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Effect of A Time-lapse Incubator (EmbryoScope®) on *in vitro* Culture of Human Embryos

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Abstract: A time-lapse embryo image monitoring system consists of an incubator with a built-in microscope and CCD camera. Embryo assessment can be performed with this type of system without removing embryos from the incubator, which eliminates the risks of stress due to temperature changes, light exposure, high oxygen exposure, and pH changes in the culture medium. We cultured embryos to the blastocyst stage using this system and compared the results with those of a normal incubator. We compared the rates of fertilization and cryopreservation of embryos cultured using EmbryoScope® and a conventional incubator. We then compared the pregnancy rates of EmbryoScope® and the conventional incubator. We finally examined the relationship between the early cleavage of embryos and blastocyst formation rate. The fertilization rates (EmbryoScope®: 74.1%, 298/402; conventional incubator: 77.4%, 202/261) and cryopreservation rates (EmbryoScope®: 57.4%, 163/284; conventional incubator: 48.7%, 91/187) of embryos were not affected by the incubator. No significant difference was observed in clinical pregnancy rates between the groups: (EmbryoScope®: 24.5%, 24/98; conventional incubator: 15.0%, 15/100). The first division time of embryos was significantly faster in the "2-cell" groups (Groups A and B) ($P < 0.01$) than in the "none 2-cell" group (Group C). The blastocyst formation rates of embryos in Groups A and B were significantly ($P < 0.05$) higher than those in Group C. These results indicated that EmbryoScope® enables a detailed and dynamic analysis of the development of human embryos.

Key words: Time-lapse, Human embryo, EmbryoScope®

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Introduction

The use of Assisted Reproductive Technology (ART) has resulted in pregnancies for many infertile patients; however, there are a number of problems with this procedure, including multiple pregnancies. Multiple pregnancies have been associated with an increased risk of perinatal morbidity and subsequent long-term health issues [1–5]. These risks have mainly been attributed to the high incidence of multiple pregnancies, which is directly related to the number of embryos transferred. Therefore, avoiding multiple pregnancies without decreasing the pregnancy rate is of importance, and the transfer of a single embryo can resolve this issue. The treatment outcome of transferring cryopreserved embryos has improved and single embryo transfer has become a popular procedure, leading to a decrease in multiple pregnancies. Therefore, cryopreserved embryo transfer has become an important technique in ART, and the number of treatment cycles has been increasing.

The development of ART has enabled the direct observation of human embryos. It is necessary to observe embryonic growth under a microscope during *in-vitro* fertilization; however, observing embryos for a long time or frequently should be avoided because of embryonic stress. Therefore, developing a new method to observe embryos under suitable culture conditions and increasing the success rate of ART is desired. A time-lapse video recording system was developed as a solution to this problem. This system allows us to non-invasively observe embryo growth and development over a long period of time. Payne et al. [6] were the first to report the development of a time-lapse video recording system mounted on the stage of an inverted microscope. Furthermore, Mio et al. developed a new system for time-lapse cinematography based on the original report by Payne et al. [7]. Using this system, Mio et al. reported

various fertilization processes [7–10].

A time-lapse incubator, EmbryoScope® (Unisense Fertilitech) has attracted widespread interest in recent years [11–13]. This time-lapse embryo image monitoring system is an incubator with a built-in microscope and CCD camera. Embryo assessment can be performed with this type of system without removing embryos from the incubator, which eliminates the risks of stress due to temperature changes, light exposure, high oxygen exposure, and pH changes in the culture medium. The CCD camera captures images at 20-min intervals during embryo culture. The present study was conducted to evaluate the operational efficiency of EmbryoScope®. More specifically, we cultured embryos to the blastocyst stage using this system and compared the results obtained with those of a normal incubator. This study revealed the results of embryo quality and morphological analysis of human embryonic development using EmbryoScope®.

Materials and Methods

Ovarian stimulation was performed using the gonadotrophin-releasing hormone antagonist protocol or GnRH agonist short protocol, in association with follicle stimulating hormone (FSH) and human menopausal gonadotrophin (hMG). Human chorionic gonadotrophin (hCG) or leuprolide was administered when the maximum diameter of two or more follicles reached 18 mm. Cumulus-oocyte complexes were retrieved by ultrasound-guided transvaginal follicle aspiration approximately 36 h after the hCG injection. Oocytes were denuded by pipetting in a hyaluronidase solution (Sigma, USA). Randomly-selected oocytes were prepared for EmbryoScope® and the conventional incubator after ICSI or *in vitro* fertilization (IVF). The culture conditions used for EmbryoScope® and the conventional incubator were 37°C, 6% CO_2, and 5% O_2. All the embryos were cultured in Global® (Life Global, USA) under mineral oil. In all experiments, no significant differences were noted in the age of patients or the number of treatment cycles. The Mann Whitney U-test was used to compare cell division times (Table 3), and X^2 analysis with continuity correction compared fertilization, cryopreservation, and pregnancy and blastocyst formation rates (Table 1, 2 and 4). We retrospectively analyzed the effects of EmbryoScope®. The methods used in this experiment are discussed below.

Experiment I

We cultured embryos to the blastocyst stage using EmbryoScope® and compared the rate of cryopreservation in the same period with that achieved by the conventional incubator. We examined the outcome of 35 retrieval cycles involving 35 women from which ≥10 eggs were retrieved between January and November, 2012. The mean age ± S.D. of patients at the retrieval cycles was 34.0 ± 3.4 years. Conventional IVF (8 retrieval cycles) embryos were cultured in EmbryoScope® or the conventional incubator 5 h after insemination. ICSI (27 retrieval cycles) embryos were cultured in EmbryoScope® or the conventional incubator immediately after ICSI. Embryos were randomized for cultivation in the conventional incubator or EmbryoScope®. All embryos were cultured for a maximum of six days to reach the blastocyst stage.

Experiment II

We compared the pregnancy rates of EmbryoScope® and the conventional incubator. We then examined the outcome of 198 retrieval cycles involving 196 women under 40 years of age from whom ≥2 eggs were retrieved between January and November, 2012. The mean age ± S.D. of patients at the retrieval cycles was 34.9 ± 3.5 years old. Oocyte retrieval and fertilization were performed by conventional IVF (45 retrieval cycles) or ICSI (153 retrieval cycles) using classical techniques. Embryos were randomized to culture in the conventional incubator or EmbryoScope®. Single embryo transfer was performed two (91 retrieval cycles), three (98 retrieval cycles), or five (9 retrieval cycles) days after oocyte retrieval. Embryo quality was assessed using Veeck's classification for cleavage stage (two or three days) embryos [14], and Gardner's classification for blastocyst stage (five days) embryos [15]. Two-day embryos had 2–4 cells, <10% fragmentation, three-day embryos had 8–12 cells, <10% fragmentation, and five-day blastocysts were defined as those with an inner cell mass and trophectoderm above 'BB' according to Gardner's criteria. Transferred embryos cultured by EmbryoScope® were selected according to growth speed and morphological evaluation. Embryos that divided quickly were chosen according to the study by Mio [9]. On the other hand, only morphological evaluations under an inverted microscope were performed for embryos cultured in the conventional incubator.

Experiment III

We examined the relationship between the early division of embryos and blastocyst formation rates to test our hypothesis that this may be used as a simple and effective selection criterion for selecting embryos. Evaluating embryo viability based on the timing of the first and second cell divisions is relatively easy. Therefore, we examined the outcomes of all vitrified blastocysts recovered from 13 single-cycle oocyte retrievals involving 13 women from

Table 1. Development of embryos Cultured in EmbryoScope® and a conventional Incubator

Type of incubator	No. of embryos cultured	Fertilization		No. of embryos transferred	*No. of embryos cultured	Cryopreservation	
		+	–			+	–
Conventional incubator	261	202 (77.4%)	59	15	187	91 (48.7%)	96
EmbryoScope®	402	298 (74.1%)	104	14	284	163 (57.4%)	121

*The number of cultured embryos after single embryo transfer.

Table 2. Clinical Pregnancy Rates According to the Number of embryos Transferred

Type of incubator	No. of embryos transferred	No. of pregnancies	
		+	–
Conventional incubator	100	15 (15.0%)	85
EmbryoScope®	98	24 (24.5%)	74

Single embryo transfer was performed two, three, or five days after oocyte retrieval.

Table 3. Cell Division Times in embryos Cultured in EmbryoScope®

No. of embryos	Group A*	Group B*	Group C*	total
	106	22	49	177
First division times (hours)	25.3 ± 2.1^{a}	25.2 ± 3.2^{a}	28.2 ± 3.6^{b}	26.1 ± 3.0
Second division times (hours)	37.8 ± 2.9^{d}	36.0 ± 4.1^{c}	41.3 ± 6.7^{e}	38.6 ± 4.7

*In this experiment, 177 embryos were fertilized checked and recorded. The first and second divisions were checked for all embryos. Embryos dividing into 2-cell embryos at the first division were classified as "2-cell" embryos. Embryos that did not divide into 2-cell embryos were classified as "none 2-cell" (Group C) embryos. Two-cell embryos dividing into 4-cell embryos were classified as "2-cell—4-cell" (Group A) embryos, and those that did not were classified as "2-cell—none 4-cell" (Group B) embryos (Fig. 1). $^{a–b}$Values with different superscripts are significantly different ($P<0.01$). $^{c–e}$Values with different superscripts are significantly different ($P<0.05$).

whom ≥10 eggs were retrieved between January and November, 2012. The mean age ± S.D. of patients at the retrieval cycles was 33.2 ± 3.1 years old. Oocyte retrieval and insemination were performed by conventional IVF (3 retrieval cycles) or ICSI (10 retrieval cycles) using standard techniques. In this experiment, 177 embryos were fertilized, checked, and recorded. The first and second cell divisions were checked for all embryos. Embryos dividing into 2-cell at the first division were classified as "2-cell" embryos, and those that did not were called "none 2-cell" (Group C) embryos. The 2-cell embryos that subsequently divided into 4-cells were classified as "2-cell–4-cell" (Group A) embryos, and those that did not were classified as "2-cell–none 4-cell" (Group B) embryos (Fig. 1). We compared the first division time, second division time, and blastocyst formation rate in each group.

Results

Experiment I

As shown in Table 1, the fertilization rates (EmbryoScope®: 74.1%, 298/402; conventional incubator: 77.4%, 202/261) and cryopreservation rates (EmbryoScope®: 57.4%, 163/284; conventional incubator: 48.7%, 91/187) of embryos were not affected by the incubator.

Experiment II

Clinical pregnancy rates were registered based on the presence of a gestational sac, visualized using ultrasound 5 weeks after embryo transfer. As shown in Table 2, no significant difference was observed in clinical pregnancy rates between the groups: (EmbryoScope®: 24.5%, 24/98; conventional incubator: 15.0%, 15/100).

Experiment III

As shown in Table 3, the first and second division times

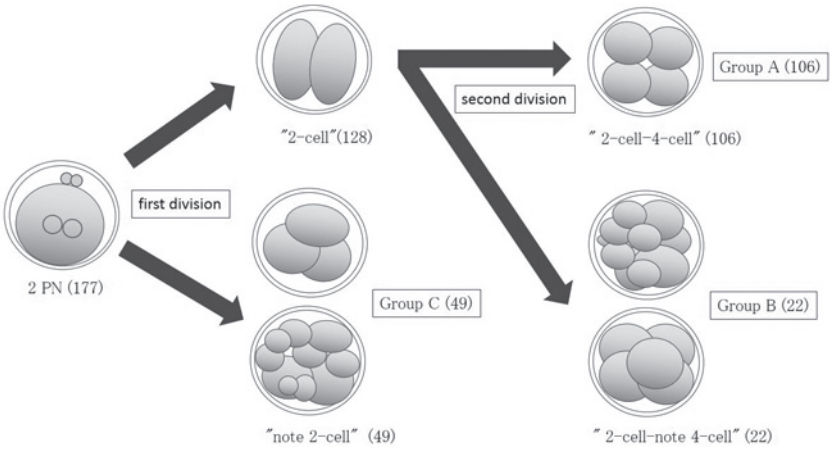

Fig. 1. Classification of divisions. In this experiment, 177 embryos were fertilized, checked, and recorded. The first and second divisions were checked for all embryos. The embryos dividing into 2-cell embryos at the first division were classified as "2-cell" embryos, and those did not were classified as "none 2-cell" (Group C) embryos. Two-cell embryos dividing into 4-cell embryos were classified as "2-cell-4-cell" (Group A) embryos, and those that did not were classified as "2-cell-none 4-cell" (Group B) embryos.

Table 4. Effects of the Cell Division Pattern on the Blastocyst Formation Rate

Cell division pattern	No. of embryos	No. of Blastocysts	
		+	–
Group A	106	91 (85.8%)[f]	15
Group B	22	14 (63.4%)[g]	8
Group C	49	5 (10.2%)[h]	44
total	177	110 (62.7%)	67

[f–h]Values with different superscripts are significantly different (P<0.05).

of 2-cell embryos (Groups A and B) were significantly (P<0.01) earlier than those of Group C. In addition, the second division time of embryos in Group B was significantly (P<0.05) faster than that in Group A.

As shown in Table 4, the blastocyst formation rate of 2-cell embryos (Groups A, and B) was significantly (P<0.05) higher than that of Group C. In addition, the blastocyst formation rate of Group A was significantly higher than that of Group B.

Discussion

Despite improvements to current procedures, the implantation rates of IVF embryos remain relatively low, with a clinical pregnancy rate of approximately 30% being reported per transfer [1]. To achieve a higher pregnancy rate, multiple embryos are often transferred simultaneously. However, the Japan Society of Obstetrics and Gynecology recommended single embryo transfer (SET) in 2008 to reduce fetal and maternal risks. Multiple embryo transfers increase the risks of multiple pregnancies and their associated neonatal complications, and maternal pregnancy-related health problems [2–5]. The elective transfer of a single embryo (e-SET) is an efficient method for reducing the risk of multiple gestations; however, the use of single embryo transfer also reduces the chance of pregnancy. Therefore, selecting the most competent embryo constitutes a major challenge in ART. However, the selection of an embryo for transfer should not be based solely on the number of cells alone, it should also depend on a morphological assessment on the day of transfer [16]. The opportunity to follow the dynamic developmental patterns of embryos using EmbryoScope® provides valuable information for embryo selection. EmbryoScope® allows a phenomenon to be observed at a specific time, which is directly linked to an embryo's developmental capacity and implantation potential. Considering all of these factors, a high quality embryo can be selected using EmbryoScope®. e-SET becomes increasingly acceptable with EmbryoScope® and the need to improve implantation rates becomes crucial. The conventional observation method mainly consisted of embryo observation at one point in time; however, embryos can now be evaluated dynamically using EmbryoScope®. It was previously difficult to evaluate the pronuclear appearance and division process by observing embryos at one point in time, and the influence anterior nucleus formation or the division process has on the subsequent growth of embryos was

unknown. EmbryoScope® enables dynamic analysis, fertilization can be precisely confirmed, and embryos can be evaluated with high precision.

Early cleavage is an established marker of developmental competence [17, 18]. However, as shown in Table 3, the second division time of embryos in Group B was significantly faster than that of Group A. Group A and B had second division times within the range reported by Mio et al. [7]. These results suggest that the most suitable division time is within a small time window. Group A showed the highest blastocyst formation rate. Therefore, the timing of the first and second divisions greatly influences embryonic selection.

In conclusion, time-lapse monitoring enables a detailed and dynamic analysis of the development of human preimplantation embryos, which suggests that improving the culture environment using a time-lapse embryo monitoring system may lead to better results. Time-lapse cinematography imaging delivers more information about human embryonic development and growth behavior than conventional practices or intermittent observations. An EmbryoScope® incubator supports embryonic development in the medium used for culture in a conventional incubator. These results indicate that EmbryoScope® enables a detailed and dynamic analysis of the development of human embryos.

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References

1) Andersen, A.N., Goossens, V., Ferraretti, A.P., Bhattacharya, S., Felberbaum, R., de Mouzon, J., Nygren, K.G. (2008): European IVF-monitoring (EIM) Consortium; European Society of Human Reproduction and Embryology (ESHRE). Hum. Reprod., 23(4), 756–771.
2) Strömberg, B., Dahlquist, G., Ericson, A., Finnström, O., Köster, M., Stjernqvist, K. (2002): Neurological sequelae in children born after in-vitro fertilisation: a population-based study. Lancet., 9;359(9305), 461–465.
3) Walker, M.C., Murphy, K.E., Pan, S., Yang Q., Wen, S.W. (2004): Adverse maternal outcomes in multifetal pregnancies. BJOG., 111, 1294–1296.
4) Pinborg, A., Loft, A., Schmidt, L., Andersen, A.N. (2003): Morbidity in a Danish national cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: health-related and social implications for the children and their families. Hum. Reprod., 18, 1234–1243.
5) Pinborg, A., Loft, A., Nyboe Andersen, A.N. (2004): Neonatal outcome in a Danish national cohort of 8602 children born after in vitro fertilization or intracytoplasmic sperm injection: the role of twin pregnancy. Acta Obstet Gynecol Scand., 83(11), 1071–1078.
6) Payne, D., Flaherty, S.P., Barry, M. F., Matthews, C.D. (1997): Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Hum. Reprod., 12, 532–541.
7) Mio, Y. (2006): Morphological analysis of human embryonic development using time-lapse cinematography. J. Mamm. Ova Res., 23, 27–35.
8) Adachi, Y., Takeshita, C., Wakatsuki, Y., Iwata, K., Kato, Y., Ueno, Y. and Mio, Y. (2005): Analysis of physiological process in early stage of human embryos after ICSI using time-lapse cinematography. J. Mamm. Ova Res., 22, 64–70.
9) Mio, Y., Maeda, K. (2008): Time-lapse cinematography of dynamic change occurring during in vitro development of human embryos. Am. J. Obstet. Gynecol., 199, 660.e1–e5.
10) Mio, Y., Iwata, K., Yumoto, K., Kai, Y., Sargant, H.C., Mizoguchi, C., Ueda, M., Tsuchie, Y., Imajo, A., Iba, Y., Nishikori, K. (2012): Possible mechanism of polyspermy block in human oocytes observed by time-lapse cinematography. J. Assist. Reprod. Genet., 29, 951–956.
11) Kirkegaard, K., Hindkjaer, J.J., Ingerslev H.J. (2012): Human embryonic development after blastomere removal: a time-lapse analysis. Hum. Reprod., 27, 97–105.
12) Kirkegaard, K., Agerholm, I.E., Ingerslev, H.J. (2012): Time-lapse monitoring as a tool for clinical embryo assessment. Hum. Reprod., 27, 1277–1285.
13) Cruz, M., Gadea, B., Garrido, N., Pedersen, K.S., Martínez, M., Pérez-Cano, I., Muñoz, M. and Meseguer, M. (2011): Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imagin J. Assist. Reprod. Genet., 28(7), 569–573.
14) Veeck, L.L. (1999): An atlas of human gametes and conceptuses. Parthenon Publishing, New York.
15) Gardner, D.K., Schoolcraft, W.B., Wagley, L., Schlenker, T., Stevens, J. (1998): A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Hum. Reprod., 13, 3434–3440.
16) Van Montfoort, A.P., Dumoulin, J.C., Kesters, A.D., Evers, J.L. (2004): Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers. Hum. Reprod., 19(9), 2103–2108.
17) Shoukir, Y., Campana, A., Farley, T., Sakkas D. (1997): Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum. Reprod., 12, 1531–1536.
18) Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, PG., 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.