CEOG Clinical and Experimental Obstetrics & Gynecology

Original Research

Separate transfer of two frozen-thawed embryos reduces multiple gestations in assisted reproductive technology

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Summary

Aim: In assisted reproductive technology (ART), there are different opinions regarding how an embryo should be transferred to the uterus. For double embryo transfer (DET) cycles in particular, there is no consensus regarding the appropriate embryo transfer (ET) method. Therefore, the present authors developed a novel ET method called separated (s-DET); with this method, two embryos are transferred to separate settings. The authors evaluated and compared pregnancy outcomes of s-DET and conventional DET (c-DET). *Materials and Methods:* In this prospective cohort study, 129 patients underwent separated ET. One hundred fifty-two patients underwent c-DET, which involves transferring two embryos together. All patients underwent ET of two frozen–thawed embryos. *Results:* The clinical pregnancy and implantation rates of the s-DET group were 20.9 % and 10.4 %, respectively; these rates were similar to those of the conventional DET group (26.3 % and 16.1 %, respectively). However, the multiple gestation rate of the D-DET group (0.0 %) was significantly lower than that of the conventional double ET group (22.5 %; p = 0.006). *Conclusions:* s-DET might make ART safer and decrease multiple gestations resulting from DET cycles.

Key words: Embryo transfer; In vitro fertilization; Obstetrics; Pregnancy; Assisted reproductive techniques.

Introduction

In assisted reproductive technology (ART), the embryo transfer (ET) method is the most important factor influencing pregnancy rates [1, 2]. Whether the implantation rate of double embryo transfer (DET) is improved compared with that of single embryo transfer (SET) twice has been a topic of much discussion. Several reports have attested to the higher clinical pregnancy rates resulting from DET compared to SET [3-5]. Furthermore, some researchers have demonstrated that the transferred embryo needs to be able to cross-talk with the uterine endometrium using decidual cytokines, such as interleukin-1, human chorionic gonadotropin (hCG), and leukemia inhibitory factor (LIF) and its ligands, in order to attach to the surface of the uterine endometrium for implantation [6-8]. Regarding implantation factors for DET, some researchers have theorized that embryo interaction, or a signal from a good embryo, helps the second embryo to implant [9]. However, the actual reason for the increased implantation rates of DET is unknown.

In rodents such as mice, transferred embryos keep a constant distance between each other which results in multiple gestation [10]. Therefore, the authors considered that pregnancy outcomes might improve if a constant distance was maintained between embryos.

In this study, the authors developed a novel ET method, namely, separated DET (s-DET), whereby two embryos are transferred to separate settings. In the present study, they evaluated and compared pregnancy outcomes of s-DET and conventional DET (c-DET).

Materials and Methods

This prospective cohort study included patients with a history of two or more implantations failures after ET cycles with thawed embryos. Patients were consecutively enrolled in the study at the Division of Reproductive Medicine, Sugiyama Clinic, Tokyo, Japan, between May and December 2015. This study was approved by the Institutional Review Board of Sugiyama Clinic. Informed consent was obtained from all patients before participation. The study patients had a history of two or more failed in vitro fertilization (IVF)/ET cycles with morphologically and developmentally average to good quality embryos transferred to an adequately prepared endometrium (endometrial thickness ≥8 mm). During the ET period, a doctor explained the difference between c-DET and s-DET to the patients before they chose their preferred method. No patient had submucosal fibroids, endometrial polyps, intrauterine adhesions, congenital uterine anomalies, or hydrosalpinx. Furthermore, no patient had current or previous autoimmune disease. Women with chronic medical or inflammatory conditions, women who miscarried after IVF, women who underwent an IVF cycle or vaccination within three months, or women with acquired or inherited thrombophilia were excluded from the study.

Ovarian stimulation and oocyte pick-up were performed according to the present authors' published protocol [11]. Their mild stimulation protocol was as follows: 50 mg of clomiphene citrate

Published: 15 April 2020

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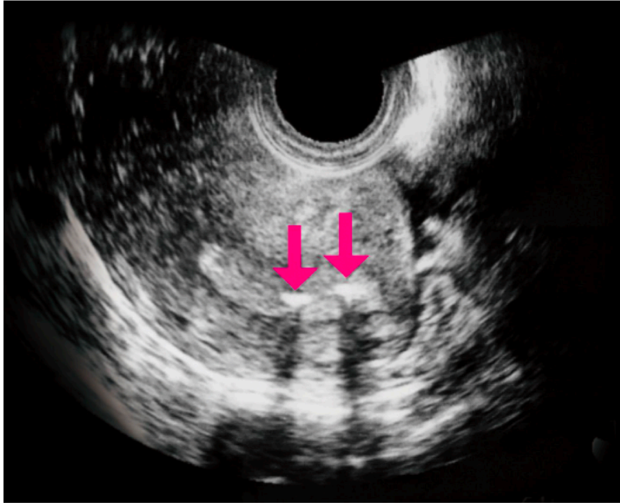

Figure 1. — Ultrasound image of the s-DET procedure. After the first embryo is transferred to the fundus of the uterus, the second embryo is transferred to a position with a 1 cm space by inserting another transfer tube. The arrows indicate air bubbles following the transfer of embryos.

was administered every day on days 3–7 of the menstrual cycle; and 225 IU of recombinant follicle-stimulating hormone (FSH) was administered on days 4, 6, and 8 of the menstrual cycle. On day 10, when dominant follicles reached ≥ 17 mm in diameter, either 10,000 IU of hCG was injected or 300 μg of buserelin acetate was nasally administered, and oocyte pickup was performed 35 hours later. Additional recombinant FSH (150 IU per day) was administered as needed based on follicular growth. Intracytoplasmic sperm injection or conventional co-culture was performed depending on the semen parameters.

Embryos were cryopreserved by vitrification and warmed using a previously published protocol [12]. All solutions used for vitrification and warming were from the vitrification kit. All transferred embryos were frozen–thawed embryos in the cleavage stage. Embryo quality was assessed on the day of transfer. On day 3, embryos in the cleavage stage were evaluated to determine the blastomere number and fragmentation. Those that were developed to at least the seven-cell stage with <10 % fragmentation were defined as morphologically good [11]. The others were classified as morpholog-ically poor. This system of embryo assessment was based on the classification system of Veeck [13].

All embryos were transcervically placed in the patient's uterus using a soft catheter. Patients were placed in the dorsal lithotomy position. Vaginal ultrasonography using a sagittal section was performed to visualize and facilitate the transfer. For c-DET, gynecologists transferred two embryos to the same site at the same time using one catheter. That site was 1 cm away from the fundus. However, for s-DET, after the first embryo was transferred to the position 1 cm away from the fundus, the second embryo was transferred to a position 1 cm away from the first by inserting another transfer tube (Figure 1). Considering the possibility of embryo damage during the procedure, the authors transferred the two embryos as quickly and gently as possible.

All three gynecologists who performed ET were qualified physicians with more than ten years of experience specializing in reproductive medicine in Japan. Endometrial preparation was achieved by a hormone replacement cycle with conjugated estrogens (0.625 mg) and transdermal estradiol (0.72 mg). These treatments were administered starting from day 3 of the menstrual cycle or the first day without bleeding until the day of the urinary test for pregnancy. Administration of progesterone (100 mg) was initiated on day 12 of the menstrual cycle. Three days after initiation of progesterone treatment, embryos were thawed and surviving embryos were transferred [11].

A positive hCG result was defined by hCG levels ≥ 40 IU/L. A clinical pregnancy was recognized when one or more gestational sacs were detected by transvaginal ultrasonography after ET. The implantation rate was determined by dividing the number of gestational sacs by the number of embryos transferred. The primary endpoints of this study were hCG positivity, implantation rates, clinical pregnancy rates, miscarriage rates, and multiple pregnancy rates. Follow-up of the fetus was continued until nine weeks of gestation using vaginal ultrasonography. Fisher's exact test was performed for categorical variables, and the Student's *t*-test was performed for continuous variables depending on distribution characteristics. Values of $p < 0.05$ was considered statistically significant. All statistical computations were carried out using SPSS version 24.0.

Results

A total of 281 patients underwent ET of two frozen–thawed embryos during this study; 152 patients underwent c-DET and 129 patients underwent s-DET. The background characteristics of the patients are shown in Table 1. The mean age of the s-DET group was 38.8 years (standard deviation [SD] ± 3.5 years), which was comparable with that of the c-DET group (38.7 ± 3.8 years). Pregnancy and delivery history of the s-DET group were similar to those of the c-DET group. The mean ± SD of the number of previous ET attempts for the s-DET group was 3.3 ± 3.3, which was comparable with that of the c-DET group (3.9 ± 3.0). The authors compared the morphologic quality of embryos of the s-DET and c-DET groups (Table 2). There were no significant differences in the total number of blastomeres between the s-DET and c-DET groups (15.3 ± 2.8 and 14.6 ± 2.3, respectively). The reproductive outcomes of the two groups are shown in Table 3. There were no significant differences in positive hCG rates of the s-DET and c-DET groups (28.7% and 29.6%, respectively). Clinical pregnancy and implantation rates of the s-DET group were 20.9% and 10.4%, respectively, and were similar to those of the c-DET group (26.3% and 16.1%, respectively). The miscarriage rate of the s-DET group (14.8%) was comparable with that of the c-DET group (22.5%). However, the multiple pregnancy rate of the s-DET group (0.0 %) was significantly lower than that of the c-DET group (22.5%; $p = 0.006$).

Table 1. — *Background characteristics of the s-DET and c-DET groups.*

	s-DET	c-DET	*p* value
Cycles, n	129	152	
Age, years[#]	38.8 ± 3.5	38.7 ± 3.8	NS
Previous ET attempts, n[#]	3.3 ± 3.3	3.9 ± 3.0	NS
Gravidity n[#]	0.9 ± 1.2	0.6 ± 0.9	NS
Parity, n[#]	0.3 ± 0.5	0.2 ± 0.4	NS
Cause of infertility			
Male factor, n (%)	66 (51.2)	78 (51.3)	NS
Tubal factor, n (%)	6 (4.7)	8 (5.2)	NS
Endometriosis, n (%)	4 (3.1)	9 (6.0)	NS
Unexplained, n (%)	53 (41.0)	57 (37.5)	NS

[#]Values are presented as mean ± standard deviation. ET: embryo transfer, c-DET: conventional double-embryo transfer, s-DET: separated double-embryo transfer, NS: not significant.

Table 2. — *Embryo quality between the s-DET and c-DET groups.*

	s-DET	c-DET	*p* value
No. of double good embryos, n (%)	35(27.1)	42(27.6)	NS
No. of good & poor embryos, n (%)	62(48.1)	60(39.5)	NS
No. of double poor embryos, n (%)	32(24.8)	50(32.9)	NS
Best no. of blastomeres, n[#]	8.2 ± 1.7	7.9 ± 1.4	NS
Total no. of blastomeres, n[#]	15.3 ± 2.8	14.6 ± 2.3	NS

[#]Values are presented as mean ± standard deviation. c-DET: conventional double-embryo transfer, s-DET: separated double-embryo transfer, NS: not significant.

Table 3. — *Reproductive outcomes in the s-DET and c-DET groups.*

	s-DET (n = 129)	c-DET (n = 152)	*p* value
Transferred embryos, n	258	304	
Positive hCG, n (%)	37 (28.7)	45 (29.6)	NS
Clinical pregnancy, n (%)	27 (20.9)	40 (26.3)	NS
GS, n	27 49		
Implantation rate, %	10.4	16.1	NS
Multiple pregnancies, n (%)	0 (0.0)	9 (22.5)	0.006
Miscarriage, n (%)	4 (14.8)	9 (22.5)	NS

hCG: human chorionic gonadotropin, GS: gestational sac, c-DET: conventional double-embryo transfer, s-DET: separated double-embryo transfer, NS: not significant.

Discussion

The authors present a novel ET method, s-DET, whereby two embryos are transferred to separate positions. They evaluated whether the s-DET affects pregnancy outcomes compared with c-DET. Contrary to their expectations, pregnancy rates of the s-DET group did not improve. However, multiple pregnancy rates of the s-DET group were significantly lower than those of the c-DET group. Moreover, the rate of miscarriage with c-DET was slightly higher than that with s-DET. From this result, it is assumed that with c-DET, both cleavage stage embryos will be just implanted, but in s-DET, one embryo will be eliminated due to natural selection, and only one will continue to grow.

It has been shown that preimplanted embryos require various factors, such as interleukin-1, hCG and vascular endothelial growth factor, in order to achieve successful implantation [6]. Goto *et al.* demonstrated that in embryo culture, these factors could induce endometrial differentiation during the cleavage stage, suggesting that cross-talk occurs between early-stage embryos and the endometrium during the stimulation of endometrium embryo transfer (SEET) method [14]. Other researchers suggested that both the control of steroid hormones and cross-talk between the endometrium and embryo are important to produce an acceptable endometrium for implantation [15]. Therefore, the present authors speculated that cross-talk between embryos being transferred to separate positions might increase endometrial receptivity. Good endometrial receptivity might allow good embryos to be implanted and poor embryos to be eliminated. During s-DET, the elimination system of the endometrium may have been successful, thereby leading to reduced multiple pregnancy rates. However, it is reasonable to think that the elimination system is not successful during c-DET.

In rodents such as mice, implantation occurs within strict distances in their long uterus, and physiological multiple gestation occurs [10]. Previous studies have suggested that approximately 80% of embryos implanted in the area where the catheter tip was situated [16, 17]. Judging from these reports and the results of this study, the authors speculated that the embryo which implants earlier during s-DET can acquire the space to create a placenta, and the second embryo might not be able to implant in that place. The second embryo might be prevented from implanting and growing, depending on the selective pressure. As a result, the multiple pregnancy rate might decrease. However, the precise mechanism remains unknown, and further research to evaluate s-DET is required.

The present authors analyzed whether the embryo quality during transfer affected pregnancy outcomes. Kaser *et al.* suggested that a lead embryo with seven cells and a sum of 14 viable cells is a risk factor for multiple pregnancy during the DET cycle [18]. In contrast, other reports have indicated that the embryo status does not significantly influence the multiple pregnancy rate [19]. A previous study also suggested that pregnancy rates were not significantly different between any combination of groups using embryos of different qualities for DET [20]. The present findings matched the results of these studies. Table 2 shows that embryo quality of any combination of two embryos from the s-DET and c-DET groups was not significantly different. Therefore, it is unlikely that embryo quality influenced these results. Even if embryos are morphologically equivalent, preimplantation genetic screening or chromosome inspection should be performed to determine the viability of the embryos [21].

One limitation of the present study involved the amount of medium injected in the uterus. Because the

amount of medium injected during s-DET is approximately twice that injected during c-DET, it is possible that the widened cavity was disadvantageous to the second implantation and growth. We also need to consider mechanical stimulation of the transfer catheter. When we enter the uterine cavity twice with the transfer catheter, we are twice as likely to disrupt the endometrium, to introduce bacteria into the uterine cavity, and to induce uterine contractions. These environmental disturbances may have influenced our results. We also have to consider the possibility that the embryo transferred first may have been damaged by the catheter during transfer of the second embryo. However, if this had occurred, then we would expect to have seen more noticeable differences in the implantation rates. Finally, because the present clinic specializes in ART, observation was performed until up to nine weeks of gestation. The authors do not know what occurred after nine weeks of gestation. In addition, if the prognosis of multiple fetuses obtained with c-DET seems to be obviously inferior to that for s-DET, then the risk of forcible implantation during c-DET with poor embryos may be revealed.

The present authors suggested that embryo selection according to the uterine endometrium should not be performed when transferring embryos during c-DET. This may be the result of the unnatural idea that transferring two embryos to the same narrow place at the same time is normal. Previous studies have indicated that the uterine endometrium may have the ability to select an embryo for implantation based on the signal from the embryo [22]. Based on these theories, it is possible that endometrial cells work as sensors of embryo quality on implantation in sufficient space.

One clinical advantage of s-DET is that it does not require a blastocyst. Blastocysts are more likely to implant than good quality embryos [23]. However, approximately half of good quality embryos do not reach the blastocyst stage, and some patients have no choice but to cancel their blastocyst transfer. Therefore, blastocyst transfer is not the best option for many patients, particularly those with few early-stage embryos. One researcher suggested that the cost-effectiveness of DET improves with age, and DET may be considered cost-effective for women 37–39 years of age [24]. In conclusion, s-DET may be both safe and efficient method without increasing dangerous multiple deliveries by choosing cases appropriately.

Acknowledgement

The authors thank the staff members of the Sugiyama Clinic for collecting some of the data used in this study.

References

[1] Salha O.H., Lamb V.K., Balen A.H.: "A postal survey of embryo transfer practice in the UK". *Hum. Reprod.,* 2001, *16*, 686.
[2] van Weering H.G., Schats R., McDonnell J., Vink J.M., Vermeiden J.P., Hompes P.G.: "The impact of the embryo transfer catheter on the pregnancy rate in IVF". *Hum. Reprod.,* 2002, *17*, 666.
[3] Berin I., McLellan S.T., Macklin E.A., Toth T.L., Wright D.L.: "Frozen-thawed embryo transfer cycles: clinical outcomes of single and double blastocyst transfers". *J. Assist. Reprod. Genet.,* 2011, *28*, 575.
[4] Kang S.M., Lee S.W., Yoon S.H., Kim J.C., Lim J.H., Lee S.G.: "Comparison of clinical outcomes between single and double vitrified-warmed blastocyst embryo transfer according to the day of vitrification". *J. Assist. Reprod. Genet.,* 2013, *30*, 779.
[5] McLernon D.J., Harrild K., Bergh C., Davies M.J., de Neubourg D., Dumoulin J.C., *et al.*: "Clinical effectiveness of elective single versus double embryo transfer: meta-analysis of individual patient data from randomised trials". *BMJ,* 2010, *341*, c6945.
[6] Dey S.K., Lim H., Das S.K., Reese J., Paria B.C., Daikoku T., Wang H.: "Molecular cues to implantation". *Endocrinol. Rev.,* 2004, *25*, 341.
[7] Chen J.R., Cheng J.G., Shatzer T., Sewell L., Hernandez L., Stewart C.L.: "Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis". *Endocrinology,* 2000, *141*, 4365.
[8] Tazuke S.I., Giudice L.C.: "Growth factors and cytokines in endometrium, embryonic development, and maternal: embryonic interactions". *Semin. Reprod. Endocrinol.,* 1996, *14*, 231.
[9] Miró F., Vidal E., Balasch J.: "Increased live birth rate in twin pregnancies resulting from embryo assistance". *Obstet. Gynecol.,* 2012, *119*, 44.
[10] Barr M. Jr., Jensh R.P., Brent R.L.: "Placental growth in the albino rat: effects of number, intrauterine position and resorptions. *Am. J. Anat.*, 1970, *128*, 413.
[11] Nakagawa K., Takahashi C., Nishi Y., Jyuen H., Sugiyama R., Kuribayashi Y.: "Hyaluronan-enriched transfer medium improves outcome in patients with multiple embryo transfer failures". *J. Assist. Reprod. Genet.,* 2012, *29*, 679.
[12] Sugiyama R., Nakagawa K., Shirai A., Nishi Y., Kuribayashi Y., Inoue M.: "Clinical outcomes resulting from the transfer of vitrified human embryos using a new device for cryopreservation (plastic blade)". *J. Assist. Reprod. Genet.,* 2010, *27*, 161.
[13] Veeck L.L.: "Atlas of the Human Oocyte and Early Conceptus". Vol. 2. Baltimore: Williams & Wilkins Co., 1991.
[14] Goto S., Kadowaki T., Hashimoto H., Kokeguchi S., Shiotani M.: "Stimulation of endometrium embryo transfer (SEET): injection of embryo culture supernatant into the uterine cavity before blastocyst transfer can improve implantation and pregnancy rates". *Fertil. Steril.,* 2007, *88*, 1339.
[15] Paria B.C., Lim H., Das S.K., Reese J., Dey S.K.: "Molecular signaling in uterine receptivity for implantation". *Semin. Cell. Dev. Biol.,* 2000, *11*, 67.
[16] Krampl E., Zegermacher G., Eichler C., Obruca A., Strohmer H., Feichtinger W.: "Air in the uterine cavity after embryo transfer". *Fertil. Steril.,* 1995, *63*, 366.
[17] Baba K., Ishihara O., Hayashi N., Saitoh M., Taya J., Kinoshita K.: "Where does the embryo implant after embryo transfer in humans?" *Fertil. Steril.,* 2000, *73*, 123.
[18] Kaser D.J., Missmer S.A., Correia K.F., Ceyhan S.T., Hornstein M.D., Racowsky C.: "Predictors of twin live birth following cryopreserved double embryo transfer on day 3". *J. Assist. Reprod. Genet.,* 2013, *30*, 1023.
[19] El-Danasouri I., Sterzik K., Rinaldi L., Pacchiarotti A., DeSanto M., Selman H.: "Effect of transferring a morphologically impaired embryo with a good quality embryo on the pregnancy and implantation rates". *Eur. Rev. Med. Pharmacol. Sci.,* 2016, *20*, 394.
[20] Wintner E.M., Hershko-Klement A., Tzadikevitch K., Ghetler Y.,

Gonen O., Wintner O., *et al.*: "Does the transfer of a poor quality embryo together with a good quality embryo affect the in vitro fertilization (IVF) outcome?" *J. Ovarian Res.,* 2017, *10*, 2.
[21] Yang Z., Liu J., Collins G.S., Salem S.A., Liu X., Lyle S.S., *et al.*: "Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study". *Mol Cytogenet* 2012, *5*, 24.
[22] Teklenburg G., Salker M., Molokhia M., Lavery S., Trew G., Aojanepong T., *et al.:* "Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation". *PLoS One*, 2010, *5*, e10258.
[23] Blake D.A., Farquhar C.M., Johnson N., Proctor M.: "Cleavage stage versus blastocyst stage embryo transfer in assisted conception". *Cochrane Database Syst. Rev.,* 2007, *4*, CD002118.
[24] Scotland G.S., McLernon D., Kurinczuk J.J., McNamee P., Harrild K., Lyall H., *et al.*: "Minimising twins in in vitro fertilisation: a modelling study assessing the costs, consequences and cost-utility of elective single versus double embryo transfer over a 20-year time horizon". *BJOG,* 2011, *118*, 1073.

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