大韓不妊學會誌:第33卷 第4號 2006
Kor. J. Fertil. Steril., Vol. 33, No. 4, 2006, 12

Efficacy of Frozen-Thawed ET in Patients with Old Age or Non-Pregnant in Fresh ET Cycles

Su Jin Choi[1], Sun Hee Lee[1], In Ok Song[2], Mi Kyoung Koong[2], Inn Soo Kang[2], Jin Hyun Jun[1]

[1]Laboratory of Reproductive Biology & Infertility, [2]Department of Ob/Gyn, Sungkyunkwan University School of Medicine 1-19, Mukjeoung-dong, Jung-gu, Cheil General Hospital & Women's Healthcare Center, Seoul, Korea

고령 환자와 신선주기 배아이식에서 임신에 실패한 환자에서 동결-융해 배아이식의 효용성

최수진[1] · 이선희[1] · 송인옥[2] · 궁미경[2] · 강인수[2] · 전진현[1]

제일병원 생식생물학 및 불임연구실[1], 성균관대학교 의과대학 산부인과학교실[2]

목 적: 동결-융해 배아이식은 보조생식술에서 환자들에게 보다 많은 임신의 기회를 제공해줄 수 있는 방법으로 이용되고 있다. 본 연구에서는 예후가 좋지 않은 환자들에서 동결-융해 배아이식의 효용성을 알아보고자 하였다.

연구방법: 나이가 많은 고령 환자군 (38~44세)과 신선주기 배아이식에서 임신 실패군을 연구대상으로 하였다. 과배란 유도를 통해 채취한 난자를 일반적인 체외수정 또는 세포질내 정자주입술을 시행하여 수정을 유도하고, 잉여의 전핵 또는 난할 시기의 배아를 완만동결법으로 동결하였다. 동결보관 배아는 급속융해법으로 융해하여 호르몬요법을 시행한 환자의 자궁에 이식하였다. 신선 배아이식과 동결-융해 배아이식 과정에서의 배아 상태, 임신율, 착상률 등을 통계적인 방법으로 분석하였다.

결 과: 나이가 많은 고령군에서 신선 배아이식을 시행한 환자들과 동결-융해 배아이식을 시행한 환자들의 평균 연령은 40.0±1.8세 (n=206)와 39.9±1.9세 (n=69)로 통계적으로 유의한 차이가 없었으나, 임상적 임신율과 착상률은 동결-융해 배아이식에서 29.0%와 11.2%로 신선 배아이식의 16.5%와 7.0%에 비해 통계적으로 유의하게 높게 나타났다 ($p<0.05$). 첫번째 신선 배아이식에서 임신 실패군의 연속되는 신선 배아이식 환자군 (31.2±2.3, n=40)과 동결-융해 배아이식 환자군 (31.9±3.1, n=119)에서의 평균 연령은 차이가 없었으며, 임상적 임신율 (42.5% vs 40.3%)과 착상률 (22.6% vs 18.8%)도 유사하였다.

결 론: 본 연구에서는 동결-융해 배아이식이 고령 환자들에서 효과적으로 임신율과 착상률을 높일 수 있음을 보여주고 있다. 이러한 결과는 과배란 유도에 따른 자궁의 착상 환경 변화가 고령 환자들에서 임신율과 착상률을 저하시키는 것과 관련이 있을 것으로 생각된다.

중심단어: 동결-융해 배아이식, 고령 환자군, 임신율, 과배란 유도, 착상 환경

Since the first reported pregnancy after a frozen-thawed embryo transfer (ET), cryopreservation of embryos has been integrated as an important procedure of assisted reproductive technologies (ART) and the safety of this procedure has been confirmed by deliveries of healthy babies.[1~3] The cryopreservation technique provides several advantages to ART in improvement of clinical outcome. It allows

주관책임자: 전진현, 우) 100-380 서울특별시 중구 묵정동 1-19, 제일병원 생식생물학 및 불임연구실
Tel: (02) 2000-7590, Fax: (02) 2265-5621, e-mail: junjh55@hanmail.net

reducing the risk of multiple pregnancies and the patients have a reservoir of excess embryos for additional transfers without ovarian stimulation and egg retrieval.[4,5] In addition, the ability to freeze all the embryos obtained and to transfer at a subsequent cycle is useful to reduce the risk of severe complication in ovarian hyperstimulation syndrome (OHSS).[6~8] Cryopreservation was successfully applied to the donor oocyte program and to ovarian tissue in chemotherapy of cancer patients as a means of preserving the patient's future fertility potential.[9~11]

Female age is an important determinant of successful clinical outcome in infertility treatment. Old age is directly associated with lower chance of pregnancy in ART program.[12] After subsequent frozen-thawed ET cycles, the cumulative clinical pregnancy rate was significantly higher in young age group (≤ 38 years; 76.1% per patient) than that of old age group (> 38 years; 46.1% per patient).[4]

Alteration of hormonal physiology by controlled ovarian hyperstimulation (COH) may affect the receptivity of uterus in fresh IVF-ET cycles. Supraphysiological levels of estradiol and increased ratio of estradiol to progesterone concentration may alter uterine environment for on-time implantation of transferred embryos in COH cycles.[13~18] It could be adjusted in frozen-thawed ET cycles by appropriate hormonal treatments for uterine receptivity.

The aim of this study was to evaluate the efficacy of frozen-thawed ET in poor prognosis patients such as old age patients (38~44 years) and the patients who did not achieve clinical pregnancy in first fresh ET cycle (non-pregnant patient).

MATERIALS AND METHODS

The subject of this study was patients with old age (OA group; 38~44 years) or non-pregnant in fresh ET cycles (NP group; less than 38 years). The OA group of this study was performed either fresh ET or frozen-thawed ET cycles. The NP group was non-pregnant in first fresh ET cycles and carried out subsequent fresh ET cycles or frozen-thawed ET cycles. Laboratory and clinical data was collected from 2004 to 2005 in our center, excluding cycles with poor response (less than 6 of retrieved oocytes) and ovarian hyperstimulation (more than 30 of retrieved oocytes) and non-obstructive azoospermia. Due to the retrospective nature of the study, Institutional Review Board approval was not required.

COH was performed using GnRH agonist or antagonist and recombinant FSH or hMG. Human chorionic gonadotropin (hCG) was administered when two or more follicles reached 18 mm or greater. Oocyte were retrieved 36 hours later and fertilized by either conventional insemination or intracytoplasmic sperm injection (ICSI). Sequential media of G1 and G2 (Vitrolife, Gothenburg, Sweden) were used for embryo culture to the cleavage stage. Embryo quality was monitored and graded by good as even or uneven blastomeres with less than 25% of fragmentation and poor as uneven blastomeres with more than 25% of fragmentation.

Supernumerary embryos were frozen at pronucleus stage or cleavage stage by the slow freezing method with propylene glycol (PROH) and sucrose. Freezing and thawing solutions were prepared in Dulbecco's phosphate-buffered saline (dPBS; Gibso-BRL, Grand Island, NY, USA) supplemented with 20% SSS (Synthetic serum substitute, Irvine). Frozen embryos were thawed by the rapid thawing method. Cryopreservation of human embryos was performed using a programmable controlled rate-freezing machine (Cryo-magic; Miraebiotech, Seoul, Korea). The embryos were exposed in a stepwise fashion to increasing concentrations of 1.5 M PROH + 0.1 M sucrose in the freezing solution. The embryos were then loaded into a 0.25 ml sterile straw (Bicef, L'Aigle, France) and then the straw was loaded into the cryo-machine and kept at 20℃.

Table 1. Clinical outcome of subsequent fresh or frozen-thawed embryo transfer cycle in old age (OA) group

	Fresh ET	Frozen-thawed ET	P values
No. of cycles	206	69	
Mean age ± SD (years)	40.0±1.8	39.9±1.9	NS
Peak E_2 (pg/ml)*	1,946.2±996.0	2,780.0±1178.1‡	< 0.01
No. of retrieved oocytes*	11.5±5.0	15.9±5.4‡	< 0.01
No. of cultured embryos*	6.2±2.3	4.1±2.0	< 0.01
Percentage of good quality embryos (number)	62.4% (796/1,276)	52.1% (149/286)	< 0.01
No. of transferred embryos*	3.7±0.7	3.4±1.0	< 0.01
Percentage of transferred embryos with good quality (number)	73.9% (562/761)	57.1% (133/233)	< 0.01
No. of clinical pregnancies†	34 (16.5%)	20 (29.0%)	< 0.05
No. of implantations†	44 (7.0%)	26 (11.2%)	< 0.01

NS: not significant (p>0.05).
*Data present as mean ± SD, †G-sac positive, ‡Data from first fresh ET cycles.

The straw was subsequently cooled from 20℃ to -7℃ at a rate of -2℃/min, held at this temperature for 5 min, and then seeded manually. It was then cooled to -40℃ by -0.3℃/min, cooled to -150℃ by -30℃/min and plunged into liquid nitrogen.

For thawing of the frozen human embryos, the straws were warmed by holding them in air for 40 sec before plunging them into a water bath at 37℃ for 1 min. The cryoprotectant was then removed by reverse stepwise dilution. In frozen cycle, a daily dose of 6 mg of oral estradiol valerate was initiated on menstrual day 2. Estrogen and progesterone were administrated and the endometrial thickness was monitored. ET was performed 3~5 days after the endometrial thickness reached more than 8 mm. After ET, hormonal supplementation was continued for 14 days until a urine pregnancy test.

Successful pregnancy was determined by more than 5 IU/ml for β-hCG on 11~13 days after ET. Clinical pregnancy and implantation were confirmed by observation on ultrasound scanning of a gestational sac between 4 and 5 weeks after the pregnancy test. Patient age and the mean number of transferred embryos per cycle were compared by Student's t-test. Pregnancy rates were compared by chi square test.

RESULTS

In OA group, all patients were ≥38 years and clinical outcome was presented in Table 1. Mean age was not significantly different between the fresh ET (40.0±1.8 years) and the frozen-thawed ET cycles (39.9±1.9 years). Peak E_2 level and number of retrieved oocytes were significantly higher in first fresh ET cycles of the frozen-thawed ET cycles than those of the fresh ET cycles ($p< 0.01$). In contrast percentages of good quality embryos and transferred embryos with good quality were significantly higher in the fresh ET cycles than those of the frozen-thawed ET cycles ($p<0.01$). However, clinical pregnancy and implantation rate of the frozen-thawed ET cycles (29.0% and 11.2%) were significantly higher than those of the fresh ET cycles (16.5% and 7.0%).

In NP group, there were no significant difference

Table 2. Clinical outcome of subsequent fresh or frozen-thawed embryo transfer in non-pregnant group

	Fresh ET	Frozen-thawed ET	P values
No. of cycles	40	119	
Mean age ± SD (years)	31.2±2.3	31.9±3.1	NS
Peak E_2 (pg/ml)*	2,478.3±923.0	3,026.7±1,338.8‡	< 0.05
No. of retrieved oocytes*	15.8±6.6	19.3±5.6‡	< 0.01
No. of cultured embryos*	7.6±1.9	4.4±1.2	< 0.01
Percentage of good quality embryos (number)	62.9% (190/302)	55.4% (288/520)	< 0.05
No. of transferred embryos*	3.6±0.5	3.5±0.8	NS
Percentage of transferred embryos with good quality (number)	80.8% (118/146)	62.8% (260/414)	< 0.01
No. of clinical pregnancies†	17 (42.5%)	48 (40.3%)	NS
No. of implantations†	33 (22.6%)	78 (18.8%)	NS

NS: not significant (p>0.05).
*Data present as mean ± SD, †G-sac positive, ‡Data from first fresh ET cycles.

in mean age and number of transferred embryos between the subsequent fresh ET and frozen-thawed ET cycles (Table 2). Peak E_2 level and number of retrieved oocytes were significantly different between the subsequent fresh ET cycles and first fresh ET cycles of the subsequent frozen-thawed ET cycles. In contrast proportion of transferred good quality embryos was significantly higher in the subsequent fresh ET cycles than that of the subsequent frozen-thawed ET cycles (80.8% vs. 62.8%, p<0.01). The clinical pregnancy and implantation rates were similar between the subsequent fresh ET cycles (42.5% and 22.6%) and the subsequent frozen-thawed ET cycles (40.3% and 18.8%).

DISCUSSION

In frozen-thawed ET cycles, pregnancy and implantation rates range from 10 to 30% and 5 to 15%, respectively, and cryopreservation provides additional chances for pregnancy without COH and oocytes retrieval. This study shows substantial improvement of clinical outcome after frozen-thawed ET in old age patients (38~44 years), especially. Also, comparable pregnancy and implantation rates were achieved by subsequent frozen-thawed ET cycles in the NP group of this study.

Previously, several reports suggested the adverse effects of cryopreservation on embryonic development and implantation potential of embryos. Levran et al. showed that cryopreservation at the 2~4 cell stage significantly reduces the capacity of human embryos to implant.[19] In addition, a significant adverse effect of cryopreservation at the 2-PN stage on embryo quality and a tendency for a lower implantation rate compared to fresh cycles was observed.[20] Our study also showed poor embryo quality after cryopreservation, however, it was not detrimental to pregnancy outcome. This results may be related that better uterine environments for implantation in physiological hormone supplementation than that of supraphysiological hormonal condition in COH.

Many reports proposed possible adverse effects of COH on uterine receptivity in fresh IVF-ET cycles.[14~18] They suggested that supraphysiological levels of estradiol may interfere with endometrial environments for successful implantation of trans-

ferred embryos. Histological observations and gene expression studies have shown that the implantation window seems to be advanced in COH cycles compared to natural menstrual cycles. Appropriate hormonal treatments in frozen-thawed ET cycles may provide better uterine environments for successful implantation than the COH cycles. Our study showed the comparable pregnancy rate of frozen-thawed ET than that of fresh ET, even though the quality of transferred embryos was better in fresh ET than that of frozen-thawed ET cycles. It seems to be related to the importance of synchronization between endometrium and transferred embryos in implantation window.

It is well known that maternal variables such as maternal age, causes of infertility and rank of the IVF cycle were critical factors on successful pregnancy and implantation.[21,22] Old age is one of the detrimental factors for successful clinical outcome in IVF-ET cycles. A trend toward a decline in delivery rates per frozen-thawed ET in line with an increase in the female age was reported when embryos were cryopreserved at the 2-PN stage and the early cleavage stage.[23,24]

This study presented significantly higher pregnancy and impanation rates in the frozen-thawed ET than the fresh ET cycles in old age patients (38~44 years). It may be related to the asynchrony between endometrial receptivity and embryo development in old age patients. Conclusively, frozen-thawed ET may be an alternative choice for poor prognosis patients for successful clinical outcome in ART program. This finding should be substantiated by large sample size and prospective randomized studies.

REFERENCES

1. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature 1983; 305: 707-9.
2. Wada I, Macnamee MC, Wick K, Bradfield JM, Brinsden PR. Birth characteristics and perinatal outcome of babies conceived from cryopreserved embryos. Hum Reprod 1994; 9: 543-6.
3. Sutcliffe AG, D'Souza SW, Cadman J, Richards B, McKinlay IA, Lieberman B. Minor congenital anomalies, major congenital malformations and development in children conceived from cryopreserved embryos. Hum Reprod 1995; 10: 3332-7.
4. Ubaldi F, Rienzi L, Baroni E, Ferrero S, Iacobelli M, Minasi MG, et al. Cumulative pregnancy rates after transfer of fresh and thawed embryos. Eur J Obstet Gynecol Reprod Biol 2004; 1 Suppl 1: S106-9.
5. Salumets A, Suikkari AM, Makinen S, Karro H, Roos A, Tuuri T. Frozen embryo transfers: implications of clinical and embryological factors on the pregnancy outcome. Hum Reprod 2006; 21: 2368 -74.
6. Wada I, Matson PL, Troup SA, Hughes S, Buck P, Lieberman BA. Outcome of treatment subsequent to the elective cryopreservation of all embryos from women at risk of the ovarian hyperstimulation syndrome. Hum Reprod 1992; 7: 962-6.
7. Wada I, Matson PL, Troup SA, Morroll DR, Hunt L, Lieberman BA. Does elective cryopreservation of all embryos from women at risk of ovarian hyperstimulation syndrome reduce the incidence of the condition? Br J Obstet Gynecol 1993; 100: 265-9.
8. Pattinson HA, Hignett M, Dunphy BC, Fleetham JA. Outcome of thaw embryo transfer after cryopreservation of all embryos in patients at risk of ovarian hyperstimulation syndrome. Fertil Steril 1994; 62: 1192-6.
9. Winkel CA, Fossum GT. Current reproductive technology; considerations for the oncologist. Oncologist 1993; 7: 40-51.
10. Hamer FC, Horne G, Pease EH, Matson PL, Lieberman BA. The quarantine of fertilized donated oocytes. Obstet Gynecol 1994; 10: 1194-6.
11. Brown JR, Modell E, Obasaju M, King YK. Natural cycle in-vitro fertilization with embryo cryopreser-

vation prior to chemotherapy for carcinoma of the breast. Hum Reprod 1996; 11: 197-9.

12. Templeton A, Morris JK, Parslow W. Factors that affect outcome of in-vitro fertilization treatment. Lancet 1996; 348: 1402-6.
13. Garcia JE, Acosta AA, Hsiu JG, Jones HW Jr. Advanced endometrial maturation after ovulation induction with human menopausal gonadotropin/ human chorionic gonadotropin for in vitro fertilization. Fertil Steril 1984; 41: 31-5.
14. Gidley-Baird AA, O'Neill C, Sinosich MJ, Porter RN, Pike IL, Saunders DM. Failure of implantation in human in vitro fertilization and embryo transfer patients: the effects of altered progesterone/estrogen ratios in humans and mice. Fertil Steril 1986; 44: 69-74.
15. Forman R, Fries N, Testart J, Belaisch-Allart J, Hazout A, Frydman R. Evidence for an adverse effect of elevated serum estradiol concentrations on embryo implantation. Fertil Steril 1988; 49: 118-22.
16. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high estradiol concentrations in high and normal responder patients. Hum Reprod 1995; 10: 2432-7.
17. Check JH, Choe JK, Katsoff D, Summers-Chase D, Wilson C. Controlled ovarian hyperstimulation adversely impacts implantation following in vitro fertilization-embryo transfer. J Assist Reprod Genet 1999; 16: 416-20.
18. Ng EHY, Yeung WSB, Lau EYL, So WWK, Ho PC. High serum oestradiol concentrations in fresh IVF cycles do not impair implantation and pregnancy rates in subsequent frozen-thawed embryo transfer cases. Hum Reprod 2000; 15: 250-5.
19. Levran D, Dor J, Rudak E, Nebel L, Ben-Shlomo I, Ben-Rafael Z, et al. Pregnancy potential of human oocytes-the effect of cryopreservation. N Engl J Med 1990; 323: 1153-6.
20. Selick CE, Hofmann GE, Albano C, Horowitz GM, Copperman AB, Garrisi GJ, et al. Embryo quality and pregnancy potential of fresh compared with frozen embryos-is freezing detrimental to high quality embryos? Hum Reprod 1995; 10: 392-5.
21. Strandell A, Bergh C, Lundin K. Selection of patients suitable for one-embryo transfer reduces the rate of multiple births by half without impairment of overall birth rates. Hum Reprod 2000; 15: 2520-5.
22. Kolibianakis EM, Zikopoulos K, Devroey P. Implantation potential and clinical impact of cryopreservation-a review. Placenta. 2003; 24: S27-33.
23. Damario MA, Hammitt DG, Session DR, Dumesic DA. Embryo cryopreservation at the pronuclear stage and efficient embryo use optimizes the chance for a liveborn infant from a single oocyte retrieval. Fertil Steril 2000; 73: 767-73.
24. Wang JX, Yap YY, Matthews CD. Frozen-thawed embryo transfer: influence of clinical factors on implantation rate and risk of multiple conception. Hum Reprod 2001; 16: 2316-9.

= Abstract =

Objective: The aim of this study was to evaluate the efficacy of frozen-thawed ET in poor prognosis patients such as the old age (38~44 years; OA group) and the patients who did not achieve clinical pregnancy with the first fresh ET cycle (non-pregnant patients; NP group).

Methods: Laboratory and clinical data were collected from fresh and frozen-thawed ET cycles of OA and NP group. Controlled ovarian hyperstimulation (COH) and conventional insemination or ICSI, in vitro culture and ET were performed by routine procedures. Supernumerary embryos were frozen by the slow freezing method, and frozen embryos were thawed by the rapid thawing method. Embryo development, pregnancy and implantation rates were statistically analyzed by Student t-test and chi square test.

Results: Mean ages were similar between fresh ET (40.0±1.8 years, n=206) and frozen-thawed ET (39.9 ±1.9 years, n=69) cycles in OA group. However, the clinical pregnancy and implantation rate of subsequent frozen-thawed ET significantly higher than those of fresh ET cycles (29.0% and 11.2% vs. 16.5% and 7.0%, $p<0.05$). In NP group, there was no difference in the mean age between fresh ET (31.2±2.3 years, n=40) and frozen-thawed ET (31.9±3.1 years, n=119) in subsequent cycles. The clinical pregnancy and implantation rates were similar between the subsequent fresh ET (42.5% and 22.6%) and the frozen-thawed ET (40.3% and 18.8%).

Conclusion: In old age patients, higher pregnancy rate of frozen-thawed ET compared to fresh ET cycles in this study. It may be related that better uterine environments for implantation in frozen-thawed ET cycles than that of non-physiological hormonal condition in uterus of fresh COH cycles.

Key Words: Frozen-thawed ET, Clinical pregnancy, COH, Implantation