Journal of Assisted Reproduction and Genetics, Vol. 18, No. 11, 2001

CLINICAL ASSISTED REPRODUCTION

Cryopreservation of Embryos, Blastocysts, and Pregnancy Rates of Blastocysts Derived From Frozen-Thawed Embryos and Frozen-Thawed Blastocysts

KOSTAS PANTOS,[1,2,6] KONSTANTINOS STEFANIDIS,[1] KOSTAS PAPPAS,[3] PADELIS KOKKINOPOULOS,[4] KONSTANTINA PETROUTSOU,[1,2] GEORGIA KOKKALI,[1,2] DIMITRIS STAVROU,[1,2] and VASILIOS TZIGOUNIS[5]

Submitted: January 10, 2001
Accepted: May 9, 2001

Purpose: *To evaluate the development of cryopreserved embryos when thawed and subsequently cultured to the blastocyst stage in comparison to transferring cryopreserved blastocysts.*
Methods: *In this retrospective clinical study, we have evaluated 170 cycles in patients undergoing IVF treatment for infertility. Cryopreserved embryos were thawed and were subsequently cultured and transferred at the blastocyst stage. Cryopreserved blastocysts (Day 6) were thawed and transferred immediately.*
Results: *Five hundred and sixty embryos and 444 blastocysts have been thawed. In the embryos group, the survival rate was 89% while in the blastocyst group the survival rate was 56%. In the embryos group the blastocyst development rate was 24.5%. The implantation rate in the embryos group was 20.6% per group blastocyst transferred compared to 5.3% in the blastocyst group.*
Conclusions: *The ability of cryopreserved embryos to develop to blastocysts and their implantation potential does not seem to be greatly affected by the cryopreservation procedure.*

KEY WORDS: blastocyst; cryopreservation; embryos; implantation rate; pregnancy rate.

[1] Centre for Human Reproduction, Athens, Greece.
[2] Centre for Human Reproduction Mitera Maternity Hospital, Athens, Greece.
[3] Mitera Maternity Hospital, Ioannina, Greece.
[4] Argos General Hospital, Argos, Greece.
[5] Department of Obstetrics & Gynecology, University of Patra, Patra, Greece.
[6] To whom correspondence should be addressed at Centre for Human Reproduction, 5 Kifissias Ave Marousi, Athens GR 15123, Greece; e-mail: pantos@iname.com.

INTRODUCTION

Blastocyst culture and transfer is a tool in Assisted Reproductive Technology. Potential advantages of this technique in human IVF include the synchronization of embryo development with the endometrium, leading perhaps to increased implantation rates, thereby reducing the need for multiple embryos transfers. Blastocyst culture also facilitates the assessment of embryo viability before transfer (1).

Human embryo cryopreservation has been shown to be a useful method in the treatment of infertility, allowing the salvage of excess embryos, the transfer of a reduced number of embryos, in order to avoid multiple gestation and to avoid the occurance of ovarian hyperstimulation syndrome. Human embryos can be cryopreserved at several developmental stages of their early life, namely as zygotes, cleaved embryos, or blastocysts (2).

Thanks to this flexibility, clinics could be able to choose the time to cryopreserve their embryos and vary their strategy of transfer. The aim of this study was to assess the development of cryopreserved embryos when cultured to be transferred at the blastocyst

1058-0468/01/1100-0579$19.50/0

stage in comparison to results of transferring cryopreserved blastocysts.

MATERIALS AND METHODS

This study included all couples undergoing embryo transfer at the blastocyst stage after cryopreservation. The study was performed retrospectively from October 1997 to 2000 and was approved by the ethics committee of the Mitera Maternity Hospital and the Hellenic Society of Obstetrics and Gynecology. Two different groups were identified. Group 1 comprised 65 cycles of women with a mean age (±SD) of 31.6 ± 3.5, who had a blastocyst transfer derived from frozen-thawed pronucleate stage embryos. Group 2 comprised 105 cycles of women with a mean age (±SD) of 34.8 ± 3.1 who had a blastocyst frozen-thawed transfer.

Controlled ovarian hyperstimulation for IVF–ET was conducted using long-protocol down-regulation with GnRH analogue followed by daily stimulation with a predetermined individual dose of subcutaneous recombinant FSH (follitropin beta, Puregon, Organon, Oss, the Netherlands). Follicular growth was monitored by using ultrasonography and measurement of estradiol concentrations. Human chorionic gonadotropin (hCG) (Pregnyl, Organon) was administered at a 10000 IU dose when there were more than three follicles measuring of more than 18 mm in diameter and an E2 concentration of more than 2000 pmol/mol.

In a recent publication, Dr Jones and coworkers, have described a cell-free culture system for the development of viable human blastocysts (3). By cooperating with Dr Jones, we have applied a replica of the same culture protocol described in the Centre of Human Reproduction in Athens and in Mitera Maternity Hospital.

After the oocyte pickup, insemination was performed with 50,000 normal motile spermatozoa per oocyte 4–6 h after collection. After 1-h exposure, oocytes were rinsed briefly and placed in preequilibrated culture medium. When the male factor was the underlying cause of infertility, oocytes were injected with a single spermatozoon. Oocytes for injection were denuded of cumulus cells following brief exposure to hyaluronidase (Sigma) and then assessed for maturity. Metaphase II oocytes were injected. On the following day, the oocytes were checked for fertilization.

Excess zygotes and embryos were frozen by a method adapted from Testart *et al.* (4). The freezing and thawing solutions were made by diluting cryoprotectants with Duldecco's phosphate-buffered saline (PBS, Biochrom) containing 10% human serum albumin (HSA, Irvine). Zygotes were placed into freezing solution with 1.0 M of 1, 2 propanediol (PROH; BDH) for 10 min at room temperature; they were then transferred into the same solution with 1.0 M of sucrose (Sigma) and immediately loaded into the plastic straws (250 L, I. M. V. L′aigle, France), which were sealed with a heat sealer. Straws were cooled at a rate of -2°C/min from room temperature down to -7°C in a Planer biological freezer (KRYO10, SERIES III). After holding at -7°C for 10 min and once seeding was performed, straws were cooled at a rate of -0.3°C/min down to -30°C and rapidly cooled at a rate of -50°C/min down to -150°C before being transferred into liquid nitrogen.

Blastocysts in excess of those required for transfer were cryopreserved (on Day 6) using a slow cooling protocol with glycerol as the cryoprotectant. Blastocysts were rinsed briefly in Dulbecco's phosphate-buffered saline (DP BS; Biochrom KG) supplemented with 2 mg/mL HSA. Blastocysts were then transferred to 10% glycerol (Sigma) in DPBS supplemented with 2 mg/mL HSA for 15–20 min at room temperature. Blastocysts were loaded into freezing straws (250 L, I.M. V. L′aigle, France), heat sealed, and placed in a cryological freezer (KRYO 10, PLANER, SERIES III) at -6°C.

Straws were manually seeded after 2 min and held at -6°C for a further 8 min, then slowly cooled at a rate of -0.5°C/min to -32°C before rapid cooling to -196°C and storage in liquid nitrogen.

The culture protocol used for growth to blastocysts is based on the protocol of Jones *et al.* Zygotes were cultured in groups of 2–3 in microdrops of Scandinavian IVF Science (SIS) IVF-50 medium under SIS Ovoil-150. The normally fertilized oocytes were placed 16–20 h after the insemination, in groups of 2–3 in 20 L microdrops of preequilibrated SIS S1 medium for 2 days. Embryos were assessed 65–76 h after the insemination, for cell number and degree of fragmentation and regrouped according to their quality. Embryos were placed in microdrops of 50 L of preequilibrated SIS S2 medium in groups of 2–3 and cultured for 2 more days. Embryos were assessed for blastocyst development in Day 5 after the insemination (Day 0 = day of insemination) and transferred to fresh preequilibrated SIS S2 drops of 50 L for 1 more day. On day 6 (approximately 130 h postinsemination) blastocysts were selected for transfer. One to three blastocysts were transferred to each patient

according to (a) patient's age, (b) previous clinical history, and (c) degree of expansion and morphology of the blastocysts. Blastocysts were transferred zona free after a brief exposure to 0.2% pronase (Sigma) in HEPES-HTF buffered medium. Blastocysts were transferred in SIS S2 medium supplemented with 10% HSA (Human Serum Albumin, Irvine Scientific CA, U.S.A.), using a Cook "Soft Trans" Embryo Transfer Set (K-Soft 3011).

To precisely control the uterine environment for embryo transfer in these women, all transfers were performed in controlled HRT cycles. The ovarian steroid replacement protocol consisted of oral estradiol valerate and intravaginal progesterone pessaries. On Day 2 of the replacement cycle, oestradiol was commenced at 2 mg daily up to Day 9 and then 6 mg daily from Days 10 to 13. From Days 14 to 28, estradiol was given at 4 mg daily. Progesterone pessaries were given initially 50 mg, and henceforth continued at 100 mg daily until Day 28. The day progesterone replacement began was arbitrarily designated as Day 15 of the cycle; embryo transfer was done on Day 21 or 22. Pregnancies were detected by the measurement of serum beta-human chorionic gonadotrophin (-hCG) concentration and confirmed by transvaginal ultrasonography. All pregnant women continued to have 4–6 mg of estradiol and 100–200 mg of progesterone, both administered daily until Week 10 of gestation.

RESULTS

During the 12-month study period, 560 embryos (Group 1) and 444 blastocysts (Group 2) have been thawed. From 560 embryos, 493 survived (88%) and were left in culture for 2–5 additional days before transfer. From 444 thawed blastocysts, 250 survived (56%) and were transferred on the same day that have been thawed. In Group 1 from a total of 493 survived embryos, 121 developed to the blastocyst stage (24.5%). Clinical pregnancies were established in 16 of 53 embryo transfers of blastocysts (30%) derived from frozen-thawed embryos and 13 of 101 of the frozen-thawed blastocyst transfers (12.8%) ($p <$ .005). The implantation rate was statistically significantly different, with 25 of 121 (20.6%) in Group 1 and 14 of 245 (5.3%) in Group 2 (Table I).

Table I. Data on Cryopreservation of Embryos and Blastocysts

Variable	Group 1 cryopreserved embryos	Group 2 cryopreserved blastocysts
No. of cycles	65	105
No. (%) of embryo transfers	53	101
No. of embryos frozen	560	444
No. (%) of survived embryos	493 (88)	250 (56)‡
No. (%) of blastocysts	121 (24.5)	245
No. of pregnancies	16	13
No. of sacs	25	14
Percentage of pregnancies per embryo transfer	30.1	12.8*
Implantation rate[a]	20.6	5.3‡

[a] No. of embryos that implanted/No. of blastocysts that transferred.
* $p < .05$ (vs. Group 1).
‡ $p < .005$ (vs. Group 1).

DISCUSSION

According to published data, embryos can be successfully frozen using propanediol and sucrose, results being similar to those obtained with cleavage embryos at 2 or 3 days postfertilization (2). Although this procedure is now well established in many IVF centers, the survival rate based on reviews from the literature, appears to be highly variable, ranging from 56 to 92% (5–9).

This discrepancy in zygote survival may be due, in part, to disparities in freeze-thaw protocols used. Our 88% cryosurvival rate in zygotes group is similar to 92% reported by Fugger *et al.* (5). In this study, 24.5% of frozen-thawed embryos developed to the blastocyst stage. This, compares favorably with the previously reported 40–52% of fresh embryos developing to blastocysts (1,3). Fugger *et al.* reported a clinical pregnancy rate of 29% and an implantation rate of 12% for frozen zygotes (5,6).

In our study, pregnancy rate and implantation rate were 30 and 20%, respectively, and compared favorably with previously reported 38 and 23%, respectively, published by Jones *et al.* (3) for fresh embryos developed to the blastocyst stage, using the same culture protocol.

In the literature some controversy has arisen over the benefits of cryopreservation of embryos at early or late developmental stages (2). Our 56% cryosurvival rate in blastocyst group is similar to 50–60% reported by Harout *et al.* (10) and Menezo *et al.* (11) while cryosurvival rate in the blastocyst group was significantly lower compared to the cryosurvival rate in embryos group (56 vs. 88%). In the literature investigators report clinical pregnancy rates of 10–19% and implantation rates of 9–18% for frozen-thawed blastocysts (1,11).

In our study the pregnancy rate for thawed blastocysts was 12.8% and compares favorably with the

previous 10% published by Menezo *et al.* (11), while our implantation rate was 5.3% and is lower than that referred in the literature (9–18%). Pregnancy and implantation rates following transfer of Day 6 cryopreserved blastocyst were significantly reduced as compared with embryos cryopreserved on Day 2 or 3.

When comparing embryo and blastocyst freezing in the literature, it is difficult to obtain conclusive data on the eventual superiority of one or other of the freezing protocols. In our study, it seems that the efficiency of blastocyst freezing in our hands is lower than that of early-stage freezing. Similar results have been reported by other study groups thus leading us to propose the cryopreservation of some of the embryos at an early stage, before starting extended embryo culture in patients with a large number of embryos (12,13). Damario *et al.* reported that the efficient use of embryo cryopreservation at the pronuclear stage and economical embryo utilization policies result in cumulative chances (exceeding 60%) for a liveborn in women <39 years of age (14). In contrast to that, other investigators have demonstrated that frozen-thawed blastocyst cycles may result in equivalent pregnancy rates as compared to fresh transfer (15,16). In our study, lower pregnancy and implantation rates of cryopreservation embryos at the blastocyst stage may be due to (a) the prior transfer of the best blastocysts within a cohort resulting in the cryopreservation of lower scoring blastocysts, (b) the cryoprotectant used, and (c) the Day 6 cryopreserved embryos as compared with embryos cryopreserved on Day 5 (17). Finally, the findings of the present study demonstrate that both procedures are efficient and complementary rather than antagonistic, and the goal in this area of research is to achieve the successful transfer of a single blastocyst.

REFERENCES

1. Gardner DK, Vella P, Lane M, Wagley L, Schlenker I, Schoolcraft WB: Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. Fertil Steril 1998;69:84–88
2. Mandelbaum J, Belaisch-Allart J, Junca AM, Antoine JM, Plachot M, Alvarez S, Alnot MO, Salat-Baroux J: Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. Hum Reprod 1998;139:161–175
3. Jones GM, Trounson AO, Gardner DK, Kausche A, Lolatgis N, Wood C: Evolution of a culture protocol for successful blastocyst development and pregnancy. Hum Reprod 1998;13:169–177
4. Testart J, Lassalle B, Belaisch-Allart J, Hazout A, Forman R, Rainhorn JD, Frydman R: High pregnancy rate after early human embryo freezing. Fertil Steril 1986;46:268–272
5. Fugger EF, Bustillo M, Katz LP, Dorfmann AD, Bender SD, Schulman JD: Embryonic development and pregnancy from fresh and cryopreserved sibling pronacleate human zygotes. Fertil Steril 1998;50:273–278
6. Veeck LL, Amundson CH, Brothman LJ, DeScisciolo C, Maloney MK, Muasher SJ, Jones HW Jr.: Significantly enhanced pregnancy rates per cycle through cryopreservetion and thaw of pronuclear stage oocytes. Fertil Steril 1993;59:1202–1207
7. Demoulin A, Jouan C, Gerday C, Dubois M: Pregnancy rates after transfer of embryos obtained from different stimulation protocols and frozen at either pronucleate or multicellular stages. Hum Reprod 1991;6:799–804
8. Cohen J, De Vane GW, Elsner CW, Fehilly CB, Kort HI, Massey JB, Turner TG Jr.: Cryopreservation of zygotes and early cleaved human embryos. Fertil Steril 1998;49:283–289
9. Pantos K, Athanasion V, Stefanidis K, Stavrou D, Vaxevanoglou I, Chronopoulou M: The influence of advanced age on the blastocyst development rate pregnancy rate and implantation rate. Fertil Steril 1999;71:1144–1146
10. Harout A, Nathan-Abbon C, Chohen-Bacrie, P: Results of replacement cycles in patients with frozen cocultured supernamerary blastocysts. J Assist Reprod Genet 1995;12,65–68S
11. Menezo Y, Nicollet B, Herbaut N, Andre D: Freezing cocultured human blastocysts. Fertil Steril 1992;58:977–980
12. Desai NN: Blastocyst stage transfer: Pitfalls and benefits. The road to blastocyst transfer. Hum Reprod 1998;13:3292–3294
13. Plachot M, Belasch Allart J, Mayenga JM, Chouragui A, Serkine AM, Tesquier L: Blastocyst stage transfer the real benefits compared with early embryo transfer. Hum Reprod 2000: 15(Suppl. 6):24–30
14. Damario MA, Hammit DG, Session DR, Dumesic DA: Embryo cryopreservation at the pronuclear stage and efficient embryo use optimizes the chance for a liveborn from a single oocyte retrieval. Fertil Steril 2000;73:767–773
15. Behr B, Pool TB, Milk AA, Moore D, Gebbardt J, Dasing D: Preliminary clinical experience with human blastocyst development in vitro without co-colture. Hum Reprod 1999;14:454–457
16. Scott L, Leondires MP, Alvero RJ, Miller B: Increased clinical pregnancy rates from frozen-thawed human blastocyst using a rapid dilution and transfer protocol. Fertil Steril 2000;74:S51–S52
17. Marek D, Langley M, McKean C, Weing L, Doody KM, Doody KJ: Frozen embryo transfer of Day 5 blastocyst embryos compared to transfer of Day 6 blastocyst embryos. Fertil Steril 2000;74:S52–S53