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Timing of first cleavage of the human embryo predicts a successful pregnancy by assisted reproductive technologies: a retrospective study

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Abstract: The absence of reliable markers to identify viable embryos for transfer at the early cleavage stage may contribute to the generally low implantation rates and high incidence of multiple births associated with IVF treatment. In the present study, we examined the relationships among the timing of the first cleavage, incidence of blastocyst formation *in vitro,* and pregnancy outcomes. All embryos (n=1,748) were examined for early cleavage at 25 h after insemination. Three groups were defined based on the persistence of two pronuclei (PN group), pronuclei breakdown (BD group), and early cleaved zygotes (EC group). Each embryo (n=1,120) was cultured until day 6 of development, and assessed for blastocyst formation. The rate of blastocysts showing good morphology was significantly higher in the EC group (44.7%) than in the BD (26.9%) or PN (13.9%) groups. The cryo-thawed good morphology blastocysts were transferred in 209 cycles. The clinical pregnancy rate was the highest and the abortion rate was the lowest in the EC group. These results indicate that early cleavage is indicative of increased developmental potential in human embryos and may be useful as an additional criterion when selecting embryos for transfer.

Key words: ART, ICSI, Embryo quality, First mitotic division, Human embryo

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Introduction

The number of infertile couples who have been treated with assisted reproductive technology, such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), has steadily increased in the past two decades. Using these techniques in combination with the vitrification method of blastocyst embryos, the successful transfer of embryos has reached more than 50% [1, 2]. However, many infertility clinics have reported a high incidence of spontaneous abortion in the range of 18–30% in assisted reproductive technology pregnancies [3–7]. Furthermore, the incidence of miscarriage has been shown to be markedly higher in assisted reproductive technology pregnancies than in normal pregnancies [7, 8].

Cytogenetic evaluations have revealed that more than 50% of sporadic spontaneous abortions are caused by embryonic aneuploidy [7, 9]. This chromosomal abnormality is frequently observed in oocytes recovered at advanced maternal ages. Oxidative stress in semen or the epididymis also induces DNA damage [10]. When damaged sperm penetrate oocyte or when sperm penetrates an oocyte with abnormal chromosome separation, the risk of spontaneous abortion is increased once the embryo is transferred. Additionally, abnormal chromosome separation frequently occurs during the first mitotic division in *in vitro* fertilized eggs, including those from ICSI. Yamagata *et al.* [11] used a live-cell imaging technique to demonstrate that abnormal chromosome segregation during the first mitotic division occurred in 26.3% mouse eggs when fresh sperm was used for ICSI. Although abnormal eggs developed blastocyst embryos with a nor-

mal morphology, their transfer resulted in a high rate of spontaneous abortion [12].

The selection of normal chromosome-separated embryos using a non-invasive technique is desired in infertility care programs to reduce spontaneous abortion following embryo transfer. The timing of the first cleavage of eggs has recently been proven useful in the selection of embryos with the highest implantation potential. Several studies have shown that the appearance of a two-cell embryo between 25 and 27 h after insemination (defined as early-cleaved embryos) yields better quality embryos with higher implantation potential than late-cleaved eggs [13–18]. Based on these findings, we hypothesized that the late or early timing of the first mitotic division might be associated with abnormal chromosome separation, which might not affect developmental competence to the blastocyst stage, but might markedly increase the risk of spontaneous abortion following embryo transfer. The relationship between the timing of the first mitotic division and the rate of successful pregnancies was investigated in previous studies using Day 2 or Day 3 fresh embryos [13–18]. Thus, it is difficult to establish whether a delay of the first mitotic division prevented the development to the blastocyst stage, decreased implantation ability, or increased the risk of spontaneous abortion. In the present study, we monitored the timing of the appearance of two-cell embryos and embryo development using a one embryo culture system. All the developed blastocyst embryos were frozen and subsequently used for embryo transfer in control cycles, and the rate of spontaneous abortion defined as miscarriage after an intrauterine pregnancy was retrospectively analyzed.

Materials and Methods

Patients

We retrospectively examined 871 ICSI cycles at the Women's Clinic Oizumi-Gakuen between November 2007 and December 2012. This study was approved by the Ethics Committee of the Women's Clinic Ooizumi Gakuen, and was performed with patient consent. All the subjects were younger than 42 years old (range: 25–42). Indications for ICSI-embryo transfer were a tubal factor (30.1%), male factor (21.3%), endometriosis (6.0%), or unexplained infertility (45.8%). The inclusion criteria were as follows: indications of ICSI for male infertility with use of fresh ejaculated spermatozoa with at least 6 million spermatozoa in the ejaculate and less than 4.0% in the Kruger test. We also performed ICSI for patients for whom fertilized eggs were not retrieved by conventional methods in a previous IVF cycle.

Stimulation and oocyte retrieval

Four stimulation protocols were used in this study: the gonadotropin-releasing hormone (GnRH) agonist protocol, the GnRH antagonist protocol, and the minimal stimulation protocol with only clomiphene citrate or a combination of clomiphene citrate and gonadotropins.

In the GnRH agonist protocol, 900 μg of a buserelin acetate nasal spray (Suprecur®; Hoescht, Frankfurt, Germany) was administered daily, commencing on day 21 of the previous menstrual cycle or on day 2 of the menstrual cycle. In the antagonist protocol, 0.25 mg s.c. daily of a GnRH antagonist (Cetrotide®; Shionogi Pharmaceutical Co., LTD., Tokyo, Japan) was started when the dominant follicle reached 15–16 mm in diameter. Ovarian stimulation was achieved in every group with 150–300 IU recombinant FSH (Follisutim®, MSD, Whitehouse Station, NJ, USA), highly purified FSH or hMG (Follirumon P®, hMG Fuji®; Fuji Pharmaceutical Co., LTD., Tokyo, Japan), daily from the third day of the menstrual cycle. Doses were adjusted according to the ovarian response, as judged by serum estradiol concentrations and ultrasound scans every 2–3 days. Human chorionic gonadotropin (HCG Mochida®, Mochida Pharmaceutical Co., LTD., Tokyo, Japan), 5,000–10,000 IU i.m., was administered to trigger ovulation when patients had two or more follicles with a mean diameter of 18 mm or greater. Oocyte retrieval was performed 36 h after ovulation had been triggered.

In the minimal stimulation protocol, patients received a daily dose of 50 mg of clomiphene citrate (Serophene®, Merck Serono, Geneva, Switzerland) from day 3. If more than three follicles were found to have grown on ultrasound scans on day 6, 100 IU recombinant FSH was also administered every other day. A total of 300 μg of a nasal GnRH analogue was administered to trigger ovulation when the dominant follicles had a mean diameter of 18 mm or greater. Oocyte retrieval was performed 36 h after ovulation had been triggered.

Fertilization and embryo culture

Oocytes were denuded to inspect the extrusion of the first polar body, and all metaphase-II (MII) oocytes were inseminated with sperm using ICSI, as described elsewhere [19]. Fertilization was determined by the presence of the two pronuclei (2PN) and polar bodies 19–21 h after ICSI. After fertilization, zygotes were cultured in microdrops of 25 μl Cleavage Medium (Cook Medical, Brisbane, Australia) under oil at 37 °C in 5% CO_2 until day 3. All zygotes that were normally fertilized (2PN) were observed again in the afternoon of day 1 for early embryo cleavage 25 h after ICSI. Three groups were defined based on persistence of 2PN (PN group), 2PN break-

Table 1. Comparison of patient backgrounds according to the developmental progress of embryos among the EC, BD, and PN groups

	EC	BD	PN	*P*-value
No. of embryos	606	495	647	
Incidence rate (%)	34.7	28.3	37.0	
Female age (y)	35.8 ± 3.4	35.8 ± 3.4	36.0 ± 3.4	N.S.
IVF attempts	3.1 ± 2.1	2.4 ± 2.1	2.6 ± 2.2	N.S.

Three groups were defined based on the observation at 25 h after ICSI: persistence of two pronuclei (PN group), two pronuclei breakdown (BD group), and early cleaved (EC group) zygotes. Data represent the mean ± standard deviation or error. N.S., Not statistically significant.

Table 2. Comparison of the incidences of early cleavage among the four ovarian stimulation protocols

	Agonist (n=604)	Antagonist (n=717)	Clomiphene (n=123)	Clomiphene + FSH (n=304)	Total (n=1,748)
Early cleavage (%)	197 (32.5)	222 (31.0)[a]	48 (39.0)	139 (45.7)[a]	606 (34.7)
Pronucleus breakdown (%)	181 (36.6)	200 (27.9)	29 (23.6)	85 (28.0)	495 (28.3)
Persistent pronucleus (%)	226 (34.9)	295 (41.1)[b]	46 (37.4)	80 (26.3)[b]	647 (37.0)

Early cleavage: cleavage was observed 25 h after ICSI. Pronucleus breakdown: pronucleus disappeared 25 h after ICSI. Persistence of two pronucleus: pronucleus persisted 25 h after ICSI. N.S.; Not statistically significant. Same letters indicate significant differences. $P<0.05$.

down (BD group), and early cleaved zygotes (EC group).

Blastocysts with an excellent morphology after 6 days culture, were frozen. Blastocysts were graded on Day 5 and 6 of culture according to Gardner and Schoolcraft's system [20]. On Day 5 and 6, an embryo of grade ≥3BB was considered an excellent morphology embryo [21]. Clinical pregnancy was assessed by three successive, positive plasma *β*-hCG concentrations and the ultrasound detection of a fetal heartbeat after 6 weeks of gestation. Miscarriage was defined as a spontaneous abortion after an intrauterine pregnancy had been detected by ultrasound. Patients with miscarriages were offered dilation and curettage with karyotype analysis. The relationships between the early occurrence of the first mitotic division and the fertilization rate, early embryo development, pregnancy rate, abortion rate, and delivery rate were determined retrospectively.

Statistical analysis

Data were compared using the χ^2- and Student's *t*-tests. The two-way ANOVA method was used for multiple groups. A *P* value of <0.05 was considered significant.

Results

Relationship between the timing of the first cleavage in the embryo and developmental ability to the blastocyst stage

The clinical records of the patients and distribution of embryos according to the developmental progress of the embryos are collectively summarized in Table 1. A total of 1,748 zygotes were classified as PN, BD, and EC in observation 25 h after ICSI. The data show that 34.7% of the zygotes belonged to the EC group, 28.3% of the zygotes in which the pronucleus disappeared, but did not divide belonged to the BD group, and 37.0% of the zygotes in which the pronucleus was still observed belonged to the PN group. The timing of the first mitotic division was not significantly affected by the age of the subjects or the number of IVF attempts. However, the early cleavage rate of the antagonist protocol group (31.0%) was significantly lower than that of the clomiphene + FSH protocol group (45.7%) (Table 2).

The categorized embryos (387 early cleavage embryos, 338 2PN breakdown embryos, and 395 two pronuclei embryos) were cultured until day 6. Two way ANOVA showed that the blastocyst formation and the quality of embryo were affected by the timing of the first division, but were not affected by the ovarian stimulation protocol (Table 3). The rate of blastocyst formation was signifi-

Table 3. ANOVA for influence of between the timing of the first cleavage and the ovalian stimulation protocol

	Source	df	*P*-value
Blastocyst formation	Timing of the first cleavage	2	0.0004
	Ovalian stimulation protocol	3	0.4507
Excellent morphology blastocyst	Timing of the first cleavage	2	0.0018
	Ovalian stimulation protocol	3	0.4207
Clinical pregnancy	Timing of the first cleavage	2	0.0043
	Ovalian stimulation protocol	3	0.6860
Abortrion	Timing of the first cleavage	2	0.0055
	Ovalian stimulation protocol	3	0.5349

df; degree of freedom.

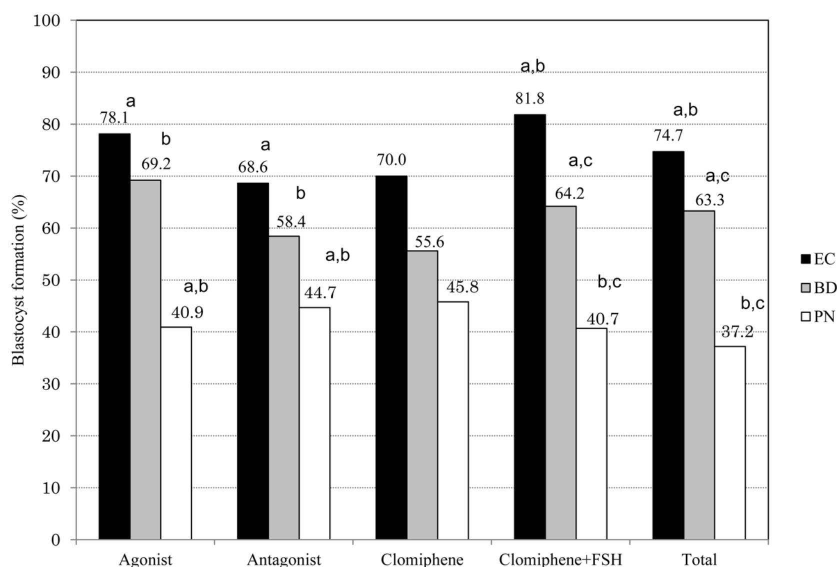

Fig. 1. Comparison of blastocyst formation rates according to the timing of the first division and ovarian stimulation.
PN, persistence of two pronuclei (PN group); BD, two pronuclei breakdown (BD group); EC, early cleaved (EC group) zygotes at 25 h after ICSI.
Same letters indicate significant differences ($P<0.05$).

cantly higher in the EC group of each ovarian stimulation protocol except for clomiphenes (Fig. 1). The same effect of early cleavage was observed on the rate of excellent morphology blastocyst (Fig. 2).

The late onset of the first cleavage of the embryo predicted a low pregnancy rate and increased abortion risk

To determine whether the early onset of the first cleavage of the embryo could be used to predict pregnancy, cryo-thawed blastocyst embryos were transferred in 209 cycles: 109 cycles were from the EC group, 57 cycles from the BD group, and 43 cycles from the PN group (Table 4). A single blastocyst embryo of good morphology was transferred in each cycle. The clinical pregnancy rate was the highest in the EC group. The clinical pregnancy rate and spontaneous abortion rate were significantly affected by the timing of first embryo cleavage, but not by the ovarian stimulation protocols (Table 3). A significantly higher rate of pregnancy was observed in the EC group than in the PN group (Table 4). The rate of spontaneous abortion of the PN group was significantly higher than those of the EC and BD groups. Villous chromosome analysis was performed on eight miscarried embryos from the PN group. The karyotypes of all embryos showed aneuploidy, as shown in Table 5.

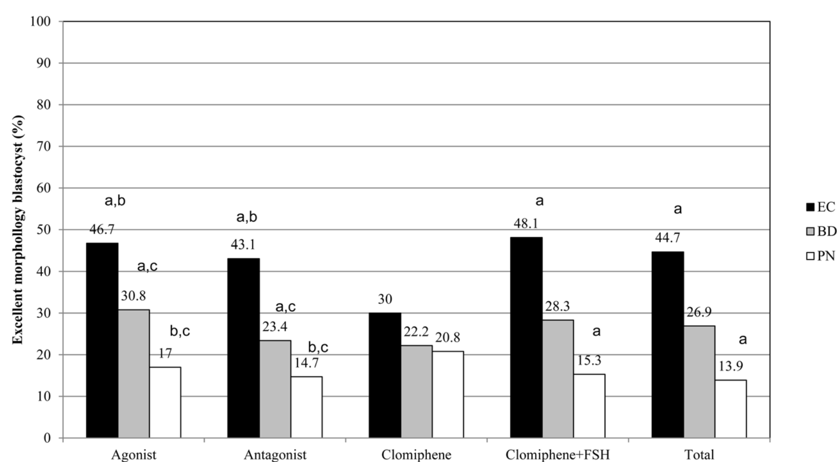

Fig. 2. Comparison of excellent morphology blastocyst formation rates according to the timing of the first division and ovarian stimulation.
PN, persistence of two pronuclei (PN group); BD, two pronuclei breakdown (BD group); EC, early cleaved (EC group) zygotes at 25 h after ICSI.
Same letters indicate significant differences (P<0.05).

Table 4. Results of cryo-thawed blastocyst transfers for EC, BD, and PN groups

		EC	BD	PN	P-value
No. of cycles		109	57	43	
Female age (y)		35.3 ± 3.3	35.6 ± 3.5	35.5 ± 4.2	N.S.
IVF attempts		3.1 ± 2.0	3.0 ± 2.0	3.4 ± 2.7	N.S.
Ovalian stimulation protocol					
Agonist	Clinical pregnancy (%)	21/32 (65.6)	13/21 (61.9)	6/15 (40.0)	N.S.
	Abortion (%)	0/21 (0)^{a,b}	4/13 (30.8)^{a}	4/6 (66.7)^{b}	$^{a,b}P < 0.01$
Antagonist	Clinical pregnancy (%)	24/37 (64.9)^{c}	10/21 (47.6)	4/14 (28.6)^{c}	$^{c}P < 0.05$
	Abortion (%)	1/24 (4.2)^{d}	0/10 (0)^{e}	3/4 (75.0)^{d,e}	$^{d,e}P < 0.01$
Clomiphene	Clinical pregnancy (%)	5/8 (62.5)	2/3 (66.7)	0/3 (0)	N.S.
	Abortion (%)	0/5 (0)	0/2 (0)	0/0 (-)	
Clomiphene+FSH	Clinical pregnancy (%)	22/32 (68.8)^{f}	8/12 (66.7)^{g}	1/11 (9.1)^{f,g}	$^{f}P < 0.01$, $^{g}P < 0.05$
	Abortion (%)	2/22 (9.1)^{h}	1/8 (12.5)^{i}	1/1 (100)^{h,i}	$^{h}P < 0.05$, $^{i}P < 0.01$
Total	Clinical pregnancy (%)	68/109 (62.3)^{j}	33/57 (57.9)^{k}	11/43 (25.6)^{j,k}	$^{j,k}P < 0.01$
	Abortion (%)	3/68 (4.4)^{l}	5/33 (15.2)^{m}	8/11 (72.7)^{l,m}	$^{l}P < 0.01$, $^{m}P < 0.05$

Three groups were defined: persistence of two pronucleus (PN group), two pronucleus breakdown (BD group), and early cleaved (EC group) zygotes at the observation 25 h after ICSI. Data represent the mean ± standard deviation or error. N.S.; Not statistically significant. Same letters indicate significant differences.

Discussion

The selection of embryos with a high developmental potential is essential for improving the success of assisted reproductive techniques. Embryo scoring systems based on embryo morphology have been developed and used to select embryos for transfer [22, 23]. However, morphological features are not a suitable quantitative method, because deciding on a strict quality rating is difficult [24, 25]. An embryo respiration measuring system has recently been developed to examine embryo quality using Scanning Electrochemical Microscopy (SECM), which assays oxygen consumption in the embryo [26, 27]. The embryos are selected based on their respiration activities and are predicted to have high implantation rates [27]; however, the instruments and skills needed to analyze respiration activities are required by each laboratory. Monitoring the early onset of the first cleavage of

Table 5. Villous chromosomal abnormality in miscarried embryos derived from PN zygote

Patient no.	age (y)	No. of IVF attempts	karyotype
1	30	3	47,XY,+22
2	35	5	47,XY,+22
3	35	4	47,XX,+8
4	36	3	47,XX,+22
5	36	2	47,XY,+22
6	38	2	47,XX,+16
7	39	2	47,XY,+22
8	39	1	47,XX,+15

the zygote has proven useful for the selection of embryos without any specialized equipment [13, 15].

The transfer of embryos from early cleaved zygotes at first mitosis has been associated with higher pregnancy and implantation rates than those of late cleaved embryos [13–18]. Moreover, a positive correlation has been reported between early cleaved zygotes, excellent quality four-cell embryos, and blastocyst development [16, 17]. Consistent with these findings, we demonstrated a significant increase in blastocyst development when embryos were derived from early cleaved zygotes than from breakdown or pronucleus zygotes.

The reason for the low developmental competence in breakdown and pronucleus embryos has not yet been elucidated in detail in human ICSI embryos. However, one possibility is that a defective fertilization process causes not only the late formation of pronuclei and delayed onset of the first mitotic division, but also results in low developmental ability to the blastocyst stage. Oocyte activation, the resumption of meiosis from metaphase II to anaphase II after the penetration of sperm, was shown to be induced by Ca^{2+} oscillation [28, 29]. The transient and repetitive induction of Ca^{2+} from the endoplasmic reticulum (ER) to the cytoplasm is known to degrade cyclin B to reduce cyclin-dependent kinase 1 (CDK1) activity and also Mos to decrease extracellular signal-regulated kinase 1/2 (ERK1/2) activity [29–31]. Because both these factors are required for the maintenance of the metaphase II stage, these reductions are initiators that emit the second polar body and form the male and female pronuclei [31]. The Ca^{2+} oscillation also selectively breaks down maternal mRNA, and starts zygotic gene transcription (zygotic activation) [32]. Marked changes are required for the alternation from oocyte to embryo specific functions, including cell division and metabolism [32]. The Ca^{2+} oscillation is induced by sperm-secreted phospholipase C zeta (PLCζ), which produces inositol 1,4,5-triphosphate (IP_3) which in turn releases Ca^{2+} from the ER via IP_3 receptors [29, 33]. The expression level of PLCζ is known to differ among sperm (30), and the IP_3 receptor is modified (phosphorylated) during oocyte maturation [34]. Thus, low activity of PLCζ or IP_3 receptors in sperm-injected oocytes may induce an insufficient Ca^{2+} oscillation, resulting in not only the delayed formation of pronuclei and first mitotic cleavage, but also impairment of the ability of fertilized eggs to develop to the blastocyst stage.

Magli *et al.* reported that the rate of chromosomal abnormalities was significantly higher in arrested or slow-cleaving embryos than in embryos with eight cells 62 h after insemination [35]. Abnormal chromosome segregation during the first mitotic division was observed in 26.3% of ICSI eggs in mouse oocytes. Yamagata *et al.* reported that even abnormal eggs could develop into blastocyst embryos with a normal morphology [11], which suggests that it is difficult to determine whether abnormal chromosome separation leads to a slow speed cleavage or the delayed timing of mitotic division induces abnormal chromosome separation. However, Yamagata *et al.* also revealed that the embryo transfer of blastocysts with abnormal chromosome copies led to a high rate of spontaneous abortion in the mouse [11]. In previous studies, 40–60% of blastocyst embryos were shown to be aneuploid [12, 36].

Cytogenetic evaluations of sporadic spontaneous abortions revealed that 50–70% of blastocyst embryos were chromosomally abnormal [37, 38]. Moreover, villous chromosome analysis was performed on eight miscarried embryos derived from PN zygotes in the present study, and all of them had chromosomal abnormalities. Since the delayed onset of the first mitotic cleavage, which might have been induced by the low activity of sperm and/or oocyte, leads to abnormal chromosome separation, monitoring the time of the first mitotic division should be useful for selecting excellent quality embryos for embryo transfer.

References

1) Gardner, D.K., Lane, M., Stevens, J., Schlenker, T. and Schoolcraft, WB. (2000): Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil. Steril., 73, 1155–1158. [Medline] [CrossRef]
2) Zhu, D., Zhang, J., Cao, S., Zhang, J., Heng, B.C., Huang, M., Ling, X., Duan, T. and Tong, G.Q. (2011): Vitrified-warmed blastocyst transfer cycles yield higher pregnancy and implantation rates compared with fresh blastocyst transfer cycles—time for a new embryo transfer strategy? Fertil. Steril., 95, 1691–1695. [Medline] [CrossRef]
3) Wennerholm, U.B., Hamberger, L., Nilsson, L., Wenner-

gren, M., Wikland, M. and Bergh, C. (1997): Obstetric and perinatal outcome of children conceived from cryopreserved embryos. Hum. Reprod., 12, 1819–1825. [Medline] [CrossRef]
4) Pantos, K., Athanasiou, V., Stefanidis, K., Stavrou, D., Vaxevanoglou, T. and Chronopoulou, M. (1999): Influence of advanced age on the blastocyst development rate and pregnancy rate in assisted reproductive technology. Fertil. Steril., 71, 1144–1146. [Medline] [CrossRef]
5) Wang, J.X., Norman, R.J. and Wilcox, A.J. (2004): Incidence of spontaneous abortion among pregnancies produced by assisted reproductive technology. Hum. Reprod., 19, 272–277. [Medline] [CrossRef]
6) Practice Committee of the American Society for Reproductive Medicine (2006): Aging and infertility in women. Fertil. Steril., 86 (Suppl 1), S248–S252. [Medline] [CrossRef]
7) Martínez, M.C., Méndez, C., Ferro, J., Nicolás, M., Serra, V. and Landeras, J. (2010): Cytogenetic analysis of early nonviable pregnancies after assisted reproduction treatment. Fertil. Steril., 93, 289–292. [Medline] [CrossRef]
8) Nybo Andersen, A.M., Wohlfahrt, J., Christens, P., Olsen, J. and Melbye, M. (2000): Maternal age and fetal loss: population based register linkage study. BMJ, 320, 1708–1712. [Medline] [CrossRef]
9) Kuliev, A., Cieslak, J., Ilkevitch, Y. and Verlinsky, Y. (2003): Chromosomal abnormalities in a series of 6,733 human oocytes in preimplantation diagnosis for age-related aneuploidies. Reprod. Biomed. Online, 6, 54–59. [Medline] [CrossRef]
10) Zini, A., Boman, J.M., Belzile, E. and Ciampi, A. (2008): Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum. Reprod., 23, 2663–2668. [Medline] [CrossRef]
11) Yamagata, K., Suetsugu, R. and Wakayama, T. (2009): Assessment of chromosomal integrity using a novel live-cell imaging technique in mouse embryos produced by intracytoplasmic sperm injection. Hum. Reprod., 24, 2490–2499. [Medline] [CrossRef]
12) Alfarawati, S., Fragouli, E., Colls, P., Stevens, J., Gutiérrez-Mateo, C., Schoolcraft, W.B., Katz-Jaffe, M.G. and Wells, D. (2011): The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertil. Steril., 95, 520–524. [Medline] [CrossRef]
13) Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P.G. and Campana, A. (1998): Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum. Reprod., 13, 182–187. [Medline] [CrossRef]
14) Bos-Mikich, A., Mattos, A.L. and Ferrari, A.N. (2001): Early cleavage of human embryos: an effective method for predicting successful IVF/ICSI outcome. Hum. Reprod., 16, 2658–2661. [Medline] [CrossRef]
15) Lundin, K., Bergh, C. and Hardarson, T. (2001): Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum. Reprod., 16, 2652–2657. [Medline] [CrossRef]
16) Fenwick, J., Platteau, P., Murdoch, A.P. and Herbert, M. (2002): Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. Hum. Reprod., 17, 407–412. [Medline] [CrossRef]
17) Neuber, E., Rinaudo, P., Trimarchi, J.R. and Sakkas, D. (2003): Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. Hum. Reprod., 18, 1307–1312. [Medline] [CrossRef]
18) Hesters, L., Prisant, N., Fanchin, R., Méndez Lozano, D.H., Feyereisen, E., Frydman, R., Tachdjian, G. and Frydman, N. (2008): Impact of early cleaved zygote morphology on embryo development and in vitro fertilization-embryo transfer outcome: a prospective study. Fertil. Steril., 89, 1677–1684. [Medline] [CrossRef]
19) Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993): High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod., 8, 1061–1066. [Medline]
20) Gardner, D.K. and Schoolcraft, W.B. (1999): In-vitro culture of human blastocyst. In: Towards reproductive certainty: fertilization and genetics beyond 1999. (Jansen, R. and Mortimer, D.,eds.), pp.378–388, Parthenon Press, London.
21) Ahlström, A., Westin, C., Wikland, M. and Hardarson, T. (2013): Prediction of live birth in frozen-thawed single blastocyst transfer cycles by pre-freeze and post-thaw morphology. Hum. Reprod., 28, 1199–1209. [Medline] [CrossRef]
22) Erenus, M., Zouves, C., Rajamahendran, P., Leung, S., Fluker, M. and Gomel, V. (1991): The effect of embryo quality on subsequent pregnancy rates after in vitro fertilization. Fertil. Steril., 56, 707–710. [Medline]
23) Steer, C.V., Mills, C.L., Tan, S.L., Campbell, S. and Edwards, R.G. (1992): The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum. Reprod., 7, 117–119. [Medline]
24) Scott, L., Alvero, R., Leondires, M. and Miller, B. (2000): The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum. Reprod., 15, 2394–2403. [Medline] [CrossRef]
25) Forman, E.J., Upham, K.M., Cheng, M., Zhao, T., Hong, K.H., Treff, N.R. and Scott, R.T Jr. (2013): Comprehensive chromosome screening alters traditional morphology-based embryo selection: a prospective study of 100 consecutive cycles of planned fresh euploid blastocyst transfer. Fertil. Steril., 100, 718–724. [Medline] [CrossRef]
26) Shiku, H., Shiraishi, T., Ohya, H., Matsue, T., Abe, H., Hoshi, H. and Kobayashi, M. (2001): Oxygen consumption of single bovine embryos probed by scanning electrochemical microscopy. Anal. Chem., 73, 3751–3758. [Medline] [CrossRef]
27) Utsunomiya, T., Goto, K., Nasu, M., Kumasako, Y., Araki, Y., Yokoo, M. and Abe, H. (2008): Evaluating the quality of human embryos with a measurement of oxygen consumption by scanning electrochemical microscopy. J. Mamm. Ova Res., 25, 2–7. [CrossRef]
28) Küpker, W., Diedrich, K. and Edwards, R.G. (1998): Prin-

ciples of mammalian fertilization. Hum. Reprod., 13 (Suppl 1), 20–32. [Medline] [CrossRef]

29) Miyazaki, S. and Ito, M. (2006): Calcium signals for egg activation in mammals. J. Pharmacol. Sci., 100, 545–552. [Medline] [CrossRef]

30) Ducibella, T., Schultz, R.M. and Ozil, J.P. (2006): Role of calcium signals in early development. Semin. Cell Dev. Biol., 17, 324–332. [Medline] [CrossRef]

31) Miao, Y.L., Stein, P., Jefferson, W.N., Padilla-Banks, E. and Williams, C.J. (2012): Calcium influx-mediated signaling is required for complete mouse egg activation. Proc. Natl. Acad. Sci. USA, 109, 4169–4174. [Medline] [CrossRef]

32) Tadros, W. and Lipshitz, H.D. (2009): The maternal-to-zygotic transition: a play in two acts. Development, 136, 3033–3042. [Medline] [CrossRef]

33) Kashir, J., Jones, C., Mounce, G., Ramadan, W.M., Lemmon, B., Heindryckx, B., de Sutter, P., Parrington, J., Turner, K., Child, T., McVeigh, E. and Coward, K. (2013): Variance in total levels of phospholipase C zeta (PLC-ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. Fertil. Steril., 99, 107–117. [Medline] [CrossRef]

34) Vanderheyden, V., Wakai, T., Bultynck, G., De Smedt, H., Parys, JB. and Fissore, RA. (2009): Regulation of inositol 1,4,5-trisphosphate receptor type 1 function during oocyte maturation by MPM-2 phosphorylation. Cell Calcium, 46, 56–64. [Medline] [CrossRef]

35) Magli, M.C., Gianaroli, L., Ferraretti, A.P., Lappi, M., Ruberti, A. and Farfalli, V. (2007): Embryo morphology and development are dependent on the chromosomal complement. Fertil. Steril., 87, 534–541. [Medline] [CrossRef]

36) Sandalinas, M., Sadowy, S., Alikani, M., Calderon, G., Cohen, J. and Munné, S. (2001): Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. Hum. Reprod., 16, 1954–1958. [Medline] [CrossRef]

37) Ogasawara, M., Aoki, K., Okada, S. and Suzumori, K. (2000): Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil. Steril., 73, 300–304. [Medline] [CrossRef]

38) Carp, H., Toder, V., Aviram, A., Daniely, M., Mashiach, S. and Barkai, G. (2001): Karyotype of the abortus in recurrent miscarriage. Fertil. Steril., 75, 678–682. [Medline] [CrossRef]