韓國家畜繁殖學會誌 24(2) : 143~154 (2000)
Korean J. Animal Reprod.

Aberrant Microtubule Assembly and Chromatin Configuration of Human Oocytes Which Failed to Complete Fertilization Following *In Vitro* Fertilization and Intracytoplasmic Sperm Injection

Chung, H.M.[†], N.H. Kim[2], J.W. Kim[1], J.M. Lim, C. Park, J.J. Ko, K.Y. Cha, J.M. Kim[3] and K.S. Chung[3]

Infertility Medical Center of CHA General Hospital, College of Medicine, Pochon CHA University

일반적 수정과 세포질내 정자주입법에 의해 수정에 실패한 인간난자의 미세소관과 염색체의 형태이상

정형민[†] · 김남형[2] · 김종욱[1] · 임정묵 · 박 찬 · 고정재 · 차광열 · 김재명[3] · 정길생[3]

포천중문의과대학교 차병원 여성의학연구소

ABSTRACT

Most eggs initiated the fertilization processes but arrested at specific stages. The stages included failure of the oocyte to exit from the meiotic metaphase-II with or without sperm penetration, failure of appropriate sperm aster formation, inability to form proper male and female pronuclei, failure of suitable pronuclear apposition, and failure to form proper number of either male or female pronuclei. Various images of defective microtubule organization and chromatin configuration during IVF and ICSI procedures were observed. We discussed the data with previous research results during normal fertilization in humans and other mammals. In conclusion, various aberrant patterns in microtubule assembly and chromatin configuration, which were assessed in the present study, could be used as criteria to improve assisted reproductive technology in clinics. However, further cellular and molecular characterization is needed to clarify these aberrant patterns of cytoskeletal assembly.

(Key words: Microtubules, *In vitro* fertilization, Intracytoplasmic sperm injection, Human eggs)

Ⅰ. INTRODUCTION

During fertilization in mammals, including humans, a series of chromosomal and cytoplasmic changes occur including second polar body extrusion, male and female pronuclear formation, pronuclear apposition, intermixing of paternal and maternal genomes, and mitotic processes. These structural changes are closely linked with changes in the organization of

* This work was supported by a grant from the Interdisciplinary Research Program of the KOSEF (No. 1999-2-205-002-5).

[†] 606-5 Yoeksam-1-dong, Gangnam-gu, Seoul 135-081, Korea.
Tel; Int.-822-3468-3421, Fax; Int.-822-501-8704, E-mail; artchung@netsgo.com

[1] 포천중문의과대학교 구미차병원 산부인과(Department of OB/GYN, Gumi CHA General Hospital, Pochon CHA University)

[2] 충북대학교 농과대학 축산학과(Department of Animal Science, Chungbuk National University)

[3] 건국대학교 축산대학 축산학과(Department of Animal Science, Konkuk University)

microtubules during specific phases following sperm penetration (for review, see Schatten, 1994). Defects of cytoskeletal assembly during fertilization processes can result in the arrest and lethality of zygotes, thus consequently leading infertility. A more complete understanding of the fundamental events that occur during each phase of fertilization would help to provide insight into strategies for improving clinical IVF and ICSI procedures.

Microtubule mediated events in human oocytes have recently been studied in either normal fertilized or arrested oocytes (Simerly et al., 1995, Asch et al., 1995). These studies demonstrated that functional microtubules are nucleated within the inseminated oocytes from the paternally contributed structure, and that a series of microtubule mediated events is necessary for the normal fertilization procedure. Several types of defects in microtubule configuration from failed fertilization were observed such as defects of maternal chromosome reconstitution, incomplete or disarrayed sperm astral organization, and mitosis failure (Asch et al., 1995).

In a relatively short time period intracytoplasmic sperm injection (ICSI) has been established as a routine procedure for the treatment of male factor infertility. Because injection of a single spermatozoon into an oocyte bypasses several steps during normal fertilization, such as sperm incorporation and egg activation procedures, comparing studies on the molecular and cytological aspects during ICSI and IVF has broadly been noted. However, little information is available on this subject in the human. Recent studies in the Rhesus monkey revealed that microtubule and DNA configurations following successful ICSI are similar to those observed during in vitro fertilization, and that the abnormalities in microtubule chromatin and configuration following ICSI are not different from those during IVF in humans (Hewitson et al., 1996; Wu et al., 1996).

Although pioneering research reports on microtubule and chromatin dynamics in humans have recently been produced by a group of researchers (Simerly et al., 1995; Asch et al., 1995), additional studies by other researchers are necessary for the full interpretation of huge amounts of images of cytoskletal and chromatin dynamics during normal and abnormal fertilization. In the present study, we extensively imaged the microtubule assembly and chromatin configuration in arrested human oocytes which were considered as failed fertilization during IVF procedures. The data obtained during IVF were then compared with those following ICSI. The images of various defects in microtubule and chromatin configurations produced in this study were compared with previous research results during normal fertilization in humans (Simerly et al., 1995), primates (Wu et al., 1996) and other large mammals (Navara et al., 1994, Kim et al., 1996a, 1997). In addition, we examined the developmental capability of eggs which have one or three pronuclei. These embryos were also imaged to determine abnormality of microtubule organization and chromatin configuration during cleavage and further development.

Ⅱ. MATRIALS AND METHODS

1. Oocyte stimulation, Oocyte Collection and Semen Preparation

Human oocytes, which failed to normally fertilized and were discarded as unfertilized, were retained from informed donors undergoing IVF cycles at the Infertility Medical Center of CHA General Hospital. Ethical approval to study human eggs which failed to fertilize was obtained from the Institutional Review Board of CHA General Hospital. Ovarian stimulation was achieved by desensitization in the previous luteal phase using GnRH agonist (Buserelin, Hoechst AG, Korea) followed by ovarian stimulation with human menopausal gonadotropin

(HMG; Pergonal, Sereno, Korea). When at least two follicles measuring > 18 m in diameter were detected, ovulation was induced with 10,000 IU human chorionic gonadotropin (Profasi, Sereno, Korea). Oocyte retrieval by transvaginal ultrasound-guided aspiration was performed 34 ~ 36 h after hCG administration. Oocytes were cultured for 4 ~ 6 h in TCM 199 medium (GIBCO/ RL, UK) containing 15% inactivated human mature follicle fluid (hFF). All oocyte preparation steps were performed at 37℃ in an atmosphere of 5% CO_2 in air. Ejaculated semen samples were obtained by masturbation. Motile spermatozoa were isolated from most samples using the swim-up method.

2. *In Vitro* Fertilization

When standard IVF was performed, the oocytes were inseminated 4 ~ 6 h after retrieval using a sperm concentration of 1.5×10^4 motile spermatozoa/ well, containing 1 ~ 4 oocytes. TCM 199 medium was used for fertilization and cleavage cultures. Fertilization was evaluated after oocyte retrieval. At 18 h after insemination, abnormal zygotes which failed to form a pronucleus, or zygotes which had single or multiple pronuclei were used for the experiments. Some single or multiple pronuclear eggs were cultured on Vero cell monolayers, and cleavage was recorded after 24 h. Further development of cleaved eggs was observed for 5 days post insemination. Chi square test was used for statistical analysis.

3. Intracytoplasmic Sperm Injection (ICSI)

ICSI was performed essentially as described by Van Steirteghem et al. (1993). Briefly, an inverted microscope equipped with Normarski DIC optics (Nikon, Diaphot, Japan) and Narshige micromanipulators (Narishige, Japan) was used for ICSI. Only morphologically normal, mature oocytes were injected. A live spermatozoon was immobilized and then drawn up into an injection pipette. An oocyte was held firmly against a holding pipette and the spermatozoon was then carefully injected into the oocyte.

4. Immunofluorescence Microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques described by Kim et al (1996b). Briefly, the eggs were permeabilized in a modified Buffer M (Simerly and Schatten, 1993) for 20 min at 39℃, fixed in methanol at −20℃ for 10 min and stored in PBS containing 0.02% sodium azide and 0.1% bovine serum albumin for 2 ~ 7 days at 4C. Microtubule and sperm axonemes localization was performed using mixture of monoclonal- tubulin (Sigma, clone no. B-5- 1-2) and acetylated tubulin (Sigma, clone no. 6-11 B-1) antibodies. Fixed oocytes were incubated for 90 min at 39℃ with antibodies diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton-X 100 and 0.5% BSA, oocytes were incubated in a blocking solution (Simerly and Schatten, 1993) at 39℃ for 1h. The blocking was followed by incubation in FITC labeled goat anti-mouse antibody (Sigma). DNA was observed by exposure to 10 μg/ml propidium iodide (Sigma). Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientic Co., Huntsville, AL, USA) to retard photobleaching. Slides were examined using a conventional immunofluorescence microscope (Olympus BX60, Japan) and all images were digitally recorded and analysed using an image analyzer (Optimas 6, Opitimas, Bothell, WA, USA). Some typical images were captured using a laser-scanning confocal microscope (BIO-RAD MRC 1024). The images were archived on an erasable magnetic optical diskette and downloaded to a dye sublimation printer (Sony, Japan) using Adobe Photoshop Software (Adobe, Mountain View, CA,

USA).

Ⅲ. RESULTS

1. Defects in Eggs Which Failed to Form Pronucleus

Fig. 1 depicts the chromatin and microtubule arrangement in human oocytes which failed to complete pronuclear formation following IVF and ICSI. Analysis of 141 human oocytes resulted in six different phases of fertilization arrest: 1) arrest at metaphases II without sperm penetration, 2) arrest at the metaphase II with sperm penetration, 3) spontaneous activation without sperm penetration, 4) activation with sperm penetration, 5) oocytes with multiple female karyomeres, and 6) oocytes with germinal vesicle (Table 1). Of the total faulty oocytes, five (5%) oocytes were neither penetrated by spermatozoa nor activated spontaneously. Microtubules were observed only in the spindle (Fig. 1A). Some oocytes (6/101, 6%) contained male chromatin, but were not activated. In four (4/6, 67%) eggs, sperm aster was not observed. In a (1/6, 17%) oocyte, sperm aster was seen incorporated with sperm tail. Paternal meiotic spindle was observed in another (1/6, 17%) oocyte (Fig. 1B). The microtubule organization and chromatin configuration of the oocytes which were not incorporated with sperm, but activated spontaneously are shown in Fig. 1C. A dense network of microtubules was observed throughout the cytoplasm during pronuclear formation (Fig. 1D). Following sperm penetration, some oocytes (48/101, 48%) were activated. Fig. 1 E, F & G show successful sperm incorporation into oocytes. While no astral microtubules were observed in connection with sperm tail in 17 of the oocytes (35%, Fig. 1E), the others had inadequate sperm aster (52%, Fig. 1F). In six oocytes (13%), cytoplasmic microtubules were also activated and male meiotic spindle observed (Fig. 1G). In other cases (9/101, 9%), multiple small female karyomeres appear in the cytoplasm (Fig. 1H). This is possibly arisen from defects during meiosis and female pronuclear reconstitution following oocyte activation (Hewison et al, 1996). A few oocytes (4/101, 31%) were arrested at germinal vesicle stage with sperm penetration. The oocytes seems to be inseminated at germinal vesicle stage. The dense microtubules were seen around pronuclear envelope in all cases. The male chromatin in all instances was in the metaphase spindle (Fig. 1I).

Nine oocytes (30%) following ICSI contained male chromatin, but were not activated (Table 1). In seven oocytes (7/9, 78%), sperm aster was seen incorporated with sperm tail and the others (2/9, 22%) had paternal meiotic spindle. Any different microtubule organization as compared with that during IVF was not observed in the eggs following ICSI.

2. Formation of One and Three or More Pronuclei

Images with microtubules and chromatin in human oocytes which had mono-pronucleus revealed four different phases of fertilization failure: 1) formation of one female pronucleus without male chromatin in the cytoplasm, 2) formation of two sets of female chromatin which were usually closed together, 3) formation of one female pronucleus and one condensed male chromatin, 4) formation of one male pronucleus and one condensed female chromatin (Table 1). Fig. 2 A, B depicts microtubule assembly and one female chromatin configuration in human oocytes following spontaneous activation. A dense microtubule network was observed in the cytoplasm of most (8/10, 80%) of these oocytes. In seven oocytes, one full grown female pronucleus and male condensed chromatin were seen (Fig. 2C). In four eggs (4/7, 57%), male condensed chromatin present near the female pronucleus and microtubules

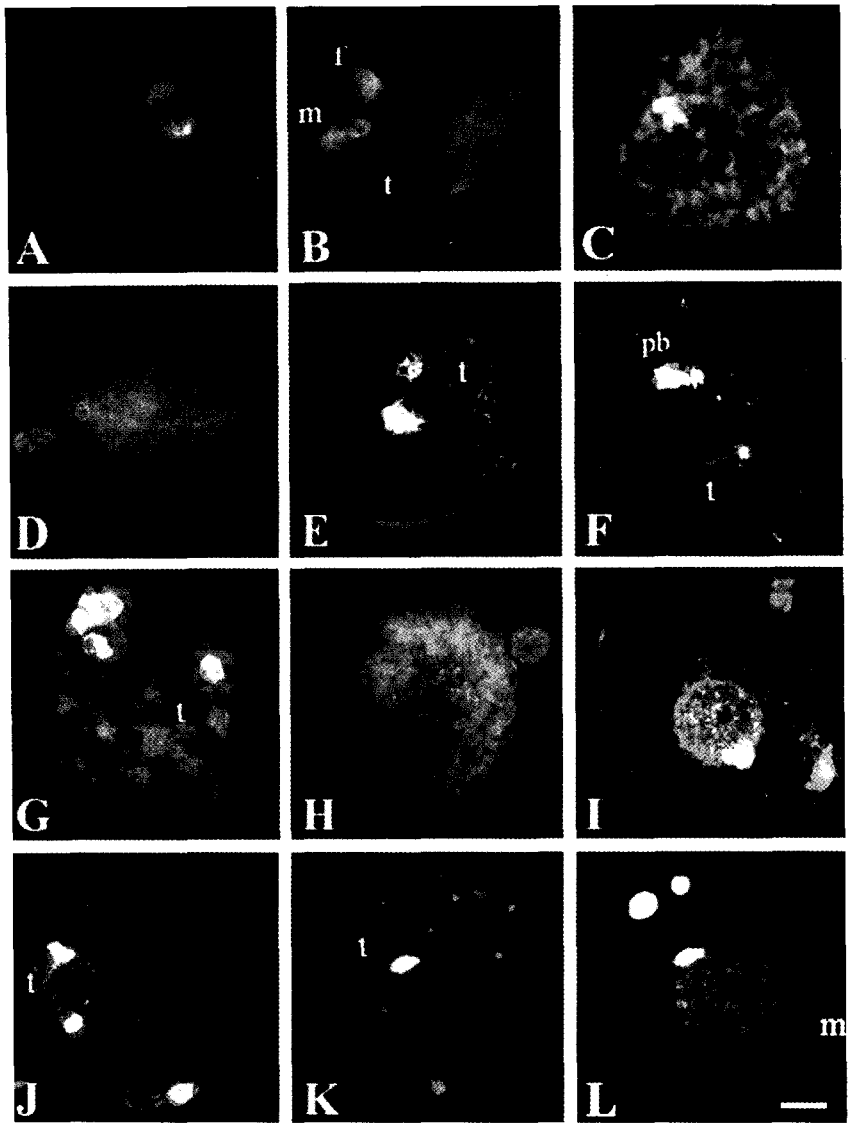

Fig. 1. Microtubule and chromatin images in human oocytes which failed to form of pronuclei following sperm insemination and ICSI. Laser scanning (A, C, E, F, G, H and I) and conventional (B, D) fluorescence microscopy were used. Green, microtubules; red, chromatin. yellow, overlap image of microtubule and chromatin, Bar = 20 μm, t, sperm tail. m, male chromatin; f, female chromatin; pb, polar body. A. Microtubules are seen in the meiotic spindle in the metaphase-II stage arrested oocytes. B. The oocytes seems to be ineffectively activated and have formed a paternal meiotic spindle. C. The oocytes were activated without sperm penetration. Numerous cytoplasmic microtubules have been induced. D. A dense microtubule network was observed in the possible parthenotes. The female structure was condensed. E. The oocytes are activated and sperm aster is not observed in the male chromatin. F. Microtubules are found in association with the incorporaterd sperm head. No microtubles are observed in the female chromatin. Two polar bodies are also seen with midbody (imaged color with yellow). G. Another ineffective egg activation following sperm penetration. Numerous cytoplasmic microtubules, along with both paternal and maternal spindles, are seen. H. Multiple maternal kayomeres are observed. I. Germinal vesicle is observed in the cytoplasm. Male chromatin (m) was not transformed to pronucleus.

Table 1. Summary of fertilization status of human oocytes which failed to normally fertilize following insemination

Fertilization stage	No. (%) of oocytes	
	IVF	ICSI
I. Failure of pronuclear formation	101	21
a. Metaphase II arrest/ no sperm in cytoplasm	5 (5)	1 (5)
b. Metaphase II arrest/ sperm in cytoplasm	6 (6)	9 (43)
c. Activation (no sperm in cytoplasm)	29 (29)	3 (14)
d. Acitivation (sperm in cytoplasm)	48 (48)	5 (24)
e. Multiple female karyomeres	9 (9)	2 (10)
f. Germinal vesicle	4 (4)	1 (5)
II. Mono pronuclear formation	22	5
a. One female pronulceus	10 (45)	3 (60)
b. Two female pronuclei	2 (9)	-
c. One female + one male condensed chromatin	7 (32)	2 (40)
d. One male chromatin + condensed female chromatin	3 (14)	-
III. Multiple pronuclear formation	67	9
a. Two female pronuclei + one male pronucleus	12 (18)	9 (100)
b. Two female pronuclei + two male pronuclei	3 (4)	-
c. One female pronucleus + multiple male pronuclei	52 (78)	-

were organized in these eggs (Fig. 2C). In some cases (2/7, 29%), sperm aster detached inappropriately from the male structure (Fig. 2D). Disarrayed microtubules were seen along detached sperm tails.

Microtubules and chromatin were also imaged in multiple pronuclear embryos. These eggs were classified by observation of sperm exonemes as follows; 1) two female pronuclei and one male pronucleus, 2) two female pronuclei and multiple male pronuclei, 3) one female pronucleus and multiple pronuclei. Most oocytes (49/67, 73%) had the pronuclei in the center. The dense microtubules were organized around the nuclei (Fig. 2F). In some instances (18/67, 27%), the nuclei were not properly apposed. Disarrayed microtubules were seen throughout the cytoplasm (Fig. 2E).

Three (3/4, 75%) mono- pronuclear eggs had one female pronucleus and the others had male condensed chromatin and one female pronucleus. All of the three pronucleated eggs had two female pronuclei and one male pronucleus. Any distinct microtubule assembly following ICSI was not observed when compared with that following in vitro fertilization.

3. *In Vitro* Development of Mono- and Tri-pronuclear Formation

Fig. 2H-L depicts images of various stages during *in vitro* development in human eggs which were developed from 1 or 3 pronuclear eggs. Structural defects were observed in fragmented blastomeres. Some blastomeres underwent cytokinesis. The DNA and microtubule images of the surviving, intact cells are shown at various stages, including late interphase or mitotic metaphase. The microtubules usually extended radially from the blastomeres to the cortex in cleaved oocytes. A total of 13 eggs were imaged for microtubule and chromatin in abnor-

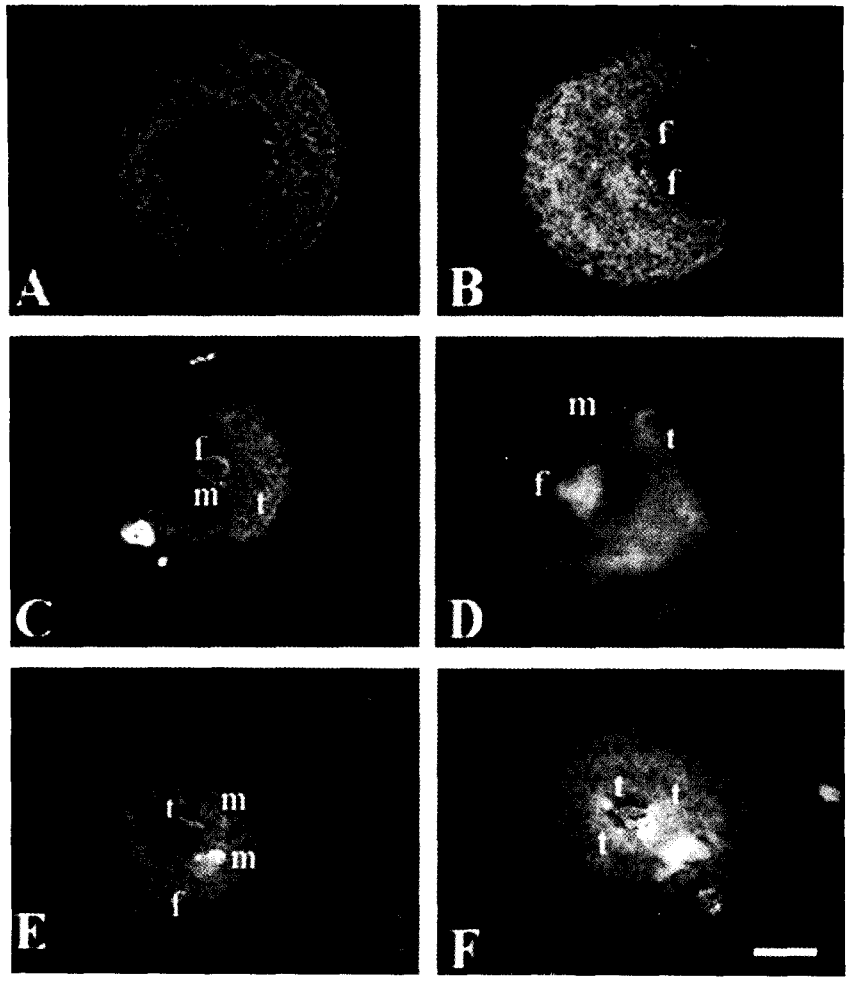

Fig. 2. Microtubule and chromatin images in human oocytes which formed mono and multiple (three or more) pronuclei following sperm insemination and ICSI. Laser scanning (A,B,C,E&F) and conventional (D) fluorescence microscopy were used. Green, microtubules; yellow, overlap image of microtubule and chromatin, red, chromatin. Bar = 20 μm, t, sperm tail. m, male chromatin; f, female chromatin; pb, polar body. A. Classifed with one pronucleus. B. Classified as three or more pronuclei. A. Dense microtubule network was observed in and around the full grown pronuclear stage parthenotes. No detectable sperm tail observed. B. Two female chromatin are closed together in the cytoplasm (no sperm tail observed). C. The inseminated oocytes have a female pronucleus and a condensed sperm chromatin separated from the nearby sperm tail (t). D. Sperm tail is detached and disarrayed microtubule is observed along sperm tail. E. Two male pronulceus and one female pronulcleus are seen. The oocytes seem to be activated during sperm incorporation. But they fail to organized proper sperm astral microtubules and pronuclear development is incomplete. F. Multiple sperm tails and spernumerary asters are seen in the cytoplasm.

mally cleaved embryos at day 5 from 3 pronuclear eggs. Nine (9/13, 69%) had multiple nuclei in the cytoplasm. In two (2/13, 15%), no interphase chromatin was observed, suggesting fragmentation. In early blastocysts which were produced from one female nuclear eggs, a network of microtubules formed around each blastomere. The developmental capability is shown in Table 2. More uni-pronuclear

Table 2. *In vitro* development of mono and tri pronuclei embryos

Pronucleus	No. of oocytes examined	No. (%) of oocytes that developed to				
		2-cell	4-cell	6-to 8-cell	Morulae	Blastocysts
Mono PN*	8	6 (75)	5 (63)	5 (63)	4 (50)	3 (38)**
Three PN	15	9 (60)	9 (60)	6 (40)	4 (27)	1 (7)

* pronucleus, ** $P < 0.05$

eggs developed to compacted morula and blastocysts as compared to those of three pronulcear eggs (Table 2, $P < 0.5$).

Ⅳ. DISCUSSION

In the present study, we explored the microtubule dynamics and chromatin configuration in human oocytes which were diagnosed in the clinic as unfertilized at 18 h following IVF and ICSI. The oocytes were classified into three categories; 1) failure of pronuclear formation, 2) formation of one pronucleus; 3) formation of three or more pronuclei. Many oocytes initiated the fertilization process, but arrested at specific stages. Various images were produced in this study. Incomplete understanding of molecular and cellular events during each phase of fertilization in mammals, including humans, make it impossible to totally interpret various aberrant forms of arrested fertilization during IVF and ICSI procedures. Previous research results during normal and abnormal fertilization and parthenogenesis in humans and other mammals were used in order to precisely interpret the numerous images.

Dense microtubules were observed in the cytoplasm of either spontaneously or sperm activated oocytes. Previous studies demonstrated that parthenogenetic stimulation induced the centrosomal material (possibly α-tubulin), which existed in the cytoplasm, to form microtubule foci which aggregated together and formed the microtubule matrix during pronuclear formation (Kim et al., 1996a,b,c; Navara et al., 1995). This microtubule matrix seems to move the male and female chromatin to the center of oocytes. In this study, we observed numerous microtubule foci in activated oocytes, which probably arose from nucleated centrosomal material found in the cytoplasm. The microtubules seemed to aggregate together and form microtubule matrixes during pronuclear formation, which was similarly observed in pig oocytes during parthenogenesis (Kim et al., 1996a). This result suggests that in humans, like in pigs, activation of oocytes induces centrosomal material to form a dense microtubule matrix for pronuclear movement and cleavage.

In the present study, we observed the defective organization of sperm-derived astral microtubules in the cytoplasm of eggs, which failed to fertilize. Abnormalities in astral microtubules may be due to defects from the sperm and/or oocyte components for the cytoskeletal and cell cycle regulation (Asch et al., 1996). Impaired microtubule motor proteins, such as dynain and kinesin may result in arrests of tubular aster development from the sperm centrosome, which could result in failure of both pronuclei to the center of eggs. Previously, Afzelius et al. (1975) reported that infertile men suffering from immobile cilia syndrome have been shown to lack the microtubule motor dynein as arms on the microtubules in their sperm tails. More recently, Navara et al. (1996) determined that bull spermatozoa following penetration into eggs vary in their ability to organize microtubules within oocytes.

The variations in sperm aster size and arrays are associated with reproductive success *in vivo* and *in vitro*. These results suggested that in assisted reproductive technologies, specifically in ICSI, factors affecting the sperm(s ability to form proper microtubules should be considered. More studies for determining the role and organization of centrosome/ centrioles during mammalian gametogenesis and fertilization are needed in both male and female aspects.

While a well developed female pronucleus is present in the cytoplasm, the male chromatin sometimes remains condensated. This phenomenon has been referred to as premature chromosome condensation (PCC, Schimiady and Kentenich, 1989; Acsh et al., 1995). This is a common feature in human oocytes, which fail to fertilize during IVF (Selva et al., 1991) and after ICSI (Bergere et al., 1995). Furthermore, during *in vitro* fertilization, the incidence of PCC was found to be generally higher than that following ICSI (Bergere et al., 1995; Plachot and Crozet, 1992). Oocyte immaturity during IVF may cause PCC (Plachot, 1992). The sperm nucleus is able to transform into swelling pronucleus in fully matured oocytes, while the spermatozoon could be condensed in metaphase II arrested oocytes. Since the morphology of PCC varies according to the stage of the interphase nucleus at the time of fusion (Johnson and Rao, 1970), PCC could be caused by lack of oocyte components, such as maturation promoting factors, cytostatic factors or mitogen activation factors, which are related to the cell cycle regulation. Schmiady and Kentennich, (1989) reported that distinct asynchronous development of male and female pronuclei of *in vitro* fertilized eggs could also result in PCC of male chromatin, leading to developmental arrest.

Sultan et al. (1995) examined the chromosomal status of one pronuclear human zygotes following IVF and ICSI. The incidence of monopronulceus among the total failure to fertilization eggs was usually higher in ICSI procedures than that in conventional IVF (Abramczuk and Lopata, 1990; Palermo et al., 1993). The majority (61.9%) of mono- pronuclear embryos formed after IVF, compared to only 9.5% mono-pronuclear embryos following ICSI (Sultan et al., 1995). These results suggest that many mono- pronuclear eggs were fertilized, in contrast to those from ICSI that were parthenogenetically activated. In the present study, about 50% of the mono-pronuclear oocytes which were examined appeared to be parthenogenetically developed. In others, condensed sperm chromatin was seen in the cytoplasm. Well organized microtubule matrix was seen around pronucleus whether sperm chromatin was present or not.

Tripronucleated eggs are capable of developing progressively through preimplantation stages (Wenz et al., 1983; Van Blerkom et al., 1987). In this study, we observed that more mono-pronuclear embryos than three pronucleated embryos developed to the morula and blastocyst stage. This is probably because most mono-pronuclear eggs seem to develop as haploid or diploid, whereas tri-pronuclear eggs form a mosaic of chromosomally normal and abnormal cells, which has been shown to possibly cause progressive developmental arrests (Van Blerkom, 1984). Previously, Kim et al. (1996b) reported that aged oocytes, following parthenogenetic activation, formed two female pronuclei which sometimes underwent mitosis independently and resulted in abnormal cleavages. In the present study, many arrested eggs up to the 8-cell stage derived from three pronuclear eggs had multiple (10～15) interphase eggs, suggesting chromatin division in the absence of cell division.

Int Veld et al. (1995) reported that a relatively high number of embryos (5/15, 33%) had serious genetic abnormalities in sex chromosomes. Karyo-

type analysis revealed that the defects seems to be of paternal origin. This suggests that ICSI for the treatment of specific male infertility may be accompanied by an increased incidence of sex chromosomal abnormalities in spermatozoa. Centrosome or centromere problems in gametes may induce defects in chromosomal segregation. In this study, most aberrant patterns of microtubule assembly and DNA configuration in failed ICSI fertilization were similar to those found in human oocytes fertilized *in vitro*. This is similar with previous results found in the Rhesus monkeys (Hewitson et al., 1996, Wu et al., 1996). These suggest that most defects following ICSI do not originate from technical procedures.

In summary, we showed various images of defective microtubule and chromatin assembly during IVF and ICSI procedures. Most eggs initiated fertilization, but arrested at specific stages. The stages include failure of the oocyte to exit from the meiotic metaphase-II with or without sperm penetration, failure of appropriate sperm aster formation, inability to form proper male and female pronuclei, failure to form suitable pronuclear apposition, and failure of the proper number of either male and female pronuclei. Much of this infertility appears to be related to improper organization of microtubules, probably due to defects in either male and female components to properly manage cytoskeletal elements. The information could be used to improve both diagnoses of different types of idiopathic infertility and therapeutic strategies for enhancing assisted reproduction techniques, such as IVF and ICSI. However, further characterization of the cellular and molecular mechanisms during and subsequent to normal or abnormal fertilization is needed to clarify the aberrant patterns of cytoskeletal assembly.

V. 요 약

본 연구는 생식보조기법을 시행한 불임환자로부터 얻은 난자를 일반적인 수정법과 세포질내 정자직접주입법으로 수정을 유도한 다음 정상수정에 실패한 난자에 대한 미세소관과 염색체의 형태학적 차이를 laser scanning confocal microscope를 이용하여 비교분석하고자 실시하였다. 일반적 수정법 혹은 세포질내 정자직접주입법 실시 후 18시간째에 해부현미경 하에서 난자를 관찰하였을 때 전핵형성에 실패한 미수정란, 한 개의 전핵 또는 3개 이상의 전핵의 형성이 관찰된 이상수정란으로 구분하여 연구를 실시하였다. 미세소관의 관찰을 위해서 (-tubulin antibody를 반응시킨 후 형광물질이 부착된 2차항체와 반응시킨 후 관찰하였으며 염색체의 관찰을 위해서는 propidium iodide로 염색한 다음 confocal microscope하에서 관찰하였다. 연구결과 대부분의 난자는 수정과정중에 있었으나 일부의 난자에서는 특정단계에서 정지되어 있는 것이 관찰되었다. 즉, 감수분열 중기에서 정자의 침입이 이루어지지 않은 경우, 정자의 침입은 이루어졌으나 sperm aster 형성이 불완전한 경우, 웅성 및 자성전핵의 형성에 실패한 경우 및 전핵의 위치가 불완전한 경우 등이 관찰되었고 이들 난자의 경우 높은 비율로 미세소관과 염색체의 이상이 관찰되었다. 이상의 연구결과로 미루어 볼 때 생식보조기법의 시술과정에서 채취되는 난자의 수정실패의 원인은 세포골격기관 특히 미세소관의 이상과 염색체의 이상에 기인되는 것으로 사료되면 이러한 세포골격 구성물질의 이상에 대해서는 추후에 세포조직학적 또는 분자생물학적 분석이 필요하다고 하겠다.

ACKNOWLEDGMENTS

We gratefully thank to anonymous patients who donated excess and discarded oocytes for the research. We extend our gratitude to Dr. Gerry Schatten for his encouragement and sending excellent reprints.

Ⅵ. REFERENCES

1. Afzelius, B.A., Eliasson, R., Johnsen, O. et al. 1975. Lack of dynein arms in immotile human spermatozoa. J. Cell Biol., 66, 225-232.
2. Abramczuk, J.W. and Lopata, A. 1990. Resistance of human follicular oocytes to parthenogenetic activation: DNA distribution and content in oocytes maintained *in vitro*. Hum. Reprod.., 5, 578-581.
3. Asch, R., Simerly, C., Ord, T. et al. 1995. The stage at which human fertilization arrests: microtubule and chromosome configuration in inseminated oocytes which failed to complete fertilization and development in humans. Hum. Reprod., 10(7), 1897-1906.
4. Bergere, M., Selva, J., Volante, M. et al. 1995. Cytogenetic analysis of uncleaved oocytes after intracytoplasmic sperm injection. J. Assist. Reprod. Gen., 12: 322-325.
5. Hewitson, L.C., Simerly, C.R., Tengowski, M.W. et al. 1996. Microtubule and chromatin configurations during rhesus intracytoplasmic sperm injection: success and failures. Biol. Reprod., 55, 271-280.
6. In't Veld, P., Brandenburg, H., Verhoeff, A. et al. 1995. Sex chromosomal abnormalies and intracytoplasmic sperm injection. Lancet, 773, 346.
7. Johnson, R.T. and Rao, P.N. 1970. Mammalian cell fusion: induction of preculture chromosome condensation in interphase nuclei. Nature, 226, 717-722.
8. Kim, N.-H., Funahashi, H., Prather, R.S., et al. 1996c. Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation. Mol. Reprod. Dev., 43, 248-255.
9. Kim, N.-H., Moon, S.J., Prather, R.S. et al. 1996a. Cytoskeletal alteration in aged oocytes and parthenogenesis. Mol. Reprod. Dev., 43, 513-518.
10. Kim, N.-H., Simerly, C., Funahashi, H. et al. 1996b. Microtubule organization in porcine oocytes during fertilization and parthenogenesis. Biol. Reprod., 54, 1397-1404.
11. Kim, N.-H., Chung, K.S. and Day, B.N. 1997. The role and distribution of microtubule and microfilaments during fertilization and parthenogenesis. J. Reprod. Fertil. in press.
12. Navara, C.S., First, N.L. and Schatten, G. 1994. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis and nuclear transfer: the role of sperm aster. Dev. Biol., 162, 29-40.
13. Navara, C.S., First, N.L. and Schatten, G. 1996. Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization: Correlations between centrosome activity and developmental success. Proc. Natl. Acad. Sci. (USA)., 93, 5384-5388.
14. Palermo, G., Joris, H., Devroey, P. et al. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 340, 17-18.
15. Palermo, G., Joris, H. and Derde, M.P. et al. 1993. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. Fertil. Steril., 59, 826-835.
16. Plachot, M. and Crozet, N. 1992. Fertilization abnormalities in human *in vitro* fertilization. Hum. Reprod., 7(1): 89-94.
17. Schatten, G. 1994. The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol., 165, 299-335.
18. Schatten, G., Simerly, C. and Schatten, H. 1985. Microtubule configurations during fertilization,

mitosis and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc. Natl. Acad. Sci. (USA), 82, 4152-4156.

19. Schatten, H., Schatten, G., Mazia, D. et al. 1986. Behavior of centrosomes during fertilization and early development of sea urchin and mice. Proc. Natl. Acad. Sci. (USA), 82, 4152-4156.
20. Simerly, C. and Schatten, G. 1993. Techniques for localization of specific molecules in oocytes and embryos. Methods Emzymol., 516-552.
21. Simerly, C., Wu, G-J., Zoran, S., et al. 1995. The paternal inheritance of the centrosome, the cell(s microtubule-organizing center, in human and the implecation for infertility. Nature Med., 1, 47-52.
22. Schmiady, H. and Kentennich, H. 1989. Premature chromosome condensation after *in vitro* fertilization. Hum. Reprod., 4, 689-695.
23. Selva, J., Martin-Pont, B., Hugues, J.N., et al. 1991. Cytogenetic study of human oocytes uncleaved after *in vitro* fertilization. Hum. Reprod., 6. 709-713.
24. Sultan, K.M., Munne, S., Palermo, G.D. et al. 1995. Chromosomal status of uni-pronuclear human zygotes following *in vitro* fertilization and intracytoplasmic sperm injection. Hum. Reprod., 10, 132-136.
25. Van Blerkom, J. and Bell, H. 1986. Regulation of development in the fully grown mouse oocyte: chromosome-mediated temporal and spatial differentiation of cytoplasm and plasma membrane. J. Embry. Exp. Morph., 93, 213-238.
26. Van Blerkom, J., Bell, H. and Henly, G. 1987. The occurrence, recognition and developmental fate of pseudo-multipronuclear eggs after *in vitro* fertilization of human oocytes. Hum. Reprod., 2, 217-225.
27. Van Steirteghem, A.C., Liu, J., Joris, H. et al. 1993. Higher success rate by intracytoplasmic sperm injection than by subzonal insertion. Report of a second series of 300 consecutive treatment cycles. Hum. Reprod., 8, 1055-1060.
28. Wenz, A.C., Rapp J.E. Maxon, W. S. et al. 1983. The problem of polyspermy in *in vitro* fertilization. Fertil. Steril., 40, 748-754.
29. Wu, G.-W., Simerly, C., Zoran, S.S. et al. 1996. Microtubule and chromatin dynamics during fertilization and early development in rhesus monkeys, and reguaration by intracellular calcium ions. Biol. Reprod., 55, 260-270.

(접수일자 : 2000. 2. 19. / 채택일자 : 2000. 5. 1.)