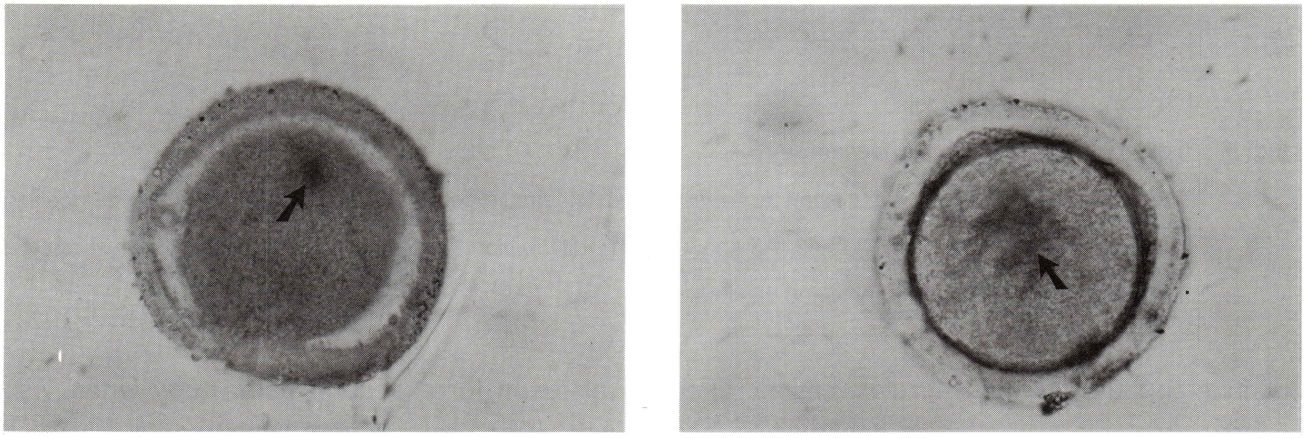
probably digestive or autophagic vacuoles, which later gave rise to residual bodies with ill-defined contents. The latter appeared to exocytose their contents at the oocyte surface. The lysosomes in both control and study groups had no difference in the character and distribution.

The endoplasmic reticulum was predominantly smooth (SER) and consisted of three types of elements: vesicular, tubular and irregular. Rough endoplasmic reticulum composed of elongated cisternae was seen only in oocytes from antral follicles, during early maturation. The predominant type of SER was vesicular and distributed randomly in both control and study groups, whereas the tubular form was rarest.

Ribosomes were generally inconspicuous and sparse in all groups. Mitochondria are oval or spherical and usually had dense stroma and

Table 1. Microtubule Distribution

Group	Normal Distribution	Abnormal Distribution
C	normal	
A1	normal	
A2		disruption
A3		disruption
A4		disruption
B		disruption



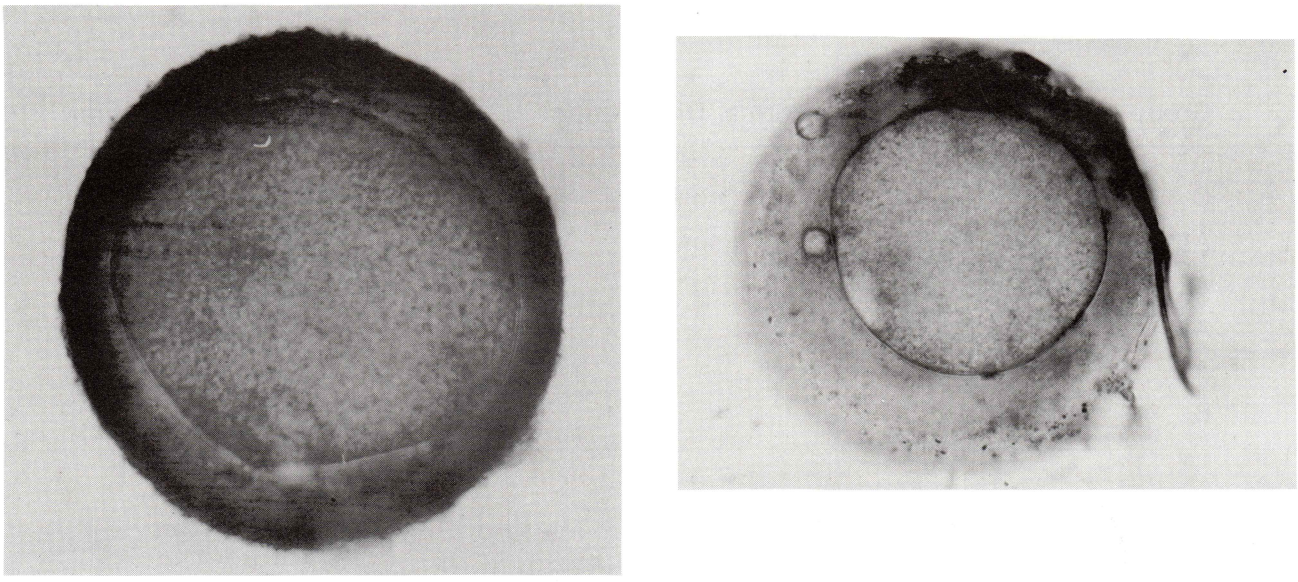
A B

Fig. 4. Light microscopic immunocytochemistry x 400.

Unfertilized human occytes show meiotic spindle visualized by immunogold-silver staining after anti-alpha tubulin labelling. The oocytes of the control group and group A1 reveal normal barrel-shaped spindles with broad anastral poles (Fig. 4a). The spindles of oocytes of of group A2, A3, A4 and B have disrupted spindles (Fig. 4b).

Table 2. Microfilament Distribution

Group	Uniform	Diminished Uniform	Non - Uniform
C	+		
A1	+		
A2	+		
A3	+		
A4		+	
B		+	



a. Control group

b. Group B.

Fig. 5. Light microscopic immunocytochemistry x400.

The unfertilized human oocytes show actin visualized by immunogold-silver staining after anti-alpha labelling. The control group and group A1, A2, A3 show uniform distribution throughout the cytocortex. But two oocytes from group A4 and B show diminished distribution.

peripheral, transverse, or reticulate cristae. Their matrixes became less electron dense while their cristae were more prominent in early embryo.[2] Mitochondria were the most conspicuous organelles and had no difference in both control and study groups.

Normally,the second meiotic division begins almost immediately after the first meiotic division. There is no interphase and nuclear formation. Prophase 2 is very short or almost nonexistent. The chromosomes align themselves on the equator of the second maturation spindle (metaphase 2), and the second arrest begins at which stage the oocyte is ovulated. Only one section cut through the chromosome level of group A1.

The first polar body contained cortical granules and isolated chromosomes associated with residual microtubules. It could easily be mistaken for a second polar body when view with phase microscopy. This would lead to a false assumption that the oocyte was fertilized, especially if the first polar body fragmented into two or three portions.

The second polar body differed from the first in that it had very few cortical granules, and its chromatin was organized into a spheroidal nucleus. There was no difference of polar bodies in both control and study groups.

Immunocytochemistry Study (Light Microscopy)

A total of twelve human oocytes were studied. Six of these (one in each group) were stained with anti-alpha tubulin by immunogold-silver enhancement technique to determine the distribution of microtubules. The other six oocytes (one in each group) were stained with anti-alpha

actin by this technique in order to determine the distribution of microfilaments.

Microtubules were detected in all the human oocytes (Table 1). Two oocytes from the control group and group A1 showed normal barrel shaped anastral spindles (Fig. 4a). The other four oocytes from group A2, A3, A4, and B revealed abnormal-shaped spindles (Fig. 4b).

Microfilaments were detected in all the human oocytes (Table 2). Four oocytes from the control group, A1, A2 and A3 groups showed uniform distribution throughout the cytocortex (Fig. 5a). But two oocytes from group A4 and B showed diminished uniform distribution (Fig. 5b).

The negative controls, in which incubation in the primary antiserum was omitted, showed no specific staining.

Immunocytochemistry Study (Electron Microscopy)

Two of the ultrathin sections from one oocyte in each group underwent post embedding immunogold staining. One ultrathin section of each group was stained with anti alpha-actin. The groups of immunogold dots could be detected at the periphery of these six oocytes (Fig. 6, 7).

The other of each group was stained with anti alpha-tubulin by this technique. Unfortunately, no chromosomes in these oocytes were sectioned and the immunogold dots found in these sections were not specific.

The negative controls, in which incubation in the primary antiserum was omitted, showed no specific staining.

Discussion

The cytoskeleton of the mammalian oocyte is essential, not only for the maintenance of chromosomal organization but also for the extrusion of the polar body and for the complex intracellular movements associated with syngamy and cleavage.[3] It has been believed that MAF(SUZI,DISCO), sucrose and sonic sword can induce spindle alterations and chromosomal anomalies.[4,5] The human oocytes from IVF and MAF were collected in order to study the effects of these procedures on the cytoskeleton by EM

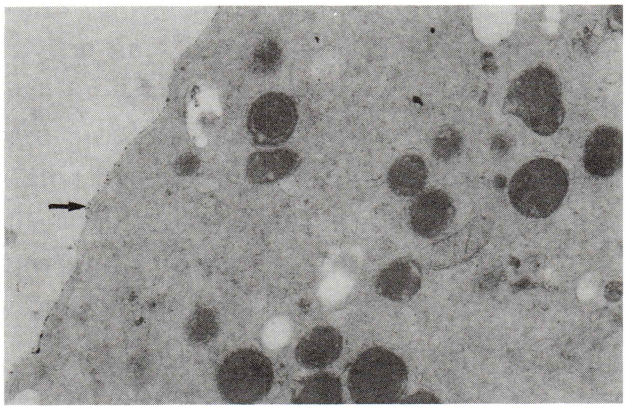


Fig. 6. Immunogold dots (control group TEM x 13,000. This micrograph shows groups of immunogold dots (arrow) along the periphery of the oocyte.

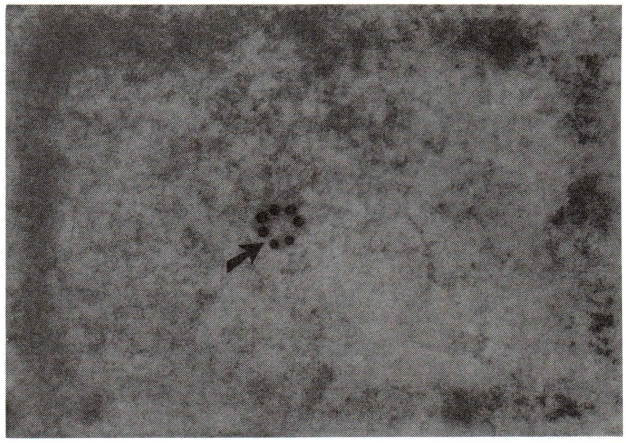


Fig. 7. Immunogold dots (control group) TEM x 130,000. High magnification of the group of immunogold dots (arrow) is shown in the periphery of the oocyte.

and immunocytochemistry.

The preparation of oocytes and embryos for light and transmission electron microscopy is complicated by the small size of the specimens and the relative ease with which they can be lost during handling. By embedding the specimens in agar, the oocytes can be routinely processed without the need for a dissecting microscope and carefully transferred from one solution to the next.[6] However, unless situated in the same plane at the surface of the agar block, locating and sectioning oocytes can be tedious and time consuming.[7] To solve these procedural difficulties, the oocytes were embedded on the face of a protein mould of a Beem capsules. This is the first application of this method to the study of the human oocytes.

Centrifugation of the oocytes and addition of the glutaraldehyde by dropping it into the capsule minimized disruption of the specimens and ensured that they would be aggregated on the surface of the mould following fixation. Shape retention was crucial for alignment of the mould in a Beem capsule for polymerization. Where care was taken to align the mould accurately in the capsule during embedding, the specimen was situated 10-130 μm below the surface of the resin block. The concentration of the specimens in a single section depended on the degree of parallelism which was attained between the knife and the face of the BSAD mould itself. Furthermore, it was critical that the dishes, micropipettes and capsules used to handle the oocytes were completely free of extraneous particles. Particles adherent to the zona pellucida occasionally cause damage to the knife during sectioning.[8]

Centrifugation of unfixed oocytes from domestic animals at forces of 10,000 g or greater leads to stratification of cytoplasmic organelle distribution was noted with the centrifugal force used in this method. This may be due to fixation prior to centrifugation and/or the ower centrifugal force required to embed the oocytes. The major advantage of this method was the reduction in time required to locate, section, stain and examine of the human oocytes.

By TEM, each microvillus has a core of microfilaments extending to the cortical ooplasm and mingles with the cortical band of microfilaments.[2] Such microfilaments are now believed to be composed of actin (probably associated with myosin) and considered to play an important role in the motility of microvilli at the cell surface.[2] In only one section of group A2, the cortical band of microfilaments could be detected beneath the oolemma. This is because this band tends to be small and disrupted in the mature oocyte. This band was first identified by Sathananthan et al (1984). It is believed to prevent the cortical granules, organized in a single layer, from migrating to the periphery at the immature stage. It disorganizes later at the germinal vesicle stage, when the granules appear to migrate to their final location, immediately beneath the oolemma. In telophase, the oocyte displays increased staining at this band in the area of the constriction furrow between the oocyte and polar body.[2] The aged human oocytes demonstrate a diminution of intensity of actin staining. Sometimes ,the bands of polymerized actin are not detected in some aged human oocytes.[3] Defects in the actin system in the oocyte are often associated with abnormal extrusion of the second polar body leading to triploidy.[3]

Microtubules were detected in the only one ultrathin section which cut through the chromosome level of group A. Microtubules could not be clearly identified because the sections were cut transversely. Normally, the oocyte is arrested at

metaphase of the second meiotic maturation, when it is ovulated. The chromosomes organize at the equator of a spindle. The spindle is more or less barrel shaped at this stage. Microtubules insert into the kinetochore (centromere) of each chromosome. The kinetochore appears as a crescent shaped dense body located in the constricted region of the chromosome. When the oocytes is activated or fertilized, the appearance of small strands or bundles of microtubules in the cytocortex can be detected and by late telophase, they become much more abundant. The organization of the meiotic spindle requires both chromosomes, which cause a local reduction in the threshold for microtubule polymerization and the pericentriolar material to nucleate microtubule polymerization. Human meiotic spindles have centrosomes but no centrioles. Centrosomes and centriole are both self-reproducing organelles and centrioles merely advertise the presence of centrosomes. In human embryos, centrioles are paternally derived.[8] Sathananthan et al (1991) demonstrated that the presence of male centrioles associated with centrosomes in the first mitotic spindle of the human fertilized oocyte.[8]

Microtubules play a central role in rapid organelle movement in animal cells in vesicle-mediated transport from Golgi to ER and in intercompartmental transport.[9] In addition, microtubules are involved in maintaining the Golgi structure since disruption of the microtubular network during mitosis or drug-induced disassembly leads to fragmentation and scattering of Golgi fragments throughout the cell.[10] Reassembly of the microtubular network results in reaggregation of the Golgi cisternae at the Microtubule Organising Centre (MTOC). During this process the Golgi elements move along microtubules; neither immediate filaments or microfilaments appear to be involved in the reassembly.[11] There are also some reports that the actin-based microfilament system is involved in intracellular vesicle transport.[12,13] However, there is no difference in the distribution of the organelles among all groups of the oocytes in this study.

In this study, the immunogold and immunogold-silver staining techniques were applied for the first time to demonstrate the cytoskeleton of the human oocyte by EM and light microscopy respectively.

With immunogold-silver staining techniques, the microtubule spindles and microfilaments could be detected in all of the oocytes by light microscopy. Only two occytes from the control group and group A1 showed normal barrel shape spindles while the other (group A2, A3, A4 and B) showed abnormal spindles. This can be explained that the occytes in group A2, A3, A4 have at least two risk factors to disturb the microtubular system ; e.g. MAF, SUZI, sucrose, and sonic sword. For the oocyte in group B, the spindle fibres may be disturbed directly from the micromanipulation (DISCO). It is possible that as the unfertilized human oocytes in this research was not fresh, the distribution and morphology of the microtubules would be abnormal. The study of Eichenlaub-Ritten (1988) revealed that the spindle of occytes aged for 48 hours was rather small and bipolar or multipolar.[14] Chromosomes were no longer aligned at the spindle equator, but were scattered all over the degenerated spindles. Four oocytes from the control group, group A1, A2, A3 have the uniform distribution of the microfilament, while those from A4 and B have diminished distribution detected by light microscopic immunocytochemistry. The reason may be that three risk factors (SUZI, sucrose and sonic sword) are present in group A4 and direct disturbance of microfilament is the risk factor of group B.

Immunogold-silver technique was compared with the FITC (fluorescein isothiocyanate-immunofluorescence) technique.[15,16] The basic principles of both techniques are similar. An immunolabelling of the spindle with a monoclonal anti-tubulin antibody is followed by an incubation with a second antibody. The difference in methods of visualization of the spindle depends on the nature of the conjugate of the second antibody, being either an FITC molecule or a gold particle. A potential disadvantage of the immunogold-silver staining technique could be the longer procedure time required compared to FITC- immunofluorescence. The obvious advantages of the immunogold-silver staining technique were the production of permanent preparation and no requirement of a special kind of microscope.

From EM immunocytochemistry study, no difference could be found among the microfilament distribution of all the occytes, while the microtubular system could not be detected in any of the groups.

The development of light and electron microscopic immunocytochemistry has provided us with a correlation between structure and function and brought a significant improvement in our knowledge of cell cytoskeleton system.

Immunogold staining was chosen in this study because it is an indirect method which is more sensitive than the direct method and providing the primary antibody host species remain the same, any number of tests can be performed using the common conjugated secondary antibody. In this method, the primary unconjugated antibody (monoclonal mouse anti-alpha tubulin or actin antisera) is allowed to bind the antigen (microtubules or microfilaments). A second tracer-conjugated antibody raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind with the primary antibody. The use of colloidal gold as a marker system for immunocytochemistry was introduced by Faulk and Taylor (1971). It has found wide usage in ultrastructural immunolocalisation. The colloidal gold is the most popular metal tracer in use for many reasons which may be summarized as follows: Firstly, their shape and intrinsic high electron density make gold particles easily recognizable under EM. Secondly, they can be produced in various sizes and therefore, using particles of different diameter, it is possible to perform multiple immunolabelling. Thirdly, they are easy to prepare and can be stored for long periods of time. The size of the colloidal gold particle is also important. They can range from 1 nm to 60 nm, depending on the method of production. If large gold particles are used, there is the possibility that it will prevent some antigen-antibody reaction. Larger particles also have greater mass and this can cause them to be pulled away from the antibody during vigorous washing. Five nm particles will give a more accurate representation of the amount of antigen present and can be used for quantitative assessment. They have one major disadvantage, however, as they are not readily visualised at low magnification. As a result, 15-20 nm particles are more convenient for general application.[17] It was not widely used in light microscope immunoctyochemistry until the advantages of silver development were reported. In this method, the gold particles are enhanced by onion-skin like layers of metallic silver.[17]

For light microscopic study, we used 0.04% Triton X-100 in PBS to facilitate contact between the hydrophobic surface of the specimens and immunoreagents and 50 mM ammonium chloride for breaking the cross-linked protein to allow the binding between immunoreagent and the epitope.

Monoclonal mouse anti-alpha actin (1 : 50) and monoclonal mouse anti alpha tubulin (1 : 1,1000) antisera were used. These concentrations of antisera were recommended by many immunological studies of mouse's oocytes.[16,18] Therefore, this may be inappropriate concentration for the human oocytes, especially with intact zona pellucida. As this is the first study to use immunogold technique in the human oocytes, there have been no previous studies about the appropriate concentration of immunogold conjugated goat antimouse IgG for the human oocytes. The concentration used in this research was suggested for the mouse's oocytes by Vander Elst et al (1988) and the silver enhancement technique was undertaken using the methodology published by Robinson et al (1990).[16,17]

In postembedding immunocytochemistry, immunoreactions are carried out after fixation and embedding, i.e. directly on ultra-thin sections, which are normally mounted on nickel or gold grids, and most workers use an indirect immunostaining procedure and a gold-labelled antibody. The main advantages of postembedding immunogold techniques are : (a) there is no penetration problems since the cells are open to the immunoreactants and these are surface reactions: (b) the antigens are marked more precisely when particulate markers, i.e. colloidal gold particles are used.[19]

The type of embedding media employed is critical in the detection of some antigens. The activity of many antigens can be drastically reduced when conventional epoxy resins are used. This has led to the reemergence of acrylic resins, especially the cross-linked resin "Lowicryl" and "LR" (white and gold), which can withstand electron interaction in the microscope.[17] This is probably why the result of our immunocytochemical postembedding technique was not successful as the epoxy resin was used in this study.

In conclusion, the first part of this work described the application of the new method to handle and prepare the human oocytes for EM. The major advantage of this method is the reduction on time required to locate, section, stain and examine of the human oocytes.

The effects of MAF (SUZI, DISCO), sucrose, and sonic sword on cytoskeleton system of the human oocytes were studied by EM and immunocytochemistry. Immunogold and immunogold-silver staining techniques were applied to study the cytoskeleton of human oocytes.

Although the sample numbers are too small to permit statistical analysis of the data, subjectively it appears that microassisted fertilization (SUZI, DISCO), sucrose, and sonic sword may be the risk factors of the human oocytes' cytoskeleton abnormality, especially those exposed to combined risk factors or the direct disturbance of the cytoskeleton system produced by DISCO.

It is possible that, in the near future, ultrastructural studies will become an integral part of new reproductive technology because there is no other techniques available at present that can provide the quality of detail on human gametes and early embryos. This will be beneficial not only in the research but also in clinical practice, as it will enable the detection and help to explain some kinds of abnormalities of human's gametes and early embryos.

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