Spindle and Chromosome Abnormality of Aged, Matured Oocytes in Human

Kaoru Yanagida[1]*, Syotaro Hayashi[1], Haruo Katayose[1], Ryota Suganuma[1], Kazuto Morozumi[1] and Akira Sato[1]

[1]Department of Obstetrics and Gynecology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan

Abstract: *There are cases in which the 1-day-old unfertilized oocytes after IVF, or in vitro matured oocytes obtained from denuded immature oocytes after ICSI, are used clinically. We investigated the morphological change of metaphase II spindle and chromosomal alignment in those oocytes. The spindles and chromosome were stained using an anti-α-tubulin antibody and Hoechst 33258 respectively. One-day-old oocytes displayed significant increases in abnormalities of spindle structure (40.4% vs. 17.6%) and chromosomal alignment (40.4% vs. 29.4%). Oocytes matured in vitro from MI and PI oocytes displayed spindle morphological abnormalities at the rates of 41.7% and 36.0% respectively, and chromosomal alignment abnormality at the rates of 45.8% and 44.0% respectively. These results suggest that 1-day-old oocytes and in vitro matured oocytes obtained from denuded immature oocytes after ICSI might have lowered developmental potential. More than 50% of those oocytes were determined to be unsuitable for use as gametes.*

Key words: *Spindle, Chromosome, 1-day-old oocyte, Matured oocyte, Mouse, Human*

One-day-old reinsemination [1, 2], or 1-day-old-ICSI [3–7] may be performed when *in vitro* fertilization (IVF) completely fails to achieve fertilization in some patients. In addition, when oocytes collected for intracytoplasmic sperm injection (ICSI) turn out to be immature oocytes, ICSI is sometimes performed on *in vitro* matured oocytes. The 1-day-old unfertilized oocytes in IVF procedures [3, 8], as well as *in vitro* matured oocytes in ICSI procedures [9–12], exhibit low developmental potential and poor clinical results. The cause of this is not clear, but disorder of cytoskeleton in aged oocytes [8] and disruption of cytoplasmic maturation in *in vitro* matured oocytes [13, 14] has been suggested.

The spindle is thought to be susceptible to damage from environmental factors, and ageing in oocytes causes spindle aberrations *in vivo* in mouse oocytes [15]. The spindle plays an important role in the segregation of chromosomes. Abnormal spindle morphology is associated with abnormal chromosomal alignment on the metaphase plate, resulting in chromosomal abnormalities in fertilized oocytes [16–18], and induces fertilization disorder according to the spindle assembly checkpoint [19].

Previous reports indicated a high incidence of spindle abnormality and chromosomal alignment abnormality in oocytes cultured in vitro for more than 24 hours post insemination with cumulus cells [20, 21]. We investigated the normality of metaphase II (MII) spindle and chromosomal alignment in unfertilized oocytes at 18–22 hours post insemination to evaluate whether these aged oocytes are suitable for clinical use. In addition, it has been reported that spindle abnormalities in matured human oocytes are obtained after in vitro culturing of immature oocytes in association with freezing [22]. However, there is no report concerned with spindle abnormalities of matured oocytes obtained from denuded immature oocytes under the conventional culturing environment. So, we investigated the normality of M II spindle and chromosomal alignment in matured oocytes obtained from denuded immature oocytes under the conventional culturing environment to evaluate whether they are also suitable for clinical use.

Received: July 14, 2002
Accepted: September 4, 2002
*To whom correspondence should be addressed.
e-mail: kyana@fmu.ac.jp

Table 1. The profile of each goup

Group of oocytes	No. of patients	Average age of patients*	No. of oocytes
Fresh	3	31.7 ± 4.1	17
1 day old	29	32.4 ± 4.3	52
P1	33	32.3 ± 4.2	25
M1	24	32.0 ± 5.2	24

*Average age of patients (mean ± SD). There were no significant differences among the groups.

Materials and Methods

Preparation of oocytes

Human oocytes were taken for consenting patients with the approval of the Ethics board of our institution. Ovarian stimulation and oocyte retrieval for IVF and ICSI were performed based on a previous report [23]. Three patients provided seventeen M II oocytes. None of the patients had undergone ICSI because of failures in sperm collection. Fifty-two unfertilized oocytes were obtained from 29 patients whose oocytes had failed to fertilize partially or totally 18–22 hours after insemination in IVF. The indications for these IVF were tubal factor (6 patients) and male factor (11 patients) and unexplained infertility (12 patients). Immature oocytes (25 prophase I oocytes and 24 metaphase I oocytes) that were retrieve from 57 ICSI treatment cycles, were used for the study. The indications for ICSI were male factor (36 patients) and fertilization disorder in IVF (21 patients). Prophase I (PI) and metaphase I (MI) oocytes were cultured until they became MII oocytes (48 hours and 24 hours maximum respectively) in human tubal fluid (HTF) (Irvine Scientific, CA, USA) supplemented with 10% synthetic serum substitute (SSS) (Irvine Scientific, CA, USA). The profiles of the patients are shown in Table 1.

Staining of the spindle and chromosomes

The oocytes were fixed in 2% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL) supplemented with 0.1% Triton X-100 and 1 μM paclitaxel (T1912, Sigma Chemical, USA) at room temperature for 60 min, then washed in PBS containing 3% bovine serum albumin (BSA) for 60 min. The oocytes were treated with a primary antibody, a monoclonal antibody against α-tubulin (1:100 in PBS; T9026, Sigma Chemical) at room temperature for 45 min, and washed in PBS containing 3% BSA for 60 min. The oocytes were then treated with FITC-conjugated secondary antibody (1:100; F2168, Sigma) and 10 μg/ml Hoechst 33258 (for counterstaining of chromosomes) for 45 min at room temperature, and washed in PBS containing 3% BSA for 60 min.

Observation of spindle and chromosomes

The stained oocytes were mounted on slides and examined with a fluorescence microscope (BH2-RFCA; Olympus, Japan). The fluorescent images were captured on a computer using a high-resolution video camera attached to a microscope. Two images were overlaid using Adobe Photoshop, and abnormalities in spindle morphology and chromosomal alignment on the metaphase plate were examined. A barrel-shaped spindle and chromosomes aligned at the spindle equator were considered to be "normal", and each abnormality was classified as follows:

a) Classification of abnormal spindle morphology

Small: smaller than normal spindle. Amorphous: spindle exhibiting a barrel shape with fibers showing some irregularity or spindle indistinct form. None: spindle not found in the oocyte. Unsatisfied: poorly stained or not discernable.

b) Classification of abnormal chromosomal alignment

Small: chromosomes clumped together. Dispersed: chromosomes imperfectly aligned at the spindle equator. None: chromosomes not found in the cytoplasm of an oocyte. Unsatisfied: poorly stained or not discernable.

Statistical evaluation for significant differences was performed using either the χ^2 test or Fisher's exact test, and results with $p<0.05$ were considered significant.

Results

The fluorescent images of stained oocytes are shown in Fig. 1.

One-day-old oocytes

Abnormal spindle morphology was observed in 17.6% of MII oocytes immediately after collection, and in 40.4% of unfertilized oocytes day 1 post IVF ($p<0.05$). One in 17 fresh oocytes displayed no spindle. The

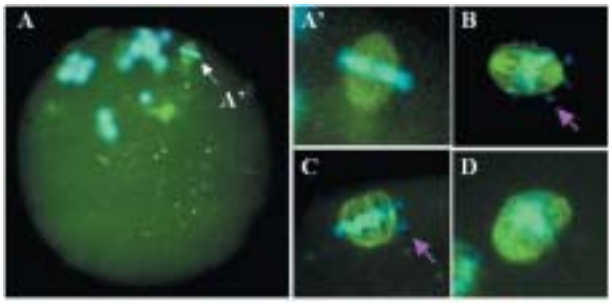

Fig. 1. Characteristics of spindle shape and chromosome alignment. (A, A') M II spindle of human unfertilized oocytes one day after IVF is shown. Spindle shape and chromosome alignment showed normal appearance. (B) Spindle shape is normal and chromosomes (arrow) are dispersed from the metaphase plate with rough alignment of chromosomes on the metaphase plate. (C) Size of the spindle (small) is small and some chromosomes (arrow) are dispersed. (D) Alignments of spindle fibers and chromosomes are rough.

primary abnormality observed in the 1-day-old oocytes was "amorphous" (11.8%) (Table 2-a). Abnormal chromosomal alignment was observed in 29.4% of MII oocytes immediately after collection. The primary abnormality observed in 1-day-old oocytes was "dispersed" (32.7%) (Table 2-b).

In vitro matured oocytes

Forty-six PI oocytes and thirty-three MI oocytes were retrieved from 34 and 31 ICSI patients respectively. Twenty-five oocytes of PI oocytes became MII oocytes after incubation, and 24 MI oocytes became MII oocytes. Observation of in vitro matured oocytes revealed abnormal spindles in 41.7% of MII oocytes matured from MI oocytes and in 36.0% of MII oocytes matured from PI oocytes (Table 3-a). Also, 45.8% of MII oocytes from MI oocytes showed abnormal chromosomal alignment, and 44.0% of MII oocytes from PI oocytes showed abnormality. The primary abnormality observed was "dispersed" (33.3%) (Table 3-b).

There were significant differences between "Fresh (Tables 2a, 2b)" and MII oocytes matured from MI or PI oocytes *in vitro* in spindle morphology and chromosomal alignment ($p<0.05$, $p<0.01$, respectively).

Discussion

Previous reports indicated the high incidence of spindle abnormality and chromosomal alignment abnormality in the oocytes cultured from 48 to 144 hours post insemination [20]. One study reported that unfertilized oocytes in IVF (18 to 22 hours post insemination) revealed normal morphology in 61% and abnormal morphology in 30%, with 9% showing no spindles [21]. The oocytes used in that study included oocytes aged for 24 to 48 hours post insemination, therefore the frequency of occurrence of spindle abnormalities in 1-day-old unfertilized oocytes was unclear. In our study, spindle and chromosomal abnormalities were observed in 40.4% and 40.4%, respectively, in aged oocytes (1-day-old), although they were observed within 22 hours after insemination. In aged oocytes, the absence of pericentriolar material (PCM) outside the meiotic spindle and the loss of γ-

Table 2-a. The morphology of metaphase II spindles in 1-day-old oocytes

Group of oocytes	No. of oocytes	No. of unclassified	No. of normal oocytes	Abnormal spindle morphology			
				Subtotal	Small	Amorphous	None
Fresh	17	0 (0)	14 (82.4)*	3 (17.6)**	0 (0)	2 (11.8)	1 (5.9)
1 day old	52	10 (19.2)	21 (40.4)*	21 (40.4)**	5 (9.6)	12 (23.1)	4 (7.7)

Statistical information is given below. There were significant differences between "Fresh" and "1 day old" only in the no. of normal oocytes ($p<0.001$) and subtotal for abnormal spindle morphology ($p<0.05$).

Table 2-b. The alignment of metaphase II chromosomes in 1 day old oocytes

Group of oocytes	No. of oocytes	No. of unclassified	No. of normal oocytes	Abnormal chromosome alignment			
				Subtotal	Small	Dispersed	None
Fresh	17	0 (0.0)	12 (70.6)*	5 (29.4)	0 (0)	5 (29.4)	0 (0.0)
1 day old	52	11 (21.2)	20 (38.5)*	21 (40.4)	2 (3.8)	17 (32.7)	2 (3.8)

There were significant differences between "Fresh" and "1 day old" only in the no. of normal oocytes ($p<0.05$).

Table 3-a. The morphology of metaphase II spindles in matured oocytes obtained from MI/PI oocytes

Group of oocytes	No. of oocytes	No. of unclassified	No. of normal oocytes	Abnormal spindle morphology			
				Subtotal	Rough	Amorphous	None
PI	25	5 (20.0)	11 (44.0)	9 (36.0)	4 (16.0)	4 (16.0)	1 (4.0)
MI	24	4 (16.7)	10 (41.7)	10 (41.7)	5 (20.8)	5 (20.8)	0 (0.0)

PI oocytes were MII oocytes cultured from PI stage oocytes. MI oocytes were MII oocytes cultured from MI stage oocytes. There was no significant difference between PI and MI in any item.

Table 3-b. The alignment of metaphase II chromosome in matured oocytes obtained from MI/PI oocytes

Group of oocytes	No. of oocytes	No. of unclassified	No. of normal oocytes	Abnormal chromosome alignment			
				Subtotal	Small	Dispersed	None
PI	25	5 (20.0)	9 (36.0)	11 (44.0)	3 (12.0)	7 (28.0)	1 (4.0)
MI	24	6 (25.0)	7 (29.2)	11 (45.8)	3 (12.5)	8 (33.3)	0 (0.0)

There was no significant difference between PI and MI in any item.

tubulin staining in the ooplasma were reported [25]. To date, only a low efficacy of 1-day-old ICSI has been obtained, as mentioned above, and only a few cases of successful pregnancy from 1-day-old ICSI have been reported [26, 27]. Spindle abnormalities appear to be one cause of the low developmental potential of 1-day-old oocytes. Spindle abnormalities can be caused by maternal age [24], but there was no significant difference between each group in the mean maternal age in this study (Table 1), so the maternal age did not affect the results acquired in this study.

In this study, denuded oocytes obtained by *in vitro* maturation under the conventional culturing environment were used. There has been no report concerning the spindle abnormalities of matured oocyte obtained from denuded immature oocytes. In MII oocytes obtained by culturing immature oocytes with cumulus cells collected from unstimulated ovaries in Eagle's medium supplemented with PMS and hCG, abnormal spindles were observed in 22.2%, including 5.6% showing loss of spindle, and chromosome analysis revealed abnormalities in 31.8% [22]. In the study by Park et al., the effect of cryopreservation on spindles was evaluated too, and the results indicated abnormal spindles in 70% and chromosomal abnormalities in 77.8% of freeze-thawed and matured oocytes [22]. A similar study conducted on immature oocytes with cumulus cells retrieved from stimulated ovaries revealed abnormal spindles in 16.2% of *in vitro* matured oocytes and in 11.1% of *in vivo* matured oocytes [28]. This study was conducted using stimulated ovaries, and revealed abnormal spindles in 41.7% of cultured MI oocytes and 36.0% of cultured PI stage oocytes. The rate of spindle abnormality in matured MI and PI oocytes was high in this study, and the rate of spindle abnormality in matured MI oocytes was similar to that in matured PI oocytes. One of the reasons for the high rates of spindle abnormalities in this study was thought to be the use of cumulus cell-free oocytes [15]. Another reason suggested was that FSH [29] and LH [30] were not added to the culture media. There are some reports that matured PI and MI oocytes produced chromosomally normal M II oocytes [31]. Boiso et al. reported that the rate of abnormalities in *in vitro* matured oocytes was 21.2%. This figure is similar to that of oocytes in *in vivo* mature. The point of difference in culture conditions of both these reports to ours was that follicular fluid was added to the culture media. The follicular fluid may be useful for cytoplasmic maturation. For example, epidermal growth factor in human follicular fluid stimulated mouse oocyte maturation *in vitro* [32]. The reason for similar rates of abnormalities in MI and PI oocytes was thought to be the lack of spindle abnormalities, despite the long culture time of PI oocytes, since spindles are not yet formed in PI stage oocytes [24]. The aged and *in vitro* matured oocytes showed the same spindle abnormalities in this study. The reason for this is not clear; perhaps the aged oocytes and matured oocytes have the same immaturity of cytoplasm.

In conclusion, abnormal spindle morphology and abnormal chromosomal alignment increased in 1-day-old unfertilized oocytes after IVF and in *in vitro* matured oocytes obtained from immature denuded oocytes in ICSI procedures. More than 50% of those oocytes were determined to be unsuitable for use as gametes. If

fertilization cannot be achieved after IVF, or with oocytes retrieved and matured for ICSI, the use of these oocytes must be considered. However, abnormalities in these oocytes leads to an increase in genetic risks. Thus, we need more debate on the clinical use of these oocytes.

References

1) Boldt, J., Howe, A.M., Butler, W.J., McDonough, P.G. and Padilla, S.L. (1987): The value of oocyte reinsemination in human in vitro fertilization. Fertil. Steril., 48, 617–623.
2) Calderon, G., Veiga, A., Penella, J. and Barri, P.N. (1993): Two years of assisted fertilization by partial zona dissection in male factor infertility patients. Fertil. Steril., 60, 105–109.
3) Nagy, Z.P., Joris, H., Liu, J., Staessen, C., Devroey, P. and Van Steirteghem, A.C. (1993): Intracytoplasmic single sperm injection of 1-day-old unfertilized human oocytes. Hum. Reprod., 8, 2180–2184.
4) Tsirigotis, M., Bennet, V., Nicholson, N., Khalifa, Y., Hogewind, G., Yazdani, N. and Craft, I. (1994): Experience with subzonal insemination (SUZI) and intracytoplasmic sperm injection (ICSI) on unfertilized aged human oocytes. J. Assist. Reprod. Genet., 11, 389–394.
5) Nagy, Z.P., Staessen, C., Liu, J., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1995): Prospective, auto-controlled study on reinsemination of failed-fertilized oocytes by intracytoplasmic sperm injection. Fertil. Steril., 64, 1130–1135.
6) Kuczynski, W., Pietrewicz, P., Grygoruk, C., Redzko, S., Grochowski, D., Szamatowicz, J., Wasilewski, T., Wolczynski, S., Syrewicz, M., Domitrz, J. and Szamatowicz, M. (2000): Reinsemination of oocytes by intracytoplasmic sperm injection after the failure of conventional fertilization. Early embryonal development. Ginekol. Pol., 71, 1053–1058.
7) Lundin, K., Sjogren, A. and Hamberger, L. (1996): Reinsemination of one-day-old oocytes by use of intracytoplasmic sperm injection. Fertil. Steril., 66, 118–121.
8) Winston, N.J., Braude, P.R. and Johnson, M.H. (1993): Are failed-fertilized human oocytes useful? Hum. Reprod., 8, 503–507.
9) Veeck, L.L., Wortham, J.Jr., Witmyer, J., Sandow, B.A., Acosta, A.A., Garcia, J.E., Jones, G.S. and Jones, H.Jr. (1983): Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. Fertil. Steril., 39, 594–602.
10) Trounson, A., Wood, C. and Kausche, A. (1994): In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil. Steril., 62, 353–362.
11) Nagy, Z.P., Cecile, J., Liu, J., Loccufier, A., Devroey, P. and Van Steirteghem, A. (1996): Pregnancy and birth after intracytoplasmic sperm injection of in vitro matured germinal-vesicle stage oocytes: case report. Fertil. Steril., 65, 1047–1050.
12) Edirisinghe, W.R., Junk, S.M., Matson, P.L. and Yovich, J.L. (1997): Birth from cryopreserved embryos following in-vitro maturation of oocytes and intracytoplasmic sperm injection. Hum. Reprod., 12, 1056–1058.
13) Thibault, C., Gerard, M. and Menezo, Y. (1975): Preovulatory and ovulatory mechanisms in oocyte maturation. J. Reprod. Fertil., 45, 605–610.
14) Thibault, C. (1977): Hammond Memorial Lecture. Are follicular maturation and oocyte maturation independent processes? J. Reprod. Fertil., 51, 1–15.
15) Eichenlaub-Ritter, U. and Chandley, A.C. (1986): Alteration to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescence study. Chromosoma, 94, 337–345.
16) Plachot, M., Veiga, A. and Montagut, J. (1988): Are clinical and biological IVF parameters correlated with chromosomal disorders in early life: a multicentric study. Hum. Reprod., 3, 627–635.
17) Ma, S., Kalousek, D.K., Zouves, C., Yuen, B.H., Gomel, V. and Moon, Y.S. (1989): Chromosome analysis of human oocytes failing to fertilize in vitro. Fertil. Steril., 51, 992–997.
18) Pellestor, F., Girardet, A., Andreo, B., Arnal, F. and Humeau, C. (1994) Relationship between morphology and chromosomal constitution in human preimplantation embryo. Mol. Reprod. Dev., 39, 141–146.
19) Rundner, A.D. and Murray, A.W. (1996): The spindle assembly checkpoint. Curr. Opin. Cell. Biol., 8, 773–780.
20) Eichenlaub-Ritter, U. and Lucian, J.M. (1988): The microtubal cytoskeleton and chromosomes of unfertilized human oocytes aged in vitro. Hum. Genet., 80, 259–264.
21) Pickering, S.J., Johnson, M.H., Braude, P.R. and Houliston, E. (1988): Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. Hum. Reprod., 3, 978–989.
22) Park, S.E.P., Lee, K.H., Son, W.Y., Lee, K., Ko, J. and Cha, K. (1997): Chromosomes and spindle configurations of human oocytes matured in vitro after cryopreservation at the germinal vesicle stage. Fertil. Steril., 68, 920–926.
23) Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Konnai, K. and Sato, A. (1998): The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans. Hum. Reprod., 14, 448–453.
24) Battaglia, D.E., Klein, N.A. and Soules, M.R. (1996): Changes in centrosomal domains during meiotic maturation in the human oocyte. Mol. Hum. Reprod., 2, 845–851.
25) George, M.A., Pickering, S.J., Braude, P.R. and Johnson, M.H. (1996): The distribution of alpha-and gamma-tubulin in fresh and aged human and mouse oocytes exposed to cryoprotectant. Mol. Hum. Reprod., 2, 445–456.
26) Lundin, K., Sjögren, A. and Hamberger, L. (1996): Reinsemination of one-day old oocytes by use of intracytoplasmic sperm injection. Fertil. Steril., 66, 118–121.

27) Bussen, S., Mulfinger, L., Sutterlin, M., Schleyer, M., Kress, W. and Steck, T. (1997): Dizygotic twin pregnancy after intracytoplasmic sperm injection of 1 day old unfertilized oocytes. Hum. Reprod., 12: 2560–2562.
28) Baka, S.G., Toth, T.L., Veeck, L.L., Jones, H.W. Jr., Muasher, S.J. and Lanzendorf, S.E. (1995): Evaluation of the spindle apparatus of in-vitro matured human oocytes following cryopreservation. Hum. Reprod., 7, 1816–1820.
29) Schroeder, A.C., Downs, S.M. and Eppig, J.J. (1988): Factors affecting the developmental capacity of mouse oocytes undergoing maturation in vitro. Ann. NY. Acad. Sci., 541, 197–204.
30) Prins, G.S., Wagner, C., Weidel, L. Gianfortoni, J., Marut, E.L. and Scommegna, A. (1987): Gonadotropins augment maturation and fertilization of human immature oocytes cultured in vitro. Fertil. Steril., 47, 1035–1037.
31) Boiso, I., Marquez, C., Veiga, A. and Munne, S. (1997): Cytogenetic and FISH analysis of in vitro matured human oocytes. Assisted. Reprod. Rev., 7, 160–164.
32) Das, K., Phipps, W.R., Hensleigh, H.C. and Tagatz, G.E. (1992): Epidermal growth factor in human follicular fluid stimulates mouse oocyte maturation in vitro. 57, 895–901.